Transmitted and Acquired HIV Drug Resistance in Viet Nam

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Abstract

The roll-out of antiretroviral therapy (ART) in Viet Nam along with limited resources for treatment monitoring are expected to be accompanied by the emergence of transmitted and acquired drug resistance. Drug resistance challenges the success of ART program and the efforts to curb the HIV epidemic in Viet Nam. Understanding factors that impact treatment outcome and prevalence and patterns of drug resistance provides imperative information for strategic and effective management. The first part of this thesis aims to study the prevalence and patterns of transmitted drug resistance (TDR) in ART-naïve patients. TDR prevalence was detected in 6.4% of ARV-naïve patients with HIV-associated tuberculous meningitis initiating ART in Ho Chi Minh City (HCMC) from 2005-2007. This rate is lower than that in developed countries and is comparable to TDR rates reported in similar resource-limited countries. Pattern of TDR reflected the standard first-line ART regimens with nucleotide and non-nucleotide reverse transcriptase inhibitors in Viet Nam. The second part of this thesis aims to investigate factors that impact treatment outcome and drug resistance in second-line ART in Viet Nam. In a cohort of adult patients on second-line ART at the Hospital for Tropical Diseases, rate of clinical and/or immunological failure was 18.2% after a median follow up of 29 months. Older age, history of injecting drug use, lower CD4 count at second-line ART initiation, suboptimal ART adherence, and previous protease inhibitor (PI) use independently

predicted treatment failure. Prevalence of virological failure (HIV RNA >1000 copies/mL as recommended by the 2013WHO guidelines) in patients who survived and were in active follow up was 9.5%, and high viral load, non-adherence and previous PI use were independent predictors for virological failure to second-line ART. 64% of patients with virological failure carried major PI mutations. Cross-resistance to third-line medications was higher than reported in other studies with cross resistance to ETR, TPV, and DRV of 55%, 45%, and 27% patients, respectively. This information informs selection of appropriate third-line ART regimen for patients failing second-line ART in Viet Nam. In conclusion, the work of this thesis provides important data on TDR in the chronically HIV-infected population in Viet Nam, provides, for the first time, data on treatment outcome to lopinavir-based second-line ART in the presence of extensive NRTI drug resistance, and identifies modifying risk factors to improve treatment outcomes in Viet Nam. Strategies to diagnose treatment failure accurately, to switch therapy timely, and to provide targeted adherence support will improve the outcomes of patients. Continued surveillance of TDR should be performed to assure the effectiveness of ART at the population level. Cost-effectiveness studies should be conducted in order to provide evidence for policy makers to decide whether to apply baseline genotypic testing and viral load monitoring in a resource limited country like Viet Nam. Prospective studies are needed to study the validity of WHO immunological/clinical criteria in defining virological treatment failure in PI-based second-line ART.

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Declaration

The work described in this thesis is my own work and was conducted under the supervision of Drs Sarah Dunstan and Thuy Le at the Oxford University Clinical Research Unit (OUCRU) – Vietnam.

Thesis result chapter 3: I was in charge of the data management, performed all the sequencing, sequence analysis, and the analysis of correlates of clinical outcomes.

Thesis result chapter 4: I designed the data collection form, enrolled all the patients, collected data from patients' clinic chart, generated the clinical databases and performed the data analysis.

Thesis result chapter 5: I enrolled and followed all patients, collected data from patients' clinic chart, generated the clinical databases, performed viral load measurement, sequencing, sequence analysis, and the analysis of resistance mutations and correlates of clinical outcome.

This thesis has not been submitted for a degree or other qualification to this or any other university.

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Abbreviations

3TC	Lamivudine
ABC	Abacavir
AIDS	Acquired immunodeficiency syndrome
ANRS	French National Agency for AIDS Research
APV	Amprenavir
ART	Antiretroviral therapy
ARV	Antiretroviral (drug)
ATV	Atazanavir
AZT	Zidovudine
PIr	Ritonavir-boosted protease inhibitor
CDC	U.S. Centers for Disease Control and Prevention
CI	Confidence interval
CRF	Circulating recombinant form
D4T	Stavudine
DDI	Didenosine
DNA	Deoxyribonucleic acid
DRM	Drug resistance mutation
DRV	Darunavir
dsDNA	Double stranded DNA
DTG	Dolutegravir
EFN	Enfuvirtide
EFV	Efavirine

EIA	Enzyme Immuno Assay
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
ETR	Etravirine
EVG	Elvitegravir
FDA	Food and Drug Administration
FPV	Fosamprenavir
FSW	Female sex worker
FTC	Emtricitabine
GSS	Genotypic sensitivity score
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B
HCMC	Ho Chi Minh City
HCV	Hepatitis C
HIV	Human immunodeficiency virus
HR	Hazard ratio
HTD	Hospital for Tropical Diseases
IAS-USA	International Antiviral Society–USA
IC50	Half maximal inhibitory concentration
IDU	Injecting drug user
IDV	Indinavir
IFA	Indirect Fluorescent Antibody
INI	Integrase inhibitor

IQR	Interquartile range
Kb	kilobases
LPV	Lopinavir
LTR	Long terminal repeat
MEGA	Molecular Evolutionary Genetics Analysis
MHC	Major histocompatibility complex
MOH	Ministry of Health
mRNA	Messenger RNA
MSM	Men who have sex with men
MVC	Maraviroc
NFV	Nelfinavir
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside(tide) reverse transcriptase inhibitor
NVP	Nevirapine
OR	Odds ratio
PCR	Polymerase chain reaction
PEPFAR	President's Emergency Plan for AIDS Response
PI	Protease inhibitor
PR	Protease
r	Ritonavir
RAL	Raltegravir
RNA	Ribonucleic acid
RPV	Rilpivirine

RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SDRM	Surveillance drug resistance mutation
SIV	Simian immunodeficiency virus
SQV	Saquinavir
TAM	Thymidine analogue mutation
TB	Tuberculosis
TBE	Tris-borate-EDTA
TBM	Tuberculosis meningitis
TDF	Tenofovir
TDR	Transmitted drug resistance
TPV	Tipranavir
UNAIDS	the Joint United Nations Programme on HIV/AIDS
VAAC	Viet Nam administration of HIV/AIDS control
VAS	Visual analogue scale
VCT	Voluntary Counseling and Testing
VL	HIV viral load
Vpr	Viral protein r
WHO	World Health Organization

List of Publications

Original publications contributing to D.Phil. thesis

Thao VP, Le T, Török EM, Yen NT, Chau TT, Jurriaans S, van Doorn HR, de Jong MD, Farrar JJ, Dunstan SJ. HIV-1 drug resistance in antiretroviral-naïve individuals with HIV-1-associated tuberculous meningitis initiating antiretroviral therapy in Vietnam. *Antiviral Therapy*. 2012;17(5):905-913. Erratum in: Antiviral Therapy. 2012;17(5):937. van Doorn, Rogier H [corrected to van Doorn, H Rogier].

Thao VP, Quang VM, Wolbers M, Anh ND, Shikuma C, Farrar JJ, Dunstan SJ, Vinh Chau NV, Day J, Guy T, Le T. Second-Line HIV Therapy Outcomes and Determminants of Mortality at the Largest HIV Referral Centre in Southern Vietnam. (submitted to Medicine HIV/AIDS: in revision).

Thao VP, Quang VM, Day J, Chinh NT, Shikuma C, Farrar JJ, Vinh Chau NV, Guy T, Dunstan SJ, Le T. High prevalence of protease inhibitor resistance in patients failing second-line antiretroviral therapy in Vietnam: risk factors, outcomes, cross-resistance and treatment implications for HIV-1 subtype CRF01_AE. (submitted to Journal of Antimicrobial Chemotherapy).

Other publication

Vi TT*, **Thao VP***, Thuy PT, Cuong DD, Hue NT, Donn C, Todd P, Le T. 2015. Prevalence and patterns of transmitted drug resistance in HIV-infected adult patients initiating antiretroviral therapy in Ha Noi, Vietnam. *Journal of Biotechnology in Viet Nam* [in press].

Conference abstracts and presentations contributing to D.Phil. thesis

Thao VP, Le T, Török EM, Yen NT, Chau TT, Jurriaans S, van Doorn RH, De Jong MD, Farrar JJ, Dunstan SJ. HIV-1 Drug Resistance in Antiretroviral-Naive Individuals with HIV-Associated Tuberculosis Meningitis in Ho Chi Minh City, Vietnam. Oral presentation. *18th Conference on Retroviruses and Opportunistic Infections*. 27 February – 2 March, Boston, MA, USA. Abstract 625.

Thao VP, Quang VM, Wolbers M, Anh ND, Chinh NT, Shikuma C, Day J, Farrar JJ, Dunstan SJ, Le T. Determinants of Treatment Failure in HIV-Infected Adults on 2nd-Line Antiretroviral Therapy in Ho Chi Minh City, Vietnam. Poster presentation. *20th Conference on Retroviruses and Opportunistic Infections*. 3 – 6 March, Atlanta, GA, USA. Abstract 1109.

1 INTRODUCTION

1.1 HIV Epidemiology

The HIV pandemic is one of the world's most serious health challenges. About 7000 people are infected with HIV per day, with 900 children aged below 15 years of age and 6000 adults. At the end of 2013 the estimated number of people living with HIV globally was 35.0 million (range 33.2 million–37.2 million), with 3.2 million (2.9 million – 3.5 million) children living with HIV. The number of people dying from Acquired Immunodeficiency Syndrome (AIDS)-related causes was 1.5 million (1.4 million–1.7 million) worldwide in 2013 (UNAIDS, 2014) and the HIV prevalence among adults was 0.8%. HIV infections have been reported in all regions of the world, yet about 97% are in low- and middle- income countries. Sub-Saharan Africa is the hardest hit region, with 24.7 million people living with HIV and HIV prevalence among adults at 4.9%. This region accounts for more than two-thirds (69%) of people living with HIV, 79% of new infections, and 70% of people dying from AIDS worldwide (UNAIDS, 2014). After sub-Saharan Africa, regions with the highest number of people living with HIV are South and South-east Asia (4 millions), Latin America (1.6 millions), Eastern Europe and Central Asia (1.1 millions), and North America (1.4 millions) (UNAIDS, 2014). The Caribbean with an adult HIV prevalence of 1.1% is the second hardest hit region after sub-Saharan Africa. South-east Asia has low overall HIV prevalence among the adult population at 0.3% and Thailand is the only country having a prevalence over 1% in the region (UNAIDS, 2014).

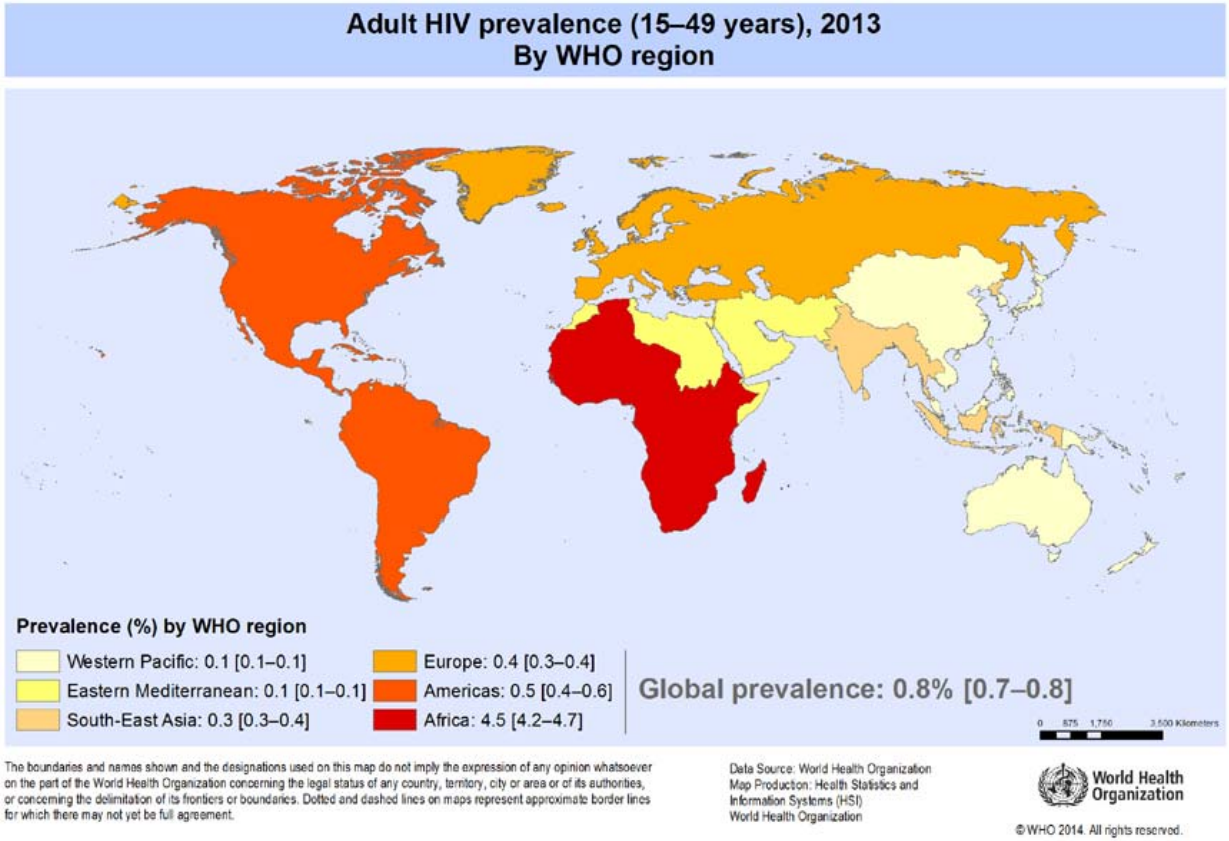


Figure 1.1: Global prevalence of HIV among adults, 2013.

Source: http://www.who.int

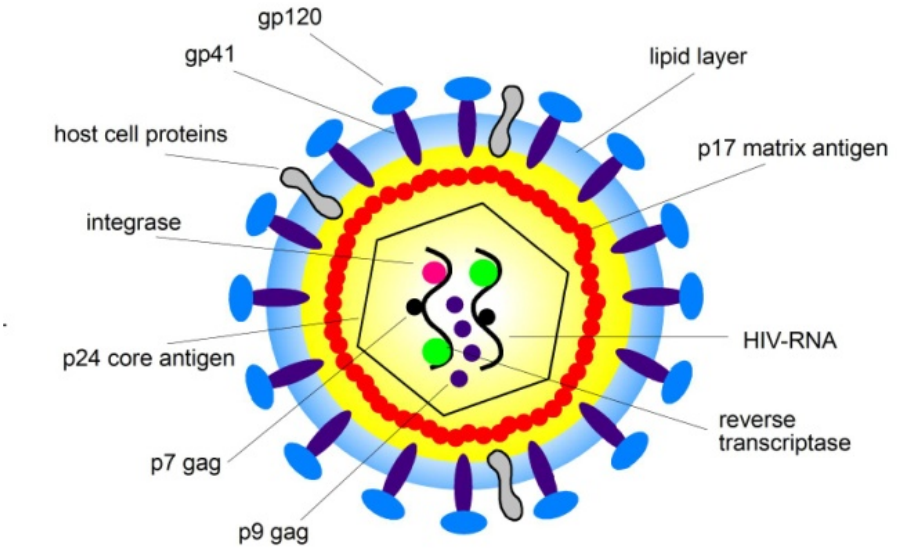
1.2 Human Immunodeficiency virus

Human immunodeficiency virus (HIV) belongs to a class known as retroviruses with single stranded RNA. Within the retrovirus family, HIV belongs to a subgroup known as lentivirus which indicates the long incubation period. There are two types of HIV, HIV-1 and HIV-2. Both HIVs are the result of multiple cross-species transmissions of simian immunodeficiency viruses (SIVs) naturally infecting African primates (Sharp & Hahn, 2011).

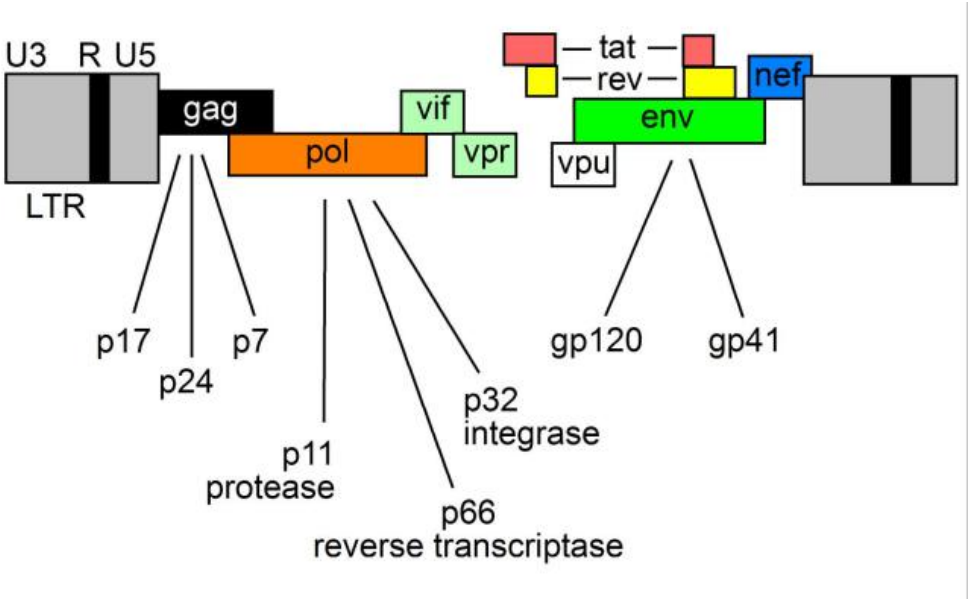
1.2.1 HIV Structure

HIV is a spherical retrovirus with a diameter of around 100-120nm (Figure 1.2a). It has an envelope which is a lipid bilayer derived from the infected human cell when a newly formed virus particle buds from the cell. Embedded from the viral envelope are host-cells proteins, such as the major histocompatibility complex (MHC) antigens and actins, and the viral protein env (Hoxie *et al.*, 1987; Tremblay *et al.*, 1998). Each env unit consists of trimeric trans-membrane glycoprotein gp41 attached to the surface glycoprotein gp120. These two viral proteins, encoded by the *env* gene of viral RNA genome, are responsible for attachment to the host cell. Beneath the envelope is the matrix protein (MA) p17 surrounding the cone-shaped capsid to ensure the integrity of the HIV virion. The capsid consists of approximately 250 hexamers and exactly 12 pentamers of the viral p24 (CA) protein (Pornillos *et al.*, 2009) . The structural proteins (p24, p7, p17) are encoded by the *gag* gene. Inside the capsid are two copies of the single stranded positive RNA genome of approximately 9.7 kilobases (kb) containing nine HIV

genes together with the viral protease, integrase and reverse transcriptase enzymes (Figure 1.2b). These enzymes are encoded by the *pol* gene. Each RNA strand is bound to a nucleocapsid protein p7 which protects the viral RNA from digestion by nucleases. Besides the three structural genes (*gag*, *pol*, and *env*) coding for structural proteins of the HIV virion, HIV has six regulatory genes (*tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*) coding for proteins that control the ability of HIV to infect a cell, produce new copies of virus, or cause disease. At the ends of each HIV RNA strand are long terminal repeats (LTRs) which control the production of new viruses.



(a)



(b)

Figure 1.2: HIV structure

Structure of the (a) HIV virion, (b) HIV genome.

Source: http://www.hivbook.com/

1.2.2 HIV Life Cycle

The time required to complete a single HIV life cycle is approximately 1.5 days (Figure 1.3). HIV begins its life cycle when gp120 surface protein binds to a CD4 receptor on the surface of host cells (CD4+ T-cells, macrophages, microglia, and dendritic cells) (Dalgleish *et al.*, 1984). After the attachment, conformation of gp120 is changed and entry of HIV to host cells is facilitated by the binding of a different domain of gp120 to another cell surface molecule called a co-receptor (such as chemokine receptor CCR5 or CXCR4) (Berger *et al.*, 1999). Virus strains that use CXCR4 as a co-receptor usually emerge later during the HIV infection, and are often associated with progression to AIDS (Moore *et al.*, 2004). The binding of co-receptors leads to conformational changes in the envelope that expose the hydrophobic fusion domain on gp41 and the formation of six-helix bundles for viral and cellular membrane fusion. Membrane fusion allows the release of the nucleocapsid into the cytoplasm of the target cell (Esté & Telenti, 2007). However there is evidence supporting another mode of virus entry in which HIV enters the cell by endocytosis followed by membrane fusion in an endosome (Uchil & Mothes, 2009). Either way, after membrane fusion HIV releases its RNA strand into the host cell cytoplasm. The viral enzyme reverse transcriptase transcribes the viral RNA into a double stranded DNA (dsDNA). After that, the preintegration complex (including linear dsDNA, integrase, matrix protein, reverse transcriptase, viral protein r (vpr) and various host proteins) is transported into the nucleus (Abbas & Herbein, 2012). The integrase enzyme catalyzes the integration of the linear dsDNA into the host chromosome. The integrated state of the viral DNA is called the provirus. Proviral DNA is replicated as part of the normal cell genome and may

persist in this form for long periods. The provirus utilizes the cell's molecular machinery for viral protein synthesis. The provirus uses a host enzyme called RNA polymerase II to create full-length RNA products which can serve either as copies of the HIV genomic material, or as messenger RNA (mRNA).

The full-length mRNA is translated into viral Gag or Gag-Pol precusor proteins. Multisplicing of full-length mRNA gives rise to the regulatory proteins (Tat, Rev, Nef, Vpr, Vif) (Briant *et al.*, 2011). The singly spliced mRNA forms *env* mRNA. *Env* mRNA is translated into the Env precursor glycoprotein gp160 at the endoplasmic reticulum. Gp160 then undergoes oligomerization to form trimers. The gp160 trimers are further transported to the host Golgi apparatus where they are cleaved into their mature subunits gp120 and gp41 by cellular proteases, not viral proteases. The gp120 and gp41 complex is transported to the cellular membrane (Hallenberger *et al.*, 1992). Viral cores (containing two copies of viral RNA, Gag, and Gag-Pol precursors) assemble at viral budding sites where gp120 and gp41 have accumulated. The assembled virion is still immature and non-infectious (Briant *et al.*, 2011; Sundquist & Kräusslich, 2012). The virus then enters the maturation stage, which involves the processing of viral proteins. The maturation process occurs during or after budding of assembled virion with the action of viral protease. The protease domains of two Gag-Pol precursors dimerize and auto-cleave to release active protease enzymes, which are responsible for further cleavage of Gag-Pol and Gag precursors into functional core proteins and viral enzymes (Bukrinskaya, 2004). This is the crucial step for the formation of infectious HIV virions.

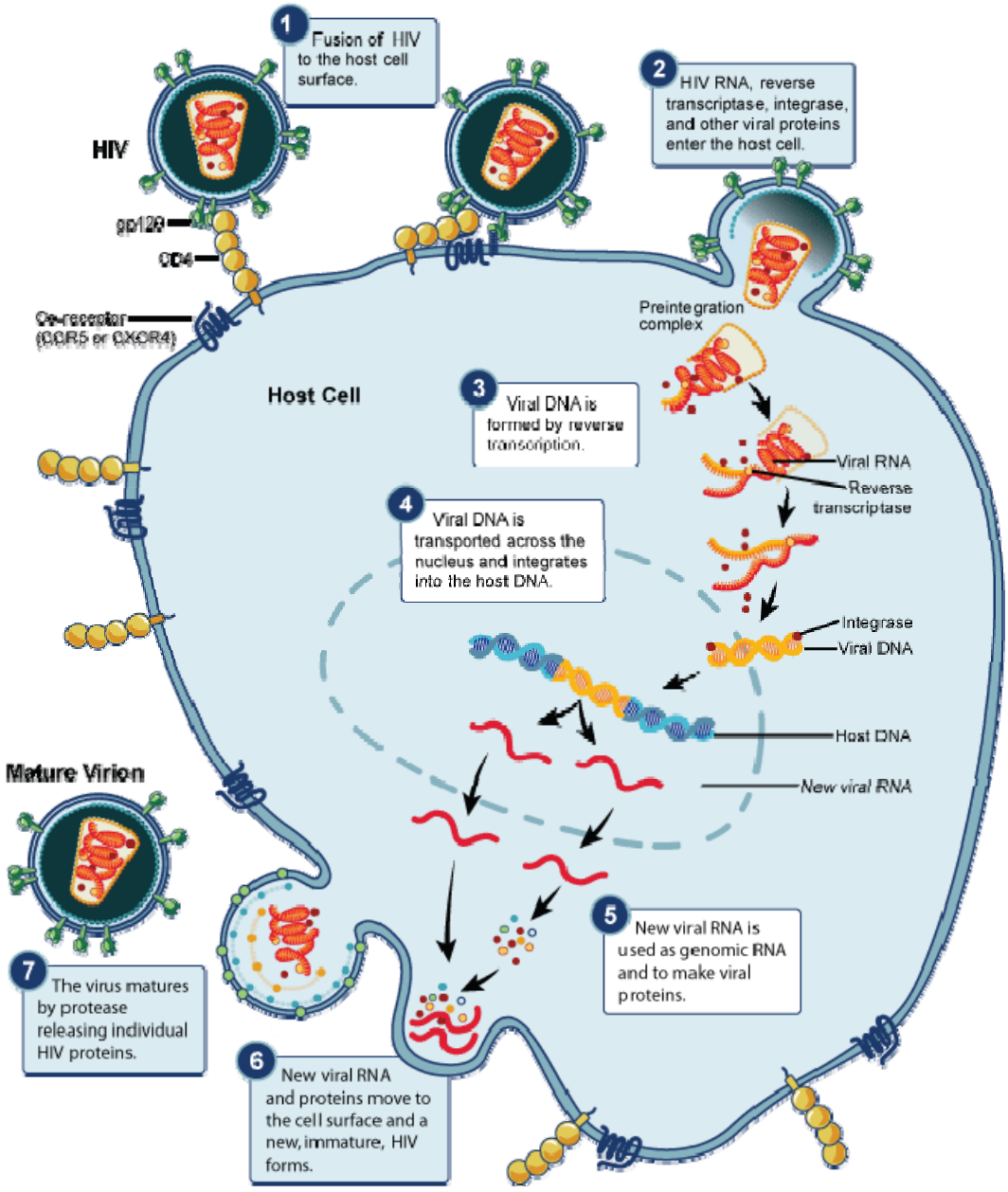


Figure 1.3: HIV life cycle.

Source: http://www.niaid.nih.gov/

1.2.3 Subtypes and diversity

The two major types HIV-1 and HIV-2 are phylogenetically distinct. HIV-2 is the result of a zoonotic infection from SIV in sooty mangabeys (*Cercocebus atys*) (Heeney *et al.*, 2006). It is believed that HIV-2 is a less virulent infection than HIV-2 with lower viral loads, higher CD4 cell counts, lower rates of vertical transmission, longer asymptomatic phase and slower progression to AIDS (Campbell-Yesufu & Gandhi, 2011). Presence of HIV-2 is confined to Western Central Africa and Southern and Western India (Berry *et al.*, 1998; Kumar, 1999) though HIV-2 infection has been reported in Europe, Australia and America (Downie *et al.*, 1992; Sullivan *et al.*, 1998; van der Ende *et al.*, 1996).

The source of HIV-1 is from the common chimpanzee (*Pan troglodytes*) (Gao *et al.*, 1999). Phylogenetic analysis classifies HIV-1 into three main genetically distinct groups or clades named M (Major group), O (Outliner group), and N (New or non-M, non-O). Group M accounts for most infections worldwide. Group O and group N infections are restricted to West-central African regions (Peeters *et al.*, 1997; Rambaut *et al.*, 2004; Yamaguchi *et al.*, 2006). In 2004 a new HIV group was discovered named group P (Pending the identification of further human cases) which is closely related to SIV found in wild gorillas (Plantier *et al.*, 2009).

Group M has been further divided into phylogenetically associated groups of HIV-1 sequences called subtypes (Robertson et al., 2000). The genetic variation within a subtype is around 15%-20% and variation between two subtypes is usually 25%-35% (Hemelaar et al., 2006). Detailed phylogenetic studies have established sub-subtype, which is a distinctive lineage that is very closely related to a particular subtype lineage,

but it is not genetically distant enough to justify calling it a new subtype (Robertson et al., 2000). Subtypes and sub-subtypes A1, A2, A3, A4, B, C, D, F1, F2, G, H, J, and K are currently recognized (Taylor et al., 2008). The recombination of different subtypes forms mosaic structures named circulating recombinant forms (CRF) (Robertson et al., 2000). Currently there are 55 different CRFs reported in the Los Alamos National Laboratory HIV database (http://www.hiv.lanl.gov). Molecular epidemiological studies show that Central Africa has greatest diversity with all subtypes and many CRFs detected and there is a specific geographic distribution pattern for HIV-1 subtypes (Hemelaar et al., 2011). The globally dominant subtype is subtype C accounting for almost 50% of all HIV-1 infections worldwide. Subtype C is predominant in Southern and Eastern Africa, and India. Subtype A is mainly found in Central and Eastern Africa, Eastern Europe and Central Asia. Subtype B is the main genetic form in Western and Central Europe, the Americas, and Australia (Buonaguro *et al.*, 2007; Hemelaar *et al.*, 2011). Subtype B is also common in several countries of South-east Asia, Northern Africa, and the Middle East and among South African and Russian homosexual men (Buonaguro et al., 2007), however it is hardly found in sub-Saharan Africa (Hemelaar et al., 2011). CRF02_AG, the fourth largest variant globally, is concentrated in Western Africa, with smaller numbers in Central Africa, the Middle East and Northern Africa. CRF01_AE, the fifth largest subtype, is found in South and South-east Asia, Eastern Asia, with a small number in Central Africa (Hemelaar et al., 2011).

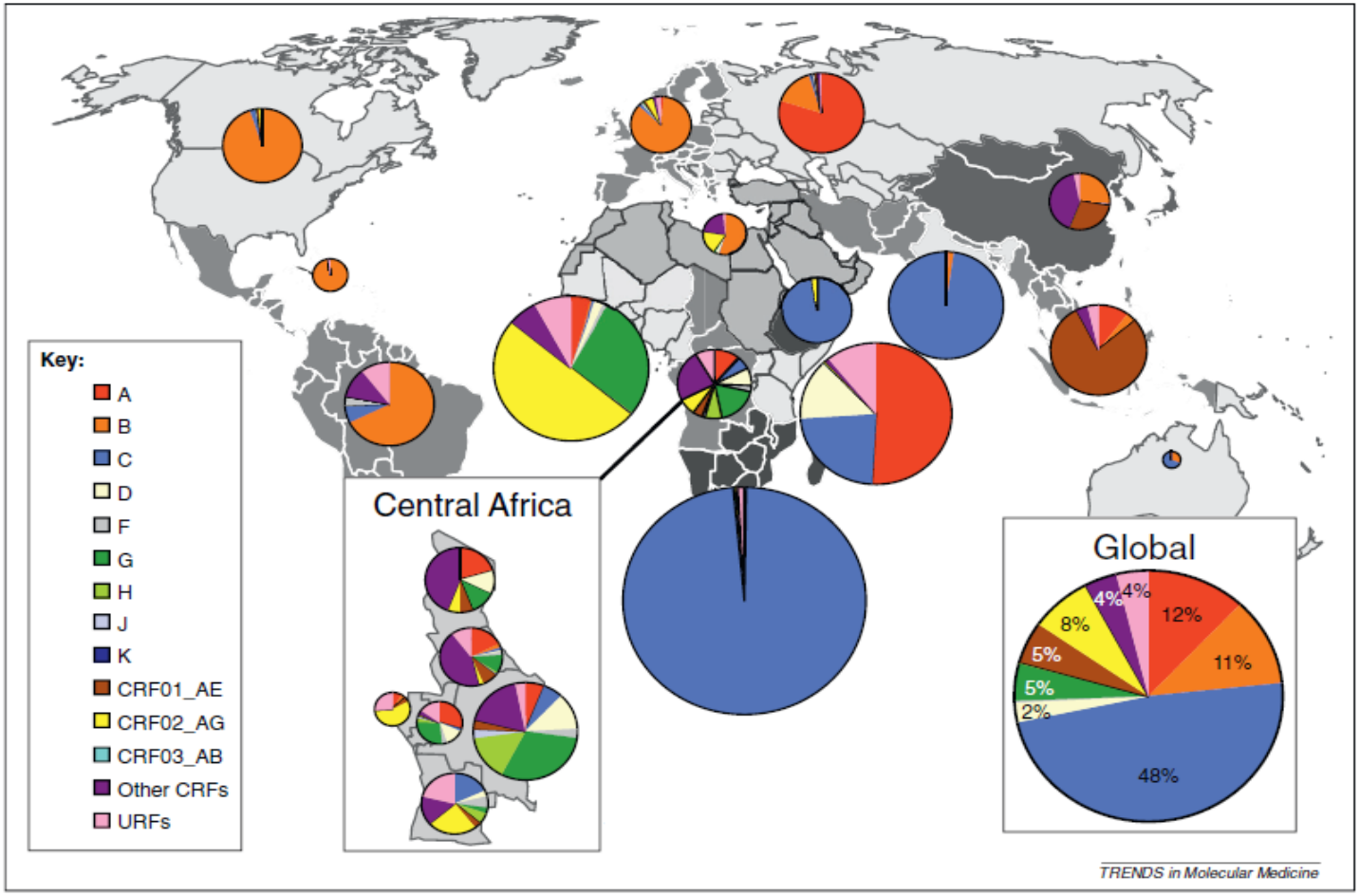


Figure 1.4: Global distribution of HIV-1 subtypes and recombinants

Source: (Hemelaar, 2012)

1.3 Course of HIV infection

1.3.1 Primary infection

The primary or acute infection starts when HIV enters the body. During this early phase, HIV infects a large amount of CD4+ T-cells and replicates rapidly in the absence of an immune response, reaching levels of plasma viremia as high as 100 million copies/mL (Altfeld & Walker, 2007; Fanales-Belasio *et al.*, 2010; Little *et al.*, 1999; Piatak *et al.*, 1993). HIV virus spreads throughout the body, disseminates into lymphoid organs such as the thymus, spleen, and lymph nodes. The peak of plasma viremia can be reached after three to four weeks of HIV infection (Fiebig *et al.*, 2003; Little *et al.*, 1999). The number of CD4+ T-cell count declines, occasionally to levels that allow opportunistic infections to develop (Gupta, 1993; Vento *et al.*, 1993). Over the following weeks after HIV infection the host generates humoral and cellular immune response that can partially control viral replication. The viremia declines by several orders of magnitude before reaching a lower steady level called the viral set-point by 6 months of infection (Kahn & Walker, 1998). CD4+ T-cell counts rebound but may not return to the normal level. At this stage antibody tests may be negative and diagnosis of primary HIV-1 infection is based on the detection of HIV-1 RNA or p24 antigen [section 1.4 Lab Diagnosis]. During this period up to 70% of HIV-infected people may suffer flu-like symptoms (Schwartz & Nair, 1999; Sudarshi *et al.*, 2008).

1.3.2 Clinical latency

At this stage HIV infected patients may remain free of HIV related symptoms yet are still able to transmit HIV to others. Without antiretroviral therapy (ART) the CD4+ T-cell count decreases at a rate of 50-90 cells/μL per year (Phillips, 1992). HIV replication is active in a relatively stable state of equilibrium with billions of virions produced and destroyed each day (Henrard *et al.*, 1995; Ho *et al.*, 1995). The level of HIV RNA has been shown to be correlated with the disease progression. A high plasma viral load is associated with rapid rates of CD4+ T-cell decline (Iuliano *et al.*, 1997; Mellors *et al.*, 1997) and rapid progression to AIDS and death (Mellors *et al.*, 1995; O'Brien *et al.*, 1996).

During this period antibody to the HIV virus is fully developed and diagnosis of HIV infection is based on antibody testing. Without treatment this stage can last up to 10 years or longer. HIV treatment can prolong this period up to 20 years or more (Knoll *et al.*, 2007; Lemp *et al.*, 1990; Moss & Bacchetti, 1989).

Around 5% of HIV infected patients named long-term non-progressors remain with high levels of CD4+ T-cells without HIV treatment for more than 5 years (Pantaleo *et al.*, 1996) whereas around 1 in 300 patients, named elite suppressors or elite controllers, maintain low or undetectable levels of HIV RNA without HIV treatment (Walker, 2007).

1.3.3 Clinical AIDS

At this stage the number of CD4+ T-cells dramatically decreases to low levels, generally to below 200 cells/μL. The immune system is badly damaged and the patient usually has one or more opportunistic infections. Without treatment, people who are diagnosed with AIDS typically survive from 6 to 19 months (Morgan *et al.*, 2002; Zwahlen & Egger, 2006).

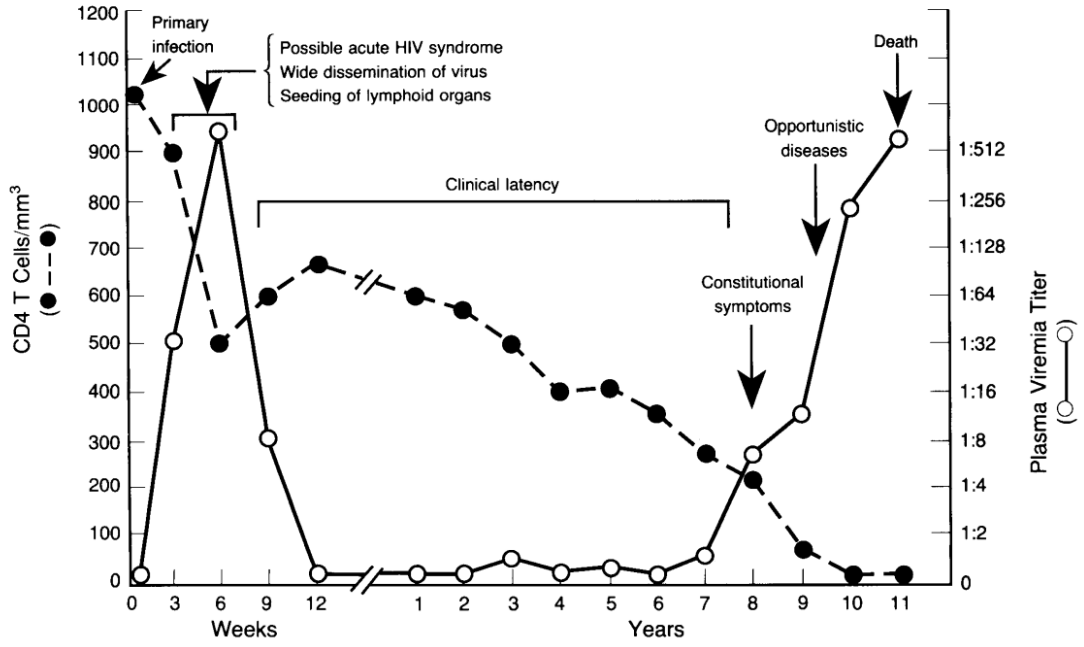


Figure 1.5: Typical course of HIV infection.

Source: Pantaleo *et al*. 1993

A clinical staging system for HIV infection was developed by World Health Organization (WHO) based on specific clinical conditions and symptoms. The system is

widely used in developing countries where sophisticated diagnostic techniques are not available. Clinical stages, as shown in table 1.1 are categorized as 1 through 4, progressing from primary HIV infection to advanced HIV/AIDS (WHO, 2007).

Table 1.1: WHO clinical staging of HIV/AIDS for adults and adolescents

Primary infection
Asymptomatic Acute retroviral syndrome
Clinical stage 1
Asymptomatic Persistent generalized lymphadenopathy
Clinical stage 2
Moderate unexplained weight loss (<10% of presumed or measured body weight)[a] Recurrent respiratory tract infections sinusitis, tonsillitis, otitis media and pharyngitis) Herpes zoster Angular cheilitis Recurrent oral ulceration Papular pruritic eruptions Seborrhoeic dermatitis Fungal nail infections

Clinical stage 3
Unexplained severe weight loss (>10% of presumed or measured body weight)
Unexplained chronic diarrhea for longer than one month
Unexplained persistent fever (above 37.6°C intermittent or constant,
for longer than one month)
Persistent oral candidiasis
Oral hairy leukoplakia
Pulmonary tuberculosis (current)
Severe bacterial infections (such as pneumonia, empyema, pyomyositis, bone or joint infection, meningitis or bacteraemia)
Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis
Unexplained anaemia (<8 g/dl), neutropaenia (<0.5 × 109 per litre)
or chronic thrombocytopaenia (<50 × 109 per litre)
Clinical stage 4[b]
HIV wasting syndrome
Pneumocystis pneumonia
Recurrent severe bacterial pneumonia
Chronic herpes simplex infection (orolabial, genital or anorectal of more than one month's duration or visceral at any site)
Oesophageal candidiasis (or candidiasis of trachea, bronchi or lungs)
Extrapulmonary tuberculosis
Kaposi's sarcoma
Cytomegalovirus infection (retinitis or infection of other organs)

Central nervous system toxoplasmosis
HIV encephalopathy
Extrapulmonary cryptococcosis including meningitis
Disseminated non-tuberculous mycobacterial infection
Progressive multifocal leukoencephalopathy
Chronic cryptosporidiosis (with diarrhea)
Chronic isosporiasis
Disseminated mycosis (coccidiomycosis or histoplasmosis)
Recurrent non-typhoidal Salmonella bacteraemia
Lymphoma (cerebral or B-cell non-Hodgkin) or other solid HIV-associated tumours
Invasive cervical carcinoma
Atypical disseminated leishmaniasis
Symptomatic HIV-associated nephropathy or symptomatic HIV-associated cardiomyopathy

[a.] Unexplained refers to where the condition is not explained by other causes.

[b.] Some additional specific conditions can also be included in regional classifications (such as reactivation of American trypanosomiasis [meningoencephalitis and/or myocarditis]) in the WHO Region of the Americas and disseminated penicilliosis in Asia).

1.4 Laboratory diagnosis

There are several tests used to diagnose HIV infection including testing for antibodies to viruses, and genetic materials of viruses. In general a highly sensitive test is used for screening and a highly specific test is used for confirmation (UNAIDS, 1997).

Enzyme Immuno Assay (EIA) or ***enzyme-linked immunosorbent assay (ELISA)*** detects the presence of antibodies to HIV viruses in serum, plasma, whole blood, dried blood spot, oral fluid and urine. First- and second-generation EIA detect IgG antibodies against HIV-1 and in most patients they can detect antibodies at 4 to 6 weeks after HIV infection , at about 3 to 4 weeks after infection (Branson, 2007). Newer generations, such as third-generation EIA assays can detect antibodies at about 3 to 4 weeks after infection. The third-generation EIAs use "antigen sandwich" techniques that can also detect IgM antibodies against HIV-1, which develop earlier after infection (Branson, 2007). The window period from HIV infection to HIV detection can be shortened by using an antigen test or nucleic acid detection method (Branson, 2007; Feinberg, 1996). The use of fourth-generation combination EIAs can identify HIV infection earlier after 16 to 18 days because they detect both HIV antibody and p24 antigen (Branson, 2007; Weber *et al.*, 1998).

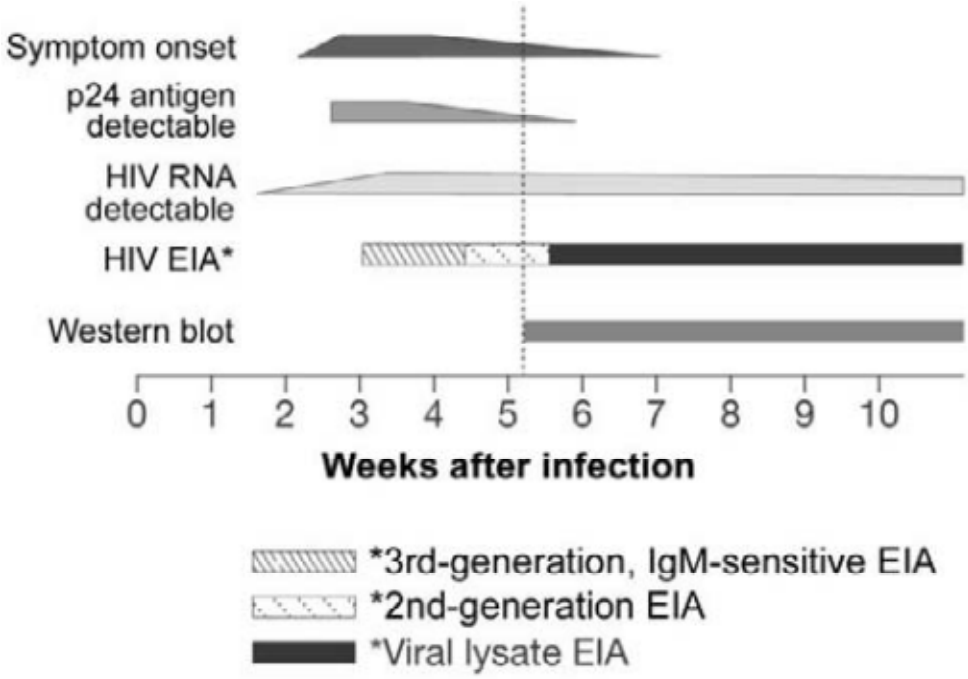


Figure 1.6: Time to HIV detection for various generations of diagnostic tests, relative to times of symptom onset and detection of p24 antigen and HIV RNA.

Source: Branson 2007

The ***Rapid test*** is an EIA test but can detect HIV antibodies much faster within 30 minutes (Branson, 2007). Rapid tests can be used for whole blood fingerstick, plasma, serum or oral fluid (saliva) specimens. However rapid tests that use oral fluid rather than blood are subjected to sampling variation, which can influence the sensitivity. In general, rapid test has a similar sensitivity and specificity as the conventional EIA test. EIA and the rapid test are very sensitive, designed to miss as few HIV infections as possible, but it may produce false positive results caused by the presence of antibodies to other diseases that the EIA mistakenly recognizes as antibodies to HIV. For this reason a confirmatory

test is required, which is less sensitive but more specific and yields a lower rate of false positive results. Western blot and Indirect Fluorescent Antibody (IFA) are used as a confirmatory test. Sometimes a rapid antibody test is confirmed with a second rapid test from a different manufacturer. If the confirmatory test result is negative or indeterminate, follow-up testing after 1 month is recommended. A negative result of rapid test and EIA is conclusive and requires no further follow-up test for confirmation (Branson, 2007; Greenwald *et al.*, 2006).

The ***Western blot*** is a solid-phase EIA with immobilized viral antigens to detect IgG antibodies to specific HIV proteins. A Western blot is interpreted as positive if bands appear at the site of two or more of the following HIV antigens: p24, gp41, or gp120/160 (U.S. Department of Health and Human Services, 2012).

Although antibody tests constitute the mainstay of HIV diagnosis, antibodies may be undetectable during the first 3–6 weeks after infection (Branson, 2007). ***RNA detection*** identifies early infection before seroconversion. Presence of RNA in the plasma of patients without antibodies to HIV-I is indicative of acute or primary HIV-1 infection. This test is not used as a stand-alone diagnostic test and should be followed by a conventional EIA to confirm the infection of HIV. In addition this test can be used as confirmatory test after positive screening results (U.S. Food and Drug Administration, 2009).

1.5 HIV treatment

1.5.1 History of HIV treatment

In 1987 the first FDA approved drug to treat HIV/AIDS was zidovudine (AZT), a nucleoside analogue (Yarchoan & Broder, 1987), which prevents HIV replication by inhibiting the activity of the reverse transcriptase enzyme. AZT was found associated with a greater survival at 24 weeks (Fischl *et al.*, 1987) but its benefits were short-lived and no longer observed by 48 weeks (Concorde Coordinating Committee, 1994; Cooper *et al.*, 1993; Fischl *et al.*, 1990, 1993; Lundgren *et al.*, 1994; Volberding *et al.*, 1990, 1995). A number of new nucleoside analogues were introduced during the next decade. The standard HIV treatment between 1986 and 1995 was "monotherapy" or treatment with a single drug. The effect of monotherapy against HIV was limited because HIV could quickly develop resistance to any single medication. In 1995 "dual therapy" with the combination of two nucleoside analogues was shown to be more effective than "monotherapy" (Delta Coordinating Committee *et al.*, 1996; Hammer *et al.*, 1996); however, none of the dual combinations, when administered without a third drug, could durably control HIV infection (Gulick *et al.*, 1997; Hammer *et al.*, 1996; Thiébaut *et al.*, 1998). The combination of several anti-HIV drugs was referred to as highly active antiretroviral therapy (HAART). The HAART era came about with the combination of the protease inhibitor based regimens (Kitchen *et al.*, 1995), and then the introduction of another drug class, non-nucleoside reverse transcriptase inhibitor (Montaner *et al.*, 1998; Staszewski *et al.*, 1999). This concept of three-drug therapy was quickly incorporated into clinical practice and rapidly showed impressive benefit with a decline in rates of

AIDS, death, and hospitalization (Egger *et al.*, 1997; Hogg *et al.*, 1997; Mocroft *et al.*, 1998, 2003; Palella *et al.*, 1998).

1.5.2 Antiretroviral drugs

Since the approval of AZT there are 25 drugs approved by the Food and Drug Administration (FDA) available for treatment of HIV-1 infections (De Clercq, 2009). There are six drug classes based on their molecular mechanism and resistance profiles.

1.5.2.1 Nucleotide (nucleoside) reverse transcriptase inhibitor

Nucleotide/nucleoside reverse transcriptase inhibitor (NRTI) is a deoxynucleotide/deoxynucleoside phosphate analogue, which lacks a free hydroxyl group at the 3' end. NRTI competes with endogenous deoxynucleotide (deoxynucleoside) and acts as a DNA-chain terminator to inhibit reverse transcription of the viral RNA genome into DNA (Sluis-Cremer *et al.*, 2000). It is administered as pro-drug and requires host cell entry and phosphorylation by cellular kinases for activation (Arts & Hazuda, 2012; De Clercq, 2010). Some nucleoside analogues include abacavir (ABC), AZT, didanosine (ddI), stavudine (d4T), and lamivudine (3TC), emtricitabine (FTC). Nucleotide analogue includes tenofovir (TDF). NRTIs are used as the backbone for ART regimens, and are given in pairs. There are three NRTI combinations available as coformulations, including TDF-FTC (Truvada), ZDV-3TC (Combivir) and ABC-3TC (Epzicom). Truvada and Epzicom are the preferred NRTI coformulations used in combination ART for treatment naïve patients (Günthard *et al.*, 2014; Panel on Antiretroviral Guidelines for Adults and

Adolescents, 2014; WHO, 2013a). Truvada in combination with a variety of different third agents was found to be effective, well tolerated, and generally superior to other NRTI combinations (Gallant *et al.*, 2006; Grant *et al.*, 2010; Sax *et al.*, 2009; Smith *et al.*, 2009). Combivir was used as a preferred choice when it was first introduced but is no longer in use because of twice-daily dosing, AZT side effects, and its inferior virologic potency compared with Truvada (Gallant *et al.*, 2006; Pozniak *et al.*, 2006).

1.5.2.2 Non-nucleoside reverse transcriptase inhibitor

Non-nucleoside reverse transcriptase inhibitor (NNRTI) blocks the reverse transcriptase enzyme by binding to the hydrophobic pocket close to the reverse transcriptase (RT) active site and altering the conformation or mobility of RT (Maga *et al.*, 2010), thereby blocking DNA synthesis. NNRTI is highly specific for HIV-1 RT and causes less adverse effects than NRTI. NNRTI drug class includes efavirenz (EFV), nevirapine (NVP), etravirine (ETR), and rilpivirine (RPV).

1.5.2.3 Protease inhibitor

Protease inhibitor (PI) competitively inhibits the action of viral protease by binding to the protease with high affinity in a non-cleavable structure (Richman, 2001; Wensing *et al.*, 2010). It prevents the proteolytic cleavage of HIV gag and gag-pol precursor proteins, resulting in the formation of immature, non-infectious virus particles. Currently there are 10 FDA-approved PIs: saquinavir (SQV), indinavir (IDV), nelfinavir (NFV), fosamprenavir (FPV), amprenavir (APV), lopinavir (LPV), atazanavir (ATV),

tipranavir (TPV), darunavir (DRV), and ritonavir (RTV) (Wensing *et al.*, 2010). Interaction between PIs and other drugs, and metabolism of PIs by CYP3A4, a liver enzyme involved in the metabolism of most protease inhibitors, can decrease the blood level of PIs (Kane, 2009). Ritonavir, in particular, is a strong inhibitor for host CYP3A4 enzyme and is administered at a low dose together with other PIs to boost the level of other PIs in the blood. Ritonavir-boosted PIs were shown to have lower rates of virological failure and development of resistance mutations compared to unboosted PIs (Walmsley *et al.*, 2002; Wood *et al.*, 2007). Ritonavir boosted LPV (LPV/r) was shown to be non-inferior to EFV in combination with two NRTI backbone (De Luca *et al.*, 2006; Domingo *et al.*, 2008). Many studies comparing other boosted PIs (e.g. FPV/r, ATV/r, and SQV/r) to LPV/r have demonstrated that these agents are non-inferior to LPV/r in term of virological efficacy (Eron *et al.*, 2006; Molina *et al.*, 2008, 2010; Walmsley *et al.*, 2009). In developing countries, the heat-stable fixed dose combinations ATV/r and LPV/r are the preferred ritonavir-boosted PI for second-line therapy (WHO, 2013a).

Studies have shown that ritonavir-boosted DRV (DRV/r) has greater efficacy compared to other PIs, even in heavily pre-treated patients (De Meyer *et al.*, 2009; Madruga *et al.*, 2007; Pellegrin *et al.*, 2008; Pozniak *et al.*, 2008). Among treatment naïve patients, DRV/r was shown to be superior to LPV/r with a significant proportion of patients achieving virological suppression and more favourable gastrointestinal and lipid profiles (Mills *et al.*, 2009). WHO recommends DRV/r as an option for third-line regimen to treat HIV infection that was resistant to other PIs in resource-limited countries (WHO, 2013a).

1.5.2.4 Integrase inhibitor

Integrase inhibitor (INI) or integrase strand transfer inhibitor was designed to block the action of viral integrase and prevent the insertion of retroviral DNA into the host cell genome. Raltegravir (RAL) is the first FDA approved drug for this class in 2007. Other INIs with a similar mechanism of action such as elvitegravir (EVG) and dolutegravir (DTG) have been clinically evaluated (Messiaen *et al.*, 2013).

1.5.2.5 Fusion inhibitor

Fusion inhibitor prevents the virus from mixing its membrane with the host cell membrane and releasing the viral core into the cytoplasm (Lobritz *et al.*, 2010). Enfuvirtide (EFN) is the only FDA approved drug for this class. EFN, a 36 amino acid peptide that is homologous to HR2 of gp41, binds to HR1 region of gp41 and blocks the formation of the six-helix bundle necessary for fusion (Eggink *et al.*, 2010; Greenberg & Cammack, 2004).

1.5.2.6 Entry inhibitor

Entry inhibitor is a co-receptor antagonist that inhibits HIV glycoprotein gp120 from binding to CD4. It binds to the co-receptor of CD4 cells (CCR5 and/or CXCR4) and changes the co-receptor conformation so that gp120 cannot recognize the co-receptor. Maraviroc (MVC) is the only CCR5 antagonist FDA-approved for the treatment of HIV infection (Kuritzkes, 2009).

1.5.3 Antiretroviral (ARV) treatment

1.5.3.1 Timing to antiretroviral therapy initiation

The goal for ARV treatment is (1) to restore and preserve immune function, (2) to delay progression of HIV disease and death, (3) to suppress HIV from replication, and (4) to prevent vertical HIV transmission. Early ARV treatment has shown to have some benefits for HIV patients by reducing mortality and AIDS defining illness, preventing HIV transmission, and restoring CD4+ T-cell count patients (Egger *et al.*, 1997; Hogg *et al.*, 1997; Mocroft *et al.*, 1998, 2003; Palella *et al.*, 1998; The Cascade Collaboration, 2000; van Sighem *et al.*, 2003). ARV treatment interruption increases the risks of opportunistic diseases and deaths of any causes (SMART Study Group *et al.*, 2006). The INSIGHT START trial has shown that patients had lower risk of developing AIDS and non-AIDS illnesses if they started ART when their CD4+ T-cell count is above 500 cells/μL (INSIGHT START Study Group, 2015). ART has been shown to prevent HIV transmission. ART reduces HIV viral load in blood, semen, vaginal fluid, and rectal fluid to a low enough level that reduces the risk of HIV transmission among individuals (Baeten *et al.*, 2011). ART used in pregnant women reduces the risk of transmitting HIV to their babies from 40% to less than 1% (Townsend et al., 2008). ART used in a HIV-positive partner in a sero-discordant couple reduces the HIV transmission risk to a HIV-negative partner by 96% for both homosexual and heterosexual couples (Cohen et al., 2011; Rodger et al., 2014).

The benefits of treating acute HIV infection are (1) to treat highly symptomatic patients who are more likely to progress rapidly (Thompson *et al.*, 2012), (2) to preserve CD4+ T cell count and reduce the viral set (Grijsen *et al.*, 2012; Le *et al.*, 2013; The

SPARTAC Trial Investigators, 2013), (3) to limit the size of viral reservoir (Ananworanich *et al.*, 2015; Buzon *et al.*, 2014; Strain *et al.*, 2005), (4) to preserve HIV-specific immunity (Oxenius *et al.*, 2000; Rosenberg *et al.*, 2000), (5) to reduce HIV transmission as early HIV infection is associated with high viral loads and increased infectiousness (Cohen *et al.*, 2011; Wawer *et al.*, 2005), and (6) not to wait until CD4+ T-cell count declines to a threshold with clinical evidence for ART benefit as this interval is short (Lodi *et al.*, 2011). However implementation for treatment at early infection is still an issue due to the financial constraint and limited resources in developing countries (Walker & Hirsch, 2013). The advances in ART are responsible for drugs that are more potent, less toxic and promote better adherence (Sabin & Phillips, 2009). There is a theoretical concern about the development of earlier onset of ART resistance in non-adherent patients. However a study showed lower rates of drug resistance development when ART was started at higher CD4 cell counts (Uy *et al.*, 2009).

In practice ART is deferred in asymptomatic patients until CD4 cell counts reach certain thresholds. These threshold have changed over time from ≤200 cells/μL, to ≤350 cells/μL, and to ≤500 cells/μL (WHO, 2006a, 2010a, 2013a). The most recent the WHO guidelines for ARV treatment recommends starting HAART as soon as the patient has (1) a CD4 cell count below 500 cells/μL, (2) WHO clinical stage 3 and 4 irrespective of CD4 count, (3) active tuberculosis (TB) and/or hepatitis B (HBV) co-infection that requires therapy (WHO, 2013a). Several cohort studies have shown that initiating ART earlier in chronically infected patients when CD4 counts are 350–500 cells/μL is associated with slower disease progression compared to initiating it later when CD4+ T-cell counts fall below 350 cells/μL (Anglemyer *et al*., 2014; CASCADE Collaboration, 2011; Kitahata *et*

al., 2009; Lodi *et al.,* 2011; Sterne *et al*., 2009). Recently, the results of a major international randomized clinical trial conducted in 215 sites in 35 countries worldwide has shown a clear-cut proof of early initiation of ART regardless of CD4 cell counts (INSIGHT START Study Group, 2015). The initiation of ART when CD4 counts are >500 cells/μL significantly reduced AIDS and non-AIDS events compared to starting ART when CD4 counts decline to 350 cells/μL.

1.5.3.2 Choice of initial treatment

The choice of initial regimen should be based on resistance testing results [please refer to section 1.6.7 on HIV drug resistance testing] and predicted virological efficacy, toxicity and tolerability, pill burden, dosing frequency, drug-drug interactions, co-morbidities, cost and availability (Thompson *et al.*, 2010). According to WHO, first-line therapy should consist of an NNRTI plus two NRTIs, one of which should be AZT or TDF (WHO, 2010a). The following regimens are considered as first-line treatment for ARV naïve HIV infected patients:

• AZT + 3TC + EFV

• AZT + 3TC + NVP

• TDF + 3TC (or FTC) + EFV

• TDF + 3TC (or FTC) + NVP

An important trial of first-line therapy (Riddler *et al.*, 2008) showed that an EFV-based regimen was superior to a LPV/r-based regimen in term of virological efficacy.

Similar effectiveness of EFV was observed over the use of ATV and RAL (Daar *et al.*, 2010; Lennox *et al.*, 2009).

NVP is not inferior to EFV in term of clinical efficacy when administered in combination regimens and triple-drug regimens with either NNRTI are valid for first-line treatment (Van Leth *et al.*, 2004). However they differ in toxicity and drug-drug interaction. EFV should not be initiated in the first trimester of pregnancy (WHO, 2012a) and should not be used for children under 3 years old (WHO, 2010b). Yet EFV is preferred in individuals taking rifampicin-containing TB treatment as rifampicin can reduce the level of NVP (Manosuthi *et al.*, 2006b). D4T was previously subscribed in WHO guided first-line therapy (WHO, 2006a) but it is no longer preferred as initial treatment due to drug toxicity and d4T-related adverse events (WHO, 2010a).

Table 1.2: WHO preferred first-line therapy in treatment-naïve adults and adolescents

Source: WHO guideline 2010 (WHO, 2010a)

Target population	Preferred options	Comments
Adults and adolescents	AZT or TDF + 3TC or FTC + EFV or NVP	Select the preferred regimens applicable to the majority of PLHIV Use fixed-dose combinations
Pregnant women	AZT + 3TC + EFV or NVP	Do not initiate EFV during first trimester TDF acceptable option In HIV women with prior exposure to PMTCT regimens, see ART recommendations in Table 11
HIV/TB coinfection	AZT or TDF + 3TC or FTC + EFV	Initiate ART as soon as possible (within the first 8 weeks) after starting TB treatment NVP or triple NRTIs are acceptable options if EFV cannot be used
HIV/HBV coinfection	TDF + 3TC or FTC + EFV or NVP	Consider HBsAg screening before starting ART, especially when TDF is not the preferred first-line NRTI Use of two ARVs with anti-HBV activity required

1.5.3.3 When to switch therapy

Therapy switching is often due to treatment failure or drug intolerance. Criteria for treatment failure according to WHO (WHO, 2010a) guidelines include:

i. Clinical failure: defined as new or recurrent WHO stage 4 condition

ii. Immunological failure: defined as

   a. fall of CD4 count to baseline (or below)

   b. 50% fall from on-treatment peak value

   c. persistent CD4 levels below 100 cells/μL

iii. Virological failure: The viral load threshold for defining is chosen for its association with clinical progression and a decline in the CD4 cell count. The optimal viral load threshold for defining has not been determined. The WHO has recommended reducing the viral load threshold for virological failure from 5000 copies/mL to 1000 copies/mL (WHO, 2013a). This level was chosen based on 2 main sources of evidence: (1) Several clinical and epidemiological data showed the risk of HIV transmission is low when HIV viral load is below 1000 copies/mL (Gale *et al.*, 2013; Loutfy *et al.*, 2013; Mocroft *et al.*, 2010); and (2) viral blips or intermittent low-level viremia, viral load ranging from 50 copies/mL to 1000 copies/mL, are not associated with increased risk of treatment failure, unless low-level viremia is sustained (Havlir *et al.*, 2001; Kanapathipillai *et al.*, 2014; Lee *et al.*, 2006).

According to WHO viral load measurement is a more sensitive indicator of treatment failure compared to clinical or immunological indicators. Routine viral load monitoring is encouraged to detect treatment failure early. In patients with good

adherence, detecting treatment failure early can reduce the risk of accumulation of drug resistance mutations and preserve the salvage therapy such as second- or third-line regimens. In resource limited settings where virological monitoring cannot be routinely accessed due to its high cost, WHO recommends targeted viral load testing to confirm clinical and/or immunological failure and thus reduce unnecessary therapy switching.

1.5.3.4 Second-line and third-line regimens

WHO recommends a boosted protease inhibitor (bPI) plus two nucleoside analogues for second-line ART. ATV/r and LPV/r are the two boosted PIs preferred for second-line ART. 3TC is maintained in the second-line regimens with argument that the M184V mutation can preserve the drug susceptibility to TDF even though Fox *et al.* reported no difference in virological outcomes among those maintaining 3TC in second-line regimens compared to those who did not (Fox *et al.*, 2006).

Third-line regimens should include new drugs likely to have anti-HIV activity, such as INIs and second-generation NNRTIs and PIs (WHO, 2010a). In patients with multiple drug resistance mutations who have few remaining treatment options, the combination of RAL, ETV, and DRV/r was associated with a rate of virological suppression similar to that expected in treatment-naïve patients and was recommended as a third-line option (Fagard *et al.*, 2012; Imaz *et al.*, 2011; WHO, 2013a; Yazdanpanah *et al.*, 2009).

Table 1.3: WHO preferred second-line options

Source: WHO guideline 2010 (WHO, 2010a)

Target population		Preferred options	Comments
Adults and adolescents (including pregnant women)	If d4T or AZT used in first-line therapy	TDF + 3TC or FTC + ATV/r or LPVr	NRTI sequencing based on availability of FDCs and potential for retained antiviral activity, considering early and late switch scenarios ATV/r and LPVr are comparable and available as heat-stable FDCs or co-package formulations
	If TDF used in first-line therapy	AZT + 3TC + ATV/r or LPVr	
TB/HIV coinfection	If rifabutin available	Same regimens as recommended above for adults and adolescents	No difference in efficacy between rifabutin and rifampicin Rifabutin has significantly less drug interaction with bPIs, permitting standard bPI dosing
	If rifabutin not available	Same NRTI backbones as recommended for adults and adolescents plus LPVr or SQV/r with superboosted dosing of RTV (LPV/r 400 mg/400 mg twice daily or LPV/r 800 mg/200 mg twice daily or SQV/r 400 mg/400 mg twice daily)	Rifampicin significantly reduces the levels of bPIs, limiting the effective options. Use of extra doses of ritonavir with selected bPIs (LPV and SQV) can overcome this effect but with increased rates of toxicity
Hepatitis B coinfection		AZT + TDF + 3TC or FTC + ATV/r or LPVr	In case of ART failure, TDF + 3TC or FTC should be maintained for anti-HBV activity and the second-line regimen should include other drugs with anti-HIV activity

1.6 HIV drug resistance

1.6.1 How does resistance develop?

Drug resistance is the consequence of genetic diversity and selective pressure. The genetic diversity is caused by its rapid replication rate with the generation of about 10^{10} virions every day, and its error-prone reverse transcriptase – which causes a high mutation rate of 3.4×10^{-5} mutations per site and generates frequent recombination events (Rambaut *et al.*, 2004). This leads to the formation of a quasispecies, which is a swarm of different but genetically related viral variants (Paredes & Clotet, 2010). As a consequence mutations, including drug resistance associated mutations, are spontaneously generated before the start of ART (Coffin, 1995). Yet when not on treatment, the wild-type variant has better ability to replicate than strains with drug resistant mutations and therefore, predominates in untreated patients (Paredes & Clotet, 2010). Sub-optimal treatment due to non-adherence, poor drug absorption or drug-drug interaction can reduce the replication of wild-type strains and favor the mutants with better fitness in the presence of HIV drugs.

1.6.2 Types of drug resistance

There are two types of drug resistance: primary (transmitted) drug resistance and secondary (acquired) drug resistance.

1.6.2.1 Transmitted drug resistance

Transmitted drug resistance (TDR) is resistance present in a treatment-naïve patient and is transmitted from the source of infection, either directly or through one or more intermediates from a person with drug resistance developed after HIV treatment (Shafer *et al.*, 2007). Replication capacity of mutant strains can affect the occurrence of TDR mutations (Brown *et al.*, 2003; Little *et al.*, 2008; Pingen *et al.*, 2014). Virus strains carrying M184V/I have lower replication capacity compared to that of virus strains carrying K103N. Hence M184V/I are rarely detected by standard population sequencing in newly-detected HIV infections whereas other mutations, such as K103N, are more frequently detected (Pingen *et al.*, 2014).

TDR most likely emerges in the regions where ART has been widely used for years. As many drug resistance mutations can impair the virus fitness (Cong *et al.*, 2007; Gonzalez *et al.*, 2004; Paredes *et al.*, 2009; Weber *et al.*, 2007), in chronically infected patients, who have never been on HIV treatment, TDR is replaced by emerging wild type variants with improved fitness. The replacement of wild type strains can be either due to the out-growth of minority wild-type variants co-existing with mutant strains in the population or due to the evolution and back-mutation from the mutant strains. Mutation M184I/V was reported to be replaced at the fastest rate with 100% replacement after 3 years of HIV infection (Jain *et al.*, 2011). Other NRTI mutations are replaced at a similar rate to NNRTI and PI mutation (Jain *et al.*, 2011). Another 2 possible evolution pathways of TDR have also been described. In the second pathway the replacement of TDR mutations by atypical amino acids that improve viral replication capacity may occur (Pingen *et al.*, 2011). In the third pathway the resistant variant can persist either because

resistant mutations have minimal reduction effect on the viral replication capacity or the reversion is inhibited by fixation through compensatory mutations (Pingen *et al.*, 2011).

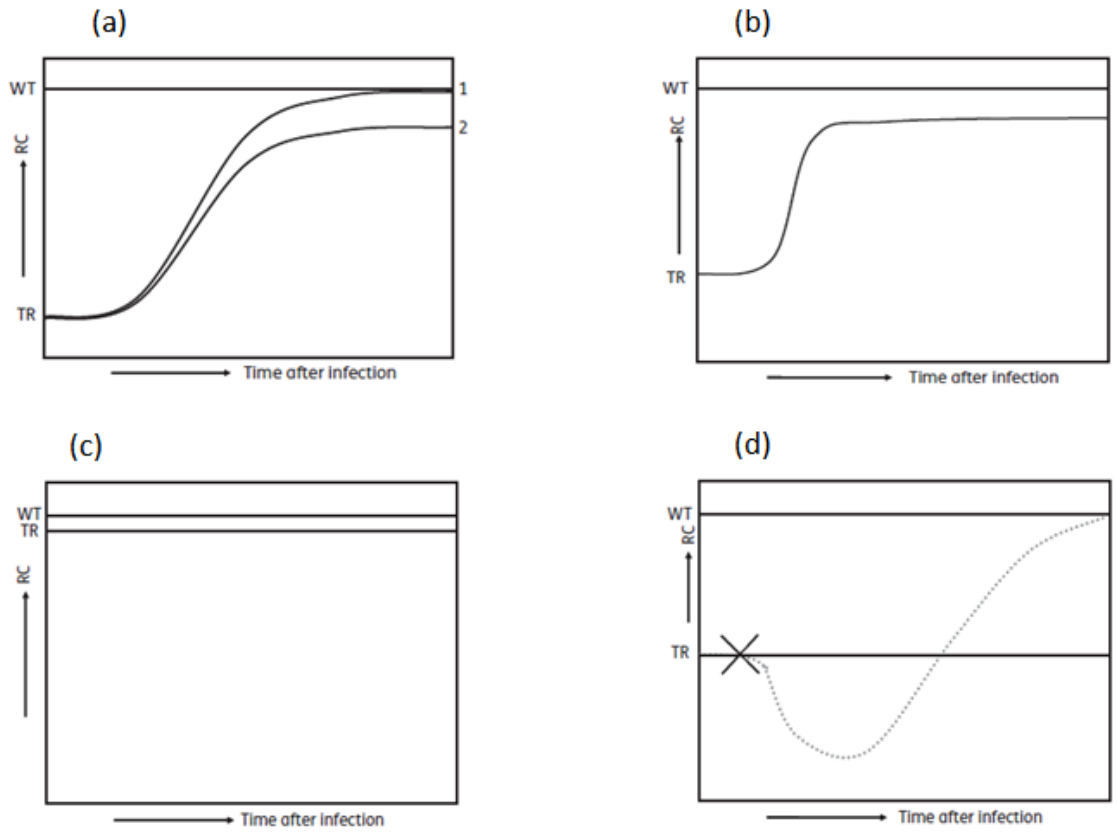


Figure 1.7: Evolutionary pathways of viral evolution after transmission

Source: (Pingen *et al.*, 2011)

(a) Evolution to wild-type with complete (line 1) or incomplete (line 2) reversion of the drug resistance mutation. (b) Evolution towards atypical mutations. (c) Persistence of TDR due to a minimal reduction in RC. (d) Persistence of TDR because reversion is blocked by compensatory fixation. WT=wild-type; RC= replication capacity; TR=transmitted resistance variants

1.6.2.2 Acquired drug resistance

Secondary drug resistance is the resistance emerging after treatment initiation under the drug selective pressure (Shafer *et al.*, 2007). The genetic barrier (which is defined as the number of mutations required to confer resistance) of an ARV drug has an important effect on the development of drug resistance. Drugs with a high genetic barrier (such as boosted PI) require multiple mutations to overcome the drug pressure whereas for drugs with a low genetic barrier (such as NNRTI, NRTI) one single mutation can cause resistance (Luber, 2005). The genetic barrier is also affected by subtype differences in nucleotide and mutation motif (Santoro & Perno, 2013). An example for impact of subtype diversity is the occurrence of mutation V106M, which is commonly selected for subtype C after the exposure of NVP and EFV. This results from the fact that V106 is encoded by GTA in subtype B viruses and GTG in subtype C viruses (Brenner *et al.*, 2003; Loemba *et al.*, 2002). A single G-to-A transition at the first position of codon 106 in subtype C viruses results in V106M, which confers high-level resistance to EFV and NVP. In contrast, in subtype B viruses, V106M requires two nucleotide substitutions (GTA-ATG) and therefore occurs infrequently (Brenner *et al.*, 2003; Grossman *et al.*, 2004).

Because of similar mechanisms of HIV drugs in each drug class, mutations conferring resistance to one drug can cause cross-resistance to other drugs in the same drug class. Cross-resistance typically occurs within a drug class. Mutations known to be selected by thymidine analogues (such as M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E) also confer reduced susceptibility to all currently approved NRTIs (Whitcomb *et al.*, 2003). Cross-resistance also occurs among first generation NNRTIs, in which

mutation patterns are similar for both NVP and EFV, and INIs, in which RAL- and EVG-resistant viruses via Q148H pathway can reduce DTG susceptibility (Kobayashi *et al.*, 2011). In some occasions resistance to one drug can lead to increased susceptibility, or hyper-susceptibility, to another drug. For example, the M184V mutation that confers 3TC resistance causes hyper-susceptibility to AZT, and the thymidine analogue mutation (TAM) T215Y is associated with EFV hyper-susceptibility (Larder *et al.*, 1995; Shulman *et al.*, 2004).

Insufficient drug-level due to poor adherence, poor drug absorption, and drug-drug interaction contribute to selective drug pressure and favor the replication of mutant strains, leading to the development of drug resistance after ART initiation. Testing for HIV drug resistance has become an integral part of HIV clinical care. Drug resistance testing can be categorised as either phenotypic or genotypic assays and is discussed in section 1.6.7.

1.6.3 Prevalence of drug resistance

1.6.3.1 Transmitted drug resistance mutations

The widespread use of HIV therapy has been associated with the reduction of mortality in HIV infected patients yet it is also paralleled with the increased transmission of antiretroviral drug resistance. Therefore, surveillance of TDR is necessary. Recent data showed that in high income countries like Europe, United States, Japan and Australia 10% to 17% of ARV naïve patients have drug resistance to at least one antiretroviral drug (Frentz *et al.*, 2012; Ross *et al.*, 2008; Wensing *et al.*, 2005; Wheeler *et al.*, 2010; WHO,

2012b; Yerly *et al.*, 2007). The prevalence of primary resistance ranges from 5% to 12% for NRTI, 2% to 8% for NNRTI and 3% to 7% for PI (Paredes & Clotet, 2010). In low- and middle-income countries the average prevalence of TDR is lower at 6.6%, with the prevalence of 5.7% in Africa and 7.6% in Asia (Stadeli & Richman, 2013; WHO, 2012b). A survey performed by WHO between 2004 and 2010 reported the prevalence of TDR in low- and middle-income countries to at least one drug class is 3.1% of the surveyed population (WHO, 2012b). The prevalence of NNRTI, NRTI and PI mutations in the survey were 1.6%, 1.3% and 0.7% respectively.

The temporal changes of the prevalence were shown not to be similar among developed countries. In Europe the prevalence of drug resistance in treatment-naïve patients was shown to decline over-time from 11.5% before 2003 to 7.7% after that year (Cane *et al.*, 2005; Frentz *et al.*, 2012; Rhee *et al.*, 2015; Vercauteren *et al.*, 2009). The decrease of overall TDR is probably driven by high prevalence of TDR before the year 2000; the time trend analysis after 2000 did not show significant change. The immigration of people from low- and middle-income countries carrying non-B subtypes may also contribute to the decrease of TDR in Europe (Rhee *et al.*, 2015). The decrease of overall TDR in Europe is accompanied with a decrease of NRTI-associated TDR and an increase in NNRTI-associated TDR. In North America TDR prevalence increased over time from 11.6% before 2001 to 14.3% after 2003 (Frentz *et al.*, 2012; Rhee *et al.*, 2015). The increase was attributable to an increase in NNRTI-associated TDR (Rhee *et al.*, 2015). A decreasing trend of prevalence was observed in high-income Asian settings like Taiwan and Hong Kong; yet in Japan this was not the case (Sohn *et al.*, 2013). In Taiwan the prevalence declined from 12.3% in 2003-2006 to 5.1% in 2007-2010 (Lai *et al.*,

2012). A 5-year study in Hong Kong reported a reduction of resistance prevalence to any drug from 11.7% in 2003 to 6.5% in 2007 (Wong *et al.*, 2010). However in Japan, the rates of patients carrying at least one resistance mutation have increased from 5.9% in 2003 to 8.3% in 2008, and to 11.9% in 2010 (Hattori *et al.*, 2010, 2012; Rhee *et al.*, 2015). The increase in overall TDR was accompanied by an increase in NNRTI and PI associated TDR (Rhee *et al.*, 2015). In low- and middle-income countries like South Africa the increase over-time in prevalence of pre-existing drug resistance associated mutations prior to ARV treatment has been observed. In South Africa the prevalence significantly increased after the ART roll-out program with an annual increase of 14% (0%-29%) (Frentz *et al.*, 2012; Gupta *et al.*, 2012; Hamers *et al.*, 2013). Due to the lack of data in developing countries in Asia, there isn't enough evidence to interpret the changes of TDR (Frentz *et al.*, 2012; Gupta *et al.*, 2012). Meta-analysis from GenBank sequences reported no significant change in overall, NRTI-associated, or NNRTI-associated TDR in South/South-east Asian countries (Rhee *et al.*, 2015). Yet Thailand reported a significant emergence of primary HIV-1 drug resistance after rapid scaling up of ART (Sungkanuparph *et al.*, 2012). TDR limits the options of HIV treatment even prior to starting therapy and it is associated with poor virological outcome (Kuritzkes *et al.*, 2008; Wittkop *et al.*, 2011). Genotypic testing in treatment naïve patients before their initial ART is highly recommended (Sax *et al.*, 2005; Wittkop *et al.*, 2011).

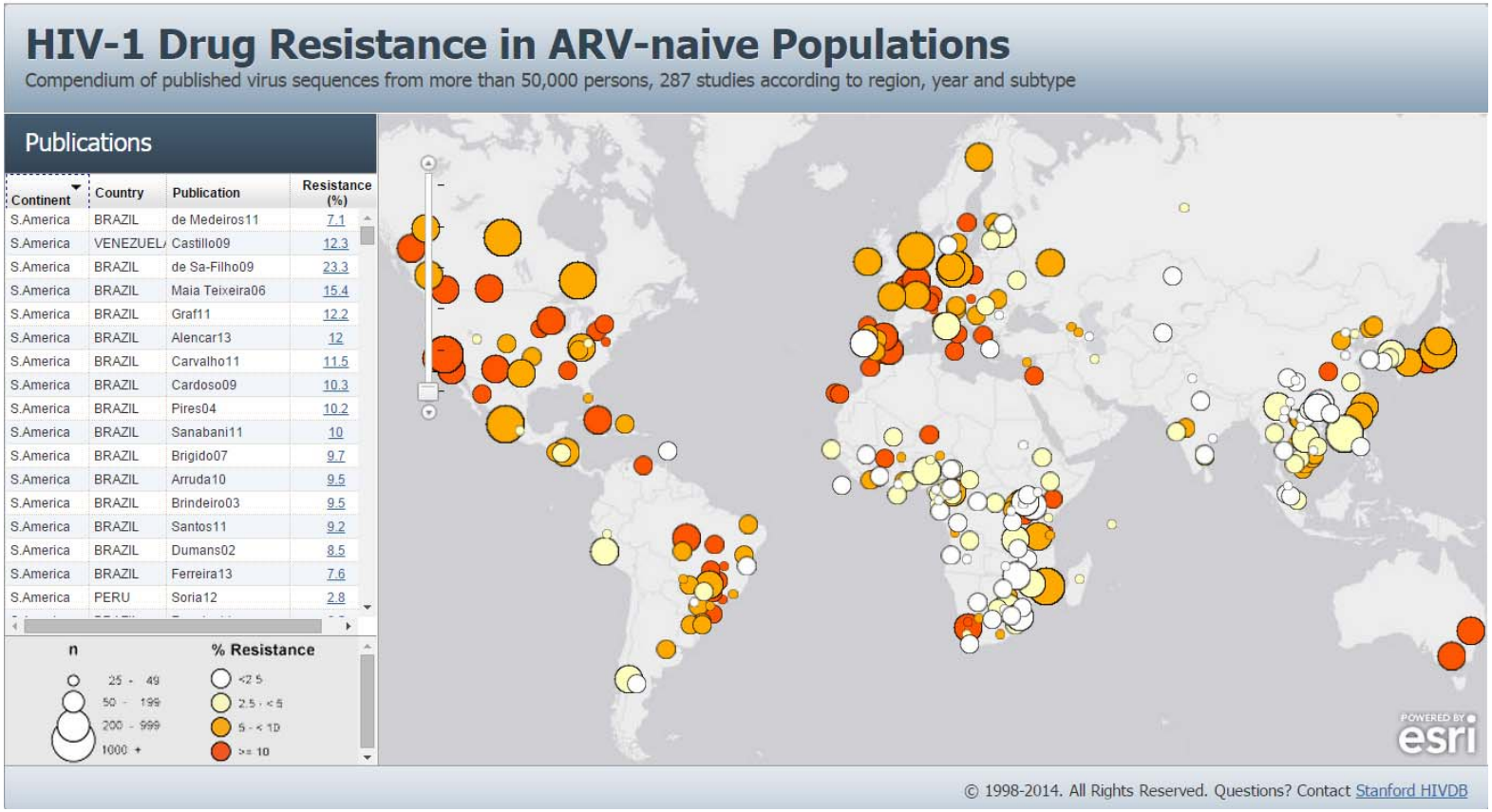


Figure 1.8: Interactive map displaying HIV-1 drug resistance in ARV-naive populations from 287 studies between 2000-2013

Source: HIV Stanford Database (http://hivdb.stanford.edu/surveillance/map/). Each study is represented by a circle. The size of the circle is proportional to the number of individuals in the study. The circle color indicates the prevalence of overall TDR in the study: white (<2.5%), pale yellow (2.5% to 4.9%), orange (5.0% to 9.9%), and red (10.0%).

1.6.3.2 Acquired drug resistance mutations:

Drug resistance development at the time of treatment failure can limit the options for next line therapy and treatment efficacy. In European countries around 81% of patients who experienced treatment failure had at least one drug resistance mutation (NRTI 67.2% - 75.5%, NNRTI 48.3% - 53.7%, PI 32.4% - 35.5%) (Prosperi *et al.*, 2011; van de Vijver *et al.*, 2010). In the United States the rate was reported at 76% to at least one drug (40.5% to NRTIs, 25.2% to NNRTIs, and 40.5% to PIs) in 1996 (Richman *et al.*, 2004). Unlike the difference in prevalence of TDR among high-income and low- and middle-income countries, the prevalence of acquired drug resistance to any HIV drug is similar. In a WHO acquired HIV drug resistance survey in low- and middle-income countries 72.1% carried HIV resistant mutations to any drug (69.5% to NNRTI, 62.5% to NRTI) (WHO, 2012b). With changes in the use of NRTIs and NNRTIs and the use of ritonavir boosted PI in replacement for non-boosted PI, the prevalence of acquired drug resistance may change over time. A study in 7 different European countries from 1997 to 2008 has shown that the proportion of patients with resistance to three drug combinations has dropped from 32% in 2000 to 1% in 2008 (De Luca *et al.*, 2013). Similar results were shown in North America with the incidence rate of any newly detected resistance decreased more than 12-fold between 1996 and 2008 (Gill *et al.*, 2010). In contrast a different observation has been reported in resource-limited settings. A WHO survey in 12 low- and middle-income countries from 2001 to 2007 showed that the prevalence of HIV drug resistance to any drug among ART experienced patients increased from 4.8% (95% CI 3.8%–6.0%) in 2007 to 6.8% (95% CI 4.8%–9.0%) in 2010 (WHO, 2012b). The proportion of patients carrying acquired resistance has been reported to go up with time

of treatment. A meta analysis of studies in resource limited settings showed that 7.2% of patients on ART for 6–11 months acquired drug resistance mutations, 11.1% at 12–23 months, 15.0% at 24–35 months, and 20.7% at ≥36 months (Stadeli & Richman, 2013).

Studies have found an U-shaped association between the number of resistance mutations developed and the plasma concentrations of HIV RNA (Brun-Vézinet *et al.*, 2004; Machouf *et al.*, 2006). In this model, propagation of wild-type virus with high replication capacity results in high HIV RNA levels. Propagation of resistant strains with lower fitness results in low plasma HIV RNA concentration. However prolonged failing therapy can lead to further accumulation of resistance mutations that can compensate the low fitness of earlier mutant strains. The increasing number of mutations will eventually overcome the reduced fitness of mutant virus, resulting in an increase in viral load level (Lohse *et al.*, 2007). Increasing prevalence of acquired drug resistance can also lead to the increasing rate of primary drug resistance and limit the treatment options for increasing numbers of HIV patients, not only in developing countries but also in developed settings.

1.6.4 Impact of resistance to treatment outcome

1.6.4.1 Transmitted drug resistance

Transmission of HIV viruses harboring resistance mutations is a major concern in both developed and developing countries with potential impact on therapeutic strategies. Virus strains carrying TDR can alter the progression of HIV disease compared to wild-type strains. In the DACSOO3 study, individuals with NNRTI-resistant or NRTI-resistant

virus had a baseline viral load that was 0.4 logs or 0.7 logs higher than those with wild-type virus (with p=0.003 and p<0.001 respectively) (Little, 2007). Rapid progression to AIDS after acute HIV-1 infection in a patient with transmitted multidrug resistant virus was reported in New York (Markowitz *et al.*, 2005). However, other reports described higher CD4 cell count and lower RNA level at the time of infection among patients carrying TDR (Bezemer *et al.*, 2004; Grant *et al.*, 2002; Poggensee *et al.*, 2007) which could be explained by the reduced fitness of resistant virus strains, and that TDR does not affect the disease progression in terms of rate of CD4 decline (Pillay *et al.*, 2006).

Nevertheless, TDR has been reported to have an impact on HIV treatment outcome (Cambiano *et al.*, 2013). TDR has been shown to be associated with virological failure in ARV naïve patients. In a case-cohort of the ACTG A5095 Trial, the risk of virological failure for subjects with baseline NNRTI resistance was higher than that for subjects without such resistance (hazard ratio 2.27 [95%confidence interval (CI): 1.15–4.49]; P=0.018) (Kuritzkes *et al.*, 2008). In a European multi-cohort study patients with transmitted resistance to at least 1 drug had a 2.6-fold higher risk of virological failure compared to patients without resistance mutations (Wittkop *et al.*, 2011). This finding is in agreement with results from a current multicenter cohort study in sub-Saharan Africa (Hamers *et al.*, 2012). The presence of drug resistance before ART usage also increased the risk of developing acquired drug resistance (OR=2.30, 95% CI 1.55-3.40; p<0.0001) and reduced the rate of CD4 recovery after treatment (Hamers *et al.*, 2012). Among patients who carried TDR mutations, the time to virological suppression after ART initiation was longer and the time to virological failure was shorter compared to those with susceptible virus at baseline (Grant *et al.*, 2002; Lai *et al.*, 2012; Little *et al.*, 2002).

Indeed some studies reported genotypic testing for optimal choice of fully active therapy could improve treatment outcome and cost effectiveness (Sax *et al.*, 2005).

The clinical significance of minority (low-level) TDR has not been clearly defined. In some studies the presence of minority TDR results in a similar impact as majority resistant variants in which pre-existing mutations before ART initiation can increase the risk of virological failure and the rate of disease progress (Johnson *et al.*, 2008; Paredes *et al.*, 2010; Simen *et al.*, 2009). However other studies have reported that the presence of minority-resistant strains did not affect the changes in viral load and CD4 cell counts after ART initiation (Lataillade *et al.*, 2010; Peuchant *et al.*, 2008; Stekler *et al.*, 2011).

1.6.4.2 Acquired drug resistance

Development of acquired ARV resistance reduces treatment efficacy. Continuing failing ART regimens leads to the accumulation of more drug resistant mutations and further resistance to multiple drugs. The emergence of drug resistance constrains subsequent ART options and gives rise to the use of more complex, more expensive and sometimes worse tolerated regimens. This in turn makes adherence more difficult and increases the chance of subsequent treatment failure (Paredes & Clotet, 2010). Acquired drug resistance has been shown to be associated with nearly 2-fold increased risk of death (Hogg *et al.*, 2006; Kozal *et al.*, 2007; Lohse *et al.*, 2007). The development of drug resistance also increases the risk of transmission of drug-resistant viruses, creating a growing public health concern.

1.6.5 Mechanisms of HIV-1 drug resistance

1.6.5.1 Nucleotide reverse transcriptase inhibitor resistance

There are two distinct mechanisms of NRTI resistance. The first mechanism is the impairment of the incorporation of the NRTI into DNA (Ammaranond & Sanguansittianan, 2012; Clavel, 2004). Mutations in the N-terminal polymerase domain of the reverse transcriptase (RT) allow the RT enzyme to discriminate against NRTI during synthesis by reducing the enzyme's ability to bind to the drug. This prevents the incorporation of a NRTI to the DNA chain. These mutations include K65R, M184V, and Q151M. The M184V mutation induces very high levels of resistance to 3TC and FTC. The group of mutations called Q151M complex including A62V, V75I, F77L, and F116Y can confer resistance to most nucleoside analogues, except for TDF (Clavel, 2004; Johnson *et al.*, 2013). K65R appears to confer resistance to most analogues, with the exception of AZT (Clavel, 2004; Johnson *et al.*, 2013). The combination of Q151M complex and K65R can dramatically decrease the susceptibility to 3TC, FTC and TDF (Ibe & Sugiura, 2011). The emergence of Q151M complex is more frequent in the viral genotype with the K65R mutation (Ibe & Sugiura, 2011). The second mechanism of NRTI resistance is mediated by mutations that increase the rate of phosphorolytic removal of NRTI from the terminated DNA chain, which in turn permits continued DNA synthesis (Ammaranond & Sanguansittianan, 2012; Clavel, 2004). Mutations involved in this mechanism are called TAM which are typically M41L, D67N, K70R, L210W, T215Y/F, and K219E/Q. These mutations are most frequently selected for after the failure of thymidine analogues AZT and d4T, but they can result in cross-resistance to almost all nucleoside and nucleotide analogues, including TDF. The response to TDF was

shown to be reduced among patients with at least 3 TAMs inclusive of either the M41L or L210W mutation (Miller & Hazuda, 2004; Miller, 2004). HIV-1 develops TAMs by two distinct pathway defined as TAM-1 (including mutations 41L, 210W and 215Y) and TAM-2 (including mutations 67N, 70R and 219E/Q). However the factors that drive the selection of the TAM1 and TAM2 pathways are currently unknown (Cozzi-Lepri *et al.*, 2005).

Other NRTI mutations such as K65R and M184V have some reverse activity on the presence of TAMs. The effect of TAMs on NRTI is decreased in the presence of M184V mutation. In patients carrying TAMs, the presence of M184V seems be associated with re-sensitization to AZT, TDF and d4T (Ross *et al.*, 2004). The presence of M184V also appears to delay or prevent emergence of TAMs (Johnson *et al.*, 2013). This effect may be overcome by an accumulation of TAMs or other mutations (Johnson *et al.*, 2013; Kuritzkes *et al.*, 1996). TAMs rarely occur in the presence of K65R due to a mutual antagonism among these mutations (Parikh *et al.*, 2006, 2007). K65R antagonizes the NRTI excision activity of RT containing TAMs; and TAMs antagonize the ability to discriminate against the NRTI in RT containing K65R. Therapies including NRTI that select for both TAMs and K65R may prolong treatment response through the mutually antagonistic interactions between these resistance mutations (Parikh *et al.*, 2007).

1.6.5.2 Non-nucleotide reverse transcriptase inhibitor resistance

Resistance mutations to NNRTI drug classes locate at the hydrophobic pocket targeted by these compounds, reducing the affinity of NNRTI drugs (Clavel, 2004). A single mutation may cause a high-level drug resistance to this drug class and can confer

significant cross-resistance among all NNRTIs (Ammaranond & Sanguansittianan, 2012). There are three different mechanisms of NNRTI resistance. In the first mechanism NRTI mutations such as K101E and K103N disrupt the contacts between the NNRTI and the NNRTI binding pocket (Alcaro *et al.*, 2011). Position 101 and 103 locates at the entrance of hydrophobic pocket. Mutation K101E and K103N form a hydrogen bond in unliganded reverse transcriptase. This additional hydrogen bond helps keep the entrance to the pocket closed, blocking the entry/binding of the drugs (Clavel, 2004). Other mutations such as Y181C and Y188L reduce the interactions between RT and the bound drugs by disrupting the important aromatic rings involved in NNRTI interactions on the inside of the pocket (Alcaro *et al.*, 2011; Das *et al.*, 1996; Ren *et al.*, 2001).

1.6.5.3 Protease inhibitor resistance

Resistance to PI is a stepwise process in which mutations in the substrate-binding site usually develop first and are called as primary or major mutations. These resistance mutations result in an overall enlargement of the catalytic site of the enzyme and decrease the binding affinity to PIs (Wensing *et al.*, 2010). Most major mutations can reduce the susceptibility to one or more PIs by two- to five-fold (Ammaranond & Sanguansittianan, 2012). Secondary or minor mutations usually occur later during continuous PI therapy at sites removed from the active site. The minor mutations by themselves only have a minor effect on drug susceptibility. They appear to be compensatory mutations which improve the replication capacity of viruses carrying major mutations (Ammaranond & Sanguansittianan, 2012; Hirsch *et al.*, 1998; Wensing *et al.*, 2010).

In regimens containing ritonavir-boosted PI, ritonavir slows the metabolism of other PIs in the liver, thereby increases the blood level of those PIs. Boosting with ritonavir can prolong a PI half-life and can increase the trough levels (i.e. the lowest level between doses), which in turn can prevent HIV replication between doses and prevent drug resistance development. This makes boosted PIs more potent against HIV. Hence ritonavir-boosted PIs have high a genetic barrier which requires more mutations to render a PI inactive. The level of PI resistance increases with the accumulation of major and minor mutations. Different mutations affect different PIs. Some mutations are major to some PIs but minor to the others. For example I50L mutation is major mutation to ATV, and D30N is a major mutation resistant to NFV (Johnson *et al.*, 2013; Martinez-Cajas & Wainberg, 2007). Due to the small and similar chemical structure among PIs, some major mutations lead simultaneously to resistance to multiple PIs, leading to cross-resistance. For example I84A/V was associated with decreased susceptibility to eight PIs (Rhee *et al.*, 2010).

PI resistance is not commonly observed in patients failing ritonavir-boosted PI based therapy (Daar *et al.*, 2012; Eron *et al.*, 2006; Kempf *et al.*, 2004; Riddler *et al.*, 2008). A computational model showed that virological failure to ritonavir-boosted PI regimens occurred soon after a decline from perfect treatment adherence even without resistance (Rosenbloom *et al.*, 2012). As boosted PIs have short half-life and high genetic barrier, treatment interruption results in a decline of PI concentration to a level that is not sufficient for a sub-optimal drug pressure. Wild-type viruses, thus, cannot be suppressed. Virological failure occurs within a short treatment interruption. Besides the mutations on the protease region that can confer resistance to PI, some studies have shown that

mutations in the *gag* and *env* gene may also reduce the PI susceptibility by either indirectly improving viral replication capacity of PI mutant strains (Maguire *et al.*, 2002; Mammano *et al.*, 2000; Parry *et al.*, 2009; Rabi *et al.*, 2013; Robinson *et al.*, 2000) or directly contributing to PI resistance (Dam *et al.*, 2009; Nijhuis *et al.*, 2007). Both mutations at either gag cleavage sites (Fun *et al.*, 2012; Larrouy *et al.*, 2010; Nijhuis *et al.*, 2007; Parry *et al.*, 2009) and non-cleavage sites (Gatanaga *et al.*, 2002; Myint *et al.*, 2004; Parry *et al.*, 2011) can contribute to PI resistance.

1.6.5.4 Integrase inhibitor resistance

INIs require multiple drug resistance mutations for the loss of clinical INI activity. INI resistance is caused by primary mutations that reduce INI susceptibility in combination with secondary mutations. These secondary mutations further reduce drug susceptibility and compensate for the decreased fitness in viruses carrying primary mutations. Indeed most RAL-resistant viruses obtained from RAL-treated patients have 2 or more RAL resistance mutations. Three main pathways of resistance to RAL involve mutations at position Q148, N155 and Y143 in combination with other mutations and act by reducing the residence time of the drug within integrase (Blanco *et al.*, 2011; Mesplède *et al.*, 2012). RAL has a modest genetic barrier to resistance development with high level resistance obtained by a single point mutation (Wainberg, 2012); however the genetic barrier to RAL resistance is lower than that of the PIs and most NRTIs (Blanco *et al.*, 2011). In *in vitro* experiments INI resistance mutations usually emerge more rapidly than resistance mutations to most NRTIs and PIs; and virological failure of an INI-

containing regimen often occurs within the first several months of therapy and is often accompanied by INI resistance mutations (Blanco *et al.*, 2011).

In addition there is extensive but incomplete cross-resistance among the INIs. The 2 most commonly occurring RAL-resistance pathways involving mutations G148 and N155 cause high-level EVG resistance yet the other pathway involving mutation Y143 does not confer EVG cross-resistance. Similarly, the common EVG associated resistance mutations do not confer RAL cross-resistance (Blanco *et al.*, 2011). Hence switching from RAL to EVG and vice versa would not be expected to have a significant antiviral effect.

Limited data is available on mutations conferring resistance to DTG. An *in vitro* study has shown that mutations at positions Y143 and N155 in combination with additional secondary mutations and mutations at position Q148 alone did not cause resistance to DTG (Quashie *et al.*, 2012). The main patterns of DTG resistance mutations are Q148R or Q148H in combination of additional substitutions including T97A, E138K, G140S and M154I (Kobayashi *et al.*, 2011; Quashie *et al.*, 2012).

1.6.5.5 Fusion inhibitor resistance

Resistance to ENF can derive with the emergence of mutations in the HR1 region of gp41 and reduces the ENF binding capacity (position G547, I548, V549) (Greenberg & Cammack, 2004; Miller & Hazuda, 2004). A single mutation at the HR1 env region results in significant loss of susceptibility to ENF. Mutations in the HR2 region, although rare, have been detected (Xu *et al.*, 2004).

1.6.5.6 Entry inhibitor resistance

The inhibitory activity of CCR5 antagonists (MVC) can be derived from two independent mechanisms. Drug resistance can be resulted from the emergence of mutations in V3 region of gp120 molecule that allows the virus to bind to the CCR5 co-receptor despite the blockage of CCR5 (MacArthur & Novak, 2008; Soriano *et al.*, 2008). Increasing concentrations of MVC do not increase the percentage of viral inhibition, because MVC-resistant HIV-1 can bind to CCR5 in both its normal conformation and its MVC-bound conformation (MacArthur & Novak, 2008). Another mechanism for drug resistance is the change in virus tropism with the outgrowth of CXCR4 and/or dual tropic viruses that were present as minority population before MVC usage (MacArthur & Novak, 2008; Soriano *et al.*, 2008).

1.6.6 Subtypes and drug resistance

Data used to define drug resistance mutations have been generated from subtype B even though this subtype only accounts for 12% of HIV-1 infection in the world (Jülg & Goebel, 2005), yet subtype B dominates in industrialized, developed countries. Drug resistance information in non-B subtypes are extrapolated from what we have known in subtype B. Some minor resistance mutations acquired in subtype B can exit as natural polymorphisms in non-B subtype which are present without selective drug pressure. These polymorphisms were shown to have an effect on drug susceptibility (Abecasis *et al.*, 2006). The genotypic drug resistance interpretation algorithms also display high levels of discordance when applied to non-B subtypes (Vergne *et al.*, 2006). In addition the nucleotide sequences of the *pol* gene (which is the target of initial HIV drugs) among

HIV-1 subtypes differ by 15% and the amino acid sequences differ by 5% (Gonzales *et al.*, 2001; Jülg & Goebel, 2005). Subtype diversity can influence the spectrum of drug resistance mutations among subtypes and different subtypes can respond differently to certain HIV drugs. Regarding to NRTI drug resistance, prevalence of T215 revertants (C/D/E/S) (24%) was reported to be higher in subtype B and prevalence of V75M (10%) was higher in subtype CRF01_AE than the remaining subtypes among treatment-naïve patients (Rhee *et al.*, 2015). The prevalence of K65R in subtype C was higher than in subtype B and in other subtypes among both treatment naïve (36%) and treatment experienced patients (70%) (Kozal *et al.*, 2011; Sunpath *et al.*, 2013). For NNRTI resistance, prevalence of the pre-existing Y181C (33%) is higher in subtype CRF01_AE compared to other subtypes. In treatment naïve patients K103N (53%) was more commonly found in subtype B; and P225H mutation (14%) was more commonly found in CRF02_AG subtypes (Rhee *et al.*, 2015). Mutation V106M was detected commonly in subtypes CRF01_AE (14%) and C (12%) after therapy of NVP or EFV compared to subtype B (Brenner *et al.*, 2003; Wainberg & Brenner, 2012). For PI resistance, a meta-analysis study in TDR showed that L23I (16%) was more common in subtype A; and proportion of F53Y (11%) was highest in subtype CRF02_AG (Rhee *et al.*, 2015). Thus subtype diversity can affect the type of drug resistance mutations, degree of resistance and timing of resistance mutation development (Martinez-Cajas & Wainberg, 2007).

1.6.7 HIV drug resistance testing

Drug resistance is a major cause of treatment failure. Drug resistance testing helps guide the optimal choice of treatment regimens. The use of HIV drug resistance testing

prior to ART initiation was shown to be associated with better virological response and improved survival (Palella *et al.*, 2009; Sax *et al.*, 2005; Wensing *et al.*, 2005). Among patients experiencing treatment failure, several studies showed that changes in therapy informed by drug resistance testing results led to better virological response compared to regimen changes guided by only clinical judgment (Baxter *et al.*, 2000; Cingolani *et al.*, 2002; Cohen *et al.*, 2002; Durant *et al.*, 1999; Tural *et al.*, 2002; Wegner *et al.*, 2004).

1.6.7.1 Genotypic testing

Genotypic testing detects known drug resistance mutations in regions that are targeted by HIV drugs (Tang & Shafer, 2012). Genotypic testing is normally performed on plasma HIV-1 RNA. Genotypic testing is based on standard population sequencing, which is direct polymerase chain reaction (PCR) dideoxynucleotide (Sanger) sequencing. Direct sequencing produces a nucleotide sequence comprising of the entire protease gene (297 nucleotides) and the N-terminal half of reverse transcriptase gene (approximately 600 nucleotides), which contain the majority of NRTI and NNRTI resistance mutations (Grant & Zolopa, 2009; Sen *et al.*, 2006; Tang & Shafer, 2012). Integrase sequencing is usually performed as a separate sequencing test. The nucleotide sequence is translated to its amino acid sequence which is then compared with a consensus (wild-type) subtype B amino acid sequence. Amino acid differences at positions that have been previously found to be associated with resistance are reported. In population sequencing only mutations present in at least 20% of the viral population can be reliably detected. There are two commercially available kits for genotypic resistance testing and interpretation: the TRUGENE_ HIV-1 Genotyping Assay (Siemens, USA) (Kuritzkes *et al.*, 2003) and

the ViroSeq_HIV-1 Genotyping System (ViroSeq, Applied Biosystems, Foster City, California) which are both FDA approved platforms (Eshleman *et al.*, 2004b). In-house method for genotypic testing is commonly used by the majority of laboratories due to its low cost.

There are two basic approaches for interpretation of genotypic testing including rule-based algorithms and "virtual phenotypes" (Grant & Zolopa, 2009; Vercauteren & Vandamme, 2006). Rule-based algorithms are developed by expert panels based on large amounts of published data detailing correlations between genotype and phenotype, and correlations with treatment history and clinical response. These findings are translated into interpretation rules, or correlate the genotypic pattern with the clinical data (Poonpiriya *et al.*, 2008; Vercauteren & Vandamme, 2006). Currently, several sophisticated rule-based interpretation systems have been developed. Commonly used web-based genotypic interpretation systems include Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu), French National Agency for AIDS Research (ANRS) drug resistance interpretation algorithm (www.hivfrenchresistance.org), Rega Institute Drug Resistance Interpretation Algorithm (www.rega.kuleuven.be) and International AIDS Society–USA drug resistance mutations list (www.iasusa.org) (Grant & Zolopa, 2009; Vercauteren & Vandamme, 2006). These algorithms need to be updated frequently. These algorithms, which differ in their sources of information, contain different rules for interpretation and there are disagreements existing among different systems (Poonpiriya *et al.*, 2008). A draw-back of these rule-based algorithms is that they evaluate the susceptibility to each drug separately, not combinations of 3 or more drugs (Vercauteren & Vandamme, 2006).

"Virtual phenotype" is an algorithm based on mathematical models to predict phenotypic drug resistance and therapy response from genotype (Vercauteren & Vandamme, 2006). It uses a large genotypic-phenotypic correlative database to infer phenotypic properties based on sequence data by comparing the query sequence with all available sequences in its database linking the query sequence to most similar drug resistance mutation pattern (Vercauteren & Vandamme, 2006). The two algorithms of this method are Geno2pheno (Beerenwinkel, 2003) and VirtualPhenotype (Vermeiren *et al.*, 2007).

1.6.7.2 Phenotypic testing

Phenotypic resistance testing measures the susceptibility of the HIV-1 variant to specific drugs by measuring the ability of this variant to grow *in vitro* in the presence of increasing concentrations of antiretroviral drugs in comparison with the wild-type variant. Two commercial tests are available: PhenoSense HIV (ViroLogic, South San Francisco, California) (Petropoulos *et al.*, 2000) and Antivirogram (Tibotec-Virco, Mechelen, Belgium) (Hertogs *et al.*, 1998). Both assays use PCR to amplify PR and RT genes from patient's plasma HIV-1 RNA. The sequence is then inserted into a viral vector which is a laboratory strain of HIV with deleted PR and RT sequences (Grant & Zolopa, 2009). A cell line is then infected with the chimeric virus and the virus's ability to replicate is measured in the presence of varying concentrations of different antiretroviral drugs. The drug concentration that inhibits viral replication by 50% (IC50) is determined and the fold change in resistance is reported as the quotient between the IC50 of the patient's chimeric virus and the reference wild type virus. The interpretation

of the phenotype is based on defined clinical cut-offs for each drug. Clinical cut-off is the level of IC50 that virologic effectiveness can still be expected. The clinical cut-off has 2 cut-off values. The lower cut-off is the fold change at which the drug susceptibility declines but the drug still has partial activity. The upper cut-off is the fold change in which all drug activity is lost. For newly approved drugs where the clinical cut-off is not defined due to limited clinical data, a biological cut-off can be reported. A biological cut-off is based on the normal variation in fold change in wild-type virus (Grant & Zolopa, 2009).

The use of phenotypic assay in combination with genotypic assay was evaluated in the ERA trial in which patients experiencing treatment failure were enrolled (Dunn *et al.*, 2005). The results showed that phenotypic resistance testing in conjunction with genotypic testing did not add benefit compared to genotypic testing alone. There is no evidence that combination phenotypic and genotypic testing should be routinely used. Phenotypic assay is only recommended in patients who have viral isolates with complex genotypic resistance patterns that cannot reliably be interpreted by genotypic assay.

1.7 HIV infection in Viet Nam

1.7.1 Epidemiology

The first case of HIV infection in Ho Chi Minh City (HCMC) was recognized in 1990. Since then HIV has spread rapidly across the country. As of September 2014, HIV cases have been reported in all 63 provinces, 98% of districts and 77% of communes (Viet Nam MOH, 2014). The last updated number of people living with HIV/AIDS by

November 2012 is 224,223 and HCMC has the highest number of HIV cases (Viet Nam MOH, 2014). The overall HIV prevalence among adults and adolescents (age 15-49) was 0.45% in 2011 (UNAIDS, 2012). With the estimated number of people living with HIV/AIDS in Viet Nam for year 2014 being 256,500, there were 32,277 (13%) left undiagnosed. Nearly half of patients present late with CD4 less than 100 cells/μL (Rangarajan *et al.*, 2014; Tran *et al.*, 2012).The HIV-infected population in Viet Nam is very young and economically active with 75% of HIV cases among those aged 20-39 (Viet Nam MOH, 2014). 67.7% of reported cases in 2014 were among men (Viet Nam MOH, 2014). This is related to the fact that intravenous drug use is the most common route of transmission in Viet Nam and most injecting drug users (IDU) are men. However, the percentage of HIV infections in women is increasing from less than 20% in 2004 to more than 30% in 2011 (UNAIDS, 2012). The increasing proportion of women infected is related to a shift in the primary mode of transmission from intravenous drug use to heterosexual transmission. The HIV epidemic in Viet Nam remains concentrated primarily among three populations at higher risk of HIV transmission: IDU, Female sex worker (FSW) and Men who have sex with men (MSM) (UNAIDS, 2011). In 2009 IDUs accounted for 53% of recorded infections, FSWs accounted for 28% of recorded infections, and surveillance did not include data on homosexual transmission (UNAIDS, 2011). Similarly, route of transmission was reported in 2011 with 41.8% cases due to sexual transmission and 46.1% cases were through IDU transmission (Viet Nam MOH, 2013). A recent national report in 2012 has recorded for the first time that sexual transmission has become more significant and accounts for more infected cases than IDU transmission (45.5% compared to 42.1%) (Viet Nam MOH, 2013). The HIV prevalence

among the IDU population is 11% (Viet Nam MOH, 2013) with the highest prevalence found in HCMC (55.1% - data in 2009) (VAAC, 2009). The average prevalence among FSWs nationwide is 2.7% (Viet Nam MOH, 2013), and like IDUs, the rate differs by province with the highest prevalence in Can Tho (19%) (VAAC, 2009). HIV/STI Integrated Biological and Behavioural Surveillance 2009 showed that 16.7% of the MSM population are HIV positive (UNAIDS, 2010).

TB is one of the most common opportunistic infections, and the leading cause of death, among HIV-1 infected population in Viet Nam, with a high mortality rate (28.7%-34.2%) (Louie *et al.*, 2004; Ngo *et al.*, 2007). Other most frequently diagnosed opportunistic infections include oropharyngeal candidiasis, oesophageal candidiasis, herpes zoster, *Pneumocystis carinii* pneumonia, Cryptococcal meningitis, and penicilliosis (Louie *et al.*, 2004; Ngo *et al.*, 2007). Co-infection of viral hepatitis in the HIV population is a concern although there is a lack of national wide prevalence data for viral hepatitis and HIV co-infection in Viet Nam. In the Hospital for Tropical Diseases (HTD) in HCMC from January 2009 to June 2009 the prevalence of HBV co-infection was 6.6%, hepatitis C (HCV) co-infection was 42.1%, and HBV/HCV co-infection was 7.7% (Tho *et al.*, 2010). Data from an out-patient clinic of Bach Mai hospital (Northern Viet Nam) showed that HCV co-infection is 44%, HBV co-infection is 13.4%, and HBV/HCV co-infection is 7.3% of the HIV population (Huong *et al.*, 2004). IDUs have a high risk of HIV and hepatitis co-infection. Among the IDU population hospitalized at HTD in the year 2000 prevalence of HBV co-infection was 22% and HCV co-infection was 46% (Louie *et al.*, 2004). Research in some provinces has shown high prevalence of

HIV/HCV co-infection among the IDU population with a frequency of up to 92.2% (Ha, 2009; Huong *et al.*, 2004).

The major HIV subtype in Viet Nam is HIV-1 CRF01_AE (Lan *et al.*, 2003). Subtype CRF01_AE is highly prevalent in South-east Asia. It was first identified in Thailand and has further spread to surrounding countries like Viet Nam, Cambodia, Myanmar and China. CRF01_AE was first described as a new subtype, subtype E, but later many studies showed that this virus is actually a recombinant of subtype A with an independent "E" region (Carr *et al.*, 1996; Gao *et al.*, 1996). The subtype "E" sequences were found mostly in the env gene, parts of vif and vpr, as well as most of the LTR, whereas the remainder of the genome is of subtype A origin (Robertson *et al.*, 2000) (Figure 1.9). The phylogenetic and evolutionary analysis of 33 nearly full-length CRF01_AE sequences from Viet Nam indicates that HIV-1 CRF01_AE was introduced from Africa to Thailand in the late 1970s (Liao *et al.*, 2009). CRF01_AE in Viet Nam originated from Thailand, which points towards the transmission of CRF01_AE from heterosexuals to IDUs in southern Viet Nam in the late 1980s and dissemination among IDUs to Northern Viet Nam in the early or mid-1990s. The genetic variations in subtype CRF01_AE can affect HIV disease progression and ARV resistance development. Longitudinal studies of military recruits in Thailand showed that, in patients infected with subtype CRF01_AE, the median time from seroconversion to AIDS or CD4<200 cells/µL was 6.5 years and the median time to death was 8 years (Rangsin *et al.*, 2004, 2007). This is compared to a median time to AIDS of 8-11 years in multi-cohort CASCADE study (Babiker *et al.*, 2000). Variations in ARV resistance profile observed among CRF01_AE infections are reported. In ART-naïve patients, the prevalence of Y181C (33%) and

V75M (10%) were higher in CRF01_AE compared to other subtypes (Rhee *et al.*, 2015). In ART-experienced patients, Y181C was present in more than half of the CRF01_AE-infected patients with NVP exposure, and higher rates of the K65R mutations (13%) were detected in patients with d4T exposure compared to other HIV subtypes (Zolfo *et al.*, 2011). A study in China showed that mutations at sites 68, 69, 75, 101, 106, 179, 190, 210, 215 and 238 in RT region were more dominant in subtype CRF01_AE compared to subtypes B and CRF08_BC (Sui *et al.*, 2014). Natural polymorphisms were observed more frequently at PR positions 20, 63, 82, and 89 in CRF01_AE compared to other subtypes (Kantor *et al.*, 2005). More data of CRF01_AE subtype are needed to identify CRF01_AE-specific pattern of drug resistance and to study their impact on treatment outcome.

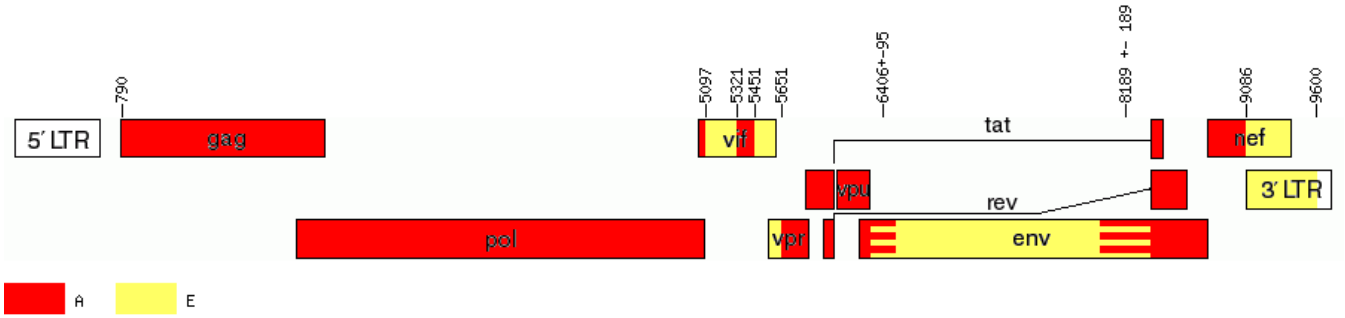


Figure 1.9: Subtype CRF01_AE structure.

Source: http://www.hiv.lanl.gov/

1.7.2 ARV Treatment and monitoring in Viet Nam

1.7.2.1 ART program in Viet Nam

Viet Nam has made significant advances in the response to HIV since the 2004 launch of "the National Strategy on HIV/AIDS Prevention and Control in Viet Nam until 2010 with a vision to 2020" and the establishment of the Viet Nam Administration for HIV/AIDS Control (VAAC) under the Ministry of Health (MOH) in 2000. Public awareness of HIV and risk behaviors, as well as of AIDS, drugs and prostitution prevention and control measures, has been improved. However, despite such encouraging indications of national commitment to funding and managing HIV-related activities, there are serious concerns regarding sustainability. Viet Nam's response to HIV/AIDS still relies heavily on international assistance. A considerable source of funding (73.7%) for the National AIDS response and majority of ARV drugs (95%) for HIV treatment in Viet Nam have been supported by international organizations (UNAIDS, 2012). The major donors are the President's Emergency Plan for AIDS Response (PEPFAR), the Global Fund to fight AIDS, TB and Malaria (GFATM); the UK Department for International Development , the Asian Development Bank and the World Bank (HIV and AIDS Data Hub for Asia Pacific, 2011). However international funds for HIV in Viet Nam have been decreased with the country's achievement of middle-income country status since 2011 coupled with global financial concerns. International donors have planned to end funding for the response or (as in the case of PEPFAR) will dramatically reduce the funds available; the Global Fund has postponed new grant applications due to a funding shortfall. There is a risk that the significant gains in HIV prevention, treatment and care will be lost (UNAIDS, 2012).

Since 2005 with the effort of national response, the ART program in Viet Nam has been rapidly scaled up. Adult ART coverage increased from an estimated 30% at the end of the 3rd quarter of 2007 to 45% in 2008 and 53.7% in 2009 (UNAIDS, 2010). By September 2012 there are 69,882 of HIV-infected people (66,167 adults and 3,715 children) receiving ART (Viet Nam MOH, 2013). Among patients who are taking ART, 96.82% are on first-line ART, 3.05% are on second-line treatment (Viet Nam MOH, 2013). Currently, WHO guidelines recommend that HIV infected patients start ART when their CD4 counts fall below 350 CD4 cells/μL (WHO, 2010a). Developed countries have changed their ART guidelines to recommend treatment irrespective of CD4 count or at treatment thresholds of 500 CD4 cells/μL (ASHM Sub-Committee for Guidance on HIV Management in Australia, 2013; Lebouche *et al.*, 2012; US Department of Health and Human Services, 2013; WHO, 2013a). In a revised version of Viet Nam - MOH guideline, the CD4 count threshold eligible for ART usage has increased from 200 cells/μL in 2005 (Viet Nam MOH, 2005) to 250 cells/μL in 2009 (Viet Nam MOH, 2009b) and to 350 cells/μL in 2011 (Viet Nam MOH, 2011).

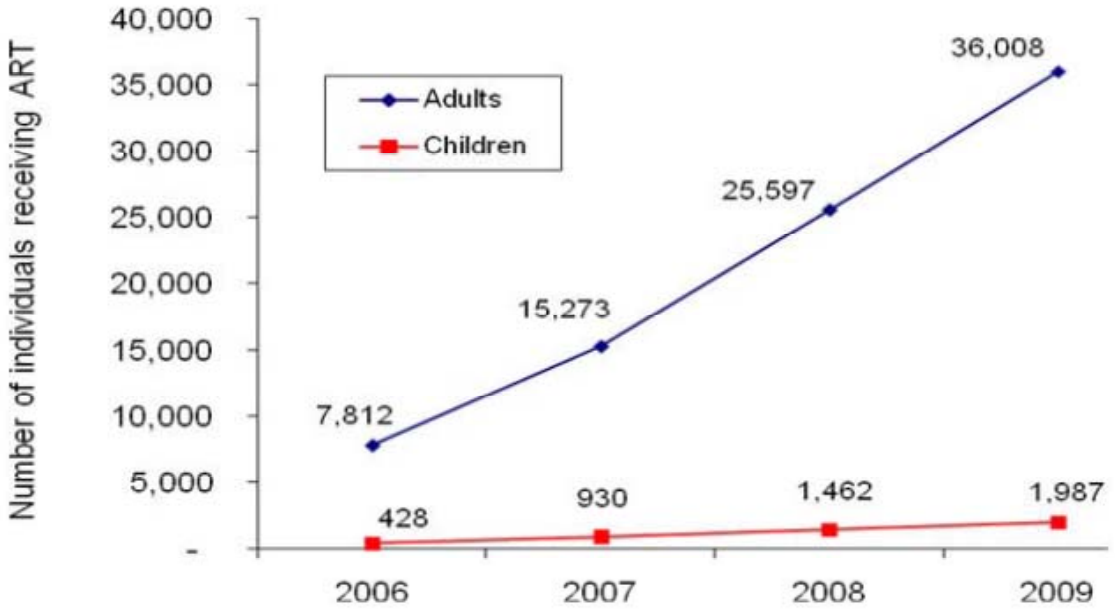


Figure 1.10: Number of adults and children on ART in Viet Nam from 2006 to 2009.

Source: National report 2009 (UNAIDS, 2010)

1.7.2.2 ART regimens in Viet Nam

First-line and second-line regimens in Viet Nam are summarized in Table 1.4. In general the first-line regimen includes 2 NRTIs + 1 NNRTI and second-line includes 2 NRTIs + 1 PI according to WHO guidelines. IDV was introduced in 2003 for patients who were intolerant to NVP as EFV was not available in Viet Nam until 2004. In 2007 NFV was withdrawn in Viet Nam and LPV/r is the only PI recommended by MOH. From 2008 a boosted dose for ritonavir was used in patients who were taking rifampicin for TB treatment. Currently there are no third-line options available in Viet Nam. D4T has been dropped from the list since 2011.

Table 1.4: Guidelines for first- and second-line regimens recommended by WHO and Viet Nam MOH

WHO recommendations			Time (year)	Viet Nam MOH guidelines		
CD4 cells/mL	First-line ART	Second-line ART		CD4 cells/mL	First-line ART	Second-line ART
≤200	Standard ART: AZT + 3TC + EFV/NVP Alternative ART: AZT + 3TC + ABC AZT + 3TC + PIr/NFV *PIr = IDVr, LPVr or SQVr*	ABC/d4T + ddI + PIr/NFV d4T + ddI + EFV/NVP/PIr d4T/ABC + ddI + EFV/NVP *PIr = IDVr, LPVr or SQVr*	2002[a] 2003[e]		AZT/d4T + 3TC + NVP AZT/d4T + 3TC + IDV	
≤200	Standard ART AZT/d4T + 3TC/FTC + EFV/NVP TDF/ABC + 3TC/FTC + EFV/NVP Triple NRTI AZT/d4T + TDF/ABC + 3TC/FTC	ddI/TDF + ABC/3TC (± AZT) + PIr ddI + PIr + EFV/NVP *PIr = ATVr, LPVr, FPVr, IDVr, SQVr*	2006[b] 2005[f]	≤200	D4T/AZT + 3TC + NVP/EFV	TDF/ABC + ddI + PIr/NFV *PIr = LPVr/SQVr*
≤350	AZT/TDF + 3TC/FTC + EFV/NVP	TDF/AZT + 3TC/FTC + PIr *PIr = ATVr, LPVr*	2010[c] 2009[g]	≤200	Standard ART: AZT/d4T + 3TC + EFV/NVP Alternative ART: TDF + 3TC + NVP/EFV	TDF + 3TC (± AZT) + LPVr ddI + ABC + LPVr ddI +ABC +LPVr AZT + 3TC + LPVr
≤500	Standard ART: TDF + 3TC/FTC + EFV Alternative ART: AZT/TDF + 3TC/FTC + EFV/NVP	AZT + 3TC + LPVr/ATVr TDF + 3TC/FTC + LPVr/ATVr	2013[d] 2011[h]	≤350	TDF/AZT + 3TC + EFV/NVP	AZT/TDF + 3TC + PIr *PIr = ATVr, LPVr*

[a](WHO, 2002); [b](WHO, 2006a); [c](WHO, 2010a); [d](WHO, 2013a); [e](Chi *et al.*, 2012); [f](Viet Nam MOH, 2005); [g](Viet Nam MOH, 2009b); [h](Viet Nam MOH, 2011)

1.7.2.3 Treatment monitoring

Viral load monitoring is not routinely performed in Viet Nam. Immunological (CD4) monitoring is accessed every 6 months and clinical monitoring is accessed every month. Immunological monitoring was shown to provide significant benefit in terms of disease progression and mortality compared to clinical monitoring alone, which resulted in earlier switching to second-line ART (DART Trial Team *et al.*, 2010; Mermin *et al.*, 2011). For patients with suspicion of immunological failure, the CD4 cell count should be re-tested after 3 months if patients are in clinical stage 1 and 2 classified by WHO, or therapy should be switched if patients are in clinical stage 3 and 4 (WHO, 2007). Targeted viral load testing may be performed for those patients. Criteria for virological/ immunological/ clinical failure follow the WHO guidelines (Viet Nam MOH, 2009b).

1.7.3 HIV drug resistance and treatment outcome in Viet Nam

From 2003-2009 the prevalence of HIV drug resistance in antiretroviral-naïve individuals in Viet Nam was reported as ranging from 2.9-7.6%. Small studies which used the WHO threshold survey method among low-risk populations reported low prevalence (< 5%) (Ayouba *et al.*, 2009; Nguyen *et al.*, 2008a), while larger studies with a more general population reported higher prevalence (6%) (Dean *et al.*, 2011; Lan *et al.*, 2003; Phan *et al.*, 2010). Ha Noi and Northern Viet Nam (Ishizaki *et al.*, 2009; Nguyen *et al.*, 2008a; Phan *et al.*, 2010) reported lower prevalence than HCMC and southern Viet Nam (Lan *et al.*, 2003). In HCMC the prevalence of HIV drug resistance was reported at 6.5% amongst 200 ART-naïve individuals (43% IDUs and 38% CSWs) from 2001-2002,

a period when ART was not yet widely available (Lan *et al.*, 2003). The pattern of pre-existing drug resistance mutations detected in Viet Nam varied. Among ARV drug classes, resistance to NRTI was more common than NNRTI and PI drug classes, ranging between 4.8% to 6.5% in 2008-2009. Prevalence of resistance to NNRTI ranged from 1.6% to 6% and resistance to PI was found less than 2% throughout the same period (Pham *et al.*, 2013).

A systematic review of first-line ART outcome among resource limited settings was conducted by WHO in 2007 (Renaud-théry *et al.*, 2010). The overall rate of treatment failure per 100 patient years of follow-up was 1.9 (95%CI 1.48-2.38) when using immunological and/or clinical criteria and 6.08 (95%CI 4.51-7.66) when using virological criteria. Rate of failure varies by regions and Asia has lowest rate of treatment failure; however, only 2 Asian countries (China and Cambodia) were included in this systematic analysis. Another observation cohort study in Asia showed that the clinical failure rate to first-line ART in Asia is 7.3 per 100 person-years (Zhou *et al.*, 2007). No data from Viet Nam were included. Data on the outcome of first-line ART in Viet Nam are limited. At the HTD in HCMC, the rate of immunological failure to first-line ART is approximately 14.5% (Chinh *et al.*, 2010). Virological suppression with viral load below limit of detection (250 copies/mL) reported in HTD was 83.1% after one year of first-line treatment (Quang *et al.*, 2011) and in public clinics in southern Viet Nam was 70% (Trinh *et al.*, 2011). Virological suppression rate in Northern Viet Nam after one year of treatment was 72%, similar to that of southern Viet Nam (Cuong *et al.*, 2012). As routine use of viral load monitoring and genotypic testing are not available in Viet Nam, decision of therapy switching is normally based on immunological and clinical failures which

have been shown to be neither sensitive nor specific for identification of virological failure. Studies in HCMC and Ha Noi (the two largest cities in Viet Nam with referral hospitals for HIV/AIDS treatment) showed that among patients who had treatment failure based on clinical/immunological criteria, approximately 60% had viral load above 1000 copies/mL (Giang *et al.*, 2008; Lien *et al.*, 2009; Nguyen *et al.*, 2008b; Nhung *et al.*, 2008; Tanuma *et al.*, 2008). The National response progress report in 2012 reported that HIV patients in Viet Nam are starting ART late, with a very low CD4 cell count, which is associated with high mortality and severe opportunistic infections. A total of 52.7% of those who started ART in 2010 had a CD4 count of less than 100 cells/μL (1,711 out of 3,247 people sampled) (UNAIDS, 2012). In a study of 889 patients by Chinh *et al*., those who started ART late when their CD4 count was <100 cells/μL or they were WHO clinical stage 3 or 4, had a 5% higher risk of death (Chinh *et al.*, 2010). The retention rate after one year of treatment in this study was high (88.6%). This result was consistent with a national survey from 30 out-patient clinics which reported the rate of HIV patients alive and receiving ART after 6, 12, 24, and 36 months in Viet Nam were 88.4% (CI: 86.8–89.9), 84.0% (CI: 81.8–86.0), 78.8% (CI: 75.7–81.6), and 74.6% (CI: 69.6–79.0) (Nguyen *et al.*, 2013). The median CD4 increased to 241 and 344 cells/μL after one and two year of treatment respectively (Chinh *et al.*, 2010). Among patients who have virological failure, more than 90% had at least one drug resistant mutation at the time of virological failure. Prevalence of resistance to NRTI is high (more than 85%) in both HCMC and Ha Noi, yet resistance to NNRTI in Ha Noi (45.5%) (Tanuma *et al.*, 2008) was less frequent than in HCMC (above 85%) (Chi *et al.*, 2008; Giang *et al.*, 2008; Lien *et al.*, 2009; Nguyen *et al.*, 2008b; Nhung *et al.*, 2008).

There have been a few number of studies published on second-line virological outcome in resource limited countries (Ajose *et al.*, 2012; Castelnuovo *et al.*, 2009; Ferradini *et al.*, 2011; Fox *et al.*, 2010; Levison *et al.*, 2012; Siripassorn *et al.*, 2010; Win *et al.*, 2011). The rate of virological failure in a resource limited setting was reported at 26.7% after 2 years of second-line ART and mortality at 12 months ranged from 5.27% (95% CI: 3.31–8.38%) to 10.49% (95% CI: 6.68–15.04%) (Ajose *et al.*, 2012; Fox *et al.*, 2010; Siripassorn *et al.*, 2010). Till September 2012 there are 2,134 patients on second-line therapy in Viet Nam (Viet Nam MOH, 2013). As virological assessment is not routinely performed, virological failure is generally identified during the later phase of treatment failure. Continuation of taking non-suppressive ART regimens results in accumulation of drug resistance mutations. The presence of such specific mutations can reduce the susceptibility of all NRTI drugs used in second-line regimens, rendering a mono-therapy situation in these patients with LPV/ritonavir being the only effective drug and leading to a higher risk of second-line ART failure. However there is no data available on the outcomes of the second-line regimes and the development of drug resistance mutations among patients experienced virological failure to current second-line ART in Viet Nam.

1.8 Study objectives

Scaling up access to ART is part of the national response to the HIV/AIDS epidemic in Viet Nam. Increasing the availability of ART leads to a rise in life quality and life span for HIV infected patients, as well as prevention of HIV transmission in developing countries like Viet Nam. However the widespread ARV drugs can also lead to

the development of drug resistant virus under selective pressure and the increase of prevalence of TDR. TDR has been a concern since the ART roll-out program in 2005 in Viet Nam. Monitoring prevalence of TDR during ARV rolling out is an important public health talk to balance between preventing the HIV transmission and increasing the burden of drug-resistant strains.

Second-line therapy is the last option of HIV treatment in Viet Nam and data on second-line treatment is not available. The efficacy of second-line treatment in Viet Nam may be different from other countries due to the difference in subtype distribution. In Viet Nam, majority of HIV-1 cases are infected by subtype HIV-1 CRF01_AE. Furthermore viral load is not routinely monitored during HIV treatment in Viet Nam, delayed therapy switching among patients who failed first-line ART is common due to late detection of treatment failure. In addition, with the planned dramatic cut-off in foreign funding for HIV care, the availability of ARV drugs will reduce and the number of patients retained on ARV treatment is estimated to drop more than 7-fold from 69,882 in 2012 to 10,000 in 2016 (Nhan, 2013). The need of having data on the second-line treatment efficacy and predictors of treatment outcome is essential for second-line regimens preservation and third-line preparation.

The primary objectives for this thesis involve:

1. To investigate the prevalence and patterns of transmitted HIV drug resistance (TDR) mutations in ART-naïve patients in HCMC, Viet Nam (Chapter 3)
2. To study immunological and clinical outcome and outcome predictors of second-line ART in HCMC, Viet Nam (Chapter 4)

3. To investigate virological outcome, predictors of virological outcome and patterns of drug resistance development to second-line ART (Chapter 5)

2 METHODS

2.1 Study sites

2.1.1 Hospital for Tropical Diseases

The main location for study enrollment in this thesis was the HTD in HCMC. This is the largest primary, secondary and tertiary hospital for infectious diseases in southern Viet Nam with 630 beds for in-patient care, serving the HCMC community, providing specialized consultative care and acting as a referral centre for HIV in southern Viet Nam. It was the first hospital to identify the first case of HIV in Viet Nam and the first hospital to provide HIV care and treatment for people living with HIV/AIDS in Viet Nam. It provides in-patient and out-patient service for approximately 5,000 people living with HIV/AIDS. More than 3,500 patients are on ART. Majority of cases suspected for treatment failure to first-line ART in southern Viet Nam are referred to HTD for failure confirmation and second-line therapy switch consultation. The number of patients receiving second-line therapy in HTD accounts for 8% of those who are on ART.

2.1.2 Pham Ngoc Thach Hospital for tuberculosis and lung diseases

The patients from the HIV-associated tuberculosis meningitis (TBM) clinical trial (chapter 3) were enrolled from both HTD and Pham Ngoc Thach Hospital. Pham Ngoc Thach Hospital is a tertiary referral center for TB, with 750 beds for in-patient and 900 for out-patient care. The hospital leads the National TB program in southern Viet Nam, directing and monitoring TB treatment and control. It also provides TB treatment for HIV positive patients referred from HTD.

2.2 Molecular-based methods

2.2.1 Quantification of HIV by Abbott Real-Time HIV-1 m2000rt

The Abbott Real-Time HIV-1 assay (Abbott Molecular, Germany) is a FDA-approved real-time nucleic acid amplification assay available for HIV-1 viral load quantitation. The target sequence for the Abbott Real-Time HIV-1 assay is a fragment of 172 nucleotides from the highly conserved region of the integrase gene of the HIV-1 genome (Tang *et al.*, 2007). This assay can detect diverse HIV-1 groups/subtypes: group M (subtypes A, B, C, D, G, H, CRF01_AE, and CRF02_AG), group O and group N (Abbott Laboratories, 2007).

200 µL of plasma or EDTA-blood samples were used as input for the assay and RNA extraction was performed using the Abbott m2000sp™ Automated Sample Preparation System (m2000sp; Abbott Molecular Inc.). Samples were treated with mLysis buffer to lyse the viral coat and release the nucleic acid. A RNA sequence that is unrelated to the HIV-1 target sequence was used as internal control and was added to the lysis buffer before the treatment of the plasma/blood samples to ensure correct extraction and non-inhibition of the reverse transcription polymerase chain reaction (RT-PCR). A negative control, a low positive control, and a high positive control were included in each run. The lysed viral and internal control RNA was captured on magnetic beads followed by several washing steps to remove contaminants and inhibitors of RNA. Pure RNA was then eluted and detected using quantitative real-time RT-PCR. 50 µL of extracted RNA was mixed with 50 µL of amplification master mix (containing thermostable rTth polymerase enzyme, MnCl2, primers, probes, and dNTPs) in m2000sp before being transferred to the Abbott m2000rt™ instrument (m2000rt; Abbott Molecular Inc.) for

reverse transcription, PCR amplification, and detection/quantitation (Tang *et al.*, 2007). The probe was a partially double-stranded complex with fluorophore 6-FAM labeled at the 5′ end of the long strand (which is complementary to HIV-1 target) and Black Hole Quencher 1 (BHQ1) labeled at the 3' end of the shorter strand (which is the quencher oligonucleotide). In the absence of the HIV-1 target, the HIV-1 specific probe fluorescence was quenched through hybridization to the quencher oligonucleotide. In the presence of the HIV-1 target sequence, the HIV-1 specific probe preferentially hybridized to the target sequence, allowing for fluorescence detection (Figure 2.1) (Swanson *et al.*, 2006; Tang *et al.*, 2007). The internal control probe was a single-stranded DNA oligonucleotide labeled with the fluorophore VIC at the 5′ end and BHQ1 at the 3′ end. Different fluorophores labeling HIV-1 and internal control probes allowed simultaneous detection of both amplified products at each cycle. Reverse transcription and PCR amplification were achieved with following thermal cycling conditions (Tang *et al.*, 2007):

i. 1 cycle at 59^{o}C for 30 minutes

ii. 4 cycles at 95^{o}C for 40 seconds and 46^{o}C for 30 seconds;

iii. 6 cycles at 92^{o}C for 30 seconds and 60^{o}C for 30 seconds;

iv. 37 cycles at 92^{o}C for 30 seconds, 56^{o}C for 20 seconds plus 2 seconds auto-increments per cycle and 35^{o}C for 40 seconds.

v. Fluorescence measurements were recorded during the 35^{o}C step of the last 37 cycles.

The assay detected a dynamic range of viral RNA ranging from 150 to 10,000,000 copies/mL.

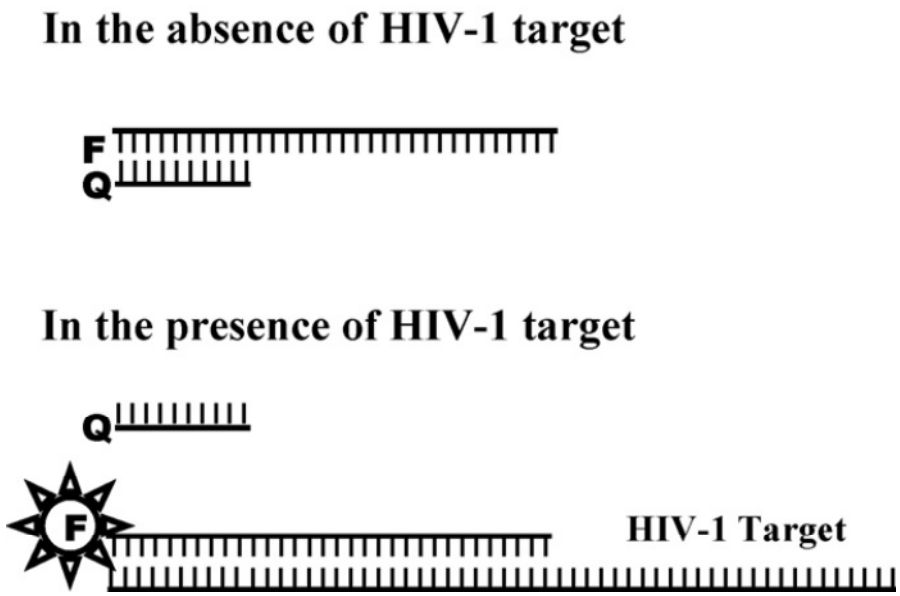


Figure 2.1: Description of the HIV-1 partially double-stranded linear probe.

The "F" on the 5'end of the probe represents the fluorophore FAM on the HIV-1 probe. The "Q" on the 3'end of the quencher oligo represents the quencher molecule BHQ1. Source: Tang *et al*. 2007

2.2.2 In-house drug resistance testing

2.2.2.1 Viral RNA extraction

Nucleic acid from plasma samples was extracted using either an automated guanidinium-thiocyanate extraction method nucliSENs EasyMAG (Boom *et al.*, 1990; Loens *et al.*, 2007) (Nuclisens, Biomerieux, Boxtel Netherlands) or Qiagen viral RNA mini kit (Qiagen, Hilden, Germany). Automated extraction by the nucliSENS EasyMAG system was performed for chapter 3 and manual extraction by Qiagen was performed for chapters 4 and 5.

The nucliSENS EasyMAG system automatically extracts HIV RNA based upon the silica extraction technology or BOOM method (also developed by Biomerieux research team). In brief, samples were lysed and nucleic acids were captured by magnetic silica particles. Then nucleic acids binding to silica were purified through several washing steps and eluted in elution buffer. RNA was finally separated from silica after a heating step.

The Qiagen viral RNA mini kit was used per manufacturer's protocol. This protocol is based on both the selective binding properties of a silica gel-based membrane and the speed of micro spin. In brief, samples were lyzed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA in a mixture of AVL lysis buffer and an RNA carrier. RNA was loaded and bound specifically to the QIAamp silica membrane. Contaminants and PCR inhibitors, such as divalent cations and proteins, were completely removed by 2 different wash buffers: guanidium chloride containing AW1 buffer and 70% Ethanol (EtOH) containing AW2 buffer. Pure RNA is then eluted in water or low-salt buffer.

2.2.2.2 Reverse transcription

RNA was eluted into 60 µL of provided elution buffer and 10 µL was used immediately for HIV genotyping. HIV genotype testing was performed according to an in-house assay described by Bezemer (Bezemer *et al.*, 2004), with some modifications.

HIV RNA was reverse transcribed into cDNA with reagents described in Table 2.1. Briefly 10 µL of reverse transcription master mix was added to 10 µL of HIV RNA.

The master mix consisted of First Strand buffer, DTT, dNTP, specific 3' RT-out primer, RNAse inhibitor (RNAse out), reverse transcriptase enzyme (SuperScript III RT) and RNA-free water. The reaction was incubated at 37^{o}C for 2 hours followed by 95^{o}C for 5 minutes to inactivate the reverse transcriptase enzyme.

Table 2.1: Reagents for reverse transcription PCR

Reagent	Stock concentration	Final concentration	Volume for 1 reaction	Supplier
First Strand Buffer	5X	1X	4 µL	Invitrogen
Primer 3'RT out	1 µM	0.1 µM	2 µL	Sigma
DTT	0.1 M	0.005 M	1 µL	Invitrogen
dNTP	25 mM each	0.5 mM each	0.4 µL	Roche
RNAse out	40 U/µL	16 U	0.4 µL	Invitrogen
SuperScript III RT	200 U/µL	60 U	0.3 µL	Invitrogen
H_2O			1.9 µL	Sigma

2.2.2.3 Amplification of complete PR and partial RT region

Nested polymerase reaction chain (PCR) was used to amplify approximately 1200 base-pairs comprising the complete 297 nucleotides in the protease (PR) region and the first 897 nucleotides in the reverse transcriptase (RT) region of *pol*. 20 µL of cDNA was added to a 30 µL mixture of first-round PCR reagents containing HiFidelity buffer (with dNTP), forward primer 5'PROT-I, reverse primer 3'ET21, HiFidelity Taq, and distilled

water. Nested PCR was done using 5 μL of the first round PCR product. Mixture of second-round PCR reagents contained HiFidelity buffer (with dNTP), forward primer 5'PROT-II, reverse primer 3'RT20, MgSO4, HiFidelity Taq, and distilled water. Details on primer sequences and concentration of each reagent were listed in Table 2.2 and Table 2.3. Thermal cycling conditions for first- and second-round PCR were listed in Table 2.4.

Table 2.2: Primers for reverse transcription and PCR amplification

RT-PCR primers	Orientation	Sequence	Position on HIV-1 BRU
RT out	R	TCTACTTGTCCATGCATGGCTTC	3953-3964
Prot-I	F	AGGCTAATTTTTTAGGGAAGATCTGGCCTTCC	1624-1655
ET-21	R	AGCTGGCTACTATTTCTTTTGCTACTACAGGTGG	3896-3929
Prot-II	F	TCAGAGCAGACCAGAGCCAACAG	1718-1740
RT-20	R	CTGCCAGTTCTAGCTCTGCTTC	3023-3044

All primers are shown 5' to 3'. F=forward primer; R=reverse primer

Table 2.3: Reagents for PCR amplification

	Reagent	Stock concentration	Final concentration	Volume for 1 reaction	Supplier
PCR 1	HiFidelity buffer	5X	1X	10 µL	Qiagen
	5'PROT-I	100 µM	1 µM	0.5 µL	Sigma
	3'ET21	100 µM	1 µM	0.5 µL	Sigma
	HiFidelity Taq	2.5 U/µL	3.33 U	1.33 µL	Qiagen
	H_2O to 50 µL			17.67 µL	Sigma
PCR 2	HiFidelity buffer	5X	1X	10 µL	Qiagen
	5'PROT-II	100 µM	1 µM	0.5 µL	Sigma
	3'RT20	100 µM	1 µM	0.5 µL	Sigma
	MgSO4	25 mM	0.5 mM	1 µL	Qiagen
	HiFidelity Taq	2.5 U/µL	1.25 U	0.5 µL	Qiagen
	H_2O to 50 µL			17.67 µL	Sigma

Table 2.4: Thermal cycles for nested PCR

(a) first- (b) second-round of PCR amplification

First-round PCR	
	5 min 95°C
35 cycles	15 sec 94°C
	1 min 55°C
	4 min 72°C
	10 min 72°C
	Hold at 10°C

(a)

Second-round PCR	
	5 min 95°C
30 cycles	15 sec 94°C
	1 min 55°C
	2 min 72°C
	10 min 72°C
	Hold at 10°C

(b)

Agarose gel electrophoresis was used to confirm the correct size of the PCR products. The PCR products mixed with 6x DNA loading dye Orange G (Sigma) and 1kb plus DNA ladder (Invitrogen) were run on 1% agarose gel (including 1% w/v DNA graded agarose dissolved in 1X Tris-borate-EDTA (TBE) buffer, together with 3% ethidium bromide for gel staining) for 45 minutes at 150Volts . Gel was then visualized under UV light on UV transilluminator and photographed using Quanlity One UVTech DNA documentation program.

2.2.2.4 Pol gene sequencing

A. Purification of PCR products

The PCR products were purified using the QIAQuick PCR Purification kit (Qiagen) following the instructions from manufacturer to remove remaining dNTPs, primers, Taq, and Mg ion. Briefly the 45 μL of PCR products were applied to QIAquick spin column and bound to silica-gel membrane through centrifugation for 1 minute at 13,000rpm. Contaminants and impurities were removed through washing with ethanol-containing buffer PE. DNA was eluted in 50 μL of distilled water.

B. DNA quantification

Concentration of purified DNA was measured by the absorbance at 260 nm using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies), a cuvette free spectrophotometer that required 2 μL of sample for quantification. Distilled water was used as a blank. The concentration of DNA was calculated by the computer based on the following formula: c = (A260 x 50)/0.1. Where c is the nucleic acid concentration in ng/μL, A260 is the absorbance, and the path length is 0.1 cm. The wavelength-dependent extinction coefficient of dsDNA is 50 ng-cm/μL.

C. PCR reaction for sequencing

Purified PCR product was used for amplification before sequencing. The DNA concentration for PCR was around 20-30 ng/μL. 0.5 μL DNA was used for 10 μL of PCR reaction. Each sequencing PCR reaction contained 5X Sequencing Buffer, 2.5X Big dye Premix, 2 μM of each primer, and distilled water up to 10 μL. Sequencing Buffer and Big dye Premix were supplied in Bigdye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). 3 forward primers and 3 reverse primers were used to generate overlapping readable regions after sequencing. Primer sequences were listed in table 2.5. The thermal cycling was performed as following program:

i. 1 cycle of 96°C for 1 minute

ii. 35 cycles of 96°C for 10 seconds, 55°C for 5 seconds, and 60°C for 4 minutes

iii. Held at 10°C

Table 2.5: Primers of PCR amplification for sequencing

Sequencing-PCR primers	Orientation	Sequence
PR5	F	AGCCAACAGCCCCACCAG
PR2	R	CTTTTGGGCCATCCATTC
RT-19new	F	CACCTGTCAACATAATTGGAAG
B-Rtrev	R	GGTGATCCTTTCCATCCC
B-RT	F	GGGATGGAAAGGATCACC
RT-20	R	CTGCCAGTTCTAGCTCTGCTTC

All primers are shown 5’ to 3’. F=forward primer; R=reverse primer

D. Ethanol precipitation and DNA sequencing

Excess reagents were removed from the sequencing PCR products by ethanol precipitation. Briefly, 2 μL of stop solution (composing 1 μL of 3M NaOAc and 1 μL of 125mM EDTA) was added to 10 μL of reaction. The solution was mixed thoroughly before and after the addition of 50 μL of cold 100% ethanol (Merck, Darmstadt, Germany). The DNA pellet was collected after centrifugation at 1800g, 4^{o}C for 45 minutes in a centrifuge Eppendorf 5810R (Eppendorf AG, Hamburg, Germany). DNA was then washed with 70 μL of cold 70% ethanol followed by centrifugation at 1800g, 4^{o}C for 15 minutes. Excess 100% and 70% ethanol was removed by brief centrifugation of the inverted plate at 185g. The DNA pellet was allowed to dry for 15 minutes and then was re-hydrated in 10 μL of sample loading buffer HiDi Formamide (Applied Biosystems, Foster City, CA, USA). DNA was incubated for at least 15 minutes at room temperature before being loaded onto a plate for sequencing in the ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

E. Sequencing analysis

DNA sequences were generated using ABI Prism 3130xl Genetic Analyzer. The sequences were aligned and analyzed using SeqScape v2.5 (Applied Biosystems, Foster City, CA, USA). Nucleotide changes were determined by comparison with the consensus sequence pNL4-3 for HIV-1 subtype B (GenBank accession number M19921).

2.2.2.5 Drug resistance mutations interpretation

A. Transmitted drug resistance mutations

HIV drug resistance mutations in ART naïve patients were determined according to the 2009 WHO surveillance drug resistance mutations (SDRMs) list (Bennett *et al.*, 2009). This is a standard list of mutations designed to provide a possibility to compare the prevalence of transmitted resistance from different times and regions and facilitate meta-analyses of surveillance data collected by different groups at different times. Some drug resistance mutations occur commonly without the presence of selective drug pressure, hence using other algorithms (such as IAS-USA, Stanford HIV database, ANRS) which include polymorphic mutations can over-estimate the levels of transmitted resistance. Transmitted HIV drug resistance in a patient was defined as the presence of at least 1 SDRM. Sequences having a mixture of wild-type and mutant residues at a given position were considered to have a mutation at that position.

B. Acquired drug resistance mutations

Mutations in followed-up samples at the time of virological failure were determined according to the International AIDS Society (IAS)-USA list (Johnson *et al.*, 2013). The IAS-USA list was developed by an independent, volunteer panel of experts. They review data on HIV drug resistance to identify and maintain a list of mutations associated with clinical resistance to HIV. This list is not developed to be used in epidemiologic analyses to identify TDR but rather for listing relevant mutations or

polymorphisms that are associated with a reduced susceptibility to antiretroviral drugs. A mutation was classified to confer resistance if it was defined as a "major" resistance-associated mutation. The drug susceptibility was estimated by Stanford HIV Drug Resistance database (Liu & Shafer, 2006). For Stanford HIV drug resistance algorithm the sequence information was entered into the database via the website of the Stanford University as nucleic acid sequence or via a mutation list (http://sierra2.stanford.edu/sierra/servlet/JSierra). The program, called HIV-SEQ (for 'HIV mutation search engine for queries') compared submitted HIV-1 sequence to a consensus subtype B reference sequence (Shafer *et al.*, 2000). The algorithm assigns a drug penalty score for each drug resistance mutation and a negative score for a mutation that causes hyper-susceptibility to a drug or is associated with reversion of resistance. The program uses the total drug score to infer the low-, intermediate- and high-level resistance to HIV drugs.

C. *HIV-1 sub-typing of protease and reverse transcriptase sequences*

HIV-1 subtype was determined by phylogenetic analysis of the RT and PR genes using the neighbor-joining algorithm integrated in the Molecular Evolutionary Genetics Analysis (MEGA4) software (Tamura *et al.*, 2007). Reference sequences of the *pol* gene from all available subtypes were obtained from the Los Alamos National Laboratory database (http://www.hiv.lanl.gov/). Phylogenetic analysis was further confirmed by the Stanford database classification system (Liu & Shafer, 2006).

3 HIV-1 drug resistance in antiretroviral-naïve individuals with HIV-associated tuberculous meningitis initiating antiretroviral therapy in Viet Nam

3.1 Abstract

Access to ART for Human Immunodeficiency Virus (HIV)-infected individuals in Viet Nam is rapidly expanding, but there are limited data on HIV drug resistance to guide ART strategies. This chapter presents data from a retrospective cohort of 220 ART-naive individuals recruited to a randomized controlled trial of immediate versus deferred ART in individuals with HIV-associated tuberculous meningitis (TBM) in HCMC from 2005-2008. HIV drug resistance testing was retrospectively performed among these individuals using frozen plasma samples. HIV drug resistance mutations were identified by population sequencing of the HIV *pol* gene and were defined based on 2009 WHO SDRMs. In this study we successfully sequenced 219/220 plasma samples from subjects prior to ART initiation. 218 were subtype CRF-01AE; one was subtype B. SDRMs were identified in 14/219 (6.4%) subjects. 8/14 were resistant to NRTI (T69D, L74V, V75M, M184V/I, K219R), 5/14 to NNRTI (K103N, V106M, Y181C, Y188C, G190A], 1/14 to both NRTI and NNRTI (D67N, Y181C), and none to PI. After 6 months of ART, 8 developed protocol-defined virological failure. HIV drug resistance mutations were identified in 5/8 subjects. All 5 had mutations with high level resistance to NNRTI; 3 had mutations with high level resistance to NRTI. Due to a high early mortality rate (58%), the effect of pre-existing HIV drug resistance mutations on treatment outcome could not be accurately assessed. In conclusion the prevalence of WHO SDRMs in ART-naive

individuals with HIV-associated tuberculous meningitis in HCMC from 2005-2008 is 6.4%. The SDRMs identified conferred resistance to NRTI and/or NNRTI reflecting the standard first-line ART regimens in Viet Nam.

3.2 Introduction

HIV is able to develop resistance to all currently-licensed antiretroviral drugs, posing threats to the sustainability of ART globally. Drug-resistant virus can be transmitted and/or acquired and can persist in the population (Barbour *et al.*, 2004; Little *et al.*, 2002; Pao *et al.*, 2004), leading to treatment failure, disease progression and death (Hogg *et al.*, 2006; Kozal *et al.*, 2007; Poggensee *et al.*, 2007; Wittkop *et al.*, 2011). Prevalence of TDR in acutely or recently infected individuals in developed countries ranges from 7-24% (Jain *et al.*, 2010; Sagir *et al.*, 2007; UK Drug Resistance database, 2003; Vercauteren *et al.*, 2008). Baseline HIV drug resistance testing has been shown to be cost effective in the United States when TDR prevalence exceeds 5% (Sax *et al.*, 2005) and is routinely performed to guide initial antiretroviral choices in resource-rich settings (Gazzard *et al.*, 2008; Panel on Antiretroviral Guidelines for Adults and Adolescents, 2014). As ART is being scaled up in low- and middle-income countries, where >90% of the world's HIV-infected population reside and ART options are limited, increased surveillance for TDR to inform ART programs in resource-limited settings is essential.

The HIV epidemic in Viet Nam started in 1990 and is still concentrated amongst the high-exposure risk groups. The overall HIV prevalence in individuals aged 15-49

years is 0.5%; however prevalence is 18.4% in IDU, 16.7% in MSM, and 3.2% in commercial sex workers (CSW) (UNAIDS, 2010). ART was initially introduced in Viet Nam in the mid-1990s through private donations with intermittent drug supply and through the black market with high prices, so treatment interruption was likely common. In addition, NNRTI and PI were not available or too expensive during the early 2000s, hence dual therapy regimens with AZT and 3TC or d4T and ddI were commonly used. With the effort of the Viet Nam National ART program supported by international funding (such as US President's Emergency Plan for AIDS Relief and the Global Fund to Fight AIDS, Tuberculosis and Malaria), the ART coverage has increased from 1% to 53.7% of individuals who meet the Viet Nam MOH criteria to initiate ART from 2003-2009 [CD4 count <200 cells/μL and WHO disease stage III or IV] (UNAIDS, 2010). Both the history of non-suppressive ART use before 2005 and the rapid ART scale up since 2005 are expected to be accompanied by the emergence of acquired and TDR in Viet Nam. From 2003-2009 6 studies have reported prevalence of HIV drug resistance in antiretroviral-naïve individuals ranging from 2.9%-7.6% in Viet Nam (Ayouba *et al.*, 2009; Dean *et al.*, 2011; Ishizaki *et al.*, 2009; Lan *et al.*, 2003; Nguyen *et al.*, 2008a; Phan *et al.*, 2010). Differences in reported prevalence may reflect differences in populations and time periods studied, in survey methods and drug resistance algorithms used, and in geographic and sample size factors, thereby making comparison and estimation problematic. For example, small studies using the WHO threshold survey method (Bennett *et al.*, 2008), which sampled relatively lower-HIV-exposure populations such as first-time pregnant women at antenatal clinics or attendees aged <25 years at HIV Voluntary Counseling and Testing (VCT) centers, reported lower prevalence (<5%)

(Ayouba *et al.*, 2009; Nguyen *et al.*, 2008a), while larger studies that sampled the general population attending HIV clinics reported higher prevalence (>6%) (Dean *et al.*, 2011; Lan *et al.*, 2003; Phan *et al.*, 2010). Studies from Ha Noi and surrounding provinces tend to report lower prevalence (Ishizaki *et al.*, 2009; Nguyen *et al.*, 2008a; Phan *et al.*, 2010) compared to a study from HCMC (Lan *et al.*, 2003). The latter study reported a prevalence of 6.5% of HIV drug resistance mutations amongst 200 ART-naïve individuals (43% IDUs and 38% CSWs) from 2001-2002, a period when ART was not yet widely available (Lan *et al.*, 2003). In this study we investigate the prevalence and impact of HIV drug resistance mutations in ART-naïve individuals who enrolled in a randomized clinical trial comparing immediate versus delayed ART for HIV-associated TBM in HCMC from 2005-2008 (Török *et al.*, 2011).

Table 3.1: Prevalence of transmitted drug resistance in Viet Nam over time

Sample year	Location	Study group	Stage of infection	Age	Samples size	Genotypes (RT/PR)	Prevalence of HIV TDR	References
2001-2002	HCMC	CSW, IDU, and STD patients	chronic infection	23	200	200/200	13 (6.5%)	(Lan *et al.*, 2003)
2006	HCMC	VCT attendees	proxy recent infection	22	63	63/63	2 (3.2%)	(Ayouba *et al.*, 2009)
2006	Ha Noi	VCT attendees	proxy recent infection	23	70	49/49	1 (<5%)	(Nguyen *et al.*, 2008a)
2007	Hai Phong	CSW, IDU, pregnant women, blood donor	chronic infection		301	273/294	8 (2.9%)	(Ishizaki *et al.*, 2009)
2008	Ha Noi, Ninh Binh, Nam Dinh		chronic infection	32	206	155/173	10 (6.5%)	(Phan *et al.*, 2010)
2008-2009	Ha Noi, Hai Phong, Da Nang, Khanh Hoa, Can Tho	CSW, IDU	chronic infection	29	122	92/92	7 (7.6%)	(Dean *et al.*, 2011)

3.3 Methods

3.3.1 Study settings and population

This study was a retrospective study and included ART-naïve individuals who enrolled in a randomized clinical trial comparing immediate versus delayed ART for HIV-associated TBM in HCMC from 2005-2007 (Török *et al.*, 2011). Briefly this clinical trial was conducted at two specialist centers for TB and HIV: Pham Ngoc Thach Hospital for TB and Lung Disease and HTD in HCMC. 253 antiretroviral-naïve subjects with HIV-associated TBM were enrolled in a randomized, double-blind, placebo-controlled trial of immediate (initiated ≤7 days after commencement of TB treatment) versus deferred (initiated after 2 months of TB treatment) ART. Subjects received standard anti-TB therapy (isoniazid, rifampicin, pyrazinamide, and ethambutol) according to Vietnamese national guidelines. Unless contraindicated, all subjects also received adjunctive dexamethasone at an initial dose of 0.3-0.4mg/kg/day, according to modified Medical Research Council TB meningitis grade at presentation, and tapered over 6 to 8 weeks as described elsewhere (Thwaites *et al.*, 2004). Antiretroviral or placebo tablets were commenced as soon as possible after randomization. The ART regimen was AZT, 3TC and EFV for all subjects. In HIV patients co-infected with TB, NVP is contraindicated due to pharmacokinetic interactions between rifampicin and NVP and due to the added hepatotoxicity (Autar *et al.*, 2005; Ribera *et al.*, 2001; Swaminathan *et al.*, 2011). EFV is therefore recommended by the WHO as first-line ART for patients co-infected with TB (WHO, 2010b). Medications were administered orally or via nasogastric tube. Subjects received directly observed therapy during their inpatient stay

(up to three months); administration was supervised by family members after discharge from hospital.

All patients enrolled in this clinical trial with available stored plasma samples were included in this study. Participants were recruited between September 2005 and December 2007 and completed follow up in December 2008.

3.3.2 Statement of ethics

The trial protocol was approved by the Scientific and Ethical Committees of the two hospitals, by HCMC Health Services, and by the Oxford Tropical Research Ethics Committee. Written informed consents were obtained from all subjects or relatives if the subject was unable to provide consent, according to standard practice in Viet Nam. For unconscious subjects with no available relatives, the consent of two independent physicians was considered acceptable.

3.3.3 HIV-1 drug resistance testing

In-house drug resistance testing was performed as described in section 2.2.2 in which HIV RNA was extracted from 200 µL of frozen plasma samples by using automated nucliSENs EasyMAG platform.

3.3.4 Statistical method

Descriptive statistics were used to summarize characteristics of the cohort and the prevalence and types of resistance mutations. In general the number and percentage in each category were determined for categorical variables, and the median, interquartile range were calculated for continuous variables.

3.4 Results

3.4.1 Presence of pre-existing HIV drug resistance mutations in chronically-infected subjects with HIV-associated tuberculous meningitis

Among the 253 subjects enrolled in the trial, stored baseline plasma samples were available for 220 participants, of whom 90% were male and 84% had history of IDU. The median baseline CD4+ T-lymphocyte count and the median baseline plasma HIV-1 RNA level were 41 cells/μL (interquartile range: 16-104) and 1.56 $x10^6$ copies/mL (interquartile range: 0.85 $x10^6$ – 2.27 $x10^6$), respectively. Both PR and RT regions were successfully sequenced in 219/220 baseline samples. Mutations that confer resistance to NRTI were found in 8/219 (3.7%) (95%CI: 1.2-6.2) subjects, and NNRTI resistance-conferring mutations in 5/219 (2.3%) (95%CI: 0.3-4.3). Resistance to both NRTI and NNRTI was observed in 1/219 (0.5%). No PI resistance mutations were identified (Table 3.2). Hence, the overall prevalence of pre-existing HIV drug resistance in this cohort in HCMC is 14/219 (6.4%) (95%CI: 3.2-9.6). In 13 of 14 subjects, the detected mutations confer resistance to the standard first-line antiretrovirals used in Viet Nam (d4T, AZT, 3TC, NVP and EFV). The remaining subject carried the mutation L74V which confers

resistance to ABC and ddI which are part of second-line antiretrovirals in Viet Nam. In 10 of 14 subjects the detected mutations are associated with high-level resistance to antiretrovirals according to the Stanford HIV drug resistance database.

Table 3.2: Pre-existing 2009 WHO surveillance drug resistance mutations in individuals with HIV-associated tuberculous meningitis in Ho Chi Minh City from 2005-2007

Subject	HIV RNA, copies/mL	Drug resistance associated mutations			CD4 (cells/µL)
		NNRTI	NRTI	PI	
1	904,780	K103N, V106M, G190A	_	_	20
2	609,200	Y181C	_	_	37
3	95,730	Y181C	_	_	49
4	1,604,510	_	V75M	_	22
5	5,600	_	M184V	_	23
6	57,235	Y181C	D67N	_	55
7	64,020	_	M184V	_	59
8	34,465	_	K219R	_	63
9	4,889,900	K103N, Y181C	_	_	24
10	333,130	_	L74V	_	40
11	315,310	_	V75M	_	65
12	1,359,330	K103N, Y181C	_	_	55
13	4,637,185	_	M184I	_	32
14	282,230	_	T69D	_	12

NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitors; PI, protease inhibitor

3.4.2 HIV-1 sub-typing and polymorphisms not associated with drug resistance in Ho Chi Minh City, Viet Nam

Phylogenetic analysis and subtype classification according to MEGA4 and the Stanford database revealed that 218/219 (99.5%) subjects were infected by HIV-1 subtype CRF01_AE; the remaining one by subtype B. Non-synonymous polymorphisms were identified at all 15 drug resistance positions described in the 2009 WHO SDRM list and were more prevalent in RT than the PR gene of the 218 CRF01_AE isolates (Table 3.2). Of note, some natural polymorphisms were present at higher frequencies in this HCMC cohort compared to a study in northern Viet Nam, both were overwhelmingly dominated by CRF01_AE subtype (Ishizaki *et al.*, 2009), i.e. T69N (9.2% versus 0.4%), V106I (8.2% versus 1.5%) and V179I (17.4% versus 0%). The polymorphism L63C, which requires changes in all three nucleotides, was present in 45.4% of samples in this cohort. This substitution was present in 27.2% of the prior 200-subject cohort in HCMC (Lan *et al.*, 2003) but has not been reported in northern Viet Nam. The two polymorphisms V106I and V179D occurred in 18/219 (8.2%) and 5/219 (2.3%) subjects, respectively. These polymorphisms are not considered TDR mutations according to the WHO SDRM list and thus were not included in our resistance prevalence analysis. However V106I and V179D have been associated with resistance to ETR (a second-generation NNRTI available only in resource-rich countries) and are included in the 2010 IAS-USA mutation list (Johnson *et al.*, 2010). If the IAS-USA algorithm is used, the prevalence of pre-existing HIV drug resistance in our study would increase from 6.4% to 16%. Other non-synonymous polymorphisms at positions not associated with the 2009 WHO SDRMs are listed in Table 3.3; similar polymorphism frequencies have been

described in subtype CRF01_AE in other studies in Asia (Ishizaki *et al.*, 2009; Liu *et al.*, 2007).

Table 3.3: Non-synonymous polymorphisms at 2009 WHO surveillance drug resistance mutation sites in 218 HIV-1 CRF-01_AE isolates from HCMC

Drug resistance positions [a]	Amino acid in B reference	Amino acid in CRF01_AE reference	Amino acid substitution in this cohort	Subjects, n (%)
Protease				
82	V	V	I	24 (11.0)
88	N	N	K	1 (0.5)
Reverse transcriptase				
67	D	D	**N**	1 (0.5)
69	T	T	**D**	1 (0.5)
_	_	_	N	20 (9.2)
_	_	_	A	4 (1.8)
_	_	_	S	3 (1.4)
74	L	L	**V**	1 (0.5)
75	V	V	**M**	2 (0.9)
_	_	_	L	2 (0.9)
101	K	K	R	1 (0.5)
103	K	K	**N**	3 (1.4)
_	_	_	R	1 (0.5)
106	V	V	**M**	1 (0.5)
_	_	_	I	17 (7.8)
179	V	V	I	38 (17.4)
_	_	_	D	5 (2.3)
_	_	_	A	2 (0.9)
_	_	_	E	2 (0.9)
_	_	_	N	1 (0.5)
181	Y	Y	**C**	4 (1.8)
184	M	M	**V**	2 (0.9)
190	G	G	**A**	1 (0.5)
210	L	L	M	7 (3.2)
_	_	_	F	1 (0.5)
219	K	K	**R**	1 (0.5)
_	_	_	T	2 (0.9)

[a] Drug resistance positions were based on 2009 WHO surveillance drug resistance mutation list. Amino acids displayed in bold font are associated with drug resistance.

Table 3.4: Non-synonymous polymorphisms at positions not associated with drug resistance based on 2009 WHO surveillance drug resistance mutation list in 218 HIV-1 CRF-01 AE isolates from HCMC

PR Position[a]	***L10***	***I13***	***K14***	***I15***	***G16***	***K20***	***E35***	***M36***	***N37***	***R41***	***K43***	***K45***	***R57***	***Q61***	***L63***
CRF01_AE	I (49) V (25) M (1)	V (148)	R (12) N (1)	V (42)	E (107) A (38) A/S (1)	R (37) I (3)	D (197) D/N (2)	I (217)	D (54) D/E (3) S (1)	K (213)	R (15)	R (15)	K (20)	E (8) H (2)	C (99) P (20) S (3)
PR Position[a]	***I64***	***H69***	***K70***	***I72***	***T74***	***V77***	***L89***	***M93***	***F99***						
CRF01_AE	L (1) M (1) V (1)	K (214) Q (1) T (1) I (1)	R (76)	V (5) R (1) T (1)	S (1)	I (3)	M (205) M/I (3) I (1)	L (41) V (2) M (1)	L (3)						
RT Position[a]	***E6***	***K11***	***V35***	***T39***	***K43***	***V60***	***Q102***	***K122***	***D123***	***I135***	***S162***	***K173***	***Q174***	***D177***	***I178***
CRF01_AE	D (191) N(4) K/N (1)	T (142)	T (211) A(1) I (1) R (1)	K (100) E (63) N (30)	E (71) R (3) Q (4) A (1)	I (32)	K (184) R (4) K/R (5) T (1)	E (212) E/V (2)	S (162) N (13) N/S (37)	T (28) V (2) L (1) R (1)	C (159)	I (125) T (33) R (26) M (10)	K (109) R (12)	E (109)	M (126)
RT Position[a]	***G196***	***T200***	***I202***	***Q207***	***R211***	***K238***	***V245***	***A272***	***V276***	***R277***	***K281***	***T286***	***E291***	***V292***	
CRF01_AE	E (12)	I (55) A (51)	V (24)	A (182) S (15) D (12)	S (208)	R (188)	E (205)	P (61)	I (34)	K (210)	R (32)	A (179)	D (187)	I (193)	

[a] Protease (PR) and Reverse Transcriptase (RT) positions are based on pNL4-3 reference. Values shown in parenthesis represent number of isolates carrying a certain amino acid substitution

3.4.3 HIV drug resistance development in subjects with virological failure

The mortality rate of TBM in HIV-infected individuals is twice as high compared to HIV-uninfected individuals (Thwaites *et al.*, 2004). Due to the early and high mortality of subjects in the trial (58%) (Török *et al.*, 2011), the impact of pre-existing drug resistance mutations on virological and clinical outcome could not be accurately assessed. Amongst the 219 trial subjects included in this study, only 69 survived and completed follow-up at month 6, 45 at month 9, and 14 at month 12. After 6 months follow-up, 8/69 surviving subjects had protocol-defined virological failure. Major 2010 IAS-USA mutations were detected in 5 of these subjects (Table 3.4). All 5 had mutations that confer high level resistance to NNRTI (K103N, Y188L, P225H and/or M230L), and 3 additionally had mutations that confer high level resistance to NRTI (M184V and/or T215Y/F/I/S). Two subjects had pre-existing mutations prior to ART initiation and accumulated further resistance mutations on non-suppressive therapy (Subjects 3 and 7). Amongst the 8 subjects with VF, 3 had virus containing V106I polymorphism both at baseline and at time of VF. Virus containing V106I and/or V179D did not develop on ART in any of the 8 subjects with virological failure.

Table 3.5: Genotypic drug resistance profile of 5 individuals with virological failure

Time, months	Subject 3		Subject 7		Subject 15		Subject 16		Subject 17	
	HIV RNA[a]	Genotype	HIV RNA[a]	Genotype	HIV RNA[a]	Genotype	HIV RNA[a]	Genotype	HIV RNA[a]	Genotype
0	76,500	Y181C	64,020	M184V	56,310	wt	4,955,200	wt	29,230	wt
1	150	_	1,540	_	490	_	4,870	_	_	_
2	150	_	37,300	M184V, K103N	3,545	Y188L	150	_	150	_
3	_	_	152,755	M184V, K103N, V108I	700	_	395	_	_	_
4	150	_	_	_	150	_	150	_	450	_
5	150	_	_	_	_	_	_	_	2,335	K103N,M184V, Y188HLFY
6	_	_	47,910	M184V, K103N, V108I, T215F/I/S, P225H, M230L	_	_	_	_	215	_
7	41,325	V179D	_	_	205	_	150	_	2,605	K103N, M184V, Y188L
8	1,295	K103N, V106I	_	_	1,975	M184V, Y188L	_	_	_	_
9	_	_	51,850	M184V, K103N, V108I, T215F, M230L	1,825	M184V, Y188L	132,495	K103N	_	_
10	_	_	_	_	_	_	95,885	K103N, P225HP	_	_
11	_	_	_	_	_	_	_	_	_	_
12	_	_	44,940	M184V, K103N, V108I, T215F/I/S, P225H, M230L	_	_	_	_	_	_

[a] Copies/mL. wt, wild type.

3.5 Discussion

We report a prevalence of 6.4% of pre-existing HIV drug resistance in 219 ART-naïve chronically-HIV-infected individuals with TBM in HCMC from 2005-2007 using the 2009 WHO SDRM list. NRTI mutations were most commonly identified, occurring in 4.1% of subjects. Most observed mutations conferred resistance to NRTIs in the standard first-line ART regimens in Viet Nam, while one subject had a mutation (L74V) that confers resistance to ABC and ddI used in second-line ART regimens in Viet Nam. NNRTI mutations were identified in 2.8% of subjects, and all confer high level resistance to NVP and EFV. No PI mutations were identified.

The prevalence of pre-existing HIV drug resistance mutations in this study appears higher than in two previous studies which used the WHO method for surveillance of TDR during 2003-2008, both of which showed TDR prevalence <5% (Ayouba *et al.*, 2009; Nguyen *et al.*, 2008a). However, aside from the small sample sets (N=63 and N=49), these studies surveyed relatively low-HIV-exposure populations (first-time pregnant women in antenatal clinics and individuals aged <25 years from VCT centers) compared to the population making up the concentrated HIV epidemic in Viet Nam, where 52% are IDUs and 4% are CSWs (International Harm Reduction Development Program, 2008). By nature of the risk behaviors and social stigmatism, these high-HIV-exposure individuals are less likely to attend VCT centers and antenatal clinics. The WHO threshold survey method - which was designed to exclude ART-experienced individuals to improve the accuracy of TDR estimation - may be counter-intuitive and underestimate TDR in countries where HIV epidemics are concentrated in high-HIV-exposure populations such as Viet Nam. This study gives a specific example of the

deficiencies of the WHO antenatal and VCT clinic-based screening method outside of sub-Saharan Africa – where the at-risk population is often made up of IDUs, CSWs and MSM. The resistance prevalence in our study is similar to studies that surveyed a more representative population attending HIV treatment clinics who report no prior use of ART, with resistance prevalence ranging from 6.2%-7.6% (Dean *et al.*, 2011; Lan *et al.*, 2003; Phan *et al.*, 2010). Our resistance prevalence falls into the range of these data during the period of 2002-2009, suggesting that TDR has remained relatively stable despite the rapid scale up of ART in Viet Nam over the past 5 years (UNAIDS, 2010). Although TDR prevalence in Viet Nam is at the level of which routine baseline resistance testing is considered cost effective in resource-rich countries (Sax *et al.*, 2005; Weinstein *et al.*, 2001), cost effectiveness studies of drug resistance testing and continuing surveillance of TDR targeting representative HIV-infected populations in resource-poor countries are clearly needed.

The prevalence of mutation M184V/I in this study is relatively higher than that reported in WHO TDR surveys in resource-limited settings and in the UK CHIC cohort study where patients were infected with multiple subtypes (1.4% compared to 0.4% and 0.9%, respectively) (Harrison *et al.*, 2010; WHO, 2012b). M184V/I is commonly selected in patients failing first-line ART but is rarely detected among newly HIV infected patients in non-AE subtype (Masquelier *et al.*, 2005; Vijver *et al.*, 2010; Wheeler *et al.*, 2010). This is thought to be a consequence of its low fitness (Back *et al.*, 1996; Pingen *et al.*, 2011, 2014; Schuurman *et al.*, 1995) and high rate of reversion and/or replacement (Jain *et al.*, 2011; Paquet *et al.*, 2011). However the rate of reversion and/or replacement of M184V/I might be different for subtype CRF01_AE, and genomic linkage to other

mutations and polymorphisms can reserve viral replication capacity (Jain *et al.*, 2011; Paquet *et al.*, 2011). This might explain the high prevalence of M184V/I seen in our patients. The other possibility is that patients in our study may not reveal their previous ART history in order to enter the study and receive ART, leading to overestimation of transmitted M184V/I in our cohort.

Non-synonymous polymorphisms were observed at all 15 drug resistance foci listed in the 2009 WHO SDRM. The V106I and V179D polymorphisms, both considered minor mutations associated with resistance to ETR according to the 2010 IAS-USA algorithm (Johnson *et al.*, 2010), were observed in 8.2% and 2.3% of subjects, respectively. If the IAS-USA algorithm is used for TDR analysis, the resistance prevalence in our study would increase from 6.4% to 16%; however this would be misleading. An example of such an analysis using the IAS-USA algorithm was recently published by the multi-center TREAT Asia study reporting a HIV drug resistance prevalence of 13.8% in 682 ART-naïve chronically-infected individuals from Hong Kong, Malaysia, and Thailand from 2007-2009 (Sungkanuparph *et al.*, 2011). In this study, 77.7% of subjects were infected with CRF01_AE subtype, and the HIV drug resistance prevalence of 13.8% included viral isolates containing the V106I (1.9%) and V179D (3.2%) polymorphisms. The resistance prevalence in this study would have substantially decreased if the WHO SDRM list was used, which would have better reflected the burden of primary resistance in the Asia Pacific region. Our study highlights the importance of appropriate use of available HIV drug resistance algorithms. The IAS-USA mutation list was not developed to be used in epidemiologic analyses to identify TDR but rather for listing relevant mutations or polymorphisms that are associated with a

reduced susceptibility to antiretroviral drugs (Johnson *et al.*, 2010). The WHO SDRM algorithm is a parsimonious list of rigorously-defined mutations only at non-polymorphic positions in 8 major HIV subtypes and should be the most appropriate algorithm for estimation and comparison of TDR in different regions and different times (Bennett *et al.*, 2009).

The two polymorphisms V106I and V179D were identified in 8.2% and 2.3% of these patients and have been associated with ETR resistance. Although ETR is not yet available outside of America and Western Europe, V106I in combination with V179D have recently been identified as a new pattern of mutations conferring resistance to NVP and EFV (Gatanaga *et al.*, 2010), which are the most common drugs used in first-line ART regimens globally. Our data is consistent with a study by Scherrer *et al.* (Scherrer *et al.*, 2009) that found a 3.2% prevalence of a similar ETR-associated-resistance mutation V179T in treatment-naive patients infected with subtype CRF01_AE. That study found that most ETR-resistance-associated mutations in drug-naïve patients are polymorphisms and not TDR mutations, and that these polymorphisms are significantly more or less common in different HIV subtypes (Scherrer *et al.*, 2009). This study highlights the need for studies to evaluate the prevalence and impact of subtype-specific polymorphisms on ART outcome and whether subtype-specific pathways to resistance occur.

In this study the most frequent polymorphism in RT region was V179I (17.4%), higher than the prevalence reported for subtype B (5.8%) and other non-B subtypes (11%), but lower than that seen in subtype A (75%) (Lambert-Niclot *et al.*, 2013; Turner *et al.*, 2004). This mutation is frequently selected in patients receiving the new generation of NNRTIs - ETR and RPV (HIV Stanford database http://hivdb.stanford.edu). The

presence of V179I in combination with E138A was reported to reduce the susceptibility of ETR by 5.2 fold (Maïga *et al.*, 2010), but the effect of V179I alone on NNRTI susceptibility has not been reported. In our study we could detect only single V179I mutation in patients but not in combination with E138A. Among five patients having virological failure, V179I was detected in only one patient at both baseline and at time of virological failure. The high mortality rate observed in our patient cohort has hampered the ability to evaluate the association of the baseline V179I on patient outcome. More data are needed to understand the impact of V179I polymorphism in subtype CRF01_AE on treatment outcome.

Due to the early and high mortality rate in subjects with HIV-associated TBM in this study (58%), it was not possible to assess the impact of pre-existing SDRMs and identified polymorphisms on clinical or virological outcome. Amongst the 8 subjects who met protocol-defined VF, HIV drug resistance mutations were detected in 5 subjects. All failed in the presence of major NNRTI mutations K103N and/or Y188L, and 3 failed with M184V mutations. Four subjects showed a pattern of accumulating resistance mutations. Interestingly, one subject had a pre-existing Y181C mutation which disappeared while the K103N emerged by month 8 on ART. Switching from Y181C to K103N may occur as K103N carrying variants have better fitness compared to Y181C carrying variants in subtype B virus (Collins *et al.*, 2004). A shift in resistance pattern from Y181C to K103N has been described in women receiving a single dose of NVP to prevent mother-to-child transmission in Uganda (Eshleman *et al.*, 2004a).

An inherent limitation of a study method using a chronically-infected population to estimate TDR is the possibility of over-estimating resistance prevalence. In this study,

the ART history was based on patients' self report, and some subjects may not have revealed their true previous ART exposure in order to be eligible for the trial. On the other hand, an opposing limitation of using a chronically-infected population to estimate TDR is the possibility of under-estimating resistance prevalence as the Sanger sequencing method used to detect HIV drug resistance does not reliably detect HIV variants present at frequencies <15-25% of viral quasispecies (Schuurman *et al.*, 1999). It is generally known that TDR strains in the absence of antiretroviral selection pressure will over time revert to wild-type virus, and thus minority resistant variants may be missed by standard genotyping methods. Nevertheless, compared to the population recommended by the WHO threshold survey method, the population in this study is more representative of the general HIV-infected population attending HIV clinics in Viet Nam (International Harm Reduction Development Program, 2008; UNAIDS, 2010). The baseline median CD4 of 41 cells/μL in the study population reflects the reality of late detection of HIV in Viet Nam. A study of etiologies of opportunistic infections in hospitalised patients with their first presentation of HIV at the HTD in 2004 reported a median CD4 count of 20 cells/μL. 62% of patients starting ART in 6 outpatient clinics in Viet Nam had CD4 counts <100 cells/μL (Tran *et al.,* 2012). Although it remains important to distinguish between TDR from acquired resistance for epidemiological purposes, particularly during the scale up of ART in the developing world, the implications of TDR and/or acquired resistance for the individual patient and/or for public health in light of ART outcome and further spread of drug resistant virus is the same.

In summary, using the 2009 WHO SDRM algorithm not in combination with the WHO threshold survey method, we report a 6.4% prevalence of pre-existing HIV drug resistance in ART-naïve chronically-infected individuals with TB meningitis in HCMC, Viet Nam from 2005-2007. The identified HIV drug resistance mutations conferred resistance to NRTIs and/or NNRTIs which are part of the standard first-line ART in Viet Nam. Our HIV drug resistance resistance data is comparable to other studies using similar sampling methodology in the general populations seen in HIV clinics and suggests that HIV drug resistance has remained relatively stable despite the rapid scale up of ART in Viet Nam.

4 Second-Line Antiretroviral Therapy Outcome in HIV-Infected Adults in Ho Chi Minh City, Viet Nam

4.1 Abstract

The number of patients on second-line ART is growing in Viet Nam, yet there are limited data on treatment outcome and predictors to inform prevention and treatment strategies. We evaluated all patients age ≥15 years who initiated second-line ART at least 6 months before January 2012 at the HTD in HCMC. Data were collected from patient charts using a standardized form. The primary outcome was time from second-line ART initiation to treatment failure based on WHO immunological and clinical failure criteria. Patients lost to follow up or transferred to other treatment centers were censored at their last visit. The Cox proportional hazards model was used to determine predictors of failure. Pre-defined variables included in the model were age, history of IDU, total time from first detection of failure to second-line ART initiation, CD4 count and viral load at regimen switch, previous protease inhibitor use and second-line adherence assessment (good ≥90% of estimated pills taken in preceding 6 months). In this study 330 patients initiated second-line ART (97.5% with a ritonavir-boosted LPV regimen in combination with at least two NRTI backbone drugs) between May 2005 and June 2011. The median follow up duration was 29 months (IQR: 15-44). Reason for switch to second-line ART was treatment failure in 326 patients (with confirmation of virological failure in 93%) and treatment intolerance in 4 patients. The median age was 32 years (IQR: 28-36). 81% were men and 44% had a history of IDU. The median time on a failing first-line ART regimen was 9 months (IQR: 5-15). The median CD4 count and viral load at time of regimen switch were 44 cells/μL (IQR: 16-84) and 5.1 log copies/mL (IQR: 4.6-5.5),

respectively. Adherence to second-line ART was good in 88% of patients. The observed failure events following second-line ART included 23 immunological failures, 44 AIDS events, and 44 deaths. Kaplan-Meier estimates of failure after 1, 2, 3, and 4 years were 12.9% (95% CI: 9.1-16.6), 18.4% (95% CI: 13.8-22.8), 20.1% (95% CI: 15.1-24.7), and 22.5% (95% CI: 16.9-27.7), respectively. The results of the Cox proportional hazards model showed older age, history of IDU, lower CD4 count at switch, history of PI usage and poor adherence independently predicted failure in both complete-case and multiple-imputation analyses. Lower CD4 count predicted failure only in the first year of second-line therapy. While treatment efficacy was similar to that reported from other resource-limited settings; mortality was higher. Deaths may be averted by prioritising second-line therapy based on CD4 counts and through improving treatment adherence support.

4.2 Introduction

Combination antiretroviral therapy has resulted in substantial reduction of morbidity and mortality for HIV-1-infected patients in resource limited countries as well as in developed countries (Bhaskaran *et al.*, 2008; Braitstein *et al.*, 2006; Floyd *et al.*, 2010; Ivers *et al.*, 2005; Laurent *et al.*, 2004; Walensky *et al.*, 2006). In resource limited settings the first-line ART regimen usually includes two NRTI and one NNRTI (WHO, 2006a). This first line ART regimen has low cost and good virological response (Bartlett *et al.*, 2006). However in order to control viral replication patients need to be on ART for the rest of their lives. Over-time first-line ART may fail to control HIV replication due to problems with medication adherence, absorption, drug-drug interactions, and/or development of drug resistance mutations (Calmy *et al.*, 2007; Gallant, 2007; Kantor,

2006; Sungkanuparph *et al.*, 2007). The rate of virological failure to first-line ART in resource limited settings in a systematic review is about 6.08 per 100 person years of follow-up (Renaud-théry *et al.*, 2010). An observational cohort study showed that the clinical failure rate to first-line ART in Asia is 7.3 per 100 person-years (Zhou *et al.*, 2007). In Viet Nam data on the treatment outcome to first-line ART are limited, but are mostly from HTD, HCMC and other public clinics in southern Viet Nam. The rate of immunological failure to first-line ART is approximately 14.5% in HTD. Virological suppression with viral load below the limit of detection (250 copies/mL) reported in HTD was 83.1% after one year of first-line treatment (Quang *et al.*, 2011) and in public clinics in southern Viet Nam was 70% (Trinh *et al.*, 2011). One report from Northern Viet Nam showed similar rate of virological suppression at 72% after one year of first-line therapy (Cuong *et al.*, 2012). The need for second-line ART is imminent and growing. It was estimated that the prevalence of second-line therapy in low and middle income countries in 2008 was 5.4% among treated patients (Galárraga *et al.*, 2007; WHO, 2008), and in 2010 the WHO forecasted the average switch rate from first-line to second-line ART was 3% per year (WHO, 2006b).

Second-line ART regimens with ritonavir boosted PIs cost six times as much as first-line ART regimens with NNRTI in low and middle income countries (Stover *et al.*, 2011), and in most resource limited countries such as Viet Nam second-line ART is the last option for patients failing ART. A multicenter study from Africa and Asia showed that the rate of either immunological, clinical or virological failure after one and two years of second-line initiation was 12% and 28% respectively (Pujades-Rodríguez *et al.*, 2010). A recent review of treatment outcome of patients taking second-line ART in

resource limited settings showed that the rate of virological failure on second-line therapy was 26.7% after two years of treatment (Ajose *et al.*, 2012). Most of the studies in the review were from Africa. Only 6 studies were from South-east Asia; two from Cambodia and 4 from Thailand had small numbers of participants (Ferradini *et al.*, 2011; Manosuthi *et al.*, 2007; Sophan *et al.*, 2010; Treebupachatsakul, 2007; Win *et al.*, 2011). The second-line regimen from Thailand was different from Viet Nam and included either ritonavir boosted IDV, ATV or SQV (Manosuthi *et al.*, 2007; Siripassorn *et al.*, 2010; Treebupachatsakul, 2007; Win *et al.*, 2011). In 2012 the number of patients on ART in Viet Nam was 69,882, and the majority of patients were on first line therapy (96.8%) (Viet Nam MOH, 2013). An estimated 3% of patients are in need of second-line therapy (VAAC, 2012). Understanding the rates and risks of second-line treatment failure is important for national programs to devise strategies that maximize treatment efficacy in limited resources and forecast the need of treatment options beyond second-line therapy.

4.3 Methods

4.3.1 Study population

All HIV-infected patients age ≥15 years who initiated second-line ART at least six months before the start of this study in January 2012 were included. Patients were identified from the electronic database at HIV out-patient clinic at HTD, HCMC.

The national ART programme began providing free antiretroviral drugs through international funding support in 2003. First-line therapy consisted of AZT or d4T in combination with 3TC and NVP. Prior to the availability of EFV in 2004, cases of NVP-

related toxicity were switched to IDV. Second-line therapy became available in 2006 initially including ABC, ddI, and NFV. In 2007 LPV/r replaced NFV, and in 2009 TDF and 3TC replaced ABC and ddI as the NRTI backbone (Viet Nam MOH, 2009a).

4.3.2 Outcome measurements

The primary outcome was treatment failure and was defined as time from second-line ART initiation to either death or immunological, and/or clinical failure, which ever occurred first, based on the WHO definition of ART failure in settings where viral load monitoring is not available (WHO, 2006a). Immunological failure was defined as a fall of CD4 count to baseline or below, a 50% fall from the on-treatment peak CD4 value, or persistent CD4 levels <100 cell/mm3 after at least 6 months on ART. Clinical failure was defined as occurrence of a new or recurrent WHO stage IV AIDS-related condition after at least 6 months on ART. The secondary outcome was time to death.

4.3.3 Data collection

Data were collected from the patients' clinic charts on a standardized case report form and included demographic information, history of IDU, first-line antiretroviral (ARV) drug history, serial CD4 counts every 6 months, WHO stage 4 AIDS-related events, deaths from all causes, HIV viral load and genotype when these values were available, and ART adherence evaluation.

4.3.4 Therapy adherence measurement and Visual Analogue Scale

Compliance to described ART has a great influence to treatment outcome. There is no gold standard for treatment adherence measurement, but there are variety of approaches to access treatment compliance among patients such as the use of MEM caps, pill counts, plasma drug concentration measurements, and self report, each with its advantages and disadvantages. MEM caps and plasma drug concentration measurement correlate well with virological outcome; however, they are costly and required special device and equipment (Alexander *et al.*, 2003; Steel *et al.*, 2007). Pill counts and self-reports are simple, inexpensive and applicable to resource-limited settings. However pill count requires significant staff time (Liu *et al.*, 2001). Self-report assessment is efficient but suffers from recall bias. The Visual Analogue Scale (VAS) is a self-report measurement of treatment adherence (Kalichman *et al.*, 2011). Several factors, such as education, stigma and discrimination, can affect VAS assessment in developing countries. Patients with low education could have difficulties in understanding questionnaires and give inaccurate estimation of treatment adherence. Healthcare providers with stigma and discrimination to certain subjects may affect data collection process. Patients might not reveal their true adherence status because of concerns about anonymity and impact of the interview on treatment (Do *et al.*, 2013). Despite those problems, VAS was shown to be as good and reliable as other assessment methods (Giordano *et al.*, 2004; Shi *et al.*, 2010) and is used in many cohorts studying level of adherence in Viet Nam (Do *et al.*, 2013; Tran & Nguyen, 2012).

ART adherence in HTD is routinely assessed by clinic doctors and is recorded in the chart either as an estimated percentage of dosages taken or as a qualitative assessment of 'optimal' or 'not optimal'. Adherence was additionally evaluated using a VAS. In VAS assessment, the patients were asked in person to estimate their adherence over the past 6 months on a 100-point VAS, where 0 indicated complete non-adherence and 100 indicated perfect adherence. People were asked if they missed taking any pills, or if they took pills 4 hours later than scheduled over the past 6 month before being asked to put a cross at the point on the scale showing their best guess of how much of each drug they had taken. For analysis, optimal adherence was defined as a score of ≥90% on the VAS assessment, an average adherence score of ≥90% or consistently 'optimal' adherence recorded at monthly visits over the preceding 6 months.

Figure 4.1: Visual analogue scales

4.3.5 Statistical analysis

Time to treatment failure was visualized using a Kaplan-Meier curve and failure rates after 1, 2, 3 and 4 years with corresponding 95% CIs were calculated. The Cox proportional hazards model was used to analyze the time to failure (primary endpoint) and the time to death (secondary endpoint). Sixty patients out of 330 (18%) were

transferred to other provincial or district clinics while on second-line treatment as part of the government's efforts to decentralize HIV care. The majority of the transferred patients (estimated 90%) had been judged by doctors to be clinically and immunologically stable before the transfer. For the analysis, event-free transferred patients were censored at the time of transfer (primary analysis). Alternatively, assuming the transferred patients were doing well (clinically and immunologically) on therapy, we treated them as censored at the time-point where their last monthly follow-up visit would have been had they not been transferred (sensitivity analysis to assess potential informative censoring).

The following pre-defined covariates were included: age at initiation of second-line therapy, history of IDU (yes/no), CD4 cell count, and (log10-transformed) HIV RNA viral load at initiation of second-line therapy, total time on a failing first-line regimen, history of PI use and an overall measure of patients' adherence (<90% vs. ≥90%). Both univariate and multivariable Cox regressions were performed.

Data was analyzed based on multiple imputations of missing data and on a complete-case analysis. To avoid bias, the imputation algorithm included the endpoints [event time T and Nelson-Aalen estimator H(T)] (White & Royston, 2009). All reported confidence intervals were two-sided at 95% and analyses were performed with the statistical software R version 2.15.0 (R Development Core Team, 2012) and the companion R package mice version 1.2.5 (for multiple imputation) (Buuren & Groothuis-Oudshoorn, 2011).

4.4 Results

4.4.1 Study population and baseline characteristics

A total of 330 patients aged ≥15 years initiated second-line therapy between May 2005 and June 2011, and all were included in this study (Figure 4.2). At the time of the study, 44 (13%) patients had died; 59 (18%) patients had been transferred to other clinics; 1 (0.3%) patient was lost to follow-up; and the remaining 226 (68%) patients were in active follow-up. 326/330 (99%) patients were switched to second-line therapy due to treatment failure to first-line therapy with confirmation of virological failure in 94%; the remaining 4 patients were switched due to severe drug-related adverse effects to first-line therapy. Intolerance to NVP is commonly observed; however most of our patients who were intolerant to NVP subsequently tolerated EFV and were able to maintain on first-line ART. The limited first-line therapy options and the high costs of second-line therapy also influence the clinical practice of reinforcing EFV when NVP fails. The characteristics of the patients at the time of second-line therapy initiation are summarized in Table 4.1. The median CD4 count was 44 cells/μL (IQR: 16 - 84) and the median HIV RNA was 5.1 log copies/mL (IQR: 4.6 - 5.5). The median time the patients had been on a failing first-line ART regimen was 9 months (IQR: 5 - 15).

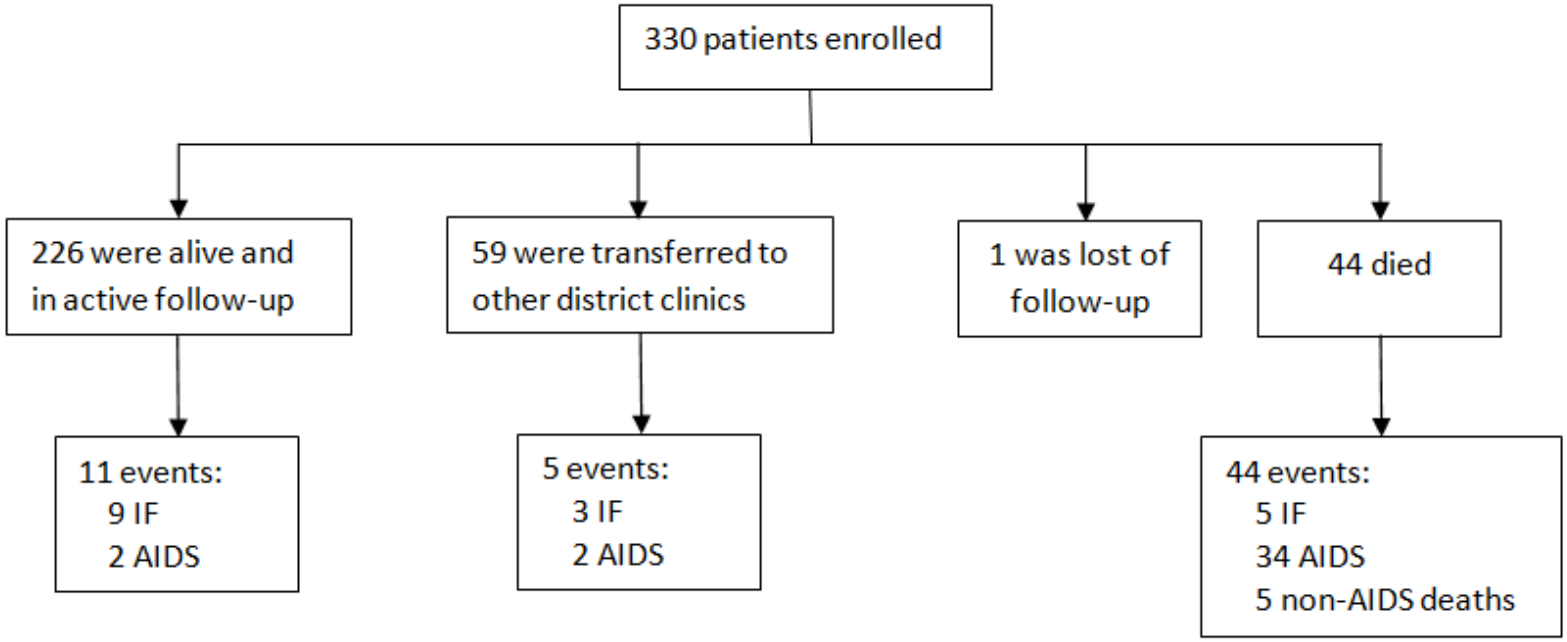


Figure 4.2: Flowchart of study population and outcome events

IF = Immunological Failure

Table 4.1: Characteristics of 330 patients starting second-line ART at the Hospital for Tropical Diseases in Ho Chi Minh

Characteristics	N=330
Sex, male (%)	271 (82%)
Age, median (IQR) (years)	32 (28-36)
Previous history of IDU (%) (n=313)	139 (44%)
CD4 count (cells/µL) before 1st line ART initiation, median (IQR)(n=308)	40 (12-93)
1st line ART regimens (%)	
d4T/3TC/NVP	116 (35.2%)
d4T/3TC/EFV	104 (31.5%)
AZT/3TC/NVP	45 (13.6%)
AZT/3TC/EFV	41 (12.4%)
Others	24 (7.3%)
Time on a failing 1st line failing ART regimen [a], median (IQR) (months) (n=324)	9 (5-15)
CD4 count (cells/µL) before 2nd line ART initiation, median (IQR) (n=324)	44 (16-84)
HIV RNA (log copies/mL) before 2nd line ART initiation, median (IQR) (n=305)	5.1 (4.6-5.6)

Characteristics	N=330
2nd line regimens (%)	
LPV/r + 3TC ± TDF ± AZT	314 (95%)
LPV/r + 3TC + other NRTIs (ddI/d4T/ABC)	10 (3%)
NFV + 2 NRTIs	6 (2%)
Adherence [b]	
≥90%	288 (87.3%)
<90%	42 (12.7%)

[a,] Time from first detection of failure to 1st line ART to time of 2nd line ART initiation; [b,] Number indicates an estimated % of pills taken in the preceding 6 months; d4T, stavudine; 3TC, lamivudine; NVP, nevirapine; EFV, efavirenz; AZT, zidovudine; TDF, tenofovir; LPV/r, ritonavir boosted lopinavir; ddI, didanosine; ABC, abacavir; NFV, nelfinavir; IQR, interquartile range

4.4.2 Second-line ART and outcome

324 (98%) patients received ritonavir-boosted LPV in combination with at least 2 NRTIs. The remaining 6 patients received a NFV with at least 2 NRTIs (Table 4.1). Optimal adherence (either by routine clinical assessment and/or by self-reported VAS) to second-line ART was observed in 87% of patients. During the median follow-up of 29 months (IQR: 15 - 44), 60 (18.2%) patients experienced treatment failure, including 34 AIDS-related deaths, 5 non-AIDS deaths, 4 clinical failures with AIDS events, and 17 immunological failures (Figure 4.2).

The Kaplan-Meier curve showing the probability of survival (i.e. treatment success) was shown in Figure 4.3. The estimated treatment failure rates after 1, 2, 3, and 4 years were 12.9% (95% CI: 9.1-16.6), 18.4% (95% CI: 13.8-22.8), 20.1% (95% CI: 15.1-24.7), and 22.5% (95% CI: 16.9-27.7) respectively. The median CD4 counts were 234 cells/µL (IQR: 166-338), 353 cells/µL (IQR: 227-465), 393 cells/µL (IQR: 255-514), 473 cells/µL (IQR: 347-574) over the four years, respectively.

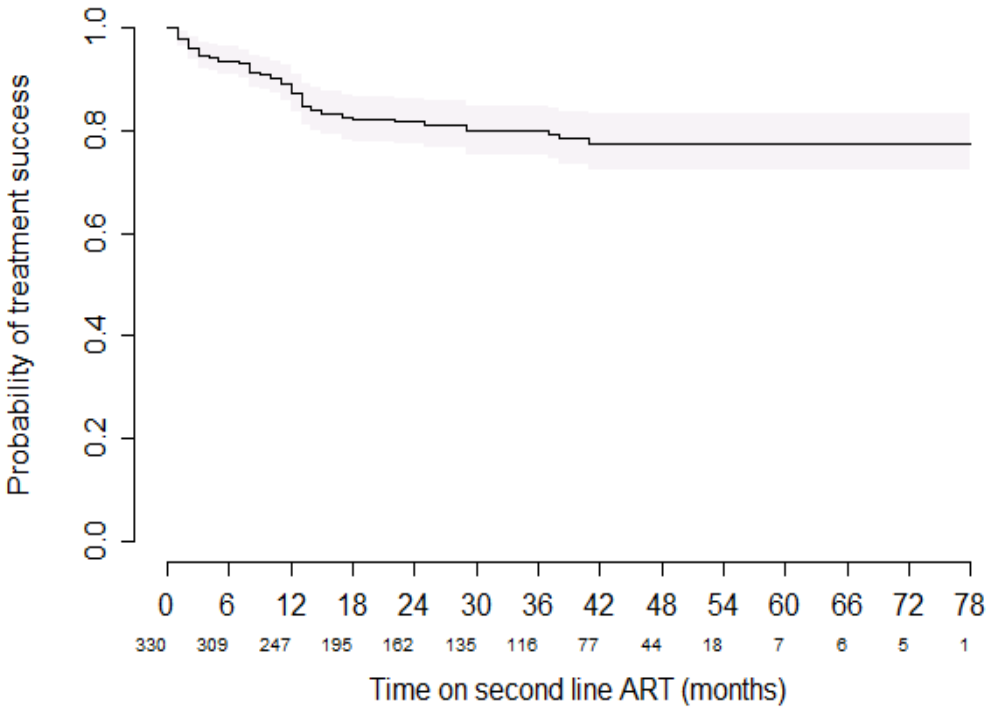


Figure 4.3: Kaplan-Meier estimates of proportion of patients with treatment failure

The numbers under the horizontal axis are numbers of patients at risk.

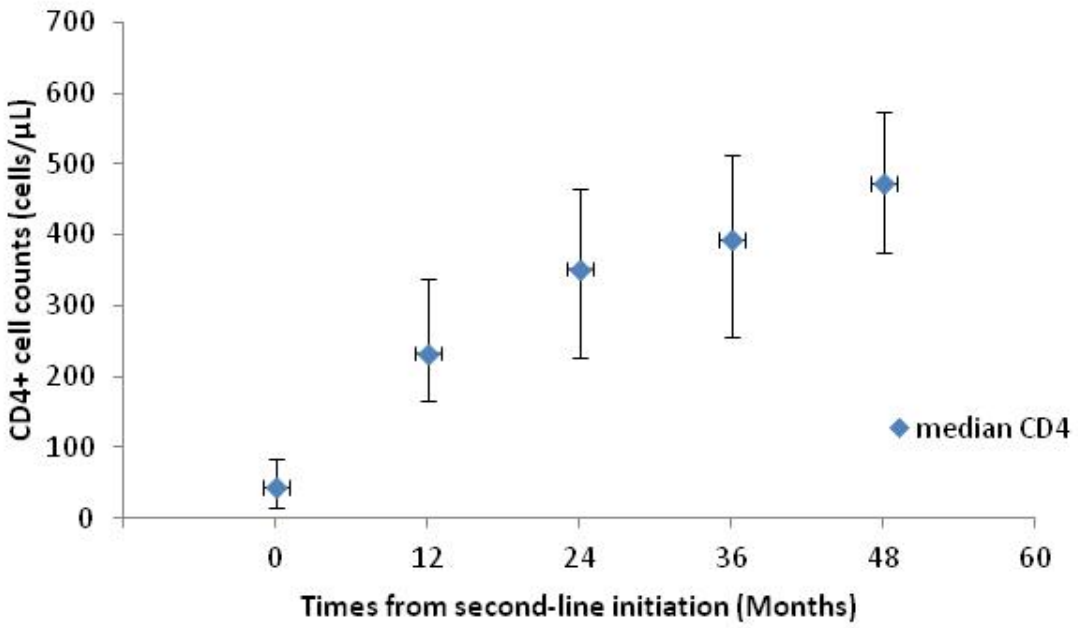


Figure 4.4: CD4+ T-cell progression after second-line treatment

4.4.3 Predictors of time to second-line ART failure

The 7 covariates used to assess prediction of time to treatment failure are listed in Table 4.2. The most frequently missing covariates were history of PI use (10% missing), viral load (8% missing), and IDU history (5% missing); other covariates were missing in ≤2% of patients. The results of the univariate and multivariate Cox regression analyses are shown in Table 4.2. Lower CD4 count at second-line ART initiation and suboptimal adherence to second-line ART predicted treatment failure in both univariate and multivariate analyses. Note that lower CD4 count predicted treatment failure during the first year of second-line therapy and not thereafter. Older age, history of IDU, and history of PI use did not predict treatment failure in the univariate analysis, but in the multivariate analysis they became statistically significant predictors of treatment failure. The association between age and IDU was evaluated ad hoc and showed that patients with a history of IDU were significantly younger than those without IDU ($p<0.0001$) (Figure 4.5). The association between PI exposure and IDU was also evaluated by using chi-square test and showed that there was a significant association between history of IDU and history of PI usage ($p=0.001$). Multivariate analysis shown in Table 4.2 was based on multiple imputations of missing data; however a complete-case analysis gave highly consistent results. A sensitivity analysis with informative censoring of the 60 transferred patients was performed (i.e. assuming the transferred patients continued to do well clinically and immunologically on therapy, and they were censored at the time-point where their last monthly follow-up visit would have been had they not been transferred), and the results were also consistent (Table 4.3).

Table 4.2: Impact of covariates on time to treatment failure

Covariate	Univariate effect		Multivariate effect [b]	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Age[a] (by +10 years)	1.36 (0.98-1.90)	0.069	**2.04 (1.34-3.11)**	**0.0009**
IDU (yes)	1.52 (0.89- 2.59)	0.127	**2.86 (1.42-5.78)**	**0.003**
CD4[a] (by +50 cells/μL)				
- effect in first year	**0.54 (0.34- 0.84)**	**0.007**	**0.52 (0.32-0.83)**	**0.006**
- effect subsequently	0.96 (0.66- 1.40)	0.820	0.95 (0.64-1.41)	0.796
log10VL[a] (by + log 10 copies/mL)	1.12 (0.75- 1.68)	0.576	1.04 (0.67-1.61)	0.847
Time on a failing 1st line therapy (by +6 months)	1.11 (0.95- 1.30)	0.199	1.07 (0.91-1.27)	0.406
Adherence < 90% (yes)	**2.00 (1.06- 3.76)**	**0.033**	**2.61 (1.33-5.10)**	**0.005**
PI exposure (yes)	0.71 (0.39- 1.31)	0.277	**2.02 (1.02-3.98)**	**0.043**

HR= hazard ratio, CI= confidence interval, [a]= at time of 2nd line ART initiation; [b]= analysis based on multiple imputation of missing covariates.

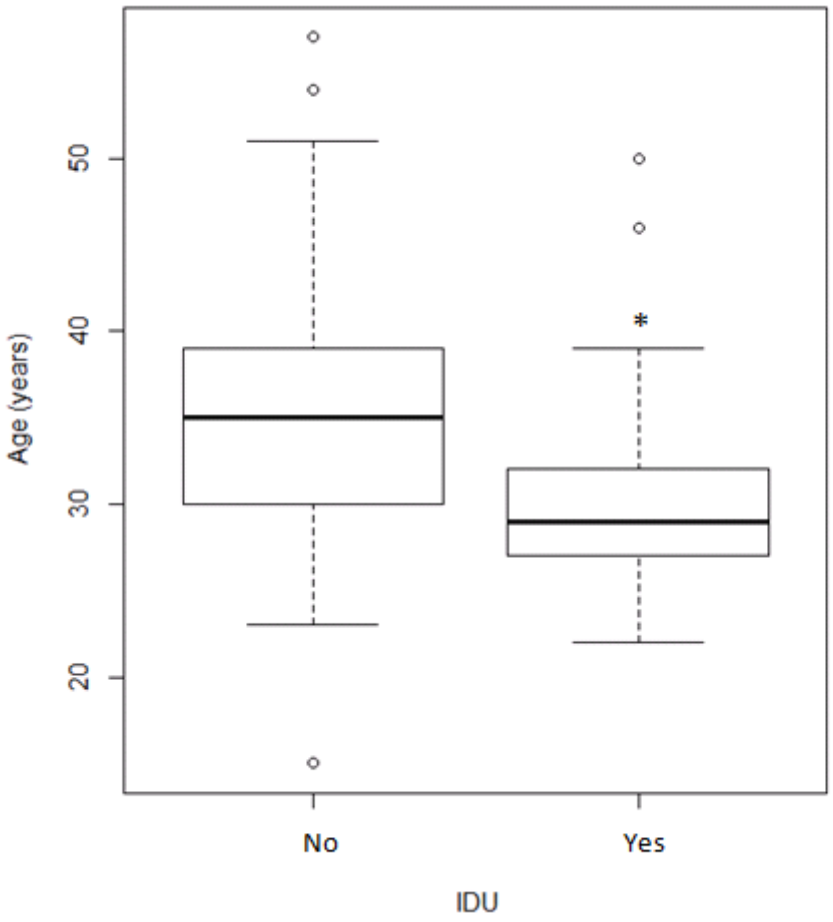


Figure 4.5: Boxplot of Age in injecting drug users (IDU) and non-IDU group.

The asterisk represents statistically significant ($p<0.0001$) difference between median age in IDU and non-IDU group (Wilcoxon rank sum test).

Table 4.3: Impact of covariates on time to treatment failure in sensitivity analysis

Covariate	Univariate effect		Multivariate effect [b]	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Age[a] (by +10 years)	1.37 (0.98-1.92)	0.066	**2.03 (1.33-3.10)**	**0.001**
IDU (yes)	1.50 (0.88- 2.55)	0.140	**2.80 (1.39-5.62)**	**0.004**
CD4[a] (by +50 cells/μL)				
- effect in first year	**0.53 (0.34- 0.84)**	**0.006**	**0.51 (0.32-0.83)**	**0.006**
- effect subsequently	0.96 (0.66- 1.40)	0.829	0.95 (0.64-1.42)	0.808
log10VL[a] (by + log 10 copies/mL)	1.11 (0.74- 1.67)	0.602	1.03 (0.67-1.59)	0.883
Time on a failing 1st line therapy (by +6 months)	1.11 (0.95- 1.30)	0.200	1.07 (0.91-1.27)	0.400
Adherence < 90% (yes)	**1.94 (1.03- 3.65)**	**0.041**	**2.55 (1.31-4.99)**	**0.006**
PI exposure (yes)	0.70 (0.38- 1.29)	0.252	**2.03 (1.03-4.01)**	**0.041**

HR= hazard ratio, CI= confidence interval, [a]= at time of 2nd line ART initiation; [b]= analysis based on multiple imputations of missing covariates.

4.4.4 Causes and predictors of death

A total of 44 patients (13.5%) died during second-line therapy; 39 were AIDS-related deaths, and 5 were unknown or non-AIDS related deaths. The median time to death were 9 months (IQR: 3-22), with 26 deaths (59.1%) occurring within the first 6 to 12 months of second-line ART initiation. The causes of AIDS-related deaths included microbiologically confirmed TB (13, 29.5%), *Pneumocystis jiroveci* Pneumonia (4, 9.1%), candida esophagitis (4, 9.1%), cryptococcal meningitis (2), *Penicillium marneffei* infection (2), herpes simplex (2), Cytomegalovirus retinitis (2),toxoplasmosis (2), HCV related liver failure (2), renal failure of unclear etiology (2), non-typhoid salmonella sepsis (1), and AIDS-associated wasting without an identified pathogen (8, 18%). The results of univariate and multivariate Cox regression analyses to determine predictors of time to death were shown in Table 4.4. Lower CD4 count at second-line ART initiation and suboptimal adherence predicted death in the univariate and multivariate analyses. Age did not predict death in the univariate analysis, but in the multivariate analysis older age became a significant predictor of death. History of IDU and PI exposure were not predictors for death.

Table 4.4: Impact of covariates on time to death

Covariate	Univariate effect		Multivariate effect [b]	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Age[a] (by +10 years)	1.41 (0.96-2.05)	0.076	**1.87 (1.17-2.99)**	**0.009**
IDU (yes)	1.21 (0.64- 2.29)	0.554	2.01 (0.90-4.48)	0.086
CD4[a] (by +50 cells/μL)	**0.61 (0.41-0.91)**	**0.016**	**0.57 (0.36-0.88)**	**0.012**
log10VL[a] (by + log 10 copies/mL)	1.00 (0.62- 1.59)	0.985	0.86 (0.51-1.44)	0.568
Time on a failing 1st line therapy (by +6 months)	1.12 (0.92- 1.36)	0.246	1.06 (0.87-1.30)	0.578
Adherence < 90% (yes)	**2.35 (1.16- 4.76)**	**0.018**	**3.06 (1.44-6.53)**	**0.004**
PI exposure (yes)	0.73 (0.35- 1.51)	0.397	1.88 (0.84-4.21)	0.122

HR= hazard ratio, CI= confidence interval, [a]= at time of 2nd line ART initiation; [b]= analysis based on multiple imputation of missing covariates.

4.4.5 Tuberculosis co-infection on second-line ART

Forty-five patients developed TB (pulmonary and extra-pulmonary) requiring concurrent rifampicin therapy. These patients received a super-boosting total daily ritonavir dose of 400mg according to the WHO recommendation for settings without access to rifabutin. TB was diagnosed and anti-TB therapy was started prior to the initiation of second-line therapy in 18 patients, 3 of whom died within a mean of 5 months (range: 1-8) of second-line initiation. The remaining 27 patients developed TB while on second-line therapy, 14 of whom died within a mean of 11 months (range: 1-35) of TB therapy initiation. The mortality in patients with TB co-infection was 38%.

4.4.6 Virological outcome in patients with protocol-defined treatment failure

Among the 60 patients who experienced treatment failure, 27 patients had HIV RNA measured using Abbott M2000 real-time HIV-1 assay (figure 4.6). 19/27 patients (70%) had HIV RNA levels above the limit of detection (>150 copies/mL); 8/27 patients had undetectable HIV RNA. Among the 19 patients with detectable viral loads, 17 had HIV RNA levels > 5,000 copies/mL. Six patients experienced immunological failure based on WHO immunological criteria yet they were virologically suppressed after 1 to 2 years of second-line ART initiation. The median CD4 count of the six patients at time of second-line failure detection was 67 cells/μL (IQR: 54-70), and in all the CD4 counts had never risen above 100 cells/μL.

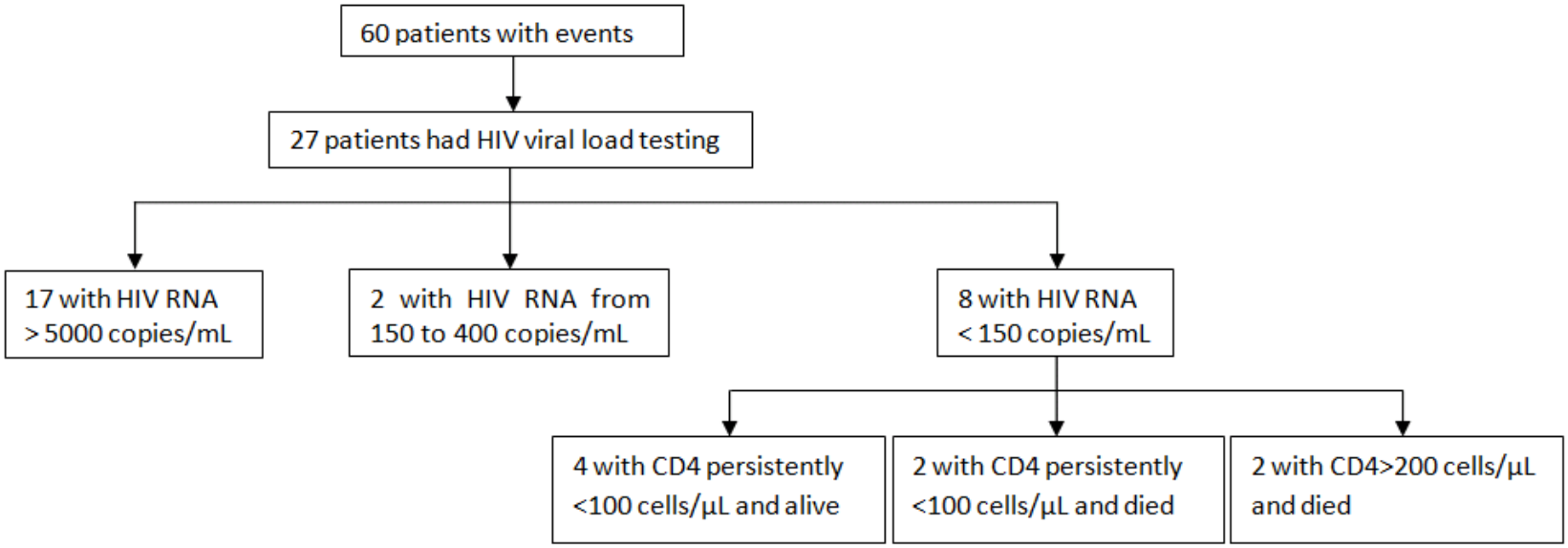


Figure 4.6: Virological outcome in patients with defined treatment failure.

4.5 Discussion

This is the first study to report the outcome of second-line ART in Viet Nam using the standard clinical and immunological assessment according the WHO recommendation for resource-constraint settings. The overall rate of treatment failure to second-line regimens was 18.2% with the median follow up of 29 months, and failure rates increased overtime.

This study showed a similar rate of treatment failure in Viet Nam compared to other countries in resource limited settings (Pujades-Rodríguez *et al.*, 2010). This multi-center study from Africa and Asia reported the average rate of treatment failure of 28% after 2 years on second-line LPV/r based regimens. The definition of treatment failure in this study was the earliest recorded event of either immunological, clinical, or virological failure whereas our definition of treatment failure was the earliest recorded event of either immunological failure, clinical failure, or death. However if considering immunological failure alone, the rate of immunological failure in our study was similar to Pujades' (7% and 8%) (Pujades-Rodríguez *et al.*, 2010).

The vast majority of immunological failure and AIDS events (53%) occurred within the first 6-12 months of second-line treatment, and 89% of deaths were AIDS related deaths. These findings reflect the late onset of therapy switching. When compared to other resource limited countries, time from first treatment failure detection to second-line switch in this study is longer, median 9 (range: 5-15) versus 5 (range: 1-8) months (Castelnuovo *et al.*, 2009; Fox *et al.*, 2010; Levison *et al.*, 2011; Win *et al.*, 2011). In this study the median CD4 count and HIV viral load at therapy switch were 44 cells/μL and 5.1 log copies/mL, respectively. These are compared to a median CD4 count of greater

than 100 cells/μL and HIV RNA ranging from 3.9 to 4.8 log copies/mL in other countries in Africa and Asia (Castelnuovo *et al.*, 2009; Hosseinipour *et al.*, 2010; Levison *et al.*, 2011; Manosuthi *et al.*, 2007; Sigaloff *et al.*, 2012; Win *et al.*, 2011). The high viral loads and low CD4 cell counts in our cohort likely reflected late therapy switching. Prolonged failing therapy leads to further accumulation of resistance mutations which can compensate the low replication capacity of mutant strains. The increased number of mutations will eventually overcome the reduced fitness of early mutant strains, resulting in an increase in viral load level (Lohse *et al.*, 2007). Late switching to second-line ART can be attributed to a numbers of reasons; some may not be unique to Viet Nam. First, in many parts of the world virological monitoring is not routinely performed due to its high cost and unavailability. It takes months to years for a patient who fails treatment virologically to manifest treatment failure immunologically and clinically. HIV viral load in this study was only performed to confirm treatment failure in patients who already met the WHO criteria for clinical and/or immunological failure after at least 6 to 12 months on continuous first line ART. Immunological failure generally has to be confirmed by a repeat CD4 measurement in another 6 month period, further delaying the onset of treatment failure detection. In Viet Nam cases of confirmed immunological and/or clinical failure from provincial and district clinics have to be referred to a major treatment center that have access to viral load and drug resistance testing. A detectable viral load has to be confirmed after demonstration of good adherence for 3 months. HIV genotyping is currently performed only at 2 major centers in the countries and takes up to 3 months for a result. Once evidence of virological failure (with or without genotyping) from a patient failing therapy is gathered, the case is presented to a selected panel of experts at

major HIV treatment centers who make the final decision on therapy switch and make recommendation on second-line ART regimen. This entire process can take one to two years from the time a patient presumably develops virological failure if routine virological monitoring is available.

In countries highly endemic for TB, co-infection with TB in HIV patients failing ART is common. The rate of treatment failure amongst patients with TB co-infection in our cohort was significantly higher (51%) compared to that from Malawi cohort (13%) (Hosseinipour *et al.*, 2010). This is likely a result of delayed ART switching due to drug-drug interaction. HIV and TB co-infection is a common reason for clinicians to delay second-line ART initiation with a ritonavir-boosted PI regimen because of the CYP450 effect of rifampicin and PI drug-drug interactions. The WHO recommends rifabutin in place of rifampicin in patients who need TB treatment while on PI therapy. Unfortunately rifabutin is not available in TB-endemic resource-limited settings, in which case a super-boosted dose of ritonavir (400mg twice daily) is recommended (WHO, 2013b). The safety data of this super-boosted ritonavir regimen (LPV/r dosages of 800mg/800mg daily) in HIV-associated TB are very limited (Colby *et al.*, 2012; Murphy *et al.*, 2012), and significant side effects and hepatic toxicity have been reported in healthy volunteers (Nijland *et al.*, 2008). Therefore many clinicians in developing countries would defer second-line therapy with PI until the initial rifampicin-containing phase of TB treatment is complete. The high mortality rate in HIV patients co-infected with TB might also be attributed to Immune Reconstitution Inflammatory Syndrome (IRIS). Up to one-third of patients with TB/HIV co-infection developed TB-IRIS after the initiation of ART (Colebunders *et al.*, 2006; Kumarasamy *et al.*, 2004; Meintjes *et al.*, 2010; Michailidis *et*

al., 2003). Given that CD4 less than 50 cells/μL as a predictor for TB-IRIS (Conesa-Botella *et al.*, 2011; Meintjes *et al.*, 2010), our patients with CD4 of 41 cells/μL have high risk of TB-IRIS development after ART initiation. However there is no evidence of increased mortality among TB-IRIS patients (De *et al.*, 2011; Manosuthi *et al.*, 2006a; Worodria *et al.*, 2011).

The main purpose of using ART is to provide long-term clinical benefit for HIV infected patients. The WHO definition of clinical failure is defined as a new or recurrent WHO stage IV event. The list of WHO disease staging forms the basic HIV treatment knowledge, is user friendly, and is available in all ART clinics all over the world. While being a relatively sensitive measure of treatment failure, it measures a very late-stage of treatment failure. By the time patients develop clinical events, it is too late, and as shown by our data, the mortality is high. Adding routine laboratory monitoring (including CD4 count) is associated with improved health and survival compared to clinical monitoring alone (DART Trial Team *et al.*, 2010; Mermin *et al.*, 2011). However the use of immunological and clinical definitions of treatment failure can still lead to over-estimation or under-estimation of virological treatment failure (Keiser *et al.*, 2009; Rawizza *et al.*, 2011; Rutherford *et al.*, 2013). Some patients were classified as treatment failure while having undetectable viral load, whereas virological failure in some patients goes undetected with false positive and false negative rates of 1.9% to 4.6%, respectively (CDC, 2006; Church *et al.*, 2011). In our study six patients experienced immunological failure based on WHO immunological criteria yet they were virologically suppressed after 1 to 2 years of second-line ART initiation. Although HIV viral load was undetectable in these patients, two patients died of AIDS, with the worst treatment

outcome overall compared to the entire patient cohort. Cases of immunological non-responders have been reported, and the pathogenesis of this is still unclear (Gazzola *et al.*, 2009). In our cohort of 330 patients, none had an undetectable viral load with a CD4 count drop more than 50% of an on-treatment peak value. Together with the fact that CD4 non-responders have worse clinical outcome, our study suggests that the WHO immunological failure criteria has a reasonably good predictive value for detection of treatment failure, thus continues to be useful in guiding care in settings where viral load monitoring is unavailable.

In our cohort of 330 patients with a median follow up period of 29 months, lower CD4 counts at therapy switch, poor adherence, history of IDU, previous PI use, and older age independently predict treatment failure both as a composite primary endpoint of immunological and/or clinical failure and as a secondary endpoint of death. Although not all previous studies of second-line ART outcome have shown consistent results (Fox *et al.*, 2010; Hosseinipour *et al.*, 2010; Levison *et al.*, 2011; Sigaloff *et al.*, 2012), our findings are consistent with established risk factors for first-line ART failure worldwide (Kamya *et al.*, 2007; Krüsi *et al.*, 2010; Lert & Kazatchkine, 2007; Maskew *et al.*, 2012; Nachega *et al.*, 2007; Vinikoor *et al.*, 2014; Zaragoza-macias *et al.*, 2010). There are conflicting data on the relationship between age and HIV treatment outcomes. A pharmacokinetic study from the UK showed that plasma PI concentration increased with increasing age, suggesting there may be a benefit on treatment outcome (Winston *et al*., 2013). A multi-cohort study in Europe showed that patients with older age (>50 years old compared to patients who were <17 years) had higher chance of virological response at 12 month; however this same study showed a worse immunological and clinical outcome

in patients older than 50 years after adjusting for CD4 cell counts among patients receiving either NNRTI- or PI-based regimens (COHERE Study Group *et al*., 2008). Older age at ART initiation has been linked to poor immunological recovery, loss to follow up and death in first-line ART cohorts in Zambia and South Africa (Maskew *et al*., 2012; Vinikoor *et al*., 2014). Our results together with the literature suggest that functional impairment of immune systems in older patients may impact HIV outcome.

In our study treatment adherence was evaluated during the preceding 6 months of the study assessment, and this window by the study design spanned the entire spectrum of time patients were on second-line ART (IQR: 15-44 months). Poor adherence at anytime during second-line ART was shown to be predictive of treatment failure. This finding was seen in other second-line cohorts from South Africa, Malawi and Thailand (Hosseinipour *et al.*, 2010; Sigaloff *et al.*, 2012; Win *et al.*, 2011), highlighting the importance of ongoing patient education and adherence support to improve treatment outcome. The impact of treatment adherence to PIs might be different from other drug classes. NNRTIs are potent drugs with long half-life in the plasma and have low genetic barrier, providing sub-optimal drug pressure during treatment interruption (Bangsberg *et al.*, 2004). PIs, however, have short half-life and high genetic barrier. Treatment interruption; therefore, results in a sharp decline of PI concentration to the level that is not sufficient for PI resistance mutations to develop (Rosenbloom *et al.*, 2012). In patients taking NRTIs and NNRTIs, strengthening treatment adherence support may not be beneficial once a single drug resistance mutation emerges; whereas in patients with virological failure to PI-based regimens, strengthening treatment adherence might still be beneficial because accumulation of resistance mutations can be prevented (von Wyl *et*

al., 2013). History of IDU predicted treatment failure independently from treatment adherence. This effect is likely multi-factorial and likely involves cofactors not measured in this study including nutritional status, social economic status and hepatitis B and/or C co-infection. The prevalence of HIV and HCV co-infection among IDU was 88.6% in northern Viet Nam (Ishizaki *et al.*, 2011). HCV co-infection has been shown to worsen ART outcome (Klein *et al.*, 2003; Zinkernagel *et al.*, 2006).

Levison *et al.* reported that for every month a patient remained on a failing first-line ART regimen, there was a 7% increase in risk of lack of virologic suppression (Levison *et al.*, 2011). The longer a patient is on a non-suppressive ART regimen, the higher the chance of developing accumulation of drug resistance mutations, which does not only impair the outcome of current regimen but also of future ART regimens. When the relationship of treatment delay and CD4 count were assessed with respect to therapy failure and death in our study, only lower CD4 count predicted therapy failure and death. The likely explanation for this is that these two variables are interdependent or have a causal relationship, i.e. treatment delay directly results in a decline in CD4 count, in which case a stronger variable will likely drive the outcomes. A clinical explanation for this is that therapy delay promotes further immune deterioration but not at equal rates in different hosts. Patients with more rapid disease process will have greater CD4 cell decline, which is a stronger predictor of AIDS-related complications and death. In our study, CD4 count but not time on a failing first-line ART regimen predicted treatment failure. The opposite is true for Levison *et al.* report, time on a failing regimen and not CD4 predicted outcome (Levison *et al.*, 2011).

Our study is not without limitations. Although HTD is the largest referral center for HIV care in the country, this is a one-center study; therefore patient population may not be representative of the true patient population on second-line therapy in Viet Nam. The HTD is one of a few centers in the country with access to both viral load and genotyping testing, thus true treatment failure rates may be underestimated. One limitation is that clinical data are not prospectively collected from the time second-line ART was initiated. However the national HIV program is designed such that all study variables (age, IDU, CD4 count, AIDS and non-AIDS events, adherence) are routinely assessed and are part of standard HIV care in Viet Nam. Patients have to come to clinic every month for a clinical evaluation and to receive medicine, thus these variables are collected unlike that of a prospective study. The only practical limitation is that 59 patients (18%) were transferred to another clinic; these patients were censored in the primary analysis of predictors of treatment failure. However clinicians generally make the transfer only when a patient is judged to have good treatment response, thus we conducted a sensitivity analysis with informative censoring (treating them as censored at the time-point where their last monthly follow-up visit would have been had they not been transferred), and the results were consistent.

In conclusion this is the first study reporting outcome of second-line ART with a PI-based regimen in Viet Nam. The clinical and immunological treatment failure rate is 18.2% after a median follow up of 29 months. Lower CD4 count at therapy switch, older age, history of IDU and poor adherence are risk factors for treatment failure. In the absence of routine virological monitoring, interventions targeting earlier detection of first-line treatment failure, initiation of second-line ART at higher CD4 counts, and

medication adherence will improve the treatment outcome of patients on second-line ART in Viet Nam.

5 Virological outcome and drug resistance development in HIV-1 infected adults receiving second-line antiretroviral therapy in Ho Chi Minh City, Viet Nam

5.1 Abstract

The number of patients on second-line ART with a ritonavir boosted protease inhibitor (PI) in Viet Nam is growing, and there is no data on virological outcome and drug resistance to guide future treatment strategies. In the absence of routine virological monitoring, we performed a cross-sectional virological assessment of all patients aged ≥15 years who had been on second-line ART for at least 6 months at the HTD in HCMC. Virological failure was defined as a repeated HIV RNA measurement of > 1000 copies/mL, performed at least one month apart and after intensive adherence counseling. Logistic regression modeling was used to identify factors associated with virological failure using a pre-defined set of variables that included age at initiation of second-line therapy, history of IDU, history of PI use, CD4 cell count, and HIV RNA viral load at second-line ART initiation, total time on a failing first-line ART regimen, and treatment adherence. Medication adherence was assessed using a standard visual analog scale. HIV drug resistance mutations were identified using standard population sequencing of reverse transcriptase and protease genes and were interpreted according to the IAS-USA 2013 mutation list. HIV drug susceptibility was interpreted according to HIV Stanford drug resistance database. Among 380 patients who had started second-line ART, 231 patients were alive and in active follow up for at least 6 months, and all were enrolled into the virological survey between June 2011 and February 2012. The median time on

second-line ART was 29 months (Interquartile range [IQR]: 16-44). 81% were male. The median age was 32 years. The median CD4 count and HIV RNA at time of second-line ART initiation was 44 cells/µL (IQR: 17-84) and 5.1 log copies/mL (IQR: 4.6-5.5), respectively. All patients received a LPV/r regimen. 22 (9.5%) patients had protocol-defined virological failure. History of prior PI use, higher viral load at second-line ART initiation, and suboptimal adherence (adherence <90% on a visual analog scale) independently predicted virological failure in both univariate and multivariate analyses. 14/22 patients (64%) had at least one major PI mutations, and 12/22 (55%) had resistance mutations to all drugs in the second-line regimen. The most common PI mutations were V82A, M46I/L, and I84V. Cross resistance to ETR, TPV and DRV was present in 55%, 45%, and 27% patients, respectively. In conclusion LPV/r-based ART is effective in re-suppressing viral replication in patients failing first-line ART. Prior PI use, higher viral load at therapy switch, and poor adherence predicted virological failure to second-line ART. Given high prevalence of cross resistance to newer antiretroviral drugs, genotype testing should be performed to guide selection of a salvage third-line regimen.

5.2 Introduction

The WHO endorses ritonavir-boosted protease-inhibitor (PI/r) based ART as efficacious second-line treatment after failure of non-nucleoside reverse transcriptase inhibitor (NNRTI) based first-line therapy in resource-limited settings (WHO, 2013a). PIr-based therapy is highly potent in ART-naïve patients participating in clinical trials (Eron *et al.*, 2006; Murphy *et al.*, 2008; Riddler *et al.*, 2008) and has high efficacy as second-line therapy in resource-limited settings (Ajose *et al.*, 2012; Pujades-Rodríguez *et*

al., 2010). Nevertheless, up to 20% of patients in resource-rich and 27% of patients in resource-limited settings develop virological failure on PI/r-based ART (Ajose *et al.*, 2012; Ortiz *et al.*, 2008; Riddler *et al.*, 2008). PI resistance is rarely observed in patients failing PIr-based therapy in clinical trials (Daar *et al.*, 2012; Eron *et al.*, 2006; Kempf *et al.*, 2004; Riddler *et al.*, 2008) and is, as well, uncommonly seen (range 0-7%) in PI-naïve patients failing second-line therapy in Sub-Saharan Africa (El-khatib *et al.*, 2010; Levison *et al.*, 2012; Reynolds *et al.*, 2012; Van Zyl *et al.*, 2011; Wallis *et al.*, 2011). However a study from Cambodia (Nerrienet *et al.*, 2012) and one from India (Saravanan *et al.*, 2012) have reported PI mutation prevalence of 30% and 70%, respectively. Data on protease inhibitor (PI) resistance developed on second-line ART in Asia are still very limited. There are many questions about the risk factors for PI resistance in various programmatic settings, the contribution of HIV-1 subtypes on mutation development, and the clinical outcomes of patents with PI resistance on long-term second-line ART.

Viet Nam is 99% dominated by HIV-1 subtype CRF01_AE (Ayouba *et al.*, 2009; Ishizaki *et al.*, 2009; Lan *et al.*, 2003; Phan *et al.*, 2010; Thao *et al.*, 2012) and is amongst the Asian countries with the highest HIV burden (UNAIDS, 2013). An estimate of 3% of a total of 88,800 people on ART are on second-line therapy (Viet Nam MOH, 2014). At the HTD in HCMC, approximately 8% of patients on ART are on a second-line regimen, and the need for second-line therapy will continue to increase the longer patients being on first-line ART. Viral load monitoring is prohibited by its costs; therefore data on virological outcome and drug resistance in patients on second-line therapy are lacking.

As the demand for second-line ART continues to increase and international funding for ART in Viet Nam is drastically being cut, knowledge of second-line ART

outcome, factors that impact outcome and resistance pattern is critical to guide treatment strategies. In chapter 4 we evaluated second-line treatment outcome using the standardized clinical and immunological criteria of treatment failure recommended by the WHO for resource-limited settings. In this chapter we aimed to generate data on antiretroviral resistance profiles of HIV-1 CRF01_AE-infected patients with viremia on second-line protease-inhibitor therapy at the largest ART centre in Viet Nam. Our objectives were to identify the risk factors for resistance development, describe the long-term clinical outcomes of patients with resistance maintaining on a failing second-line regimen, and to investigate cross-resistance to second-generation NNRTIs and PIs to inform national policy on third-line therapy.

5.3 Methods

5.3.1 Study population and data collection

This is a cross sectional virological assessment of all patients on second-line ART for at least 6 months at the HTD in HCMC. The study included all patients in the second-line ART cohort presented in chapter 4 who were alive and in active follow up at the time of study assessment. Routine clinical data were collected from the patients' clinic charts on a standardized case report form and included demographic data, history of IDU, ART drug history, serial CD4 counts, HIV viral load and genotype at time of treatment failure to first-line ART (if available). Treatment adherence was evaluated by using the visual analog scale (VAS) score as described in chapter 4.

5.3.2 HIV viral load measurement and genotype testing

Blood samples were collected at the time of enrollment for HIV viral load measurement using the Abbott RealTime HIV-1 assay (*m*2000, Abbott Molecular, IL, USA) as described in chapter 2. With 200 µL of plasma input, the limit of detection for this assay was 150 copies/mL. Virological failure was defined as a repeated HIV RNA level (performed at least 1 month apart after intensive adherence counseling) above 1000 copies/mL after at least 6 months of second-line ART. Drug resistance testing was performed as described in chapter 2. HIV RNA was extracted from 140 µL of plasma samples by using manual extraction with Qiagen viral mini kit. Drug resistance mutations were assessed using International AIDS Society (IAS)-USA list (Johnson *et al.*, 2013). The drug susceptibility was estimated by Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu/).

5.3.3 Statistical analysis

A logistic regression model was used to identify factors associated with virological failure. The following pre-defined covariates were included in the model: age at initiation of second-line therapy, history of IDU, history of PI use, CD4 cell count, and (log10-transformed) HIV RNA viral load at second-line ART initiation, total time on a failing first-line ART regimen, and treatment adherence based on the VAS score (<90% vs. ≥90%). Both univariate and multivariable logistic regression analyses were performed.

Among patients whose HIV genotype was performed at time of treatment failure to first-line ART, we investigated the impact of the existing NRTI resistance to

virological outcome of second-line ART. The genotypic level of resistance for each NRTI drug in the second-line regimen (TDF, 3TC, ±AZT) was defined by the Stanford HIV Resistance Database (http://hivdb.stanford.edu/). For each drug a score is given based on the interpretation of virus genotype: 0 is assigned to high-level resistance, 0.5 is assigned to low- or intermediate-level resistance, and 1 is assigned to full susceptibility or potential low-level resistance. The sum of the scores for all NRTI drugs in the second-line ART backbone is the Genotypic Sensitivity Score (GSS) score for a patient. The association of having a GSS of 0, 0.5, or ≥1 to virological outcome was analyzed using logistic regression analyses.

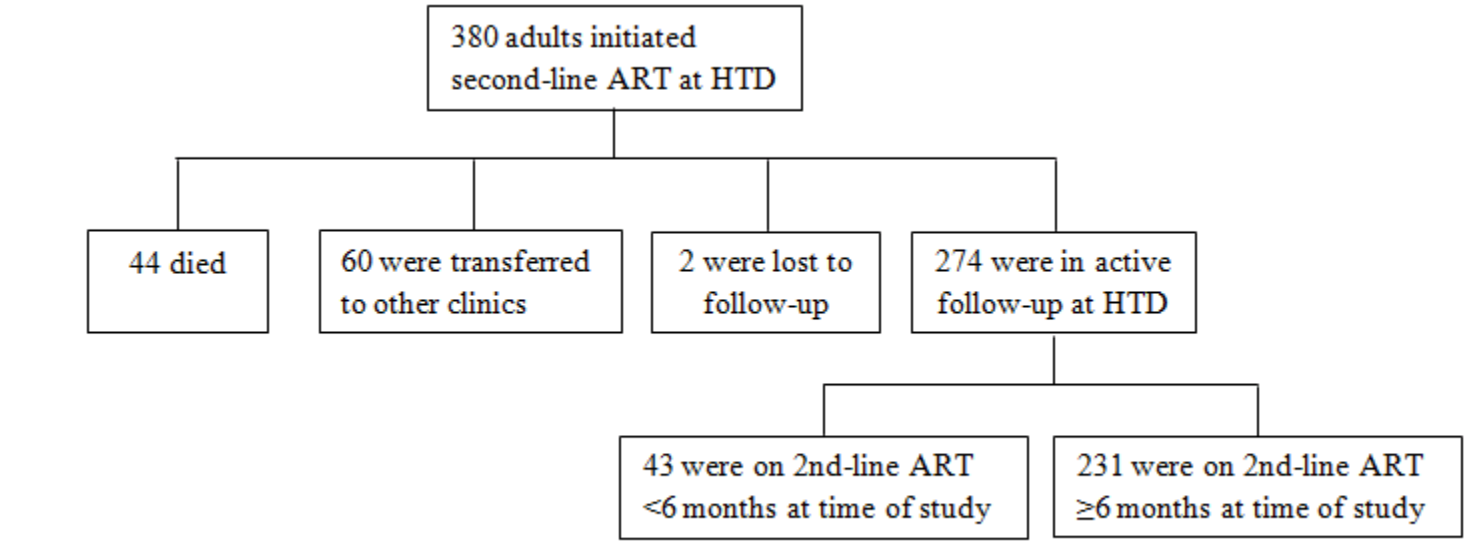
All reported confidence intervals were two-sided 95% confidence intervals and analyses were performed with the statistical software R version 2.15.0 (R Development Core Team, 2012).

5.4 Results

5.4.1 Study population and baseline characteristics

Among 380 adult patients initiated second-line ART at the HTD, 231 (61%) patients were alive, in active follow up, and had been on therapy for at least 6 months. All were enrolled into the virological survey study, conducted between June 2011 and February 2012 (Figure 5.1). The characteristics of the patients at the time of second-line initiation were summarized in Table 5.1. The median CD4 cell count was 44 cells/µL (IQR: 17–84 cells/µL) and median HIV RNA was 5.1 log copies/mL (IQR: 4.6–5.5). The median time the patients had been on a failing first-line ART regimen was 9 months (IQR: 5 - 15). All patients received a second-line ART regimen that included TDF, 3TC,

and LPV/r. One-third of patients received a 4th drug AZT as some clinicians believe presence of AZT will reduce the likelihood of developing TDF resistance mutation K65R. Clinical studies showed that the lack of TAMs is a predictor for K65R selection (Valer *et al.,* 2004; von Wyl *et al.,* 2008). Kinetic analyses of reverse transcriptase showed that TAMs antagonize the ability of reverse transcriptase carrying K65R to discriminate nucleotide analogue (Parikh *et al.,* 2006, 2007). AZT selects for TAMs; hence, reduces the likelihood of K65R development. Treatment adherence was optimal (≥90% on a VAS) in 88%.



HTD = Hospital for Tropical Diseases

Figure 5.1: Flow-chart of study population

Table 5.1: Characteristics of patients at the time of second line ART initiation at the Hospital for Tropical Diseases in Ho Chi Minh City

Characteristics	N=231
Sex, male (%)	187 (81%)
Age, median (IQR) (years)	32 (28-36)
Previous history of IDU (%)[(n=230)]	93 (40%)
Time on a failing 1st line failing ART regimen [a], median (IQR) (months)[(n=228)]	9 (5-15)
CD4 count (cells/μL) at 2nd line ART initiation, median (IQR)[(n=227)]	44 (17-84)
HIV RNA (log copies/mL) before 2nd line ART initiation, median (IQR)[(n=215)]	5.1 (4.6-5.5)
2nd line regimens (%)	
TDF/3TC/ LPV/r	128 (55.4%)
TDF/3TC/ LPV/r + AZT	88 (38.1%)
LPV/r + other NRTIs (ABC/ddI/AZT/TDF/3TC/d4T)	15 (6.5%)
Adherence [b]	
≥90%	203 (88%)
<90%	28 (12%)

[a,] Time from first detection of treatment failure to 1st line ART to time of 2nd line ART initiation; [b,] Adherence to ART was evaluated by using visual analog scale from 0%-100%; Abbreviations: IQR, Interquartile range; IDU, injecting drug users; LPV/r, ritonavir-based lopinavir; NFV, nelfinavir; TDF, tenofovir; 3TC, lamivudine; AZT, zidovudine.

5.4.2 Drug resistance mutations to first-line ART

Among 231 patients with treatment failure to first-line ART, 173 patients (75%) had drug resistance testing performed. All had at least one major drug resistance mutation to first-line ART drugs. Mutations conferring resistance to NRTI, NNRTI and PI were present in 170/173 (98%), in 159/173 (92%), and in 9/173 (5.2%) patients, respectively. The prevalence and patterns of drug resistance mutations in 173 patients at time of second-line ART initiation are shown in Figure 5.2. The most common NRTI mutations were M184I/V (N=149, 86.1%) and TAMs (N=117, 67.6%). The most common NNRTI mutations were Y181C/I/V (N=84, 48.6%), G190A/S (N=74, 42.8%), K103N (N=52, 30.1%), and K101E/P (N=46, 26.1%). The most frequent major PI resistance mutations were M46I/L (N=5), I54V (N=5), L90M (N=3), and V82A (N=2). Overall 97% (168/173) patients did not have fully active NRTI options for second-line ART with 69.4% (120/173) exhibiting intermediate to high level resistance to TDF and 95% (165/173) exhibiting intermediate to high level resistance to 3TC. Four percent (7/173) were not fully susceptible to the main drug in the second-line regimen LPV/r.

Among these 173 patients with available genotypic testing after first-line ART failure, GSS score of second-line NRTI back-bone was calculated for each patient. Forty-five (26.0%) patients had GSS ≥1; 77 (44.5%) patients had GSS ranging from above 0 to less than 1; 51 (29.5%) patients had GSS=0.

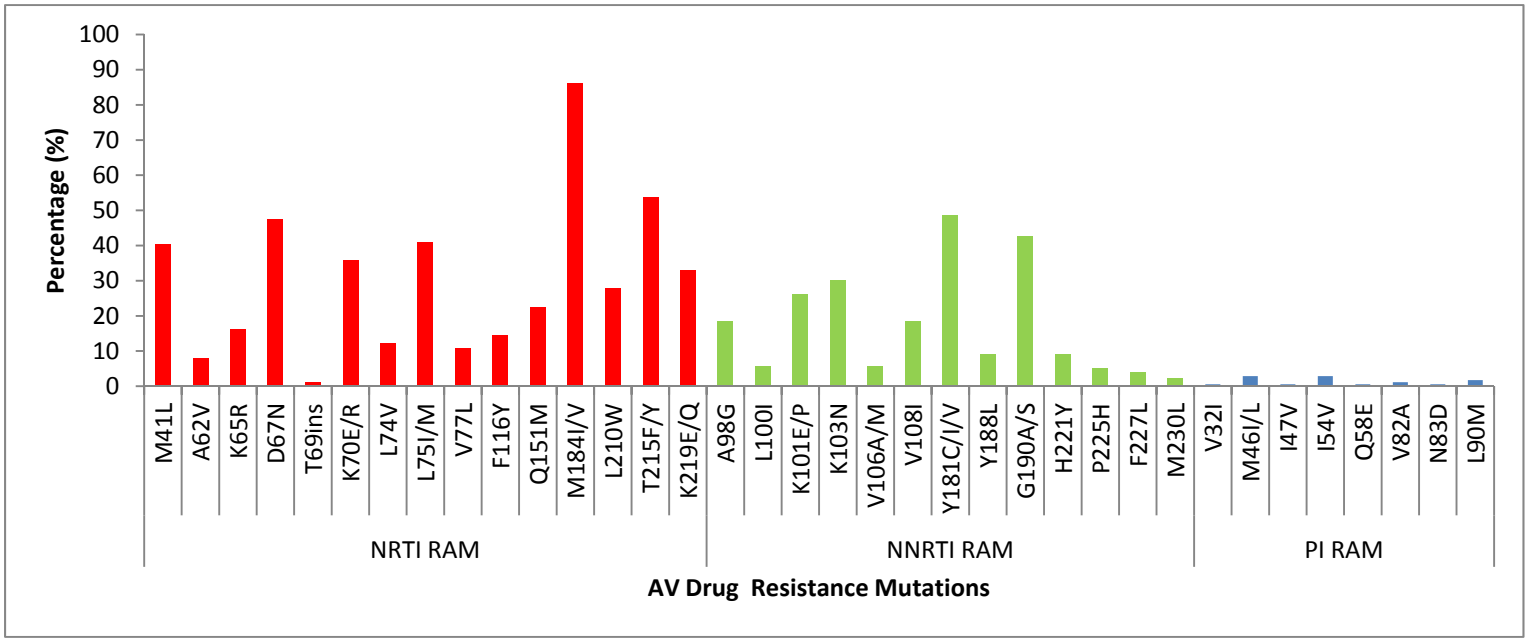


Figure 5.2: Prevalence and patterns of ARV resistance mutations in 173 patients at time of second-line ART initiation in Ho Chi Minh City.

NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleotide reverse transcriptase inhibitor; PI, protease inhibitor; RAM, resistance associated mutation; ARV, antiretroviral.

5.4.3 Virological outcome and factors associated with virological failure

A total of 22 out of 231 (9.5%) patients met protocol-defined virological failure with HIV RNA above 1000 copies/mL, with a median HIV RNA of 4.35 log10 copies/mL (IQR: 3.39-5.01). 27/231 (11.7%) patients had a HIV RNA above 400 copies/mL, and 200/231 (86.6%) patients had undetectable viral load (<150 copies/mL). The 7 pre-defined covariates used to assess prediction of virological failure and the results of the logistic regression analyses are listed in Table 5.2. Higher viral load (+log 10 copies/mL higher) at second-line ART initiation, suboptimal adherence (less than 90%), and previous PI use significantly increased the risk of virological failure in both univariate and multivariate analyses (Table 5.2). Sensitivity analyses were conducted with virological failure being defined as HIV RNA above 400 copies/mL or HIV RNA above limit of detection (Table 5.3). The effect of higher viral load, suboptimal adherence and previous PI use remained consistent. In addition, time on a failing first-line ART regimen became an independent predictor of suboptimal virological suppression.

There was no association between GSS and virological failure (Table 5.4). An exploratory analysis of the addition of AZT as a 4th drug to second-line regimen did not show a significant association with virological outcome (OR=1.70, 95% CI: 0.65 - 4.45; p=0.274).

Table 5.2: Factors associated with protocol-defined virological failure (HIV RNA ≥ 1000 copies/mL) in 231 patients on second-line ART in Ho Chi Minh city

Covariate	Univariate effect		Multivariate effect	
	OR (95% CI)	**p-value**	**OR (95% CI)**	**p-value**
Age* (by +10 years)	1.32 (0.66-2.47)	0.409	1.53 (0.50-4.47)	0.437
History of IDU (yes) (N=230)	0.72 (026-1.79)	0.488	3.61 (0.76-20.15)	0.118
CD4* (by +50 cells/μL) (N=227)	0.72 (0.42-1.09)	0.184	0.70 (0.30-1.27)	0.328
log10VL* (by + log 10 copies/mL) (N=215)	**3.14 (1.56-6.69)**	**0.002**	**3.66 (1.38-11.01)**	**0.013**
Time on a failing 1st line therapy (by +6 months) (N=228)	1.08 (0.82-1.38)	0.537	1.09 (0.74-1.56)	0.662
Adherence < 90% (yes)	**4.23 (1.47-11.37)**	**0.005**	**7.67 (1.76-14.99)**	**0.006**
PI use (yes) (N=211)	**5.50 (2.02-14.91)**	**<0.001**	**23.47 (5.31-103.43)**	**<0.001**

OR=odds ratio, CI=confidence interval, *=at time of 2nd line ART initiation, N = numbers of patients without missing values

Table 5.3: Factors associated with virological failure (HIV RNA ≥ 400 copies/mL or HIV RNA ≥ 150 copies/mL) in 231 patients on second line ART in Ho Chi Minh City (multivariate sensitivity analyses)

Covariate	HIV RNA ≥ 400 copies/mL		HIV RNA ≥ 150 copies/mL	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Age* (by +10 years)	1.40 (0.51-3.66)	0.493	1.76 (0.69-4.41)	0.227
History of IDU (yes) (N=230)	1.79 (0.44-7.55)	0.415	1.79 (0.48-6.94)	0.384
CD4* (by +50 cells/µL) (N=227)	0.61 (0.27-1.09)	0.161	0.54 (0.23-1.00)	0.102
log10VL* (by + log 10 copies/mL) (N=215)	**2.94 (1.21-7.80)**	**0.021**	**3.73 (1.57-9.86)**	**0.005**
Time on a failing 1st line therapy (by +6 months) (N=228)	**1.44(1.10-1.96)**	**0.022**	**1.43 (1.06-1.95)**	**0.020**
Adherence < 90% (yes)	**4.53 (1.09-18.26)**	**0.033**	**4.77 (1.24-18.06)**	**0.020**
PI use (yes) (N=211)	**20.11 (5.34-90.29)**	**<0.001**	**15.79 (4.38-65.27)**	**<0.001**

OR=odds ratio, CI=confidence interval, *=at time of 2nd line ART initiation, N = numbers of patients without missing values

Table 5.4: Impact of genotypic sensitivity score (GSS) on virological outcome

	OR (95% CI)	p-value
GSS ≥ 1	1	-
GSS = 0.5	2.26 (0.66-10.44)	0.229
GSS = 0	0.91 (0.16-5.17)	0.914

5.4.4 Genotypic resistance patterns of patients failing second-line ART

The treatment history and drug resistance mutations identified in the 22 patients with virological failure are shown in Table 5.5. The patterns of ARV susceptibility predicted based on the Stanford HIV Drug Resistance database (version 7.0 updated on 27 February 2014) are shown in Figure 5.3.

Major mutations conferring resistance to NRTI, NNRTI and PI drugs were present in 19 (86%), 17 (77%) and 14 (64%) patients, respectively. In twelve patients (55%) the virus was resistant to all drugs in the second-line regimen (TDF, 3TC, and LPV/r). Two patients (patients # 14 and 18) had no major resistance associated mutations detected and were fully susceptible to all drugs in the second-line regimen. Eight patients did not have any major PI mutations detected.

Among the 14 patients carrying major PI resistance associated mutations, 10 had a history of prior PI use, compared to only 2 of 8 patients without PI resistance associated mutations. The most common PI used prior to the availability of LPV was IDV (N=11) and NFV (N=2). The most common major PI mutations were V82A/F (N=9), M46I/L

(N=8), and I84V (N=4) (Table 5.5). Intermediate to high level cross resistance to ETR, TPV and DRV was present in 55%, 45%, and 27% patients, respectively.

Table 5.5: Antiretroviral history, drug resistance profile and two-year outcomes of 22 patients with virological failure on second-line antiretroviral therapy in Ho Chi Minh City

Patient	Time on 2nd-line ART (months)	At time of therapy switch		Prior PI use	Mutations at time of therapy switch			At time of virological failure		Mutations at time of virological failure			2-year outcomes
		CD4 (cells/µl)	Viral load (copies/ml)		NRTIs	NNRTIs	PIs	CD4 (cells/µl)	Viral load (copies/ml)	NRTIs	NNRTIs	PIs	
1	15	79	4,720,000	No	L74V, M184I	K101E, K103N, G190A, M230L		191	289,000	D67N, K70R, L74V, M184I, K219Q	K101E, K103N, E138G, G190A, M230L	L10I, V82A	Virological re-suppression
2	18	2	7,590,000	__	_____	_____	_____	2	1,630,000	M184V	K103N, V108I, Y181C		Death
3	19	13	170,000	IDV	_____	_____	_____	50	402,194	T215S		G16E, K20I, M36I, M46L, I54V, H69K, V82A, L89M	Death
4	18	74	118,000	IDV	M41L, D67N, K70R, V75M, M184V, T215F, K219Q	K101P, K103N		303	1,574	M41L, D67N, K70R, V75M, M184V, T215F, K219Q	K101P, K103N	L10I, G16E, K20I, M36I, H69K, L89M	Transferred to other clinic
5	18	45	435,000	IDV	A62V, K65N, T69S, V75M, F77L, Q151M, M184V	V106I, Y181C, Y188L, H221Y	I54V, N83D, I84R	152	1,184	V75M, M184V, T215F	V106I, Y181C, Y188L, H221Y	L10I, K20I, M36I, M46L, F53L, I54V, H69K, V82A, L89I	Worsening HIV control

5. Virological outcome and drug resistance development to second-line ART

Patient	Time on 2nd-line ART (months)	At time of therapy switch		Prior PI use	Mutations at time of therapy switch			At time of virological failure		Mutations at time of virological failure			2-year outcomes
		CD4 (cells/μl)	Viral load (copies/ml)		NRTIs	NNRTIs	PIs	CD4 (cells/μl)	Viral load (copies/ml)	NRTIs	NNRTIs	PIs	
6	29	5	365,000	No	K65R, Q151M	Y181C, G190A	L33F, I84L	143	5,490	K65R, Q151M	Y181C, G190A	K20R, L33F, M36I, M46I, I62V, H69K, L76V, I84V, L89M	Worsening HIV control
7	6	6	190,000	IDV	A62V, D67N, T69P, V75I, F77L, F116Y, Q151M, M184I, T215S, K219Q	K101E, Y181C, G190A	L10V	293	3,910	D67N, V75I, F77L, F116Y, Q151M, M184I, K219Q	K101E, Y181C, G190A	L10V, G16E, M36I, H69K, V82A, L89M	Virological re-suppression
8	31	40	184,000	No	M41L, D67N, T69N, K70R, L74I, M184V, T215F, K219Q	V108I, G190A	L10IV	546	1,520	M41L, D67N, K70R, L74I, M184V, T215F, K219Q	G190A	L10V, G16E, L33F, M36I, I54V, V82A, L89I	Virological re-suppression
9	47	8	253,000	IDV	M41L, D67N, T69N, K70R, L74I, M184V, T215F, K219Q	A98G, K103N, G190A		171	37,379	M41L, D67N, K70R, M184V, T215F, K219Q	K103N, G190A	L10V, K20I, L33F, M36L, M46I, I47V, I54V, H69K, T74P, V82F, L89M	Worsening HIV control
10	8	21	752,000	__	_____	_____	_____	152	16,582	K65R, V75M	V179F, Y181C, H221Y	M36I, H69K, L89M	Virological re-suppression

5. Virological outcome and drug resistance development to second-line ART

Patient	Time on 2nd-line ART (months)	At time of therapy switch		Prior PI use	Mutations at time of therapy switch			At time of virological failure		Mutations at time of virological failure			2-year outcomes
		CD4 (cells/μl)	Viral load (copies/ml)		NRTIs	NNRTIs	PIs	CD4 (cells/μl)	Viral load (copies/ml)	NRTIs	NNRTIs	PIs	
11	45	21	867,000	No	M41L, E44AD, D67N, L74V, V75M, V118I, M184V, L210W, T215Y, K219N	A98G, L100I, K101P, G190A		121	96,147	M41L, D67N, V75M, M184V, L210W	A98G, G190A	M36I, H69K, V82I, L89M	Death
12	43	1	132,000	No	D67N, K70R, M184V, L210W, T215F, K219W	K103N, V108I, Y181C, G190A		11	22,600	M184V			Death
13	50	113	38,238	IDV	M41L, D67N, T69P, K70R, M184V, L210W, T215F, K219E			57	319,798	M41L, D67N, K70R, M184V, L210W, K219QE		L10V, G16E, K20V, L33F, M36I, I47V, I54V, H69K, A71V, I84V, L89M	Worsening HIV control
14	12	25	693,000	No	_____	_____	_____	48	875,664			L10I, K20R, M36I, H69K, L89M	Virological re-suppression

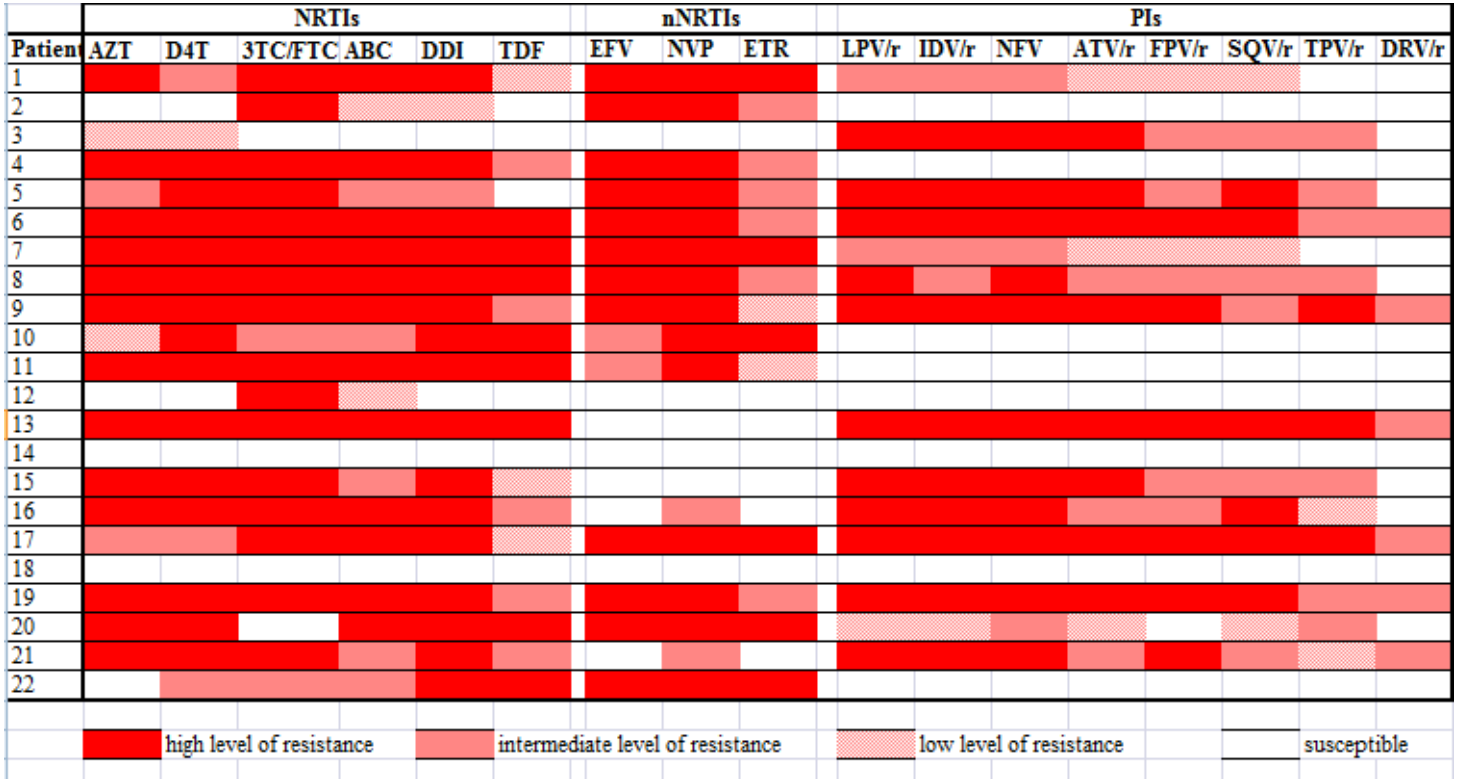
5. Virological outcome and drug resistance development to second-line ART

Patient	Time on 2nd-line ART (months)	At time of therapy switch		Prior PI use	Mutations at time of therapy switch			At time of virological failure		Mutations at time of virological failure			2-year outcomes
		CD4 (cells/μl)	Viral load (copies/ml)		NRTIs	NNRTIs	PIs	CD4 (cells/μl)	Viral load (copies/ml)	NRTIs	NNRTIs	PIs	
15	29	211	189,000	IDV	T69N, V75M		V32I, M46I, Q58E	253	64,262	K70R, V75M, M184V, K219E	V90I	L10I, G16E, K20I, M36I, M46I, I54A, Q58E, H69K, K70R, V82A, L89I	Worsening HIV control
16	47	44	139,000	IDV	M41L, T69N, V75M, F77L, F116Y, Q151M, M184V, T215Y	A98G, L100I, K103N		154	34,900	M41L, V75M, F77L, M184V, T215Y	A98G	L10I, G48A, I54V, A71V, V82A	RAL + DRV/r + 3TC
17	43	64	174,550	IDV	M41L, D67N, K70R, L74V, M184V, T215F, K219Q	Y181C, G190S	M36I	67	47,500	M41L, D67N, K70R, L74V, M184V, T215F, K219Q	A98G, Y181C, G190S	L10I, L33F, M46I, I54M, A71V, G73S, I84V	Death
18	23	41	377,000	No	K65R, V75M	K103N		6	67,934			M36I, H69K, V82I, L89M	Worsening HIV control
19	17	40	590,000	No	D67N, T69N, K70R, L74I, V75M, M184V, T215F, K219E	K101P, Y181C, G190S		352	3,776	D67N, K70R, V75M, M184V, T215F, K219E	K101Q, Y181C, G190A	K20R, M36I, M46I, L63P, H69K, A71V, L76V, I84V, L89M	Transferred to other clinic

5. Virological outcome and drug resistance development to second-line ART

Patient	Time on 2nd-line ART (months)	At time of therapy switch		Prior PI use	Mutations at time of therapy switch			At time of virological failure		Mutations at time of virological failure			2-year outcomes
		CD4 (cells/μl)	Viral load (copies/ml)		NRTIs	NNRTIs	PIs	CD4 (cells/μl)	Viral load (copies/ml)	NRTIs	NNRTIs	PIs	
20	30	3	948,909	__	_____	_____	_____	98	103,000	K70R, T215F, K219E	A98G, K101E, Y181C, Y188L, G190A	L10I, I54V, N83D	Death
21	45	113	2,470,000	IDV	M41L, D67N, V75M, V118I, M184V, L210W, T215F, K219W	K101P, V108I, G190A	L10F	173	79,800	M41L, D67N, V75M, M184V, T215Y	A98G	L10F, M46L, I54V, L76V, V82A, L89V	RAL + DRV/r + 3TC
22	26	14	81,422	IDV	_____	_____	_____	28	83,300	K65R	K101E, Y181C, G190A		Death

ART, antiretroviral therapy; NRTIs, nucleoside/tide reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors; IDV, indinavir; DRV/r, darunavir-ritonavir; RAL, raltegravir; 3TC, lamivudine. Bold: major drug resistant mutations according to the IAS-USA 2014. Dash: data is unknown.



NRTIs, nucleoside reverse transcriptase inhibitors; nNRTIs, non-nucleotide reverse transcriptase inhibitors; PI, protease inhibitors; AZT, zidovudine; d4T, stavudine; 3TC, lamivudine; FTC, emtricitabine; ABC, abacavir; ddI, didenosine; TDF, tenofovir; EFV, efavirenz; NVP, nevirapine; ETR, etravirine; LPV, lopinavir; IDV, indinavir; NFV, nelfinavir; ATV, atazanavir; FPV, fosamprenavir; SQV, saquanavir; TPV, tipranavir; DRV, darunavir; /r, ritonavir-boosted.

Figure 5.3: ARV Drug susceptibility among 22 patients experiencing virological failure to second-line ART in Ho Chi Minh City

5.4.5 Management and follow up of patients with virological failure to second-line ART

The 22 patient who had virological failure to second-line ART were followed up over the subsequent 2 years, and their outcome are summarized in Figure 5.4. Seven patients died: 4 were due to TB co-infection and 3 were from wasting syndrome. Two patients who could afford the treatment were switched to RAL and DRV purchased from Thailand. The remaining 13 patients were alive and continued taking second-line ART. A follow up blood sample was collected from 11 of these 13 patients for viral load testing (two patients were transferred to other clinics and refused blood test). 5/11 patients had virological re-suppression and CD4 cell count increasing to above 380 cells/μL (average CD4: 611 cells/μL) upon continuation of second-line regimen. At the time of virological failure to second-line therapy, two of the five patients had no PI resistance mutation. The other three patients carried a single major PI mutation V82A which confers intermediate-level resistance to LPV/r. 6/11 patients continued to have viral load above 1000 copies/mL (median HIV RNA: 5.10 log copies/mL; range: 4.97-5.39). Five of them continued to carry viruses with multiple major PI resistance mutations. One patient had wild-type virus with a CD4 count below the limit of detection, suggesting the patient had not adhered to his medications. All 6 patients had immunological failure with decreasing CD4 counts to below 200 cells/μL; however they had not developed any AIDS events.

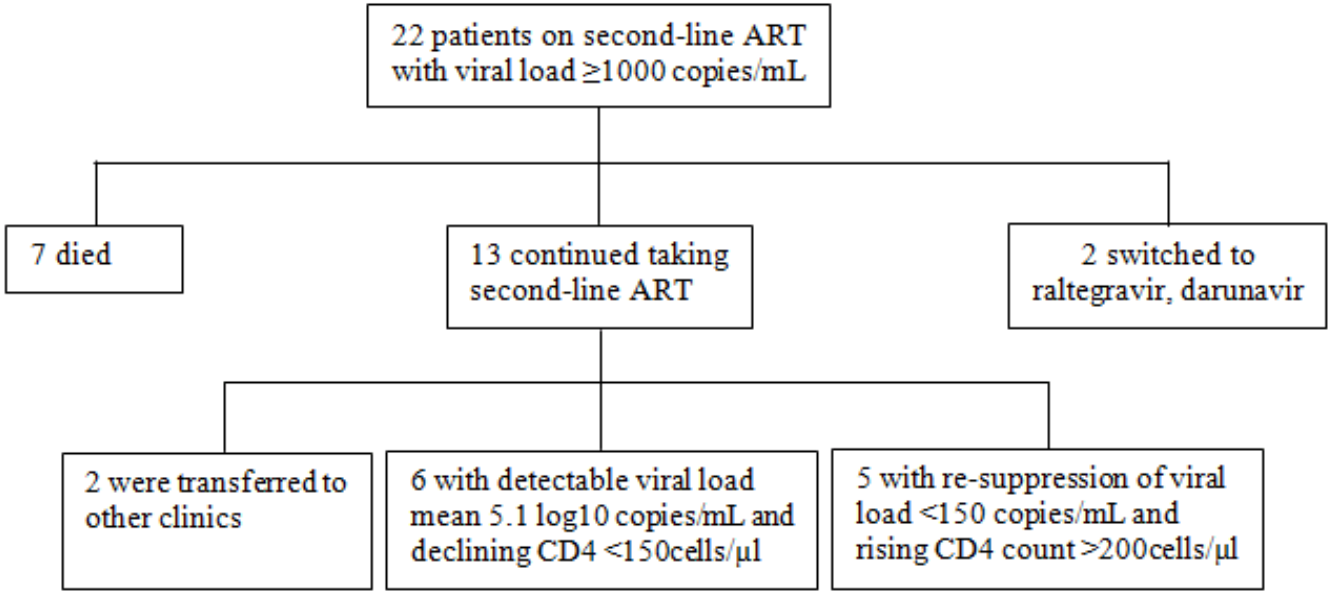


Figure 5.4: Two year-follow-up for 22 patients with virological failure to second-line ART

VL, viral load; ART, antiretroviral therapy

Table 5.6: The evolution of resistance mutations of 6 patients with worsening HIV control maintaining on a failing second-line regimen

Patient	Time on 2nd-line ART (months)	At time of virological failure		Mutations at time of virological failure			At 2-year follow-up		Mutations at 2-year follow-up		
		CD4 (cells/µl)	Viral load (copies/ml)	NRTIs	NRTIs	PIs	CD4 (cells/µl)	Viral load (copies/ml)	NRTIs	NNRTIs	PIs
5	18	152	1,184	V75M, M184V, T215F	V106I, Y181C, Y188L, H221Y	L10I, K20I, M36I, M46L, F53L, I54V, H69K, V82A, L89I	77	93,500	A62V, K65N, V75M, F77L, Q151M, M184V	V106I, Y181C, Y188L, H221Y	L10F, K20I, M36I, M46L, F53L, I54V, H69K, V82A, L89I
6	29	143	5,490	K65R, Q151M	Y181C, G190A	K20R, L33F, M36I, M46I, I62V, H69K, L76V, I84V, L89M	177	164,000	K65R, D67N, T69d, Q151M, K219E	Y181C, G190A	L10F, K20R, L33F, M36I, M46I, I62V, H69K, L76V, V82A, T74S, I84V, L89M
9	47	171	37,379	M41L, D67N, K70R, M184V, T215F, K219Q	K103N, G190A	L10V, K20I, L33F, M36L, M46I, I47V, I54V, H69K, T74P, V82F, L89M	159	61,100	M41L, D67N, T69N, K70R, V75M, M184V, L210W, T215F, K219Q	V106I, G190A	L10V, K20I, L33F, M36L M46I, I47V, I54V, H69K, T74P, V82F, L89M
13	50	57	319,798	M41L, D67N, K70R, M184V, L210W, K219QE		L10V, G16E, K20V, L33F, M36I, I47V, I54V, H69K, A71V, I84V, L89M	10	498,000	M41L, D67N, K70R, M184V, L210W, T215Y, K219D		L10V, G16E, K20V, L33F, M36I, M46I, I47V, I54V, H69K, A71V, G73T, L76M, I84V, L89T

5. Virological outcome and drug resistance development to second-line ART

15	29	253	64,262	K70R, V75M, M184V, K219E	V90I	L10I, G16E, K20I, M36I, M46I, I54A, Q58E, H69K, K70R, V82A, L89I	159	97,100	D67H, T69G, K70R, V75M, M184V, T215I, K219E	V90IV	L10I, G16E, K20I, L33F, M36I, M46I, I54A, Q58E, H69K, K70R, V82A, L89I
18	23	6	67,934			M36I, H69K, V82I, L89M	0	279,000		V106I	M36I, H69K, V82I, L89M

ART, antiretroviral therapy; NRTIs, nucleotide/side reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors; Bold: major drug resistant mutations.

5.5 Discussion

This is the first survey of virological response to second-line ART regimen with a LPV/r based regimen in Viet Nam. The prevalence of virological failure (HIV RNA ≥1,000 copies/mL) after a median follow up of 29 months was 9.5%. Virological failure prevalence in Viet Nam is lower compared to other developing countries using a LPV/r based second-line regimen: 14-28% after 1 year in developing countries (Castelnuovo *et al.*, 2009; Fox *et al.*, 2010); 11-15% after 1 year and 13% after 2 years in developed countries (Arribas *et al.*, 2009; Waters *et al.*, 2013). Due to the nature of a cross-sectional study we only were able to enroll patients who were alive and in active follow up at the time of the survey. The exclusion of patients who died, who were lost to follow up, and who were transferred to other out-patient clinics (N=44, N=2, N=60, respectively) would result in an underestimation of true virological failure prevalence. If death and loss-to-follow-up were considered failure, and that rate of failure was assumed to be similar in the patients who were transferred to other clinics, the prevalence of virological failure in our study would be higher at 22% and would be more consistent with the existing literature (Figure 5.1).

A history of previous PI use, high viral load at second-line ART initiation, and an adherence level of less than 90% on a visual analog scale were identified as independent predictors for virological failure to second-line therapy. Time on a failing first-line ART regimen became an additional independent predictor of failure when virological failure was defined as a detectable HIV RNA (>150 copies/mL) or HIV RNA >400 copies/mL. Regarding history of previous PI use, IDV were available and were prescribed unboosted both in private and public sectors for patients who were intolerant to NVP prior to the

availability of EFV in Viet Nam, increasing their risk of virological failure with subsequent PI based regimen. It has been shown that the risk of virological failure is higher with an unboosted PI regimen versus a ritonavir boosted PI regimen in patients who are less adherent to their medications (Martin *et al.*, 2008). Low plasma level of IDV due to intermittent use of unboosted IDV was shown to increase the risk of developing PI mutations in patients experiencing virological failure (González de Requena *et al.*, 2003). Switching to EFV regimens removed PI drug pressure. Thus virus strains carrying PI resistance associated mutations became outnumbered by wild-type strains and could not be detected by population sequencing. However, there are limited data on the impact of minority PI mutations on treatment outcome. High viral load at second-line ART initiation indicates a delay in diagnosis of failure to first-line ART and late onset of switching to second-line ART. Improving the detection of first-line ART failure and streamlining the process of switching patients to second-line therapy when resistance to treatment is confirmed will reduce to the risk of virological failure to second-line therapy. Treatment adherence continues to play a vital role in the long-term success of patients during any stages of ART. Among 22 patients with protocol-defined virological failure, 8 patients (36%) did not have any major PI mutations, suggesting lack of adherence was driving virological failure (Table 5.5). PI resistance is uncommonly observed in patients failing ritonavir-boosted PI therapy (Daar *et al.*, 2012; Eron *et al.*, 2006; Kempf *et al.*, 2004; Riddler *et al.*, 2008). As boosted PIs have short half-life and high genetic barrier, lack of treatment adherence decreases PI concentration to the level that is not sufficient for PI resistance mutations to develop. Virological failure, thus, occurs in the absence of PI resistance (Rosenbloom *et al.*, 2012). In addition several studies have found that

mutations present in the *gag* and *env* gene may compromise PI activity (Fun *et al.*, 2012; Gatanaga *et al.*, 2002; Larrouy *et al.*, 2010; Myint *et al.*, 2004; Nijhuis *et al.*, 2007; Parry *et al.*, 2009, 2011; Rabi *et al.*, 2013). As sequencing *gag* and *env* gene is not routinely performed in genotypic resistance assays, this PI resistance pathway has not been adequately studied. Among 8 patients without any major PI mutations, five of these patients had already developed several minor or accessory PI mutations, and eventually major PI mutations would ensue if these patients continued to struggle with adherence. Upon further follow-up, five of the 13 patients who continued the failing second-line regimen achieved immunological and virological response 36 months later. At the time of virological failure to second-line therapy, two of the five patients had no PI resistance mutation. The other three patients carried a single major PI mutation V82A which confers intermediate-level resistance to LPV/r. All 5 patients reported good adherence at the time, but upon further adherence support over 2 years, they were able to achieve re-suppression of viral load and rising CD4 counts (mean CD4 of 611 cells/μL). This further emphasizes the importance of ongoing adherence support at all stages of treatment to treatment outcome.

The prevalence and level of drug resistance to first-line ART in our cohort is extensive. Among the 75% of patients who had a genotype performed prior to switching to second-line ART, resistance to NRTI, NNRTI and PI were present in 98%, 92%, and 5.2%, respectively. A total of 97% did not have a fully active NRTI backbone choice with intermediate to high level resistance to 3TC at 95% and TDF at 69%. The prevalence of K65R in our study (16%) is higher than that reported in subtype B (Margot *et al.*, 2006) and has been observed in other subtype CRF01_AE cohorts failing first-line

ART (Giang *et al.*, 2008; Ngo-Giang-Huong *et al.*, 2011; Nouhin *et al.*, 2013; Somnuek *et al.*, 2007; Zolfo *et al.*, 2011). A meta-analysis of sequences obtained from HIV Stanford database showed that subtype CRF01_AE is an independent predictor of K65R development among patients failing d4T regimens (Tang *et al.*, 2013). The GSS for second-line NRTI backbone in our study is lower than that in the UK (26% with GSS ≥1 in our study vs. 57% with GSS >1) (Waters *et al.*, 2013). LPV/r was therefore the only fully active drug in a significant proportion of our patients. However in our study non-active NRTI backbone with GSS=0 and partially active NRTI backbone with GSS=0.5 did not increase the risk of virological failure. Despite non-fully active NRTI backbone, second-line ART with LPV/r based regimens was highly effective (86.6% with viral load undetectable) in re-suppressing virological replication in our patients. This is consistent with reports of cohorts from Africa and UK in which rate of virological failure was not significantly different among patients taking fully active ART and non-fully active ART (Osinusi-Adekanmbi *et al.*, 2014; Sigaloff *et al.*, 2012; Waters *et al.*, 2013). This suggests that LPV/r based second-line ART is very potent even in combination with a non-fully active NRTI backbone. The use of non-fully active NRTI may contribute partially to the antiviral activity of LPV/r based regimen. The partial control of viral replication might be maintained by the selection of less-fit virus population and the residual activity of NRTI against the drug resistant strains (Deeks *et al.*, 2005). An overall high level of adherence in our patients (i.e. 88% with optimal adherence) likely plays a role as well. This is consistent with the literature showing higher level of adherence in developing countries compared to developed countries (Mills *et al.*, 2006). In this meta-analysis of pooled adherence data of 17,573 patients (31 studies) from North America and 12,116 patients

(27 studies) from Africa, median optimal adherence (>90%) was 77% (IQR: 68%-85%) in Africa compared to 55% (IQR: 49%-62%) in North America.

In our study addition of AZT to the recommended second-line regimen (TDF+3TC+LPV/r) was not associated with better virological outcome. Our result was consistent with 2 reports from India in which standard second-line therapy with and without AZT was compared (Guha *et al.*, 2011; Patel *et al.*, 2013). Both studies (N=126 and N=68, with 65-77% patients having additional AZT) showed that the addition of AZT did not have any significant benefit in term of CD4 increase or HIV viral load suppression. Our study with larger number of patients confirmed that the addition of AZT aiming to prevent the development of K65R on TDF therapy did not improve virological outcome. Omission of AZT reduces the cost of HIV treatment and reduces well-known side effect of AZT induced anemia.

Cross-resistance to potential salvage drugs is a concerning issue in this cohort. Candidate PIs for salvage therapy are DRV and TPV, and candidate NNRTI is ETR. Cross resistances to TPV (45%) and to ETR (55%) are more common than to DRV (27%). The prevalence of cross resistance to these drugs is much higher than a study from Cambodia where cross resistance to ETR, TPV, and DRV were 20%, 0%, 7% respectively (Nerrienet *et al.*, 2012). Our data suggests that the use of a new drug class, an INI, such as RAL +/- ritonavir boosted DRV and/or ETR, based on genotyping data would be a reasonable salvage third-line regimen option for Viet Nam. In patients with multiple cross resistance, a regimen of RAL, DRV/r and ETR has been shown to have similar rates of virological suppression and tolerance compared to that expected among treatment-naïve patients (Fagard *et al.*, 2012; Imaz *et al.*, 2011).

In conclusion LPV/r based regimen is a highly effective second-line ART for patients who fail first-line ART with NRTI and NNRTI therapy in Viet Nam. Prior PI use, high viral load and suboptimal adherence independently predict virological failure. PI resistance was detected in 64% of patients failing second-line ART. Cross-resistance to ETR and TPV were more common than DRV, which informs options for third-line ART in Viet Nam.

6 GENERAL DISCUSSION

Development of drug resistance is a major challenge of HIV treatment. With the roll-out of ART in resource-limited settings via support from international funding, prevalence of primary and secondary drug resistance is expected to increase. Viet Nam, like other resource-limited countries, has had a sharp increase in the number of patients taking ART, from 1% in 2003 to 54% in 2011 among individuals who meet the Viet Nam MOH criteria to initiate ART (UNAIDS, 2012). Unlike other developed countries where many treatment options are available, HIV treatment in Viet Nam is limited to AZT/d4T/TDF plus 3TC plus NVP/EFV for first-line ART and TDF (with or without AZT) plus 3TC plus ritonavir-boosted LPV for second-line ART. Due to financial constraints routine treatment monitoring in Viet Nam is based on immunological and clinical assessment. The history of dual NRTI use, limited options of treatment, increasing number of patients on ART, along with unavailability of routine viral load monitoring and baseline genotypic testing could escalate the rate of drug resistance in Viet Nam. The development of secondary drug resistance not only affects an individual treatment outcome but also leads to transmission of drug resistance within the population, threatening the success of ART program. This is particularly important in Viet Nam where ART is being rapidly scaled up, and drug resistance could go undetected without routine viral load monitoring and genotype testing.

The HIV epidemic in Viet Nam is dominated by HIV-1 subtype CRF-01_AE (>95%), which differs genetically by 15% in the reverse transcriptase gene and 30% in the envelope gene of HIV-1 compared to the well-studied subtype B. In South and South-east Asia subtype AE accounts for 84% of total cases (except India) (Hemelaar *et al.*,

2006). The genetic diversity could alter the patterns of HIV resistance and treatment outcome in those infected with subtype CRF01_AE compared to those carrying the well-studied subtype B. As HIV drug susceptibility of non-B strains cannot be extrapolated from that of B strains, there is a need to study drug efficacy and development of drug resistance in subtype CRF01_AE in Viet Nam. Understanding the epidemiology of drug resistance, efficacy of antiretroviral treatment, and factors that impact treatment can support therapy management during the ART roll-out in Viet Nam. The purpose of this thesis is to study transmitted and acquired HIV drug resistance, treatment outcome and predictors of outcome in patients starting second-line ART in Viet Nam.

Mathematical modeling and experiences in resource-rich settings have shown that TDR increases as ART coverage increases (Blower *et al.*, 2001). A meta-analysis conducted after ART rollout in resource-limited countries showed a significant increase in prevalence of TDR over time in Sub-Saharan Africa (Gupta et al., 2012). However TDR was not increased with increased ART coverage in Latin America and the Caribbean. The result was inconclusive for Asia as there were too few studies to perform formal country-level meta-analysis. In a cohort of ART-naïve patients with HIV-associated tuberculous meningitis enrolling into a clinical trial of immediate versus delayed ART study conducted between 2005-2007 in HCMC, prevalence of TDR prior to first-line ART initiation was 6.4%, which was similar to a prior study conducted in HCMC in 2003 before the ART roll-out program (Lan *et al.*, 2003) and other reports Viet Nam (Dean *et al.*, 2011; Phan *et al.*, 2010). TDR remained relatively stable during the implementation of ART roll-out in Viet Nam, with prevalence ranging from 6.2% to 7.6% (Dean *et al.*, 2011; Lan *et al.*, 2003; Pham *et al.*, 2013; Phan *et al.*, 2010). This level

of TDR is considered moderate by the WHO (Bennett *et al.*, 2008), with <5% being low, 5-10% being moderate and >10% being high. At this level of TDR, routine baseline resistance testing is considered cost effective in developed countries (Sax *et al.*, 2005; Weinstein *et al.*, 2001). A recent study from a middle income country of Brazil showed that baseline genotypic testing for NNRTI mutations was cost saving even when NNRTI resistance prevalence was as low as 2.4%, and baseline genotypic testing was shown to increase life-expectancy (Luz *et al.,* 2015*).* There is no study of cost effectiveness of baseline genotype testing in low-income countries. Several low-cost tests for drug resistance have been developed for use in low-income countries, including point mutation assay (multiplex-allele specific assay MAS, oligonucleotide ligation assay OLA) and RT specific assay (ART-A test) (Aitken *et al.*, 2013; Ellis *et al.*, 2013; Panpradist *et al.*, 2015a; b; Zhang *et al.*, 2013). However these assays can only detect a subset of known drug resistance mutations, and is only applicable for certain subtypes. Even though TDR was shown to be stable from 2003 to 2008 during the beginning years of the ART roll-out program, continuing expansion of ART coverage and evolving transmission dynamic in Viet Nam may change the epidemiology of TDR, therefore the long-term effect of ART expansion need to be studied and the epidemiology of TDR in Viet Nam should continue to be monitored to contribute to ART policy decisions, including guidelines on ART regimens and HIV prophylaxis. TDR mutations detected by population sequencing in this thesis showed resistance to NNRTI and NRTI drug classes, corresponding to the National first-line ART regimen in Viet Nam. No PI resistance mutations were found.

Viet Nam has had an impressive response to HIV, owning to the concerted efforts and commitment by the government and international support. The country went from a

capacity of a few central clinics to having at least one HIV outpatient clinic in each of the 64 provinces of Viet Nam over 5-7 year time. Despite remarkable programmatic response, there are limited data on the treatment efficacy of HIV therapy in Viet Nam. Data on treatment outcome of first-line ART are limited, with three studies reporting virological response rates after 1-2 years of 70-85% (Chinh *et al.*, 2010; Quang *et al.*, 2011; Trinh *et al.*, 2011). There are no data on outcome of second-line ART in Viet Nam. The estimated proportion of patients on second-line ART is 3.05%, and this is estimated to cover 55% of patients needed second-line therapy (Viet Nam MOH, 2013). With Viet Nam achieving the low middle income status in 2011, the US PEPFAR program and Global Fund are making drastic funding cuts to HIV program in Viet Nam. The country is confronted with the need to maintain current ART coverage and the need to optimize care for as little resource as possible. Preventing treatment failure in patients on ART is crucial for public health management. Understanding the efficacy, prognosis and drug resistance development on second-line therapy is crucial as this is the last resource for patients failing first-line ART in Viet Nam. In our cohort of 330 adult patients on second-line ART with a ritonavir boosted LPV regimen at the HTD with a median follow up of 29 months, the rate of immunological and/or clinical outcome, including death was estimated at 18.2%, which was comparable to other resource limited countries (18.2% vs. 28%) (Pujades-Rodríguez *et al.*, 2010). Among the identified risk factors for treatment failure, older age and history of IDU at second-line switching are non-modifiable; however, closely monitoring treatment response in older patients and those with a history of IDU has the potential to improve treatment outcomes in these patients. For both immunological/clinical and virological outcomes, ART adherence and late therapy

switching leading to lower CD4 cell counts and higher viral load level are risk factors that can be modified to improve the HIV care in Viet Nam. Interventions targeting earlier detection of first-line treatment failure, either by more frequent CD4 measurements in patients at risk or by a more strategic, cost effective use of viral load monitoring can improve the outcomes of second-line ART. The process of therapy switching can be streamlined to prioritise second-line therapy in patients with lower CD4 counts (e.g. lower than 100 cells/μL) by (1) improving the access for viral load testing to confirm immunological/clinical failure, particularly in the more rural communities, (2) optimizing the use of dried blood spot for viral load and genotyping testing, thereby limits the time lost for plasma transport and the time lost to patient referral, and (3) the use of on-line tools for consultations and communications between clinicians from major referral centres and those from rural communities, limiting the time lost to patient referral. Interventions to improve medication adherence in patients at risk can maximize the efficacy of second-line ART. Promoting treatment adherence via ongoing patient education from health care providers, mobile phone reminders and reminders from family members have been shown to improve adherence in a multicentre study in Viet Nam (Tran et al., 2013). Such interventions are simple, and should be feasible to incorporate into routine HIV care in Viet Nam.

Among the 22 patients who had virological failure to second-line ART, 14 patients had at least one major PI mutation conferring resistance to LPV. The most common mutations were V82A, M46I/L, and I84V. These mutations are also the most common mutations developed among HIV-1 subtype B infected patients failing LPV/r based second-line regimens (Grossman *et al.*, 2014). Cross resistance to ETR was

detected in 55%, to TPV in 45% and to DRV in 27%. This information is critical for the selection of third-line ART regimen for Viet Nam. Amongst the patients who were maintained on the failing LPV/r regimen, virological re-suppression and good immune response were achieved in 38% of patients; these patients either had no or only one major PI mutation at virological failure detection. This suggests that a strategy combining adherence intervention and close monitoring of patients failing second-line therapy before switching to third-line therapy would be cost saving yet effective in resource-poor settings.

Before 2013, the WHO guidelines recommended using viral load testing only to confirm treatment failure (WHO, 2010a). In 2013, the WHO recommended routine viral load monitoring for earlier identification of ART failure and confirmation of treatment failure among patients with evidence of immunological and/or clinical failure (WHO, 2013a). Viral load monitoring helps to discriminate treatment failure and non-adherence (Orrell et al., 2007). A systematic review showed that WHO guidelines on immunological and clinical monitoring for treatment failure have poor sensitivity and predictive value for identifying virological failure (Rutherford *et al.*, 2013). However viral load monitoring is still not affordable in Viet Nam. In our study six patients were classified as immunological failure while having undetectable viral load; however two of these patients died of AIDS, and this immunological failure group had an overall worst clinical outcome compared to the patients in the entire cohort. None of the patients who had a CD4 count drop more than 50% of an on-treatment peak value had an undetectable viral load. Hence, our data suggest that the WHO immunological failure criteria have a

reasonably good predictive value for detection of treatment failure, thus continues to be useful in guiding care in settings where viral load monitoring is unavailable.

In conclusion, despite the rapid ART scale up in Viet Nam over the past 10 years, TDR remains stable at the intermediate levels of 5-10%. Surveillance of TDR should be continued, particularly during the critical transition of the HIV health system from a predominantly international donor approach to a national funding approach. Close monitoring of older patients, of those with a history of IDU, and providing treatment adherence support for patients at higher risk for treatment failure, such as those with a history of previous IDV use, can improve the outcomes of patients on second-line therapy in Viet Nam. Starting second-line therapy before the CD4 deteriorates will improve treatment outcomes and requires accurate and early detection of treatment failure. Our data call for policy makers to improve the access to viral load testing and monitoring in Viet Nam. We systematically linked the high prevalence of PI resistance seen in patients virologically failing second-line therapy to previous exposure to locally produced IDV, highlighting the issue that has not received adequate global attention. The high prevalence of cross-resistance to ETR seen in our cohort suggests that ETR should not be used as a third-line drug choice for Viet Nam. Lastly, the 24 month follow up of patients with virological failure showed that 38% of patients were virologically re-suppressed despite no change in therapy. These patients either had no or a maximum of one major PI mutation. A strategy combining adherence intervention and close monitoring of patients failing second-line therapy before switching to third-line therapy would be cost saving yet effective in resource-poor settings.

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8 APPENDICES

8.1 Case report form for clinical data collection

2nd-Line HIV Drug Resistance Cohort – Case Report Form **Study Code _____**

Patient Information

Patient Name: _____ 1st Phone _____ 2nd Phone _____

Year of birth: _____ □ Male □ Female

Education (years) _____ Address during 2nd-line ART treatment: _____

IVDU □ yes □ no

Current status □ Death □ Alive □ Lost to follow-up □ Transferred out □ Others, specify _____

Date of death _____ Cause of death: □ AIDS events, specify _____

□ Others, specify _____ □ Unknown

Clinical history

TB □ positive □ negative. □ Unknown

If positive, time of therapy start (month/year) _____

time of rifampicin stop (month/year) _____

ARV history prior to 1st-line ART

Has patient ever used/exposed to ARV for HIV or HBV before starting 1st line ART? □ Yes □ No □ Unknown

If Yes, list medication: _____

1st-line ART

Time of start of 1st-line ART (month/year) _____

1st-line ART regimens: First regimen: _____
Second regimen: _____, Date (month/year) _____ , Reason: _____
3rd regimen: _____, Date (month/year _____, Reason:_____
4th regimen: _____, Date (month/year _____, Reason:_____

CD4 count prior to 1st-line ART: _____ cells/mm^3 Date (month/year)_____

Indication for 2nd-line ART: □ Clinical failure _____ Date of first detection (month/year)_____
□ Immunological failure Date of first detection (month/year)_____
□ Virological failure HIV RNA level: _____ copies/mL
Date (month/year): _____
□ Intolerance, please specify _____

Time of first detection of clinical/immunological/virological failure (month/year) _____

Was Genotype done? □ Yes □ No

1st test Date (month/year) _____
Results: □ No mutations □ Yes mutations
List of mutations: Major PI _____
Major NNRTI _____
Major NRTI _____
Minor PI _____

2nd test Date (month/year) _____
Results: □ No mutations □ Yes mutations
List of mutations: Major PI _____
Major NNRTI _____
Major NRTI _____
Minor PI _____

2nd line ART

Time to start 2nd-line ART: _____

ARV regimens: First regimen: _____
Second regimen: _____, Date (month/year) _____ , Reason: _____
Third regimen: _____, Date (month/year) _____ , Reason: _____

CD4 prior to 2nd-line ART _____cells/mm^3 Date (month/year) _____

HIV RNA prior to 2nd-line ART (if available) _____ copies/ml Date (month/year) _____

Has patient had any side effect? _____

Has patient had any AIDS events (recurrent/new)? _____

How much medications has patient taken in during 2nd line treatment?

If virological failure was established, HIV RNA level: _____ copies/ml, Date (month/year): _____

Was Genotype done? □ Yes □ No
Date of Genotyping test (month/year) _____
Results: □ No mutations □ Yes mutations
List of mutations: Major PI _____
Major NNRTI _____
Major NRTI _____
Minor PI _____

2nd-Line HIV Drug Resistance Cohort – Case Report Form **Study Code _____**

Date														
CD4														
VL														

Date														
CD4														
VL														

Date of visit	CD4 (cells/µL)
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____

Date of visit	CD4 (cells/ µL)
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____

Date of visit	RNA (copies/mL)
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____

Date of visit	RNA (copies/mL)
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____

TB co-infection

Does patient have TB co-infection? □ Yes □ No

Time of TB treatment

Date start	Date stop	Regimens	Dose of Rifampicin
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____

Ritonavir boosted □ Yes □ No

Dose _____ Time _____

8.2 Publications resulting from work conducting during DPhil

HIV-1 Drug Resistance in Antiretroviral-Naïve Individuals with HIV-Associated Tuberculous Meningitis Initiating Antiretroviral Therapy in Viet Nam

Journal:	*Antiviral Therapy*
Manuscript ID:	AVT-11-OA-2195
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Keywords:	Antiretroviral therapy, Asia, Tuberculosis, HIV drug resistance/resistance mutations

Title:

HIV-1 Drug Resistance in Antiretroviral-Naïve Individuals with HIV-Associated Tuberculous Meningitis Initiating Antiretroviral Therapy in Viet Nam

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Running head:

HIV-1 Drug Resistance in Viet Nam

Key words:

HIV drug resistance, antiretroviral therapy, tuberculous meningitis, Viet Nam

Meeting:

Data were partially presented at the 18th Conference of Retroviruses and Opportunistic Infections, Boston, Feb 27 – Mar 2, 2011

Abstract

Background: Access to antiretroviral therapy (ART) for Human Immunodeficiency Virus (HIV)-infected individuals in Viet Nam is rapidly expanding, but there are limited data on HIV drug resistance (HIVDR) to guide ART strategies.

Methods: We studied HIVDR in 220 ART-naive individuals recruited to a randomized controlled trial of immediate versus deferred ART in individuals with HIV-associated tuberculous meningitis in Ho Chi Minh City (HCMC) from 2005-2008. HIVDR mutations were identified by population sequencing of the HIV *pol* gene and were defined based on 2009 WHO surveillance drug resistance mutations (SDRMs).

Results: We successfully sequenced 219/220 plasma samples of subjects prior to ART. 218 were subtype CRF-01AE; one was subtype B. SDRMs were identified in 14/219 (6.4%) subjects. 8/14 were resistant to nucleoside/tide reverse transcriptase inhibitors [(NRTI) T69D, L74V, V75M, M184V/I, K219R], 5/14 to non-nucleoside reverse transcriptase inhibitors [(NNRTI) K103N, V106M, Y181C, Y188C, G190A], 1/14 to both NRTI and NNRTI (D67N, Y181C), and none to protease inhibitors. After 6 months of ART, 8 developed protocol-defined virological failure. HIVDR mutations were identified in 5/8 subjects. All 5 had mutations with high level resistance to NNRTI; 3 had mutations with high level resistance to NRTI. Due to a high early mortality rate (58%), the effect of pre-existing HIVDR mutations on treatment outcome could not be accurately assessed.

Conclusions: The prevalence of WHO SDRMs in ART-naive individuals with HIV-associated tuberculous meningitis in HCMC from 2005-2008 is 6.4%. The SDRMs identified conferred resistance to NRTI and/or NNRTI reflecting the standard first-line ART regimens in Viet Nam.

Introduction

Human Immunodeficiency Virus (HIV) is able to develop resistance to all currently-licensed antiretroviral drugs, posing threats to the sustainability of antiretroviral treatment (ART) globally. Drug-resistant virus can be transmitted and/or acquired and can persist in the population [1-3], leading to treatment failure, disease progression and death [4-6]. Prevalence of transmitted drug resistance (TDR) in acutely or recently infected individuals in developed countries ranges from 7-24% [7-10]. Baseline HIV drug resistance (HIVDR) testing has been shown to be cost effective in the United States when TDR prevalence exceeds 5% [11] and is routinely performed to guide initial antiretroviral choices in resource-rich settings [12, 13]. As ART is being scaled up in low- and middle-income countries, where >90% of the world's HIV-infected population reside and ART options are limited, increased surveillance for TDR to inform ART programmes in resource-limited settings is essential.

The HIV epidemic in Viet Nam started in 1990 and is still concentrated amongst the high-exposure risk groups. The overall HIV prevalence in individuals aged 15-49 years is 0.5%; however prevalence is 18.4% in injection drug users (IDU), 16.7% in men who have sex with men (MSM), and 3.2% in commercial sex workers (CSW) [14]. ART was initially introduced in Viet Nam in the mid-1990s through private donations with intermittent drug supply and through the black market with high prices, so treatment interruption was likely common. In addition, non-nucleoside reverse transcriptase inhibitors (NNRTI) and protease inhibitors (PI) were not available or too expensive during the early 2000s, hence dual therapy regimens with zidovudine and lamivudine or stavudine and didanosine were commonly used [personal communication with Dr. Donn

Colby, Harvard Medical School AIDS Initiative in Viet Nam]. The Viet Nam National ART programme was commenced in 2005 through international support, primarily from the US President's Emergency Plan for AIDS Relief and the Global Fund to Fight AIDS, Tuberculosis and Malaria. ART coverage has increased from 1% to 53.7% of individuals who meet the Viet Nam Ministry of Health criteria to initiate ART from 2003-2009 [CD4 count <200 cells/µl and World Health Organization (WHO) disease stage III or IV] [14]. Both the history of non-suppressive ART use before 2005 and the rapid ART scale up since 2005 are expected to be accompanied by the emergence of acquired and TDR in Viet Nam. From 2003-2009 6 studies have reported prevalence of HIVDR in antiretroviral-naïve individuals ranging from 2.9%-7.6% in Viet Nam [15-20]. Differences in reported rates may reflect differences in populations and time periods studied, in survey methods and drug resistance algorithms used, and in geographic and sample size factors, thereby making comparison and estimation problematic. For example, small studies using the WHO threshold survey method [21], which sampled relatively lower-HIV-exposure populations such as first-time pregnant women at antenatal clinics or attendees aged <25 years at HIV Voluntary Counseling and Testing (VCT) centers, reported lower rates (<5%) [16, 18], while larger studies that sampled the general population attending HIV clinics reported higher rates (>6%) [15, 19, 20]. Studies from Ha Noi and surrounding provinces tend to report lower rates [16, 17, 19] compared to a study from Ho Chi Minh City (HCMC) [15]. The later study reported a 6.5% rate of HIVDR mutations amongst 200 ARV-naïve individuals (43% IDUs and 38% CSWs) from 2001-2002, a period when ART was not yet widely available [15]. In this study we investigate the prevalence and impact of HIVDR mutations in ARV-naïve individuals

who enrolled in a randomized clinical trial comparing immediate versus delayed ART for HIV-associated tuberculous meningitis (TBM) in HCMC from 2005-2008 [22].

Methods

Study setting and population

TBM is the most severe form of extra-pulmonary tuberculosis (TB) and is the most common central nervous system complication in HIV-infected people in Viet Nam [Le T. et al. unpublished]. This study was conducted at two specialist centers for TB and HIV: Pham Ngoc Thach Hospital for TB and Lung Disease (PNT) and Hospital for Tropical Diseases (HTD) in HCMC. All antiretroviral-naïve subjects with HIV-associated TBM who enrolled in a randomized, double-blind, placebo-controlled trial of immediate versus deferred initiation of ART, were included in this study. Participants were recruited between September 2005 and December 2008 and completed follow up in December 2008 [22]

Statement of ethics

The trial protocol was approved by the Scientific and Ethical Committees of the two hospitals, by HCMC Health Services, and by the Oxford Tropical Research Ethics Committee. Written informed consent was obtained from all subjects, or a relative if the subject was unable to provide consent, according to standard practice in Viet Nam. For unconscious subjects with no available relatives, the consent of two independent physicians was considered acceptable.

Treatment

Subjects received standard anti-tuberculosis therapy (isoniazid, rifampicin, pyrazinamide, and ethambutol) according to Vietnamese national guidelines.

Antiretroviral or placebo tablets were commenced as soon as possible after randomization. The ART regimen was zidovudine, lamivudine and efavirenz for all subjects. Medications were administered orally or via nasogastric tube. Subjects received directly observed therapy during their inpatient stay (up to three months); administration was supervised by family members after discharge from hospital.

Laboratory procedures

Plasma HIV RNA was measured at baseline and at months 2, 3, 6, 9 and 12 using the Abbott M2000 real-time polymerase chain reaction (PCR) platform. Subjects with confirmed virological failure (VF, HIV RNA >1000 copies/ml) at months 6, 9, and 12 were selected for drug resistance evaluation. Genotypic drug resistance analysis was performed on all available plasma samples for subjects in this trial at baseline and at the time of VF.

Genotypic drug resistance evaluation

HIV RNA was extracted from 200µl plasma samples using an automated guanidinium-thiocyanate extraction method described elsewhere [23, 24]. HIV genotype testing was performed on the ViroSeq platform (N=23 samples) and according to an in-house assay described by Bezemer [25], with the following modifications: the SuperScriptIII RT (Invitrogen, Carlsbad, CA) instead of the MMLV-RT enzyme were used during reverse transcription, 45 instead of 25 cycles and 30 instead of 25 cycles were used during the first- and second-rounds of PCR amplification. Approximately 1200 base-pairs comprising the complete 297 nucleotides protease (PR) region and the first 897 nucleotides in the reverse transcriptase (RT) region of *pol* were checked by agarose gel

electrophoresis. Amplification products were purified using QIAquick PCR purification kits (Qiagen, Hilden, Germany) and were subjected to direct sequencing using the ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City CA). The gene sequences were analyzed using SeqScape (Applied Biosystems). Nucleotide changes were determined by comparison with the consensus sequence pNL4-3 for HIV-1 subtype B (GenBank accession number M19921). HIVDR mutations in baseline samples were determined according to the 2009 WHO drug resistance surveillance mutations (SDRMs) list [26]. Mutations in samples at VF were determined according to the 2010 International AIDS Society (IAS)-USA list [27]. For identification of polymorphisms, the pNL4-3 subtype B amino acid sequence was used as a reference sequence in the PR and RT regions of the 218 CRF01_AE isolates.

HIV-1 Sub-typing of protease and reverse transcriptase sequences

HIV-1 subtype was determined by phylogenetic analysis of the RT and PR genes using the neighbor-joining algorithm integrated in the Molecular Evolutionary Genetics Analysis (MEGA4) software [28]. Reference sequences of the *pol* gene from all available subtypes were obtained from the Los Alamos National Laboratory database [29]. Phylogenetic analysis was further confirmed by the Stanford database classification system (http://sierra2.stanford.edu/sierra/servlet/JSierra)

Results

Presence of pre-existing HIVDR mutations in chronically-infected subjects with HIV-associated TBM

Among the 253 subjects enrolled in the trial, stored baseline plasma samples were available for 220 participants, of whom 90% were male and 84% had history of IDU. The median baseline CD4+ T-lymphocyte count and the median baseline plasma HIV-1 RNA level were 41 cells/µL (interquartile range: 16-104) and 1.56 $x10^6$ copies/ml (interquartile range: 0.85 $x10^6$ – 2.27 $x10^6$), respectively. Both PR and RT regions were successfully sequenced in 219/220 baseline samples. Mutations that confer resistance to nucleoside/tide reverse transcriptase inhibitors (NRTI) were found in 8/219 (3.7%) subjects, and NNRTI resistance-conferring mutations in 5/219 (2.3%). Resistance to both NRTI and NNRTI was observed in 1/219 (0.5%). No PI resistance mutations were identified (Table 1). Hence, the overall prevalence of pre-existing HIVDR in this cohort in HCMC is 14/219 (6.4%). In 13 of 14 subjects, the detected mutations confer resistance to the standard first-line antiretrovirals used in Viet Nam (stavudine, zidovudine, lamivudine, nevirapine and efavirenz). The remaining subject carried the mutation L74V which confers resistance to abacavir and didanosine which are part of second-line antiretrovirals in Viet Nam. In 10 of 14 subjects the detected mutations are associated with high-level resistance to antiretrovirals according to the Stanford HIV drug resistance database.

HIV-1 sub-typing and polymorphisms not associated with drug resistance in Ho Chi Minh City, Viet Nam

Phylogenetic analysis and subtype classification according to MEGA4 and the Stanford database revealed that 218/219 (99.5%) subjects were infected by HIV-1 subtype CRF01_AE; the remaining one by subtype B. Non-synonymous polymorphisms were identified at all 15 drug resistance positions described in the 2009 WHO SDRM list and were more prevalent in RT than PR gene of the 218 CRF01_AE isolates (Table 2). Of note, some natural polymorphisms were present at higher frequencies in this HCMC cohort compared to a study in northern Viet Nam, both were overwhelmingly dominated by CRF01_AE subtype [17], i.e. T69N (9.2% versus 0.4%), V106I (8.2% versus 1.5%) and V179I (17.4% versus 0%). The polymorphism L63C, which requires changes in all three nucleotides, was present in 45.4% of samples in this cohort. This substitution was present in 27.2% of the prior 200-subject cohort in HCMC [15] but has not been reported in northern Viet Nam. The two polymorphisms V106I and V179D occurred in 18/219 (8.2%) and 5/219 (2.3%) subjects, respectively. These polymorphisms are not considered TDR mutations according to the WHO SDRM list and thus were not included in our resistance prevalence analysis. However V106I and V170D have been associated with resistance to etravirine (a second-generation NNRTI available only in resource-rich countries) and are included in the 2010 IAS-USA mutation list [27]. If the IAS-USA algorithm is used, the prevalence of pre-existing HIVDR in our study would increase from 6.4% to 16%.

Other non-synonymous polymorphisms at positions not associated with the 2009 WHO SDRMs are listed in Table 3; similar polymorphism frequencies have been described in subtype CRF01_AE in other studies in Asia [17, 30].

HIV drug resistance development in subjects with virological failure

The mortality rate of TBM in HIV-infected individuals is twice as high compared to HIV-uninfected individuals [31]. Due to the early and high mortality of subjects in the trial (58%) [22], the impact of pre-existing drug resistance mutations on virological and clinical outcome could not be accurately assessed. Amongst the 219 trial subjects included in this study, only 69 survived and completed follow-up at month 6, 45 at month 9, and 14 at month 12. After 6 months follow-up, 8/69 surviving subjects had protocol-defined VF. Major 2010 IAS-USA mutations were detected in 5 of these subjects (Table 4). All 5 had mutations that confer high level resistance to NNRTI (K103N, Y188L, P225H and/or M230L), and 3 additionally had mutations that confer high level resistance to NRTI (M184V and/or T215Y/F/I/S). Two subjects had pre-existing mutations prior to ART initiation and accumulated further resistance mutations on non-suppressive therapy (Subjects 3 and 7). Amongst the 8 subjects with VF, 3 had virus containing V106I polymorphism both at baseline and at time of VF. Virus containing V106I and/or V179D did not develop on ART in any of the 8 subjects with VF.

Discussion

We report a 6.4% rate of pre-existing HIVDR in 219 ARV-naïve chronically-HIV-infected individuals with TBM in HCMC from 2005-2007 using the 2009 WHO SDRM list. NRTI mutations were most commonly identified, occurring in 4.1% of subjects. Most observed mutations conferred resistance to NRTIs in the standard first-line ART regimens in Viet Nam, while one subject had a mutation (L74V) that confers resistance to abacavir and didanosine used in second-line ART regimens in Viet Nam. NNRTI mutations were identified in 2.8% of subjects, and all confer high level resistance to nevirapine and efavirenz. No PI mutations were identified.

The prevalence of pre-existing HIVDR mutations in this study appears higher than in two previous studies which used the WHO method for surveillance of TDR during 2003-2008, both of which showed TDR rates <5% [16, 18]. However, aside from the small sample sets (N=63 and N=49), these studies surveyed relatively low-HIV-exposure populations (first-time pregnant women in antenatal clinics and individuals aged <25 years from VCT centers) compared to the population making up the concentrated HIV epidemic in Viet Nam, where 52% are IDUs and 4% are CSWs [32]. By nature of the risk behaviors and social stigmatism, these high-HIV-exposure individuals are less likely to attend VCT centers and antenatal clinics. The WHO threshold survey method, which was designed to exclude ARV-experienced individuals to improve the accuracy of TDR estimation, may under-estimate TDR in countries where HIV epidemics are concentrated in high-HIV-exposure populations. The resistance rate in our study is similar to studies that surveyed a more representative population attending HIV treatment clinics who report no prior use of ART, with resistance rates ranging from

6.2%-7.6% [15, 19, 20]. Our resistance rate falls into the range of these data during the period of 2002-2009, suggesting that TDR has remained relatively stable despite the rapid scale up of ART in Viet Nam over the past 5 years [14]. Although TDR prevalence in Viet Nam is at the level of which routine baseline resistance testing is considered cost effective in resource-rich countries [11, 33], cost effectiveness studies of drug resistance testing and continuing surveillance of TDR targeting representative HIV-infected populations in resource-poor countries are clearly needed.

Non-synonymous polymorphisms were observed at all 15 drug resistance foci listed in the 2009 WHO SDRM. The V106I and V179D polymorphisms, both are considered minor mutations associated with resistance to etravirine according to the 2010 IAS-USA algorithm [27], were observed in 8.2% and 2.3% of subjects, respectively. If the IAS-USA algorithm is used for TDR analysis, the resistance rate in our study would increase from 6.4% to 16%; however this is misleading. An example of such an analysis using the IAS-USA algorithm was recently published by the multi-center TREAT Asia study reporting a HIVDR prevalence of 13.8% in 682 ARV-naïve chronically-infected individuals from Hong Kong, Malaysia, and Thailand from 2007-2009 [34]. In this study, 77.7% were infected with CRF01_AE subtype, and the 13.8% prevalence included viral isolates containing the V106I (1.9%) and V179D (3.2%) amino acid changes. The resistance rate in this study would have substantially decreased if the WHO SDRM list was used and would have better reflected the burden of primary resistance in the Asia Pacific region. This highlights the importance of the WHO SDRM algorithm, a parsimonious list of rigorously-defined mutations at non-polymorphic positions in 8 major HIV subtypes, as the most appropriate algorithm for estimation and comparison of

TDR in different regions and different times [26]. This study also highlights the need for studies to evaluate the impact of these subtype-specific polymorphisms on ART outcome, particularly as V106I in combination with V179D has recently been identified as a new pattern of mutations conferring resistance to nevirapine and efavirenz, which are used in first-line ART regimens in Asia and globally [35].

Due to the early and high mortality rate in subjects with HIV-associated TBM in this study (58%), it was not possible to assess the impact of pre-existing SDRMs and identified polymorphisms on clinical or virological outcome. Amongst the 8 subjects who met protocol-defined VF, HIVDR mutations were detected in 5 subjects. All failed in the presence of major NNRTI mutations K103N and/or Y188L, and 3 failed with M184V mutations. Four subjects showed a pattern of accumulating resistance mutations. Interestingly, one subject had a pre-existing Y181C mutation which disappeared while the K103N emerged by month 8 on ART. Switching from Y181C to K103N may occur as K103N carrying variants have better fitness compared to Y181C carrying variants in subtype B virus [36]. A shift in resistance pattern from Y181C to K103N has been described in women receiving a single dose of nevirapine to prevent mother-to-child transmission in Uganda [37].

An inherent and opposing limitation of a study method using a chronically-infected population to estimate TDR is the possibility of underestimating while simultaneously overestimating resistance prevalence. This study may overestimate TDR as ART history was based on patients' self report, and some subjects may have not revealed their true ART history in order to be eligible for the trial. This study may also underestimate TDR as the Sanger sequencing method used to detect HIVDR does not reliably detect HIV

variants present at frequencies <15-25% of viral quasispecies [38]. It is generally known that TDR strains in the absence of antiretroviral selection pressure will overtime revert to wild-type virus, and thus minority resistant variants may be missed by standard genotyping methods. Nevertheless, compared to the population recommended by the WHO threshold survey method, the population in this study is more representative of the general HIV-infected population attending HIV clinics in Viet Nam [14, 32]. The implications of TDR and/or acquired resistance for the individual patient and/or for public health in light of ART outcome and further spread of drug resistant virus is the same, perhaps making the academic distinction between TDR and acquired resistance no longer relevant.

In summary, using the 2009 WHO SDRM algorithm not in combination with the WHO threshold survey method, we report a 6.4% prevalence of pre-existing HIVDR in ARV-naïve chronically-infected individuals with TB meningitis in HCMC, Viet Nam from 2005-2007. The identified HIVDR mutations conferred resistance to NRTIs and/or NNRTIs which are part of the standard first-line ART in Viet Nam. Our HIVDR resistance data is comparable to other studies using similar sampling methodology in the general populations seen in HIV clinics and suggests that HIVDR has remained relatively stable despite the rapid scale up of ART in Viet Nam.

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Potential conflicts of interest

All authors declare no conflicts of interest.

Contributors

T.L., M.E.T., M.D.J, J.F. and S.D. designed and obtained funding for this study. V.P.T., S.J. and R.V.D. performed the laboratory work. M.E.T T.T.H.C., N.T.B.Y., M.D.J. and J.F. designed and conducted the clinical trial. V.P.T., T.L. and S.D. analyzed the data. V.P.T, T.L. and S.D. wrote the first draft. All authors contributed to and approved the final manuscript.

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Table 1: Pre-existing 2009 WHO Surveillance Drug Resistance Mutations in individuals with HIV-associated tuberculous meningitis in Ho Chi Minh City from 2005-2007

Subjects	HIV RNA (copies/ml)	Drug resistance associated mutations		
		NNRTI	NRTI	PI
1	904780	K103N, V106M, G190A		
2	609200	Y181C		
3	95730	Y181C		
4	1604510		V75M	
5	5600		M184V	
6	57235	Y181C	D67N	
7	64020		M184V	
8	34465		K219R	
9	4889900	K103N, Y181C		
10	333130		L74V	
11	315310		V75M	
12	1359330	K103N, Y181C		
13	4637185		M184I	
14	282230		T69D	

Table 2: Non-synonymous polymorphisms at 2009 WHO Surveillance Drug Resistance Mutation sites in 218 HIV-1 CRF-01_AE isolates from Ho Chi Minh City

	Drug resistance positions [a]	Amino acid in B reference	Amino acid in CRF-01 AE reference	Amino acid substitution in this cohort	Number of subjects (%)
Protease	82	V	V	I	24 (11.0%)
	88	N	N	K	1 (0.5%)
Reverse transcriptase	67	D	D	**N**[b]	1 (0.5%)
	69	T	T	**D**	1 (0.5%)
				N	20 (9.2%)
				A	4 (1.8%)
				S	3 (1.4%)
	74	L	L	**V**	1 (0.5%)
	75	V	V	**M**	2 (0.9%)
				L	2 (0.9%)
	101	K	K	R	1 (0.5%)
	103	K	K	**N**	3 (1.4%)
				R	1 (0.5%)
	106	V	V	**M**	1 (0.5%)
				I	17 (7.8%)
	179	V	V	I	38 (17.4%)
				D	5 (2.3%)
				A	2 (0.9%)
				E	2 (0.9%)
				N	1 (0.5%)
	181	Y	Y	**C**	4 (1.8%)
	184	M	M	**V**	2 (0.9%)
	190	G	G	**A**	1 (0.5%)
	210	L	L	M	7 (3.2%)
				F	1 (0.5%)
	219	K	K	**R**	1 (0.5%)
				T	2 (0.9%)

[a] Drug resistance positions based on 2009 WHO Surveillance Drug Resistance Mutation; [b] Bold, amino acids associated with drug resistance

Table 3: Non-synonymous polymorphisms at positions not associated with drug resistance based on the 2009 WHO Surveillance Drug Resistance Mutation list in 218 HIV-1 CRF-01 AE isolates from Ho Chi Minh City

PR Position [a]	*L10*	*I13*	*K14*	*I15*	*G16*	*K20*	*E35*	*M36*	*N37*	*R41*	*K43*	*K45*	*R57*	*Q61*	*L63*
CRF01-AE	I (49) [b]	V (148)	R (12)	V (42)	E (107)	R (37)	D (197)	I (217)	D (54)	K (213)	R (15)	R (15)	K (20)	E (8)	C (99)
	V (25)		N (1)		A (38)	I (3)	D/N (2)		D/E (3)					H (2)	P (20)
	M (1)				A/S (1)				S (1)						S (3)
PR Position	*I64*	*H69*	*K70*	*I72*	*T74*	*V77*	*L89*	*M93*	*F99*						
CRF01-AE	L (1)	K (214)	R (76)	V (5)	S (1)	I (3)	M (205)	L (41)	L (3)						
	M (1)	Q (1)		R (1)			M/I (3)	V (2)							
	V (1)	T (1)		T (1)			I (1)	M (1)							
		I (1)													
RT Position [a]	*E6*	*K11*	*V35*	*T39*	*K43*	*V60*	*Q102*	*K122*	*D123*	*I135*	*S162*	*K173*	*Q174*	*D177*	*I178*
CRF01-AE	D (191)	T (142)	T (211)	K (100)	E (71)	I (32)	K (184)	E (212)	S (162)	T (28)	C (159)	I (125)	K (109)	E (109)	M (126)
	N(4)		A(1)	E (63)	R (3)		R (4)	E/V (2)	N (13)	V (2)		T (33)	R (12)		
	K/N (1)		I (1)	N (30)	Q (4)		K/R (5)		N/S (37)	L (1)		R (26)			
			R (1)		A (1)		T (1)			R (1)		M (10)			
RT Position	*G196*	*T200*	*I202*	*Q207*	*R211*	*K238*	*V245*	*A272*	*V276*	*R277*	*K281*	*T286*	*E291*	*V292*	
CRF01-AE	E (12)	I (55)	V (24)	A (182)	S (208)	R (188)	E (205)	P (61)	I (34)	K (210)	R (32)	A (179)	D (187)	I (193)	
		A (51)		S (15)											
				D (12)											

PR, Protease; RT, Reverse Transcriptase; [a] Positions based on pNL4-3 reference; [b] Number in brackets represents number of isolates carrying a certain amino acid substitution

Table 4: Genotypic drug resistance profile of 5 individuals with virological failure and major 2010 International AIDS Society-USA Mutations

	Subject 3		Subject 7		Subject 15		Subject 16		Subject 17	
Time (months)	HIV RNA [a]	Genotype	HIV RNA	Genotype	HIV RNA	Genotype	HIV RNA	Genotype	HIV RNA	Genotype
0	76500	Y181C	64020	M184V	56310	wt	4955200	wt	29230	wt
1	150		1540		490		4870			
2	150		37300	M184V, K103N	3545	Y188L	150		150	
3			152755	M184V, K103N, V108I	700		395			
4	150				150		150		450	
5	150								2335	K103N,M184V, Y188HLFY
6			47910	M184V, K103N, V108I, T215F/I/S, P225H, M230L					215	
7	41325	V179D			205		150		2605	K103N, M184V, Y188L
8	1295	K103N, V106I			1975	M184V, Y188L				
9			51850	M184V, K103N, V108I, T215F, M230L	1825	M184V, Y188L	132495	K103N		
10							95885	K103N, P225HP		
11										
12			44940	M184V, K103N, V108I, T215F/I/S, P225H, M230L						

[a] HIV RNA (copies/ml); wt, wildtype

Medicine

Second-Line HIV Therapy Outcomes and Determinants of Mortality at the Largest HIV Referral Centre in Southern Vietnam

--Manuscript Draft--

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Abstract:	Background: The growing numbers of HIV-infected patients requiring second-line antiretroviral therapy (ART) in Vietnam makes essential the evaluation of treatment efficacy to guide treatment strategies. Methods: We evaluated all patients aged ≥15 years who initiated second-line ART after documented failure of first-line therapy at the Hospital for Tropical Diseases in Ho Chi Minh City. The primary outcome was time from second-line ART initiation to death, or to a new or re-occurrence of a WHO-defined immunological or clinical failure event, whichever occurred first. Risks of treatment failure and death were evaluated using Cox proportional hazards modeling. Results: Data from 326 of 373 patients initiating second-line ART between November 2006 and August 2011 were included in this analysis. The median age was 32 years (IQR: 28-36). 81% were men. The median CD4 count was 44 cells/μL (IQR: 16-84). During a median follow-up of 29 months (IQR: 15-44), 60 (18.4%) patients experienced treatment failure, including 12 immunological failures, 4 WHO stage IV AIDS events, and 44 deaths (13.5%). 60% of deaths occurred during the first 6-12 months. The Kaplan-Meier estimates of treatment failure after 1, 2, 3, and 4 years were 13.1% (95%CI: 9.2-16.8), 18.6% (95%CI: 14.0-23.1), 20.4% (95%CI: 15.4-25.1), and 22.8% (95%CI: 17.2-28.1), respectively. Older age, history of injection drug use, lower CD4 count, medication adherence <95%, and previous protease inhibitor use independently

	predicted treatment failure. Conclusions: While treatment efficacy was similar to that reported from other resource-limited settings; mortality was higher. Deaths may be averted by prioritizing second-line therapy based on CD4 counts and through improving treatment adherence support.

23rd July, 2015

Dear Editor of Medicine HIV/AIDS,

Re: the manuscript entitled: "Second-Line HIV Therapy Outcomes and Determinants of Mortality at the Largest HIV Referral Centre in Southern Vietnam"

We appreciate your and the reviewers' comments and suggestions and we believe that addressing them has significantly improved the clarity and quality of the manuscript. We want to emphasize that viral load monitoring is still, very unfortunately, not the standard of care in Vietnam as well as in many resource-poor settings in 2015. For this reason, aside from the scientific reason, we believe this programmatic analysis should be published and disseminated to the world community. Below are our point-by-point responses to your and the reviewers' comments:

RESPONSES TO EDITOR'S COMMENTS

COMMENT 1:
This study has a serious limitation, who is, that it took place in a single center, which, as the authors say, does not allow to generalize the results to the whole country.
Secondarily it should be noted that in the latest WHO recommendations, "Viral load testing is recommended as the preferred approach for monitoring ART response to provide an early and more accurate indication of treatment failure and more appropriate switching to second-line drugs, reducing the accumulation of drug-resistance mutations, and improving clinical outcomes. A pooled analysis showed the current WHO immunological and clinical criteria for treatment failure have poor sensitivity and positive predictive value for identifying virological failure in adults and children." Although the WHO immunological criteria can be used in settings where viral load routine is not available; here the authors report that 94% of patients had a test of the available viral load in the antiretroviral therapy first line failure.

I therefore recommend that the definition of antiretroviral treatment failure based on a threshold value (50, 500, 1,000 or 10,000 copies / ml) of viral load that the authors themselves have chosen, they can be limited to a one measure or two of the viral load, as recommended by WHO.
Failure to antiretroviral therapy in first and second line can be based only on the measurement of viral load.
It is difficult to retain immunological and clinical criteria for defining the failure to antiretroviral therapy.

→RESPONSE:
To respond to your valid criticism that this is a one-centre study, we have added a section in the discussion of study limitation in the revised manuscript, page 13, lines 18-22, defending the validity of this study, as follows:
…"However the HTD is the largest centre for HIV care in Vietnam and is the primary provider of second-line therapy for patients in southern Vietnam. Half of the patients in this cohort come from the 17 southern provinces of Vietnam, representing a wide selection of patients on second-line therapy in Vietnam. Further this study is the largest

second-line therapy cohort ever conducted in Southeast Asia, allowing for robust analyses of clinical outcome."

We absolutely agree with the Editor that viral load testing should be used to monitor antiretroviral therapy and to identify treatment failure. However, despite the current WHO's recommendations, and despite Vietnam being a US PEPFAR's supported country, viral load monitoring is still not the reality in 2015! Antiretroviral therapy in Vietnam and in many countries in resource-limited settings is still being monitored by CD4 count testing every 6 months and by clinical evaluation. Viral load testing in Vietnam is indicated only to confirm/document treatment failure in patients who meet the WHO criteria for clinical and/or immunological failure. This is in accordance with the newest national guidelines in 2013. The 94% of patients in this study who had a viral load test performed had it done to document treatment failure prior to switching to second-line therapy. As mentioned in our discussion, in a meta-analysis of 27-cohort study from Africa and Asia [Pujades-Rogiguez et al, JAMA 2010, ref. #16], only 4 of 27 centres had access to routine viral load monitoring. The HIV care in Vietnam is still dependent on clinical/immunological monitoring, very similar to that in other resource-poor settings worldwide.

Without viral load monitoring, an analysis of viral load as an outcome measure of second-line therapy cannot be performed. A cross-sectional evaluation of virological outcome could be done. However such an analysis is an entirely different study and cannot be compared to this longitudinal analysis of time to event over a median of 29 months follow up. A cross-sectional analysis cannot account for those who have died in our cohort, and death was responsible for the majority (73%) of the combined clinical/immunological outcome in our study.

COMMENT 2: In chapter Methods

In section "ART monitoring", the first sentence and references do not seem suited to the current context, I suggest that you use the 2013 WHO recommendations. Also in this chapter identify the defining elements of your virologic failure, the details I put above.

→RESPONSE: This was a retrospective study analyzing patients in care between November 2006 and August 2011. The care was in accordance with Vietnam and international guidelines during that window of time.

COMMENT 3: In Section outcome measurements, note a lack of realism and not confuse the practice every day with clinical research, first in the criteria to switch on second line of antiretroviral therapy is clinical and immunological, which cannot be accepted, and the judgment criteria can be death and virological failure, but not clinical and immunological alone especially in the first 6 months, data reported a weak performance WHO immunological criteria in patients in second line antiretroviral treatment

→RESPONSE: Please refer to our response to comment 1 above, that antiretroviral therapy is monitored using WHO-defined immunological and clinical failure criteria, and

not by viral load monitoring. Viral load test was performed only to confirm/document treatment failure and was not done to monitor patients on therapy.

COMMENT 4: This sentence is very misplaced in the methodology '52 of 326 patients (16.0%) were transferred to other provincial or district clinics while on second-line therapy as part of the government's efforts to decentralize HIV care' his place is in Chapter results.

→RESPONSE: Thank you for this suggestion. The numbers of transferred patients are now removed from the Methods and emphasized in the Result section, page 9, line 7-8.

COMMENT 5: I also noted the presence of the references in the chapter Results.

→RESPONSE: Thank you. References cited in the Results section are now removed.

RESPONSE TO REVIEWER #2's COMMENTS

COMMENT: It's the first manuscript describing the effect of second-line therapy in Vietnam. Some important information was not shown. Such as, the duration of patients on second-line therapy, the number of clinical visits and CD4 counts, the number of subjects on risk in Kaplan-Meier analyses.

→RESPONSE: Thank you for the opportunity to clarify and improve the Kaplan-Meier summary. The duration of second-line therapy was 29 months (interquartile range: 15-44). This information was reported in the Results on page 9, lines 6-7. The patients were required to come to the clinic monthly for clinical and adherence evaluation, and CD4 count was performed 6 monthly. This information was reported in the Methods, page 5, lines 16-18. As such, the median number of clinical visits was 29 visits per patient, and the median number of CD4 tests performed per patient was 5. The numbers of patients at risk in all cumulative incidence curves are now added in Figure 2.

RESPONSE TO REVIEWER #3's COMMENTS

COMMENT: Though it is the first study on second line ART treatment in clinical failures. As being a retrospective study can you clarify on inter/ intra-obsever bias in data collection? This the major and key limitation which might have produced biased result which you have highlighted in discussion part also.???

→RESPONSE: The study outcomes were very clearly defined on page 6, lines 2-8, that: "*The primary outcome was treatment failure and was defined as time from second-line ART initiation to death, or to a new or re-occurrence of an immunological or a clinical failure event, whichever occurred first. Immunological failure was defined by the WHO as a decrease of CD4 count to or lower than baseline, a decrease of >50% of peak CD4 value while on treatment, or a persistent CD4 count of <100 cells/μL after at least 6 months of continued ART. Clinical failure was defined as new occurrence or re-occurrence of a WHO stage IV disease.[10] The secondary outcome was time to death.*"

These outcomes have been standardized by the WHO and are routinely used in clinical practice in resource-poor settings to monitor patients on therapy. There is little room

for variability that inter/intra-observer variability has not been recommended for this type of assessment.

RESPONSE TO REVIEWER #4's COMMENTS

COMMENT: This is a first study conducted in your Field with validity and reliability.

→RESPONSE: Thank you.

RESPONSE TO REVIEWER #5's COMMENTS

COMMENT 1: The study reflects the management of HIV-infected patients failing to a first combination treatment consisting of a backbone of two nucleoside/nucleotide reverse transcriptase inhibitors (NTRI) and non-nucleoside reverse transcriptase inhibitor (NNRTI) in a country with low income and limited access to current HAART therapy.
It is a retrospective and cross-sectional study (no longitudinal as the authors described in materials and methods section) that evaluated treatment with lopinavir / ritonavir plus two NRTIs, mostly with tenofovir disoproxil fumarate (TDF).

→ RESPONSE: Thank you. We have removed the word "longitudinal" as suggested.

COMMENT 2: The first striking fact is that the therapeutic rescue occurs in patients with low CD4 T-cells (median < 50/mcL) and very high viral load (>105 c/mL). This indicates that patients were for many months with an ineffective antiretroviral therapy or were not taking any antiretroviral treatment (no adherent and inmates patients). Thus it is not surprising the high rate of viral quasispecies with high levels of resistance to NNRTI and NRTI observed in the analysis conducted in the reference center.
In fact it could almost be assumed as a study to assess the efficacy of monotherapy with lopinavir / ritonavir, as the high-level resistance to NRTIs and TDF was 97% and 65% respectively.
The results are similar in terms of effectiveness to those reported by other authors in similar circumstances, except for early mortality from AIDS-related causes, perhaps it is reflecting that patients had severe immunosuppression when they started rescue therapy. A fact that draws attention to the reliability of the reported causes of death is that 9% die from esophageal candidiasis (¿?).
The discussion is correct and factors found in multivariate analysis related to therapeutic failure are already known and previously reported in other studies: low adherence, history of intravenous drug use and low CD4 T-cells count and previous protease inhibitor use. Older age was also associated with a worse outcome (although the median age of patients was only 32 years) finding poorly justified or explained in the discussion.
In summary, this study provides little relevant information for the management of HIV-infected patients failing to a first regimen HAART consisting of a NNRTI and two NRTIs and they are rescued with HAART based on IP, even in countries with low income.

→RESPONSE: First, thank you for the suggestion to discuss older age as a risk factor of poor ART outcome. As suggested, we have added the following point in the Discussion,

page 12, lines 21-22: "*Older age at ART initiation has been linked to poor immunological recovery, loss to follow up and death in ART cohorts in Zambia and South Africa.*[31,32]"

With respect to cadida esophagitis being a cause of death in 4 patients, these patients very likely had died from other causes, but candida esophagitis was the only diagnosis recorded in their outpatient medical charts at the time of death.

We very respectfully disagree with the reviewer about the clinical relevance of the study in clinical care of patients in Vietnam and in similar resource-poor settings. The study is a programmatic evaluation of the real-life HIV care in Vietnam. It is the largest second-line cohort ever conducted in Southeast Asia and is the first study to document second-line treatment outcomes in Vietnam after 10 years of ART scale up in this country. The data are extremely relevant to clinical practice, and we believe should be urgently shared so that clinicians and policy makers in Vietnam, in Southeast Asia, and in similar resource-poor settings are made aware of the treatment outcomes, the high mortality, and what can be done to avert those early deaths. While little can be done about non-modifiable risk factors of older age and history of injection drug use, interventions to prioritise treatment based on CD4 count and adherence support are critical and are modifiable risk factors that can be implemented immediately to prevent early death and to improve the outcomes of patients.

Thank you for your consideration of your manuscript for publication in Medicine,

Yours Sincerely,

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TITLE PAGE

Title

Second-Line HIV Therapy Outcomes and Determinants of Mortality at the Largest HIV Referral Centre in Southern Vietnam

Authors

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Abbreviation

3TC = lamivudine, ABC = abacavir, ART = Antiretroviral therapy, AZT = zidovudine, D4T = stavudine, ddI = didanosine, HCMC = Ho Chi Minh City, HTD = Hospital for Tropical Diseases, IDU = injection drug use, IDV = indinavir, LPVr = Ritonavir-boosted lopinavir, NFV = nelfinavir, NNRTI = non-nucleotide reverse transcriptase inhibitors, NRTI = nucleotide reverse transcriptase inhibitor, NVP = nevirapine, PI = protease inhibitor, TAM = thymidine analog mutation, TDF = tenofovir, VAS = Visual Analogue Scale, WHO = World Health Organization

Running head

Meeting

Data were partially presented at the 20th Conference of Retroviruses and Opportunistic Infections, Atlanta, GA, USA, 3 – 6 March, 2013. Abstract #1109.

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Conflict of interest

All authors declare no conflicts of interest.

ABSTRACT

Background:

The growing numbers of HIV-infected patients requiring second-line antiretroviral therapy (ART) in Vietnam makes essential the evaluation of treatment efficacy to guide treatment strategies.

Methods:

We evaluated all patients aged ≥15 years who initiated second-line ART after documented failure of first-line therapy at the Hospital for Tropical Diseases in Ho Chi Minh City. The primary outcome was time from second-line ART initiation to death, or to a new or re-occurrence of a WHO-defined immunological or clinical failure event, whichever occurred first. Risks of treatment failure and death were evaluated using Cox proportional hazards modeling.

Results:

Data from 326 of 373 patients initiating second-line ART between November 2006 and August 2011 were included in this analysis. The median age was 32 years (IQR: 28-36). 81% were men. The median CD4 count was 44 cells/μL (IQR: 16-84). During a median follow-up of 29 months (IQR: 15-44), 60 (18.4%) patients experienced treatment failure, including 12 immunological failures, 4 WHO stage IV AIDS events, and 44 deaths (13.5%). 60% of deaths occurred during the first 6-12 months. The Kaplan-Meier estimates of treatment failure after 1, 2, 3, and 4 years were 13.1% (95%CI: 9.2-16.8), 18.6% (95%CI: 14.0-23.1), 20.4% (95%CI: 15.4-25.1), and 22.8% (95%CI: 17.2-28.1), respectively. Older age, history of injection drug use, lower CD4 count, medication adherence <95%, and previous protease inhibitor use independently predicted treatment failure.

Conclusions:

While treatment efficacy was similar to that reported from other resource-limited settings; mortality was higher. Deaths may be averted by prioritizing second-line therapy based on CD4 counts and through improving treatment adherence support.

INTRODUCTION

The availability of low-cost fixed-dose combination antiretroviral drugs has enabled rapid scale-up of antiretroviral therapy (ART), resulting in substantial reduction in morbidity and mortality due to HIV in resource limited countries.[1–3] The World Health Organization (WHO) estimates that 16.8 million adults and children in low and middle-income countries will be on ART in 2016; among them 5% will be on second-line therapy.[4] This represents a more than 50% increase in ART coverage over the past five years. Despite generic production for resource-limited countries, a second-line regimen containing ritonavir-boosted lopinavir (LPVr) costs six times that of a first-line regimen.[5] In most low and middle-income countries second-line therapy is the last option for patients failing treatment with drug resistance. As third-line therapy is forbiddingly expensive and is unavailable in resource-limited countries, it is imperative for national programmes in these settings to maximize the efficacy and durability of second-line therapy.

Vietnam is among the countries with the highest HIV burden in Asia with an estimate of 280,000 people living with HIV.[6] Nearly 90,000 people were on ART as of 2014, and an estimated 3% were on second-line therapy.[6] The HIV system in Vietnam is undergoing a critical transition from an international-donor to a national-funding approach that integrates with the national health insurance programme.[7] Outcome data on second-line therapy in Vietnam are lacking, but are important for the national programme to devise treatment strategies and to forecast treatment options beyond second-line therapy. In this study we investigate second-line therapy outcomes and factors that determine therapy failure and death at the largest HIV referral centre in southern Vietnam.

METHODS

Study design and setting

This was a retrospective ~~longitudinal~~ analysis of adult patients who switched to second-line therapy in a cohort of over 4000 patients on the national ART programme at the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City (HCMC). This is the largest primary and referral centre for HIV care in southern Vietnam (population around 45 million). The national ART programme began providing free antiretroviral drugs through international funding support in 2003. First-line therapy consisted of zidovudine (AZT) or stavudine (d4T) in combination with lamivudine (3TC) and nevirapine (NVP). Prior to the availability of efavirenz in 2006, cases of NVP-related toxicity were switched to indinavir (IDV). Second-line therapy became available in 2006 initially including abacavir (ABC), didanosine (ddI), and nelfinavir (NFV). In 2007 LPVr replaced NFV, and in 2009 tenofovir (TDF) and 3TC replaced ABC and ddI as the nucleotide reverse transcriptase inhibitor (NRTI) backbone.[8]

ART monitoring

ART was monitored using immunological and clinical failure criteria based on the WHO's guidelines for settings without routine viral load monitoring.[9,10] Patients were required to come to the clinic monthly for clinical evaluation and medication pick up. CD4 count was measured every 6 months. HIV viral load was tested at the time patients were diagnosed with immunological or clinical failure and was confirmed with repeat testing. HIV viral load was performed using a generic real-time PCR assay (Biocentric, Bandol, France) with a limit of detection of 250 copies/mL.[11] HIV genotyping was performed to evaluate for drug resistance prior to therapy switch using a published in-house assay[12] on the Beckman Coulter CEQ 8000 platform. Both HIV viral load and genotyping tests were performed at the Pasteur Institute, a WHO accredited HIV reference laboratory, in HCMC.

Study population

We included all HIV-infected patients aged ≥15 years who initiated second-line therapy due to documented immunological and/or clinical failure of first-line therapy. Patients who were alive and well but had been on second-line therapy for <6 months, and those without documented treatment failure to first-line therapy, were excluded. The study was approved by the Scientific and Ethical Committee of the HTD.

Outcome measurements

The primary outcome was treatment failure and was defined as time from second-line ART initiation to death, or to a new or re-occurrence of an immunological or a clinical failure event, whichever occurred first. Immunological failure was defined by the WHO as a decrease of CD4 count to or lower than baseline, a decrease of >50% of peak CD4 value while on treatment, or a persistent CD4 count of <100 cells/μL after at least 6 months of continued ART. Clinical failure was defined as new occurrence or re-occurrence of a WHO stage IV disease.[10] The secondary outcome was time to death.

Data collection

Routinely collected clinical and laboratory data were recorded on a standardized form and included demographic information, history of injection drug use (IDU), antiretroviral drug timeline, WHO stage 4 AIDS events, 6-month serial CD4 counts, HIV viral load and HIV genotype when these were available, ART adherence evaluation, deaths, and causes of death.

Antiretroviral therapy adherence evaluation

ART adherence counseling was provided to patients pre- and post-second-line ART initiation according to standard of care. Adherence was routinely assessed by the clinicians according to the MoH guidelines and was recorded either as an estimated percentage of pills taken, or as a qualitative assessment of 'good', 'average', or 'poor', corresponding to ≥95%, 80-94%, or <80% adherence, respectively.[13] Additionally in patients who were in active follow up, adherence was prospectively evaluated using a simple self-reported Visual Analogue Scale (VAS).[14] This VAS has been shown to be as reliable as other methods such as pill-count and 3-day recall self-report, yet much simpler to administer.[15]

For analysis, sub-optimal adherence was defined as having at least one adherence score of <95% by pill count, by the VAS, and/or receiving at least one qualitative adherence assessment of 'average' or 'poor' over a 6-month period preceding an outcome event or preceding the time of study assessment in patients who had not had an event.

Statistical analysis

The cumulative incidence of treatment failure and failure rates after 1, 2, 3, and 4 years and corresponding 95% confidence intervals were calculated using the Kaplan-Meier method. The

Cox proportional hazards model was used to analyse the time to treatment failure (composite primary endpoint) and the time to death (secondary endpoint). Patients who were transferred to other provincial or district clinics while on second-line therapy had been judged by doctors to be clinically and immunologically stable before the transfer. For the analysis, event-free transferred patients were censored at the time of transfer (primary analysis). Alternatively, assuming the transferred patients were doing well clinically and immunologically on therapy, we treated them as censored at the time-point where their last monthly follow-up visit would have been had they not been transferred (sensitivity analysis to assess potential informative censoring).

The following pre-defined covariates were included in the model: age at second-line therapy initiation, history of IDU (yes/no), CD4 cell count, and (log10-transformed) HIV RNA viral load at second-line therapy initiation, second-line therapy delay (defined as time from first detection of immunological or clinical failure to time of second-line therapy initiation), history of protease inhibitor (PI) use, and an overall measure of therapy adherence (<95% vs. ≥95%). The chosen covariates have been shown to be associated with poor ART outcome.[16–21] The proportional hazards assumption was assessed by examining plots of weighted Schoenfeld residuals and by formal testing. There was strong evidence of non-proportional hazards for the effect of CD4 cell count at second-line therapy initiation on treatment failure (p=0.001 univariate analysis, p=0.0002 multivariable analysis). To account for this, we decided to model a time-varying effect on the hazard of treatment failure with separate effects for the first year of follow-up and subsequently. There was no clear evidence for non-proportional hazards between any other covariates and the primary or secondary endpoint (all univariate p>0.13). Both univariate and multivariable Cox regressions were performed.

Data were analysed based on multiple imputations of missing data and on a complete-case analysis. To avoid bias, the imputation algorithm included the endpoints [event time T and Nelson-Aalen estimator H(T)].[22] All reported confidence intervals are two-sided 95% intervals and analyses were performed with the statistical software R version 2.15.0,[23] and the companion R package mice version 1.2.5 (for multiple imputation).[24]

RESULTS

Study population and baseline characteristics

A total of 373 patients aged ≥15 years initiated second-line therapy between November 2006 and August 2011. 47 patients were excluded from the analysis, including 43 who had received second-line therapy for <6 months and 4 who had switched to second-line therapy because of treatment intolerance. The remaining 326 patients had documented treatment failure to first-line therapy (with confirmation of virological failure in 94%) and were included in this study. Approximately 50% of patients came from HCMC; the rest from the remaining 17 southern provinces of Vietnam. The median duration of first-line ART treatment was 33 months (IQR: 21-44). The characteristics of the 326 patients at the time of initiation of second-line therapy are summarized in Table 1. The median CD4 count was 44 cells/μL (IQR: 16-84) and the median HIV RNA was 5.1 log copies/mL (IQR: 4.6-5.6). The median time of second-line therapy delay was 9 months (IQR: 5-15).

Drug resistance patterns in patients failing first-line ART

HIV genotyping was performed for 246/326 (75.5%) patients who failed first-line therapy. Mutations conferring high-level resistance to NRTIs were detected in 238/246 patients (96.7%), to non-nucleotide reverse transcriptase inhibitors (NNRTIs) in 229/246 (93.1%), and to PIs in 6/246 (2.4%). Resistance mutations to both NRTIs and NNRTIs were present in 226/246 patients (91.9%) and to all three drug classes in 5/246 patients (2.0%). The most common NRTI mutations were M184I/V (85.4%), thymidine analog mutations (TAMs) M41L, D67N, K70E/R, T215F/Y, and K219E/Q (30-55%), Q151M (21.1%), and K65R (14.6%). Two patients had a T69 insertion mutation. The most common NNRTI mutations were Y181C/I/V (45.5%), G190A/S (41.9%) and K103N (31.3%). The most common PI mutations were I54V (2.4%), M46I/L (2.8%), V82A (2.0%), and L90M (1.2%).

Predicted resistance to second-line ART regimen

The predicted susceptibility to the national second-line regimen containing TDF, 3TC, and LPVr were evaluated for the 246 patients who had genotype results using the Stanford HIV Drug Resistance Database (access date: 09 April 2015). Intermediate to high-level resistance to TDF was present in 161/246 (65.4%), to 3TC in 230/246 (93.5%), and to LPVr in 5/246 (2.0%).

Second-line ART outcome

320 patients (98.2%) received LPVr in combination with 2 NRTIs and 6 patients received NFV with 2 NRTIs (Table 1). 121 patients (37.1%) also received AZT; this was chosen by clinicians who believed that an AZT-containing regimen might reduce the likelihood of developing the TDF-signature-resistance-mutation K65R,[25-27] and thereby preserve the potency of the second-line regimen. Sub-optimal adherence was observed in 43 patients (13.2%). During a median follow-up of 29 months (IQR: 15-44), 52 patients (16.0%) were transferred to other clinics as part of the government's efforts to decentralize HIV care; 1 was imprisoned and was lost to follow-up, 44 (13.5%) died and the remaining 229 (70.2%) were in active follow-up. 60 (18.4%) patients experienced treatment failure, including 12 immunological failures, 4 WHO stage IV AIDS events, 39 AIDS-related deaths, and 5 non-AIDS deaths (Figure 1). The cumulative incidence of treatment failure and corresponding 95% confidence interval are shown in Figure 2a. The Kaplan-Meier estimates of the risk of treatment failure by 1, 2, 3, and 4 years were 13.1% (95%CI: 9.2-16.8), 18.6% (95%CI: 14.0-23.1), 20.4% (95%CI: 15.4-25.1), and 22.8% (95%CI: 17.2-28.1) respectively. The median CD4 counts after 1, 2, 3, and 4 years were 234 cells/μL (IQR: 166-338), 353 cells/μL (IQR: 227-465), 393 cells/μL (IQR: 255-514), 473 cells/μL (IQR: 347-574), respectively.

Predictors of second-line ART failure

The 7 covariates entered in the Cox model are listed in Table 2. The most frequently missing covariates were history of PI use (10% missing), viral load (6% missing), and IDU history (5% missing); other covariates were missing in ≤2% of patients. The results of the univariate and multivariate Cox regression analyses are shown in Table 2. Lower CD4 count and sub-optimal adherence predicted treatment failure in both univariate and multivariate analyses. However, lower CD4 count affected the rate of treatment failure only during the first year of second-line therapy and not thereafter. Older age, history of IDU, and history of PI use did not predict treatment failure in the univariate analysis, but in the multivariate analysis they became statistically significant predictors of treatment failure. Multivariate analysis shown in Table 2 was based on multiple imputations of missing data; however a complete-case analysis gave highly consistent results (data not shown). A sensitivity analysis with informative censoring of the 52 transferred patients was performed (i.e. assuming the transferred patients continued to do well clinically and immunologically on therapy, with censoring occurring at the time-point where their last monthly follow-up visit would have been had they not been transferred), and the

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results were also consistent (data not shown). The cumulative incidence of treatment failure among patients according to IDU, treatment adherence, and PI is shown in Figures 2b, 2c, and 2d, respectively.

We performed an exploratory univariate analysis of the impact of adding AZT as a 4th drug to second-line regimen using Cox proportional hazard modeling, and a statistically significant effect on treatment outcome was not observed (HR: 1.12, 95% CI: 0.50-1.62, p=0.715).

Causes and predictors of death

Deaths occurred in 44 patients (13.5%) and accounted for 73.3% of failure events; 39 were AIDS-related deaths, and 5 were unknown or non-AIDS related deaths. The median time to death was 9 months (IQR: 3-22), with 26 deaths (59.1%) occurring within the first 6 to 12 months. The causes of AIDS-related deaths included microbiologically-confirmed tuberculosis (13, 29.5%), *Pneumocystis jiroveci* pneumonia (4, 9.1%), candida esophagitis (4, 9.1%), cryptococcal meningitis (2), *Penicillium marneffei* infection (2), herpes simplex (2), cytomegalovirus (2), toxoplasmosis (2), hepatitis C related liver failure (2), renal failure of unclear etiology (2), non-typhoid salmonella sepsis (1), and AIDS-associated wasting (8, 18.2%). Univariate and multivariate Cox regression analyses were performed to determine predictors of death. Consistent with the results of the primary outcome analysis, lower CD4 count (multivariate HR: 1.75, 95% CI: 1.11-2.70, p=0.015) and sub-optimal adherence (multivariate HR: 3.41, 95%CI: 1.59-7.30, p=0.002) predicted death in both univariate and multivariate analyses. Age and history of IDU did not predict death in the univariate analysis, but in the multivariate analysis older age and history of IDU became significant predictors of death (HR: 1.95, 95% CI: 1.22-3.11, p=0.005 and HR: 2.30, 95% CI: 1.05-5.05, p=0.037, respectively).

DISCUSSION

To our knowledge this is the first study to systematically evaluate the outcomes of second-line ART in patients who fail first-line therapy in Vietnam according to the WHO's immunological and clinical criteria. Despite the profound level of immune deficiency in this patient cohort (median CD4 count: 44 cells/μL), high viral replication (median HIV RNA: 5.1 log copies/mL), and extensive resistance to second-line NRTI backbone (93.5% resistance to 3TC, 65.4% resistance to TDF) at the time of treatment switch, treatment failure rates are similar to studies in comparable settings.[16,18,19,21,25] A 27-cohort study comprising 632 patients from Africa and Asia reported a failure rate of 28% over two years.[16] Treatment failure in that study was defined as the first diagnosis of clinical, immunological or virological failure, or death. Only 4 of these 27 centres had routine viral load monitoring. These failure rates reflect the reality of HIV care in resource-limited settings. Our treatment failure rates are lower when compared to studies that use virological failure as the measure of outcomes. A meta-analysis of 2035 patients from 19 cohorts across low-income and middle-income countries reported failure rates of 22-38% after 6-36 months on second-line ART.[25] Although an outcome measure relying on CD4 count and clinical evaluation can underestimate virological failure rates, the long-term clinical and health economic benefits of routine viral load monitoring still need to be determined in resource-poor settings.

While the overall treatment failure rates were similar to equivalent settings, the mortality in our cohort was higher: 13.5% versus an estimate of 5% in the 27-cohort study from Africa and Asia.[16] The median time to death was shorter, 9 months (IQR: 3-22) versus 15 months (IQR: 12-26).[16] Death accounted for 73% of failure events, and 90% of deaths were due to AIDS-related infections. Using multivariate analysis, death during the first year was predicted by lower CD4 counts at second-line ART initiation. The median CD4 count and HIV viral load at second-line ART initiation were 44 cells/μL and 5.1 log copies/mL, respectively. This compares with median CD4 counts >100 cells/μL and HIV viral load ranging from 3.9 to 4.8 log copies/mL in other African and Asian cohorts.[17,19–21,26,27] The lower CD4 count likely explains the higher mortality observed in our patients and suggests second-line therapy delay plays a role. When comparing to studies from other resource limited countries, therapy delay in our cohort was longer, median 9 (range: 5-15) versus 5 (range: 1-8) months.[17,18,20,26]

Therapy delay can be explained by programmatic reasons not unique to Vietnam. Without viral load monitoring, it can take months to years for a patient who fails treatment virologically to

manifest failure immunologically and clinically. The process of defining treatment failure in Vietnam may be too conservative. Immunological failure is usually confirmed with a repeat CD4 measure in 3-6 months. Cases of confirmed immunological and/or clinical failure are referred to a panel of experts located in centres that have access to viral load and drug resistance testing. The referral process, albeit necessary in some circumstances, further delays the onset of therapy switch. When the relationship of treatment delay and CD4 count were assessed with respect to therapy failure and death in our study, only lower CD4 count predicted therapy failure and death. The likely explanation for this is that these two variables are interdependent or have a causal relationship, i.e. treatment delay directly results in a decline in CD4 count. In our cohort the CD4 count is likely the stronger variable that drives the outcomes. When these same variables were evaluated by Levison *et al.* in a study using virological failure as outcome, they found that therapy delay and not CD4 count predicted virological failure, and for every month a patient remained on a failing first-line ART regimen there was a 7% increase in risk of lack of virological suppression.[20] The longer a non-suppressive ART regimen is given, the higher the chance of developing accumulation of drug resistance mutations, impairing the efficacy of current as well as future ART options.

In our cohort older age, history of IDU, PI exposure, lower CD4 count, and suboptimal adherence were independent predictors of treatment failure in both complete-case and multiple imputation analyses. With the exception of PI exposure, these variables remained independent predictors of death. These findings are consistent with published literature on first-line ART failure worldwide.[28–34] Older age at ART initiation has been linked to poor immunological recovery, loss to follow up and death in ART cohorts in Zambia and South Africa.[31,29] CD4 count and treatment adherence are established risk factors for first-line ART failure and are also found to be independent predictors of second-line ART failure in the analysis of 27 ART programmes across Africa and Asia.[16] PI exposure has been associated with risks of virological failure in Cambodia and India.[35,36] Treatment adherence in our study was assessed at multiple time points, suggesting that sub-optimal adherence at anytime during therapy predicts treatment failure. This finding is similar to other second-line cohorts from South Africa, Malawi and Thailand,[17,19,21] highlighting the importance of ongoing patient education and adherence support in improving treatment outcomes. The history of IDU predicted treatment failure independently from treatment adherence. This effect is likely multi-factorial and likely involves cofactors that

are not measured in this study including nutritional status, social economic status, and hepatitis B and/or C co-infection.

Co-infection with tuberculosis in patients failing ART is common in countries where tuberculosis is highly endemic. In our study, microbiologically-confirmed tuberculosis accounted for the majority of AIDS-related deaths (30%). HIV and tuberculosis co-infection is a common reason for clinicians to delay second-line ART initiation because of the rifampicin and PI drug-drug interactions via CYP450 metabolism pathway. The WHO recommends rifabutin in place of rifampicin for patients who need tuberculosis treatment while on PI therapy. Unfortunately rifabutin is not yet available in Vietnam. A super-boosted dose of ritonavir (400mg twice daily) is therefore recommended.[37] The safety data of this super-boosted ritonavir regimen in HIV-associated tuberculosis are very limited,[38,39] and significant side effects and hepatic toxicity have been reported in healthy volunteers.[40] Therefore many clinicians in developing countries defer second-line therapy until the rifampicin-containing phase of tuberculosis treatment is complete. This delay may explain the significantly higher treatment failure rate (51%) in patients with tuberculosis co-infection in our cohort.

Our study has limitations. This is a single-centre study; therefore the criticism is that the patients may not be representative of the entire adult population on second-line therapy in Vietnam. However the HTD is the largest centre for HIV in Vietnam and is the primary provider of second-line therapy for patients in southern Vietnam. Half of the patients in this cohort come from the 17 southern provinces of Vietnam, representing a wide selection of patients. Further this study is the largest second-line therapy cohort in Southeast Asia, allowing for robust analyses of clinical outcomes. Another limitation is that clinical data are not prospectively collected. However all study variables were consistently assessed in a standardized way, in accordance with the national guidelines. We expect the quality of data is approaching that of a prospective study. Lastly the attrition rate was high (16%); however this is largely due to transfer of care (52 patients). Only one patient was lost to follow up.

In summary this is the first study to report the outcomes of second-line ART with a LPVr-based regimen in Vietnam. The overall treatment failure rate using immunological and clinical criteria is 18.4% after a median follow up of 29 months. Early AIDS-associated death is the main result of treatment failure and is predicted by older age, history of IDU, lower CD4 count at therapy switch, and medication adherence levels <95%. In the absence of routine virological monitoring,

interventions to prioritize timing of second-line ART based on CD4 counts and to support medication adherence will improve the treatment outcomes of patients on second-line ART in Vietnam.

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AUTHOR CONTRIBUTIONS

Study concept and design: Le, Quang, Shikuma, Dunstan, and Farrar. Obtaining funding: Le, Dunstan, Day, and Farrar. Acquisition of data: Thao, Quang, Vinh Chau, and Le. Analysis and interpretation of the data: Wolbers, Thao, Duc Anh and Le. Drafting the manuscript: Thao, Wolbers and Le. Critical revision of the manuscript for important intellectual contents: Quang, Duc Anh, Shikuma, Farrar, Dunstan, Day, Vinh Chau and Thwaites. All authors contributed to and approved the final manuscript.

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Figure 1: Flowchart of study population and outcome events

Figure 2: The cummulative incidence of treatment failure on second-line antiretroviral therapy over time

(a) Treatment failure in all patients in the cohort. The dotted lines represent the point-wise 95% confidence interval (b) Treatment failure in patients with and without history of injection drug use, (c) Treatment failure in patients with sub-optimal and optimal antiretroviral adherence, and (d) Treatment failure in patients with previous and no protease inhibitor exposure

TITLE PAGE

Title

Second-Line HIV Therapy Outcomes and Determinants of Mortality at the Largest HIV Referral Centre in Southern Vietnam

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Abbreviation

3TC = lamivudine, ABC = abacavir, ART = Antiretroviral therapy, AZT = zidovudine, D4T = stavudine, ddI = didanosine, HCMC = Ho Chi Minh City, HTD = Hospital for Tropical Diseases, IDU = injection drug use, IDV = indinavir, LPVr = Ritonavir-boosted lopinavir, NFV = nelfinavir, NNRTI = non-nucleotide reverse transcriptase inhibitors, NRTI = nucleotide reverse transcriptase inhibitor, NVP = nevirapine, PI = protease inhibitor, TAM = thymidine analog mutation, TDF = tenofovir, VAS = Visual Analogue Scale, WHO = World Health Organization

Running head

Meeting

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Conflict of interest

All authors declare no conflicts of interest.

ABSTRACT

Background:

The growing numbers of HIV-infected patients requiring second-line antiretroviral therapy (ART) in Vietnam makes essential the evaluation of treatment efficacy to guide treatment strategies.

Methods:

We evaluated all patients aged ≥15 years who initiated second-line ART after documented failure of first-line therapy at the Hospital for Tropical Diseases in Ho Chi Minh City. The primary outcome was time from second-line ART initiation to death, or to a new or re-occurrence of a WHO-defined immunological or clinical failure event, whichever occurred first. Risks of treatment failure and death were evaluated using Cox proportional hazards modeling.

Results:

Data from 326 of 373 patients initiating second-line ART between November 2006 and August 2011 were included in this analysis. The median age was 32 years (IQR: 28-36). 81% were men. The median CD4 count was 44 cells/μL (IQR: 16-84). During a median follow-up of 29 months (IQR: 15-44), 60 (18.4%) patients experienced treatment failure, including 12 immunological failures, 4 WHO stage IV AIDS events, and 44 deaths (13.5%). 60% of deaths occurred during the first 6-12 months. The Kaplan-Meier estimates of treatment failure after 1, 2, 3, and 4 years were 13.1% (95%CI: 9.2-16.8), 18.6% (95%CI: 14.0-23.1), 20.4% (95%CI: 15.4-25.1), and 22.8% (95%CI: 17.2-28.1), respectively. Older age, history of injection drug use, lower CD4 count, medication adherence <95%, and previous protease inhibitor use independently predicted treatment failure.

Conclusions:

While treatment efficacy was similar to that reported from other resource-limited settings; mortality was higher. Deaths may be averted by prioritizing second-line therapy based on CD4 counts and through improving treatment adherence support.

INTRODUCTION

The availability of low-cost fixed-dose combination antiretroviral drugs has enabled rapid scale-up of antiretroviral therapy (ART), resulting in substantial reduction in morbidity and mortality due to HIV in resource limited countries.[1–3] The World Health Organization (WHO) estimates that 16.8 million adults and children in low and middle-income countries will be on ART in 2016; among them 5% will be on second-line therapy.[4] This represents a more than 50% increase in ART coverage over the past five years. Despite generic production for resource-limited countries, a second-line regimen containing ritonavir-boosted lopinavir (LPVr) costs six times that of a first-line regimen.[5] In most low and middle-income countries second-line therapy is the last option for patients failing treatment with drug resistance. As third-line therapy is forbiddingly expensive and is unavailable in resource-limited countries, it is imperative for national programmes in these settings to maximize the efficacy and durability of second-line therapy.

Vietnam is among the countries with the highest HIV burden in Asia with an estimate of 280,000 people living with HIV.[6] Nearly 90,000 people were on ART as of 2014, and an estimated 3% were on second-line therapy.[6] The HIV system in Vietnam is undergoing a critical transition from an international-donor to a national-funding approach that integrates with the national health insurance programme.[7] Outcome data on second-line therapy in Vietnam are lacking, but are important for the national programme to devise treatment strategies and to forecast treatment options beyond second-line therapy. In this study we investigate second-line therapy outcomes and factors that determine therapy failure and death at the largest HIV referral centre in southern Vietnam.

METHODS

Study design and setting

This was a retrospective analysis of adult patients who switched to second-line therapy in a cohort of over 4000 patients on the national ART programme at the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City (HCMC). This is the largest primary and referral centre for HIV care in southern Vietnam (population around 45 million). The national ART programme began providing free antiretroviral drugs through international funding support in 2003. First-line therapy consisted of zidovudine (AZT) or stavudine (d4T) in combination with lamivudine (3TC) and nevirapine (NVP). Prior to the availability of efavirenz in 2006, cases of NVP-related toxicity were switched to indinavir (IDV). Second-line therapy became available in 2006 initially including abacavir (ABC), didanosine (ddI), and nelfinavir (NFV). In 2007 LPVr replaced NFV, and in 2009 tenofovir (TDF) and 3TC replaced ABC and ddI as the nucleotide reverse transcriptase inhibitor (NRTI) backbone.[8]

ART monitoring

ART was monitored using immunological and clinical failure criteria based on the WHO's guidelines for settings without routine viral load monitoring.[9,10] Patients were required to come to the clinic monthly for clinical evaluation and medication pick up. CD4 count was measured every 6 months. HIV viral load was tested at the time patients were diagnosed with immunological or clinical failure and was confirmed with repeat testing. HIV viral load was performed using a generic real-time PCR assay (Biocentric, Bandol, France) with a limit of detection of 250 copies/mL.[11] HIV genotyping was performed to evaluate for drug resistance prior to therapy switch using a published in-house assay[12] on the Beckman Coulter CEQ 8000 platform. Both HIV viral load and genotyping tests were performed at the Pasteur Institute, a WHO accredited HIV reference laboratory, in HCMC.

Study population

We included all HIV-infected patients aged ≥15 years who initiated second-line therapy due to documented immunological and/or clinical failure of first-line therapy. Patients who were alive and well but had been on second-line therapy for <6 months, and those without documented treatment failure to first-line therapy, were excluded. The study was approved by the Scientific and Ethical Committee of the HTD.

Outcome measurements

The primary outcome was treatment failure and was defined as time from second-line ART initiation to death, or to a new or re-occurrence of an immunological or a clinical failure event, whichever occurred first. Immunological failure was defined by the WHO as a decrease of CD4 count to or lower than baseline, a decrease of >50% of peak CD4 value while on treatment, or a persistent CD4 count of <100 cells/μL after at least 6 months of continued ART. Clinical failure was defined as new occurrence or re-occurrence of a WHO stage IV disease.[10] The secondary outcome was time to death.

Data collection

Routinely collected clinical and laboratory data were recorded on a standardized form and included demographic information, history of injection drug use (IDU), antiretroviral drug timeline, WHO stage 4 AIDS events, 6-month serial CD4 counts, HIV viral load and HIV genotype when these were available, ART adherence evaluation, deaths, and causes of death.

Antiretroviral therapy adherence evaluation

ART adherence counseling was provided to patients pre- and post-second-line ART initiation according to standard of care. Adherence was routinely assessed by the clinicians according to the MoH guidelines and was recorded either as an estimated percentage of pills taken, or as a qualitative assessment of 'good', 'average', or 'poor', corresponding to ≥95%, 80-94%, or <80% adherence, respectively.[13] Additionally in patients who were in active follow up, adherence was prospectively evaluated using a simple self-reported Visual Analogue Scale (VAS).[14] This VAS has been shown to be as reliable as other methods such as pill-count and 3-day recall self-report, yet much simpler to administer.[15]

For analysis, sub-optimal adherence was defined as having at least one adherence score of <95% by pill count, by the VAS, and/or receiving at least one qualitative adherence assessment of 'average' or 'poor' over a 6-month period preceding an outcome event or preceding the time of study assessment in patients who had not had an event.

Statistical analysis

The cumulative incidence of treatment failure and failure rates after 1, 2, 3, and 4 years and corresponding 95% confidence intervals were calculated using the Kaplan-Meier method. The

Cox proportional hazards model was used to analyse the time to treatment failure (composite primary endpoint) and the time to death (secondary endpoint). Patients who were transferred to other provincial or district clinics while on second-line therapy had been judged by doctors to be clinically and immunologically stable before the transfer. For the analysis, event-free transferred patients were censored at the time of transfer (primary analysis). Alternatively, assuming the transferred patients were doing well clinically and immunologically on therapy, we treated them as censored at the time-point where their last monthly follow-up visit would have been had they not been transferred (sensitivity analysis to assess potential informative censoring).

The following pre-defined covariates were included in the model: age at second-line therapy initiation, history of IDU (yes/no), CD4 cell count, and (log10-transformed) HIV RNA viral load at second-line therapy initiation, second-line therapy delay (defined as time from first detection of immunological or clinical failure to time of second-line therapy initiation), history of protease inhibitor (PI) use, and an overall measure of therapy adherence (<95% vs. ≥95%). The chosen covariates have been shown to be associated with poor ART outcome.[16–21] The proportional hazards assumption was assessed by examining plots of weighted Schoenfeld residuals and by formal testing. There was strong evidence of non-proportional hazards for the effect of CD4 cell count at second-line therapy initiation on treatment failure (p=0.001 univariate analysis, p=0.0002 multivariable analysis). To account for this, we decided to model a time-varying effect on the hazard of treatment failure with separate effects for the first year of follow-up and subsequently. There was no clear evidence for non-proportional hazards between any other covariates and the primary or secondary endpoint (all univariate p>0.13). Both univariate and multivariable Cox regressions were performed.

Data were analysed based on multiple imputations of missing data and on a complete-case analysis. To avoid bias, the imputation algorithm included the endpoints [event time T and Nelson-Aalen estimator H(T)].[22] All reported confidence intervals are two-sided 95% intervals and analyses were performed with the statistical software R version 2.15.0,[23] and the companion R package mice version 1.2.5 (for multiple imputation).[24]

RESULTS

Study population and baseline characteristics

A total of 373 patients aged ≥15 years initiated second-line therapy between November 2006 and August 2011. 47 patients were excluded from the analysis, including 43 who had received second-line therapy for <6 months and 4 who had switched to second-line therapy because of treatment intolerance. The remaining 326 patients had documented treatment failure to first-line therapy (with confirmation of virological failure in 94%) and were included in this study. Approximately 50% of patients came from HCMC; the rest from the remaining 17 southern provinces of Vietnam. The median duration of first-line ART treatment was 33 months (IQR: 21-44). The characteristics of the 326 patients at the time of initiation of second-line therapy are summarized in Table 1. The median CD4 count was 44 cells/μL (IQR: 16-84) and the median HIV RNA was 5.1 log copies/mL (IQR: 4.6-5.6). The median time of second-line therapy delay was 9 months (IQR: 5-15).

Drug resistance patterns in patients failing first-line ART

HIV genotyping was performed for 246/326 (75.5%) patients who failed first-line therapy. Mutations conferring high-level resistance to NRTIs were detected in 238/246 patients (96.7%), to non-nucleotide reverse transcriptase inhibitors (NNRTIs) in 229/246 (93.1%), and to PIs in 6/246 (2.4%). Resistance mutations to both NRTIs and NNRTIs were present in 226/246 patients (91.9%) and to all three drug classes in 5/246 patients (2.0%). The most common NRTI mutations were M184I/V (85.4%), thymidine analog mutations (TAMs) M41L, D67N, K70E/R, T215F/Y, and K219E/Q (30-55%), Q151M (21.1%), and K65R (14.6%). Two patients had a T69 insertion mutation. The most common NNRTI mutations were Y181C/I/V (45.5%), G190A/S (41.9%) and K103N (31.3%). The most common PI mutations were I54V (2.4%), M46I/L (2.8%), V82A (2.0%), and L90M (1.2%).

Predicted resistance to second-line ART regimen

The predicted susceptibility to the national second-line regimen containing TDF, 3TC, and LPVr were evaluated for the 246 patients who had genotype results using the Stanford HIV Drug Resistance Database (access date: 09 April 2015). Intermediate to high-level resistance to TDF was present in 161/246 (65.4%), to 3TC in 230/246 (93.5%), and to LPVr in 5/246 (2.0%).

Second-line ART outcome

320 patients (98.2%) received LPVr in combination with 2 NRTIs and 6 patients received NFV with 2 NRTIs (Table 1). 121 patients (37.1%) also received AZT; this was chosen by clinicians who believed that an AZT-containing regimen might reduce the likelihood of developing the TDF-signature-resistance-mutation K65R, and thereby preserve the potency of the second-line regimen. Sub-optimal adherence was observed in 43 patients (13.2%). During a median follow-up of 29 months (IQR: 15-44), 52 patients (16.0%) were transferred to other clinics as part of the government's efforts to decentralize HIV care; 1 was imprisoned and was lost to follow-up, 44 (13.5%) died and the remaining 229 (70.2%) were in active follow-up. 60 (18.4%) patients experienced treatment failure, including 12 immunological failures, 4 WHO stage IV AIDS events, 39 AIDS-related deaths, and 5 non-AIDS deaths (Figure 1). The cumulative incidence of treatment failure and corresponding 95% confidence interval are shown in Figure 2a. The Kaplan-Meier estimates of the risk of treatment failure by 1, 2, 3, and 4 years were 13.1% (95%CI: 9.2-16.8), 18.6% (95%CI: 14.0-23.1), 20.4% (95%CI: 15.4-25.1), and 22.8% (95%CI: 17.2-28.1) respectively. The median CD4 counts after 1, 2, 3, and 4 years were 234 cells/μL (IQR: 166-338), 353 cells/μL (IQR: 227-465), 393 cells/μL (IQR: 255-514), 473 cells/μL (IQR: 347-574), respectively.

Predictors of second-line ART failure

The 7 covariates entered in the Cox model are listed in Table 2. The most frequently missing covariates were history of PI use (10% missing), viral load (6% missing), and IDU history (5% missing); other covariates were missing in ≤2% of patients. The results of the univariate and multivariate Cox regression analyses are shown in Table 2. Lower CD4 count and sub-optimal adherence predicted treatment failure in both univariate and multivariate analyses. However, lower CD4 count affected the rate of treatment failure only during the first year of second-line therapy and not thereafter. Older age, history of IDU, and history of PI use did not predict treatment failure in the univariate analysis, but in the multivariate analysis they became statistically significant predictors of treatment failure. Multivariate analysis shown in Table 2 was based on multiple imputations of missing data; however a complete-case analysis gave highly consistent results (data not shown). A sensitivity analysis with informative censoring of the 52 transferred patients was performed (i.e. assuming the transferred patients continued to do well clinically and immunologically on therapy, with censoring occurring at the time-point where their last monthly follow-up visit would have been had they not been transferred), and the

results were also consistent (data not shown). The cumulative incidence of treatment failure among patients according to IDU, treatment adherence, and PI is shown in Figures 2b, 2c, and 2d, respectively.

We performed an exploratory univariate analysis of the impact of adding AZT as a 4th drug to second-line regimen using Cox proportional hazard modeling, and a statistically significant effect on treatment outcome was not observed (HR: 1.12, 95% CI: 0.50-1.62, p=0.715).

Causes and predictors of death

Deaths occurred in 44 patients (13.5%) and accounted for 73.3% of failure events; 39 were AIDS-related deaths, and 5 were unknown or non-AIDS related deaths. The median time to death was 9 months (IQR: 3-22), with 26 deaths (59.1%) occurring within the first 6 to 12 months. The causes of AIDS-related deaths included microbiologically-confirmed tuberculosis (13, 29.5%), *Pneumocystis jiroveci* pneumonia (4, 9.1%), candida esophagitis (4, 9.1%), cryptococcal meningitis (2), *Penicillium marneffei* infection (2), herpes simplex (2), cytomegalovirus (2), toxoplasmosis (2), hepatitis C related liver failure (2), renal failure of unclear etiology (2), non-typhoid salmonella sepsis (1), and AIDS-associated wasting (8, 18.2%). Univariate and multivariate Cox regression analyses were performed to determine predictors of death. Consistent with the results of the primary outcome analysis, lower CD4 count (multivariate HR: 1.75, 95% CI: 1.11-2.70, p=0.015) and sub-optimal adherence (multivariate HR: 3.41, 95%CI: 1.59-7.30, p=0.002) predicted death in both univariate and multivariate analyses. Age and history of IDU did not predict death in the univariate analysis, but in the multivariate analysis older age and history of IDU became significant predictors of death (HR: 1.95, 95% CI: 1.22-3.11, p=0.005 and HR: 2.30, 95% CI: 1.05-5.05, p=0.037, respectively).

DISCUSSION

To our knowledge this is the first study to systematically evaluate the outcomes of second-line ART in patients who fail first-line therapy in Vietnam according to the WHO's immunological and clinical criteria. Despite the profound level of immune deficiency in this patient cohort (median CD4 count: 44 cells/μL), high viral replication (median HIV RNA: 5.1 log copies/mL), and extensive resistance to second-line NRTI backbone (93.5% resistance to 3TC, 65.4% resistance to TDF) at the time of treatment switch, treatment failure rates are similar to studies in comparable settings.[16,18,19,21,25] A 27-cohort study comprising 632 patients from Africa and Asia reported a failure rate of 28% over two years.[16] Treatment failure in that study was defined as the first diagnosis of clinical, immunological or virological failure, or death. Only 4 of these 27 centres had routine viral load monitoring. These failure rates reflect the reality of HIV care in resource-limited settings. Our treatment failure rates are lower when compared to studies that use virological failure as the measure of outcomes. A meta-analysis of 2035 patients from 19 cohorts across low-income and middle-income countries reported failure rates of 22-38% after 6-36 months on second-line ART.[25] Although an outcome measure relying on CD4 count and clinical evaluation can underestimate virological failure rates, the long-term clinical and health economic benefits of routine viral load monitoring still need to be determined in resource-poor settings.

While the overall treatment failure rates were similar to equivalent settings, the mortality in our cohort was higher: 13.5% versus an estimate of 5% in the 27-cohort study from Africa and Asia.[16] The median time to death was shorter, 9 months (IQR: 3-22) versus 15 months (IQR: 12-26).[16] Death accounted for 73% of failure events, and 90% of deaths were due to AIDS-related infections. Using multivariate analysis, death during the first year was predicted by lower CD4 counts at second-line ART initiation. The median CD4 count and HIV viral load at second-line ART initiation were 44 cells/μL and 5.1 log copies/mL, respectively. This compares with median CD4 counts >100 cells/μL and HIV viral load ranging from 3.9 to 4.8 log copies/mL in other African and Asian cohorts.[17,19–21,26,27] The lower CD4 count likely explains the higher mortality observed in our patients and suggests second-line therapy delay plays a role. When comparing to studies from other resource limited countries, therapy delay in our cohort was longer, median 9 (range: 5-15) versus 5 (range: 1-8) months.[17,18,20,26]

Therapy delay can be explained by programmatic reasons not unique to Vietnam. Without viral load monitoring, it can take months to years for a patient who fails treatment virologically to

manifest failure immunologically and clinically. The process of defining treatment failure in Vietnam may be too conservative. Immunological failure is usually confirmed with a repeat CD4 measure in 3-6 months. Cases of confirmed immunological and/or clinical failure are referred to a panel of experts located in centres that have access to viral load and drug resistance testing. The referral process, albeit necessary in some circumstances, further delays the onset of therapy switch. When the relationship of treatment delay and CD4 count were assessed with respect to therapy failure and death in our study, only lower CD4 count predicted therapy failure and death. The likely explanation for this is that these two variables are interdependent or have a causal relationship, i.e. treatment delay directly results in a decline in CD4 count. In our cohort the CD4 count is likely the stronger variable that drives the outcomes. When these same variables were evaluated by Levison *et al.* in a study using virological failure as outcome, they found that therapy delay and not CD4 count predicted virological failure, and for every month a patient remained on a failing first-line ART regimen there was a 7% increase in risk of lack of virological suppression.[20] The longer a non-suppressive ART regimen is given, the higher the chance of developing accumulation of drug resistance mutations, impairing the efficacy of current as well as future ART options.

In our cohort older age, history of IDU, PI exposure, lower CD4 count, and suboptimal adherence were independent predictors of treatment failure in both complete-case and multiple imputation analyses. With the exception of PI exposure, these variables remained independent predictors of death. These findings are consistent with published literature on first-line ART failure worldwide.[28–34] Older age at ART initiation has been linked to poor immunological recovery, loss to follow up and death in ART cohorts in Zambia and South Africa.[31,29] CD4 count and treatment adherence are established risk factors for first-line ART failure and are also found to be independent predictors of second-line ART failure in the analysis of 27 ART programmes across Africa and Asia.[16] PI exposure has been associated with risks of virological failure in Cambodia and India.[35,36] Treatment adherence in our study was assessed at multiple time points, suggesting that sub-optimal adherence at anytime during therapy predicts treatment failure. This finding is similar to other second-line cohorts from South Africa, Malawi and Thailand,[17,19,21] highlighting the importance of ongoing patient education and adherence support in improving treatment outcomes. The history of IDU predicted treatment failure independently from treatment adherence. This effect is likely multi-factorial and likely involves cofactors that

are not measured in this study including nutritional status, social economic status, and hepatitis B and/or C co-infection.

Co-infection with tuberculosis in patients failing ART is common in countries where tuberculosis is highly endemic. In our study, microbiologically-confirmed tuberculosis accounted for the majority of AIDS-related deaths (30%). HIV and tuberculosis co-infection is a common reason for clinicians to delay second-line ART initiation because of the rifampicin and PI drug-drug interactions via CYP450 metabolism pathway. The WHO recommends rifabutin in place of rifampicin for patients who need tuberculosis treatment while on PI therapy. Unfortunately rifabutin is not yet available in Vietnam. A super-boosted dose of ritonavir (400mg twice daily) is therefore recommended.[37] The safety data of this super-boosted ritonavir regimen in HIV-associated tuberculosis are very limited,[38,39] and significant side effects and hepatic toxicity have been reported in healthy volunteers.[40] Therefore many clinicians in developing countries defer second-line therapy until the rifampicin-containing phase of tuberculosis treatment is complete. This delay may explain the significantly higher treatment failure rate (51%) in patients with tuberculosis co-infection in our cohort.

Our study has limitations. This is a single-centre study; therefore the criticism is that the patients may not be representative of the entire adult population on second-line therapy in Vietnam. However the HTD is the largest centre for HIV in Vietnam and is the primary provider of second-line therapy for patients in southern Vietnam. Half of the patients in this cohort come from the 17 southern provinces of Vietnam, representing a wide selection of patients. Further this study is the largest second-line therapy cohort in Southeast Asia, allowing for robust analyses of clinical outcomes. Another limitation is that clinical data are not prospectively collected. However all study variables were consistently assessed in a standardized way, in accordance with the national guidelines. We expect the quality of data is approaching that of a prospective study. Lastly the attrition rate was high (16%); however this is largely due to transfer of care (52 patients). Only one patient was lost to follow up.

In summary this is the first study to report the outcomes of second-line ART with a LPVr-based regimen in Vietnam. The overall treatment failure rate using immunological and clinical criteria is 18.4% after a median follow up of 29 months. Early AIDS-associated death is the main result of treatment failure and is predicted by older age, history of IDU, lower CD4 count at therapy switch, and medication adherence levels <95%. In the absence of routine virological monitoring,

interventions to prioritize timing of second-line ART based on CD4 counts and to support medication adherence will improve the treatment outcomes of patients on second-line ART in Vietnam.

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AUTHOR CONTRIBUTIONS

Study concept and design: Le, Quang, Shikuma, Dunstan, and Farrar. Obtaining funding: Le, Dunstan, Day, and Farrar. Acquisition of data: Thao, Quang, Vinh Chau, and Le. Analysis and interpretation of the data: Wolbers, Thao, Duc Anh and Le. Drafting the manuscript: Thao, Wolbers and Le. Critical revision of the manuscript for important intellectual contents: Quang, Duc Anh, Shikuma, Farrar, Dunstan, Day, Vinh Chau and Thwaites. All authors contributed to and approved the final manuscript.

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Figure 1: Flowchart of study population and outcome events

Figure 2: The cummulative incidence of treatment failure on second-line antiretroviral therapy over time

(a) Treatment failure in all patients in the cohort. The dotted lines represent the point-wise 95% confidence interval (b) Treatment failure in patients with and without history of injection drug use, (c) Treatment failure in patients with sub-optimal and optimal antiretroviral adherence, and (d) Treatment failure in patients with previous and no protease inhibitor exposure

Table 1: The characteristics of 326 patients starting second-line ART at the Hospital for Tropical Diseases in Ho Chi Minh City

Characteristics	**N=326**
Sex, male (%)	268 (82%)
Age, median (IQR) (years)	32 (28-36)
Previous history of IDU (%)[(n=309)]	136 (44%)
CD4 count (cells/µL) before 1st line ART initiation, median (IQR)[(n=303)]	39 (12-92)
1st line ART regimens (%)	
d4T/3TC/NVP	116 (35.6%)
d4T/3TC/EFV	102 (31.3%)
AZT/3TC/NVP	43 (13.2%)
AZT/3TC/EFV	41 (12.6%)
Others	24 (7.4%)
Time of 2nd line therapy delay ART[a], median (IQR) (months)[(n=320)]	9 (5-15)
CD4 count (cells/µL) before 2nd line ART initiation, median (IQR)[(n=323)]	44 (16-84)
HIV RNA (log copies/mL) before 2nd line ART initiation, median (IQR)[(n=305)]	5.1 (4.6-5.6)
2nd line regimens (%)	
LPVr + 3TC + TDF	180 (55.3%)
LPVr + 3TC + TDF + AZT	121 (37.1%)
LPVr + 3TC + other NRTIs (ddI/d4T/ABC)	19 (5.8%)
NFV + 2 NRTIs	6 (1.8%)
Adherence[b]	
≥95%	286 (88%)
<95%	40 (12%)

[a,] Time from first detection of failure to first-line to second-line ART initiation; [b,] Number indicates an estimated proportion of pills taken in the preceding 6 months; d4T, stavudine; 3TC, lamivudine; NVP, nevirapine; EFV, efavirenz; AZT, zidovudine; TDF, tenofovir; LPVr, ritonavir boosted lopinavir; ddI, didanosine; ABC, abacavir; NFV, nelfinavir; IQR, interquartile range

Table 2: The impact of covariates on treatment failure

Covariate	Univariate effect [b]		Multivariate effect [c]	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Age [a] (by +10 years)	1.37 (0.98-1.92)	0.066	2.13 (1.40-3.24)	**<0.001**
IDU (yes)	1.68 (0.97- 2.90)	0.063	3.15 (1.57-6.35)	**0.001**
CD4 [a] (by +50 cells/µL)				
- effect in first year	0.54 (0.34- 0.84)	**0.007**	0.52 (0.33-0.84)	**0.007**
- effect subsequently	1.00 (0.68- 1.46)	0.989	0.97 (0.65-1.44)	0.873
log10VL [a] (by + log 10 copies/mL)	1.15 (0.77- 1.72)	0.502	1.10 (0.70-1.72)	0.683
Time on a failing 1st line therapy (by +6 months)	1.10 (0.93- 1.29)	0.277	1.05 (0.89-1.25)	0.546
Adherence < 95% (yes)	2.15 (1.14- 4.05)	**0.018**	2.76 (1.41-5.40)	**0.003**
PI exposure (yes)	0.71 (0.38- 1.34)	0.293	2.12 (1.05-4.28)	**0.035**

HR= hazard ratio from Cox regression analysis, CI= confidence interval, [a] = at time of second-line ART initiation; [b] = complete case analysis; [c] = analysis based on multiple imputations of missing covariates

OA_Figure 1
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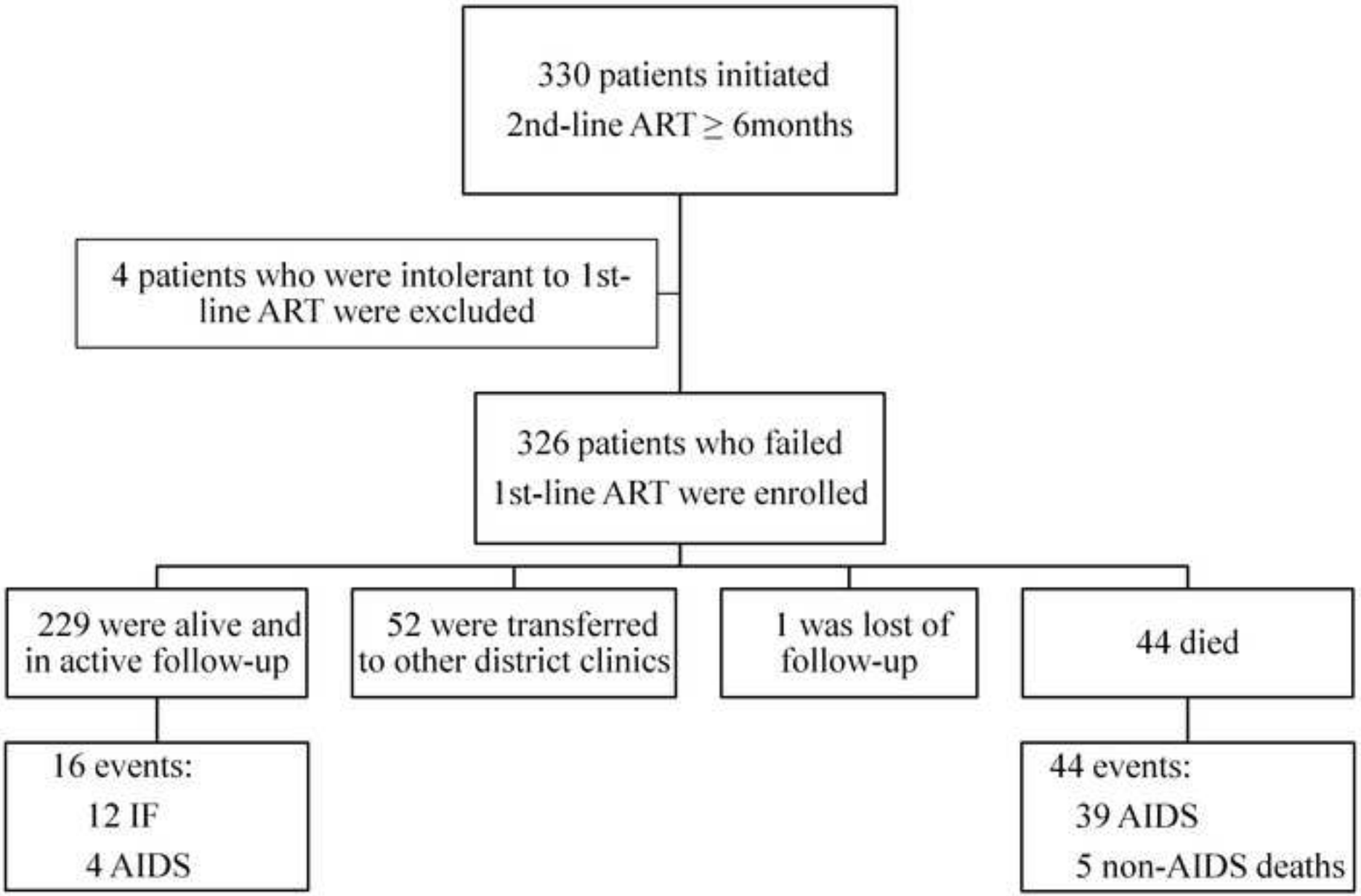

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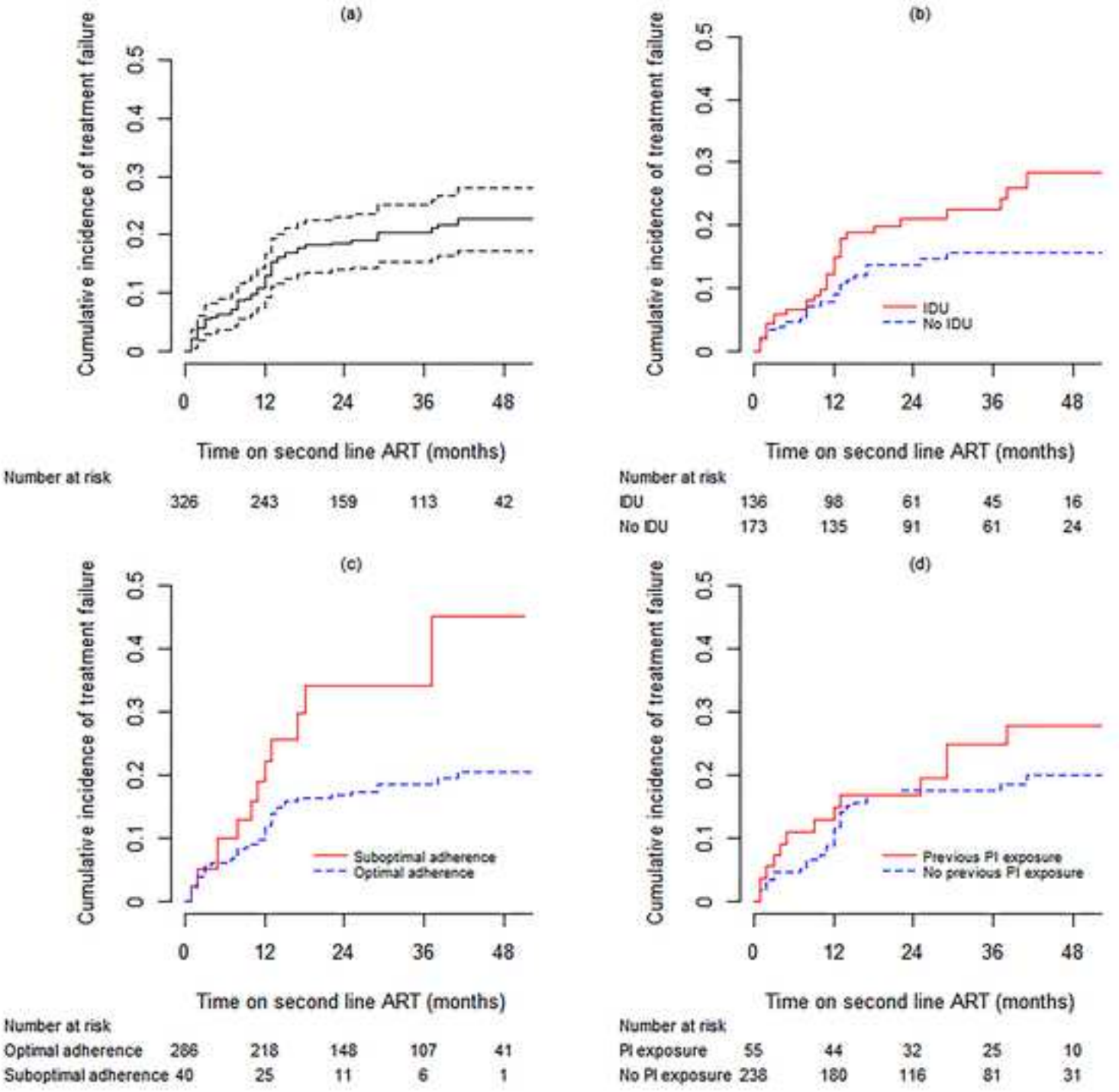

STROBE Statement

Checklist of items that should be included in reports of observational studies

Section/Topic	Item No	Recommendation	Reported on Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	3
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5-6
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5-6
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6-7
Data sources/measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5-6
Bias	9	Describe any efforts to address potential sources of bias	6-7
Study size	10	Explain how the study size was arrived at	5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6-7
		(*b*) Describe any methods used to examine subgroups and interactions	6-7
		(*c*) Explain how missing data were addressed	6-7
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	6-7
		(*e*) Describe any sensitivity analyses	6-7

Section/Topic	Item No	Recommendation	Reported on Page No
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	8
		(b) Give reasons for non-participation at each stage	8
		(c) Consider use of a flow diagram	Figure 1
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	8
		(b) Indicate number of participants with missing data for each variable of interest	Table 1
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	9
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	9
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	9-10
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	9-10
Discussion			
Key results	18	Summarise key results with reference to study objectives	11
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	13
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	11-13
Generalisability	21	Discuss the generalisability (external validity) of the study results	13
Other Information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	2

**Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.*

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Keywords:	HIV, Antiretroviral resistance mutations, second-line antiretroviral therapy, third-line antiretroviral therapy, Vietnam

TITLE PAGE

Title:

High prevalence of protease inhibitor resistance in patients failing second-line antiretroviral therapy in Vietnam

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Running head:

Antiretroviral resistance on 2nd-line ART

Key words:

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ABSTRACT:

Objectives:

There are limited data from resource-limited settings on antiretroviral resistance mutations that develop in patients failing second-line protease-inhibitor ART.

Methods:

We performed a cross-sectional virological assessment of adults on second-line ART for ≥6 months between November 2006 and December 2011, followed by a prospective follow-up over two years of patients with virological failure (VF) at the Hospital for Tropical Diseases, Vietnam. VF was defined as HIV RNA levels ≥1000copies/mL. Resistance mutations were identified by population sequencing of the *pol* gene and interpreted using the 2014 IAS-USA mutation list and the Stanford algorithm. Logistic regression modeling was performed to identify predictors of VF.

Results:

231 patients were enrolled in the study. The median age was 32 years, 81.0% were male. 95.7% were on a lopinavir-ritonavir containing regimen. 22 (9.5%) patients had VF. Of these, 14 (64%) carried at least 1 major protease mutation [median:2(IQR:1-3)]; 13 (59%) had multiple protease mutations conferring intermediate- to high-level resistance to lopinavir-ritonavir. Mutations conferring cross-resistance to etravirine, rilpivirine, tipranavir and darunavir were identified in 55%, 55%, 45%, and 27% patients, respectively. Higher viral load, adherence <95%, and previous indinavir use were independent predictors of VF. The two-year outcomes of the patients maintaining on

lopinavir-ritonavir included death, 7 (35%); worsening virological/immunological control, 6 (30%); and virological re-suppression, 5 (25%). Two patients were switched to raltegravir and darunavir-ritonavir with good HIV control.

Conclusions:

High-prevalence PI resistance was associated with previous indinavir exposure. Darunavir plus an integrase inhibitor and lamivudine might be a promising third-line regimen in Vietnam.

Introduction

The WHO endorses ritonavir-boosted protease-inhibitor (PIr) based ART as efficacious second-line treatment after failure of NNRTI based first-line therapy in resource-limited settings.[1] PIr-based therapy is highly potent in ART-naïve patients participating in clinical trials[2–4] and has high efficacy as second-line therapy in resource-limited settings.[5,6] Nevertheless, up to 20% of patients in resource-rich and 27% of patients in resource-limited settings develop virological failure (VF) on PIr-based ART.[4,6,7] PI resistance is rarely observed in patients failing PIr-based therapy in clinical trials[3,4,8,9] and, similarly, is uncommon (range 0-7%) in PI-naïve patients failing second-line therapy in Sub-Saharan Africa.[10–14] However, studies from Cambodia[15] and India[16] have reported PI resistance mutation prevalences of 40% and 70% in patients failing second-line ART, respectively. There are few data regarding the prevalence of and risk factors for PI resistance developed on second-line ART in Asia. Significant uncertainty exists regarding the risk factors for PI resistance in programmatic settings, the contribution of HIV-1 subtypes on mutation development, and the clinical outcomes in patients with PI resistance on long-term second-line ART. HIV-1 subtype CRF01_AE accounts for 99% of HIV infections in Vietnam,[17–21] which is amongst the Asian countries with the highest numbers of HIV infections.[22,23] 3% of the 90,000 people on ART are on second-line therapy.[23] Due to its costs HIV viral load monitoring is not performed routinely. Therefore, data on virological outcome and drug resistance in patients on second-line therapy are lacking. To this end, we aimed to generate data on antiretroviral resistance profiles of HIV-1 CRF01_AE-infected patients with viremia on second-line PI therapy at the largest HIV treatment centre in Vietnam. Our objectives were i) to identify the risk

84 factors for resistance development, ii) to describe the long-term clinical outcomes of
85 patients with resistance maintaining on a failing second-line regimen, and iii) to
86 investigate cross-resistance to second-generation NNRTIs and PIs to inform national
87 policy on third-line therapy.

88

Method

Study setting and design

The study was conducted at the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City (HCMC). The HTD is the largest centre for HIV care in southern Vietnam, providing ART for more than 5,000 patients according to the national ART programme. Until the de-centralisation of care in 2011-2012 the HTD had been the primary provider of second-line ART for patients living in the 17 southern provinces of Vietnam. First-line therapy is according to national and international guidelines and at the time of the study consisted of 2 NRTIs (lamivudine in combination with either zidovudine or stavudine) and one NNRTI - either nevirapine or efavirenz. Indinavir was generically and locally produced (STADA, Vietnam) during this time and was prescribed (without ritonavir boosting) in public and private settings for patients with treatment failure or intolerance on nevirapine before efavirenz became available in 2004.[24] In 2011 tenofovir replaced stavudine as a preferred NRTI backbone drug. Patients in the national programme were required to attend monthly appointments for clinical and adherence evaluation. CD4 cell count was performed 6 monthly. HIV viral load was performed to confirm treatment failure when the WHO's defined clinical and/or immunological failure criteria were met.[25,26] HIV genotyping was performed to diagnose antiretroviral resistance prior to therapy switch and reported to treating clinicians. Second-line therapy included nelfinavir prior to 2006 and lopinavir-ritonavir thereafter, in combination with tenofovir and/or zidovudine plus lamivudine.[27,28]

This study consisted of a cross-sectional survey of adult patients (aged ≥15 years) who had been on second-line ART for at least 6 months and were on active care to identify

those with VF and their drug resistance development, followed by a prospective follow-up over 2 years of patients with VF. VF was defined as at least 2 viral loads ≥1000 copies/mL measured 1-3 months apart after intensive adherence counseling. The patients who had been on second-line therapy for <6 months at the time of study assessment, who died and those who switched therapy due to drug intolerance were excluded. The study was conducted between December 2011 and June 2014.

Data collection

Clinical data were obtained both retro- and prospectively from patients' charts and from one-on-one interview including demographic information, HIV risk factors, ART history, CD4 counts, HIV viral load, genotyping results at the time of therapy switch (if available), AIDS events, and therapy adherence.

Antiretroviral therapy adherence evaluation

Adherence is routinely assessed by treating clinicians according to the national guidelines at all clinic visits and is recorded either as an estimated percentage of pills taken, or as a qualitative assessment of 'good', 'average', or 'poor', corresponding to ≥95%, 80-94%, or <80% adherence, respectively.[26] Additionally, for this study, adherence was evaluated over the 6 months preceding the time of study assessment using a simple self-reported Visual Analogue Scale (VAS).[29] For analysis, sub-optimal adherence was defined as having at least one adherence score of <95% by pill count, by the VAS, and/or receiving at least one qualitative adherence assessment of 'average' or 'poor' over the preceding 6 months prior.

HIV RNA measurement and antiretroviral resistance testing

5mL of EDTA blood was collected at the time of enrolment for viral load measurement using the Abbott Real Time HIV-1 assay (limit of detection of 150 copies/mL) (*m*2000, Abbott Molecular, IL, USA). Antiretroviral resistance testing was performed for patients with VF using an in-house population sequencing and sequence analysis protocol as previously described, with bidirectional coverage of the complete protease gene and reverse transcriptase codons 1-300.[18] The sequences were analysed using SeqScape (Applied Biosystems). Nucleotide changes were determined by comparison with the consensus sequence pNL4-3 for HIV-1 subtype B (GenBank accession number M19921). Antiretroviral resistance mutations were identified based on the 2014 International AIDS Society (IAS)-USA mutation list.[30] The antiretroviral resistance profile of each patient was predicted using the Stanford resistance interpretation algorithm (http://hivdb.stanford.edu). The Rega HIV-1 Subtyping Tool was used to determine the HIV-1 subtypes of each patient sample.[31]

Statistical analysis of predictors of virological failure

The following pre-defined covariates were included in the logistic regression model: CD4 cell count and (log10-transformed) HIV RNA viral load at therapy switch, history of indinavir use, second-line therapy delay (defined as time in months from first detection of failure to first-line ART to time of second-line therapy initiation), and an overall measure of therapy adherence (<95% versus ≥95%). The chosen covariates were either established risk factors for ART outcome[5,32–36] or were based on clinicians' observations (i.e., previous indinavir use). Both univariate and multivariable analyses were performed.

Follow up of patients with virological failure

The results of viral load and resistance testing were reported to the treating clinicians. Patients with VF then received intensive adherence counseling. As third-line therapy was not available through the national programme, these patients were continued on the current treatment according to national guidelines. The clinical and immunological outcomes of these patients over the following 24 months were evaluated. HIV RNA was re-tested at month 24, and repeat genotype testing was performed if HIV RNA were ≥1,000 copies/mL to evaluate the evolution of resistance mutations in these patients.

Ethics

The study was approved by the Scientific and Ethical Committee of the HTD. All patients gave written consent prior to study enrolment.

Results

Study population and characteristics

Figure 1 describes the study participants, virological outcome, and follow-up of the patients with VF maintained on the failing second-line regimen. Of 373 patients who started second-line ART between November 2006 and December 2011, 44 (11.8%) had died, and 51 (13.7%) had been transferred to other provincial clinics by the time of the study. Forty one (11.0%) patients who had been on second-line ART for <6 months were excluded. The remaining 231 patients were enrolled into the study. Table 1 shows the characteristics of the 231 patients. The median age was 32 years; 81% were men. The median CD4 cell count and HIV RNA at time of therapy switch were 44 cells/mm^3 and 5.1 log10 copies/mL, respectively. The median time on second-line ART was 29 months (IQR: 16-43). Nelfinavir was the starting PI in 10 (4.3%) patients but was replaced by lopinavir-ritonavir within 12 months for all patients. 36 (17.1%) patients had a history of indinavir use, frequently prescribed at 800mg twice daily or 400mg three times daily. Sub-optimal adherence was identified in 12.1% patients.

Antiretroviral resistance mutations detected in patients prior to second-line therapy switch

Due to cost constraint, HIV genotyping was only performed for 173/231 (74.9%) patients prior to therapy switch. Figure 2 shows the mutations and prevalences detected in these patients. Mutations conferring high-level resistance to NRTIs were detected in 168/173 (97.1%) patients, and to NNRTIs in 163/173 (94.2%). High-level resistance to PIs was detected in 4/173 (2.3%) patients. Resistance mutations to both NRTIs and NNRTIs were present in 161/173 (93.1%) patients and to all three drug classes in 6/173 (3.5%) patients.

The most common NRTI resistance mutations were M184I/V (86.1%), thymidine analogue mutations (TAMs) M41L, D67N, K70E/R, T215F/Y, and K219E/Q (33-57%), Q151M (22.5%), and K65R (16.2%). 142 (82.1%) patients harboured multiple thymidine analogue mutations and multiple NRTI resistance mutations (Q151M complex). Two patients had a T69 insertion mutation. The most common NNRTI resistance mutations were Y181C/I/V (48.6%), G190A/S (42.8%), and K103N (30.1%). At least 3 major NNRTI resistance mutations were present in 55/173 (31.8%) patients. Eight patients carried at least one major PI resistance mutation. The most common protease mutations were M46I/L (2.9%), L90M (1.7%), and V82A (1.2%).

Predicted resistance to second-line ART regimen

The predicted susceptibility to the national second-line regimen containing tenofovir, lamivudine, and lopinavir-ritonavir were evaluated for the 173 patients who had genotype results using the Stanford HIV Drug Resistance algorithm. Intermediate- to high-level resistance to tenofovir was present in 120/173 (69.4%), to lamivudine in 165/173 (95.4%) and to lopinavir-ritonavir in 2/173 (1.2%).

The number of patients predicted to receive one, two and three fully active drugs were 138/173 (79.8%), 25/173 (14.5%), and 4/173 (2.3%), respectively.

Virological outcome

The virological outcomes of the 231 patients were shown in figure 1. 22 (9.5%) patients had confirmed VF with a median HIV RNA of 4.75 log10 copies/mL (IQR: 3.92-5.01). 5/231 (2.2%) patients had HIV RNA levels between 400 and <1000 copies/mL; 4 (1.7%) patients had HIV RNA levels between 150 and 400 copies/mL, and the remaining 200 (86.6%) patients had undetectable viral loads.

HIV subtypes, antiretroviral resistance mutations and predicted susceptibility of the 22 patients with virological failure

21 (95%) patients were infected with HIV-1 subtype CRF01_AE; a single patient was infected with HIV-1 CRF01_AE/B recombinant. Table 2 shows the mutation profiles of the 22 patients prior to therapy switch and at VF. The majority of the NRTI and NNRTI resistance mutations detected prior to therapy switch remained detectable at therapy failure, with the NNRTI resistance mutations persisting up to 45 months off NNRTI therapy. Major PI resistance mutations developed in 14 (64%) patients; the median number of PI resistance mutations was 2 (IQR: 1-3). The most common PI resistance mutations were V82A/F (64%), M46I/L (57%), I84V (29%), and L76V (21%). Five patients had only one PI resistance mutation; the remaining 9 had multiple PI resistance mutations. Minor or accessory PI resistance mutations developed in 5 patients. Three patients did not have any PI resistance mutations.

Figure 3 shows the predicted resistance profiles of the 22 patients based on their individual genotype profiles. Mutations conferring intermediate- to high-level resistance against the second-line drugs tenofovir, lamivudine and lopinavir-ritonavir were detected in 13 (59%), 18 (82%), and 13 (59%) patients, respectively. Cross-resistance to the second-generation NNRTIs etravirine and rilpivirine were intermediate- to high-level and were both present in 12 (55%) patients. Cross-resistance to the second-generation PIs tipranavir and darunavir were present in 10 (45%) and 6 (27%) patients, respectively. Cross-resistance to darunavir was only at intermediate level.

Predictors of second-line virological outcome

Table 3 lists the data for the 5 covariates entered into the logistic regression model and the results of the univariate and multivariate analyses. The most frequently missing covariates were history of indinavir use (8.7%), viral load (6.9%); other covariates were missing in ≤2% of patients. Higher viral load, sub-optimal adherence, and previous indinavir use predicted VF in both univariate and multivariate analyses [multivariate ORs 2.7 (95% CI: 1.1-7.4), p=0.039; 7.8 (95% CI: 2.1-31.0), p=0.002; 12.8 (95% CI: 3.7-49.8, p<0.001); respectively]. Multivariate analysis shown in Table 3 was based on an analysis excluding missing data.

We performed *ad hoc* univariate and multivariate analyses of factors associated with the development of PI resistance. The three covariates identified to be independent predictors of VF were entered into the logistic regression model. Higher viral load and previous indinavir exposure remained independent predictors of PI resistance in both univariate [ORs: 2.7 (95% CI: 1.1-6.5), p=0.03 and 9.7 (95% CI: 3.0-34.2), p<0.001, respectively] and multivariate analyses [ORs: 4.3 (95% CI: 1.5-14.8), p=0.01 and 13.6 (95% CI: 3.6-61.3), p<0.001, respectively]. Adherence did not predict PI resistance in the univariate or multivariate analyses.

Two year follow-up of patients with virological failure maintained on the failing second-line therapy

The clinical outcomes of the 22 patients with VF are shown in Figure 1 and are also reported along with their treatment history and resistance profiles in Table 2. Seven patients died after a median duration of 8 (IQR: 8-16) months from the time of study enrolment: 4 due to tuberculosis and 3 due to severe wasting syndrome. Of the 15 patients who were alive, two patients were transferred to provincial clinics and declined

study follow-up visits. Two patients were switched to raltegravir and darunavir-ritonavir (purchased privately from Thailand for $600 US/month) and remained on lamivudine. The remaining 11 patients were maintained on the lopinavir-ritonavir-based regimen. Viral load test was performed at 24 month in the 13 patients in active follow-up. Virological re-suppression was achieved in the 2 patients switching to raltegravir and darunavir-ritonavir and in 5 patients maintaining on lopinavir-ritonavir. Their CD4 cell counts increased to a mean of 611 cells/mm^3 (range: 384-942). Of the 5 patients with virological re-suppression, 2 had no major PI resistance mutations and 3 had only one major mutation - V82A - at VF. The remaining 6 patients had persistent viral replication (mean HIV RNA: 5.2 log10 copies/mL; range: 4.79-5.70). Table 4 shows the evolution of antiretroviral resistance mutations of these 6 patients. With the exception of patient #18 who had no major drug resistance mutations, the other 5 had multiple major PI resistance mutations at VF and continued to accumulate NRTI resistance mutations (in 5 patients) and PI resistance mutations (in 2 patients). All 6 patients had worsening immunological control (mean CD4 count: 97 cells/mm^3, range 0-177); however, there were no AIDS events over the 24 months of follow-up.

Discussion

We report the antiretroviral resistance profiles of patients failing second-line PI-based therapy in Vietnam. The major finding was that 64% of patients experiencing VF harbored at least one major PI resistance mutation, and 60% patients had mutations that conferred intermediate- to high-level resistance to lopinavir-ritonavir. This level of PI resistance is significantly higher than has been reported in either resource-rich or resource-poor settings.[3,4,8–15,37] Ritonavir-boosted PIs are known to have a high genetic barrier to resistance.[8,38] The minimum plasma concentrations of ritonavir-boosted PIs far exceed the levels required to inhibit wild type virus replication,[39,40] making PIs a durable class of antiretroviral drug to be used across different patient populations. High prevalence of PI resistance has been reported in four studies; two of which were from Asia: Cambodia (N=71, 40%)[15] and India (N=45, 73%).[16] The other two were from West Africa, Mali (N=93, 25%)[37] and Nigeria (N=61, 62%).[41] Except for the study from India where indinavir-ritonavir and atazanavir-ritonavir were commonly used, the other three countries used lopinavir-ritonavir for second-line therapy. Previous exposure to generically-produced un-boosted indinavir and nelfinavir was implicated in the reports from Asia and Nigeria, although formal analyses were lacking. Our study is the first to systematically link previous PI exposure to VF and PI resistance.

Indinavir was generically produced in Vietnam during the early 2000s. The correct dosing was 800mg three times daily; however because of the high rate of side effects many Vietnamese clinicians prescribed it at 400mg three times daily or 800mg twice daily. A combination of high pill burden short half-life, food restriction, high rate of side effects, and inadequate dosing likely led to inadequate plasma drug concentrations and

increased the risk of PI resistance in patients. Low plasma indinavir concentrations has been shown to increase the risk of developing PI resistance mutations in patients experiencing early VF.[42] Further, the most common PI resistance mutations detected in our cohort - the M46L/I and V82A - were shown to be the first mutations to be sequentially selected by indinavir therapy.[43] Cheap generically-made indinavir, nelfinavir and saquinavir were available in India, China, and Southeast Asian countries during the same time.[15,44] This likely explains the higher prevalence of PI resistance reported in the studies from Cambodia and India and suggests that the scope of PI exposure and resistance in Asia might be larger than is currently appreciated. Another reason for the high level of PI resistance observed in our and these studies is the lack of viral load monitoring leading to late detection of virological failure and accumulation of PI resistance mutations. Better understanding of the extent and determinants of PI resistance in developing countries is needed.

Amongst the next-generation NNRTI and PIs potentially available as third-line drugs, there was evidence of probable intermediate- or high-levels of cross-resistance to etravirine, rilpivirine and tipranavir in approximately 50% of patients. Cross-resistance to darunavir was less frequent (27%) and was only observed at the intermediate level. These prevalences are noticeably higher compared with studies in similar settings.[15,16,37,41] One reason for the observed high-level etravirine cross-resistance is programmatic. The lack of virological monitoring lead to prolonged periods of undetected VF in presence of low-genetic-barrier drugs nevirapine and efavirenz and accumulation of resistance mutations. This was shown by the extensive NNRTI resistance mutations in our cohort (94% patients with ≥1 and 32% with ≥3 major NNRTI resistance mutations). NNRTI resistance

mutations have been shown to persist up to 45 months after discontinuation of NNRTI therapy. This is due to the low fitness costs of these mutations on viral replication, thus explaining the slow reversion of these mutant virus to wild-type in the absence of drug pressure.[45,46] The presence of ≥3 IAS-USA-defined NNRTI resistance mutations has been associated with decreased virological response to etravirine in the DUET trials.[47,48] Another reason for high etravirine cross-resistance is the inherent genetic variability of the HIV-1 subtype CRF01_AE in Southeast Asia. Etravirine was designed to work against HIV containing the NNRTI signature mutation - K103N - which is highly prevalent in HIV-1 subtype B.[49] However the most frequent NNRTI resistance mutations selected in subtype CRF01_AE virus by nevirapine and efavirenz exposure are Y181C and G190A/S, rather than K103N.[50,51] In the DUET trials, the presence at baseline of these substitutions was associated with impaired virological response to etravirine.[47,48] High-prevalence of cross-resistance to etravirine (60%) has been reported in several studies of CRF01_AE-infected patients failing first-line NNRTI-based therapy in Thailand.[52–54] As efficacy data of etravirine use in Southeast Asia are lacking, phenotypic assays investigating the *in vitro* susceptibilities of these clinical isolates would be helpful. Until then etravirine and rilpivirine should probably be avoided as third-line drugs for patients infected with subtype CRF01_AE in Southeast Asia. Our data does not support the 2010 and 2013 WHO's recommendations to use etravirine in a third-line ART regimen in resource-limited settings.[1,25] The phenotypic susceptibility of tipranavir is not as well predicted compared to darunavir by most genotypic interpretation algorithms, in particular for non-B subtypes.[55] However, based on our predicted cross-resistance data, darunavir-ritonavir plus a brand new class of antiretroviral drug, such as integrase strand

transfer inhibitors (INSTIs), combined with lamivudine is a reasonable third-line option for Vietnam. As the need for third-line therapy is imminent in the developing world, clinical trials evaluating cost-effective third-line treatment strategies and regimens are needed.

Amongst the 20 patients who were maintained on the failing lopinavir-ritonavir regimen, death or worse virological/immunological control ensured in 13 patients, with accumulation of resistance mutations occurring in those who stayed alive at 24 months. This is consistent with a study from Nigeria showing accumulation of PI resistance mutations in patients maintaining on failing second-line therapy.[41] However, virological re-suppression and good immune response were achieved in 5 patients; these patients either had no or only one major PI resistance mutation at VF detection. A strategy combining adherence intervention and close monitoring of patients failing second-line therapy before switching to third-line therapy would be cost saving yet effective in resource-poor settings.

Our study has limitations. The study captured VF at one point in time and only in patients who were in active follow up. The unavoidable exclusion of the 12% who had died and the 14% who had been transferred to their respective resident provinces reduces the power of our observations. Further, PI resistance might be underestimated due to the lack of data from those who had died. Nevertheless, the study site is the largest centre for second-line therapy in Vietnam. The highly uniform HIV care system along with standardised ART regimens in the national programme allow for reasonable generalisability of our findings. We did not sequence the integrase gene in this cohort as INSTIs are not yet available in Vietnam.

In conclusion, we identified significantly higher prevalence of PI resistance in patients failing second-line therapy in Vietnam, which was associated with previous indinavir exposure. The widespread availability of generically-made PIs in Asia suggests that the scope of PI resistance might be underestimated in this region. Our data emphasize the need for viral load monitoring to limit the accumulation of NRTI and NNRTI resistance mutations, thus improving second-line treatment outcome and preserving the limited third-line therapy options. Significant cross-resistance to etravirine is common in subtype CRF01_AE-infected patients failing NNRTI therapy, suggesting that etravirine should be avoided as a third-line therapy drug. Research on cost-effective strategies and timing of third-line therapy switch are now needed.

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Transparency declarations

None to declare

Author contributions:

Study concept and design: Le, Quang, Shikuma, Dunstan, and Farrar. Obtaining funding: Le, Dunstan, Day, and Farrar. Acquisition of data: Thao, Quang, Chinh, Vinh Chau, and Le. Analysis and interpretation of the data: Thao and Le. Drafting the manuscript: Thao and Le. Critical revision of the manuscript for important intellectual contents: Quang, Chinh, Shikuma, Farrar, Day, Vinh Chau, Dunstan, and Thwaites. All authors contributed to and approved the final manuscript.

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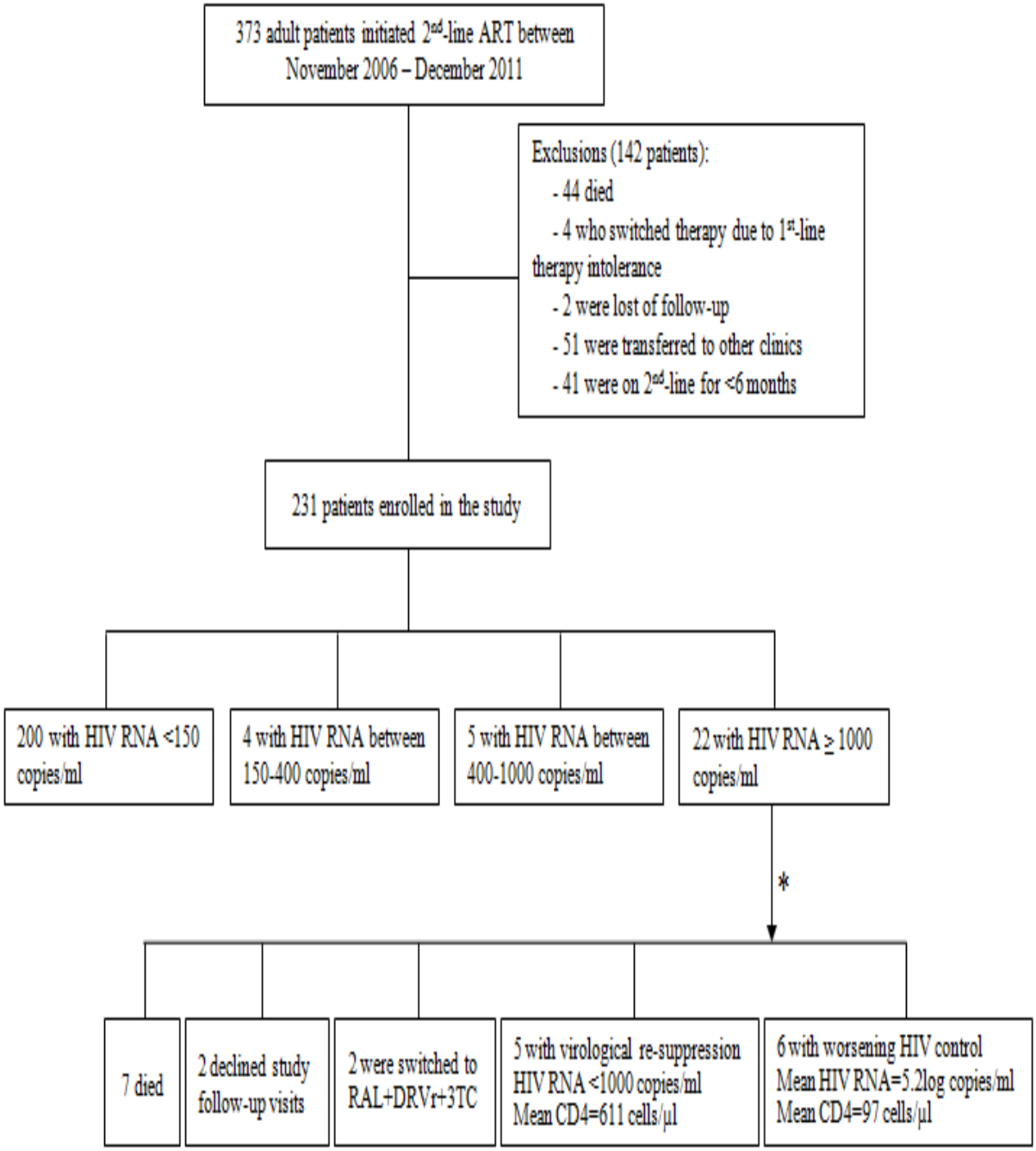


Figure 1: Flow chart of the study participants, virological outcome, and follow-up of patients with virological failure maintained on the failing second-line antiretroviral therapy

* 24 months follow-up; DRVr=ritonavir-boosted darunavir; RAL=raltegravir; 3TC=lamivudine

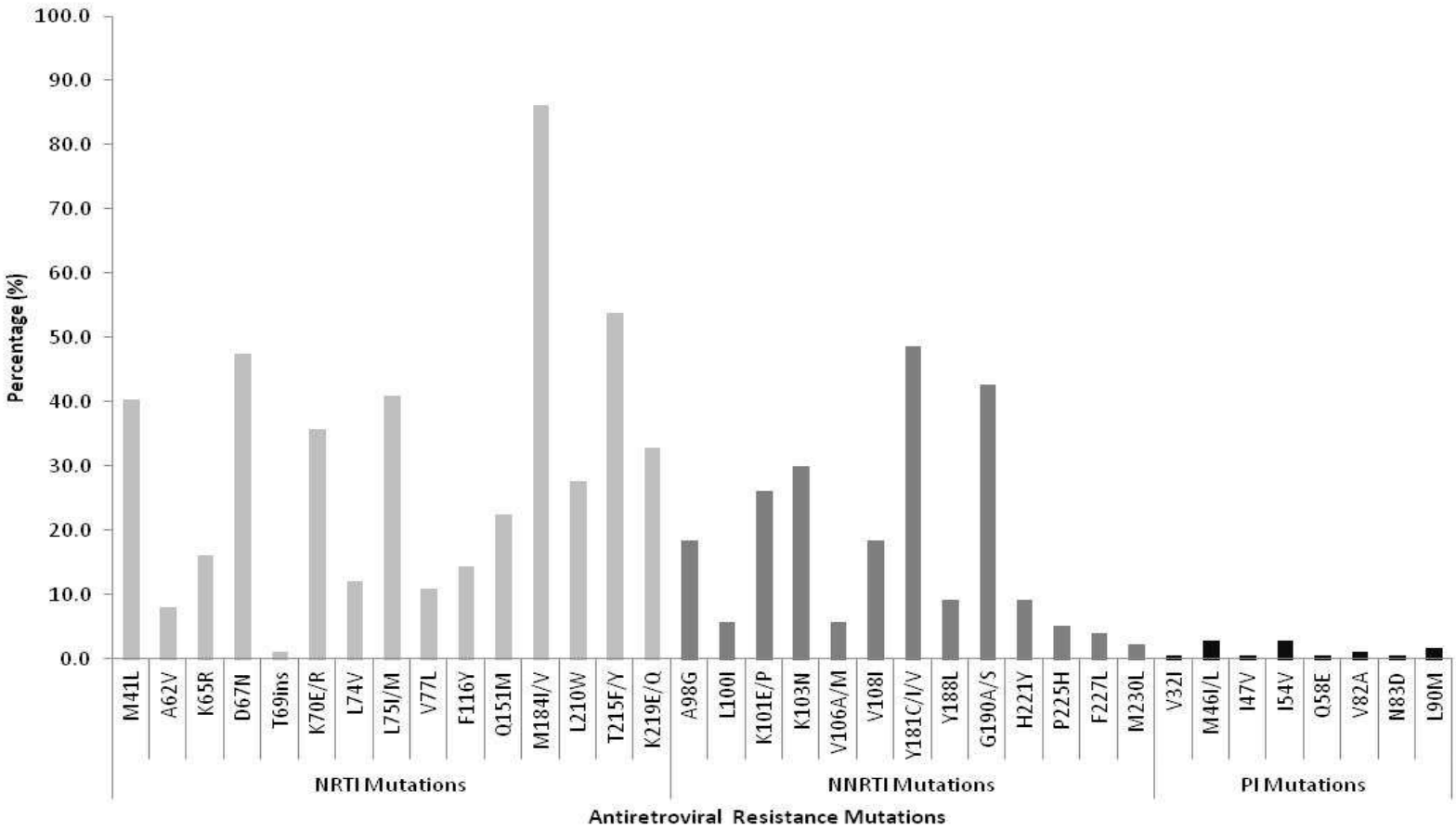
560 **Table 1: The characteristics of 231 patients on second-line antiretroviral therapy in**

561 **Ho Chi Minh City**

562

Characteristics	N=231
Sex, male (%)	187 (81.0%)
Age (years), median (IQR)	32 (28-36)
Previous history of IDU (%)$^{(n=230)}$	93 (40.4%)
CD4 count (cells/mm^3), median (IQR)$^{(n=227)}$	44 (17-84)
HIV RNA (log10 copies/mL), median (IQR)$^{(n=215)}$	5.1 (4.6-5.5)
Previous IDV use (%)$^{(n=211)}$	36 (17.1%)
Time on 2nd line therapy (months), median (IQR)	29 (16-43)
2nd line regimens (%)	
Initial regimens	
TDF/3TC/ LPVr	112 (48.5%)
TDF/3TC/ LPVr + AZT	82 (35.5%)
LPVr + other NRTIs [a]	27 (11.7%)
NFV + other NRTIs [a]	10 (4.3%)
Regimens at time of study assessment	
TDF/3TC/ LPVr	128 (55.4%)
TDF/3TC/ LPVr + AZT	88 (38.1%)
LPVr + other NRTIs [a]	15 (6.5%)
Adherence	
≥95%	203 (87.9%)
<95%	28 (12.1%)

IQR, interquartile range; IDU, injecting drug use; TDF, tenofovir; 3TC, lamivudine; AZT, zidovudine; LPVr, lopinavir-ritonavir; NFV, nelfinavir; NRTI, nucleotide/side reverse transcriptase inhibitor; [a,] other NRTIs include 2 or 3 of the following drugs: abacavir, didanosine, zidovudine, lamivudine, stavudine, or tenofovir.



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564 **Figure 2: Prevalence of antiretroviral resistance mutations in 173 patients at the time of switch to second-line therapy in Ho**

565 **Chi Minh City.**

566 NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleotide reverse transcriptase inhibitor; PI, protease inhibitor.

Table 2: Antiretroviral history, drug resistance profile and two-year outcomes of 22 patients with virological failure on second-line antiretroviral therapy in Ho Chi Minh City

Patient	Time on 2nd-line ART (months)	At time of therapy switch		Prior PI use	Mutations at time of therapy switch			At time of virological failure		Mutations at time of virological failure			2-year outcomes
		CD4 (cells/mm^3)	Viral load (copies/mL)		NRTIs	NNRTIs	PIs	CD4 (cells/mm^3)	Viral load (copies/mL)	NRTIs	NNRTIs	PIs	
1	15	79	4,720,000	No	**L74V, M184I**	**K101E, K103N, G190A, M230L**		191	289,000	**D67N, K70R, L74V, M184I, K219Q**	**K101E, K103N**, **E138G, G190A**, **M230L**	L10I**, V82A**	Virological re-suppression
2	18	2	7,590,000	NA	NA	NA	NA	2	1,630,000	**M184V**	**K103N, V108I, Y181C**		Death
3	19	13	170,000	IDV	NA	NA	NA	50	402,194	T215S		G16E, K20I, M36I, **M46L**, I54V, H69K, **V82A**, L89M	Death
4	18	74	118,000	IDV	**M41L, D67N, K70R**, V75M, **M184V, T215F, K219Q**	**K101P, K103N**		303	1,574	**M41L, D67N, K70R,** V75M**, M184V, T215F, K219Q**	**K101P, K103N**	L10I, G16E, K20I, M36I, H69K, L89M	Transferred to other clinic
5	18	45	435,000	IDV	**A62V, K65N**, T69S, V75M**, F77L, Q151M, M184V**	V106I, **Y181C, Y188L, H221Y**	I54V, **N83D**, I84R	152	1,184	V75M**, M184V, T215F**	V106I, **Y181C, Y188L, H221Y**	L10I, K20I, M36I, **M46L**, F53L, I54V, H69K, **V82A**, L89I	Worsening virological/ immunological control

Patient	Time on 2nd-line ART (months)	At time of therapy switch		Prior PI use	Mutations at time of therapy switch			At time of virological failure		Mutations at time of virological failure			2-year outcomes
		CD4 (cells/mm3)	Viral load (copies/mL)		NRTIs	NNRTIs	PIs	CD4 (cells/mm3)	Viral load (copies/mL)	NRTIs	NNRTIs	PIs	
6	29	5	365,000	No	**K65R, Q151M**	**Y181C, G190A**	L33F, I84L	143	5,490	**K65R, Q151M**	**Y181C, G190A**	K20R, L33F, M36I, **M46I**, I62V, H69K, **L76V, I84V**, L89M	Worsening virological/ immunological control
7	6	6	190,000	IDV	**A62V, D67N,** T69P, **V75I, F77L, F116Y, Q151M, M184I,** T215S, **K219Q**	**K101E, Y181C, G190A**	L10V	293	3,910	**D67N, V75I, F77L, F116Y, Q151M, M184I, K219Q**	**K101E, Y181C, G190A**	L10V, G16E, M36I, H69K, **V82A**, L89M	Virological re-suppression
8	31	40	184,000	No	**M41L, D67N,** T69N, **K70R,** L74I**, M184V, T215F, K219Q**	**V108I**, **G190A**	L10IV	546	1,520	**M41L, D67N, K70R**, L74I, **M184V, T215F, K219Q**	**G190A**	L10V, G16E, L33F, M36I, I54V, **V82A**, L89I	Virological re-suppression
9	47	8	253,000	IDV	**M41L, D67N,** T69N, **K70R,** L74I**, M184V, T215F, K219Q**	A98G, **K103N, G190A**		171	37,379	**M41L, D67N, K70R, M184V, T215F, K219Q**	**K103N, G190A**	L10V, K20I, L33F, M36L, **M46I**, **I47V**, I54V, H69K, **T74P**, **V82F**, L89M	Worsening virological/ immunological control
10	8	21	752,000	NA	NA	NA	NA	152	16,582	**K65R,** V75M	V179F, **Y181C, H221Y**	M36I, H69K, L89M	Virological re-suppression

Patient	Time on 2nd-line ART (months)	At time of therapy switch		Prior PI use	Mutations at time of therapy switch			At time of virological failure		Mutations at time of virological failure			2-year outcomes
		CD4 (cells/mm3)	Viral load (copies/mL)		NRTIs	NNRTIs	PIs	CD4 (cells/mm3)	Viral load (copies/mL)	NRTIs	NNRTIs	PIs	
11	45	21	867,000	No	**M41L,** E44AD, **D67N, L74V,** V75M, V118I, **M184V, L210W, T215Y,** K219N	A98G, **L100I, K101P, G190A**		121	96,147	**M41L, D67N,** V75M, **M184V, L210W**	A98G, **G190A**	M36I, H69K, V82I, L89M	Death
12	43	1	132,000	No	**D67N, K70R, M184V, L210W, T215F,** K219W	**K103N, V108I, Y181C, G190A**		11	22,600	**M184V**			Death
13	50	113	38,238	IDV	**M41L, D67N,** T69P, **K70R, M184V, L210W, T215F, K219E**			57	319,798	**M41L, D67N, K70R, M184V, L210W, K219QE**		L10V, G16E, K20V, L33F, M36I, **I47V**, I54V, H69K, A71V, **I84V**, L89M	Worsening virological/ immunological control
14	12	25	693,000	No	NA	NA	NA	48	875,664			L10I, K20R, M36I, H69K, L89M	Virological re-suppression
15	29	211	189,000	IDV	T69N, V75M		**V32I, M46I, Q58E**	253	64,262	**K70R,** V75M, **M184V, K219E**	V90I	L10I, G16E, K20I, M36I, **M46I**, I54A, **Q58E**, H69K, K70R, **V82A,** L89I	Worsening virological/ immunological control

Patient	Time on 2nd-line ART (months)	At time of therapy switch		Prior PI use	Mutations at time of therapy switch			At time of virological failure		Mutations at time of virological failure			2-year outcomes
		CD4 (cells/mm3)	Viral load (copies/mL)		NRTIs	NNRTIs	PIs	CD4 (cells/mm3)	Viral load (copies/mL)	NRTIs	NNRTIs	PIs	
16	47	44	139,000	IDV	**M41L,** T69N, V75M**, F77L, F116Y, Q151M, M184V, T215Y**	A98G, **L100I, K103N**		154	34,900	**M41L,** V75M, **F77L, M184V, T215Y**	A98G	L10I, G48A, I54V, A71V, **V82A**	RAL + DRVr + 3TC
17	43	64	174,550	IDV	**M41L, D67N, K70R, L74V, M184V, T215F, K219Q**	**Y181C, G190S**	M36I	67	47,500	**M41L, D67N, K70R, L74V, M184V, T215F, K219Q**	A98G, **Y181C, G190S**	L10I, L33F, **M46I, I54M**, A71V, G73S, **I84V**	Death
18	23	41	377,000	No	**K65R,** V75M	**K103N**		6	67,934			M36I, H69K, V82I, L89M	Worsening virological/ immunological control
19	17	40	590,000	No	**D67N,** T69N, **K70R,** L74I, V75M, **M184V, T215F, K219E**	**K101P, Y181C, G190S**		352	3,776	**D67N, K70R,** V75M, **M184V, T215F, K219E**	K101Q, **Y181C, G190A**	K20R, M36I, **M46I**, L63P, H69K, A71V, **L76V**, **I84V**, L89M	Transferred to other clinic
20	30	3	948,909	NA	NA	NA	NA	98	103,000	**K70R, T215F, K219E**	A98G, **K101E, Y181C, Y188L, G190A**	L10I, I54V, **N83D**	Death
21	45	113	2,470,000	IDV	**M41L, D67N,** V75M, V118I, **M184V, L210W, T215F,** K219W	**K101P, V108I, G190A**	L10F	173	79,800	**M41L, D67N,** V75M, **M184V, T215Y**	A98G	L10F, **M46L**, I54V, **L76V**, **V82A**, L89V	RAL + DRVr + 3TC

Patient	Time on 2nd-line ART (months)	At time of therapy switch		Prior PI use	Mutations at time of therapy switch			At time of virological failure		Mutations at time of virological failure			2-year outcomes
		CD4 (cells/mm3)	Viral load (copies/mL)		NRTIs	NNRTIs	PIs	CD4 (cells/mm3)	Viral load (copies/mL)	NRTIs	NNRTIs	PIs	
22	26	14	81,422	IDV	NA	NA	NA	28	83,300	**K65R**	**K101E, Y181C, G190A**		Death

ART, antiretroviral therapy; NRTIs, nucleoside/tide reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors; IDV, indinavir; DRVr, darunavir-ritonavir; RAL, raltegravir; 3TC, lamivudine. Bold: major drug resistant mutations according to the IAS-USA 2014. NA: non-applicable as data are unknown.

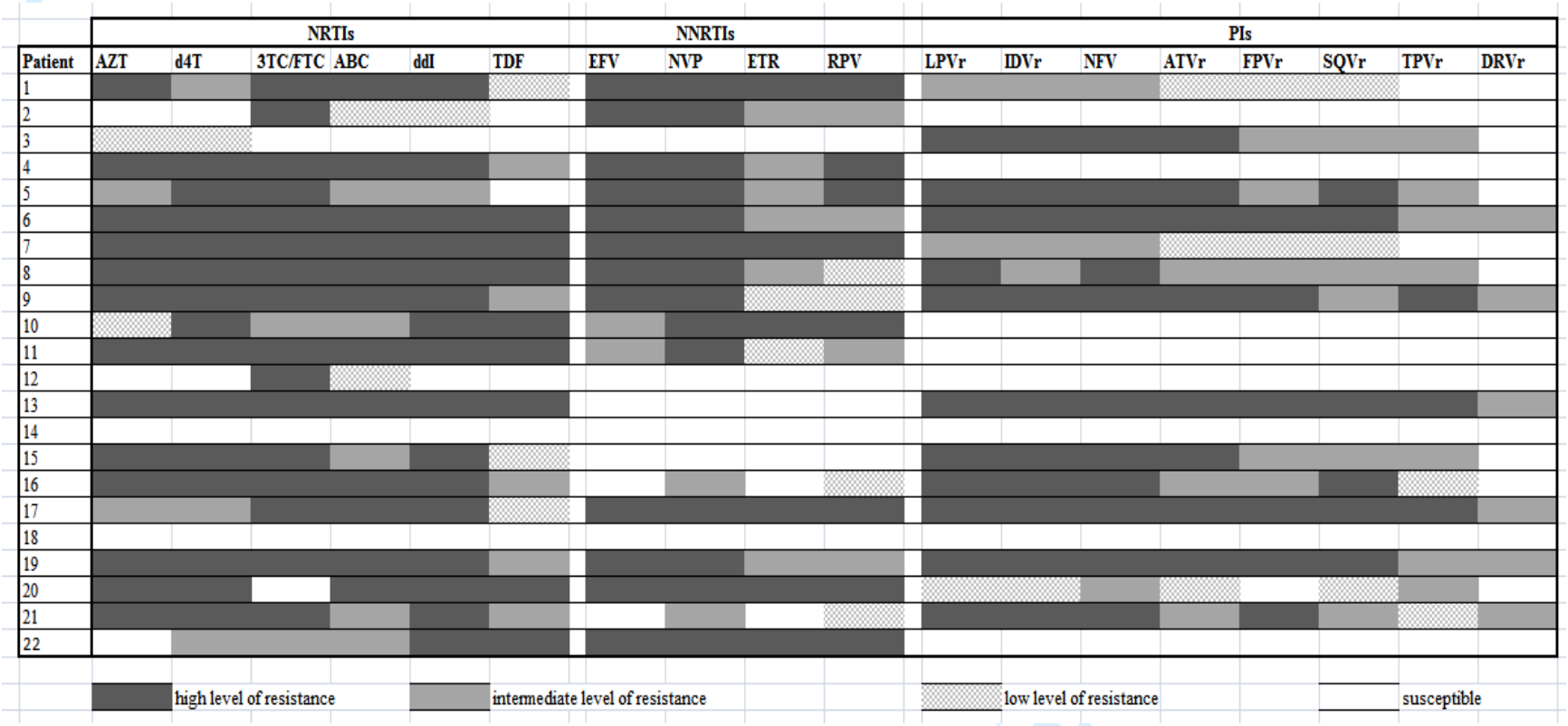

Figure 3: Predicted antiretroviral susceptibility among 22 patients experiencing virological failure on second-line antiretroviral therapy in Ho Chi Minh City using the Stanford's algorithm.

NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, non-nucleotide reverse transcriptase inhibitors; PIs, protease inhibitors; AZT, zidovudine; d4T, stavudine; 3TC, lamivudine; FTC, emtricitabine; ABC, abacavir; ddI, didenosine; TDF, tenofovir; EFV, efavirenz; NVP, nevirapine; ETR, etravirine; RPV, rilpivirine; LPV, lopinavir; IDV, indinavir; NFV, nelfinavir; ATV, atazanavir; FPV, fosamprenavir; SQV, saquanavir; TPV, tipranavir; DRV, darunavir; r, ritonavir-boosted.

582 **Table 3: Factors associated with virological failure in 231 patients on second-line antiretroviral therapy in Ho Chi Minh city**

Covariates	Patients without virological failure (N=209)	Patients with virological failure (N=22)	Univariate effect		Multivariate effect	
			OR (95% CI)	p-value	OR (95% CI)	p-value
CD4 count[a] (by -50 cells/µl)	47 (17-88)[(n=205)]	33 (9-59)	1.39 (0.92-2.38)	0.184	1.52 (0.84-3.45)	0.248
HIV RNA[a] (by + log10 copies/ml)	5.1 (4.6-5.5)[(n=194)]	5.6 (5-5.9)[(n=21)]	**3.14 (1.56-6.69)**	**0.002**	**2.70 (1.08-7.35)**	**0.039**
Time on failing 1st-line ART (months)	9(5-15)[(n=206)]	9(3-21)	1.01 (0.97-1.05)	0.537	1.01 (0.95-1.07)	0.786
Adherence <95% (yes)	21 (10%)	7 (32%)	**4.18 (1.46-11.16)**	**0.005**	**7.81 (2.06-31.00)**	**0.002**
Prior IDV use (yes)	27 (14%)[(n=192)]	9 (47%)[(n=19)]	**5.50 (2.02-14.91)**	**<0.001**	**12.80 (3.69-49.80)**	**<0.001**

583 Data are absolute numbers (%) for categorical variables and median (interquartile range) for continuous variables; n=numbers of patients with complete data on a
584 covariate; OR=odds ratio; CI=confidence interval; [a]=at time of therapy switch; IDV=indinavir.

Table 4: The evolution of resistance mutations in 6 patients with worsening HIV control who were maintained on a failing second-line regimen

Patient	Time on 2nd-line ART (months)	At time of virological failure		Mutations at time of virological failure			At 2-year follow-up		Mutations at 2-year follow-up		
		CD4 (cells/mm^3)	Viral load (copies/mL)	NRTIs	NNRTIs	PIs	CD4 (cells/mm^3)	Viral load (copies/mL)	NRTIs	NNRTIs	PIs
5	18	152	1,184	V75M, **M184V, T215F**	V106I, **Y181C, Y188L, H221Y**	L10I, K20I, M36I, **M46L**, F53L, I54V, H69K, **V82A**, L89I	77	93,500	**A62V, K65N,** V75M**, F77L, Q151M, M184V**	V106I, **Y181C, Y188L, H221Y**	L10F, K20I, M36I, **M46L,** F53L, I54V, H69K, **V82A,** L89I
6	29	143	5,490	**K65R, Q151M**	**Y181C, G190A**	K20R, L33F, M36I, **M46I**, I62V, H69K, **L76V, I84V**, L89M	177	164,000	**K65R**, **D67N**, T69d, **Q151M, K219E**	**Y181C, G190A**	L10F, K20R, L33F, M36I, **M46I,** I62V, H69K, **L76V, V82A,** T74S, **I84V,** L89M
9	47	171	37,379	**M41L, D67N, K70R, M184V, T215F, K219Q**	**K103N, G190A**	L10V, K20I, L33F, M36L, **M46I**, **I47V**, I54V, H69K, **T74P**, **V82F**, L89M	159	61,100	**M41L, D67N,** T69N**, K70R,** V75M**, M184V, L210W, T215F, K219Q**	V106I**, G190A**	L10V, K20I, L33F, M36L **M46I, I47V,** I54V, H69K, **T74P, V82F,** L89M
13	50	57	319,798	**M41L, D67N, K70R, M184V, L210W, K219QE**		L10V, G16E, K20V, L33F, M36I, **I47V**, I54V, H69K, A71V, **I84V**, L89M	10	498,000	**M41L, D67N, K70R, M184V, L210W, T215Y,** K219D		L10V, G16E, K20V, L33F, M36I, **M46I, I47V,** I54V, H69K, A71V, G73T**,** L76M**, I84V,** L89T
15	29	253	64,262	**K70R,** V75M, **M184V, K219E**	V90I	L10I, G16E, K20I, M36I, **M46I**, I54A, **Q58E**, H69K, K70R, **V82A,** L89I	159	97,100	D67H, T69G, **K70R,** V75M**, M184V,** T215I**, K219E**	V90IV	L10I, G16E, K20I, L33F, M36I, **M46I,** I54A**, Q58E,** H69K, K70R, **V82A,** L89I
18	23	6	67,934			M36I, H69K, V82I, L89M	0	279,000		V106I	M36I, H69K, V82I, L89M

ART, antiretroviral therapy; NRTIs, nucleotide/side reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors; Bold: major drug resistant mutations.

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PREVALENCE AND PATTERNS OF TRANSMITTED DRUG RESISTANCE IN HIV-INFECTED ADULT PATIENTS INITIATING ANTIRETROVIRAL THERAPY IN HA NOI, VIET NAM

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SUMMARY

As antiretroviral therapy (ART) coverage for HIV-infected patients in Vietnam continues to increase, data on the prevalence and patterns of transmitted drug resistance (TDR) mutations are important to guide national ART strategies. TDR was evaluated in 345 antiretroviral-naïve patients consecutively initiating first-line ART in the clinical trial of Virological Monitoring in Vietnam (VMVN) at Bach Mai Hospital in Hanoi between April 2011 and October 2013. TDR mutations were identified by Sanger sequencing of HIV pol gene and were defined based on the 2009 World Health Organization surveillance drug resistance mutation (SDRM) list. 330 plasma samples were successfully sequenced in both protease and reverse transcriptase regions of HIV pol gene. 323 samples were subtype CRF01_AE; two were subtype A/CRF01_AE, two were subtype BC, one was subtype C; one was subtype F/C recombinant. SDRMs were identified in 16 (4.8%) patients. Among them 6 (38%) patients carried mutations conferring resistance to nucleoside/tide reverse transcriptase inhibitors (NRTIs) (K70E, V75M, K219N/E, T215S), 5 (31%) to non-nucleoside reverse transcriptase inhibitors (NNRTIs) (K101E, K103N, Y181C, G190A), 4 (25%) to protease inhibitors (PIs) (M46I/L, I54L, L90M), and one to both NRTIs and NNRTIs (L74I, V75M, M184V, T215F, K101E, G190A). The level of TDR remains low despite the rapid scale up of ART in Vietnam over the past 10 years. TDR to PIs was identified in 4 patients for the first time in Ha Noi. As PIs are the main component of 2nd-line therapy and the last resort for patients with drug-resistant virus in Vietnam. The detection of TDR to PIs is of concern and requires further investigation.

***Keywords**: Human Immunodeficiency Virus, Acquired Immunodeficiency Syndrome, Antiretroviral therapy, Transmitted drug resistance, Vietnam*

INTRODUCTION

Human Immunodeficiency Virus (HIV) has infected thirty five million people worldwide. Each year approximately 3 million are infected, and nearly 2 million die of Acquired Immunodeficiency Syndrome (AIDS) (UNAIDS 2013). Antiretroviral drugs are available and are effective in suppressing HIV from replication in an infected person and in reducing the probabilities of HIV to be transmitted from one person to another. HIV is a retrovirus that replicates without a proof reading and repair mechanism and is thus error prone. Another feature is its high replication capacity, producing a billion of new virions a day within an infected individual (Perelson *et al.*, 1996). The above characteristics allow HIV to evolve rapidly, capable of becoming resistant to all currently available antiretroviral drugs ever produced on the market. As a cure for HIV is still a far reach, life-long ART remains the main tool against HIV, and the development of HIV drug resistance (HIVDR) would threaten the sustainability of ART worldwide.

Drug resistant virus can develop *de novo* or be transmitted from persons to persons within a population; this is called primary or transmitted drug resistance (TDR). Drug resistant virus can develop in an individual taking ART; this is called secondary or acquired resistance. Harbouring drug resistant virus (whether by primary or secondary resistance pathway) can lead to treatment failure, disease progression and death (Hogg *et al.,* 2006, Kozal *et al.,* 2007, Poggensee *et al.,* 2007). Antiretroviral drugs have been available for much longer in the United States and Western Europe; as such the prevalence of TDR are higher (up to 24% in some cities) (Sagir *et al.,* 2007, Vercauteren *et al.,* 2008, Jain *et al.,* 2010). In these countries, drug resistance testing prior to initiation of ART is therefore routinely performed and has been shown to be cost effective (Sax *et al.,* 2005). The cost of HIV genotype testing is still prohibitively expensive for routine clinical care in the developing countries. Patients in these settings do not receive HIV drug resistance testing prior to ART initiation. TDR that goes undetected in settings where ART is being scaled up can have a major impact on the global control of HIV. Increased surveillance of TDR in resource-limited settings is therefore essential and is recommended by the World Health Organization (WHO) (Bennett *et al.,* 2008).

The first case of HIV infection was identified in Vietnam in 1990. HIV has since spread to all 64 provinces of the country. New cases more than doubled in numbers from 112,000 in 2000 to 256,000 in 2014 (Viet Nam MOH 2014). The epidemic is still considered to be in its concentrated phase, meaning HIV is concentrated in intravenous drug users (IDU), female sex workers (FSW) and men who have sex with men (MSM) (UNGASS 2010). However the increasing numbers of women infected with HIV means that the epidemic is beginning to spread to the general population (National Committee for AIDS, Drugs, and Prostitution Prevention and Control, 2012). HIV-1 CRF01_AE subtype remains the predominant subtype seen in Vietnam over the years with >95% prevalence (Hemelaar *et al.,* 2006, Nouhin *et al.,* 2011, Thao *et al.,* 2012). ART was first introduced in Vietnam by private donations and through the black markets in the beginning of 2000s. During this time treatment interruption was common due to unstable drug supply and lack of national treatment guidelines. The Vietnam national ART program began in 2005 with international donations, primarily the US President's Emergency Plan for AIDS Relief (PEFFAR) and the Global Fund to Fight AIDS, Tuberculosis and Malaria. As of September 2014, 88,800 adults and children were on ART (MOH 2014) and ART coverage for patients who meet treatment criteria increased from 1% to 53% in adults and to 83% in children from 2003 to 2011 (UNGASS 2012). Both the history of treatment interruptions and the rapid rise in ART coverage are expected to be accompanied by the emergence of acquired and TDR in Vietnam.

Data on TDR in Vietnam is limited and have shown prevalence ranging from <5% in low-risk populations (e.g. women attending antenatal care centers and individuals diagnosed from volunteer counselling and testing centers (VCT) (Nguyen *et al.,* 2008, Ayouba *et al.,* 2009) to levels of 5-10% in a more general population of patients from the HIV outpatient clinics in Vietnam (Lan *et al.,* 2003, Phan *et al.,* 2010, Dean *et al.,* 2011). These data reflect the TDR situation in Vietnam from 2003 to 2009. More recent data is needed and in this study, we investigate the prevalence and patterns of TDR in patients initiating ART at Bach Mai Hospital in Ha Noi, Vietnam from 2011 to 2013.

MATERIALS & METHODS

Study population

Consecutive baseline plasma samples from patients participating in the Virological Monitoring in Vietnam (VMVN) clinical trial were analysed in this study. VMVN is a randomized trial comparing the impact of routine viral load monitoring versus standard clinical and immunological monitoring on clinical outcome of patients initiating first-line ART in Hanoi, Vietnam over three years of follow up. This trial was registered at Clinicaltrial.gov, number: NCT01317498. HIV-infected patients aged 18 years or older who were not currently taking ART, met Vietnam Ministry of Health (MOH) criteria to initiate ART (CD4 $\leq$350 cells/mm^3, and/or WHO Clinical Stage III or IV), were eligible to enter the trial (Viet Nam MOH 2011, WHO 2010). Patients who had been on ART in the past but stopped for whatever the reason for more than 3 months were still considered eligible to enter the trial as long as they did not have evidence of treatment failure to first-line ART in the past. For this drug resistance sub-study, only patients who were ART-naive were included in the analysis of TDR. The VMVN clinical

trial and this sub-study were approved by the Scientific and Ethical Committees of Bach Mai Hospital and the Beth Israel Deaconess Medical Center.

Specimen collection

5 ml of blood was collected at study entry. Plasma were obtained, stored at -80°C, and these samples were subsequently transported on dry ice to the Oxford University Clinical Research Unit (OUCRU) in Ho Chi Minh City for drug resistance analysis.

Extraction and amplification of plasma HIV-1 viral RNA

HIV-1 RNA was extracted from 200 µl of plasma using a either MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche, Vedbaek, Denmark) or QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany). HIV-1 *pol* gene, which encodes reverse transcriptase and protease, was amplified by reverse transcription PCR and nested PCR according to an in-house assay described initially by Bezemer with some modifications by Thao VP (Thao, Le *et al.*, 2012). MMLV-RT enzyme was substituted by SuperScriptIII RT (Invitrogen, Carlsbad, CA) during reverse transcription; HiFidelity Tag (Qiagen, Center Mainz, Germany) was used for PCR amplification; and 30 instead of 25 cycles were used for second-round PCR. Primers specific for subtype AE were modified from subtype B primer set based on subtype AE consensus sequences.

Reverse transcription PCR

Reverse transcription was performed using primer 3'RT-outAE (TCCACTTGTCCATGCATA GCTTC). 10 µl of RNA was reverse-transcribed in a total volume of 20 µl with 2 mM of dNTP, 0.1 mM of primer, 1x First Strand Buffer, 5 mM of DTT, 16U of RNAse-out (Invitrogen, Carlsbad, CA), and 60U of SuperScriptIII RT. The reaction was incubated at 37^{o}C for 2 hours followed by 95^{o}C for 5 minutes to inactivate the enzyme.

Nested PCR

The *pol* gene was amplified with nested PCR. The entire 20 µl of cDNA was added to the first PCR reaction. The primers used for the first PCR reaction were 5'Prot-IAE (AGGCTAATTTTTA GGGAAAATTTGGCCTTCC) and 3'ET-21AE (AGCTGGCTACTATTTCCTTTGCTACTAYAGG TG). The reaction volume was 50ul, containing 1xPCR buffer, 1 µM of each primer, and 3.33U of HifidelityTaq. The first PCR involved in initial denaturation at 95°C for 5 minutes; 35 cycles of denaturation at 94°C for 15s, annealing at 55°C for 60s, and polymerisation at 72°C for 4 minutes; and final elongation at 72°C for 10 minutes.

A nested PCR contained 5 µl from first amplicon, 1xPCR buffer, 1 µM of each primer, 0.5 mM $MgSO_4$, and 1.25U of HifidelityTaq. Primers for second PCR were 5'Prot-II (TCAGAGCAGACC AGAGCCAACAG) and 3'RT-20AE (CTGCCAGTT CTAATTCTGCTTC). The cycling conditions were 95^{o}C for 5 minutes, followed by 30 cycles of 94^{o}C for 15 s, 55^{o}C for 1 minute, and 72^{o}C for 2 minutes, and then followed with a final extension at 72^{o}C for 10 minutes.

Five microliters of the PCR products containing 1194 base-pairs of the complete 297 nucleotides protease (PR) region and the first 897 nucleotides in the reverse transcriptase (RT) region of *pol* was checked by agarose gel electrophoresis.

Genotyping and drug resistance determination

Forty-five microliters of amplification products were purified using QIA quick PCR purification kits (Qiagen, Hilden, Germany) and were subjected to direct sequencing with 6 primers 5' PR5 (AGCCAACAGCCCCACCAG), 3' PR2 (CTTTTGGGCCATCCATTC), 5' RT-19new (CACCTGTCAACATAATTGGAAG), 3' B-RTrev (GGTGATCCTTTCCATCCC), 5' B-RT (GGGATGGAAAGGATCACC), 3' RT-20AE (CTGCCAGTT CTAATTCTGCTTC) using the ABI 3130xl Genetic Analyser (Applied Biosystems, Foster City, CA). The gene sequences were analysed using SeqScape (Applied Biosystems, Foster City, CA). The consensus sequence pNL4-3 for HIV-1 subtype B (GenBank accession number M19921) was used as the reference sequence for assessing nucleotide changes in the sample sequences. HIV drug resistance mutations in ART naïve patients were determined according to the 2009 WHO drug resistance surveillance mutations (SDRMs) list (Bennett et al., 2009). HIV subtyping using RT and PR genes were done by the Stanford database system (http://sierra2.stanford.edu/sierra/servlet/JSierra).

RESULTS

Study population

Between April 2011 and October 2013, 370 patients met the entry criteria and were enrolled in the VMVN trial. Plasma samples from 345 patients who were ART-naïve were analysed for this drug resistance sub-study. Among them 330 samples were successfully sequenced in both PR and RT regions. The characteristics of the 330 patients at study enrolment are listed in table 1.

Table 1. The characteristics of 330 antiretroviral-naïve HIV-infected individuals starting antiretroviral therapy in Ha Noi, Vietnam.

Characteristics	Study population (n=330)
Male gender, n (%)	217 (66%)
Age, median year (IQR)	37 (32-42)
CD4 cell counts, median cell/mm3 (IQR)	114 (32-258)
Viral load, median log10copies/ml (IQR)	5.15 (4.67-5.55)
Transmission routes	
Heterosexual route	260 (79%)
Injection drug use	59 (18%)
Others	11 (3%)

HIV-1 phylogenetic analysis

Phylogenetic analysis of 330 HIV-1 *pol* sequences revealed that 323 (98%) sequences were subtype CRF01_AE, two (0.6%) were subtype A and CRF01_AE recombinant, two (0.6%) were subtype B and C recombinant, one (0.3%) was subtype B and CRF01_AE recombinant, one (0.3%) was subtype F and C recombinant, and one (0.3 %) was subtype C.

Prevalence and patterns of SDRMs

According to the WHO SDRM list, mutations that confer resistance to nucleoside/tide reverse transcriptase inhibitors (NRTIs) were detected in 6/330 (1.7%) patients and to non-nucleotide reverse transcriptase inhibitors (NNRTIs) in 5/330 (1.5%) patients. One patient (0.3%) had a viral strain that carried resistance mutations to both NRTIs and NNRTIs. Resistance to protease inhibitors (PIs) was detected in 4/330 (1.2%) patients. We did not identify any strain that harboured both PI resistance and NRTI or NNRTI resistance mutations. Hence, the overall prevalence of TDR in this cohort in Ha Noi is 16/330 or 4.8%.

The patterns of SDRM are shown in table 2. Patients 12, 13 carried the NRTI mutation V75M which confers resistance to stavudine (d4T). Patient 8 carried the NRTI mutation K219E which confers resistance to zidovudine (AZT). AZT and d4T were the most common NRTI drugs used in Vietnam from 2003 to 2013. Patient 9 carried the NRTI mutation K70E which confers resistance to tenofovir (TDF), abacavir (ABC) and didanosine (ddI); all these drugs had been used either in the first-or second-line ART regimens, except for ddI which came off the national guidelines in 2011. Patient 4 carried the NRTI mutation K219N, an accessory mutation usually occurring in combination with other thymidine analog mutations (TAMs) that are selected by d4T and AZT. Patient 11 carried the revertant NRTI mutation T215S, which does not confer resistance to NRTIs but its presence suggests that the patient had harbored the major mutation T215Y/F in the past. Six patients carried at least one major NNRTI mutation (Y181C, G190A, K101E); each confers high-level resistance to nevirapine (NVP) and/or efavirenz (EFV). These have been the most common NNRTIs used in Vietnam and in other developing countries. Patient 7 had multi-drug class resistance mutations (NRTI mutations: L74I, V75M, M184V, T215F and NNRTI mutations: K101E, G190A). This patient carried a viral strain that confers resistance to all drugs used in the standard first-line ART regimens in Vietnam. Four major PI resistance mutations were detected in four different patients. Each of these mutations, M46I/L, L90M and I54L, reduces

susceptibility to PIs, including lopinavir/ritonavir (LPV/r) which is the standard PI used in the second-line ART regimens in Vietnam.

DISCUSSION

In this study we report a TDR prevalence of 4.8% in a cohort of 330 ART-naïve HIV-infected adults initiating ART in Ha Noi from 2011 to 2013. The detected NRTI and NNRTI mutations confer resistance to the standard first-line ART regimens used in Vietnam. Major PI mutations were detected in four patients (1.2%). These are associated with high-level resistance to many PIs, including LPV/r, the key drug in the second-line ART regimens in Vietnam.

TDR prevalence of 4.8% found in this study is consistent with studies published previously from Vietnam (Dean, Ta Thi *et al.,* 2011, Bontell *et al.,* 2012, Duc *et al.,* 2012). When comparing TDR prevalence among studies, it is important to take into account differences in the study populations, study time periods, geographic locations, study sample sizes, clinical and laboratory methods, and finally drug resistance algorithms used. Published studies in Vietnam using the WHO's recommended threshold survey method, which sampled TDR in low-HIV-risk exposure populations such as women who attend antenatal clinics and people who come to VCTs for HIV testing, reported TDR prevalence of <5% (Nguyen, Duc *et al.,* 2008, Ayouba, Lien *et al.,* 2009). While studies that sampled TDR from patient populations attending HIV clinics reported higher TDR prevalence of 5-10% (Lan, Recordon-Pinson *et al.,* 2003, Phan, Ishizaki *et al.,* 2010, Dean, Ta Thi *et al.,* 2011, Thao, Le *et al.,* 2012). The Vietnam HIV epidemic is still concentrated in the high-HIV-exposure populations; therefore the WHO algorithm which samples from antenatal clinics and VCTs may under-estimate levels of TDR in Vietnam. However studying TDR in chronically-infected patients in HIV clinics are not without disadvantages. First, we rely on patients' self-reported history of ART use. This is not optimal, as some patients might decide to withhold their history of previous ART use for perceived fear that they might not qualify for ART. This can result in overestimation of TDR. On the other hand, TDR might be underestimated in chronically-infected patient populations using standard population sequencing. In the absence of antiretroviral selection pressure TDR strains will overtime revert to minority resistant variants or wild-type virus, and may be missed by the standard population sequencing method which only reliably detects resistant variants present in more than 20% of the viral quasispecies. Our study is not immune to these methodological limitations. The detected TDR prevalence of 4.8% suggests that TDR levels remains relatively stable despite the rapid scale up of ART in Vietnam over the past 10 years.

The observed RT mutations in our study conferred resistance to antiretroviral drugs in the standard first-line ART regimens in Vietnam. One patient has a L74I mutation which confers resistance to abacavir (ABC) and didanosine (ddI). L74I has a moderate fitness cost to the virus, meaning a virus that carries such a mutation has lower fitness compared to a wild type virus in replication. In the absence of drugs that select for such a mutation, the resistant virus will be outcompeted by wild type virus overtime (Martinez-Picado and Martinez 2008). The fitness costs of carrying the L74I and the M184V mutations (M184V is a mutation with high fitness cost in itself) are additive (Martinez-Picado and Martinez 2008). The fact that the L74I was found in patient 7 (Table 2) together with the M184V and multiple other NRTI and NNRTI mutations suggests that: i) the patient may have been recently infected by a person who was highly ART-experienced and had been on ddI or ABC in the past, or ii) the patient was not honest about his previous ART exposure. The latter means that the patient may have recently been on ART and has acquired resistance rather than being infected with a multi-drug resistant strain. A throughout review of the patient treatment history might assist in the differentiation of the two possibilities.

Patient 11 has a revertant T215S mutation, which does not reduce NRTI susceptibility but arises from viruses that once contained the mutations T215Y and F (Garcia-Lerma *et al.,* 2001). The mutations T215Y/F cause intermediate to high level resistance to AZT and d4T. These mutations have a fitness cost to the virus (Cong *et al.,* 2007). Thus when AZT/d4T drug pressure is removed, T215F/Y-containing viral strains will reverse-mutate to wild type viruses which confer better survival in the absence of treatment. The viral strains containing the T215F/Y mutations would have to back mutate in two different nucleotides to return to the wild-type viruses, the process which takes time. This likely

explains why T215 revertant mutations (which require only one nucleotide change) are among the most commonly reported transmitted drug resistance mutations (Garcia-Lerma, Nidtha *et al.*, 2001, Wensing *et al.*, 2005, Wheeler *et al.*, 2010). Comparing to the T215F/Y and other TAM mutations, T215 revertant mutations have been shown to persist much longer, consistent with the fitness advantages associated with this resistance pathway (Yerly *et al.*, 1998).

Table 2. The surveillance drug resistance mutations detected in 16 antiretroviral-naïve HIV-1 infected individuals in Ha Noi from 2011-2013.

Subject	Transmission route	CD4 at baseline (cells/µL)	Viral Load (copies/mL)	Drug resistance associated mutations		
				Protease inhibitors (PIs)	Nucleoside/tide reverse transcriptase inhibitors (NRTIs)	Non-nucleotide reverse transcriptase inhibitors (NNRTIs)
1	Heterosexual	215	354,000	L90M		
2	Heterosexual	24	54,200			Y181C, G190A
3	Heterosexual	306	18,400	M46L		
4	Injection drug use	82	99,300		K219N	
5	Heterosexual	22	54,100	I54L		
6	Heterosexual	480	13,200			K101E
7	Other	7	1,540,000		L74I, V75M, M184V, T215F	K101E, G190A
8	Injection drug use	11	615,000		K219E	
9	Heterosexual	144	272,000		K70E	
10	Heterosexual	120	154,000	M46I		
11	Injection drug use	13	12,900		T215S	
12	Heterosexual	20	130,000		V75M	
13	Heterosexual	33	898,000		V75M	
14	Heterosexual	35	189,000			Y181C
15	Heterosexual	303	22,500			K101E
16	Heterosexual	173	42,600			K103N

TDR mutations that confer resistance to PIs have been identified in the past in Northern Vietnam (Phan, Ishizaki *et al.*, 2010) and in southern Vietnam (Lan, Recordon-Pinson *et al.*, 2003) but are quite rare. In this study, PI mutations were found in 4 of 16 patients with TDR mutations. Each of the identified PI mutations M46L, M46I, I54L, and L90M was detected as a single mutation in a different individual. The M46L/I mutations confer low-level resistance to nelfinavir. The I54L mutation confers high-level resistance to fosamprenavir and low-level resistance to indinavir, atazanavir, and lopinavir. The L90M mutation confers high-level resistance to nelfinavir, intermediate level resistance to saquinavir, and low level resistance to most PI drugs except the second-generation PI drugs darunavir and tipranavir. The PI mutation pattern is consistent with the history of PI use in Vietnam. Nelfinavir and indinavir were available in public private settings for patients who developed treatment intolerance to nevirapine before efavirenz and lopinavir became available in Vietnam in 2006. It has been shown that these mutations have low fitness costs and therefore can persist in the viral

populations in the absence of PI therapy (Martinez-Picado *et al.,* 1999). The detection of these as TDR mutations is concerning as LPV/r is the last treatment option for patients who fail first-line therapy with drug resistance HIV in Vietnam. These patients are participants in the VMVN clinical trial. The trial will follow these patients for 3 years and will provide the opportunity to study both the impact and the evolution of these primary resistance mutations during long-term ART.

As mentioned previously an inherent limitation of this study is that TDR might be underestimated in our study population of chronically-HIV-infected patients. The standard Sanger sequencing method used to detect drug resistance may miss minority drug resistance population present in <15-20% of the viral population as resistant viral population revert to wild type in the absence of antiretroviral drug pressure overtime. Newer high throughput sequencing methods that allow detection of resistant viral populations down to 1% will allow more accurate detection of TDR in chronically infected patients.

CONCLUSION

In summary, we report a TDR prevalence of 4.8% in 330 antiretroviral- naive individuals initiating antiretroviral therapy in Ha Noi, Vietnam from 2011 to 2013 using the 2009 WHO SDRM algorithm. The identified drug resistance mutations confer resistance to the NRTIs and/or NNRTIs used in the first-line ART regimens but also to PIs, the main component of second-line ART in Vietnam. TDR level in this study is consistent with TDR prevalence reported in previous studies, suggesting that TDR remains relatively stable despite the rapid ART scale up in Vietnam over the last 10 years.

As ART continues to be scaled up in Vietnam, it is likely that the prevalence of TDR will increase, and surveillance of TDR continues to be important to inform treatment strategies. Prevention of transmission of drug resistance HIV during ART scale up is a national priority. This highlights the needs to continue to strengthen the HIV care delivery system, the antiretroviral adherence support, and ensuring a continuous supply of antiretroviral drugs in Vietnam.

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TỶ LỆ VÀ KIỂU HÌNH KHÁNG THUỐC TRÊN BỆNH NHÂN HIV NGƯỜI LỚN CHƯA ĐIỀU TRỊ ARV TẠI HÀ NỘI, VIỆT NAM

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*** Ñoàng taùc giaû**

TÓM TẮT

Tại Việt Nam, do nhu cầu điều trị kháng virut cho bệnh nhân HIV (điều trị ARV) ngày càng gia tăng, những dữ liệu về tần suất và kiểu hình đột biến kháng thuốc trên bệnh nhân chưa điều trị ARV có vai trò quan trọng trong việc định hướng cho các chiến lược điều trị ARV trong nước. Vì vậy chúng tôi thực hiện nghiên cứu về tỷ lệ kháng thuốc và đột biến kháng thuốc trên 345 bệnh nhân nhiễm HIV chưa được điều trị ARV. Các bệnh nhân này đủ tiêu chuẩn điều trị phác đồ bậc 1 và tham gia vào nghiên cứu thử nghiệm lâm sàng "Theo Dõi Tải Lượng virut tại Việt Nam" (VMVN) tại bệnh viện Bạch Mai, Hà Nội, từ tháng 4 năm 2011 đến tháng 10 năm 2013. Để xác định đột biến kháng thuốc, một phần đoạn gen HIV *pol* được giải trình tự bằng phương pháp Sanger và đột biến kháng thuốc sau đó được xác định dựa vào "Danh sách giám sát đột biến kháng thuốc" của Tổ chức Y tế Thế giới ban hành năm 2009. Vùng protease (PR) và reverse transcriptase (RT) của gen HIV *pol* phân lập từ 330 mẫu huyết tương đã được giải trình tự thành công. 323 mẫu được xác định thuộc thứ týp

CRF01_AE; 2 mẫu thuộc thứ týp A/CRF01_AE, 2 mẫu thuộc thứ týp BC, 1 mẫu thuộc thứ týp C; 1 mẫu thuộc thứ týp F/C. Tổng cộng 16/330 (4.8%) bệnh nhân mang đột biến kháng thuốc. Trong soá ñoù, 6 (38%) bệnh nhân có đột biến kháng thuốc ức chế phiên mã ngược tương tự nucleosid NRTIs (K70E, V75M, K219N, K219E, T215S); 5 (31%) bệnh nhân có đột biến kháng thuốc ức chế phiên mã ngược phi nucleosid NNRTIs (K101E, K103N, Y181C, G190A); 4 (25%) bệnh nhân có đột biến kháng thuốc ức chế protease PI (M46I, M46L, I54L, L90M); và 1 bệnh nhân kháng cả 2 thuốc NRTI và NNRTI (L74I, V75M, M184V, T215F, K101E, G190A). Kết quả trên cho thấy ở Việt Nam mặc dù tỷ lệ bệnh nhân được điều trị ARV tăng nhanh chóng trong 10 năm qua, tỷ lệ kháng thuốc trên bệnh nhân nhiễm HIV chưa điều trị vẫn duy trì ở mức thấp. Tuy nhiên sự xuất hiện những đột biến kháng PIs trên 4 bệnh nhân chöa điều trị ARV là vấn đề đáng quan tâm và cần được nghiên cứu thêm.

Từ khoá: *Vi rút gây suy giảm hệ miễn dịch ở người, Hội chứng suy giảm miễn dịch, điều trị ARV , kháng thuốc trước điều trị ARV, Việt Nam.*

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GIẤY XÁC NHẬN

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Ban biên tập **Tạp chí Công nghệ Sinh học** xin thông báo tới nhóm tác giả về bài báo nhan đề: "**Prevalence and patterns of transmitted drug resistance in HIV-infected adult patients initiating antiretroviral therapy in Ha Noi, Vietnam**" đã xong thủ tục phản biện và chờ xếp đăng trong thời gian tới.

Xin chân thành cảm ơn và mong tiếp tục nhận được sự hợp tác!

Hà Nội, ngày 01 tháng 7 năm 2015

Tổng biên tập

VIỆN HÀN LÂM KHOA HỌC VÀ CÔNG NGHỆ VIỆT NAM
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LÊ TRẦN BÌNH