DETECTION AND SIGNIFICANCE
OF DRUG-RESISTANT HIV-1
MINOR POPULATIONS

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ABSTRACT

The human immunodeficiency virus (HIV) is the cause of the HIV/AIDS pandemic affecting millions of people around the world. Treatment with antiretroviral drugs, if used properly, leads to a dramatic decrease in virus production in the patient and prevents the onset of AIDS. However, treatment is not always successful, mainly due to the extraordinary ability of the virus to change and become resistant to the antiretroviral drugs.

The general aim of this thesis was to study the development of HIV-1 drug resistance, specifically the detection and significance of small populations of drug-resistant virus in infected individuals that can go undetected by the standard resistance tests used in the clinics today. In study I, we developed and evaluated an assay called selective real-time PCR (SPCR), which can detect and display the proportions of different populations of drug-resistant virus carrying the clinically relevant M184I/V mutations in the HIV-1 reverse transcriptase enzyme. These mutations confer high-level resistance to one of the most commonly used drugs today, lamivudine (3TC). The SPCR assay was then applied to several different clinical cohorts in the subsequent papers.

We found SPCR to be a well-functioning, sensitive and reliable method to study the kinetics of antiretroviral resistance development in HIV-1, which was able to detect resistance mutations not seen by conventional genotyping assays.

In study II, we evaluated the presence of M184I/V-containing minor viral populations and their potential role in treatment failure in didanosine (ddI)-treated patients experienced with multiple nucleoside reverse transcriptase inhibitors. We found that the survival advantage of virus strains having the M184I/V mutations were limited during ddI treatment, suggesting that the presence of these mutations should not preclude the use of ddI in nucleoside-experienced patients.

In study III, we investigated to which extent unique drug resistance patterns appear in cerebrospinal fluid as compared to blood in patients who had failed 3TC-containing therapy. We found differences in drug resistance patterns in both minor and major populations, which were likely to be related to differences in the selective pressure of antiretroviral drugs within the two compartments rather than unique evolutionary pathways. Minor populations were not seen to play a major role in resistance development in cerebrospinal fluid in the patients we tested.

In study IV, we studied to which extent selection of M184I/V mutations occurs in treatment-naïve patients during the first months after initiation of antiretroviral therapy containing two, three or four drugs. We found the selection of drug-resistant variants to be rare in the three- and four-drug cohorts, but common in the two-drug cohort, suggesting that current treatment regimens initiated in treatment-naïve patients should be sufficient to stop resistance development during the first phase of viral decay, as long as the patients are adherent.

The clinical significance of minority populations in the development of resistance to antiretroviral drugs is an important topic to be defined. SPCR and similar assays will provide valuable tools to further expand the current knowledge in this field.
LIST OF PUBLICATIONS


II. Veronica Svedhem, Tobias Bergroth, Knut Lidman, Anders Sönnerborg. Presence of M184I/V in minor HIV-1 populations of patients with lamivudine and/or didanosine treatment failure. *HIV Medicine* 2007, 8:504-510

III. Tobias Bergroth, Halime Ekici, Magnus Gisslén, Lars Hagberg, Anders Sönnerborg. Comparison of drug resistance patterns in minor and major HIV-1 populations in plasma and cerebrospinal fluid. *Submitted*

IV. Tobias Bergroth, Halime Ekici, Magnus Gisslén, Sabine Kinloch-de Loes, Li-Ean Goh, Andrew Freedman, Margaret A Johnson, Anders Sönnerborg. Drug-resistant HIV appears seldom during the early phase of viral decay in patients initiated on three- or four-drug ART. *Submitted*
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Acknowledgements

References
LIST OF ABBREVIATIONS

3TC Lamivudine
ABC Abacavir
AIDS Acquired immunodeficiency syndrome
ART Antiretroviral therapy
ATV Atazanavir
AZT/ZDV Zidovudine
BBB Blood-brain barrier
CNS Central nervous system
CRF Circulating recombinant form
CSF Cerebrospinal fluid
CTL Cytotoxic T-lymphocyte
d4T Stavudine
ddI Didanosine
DLV Delavirdine
DNA Deoxyribonucleic acid
DRV/TMC-114 Darunavir
EFV Efavirenz
ENF Enfuvirtide
fAPV Fosamprenavir
FTC Emtricitabine
HAART Highly active antiretroviral therapy
HIV-1/2 Human immunodeficiency virus type 1/2
IDV Indinavir
LPV Lopinavir
MK-0518 Raltegravir
NFV Nelfinavir
NNRTI Non-nucleoside reverse transcriptase inhibitor
NRTI Nucleoside reverse transcriptase inhibitors
NVP Nevirapine
PCR Polymerase chain reaction
PHI Primary HIV-1 infection
PI Protease inhibitor
RNA Ribonucleic acid
RT Reverse transcriptase
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INTRODUCTION

The human immunodeficiency virus (HIV) is the cause of the HIV/AIDS pandemic affecting millions of people around the world. Treatment with antiretroviral drugs, if used properly, leads to a dramatic decrease in virus production in the patient and prevents the onset of AIDS. However, treatment is not always successful, mainly due to the extraordinary ability of the virus to change and become less susceptible to the antiretroviral drugs it is exposed to. This resistance often decreases the sensitivity of the virus to several drugs of the same class at once and limits future treatment options.

The work included in this thesis has focused on the development of HIV-1 resistance against antiretroviral drugs, specifically the detection and significance of small populations of drug-resistant virus in infected individuals that are undetected by the standard resistance tests used in the clinics today.

DISCOVERY

In 1981, AIDS was described for the first time when an increased incidence of opportunistic diseases in previously healthy young homosexual men was identified. Two years later, a virus, which we now know is the causative agent of AIDS, was isolated by a French research group that named it lymphoadenopathy-associated virus (LAV). The following year, researchers in USA isolated an identical virus and named it AIDS associated virus or human T-cell lymphotrophic virus (HTLV III). The virus was subsequently renamed human immunodeficiency virus (HIV) due to its biological properties. The earliest documented HIV-1 case is a frozen plasma sample from 1959, found in the Democratic Republic of Congo.

In 1986, a similar immunodeficiency virus was discovered that was named HIV-2, and the former strains of HIV were referred to as HIV-1. Compared to HIV-1, HIV-2 is less contagious and it takes longer time for HIV-2-infected individuals to develop AIDS.

HIV TYPES AND SUBTYPES

The consensus among researchers today is that HIV-1 and HIV-2 have been introduced to humans from other primates by transmission of simian immunodeficiency viruses (SIVs), probably during hunting and butchering of wild primates or through bites from captured primates kept as pets. The SIVs are species-specific and do not seem to cause any disease in their natural host. HIV-1 is thought to have originated from chimpanzee SIV, while HIV-2 is thought to have originated from SIV from the Sooty Mangabey monkey.

By comparing the genetic sequences of different viral strains, HIV-1 has been divided into three main groups; M (major), O (outlier) and N (non-M-non-O), which are believed to have resulted from three separate SIV transmissions. Group M, in turn, is divided into nine further subgroups, A, B, C, D, F, G, H, J and K, including two sub-subtypes (A1, A2 and F1, F2).Viruses from different lineages within group M have also recombined to form various recombinant viruses known as circulating recombinant forms (CRFs). The O and N groups still contain very few identified strains and are not divided into subtypes. HIV-2 is divided into 7 subtypes (A-G), where A and B are the most common.

HIV-1 subtypes and CRFs are unevenly distributed around the globe, with four subtypes and two CRFs being most prevalent; A, B, C, D, CRF01_AE and CRF02_AG. Subtype B is commonly found in Europe and North America while subtype C is the most spread subtype globally.
Subtype C was originally described by our research group\textsuperscript{16}. All subtypes are found in Africa. Aside from some sporadic infections, HIV-1 groups N and O never established epidemics outside of West Africa\textsuperscript{17-20}.

\begin{figure}
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Global adult HIV prevalence. Image based on data from UNAIDS.}
\end{figure}

Today, it is estimated that approximately 33 million people are infected with HIV. 2.5 million of them were infected and 2.1 million people died due to HIV/AIDS\textsuperscript{21} during this year alone. In Sweden, the prevalence of HIV infection is still low. Around 7500 people with HIV infection have been reported to the Swedish Institute for Infectious Disease Control up to the year 2006, with more than 4000 of them still living. 390 new cases were reported during 2006\textsuperscript{22}.

Infection with HIV occurs by exposure to infected blood and blood products, semen, vaginal fluid, pre-ejaculate or breast milk. HIV is present within these body fluids both as free virus particles and virus within infected cells. The major routes of transmission are unprotected sexual intercourse, contaminated needles and mother-to-child transmission at birth or by breast feeding. Worldwide, sexual transmission is the most common route of transmission.
THE HIV-1 VIRUS

HIV-1 belongs to the *Lentivirus* genus and the *Retroviridae* family. It is a spherical virus with a diameter of approximately 110 nm. Inside the virus, two genomic positive stranded RNA molecules of approximately 9,200 base pairs are embedded in a protein capsid together with viral enzymes necessary for successful infection. The capsid is surrounded by a matrix protein layer that in turn is enclosed by a lipid bilayer, the envelope, which the virus acquires from the infected host cell. The envelope is equipped with viral glycoproteins called gp41 and gp120 that protrude from the membrane.

![Morphologic and genetic structure of HIV-1](image)

*Figure 2.* Morphologic and genetic structure of HIV-1.

The HIV genome consists of three major genes common to all retroviruses; *gag*, *pol* and *env*, which encode structural proteins and three viral enzymes; reverse transcriptase, protease, and integrase. Besides the three major genes, six other genes are found that code for the regulatory proteins *Tat* and *Rev* as well as the accessory proteins *Vif*, *Vpr*, *Vpu* and *Nef*. All of these genes are essential for viral replication and infection.

The nine genes are flanked by two repetitive regions called non-coding long terminal repeats (LTRs), which are important for both integration and transcription of the provirus once it is integrated. To utilize the genome size as efficiently as possible, all three reading frames are used as well as differential splicing.
THE HIV-1 LIFE CYCLE

The first step in the HIV-1 life cycle involves binding of the viral envelope glycoprotein 120 (gp120) to a host cell CD4 receptor. After binding, it interacts with a chemokine co-receptor, mainly CCR5 or CXCR4, which triggers a fusion between the membranes of the virus and the cell, releasing the viral nucleocapsid into the cell.

Once inside the cell the viral nucleocapsid is dismantled, releasing its contents into the cell cytoplasm. The two copies of viral positive single stranded RNA are then exposed and reverse transcription of the viral RNA to DNA is started by the reverse transcriptase (RT) enzyme. During this process, the single stranded viral RNA is converted into double stranded DNA and acquires long terminal repeats (LTRs) flanking the genome at both ends.

The newly synthesized double stranded DNA, together with integrase and viral matrix proteins, forms a pre-integration complex that is translocated into the nucleus of the infected cell, where
it is integrated into the host genome by the viral integrase enzyme. HIV DNA integrated into the chromosome is called a provirus, which later is used as a template for production of viral progeny.

Frequently transcription of the integrated DNA (called proviral DNA) is initiated immediately, but in some cells it remains latent for a variable length of time until the host cell is activated. The cellular activation initiates transcription of the structural genes into mRNA, which are then transported out of the nucleus into the cytoplasm. Following translation, the viral mRNA and proteins begin to assemble at the host cell surface, and start the process of budding where it acquires the modified host plasma membrane as its envelope. During this process the viral gag and gag-pol polyproteins are cleaved by the viral protease enzyme, generating mature infectious virus particles.

THE CLINICAL COURSE OF HIV-1 INFECTION

The HIV-1 virus targets all cells that express the CD4 molecule on their surface. These cells include CD4+ T-lymphocytes, monocytes, macrophages, follicular dendritic cells and microglial cells in the brain. The primary targets are the CD4+ T-lymphocytes, and destruction of these cells is the main cause of AIDS.

![Figure 4](image_url) The clinical course of HIV-1 infection.

Although the rate of disease progression is highly variable among HIV patients, most infections follow a typical course that can be divided into three stages.

The first stage is the primary infection that occurs a few weeks after the initial infection. At this stage a very high level of viral replication takes place, referred to as the acute phase viremia. The clinical signs are similar to those that occur in many acute viral infections, for example fever, rash, sore throat, lymph node enlargement and headache. The acute phase viremia subsides alongside the clinical symptoms after a few weeks.
The second stage is a clinically latent, chronic phase that may last up to ten years or more\textsuperscript{39}. In general the patient remains relatively healthy during this period. During this stage the viral load drops to a stable level, which is termed the viral set-point. The set-point is a predictive marker for the course of infection; the higher the viral set-point, the more rapid the progression to AIDS\textsuperscript{40}. This period is characterized by low but persistent levels of virus replication, predominantly in lymph nodes, and a slow, continuous loss of CD4\textsuperscript{+} cells\textsuperscript{41}.

The third stage is AIDS, which occurs through an accelerated loss of CD4\textsuperscript{+} cells and a rise in virus replication. As a result of the weakening immune system, the body becomes progressively more susceptible to AIDS-associated opportunistic infections and tumors. Without treatment, the patient usually dies within a few years.

**GENETIC VARIABILITY**

HIV-1 is one of the fastest evolving organisms known to exist today. Compared to humans, the evolutionary rate of HIV-1 is about one million times faster\textsuperscript{42}. The major natural selective pressure is the human immune system, which drives HIV-1 evolution to select for viral variants with reduced sensitivity to neutralizing antibodies and cytotoxic T-cells (CTLs). The other major selective pressure is antiretroviral treatment, where only viruses that successfully replicate during treatment are selected. Although the selection forces imposed by these two pressures act on different regions of the viral genome, the basic principles of viral adaption are the same: creating a vast number of viral variants so that some of these will contain changes necessary for survival and further reproduction when faced with a change in the environment. Upon infection with HIV-1, the patient harbors a relatively homogenous population of the virus\textsuperscript{43-45}. Over time, this population evolves and becomes more diversified and several new sub-populations may be created. These populations of genetically related but non-identical viruses are called quasispecies\textsuperscript{46}.

Except for the selective pressures, viral high evolutionary rate over time is also determined by the enormous amount of viral progeny created during a replication cycle as well as the remarkably fast turnover of the evolving viral population. The number of newly created virus particles has been estimated to be \(10^{10}\) each day, and the half-life of an evolving population \textit{in vivo} has been shown to be about 6 hours\textsuperscript{47}.

The high variability of HIV-1 is mainly due to the low fidelity of the viral reverse transcriptase enzyme. This enzyme lacks proof-reading activity and is therefore very error prone. Most of these errors are base substitutions, but duplications, insertions, and recombination events can also occur\textsuperscript{48, 49}, which may allow for the escape from the immune system as well as from antiretroviral treatment. The rate of nucleotide errors caused by the RT enzyme has been estimated to be about one error per virus genome per cycle of replication\textsuperscript{50, 51}. In addition, host RNA-polymerase II, the enzyme responsible for RNA synthesis from the proviral DNA, also lacks proof-reading activity, which may result in further addition of misincorporations into the newly synthesized viral mRNA.

The genetic sequence variation is due to nucleotide substitutions that are either silent/synonymous (ds), which do not cause amino acid changes, or non-synonymous (dn), which cause amino acid changes. Generally, synonymous substitutions occur in the third position of a codon. The ds/dn ratio varies between different genomic regions and is in part influenced by the selective pressure of the immune system\textsuperscript{52, 53} and the function of the protein that the gene is coding for. The \textit{env} gene allows extensive misincorporations since high variability of the proteins that it encodes results in potential escape from the host immune system. The \textit{pol} gene, on the other hand, shows considerably lower variability since it encodes enzymes essential for the viral
replication. These enzymes have to maintain their function and therefore allow fewer changes in their functional domains.

At the late AIDS stage, the genetic diversity diminishes, probably as a result of immune system failure\textsuperscript{54-56}.
TREATMENT OF HIV-1 INFECTION

The first antiretroviral drug, zidovudine (ZDV), was introduced in 1987 and was later followed by didanosine (ddI) and zalcitabine (ddC). Treatment was initially started as mono-therapy, but was later replaced by combination therapy, which was proven to have higher clinical benefit. Today, three or more drugs from at least two different drug classes are used simultaneously, to minimize the risk of viral replication and thus developing drug resistance, which was seen to be common with mono- or dual therapy. This is sometimes called “HAART”, which stands for highly active antiretroviral therapy. The introduction of combination antiretroviral therapy has dramatically decreased both number of deaths and increased the quality of life of HIV-infected individuals.

Antiretroviral therapy results in a rapid decrease in viral load (the amount of virus in the patient) and is usually followed by an increase in CD4+ T-cell counts. However, successful suppression of the viral load is not immediately followed by normalization of T-cell levels, which may take many years and is not always achieved.

For a while there was optimism that HIV could be completely eradicated with a sufficiently prolonged course of antiretroviral combination therapy. However, after discovering that HIV also infects long-lived cells like microglia, and establishes latency in resting memory CD4+ T-cells, these hopes were abandoned. Today the prospect is that the treatment will be life-long.

There are 22 antiretroviral drugs available today for clinical use. They are of five different classes; nucleoside analog reverse transcriptase inhibitors (NRTI), non-nucleoside analog reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), integrase inhibitors and entry inhibitors, all with different modes of action.

NUCLEOSIDE ANALOG REVERSE TRANSCRIPTASE INHIBITORS

Seven drugs belong to the NRTI class; abacavir (ABC), didanosine (ddI), emtricitabine (FTC), lamivudine (3TC), stavudine (d4T), tenofovir (TDF) and zidovudine (AZT/ZDV).

NRTIs target the viral enzyme reverse transcriptase (RT), and stop the enzyme from copying the viral RNA genome into DNA once the virus has entered the cell. They do this by mimicking the normal building blocks of DNA (the deoxyribonucleotide triphosphates or dNTPs), and competing with them for incorporation into the same “slots” in the growing DNA chain. The NRTIs, however, lack the 3’ hydroxyl group on normal dNTPs that is required for the continuation of DNA synthesis, resulting in an unfinished DNA strand that cannot be used to produce viable virus.

All NRTIs except tenofovir are prodrugs that need to be phosphorylated to triphosphate forms by cellular enzymes to become active, while tenofovir is intracellularly phosphorylated to its diphosphate form.

NON-NUCLEOSIDE ANALOG REVERSE TRANSCRIPTASE INHIBITORS

Three current drugs belong to the NNRTI class; efavirenz (EFV), delavirdine (DLV, not approved in EU) and nevirapine (NVP). In addition, a fourth drug, etravirine (TMC-125), has shown good results and is likely to become approved during 2008.

NNRTIs are a diverse set of compounds that also target the reverse transcriptase enzyme, but by a different mode of action than the NRTIs. They act by binding to a hydrophobic pocket in
close proximity to the active site of the enzyme, which is thought to interfere with the precise alignment between the 3’ end of the primer and the incoming nucleotide, thereby diminishing the efficiency of the catalytic step.

PROTEASE INHIBITORS
Nine current drugs belong to the protease inhibitor (PI) class; atazanavir (ATV), darunavir (DRV), fosamprenavir (fAPV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV) and tipranavir (TPV).

PIs prevent the HIV-1 protease enzyme from cleaving the gag precursor protein. This stops newly produced viruses from maturing after leaving the cell, thereby making them non-infectious.

All PIs, except nelfinavir, are commonly given together with a low dose of ritonavir. Ritonavir is only used together with other PIs, creating the term “boosted PI” or “PI/r”. The low-dose ritonavir keeps the cellular enzymes normally responsible for breaking down PIs in the liver busy, thereby keeping the concentration of the other protease inhibitor higher in the blood. Nelfinavir, however, is broken down by other cellular enzymes, negating the use of ritonavir.

INTEGRASE INHIBITORS
Only one drug belongs to the integrase inhibitor class; raltegravir (MK-0518). This drug targets the HIV-1 integrase enzyme and stops viral DNA from integrating in the host genome after reverse transcription.

ENTRY INHIBITORS
Two drugs belong to the entry inhibitor class; enfuvirtide (T-20/ENF) and maraviroc. These entry inhibitors are designed to stop the virus from entering and infecting the cell, but do so by different mechanisms.

Enfuvirtide is an injectible peptide that works by inhibiting the fusion of the virus with the target cell by binding to the viral gp41 protein.

Maraviroc is the first antiretroviral drug directed against a human target. It blocks the attachment of the virus to the target cell by binding the CCR5 co-receptor on the host membrane, which HIV-1 normally uses for entry into the cell together with the CD4 receptor. Maraviroc does not, however, work on viruses that use the CXCR4 co-receptor, and thus require a test of which co-receptor the patient virus prefers before it can be used.

The fusion inhibitors are often used when regular treatment has failed due to resistance development.

SIDE EFFECTS OF ANTIRETROVIRAL THERAPY
Besides being very expensive, antiretroviral drugs can also cause various side effects such as loss of subcutaneous fat in the face, extremities and buttocks, muscle or nervous system diseases, gastrointestinal intolerance, diabetes, and increased risk for myocardial infarctions. The side effects vary from different drugs and in different patients.
VIRAL DECAY CHARACTERISTICS DURING TREATMENT

Following the initiation of antiretroviral therapy, the virus decays in two phases\(^7\). The initial phase includes a rapid drop in viremia, which is thought to be due to a very short half-life of plasma virus (minutes to hours)\(^47, 73\) and the half-life of the activated CD\(_4^+\) T cells that produce almost the majority of the plasma virus\(^74\), probably <1 day\(^47, 75, 76\). After the initial drop, the plasma virus levels off to a slower decay. This probably reflects the decay of a different population of infected cells, which are not as readily killed when productively infected by HIV-1. In untreated patients, these cells contribute only a small proportion of the plasma virus, and the contribution of this compartment becomes apparent only when replication is suppressed by antiretroviral drugs. The half-life of the compartment responsible for this second phase of decay is estimated to be 1–4 weeks\(^77\).

TREATMENT RECOMMENDATIONS

The current Swedish recommendations for first-line therapy in treatment-naïve patients is the use of 2 NRTIs together with 1 NNRTI, or 2 NRTIs and one PI/r\(^77\). With regard to their virological effect, NNRTI and PI/r are considered equally potent. Using a fourth active drug has been shown not to confer any additional benefit\(^78, 79\). Considerations of factors such as adverse effects profile, dosing regimen requirements, expected adherence levels and the possibility of pregnancy are the decisive factors for the choice of particular drugs. Some NRTI combinations are not recommended due to antagonism, lack of additive or synergistic effect, risk for treatment failure or additive side effects.

Before initiation of therapy, an individual decision in each case is made based on the appearance of symptoms, CD\(_4^+\) T-cell count, the progression of loss of CD\(_4^+\) T-cells, viral load, age, social situation and possible drug abuse. Special drug regimens are available for treatment of HIV in pregnant women and for post-exposure prophylaxis\(^77, 80\).
HIV-1 DRUG RESISTANCE

Successful antiretroviral therapy reduces the plasma viral load to undetectable levels. If the virus rebounds to detectable levels, it is seen as treatment failure, which could be due to adherence problems, low potency of the drugs, disturbance in drug metabolism or absorption, or emergence of mutated, drug-resistant virus.

Suboptimal ART, which allows the viral replication to continue, is known to select for virus variants with reduced sensitivity to the antiretroviral drugs, and drug resistance is therefore a common problem.

HIV-1 RESISTANCE TESTING

Resistance testing is best performed when patient is on treatment, since resistance mutations in the dominating viral population easily revert when off treatment. Testing is today recommended in the situation of primary HIV infection, treatment failure and pregnancy, and may also be considered before starting first line treatment.

Currently two types of resistance tests are used: genotypic assays (i.e., sequencing to detect mutations that confer drug resistance) and phenotypic assays (i.e., drug susceptibility testing by growing patient-derived virus in different concentrations of the drug). Genotyping is faster and less expensive and offers the possibility to detect transitional mutations that may predict emerging drug resistance. In Sweden, genotypic testing is used for clinical practice due to practical advantages and less cost.

SELECTION OF DRUG-RESISTANT VIRUS

Although resistance-conferring mutations may precede the initiation of therapy, due to both spontaneous mutagenesis and the spread of resistant viruses by sexual and other means of transmission, it is generally believed that multiple drug mutations to any single or combination of antiretroviral drugs are selected during continued viral replication in the presence of incompletely suppressive drug regimen. Because these mutated strains represent only a small proportion of the total viral load, they presumably have a replicative or other competitive disadvantage compared to wild-type virus. The selective pressure of antiretroviral therapy provides these drug resistant mutants with a competitive advantage and under such circumstances they eventually become the dominant population.

The patterns of mutations accumulated by HIV-1 under drug pressure in vivo are quite complex and variable, depending not only on the intrinsic biochemical properties and mechanism of action of each drug, but also on the level of pharmacologic pressure, length of therapy, and the backbone of virus strains. Moreover, such variability is further increased in vivo by host selective pressure such as the immune system.

Two factors influence the rate of replacement of the non-resistant viral population by a resistant one.

- The number of mutations needed to reach a high level of resistance or overcome the genetic barrier. This phenomenon can be very fast with drugs having a low genetic barrier, such as NVP and 3TC, where only one nucleotide change can induce high level of resistance. In contrast, other drugs with a higher genetic barrier, such as...
zidovudine or protease inhibitors, need a sequential accumulation of multiple mutations at different positions to reach a high level of resistance91,92.

- Kinetic cost. Some mutations are responsible for a decrease in the replication capacity (or loss of “fitness”) in the absence of drug pressure. When treatment is interrupted, mutant viral variants are rapidly substituted by wild-type virus, which has higher fitness93. However, after reinitiating therapy, drug-resistant viruses can rapidly re-appear94-96.

Resistance is most easily developed when the level of potency of the given combination of drugs is intermediate. If the drug activity is too low, non-mutated virus can still replicate and the competitive advantage of the drug-resistant viruses is not sufficient to outgrow the wild-type viral variants. If the drug activity is high, but the viral replication still continues, the appearance of mutations is delayed and viruses with drug resistance are rarely seen or develop more slowly.

Depending on which protein is affected, mutations may be unfavorable or beneficial for the virus. Primary resistance mutations are generally selected early in the process of resistance mutation accumulation and tend to be relatively specific for each drug. They usually appear as single mutations giving rise to considerably high decrease in drug susceptibility97, but often the replicative capacity decreases at the same time. If the viral replication is allowed to continue, secondary (also called compensatory) mutations can appear which confer little or no reduction in drug susceptibility. Instead, these mutations compensate the viral fitness that is lost with the appearance of the primary mutations98. Secondary mutations can pre-exist in untreated individuals as natural polymorphisms99. These do not interfere with treatment in the absence of primary mutations.

If resistant HIV is transmitted to new individuals where no antiretroviral pressure is present, the virus tends to lose its resistance mutations because of the cost the resistance brings for viral fitness and replication capability100,100. However, the reversion of resistance mutations may not always be to wild type, but to an intermediate strain, which has better fitness and a greater likelihood of developing drug resistance100-102.

VIRAL RESERVOIRS

Successful ART reduces the viral load and at the same time diminishes the diversity of viral quasispecies. However, diverse viral variants can persist in so-called viral reservoirs, which could be cellular or anatomical.

Cellular reservoirs represent cells where the virus can hide from both the immune system and from antiretroviral therapy. The most important reservoirs for HIV-1 are the resting memory CD4+ T cells103,104. Most infected CD4+ T cells generally die within a few days, but some cells survive long enough to revert to a resting state, thereby establishing a stable latent reservoir of resting memory cells carrying an integrated form of the HIV-1 genome103,104. The virus in this reservoir appears to be latent, since no virus is produced by these cells unless they are activated by antigen. They are not detected by the immune system or affected by antiretroviral therapy.

Since this population of latently infected cells persists in individuals receiving antiretroviral therapy65-67,105,106, these cells can also act as a reservoir for drug-resistant virus. During conditions of high-level viremia, viruses with drug resistance mutations can enter the latent reservoir, and persist there undetected for a long time, essentially archiving the drug resistance for life. The resistant variants can then re-appear at a later time. The same applies for wild-type virus, which can rapidly re-appear during treatment cessation107,108. Once suppression to <50 copies/ml has been achieved, there appears to be little additional turnover in the latent reservoir106,109,110, despite low levels of ongoing virus production111-115.
Other cellular reservoirs of lesser importance have been suggested to be naive CD4+ T-cells, CD8+ T-cells, monocytes, B cells, dendritic cells, and NK cells\textsuperscript{116}. Anatomical reservoirs correspond to sanctuary sites in the body, where access for antiretrovirals and/or the immune system are restricted. Examples of such sites are the central nervous system, the genitourinary tract and the lymphoid organs. In these sites, resistant strains can be selected due to suboptimal drug concentrations, which can later spread into the bloodstream, potentially causing treatment failure.

THE HIV-1 RESISTANCE MUTATIONS

NRTI RESISTANCE MUTATIONS

Most nucleoside reverse transcriptase inhibitor (NRTI)-resistance mutations appear in the so called “fingers” and “palm” subdomains of the RT enzyme, and confers resistance by two known mechanisms\textsuperscript{117};

- enhanced ability to discriminate between the drug and its natural dNTP counterpart before it binds. The mutations K65R, L74V, Q151M and M184V use this mechanism.

- enhanced removal of the NRTI once it is incorporated. This mechanism is known as primer unblocking or pyrophosphorolysis. The thymidine analog mutations (TAMs); M41L, D67N, K70R, L210W, T215Y/F and K219Q/E, use this mechanism.

Which mechanism is used depends on where the mutations are located on the RT enzyme.

<table>
<thead>
<tr>
<th></th>
<th>K</th>
<th>L</th>
<th>Y</th>
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<tr>
<td>Abacavir</td>
<td>65</td>
<td>74</td>
<td>115</td>
<td>184</td>
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<tr>
<td>Didanosine</td>
<td>K</td>
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<td>Emtricitabine</td>
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<td>Lamivudine</td>
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<td>67</td>
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<td>67</td>
<td>70</td>
<td>210</td>
<td>215</td>
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Table 1. The major NRTI mutations\textsuperscript{118}.  

13
NRTI MULTI-DRUG RESISTANCE MUTATIONS

Resistance mutations selected for one drug can also cause cross-resistance for some, or all, the other drugs in the same class. There are three known pathways to multi-drug NRTI resistance; the TAM pathway, the 69 insertion complex pathway and the 151 complex pathway.

The TAMs are selected by ZDV and d4T and accumulate in step-wise fashion and can cause cross-resistance to the other NRTIs if three or more of them are expressed. The M41L and T215Y/F are defined as primary mutations, and D67N, K70R, L210W and K219Q are defined as secondary mutations. The TAMs are also sometimes called nucleoside-associated mutations (NAMs), to take into account that such changes can also reduce susceptibility to non-thymidine analogs.

The 69 insertion complex consists of a substitution at codon 69 (typically T69S) and an insertion of 2 or more amino acids. It is associated with resistance to all NRTIs when present with one or more TAMs at codons 41, 210, or 215119.

The 151 pathway, consisting of the Q151M mutation and mutations A62V, V75I, F77L, and F116Y, confers resistance to all NRTIs except TDF119.

<table>
<thead>
<tr>
<th>69 Insertion Complex</th>
<th>41 62 69 70</th>
<th>210 215 219</th>
</tr>
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<tbody>
<tr>
<td>TAMs</td>
<td>M A K</td>
<td>L T K</td>
</tr>
<tr>
<td>151 Complex</td>
<td>A V F</td>
<td>W Y Q</td>
</tr>
<tr>
<td>210 215 219</td>
<td>L T K</td>
<td>W Y Q</td>
</tr>
<tr>
<td>TAMs</td>
<td>M D K</td>
<td>L T K</td>
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Table 2. The NRTI multi-drug resistance pathways118.

NNRTI RESISTANCE MUTATIONS

NNRTIs resistance mutations are clustered around the hydrophobic binding pocket in close proximity to the active site120, which diminishes binding or restricts access of the drug. The most prominent mutations include the K103N, Y181C/I, and Y188C changes.

Cross-resistance is particularly prevalent amongst the NNRTIs; a single mutation can give cross resistance to all drugs used in the class121-123.

<table>
<thead>
<tr>
<th>Efavirenz</th>
<th>100 103 106 110</th>
<th>181 188 150</th>
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<tbody>
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<td></td>
<td>I N M I</td>
<td>C L S H</td>
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<table>
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<tr>
<th>Nevirapine</th>
<th>100 103 106 108</th>
<th>181 188 150</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I N M I</td>
<td>C L A M I</td>
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</table>

Table 3. The major NNRTI mutations118.

PROTEASE INHIBITOR RESISTANCE MUTATIONS

Resistance to PI is due to amino acid changes in the active site of the HIV-1 protease or in surrounding regions involved in the binding of the PI. The PIs have multiple, overlapping
patterns of resistance. High level resistance to most PIIs requires the accumulation of multiple mutations, either by a complete change of the resistance profile or the successive addition of mutations on an unchanged background\textsuperscript{124,125}.

**INTEGRASE INHIBITOR RESISTANCE MUTATIONS**

Since this drug class is very new, little is known about the resistance development. However, preliminary resistance data indicates two mutational pathways associated with mutations on positions 148 or 155 on the integrase gene\textsuperscript{126}.

**FUSION INHIBITOR RESISTANCE MUTATIONS**

Enfuvirtide is associated primarily with mutations in the HR1 region of the gp41 envelope gene\textsuperscript{127}, while maraviroc mutations are clustered in the V3 region of the gp120 enzyme\textsuperscript{128}.

**THE M184I/V MUTATIONS**

The M184 mutations are among the most studied resistance mutations in HIV-1. They appear in the methionine residue at the highly conserved YMDD motif at codon 184 of the RT gene, right in the catalytic center of the enzyme\textsuperscript{68,129}. Both mutations require only a single base substitution and appear in two variants, M184I (isoleucine; ATG\textarrowrightATA) and M184V (valine; ATG\textarrowrightGTG). A two-step variant of M184V (ATG\textarrowrightGTA) may also appear through an intermediate ATA or GTG mutation. In addition, a M184T (threonine; ATG\textarrowrightACG) mutation has been described, but occurs rarely. No compensatory mutations have so far been described for M184I/V.

The M184I mutation is usually the first one to appear\textsuperscript{130}, which is thought to be because RT is more prone to perform G\textarrowrightA than A\textarrowrightG transitions\textsuperscript{131}. It is, however, most often rapidly replaced by viruses harboring the M184V mutation (ref), due to the lower enzymatic efficiency of viruses carrying the M184I mutation compared to the M184V mutation\textsuperscript{93,132,133}. The M184T mutation results in an even further reduction of fitness\textsuperscript{134}.

The M184I/V mutations emerges rapidly\textsuperscript{135} and are selected by 3TC, FTC and ABC. They confer high-level resistance to 3TC and FTC\textsuperscript{122,136} and low-level phenotypic resistance to ABC and ddI\textsuperscript{137-140}. In isolation the mutations do not compromise virologic responses to ABC or ddI, but in combination with \textgeq 3 TAM or with mutations at positions 65, 74, or 115 M184V causes significant resistance to ABC\textsuperscript{141}. The M184I/V mutations do not confer significant cross-resistance to other NRTIs.

If 3TC is withdrawn from therapy, viruses harboring M184I/V mutations will be overgrown by wild-type viruses due to fitness advantages, and probably will not be detected by genotypic analysis. If 3TC is reintroduced, however, they can be selected again and become the dominating quasispecies.

If not all antiviral effect is lost following the emergence of M184I/V mutations. Residual antiviral effect and clinical benefit have been seen with continued use of 3TC in combination therapy regimens. This could be explained by that the presence of M184V have been seen to be associated with alteration of several mechanisms relating to RT function, including decreased RT processivity, reduced nucleotide-dependent primer unblocking, increased fidelity, hypersensitization to other NRTIs, impaired viral fitness, and delayed appearance of TAM mutations in RT\textsuperscript{142-147}. Unlike the situation with the NNRTIs, these factors suggest that 3TC may continue to contribute to the effectiveness of antiretroviral combination therapy regimens,
even after the appearance of the M184V mutation. This is however only applicable for patients with very limited other treatment options.

MINOR POPULATIONS

In patients undergoing treatment and harboring resistant viral strains, the dominant quasispecies are accompanied by minority populations, which can express distinct resistance genotypes. Because these viral species can evolve independently, a minority population, having followed a distinct evolutionary pathway, could emerge as the dominant population. This could be either because it had evolved higher resistance or because a change in drug pressure gave that population a growth advantage over the prior majority population. These minority populations can represent vestiges of previously dominant populations, representing earlier stages of HIV-1 evolution, alternatively originate from tissue compartments with lower selective pressure, and may therefore serve as a reservoir of diversity and possibly accelerating the development of drug resistance.

This has important implications for the treatment of patients experiencing drug resistance. Even if the resistance profile of the currently dominant genotype is known and serves as the basis for the choice of a new therapeutic regimen, the possibility remains that minority species may exist against which the treatment will prove less effective. The existence of minority populations may also create important obstacles for the accurate assessment of viral resistance, further complicating the choice of alternate regimens for patients experiencing treatment failure with antiretroviral drugs.

Genotypic resistance tests used in the clinics report the consensus sequence at each nucleotide position. Although these tests can detect the presence of mixed populations, they provide only a rough estimate of the relative proportions of wild-type and mutant species in the population. In addition, standard genotypic tests only detect resistance mutations present in more than 20-25% of the viral population, likely underestimating the prevalence of drug resistance mutations at any given time point.

The study of minor viral variants in HIV-1 infection is relevant to understand the mechanisms of viral persistence, escape from pharmacologic and immunologic pressure, and co-receptor usage. Detection of drug-resistant minority variants may help predict virological failure in patients with HIV-1 that appears to be wild-type by standard sequencing methods.

Several methods to detect and quantify minor populations of drug-resistant HIV-1 have been described during the recent years. Among these methodologies, one of the most powerful is the selective real-time PCR (SPCR) or allele-specific real-time PCR (AS-PCR), which allows detection of minority quasispecies with a sensitivity of down to 0.01% for certain mutations and currently quantifies them with the best accuracy, however just few mutations at a time. SPCR/AS-PCR is less expensive and time-consuming than single-genome sequencing or clonal sequence analysis, and due to its high throughput it can also be used as a way to rapidly screen very large pooled populations. It has been applied successfully to resistance mutations in the protease (D30N, V82A, L90M), reverse transcriptase (NRTI: M41L, K65R, K70R, M184I/V, T215Y/C), NNRTI: K103N, Y181C), and env (V38A) genes.

Using SPCR/AS-PCR and other similar methodologies, the prevalence and persistence of minority populations of drug-resistant viruses has been shown in acute seroconverters, chronically infected patients, and in treatment-naive as well as treatment-experienced patients. These studies have shown evidence of emergence of minority populations into
major populations in patients failing antiretroviral therapy\textsuperscript{154, 184}, correlations between minority species and therapy failure\textsuperscript{185}, minor populations in the early phase of therapy failure not seen by direct sequencing\textsuperscript{154, 180, 184, 186}, and minority species that could compromise the use of salvage regimens\textsuperscript{167, 186}. 
AIMS OF THE STUDY

The general aim of this thesis was to study the development and kinetics of HIV-1 resistance to antiretroviral drugs, with specific regard to minor drug-resistant viral populations. More specifically,

- to evaluate a real-time PCR based method to study minor drug-resistant viral populations carrying the frequently occurring lamivudine-induced M184I/V mutations (Paper I)
- to investigate the presence of M184I/V in minor viral populations of patients who failed didanosine-containing treatment and did not have detectable M184I/V in the major viral population (Paper II)
- to determine to which extent unique drug resistance patterns appear in major and minor viral populations in plasma and cerebrospinal fluid (Paper III)
- to study the extent to which selection of the M184I/V mutations occurs in treatment-naïve HIV-1 infected patients during the initial months of antiretroviral therapy (Paper IV)
MATERIAL AND METHODS

The studies performed in this thesis center around a method called selective real-time PCR (SPCR), which has been used to study frozen body fluid samples from HIV-1 infected patients collected during different phases of infection.

PATIENTS

In the four studies performed in this thesis, patient samples from several different cohorts have been used. Ethical permissions were obtained for all studies. For details, see the individual papers.

In paper I, 15 samples were collected from the Infections Disease Department at the Karolinska University Hospital in Stockholm, Sweden. These samples all contained M184 mutations and were used to test the SPCR assay on clinical material.

In paper II, 90 samples from 16 patients were collected from the Infections Disease Department at the Karolinska University Hospital. These belonged to 3TC-experienced patients who, after switching therapy to a ddI-containing regimen, developed a new treatment failure without detectable M184I/V mutations in the major viral population.

In paper III, 44 plasma and cerebrospinal fluid samples were collected from 13 multi-therapy experienced patients, who had developed virological failure during 3TC-containing antiretroviral treatment as a part of a prospective longitudinal study at the Sahlgrenska University Hospital in Göteborg, Sweden.

In paper IV, three different patient cohorts were used. Cohort #1 consisted of 315 samples from 43 patients with early primary HIV-1 infection from the QUEST study187, in whom quadruple ART had been initiated shortly after diagnosis. Baseline samples and samples taken 1-16 weeks into therapy were collected, as well as samples taken after treatment cessation, which occurred after at least two years of therapy. Cohort #2 consisted of 14 chronically HIV-1 infected patients from a Nordic randomized multicenter study called NORTHIV. These patients had been randomized to a regimen consisting of lamivudine, one other NRTI and a PI/r or efavirenz. Twenty-six samples were collected from this cohort; 12 baseline samples and 14 samples taken 4-12 weeks into therapy. Cohort #3 consisted of 15 chronically HIV-1 infected patients that had been followed during routine clinical care at the Department of Infectious Diseases at the Karolinska University Hospital. These patients had been given dual therapy with ZDV and 3TC between the years 1995-1998. The selection of the patients was done consecutively among those who had initiated this dual therapy at our clinic and had available stored plasma samples. Thirty-six samples were collected; 15 baseline samples and 21 samples taken 2-28 weeks into therapy. All patients in these three cohorts were treatment-naïve.

All samples were analyzed by SPCR and/or sequencing, as described below.

RNA EXTRACTION

Our interest lies in the genetic material of HIV-1, its RNA. In order to obtain the HIV-1 RNA, it has to be extracted from the patient blood or CSF samples. Viral RNA is extracted with commercially available specialized kits that catch the viral RNA in filters or on small beads, while unwanted waste products are washed off with different washing solutions. In the end the clean genetic material is eluted from the filters or beads with a small amount of liquid, and is then
either experimented on directly or frozen in -70°C for later use to minimize degradation. In our studies, we used three different kits to extract RNA; the Viral RNA Mini Spin Kit (Qiagen), the NucliSens Isolation Kit (BioMérieux) and the Total Nucleic Acid Isolation Kit (Roche).

RT-PCR AND PCR

The amount of RNA extracted from blood samples is very small and needs to be amplified in order to be successfully analyzed. In order to convert RNA to complementary DNA (cDNA), RT-PCR was performed. The cDNA product was then either used directly for SPCR, or was further amplified in a nested PCR to obtain enough cDNA products to enable successful sequencing. A nested PCR was also performed in some patient samples where polymorphisms were found that could affect primer and probe binding in the SPCR assay (described later in this section).

All nested PCR products were run on a 1.5% agarose gel for verification and purified using the PureLink PCR Purification Kit (Invitrogen) before use in subsequent SPCR or sequencing.

DNA SEQUENCING

To study the occurrence of resistance-associated mutations outside the M184I/V mutations, cDNA obtained from the PCR was analyzed by sequencing, which is a PCR-based method for determining the order of the nucleotide bases in genetic material. This was performed with primers targeted to the reverse transcriptase gene of HIV-1, to obtain all NRTI and NNRTI resistance mutations present in our patient material. The HIV-1 subtype B HXB2 strain sequence was used as reference.

SELECTIVE REAL-TIME PCR (SPCR)

The SPCR methodology is based on a modification of the amplification refractory mutation system (ARMS)\(^{28}\), combined with real-time PCR technology. It uses mutation-specific primers to selectively amplify resistant viral variants, which could then be compared to the total amount of viral variants in the sample, thus generating a value of the proportion of resistant virus. If longitudinal clinical samples are available, this allows kinetic studies of resistance development over time. SPCR can, however, only study few mutations at a time.

Real-time PCR enables the monitoring the progress of the PCR “in real time”, as it occurs, by detecting the accumulation of amplified DNA (also called "amplicon") during the reaction. The data is measured at the exponential phase of the PCR reaction by the detection of fluorescence after each PCR cycle, which corresponds to the amount of DNA that has been copied in the reaction.

There are two types of real-time PCR chemistries, TaqMan\(^{®}\) and SYBR\(^{®}\) Green I dye. The TaqMan\(^{®}\) chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles, while SYBR\(^{®}\) green I chemistry uses a highly specific, double-stranded DNA binding dye. The most important difference between the TaqMan\(^{®}\) and SYBR\(^{®}\) Green I dye chemistries is that the SYBR\(^{®}\) Green I dye chemistry will detect all double-stranded DNA, including non-specific reaction products, while TaqMan\(^{®}\) chemistry will only detect DNA which the fluorogenic probe binds to. A well-optimized reaction is therefore essential for accurate results if SYBR\(^{®}\) Green I dye chemistry is used.
We have used a TaqMan®-based approach in our studies. However, SPCR-based methods can also be constructed using SYBR Green I dye chemistry.

Figure 5. SPCR primers and probe. To the left are the forward primer and probe, to the right are the selective and universal primers. Mismatches in the selective primers are underlined.

THE SPCR REACTION

An SPCR reaction contains the following elements:

- An amplicon, in our case patient-derived HIV-1 cDNA.
- A forward primer, not different from any normal PCR primer.
- A fluorogenic probe. This is a DNA oligonucleotide with a fluorescent reporter dye (FAM) in the 5' end, which emits light when hit by a certain wavelength, and a "quencher" molecule the 3’ end, which absorbs energy from the reporter dye when they are in close proximity to each other. In addition, it contains a minor groove binder (MGB) molecule, which increases the binding between the probe and the corresponding DNA. This enables the use of shorter probes and lessens potential problems with polymorphisms that could otherwise disrupt binding.

- A universal reverse primer. A normal PCR primer which will amplify all HIV-1 cDNA in a sample.
- Mutant-specific selective reverse primers. These primers are modified by intentional nucleotide mismatches at positions -1 to -3 from the 3’ end of the primer, which allow them to bind only when a specific target mutation is present, in our case the M184I/V mutations. They do not bind wild-type or other mutant HIV-1 cDNA.
- A DNA polymerase enzyme with 5’ exo-nuclease activity, which can remove obstacles downstream of the enzyme (i.e. the probe).
- Other necessary ingredients, such as buffers and dNTPs.

All SPCR reactions were performed in 96-well plates in an ABI Prism 7700 Sequence Detection System (Applied Biosystems), combining standard control, positive and negative control and purified patient-derived HIV-1 cDNA. Six wells were used per patient sample, where the contents of the wells are identical except for the reverse primers. Duplicates of universal reverse primer and the M184I (ATA) and M184V (GTG) mutant primers were used in these six wells,
with only one reverse primer per well and reaction. The M184T (ACG) primer was not used after paper I, due to low clinical relevance.

Once placed in the machine, the following steps occur:

1. The temperature is raised to 50°C to activate the polymerase enzyme, followed by a raise to 95°C in order to separate the DNA strands.

2. The temperature is now lowered to 60°C, to allow the non-specific primers and probe to bind to both strands of the sample DNA in each individual well. The temperature is intentionally kept high to prevent non-specific binding. The reverse selective primer may or may not bind, depending on the presence of mutated viral cDNA in the sample. The universal reverse primer binds irrespective of the presence of mutant virus.

3. The polymerase starts working, cleaving the probe in front of it (which separates the reporter molecule from the quencher molecule) and amplifies a new DNA strand. If the reverse primer has bound, DNA from this strand is also amplified.

4. The machine emits light into the sample, which is absorbed by the reporter dye on the probe. If the reporter is cleaved, it emits light back by a different wavelength, which is then registered by the machine. However, reporters on non-cleaved probes do not emit light; that energy is instead transferred to the quencher.

5. The temperature is again raised to 95°C, to allow strand separation. Steps 2-5 are then repeated for 40-50 cycles.

For each successful PCR cycle, one additional cleaved reporter is able to emit light, which enables counting of every successful amplification. In the wells with selective primers the amplification is dependent on the binding of the respective primers. If no mutant is present, the DNA strand the reverse primer binds to is not amplified. This creates a bottleneck for the reaction, since only one strand (the one where the forward primer and probe bind) can be amplified. If mutant virus is present, amplification can occur, but this is then dependent on the amplification of the selective primer strand.

By comparing the amount of DNA amplified in the wells with the mutant-specific selective primers with that from the wells with the universal primer, you can calculate the proportion of mutated virus vs. the total viral population with the help of standards.

In our assay, we used two mutant standard controls from purified plasmid viral cDNA with a subtype B HXB2 strain background, where either a M184V GTG mutation or a M184I ATA mutation had been introduced, respectively. These mutant amplicons were then used to generate both specific (mutant) and non-specific (total viral population) standard curves for clinical specimen analysis. This ensured identical starting DNA copies for both standard reactions and enabled parallel, comparable curves. Only one mutant standard (M184V GTG per default) was used per individual assay due to cost and time issues. However, when reactivity from the SPCRATA primer was detected, those samples were re-run with M184I ATA mutant standard to obtain correct results.

Since we are only interested in the ratio of mutant vs. total viral population, we used a so called relative standard curve. Because the amount of mutant cDNA is divided by the total amount of viral cDNA, the unit from the standard curve becomes irrelevant. Thus, all that is required of the standards is that their relative dilutions be known, meaning that any HIV-1 cDNA can be used to prepare standards. However, it is very important that the standards are accurately diluted to obtain correct results.
The same standard amplicons, which had been prepared in large volumes, aliquoted for single use and frozen, were used for all assays throughout all four studies.

**Figure 6.** Typical SPCR plot. From left to right; duplicates of universal primer, SPCRGTG primer and SPCRATA primer curves. The results correspond to 71% M184V GTG in the total viral population. The ATA curves are below the cut-off of 0.2%.

Results are obtained in a plot, which is presented as curves representing the amount of fluorescence in each cycle in each well. The curve is measured where the fluorescence crossed the so called threshold, which is set in the exponential phase of the amplification for the most accurate reading. This generates a cycle threshold (Ct) value. By comparing the Ct values generated by the reactions with universal, SPCRGTG and SPCRATA primers with the standard curves, you can calculate the proportion of mutated virus vs. the total viral population, from a value of 0.2-100%. The assay cut-off was set to 1.0% for the SPCRGTG primer and 0.2% for the SPCRATA primer when running clinical samples.

**PRIMER CROSS-REACTIVITY**

In addition to binding their specific mutations, the selective primers also display cross-reactivity with certain other mutations.

Besides binding the M184V GTG mutation, the SPCRGTG primer also binds, and thus amplifies, sequences carrying the M184V GTA mutation. In addition, it also binds to M184I ATC mutation-containing sequences, since they do not present any mismatch at the -3 position of the primer. However, this specific mutation is very rare, since it has to develop through a M184I ATA intermediate. We found the ATC mutation at one occasion in clones from one patient sample in paper I. This sample contained a mixture of M184I ATA and ATC mutations as well as wild-type M184 ATG. No literature has been published of this mutation, and it could be assumed to have very limited clinical relevance.

The SPCRATA primer can bind to sequences carrying the M184I ATA mutation as well as the M184V GTA mutation. In some samples, this cross-reactivity can result in uncertainty of the specific contribution of different resistant viral populations. For example, mixes of the GTG and...
ATA mutations can sometimes be indistinguishable from mixes of GTG and GTA mutations or mixes of all three.

However, reactivity of the selective primers always indicates presence of resistant virus, since wild-type virus is not amplified.

POLYMORPHISMS IN THE PRIMER AND PROBE BINDING AREAS

Polymorphisms in the primer and probe binding areas cause mismatches between primers and amplicon and can pose a problem for the discriminatory ability of the SPCR assay. However, this is highly dependent on the amount of mismatches and their individual positions. In general, mismatches were seen to be more disruptive towards the 3' end of the primers.

Mismatches in the forward primer binding area are quite harmless, especially if they are located near the 5’ end of the primer. They can result in a slight delay of Ct for all reactions, but do not affect the discriminatory ability of the assay, since all reactions, universal or selective, are affected by these mismatches.

Mismatches in the probe binding area can be directly fatal if present near the 5’ end of the probe. Mutations clustered there can cause dislocation of the probe instead of cleavage when the polymerase starts working, which results in no fluorescence despite successful amplification. Mismatches in the middle or 3’ end of the probe still allow it to bind, but to a lesser degree, which results in a more level curve in the plot but does not affect the discriminatory ability of the assay. However, >3 mismatches in a row, or two mismatches within a four base pair region can affect binding to such a degree that no fluorescence will be registered.

In the reverse primer binding area, mismatches do affect the discriminatory ability of the assay. This is because the positions of the 3’ ends of the selective and universal primers are different. For example, the SPCRGTG primer has its 3’ end three bases downstream of the universal primer. A T→C substitution at the third base of codon 186 (which is very common) would then be more disruptive for the universal primer than the SPCRGTG primer, since the 3’ end of the universal primer is much closer to the mismatch. This will result in an overrepresentation of samples amplified with the SPCRGTG primer due to the replicative advantage, thus giving an inaccurate presentation of the proportion of resistant virus in the sample. The effect in much less pronounced between the SPCRATA and universal primers, since the position of their 3’ ends only differ by one base.

This problem can be circumvented by introducing wobble bases into the primers at the specific site of the mismatch. However, this requires knowledge of the specific mutations beforehand, in other words requires prior sequencing. It also requires an incorporation of the wobble bases in the standards.

Another solution is to introduce an additional PCR step in-between RT-PCR and SPCR (as described below) to clear the primer and probe area of mismatches. This requires more work, and all traces of the primers used in this PCR have to be removed post-PCR to not affect the results in the subsequent SPCR assay.

The problem can also be minimized by putting the 3’ end of the universal and selective primers as close as possible when designing them. However, this may not be possible for some resistance mutations that require more than a single nucleotide change.
SPECIFICITY VS. VIRAL LOAD IN THE ORIGINAL SAMPLE

As with any method for assessing minor HIV-1 variants, true assay sensitivity is directly dependent on the number of RNA molecules in the original sample, which can be problematic when samples with low viral load and limited sample volumes are to be tested. To ensure a sensitivity of at least 0.1%, a minimum input of 1000 RNA copies in the initial RT-PCR step is required (1 mutant/1000 total variants = 0.1%). In reality, however, more than that is needed. The RNA copy in the assay depends on plasma HIV-1 RNA concentration, the volume of plasma used and the efficiency of the RNA extraction process. The efficiency of the reverse transcriptase step also must be taken into account.

CHANGES IN METHODOLOGY BETWEEN PAPER I-IV

After paper I, the M184T primer SPCRACG was dropped due to the low clinical relevance of this mutation.

In paper I and II, mixtures of the selective primers SPCRGTG/SPCRGTG2 as well as SPCRATA/SPCRATA2 were used at a concentration of 200 nM, to accommodate a common polymorphism at the third base at codon 186 (GAT → GAC). The universal primer in paper I, SPCRWDx, had its 3’ end outside the 186 codon and was not affected by this polymorphism. In paper II, the universal primer was changed to SPCRWD30, which lies closer to the M184 site. This primer also accommodated the 186 GAC polymorphism.

In paper II, we introduced an additional PCR step in-between the RT-PCR and SPCR to remove unwanted polymorphisms at the SPCR primer and probe binding sites which could affect the results of the SPCR assay. This was performed with primers SPCRWU34 and SPCRWD34, which covered the entire selective primer binding site (outside 184), all of the upstream primer site and most of the probe binding site, in a PCR using low annealing temperatures. In paper III and IV, the primers were changed to new, shorter primers; SPCRWU30 and SPCRWD30v2, which were seen to have less unspecific binding.

The additional PCR step was only performed on patient samples where sequencing had revealed disruptive polymorphisms on the selective primer binding site or in the 5’ end of the probe binding site. This was seen in about 40% of all samples tested during our studies. All other samples were run directly from RT-PCR products. During paper II, assay cut-off was changed to 1.0% for the SPCRGTG primer and 0.2% for the SPCRATA primer.

In paper III and IV, only SPCRGTG and SPCRATA primers were used, at a concentration of 100 nM, since the accommodation of the 186 GAC mutation wasn’t longer necessary. The universal primer was at the same time changed to SPCRWD30v2. The reduction in concentration of the specific primers was done to lower the unspecific primer binding during SPCR.

No changes were made in the sequence or concentration of the upstream primer SPCRWU or probe SPCRPROBE during the studies. Different primer annealing temperatures as well as changes in assay total volumes were tested between paper II and III, but were in the end not changed.
RESULTS AND DISCUSSION

In this thesis, we have investigated the role of minority drug-resistant HIV-1 populations in different clinical settings. In paper I, we developed a method to detect and display the proportions of different populations of drug-resistant virus carrying the clinically relevant M184I/V mutations in the HIV-1 reverse transcriptase enzyme. This method was then applied to several different clinical cohorts in the subsequent papers, in order to expand the current knowledge of the clinical significance of minority populations in the development of resistance to antiretroviral drugs.

PAPER I - DISCRIMINATION OF LAMIVUDINE RESISTANT MINOR HIV-1 VARIANTS BY SELECTIVE REAL-TIME PCR

STUDY BACKGROUND

The standard HIV drug resistance genotyping tests used in the clinics today are not able to detect viral populations below a level of 20-30%, which may have clinical relevance. To detect these minority populations other methods must be used. The aim of this study was to develop and evaluate such a method.

We developed a real-time PCR based method that could detect and display the proportions of specific point-mutations at the M184 site in the HIV-1 reverse transcriptase gene. Three M184 mutations had been described at that time; M184V (ATGÆGTG), M184I (ATGÆATA) and M184T (ATGÆACG), and these were selected for study. Mutations in this site result in high-level resistance to the antiretroviral drugs lamivudine (3TC) and emtricitabine (FTC), as well as low-level resistance to abacavir (ABC) and possibly didanosine (ddI). The M184 mutations were chosen as a model system due to three reasons:

- they only require a single nucleotide change to develop resistance (low genetic barrier)
- they develop more rapidly than most other resistance mutations in the setting of incomplete viral suppression
- they are highly clinically relevant, since a majority of HIV-1 treated patients today are, or have been, treated with lamivudine

RESULTS

Our method, which we called selective real-time PCR (SPCR), was found to be very sensitive and reliable. It allowed detection of sequences containing specific mutations from 0.1% (ATA mutation) and 0.5% (GTG mutation) up to 100% of the total population in the site-directed mutants tested. The concordances with direct sequencing as well as cloning results were found to be very good. The SPCR assay was also found to be easy to construct and to perform, making it suitable for large screening of patient samples.

To study the distribution of the different M184 mutations in Sweden, we obtained 241 patient-derived sequences, previously analyzed as part of routine clinical care and all containing M184 mutations, from a sequence database at the Division of Clinical Virology at the Karolinska University Hospital in Stockholm. Among these sequences, we saw that the M184V mutations were the far most prevalent, found in 95.9% of the samples (88.6% GTG, 7.3% GTA). The
remaining 4.1% consisted of M184I ATA mutants. Other M184 mutations, such as the M184T ACG mutation, were not observed.

We also looked at possible polymorphisms which could interfere with binding at the primer and probe binding sites in these sequences. Most of the individual base positions proved to be very stable, displaying the same nucleotide as the HXB2 reference strain in >99% of all sequences that were compared. When polymorphisms were found, they appeared in the third base of the codon and would not lead to amino acid changes. These were found in highest frequency (>10%) at the F160, Q161 (probe) and D186, L187, G190, D192, G196 and Q197 (reverse primer) positions. The S162 and G196 positions were found to have changes in the first (S162 only) and second (S162 and G192) bases, leading to amino acid substitutions. When running patient samples with SPCR, we found that polymorphisms could influence the discriminatory effect of the assay, depending on their individual positions (discussed in the Material and Methods section).

Cross-reactivity of the SPCRGTG primer was observed for the M184I ATC mutation in clones from one patient, who had a mixture of two M184I mutations (ATA and ATC) and wild-type M184 (ATG) in his/her viral population (also discussed in the Material and Methods section).

When starting this study no SPCR-based assay was available to study the M184 mutations, only one aimed for protease inhibitor mutations151, 166. However, in 2003, Metzner and colleagues published an article152 describing a real-time PCR assay they called allele-specific PCR (AS-PCR). This method was based on SYBR® Green I dye chemistry, and targeted the M184V mutation and the protease inhibitor mutation L90M. Since then, other groups have successfully applied SPCR-based assays targeting at least eleven different NRTI, NNRTI, PI and entry inhibitor mutations151, 152, 154, 157, 161-163, 165-178, proving it to be a relevant methodology to detect minority populations of drug-resistant virus in clinical settings.

After finishing paper I, the SPCRACG primer was abandoned due to the low clinical relevance of the M184T mutation.

PAPER II - PRESENCE OF M184I/V IN MINOR HIV-1 POPULATIONS OF PATIENTS WITH LAMIVUDINE AND/OR DIDANOSINE TREATMENT FAILURE

STUDY BACKGROUND

The M184V mutation can be selected in vitro by abacavir (ABC)138 and didanosine (ddI)137. However, studies report that a single M184V mutation may not affect the clinical response to ABC190 and ddI191. Hence, the resistance effect of the M184V mutation may be present only in the presence of additional mutations in the RT gene192, such as K65R, L74V and Y115F. Many resistance algorithms still consider the M184V mutation to confer some degree of resistance to ddI193. However, most in vivo data have been collected from studies of the major viral population using direct sequencing.

The aim of our study was to evaluate the presence of M184V-containing minor viral quasispecies and their potential role in treatment failure in ddI-treated patients experienced with multiple nucleoside inhibitors. We therefore selected 3TC-experienced patients, who after switching therapy to a ddI-containing regimen developed a new treatment failure without detectable M184V mutation in the major viral population, as determined by routine genotypic resistance testing. Treatment failure was defined as >50 HIV-RNA copies/ml in two consecutive plasma samples after at least six months of the new therapy.
16 patients were studied, including two positive controls where M184V had been detected by routine genotyping. From these patients, 90 plasma samples, of which 13 samples were obtained before the study period to investigate prior presence of M184I/V mutations, were tested by SPCR and sequencing.

RESULTS

In SPCR-positive samples, SPCRGTG primer reactivity dominated, while SPCRATA reactivity was uncommon, consistent with the theory that M184I is a transient mutation with lower fitness than M184V. Cross-reactivity of the SPCRATA primer was observed for the M184V GTA mutation (discussed in the Material and Methods section).

The SPCR results corresponded with sequencing results except in five samples where SPCR detected resistant virus while direct sequencing did not, indicating that patients may harbor M184I/V resistant virus in minor populations which are missed by standard genotyping tests in the clinics.

M184I/V was not the preferred route to ddI resistance in our patient population. In ten patients who all had ddI failure, no M184I/V mutations were found. In addition, after termination of 3TC treatment despite continuation of ddI treatment, M184I/V mutations were detected in the minor viral populations in only one patient sample. This represented a small M184I ATA population, which disappeared in the subsequent sample. These findings suggest that the survival advantage of virus strains having the M184I/V mutations is none or limited during ddI treatment. However, the mutation still may contribute to a more rapid failure to ddI, e.g. due to an easier appearance of K65R or L74V related to a decrease in the genetic barrier.

However, our data strengthen the view that the presence of the M184I/V mutations should not preclude the use of ddI in nucleoside-experienced patients.

PAPER III - COMPARISON OF DRUG RESISTANCE PATTERNS IN MINOR AND MAJOR HIV-1 POPULATIONS IN PLASMA AND CEREBROSPINAL FLUID

STUDY BACKGROUND

The central nervous system can, together with the lymphoid organs and the genitourinary tract, act as a viral reservoir for HIV-1. This is partly due to the restricted access of some antiretroviral drugs through the blood-brain-barrier, leading to suboptimal drug levels that could facilitate the evolution of drug resistance in the cerebrospinal fluid (CSF). These resistant strains may possibly result in later treatment failure if drug resistant strains enter the blood from the CSF.

Differences in the occurrence of M184 mutations between in plasma and CSF have been observed previously, but the pattern in the minor populations below the detection level of sequencing has not been investigated. The aim of the study was to determine to which extent unique drug resistance patterns appear in the minor viral populations in the separate compartments.

44 plasma and CSF samples were collected from 13 multi-therapy experienced patients who had developed virological failure during antiretroviral treatment including 3TC. Seven patients had longitudinal samples. The paired CSF/plasma samples were tested by both SPCR and sequencing.
RESULTS

Differences in the pattern of drug resistance mutations were found frequently between plasma and CSF viruses, both in the major and the minor viral populations. However, SPCR showed discrepancies mainly in the proportion of mutated virus, suggesting that the role of minority populations for resistance development in the CSF compartment is limited.

SPCR detected resistant virus in one patient where sequencing showed wild-type M184. It represented a small M184V population that was present in the plasma sample but not in the CSF sample. Since M184V had been present in both compartments in the previous sample, this most likely represented a re-emergence of wild-type virus due to drug adherence problems, which had a slower progression in the CSF compartment.

Sequencing revealed differences in resistance at other RT positions between viruses from the two compartments in several patients, detecting major resistance mutations present either in plasma only (n=3) or CSF only (n=4). However, in all patients with longitudinal samples, all mutations appeared or disappeared from the compartments, respectively, during the study, which is likely to be related to differences in the selective pressure of the drugs within the two compartments.

In one patient, after having been present in both compartments before the change in therapy, the L74V mutation was found in CSF but not in plasma five months after treatment had changed to a non ddI-containing regimen. Thus, the CSF constituted a detectable reservoir for ddI resistance during this time period. In another patient we observed the K70R mutation in CSF only, which could possibly indicate that resistance to zidovudine started to develop within this compartment.

One patient, from which we had four consecutive paired samples, proved to be very interesting. This patient had been exposed to several treatment periods over the years, although with adherence problems, resulting in very high HIV RNA levels and low CD4+ T-cell values. The patient also had several treatment interruptions between samples one to three. These factors contributed to a frequent change of antiretroviral pressures in this patient at the different sampling time points.

SPCR revealed differences in M184I/V populations in six out of the eight samples. No M184I/V was found at the first time point. However, in the second paired samples, it appeared in a dominating population in the plasma, while in a smaller population in the CSF. In the subsequent samples, M184V stayed in the dominant population in plasma, but gradually disappeared from the CSF and was undetectable in the last sample. Sequencing revealed one NRTI and one NNRTI mutation in plasma, but none in the CSF compartment in the first paired samples. In the subsequent samples, the patient obtained several more NRTI and NNRTI mutations in plasma, where most did not appear in the CSF until the third sample, but there in a mix with the wild type amino acids. Between the third and forth samples, treatment was changed, resulting in a drastic decrease in viral load and a raise in CD4+ T-cell values. At this time all except one mutation (K70E) reverted to wild type in the CSF, but no change was seen in the plasma compartment. This pattern suggests that differences in viral evolution and exchange between the compartments may be more frequent that earlier anticipated.

Altogether, our results confirm earlier findings that drug resistance can develop differently between the two compartments and that the CSF can act as a viral reservoir for resistance mutations. In addition, differences in the relative proportion of the M184I/V mutation were found to a high degree between CSF and plasma virus, possibly due to differences in selective drug pressure. However, our analysis of the longitudinal paired plasma and CSF samples suggest
that these discrepancies were due to differences in the kinetics of the mutations rather than unique evolutionary pathways.

PAPER IV - DRUG-RESISTANT HIV APPEARS SELDOM DURING THE EARLY PHASE OF VIRAL DECAY IN PATIENTS INITIATED ON THREE- OR FOUR-DRUG ART

STUDY BACKGROUND

Current standards of care for the treatment of human immunodeficiency virus 1 (HIV-1) infection include the combination of three different antiretroviral drugs. The use of >4 drugs has been shown to further enhance antiviral activity and to increase the rate of viral decay after initiation of ART, but with the cost of additional side effects. However, the long-term potential clinical advantage of a four-drug ART regimen has not been confirmed.

In treated patients with undetectable viremia and in those treated with as many as four antiretroviral drugs, low-level ongoing viral replication has been described. However, it is still not clear to which extent infection of new cells occurs during the phase of viral decay after initiation of suppressive ART, and whether viral replication can lead to the early selection of drug-resistant viruses.

The aim of this study was to investigate the extent to which selection of the M184I/V mutations occur in treatment-naïve HIV-1 infected patients during this initial phase after initiation of antiretroviral therapy.

We compared three cohorts of treatment-naïve patients initiating quadruple (n=43), triple (n=14) or dual (n=15) lamivudine-containing ART. All patients on quadruple ART were treated at primary HIV-1 infection (PHI), whereas the other cohorts consisted of patients with chronic HIV-1 infection. Plasma virus was tested for M184I/V up to six months of after initiation of therapy, using SPCR and direct sequencing. PHI patients were also re-tested for M184I/V after cessation of long-term ART.

RESULTS

Among the patients included in the three- or four-drug treated cohorts, only a single sample from one of the patients in the four-drug cohort developed a M184V mutation during the initial phase of viral decay, despite advanced immunodeficiency in the chronically infected patients and an initial very high rate of viral replication in those with PHI. This sample was taken 6 weeks after start of treatment and contained a 2.5% M184V population, which then disappeared in the following samples up to 16 weeks after therapy initiation. It was not present in any of the samples taken at baseline or before week 6. The lack of evolution of this mutant virus, including that present in the minor viral quasispecies population, is in line with the concept that viremia during this time period originates to a large extent from the proviral DNA in long-lived infected cells or from trapped virus in follicular dendritic cells.

In contrast, a large proportion of patients (8/15) on a two-drug regimen were found to develop resistance early. The selection of the resistant strains occurred after about 5 weeks of treatment and was completed after 20 weeks. Since the antiviral potency of this combination was limited, lamivudine-resistant virus was allowed to evolve. No zidovudine-associated mutations were found by sequencing of any sample taken from this cohort.
The appearance of minor mutant virus quasispecies was generally not associated with a significant increase in plasma viral load. In fact, in 2 patients viremia levels declined even further despite a dominant mutant viral population (>90%), but this did not prevent therapy failure in all patients at a later time point. This viremia pattern suggests that a lack of ART potency leads to the subsequent selection of mutant virus due to the remaining viral replication and to a further decrease in antiviral efficacy. Persistent viral replication over time is associated with the development of further resistance to other components of the drug regimen and limits future options.209

In two patients from the four-drug and two-drug cohorts, pre-existing minor resistant viral populations were found. These represented small M184I ATA populations (0.3% and 0.5% of the total viral population). In the patient from the four-drug cohort, this mutation was not selected during the first six months of treatment, probably due to sufficient drug pressure and the low fitness of the M184I-containing virus, but was, however, detected after cessation of therapy (at about 1% of the viral population). The other patient, who had significantly lower drug pressure, selected the mutation in the subsequent samples.

In contrast to our findings, an early selection of resistance in minority populations with the occurrence of the L90M (PI), K103N (NNRTI) and/or M184V mutations has been reported in 6/15 treatment-naïve patients initiated on a three-drug regimen consisting of 3 NRTI, 2 NRTI + 1 NNRTI, 2 NRTI + PI or 2 NRTI + PI/r165, in whom the minority viral variants were able to replicate within the initial months of suppressive ART despite viral decline. 8/15 patients were also seen to have detectable resistance mutations in the minor populations at, or prior to, baseline. A correlation was found in this study between early selection of drug-resistant viruses and time to undetectable viremia. In our study, none of the 57 patients on a triple or quadruple regimen had detectable viremia after 16 weeks of ART and in only one of these patients resistance was noted in the minority viral population.

In two patients in the four-drug cohort, we saw selection of M184I/V after cessation of therapy, which may have been caused by suboptimal levels of lamivudine due to its long half-life in blood152, 210. These were present as a major M184V population (>90%) in the first patient, and a minor M184I population (1.0%) in the second. However, these resistant strains may have been selected at earlier time points (i.e. during therapy), since the M184V consisted of >90% of the population in the first patient only one week after cessation of therapy, and M184I-containing virus in the second patient had been detected in a low proportion at baseline.

In conclusion, even though resistant virus can appear and be selected during the initial months of treatment, it appears to be a rare occurrence in adherent patients with PHI or advanced infection receiving a three- or four-drug ART regimen. The current treatment strategies using three drugs in combination seem to be able to prevent the development of early resistance during the initial viral decay after initiation of ART in most patients.
CONCLUDING REMARKS

Drug-resistant HIV-1 minor populations, either pre-existing variants or quasispecies selected during antiretroviral therapy, can emerge as the dominant population and cause therapy failure. The mutations appear in minor populations before they are seen in the major population and may be undiagnosed by standard genotyping methods. The clinical impact of resistance in minor viral variants is an important topic to be defined.

In our studies, we found that SPCR is a well-functioning, sensitive and reliable method to study antiretroviral resistance development in HIV-1, which is able to detect resistance mutations not seen by conventional genotyping assays. SPCR-based assays have at present been successfully applied for at least eleven different mutation sites in the reverse transcriptase, protease and envelope genes, proving it to be a relevant methodology to study minority populations of drug-resistant virus in clinical samples. However, accurate results depend on the amount of original source material and the presence of polymorphisms in the primer and probe binding areas, which can affect the discriminatory ability of the assay. These problems can be overcome to some extent by introducing wobble bases in the primers or probes, or introducing an additional PCR step to remove polymorphisms prior to the SPCR assay.

SPCR-based methods are not well suited for clinical use, due to the limited amount of resistance mutations that can be studied simultaneously. However, they are useful for studying changes within viral populations and their impact on antiretroviral therapy and the pathogenesis of HIV-1.

As expected, the M184V GTG mutation was the most frequently occurring M184 mutation in all patient samples tested, followed by the M184V GTA and M184I ATA mutations, which were found only in a small number of patients.

In 3TC-experienced patients who failed ddI-containing therapy, we found the survival advantage of virus strains having M184I/V mutations to be limited, suggesting that the presence of these mutations should not preclude the use of ddI in nucleoside-experienced patients.

In plasma and cerebrospinal fluid samples from patients who had failed 3TC-containing therapy, differences in drug resistance patterns between the two compartments were found in both minor and major populations. These were likely related to differences in the selective pressure of antiretroviral drugs within the two compartments rather than unique evolutionary pathways. Minor populations were not seen to play a major role in resistance development in cerebrospinal fluid in the patients we tested.

Selection of drug-resistant virus in treatment-naïve patients during the first months after initiation of antiretroviral therapy was found to be rare in patients receiving a combination of three or four drugs, but common in patients receiving two drugs. This suggests that current treatment regimens initiated in treatment-naïve patients should be sufficient to stop the development of resistance during the first phase of viral decay, as long as the patients are adherent.
M184I/V innebär att aminosyran metionin (M) som icke resistent virus har, har bytts ut mot isoleucin (I) eller valin (V) på position 184 i det virusprotein datorn är. Mutationen uppstår tills sjukdomen upptäcks. Skadan på immunförsvar vet utvecklas oftast långsamt och efter fem till tio års obehandelad infektion kan immunförsvar inte längre skydda kroppen mot bakterier, virus, svampar och parasiter. Det är dessa sekundära infektioner som ligger bakom det tillstånd som kallas AIDS.

Den behandling mot HIV som finns idag utgörs av s.k. bromsmediciner, vilka har mycket kraftigt ökat överlevnad för HIV-infekterade personer i de länder behandlade. Läkemedlen minskar påtagligt mängden virus i kroppen och motverkar sjukdomsutvecklingen som annars skulle ske. Behandlingen botar dock inte infektionen som förväntas för närvarande att bli livsläng.

När HIV får föröka sig fritt bildas många miljoner nya viruspartiklar varje dag. Som hos alla levande organismer sker små förändringer, mutationer, hela tiden i arvsmassan. Om en förändring av arvsmassan ökar överlevnadsförmågan kommer den nya, mer livskraftiga varianten att leva vidare och konkurrera ut andra varianter. Detta sker hela tiden under en obehandle HIV-infektion och är centralt för att viruset ska kunna undkomma kroppens immunförsvar.

Vid en fungerande HIV-behandling föröka sig inte viruset eller mycket lite. Om behandlingen inte är tillräckligt effektiv av olika skäl och viruset kan föröka sig, får det en möjlighet att förändra sig för att kunna stå emot läkemedlen. Detta fenomen kallas resistens.

Användningen av flera HIV-läkemedel på samma gång, s.k. kombinationsbehandling, vilket är standard idag, ger både bättre effekt och minskar markant risken för resistensuppkomst. Läkemedelsresistens förebyggs både av den goda behandlingseffekten – infektionen trycks ned och viruset kan inte föröka sig – och att det blir svårare för viruset att skaffa sig motståndskraft mot flera olika medel samtidigt.


Den kliniska relevansen av små resistenta viruspopulationer är oklar. I denna avhandling har vi utvecklat en metod, kallad selektiv realtids-PCR (SPCR), som möjliggör studier av dessa. SPCR-metoden är riktad mot två vanligt förekommande resistensmutationer, kallade M184I/V, som ger höggradig resistens mot lamivudin, en av de mest använda HIV-läkemedlen idag. Termen M184I/V innebär att aminosyran metionin (M) som icke resistent virus har, har bytts ut mot antingen isoleucin (I) eller valin (V) på position 184 i det virusprotein där mutationen uppställt.

I studie I beskriver vi SPCR-metoden, och fann att den var välfungerande och pålitlig, med hög känslighet för M184I/V-mutationerna. Test på blodprov från 15 patienter som utvecklat M184I/V-mutationer visade att den fungerade väl på kliniskt material.
I studie II-IV applicerade vi sedan SPCR-metoden på flera olika kliniska material. I dessa studier fann vi att:

- M184I/V-mutationerna ej selekteras vid sviktande (misslyckad) behandling innehållande HIV-läkemedlet didanosin, även om de uppkommit vid en tidigare tidpunkt. Detta indikerar att didanosin fortfarande kan användas i patienter som utvecklat M184I/V-mutationer.

- Mindre resistenta viruspopulationer ej har någon större betydelse för resistensuppkomst i cerebrospinalvätska.

- Resistensuppkomst under de första månaderna av HIV-behandling är begränsad hos patienter som behandlas med en kombination av tre eller fyra läkemedel, medan den är vanlig hos patienter som behandlas med två läkemedel. Detta tyder på att dagens kombinationsbehandling är tillräcklig för att undvika resistensuppkomst hos patienter som ej behandlats tidigare.

Sammanfattningsvis sa har vi i denna avhandling studerat små resistenta viruspopulationer i flera olika kliniska frågeställningar som tidigare varit obesvarade, och förhoppningsvis utökat kunskapen om den kliniska roll de spelar. Men för att slutgiltigt definera den kliniska roll mindre resistenta viruspopulationer har behövs många fler studier. SPCR och liknande metoder är värdefulla verktyg för att åstadkomma just detta.
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