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KINETICS OF HIV-1 DRUG RESISTANCE MUTATIONS IN VIVO

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ABSTRACT

Antiretroviral therapy (ARV) exert a selective pressure on HIV-1 and may select viral drug resistant strains which have competitive advantage in the presence of the drug. Knowledge about the appearance and disappearance of these mutations is important when choosing ARV. The aim of this thesis was to study the kinetics of drug induced resistance mutations and the relevance of the mutational patterns in minor and major viral populations at ARV cessation, change and failure in a clinical setting. Paper 1. To study the kinetics if HIV-1 RNA and drug-induced mutations after cessation of ARV therapy in 26 patients. After cessation of ARV a phase of varying length with undetectable virus was followed by a rapid viral increase before a plateau, corresponding to pre-treatment levels, was reached. A significant larger number of primary protease inhibitor (PI) associated mutations reverted to wild-type, as compared with primary reverse transcriptase inhibitor (RTI) associated mutations, suggesting that they may cause a more impaired viral fitness. During the phase of rapid viral increase mutated and wild-type virus replicated equally good, indicating that mutated stains still may replicate efficiently. Paper 2. To study the reappearance of drug resistance mutations detected earlier at a new virological failure during second or third line antiretroviral therapy. Genotypic resistance testing was carried out in 66 patients before change of therapy and at the next treatment failure. The majority of primary and secondary resistance mutations persisted at new failures with modified treatment in both the RT and the PI genes. The results suggest that clinical cross-resistance may develop via common pathways within all categories of drugs in heavily treated patients. Paper 3. To describe the pattern of resistance mutations in PI-naive HIV-1 infected patients experiencing their first treatment failure on nelfinavir(NFV) containing therapy. 172 patient were studied. Virus from base-line was sequenced in 29 patients, the V82A mutation was found in four patients without any epidemiological connections. Treatment-naïve patients in Sweden may thus harbour PI-resistant virus and resistance testing should be considered before treatment. 43 patients failed treatment. A diverse pattern of primary PI mutations was seen: 46I (n= 7), 30N (n= 6), 90M (n= 5) and 82A (n= 4). The finding of 46I was unexpected and is associated with a higher degree of PI cross-resistance than the common 30N. Paper 4. To investigate the kinetics of M184I/V in the minor HIV-1 populations. Sixteen HIV-1 infected patients with treatment failure on 3TC and/or ddI containing regimens were analysed by direct sequencing and selective PCR (SPCR). In five samples, SPCR detected mutated virus, at low proportions, when direct sequencing showed a wild-type sequence. The good correlation between direct sequencing and SPCR is in line with that M184V mutants rapidly become dominating during 3TC failure, although a few patients may harbour 3TC resistant virus in minor populations only. Eleven patients with treatment failure on ddI containing treatment exhibited wild-type virus by both methods suggesting M184V does not cause any clinical significant decrease in sensitivity to ddI in vivo.

Keywords: HIV-1, anti-retroviral therapy, drug resistance, therapy failure, rebound, persistence, wild-type virus, SPCR, minor population

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**Original manuscripts**

This thesis is based on the following original articles and manuscripts, which will be referred to in the text by their numerals:


4. Svedhem V, Bergroth T, Lidman K, Sönnerborg A. Kinetics of M184I/V in minor HIV populations of patients with 3TC and/or ddI treatment failure. Submitted
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Abbreviations

ABC  abacavir
AIDS acquired immunodeficiency syndrome
APC antigen presenting cells
APV Amprenavir
ARV antiretroviral treatment
RT reverse transcriptase
AZT, ZDV zidovudine
CCR5 cystein-cystein linked chemokine receptor
CTL cytotoxic T-lymphocyte
CXCR4 cystein-x-cystein linked chemokine receptor
ddi Didanosine
d4T Stavudine
DNA deoxyribonucleic acid
EFV Efavirenz
fAPV Fosamprenavir
HIV-1/2 human immunodeficiency virus type 1 / 2
IDV Indinavir
LPV Lopinavir
MSM Men who have sex with men
NFV Nelfinavir
NNRTI non nucleoside reverse transcriptase inhibitors
NRTI nucleoside reverse transcriptase inhibitors
NVP Nevirapine
PCR polymerase chain reaction
PHI primary HIV infection
PI protease inhibitor
Pol HIV-1 polymerase gene
RNA ribonucleic acid
RT reverse transcriptase
RTV Ritonavir
SIV simian immunodeficiency virus
STI sexually transmitted infection
SQV Saquinavir
TAM thymidin analogue mutation
TDF Tenofovir
TDM therapeutic drug monitoring
3TC Lamivudine
VL viral load
General introduction

The first cases of the acquired immunodeficiency syndrome (AIDS) were reported in 1981. The causative agent HIV-1 was isolated in 1983. HIV-1 uses the enzyme reverse transcriptase (RT) for transcription of its genomic RNA into DNA. The RT is a very error-prone enzyme. \(10^{10}\) virions are considered to be produced daily in a HIV-1 infected person. The combination of these two characteristics is of major importance for the development of drug induced resistance mutations.

Since combination antiretroviral therapy including protease inhibitors (PI) became available in 1996 the prognosis for HIV-infected individuals has substantially improved. Antiretroviral drugs exert selective pressure on naturally mutating virus and quasispecies with a competitive advantage are selected in the presence of therapy and incomplete inhibition of the viral replication. Drug resistant mutants thereafter become the dominant strains and one important reason for therapy failure. Resistant virus may however obtain a decreased fitness. Resistance develops easily when the level of potency of the given combination of drugs is intermediate. A good adherence of the patient to the therapy seems to be the most important factor for a successful long-term antiretroviral treatment since resistance then can be avoided and the drugs can exert their maximal potency.

Pandemic

By the end of 2005, 40.3 million people were estimated to live infected with HIV [1]. More than 2.3 million of them are children below 15 year of age. During that year 3.1 million have died in AIDS, 2.4 millions of them are from sub-saharan. 4.9 million people were estimated to become newly infected during the year. Furthermore there were more than 14 million orphans, defined as children 0-14 year, who have lost one or both parents due to AIDS. The majority of the infected population is found in Sub-Saharan Africa. The HIV epidemic started later in Asia and Eastern Europe but is now spreading rapidly there. However in Western Europe and in the U.S. the infection rates are now stable. Resources for treatment with antiretroviral drugs and monitoring are for financial reasons mostly concentrated to the industrialised world. In Sweden up to December 2004, 6705 cases have been registered. 1289 of them have died because of AIDS, 58 during the years 2003-2004. 427 new cases were reported in 2004 [2].
Transmission

HIV is transmitted sexually, parentally and from the mother to the child. Following sexual contact HIV passes through the mucosal epithelium to the submucosa using the Langerhans cells. Cells most likely to acquire HIV are dendritic cells (DC), macrophages and T cells, which all express the CD4+ receptor and co-receptors for viral entry. The virus penetrates more easily if the epithelium is ulcerated. The process of transmission occurs within 30-60 minutes following exposure of the mucosa. The virus then passes to regional lymphatic nodes and further systemically. Biological factors that influence the transmission include; male circumcision [3], the presence of other STIs and most important the concentration of HIV in the blood and genital secretions [4].

Transmission of virus does not always happen even though some persons are highly exposed. In a few cases there is a genetic marker that has a correlation to protection: a genetic deletion (delta 32) of the second receptor required for HIV entry, CCR5. Up to 1% of the Caucasian population is homozygote for this deletion, which is not present in Africans or Asians [5]. An interesting discovery is an HIV specific secretory Ig A antibody with broad specificity, which has been found in a group of African prostitutes [6], it may have a protective function. Other studies have shown the presence of anti-HIV CTL in mucosal tissues of persons who remain seronegative despite repeated high-risk exposures [7].

Clinical spectrum

There are differences between different transmission categories in the prevalence of symptoms of the sero-conversion illness, the primary HIV infection (PHI). Higher frequencies are reported among those who acquire the virus through sexual transmission, [8] than among injecting drug users, [9]. The median incubation period is 14 (5-29) days after the transmission [10].

The clinical signs are similar to those that occur in many acute virus infections e.g. fever, rash, pharyngitis, lymphadenopathy, and headache. The duration of symptoms is approximately 1-2 weeks [10]. After primary HIV infection (PHI) most persons enter an asymptomatic phase which can last from months to more than ten years [11]. During this time there is a gradual destruction of the immune system. A decline in the number of CD4+ T-cells and an increasing level of viral HIV-1 RNA is observed in parallel. This increases the risk of developing opportunistic diseases and malignancies associated with HIV and AIDS. If
the patients don’t get access to antiretroviral treatment (ARV) the infection will in most cases lead to death.

**Viral structure**

HIV-1 belongs to the *lenti* viruses, a genus in the retrovirus family, most likely originating from a chimpanzee virus named SIV<sub>cpz</sub> in West Africa. The HIV-1 genome consists of three major genes (*gag, pol, env*) that code for structural proteins and the viral enzymes, reverse transcriptase (RT), protease and integrase. The ARV drugs from the classes: nucleoside analogue reverse transcriptase inhibitors (NRTI), nonnucleoside analogue reverse transcriptase inhibitors (NNRTI), and protease inhibitors (PI) interfere with these enzymes. There are also six minor genes that encode for regulatory (*tat, rev*) and accessory (*nef, vif, vpr, vpu*) proteins. These last mentioned proteins are essential in the viral life cycle including viral infectivity (*vif*) and down regulation of viral replication (*nef*).

Through genetic characterisation based on phylogenetic analysis of sequences derived from *env* and *gag* genes, HIV-1 can be classified into various HIV-1 subtypes. The majority of strains belong to the major M group while a small proportion belong to the outliers, group O and group N [12]. The major M group can be divided in to nine genetic sequences clusters, referred to as subtypes, A, B, C, D, F, G, H, J, and K. During spread in man each subtype have further diversified, and today subtype B is as diverse as the entire group M at the beginning of the epidemic [13] Recombinants of at least two gene segments from separate viral clones in an individual are named circulating recombinant form (CRF). In the pandemic, four subtypes and two CRFs are most prevalent: A, B, C, D, CRF01_AE and CRF02_AG [14]. They have different geographical distribution. Subtype B is commonly found in Europe and North America while C is the mostly spread subtype globally [15]. All subtypes can be found in Africa.
Viral Replication

HIV-1 replicates through a DNA intermediate which is created when the viral RNA is transcribed by the enzyme RT to DNA (unintegrated provirus). The viral DNA is then be transported into the cell nucleus and becomes integrated with the host genome (integrated provirus).

RT is a very error-prone enzyme [16]. Due to the lack of a proof-reading function during reverse transcription, there is a considerable inter- and intra-individual variability of molecular sequences in different HIV-1 strains [17]. The average half-life of free plasma virus has been estimated to be only six hours and the total production of HIV-1 has been calculated to $10^{10}$ virions per day [18]. The main reason why the HIV-1 infection cannot be cured with available antiretroviral therapy is the capacity of the virus to establish latency. There are two types of viral latency; 1. preintegration and 2. postintegration latency. The former is extrachromosomal, unintegrated and labile, consisting of partially or completely reverse transcribed HIV-1 DNA in the cytoplasm. The latter is more stable and integrated in the host chromosome [19]. If HIV-1 enters the CD4+ T-cell in the quiescent phase of the cell cycle(G₀) it will result in unintegrated provirus and integration will not occur unless the cell becomes activated in a few days i.e. integration will only take place if the virus enters the cell just prior to its entering the G₁ phase. CD4+ T-cells exist mainly in G₀ phase, but can proliferate after contact with HIV-1. After several rounds of division, some of these cells return to resting memory state carrying provirus. Only 1% of the population of free HIV -1 in plasma originate from latent provirus.

Viral adaptation

Three factors allow HIV to adapt rapidly to selective pressures such as the presence of ARV. First, the viral reverse transcriptase is, as previously mentioned, an error prone enzyme that makes, on average, one mistake every $0.3 \times 10^5$ nucleotides in vivo [20].

Secondly, approximately $10^{10}$ cells are infected every day, which allow the production of many different genotypes [18]. Third, recombination between viruses may accelerate evolution [21]. This together, leads to a generation of new viral genotypes with approximately one in three progeny viruses containing a mutation.
Treatment

Since potent combination ARV became available in 1996 the prognosis for HIV-infected individuals has been substantially improved. The mortality among HIV infected persons declined in Stockholm from a maximum 149 persons in 1995 to 28 in the year 2004. Before effective ARV was available there were several wards in Stockholm for the treatment. Ward 52 at Södersjukhuset, ward 3 at Danderyds Hospital and ward 154 at Huddinge Hospital. I have worked in all of them. All three wards were usually full of HIV infected patients with opportunistic infections or tumors, and many of the patients were dying with AIDS.

1987 the first antiretroviral drug, the NRTI zidovudine (ZDV) was introduced and later didanosine (ddI) and zalcitabine (ddC). Mono-therapy with one of these drugs became the standard of HIV treatment for many years. 1996 the Delta trial [22] showed that combination therapy was superior and became the treatment of first choice.

Today, the drugs used in the treatment of HIV-1 belong to four classes: NRTI, NNRTI, PI and fusion inhibitors (Table 1). All together there are 18 generics and 5 combination products ZDV+Lamivudine (3TC), ZDV+3TC+abacavir (ABC), ABC+3TC, Tenofovir (TDF)+emtricitabine (FTC) and Lopinavir (LPV) +ritonavir (RTV).

Combination therapy with these drugs may suppress the replication of HIV-1 in infected persons to such an extent that virus become undetectable in plasma, but it does not cure. Today the prospect for the treatment is that it is going to be lifelong.

After the introduction of combination therapy including PI’s in the later part of the nineties the leading idea was to “hit early and hard” [23]. Later it was shown that the use of ARV drugs was associated with many side effects and the treatment called for strong self discipline among the patients to be able to adhere to the drug regimens. The recommendations for initiation of therapy today is to do an individual decision in each case based on the appearance of symptoms, CD4+ T-cell count, the progression of loss of CD4+ T-cells, viral load (VL), age, social situation, including possible drug abuse. Usually start of therapy is considered when the CD4+ T-cell count of the patient is between 200-350 x 10^6/ml [24].

HIV-associated symptoms such as thrombocytopenia and Kaposi’s sarcoma and symptomatic primary infections can be options to treat. In case of opportunistic infection there is a delicate clinical challenge to decide when to start ARV treatment in relation to CD4+ T-cells, opportunistic treatment, side effects and risk for immune reconstitution inflammatory syndrome. Special programs are available for treatment of HIV in pregnant women and for post-exposure prophylaxis [24, 25].
Out of the available ARV drugs NRTI and NNRTI are inhibiting the HIV RT enzyme. PI’s act upon the HIV enzyme protease and the fusion inhibitors block the virus entry into the cell. There is not, until now, any specific combination of ARV which has definitively been shown to be superior in the long term on previously untreated patients [EACS, 2003. #4], therefore various combinations for first line treatment may be considered. Comparative studies of different drugs often show a similar effect of different combinations [26]. The ultimate decision concerning the patients first line treatment must be based on the individual conditions, but the national guidelines in Sweden recommend for treatment of a naïve patient: 2 NRTI + 1 NNRTI or 2 NRTI + 1 PI/r. PI/r means a PI together with a low dose of RTV i.e. boosted PI. RTV are first choices, alternative atazanavir. Efavirenz (EFV) are preferred before nevirapine (NVP). First line NRTI: ZDV + 3TC, were ZDV can be replaced by TDF or ABC and 3TC by FTC. [24]

Some NRTI combinations are not recommended to use: stavudine (d4T) + ZDV are antagonistic, FTC + 3TC are not additive or synergistic, TDF + ABC and TDF + ddI: risk for treatment failure, ABC + ddI: not well documented, d4T + ddI: has additive side effects.

It is preferable that treatment naive patients are encouraged to participate in a clinical trial. Presently the Nordic trial North-HIV, which compares some of the recommended standard regimens, is open for recruitment. Mostly there is time fore careful preparation of the patient, all staff in the HIV-team is needed to consider the patients situation concerning medical history with concurrent illness, interactions with other medications, history of exposure to resistant virus and/or result from resistance testing, dosing issues, psycho-social situation and the patient’s ability to adhere to the drug regime. Often it demands several visits to doctor/nurse/social worker before the patient is prepared. Treatment simplification is possible in patients who have had viral load (VL) below 50 HIV-1 RNA copies for at least 6 months [24]. In case of failure of the first line therapy, resistance testing should be performed if more than 1000 HIV-1 RNA copies are present in the blood. If resistance mutations are found one can either change only the drug for which resistance is documented or change the whole regimen because minor resistant variants could be present which are not detected by used assay systems.

When patients fail with \( >10^4 \) HIV-1 RNA copies/ml it is recommended that all drugs in the regimen should be is exchanged irrespective of the result in test [24]. For patients failing on
second line therapy the general recommendations are the same as for failure on first line. One could consider to keep 3TC even though mutation M184V/I developed, since there are data that the resistant virus exhibits lower IC50 to other NRTI (ZDV) and also that the mutation contributes to lower viral fitness expressed as less replication [27]. *In vitro* data also report M184V has a positive effect on HIV-1 RT fidelity, reducing spontaneous HIV mutagenesis[28]. Usually the options left are less considering all possible cross-resistance. Patients without any new options may still have clinical benefit from keeping a regimen even in a situation of virological failure. We have seen a CD4+ T-cell count that stays stable for years and VL that is half or less of the initial value in spite of virological failure. This could be related to decreased fitness of the virus during continued drug pressure [29].
<table>
<thead>
<tr>
<th>Nucleosides RT-inhibitors (NRTI)</th>
<th>Non-nucleoside RT-inhibitors (NNRTI)</th>
<th>Protease inhibitors (PI)</th>
<th>Fusion inhibitor</th>
</tr>
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<tbody>
<tr>
<td>zidovudine (AZT, ZDV)</td>
<td>nevirapine (NVP)</td>
<td>ritonavir (RTV)</td>
<td>enfuvirtide (T-20)</td>
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<td>lamivudine (3TC)</td>
<td>efavirenz (EFV)</td>
<td>indinavir (IDV)</td>
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<td>didanosine (ddI)</td>
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<td>saquinavir (SQV)</td>
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<td>stavudine (d4T)</td>
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<td>nelfinavir (NFV)</td>
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<td>abacavir (ABC)</td>
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<td>tenofovir (TDF)</td>
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<td>tipranavir (TPV)</td>
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**Adherence**

Patient’s ability and willingness to keep to the prescription seems to be the most important factor for a successful treatment. Adherence is related to several factors, both to the patient; such as cognitive, psycho-social factors, fear of side-effects, and the medical regimen; treatment related side-effects, pill burden, food restrictions, as well as the amount of support that is available from relatives and medical advisers.

Self-reported non-adherence from patients on treatment are associated with higher risk of virological rebound [30]. The relationship is thought to be bell shaped such that complete adherence and total nonadherence to ART are associated with low probabilities of resistance, whereas intermediate levels of adherence increase the risk of resistance. The level of adherence associated with the highest risk of resistance is not yet known, it has been suggested that only marginally suboptimal adherence can lead to resistance [31]. Most probably there is a difference between the drugs [32]. A number of measurement strategies for registrating adherence exist, including pill counts, electronic monitors, diaries and
interviewer-administered or self-report questionnaires. Each of these approaches has strengths and weaknesses. Self report approaches tend to over-estimate adherence, but are often used because they are inexpensive, feasible in a wide variety of settings and clinically applicable [33]. Most important, self-reported assessments of adherence are significantly associated with plasma HIV concentration [34]. Evolution of drug resistance does need non-adherence over time.

**Monitoring**

The level of CD4+ T-lymphocytes indicates when to start treatment but is also important to measure when the ongoing treatment regimen is monitored. Treatment outcome is also measured by VL, quantification of HIV-1 RNA in plasma. An ultrasensitive method is used if a result between 50 and 75000 HIV-1 RNA copies/ml is expected. The tests can not be used for quantification of HIV-2 or HIV-1 group N and O. After starting a new treatment the goal is undetectable VL (< 50 HIV-1 RNA copies/ml) after three to four months.

Increasing VL in plasma during treatment is a sign of treatment failure. The reason could be lack of adherence, that the ARV-therapy is not potent enough, disturbance in metabolism or absorption of drug or emergence of mutated virus. Therapeutic drug monitoring (TDM) is used to optimise drug efficacy, reduce side-effects and evaluate adherence. In patients who are on a successful treatment the infection has usually been monitored two to three times a year.

**Drug resistance**

The emergence of drug resistance are likely to develop when selection pressure is suboptimal and permits replication. $10^{10}$ virions are estimated to be produced in an HIV-1 infected person daily. The RT has an error rate of 1 nucleotide/10000 nucleotides. The genomic size of HIV-1 is about 10000 nucleotides. Mathematical modelling then suggests that every possible single-point mutation may occur up to 10000 times per day in an infected individual [35]. So all single mutations, that can contribute to resistance to ARV exist before the person has been exposed to the drugs. However 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 [35]. The selective pressure of antiretroviral therapy provides these drug resistant mutants with a competitive advantage and under such
circumstances they eventually become the dominant quasispecies [36]. Resistance is present when the virus has reduced drug susceptibility. The phenotypic feature is mostly expressed as x-fold increase in the observed IC$_{50}$ compared to wild-type virus, i.e. an in vitro construction. A $>3$-8-fold increase in IC$_{50}$ may be clinically relevant for certain drugs (e.g. abacavir) but for others (e.g. 3TC and NNRTI) the dynamic range in susceptibility could differ 1000-fold between wildtype and resistant quasispecies [37]. For some agents with low genetic barrier the change of susceptibility can occur very rapidly. For 3TC and the NNRTIs, a single mutation is enough to cause full resistance. A replacement of wild-type virus by drug-resistant variants can e. g take place if only a single-dose of nevirapine is used [38]. Other drugs have a higher genetic barrier e.g. abacavir.

For ZDV and LPV/r, several mutations are required for full resistance. Resistance is most easily developed when the level of potency of the given combination of drugs is intermediate.

**Suboptimal antiretroviral therapy**, which allows the viral replication to continue, is known to select for viral variants with reduced sensitivity to the antiretroviral agents and there is frequently a wide cross-resistance within the three major groups [39]. If on the other hand, the drug activity is low, the competitive advantage of the viruses with drug-resistance would be too low to outgrow the wild-type viral variants. In presence of high drug-activity but the viral replication still continues, the appearance of mutations is delayed, and viruses with drug-resistance are rarely seen or develop more slowly.

The most important reason for suboptimal treatment is low adherence. Another explanation to inadequate drug concentrations in plasma are pharmacokinetic reasons, such as concomitant interacting medication. Therapeutic drug monitoring (TDM) is possible to do on NNRTIs and PIs. It is recommended two to four weeks after starting up or changing therapy. One always need to take into account the patients earlier drug experience and the possibility of acquired resistance. Thus, latent drug resistant replication competent virus persist within memory CD4+ T-lymphocytes and may later start to replicate despite long-term effective treatment [40, 41]. A reservoir of the transmitted HIV-1 is established already early in infection and has a long lifespan [42, 43]. It seems however that no significant evolution of the virus takes place if the viral suppression in blood is efficient [41] [44].

Of all HIV-1 quasispecies, the dominant variant may vary over time according to the selective pressure of the antiretroviral drugs [45]. Depending on which protein is affected, the mutation may be unfavourable or beneficial for the virus. Primary mutations (key mutations)
often develop early; these alter the binding of the drug to the virus and make it thereby less sensitive for the drug, but often the replicative capacity decreases at the same time [46]. If the viral replication is allowed to continue, secondary mutations (compensatory mutations) frequently make virus with primary mutation fit again through further lowering the binding affinities to e.g. a protease inhibitor (PI) and significantly amplify the effects of the primary drug resistant mutation and also by improving the decreased replicative capacity [47]. Secondary mutations also pre-exist in untreated individuals as natural polymorphisms and will not interfere with treatment in the absence of primary mutations.

The mechanism of resistance to the NRTI is alterations of the structural conformation of the RT in two major ways: A, nucleotide dependent primer unblocking or B, pyrophosphorolysis. The mutant enzyme either preferentially takes up natural deoxynucleotide triphosphates or selectively excludes NRTI. 3TC is using the A alternative and ZDV, d4T and ddI alternative B [48]. For NNRTI, aminoacid changes associated with resistance cluster around the binding pocket which gives a pronounced 3-D steric effect [49].

Resistance to PI is due to aminoacid changes in the active site of the HIV-1 protease or in surrounding regions involved in the binding of the PI. The PI inhibits the substrate binding and the catalysis of the enzymes needed for viral maturation and thereby processing an immature progeny which is non-infectious. These effects reduce also viral fitness [50, 51].

Drug resistance may also develop at a low viremia. In one study (Alemanset al) RT mutations appeared at a mean VL of 500 copies/ml after a mean time of 9 months and primary PI mutations developed at a viremia of 200 copies/ml after mean of 11 months. There was a trend for patients receiving second/third line therapy to develop mutations at lower VL than patients receiving first line therapy [52].

**Comments on special mutations.**

During mono-therapy the level of resistance correlates with the number of mutations present depending on the drug, but in combination therapy the picture is more complicated.

The thymidine analogue mutations (**TAM**): 41L, 210W, 70R, 215Y, 219Q/E/N are selected predominantly by ZDV and d4T but can cause cross-resistance to the other NRTIs if three or more of them are expressed, including the 41L or 210W mutations. The 41L and 215YF are defined as primary RT mutations, and 67N, 70R, 219Q, 210W are secondary mutations. TAM associated mutations: 69D/N, 69_ins, 75T/M/A, 118IQ, 44A/D, the latter two may also be found as polymorphisms in wildtype virus.
Discriminatory mutations 65R, 74VI, 115F, 184V/I, prevent the addition of a NRTI to growing viral DNA chain. Nucleotide dependent primer unblocking mutation, 65R, may seen when TDF and less frequently and ABC. It have now become more prevalent. The 184V mutation, which is one of the most studied mutations, is associated with high level, 1000 fold, resistance to 3TC [53, 54], increased susceptibility to ZDV \textit{in vitro} and with decreased viral fitness [28]. The 184 mutation also has a positive effect on HIV-1 RT fidelity, reducing spontaneous HIV mutagenesis. The 74V causes reduced susceptibility for ddI. Together with M184V it also has impact on ABC. The 74V also been noted to be antagonistic to the 215Y mutation \textit{in vitro} and decrease the viral fitness, the later effect is additive to 184V when the two mutations are together [55]. Q151M pathway mutations: 62V, 75I, 77L, 116Y, 151M. The mutations always accompany the 151M and cause multiresistance to all NRTI, except TDF.

The PIs have multiple, overlapping patterns of resistance. High level resistance to most PI requires the accumulation of multiple mutations. PI-resistant virus exhibits four categories of mutations: The \textbf{primary, major mutations}: 30N, 48V/M, 50V, 50L, 82A/T/F/S, 84V/A/C, and 90M. All except the last are situated in the substrate cleft. They cause resistance by themselves. 82A/F/S/T is found during e.g. IDV monotherapy and is also the first that might develop with TPV treatment. 90M results in a ten-fold change in susceptibility to all PI [56]. 48V itself is primarily associated with SQV resistance. The 50V mutation emerges exclusive for APV. If present as a single mutation, it results in a significant growth impairment but in only a small decrease of susceptibility to the drug. 50L has been isolated from virus obtained from patients failing ATV.

The “flap” mutations are named after their place in the 3-dimentional protease, and are second in importance, 46I/L, 47A/V, 53L, 54VTAS, 54ML. 46IL causes resistance to NFV and together with 84V it contributes to one of the LPV pathways for developing decreased susceptibility. The remaining flap mutations are more often accessory.

\textbf{Polymorphic secondary mutations} 10I/V/F/R, 20M/R/I/T,M36I/V, 63P, 71V/T/I, 77I, all except 10I/V/F/R are polymorphic. They contribute to resistance in combination with other mutations.

\textbf{Other nonpolymorphic mutations} 23I, 24I, 32I, 33F, 73C/S/T/A, 76V, 88S, 88D. They indicate past PI exposure and may cause resistance themselves but are more often accessory.

In patients failing their non-first line of therapy, the same mutations emerge whether or not the PIs are boosted with low-dose ritonavir, although there is some difference in the relative
frequency of various mutations. However, with regimens that include boosted PIs, multiple mutations may be required to result in less virological activity. Also, patients failing a boosted PI in the first line of therapy frequently exhibit wild-type virus.

The mutation associated with failure on LPV/r have now been designated as minor mutations. Previously, the mutations had not been assigned as either major or minor. The initial data analysis for this drug focused on the number of mutations associated with resistance rather than impact of individual mutations [57].

I50L is the most common protease mutation to develop in PI-naive patients with virological failure while receiving unboosted ATV. 50L reduces ATV susceptibility by ~8-fold. 50L occurs less frequently in previously PI-treated patients receiving ATV. There are no data on the frequency of 50L in patients failing ATV/r. The effect of 48V alone on ATV susceptibility is not known, but in combination with mutations at positions 54 and 82, ATV susceptibility is reduced >20-fold. In addition, the genotypic correlates of phenotypic resistance are less well known for ATV than for other PIs [58].

The main NNRTI mutations are K103N and Y181C/I. They both confer high-level resistance and there is a substantial cross-resistance between NVP and EFV.

Enfuvirtide is associated primarily with mutations in the gp41 envelope gene.
### TABLE 2a. Nucleosides, nucleotide and non-nucleoside reverse transcriptase inhibitors

<table>
<thead>
<tr>
<th>Drug</th>
<th>Codon mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>3TC, FTC</td>
<td>44D, 65R, 118I, 184V.</td>
</tr>
<tr>
<td>ddI</td>
<td>65R, 74V, 184V</td>
</tr>
<tr>
<td>ABC</td>
<td>65R, 74V, 115F, 184V</td>
</tr>
<tr>
<td>TDF</td>
<td>65R</td>
</tr>
<tr>
<td>Multinucleoside Q151M</td>
<td>62V, 75I, 77L, 116Y, 151M,</td>
</tr>
<tr>
<td>Nevirapine</td>
<td>100I, 103N, 106M, 108I, 181CI, 188CLH, 190A</td>
</tr>
<tr>
<td>Efavirenz</td>
<td>100I, 103N, 106AM, 108I, 181CI, 188L, 190SA, 225H</td>
</tr>
</tbody>
</table>

Q151M cause multi-resistance to all NRTI, except TDF. TAM cause cross-resistance to the other NRTIs if three or more of them are expressed.

### TABLE 2b Protease inhibitors

<table>
<thead>
<tr>
<th>DRUG</th>
<th>Major</th>
<th>Minor</th>
</tr>
</thead>
<tbody>
<tr>
<td>APV, FPV</td>
<td>50V, 84V</td>
<td>10FIRV, 32I, 46I, 47V, 54LVM, 73S, 90M</td>
</tr>
<tr>
<td>ATV</td>
<td>50I, 84V</td>
<td>10FIRV, 20RMI, 24T, 32I, 33IFV, 36ILV, 46I, 48V, 54V, 71V, 73 CSTA, 82A, 88S, 90M</td>
</tr>
<tr>
<td>IDV</td>
<td>46IL, 82AFT, 84 V</td>
<td>10IRV, 20RM, 24i, 32I, 36I, 46I, 54V; 71VT, 73SA, 77I, 90M</td>
</tr>
<tr>
<td>LPV/r</td>
<td></td>
<td>10FIRV, 20RM, 24I, 32I, 33IF, 36ILV, 46IL, 47VA, 50V, 53L, 54 VLMAMTS, 63P, 71VT, 73S, 82AFTS, 84V, 90M</td>
</tr>
<tr>
<td>NFV</td>
<td>30N, 90M</td>
<td>10FIRV, 36I, 46IL, 71V, 77I, 82AFTS, 84V, 88I.</td>
</tr>
<tr>
<td>RTV</td>
<td>82AFTS, 84V</td>
<td>10FIRV, 20RM, 32I, 33F, 36I, 46IL, 54VI, 71VT, 77I, 90M</td>
</tr>
<tr>
<td>SQV</td>
<td>48V, 90M</td>
<td>10FIRV, 54VL, 71VT, 73S, 77I, 82A, 84V</td>
</tr>
<tr>
<td>TPV</td>
<td>33IF, 82AFLT, 84 V</td>
<td>10FIRV, 32I, 54LVM</td>
</tr>
<tr>
<td>Multi PI resistant</td>
<td>46IL, 82AFTS, 84VAC, 90M</td>
<td></td>
</tr>
</tbody>
</table>

Modified from Johns Hopkins University 2005
Cross-resistance

Virus isolates with reduced sensitivity to one drug may display cross-resistance to additional drugs in the same class[59]. In fact, all currently approved and experimental drugs belonging to the same class share some degree of overlapping resistance. Some mutations are selected by more than one drug. The degree of resistance they cause may vary between agents [60]. A particular mutation or patterns of mutations selected by one drug can sometimes confer resistance to another drug even though the other drug does not select for it. The probability of cross-resistance is likely to increase with the level of resistance to a single drug. As the number of mutations and the level of resistance frequently increases with time after the initial failure, cross-resistance becomes more probable in strains that are highly resistant to one inhibitor [61]. Therefore, the termination of a failing regimen should be rapid in order to maximize chances of finding a suitable second line or salvage regimen. On the other hand for patients receiving salvage therapy with a detectable viral load and with limited or no remaining drug options, continuing the current regimen may be a useful although drug resistance is associated with impaired viral fitness compared to wild-type and thus a strategy to delay disease progression [62].

Even though the amount of registered generic drugs for ARV now is 18 (Table 1) and five combination products exist, the options are anyhow limited for a patient failing his/her second or subsequent therapy because of the cross-resistance.

Cross-resistance frequently occurs among NRTIs and are of clinical relevance particularly when three or more TAM have accumulated, if 41L or 215YF is present. TAM are selected predominantly by ZDV and d4T but can cause cross-resistance to the other NRTIs if 3 or more of the TAM are present, including the 41 or 210. TDF gives cross-resistance to ABC and ddI, ABC to TDF, ddI, 3TC and FTC. There is total cross-resistance between 3TC and FTC. Viral strains that are fully resistant to all NRTI’s have been identified but they are still rare. 151M, included in the NRTI multiresistance complex, is a two base-pair change in a conserved RT region and it can alone cause intermediate levels of resistance to ZDV, ddI, d4T and ABC [63, 64]. Additional mutations at codons 62, 75, 77 and 116 increase the level of resistance in the multiresistance complex [60]. The 69S-S-S and T69S-S-A are other mutations which cause multiresistance to NRTI. These mutations also reduce the sensitivity for TDF.
Cross-resistance is particularly prevalent amongst the NNRTIs, a single mutation gives cross resistance to all drugs used in the class, due to interaction at a common site in the binding pocket of RT.

For the first generation of PI a broad pattern of cross-resistance is found among most members [65]. Some primary mutations are known to create decreased susceptibility to all PI, as the mutation L90M, but some mutations have effects only on single PI, e.g. 30N for NFV and 50V for APV.

**Resistance testing**

There are two categories of methods available for resistance testing, genotypic and phenotypic methods. Phenotypic tests measure the ability of viruses to grow in various concentrations of drugs in culture assays. The result is commonly expressed as the IC$_{50}$ - the concentration of drug needed to inhibit the growth of virus by 50%. It provides quantitative data for any drug added to the test, including cross-resistance.

For clinical practice in Sweden genotypic testing is in use due to practical advantages and less cost. The genotypic method uses patient plasma from which viral RNA is extracted. This is then reverse transcribed to DNA and amplified through the use of PCR. After this, the amplified product, corresponding to the *pol* gene, is sequenced. By comparing the obtained sequence against a consensus gene HIV-1*~hxb2* sequence, substitutions or other mutations in amino acids that have taken place in the protease or the reverse transcriptase (RT) gene are identified. A genotypic change is described by an aminoacid codon letter indicating the non-mutated amino acid found in the wild/consensus sequence, followed by the specific position of the mutant aminoacid and last, the mutant aminoacid. Using mathematical algorithms a ranking of the impact of the given mutation on the sensitivity of the virus for a drug is determined.

With the method, direct sequencing, used today, the sample must contain 500-1000 copies/ml plasma and the virus-strain must represent more than 25% of the HIV-population in the specimen to be detected. Detection of drug resistance by tests can predict which drug the virus will not respond to but not which drug the patient will respond to. Previously developed resistant virus in minor population and memory cells may rapidly emerge once the drug or a cross-resistant drug from the same group is re-introduced. Testing is best done when patient is on treatment since resistance mutations in the dominating viral population easily revert when off treatment [66]. Resistance testing is today recommended in the situation of a PHI,
treatment failure and pregnancy. It may also be consider before starting first line treatment [24] [67].

**Minor population**

The nondominating population among the quasispecies are called the minor population. The knowledge about the kinetics of the mutational pattern have lately increased by the development of real-time PCR which allows easier studies on the minor population.

The emergence of new dominating genotypes follows two possible mechanisms, either new genotypes derived from minor population or emerge of new mutations in a current dominating genotype. A theory is that minor population which evolve independently of major population, can eventually become dominating, there by serving as a reservoir of diversity and possibly accelerating the development of drug resistance. It happens either because it had evolved higher resistance or because a change in drug pressure gave that population a growth advantage over prior major population. In some cases the minority populations corresponded to strains with fewer resistance mutations and lower resistance than the dominant species. Possibly these populations represented vestiges of previously dominant populations, representing earlier stages of HIV-1 evolution, alternatively originate from tissue compartments with lower selected pressure [68].

**Transmission of resistant virus**

The prevalence/incidence of transmission of mutant resistant virus has been reported to either decrease [69] or increase [70], according to different studies from different places. In cohort studies from Europe and USA, 5-20 % resistance was reported in 2001. Frequencies in Sweden 1993 have been reported to be <2% [71] or <5% [72]. Recent studies have reported somewhat higher figures [67, 73].

Potential factors causing any decreased transmission of drug-resistance variants are increased immigration of untreated HIV infected persons from non-European countries where ARV is not available and increase of patients with undetectable viremia due to treatment. Factors related to increased frequency of transmission of drug-resistant species can be related to the sequential use of antiretroviral drug regimes or poor adherence to the therapy schedules and the incomplete suppression of viral replication that follows.

It is of importance when reading reports of transmission of drug-resistant virus to distinguish between aminoacid substitutions, particular secondary mutations, representing
polymorphisms and primary mutations that reflect prior drug selective pressure or reduced drug susceptibility. Transmitted resistance is best detected during a primary infection, since the mutation later on can disappear from the dominant viral population. However the mutation can reappear later during treatment [74].
Overall aim of the study

To study drug resistance patterns in minor and major HIV-1 populations at antiretroviral-therapy (ARV) cessation, change and failure in a clinical setting.

Specific aims

- To analyse the kinetics of HIV-1 RNA and the evolution of drug induced resistance mutations, at cessation of ARV (Paper 1).
- To study the kinetics of earlier detected resistance mutations at a new virological failure during second or third line ARV therapy and obtain information about any persistence of these mutations, and possible cross-resistance patterns (Paper 2).
- To identify the patterns of resistance mutations in patients with first-line protease inhibitor failure in nelfinavir-containing ARV (Paper 3).
- To investigate the kinetics of M184I/V minor HIV-1 populations in patients with 3TC and/or ddI treatment failure (Paper 4).
Materials

All patients in Papers 1-4 have been recruited among the patients attending the Clinics of Infectious Diseases at Karolinska University Hospital, Huddinge and Solna, Stockholm, Sweden. Twenty-six consecutive patients were prospectively followed in the first paper. Sixty-six, 172 and 16 were retrospectively included in paper two, three and four. Mean age was 43, 40, 40 and 43 years (range: 18-72), respectively. The three major transmission routes, MSM, intravenous drug use and heterosexual are represented in all papers. The percentage of males was 77%, 80%, 53% and 62%, reflecting that the dominating patient group in the early treatment era were men who have sex with men, but at the time of Paper 3 and 4 immigrants from highly endemic areas dominate the study population. The majority of patients in these studies were treatment experienced, especially in Paper 2 and 4. Many patients had earlier been on single and double therapy with NRTI, later a combination that included a PI or a NNRTI were tried but adherence was not particularly emphasised. Thus, most patients had several mutations before these studies started.

Methods

In Paper 2, the definition of failure was a viral load >500 HIV-1 RNA copies/ml and in Papers 1, 3 and 4 >50c/ml was used, reflecting the development of the technique that made a more sensitive assay available.

Quantification of plasma HIV-1 RNA was performed using a commercial kit for PCR (Amplicor HIV-1 Monitor Test, Roche Diagnostic, Inc, Branchburg, NJ or later the COBAS AmpliPrep/Cobas Amplicor HIV-1 Monitor Test). In brief, HIV-1 RNA was extracted from 0.5 ml plasma by the QIAamp Viral RNA Kit, and was transcribed to cDNA by reversed transcription, followed by nested PCR for amplification. If the viral load was <500 copies/ml the sample was re-examined using a ultrasensitive test with a detection limit of 50 copies/ml (Papers 1 and 3).

Drug resistance testing was done by direct sequencing as described in Paper 1 by sequencing of the pol gene. The DNA products were sequenced using ALFexpress and the Cy5 Autored sequencing kit. Samples obtained during 2000 to 2002 were analysed using the TRUGENE HIV-1 Genotyping KIT (Visible Genetics Inc.). Gene sequences were analyzed with DNAsis software and were related to the HIV-1_hab2 sequence (Genebank accession number Ko3455). Details of mutations in the pol gene associated with reduced sensitivity to antiretroviral drugs were obtained from the literature.
**Subtyping:** Part of the *pol* gene was amplified and directly sequenced in order to determine the subtype within this region. The genetic relationship was analysed by constructing a phylogenetic tree with the TREECON software. Distance estimation was done with the Kimura 2-parameter algorithm and the tree topology was inferred by Neighbor-joining. The relative support for the various clades was analyzed with Bootstrap analysis, replicates set to 500.

**SPCR:** HIV-1 cDNA to be used for SPCR was extracted and amplified from patient plasma samples. The resulting PCR fragments were purified and run on gels for verification. SPCR was performed by using one upstream primer, three different downstream primers and a 5'-FAM florescent MGB probe situated right after the upstream primer. The three downstream primers consisted of one universal primer, which is non-discriminatory and amplifies all cDNA in the sample, and two mutation-specific primers, which only amplifies cDNA with the corresponding mutation (GTG or ATA) in position 184 of HIV-1 RT. In each SPCR run, all cDNA samples were run in duplicates with the upstream primers and either the universal primer or one of the mutant primers (i.e. 2 wells per individual primer, 6 wells in total), as well as mutant standard control, a negative control and a wild-type HIV-1 Hxb2 cDNA positive control. All samples were run at least twice. Amplification, data acquisition and analysis were performed using the ABI Prism 7700 sequence detector system. The threshold value was determined by the SDS software, and the cycle threshold (Ct) values (i.e. at which cycle the individual samples cross the threshold) could be extracted. The ratio of mutant versus total viral population was then calculated by dividing the amount of mutant cDNA with the total amount of viral cDNA in the sample (as determined by sample mutant primer Ct value, universal primer Ct value and corresponding Ct values in standard curve).
SPCRGTG primer 71% in relation to universal primer curve to the left and ATAmutation primer to the right.

**CD4+ T-cells:** were analysed by routine flow cytometry in all four papers at the Departments of Immunology, Karolinska University Hospital or Swedish Institute of Infectious Disease Control.

**Results**

**Paper 1** After cessation of ARV a phase when HIV was still undetectable ranging from 6-29 days (phase 1), was followed by a rapid viral increase (phase 2), which later slowed down (phase 3), before a plateau was reached (phase 4), corresponding to pre-treatment levels or higher in most cases (14/19). A significantly larger number of primary PI-associated mutations reverted to wild-type (10/11) as compared with secondary PI (10/21) and primary RTI-associated mutations (6/16), but all mutations at position 184 disappeared. Resistance associated mutations did not seem to interfere with the doubling time, as we saw a rapidly increasing HIV-1 RNA load corresponding to phase 2 despite persistence of resistance mutations. We could observe three distinct patterns:

First pattern, rapidly increasing HIV-1 RNA load, 2 log<sub>10</sub> - 3 log<sub>10</sub> even though all mutations remained. Second pattern, less pronounced changes in the HIV-1 RNA load, <1 log<sub>10</sub> and concomitant disappearance of resistance-associated mutations.
Third pattern, no drug related mutations were present and the viral load was low before cessation of ARV. Thereafter the viral load increased immediately and no drug-related mutations were observed.

**Paper 2.** The majority of the 255 resistance mutations found in the RT genes were also found in the sample drawn during the new treatment failure (79%). A substantial number of the patients had developed TAM during earlier ZDV treatment in mono or double therapy. After changing from ZDV to a d4T containing regimen the TAM persisted in almost all patients (90%). The M184V mutation persisted in nine non-3TC treated patients, who had been given ddi (n= 7) or ABC (n= 2). The L74V was found in four out of 13 cases with a ddi-containing base-line therapy. At failure the mutation had disappeared in two cases despite that ddi or ABC, respectively, was given.

At base-line, 164 resistance mutations were found in the protease gene. The majority of primary (24/41; 59%) and secondary mutations (99/123; 80%) were found also during failure of the new treatment, which in most cases (60/66) contained a PI.

The V82A/F/S/T was detected in 15 cases who had received IDV (n= 14) or RTV (n= 1). At failure, the mutation was found in nine (60%) subjects of whom seven were still given a PI (NFV:4, IDV:2, SQV+RTV:1). Among the six cases who lost the mutation, five were still given a PI (SQV:3; NFV:1; SQV+RTV:1).

At baseline, L90M was found in nine cases (IDV:3, SQV:1, SQV+RTV:1, SQV+NFV:1, NFV:1, RTV:1, APV:1). At failure, the mutation persisted in five (SQV:4, no PI:1). Among the four cases who lost the mutation, three were still given a PI (APV:1; NFV: 1; SQV+RTV:1).

In three patients, primary PI mutations V82A/F/S/T and L90M persisted although no PI was given. Most secondary mutations persisted (80%) except for the N88D.

**Paper 3.** Most patients 111/172 (65%) had a treatment success, forty-six (27%) patients developed failure and fifteen (9%) patients discontinued treatment without any treatment failure. Patients that were treatment naïve at base-line had more often treatment success (96/126; 76%) as compared to treatment experienced patients (15/46; 32%) (p< 0.001). At base-line, four PI-naive patients had V82A mutation. At treatment failure, 26 patients exhibit primary PI and RT mutations, the primary PI mutations found were M46I (n= 7), D30N (n= 6), L90M (n= 5) and V82A (n= 4). The remaining 17 patients failed with wild-type virus.
Secondary PI mutations were found in 14 out of 29 tested patients at baseline and in 29 out of the 43 patients at end point (L63P: 16; M36I: 11; A71V: 6; V77I: 5). Secondary PI mutations at start of treatment did not predict bad outcome.

**Paper 4:** There was a good correlation between direct sequencing and SPCR. However in five samples, SPCR detected mutated virus, at low proportions, when direct sequencing showed a wild-type sequence (SPCRGTG primer only n= 3, SPCRATA primer only, n= 2). In 17 samples of nine patients, the GTG sequence was found by direct sequencing. In these samples, the SPCRGTG primer revealed proportions of mutated virus ranging from 68% to >90% (median: >90 %). An additional six samples of four patients showed a mixed population by direct sequencing, the proportion of mutated virus was in median 30% by SPCR. SPCRGTG primer reactivity dominated while SPCRATA reactivity only was uncommon. Most of these the SPCRATA samples were also positive to SPCRGTG.

At sixteen occasions in eleven patients, the M184V/I mutation identified by direct sequencing, SPCR or both, disappeared from plasma during the observational time. Among five of these patient the treatment of 3TC and/or ddI were not changed, the other six were still on ddI containing therapy. Eleven patients failed ddI treatment with a wild-type virus, three of the patient also had 3TC in the combination.


Discussion

The development of HIV drug resistance is a large problem among patients with treatment failure. For example, in a selected patient material at the Clinic of Infectious Diseases, Karolinska University Hospital, 95 out of 107 (89 %) patients, who had been tested with one or more genotypic resistance routine tests during treatment failure, exhibited primary resistance mutations, during the years 2000-2002 (unpublished data). There are several factors influencing the development of resistance (page 15). Briefly they could be referred into two categories which obviously interact. 1: the genetic barrier, characteristics of the drug determine with how easy and rapidly the virus develops resistance mutations to the drug. 2: selective pressure, if the regimen is not optimal because of lack of potency of a regimen, residual replication continues. The latter could of course have different reasons, the most significant being insufficient adherence (page 14).

Because there were so few antiretroviral drugs available before 1996, a patient in need of anti-HIV-1 treatment, received sequential mono- and/or dual therapy. In 1996, it was common that the patient received a PI added to a failing regimen, and a complete suppression of the viral replication was seldom obtained. Although it was clearly shown that such a suboptimal procedure gave a clinical benefit and prolonged survival [75], such a patient could never benefit from the full advantage that ARV nowadays offers since their virus population already had accumulated several resistance mutations. Thus, mutations to the PI accumulated in addition to the preexisting NRTI-mutations. Such patients and some other patients with extensive treatment experience, thus having problems with multiple drug induced mutations of several ARV-classes, participated in our studies. Our results can therefore not uncritically be implemented on treatment naive patients on their first line treatment or patients with less advanced treatment history. The vast majority of patients who are given first line therapy today at our clinic, do not develop treatment failure. However, since the death rate among HIV-1 patients declined remarkable rapidly to very low figures, patients who received suboptimal therapy during the 1990-ties still consist of the majority of patients in whom treatment failures are seen [76]. Papers 2-4 are based on retrospective data and plasma samples, frozen in -70 °C, which had been collected for clinical purposes. Therefore, the time-points of sampling are not always optimally coordinated. Furthermore, the patients are not equally distributed concerning gender since homo- and bisexual men dominated initially the HIV-1 epidemic. There is however to my knowledge no data showing different pattern of resistance mutation related to gender or age. Also, only limited differences have been found
between different HIV-1 subtypes. Thus, our patient cohort was then dominated by homo-
bisexual men and intravenous drug addicts, both infected with subtype B [77]. Eight years
later immigration from highly HIV-1 endemic countries had brought more women and other
HIV-1 subtypes to our patient cohort [67].

The aim of our studies was to describe the kinetics of drug resistance patterns in patients who
changed or terminated their therapy. Since we did it on retrospective data, in all studies
except Paper I, it was not possible to control several factors known to influence development
of resistance (page 15). The ideal design, if such factors should also be analysed, would be to
combine prospective virological data from several compartments with pharmacological data
including therapeutic drug monitoring (TDM), and clinical data including importantly
adequate adherence data (page 14). With the coming establishment of a Swedish clinical
cohort using a common database such studies will be facilitated [78]

The unexpected finding of the appearance of mutation M46I in patients failing nelfinavir
(NFV) treatment (Paper 3) demonstrates, however, that studies on patients, in whom the
clinical follow-up and the documentation is of high quality, could come up with new data
earlier not reported in e.g. trials run by pharmaceutical companies. Some of the patients in our
studies should thus never have been eligible in clinical trials. In contrast, our study included
all patients failing NFV during the defined time period at our hospital and any bias in
selection can therefore not occur. So studies in a clinical setting can contribute with highly
relevant non-selected data that can influence the clinical use of the approved drugs. In all
studies of NFV-failing patients published so far, the D30N mutation has been the primary PI
mutation in subtype B patients, which has almost exclusively been found [79]; [69] [80], or
dominated substantially [81] [82, 83]. As expected both the D30N and the L90M were found
among our failing patients. More surprisingly, the M46I occurred frequently and was the only
primary PI mutation in five out of 17 (29.5%) subjects. One argument for using NFV as an
option in the first-line therapy at the time of the study, was the advantageous mutation pattern
with D30N in case of treatment failure, because this mutation is known not to give rise to
cross-resistance to other PI. The findings of M46I, however, indicate that cross-resistance
patterns may develop when NFV is used. However, NFV is not a first line drug anymore
mainly due to another reasons. For example, boosting of NFV is not used since no substantial
increase in plasma concentration is seen [84, 85], as compared to other PI where boosting is
frequently used. Thus, the higher plasma concentration of other PI due to boosting with
ritonavir, make these PI less sensitive to pre-existing mutations.
The rapid rate of development of new ARV, and new knowledge about the established drugs have also made stavudine (d4T), another drug to which we have put focus on in our studies (Paper 2), to a second line drug. Side effects with mitochondria toxicity and lipoatrophy are the main reasons [86].

In all Papers, we used genotypic resistance testing, i.e. direct sequencing as a method for detection of resistance mutations. It is also the method used for clinical purpose and therefore our results are of direct clinical relevance. Direct sequencing is partly an indirect method, comparing the obtained sequence with data prototype HIV-1 sequence where after the impact of a given mutation on the virus in vivo is predicted using algorithms in which phenotypic methods commonly are the golden standard. These phenotypic methods provide quantitative data for any drug added to the test including cross-resistance, and are from a theoretically point of view attractive. However, the usefulness and the precision of these assays for determining decreased sensitivity varies substantially between drugs [37]. Therefore, and also due to costs and the long turn-over time the phenotypic assays are seldom used. Thus, our data should only be interpretated with these facts in mind. Data from Paper 1, demonstrate a rapid disappearance of primary PI and most RTI mutations (all mutations at position 184) after cessation of ARV, stressing the importance of providing the resistance test while the patient still is on treatment.

Both in clinical practice and when analysing results from studies we have only access to information concerning quasispecies, representing >25% of the total viral population. This fact is clearly limiting. In order to get a more complete picture of the kinetics of mutated viral populations, we used a newly developed SPCR method for resistance testing with which we were able to analyse minor HIV-1 populations. In Paper 4 we found good correlation between direct sequencing and SPCR. All samples with virus exhibiting M184I/V by direct sequencing were reactive by the SPCRGTG, at very high proportions. When direct sequencing reported a mixed population, the SPCRGTG were positive in a lower proportion (90 vs. 30%). The SPCRATA primer yielded more seldom a positive signal and with lower reactivity. These results are well in line with the knowledge that M184I is a transient form preceding M184V during therapy failure [87]and suggest that M184I is uncommon to find, also in minor plasma viral populations after 3TC failure. In almost all cases of positive SPCRATA, a concomitant SPCRGTG reactivity dominated. It is likely that the SPCRATA reactivity frequently represented cross-reactivity with the SPCRGTA.
Direct sequencing did thus not underestimate the presence of drug-resistant virus in minor population to any large extent. Mutant viruses were found at low proportions (0.4%-5%) by SPCRG/TG /ATA in only five out of 67 samples, when direct sequencing reported wild-type. This data confirm the relevance of genotypic testing for mutation 184 by direct sequencing in a clinical setting. SPCR on the other hand can be useful in situations of certain needs as when studying transmitted resistance [88] or events during structured treatment interruption [89].

The aims of our studies did not include studies on drug resistance in newly infected or newly detected HIV-1 infected patients. Earlier Swedish studies have shown that the rate of transmission of drug resistant virus is low. [72] [71]. Maljkovic et al showed higher frequency on a selected patient material [73]. However, our data have still bearing on this issue. The results from Paper 3 with four PI-naive patients having the V82A mutation at baseline, is a surprisingly high figure, although transmission of resistant strains in Sweden was reported already in the beginning of the 1990ties [71] [72]. No epidemiological connection between the subjects was found, nor any molecular epidemiological relationship between their viruses (data not shown). The finding supports the routine of having a resistance testing done before starting first line treatment, now also recommended by the national guidelines [24].

The impact of changes of the selective antiretroviral drug pressure on the development, disappearance and reappearance of the resistance mutations is demonstrated in Paper 1, 2 and 4.

Paper 1 illustrates an initial rapid increase from low viral load to high viremia levels after cessation of therapy, irrespective of the presence of mutated or wild type virus. Followed by disappearance of all the primary and half of the secondary PI mutations, and 38% of the RT mutations. This finding indicates that mutated strains may still replicate sufficiently under certain circumstances and that resistance pattern changes due to selective pressure. Primary PI mutations in general severely impair the viral fitness resulting in less viral replication [46] that’s why they tend to disappear early in drug free environment as described above. In Paper 2, the primary PI resistance mutations present during base-line therapy disappeared, as expected, in most of the patients who did not receive PI in the new treatment [41]. However some primary PI mutations, V82A/F/S/T and L90M, persisted although no PI was given. It is possible that this persistence was due to the fact that the fitness of the virus harbouring these mutations (together with secondary mutations) was not decreased [50]. Also, we confirmed
that the clinically important M184I/V mutation disappears rapidly from the major virus population in plasma when failing 3TC-therapy is stopped (Paper 1). In Paper 4 we expanded this knowledge to be true also for minor viral populations. Thus, our data support that the pattern of resistance mutations in plasma changes rapidly when the selective pressure on the virus is changed. Our data also show that the kinetics may differ between resistance mutations induced by different drugs which is most likely due to their impact on viral fitness in a specific drug-free environment [50]. However, mutations may persist due to increased fitness related to development of secondary mutations, together with the primary mutations, as discussed above. In addition, even if treatment with a specific drug is terminated, the selective pressure on the virus may not change due to cross-resistance, as discussed below.

In several patients drug-resistant mutants disappeared from plasma despite that therapy was not changed. Paper 2 demonstrated L74V in four out of 13 cases with a ddI-containing base-line therapy. At treatment failure the mutation had disappeared in two cases although no change in therapy had been done. In that study we did not have the opportunity to analyse the minor viral population. In Paper 4, another RT mutation, the M184V/I, disappeared from five patients without any changes in therapy. Using a recently developed sensitive real-time selective PCR (SPCR), we could demonstrate that the mutation had disappeared also from the minor viral populations. The cause to this phenomena was not identified. It is possible that the disappearance was related to non-self reported changes in adherence. However, it is also possible that a selection bias of viral strains due to a low viral load occurred. Independent on that, we have to remember that the mutations are likely to still persist in cellular reservoirs.

M184V/I causes various degrees of decreased sensitivity in vitro to didanosine (ddI) [90], although the clinical relevance in vivo is questioned [90]. In Paper 4, the M184I/V was not found by any method in eleven patients when failing ddI containing therapy. In addition, the drug resistant mutants disappeared in three subjects, when 3TC was stopped but ddI treatment was continued. These findings suggest that the survival advantage of virus strains having the M184I/V was none or limited during the selective pressure of ddI. This finding supports the view that M184V does not cause any clinically significant decrease in sensitivity to ddI [91]. This notion is further supported by our findings in Paper 2, where M184V persisted in less than half of the subjects, who received ddI (but not 3TC) after change of therapy.

Cross-resistance (page 21) between drugs within the four classes is of major clinical importance since it dramatically reduces the possible number of potent drug combinations once resistance mutations have developed. At the time when the studies in Paper 2 were
performed, this was not a common knowledge. Patients with previous ZDV treatment in mono or double therapy, who had developed TAM, especially the M41L and the T215Y/F, were studied. After changing the treatment to d4T-containing regimens, we identified that the mutations persisted in the major viral population during the new treatment in almost all patients. These results are in line with the assumptions of a frequent cross-resistance \textit{in vivo} between ZDV and d4T [92, 93]. For ABC, a single L74V or M184V cause a minor decrease in susceptibility \textit{in vitro} [94], which is claimed not to influence the result of ABC containing treatment \textit{in vivo} [95] Among our patients the M184V persisted at new treatment failure together with L74V and TAM, respectively, in two patients. This is in line with data showing that viruses carrying both the L74V or TAM and M184V mutations are cross-resistant to ABC [96].

Also for the PI, our \textit{in vivo} data supports the clinical importance of cross-resistance. In Paper 2, after changing IDV to SQV or NFV only 9 out of 21 mutations disappeared at the new treatment failure, which might suggest that clinical cross–resistance may develop via common pathways within all categories of drugs in heavily treated patients. In Paper 3, 17 of the 43 (40%) tested patients with first line PI therapy failure on NFV, no mutations were found in the PI sequence. Treatment naive patients failing unboosted PI therapy with wildtype virus have earlier been reported[97, 98] also for NFV [80, 82]. The cause of the high frequency of \textbf{wild-type virus} in failing patients was not investigated in these studies. However, it has been suggested that virological failure of NFV-containing therapy can be explained, to a large extent, by low plasma levels of NFV [99]. It is however possible that PI- mutations could have been recovered in the minor viral populations, if a more sensitive technique had been used, such as that demonstrated in Paper 4.
Closing remarks on particular clinical relevance

The mutations emerging \textit{in vitro} usually represent only a subset of mutations that may emerge \textit{in vivo}. The \textit{in vivo} situation is far more complex than the \textit{in vitro} because of the much larger number of viruses \textit{in vivo} and their greater heterogeneity. For the clinician, predicting which ARV combination that is going to be the best for a certain patient is an even greater challenge, since not all facts are known and humans are even more unpredictable than the virus. But we have identified some data to rely on.

The rebound of HIV-1 RNA levels after cessation of ARV is an inevitable fact but in the clinic we have got a routine to monitor this situation. We follow VL and CD-4 cells closely untill treatment is readmission. Förstå inte vad du syftar på?. Cross–resistance is a severe problem in the clinic when a combination for third-line therapy is to be chosen. Our results concerning NRTI- and PI-mutations contribute to the knowledge that cross-resistance may develop via common pathways within all categories of drugs in heavily treated patients. Those findings are the base for the routine procedure, if possible, never to start a new ARV-combination when there is full sensitivity to only one drug.

The unexpected findings of the appearance of mutation V82A in PI- naive patients and of M46I in patients failing NFV-treatment demonstrate, that studies close to patients, when the clinical follow-up and the documentation are of high quality, could come up with new earlier not reported data. Possibly because no selection bias occur, in our retrospective study, all patients were included, . The findings of V82A support the routine of having a resistance testing done before starting first line treatment, which is now also recommended by the national guidelines.

There are several difficulties when the results of a genotypic resistance test are to be applied by the clinician. One is the lack of knowledge about what is going on in minor virus populations. Our studies demonstrated however that direct sequencing did not to any large extent underestimate the presence of mutation M184I/V in minor viral populations.

Our data suggest that the survival advantage of virus strains having the M184I/V is limited or none during the selective pressure of ddI. It thus supports the use of ddI in patients having M184V.

From time to time in the literature and at conferences there have been suggestions of a possible advantage for the patient having low-grade viremia during treatment. The discussion is based on a theory of possible stimulation of the immunological defence in the presence of virus. In addition there are observations from some patients, who have continued a failing
ARV-combination with a viremia with multi-resistance mutations, that the CD4+ T-cell levels may still be maintained or even rising. One explanation for this could be that mutated virus is less fit and less virulent. Our findings however show that mutated virus can eventually replicate just as well as wild-type virus and that resistance mutations related to different drugs contribute to fitness in an unequal way. Decreased fitness can easily be lost if an additional mutation is acquired.

These facts mean that keeping a failing treatment is not beneficial for the patient if there is an option left to decrease the viremia to undetectable levels in the immunocompromised patient.
Conclusions

- After cessation of ARV, viral load increases to pre-treatment levels or higher in most patients.

- The complete loss of primary PI mutations after cessation of ARV suggests they impaired the viral fitness more than the primary RT mutations did.

- The rapid increase of viral load after cessation of therapy of both mutated strains and wild-type indicates that mutated virus may replicate efficiently.

- In patients with multiple treatment failures, most NRTI mutations persist at new failures with a modified NRTI-containing treatment. A similar pattern is seen for protease inhibitors. This suggest that a high frequency of cross-resistance exists within these classes of drugs.

- Patients on first-line PI treatment can fail nelfinavir treatment not only with the common mutation 30N, but also 46I, 82A and 90M. These latter mutations are associated with an increased risk for therapy failure of a second PI-containing regimen.

- Treatment-naive patients in Sweden may harbour PI-resistant virus and resistance testing should be considered before treatment. Direct sequencing does not underestimate the presence of mutation 184 in minor plasma virus population to any large extent and is adequate to use for clinical purpose.

- Using a sensitive real-time selective PCR (SPCR), we could demonstrate that M184I/V disappears rapidly from the minor viral populations in plasma when failing 3TC-therapy is stopped.

- M184V does not seem to cause any clinical significant decrease in sensitivity to ddI in vivo.
Ethics

Ethical permission was obtained from the ethical committee at Karolinska Institute

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