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STUDIES OF DRUG RESISTANCE IN MINOR HIV QUASISPECIES

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STUDIES OF DRUG RESISTANCE IN MINOR HIV
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ABSTRACT

The main objective was to study drug resistance mutations (DRM) in the HIV-1 reverse transcriptase (RT) gene of minor HIV-1 *quasispecies*, not detectable with standard techniques. Sensitive allele-specific PCR (AS-PCR) and next-generation sequencing (NGS) were developed to study resistance to drugs of relevance in low- and middle-income countries (LMIC); the nucleoside analogue RT inhibitor (NRTI) lamivudine (3TC) and the non-nucleoside RT inhibitors (NNRTIs) efavirenz (EFV) and nevirapine (NVP).

In **Paper I** and **II**, AS-PCR was used to detect M184I/V mutations which confer high-level resistance to 3TC. We addressed the selection of drug-resistant HIV *quasispecies* occurs during the initial phase of viral decay after treatment initiation and their emergence in two viral reservoirs, blood plasma and cerebrospinal fluid (CSF). Selection of M184I/V was found to be rare during the first phase of viral decay in patients with primary HIV-1 infection (PHI) or advanced chronic infection initiated on a three- or four-drug antiretroviral treatment (ART), containing 3TC. In contrast, drug-resistant *quasispecies* were more commonly detected in patients given dual ART, implicating that highly potent ART is necessary to avoid drug resistance during the early phase of viral decay. In patients who had ART-failure during 3TC containing therapy differences in drug resistance patterns, in both minor and major viral populations, were observed in the blood and CSF. However, the differences observed were most likely a result of differences in the selective pressure of ART rather than unique evolutionary pathways.

In **Paper III**, in order to study transmitted drug resistance (TDR) AS-PCR was used to detect K103N and Y181C mutations, which confer high-level resistance NVP and EFV, in treatment-naïve patients from Ethiopia, from East Africans and Caucasians living in Stockholm. The AS-PCR was highly sensitive and detected K103N and Y181C in minor *quasispecies* in

both subtype B (HIV-1B) and subtype C (HIV-1C) infected patients. These NNRTI mutations were found in the minor HIV-1 populations in all three patient groups.

In **Paper IV**, we developed a feasible, cost-efficient and easy-to-use high throughput NGS protocol for detection RTI mutations, including M184V, K103N and Y181C, to be applied in large scale surveillance of DRM in LMIC. The NGS assay was applicable to both HIV-1 C and HIV-1 B. It showed good concordance with standard population sequencing in detecting major DRMs and was also able to detect additional low abundance DRMs.

In summary, standard population sequencing assays underestimate the prevalence of important DRM in ART naïve and ART experienced patients. AS-PCR and easy to use high throughput assays can be useful in large scale surveillance in LMIC and to address the clinical significance of drug resistance in minor HIV-1 *quasispecies*.

LIST OF SCIENTIFIC PAPERS

- I. Bergroth T, **Ekici H**, Gisslén M, Kinloch-de Loes S, Gog LE, Freedman A, Lampe F, Johnson MA, Sönnernborg A. Selection of drug-resistant HIV-1 during the early phase of viral decay is uncommon in treatment-naïve patients initiated on a three- or four-drug antiretroviral regimen including lamivudine. *Journal of Medical Virology* 2009;81(1):1-8
- II. Bergroth T, **Ekici H**, Gisslén M, Hagberg L, Sönnernborg A. Difference in drug resistance patterns between minor HIV-1 populations in cerebrospinal fluid and plasma. *HIV Medicine* 2009;10(2):111-115
- III. **Ekici H**, Amogne W, Aderaye G, Lundquist L, Sönnernborg A, Abdurahman A. Minority drug resistant HIV-1 variants in treatment-naïve East-African and Caucasian patients detected by allele-specific real-time PCR. *Submitted*
- IV. **Ekici H**, Rao S, Sönnernborg A, Ramprasad VL, Gupta R, Neogi U. Cost-efficient HIV-1 drug resistance surveillance using tagged pooled high throughput amplicon sequencing: Implications for use in low and middle income countries. *Submitted*

CONTENTS

1	A glance at the HIV pandemic today.....	10
1.1	The heart of the HIV pandemic: sub-Saharan Africa.....	12
1.1.1	Ethiopia.....	14
1.2	HIV/AIDS in India	15
1.3	The status of the epidemic in Sweden	16
2	The subtypes of HIV-1 and distribution worldwide.....	18
2.1	The viral structure.....	19
2.2	The viral life-cycle	20
3	Antiretroviral therapy	23
3.1	Treatment of HIV-1 infection in Sweden: Individualized management.....	26
3.2	Treatment strategies in low- and middle-income countries: A public health approach	26
3.3	ART-Prophylaxis in the prevention of vertical transmission of HIV-1	27
4	Development of drug resistance	29
4.1	HIV-1: A highly diverse virus.....	29
4.2	Acquired and transmitted drug resistance.....	30
4.3	HIV-1 minor populations	31
4.4	Viral reservoirs	32
4.5	Drug resistance mutations	33
4.5.1	The NRTI drug resistance mutations M184I/V	33
4.5.2	The NNRTI drug resistance mutations K103N and Y181C	35
5	Methods to detect drug resistance.....	37
5.1	Population based sequencing	37

5.2	Allele-specific real-time PCR	38
5.3	Next-generation sequencing.....	40
6	Aims of the study	42
7	Material and methods	43
7.1	Patients.....	43
7.2	RNA extraction.....	44
7.3	Reverse transcription and nested PCR.....	45
7.4	Sequencing.....	45
7.5	Subtype classification and phylogenetic analysis.....	46
7.6	Allele-specific real-time PCR (Papers I-III).....	46
7.6.1	Detection and quantification of the M184I/V mutations	47
7.6.2	The detection and quantification of the K103N and Y181C mutations.....	49
7.7	Tagged pooled high throughput amplicon sequencing (Paper IV) .	53
7.7.1	Targeted amplicon sequencing	53
7.7.2	Bioinformatics analysis.....	53
8	Results	55
8.1	Selection of drug-resistant HIV-1 during the early phase of viral decay is uncommon in treatment-naïve patients initiated on a three- or four-drug antiretroviral regimen including lamivudine (Paper I)	55
8.2	Difference in drug resistance patterns between minor HIV-1 populations in cerebrospinal fluid and plasma (Paper II)	58
8.3	Minority drug resistant HIV-1 variants in treatment-naïve Ethiopian, East African and Caucasian patients detected by allele- specific real-time PCR (Paper III)	62

8.4	Cost-efficient HIV-1 drug resistance surveillance using tagged pooled high throughput amplicon sequencing: Implications for use in low- and middle-income countries (Paper IV)	66
9	Discussion.....	70
10	Concluding remarks	79
11	Future considerations	80
12	Acknowledgements	81
13	References	88

LIST OF ABBREVIATIONS

ABC	Abacavir
AIDS	Acquired immunodeficiency syndrome
AS-PCR	Allele-specific PCR
ART	Antiretroviral therapy
AZT	Zidovudine
bp	Base-pair
CRF	Circulating recombinant form
Ct	Threshold cycle
ddC	Zalcitabine
ddI	Didanosine
DLV	Delavirdine
DNA	Deoxyribonucleic acid
DRM	Drug resistance mutations
EFV	Efavirenz
Env	Envelope
FDA	Food and Drug Administration
FDC	Fixed-dose drug combination
FHAPCO	Federal HIV/AIDS Prevention and Control Office
Gag	Group specific antigen
GRT	Genotypic resistance testing
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus

HIV-1 B	Human immunodeficiency virus type 1 subtype B
HIV-1 C	Human immunodeficiency virus type 1 subtype C
HIVResNet	Global HIV Drug Resistance Network
IDU	Intravenous drug user
IN	Integrase
INI	Integrase inhibitor
LMIC	Low-and middle-income countries
LTR	Long terminal repeat
M-Mulv	Moloney murine leukemia virus
MSM	Men who have sex with men
mRNA	Messenger ribonucleic acid
NACO	National AIDS Control Organization
NACP	National AIDS Control Programme
Nef	Negative regulatory factor
NGS	Next-generation sequencing
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
PEPFAR	The US President's Emergency Plan for AIDS Relief
PHI	Primary human immunodeficiency virus type 1 infection
PI	Protease inhibitor
PI/r	Ritonavir-boosted protease inhibitor
Pol	Polymerase
PR	Protease

PS	Population sequencing
Rev	Regulator of virion gene
RNA	Ribonucleic acid
RT	Reverse transcriptase
SBS	Sequencing by synthesis
SD-NVP	Single-dose nevirapine
Tat	Transcriptional transactivator
TDR	Transmitted drug resistance
URF	Unique recombinant form
Vif	Virion infectivity
Vpr	Viral protein R
Vpu	Viral protein U
WHO	World Health Organization

1 A GLANCE AT THE HIV PANDEMIC TODAY

When the antiretroviral drug *zidovudine* (AZT), was introduced as the first therapy of human immunodeficiency virus type 1 (HIV-1) infection in 1987 (1), it gave hope of finding a cure within a near future. The antiviral activity of AZT, did not only indicate that the infection could be controlled, it also evoked optimism to search possible strategies to eradicate the virus from the body. Today, more than 30 years later after the HIV-1 discovery (2-4) and despite that tremendous scientific advances have been gained, the hope of a possible cure is still only a hope. In the absence of a potent cure, antiretroviral therapy (ART) has become a very important achievement and breakthrough in controlling the HIV disease progression and thereby has saved countless of lives over the years in the fight towards HIV and acquired immunodeficiency syndrome (AIDS).

Currently, about 35.3 million people are living with HIV worldwide (5). As a result of the *2000 United Nations Millennium Declaration* to initiate a global response to the HIV/AIDS- crisis (6, 7), major advances have been done to halt the pandemic. Together with the establishment of the health initiatives, such as *The United States President's Emergency Plan for AIDS Relief* (PEPFAR) (8) and the *Global Fund to Fight AIDS, Tuberculosis and Malaria* (9), a rapid scale-up of ART globally has been accomplished and a decline in new HIV infections as well as AIDS-related deaths have been observed during the recent years (5).

At the end of 2012, approximately 10.6 million people living with HIV were receiving ART. The biggest change in access to ART has occurred in low-and middle-income countries (LMIC), where the HIV prevalence is also the highest (Figure 1). The number of people receiving ART increased from 300 000 in 2002 to 9.7 million at the end of 2012 in LMIC (10). Despite the major developments in the response to the HIV/AIDS endemic, the same expansion in strategies to monitor and control the disease progression in patients before and during initiation of ART has not

equally advanced in these settings. In the World Health Organization's (WHO) report on drug resistance from 2012, an increase of transmitted drug resistance mutations (TDR) were observed at the end of 2010 in LMIC. The increase of TDR was associated with greater availability of ART (11). As one of the targets in the *2011 United Nations Political Declaration on HIV and AIDS*, 15 million people with HIV are planned to be on ART by 2015 (12). In settings with inadequate monitoring systems, the wider use of ART increases the risk of TDR (13-16), which may lead to treatment failure and imperil available therapy regimen options.

In clinical settings and surveys, the methods utilized to analyze drug resistance mutations (DRM) are restricted and can only detect mutations present in more than 20% of the total viral population (17-19), which may underestimate the existing prevalence since drug-resistant strains may be present in viral *quasispecies* consisting of less than 20% of the population. In the beginning of this thesis, the role and the impact on treatment outcome of such undetectable drug-resistant viral populations was unclear. Therefore there was a need to explore more sensitive assays that could be used for this purpose. As ART is becoming more available globally, the demand on feasible and sensitive methods to detect drug-resistant viral strains is more urgent than ever. For this purpose we have used allele-specific PCR (AS-PCR) assays, which have the capacity to detect viral populations that are present down to 0.1% of the total population (20-22) in **Papers I-III**, and a more recent approach, next generation sequencing (NGS) (23, 24) in **Paper IV**. Consequently, the overall aim of this thesis was to address these issues further and to investigate the potential usefulness of these sensitive assays in detecting drug resistance.

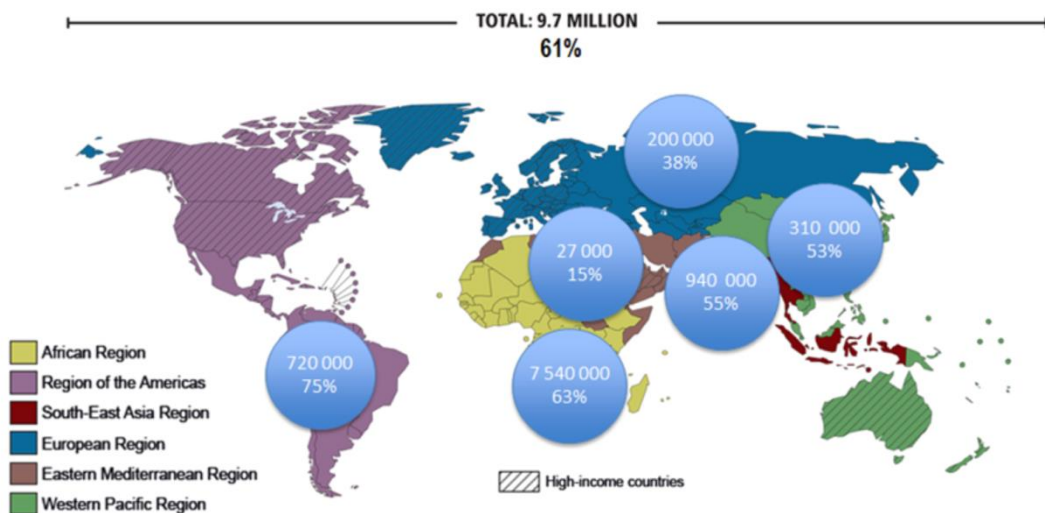


Figure1. Illustrating the progress in people receiving treatment in low- and middle-income countries and percent of eligible people receiving antiretroviral therapy at the end of 2012 (25).

1.1 THE HEART OF THE HIV PANDEMIC: SUB-SAHARAN AFRICA

Two-thirds of the world's HIV infected population lives in sub-Saharan Africa, which makes it the hardest hit region of the pandemic. Women constitute more than half of the infected and more than 90% of the pregnant women with HIV reside in this region (26). sub-Saharan Africa has also the highest burden of HIV-infected children, accounting for at least 90% of the known 3.3 million children living with HIV today (27). The HIV treatment programs in Africa are mainly funded externally and the main focus is to provide affordable ART for both treatment and prevention (28, 29). Due to the wider access of ART, 7.5 million people received treatment at the end of 2012 compared to only 50 000 a decade earlier (29). This has resulted in a decline in both new HIV-infections and AIDS-related deaths by 40% and 22% respectively (5). However, the coverage between the countries varies markedly; in Botswana, Namibia, Rwanda, Swaziland and Zambia the ART coverage was more than 80%

while it was less than 20% in Madagascar, Somalia and South Sudan at the end of 2011 (26).

Initiation of ART is a life-long commitment and needs to be monitored regularly. In high-income countries monitoring of patients was introduced early (30-33) and is frequently updated in the routine health care system (34, 35). Monitoring strategies include measurement of viral parameters that are essential to determine individually for each patient to obtain an optimal treatment outcome. Among these parameters, viral load assessment and drug resistance profiling of the patient's virus is vital to avoid treatment failure and onward transmission of resistant viral strains. In LMIC, the management of ART is based on WHO's guidelines and recommendations. Due to the absence of viral load and drug resistance testing in these settings, the guidelines comprise clinical manifestations and measurement of immunological markers as therapeutic monitoring strategy. Even though WHO's guidelines from 2010 recommends viral load testing as the preferred mode for treatment monitoring (36) the implementation of it in the health-care system is challenging for many high-burden countries. In high-income countries, drug resistance testing is usually highly recommended and carried out for each patient as a guide to select the most appropriate therapy option. Because initiation of ART is life-long, drug resistance testing is also an important clinical tool to monitor the emergence of possible drug resistance mutations during treatment. In resource-limited countries, where drug resistance testing is either generally not available or too costly to be implemented in the routine monitoring, WHO recommends the testing to be applied for public health assessments. The rapid scale-up of ART poses a risk for the emergence of drug resistance. Through the *Global HIV Drug Resistance Network* (HIVResNet) (37), WHO has initiated a global surveillance of the emergence and transmission of drug resistance and several African countries have participated in the surveys since 2004 (11). As mentioned earlier the results of the surveys already indicate an increase of TDR which in the longer term may create severe effects on existing therapy options in

the continuation of sub-optimal monitoring systems as ART is becoming more available.

1.1.1 Ethiopia

To investigate whether TDR is present after the roll-out of ART and the relevance of sensitive assays, we have studied HIV-1 infected patients living in Ethiopia. Ethiopia is located in the horn of East-Africa and is among the countries heavily affected by the HIV epidemic today. The epidemic is believed to have begun in the late 1970s or early 1980s in the country. The first cases of AIDS was reported in 1986 and currently about 800.000 people are estimated to be living with HIV (38). Even though the number of infected people is still high a significant drop in new HIV cases, from 130 000 in 2001 to 20 000 at the end of 2012, have been reported since the beginning of the ART scale-up (10).

In order to respond to the accumulating HIV epidemic in the country, Ethiopia approved a National HIV/AIDS policy in 1998 (39). The policy was a statement over HIV/AIDS-crisis as both a health and development problem with severe effects on the society that needed urgent interventions on a national level with the objective to provide environment for the prevention and the control of the disease. Later in 2002, when AIDS was declared as a national public health emergency by the government, the Federal HIV/AIDS Prevention and Control Office (FHAPCO) was established to organize and facilitate the implementation of the HIV/AIDS policy. Today, FHAPCO is the main coordinator of the national response to HIV/AIDS. Since its establishment, FHAPCO has developed several strategies and guidelines to implement the policy and to maintain the commenced progress towards universal access of treatment and care for people living with HIV (38). An important step towards this goal was the initiation of the *Antiretroviral Treatment program* consisting of guidelines on the use of ART in 2003 (40). Shortly after in 2004, the Ethiopian

government launched a free ART program which has resulted in 743 public and private health facilities providing HIV treatment and care services through the country today. Currently, the ART coverage has reached 71% of eligible HIV-infected people. This rapid expansion of ART access, with 265 000 people on therapy at the end of 2011 compared to only 11 000 in 2004 (41), has mainly been accomplished through major donors as the Global Fund, PEPFAR, the World Bank and the UN system (38).

1.2 HIV/AIDS IN INDIA

India is currently the home of the third largest HIV population in the world and with its high-burden epidemic it is among the 22 top priority countries in the global response to the HIV/AIDS pandemic (28). The first HIV/AIDS case was reported in 1986 and presently about 2.4 million people are living with HIV. A national response to HIV/AIDS epidemic was initiated shortly after the reported first incidences by the establishment of the National AIDS Control Programme (NACP) under the Ministry of Health and Family Welfare. The major objectives of the programme included preventive interventions among high-risk groups, increase awareness of HIV/AIDS in the country as well as amend surveillance strategies. The national commitment, together with the contribution of non-governmental and community based organizations, has resulted in a decline of the HIV prevalence from 0.39% in 2004 to 0.31% in 2009 (42, 43).

The HIV/AIDS prevention and care is supported by major donors as the World Bank, PEPFAR, the UN system and private donors like the Clinton Foundation (42). In 2004, free ART services were introduced as a part of the ART programme under the initiation of National AIDS Control Organizations (NACOs), which has at the present resulted in 292 health care centers providing treatment for HIV infected individuals (43). At the end of 2012 the ART coverage had reached 51%, corresponding to

570 620 people. However, an estimated 1000 000 HIV infected people are still in need of therapy (10).

1.3 THE STATUS OF THE EPIDEMIC IN SWEDEN

Compared to other regions in the world, Sweden has a low prevalence of HIV-1, corresponding to about 60 individuals per 100 000 inhabitants (44). The first cases of AIDS in the country were reported contemporary with the first incidences observed globally in the beginning of the 1980s (45). Later in 1985, HIV/AIDS was declared as a notifiable disease and brought under the regulation of the *Communicable Disease Act* (46). The act applies to all diseases that can be transmitted among people and that constitute a threat to all individuals' health. A total of 9 891 HIV positive cases have been reported so far (47) and today (8th of May 2014) 6477 people are known to live with HIV (Swedish InfCare HIV national quality assurance registry). The Swedish HIV/AIDS response is controlled through the *National strategy against HIV/AIDS and Certain Other Communicable Diseases* (Prop.2005/06:60) which was issued by the Swedish government in 2005 (48), as a result of the *United Nations Millennium Declaration on HIV/AIDS*(6, 7). The strategy formed a framework of measures and targets to prevent the spread of HIV-infection in the Swedish society. From an international perspective the domestic HIV prevalence has been low and stable in Sweden but because of the prevailing circumstances, the global HIV/AIDS-crisis poses a threat also in countries that have established a control of the epidemic at an early stage. In the beginning, the epidemic was driven by men who have sex with men (MSM) and intravenous drug users (IDUs) infected in Sweden. During the years, the reported new HIV cases among these two risk groups in the Swedish epidemic has decreased and since the 1990s the highest number of new cases is consisting of migrants infected heterosexually before arrival to Sweden. During 2010-2011, a total of 516 new heterosexually acquired HIV cases were reported and of these the most common country of birth was Thailand (60 cases), Eritrea (44 cases) and Ethiopia (42 cases)

(47). This indicates that the majority of new cases in Sweden are presently associated to high-endemic countries with insufficient monitoring systems and thereby at higher risk of acquiring drug resistance.

2 THE SUBTYPES OF HIV-1 AND DISTRIBUTION WORLDWIDE

HIV is categorized into HIV-1 and HIV-2 and both types are proposed to have passed to humans from chimpanzee (49, 50) and Sooty Mangabey monkey (51), respectively. HIV-1 is further divided into three main groups; group M (main), group O (outlier) and group N (non-M/non-O) (52-56) . In addition, a new HIV-1 lineage, denoted as group P, has recently been discovered in two individuals originating from Cameroon (57, 58). While the prevalence of HIV-2 is mainly restricted to West Africa at low levels, HIV-1 group M is the major causative virus type of the HIV-1 pandemic. HIV-1 group M is further classified into nine subtypes from A –D, F-H, J, and K. Recombination between the different subtypes within this group has resulted in additional subtypes known as circulating recombinant forms (CRFs) and unique recombinant forms (URFs). Among the HIV-1 group M, subtype C (HIV-1C) is the most prevalent worldwide and is responsible for half of the infections globally (59, 60) (Figure 2). HIV-1C was described for the first time in the end of the 1980-thies, isolated from Ethiopian patients, by our research group (61).

HIV-1C is common in sub-Saharan Africa, India, and Brazil while HIV-1B, which is the third dominating subtype globally (11%), is common in Europe and North America (60, 62). In Ethiopia HIV-1C is predominating (61). HIV-1B is the most common strain in Sweden, although due to migration of HIV-infected people from high-endemic sites non-B subtypes have begun to accumulate as well (44, 63, 64). Thus, research results from our group suggest that CRFs will become dominating in Sweden within a few years of time (Personal communication, Ujjwal Neogi and Amanda Häggblom).

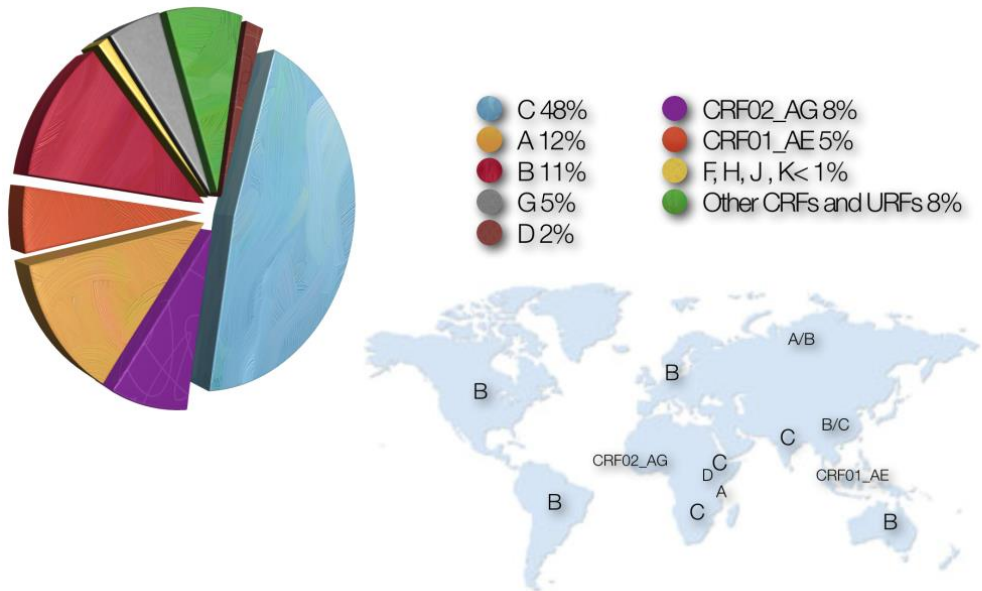


Figure 2. Prevalence of HIV-1 subtypes and distribution worldwide. Adapted from (60).

2.1 THE VIRAL STRUCTURE

HIV-1 is a retrovirus which belongs to the *Lentivirus* genus of the *Retroviridae* family. The virus is 100 nm in diameter and has a spherical shape (Figure 3). It contains two single stranded RNA molecules which are together with other viral enzymes surrounded by a capsid (p24) and a matrix (p17). These enzymes comprise the reverse transcriptase (RT), integrase (IN) and protease (PR). All enzymes have three distinct functions in the replication process, which is detailed in the next paragraph. The envelope, which encloses the capsid and the matrix, consists of a lipid bilayer derived from the infected host cell. The envelope contains two glycoproteins, gp41 and gp120, which are used in the virus-cell attachment upon infection. The genetic material of HIV-1 comprises 9 genes (Figure 3). Of these, three major genes (*gag*, *pol* and *env*) encode the structural proteins and the enzymes while the remaining six genes code for

regulatory proteins (*tat* and *rev*) and accessory proteins (*vif*, *vpr*, *vpu* and *nef*) all required for a successful replication (65).

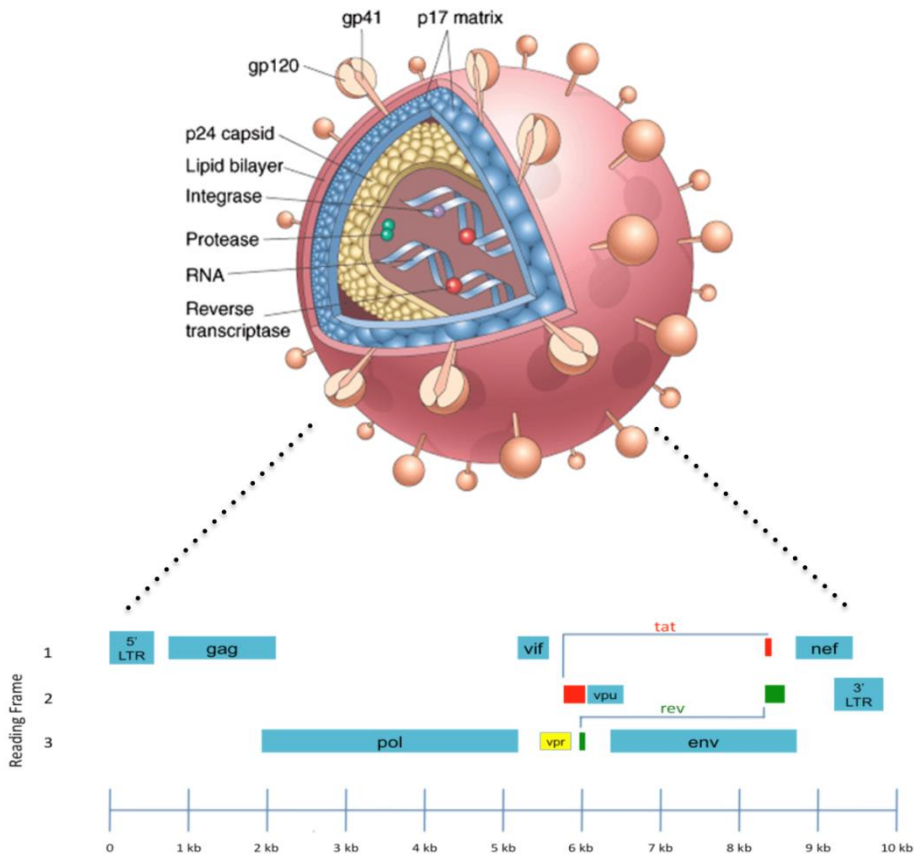


Figure 3. HIV-1 structure (66) and genomic organization (67). The virus is 9kb long and has three open reading frames; *gag*, *pol* and *env*.

2.2 THE VIRAL LIFE-CYCLE

The HIV life-cycle involves several steps and is dependent on both viral and host-cell factors (Figure 4). Upon infection, the viral life-cycle is initiated through the attachment of the gp41 and gp120 unit complex on the viral envelope to the CD4 receptor (68, 69) and the chemokine co-receptors CXCR4 or CCR5 on the host cell (70, 71). This virus-cell

interaction induces a fusion between the viral envelope and the cell membrane which is followed by the subsequent release of the nucleocapsid into the target cell. Through a disassembly mechanism of the nucleocapsid, the viral contents are released into the surrounding cytoplasm of the target cell (72, 73). The exposure of the viral genome and enzymes initiates the reverse transcription process. This event occurs through a cellular tRNA molecule, which base pair with the viral RNA and forms the starting site for the reverse transcription, and the RT enzyme which transcribes the viral RNA genome into a double stranded DNA (74, 75). During this process two identical sequences of DNA called as long terminal repeats (LTRs) are added at the both end of the dsDNA. After the reverse transcription the dsDNA is transported into the nucleus and integrated into the host chromosome by the IN enzyme through the LTRs (76, 77). As integrated the LTR regions act as a promotor for host cellular transcription factors. Subsequently the provirus DNA is transcribed to mRNA which is followed by splicing and transportation out to the cytoplasm from the nucleus (78). In the cytoplasm the mRNA fragments are translated to precursor proteins. After the translation the full-length mRNA, viral enzymes and proteins assemble at the host cell surface through the *env* proteins, which are inserted into the plasma membrane of the host cell after the translation and used as an envelope (79). From here the viral particle-complex buds from the host cell and subsequently the PR enzyme cleaves the *gag* and *gag-pol* polyproteins which engage the production of new infectious viral particles. At some occasions the provirus can become inactivated and remain in a latent state in the host cell. This can either occur during pre-integration or post-integration of the dsDNA into the host cell genome (80). This feature of the HIV pathogenesis is an important obstacle for the complete eradication of the infection from the infected body.

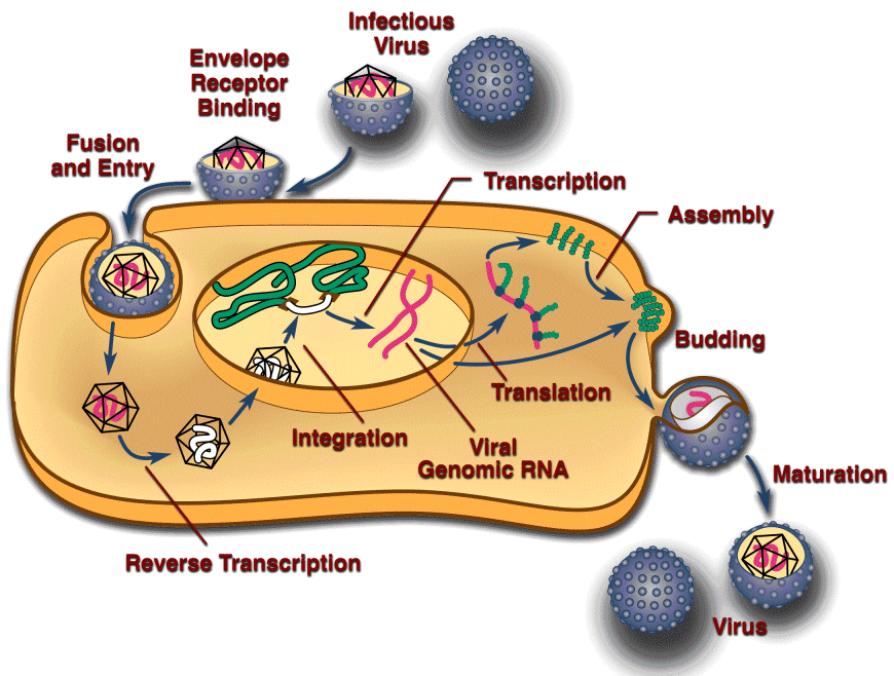


Figure 4. The HIV life-cycle involves several steps and is dependent on both viral and host cell factors (81).

3 ANTIRETROVIRAL THERAPY

In the beginning of the HIV pandemic, only one drug (AZT) was available and used as monotherapy for the treatment. Subsequently additional drugs as didanosine (ddI) and zalcitabine (ddC) were approved and together with AZT they introduced the first HIV-1 drug class, *nucleoside reverse transcriptase inhibitors* (NRTIs) (82). Later the drug lamivudine (3TC) was approved and rapidly became one important component in combination antiretroviral therapy (ART). The drugs within this class compete with the host cellular deoxy-nucleotides to be incorporated into the growing viral DNA by the RT during synthesis. In contrast to the host cellular deoxynucleotides, the nucleoside analogues lack 3'-hydroxyl group which prevents the incorporation of other deoxynucleotides and thereby terminating the synthesis of the viral DNA (83).

However, due to the rapid development of drug resistance with monotherapy (84, 85), a second class of antiretroviral regimens, *protease inhibitors* (PIs), were introduced for the treatment of HIV-1 infection in 1995. Thus, the treatment of HIV-1 became combination therapy and the beginning of the Highly Active Antiretroviral Therapy era (HAART). Saquinavir, zidovudine and didanosine were the first drugs approved within this class (82) and their chemical structure resembles the structure of viral peptides which are recognized and cleaved by the protease. Through their binding to the active site of the enzyme the proteolytic cleavage of the *gag* and *gag-pol* remains inactivated which in turn prevent the maturation of viral particles to infectious viruses (83). Combination therapy proved to be beneficial in terms of suppressing the viral load, preventing the rapid emergence of drug resistance and reducing the incidence of AIDS-related deaths among HIV infected patients. Consequently, a third drug class, *non-nucleoside reverse transcriptase inhibitors* (NNRTIs), were introduced in 1996 with nevirapine (NVP) as the first approved drug. Shortly after, delavirdine (DLV), which is not approved in Europe due to severe side effects and efavirenz (EFV) were added to this new drug class (86). In

contrast to the nucleoside analogues described above, the non-nucleoside analogues are non-competitive compounds which bind to the hydrophobic domain near the active site of the RT. This event leads to a conformational change of the enzyme which becomes less flexible and unable to continue polymerization of the viral DNA (83).

As a result of monotherapy in the early days of the HIV pandemic many patients could not benefit completely from the HAART because of drug resistance to the available regimens. Due to this dilemma there has been a constant need of improving the antiretroviral treatment strategies and the drugs. Recently, second generation drugs with high genetic barrier have become available within the existing drug classes to overcome this challenge. Additional drug classes have also been introduced for the treatment of HIV-1, such as *entry inhibitors*, *fusion inhibitors* (87) and *integrase inhibitors* (INIs) (88) all targeting distinct stages of the viral life-cycle. The different ART drug classes and their inhibitory mechanisms are illustrated in figure 5.

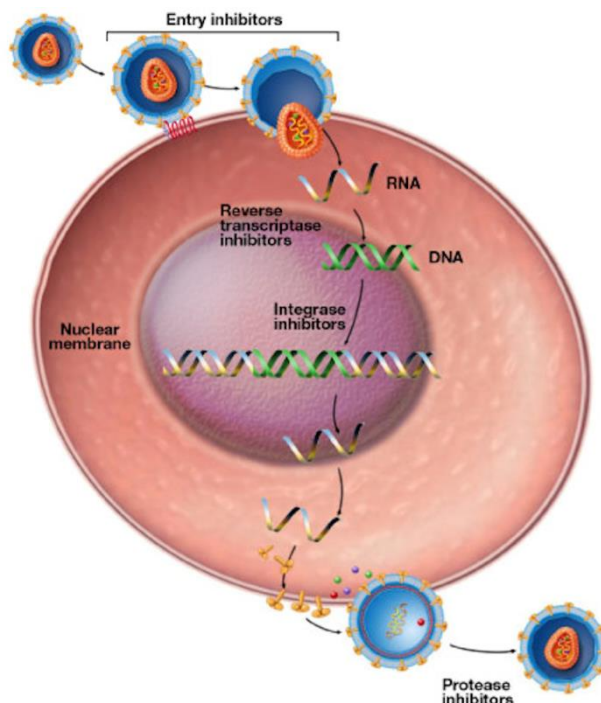


Figure 5. Antiretroviral drug classes and their inhibitory mechanisms in an HIV-1 infected host cell (89).

The NRTI drug class is the cornerstone in combination therapy and the current guidelines recommend the use of two NRTIs together with one NNRTI or together with one PI/r. These guidelines are well implemented in high-income countries and at the present, FDA approved 27 single anti-HIV pharmaceuticals and seven fixed-dose drug combinations (FDCs) belonging to the existing six different antiretroviral drug classes are in the use for HIV therapy (88). Although, access to all drug classes and the drugs within each class, varies markedly worldwide with the lowest availability in low- and middle- income countries.

3.1 TREATMENT OF HIV-1 INFECTION IN SWEDEN: INDIVIDUALIZED MANAGEMENT

All drug classes are available in Sweden with 23 single drugs and six FDCs. Several laboratory parameters, such as CD4+ T cell counts, HIV RNA viral load and drug resistance profiling, are important to assess the clinical care in order to maintain good adherence and optimal outcome of the treatment. CD4+ T cell count is a clinical parameter which is used to determine the disease state of the infected individual and is also a predictor of when to start therapy. In Sweden, ART is recommended to be initiated in HIV-1 infected patients with CD4+ T cells counts < 500 cells/ μ L. During therapy, HIV RNA viral load is the most important marker to evaluate the outcome of the treatment. A suppressive and effective ART is achieved when the viral load level is < 50 copies/mL in the infected individual and is usually combined with measurement of the CD4+ T cell count. Genotypic resistance testing (GRT) is an essential tool used to select the most effective treatment option. Earlier, GRT was carried out at ART failure only, but since 2002 it is performed routinely in Sweden also before at diagnosis, or if this has not been done, before initiation of ART in infected patients in order to identify drug resistance and thereby avoid treatment failure. Monitoring of patients is advised to occur twice-three times a year, depending on the clinical situation, with measurement of the above described parameters and other necessary blood markers but can be maintained more frequently, depending on special circumstances such as drug adherence problems or emergence of drug resistance (90).

3.2 TREATMENT STRATEGIES IN LOW- AND MIDDLE- INCOME COUNTRIES: A PUBLIC HEALTH APPROACH

In contrast to Sweden and other high-income countries with an individualized management of ART and monitoring interventions, the standard of care in LMICs is based on the WHO's public health approach (91). In the beginning, ART was mainly available in high-income

countries due to their expensiveness as a result of patent rights of the drug companies. However, the manufacturing of generic ARVs has made it possible to produce affordable drugs in a larger scale in the reach of HIV-1 infected people living in resource-limited settings (92, 93). Even though, ART scale up has increased significantly in LMICs mainly, not all drug classes are available in these settings. The most accessible drugs belong to the NRTI [tenofovir (TDF), 3TC, ZDV, and emtricitabine] and NNRTI (EFV and NVP). Current recommendation is the use of 2 NRTIs together with one NNRTI in first-line therapy, which is administered as a once-daily FDC (29, 91). Second-line and third-line regimens are not widely accessible because of high-cost, however the PI/r lopinavir/r and atazanavir/r are becoming more available and are recommended to be used together with two NRTIs as second-line ART.

Recently, the treatment recommendations by WHO were revised and at the present the initiating of therapy is advised to begin at CD4+ T cell counts ≤ 500 cells/mm³ in adult and adolescents, and regardless of CD4+ T cell counts in pregnant women. In monitoring of ART, viral load is the recommended mode and in the absence of access, the approach is to assess CD4+ T cell counts and to use clinical criteria's (91). GRT is only performed for surveillance purposes and is not recommended to be implemented routinely (37).

3.3 ART-PROPHYLAXIS IN THE PREVENTION OF VERTICAL TRANSMISSION OF HIV-1

A major event that has led to a decrease of new infections worldwide are attributed to preventive treatment (ART-prophylaxis) in HIV-1 infected mothers and their children during pregnancy, labour and after delivery. Before ART-prophylaxis was introduced, the transmission risk from infected mothers to babies was estimated to vary between 14% (in Europe) and up to 50% (in Africa) (94). With the introduction of HAART, more potent drugs with less toxic effects became available and were therefore evaluated to be used as ART-prophylaxis. The first study to show a

decrease in the risk was conducted 1994 (ACTG 076) (95). In the study, the vertical transmission risk was reduced from 25.5% to 8.3% by administering the ZDV to non-breast feeding mothers during the second and third trimester of pregnancy and their infants during the first six weeks of life. However, due to its complex administration and expensiveness it was not a feasible therapy option in resource-limited settings. A few years later, another study published an alternative strategy in which single-dose nevirapine (SD-NVP) was administered at the onset of labour to the mothers and to their infants within 72 hours of birth, resulting in a decrease of the transmission risk by 50% (96, 97). Because of its simple use and cost effectiveness this intervention was recommended to be used in resource-limited settings by WHO. As a monotherapy, this strategy also increased the risk of developing drug resistance and several studies have, since the implementation of it, reported the emergence of drug resistance mutations in these patient groups (98-102). In the studies, the NNRTI drug resistance mutations Y181C and K103N was shown to be quickly selected in a large proportion of the women after exposure to SD-NVP. Currently, the recommendation in the prevention of vertical transmission is the use of combination therapy consisting of two or three drugs depending on the accessibility of treatment regimens. In the revised treatment guidelines from WHO a once-daily FDC consisting of TDF, 3TC and EFV is recommended in pregnant and breastfeeding women (91). However in settings with limited access to ART, SD-NVP is still widely used (28). In **Paper III** and **Paper IV** in which the focus were on LMICs, women who had received ART-prophylaxis were excluded from the studies.

4 DEVELOPMENT OF DRUG RESISTANCE

The therapy failure that were observed in patients treated with AZT in the early days of the pandemic revealed that monotherapy was insufficient in preserving the viral load suppressed and as a consequence, viruses with decreased sensitivity to the AZT emerged quickly in these patients. Today, the development of drug resistance to HIV-1 is ascribed to the highly genetic variability of the virus.

4.1 HIV-1: A HIGHLY DIVERSE VIRUS

The expression “survival of the fittest” is very well applied to HIV-1. The features of the virus create an advantage for its rapid adaptation and survival even under the most unbeneficial environments. The viral population in an infected subject is consisting of a pool of variants, called *quasispecies*, which have been generated from one or a few virus upon infection. This variability of the virus is attributed to three important characteristics; error prone replication, high turnover, and large population size. It is estimated that in untreated patients, the number of infected cells is about 10^8 (103) and due to the short half-life (~2 days) of these cells, HIV-1 is dependent on infecting new cells at a very high rate. Due to very high replicative capacity, 10^{10} - 10^{11} viral particles are generated on daily basis (104). However, amongst these characteristics the error prone replication is the most critical for the emergence of drug resistance. The RT, which replicates the viral RNA into dsDNA, lacks proof reading activity and is responsible for one misincorporation per 10^4 nucleotide incorporations. On average, one error per genome per replication cycle is introduced (105) and with each viral strain that replicates, the pool of viruses will expand further and result in a highly heterogeneous HIV-1 population (106). Within this pool of viruses the wild type strain is the most adapted to the host environment and therefore predominates the HIV-1 population. *Quasispecies* on the other hand, which are less adapted

because of mutations, will be inferior and not equally fit to replicate at the same rate as the wild type strain (107).

When the host environment change, e.g. during drug pressure, the conditions for replication capacity and survival will also shift within the viral population. If the mutation made by the RT is introduced in important drug target sites, it will lead to a selective advantage for the *quasispecies* carrying the mutation compared to the wild type virus, which will be sensitive to the drug. In prolongation of the drug pressure, the mutant *quasispecies* will outgrow the wild type virus and dominate the viral population with time. The replicative capacity of the mutant *quasispecies* is not as effective compared to the wild type virus, since mutation impairs protein function but in the continuation of drug pressure this is compensated by the accumulation of additional mutations which will improve the fitness and replication ability of the virus. However, if the drug pressure is interrupted, the wild type virus will become the dominant virus (83, 108).

4.2 ACQUIRED AND TRANSMITTED DRUG RESISTANCE

The introduction of HAART made it possible to target the virus at different sites of the viral replication at the same time, thus preventing the emergence of drug resistance and subsequent treatment failure. However, DRM can still be acquired in some patients, due to several factors but incomplete therapy adherence and sub-optimal treatment are usually common factors that contribute. In both cases, the levels of drugs will be too low to prevent the viral replication completely but sufficient to promote the emergence of mutant strains. The level of resistance that may arise is dependent on the genetic barrier, which is the number of mutations required to induce drug resistance. Some drugs have a high genetic barrier, requiring the accumulation of several mutations to induce high-level drug resistance like PIs/r, while other drugs have a low genetic barrier for which

only a single mutation is sufficient for the emergence of drug resistance as NRTIs and NNRTIs. In the context of treatment outcome, the appearance of secondary mutations which may emerge to increase viral fitness, do not affect the drug susceptibility. Cross-resistance can also occur within drug classes, which may compromise alternative regimens options for continued therapy.

HIV strains carrying DRMs can be transmitted between individuals, so called primary drug resistance. Transmission of drug resistant-virus has been observed to be less efficient compared to wild type virus because of loss of fitness in the absence of ART (109). The stability and reversion of transmitted DRMs has been shown to vary in when ART is not present (110). Some mutations, that decrease viral fitness, seem to revert back to wild type quickly (111) while others with little impact on the fitness can sustain for longer time (112).

4.3 HIV-1 MINOR POPULATIONS

Within the pool of *quasispecies* in treatment experienced patients with drug resistant viral strains, the viral populations are divided into major and minor populations. Like in treatment-naïve patients, the pool of *quasispecies* presented in these subjects is heterogenous (113). The major population is the dominant *quasispecies* and co-exists with minor (non-dominant) *quasispecies* consisting of different resistant genotypes (113, 114). As the evolution of both populations occurs independently from each other, the minor *quasispecies* can become the dominant population under circumstances that are beneficial for its outgrowth. As such, minor *quasispecies* may constitute a reservoir consisting of a mixture of various viral populations which may enhance the development of drug resistance (115). The emergence of drug resistance in minor HIV-1 populations is clearly a field that has been under intense investigation lately. Earlier, the limitation of techniques used to detect these has made it difficult to

understand their implications in clinical context. However, with recent more sensitive methodological approaches that are becoming more available, it has become possible to investigate their clinical significance further. Although the availability of such assays are restricted to high-income countries and as a result the implication of minority populations in non-B subtype infections have been less investigated as in comparison to the HIV-1B.

4.4 VIRAL RESERVOIRS

One of the major obstacles to eradicate HIV-1 in infected individuals is the persistence of the virus as a latent form in viral *reservoirs*. A viral reservoir can be defined as a cell type in which a replication-competent virus can persist even under prolonged suppressive HAART (116). Resting memory CD4⁺ T cells were the first reservoirs that were isolated for HIV-1 (117). Normally, activated CD4⁺ T cells have a short survival after infection and dies rapidly (118). However, resting memory CD4⁺ T cells seldom get infected by HIV and therefore it is proposed that latency of HIV-1 is established through the transition of activated CD4⁺ T cells into resting memory cells which can serve as a viral reservoir (119). Within these cells HIV-1 is integrated into the host genome and becomes transcriptionally silent. During this resting state there is no virus production from these cells but upon activation the production will be induced. A major consequence of such latency is that it constitutes a barrier for HAART to reach and affect these cells since the drugs are not able to eliminate integrated virus. Another consequence is the possibility of drug-resistant viruses becoming archived in the cells during ART and remain in a latent form until activated.

HIV-1 can also reside in anatomical sites, which may act as viral reservoirs because of the limited penetration of antiretroviral drugs to these locations. As a result of sub-optimal drug concentration, drug-resistant strains can

emerge and evolve in these sites despite drug pressure (116). If exchange of drug-resistant viruses occurs to other sites of the body it may lead to treatment failure. Since HIV-1 invades the central nervous system (CNS) and the access of some antiretrovirals may be restricted to this site it can act as a viral reservoir (120). We have therefore in this thesis investigated to which extent distinct drug resistance patterns in the cerebrospinal fluid (CSF) differ compared to blood, in both major and minor HIV-1 populations.

4.5 DRUG RESISTANCE MUTATIONS

In this thesis, we have focused on the NRTI associated mutations M184I/V and the NNRTI associated K103N and Y181C mutations. These mutations are key mutations within their respective drug class and can confer high-level resistance to regimens which are frequently used in first-line and second-line therapy. In the WHO drug resistance report from 2012 an increase of TDR was observed in LMICs over time and the most commonly observed TDRs were the K103N, M184V, and Y181C (11). In high-income countries, while NNRTI associated TDRs have increased, NRTI associated TDRs have declined over time (121).

4.5.1 The NRTI drug resistance mutations M184I/V

The M184I/V mutations occur in the catalytic site of the RT and involve a single base substitution at codon 184 (Figure 6). This substitution results in two alleles, M184I where the amino acid methionine is replaced by the isoleucine (ATG → ATA) and M184V where the methionine is replaced by the amino acid valine (ATG → GTA) (Figure 7). Because these amino acids have different side chains compared to the methionine they interfere with the incorporation of the nucleoside analogues within the catalytic site (122, 123). The M184I mutation is the first one to appear but is quickly replaced by the M184V since this mutation has greater ability to induce higher replicative capacity (124). Both mutations arise rapidly and confer

high-level resistance to 3TC, FTC and low-level resistance to abacavir (ABC) and ddI (125) (Figure 6). However, these mutations do not confer cross-resistance to other NRTIs and is replaced by the wild type virus when treatment is interrupted.

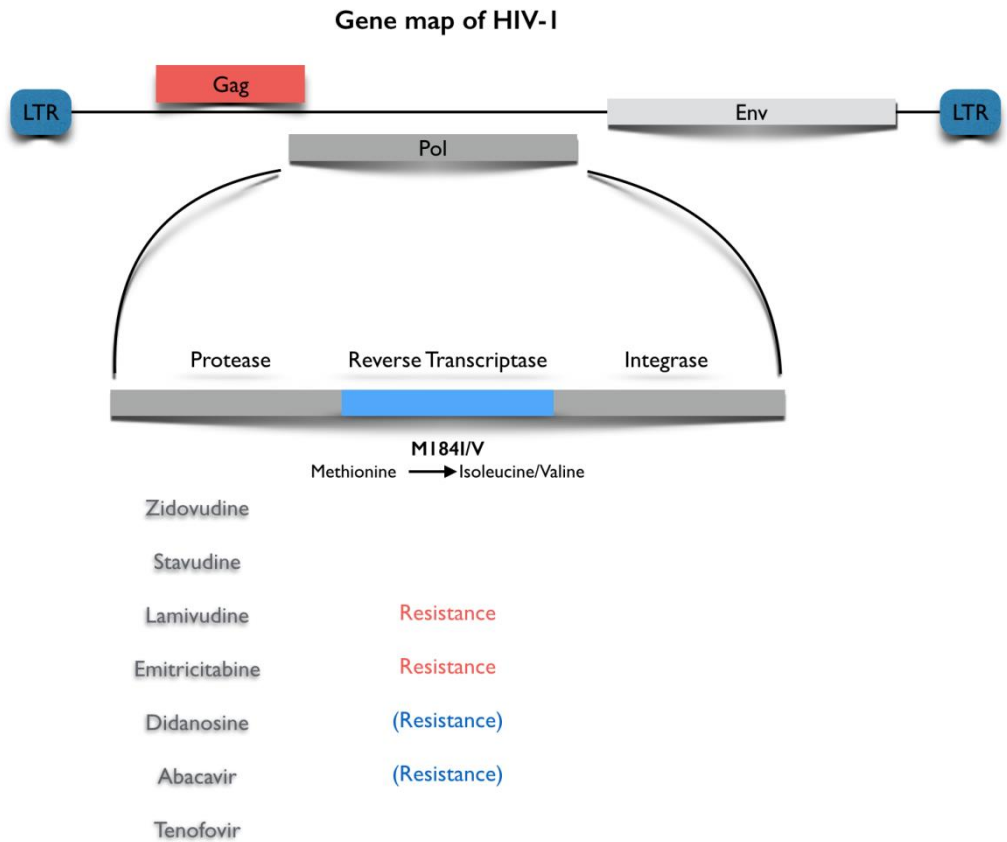


Figure 6. The gene map of HIV-1. Reverse transcriptase gene and associated mutations M184I/V. The emergence of these mutations confers high-level resistance to the nucleoside reverse transcriptase inhibitors lamivudine and efavirenz, and low-level resistance to didanosine and abacavir.

4.5.2 The NNRTI drug resistance mutations K103N and Y181C

The NNRTI drug resistance mutations occur in the hydrophobic binding pocket close to the active site of RT (Figure 7). The K103N and Y181C occur through a single base substitution. The K103N appears in codon 103 in the RT gene as two alleles (AAA → AAC, AAA → AAT) where the amino acid lysine is replaced by the amino acid asparagine, while the Y181C occurs in codon 181 as one allele (TAT → TGT) where the amino acid tyrosine is replaced by the amino acid cysteine (Figure 8). The appearance of Y181C and K103N reduce the affinity of NNRTIs (126). Both mutations can emerge within a few weeks (127, 128) and have minor influence on the viral fitness (128, 129). In general, the NNRTI drug resistance mutations are highly cross-reactive to the first generation regimens within the drug class. The K103N can confer high-level resistance to EFV and NVP while Y181C confers intermediate to high-level resistance to all regimens within the drug class (Figure 7).

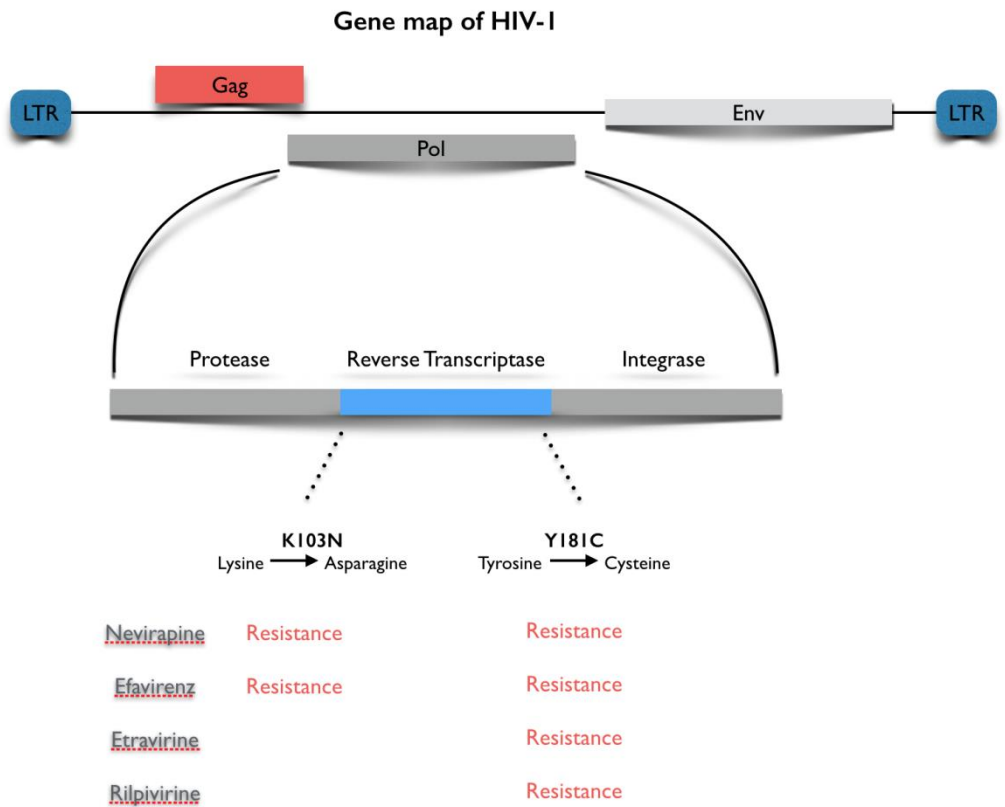


Figure 7. The gene map of HIV-1. Reverse transcriptase and associated mutations K103N and Y181C. The K103N mutation confers high-level resistance to the non-nucleoside reverse transcriptase inhibitors nevirapine and efavirenz. The Y181C mutation confers wide-class resistance.

5 METHODS TO DETECT DRUG RESISTANCE

Both genotypic and phenotypic tests are available to study drug resistance. In this thesis we have used genotypic methods. There are different genotypic approaches available for detection of DRM. We have used direct population based sequencing, AS-PCR and NGS. GRT involves detection of known DRM in the genes of HIV-1, which confer reduced susceptibility to ART.

5.1 POPULATION BASED SEQUENCING

The most conventional GRT approach is based on direct polymerase chain reaction (PCR) dideoxynucleotide sequencing (Sanger) (17). This approach is the preferred method as part of standard-of-care to guide ART and monitor DRMs in HIV-1 infected patients. Direct sequencing can be carried out using either *in house* methods or commercially available assays such as TRUEGENE® HIV-1 Genotyping Assay (Siemens) (130) and Celera ViroSeq® HIV-1 Genotyping System (Abott) (131). These assays produce a nucleotide sequence of the *pol* gene, covering the protease and the RT coding regions in the clinical sample. In addition, the integrase and the part of the *env* gene coding for the glycoprotein (gp) 41 can also be sequenced. The obtained clinical sequence is a consensus sequence generated from a population of viral genomes, hence the name population sequencing. The consensus clinical sequence is then aligned and compared to a reference sequence of laboratory wild type strain to determine the presence of possible mutations. The identification of which mutations are clinically relevant is performed by interpretation systems that determine/predict the level of reduced susceptibility to ARV regimens (132, 133). Even though standard GRT is the recommended approach to monitor DRMs by international guidelines (34, 35), these assays have their disadvantages. First, they are complex in the context of interpreting the many distinct mutations identified within a clinical sequence, which can be

time consuming as well as difficult to apply into ART management. Second, they are not sensitive enough to detect viral populations below 20% of the total viral population, which may allow clinically important minor variants to become undetected. Although these assays are relatively inexpensive assays to monitor DRMs in high-income countries, they are not cost-effective and easy to implement in resource-limited settings.

Recently more sensitive GRT methods have been developed, such as point-mutation assays (AS-PCR) and deep sequencing (NGS), which could be an alternative to study drug resistance and as well as an option to use in resource-limited settings by targeting key DRMs.

5.2 ALLELE-SPECIFIC REAL-TIME PCR

AS-PCR is a point-mutation assay that allows the detection and amplification of different alleles of a gene simultaneously. Although a few mutations can be analyzed at a time, it is currently one of the most sensitive assays that can be used to study drug resistance. In comparison to standard sequencing, the sensitivity of point-mutation assays is quite high, enabling the detection of viral populations down to 0.01% (21, 22). In addition, AS-PCR is less expensive and time-consuming as compared to other sensitive assays like single-genome sequencing and clonal sequence analysis (134).

Distinguishable for AS-PCR is the use of specific primers targeted for the allele of interest in DNAs of unknown genotype. These primers are modified intentionally and can specifically amplify the target allele within a sample by forming a 3'mismatch with the DNA template, which is described more in detail in chapter 7. In our AS-PCR methodology, we have used real-time PCR, which allows the detection and measurement of the amount of amplicon generated at each PCR cycle as it occurs in real time. The amount of target DNA is quantified using fluorescent probes or DNA-binding dyes which is incorporated to the amplicon during the PCR

cycles. The real-time PCR instrument measures this fluorescence signal as it increase during each cycle, which is proportional to the amount of amplicon generated in the exponential phase of the reaction. The real-time PCR reaction is displayed as an amplification plot in which the fluorescent signal is plotted against cycle number for each sample (Figure 8). For each sample a threshold cycle value (Ct) is obtained in the exponential phase where the florescent signal crosses threshold signal of the assay used to distinguish relevant amplification signal from the background. With each amplification a standard curve consisting of known concentrations of DNA is also amplified with the specific primers and run in parallel with the unknown samples. The obtained Ct values for the unknown samples are then compared with the Ct values of the standards to calculate the amount of the target allele. By comparing the amount of different alleles it is possible to calculate their relative proportion within a sample (135).

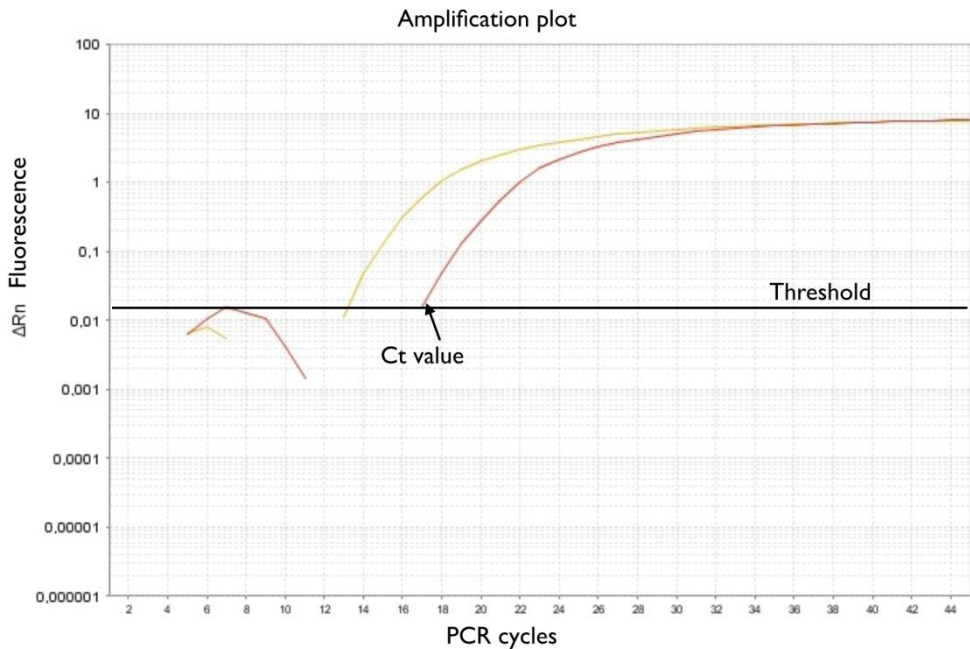


Figure 8. Allele-specific real-time PCR amplification plot. The measurement of the curve occurs where the fluorescence signal crosses the threshold signal of the assay.

In this thesis different AS-PCR assays with distinct detection methodologies were utilized to study drug resistance in HIV-1 infected patients. In **Paper I** and **II**, we used an AS-PCR assay that was developed by us to detect the NRTI M184I/V mutations by using fluorescent probes. In **Paper III** we designed two AS-PCR assays for the detection of the NNRTI mutations K103N and Y181C using DNA-binding dyes.

5.3 NEXT-GENERATION SEQUENCING

The field of sequencing technologies has been revolutionized by the recent approach with NGS platforms. The high throughput ability of these platforms has made it possible to generate massive sequence data from different biological systems in a single run. There are currently several options of platforms accessible for deep sequencing, such as Illumina, Roche 454, Ion Torrent. However, due to their expensiveness they have not been available on a wider scale until recently. The introduction of bench-top sequencing platforms with high throughput have been shown to reduce the running cost and time and therefore in comparison to conventional methods (*136*) may be more suitable option to be used in the clinical diagnostics.

In **Paper IV**, we used amplicon sequencing approach with Illumina MiSeq to develop a NGS protocol for the identification of the key DRMs, including K103N, Y181C and M184V, to be used in large scale sequencing and surveillance of DRMs. Compared to other NGS platforms, MiSeq has been shown to be more advantageous in terms of reducing hands-on-time, providing simpler laboratory workflows as well as deeper sequencing capacity to detect single nucleotide polymorphisms with the lowest error rate (*24, 137*).

The methodology in Illumina platform is characterized by the approach sequencing by synthesis technology (SBS) which enables tracking of fluorescently labeled nucleotides as they are being added to massively DNA strands in a parallel mode. In our protocol we used targeted sequencing through which only small selected or defined regions of genes are sequenced. The target amplicon was amplified using barcoded primers followed by multiplexing of 24 samples together that makes it possible to analyze and sequence a large number of samples simultaneously. The gene specific primers with platform-specific oligonucleotide adapters and individual index containing specific nucleotide stretch are used as the final primer for nested PCR amplification and thereby making each sample distinguishable during the process of sequencing. As such, this approach enables pooling of samples which drastically increases the number of samples that can be processed and analyzed in a single run. Following the sequencing, the raw reads are quality controlled and aligned to a reference sequence for variant calling (138). The downstream analysis used in our protocol is described in more detail in chapter 7.

6 AIMS OF THE STUDY

The main aim of this thesis was to study drug resistance in minor HIV-1 *quasispecies* and evaluate three assays based on allele-specific PCR (AS-PCR) and next generation sequencing (NGS), respectively.

This was done through the specific aims stated below:

- To study by AS-PCR to which extent M184I/V mutations emerge during the first phase of viral decay in therapy-naïve patients initiated on lamivudine-containing antiretroviral therapy (ART) of various potency.
- To investigate by AS-PCR to which extent distinct drug resistance patterns, including M184I/V, appear in major and minor viral populations in the cerebrospinal fluid and blood compartments in patients failing lamivudine-containing ART.
- To assess by AS-PCR, the occurrence of the K103N and Y181C mutations in minor populations of treatment-naïve Ethiopian patients living Ethiopia, East African patients who have migrated to Sweden and Caucasian patients living in Sweden.
- To develop and evaluate a feasible and easy-to-use high throughput NGS protocol for the detection of DRMs in the RT-gene, including K103N, Y181C and M184V, that can be applied in large scale surveillance in low- and middle-income countries.

7 MATERIAL AND METHODS

7.1 PATIENTS

The samples that have been used in the different sub-studies have been obtained from both treatment-naïve and treatment-experienced HIV-1 infected patients. Ethical clearance was obtained for all studies. For more detailed information about patients, ethical permits and statistical analysis, see respective papers. Below follows a brief description of the patients.

In **Paper I**, patients from three different cohorts were used. Cohort 1 consisted of 315 samples which were obtained from 43 patients with primary HIV-1 infection (PHI) enrolled in the QUEST study (139). The patients were initiated on quadruple-regimen containing 3TC very early after diagnosis. Samples were collected at baseline, 1-6 weeks into therapy and after treatment cessation, which occurred after a median of 2.5 years of therapy. Cohort 2 consisted of 14 chronically HIV-1 infected patients from a Nordic randomized multicenter study (NORTHIV). The patients were randomized to a triple-regimen containing 3TC, one other NRTI, and a PI/r or efavirenz. A total of 26 samples were included from this study, of which 12 samples were taken at baseline and 14 samples were taken 4-12 weeks into therapy. Cohort 3 consisted of 15 chronically HIV-1 infected patients followed during routine clinical care at the Department of Infectious Diseases at the Karolinska University Hospital, Sweden. The patients were initiated on dual-regimen containing ZDV and 3TC during 1995-1998. From these patients 36 samples were obtained, of which 15 were baseline samples and 21 samples were taken 2-28 weeks into therapy. All patients in the three cohorts had no previous treatment experience when ART was initiated.

In **Paper II**, 13 multi-therapy experienced patients were included. A total of 44 plasma and CSF samples were obtained. These patients, who had developed virological failure during 3TC-containing therapy, were a part

of a prospective longitudinal study conducted at the Sahlgrenska University Hospital in Gothenburg, Sweden (140).

In **Paper III**, plasma samples from 191 treatment-naïve patients belonging to three different HIV-1 infected cohorts were included. Cohort 1 consisted of 92 Ethiopian patients attending various clinics in Addis Ababa, Ethiopia, during 2008-2009 before initiation of ART as a part of a clinical research cohort (141). Cohort 2 consisted of 55 treatment-naïve East African patients who had migrated to Sweden from following countries: Ethiopia (n=26), Eritrea (n= 23), Somalia (n= 2), Zimbabwe (n=2), Tanzania (n=1), and Kenya (n= 1). Cohort 3 consisted of 44 Caucasians living in Sweden. The patients residing in Sweden were followed at the Department of Infectious Diseases at the Karolinska University Hospital, Sweden during 2002-2013. Women who had received prophylaxis for the prevention of vertical transmission were excluded from the study.

In **Paper IV**, single peripheral blood plasma samples obtained from treatment-naïve HIV-1 infected patients belonging to three different cohorts were used. A total of 96 patients were included, of which 49 were Indian patients that had been followed during 2010 to 2013 at St. John's Medical College and Hospital, Bangalore, India, 17 were East African migrants and 25 were Caucasians living in Sweden that had been followed during 2003-2013 at the Infectious Disease Clinic at Karolinska University Hospital. The selection of the samples was done retrospectively and randomly among those who had sufficient amounts of frozen plasma available.

7.2 RNA EXTRACTION

The RNA extraction was carried out by using the QIAmp Viral RNA Mini Kit (Qiagen) for all patients included with exception of the patients described in Cohort 1 in **Paper I** for which RNA was isolated by using the

Total Nucleic Acid Isolation Kit (TNAI) on a COBAS Ampliprep instrument (Roche).

7.3 REVERSE TRANSCRIPTION AND NESTED PCR

In **Paper I and II**, the Moloney Murine Leukemia Virus (M-Mulv) RT and the Expand High Fidelity PCR System (both Roche) was used to synthesize the cDNA from the extracted RNAs. The cDNA was subsequently amplified and nested in several rounds by using the Expand High Fidelity PCR System (Roche) and the Applied Biosystem PCR reagents.

In **Paper III and IV**, the extracted RNAs were converted to cDNA by using the Superscript III First Strand Synthesis Supermix (Invitrogen) and Random Primers (Promega) for the Indian samples. The cDNA was further amplified and nested by using the Expand High Fidelity Plus PCR System (Roche Diagnostics) and high fidelity Taq DNA polymerase with proof reading activity Platinum® Taq DNA Polymerase, High Fidelity (Invitrogen, US) for the Indian samples in **Paper IV**.

7.4 SEQUENCING

Sequencing was conducted to investigate the presence of other DRM in the *pol* gene. In **Paper I and II**, the 1315 bp long cDNA fragment was purified with PureLink™ PCR Purification Kit (Invitrogen) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kits on the ABI PRISM 3100 Genetic Analyzer (both Applied Biosystems). The obtained nucleotide sequences were aligned by using SEQUENCHER software (Gene Codes Corporation, USA) and edited manually.

In **Paper III**, nested PCR products, which corresponded to a 1203 bp fragment in the 5' half of the *Pol* gene, were purified with QIAquick PCR

purification Kit (Qiagen) and sent for sequencing to MWG operon (MWG operon, Germany). Sequences were edited by using the BioEdit software v7.2.5 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). The identification of TDR was assessed according to the WHO 2009 list of mutations for surveillance of TDR (142) as implemented in the Calibrated Population Resistance tool (v5.0 beta) (143) available at the Stanford HIV Drug Resistance Database (hivdb.stanford.edu).

In **Paper IV**, sequencing was performed using an *in house* method described in (144) for the Indian samples. The obtained 680 bp long fragment after the first round PCR was sequenced and edited using an automated web based sequence editor, RECall (beta v2.9). For the Swedish samples sequencing and editing were carried out as described in **Paper III**. The interpretation of TDR was performed using the WHO 2009 list of mutations for surveillance of TDR, as described above.

7.5 SUBTYPE CLASSIFICATION AND PHYLOGENETIC ANALYSIS

In **Paper IV**, HIV-1 subtyping was performed using partial *pol* sequences. Phylogenetic analysis was conducted using the subtype reference downloaded from Los Alamos database and the PhyML version 3.0 with the best fitted model predicted in FindModel (www.hiv.lanl.gov).

7.6 ALLELE-SPECIFIC REAL-TIME PCR (PAPERS I-III)

Different detection methods can be used in AS-PCR. In this thesis both TaqMan® and SYBR® Green I dye chemistries have been utilized for the investigation of the M184I/V, K103N and Y181C DRM. The principle in TaqMan® chemistry is based on the application of a fluorogenic probe which is specifically designed to detect the accumulation of the sequence of interest while in the SYBR® Green I dye chemistry the use of a specific

dsDNA binding dye is applied. The major difference between the both detection assays is that the fluorophore dye in SYBR® Green I dye chemistry will detect all dsDNA that are present, including non-specific PCR products, compared to the fluorogenic probe in TaqMan® chemistry which only allows the detection of specific DNA targets.

7.6.1 Detection and quantification of the M184I/V mutations

The quantification and detection of the M184I/V mutations were performed as described earlier in (145) and was carried out using the TaqMan® chemistry. A typical reaction set-up includes forward and reverse primers, fluorogenic probe and a DNA polymerase with a 5' exonuclease activity.

In the amplification one forward primer and three reverse primers were used (Figure 9). Among the reverse primers two were mutant specific and modified through the introduction of mismatches at the -1 and -3 nucleotide positions from 3' end of the primer. These mismatches enable the amplification of the corresponding mutant (M184I/V) and prevent the amplification of wild type as well as other mutant HIV-1 DNA. The forward primer and the third reverse primer, which are non-specific, amplify all HIV-1 DNA that are present.

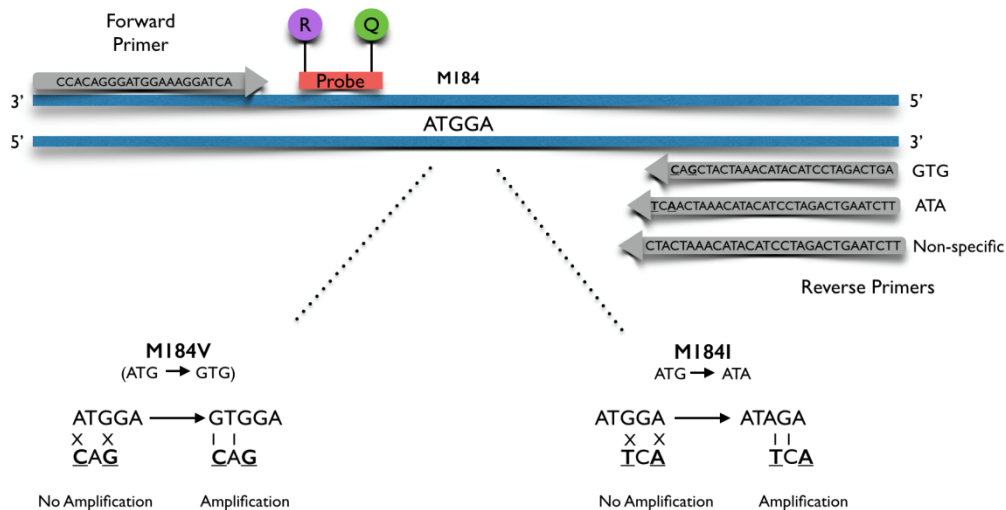


Figure 9. For the amplification of M184I/V one forward primer and two mutant specific primers were used. The mutant specific primers were modified by mismatches introduced at the -1 and -3 nucleotide positions from the 3' end of the primer. These mismatches enable the amplification of the corresponding mutant sequence (M184V: ATG → GTG; M184I: ATG → ATA) and prevent the amplification of the wild type sequence (M184: ATG). The mismatches are indicated in bold and underlined. The forward primer and the non-specific third reverse primer amplify all sequences that are present. R, reporter; Q, quencher.

The fluorogenic probe used in the amplification reaction is a DNA oligonucleotide which has two distinct functions carried out through a fluorescent reporter dye in the 5' end, which fluorescence at a specific wavelength, and through a quencher molecule in the 3' end, which quenches the fluorescence emitted from the reporter dye. During the reaction the probe hybridize to the complementary target DNA downstream of the primer site. As the elongation of the new DNA strand continues the DNA polymerase will reach the probe and cleave it through the 5' exo-nuclease activity. This results in the dissociation of the reporter dye from the quencher and increased fluorescence emission which is registered by the thermal cycler. With each cycle more reporter dyes will be dissociated, leading to an increase in fluorescence signal proportional to the amount of amplification products. If the target DNA is absent the probe

will remain intact and will not emit light due to the close proximity of the quencher which will absorb the light.

All reactions, including patient sample and standard controls, were carried out in an ABI Prism 7700 Sequence Detection System (Applied Biosystems) which generated an amplification plot displaying the amount of fluorescence in each cycle for each sample and standards. The amount of HIV total population and mutant of each patient sample was calculated through non-specific (total population) and specific (mutant population) standard curves, consisting of plasmid viral DNA subtype B HXB2 strain introduced with M184V GTG mutation or M184I ATA mutation, as described in (20). By comparing the cycle threshold (Ct) of the patient samples with the Ct values for the standards the proportion of mutated virus in correlation to the total viral population was determined. The cut-off for the M184V mutation was set to 1.0% and 0.2% for the M184I mutation.

7.6.2 The detection and quantification of the K103N and Y181C mutations

For the quantification and detection of the K1013N and Y181C mutations the SYBR® Green I dye chemistry was used. A typical reaction set-up includes similar contents as described for the TaqMan® chemistry but instead of a probe a reporter dye is used instead. During the reaction, when the extension phase begins and the new DNA strand is synthesized, the SYBR® Green I dye will bind specifically to dsDNA by intercalating between the base pairs. As the extension phase will continue the more dyes will bind to the dsDNA and lead to an increased fluorescence signal. During the accumulation of the PCR products in each PCR cycle more fluorescence will be detected and this increased signal is proportional to the amount of amplification products.

Mutant specific and non-specific primers were designed for the amplification of the K103N (AAC and AAT) and Y181C (TGT) mutations and for the total viral population, respectively. The primers were designed by choosing the maximum consensus sequence from alignments of 598 HIV-1 subtype B and C sequences available from the Los Alamos National Laboratories HIV sequence database (www.hiv.lanl.gov). For the amplification of the K103N mutants the same reverse primer was used for all reactions. The forward primers on the other hand contained a mutagenic sequence at the 3' end which enabled specific amplification of respective mutant (Table 1). In contrast, for the amplification of the Y181C, the same forward primer was used for all reactions and the reverse primers contained the mutagenic sequence which amplified the respective mutant (Table 1). In addition, each mutant specific primer was anchored at position -1 of the 3'end, creating a mismatch which increased the selectivity of the primers (depicted as bold and italic "I" in Table I).

Application	Denomination	Sequences (5'→3')	Nucleotides
Nested-PCR	A1	GAAAGAYTGTACYGAGAGACAGGCTAAT	2058-2081
	A2	TTAATCCCTGGGTAAATCTGACTTG	3350-3373
	B1	AGCAGGAGCYGAARGACAGGG	2135-2158
	B2	TYCCACTAACTTYTGTATRTCATTG	3315-3338
K103N-ASPCR	D1-AAC	CMGCAGGGTTAAAAAGA <i>I</i> <u>C</u>	2839-2858
	D1-AAT	CMGCAGGGTTAAAAAGA <i>I</i> <u>T</u>	2839-2858
	D1-ALL	CAYCCMGCAGGGTTAAAAAAG	2835-2856
	D2	CTGTGGRAGSACATRTTAYTG	2982-3002
Y181C-ASPCR	E1	TCARTAYAATGTSCTYCCACAGG	2981-3003
	E2-TGT	ATACAARTCATCCATRTATTG <i>I</i> <u>C</u>	3091-3113
	E2-ALL	CTACATACAARTCATCCATRTATT	3094-3117

Table I. Primer sequences and their binding sites.* Sequence numbering is relative to the HXB2 laboratory reference strain. The mutagenic nucleotide is shown in boldface and underlined in allele-specific real-time PCR (AS-PCR) primers. The incorporated intentional mismatch (A→I in K103 and T→I in Y181) at the -1 position of the 3'-end is shown in boldface and italic. This intentional mismatch was incorporated only in the mutant specific primer in order to increase primer specificity.

Plasmid controls were constructed as described earlier (22) and run in parallel with clinical samples. By using the primer pairs B1 and B2 (Table 1) a 1203 bp DNA fragment of the reverse transcriptase gene of HIV-1NL4-3 strain was amplified and purified before cloned into pCR4-TOPO cloning vector (Invitrogen). The obtained HIV-1 RT clone, pCR4-RT, was used as a template for QuickChange II Site-directed PCR mutagenesis (Stratagene) to create the K103N (AAA→AAC, AAA→AAT) and Y181C (TAT→TGT) mutant plasmids used for generating standard curves. All AS-PCR reactions were carried out in an ABI sequence Detector System 7500 (Applied Biosystems). AS described under section 7.5.2, the amount of total population and mutant of each clinical sample was determined by using specific and non-specific standard curves. The proportion of mutant in each sample was calculated by comparing the obtained Ct values with Ct values for standards. The cut-off for detecting K103N (AAC and AAT) was set to 0.1% and for Y181C (TGT) was set to 0.25%. An amplification with AS-PCR mutant specific and non-specific primers is illustrated in Figure 10.

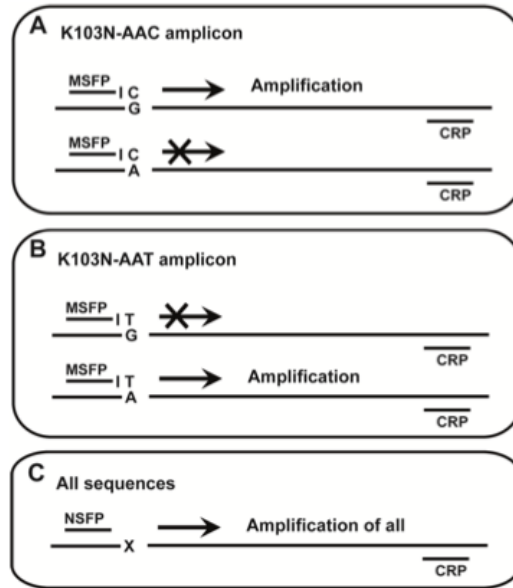


Figure 10. Schematic overview of primers design used in the allele-specific PCR assays. For K103N analysis, each patients sample was analyzed with mutant specific AAC (A) and AAT (B) primers as well as non-specific primers that amplified the total populations (C). In presence of K103N-AAC (A) and K103N-AAT (B) mutants, only specific mutant amplicons will be amplified when using mutant specific forward (MSFP) and a common reverse primer (CRP), respectively. The total viral populations in each sample were amplified using non-specific forward primer (NSFP) and the CRP (C). MSFP, mutant specific forward primer; CRP, common reverse primer; NSFP, non-specific forward primer.

The selectivity and accuracy of the AS-PCR assay was evaluated by mixing various proportions of mutant and wild type DNAs. The mixtures, in which the proportion of mutant ranged from 0.01 to 100%, were analyzed with each mutant specific (AAC, AAT, or TGT) and non-specific primer pairs.

The cut-off for the assay background was determined as a mean Ct value from eight independent runs of 100% wild type template with mutant specific primers plus three standard deviations.

7.7 TAGGED POOLED HIGH THROUGHPUT AMPLICON SEQUENCING (PAPER IV)

7.7.1 Targeted amplicon sequencing

NGS was performed using Illumina amplicon sequencing with MiSeq at SciGenom Lab Pvt. Ltd, Cochin, India. First round PCR products described in section 7.3 were amplified and ligated to barcoded primers and Illumina specific sequence adaptors with P7 and P5 (Figure 11) using Phusion High-Fidelity DNA Polymerase (New England Biolab, MA). After gel separation and elution, 24 consecutive samples were pooled in each run with 30% PhiX control and subjected to pair end 2x300 base-pair reads.

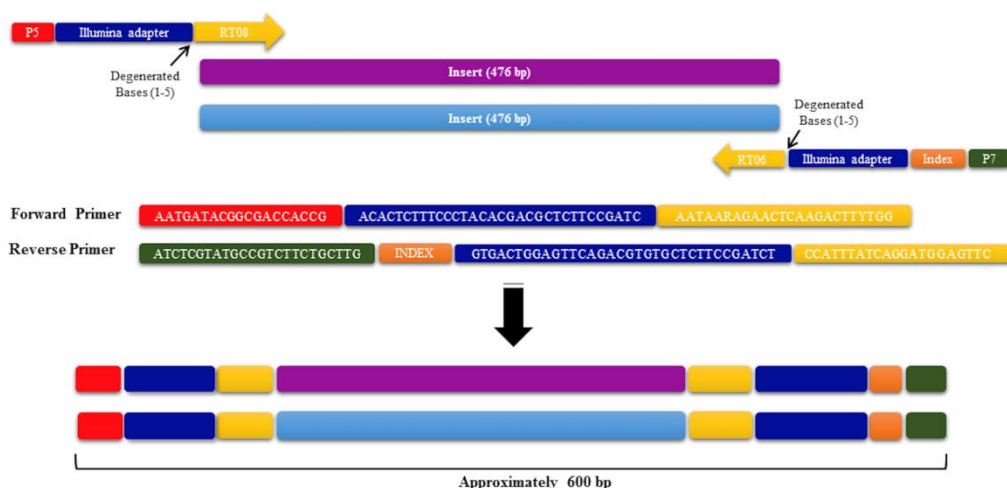


Figure 11. Schematic overview of the primers, Illumina specific adapters with P5 and P7 used in the next-generation sequencing analysis. Twenty-four index sequences were used in the pooling of 24 samples and run in a single lane of MiSeq.

7.7.2 Bioinformatics analysis

The bioinformatics pipeline for NGS is illustrated in Figure 12. In order to assure the quality of the raw fastq files, the FastQC program

(<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) was used. Low quality bases from the end of the read were removed based on a cut-off value of $>Q30$ of the Phred value score. The Cutadapt program (146), was used to remove any adapter sequences, if present, from the read sequence. Using the Bowtie2 version 2.0.5 program (147), the trimmed reads were thereafter aligned to the reference gene segment (HXB2 position 2813 to 3246). Samtools was used to identify the properly-paired aligned reads, which were taken for further analysis (148). The UnifiedGenotype caller in GATK-Lite program (<http://www.broadinstitute.org/gatk/>) was used to perform variant calling. Variants with a quality score $>50\%$ and a read depth of at least 5000X were taken further for annotation. An *in house* Perl script was used to calculate amino acid frequencies. Mutations, which had a frequency of $>1\%$, were considered for interpretation.

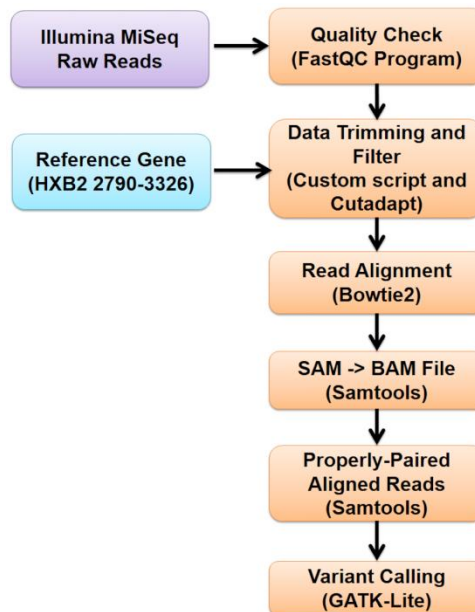


Figure 12. Illustrating the bioinformatics pipeline for next-generation sequencing using Illumina MiSeq platform.

8 RESULTS

8.1 SELECTION OF DRUG-RESISTANT HIV-1 DURING THE EARLY PHASE OF VIRAL DECAY IS UNCOMMON IN TREATMENT-NAÏVE PATIENTS INITIATED ON A THREE-OR FOUR-DRUG ANTIRETROVIRAL REGIMEN INCLUDING LAMIVUDINE (PAPER I)

Treatment of HIV-1 infection comprises the use of combination therapy consisting of three different antiretroviral regimens (*149*). The use of more regimens (≥ 4) has been shown to be beneficial in terms of enhanced antiviral activity and increased viral decay after start of therapy (*150, 151*), but these advantages are nevertheless accompanied by additional side effects (*152*). Although, the clinical benefits with long-term four-drug ART has not been confirmed. Low-level ongoing viral replication has been described in patients with undetectable viremia (*153, 154*) and in patients treated with as many as four-drug ART (*155*). To which extent infection of new cells occurs during the first period of viral decay after start of suppressive therapy and if viral replication can result in the early selection of drug-resistant viral populations is not completely understood. To address these questions further, the main objective of this study was to explore to which extent the M184I/V mutations are selected during the first phase of initiation of ART in treatment-naïve HIV-1 infected patients.

In this study, three different cohorts consisting of treatment-naïve patients initiated on quadruple ($n=43$), triple ($n=14$) or dual ($n=15$) 3TC-containing therapy was included. All patients in the first group with quadruple therapy were treated at PHI while the patients in the remaining two groups were chronically HIV-1 infected at start of therapy. By using AS-PCR and sequencing the patients were investigated for the M184I/V mutations up to six months after therapy initiation. Patients with quadruple therapy were also re-analyzed for the M184I/V mutations after ART cessation.

One patient developed the M184V mutation during the first phase of viral decay among the patients in the quadruple and triple ART groups. The

mutant proportion, consisting of 2.5%, was not detected until 6 weeks after start of therapy and disappeared in subsequent samples taken up to 16 weeks.

In the group with dual therapy, a higher proportion of patients (8/15) were found to develop resistance early compared to the other two groups (Table II). Resistant strains were selected after about 5 weeks of ART and was completed after 20 weeks (Figure 13).

The appearance of mutant populations was found not to be associated with a significant increase in plasma viral load. In two patients viremia levels was observed to decline even further despite a dominant mutant population present as >90%, however it did not prevent therapy failure in all patients at a later time point.

Patient/ sample	Sex	Age	Duration of treatment (weeks)	CD4+ T-cells/ μ l at baseline	HIV-1 RNA copies/ml at baseline	SPCRGTG reactivity	SPCRATA reactivity	RT resistance mutations by direct sequencing
1:1	M	42	Baseline	410	8,100	<d.l.	<d.l.	None
1:2			10	430	4,000	25.8 \pm 4.5%	<d.l.	None
2:1	M	40	Baseline	480	130,000	—	—	—
2:2			3	580	900	<d.l.	<d.l.	None
3:1	M	39	Baseline	220	62,000	<d.l.	0.3 \pm 0.0%	None
3:2			5	320	1,000	9.3 \pm 1.0%	<d.l.	None
3:3			16	330	2,000	35.0 \pm 0.7%	8.4 \pm 1.0%	M184V (GTG)
4:1	M	34	Baseline	120	1,800,000	—	—	—
4:2			2	310	9,000	<d.l.	<d.l.	None
5:1	M	56	Baseline	180	390,000	<d.l.	<d.l.	None
5:2			6	510	9,000	>90%	<d.l.	M184V (GTG)
5:3			28	320	26,000	>90%	<d.l.	M184V (GTG)
6:1	F	47	Baseline	380	72,000	—	—	—
6:2			5	530	400	<d.l.	<d.l.	None
7:1	M	36	Baseline	380	160,000	—	—	—
7:2			5	460	4,000	<d.l.	<d.l.	None
8:1	M	28	Baseline	200	12,000	—	—	—
8:2			2	290	23,000	<d.l.	<d.l.	None
9:1	F	26	Baseline	10	160,000	—	—	—
9:2			4	10	4,000	<d.l.	<d.l.	None
10:1	M	46	Baseline	370	71,000	—	—	—
10:2			5	530	700	<d.l.	<d.l.	None
11:1	M	64	Baseline	840	460,000	<d.l.	<d.l.	None
11:2			5	1,196	120,000	11.0 \pm 1.7%	<d.l.	None
12:1	F	36	Baseline	512	160,000	<d.l.	<d.l.	None
12:2			4	569	16,000	<d.l.	<d.l.	None
12:3			22	529	2,000	>90%	<d.l.	M184V (GTG)
13:1	M	39	Baseline	205	46,000	<d.l.	<d.l.	None
13:2			8	400	8,300	12.1 \pm 1.3%	<d.l.	None
13:3			14	339	1,000	>90%	<d.l.	M184V (GTG)
14:1	F	32	Baseline	479	17,000	<d.l.	<d.l.	None
14:2			5	665	5,000	24.2 \pm 0.7%	<d.l.	M184V (RTG)
14:3			20	580	12,000	>90%	<d.l.	M184V (GTG)
15:1	M	37	Baseline	282	180,000	<d.l.	<d.l.	None
15:2			14	389	13,000	37.1 \pm 4.6%	<d.l.	M184V (RTG)
15:3			21	Unknown	12,000	>90%	<d.l.	M184V (GTG)

—, Not performed; <d.l., below detection limit of minor variants; RTG/GTG, bases on the M184 site as determined by sequencing, R corresponds to A or G.

Table II. Patients initiated on dual treatment containing zidovudine and lamivudine.

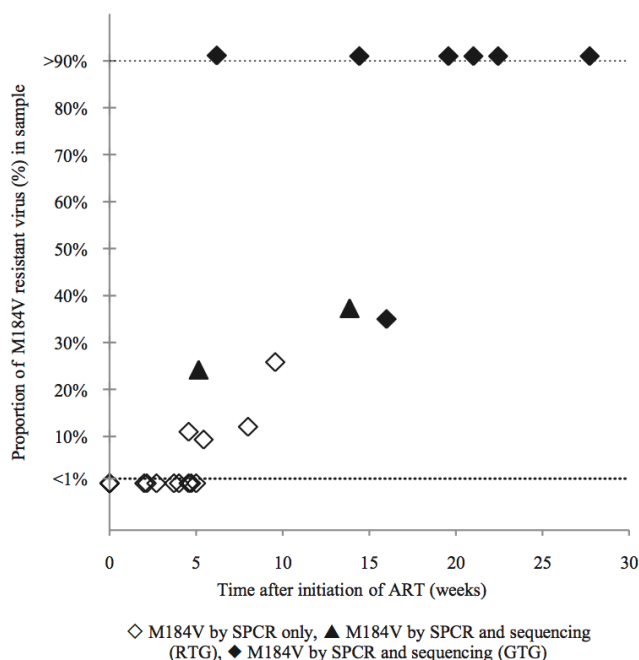


Figure 13. Proportion of M184Vmutants in samples from patients during the first 30 weeks of dual drug treatment. ART, antiretroviral therapy; RTG/GTG, bases on the M184 site as determined by sequencing.

Two patients were found to harbor M184I as minor population at baseline. In the first patient belonging to the quadruple therapy group, the mutant population was estimated to 0.5% and was not selected during the first 6 months of therapy. However, after cessation of therapy it re-appeared again at about 1% of the total population. In the other patient included in the dual therapy group, the mutation was selected in samples taken 5 and 16 weeks after therapy start as 0.3 and 8.4% of the total population, respectively.

Compared to previous findings of the early selection of resistance in minor populations in treatment-naïve patients initiated on triple ART, in whom minority viral variants were able to replicate within the first months of suppressive therapy despite viral decline (156), none of the 57 patients on

triple or quadruple therapy in this study had detectable viremia after 16 weeks of treatment and only one patient was found to have resistance in the minor population.

M184I/V mutants were selected after cessation of therapy in two patients included in the quadruple therapy group. In one patient M184V was detected in the major population consisting of >90% only one week after therapy cessation and in the other patient M184I was detected as 1.0% 8 weeks after therapy cessation.

8.2 DIFFERENCE IN DRUG RESISTANCE PATTERNS BETWEEN MINOR HIV-1 POPULATIONS IN CEREBROSPINAL FLUID AND PLASMA (PAPER II)

The CSF can constitute a viral reservoir for HIV-1, in which the virus can continue to replicate despite suppression of blood viremia (157). The passage of some antiretroviral drugs is restricted through the blood-brain barrier (158), which in turn could facilitate the evolution of drug resistance in the CSF compartment (158-160). Resistant viral strains can also be exchanged between the two compartments (161) and lead to treatment failure. Earlier studies have reported differences in the occurrence of M184 mutations between the CSF and blood compartments (162-164), but only in the major populations. No study has investigated the pattern in minor populations below the detection limit of standard sequencing. Therefore, the aim of this study was to investigate to which extent unique resistance patterns appear in both minor and major populations in CSF and blood.

Forty-four plasma and CSF samples were collected from 13 multi-therapy experienced patients. All patients had developed virological failure during ART containing 3TC. Of these, seven patients provided longitudinal samples. Both plasma and CSF samples were analyzed by AS-PCR specifically for the M184I/V mutations and direct sequencing for the

presence of other drug resistance mutations in the RT-coding gene. All results are presented in Table III.

In one patient (patient 2) the M184V mutant was detected in the major populations in both compartments but declined in subsequent samples and was only detected as a small population (5.6%) in the plasma compartment only.

In seven patients (1:1, 2:1, 4:2, 6:1, 7:1, 8 and 13:1-4), differences in resistance at other RT positions between viruses from the two compartments were detected by direct sequencing. In four of these patients, a major drug resistance mutation was detected in the CSF but not in the plasma while in the remaining three patients it was detected in the plasma but not in the CSF. All mutations were however observed to appear or disappear from the compartments in all patients with longitudinal samples.

Another patient (patient 4) was found to harbor several thymidine-associated mutations (TAMs) and the L74V mutation in both compartments. Five months after therapy switch to a non-ddI containing regimen all TAMs persisted in both compartments while the L74V only persisted in the CSF compartment. In patient 8, the NNRTI mutation K70R was detected in the CSF only.

For patient 13, the differences between the viruses in both compartments were found to be more diverse. The patient had been exposed to several treatment regimens over the years but with adherence problems, which contributed to high HIV RNA values and low CD4 T-cell counts. In addition, the patient also had treatment interruption between sample points 2 and 3. The M184V mutation was not initially detected in the first paired sample for this patient by either AS-PCR or direct sequencing but was detected in the second sample as dominating population in the plasma and as a smaller population in the CSF. In subsequent samples M184V mutant persisted in the dominant population in the plasma compartment but

disappeared from the CSF. Direct sequencing showed also the presence of NRTI (T215S) and NNRTI (K103N) mutations in the first paired plasma sample but not in the CSF. In subsequent samples for the patient additional NRTI and NNRTI mutations were detected in plasma, of which most did not appear in the CSF compartment until the third sample in a combination with wild type amino acids. As a result of regimen change between the third and fourth samples all mutations, with the exception of K70E, reverted to wild type in the CSF but not in the plasma.

Patient/ sample	Treatment at sampling	Duration of treatment	CD4 count (cells/ μ L)	Plasma				CSF			
				HIV RNA (copies/mL)	SPCRGTG (mean \pm SD)	SPCRATA (mean \pm SD)	RT resistance mutations by direct sequencing	HIV RNA (copies/mL)	SPCRGTG (mean \pm SD)	SPCRATA (mean \pm SD)	RT resistance mutations by direct sequencing
1:1	d4T/3TC	11 months	262	32 316	>90%	<dl	M41L, D67N, K70R, M184V (GTG), T215F, K219Q	888	>90%	<dl	M41L, D67N, K70R, M184V (GTG), T215F, K219H
1:2	No change	17 months	247	47149	>90%	<dl	M41L, D67N, M184V (GTG), T215F, K219Q	3558	>90%	<dl	M41L, D67N, M184V (GTG), T215F, K219Q
2:1	ZDV/3TC	17 months	216	3665	>90%	<dl	M184V (GTG)	53	>90%	<dl	V118I, M184V (GTG)
2:2	No change*	43 months	230	39 200	5.6 \pm 0.5%	<dl	None	14 900	<dl	<dl	None
3	ZDV/3TC*	7 months	112	583	<dl	<dl	None	4113	<dl	<dl	None
4:1	ddl/3TC/loviride [†]	12 months	54	32 172	>90%	<dl	M41L, L74V, A98G, M184V (GTG), L210W, T215Y	668	74.3 \pm 0.9%	<dl	M41L, L74V, A98G, M184V (GTG), L210W, T215Y
4:2	d4T/3TC/IDV	17 months	117	23 063	>90%	<dl	M41L, A98G, M184V (GTG), L210W, T215Y	1046	>90%	<dl	M41L, L74V, A98G, M184V (GTG), L210W, T215Y
5	d4T/3TC/ddC*	1 week	140	12 700	<dl	<dl	None	6250	<dl	<dl	None
6:1	d4T/ddl/3TC/SQV	12 months	193	22 364	>90%	<dl	D67N, T69N, K70R, A98G, M184V (GTG), T215F, K219Q	278	>90%	<dl	D67N, T69N, K70R, A98G, V118I, M184V (GTG), T215F, K219Q
6:2	No change	21 months	136	54 137	>90%	0.3 \pm 0.0%	D67N, T69N, K70R, A98G, M184V (GTG), T215F, K219Q	1838	>90%	<dl	D67N, T69N, K70R, A98G, M184V (GTG), T215F, K219Q
7:1	ZDV/ddl/3TC/loviride [‡]	11 months	25	8659	<dl	<dl	M41L, E44D, D67N, V106A, V118I, L210W, T215Y	10 280	<dl	<dl	M41L, D67N, T215Y
7:2	d4T/ddC/IDV	7 months	80	44 277	84.4 \pm 4.2%	18.7 \pm 3.7%	M41L, E44D, D67N, V118I, M184V (GTG), L210W, T215Y	7868	>90%	<dl	M41L, E44D, D67N, V118I, M184V (GTG), L210W, T215Y
8	ZDV/3TC	51 months	210	24 100	71.6 \pm 10.1%	0.7 \pm 0.2%	M184V (GTG)	7240	56.2 \pm 16.6%	<dl	K70KR, M184V (GTG)
9:1	d4T/3TC	17 months	420	76 720	>90%	2.2 \pm 0.5%	M184V (GTG)	792	>90%	<dl	M184V (GTG)
9:2	No change	28 months	261	85 900	>90%	1.1 \pm 0.4%	M184V (GTG)	921	>90%	<dl	M184V (GTG)
10	d4T/3TC/SQV/NFV	21 months	325	89 000	65.7 \pm 8.2%	>90%	M41L, E44D, V118I, M184V (GTA), L210W, T215Y	24 500	72.6 \pm 6.3%	>90%	M41L, E44D, V118I, M184V (GTA), L210W, T215Y
11	d4T/3TC/NFV	3 months	270	43 300	>90%	1.1 \pm 0.5%	A62V, D67N, K70R, V75I, F77L, F116Y, Q151M, M184V (GTG), K219Q	5860	>90%	<dl	A62V, D67N, K70R, V75I, F77L, F116Y, Q151M, M184V (GTG), K219Q
12	ZDV/3TC/LPV/r	3 months	53	24 800	<dl	<dl	None	27 900	<dl	<dl	None
13:1	TDF/3TC/EFV*	3 days	20	2 030 000	<dl	<dl	K103KN, T215S	151 000	<dl	<dl	None
13:2	No change*	3 months	30	5 460 000	>90%	0.6 \pm 0.1%	D67N, K70E, K101E, Y181C, M184V (GTG), G190S, T215S	4930	29.9 \pm 2.0%	0.7 \pm 0.1%	Y181YC, M184MV (RTG), G190GR
13:3	No change*	3 weeks [§]	30	4 780 000	>90%	0.3 \pm 0.0%	D67N, K70E, K101E, Y181C, M184V (GTG), G190S, T215S	2060	5.5 \pm 0.8%	<dl	D67DN, K70KE, K101KE, Y181YC, M184MV (RTG), G190GS, T215TS
13:4	ZDV/3TC/ABC/LPV/r*	3 weeks	70	6810	>90%	<dl	D67N, K70E, K101E, Y181C, M184V (GTG), G190S, T215S	3220	<dl	<dl	K70KE

Table III AS-PCR and direct sequencing results for the blood and CSF compartments. Differences between paired samples are shown in bold. *Suspicion of low adherence.

[†]Treatment included the investigational non-nucleoside reverse transcriptase inhibitor loviride. [‡]3TC and loviride were introduced 5 weeks before the sample date. [§]Preceded by 4-week treatment interruption. ABC, abacavir; CSF, cerebrospinal fluid; ddC, zalcitabine; ddl, didanosine; <dl, below detection limit of minor variants; d4T, stavudine; EFV, efavirenz; IDV, indinavir; LPV/r, lopinavir + ritonavir; NFV, nelfinavir; RT, reverse transcriptase; SD, standard deviation; SQV, saquinavir; 3TC, lamivudine; TDF, tenofovir; ZDV, zidovudine.

8.3 MINORITY DRUG RESISTANT HIV-1 VARIANTS IN TREATMENT NAÏVE ETHIOPIAN, EAST AFRICAN AND CAUCASIAN PATIENTS DETECTED BY ALLELE-SPECIFIC REAL-TIME PCR (PAPER III)

Transmission of drug-resistant virus has begun to accumulate in resource-limited settings (11, 16, 165-168). In WHO's report on drug resistance this increase was found to be associated with the wider availability of ART in these settings (11). According to the report the drug resistance mutations at the positions K103 and Y181 of the RT-genome were more commonly observed. In the absence of adequate monitoring interventions this prevailing outcome may compromise available treatment strategies in the future. Lately, a slowly increasing trend among reported new HIV infections has been observed in Sweden (47). The majority of the cases were related to immigrants infected prior arrival to Sweden from high-endemic countries as Eritrea and Ethiopia. The methods for detecting drug resistance mutations in surveys are based on standard genotypic testing, which is not able to detect drug-resistant viral populations in the minor population. For this purpose we aimed at studying the prevalence of the two NNRTI key mutations, K103N and Y181C, in the minor population by designing sensitive AS-PCR assays.

Three different patient groups consisting of 92 treatment-naïve Ethiopian patients living in Ethiopia, 55 East African migrant patients and 44 Caucasian patients residing in Sweden were included in the study.

Each mutant-specific primer was tested for specificity and cross-reactivity by using wild type plasmids as a template. No cross-reactivity was found as determined by differences in Ct values between the wild type and mutant plasmid DNAs. The amplification efficiency of mutant and wild type standard controls with their corresponding mutant and non-specific primers demonstrated a high correlation ($r^2 > 0.99$) and were run in parallel with each sample (Figure 14).

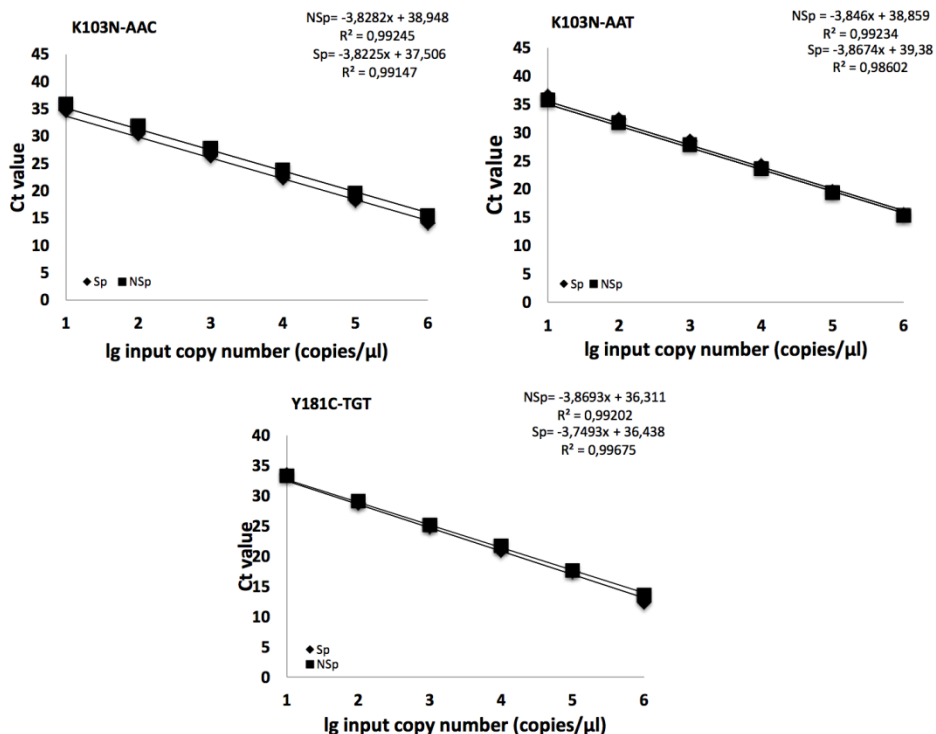


Figure 14. Allele-specific real-time PCR standard curves. Mutant specific (Sp) and non-specific (NSp) standard curves of K013N AAC allele, K103N AAT allele and Y181C TGT allele. These standard curves were run in duplicate, parallel with each sample and used to determine the copy number of each mutant specific and total population of sequence amplifications of clinical samples. By comparing the samples Ct values with those of the specific and non-specific standard curves derived from the standard plasmid controls using the corresponding primers, the quantity of the patients' mutant specific and the total population of sequences (amplified with non-specific primer) was determined. By dividing the quantity of mutant specific sequence by the quantity of the total sequences and multiplying by 100 the percentage of mutant specific sequences was obtained for each sample. Positive samples were repeated at least twice. Correlation coefficients (r^2) were higher than 0.99 for respective standard curve. Sp: mutant specific amplification. NSp: non-specific amplification (amplify the total population of sequences).

The specificity and selectivity of the AS-PCR assays was determined by mixing mutant plasmid DNA controls ranging from, 0.01% to 100%, with wild type plasmid DNA control. Amplification of the mixtures with mutant specific

primers in the background of wild type sequence allowed the detection of mutants (AAC, AAT and TGT) down to 0.01%, which was also confirmed by melting curve analysis (Figure 15). The cut-off for K103N (AAC and AAT) was set to 0.1% and for Y181C (TGT) 0.25% from replicates of two independent experiments.

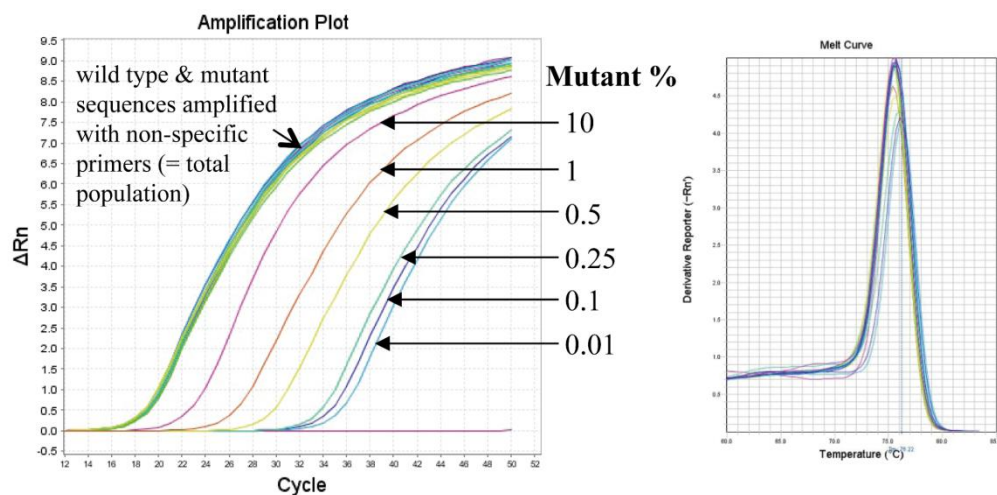


Figure 15. (A) Example of allele-specific real-time PCR amplification curves of cloned wild type and mutant TGT plasmid DNAs at different frequencies (raw data). The specificity and accuracy of the AS-PCR assay was determined by analyzing mixtures of mutant and wild type DNA standards ranging from 0.01 to 100% amplified with mutant specific primers in the background of wild type sequence. Amplification of the total population results always in the same C_t values, regardless of the amount of mutant DNAs present in the reaction. (B) Melting curve analysis. WT, wild type.

Of 92 treatment-naïve Ethiopian patients living in Ethiopia AS-PCR detected six individuals harboring the major NNRTI mutation Y181C (6.5%) (Table IV). The proportions of Y181C mutants that were detected ranged from 0.25-4.5%. None of the patients harbored the K103N mutation. Because of the low number of patients with DRM at baseline, it was not possible to study any impact on the outcome of ART in this patient group. However, two patients with the highest mutant proportions developed treatment failure and died within three months after initiation of therapy. Direct sequencing was performed to investigate the

presence of other mutations in the *pol* gene. Two patients were found to have additional DRM (NRTI: L100IL and PI: M46L) (Table IV).

AS-PCR detected NNRTI mutations in 2 individuals (3.6%) among the East African patient group who had migrated to Sweden (Table IV). One was a female with the Y181C mutation in the proportion 0.8% and the other was a male with the K103N mutation corresponding to 17.5% which was also detected by direct sequencing. Using direct sequencing, additional drug resistance mutations were detected in four patients (Table IV). Among these two harbored the NRTI mutation M184V, two had NNRTI mutations (1 K101E, 1 Y188L) and one patient had the PI mutation N88S.

In the Caucasian patient group living in Sweden AS-PCR detected the Y181C mutation in two male individuals (4.5%). The proportions of the Y181C mutants were 3% and 10.3%, respectively (Table IV). None of the patients in this group harbored the K103N mutation. Direct sequencing revealed the presence of additional DRM in two patients, one case with T215S and one case with L90M.

							AS-PCR		Direct sequencing	
ID	Gender	Subtype	Year	Origin	CD4	VL log	Y181C (%)	K103N (%)	RT-region	PI-region
East Africans living in Sweden										
1	M	C	2003	Ethiopia	190	4.02			M184V	
2	M	C	2003	Eritrea	270	5.57			M184V	N88S
3	M	C	2005	Eritrea	281	5.15			K101E, Y188L	
8	M	C	2009	Eritrea	186	5.13		17.5	K103N	
10	F	B	2006	Tanzania	189	5.03	0.8			
Caucasians living in Sweden										
12	M	B	2011	Belarus	530	2.75	10.3		N.D ^a	
13	M	B	2008	Russia	14	5.22			V106I	
19	M	B	2009	Sweden	330	3.57	3.0			
20	M	B	2011	Sweden	230	5.29			T215S	
21	M	B	2011	Lebanon	960	3.87				L90M
Ethiopians living in Ethiopia										
24	F	C	2008-2009	Ethiopia	48	6	0.28		L100IL	
26	M	C	2008-2009	Ethiopia	184	5.80	4.46		N.D ^a	
27	F	C	2008-2009	Ethiopia	37	5.76	0.85			
29	F	C	2008-2009	Ethiopia	115	6	0.53		N.D ^a	
43	M	C	2008-2009	Ethiopia	77	5.50				M46L
44	F	C	2008-2009	Ethiopia	90	5.40	0.25			

^aND, not done

Table IV. Characteristics of East African, Caucasian and Ethiopian patients with drug resistance mutations.

8.4 COST-EFFICIENT HIV-1 DRUG RESISTANCE SURVEILLANCE USING TAGGED POOLED HIGH THROUGHPUT AMPLICON SEQUENCING: IMPLICATIONS FOR USE IN LOW- AND MIDDLE-INCOME COUNTRIES (PAPER IV)

DRMs is presently increasing among therapy-naïve HIV-1 infected individuals in LMICs, where standard genotypic testing is not a part of standard healthcare system due to its high cost. In addition, these assays are not sensitive enough to detect DRMs in the minor populations. New approaches with high throughput, like NGS, have become available. These methods have been found to reduce both cost and time in comparison to conventional methods (136) .

Therefore, the main objective of the study was to design a feasible and simple-to-use high throughput drug resistance protocol for the use in large scale surveillance in LMICs by using the MiSeq (Illumina) platform.

Ninety-six ART-naïve patients with different ethnic origin were included in the study. Of these 49 were Indian patients residing in India, 17 were East African immigrants and 25 were Caucasians residing in Sweden.

Subtype classification revealed 73% of the patients harboring HIV-1 C and the rest of the patients harboring HIV-1 B. Two separate clusters observed within the HIV-1 C cluster corresponded distinguishably to the East African and Indian patients (Figure 16).

Both GRT-PS and GRT-NGS detected DRMs in 6% (6/96) of the subjects, of which two had NRTI (T215S) and four had NNRTI (K103KN, K101E, M230L and Y181C) mutations respectively (Table V). Hence, GRT-NGS detected all mutations detected by GRT-PS. GRT-NGS detected additional DRMs in 7% of the subjects (7/96) with a conclusive prevalence of 13% (13/96) (Table VI). The prevalence of low abundance mutations ($< 20\%$) detected only by GRT-NGS were 19% (4/21) in East African individuals residing in Sweden, 6% (3/49) in Indian patients and 3.8% (1/26) in Caucasian patients residing in Sweden.

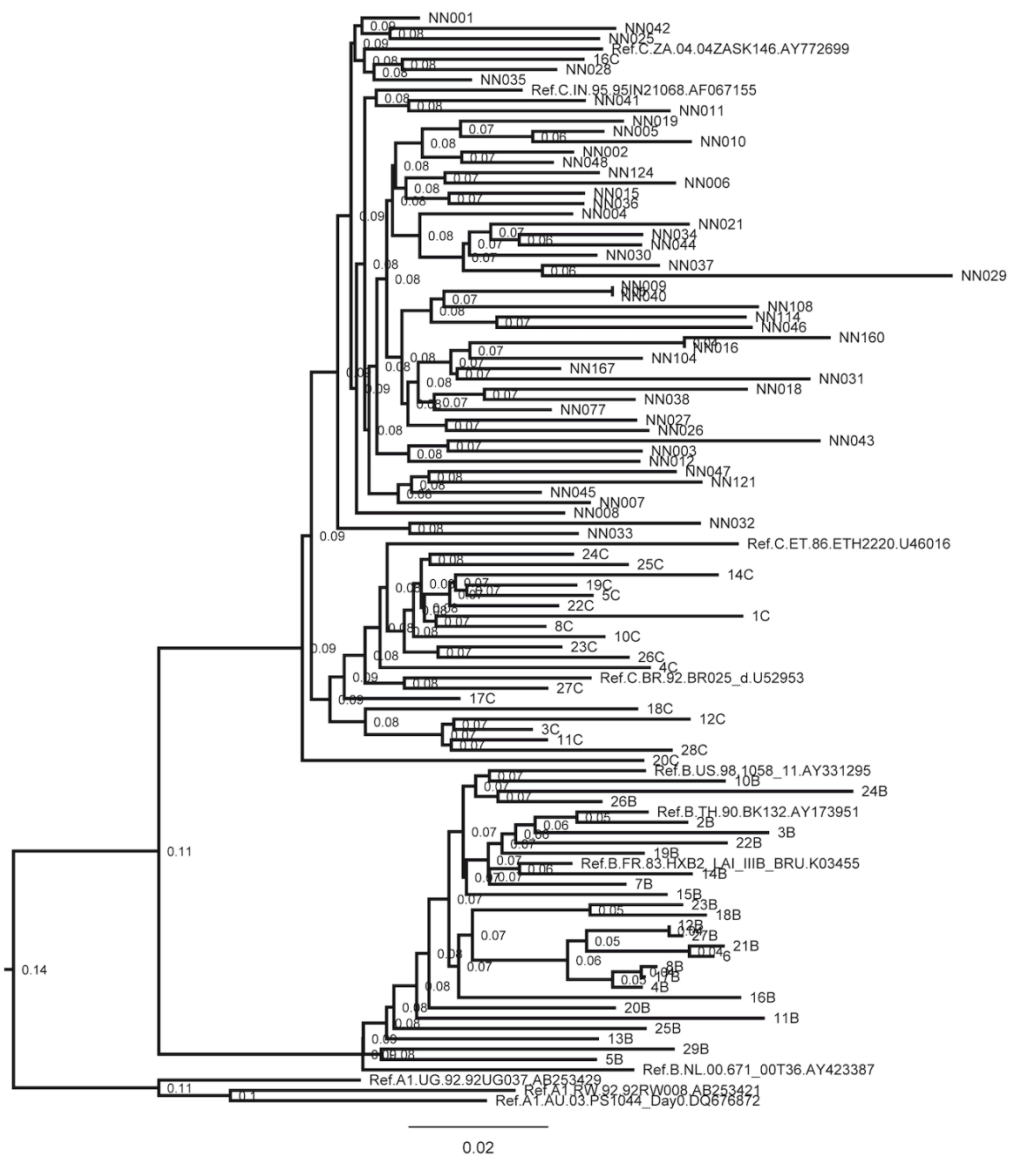


Figure 16 . Phylogenetic analysis. Two separate clusters were identified within the HIV-1 C cluster corresponding to the East African and Indian patients, respectively and one HIV-1 B cluster for the Caucasian group.

Subtype	Ethnicity	Year of sampling	Average Depth	GRT-NGS		GRT-PS
				DRM	Frequency	
C	East African	2008	56902X	Y181C	28.6%	No
				M184V	28.5%	No
C	East African	2009	7472X	K103N	14.2%	No
				M184V	14.1%	No
C	East African	2009	17374X	K103N	6.2%	No
C	East African	2012	23379X	K103N	4.2%	No
B	Caucasian	2009	12296X	Y181C	37.4%	No
				Y188C	22.2%	No
C	Indian	2013	146186X	K101E	17.1%	No
C	Indian	2010	15595X	K103N	13.9%	No
				Y181C	79.6%	Y181C
C	Indian	2011	18993X	K219Q	22.6%	No
B	Caucasian	2011	18941X	T215S	99.7%	T215S
C	East African	2013	22376X	M230L	99.8%	M230L
B	Caucasian	2006	359396X	K101E	98.9%	K101E
C	Indian	2011	184849X	K103N	68.7%	K103KN
C	Indian	2012	12846X	K103N	28.4%	K103KN

Table V. Primary drug resistance mutations (DRMs) detected by genotypic resistance testing, by next generation sequencing (GRT-NGS) and by population sequencing (GRT-PS).

9 DISCUSSION

I have studied drug resistance in minor HIV-1 populations in treatment-naïve and treatment-experienced patients in various clinical contexts. In the first part of this thesis, we investigated to which extent selection of the important M184I/V mutation occurred during the early phase of viral decay in ART-naïve patients initiated on lamivudine-containing combination therapy of various potency. The selection of the M184I/V mutations were frequently detected in patients receiving dual therapy but was a rare event in those treated with more than two drugs. This implicates that the use of a highly potent drug combination is sufficient to prevent the emergence of DRM in the first phase of viral decay in adherent patients. We also investigated to what extent specific resistance patterns emerge in the CSF compartment in comparison to blood in multi-therapy experienced patients with virological failure during lamivudine-containing treatment. The pattern of DRM suggested that drug resistance can develop differently in different compartments and that anatomic sites like CSF can constitute as a viral reservoir for resistant viral strains. Although I did not specifically analysed blood samples obtained from patients living in LMIC, the methodology is likely to be of clear relevance of such studies sine M184I/V is one of the most commonly occurring DRM in such settings.

In the latter part of the thesis we instead focused on the emergence of DRM in treatment-naïve patients originating from LMIC, where ART has rapidly expanded in contrast to monitoring interventions. We developed sensitive AS-PCR assays to study TDR in an African population and investigated the prevalence of K103N and Y181C in treatment-naïve Ethiopian patients living in Ethiopia and East African migrants in Sweden in comparison to Caucasians living in Sweden. We also developed a feasible high throughput NGS protocol that can be used for the detection of key mutations as K103N, Y181C and M184V as well as any other DRM in the *pol* gene, in the surveillance of drug resistance in LMIC. Using NGS, patients originating from East Africa and India were compared to Caucasian patients living in Sweden. Both AS-PCR and NGS detected clinically important DRMs in minor *quasispecies* in the patients

suggesting that conventional direct population sequencing assays can underestimate the prevalence of DRMs and thereby the extent of TDR in such settings. It shall also be noted that the included patients were infected by either HIV-1B or HIV-1C showing the feasibility to develop methods for different subtypes.

In this thesis drug resistance to only two drug classes, NRTI and NNRTI, were investigated because dual NRTI backbone together with one NNRTI regimen is the most recommended first-line therapy worldwide. In addition, in LMICs ART is being scaled up and the treatment of HIV-1 infected patients is only restricted to a few regimens where 3TC, EFV and NVP are key components of first-line therapy and other options are currently not available on a wider scale. In our studies of patients from LMICs we have focused on treatment-naïve patients since global trends of TDR in this category of patients have increased over time (121). In particular an increase of NNRTI drug-resistance has been observed in both LMIC and in Europe. The prevalence of TDR in Africa has increased from 2.8% (2001) to 4.7% and is mostly driven by NRTI and NNRTI drug resistance. In Europe, the prevalence of TDR has declined over time from 11.5% to 10.9%. This decrease was associated with a decline in NRTI drug resistance. In contrast, the prevalence of NNRTI resistance increased over time in Europe but a slight decline has been observed lately (121, 169). However, the methodologies in these reports comprise standard GRT which may underestimate the prevalence of TDR. Even though a slightly declining trend of TDR has been observed in Europe it can revert due to migration of people from high-endemic settings with increasing TDR.

The AS-PCR assays used in this thesis were designed and developed for the detection of only three major mutations; M184I/V, K103N and Y181C. These mutations are clinically important to assess since they confer high-level resistance to the most commonly used anti-HIV drugs presently and are therefore key mutations that can compromise the treatment management of a large number of HIV-1 infected individuals. Initially we focused on the M184I/V mutation in the beginning of this thesis because this mutation confers high-level resistance to

3TC, which is a preferred regimen in HIV therapy. Monotherapy with 3TC results in the rapid selection of M184I/V (170). This was also observed in 8 of 15 treatment-naïve patients initiated on dual therapy with 3TC in **Paper I**, in whom selection of drug-resistant strains carrying M184I/V occurred after approximately 5 weeks and completed after 20 weeks. In monotherapy with 3TC, the selection of M184I/V is associated with viral rebound (171). In contrast, the emergence of minor mutant strains in the dual-regimen group in our study was not associated with a significant increase in viral load, despite an increase in the proportion of mutants in five patients with serial samples. Subsequently all patients experienced treatment failure at a later time point most likely due to insufficient ART. The selection of M184I/V was in contrast rare in patients initiated on three or four drugs with 3TC. Only one patient developed this mutation during the first phase of viral decay. This patient had a minor M184V mutant after 6 weeks of ART and wild type during the following 10 weeks. This suggest that low-level viremia during the first phase of treatment initiation originates from proviral DNA in long lived infected cells or trapped virus in follicular dendritic cells (118). Our findings imply that ART consisting of three or four drugs is necessary to avoid the emergence of drug resistance in the early phase of viral decay after therapy initiation in adherent patients.

In **Paper II** we investigated the M184I/V mutation further by studying to what extent distinct resistance patterns occur in CSF compared to blood in both major and minor *quasispecies* as earlier studies using conventional assays have shown differences in the occurrence of the M184I/V mutation in both compartments (162-164). In our study differences in the pattern of resistance were also observed frequently in CSF and blood in both minor and major populations. Using AS-PCR, differences were observed mainly in the proportion of mutated virus. Some samples though had low viral load, which may have contributed to a decrease in sensitivity of the AS-PCR assay, and could be an explanation for some of the differences in the proportion of the M184I/V mutations that were observed. In twelve paired samples from eight patients, differences were found in both major and minor populations between plasma and CSF. Of these, eleven samples showed a mixed population with semi-quantitative differences in M184

mutants and wild type virus. Only one patient harboured a minor resistant M184V population using AS-PCR and wild type using sequencing in the plasma. Although, this patient had this mutation as detected in the major population in the previous samples in both compartments but declined during following time points, likely as a result of low adherence. However, in overall there was a good correlation between the results obtained by AS-PCR and sequencing. Differences in resistance between viruses in the two compartments were also observed in other RT positions in seven patients using sequencing. In four of the patients a major mutation was detected in the CSF but not in the plasma, while in the remaining three patients a major mutation was found in plasma but not in the CSF. In serial samples belonging to six of these patients, all mutations were found to appear or disappear from the compartments, suggesting that the differences most likely were related to the selective drug pressure than distinct evolutionary pathways. The differences in viral resistance pattern were even more frequently observed in the patient who had been exposed to several treatment regimens as a result of adherence problems. This patient, who provided longitudinal samples, developed NRTI and NNRTI mutations initially in the plasma compartment, and in subsequent samples they also appeared in the CSF compartment in combination with wild type amino acids. However, when treatment was changed almost all mutations reverted to wild type in the CSF but not in the plasma. The M184V mutation followed a similar pattern, from being absent in the first paired samples it occurred as a dominant population in the plasma and as minor population in the CSF in the subsequent samples. As treatment switched, the mutant remained as dominant population in the plasma but disappeared from the CSF. This suggests that the differences in drug resistance development and exchange between the CSF and blood compartments can occur more frequently than anticipated.

In addition to the M184I/V mutation we chose to study the K103N and Y181C in the latter part of this thesis because there is strong evidence of their accumulation in resource-limited settings currently. In WHO's drug resistance report these mutations were the most common mutations found in ART-naïve patients in LMICs (11). A global collaborative study to assess the prevalence of

HIV drug resistance in therapy-naïve patients in LMICs reported the K103N mutation in more than half of the HIV infected African patients with NNRTI resistance (168). In addition, the study also reported on the urgent need of feasible and sensitive assays for the detection of key mutations that could be implemented to monitor key DRMs at the population level in settings with inadequate resources. Standard GRT is not a feasible option in these settings, because it is complex due to extensive labor and as well as to costly to maintain regularly. We therefore designed a sensitive AS-PCR assay for the detection of K103N and Y181C in **Paper III**, and a cost-effective, easy-to-use high throughput NGS protocol for the detection of K103N, Y181C and M184V mutations that could be used to monitor key DRMs in **Paper IV**.

Our AS-PCR assay in **Paper III** was found to be highly sensitive and selective with the capacity of detecting HIV-1 mutants down to 0.01%. A conservative accuracy of 0.1% for K103N and 0.25% for Y181C was obtained by mixing mutants in the background of wild type. The assay procedure is simple and easy to conduct but the design of mutant specific primers and standard curves needed careful handling to avoid underestimation because of polymorphisms at the primer binding sites in the target sequences. The primers were designed for amplification of only HIV-1C and HIV-1B, however these subtypes are the most dominant globally, and the primers were able to successfully amplify sequences of East African and Caucasian patients respectively. Using AS-PCR, we investigated the prevalence of TDR in Ethiopians infected with HIV-1C living in Sweden, East Africans with HIV-1C living in Sweden and compared the findings with the results of Caucasian patients with HIV-1 B living in Sweden. In the Ethiopian group, AS-PCR detected the Y181C mutation in 6.5% (6/92) of the patients with mutant frequencies ranging from 0.25% to 4.5%. All patients were newly diagnosed and confirmed no prior use of ART as well as prophylaxis for the prevention of vertical transmission. Additional DRMs were found by direct sequencing in the RT- and PR-coding region. The AS-PCR detected a higher prevalence of DRM compared to earlier studies in Ethiopia (172, 173), in which the prevalence ranged from 0% to 2.2% using conventional methods. In comparison, our result suggests that transmission of NNRTI-resistant virus

occurs in Ethiopia and standard GRT underestimates the prevalence of drug resistance. DRMs were also found in East African immigrants and Caucasian patients living in Sweden. In overall AS-PCR showed a good concordance with results obtained by direct sequencing in these two groups, however NNRTI mutations were detected in the minor *quasispecies* of both patient groups using AS-PCR. The prevalence of these minor mutants were found to be at the same level as the prevalence of resistance to NNRTIs (3.4%) across Europe obtained by conventional GRT (121).

We developed a NGS protocol in **Paper IV**, which is an assay approach that has gained ground lately due to high throughput and time efficient properties. The implementation of routine GRT in the health-care system and surveillance of DRMs in high-endemic countries with poor recourses is still a challenge due to economical and infrastructure barriers. Conventional GRT assays are high-labor and less sensitive methods that further diminish the possibility to use them on a population level in LMICs. We therefore designed and evaluated a cost and labor efficient tagged-pooled NGS genotypic resistance testing approach for the detection of key DRMs. The NGS platform has mostly been available in high-resource settings and has not been a feasible option for the use in LMICs due to its expensiveness. Our NGS protocol reduced the cost of running by multiplexing of samples. The cost of preparation of samples in NGS does not exceed the cost with conventional assays using *in house* techniques or commercial kits and is in addition further reduced in both cost and in labor per sample by pooling samples. In our protocol we used the MiSeq (Illumina) platform for which a run yielded around \$750 to \$1000 and pooling of samples yielded between \$31 and \$42 per sample. A significant additional reduction in cost can be obtained in large scale multiplexing, which makes this approach suitable for handling a large number of samples at the same time for a reasonable cost and without affecting the quality of the data (174). The preparation of the first round PCR for the Swedish and Indian samples were performed differently but the NGS analysis conditions with primers and amplification were the same for all of them which makes our protocol easy to adopt and applicable in different protocol set-ups.

The method is very sensitive and therefore careful handling and precise preparation of samples is required to avoid highly variant read numbers as well as detection of very minute cross-contaminations, which are usually not detected by direct population sequencing. We were able to amplify and analyze three different patient populations with HIV-1 C and HIV-1 B which were confirmed by subtyping analysis showing distinct clusters of HIV-1 strains for respective patient group. Our assay was also found to be applicable to a broad range of HIV subtypes as the primers had > 97% sequence identity to all major pure and recombinant HIV-1 strains when aligned for nucleotide analysis in Los Alamos database. In comparison, the NGS assay was able to detect all DRMs detected by standard population sequencing. NGS identified also additional important low abundance DRMs (K101E, K103N, Y181C and M184V) that were undetected by standard sequencing. It is noteworthy that while DRMs, at frequencies >20%, in three out of four patients was detected by NGS they were undetected using standard population sequencing, which usually has a limit of detection >20%. However, an under-detection of DRMs using direct population sequencing has also been observed in comparison to deep sequencing in earlier studies (175, 176). We detected the clinically important DRMs K103N and M184V as low abundance (<20% of the viral population) in five patients. The proportion of low frequency DRMs among the East African patients was found to be higher compared to Caucasian and Indian patients. Our results are in line with global trends of primary DRMs. While the prevalence of primary DRMs is low in Sweden (169), a significant increase has been observed in India (177) and in sub-Saharan Africa, particular in East Africa (168). With respect to drug classes, the increase of DRMs to the NNRTI class has been mostly observed, were K103N and Y181C were the most common mutations occurring in these settings over time since the roll-out of ART.

We have detected DRMs in the minor *quasipsecies* of HIV-1 infected patients with different clinical backgrounds. We found that the emergence of drug-resistant variant carrying the M184I/V mutations can be prevented in the early phase of viral decay when potent ART is used. Further, drug resistance can develop differently in the blood and CSF, in which we found the M1814I/V in

different proportions most likely as result of different kinetics of the mutations rather than distinct evolutionary pathways. We also designed a sensitive AS-PCR assay specifically for the detection of two key NNRTI mutations K103N and Y181C to investigate the prevalence of TDR in Ethiopian patients living in Ethiopia and East African migrants residing in Sweden as compared to Caucasian patients living in Sweden. We found that the prevalence of TDR was higher in Ethiopian patients, suggesting that transmission of resistance strains occur in Ethiopia. In addition, we also developed a feasible easy-to-use high throughput NGS assay for detection of the key mutations K103N, Y181C and M1814V. Using NGS, East African migrants living in Sweden and Indian patients living in India was compared to Caucasian patients living in Sweden. The NGS detected a higher prevalence of low abundance NNRTI mutations in East African patients, which is in line with recent observations showing accumulating NNRTI resistance in these settings since the roll-out of ART.

Although, the clinical cut-off and relevance of low abundance DRMs for treatment outcome is yet not defined there is studies which have shown that patients harboring drug-resistant viruses with a frequency ranging from as low as 0.07% to 2.0% before therapy initiation has experienced treatment failure to first-line regimen containing 3TC and TFV in combination with EFV or NVP (178). In a systematic review and pooled analysis study, in which a strong association was observed between minority drug-resistant variants involving NNRTI resistance in particular and a dose-dependent increased risk of virological failure with first-line ART, found that this increased risk was also significant even at low minority frequencies presented as <0.5% (179).

The widespread use of ART globally is necessary and urgent, especially in low- and middle-income countries (LMIC) where the HIV pandemic has its epicenter. The recently observed gains in lives and the decrease in new infections is an important step towards the right direction in combating this devastating illness. Unfortunately, these breakthroughs can be challenged in the future due to unequally expanded monitoring strategies globally. We have in this thesis presented sensitive assays that can detect DRMs in the minor populations of

HIV-1 infected patients that are not identified with conventional GRT, which may compromise available regimens. Therefore, the question is not whether they exist or not and at what proportions, because as long as they emerge as a result of selective advantage under drug pressure they will most likely influence treatment outcome in HIV-1 infected patients independently if they pre-exist as *de novo* or due to transmission, the question is rather how we can optimize the management of ART globally. There is already evidence of virological failure within the first year in patients receiving first-line regimen in LMICs after the roll-out of ART (180) which are mostly driven by NNRTI and NRTI resistance (181, 182). As such, this poses a risk for the affected individual in settings with limited regimen alternatives as well as it is increasing the risk of transmission of clinically important DRMs in the absence of sufficient monitoring systems. In addition, infrequent monitoring of patients on ART has been associated with higher genotypic resistance to first-line regimens in these settings (181). The implementation of standard GRT is still a challenge for many high-burden countries, which influence the inadequate ART management. Therefore, the accesses of more feasible sensitive as well as easy to use techniques could simplify the monitoring DRMs and the guidance of treatment in HIV-1 infected patients in these settings.

10 CONCLUDING REMARKS

The main findings of this thesis were:

- Three sensitive AS-PCR assays were developed and found to detect point-mutations not identified by standard population direct sequencing which is the present routine method in clinical care.
- Using AS-PCR, selection of the important M184I/V mutations were found to be rare in the first phase of viral decay in patients with primary HIV infection or in patients with chronic infection initiated on potent three- or four-drug lamivudine-containing therapy.
- In contrast, the M184I/V were selected in the decay phase in patients given less potent dual therapy, lamivudine and zidovudine, showing that highly potent therapy is necessary to avoid the emergence of drug resistance already during this early phase.
- Distinct drug resistance patterns, in both minor and major HIV-1 populations, can be found in the blood and CSF during therapy failure, showing that the CSF compartment can act as a reservoir for drug resistant strains, not found in blood.
- Using AS-PCR, the clinically important K103N and Y181C mutations, can be found in minor viral populations of ART-naïve patients from low- and middle-income countries and from Sweden, not found by standard sequencing technique showing that transmission of drug-resistant strains occurs today.
- A cost-efficient high-throughput NGS method using multiplexed amplicons can detect DRM in minor quasispecies and is a potential suitable approach for the surveillance of DRMs in LMICs.

11 FUTURE CONSIDERATIONS

- The clinical significance and the clinical cut-off for minor DRMs are yet not defined and need to be established.
- Using sensitive and cost efficient NGS assays with high throughput using multiplexed amplicons allows analysis of a larger number of samples and will thereby contribute significantly to the studies of the presence of drug resistance.
- Implementation of such cost-effective and sensitive assays in the surveillance of DRMs should be considered in LMICs.

12 ACKNOWLEDGEMENTS

Looking back on the beginning of this tremendous experience as a PhD student brings back many memories...my first clinical course during my time as an undergraduate was at the routine Clinical Microbiology Division. The second week of the course I got the opportunity to observe Anne Quist who was screening blood samples obtained from children of HIV positive mothers. Luckily, none of the children during that particular analysis were found to have HIV. I remember that I was so amazed by this fact that the transmission of such an infectious illness could be prevented during such a critical time as labor. I was a rookie and didn't know so much and now I have spent years to learn more about this illness. Sitting next to Anne and listening to her while she was telling me about the success with the preventive work of vertical transmission filled me with hope and during the course I decided to perform my bachelor's degree project on HIV, which led me to *Group Sönnernborg*. Being a PhD student requires a lot of commitment and can be challenging at some times and in my case it became a double challenge when I became ill. The hardest part was that I had to interrupt my studies and for some time it was unclear if I was going to make my way back. Thanks to the enormous support and the dedication of you; my family, research group, and friends I made it back. For that I am forever in your debt.

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