ABSTRACT

Objective

Highly active antiretroviral therapy (HAART) has led to a dramatic decrease in AIDS-related morbidity and mortality, but can be compromised by the development of HIV drug resistance. The objective of this thesis is to explore issues important to the understanding of HIV drug resistance at the populational level in British Columbia, Canada.

Methods

HIV drug resistance was analyzed via retrospective, observational analyses of the population of HIV-infected individuals receiving treatment through the HIV/AIDS Drug Treatment Program in British Columbia, Canada. Analyses were largely based upon viral protease and reverse transcriptase genotypic sequences obtained from archived plasma samples as well as information in the BC Centre for Excellence in HIV/AIDS’ monitoring and evaluation system.

Results

One analysis demonstrated a drastic, exponential decrease in the incidence of new cases of HIV-1 drug resistance over time in individuals followed from 1996-2008 that has been concomitant with linear increases in the proportion of individuals with undetectable plasma viral loads. An analysis of the probability of developing resistance, adjusted for several factors, between different initial antiretroviral regimens showed 2.4-fold lower odds of developing HIV drug resistance among individuals initiating HAART with a boosted protease-inhibitor-based regimen versus other common regimens, as well as a decreased
likelihood of developing resistance based upon initiating more modern HAART regimens. Finally, a third analysis focussed on specific K65K and K66K silent mutations in HIV reverse transcriptase, which are strongly co-selected with known thymidine analogue mutations, in particular D67N. In steady-state kinetic assays, the presence of these mutations was shown to alleviate replicative pausing and/or dissociation events of HIV-1 reverse transcriptase on RNA reverse transcriptase templates that contained the D67N and K70R mutations.

**Conclusion**

Changes in HIV drug resistance have occurred at the populational level in BC over the period of 1996-2008. The superiority of modern HAART regimens in regard to the development of resistance, and overall drastic exponential decreases in the levels of incident drug resistance over time, provide new benchmarks for the analysis of the efficacy of current and future antiretroviral regimens. Furthermore, the identification of novel silent mutations co-selected with therapy exposure may have clinical implications.
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ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Richard Harrigan, for providing me the incredible opportunity to work and learn in the BC Centre for Excellence in HIV/AIDS laboratory; his guidance, patience, and understanding over the past few years have been more valuable than I can adequately express with words. I would also like to thank my fellow students and co-workers who have provided expertise, advice, support, and encouragement. Everyone I have worked with over the past few years has earned my gratitude and deserves recognition, but in this limited space I would especially like to acknowledge the following individuals: Dr. Zabrina Brumme for being one of my mentors and an exemplary role model, Andrew Low for being a good man to drink with and an even better man to look up to, and Graham Pollock for being my best friend. Furthermore, I would like to thank the members of my thesis committee, Dr. Mark Tyndall and Dr. Anita Palepu, for their counsel and valued commentary on my work. Finally, I gratefully acknowledge the financial support of the British Columbia Centre for Excellence in HIV/AIDS through a graduate stipend.
For everyone who ever loved or believed in me
CO-AUTHORSHIP STATEMENT

Each of chapters 2-4 consists of a manuscript has been accepted for publication in an international, peer-reviewed journal. The candidate is the first author of chapter 2, the second author of chapter 3, and the third author of chapter 4. The candidate was a major contributor to the collection of laboratory data and data analysis. Furthermore, the candidate also was the lead contributor to the drafting of each of the manuscripts.

Primary co-authors of these manuscripts include the thesis supervisor (Dr. P. Richard Harrigan), statistical analysts (Dr. Viviane D. Lima, Wen Zhang), authors who provided data access and assistance with data analysis (Brian Wynhoven, Benita Yip, Dr. Robert S. Hogg, Dr. Julio SG Montaner), as well as external collaborators.
CHAPTER 1
THESIS OBJECTIVES AND GENERAL INTRODUCTION

1.1. THESIS OBJECTIVES AND ORGANIZATION

The general aim of this thesis is to address issues pertinent to antiretroviral drug resistance in Human Immunodeficiency Virus Type 1 (HIV-1). It is divided into five chapters. This first chapter provides a general introduction to HIV-1 pathogenesis, treatment with antiretroviral agents, and viral resistance to antiretroviral therapy. Chapter 2 provides an analysis of the incidence of HIV-1 drug resistance over time in a populational setting. Chapter 3 shows the results of an analysis which used a logistic regression model to predict the probability of the emergence of antiretroviral resistance in treated individuals and demonstrated the unequal risk of developing drug resistance among different types of initial antiretroviral therapy regimens. Chapter 4 shows the results of a retrospective observational analysis and accompanying biochemical assays that identified the location, and provided insight into the mechanistic importance, of newly-identified silent mutations in the HIV-1 genome that are associated with antiretroviral therapy. Chapter 5 provides a discussion and summary of the research results in the context of HIV-1 antiretroviral resistance and comments on the implications of the work as well as current advances and future research that may build upon it.

1.2. SCALE OF THE HIV EPIDEMIC

Since 1981, when inexplicable clusters of previously rare *Pneumocystis carinii* pneumonia (PCP) began to be reported in young homosexual males [1, 2], the causative
disease, Acquired Immunodeficiency Syndrome (AIDS), and its etiological agent, Human Immunodeficiency Virus (HIV) [3-7], have become health issues of a pandemic proportion. As of 2008, an estimated 33.4 million (31.1 million – 35.8 million) people worldwide are living with HIV, with 2.7 million (2.4 million – 3.0 million) new infections that year [8]. Approximately 67% of all HIV-infected individuals live in Sub-Saharan Africa, and in 2008 the same population accounted for 72% of all AIDS-related deaths [8]. In Canada, as of the end of 2007, an estimated 58,000 (48,000 – 68,000) people were living with HIV, approximately 27% of whom were undiagnosed [9].

1.3. HUMAN IMMUNODEFICIENCY VIRUS: PATHOGENESIS

HIV targets human T-lymphocytes that express the cluster of differentiation 4 (CD4) glycoprotein on their surfaces [10, 11]. Due to viral replication and indirect mechanisms that are not entirely understood, HIV causes depletion of CD4+ T-lymphocytes over time. The cellular destruction caused by HIV leads to progressive weakening of the host immune response, and untreated individuals eventually become susceptible to an array of opportunistic viral, fungal, and bacterial infections. The end stages of this immune system destruction are what define the transition from clinically asymptomatic HIV infection to AIDS. The U.S. Centers for Disease Control and Prevention currently describes the surveillance case definition of AIDS for HIV-infected individuals ≥13 years of age as having had: a) <200 CD4+ T-lymphocytes/μL; b) a CD4+ T-lymphocyte percentage of total lymphocytes of less than 14%; or c) one or more predetermined AIDS-defining illnesses. AIDS-defining illnesses include opportunistic infections (e.g. \textit{Pneumocystis carinii} pneumonia), diseases affecting the central and peripheral nervous systems (e.g. encephalopathy), malignancies (e.g. Kaposi’s sarcoma), and wasting syndrome [12].
addition to the development of AIDS, there is emerging evidence that HIV infection may be contributing to the development and progression of serious non-AIDS diseases including cancer, liver cirrhosis, renal disease, and cardiovascular diseases [13].

1.4. HIV GENETIC DIVERSITY

There are two known types of HIV: HIV-1 and HIV-2. HIV-1 is the virus that was initially discovered and it is more virulent and prevalent than HIV-2. HIV-2 was originally isolated from individuals in Western Africa and is largely confined to that region [14]. HIV-1 infection in humans most likely arose through cross-species transmission of a simian immunodeficiency retrovirus (SIV$_{cpz}$) found in chimpanzees [15, 16]. HIV-1 phylogenetic classifications are currently based either on nucleotide sequences derived from the gag, pol, and env genes of the same viral isolates, or on full-length genome sequence analysis. There has been an established classification of HIV-1 into three genetic groups: M (major), O (outlier) and N (non-M, non-O) [16, 17]. Recently, a new viral sequence closely related to gorilla simian immunodeficiency virus was isolated from a Cameroonian woman. This new virus seems to be distinct from HIV-1 groups M, N and O and has been proposed to be the first evidence of a new HIV-1 group, termed “group P”, pending its identification in more individuals [18].

Globally, more than 90% of HIV-1 infections are caused by group M viruses. Within group M, there are nine designated subtypes: A, B, C, D, F, G, H, J and K [19]. Genetic variation within a subtype can be on the order of 15 to 20%, whereas variation between subtypes is usually 25 to 35%, depending on the subtypes and genome regions being compared [20]. Inter-subtype recombinants, that are thought to have originated in individuals simultaneously infected by two or more subtypes, are also prevalent.
Recombinant viruses identified in at least three epidemiologically unlinked individuals and characterized by full-length genome sequencing are designated as circulating recombinant forms (CRFs) [19]. More than 40 CRFs have been reported [21].

The various HIV-1 subtypes and recombinants have distinct global patterns of distribution [20, 22]. Most antiretroviral drugs were designed to be active against HIV-1 subtype B, the predominant subtype in Western nations, including western and central Europe, the Americas, and Australia [22, 23]. However, the global prevalence of HIV-1 subtype B has been estimated to be only around 10% [20]. Subtype C virus accounts for almost 50% of global HIV-1 infections and it is the predominant subtype in countries which account for over 80% of global HIV-1 infections, including southern African nations and India [22]. The analyses in this thesis focus on studies of HIV-1.

1.5. BRIEF OVERVIEW OF STRUCTURE AND GENOMIC ORGANIZATION OF HIV

HIV is taxonomically classified as a member of the genus Lentiviridae, within the family of Retroviridae. Individual HIV-1 virions are approximately 110 nm in diameter and consist of a capsid core that is surrounded by a lipid bilayer envelope (Illustration 1.1). The HIV-1 genome is made up of two linked copies of single-stranded positive-sense RNA, each approximately 9 kilobases long [24]. HIV-1 RNA encodes nine open reading frames which produce 15 distinct structural, enzymatic, regulatory, and accessory proteins (for reviews of the genomic structure and lifecycle of HIV-1 see [25, 26]). Three of the open reading frames in its genome encode regions that share a common genomic structure and organization with other members of Retroviridae; specifically, \textit{gag} (group-specific antigen), \textit{pol} (polymerase) and \textit{env} (envelope) genes (Illustration 1.2).
The *gag* gene encodes the Gag polyprotein precursor (p55). The Gag polyprotein precursor contains several functional domains, which are cleaved by the viral protease to produce the major structural proteins of the virus: matrix (p17), capsid (p24), nucleocapsid (p7), and p6. Two additional interdomain peptides, p1 and p2, are also released during Gag cleavage. The *pol* gene is encoded in a different reading frame to *gag*, but both the *gag* and *pol* genes overlap by 241 nucleotides. Ribosomal frameshifting allows translation to continue beyond *gag* in order to create a gag-pol polyprotein precursor (p160) [27, 28]. The *pol*-encoded region is then cleaved by the viral protease to liberate the viral enzymes: protease, reverse transcriptase, and integrase. The *env* gene also encodes a polyprotein precursor (gp160), which is cleaved by a cellular protease [29]. The *env* gene is transcribed to produce the surface (gp120) and transmembrane (gp41) glycoprotein components of HIV envelope [29]. In addition to the *gag*, *pol*, and *env* genes, the HIV genome encodes reading frames for regulatory proteins (Tat and Rev), and accessory proteins (Vif, Vpr, Vpu, and Nef).

1.6. VIRAL LIFE CYCLE

The HIV life cycle can be described by the following series of steps: binding and fusion, reverse transcription, integration, transcription, assembly, and budding and maturation (Illustration 1.1).

**Binding and fusion**

The two viral envelope proteins, gp120 and gp41, are conformationally associated to form a trimeric functional unit consisting of three molecules of gp120 exposed on the virion
surface and associated with three molecules of gp41 inserted into the viral lipid membrane. Trimeric gp120 on the surface of the virion binds to CD4 receptors on the surface of target cells, specifically, helper T lymphocytes and macrophages [10, 11]. The binding of gp120 to CD4 induces a conformational change in the envelope proteins [30] that allows binding of the virion to one of two human chemokine co-receptors necessary for successful viral entry: CXC motif chemokine receptor 4 (CXCR4) [31] or CC motif chemokine receptor 5 (CCR5) [32-36]. The binding of surface gp120, CD4, and the chemokine co receptors allow for the insertion of the fusion peptide, located at the N-terminus of gp41, into the target cell membrane [30]. The lipid membrane of the virus fuses with the host cell and the viral core is inserted into the host cell. During entry, in what is a poorly understood process, the viral core uncoats, releasing the contents of the viral core into the host cell’s cytoplasm [26].

Reverse transcription

The conversion of viral RNA to DNA is catalyzed by the reverse transcriptase (RT) enzyme. RT is multifunctional enzyme, with two types of DNA polymerase activity: 1) RNA-dependent DNA polymerase to synthesize a DNA strand copy of the viral genomic RNA template, and 2) DNA-dependent DNA polymerase to complete the synthesis of double-strand viral DNA. In addition to its polymerase functions, RT also possesses an intrinsic RNase H activity which allows it to degrade genomic RNA. Reverse transcription proceeds in a series of steps that are described briefly below (for reviews and diagrammatic representations of reverse transcription, please see [26, 37]):
1. RT requires both a primer and a template. DNA synthesis is initiated from a host tRNA primer which binds to a region near the 5’ end of the viral genome called the primer binding site (PBS).

2. The template viral RNA genome is plus-strand. Minus-strand DNA synthesis is initiated from the tRNA primer, allowing RT to copy the 5’ end of the viral RNA genome. Synthesis of the minus-strand DNA generates an RNA/DNA hybrid that is a substrate for RNase H, which degrades the RNA strand, leaving the nascent single-stranded DNA.

3. The sequences at the 5’ and 3’ ends of the viral RNA genome, termed the “repeats”, are identical. The minus-strand DNA jumps from the 5’ repeat to the 3’ repeat of the genome. This step is referred to as the first strand transfer.

4. After the nascent DNA hybridizes to the 3’repeat region, minus-strand synthesis can continue along the viral RNA using the 3’ end of the minus-strand DNA as a primer. As DNA synthesis continues, RNase H degrades the RNA strand.

5. A purine-rich sequence (called the polypurine tract or PPT) near the 3’ end of the viral RNA is relatively resistant to cleavage by RNase H. The PPT serves as the primer for plus-strand DNA synthesis. Plus-strand DNA synthesis proceeds until RT starts to copy the tRNA primer.

6. Once the 3’ end of the tRNA has been reverse transcribed, an RNA/DNA hybrid that is a substrate for RNase H is created. RNase H removes the remaining RNA of the PPT and the tRNA primer.

7. The removal of the tRNA primer exposes a single-stranded portion of the plus-strand DNA that has the same sequence as the PBS. Exposure of the 3’ end of the plus-strand DNA allows the 5’ end of the minus-strand (once the PBS has been copied) to be hybridized
to the plus-strand, resulting in a circularization of the DNA. This step is called the second strand transfer.

8. Once this second transfer happens, both the minus and plus strands are extended until the entire DNA is double stranded. The resultant double-stranded DNA that has the same sequences at both ends (these repeats are called long terminal repeats or LTRs).

The RT and template interaction is of a relatively low affinity [38] and template switches during reverse transcription can occur. If the two RNA molecules in a particular virion are not genetically identical, template switching will result in the generation of a novel recombinant DNA genome containing sequences from both of the parental RNAs.

**Integration**

Following reverse transcription of the viral genomic RNA, the newly synthesized double-stranded viral DNA undergoes two reactions catalyzed by integrase: 3’-processing and strand transfer. 3’-processing begins in the cytoplasm, where integrase recognizes and binds to a specific sequence in the long terminal repeats (LTR) of the viral DNA [39]. Integrase then catalyzes the removal of a GT dinucleotide at the 3’ end of both strands of the viral cDNA [40, 41]. Integrase remains bound to the LTR, forming a structure called the preintegration complex (PIC). The PIC contains viral proteins (including, matrix, Vpr, p7/nucleocapsid, and reverse transcriptase), the newly transcribed viral DNA, and various host proteins [42, 43].

The PIC is actively transported to the nucleus, where the strand transfer reaction occurs [44]. The strand-transfer reaction consists of a direct nucleophilic attack on the host chromosome by the 3’-hydroxyl recessed viral DNA ends. Both ends of the viral DNA are
kept in close proximity and integrate at the 5′-ends of the host chromosomal DNA with a 5-base pair stagger [45, 46]. The two nucleotides at the 5′end of the viral DNA form a flap, which is trimmed. In order to complete integration, gaps at the 3′-end of the host genomic DNA are filled by yet-to-be identified cellular DNA repair enzymes [47]. The strand transfer reaction completes the viral DNA integration into the host chromosome and the resulting integrated viral DNA is known as a provirus, which serves as a template for the synthesis of viral RNAs by host cellular machinery.

**Transcription**

The synthesis of new virions begins with the transcription of the proviral DNA into RNA. HIV RNA transcription begins at the 5′Long-Terminal Repeat, which is a regulatory region located at the 5′ end of the viral genome. Transcription of the viral genome results in more than a dozen different HIV-specific transcripts. Initially, multiply spliced transcripts are transported to the cytoplasm and encode Nef, Tat, and Rev. Once synthesized, Tat and Rev help regulate the expression of the remaining viral RNAs. This leads to the production of singly spliced or unspliced viral transcripts that remain in the nucleus and are relatively stable. These viral transcripts encode the structural, enzymatic, and accessory proteins and represent viral genomic RNAs that are needed for the assembly of fully infectious virions [48]. The spliced viral RNA codes for envelope and other auxiliary proteins. Envelope proteins are produced in the rough endoplasmic reticulum and then move through the Golgi apparatus before arriving at the cell surface [49].
Assembly

After synthesis, the Gag (pr55) and Gag-Pol (pr160) polyproteins are targeted to the host cell plasma membrane by the MA (matrix) domain of gag [26] and help mediate the assembly of new virions as well as packaging several components of the virus particle, including viral RNA and Vpr protein.

Budding and maturation

The newly assembled virus buds from the host cell as an immature virion. During budding, the new virus acquires part of the cell's outer membrane. In HIV, the p6 protein, located at the 3’end of Gag, contains a specific sequence called the “late (L) domain”, which is responsible for mediating the release of new virus particles from the plasma membrane [50]. After the release of immature progeny virions, a final maturation step begins as the protease domains of the Gag-Pol precursor proteins become active and cleave themselves from the Gag-Pol precursor proteins [51]. The HIV protease subsequently cleaves the remainder of the Gag and Gag-Pol polyproteins, resulting in the release of the individual structural and enzymatic proteins. The resultant mature virions are able to infect new cells [26].

1.7. ANTIRETROVIRAL THERAPY

Mono/dual therapy

Antiretroviral medications work by inhibiting the replication of HIV virions, thereby mitigating their pathogenic effects. The treatment of HIV infection through antiretroviral therapy is a strikingly successful example of drug development in medicine. Before antiretroviral medications were available, the median time from infection to AIDS diagnosis
was found to range from approximately 5 to 11 years, depending on age at seroconversion with older individuals progressing faster [52]. The median survival time following seroconversion, dependent upon age at infection, was approximately 4.0 to 12.5 years, with older individuals dying sooner [52]. In the late 1980s zidovudine, a Nucleoside Reverse Transcriptase Inhibitor (NRTI), was the first antiretroviral agent which showed modest short-term improvements in the prognosis of HIV-infected individuals [53-56]. Unfortunately, the clinical benefits of zidovudine monotherapy were not robust or long-term, as symptom-free individuals infected with HIV who were immediately given zidovudine monotherapy during symptom-free HIV-infection were found to have higher CD4 counts and lower CD4 cell decline, but no significant difference in disease progression after 3 years compared to individuals who were given zidovudine at the onset of AIDS or persistently low CD4 counts [56]. Shortly after zidovudine came the additional NRTIs didanosine, zalcitabine, and lamivudine, which allowed for the use of dual-combination therapies. The use of dual combinations of antiretroviral drugs resulted in improved clinical responses and survival when compared to zidovudine monotherapy [57-63]. However, mono and dual combination therapies still only showed a relatively short period of effectiveness, largely due to the rapid selection of drug-resistant HIV variants [64-66].

**Highly active antiretroviral therapy**

It was not until the development of additional classes of antiretroviral agents and the universal recommendation of triple combination therapy in the mid-1990s, known as Highly Active Antiretroviral Therapy, or HAART, that dramatic and sustained reductions in HIV-related morbidity and mortality were observed on a population basis, resulting in a complete change in treatment outcomes for HIV-positive individuals [67-69]. There are currently 26
antiretroviral drugs and 6 fixed-dose combinations approved for the treatment of HIV by the United States Food and Drug Administration (Table 1.1). The majority of current first-line antiretroviral combination therapies include a backbone of two NRTIs in combination with either a protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI). The current recommendations of the International AIDS Society-USA, an independent body of experts in the field of HIV treatment, call for two NRTIs plus either an NNRTI or a ritonavir-boosted protease inhibitor for initial therapy [70].

**Classes of drugs**

**a) Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs)**

Nucleoside analogues are the oldest class of antiretrovirals. NRTIs mimic the structure of natural deoxynucleotide triphosphates, but lack a 3’-hydroxyl group (Illustration 1.3). This class of HIV medications works by inhibiting reverse transcription when the NRTIs are incorporated into the newly synthesized viral DNA and preventing its further elongation by acting as chain terminators [71-73]. In order to be active, NRTIs must first be phosphorylated by host enzymes to be converted to NRTI-triphosphates [71].

**b) Protease inhibitors (PIs)**

Protease inhibitors were the class of antiretroviral drugs developed to target the HIV protease enzyme (Illustration 1.4). Protease enzymes are proteins that cut other proteins at specific locations. The HIV protease is an aspartyl protease enzyme similar to other retroviral proteases [74]. PIs target the viral protease through direct interactions that disturb its ability to cleave nascent viral proteins into functional products. Through a “lock-and-key” mechanism, PIs bind to the protease enzyme and disable it before it can cleave the gag-pol polyprotein into its products that are essential for virion production [74].
c) **Boosted versus non-boosted PIs**

The dosing of protease inhibitors has been improved by reductions in their pill burdens and improvements in pharmacokinetics through the concomitant administration of low-dose ritonavir as a boosting agent [75-77]. Boosted PIs work by taking advantage of the fact that ritonavir inhibits the liver enzyme cytochrome P450 3A, which metabolizes protease inhibitors. Therefore, low-dose ritonavir is used to maintain higher concentrations of PIs for longer periods of time. Ritonavir-boosting has been shown to have several positive effects on the pharmacokinetics of PIs, including raising their minimum plasma concentration during dosing intervals \( (C_{\text{min}}) \), reducing interpatient variability in drug exposure, and prolonging the half-life of PI elimination [77]. Improvements in \( C_{\text{min}} \) correlate with maintaining higher effective concentrations of PIs that are able to inhibit viral replication [78], reductions in interpatient variability of drug exposure increase the reliability of PIs for different individuals. Prolonging the half-life allows for less frequent dosing of PIs and diminishes the requirement of taking them with food, thus making dosing more convenient for patients.

Attempts to improve PI efficacy by increasing dosages would decrease the convenience of PI regimens by requiring more frequent dosing or larger tablet volumes. Furthermore, for patients who metabolize PIs quickly, or experience an inherently low bioavailability to the drugs, increased dosing would not be expected to create substantial improvements in \( C_{\text{min}} \) [77].

In a double-blinded, randomized, controlled study of lopinavir boosted with ritonavir versus nelfinavir, each administered with stavudine and lamivudine, the results indicated a strong superiority for the boosted lopinavir regimen in regard to resistance [79]. For nelfinavir-treated patients, a maximum 20% probability of nelfinavir resistance was observed
with 85%-90% adherence levels. Patients treated with boosted lopinavir, in contrast, showed no lopinavir resistance. Furthermore, each regimen showed an effect upon lamivudine resistance, with a maximum probability of 50% at 75%-80% adherence to the nelfinavir regimen and of 15% at 80%-85% adherence to the boosted lopinavir regimen [79].

d) Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

Like the NRTIs, NNRTIs inhibit viral replication by targeting reverse transcription in the viral lifecycle (Illustration 1.5). The NNRTIs non-competitively inhibit viral replication by directly targeting and interacting with the viral reverse transcriptase at a hydrophobic pocket which lies adjacent to the active site of the reverse transcriptase enzyme [80]. The binding of NNRTIs results in a disabling conformational change in the reverse transcriptase enzyme.

e) Entry inhibitors

Entry inhibitors are unique from the other approved classes of antiretroviral medications in that they do not target viral enzymes. There are currently two FDA-approved antiretroviral medications which block the entry of HIV into target cells. Enfuvirtide is a thirty-six amino acid peptide that binds to gp41 and blocks HIV entry by acting as a steric inhibitor of the conformational change in the Env protein that allows for fusion of the virions and target cell [81, 82]. Maraviroc is a CCR5-antagonist which blocks HIV entry by antagonistically binding the CCR5 co-receptor [83].

f) Integrase inhibitors

This class of drugs inhibits the strand transfer function of the enzyme integrase, which is responsible for integration of viral DNA into the DNA of the infected cell [84]. By blocking integration, integrase inhibitors block incorporation of HIV-1 DNA into the host
genome and prohibit the establishment of viral latency within the host cell, preventing high-level HIV-1 replication and infection of new cells by competent virus. There are several integrase inhibitors currently under clinical trial, and in October 2007, the United States Food and Drug Administration approved raltegravir, the first drug in the integrase-inhibitor class [85], for the treatment of HIV-1 as part of HAART in treatment-experienced patients.

g) Maturation inhibitors: A new drug class in late-stage development

There are currently no approved maturation inhibitors, though a candidate drug, bevirimat, is undergoing Phase II clinical development. Bevirimat inhibits the processing of the Gag polyprotein by blocking the cleavage of the spacer peptide (p2) from p25 through a direct interaction between bevirimat and the Gag protein [86]. This inhibition prevents the conversion of the polyprotein into the mature capsid protein (p24), and results in noninfectious viral progeny with aberrant cores [86].

1.8. ANTIRETROVIRAL RESISTANCE

The advent of highly active antiretroviral therapy (HAART) has resulted in marked improvements in clinical outcomes for patients infected with human immunodeficiency virus type 1 (HIV-1) [67, 87]. However, a major hindrance to the effectiveness of HAART is the development of viral resistance to antiretroviral agents [88, 89]. Antiretroviral resistance is a result of mutations occurring in the wild-type viral genome that produce variants that can efficiently replicate in the presence of antiretroviral drugs. HIV antiretroviral resistance has negative implications for treatment outcome, including poorer virologic response to a new treatment regimen when prior therapy has failed [90], the limitation of future therapeutic options [90], and a higher risk of death for people initiating their first HAART regimen [89].
Resistance of HIV to antiretroviral agents was first identified for zidovudine. Larder et al. analyzed the zidovudine susceptibility of 46 HIV isolates from 33 individuals via an *in vitro* plaque reduction assay [91]. This study showed several novel findings, including that sequential isolates from several individuals receiving zidovudine displayed step-wise increases in resistance and several isolates of those isolates had large (>100-fold) increases in the IC₅₀ of zidovudine compared to viral isolates from untreated patients. Furthermore, isolates which showed relatively small increases in their IC₅₀, but large increase in their IC₉₅, provided evidence of minority resistant viral strains existing in an otherwise zidovudine-sensitive viral population. Subsequent studies were conducted to characterize the mechanism of zidovudine resistance [92, 93], and the first correlation of mutations in HIV-1 reverse transcriptase gene with antiretroviral resistance was reported [93]. *In vitro* studies identifying zidovudine resistant virus continued [94, 95], and were also supplemented by studies which aimed to find the clinical significance of zidovudine resistance [64, 96, 97]. The direct causal link between *in vivo* zidovudine resistance, while on zidovudine, and poor clinical outcomes was not as robustly characterized as it would be for future regimens, but evidence did correlate zidovudine resistance and poor patient outcomes.

Antiretroviral resistance is caused by mutations selected in the viral proteins targeted by antiretroviral agents. Three characteristics of HIV ensure that patients have a complex and diverse mixture of viral quasispecies, each differing by one or more mutations, which ease the development of antiretroviral resistance: 1) high levels of virus production and turnover, 2) the reverse transcription of viral DNA to RNA is extremely error-prone, and 3) HIV infection is life-long, with latently infected cells and viral reservoirs preventing even the most efficient antiretroviral regimens from entirely clearing infection [98-100].
place the number of infected cells in the lymphatic systems of untreated individuals at $10^7$ to $10^8$ cells [101]. The propensity of the viral reverse transcriptase to introduce errors results in an average one mutation for each viral genome [102, 103], with most of these errors being base substitutions. However, duplications, insertions, and recombination can also occur.

1.9. RESISTANCE TESTING: GENOTYPING AND PHENOTYPING

There are two main methods to test for HIV drug resistance. The phenotypic method involves using tissue-culture systems to grow virus in the presence of different drugs, measuring its susceptibility to them by comparing their replication to that of wild-type strains. The interpretation of the phenotypic method is based upon culturing viral isolates in the presence of different drug concentrations in order to ascertain the IC$_{50}$ or IC$_{90}$ (the concentrations required for a 50% and 90% reduction in viral replication, respectively) of that drug for the isolate and comparing that value to the IC$_{50}$ or IC$_{90}$ for a drug-susceptible control virus in order to generate a fold-change in the IC$_{50}$ or IC$_{90}$ for the patient virus [88, 90]. The genotypic method involves sequencing portions of the viral genome and looking for the presence of pre-defined mutations known to cause antiretroviral resistance. Genotyping results can be interpreted by consulting lists of drug resistance mutations, computerized rules-based algorithms, or the use of a “virtual” phenotype. The virtual phenotype compares patient viral genotypes with large databases of linked genotypic and phenotypic data from other viruses in order to look for similarities between patient virus and known drug resistant isolates [88]. Phenotyping is both laborious and relatively expensive. Genotyping, on the other hand, is relatively inexpensive and less time-consuming than phenotyping but it does not provide a direct measure of viral susceptibility to drugs, and therefore the interpretation of the sequences requires that they be more indirectly linked to clinical outcomes.
Since the goal of genotyping and phenotyping is to determine to what degree a patient is likely to respond to a particular drug, it is useful to interpret results from resistance assays in the context of cut-offs or break points. The interpretation of phenotypic data is based on the measurement of the fold change for each antiretroviral drug tested against pre-defined cut-offs. In order to interpret phenotypic results, the appropriate cut-offs for defining a clinical isolate as either drug susceptible or drug resistant need to be determined. Previously, “technical” cut-offs were used, to interpret phenotypic resistance tests. These cut-offs were based on the limits of the technical reproducibility of phenotypic assays, were uniform for all drugs investigated, and were not clinically derived [104, 105]. An improvement over technical cut-offs was the introduction of “biological” cut-offs, which are based on the distribution of the drug susceptibility of isolates from thousands of treatment-naive patients [106, 107]. Using the biological cut-off, clinical isolates are scored as susceptible to a certain drug if the fold change falls within the mean fold change observed with samples from treatment-naive patients, plus two standard deviations. Neither technical cut-offs nor biological cut-offs provide a link between drug susceptibility measured \textit{in vitro} and the virological response observed \textit{in vivo}.

“Clinical” cut-offs, use data from clinical trials or cohort studies to determine the changes in susceptibility that lead to compromised virologic response [108, 109]. Clinical cut-offs are derived by determining the relationship between fold changes measured at baseline and the reduction in viral load after a defined period of treatment. The two values in fold-change that comprise the clinical cut-offs are the value at which clinical responses diminish compared with wild-type virus (lower clinical cut-off), and the value at which no clinical response can be expected (upper clinical cut-off) [88]. Clinical cut-offs correlate fold
changes in viral susceptibility with clinical outcomes, but require large samples of data in order to be generated [104, 108, 109]. Furthermore, it is difficult to extrapolate the activity of individual drugs to how they will behave in the context of ever-changing combination therapy. Therefore, as data from cohorts and clinical trials continues to become available, clinical cut-offs will need to be continually refined and validated [108, 109].

1.10. RESISTANCE MUTATIONS

There are several different mutations which confer resistance to the different classes of drugs. Resistance mutations can be characterized as either being major or minor, with major resistance mutations usually being those that are those shown at the biochemical or virologic level to lead to an alteration in drug binding or an inhibition of viral activity or replication [110]. Minor mutations usually come about later than major mutations and on their own do not confer a major effect on phenotype [110]. It should be noted that the definition of “major” versus “minor” mutations is not static and can change over time. With the accumulation of data over time, mutations can be reclassified or removed from consideration altogether [110-112].

Mutations are annotated by the one-letters code for wild-type amino acid, followed by the position number of the amino acid, and finally by the mutant amino acid (e.g. the D30N PI mutations indicates a change from an aspartic acid residue to an asparagine at position 30 of HIV-1 protease) [112]. For the PIs, major mutations of note include D30N, I50L/V, V82A/F/S/T/L, and L90M. Major mutations of note for the NNRTIs include K103N, V106A/M, Y181C, and Y188C/L/H. For NRTIs, resistance can come about via two distinct methods: discrimination and excision. Discrimination mutations allow the HIV-1 reverse transcriptase to preferentially add the natural dNTP as opposed to the NRTI. Discrimination
mutations of note include K65R, K70E, L74V, M184V, and Q151M. Excision mutations allow the excision of a chain-terminating NRTI, and are brought about by a class of mutations called the thymidine analogue-associated mutations (TAMs), so-named because of their common selection in regimens which contain the thymidine analogues zidovudine or stavudine. The TAMs include M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E.

1.11. RESISTANCE IN UNTREATED PATIENTS

A distinction can be made between transmitted resistance and selected resistance. Transmitted resistance occurs when an individual receives drug resistant virus during initial infection, while selected resistance occurs when the viral population in a person evolves due to the selective pressure of drug use. Awareness of transmitted drug resistance is important, because recent studies have shown that transmitted drug-resistant strains can persist for several years as dominant quasispecies and possibly longer as minority strains which have the potential to impact antiretroviral therapy in the long-term [113-117].

1.12. RESISTANCE IN TREATED PATIENTS: IMPORTANCE OF THE RESISTANCE-ADHERENCE RELATIONSHIP

Among individuals who develop resistance as a consequence of exposure to antiretroviral medications, the level of adherence to those drugs has been shown to be one of the strongest predictors of the development of resistance [118-120]. A retrospective study of the HAART Observational Medical Evaluation and Research Cohort (HOMER), a large cohort of antiretroviral-naïve individuals initiating HAART in British Columbia, found low CD4 cell count, high baseline plasma viral load (pVL), and substantial but imperfect levels of adherence to HAART were determinants of the development of resistance [118]. Adherence is defined as the level at which an individual takes their antiretroviral drugs as prescribed,
where a 100% adherence level would indicate never missing a dose of medication. The results of this study indicated that patients who refilled their antiretroviral prescriptions 80% to <90% of the time, as prescribed, were at an elevated risk for harboring drug resistance mutations. It should be noted that prescription refill percentage is a calculation of the amount a patient refills prescriptions as they are prescribed, and serves as a surrogate marker of adherence rather than a direct measure. Individuals that had ≥95% adherence and no abnormally low plasma drug concentrations were at a substantially lower risk for developing resistance than these other groups. The plausible biological explanation for this is that at these adherence levels, enough drug is provided to create a strong selective pressure for drug resistant strains, however not enough drug is available to keep viral replication suppressed to a level that prevents the virus from actively reproducing.

In a treatment-experienced population, the effects of adherence were found to vary from those found in the treatment-naïve individuals of HOMER. Analysis of the Research in Access to Care for the Homeless Cohort (REACH), a cohort of HIV-positive urban poor individuals in San Francisco that are not necessarily antiretroviral-naïve at enrolment, has shown that high levels of adherence are not helpful in preventing the accumulation of drug resistance mutations [121]. This study found that 23% of all drug resistance occurred in individuals who were 92% to 100% adherent, as measured by unannounced pill counts, and over 50% of all drug resistance occurs in individuals who are adherent 79% to 100% of the time.

A computer simulation was performed to investigate the heterogeneity between the adherence-resistance curves for treatment-naïve and treatment-experienced populations [122]. This study found that a simulated cohort which starts out with no drug resistance
mutations has an adherence-resistance curve (i.e., a plot of the level of resistance versus increasing levels of adherence to antiretroviral medications) that is high in amplitude and shows a relatively steep downward slope over commonly observed adherence levels, much like the HOMER cohort. As prior exposure to antiretrovirals and initial presence of drug resistance mutations were increased in the simulation, the curve showed a lower amplitude peak and a shallower downward slope over commonly observed adherence levels, thereby resembling the curve for the REACH cohort. These observations show that adherence effects are different for treatment-naïve and experienced individuals.
Table 1.1. United States Food and Drug Administration-approved antiretroviral medications for the treatment of HIV infection

<table>
<thead>
<tr>
<th>Class and Generic Name</th>
<th>Brand Name</th>
<th>Approval Date</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleoside Reverse Transcriptase Inhibitors (NRTIs)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>zidovudine, azidothymidine, AZT, ZDV</td>
<td>Retrovir</td>
<td>March 1987</td>
</tr>
<tr>
<td>didanosine, dideoxyinosine, ddI</td>
<td>Videx</td>
<td>October 1991</td>
</tr>
<tr>
<td>zalcitibine, dideoxycytidine, ddC</td>
<td>Hivid</td>
<td>June 1992</td>
</tr>
<tr>
<td>stavudine, d4T</td>
<td>Zerit</td>
<td>June 1994</td>
</tr>
<tr>
<td>lamivudine, 3TC</td>
<td>Epivir</td>
<td>November 1995</td>
</tr>
<tr>
<td>lamivudine and zidovudine</td>
<td>Combivir</td>
<td>September 1997</td>
</tr>
<tr>
<td>abacavir, ABC</td>
<td>Ziagen</td>
<td>December 1998</td>
</tr>
<tr>
<td>enteric coated didanosine, ddI EC</td>
<td>Videx EC</td>
<td>October 31 2000</td>
</tr>
<tr>
<td>abacavir, zidovudine, and lamivudine</td>
<td>Trizivir</td>
<td>November 2000</td>
</tr>
<tr>
<td>tenofovir, TDF</td>
<td>Viread</td>
<td>October 2001</td>
</tr>
<tr>
<td>emtricitabine, FTC</td>
<td>Emtriva</td>
<td>July 2003</td>
</tr>
<tr>
<td>tenofovir disoproxil fumarate and emtricitabine</td>
<td>Truvada</td>
<td>August 2004</td>
</tr>
<tr>
<td><strong>Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nevirapine, NVP</td>
<td>Viramune</td>
<td>June 1996</td>
</tr>
<tr>
<td>delavirdine, DLV</td>
<td>Rescriptor</td>
<td>April 1997</td>
</tr>
<tr>
<td>efavirenz, EFV</td>
<td>Sustiva</td>
<td>September 1998</td>
</tr>
<tr>
<td>etravirine, ETV</td>
<td>Intelence</td>
<td>January 2008</td>
</tr>
<tr>
<td><strong>Protease Inhibitors (PIs)</strong></td>
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<td></td>
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<tr>
<td>saquinavir mesylate, SQV</td>
<td>Invirase</td>
<td>December 1995</td>
</tr>
<tr>
<td>ritonavir, RTV</td>
<td>Norvir</td>
<td>March 1996</td>
</tr>
<tr>
<td>indinavir, IDV</td>
<td>Crixivan</td>
<td>March 1996</td>
</tr>
<tr>
<td>nelfinavir mesylate, NFV</td>
<td>Viracept</td>
<td>March 1997</td>
</tr>
<tr>
<td>saquinavir, SQV</td>
<td>Fortovase</td>
<td>November 1997</td>
</tr>
<tr>
<td>amprenavir, APV</td>
<td>Agenerase</td>
<td>April 1999</td>
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<tr>
<td>lopinavir and ritonavir, LPV/RTV</td>
<td>Kaletra</td>
<td>September 2000</td>
</tr>
<tr>
<td>atazanavir sulfate, ATV</td>
<td>Reyataz</td>
<td>June 2003</td>
</tr>
<tr>
<td>fosamprenavir calcium, FOS-APV</td>
<td>Lexiva</td>
<td>October 2003</td>
</tr>
<tr>
<td>tipranavir, TPV</td>
<td>Aptivus</td>
<td>June 2005</td>
</tr>
<tr>
<td>darunavir, DRV</td>
<td>Prezista</td>
<td>June 2006</td>
</tr>
<tr>
<td><strong>Entry Inhibitors</strong></td>
<td></td>
<td></td>
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<tr>
<td>enfuvirtide, T-20, ENF</td>
<td>Fuzeon</td>
<td>March 2003</td>
</tr>
<tr>
<td>Maraviroc, MVC</td>
<td>Selzentry</td>
<td>August 2007</td>
</tr>
<tr>
<td><strong>Integrase Inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raltegravir, RGV</td>
<td>Isentress</td>
<td>October 2007</td>
</tr>
<tr>
<td><strong>Multi-class Combinations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>efavirenz, emtricitabine, and tenofovir disoproxil fumarate</td>
<td>Atripla</td>
<td>July 2006</td>
</tr>
</tbody>
</table>

Note: FDA data is provided because it is easily available [123] and because it is usually one of the first agencies pharmaceutical companies will apply to for approval of their drugs.

FDA approval for antiretroviral agent will therefore usually precede approval from Health Canada.
Illustration 1.1. Structure and lifecycle of HIV-1

Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, [124], copyright 2003
HIV-1 genome, HXB2 strain (above). Open reading frames are shown as rectangles. The gene start, indicated by the small number in the upper left corner of each rectangle, normally records the position of the a in the ATG start codon for that gene, while the number in the lower right records the last position of the stop codon. For pol, the start is taken to be the first T in the sequence TTTTTTAG, which forms part of the stem loop that potentiates ribosomal slippage on the RNA and a resulting -1 frameshift and the translation of the Gag-Pol polyprotein. The tat and rev spliced exons are shown as shaded rectangles. In HXB2, *5772 marks position of frameshift in the vpr gene caused by an "extra" T relative to most other subtype B viruses; †6062 indicates a defective ACG start codon in vpu; †8424, and †9168 mark premature stop codons in tat and nef. Image used with permission from HIV Sequence Database Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM (http://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html)
Illustration 1.3. Chemical structure of cytidine, and the corresponding NRTI analogue, Lamivudine
Illustration 1.4. Chemical structure of the PIs Atazanavir and Ritonavir
Illustration 1.5. Chemical structure of the NNRTIs Efavirenz and Nevirapine
1.13. REFERENCES


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CHAPTER 2

IMPROVED VIROLOGICAL OUTCOMES IN BRITISH COLUMBIA CONCOMITANT WITH DECLINING INCIDENCE OF HIV-1 DRUG RESISTANCE DETECTION¹

2.1. INTRODUCTION

Since the introduction of combination drug regimens to treat HIV infection, known as highly active antiretroviral therapy (HAART), the rates of HIV-related morbidity and mortality have been markedly reduced [1, 2]. Over time, there has been an increased variety and availability of antiretroviral drugs. However, sub-optimal therapy response can lead to the development of antiretroviral drug resistance, which is a significant barrier to the future success of therapy. Virological failure while on HAART is dependent upon several factors, including drug toxicity, insufficient adherence to therapy, and problems with drug pharmacokinetics.

The prevalence of drug resistance can be defined as the amount of drug resistance present in an investigated population in a given period of time. Several cross-sectional studies have investigated the prevalence of HIV drug resistance in relatively small populations [3-5], as well as in larger populations [6-8]. Some of these studies have investigated the prevalence of transmitted resistance, while others have addressed the prevalence of acquired drug resistance in patients on treatment. While studies that determine prevalence are important for assessing the current impact of HIV drug resistance, a critical indicator of the future success of HAART is the incidence of

resistance over time on therapy, which remains relatively poorly defined. The incidence of drug resistance is defined as the number of new cases of drug resistance that develop in a population at risk for developing drug resistance during a given period of time. Thus far, one of the few studies that has investigated HIV drug resistance incidence on the scale of a large treatment population occurred in Portugal and showed that the crude incidence of both multi-drug and full drug class resistance has decreased over time [9]. We undertook this study to evaluate the incidence rate of HIV drug resistance after starting therapy, reflecting the entire population of individuals being treated for HIV-1 infection in British Columbia from July 1996 to December 2008.

2.2. METHODS

HIV/AIDS drug treatment program

The British Columbia Centre for Excellence in HIV/AIDS (the Centre) distributes antiretroviral agents at no cost to all eligible HIV-infected individuals through its Drug Treatment Program (DTP). This program has been described in detail elsewhere [10]. The Centre's HIV/AIDS Drug Treatment Program has received ethical approval from the University of British Columbia Ethics Review Committee at its St. Paul's Hospital site.

Study population

Longitudinal plasma viral load (pVL) and genotypic resistance data were obtained from all archived plasma samples of patients receiving antiretroviral therapy from the DTP from July 1996 to December 2008 (N = 8016), regardless of when they initiated therapy and whether they started on mono/dual drug regimens or HAART. A total of 7730 (96%) had at least one pVL measured. In total, 24,652 resistance tests were available from 5422 (70%) individuals. The prevalence of successful pVL suppression
and the incidence rate of detection of resistance to each of three antiretroviral categories - nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) - were calculated for the population receiving therapy. The incidence rates of the most common individual drug resistance mutations in each class were also investigated. These were: V82A/F/T/S and L90M in the protease, associated with PI resistance; M41L, M184V/I and T215F/Y in reverse transcriptase, associated with resistance to the NRTIs; and K103N, Y181C/I in reverse transcriptase, associated with NNRTI resistance [11]. For all incidence calculations, individuals were included in the denominator (i.e., they were considered at risk for developing resistance) until the first detection of the resistance of interest. From this point, subjects were considered resistant to that drug class until the end of follow-up, but still at risk for developing further mutations and resistance to other antiretroviral classes. Time at risk, in a given year, was defined in terms of total person-months of exposure to each of the antiretroviral categories investigated, with the exception of 1996, where all available time on antiretrovirals prior to the end of 1996 was used in the incidence calculation for that year. Time on therapy was recorded from 1992 onwards, and therefore time prior to 1992 was not available. A total of 338 individuals (4%) have missing data for their time on therapy prior to 1992. Detection of resistance was only considered from July 1996 onwards, when samples became available for resistance testing. Therefore, the incidence rates calculated for the year 1996 include the cumulative number of resistance cases developed, and cumulative available exposure time from 1992 to the end of 1996.
Data collection

HIV-positive individuals receiving antiretroviral treatment in British Columbia are entered into an Oracle-based monitoring and evaluation system that uses standardized indicators to prospectively track their antiretroviral use and clinical health status [12]. The Centre’s Treatment Guidelines recommend that pVLs and CD4 cell counts be monitored at baseline, at four weeks after starting antiretroviral therapy, and every three months thereafter. pVLs are determined with the Roche Amplicor Monitor assay (Roche Diagnostics, Laval, Quebec, Canada), using the standard method until April 1999 and the ultrasensitive adaptation thereafter.

Resistance testing was performed on stored pVL samples extracted manually or automatically using guanidinium-based buffer, followed by ethanol washes. Genotyping does not yield consistently successful results on samples with low pVL; therefore, samples with pVL <50 copies/mL could not be genotyped and those with pVL ≥50 copies/mL to <250 copies/mL were rarely genotyped. Protease (PR) and reverse transcriptase (RT) genes were amplified from plasma HIV-1 RNA using nested RT-PCR as described previously [13]. PCR products were sequenced in both the 5’ and 3’ directions using an ABI automated sequencer, and a consensus sequence was generated. Results of the genotyping analysis are reported here as amino acid changes in the HIV-1 PR and RT with respect to a wild-type reference sequence (HIV-1 HXB2). Samples were considered resistant if they displayed one or more major resistance mutations in any of the three categories: any NRTI, any NNRTI, or any PI. These resistance categories are based upon the key resistance mutations from the IAS-USA table [14].
Of note, a specific group of patients in our study were systematically genotyped more frequently than would have occurred due to clinical indications alone. These individuals were members of the HAART Observational Medical Evaluation and Research (HOMER) cohort who were antiretroviral-naïve before beginning a HAART regimen between 1 August 1996 and 30 September 1999. More detailed descriptions of this study population have been published elsewhere [10, 15]. For this particular subset of patients, HIV drug resistance genotyping was systematically attempted on all plasma samples with pVL \( \geq 1000 \) copies/mL collected in the first 30 months of treatment.

We conducted two sensitivity analyses, the first of which addressed the impact of the time of therapy initiation on the incidence rate. For this sensitivity analysis we repeated the original analysis, stratifying patients by the date they began antiretroviral therapy in the following groups: 1987-1995, 1996-1999, 2000-2004, and 2005-2008. We also conducted a sensitivity analysis to assess the impact of patients with no baseline (pre-therapy) genotypes, and potential transmitted resistance they may harbor, on our calculation of the incidence rate. For this sensitivity analysis, we repeated the original analysis only on those individuals who had an available baseline genotype.

**Outcome measures**

The primary outcome in this analysis was emergence of drug resistance in any of the three resistance categories described previously (yes vs. no) and the on-therapy variable investigated was pVL (log_{10} transformed). In 1996, the lower limit of the viral load assay was changed from 500 to 400 copies/mL. In April 1999, the lower limit of the viral load assay changed again from 400 to 50 copies/mL with the introduction of the ultra-sensitive adaptation. Since pVL was measured over time starting in 1996, our
earliest pVLs were obtained based on the standard pVL assay and our most recent measurements were obtained based on the ultrasensitive pVL assay. Thus our lower and upper limits of pVL ranged over time from 500 and 1x10^6, to 400 and 7.5x10^5, and then to 50 and 1x10^5 copies/mL, respectively.

**Statistical analyses**

Calculations for the incidence rate of resistance detection in each antiretroviral category were conducted using the number of new cases of resistance detected in each year (i.e., excluding previously identified resistance) divided by the number of person-months of exposure to antiretroviral drugs within each category. Incidence rates were natural log-transformed and plotted against calendar year. The proportion of individuals achieving a plasma viral load less than 50 copies/mL within a given year was calculated from 2000 to 2008. Trend analyses were conducted with linear or log-linear regressions and reported in order to estimate the slope of any change in the incidence rate. $R^2$ values were generated for the regressions.

**2.3. RESULTS**

The raw numbers of patients with newly detected resistance suggest that there have been significant declines in the occurrence of new HIV-1 drug resistance over the period from 1996-2008 (Table 2.1). The number of incident cases of new resistance to any category of antiretrovirals was 571 in 1996, considerably more than the 71 cases detected in 2008, despite increased exposure to antiretroviral therapy in 2008 and a cumulative exposure of 461,787 patient-months of therapy (Table 2.1). These results were broadly similar across all the drug classes after the year 2000. Note that resistance
to both the PI and NNRTI classes showed an initial rise in the absolute number of cases period soon after their introduction in 1996 (Table 2.1), but the number has subsequently declined. As an illustration, only 14 new cases of PI resistance were detected in the entire province of British Columbia in 2008, despite over 30,000 patient-months of PI therapy in 2008 and a cumulative total of over 269,000 patient-months of PI therapy by the end of 2008.

When expressed as the incidence rate per 100 patient-months of therapy for resistance to any drug category, there has been an exponential decay in resistance incidence since 1996, with a half-time (the time it takes for the incidence rate to drop to half its original value) of approximately 3.2 years ($R^2 = 0.98$, $p<0.001$) (Figure 2.1a). Overall, the incidence rate dropped approximately 12-fold, from 1.73 cases per 100 person-months in 1997 (the first year with complete exposure time and incidence available) to 0.13 cases per 100 person-months in 2008. This trend toward exponential decrease was consistent across the drug classes (Figure 2.1b). The incidence rate of resistance to the NRTIs decreased exponentially with a half-time of 2.9 years ($R^2 = 0.98$), while for the PI class the half-time was 2.0 years ($R^2 = 0.98$; see Figure 2.1b). The incidence of NNRTI resistance per 100-patient months of therapy has been consistently higher than the other drug classes, and has been decreasing with a half-time of 2.5 years ($R^2 = 0.94$; Figure 2.1b). The incidence of PI resistance is now the lowest of the three classes (Figure 2.1b).

When the commonly occurring major NRTI resistance mutations were investigated individually, each showed broadly similar decreasing trends over time (Figure 2.2a). M41L and T215F/Y are the most commonly occurring thymidine analog-
associated mutations ("TAMS"), while M184V/I are associated with resistance to
3TC/FTC and are the most common NRTI mutations. The incidence of M41L and
T215F/Y RT mutations decreased with a half-time of 2.0 years and 1.8 years,
respectively. The incidence of M184V/I mutations decreased more slowly in comparison
(half-time = 2.8 years, $R^2 = 0.96$). The incidence of the NNRTI mutations K103N and
Y181C/I decreased with a half-time of 2.9 years and 2.4 years, respectively ($R^2 = 0.96$ for
both mutations), while the incidence of the PI resistance mutations V82A/F/T/S and
L90M each decreased with a half-time of 1.9 ($R^2 = 0.86$) and 1.7 years ($R^2 = 0.96$),
respectively. Note that although the incidence of the most common mutations is
decreasing, it is unlikely that all individual mutations are decreasing. Some may even be
increasing as different therapies are introduced. For example, the incidence of the RT
mutation K65R has not shown a clear trend towards decreasing incidence over the period
1996-2008 (data not shown).

The declines in the incidence of resistance can be partially explained by higher
rates of virological suppression over time. The lowest (Figure 2.3a) and highest (Figure
2.3b) recorded median pVL for each patient on therapy in BC have decreased
dramatically since 1996. In 1996, the median lowest pVL recorded was $3.76 \log_{10}$
copies/mL, and by 2007 it was below the lower limits of detection for the assay (<50
copies/mL). The range of values recorded for the lowest patient pVL has also decreased
over time, as indicated by the shrinking interquartile range (Figure 2.3a). There has been
a roughly linear increase in the proportion of individuals with pVL suppression over time
below the limit of detection of the viral load assay (64.7% <50 copies/mL in 2000 to
87.0% in 2007; $R^2 = 0.97$, p<0.001) (Figure 2.3c).
When stratified by time of therapy initiation, the trend of an exponentially decreasing incidence rate is confirmed (Figure 2.4). This is consistent across all four periods of therapy initiation, and all the drug classes investigated. There is a temporal trend towards a faster decline in incidence rate by the period of therapy initiation (see slopes of regression lines for 2000-2004 and 2005-2008 vs. 1987-1995 and 1996-1999) (Figure 2.4). Also, there have been smaller trends for improvement even within the first year of therapy, as the first two incidence rates for initiation from 2000-2004 and 2005-2008 are lower than the first two rates for those who initiated therapy between 1987-1995 and 1996-1999. Finally, we also performed a sensitivity analysis restricting the analysis to individuals with available pre-therapy genotypes (N=2571), and confirmed the declining incidence to any category of resistance over time ($R^2=0.89$, half-time = 3.0 years). Note, however, that this group appeared to show small trends towards a levelling off in incidence decline in later calendar years (data not shown).

### 2.4. DISCUSSION

Based on a province-wide cohort of individuals followed longitudinally, our results demonstrate that there has been a drastic decrease in the incidence of new cases of HIV-1 drug resistance, despite increases in annual (and especially cumulative) exposure to antiretrovirals. This has occurred alongside a steady increase in the proportion of treated patients achieving virological suppression. Remarkably, the incidence of resistance per person-month of therapy appears to decline with increasing duration of therapy (Figure 2.4). This is consistent across all drug classes and years of initiation. Improvements over time in HAART, including the periodic introduction of new therapeutic agents and continual assessment and improvement of how those agents are
prescribed, have most likely contributed to decreases in the incidence rate of HIV-1 drug resistance detection. Before 1996, drugs were prescribed as monotherapy or dual-therapy combinations, which resulted in the rapid selection of drug resistance. Initial increases in the incident cases of drug resistance to NNRTIs and PIs are most likely linked to patients previously exposed to mono/dual therapy who had developed NRTI resistance and were therefore more likely to develop further resistance because of compromised HAART regimens (Table 2.1). The relative “fragility” of the NNRTI class likely accounts for both the higher incidence of NNRTI resistance per patient-month of therapy, as well as the slower decline in the rate of selection of NNRTI resistant virus. However, even for this drug class the decrease in resistance incidence has been remarkable, with an over 40-fold decrease in NNRTI resistance per 100 person-months of NNRTI exposure from 1996 to 2008.

Our results complement those found by Vercauteren et al. [8], which is most likely a reflection of both studies being carried out on large populations with free access to HAART. However, because we had detailed information about each patient’s antiretroviral regimen and time on therapy, we were able to analyze the incidence in terms of rate per person-months of drug exposure. Particular strengths of this analysis are its size and the ability to monitor an entire HIV treated population. The centralized system allows monitoring of both the amounts of therapy dispensed as well as the laboratory measures of viral load and resistance. In addition, resistance testing is widely used in BC, with almost 25,000 resistance tests performed on 5422 individuals.

While the data shown are encouraging, there are limitations. This analysis is observational and, therefore, cannot definitively establish a causal relationship between
reductions in the incidence of resistance detection and concomitant decreases in pVL. Another limitation is that not every person on therapy is monitored clinically in exactly the same way (e.g., some will be missing baseline resistance data, some will be monitored more heavily than others, etc.). The frequency of genotyping due to clinical indications varies between individual patients in the population and depends upon several factors, including date of therapy initiation, differences between physicians in the number of tests requested, and whether or not a particular patient sample has a sufficiently high pVL to genotype. These factors could contribute to producing a detection bias. Approximately 50% of patients with at least one viral load $\geq 250$ copies/mL have been tested for resistance each year since 1996 (Figure 2.1). However, practices in genotyping have changed over time, such that genotyping is now performed prior to the initiation of antiretroviral therapy. This could lead to a conservative bias when measuring the incidence of resistance over time, as patients who initiated therapy in later calendar years would be more likely to have transmitted resistance detected. It should be noted that this analysis shows trends in the detection of resistance, and the results are therefore dependent upon the frequency of genotyping and cannot tell us about the development of resistance below the pVL limits of genotype testing. However, such low viral loads suggest that any resistance which is evolving may have little clinical significance. Although there is missing exposure data for 338 (4%) individuals who were on therapy prior to 1992, this missing data only impacts the incidence rates calculated for 1996, resulting in a probable slight overestimate of the cumulative rates reported for that year.

These results provide a benchmark for monitoring HIV treatment programs. Efforts to improve accessibility to HAART have the potential to greatly reduce HIV-1
levels in populations without increased risk of drug resistance. If current trends persist, the continued improvement of HAART and the increased availability of new drugs could potentially make the development of new HIV drug resistance a rare event.
Table 2.1. The annual number of incident cases of drug resistance and corresponding patient-months of exposure within any antiretroviral (ARV) category, or for the NRTIs, NNRTIs, and PIs individually

Cumulative patient-months of antiretroviral exposure is also indicated for any resistance category.

<table>
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<tr>
<th>Year</th>
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<th>NRTI Resistance</th>
<th>NNRTI Resistance</th>
<th>PI Resistance</th>
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<td>Patient-months</td>
<td>Cumulative</td>
<td>Patient-months</td>
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<td>Patient-months</td>
<td>of NRTI</td>
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<td>of Any ARV</td>
<td>Exposure per</td>
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<tr>
<td></td>
<td></td>
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Figure 2.1. The annual incidence rate of drug resistance detected for any resistance category and individual drug classes

(A) The annual incidence rates for any resistance category. (B) The annual incidence rates for the NRTIs (triangles), NNRTIs (diamonds), and PIs (circles), separately. Incidence rates are natural-log transformed. Dashed and solid lines represent corresponding linear regressions. The number of patients on antiretroviral therapy, the number of patients tested for resistance, the number of samples genotyped, and the number of patients with at least one pVL $\geq 250$ copies/mL within each year is indicated below the graphs.
Any Resistance

A

B

NRTI, NNRTI, and PI Resistance

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<td>2861</td>
<td>3089</td>
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<td>1909</td>
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Figure 2.2. The annual incidence rate of drug resistance detected for the most commonly occurring mutations in HIV-1 protease and reverse transcriptase as a function of calendar year

(A) Incidence rate for the major NRTI resistance mutations M41L, M184V/I, and T215F/Y. (B) Incidence rate for the major NNRTI resistance mutations K103N and Y181C/I. (C) Incidence rate for the major PI resistance mutations V82A/F/T/S and L90M. Incidence rates are natural-log transformed. Dashed and solid lines represent corresponding linear regressions. The number of people exposed to each therapy, by calendar year, is indicated below the graphs.
Figure 2.3. Distribution of the lowest (A) and highest (B) plasma HIV-1 viral loads and (C) the percentage of individuals achieving a plasma HIV-1 RNA level below the limit of detection of the RNA assay for all patients in British Columbia

The box plot includes the median (solid horizontal bar), interquartile range (box), and the lower of 1.5 times the interquartile range or the most extreme value (dashed line). The number of patients who received therapy within a given year, and had an available pVL test, is indicated above the bars. These data include patients recently starting antiretrovirals. Note that the reporting of pVL values is capped at the limits of the pVL assay. The lower limit of the viral load assay was changed in 1999. Therefore, the newer assay would report lower values for the lower quartile and 1.5 times the interquartile range for pVLs in 1996-1998. Except for 1998, median values could not be affected, but IQR values could be affected. For the percentage of individuals achieving a plasma HIV-1 RNA level below the limit of detection, data is shown from the year 2000 onwards due to the decrease of the lower limit of the viral load assay from 500 to 50 copies/mL in April 1999. Annual percentages were based upon the lowest available pVL from individuals, regardless of whether or not they were on therapy at the time of testing.
A: Graph showing the change in Log10 Plasma Viral Load (copies/mL) from 1996 to 2008.

B: Graph showing the same data as in A, with a trend line indicating a decrease in viral load over time.

C: Graph showing the percentage of patients with pVL <50 copies/mL from 2000 to 2008, with a regression line $R^2 = 0.97$. 

N= 2099 2861 3089 3275 3265 3231 3280 3388 3554 3857 4170 4582 5004
Figure 2.4. The annual incidence rate of drug resistance detected for any resistance category and individual drug classes, stratified by period of therapy initiation.

Annual incidence rate of drug resistance detected for each of (A) any resistance category, and (B) the NRTIs, (C) NNRTIs, and (D) PIs. Incidence rates are natural-log transformed. Dashed and solid lines represent corresponding linear regressions. Patients were grouped into the following periods of therapy initiation: 1987-1995, 1996-1999, 2000-2004, and 2005-2008.
2.5. ACKNOWLEDGMENTS

This work was supported by the Michael Smith Foundation for Health Research and by the Canadian Institutes of Health Research (CIHR) through fellowships for Dr. Lima, and through a GlaxoSmithKline/CIHR Chair in Clinical Virology for Dr. Harrigan. Dr. Montaner is a recipient of an Avant-Garde Award from the National Institute on Drug Abuse. The authors wish to thank an anonymous reviewer for the suggestion of including drug exposure data in this manuscript.
2.6. REFERENCES


CHAPTER 3
INCREASED RESILIENCE TO THE DEVELOPMENT OF DRUG RESISTANCE
WITH MODERN BOOSTED PROTEASE INHIBITOR-BASED
HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

3.1. BACKGROUND

Highly active antiretroviral therapy (HAART) has led to a dramatic decrease in AIDS-related comorbidity and mortality [1–3]. However, the success of HAART can be compromised by the development of HIV drug resistance [4, 5]. Antiretroviral resistance is an independent risk factor for virologic failure in HIV-1-infected populations [4, 6, 7]. Drug-resistance testing is now widely recommended in HIV therapy monitoring to detect the development of resistance to antiretrovirals and make appropriate regimen changes [8].

Several individual factors have been associated with the development of HIV-1 drug resistance during HAART, including incomplete adherence to therapy [9–12], high baseline plasma viral load (pVL), and low CD4 cell count [12, 13]. However, there has been limited research on how the simultaneous presence of these factors and their interactions affect the development of antiretroviral resistance [7, 12]. Recent studies have shown that the relationship between adherence and resistance is complex [12, 14]. The accumulation of resistance mutations across all levels of adherence was greater in treatment-naive individuals beginning HAART than in those who were treatment experienced [12, 15]. The heterogeneity

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1 A version of this chapter has been published. Increased resilience to the development of drug resistance with modern boosted protease inhibitor-based highly active antiretroviral therapy. Lima VD, Gill VS, Yip B, Hogg RS, Montaner JS, Harrigan PR. J Infect Dis 2008;198:51-8.
in the relationship between adherence and resistance in antiretroviral-exposed and -naive populations has been explored via computer simulations [16].

The relationship between adherence and resistance is also dependent on the individual drug classes used in combination therapy [14, 17, 18], with discrepancies between the adherence-resistance relationships for nonnucleoside reverse-transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) being at least partially explained by differences in the replicative capacity of drug-resistant versus wild-type virus in the presence of clinically relevant drug levels for each drug, respectively [14]. Recommendations for the optimal application of HAART have varied over time [19–21] as therapies have evolved to become more convenient and tolerable. One particularly relevant change has been the widespread shift of international guidelines in favor of low-dose ritonavir as a PI “boosting” agent when PI-based HAART is used [22, 23]. The use of boosted PIs have led to improved virological suppression, as detailed in clinical trials [22, 24, 25] and cohort studies [26], as well as improved clinical outcomes in cohort studies in observational settings [27]. However, the impact of boosted PI-based regimens versus nonboosted PI-based and NNRTI-based regimens on the development of HIV drug resistance mutations remains to be defined in a population-based setting.

The objective of the present study was to characterize the probability of the development of drug resistance in drug-naive individuals starting HAART in the modern era, adjusting for the simultaneous effects of adherence, pVL, and initial HAART regimen. We based our analysis on data from a large population-based cohort of HIV-1-infected antiretroviral-naive adults initiating HAART in British Columbia, Canada, between 1 August 1996 and 30 November 2004.
3.2. METHODS

HIV/AIDS drug distribution program

The British Columbia Centre for Excellence in HIV/AIDS (BC-CfE) distributes antiretroviral agents at no cost to all eligible HIV-infected individuals through its drug distribution program, the HIV/AIDS Drug Treatment Program (BC-CfE DTP). This program has been described in detail elsewhere [28]. ART is distributed according to the specific guidelines generated by the Therapeutic Guidelines Committee. The BC-CfE’s guidelines have been regularly updated and remain consistent with those of the International AIDS Society-USA [19, 20, 29]. The BC-CfE DTP has received ethical approval from the University of British Columbia Ethics Review Committee at its St. Paul’s Hospital site.

Study participants

Eligible study participants were ≥18 years old and were naive to ART when they started HAART (consisting of 2 nucleosides/nucleotides plus either a nonboosted PI, an NNRTI, or a PI plus <800 mg of ritonavir [boosted PI]). Participants started treatment between 1 August 1996 and 30 November 2004 and were followed up until 30 November 2005 (n=2350, with median follow-up of 4.8 years and a total of 6066 tests). Participants must have had a CD4 cell count and pVL measurement within 6 months of the first antiretroviral start date. Study data from eligible participants were extracted from the BC-CfE’s monitoring and evaluation system to form the HOMER (HAART Observational Medical Evaluation and Research) cohort. The characteristics of this study population have been extensively described elsewhere [28, 30].
**Data collection**

HIV-positive individuals receiving ART in British Columbia are entered into an Oracle-based monitoring and evaluation system that uses standardized indicators to prospectively track antiretroviral use and the clinical health status of these individuals. Physicians enrolling an HIV-infected individual into the system must complete a drug request enrollment prescription form, which compiles information on the participant’s address, past HIV-specific drug history, CD4 cell counts, pVL, current drug requests, and the enrolling physician. A qualified practitioner reviews all requests to verify that they follow the therapeutic guidelines outlined by the BC-CfE. Approved prescriptions are renewed every 1 to 3 months.

The BC-CfE recommends that pVLs and CD4 cell counts be monitored at baseline, at 4 weeks after the start of ART, and every 3 months thereafter. pVLs are determined using the Roche Amplicor Monitor assay (Roche Diagnostics) by either the standard method or the ultrasensitive adaptation (since 1999). CD4 cell counts are measured by flow cytometry, followed by fluorescent monoclonal antibody analysis (Beckman Coulter).

Resistance testing was performed on stored pVL samples extracted manually or automatically using guanidinium-based buffer, followed by ethanol washes. Protease (PR) and reverse-transcriptase (RT) genes were amplified from plasma HIV-1 RNA by nested RT polymerase chain reaction (PCR), as described elsewhere [12]. PCR products were sequenced in both the 5’ and 3’ directions using an ABI automated sequencer, and a consensus sequence was generated. Results of the genotyping analysis are reported here as amino acid changes in the HIV PR and RT genes with respect to a wild-type reference sequence (HIV HXB2; GenBank accession number K03455). Samples were assigned to 1 of
4 resistance categories on the basis of a modification of the International AIDS Society-USA table [31]. Samples were considered to be resistant if they displayed 1 or more major resistance mutations in any of the following 4 categories: lamivudine (184I/V); any other nucleoside reverse-transcriptase inhibitors (NRTIs; 41L, 62V, 65R, 67N, 69D or insertion, 70R, 74V, 75I, 151M, 210W, 215F/Y, or 219E/Q); any NNRTIs (100I, 103N, 106A/M, 108I, 181C/I, 188C/H/L, 190A/S, P225H, M230L, or 236L); and any PIs (30N, 46I/L, 48V, 50L/V, 54V/L/M, 82A/F/S/T, 84V, or 90M). Lamivudine resistance was analyzed as a separate category because of the very common appearance of this mutation and the lack of cross-resistance conferred to other NRTIs. The percentage of samples that were obtained while individuals were being actively prescribed any ART were as follows: 82% for first lamivudine resistance, 78% for other NRTIs, 84% for NNRTIs, and 81% for PIs. Because genotyping does not yield consistently successful results for samples with low pVL, samples with a pVL <1000 copies/mL were not systematically genotyped and were assumed to have no drug-resistance mutations. We conducted a sensitivity analysis to assess the impact of our assumption that samples with a pVL <1000 copies/mL harbored no drug-resistance mutations. For the sensitivity analysis, we repeated the original analysis only for those individuals with at least 1 sample that had a pVL ≥1000 copies/mL. Resistance data from those who started therapy between August 1996 and September 1999 have been reported elsewhere [12].

Of the 2350 individuals included in this analysis, at least 1 available pretherapy genotype was available for 1426 (61%). Primary resistance was assessed using a standardized list of mutations suitable for transmitted resistance surveillance [32]. From the earliest available pretherapy genotypes, 131 (9.2 %) showed some evidence of transmitted
resistance. A sensitivity analysis was conducted that eliminated those individuals who were known to have transmitted resistance, leaving 1295 patients.

For each individual, the median number of genotypes that could possibly have been included in the analysis for which data was unavailable was 0 (interquartile range [IQR], 0–1) and the median number completed was 1 (IQR, 0–2). A total of 1433 (61.0%) of individuals had all possible genotypes completed.

**Outcome measures and predictor variables**

The primary outcome in this analysis was the emergence of drug resistance in any of the four resistance categories described previously (yes vs. no). The following baseline predictor variables were investigated: age, sex, CD4 cell count, pVL (log_{10} transformed), first regimen, AIDS diagnosis, history of injection drug use, year of first therapy, and adherence. Estimates of adherence to ART were based on medications actually dispensed, not prescribed. For this study, we limited our measure of adherence to the first year of therapy, estimated by dividing the number of months of medications dispensed by the number of months of follow-up. This measure of adherence has been found to be independently associated with HIV suppression and survival among HIV-infected persons enrolled in the BC-CfE DTP [33, 34]. Adherence was categorized as 0% to <40%, 40% to <80%, 80% to <95% and ≥95%. Because pVL was measured over time starting in 1996, our baseline pVLs were obtained on the basis of the standard pVL assay, and our last measurements were obtained on the basis of the ultrasensitive pVL assay; thus, our upper and lower limits of pVLs ranged over time from 500 and 1 \times 10^6 to 50 and 1 \times 10^5 copies/mL. Therefore, our pVL measurements were recoded to range from 500 to 1 \times 10^5 copies/mL in order to standardize the viral load range over time.
Statistical analyses

An exploratory logistic regression model was developed for identifying which patient characteristics were most influential in the development of drug resistance during ART. A backward stepwise technique was used in the selection of covariates. The area under the receiver operating characteristic curve was used to measure the model’s ability to discriminate between those in whom resistance developed versus those in whom it did not [35]. Categorical variables were compared using the $\chi^2$ or Fisher's exact test, and continuous variables were compared using the Wilcoxon rank-sum test. For the purposes of analysis, we followed the intent-to-treat principle, with subjects retained in their initial treatment groups irrespective of whether participants subsequently switched to regimens that were available later. This approach provides a conservative estimate of the true treatment effect. All analyses were performed using SAS software (version 9.1.3, service pack 3).

3.3. RESULTS

Demographic characteristics

Between August 1996 and November 2004, a total of 2350 antiretroviral-naive participants (81.6% males) at least 18 years old started triple-combination therapy in British Columbia and were eligible to participate in this study. Of these patients, 991 (42%) individuals initiated nonboosted PI-based regimens, 475 (20%) initiated boosted PI-based regimens, and 884 (38%) initiated NNRTI-based regimens. A further breakdown of patients’ antiretroviral regimens, stratified by years of initiation, is provided in table 3.1. The temporal change toward increasing prevalence of ritonavir-boosted regimens in recent years reflects the changes in treatment guidelines over time. During a median of 4.8 years (IQR, 2.7–10.0) of follow-up, a total of 6066 resistance tests were done, and resistance to at least one drug
category developed in 650 (28%) patients. As shown in table 3.2, the development of drug resistance was associated with a higher pVL, history of injection drug use, NNRTI-based regimens, starting therapy in 1996–1998, age, and higher adherence levels ($P<0.0001$). Sex, AIDS diagnosis, and CD4 cell count were not significantly associated with the development of drug resistance.

**Probability of drug-resistance development**

Table 3.3 shows the results for univariate and multivariate associations between the emergence of drug resistance and several baseline factors. The univariate analysis for baseline characteristics showed that age, pVL, history of injection drug use, first regimen, year of first therapy, and adherence were associated with the development of drug resistance. The multivariate model predicted no difference in the odds of the development of key resistance mutations between nonboosted PI-based regimens (reference group) and NNRTI-based HAART regimens (odds ratio [OR], 1.09 [95% confidence intervals {CI}, 0.84–1.42]) but greatly reduced odds for boosted PI-based regimens (OR, 0.42 [95% CI, 0.28–0.62]). A skewed, nonlinear relationship with adherence was confirmed [12], as was a strong association of resistance with increasing pVL. A weaker association with decreasing CD4 cell count, late start of therapy, and history of injection drug use was also observed. Of interest, after adjusting for other parameters, there was a reduction in the risk of detecting resistance in those who started HAART during 1999–2001 (OR, 0.83) or 2002–2004 (OR, 0.43), compared with those who started HAART during 1996–1998 (reference group) (table 3.3).

Figures 3.1 and 3.2 show estimated model-based probabilities of the development of resistance by adherence level and several covariates of interest. Figure 3.1a demonstrates a
nonlinear relationship between adherence and drug resistance, with the development of resistance being more likely in those individuals with adherence levels in the 40% to <80% and 80% to <95% strata. There were no visible differences in the relationship when the data were stratified by CD4 cell count (figure 3.1b); however, when stratified by year of first therapy, the data indicated that individuals starting therapy during 1996–1998 presented the highest probabilities for the development of drug resistance, and this probability decreased linearly until 2002–2004 (figure 3.1c). Furthermore, stratifying by pVL (figure 3.1d) demonstrated that individuals in the baseline pVL <5 log_{10} copies/mL stratum had a considerably lower probability for the development of resistance than those with baseline pVL values ≥5 log_{10} copies/mL at equivalent levels of adherence.

Of particular interest is the relationship between emergence of resistance, pVL, and adherence stratified by regimen type (figure 3.2). The dependence of resistance selection on baseline pVL and patient adherence was markedly decreased for boosted PI-based regimens. The estimated probability of resistance for the worst adherence-pVL stratum for boosted PI-based regimens was equal to or lower than that observed for any adherence stratum for nonboosted PI-based regimens and was very similar to that of NNRTI-based regimens for adherence levels <80%. For those patients with a pVL in the <5 log_{10} copies/mL range, the risk of the development of drug resistance varied greatly depending on the first regimen.

**Sensitivity analyses**

In the central analysis, samples with a pVL <1000 copies/mL were not genotyped and were assumed to not carry resistance mutations. We addressed the impact of this assumption by conducting a sensitivity analysis, eliminating those individuals with viral suppression (pVL <1000 copies/mL) during the entire follow-up period (n=541 [23%]). An explanatory
logistic regression model was developed for identifying which patient characteristics were the most influential in the development of drug resistance during antiretroviral treatment. The results from the new univariate analyses were consistent with the previous ones, and the multivariate analysis also yielded results similar to those presented before. Based on the multivariate model, we also observed no difference in the odds of the development of key resistance mutations between nonboosted PI-based regimens (reference group) and NNRTI-based regimens (OR, 1.27 [95% CI, 0.95–1.68]) but greatly reduced odds for boosted PI-based regimens (OR, 0.36 [95% CI, 0.24–0.54]). Similar results were observed regarding associations between resistance and adherence, pVL, CD4 cell count, start of therapy, and history of injection drug use. In addition, we observed a similar reduction in the risk of detecting resistance in those who started HAART during 1999–2001 (OR, 0.79 [95% CI, 0.60–1.05]) or 2002–2004 (OR, 0.43; 0.30–0.63), compared with those who started HAART during 1996–1998 (reference group).

To address the potential impact of transmitted resistance on resistance that develops during therapy, we conducted another sensitivity analysis examining only those 1426 individuals for whom pretherapy genotypes were available and excluding those who exhibited transmitted resistance (n=131), leaving a total of 1295 patients. As with the other sensitivity analysis, the results from the new univariate and multivariate analyses yielded similar results as the original. Based on the multivariate model, we observed no difference in the odds of the development of key resistance mutations between nonboosted PI-based regimens (reference group) and NNRTI-based regimens (OR, 1.24 [95% CI, 0.87–1.77]) but greatly reduced odds for boosted PI-based regimens (OR, 0.44 [95% CI, 0.24–0.83]). Similar results to those of the original analysis were observed regarding associations between
resistance and adherence, pVL, CD4 cell count, start of therapy, and history of injection drug use. Once again, we observed a similar reduction in the risk of detecting resistance in those who started HAART during 1999–2001 (OR, 1.01 [95% CI, 0.72–1.44]) or 2002–2004 (OR, 0.46 [95% CI, 0.28–0.79]), compared with those who started HAART during 1996–1998 (reference group).

To address the potential effects of wild-type virus outgrowth in patients who ceased active therapy, we conducted another sensitivity analysis eliminating the 117 individuals who were not receiving any therapy at the time of resistance detection. Overall, we obtained results similar to those of the original analysis and of other sensitivity analyses. Of particular note, the effect of boosted PIs on the OR for resistance was even greater than that in the original analysis (OR, 0.37 [95% CI, 0.24–0.56]).

3.4. DISCUSSION

Our results demonstrate that the probability of emergent drug resistance decreased steadily during 1996–2004. Incomplete adherence and nonboosted PI-based or NNRTI-based antiretroviral regimens were associated with a greater probability of the development of drug resistance. In contrast, boosted PI-based regimens were significantly associated with a lower emergence of resistance, even after adjustment for pVL and CD4 cell count. Of note, the latter remained the case at all levels of adherence.

Suboptimal adherence levels (80% to <95%) were associated with the highest risk of resistance in any drug category (hazard ratio [HR], 4.15; \( P < 0.001 \)) or in multiple resistance categories (HR, 6.99; \( P = 0.010 \)) (survival analysis data not shown). Recent studies have also shown that different ART classes have unique adherence-resistance relationships [17, 18]. Bangsberg et al. [18], gathering data from several studies, demonstrated that, with regimens
containing nonboosted PIs, most drug resistance occurred in patients with adherence levels between 70% and 80%.

The data obtained here show that boosted PI-based regimens are associated with relatively low levels of resistance development across all adherence strata, consistent with previous observations that boosted PI-based regimens may have a more “forgiving” profile in terms of virological suppression [22, 36]. Also, it is interesting to note that we observed a 2-fold increase in the risk of resistance in patients with a history of injection drug use; this contrasts with an only slightly increased risk noted previously [12]. The reasons for this difference are not clear.

There are several features of the present study that should be highlighted. First, this study was based on a large sample of patients within a provincewide treatment program, in which all individuals had free access to medical attention, combination ART, and laboratory monitoring. We are confident, therefore, that our results are not influenced by access to therapy, a factor that has often compromised the interpretation of similar population- and cohort-based studies. Second, the very simple methodology of logistic regression was sufficient to show the simultaneous effects of adherence, pVL, and antiretroviral regimen on the development of drug resistance. Third, this study was based on individuals who were initially naive to ART, ensuring that our results were not confounded by previous therapy use.

There are some important potential limitations in our study. First, study participants who had samples with a pVL <1000 copies/mL were assumed to have no drug-resistance mutations. To assess whether this introduced a possible source of bias, we conducted a sensitivity analysis excluding these individuals. This analysis showed that this assumption
did not bias our results. Second, pretherapy genotypes were not available for the assessment of transmitted resistance in all study participants. We conducted a sensitivity analysis in the subset of individuals who had pretherapy genotypes without transmitted resistance and found that our original findings still held. Third, we used pharmacy-refill compliance as a surrogate for adherence; however, this measure of adherence has been found to be independently associated with HIV suppression and survival among HIV-infected individuals enrolled in the BC-CfE DTP [11, 37]. Fourth, the measure of adherence used in this study was that obtained at the end of the first year of therapy, which is a conservative measure of adherence. Some recent studies have shown that patients change their level of adherence over time, with longer treatments being associated with lower adherence levels [38–42]. Therefore, it is possible that if we used time-varying adherence we would see an even stronger effect of adherence on the emergence of resistance than that found here. Further investigation of the role played by longitudinal adherence in predicting drug resistance is important in understanding this major contributor to drug resistance. Finally, although we adjusted our analyses for pertinent demographic and clinical characteristics, residual confounding may exist among observational study populations, and for this reason caution is warranted.

In conclusion, we found a complex relationship among adherence, first antiretroviral regimen, pVL, and the probability of the development of drug resistance among naive individuals initiating their first HAART regimen. The data show a clear improvement in the populational levels of HIV drug resistance as patients start more modern HAART regimens. More importantly, our results demonstrate increased resilience to the development of drug resistance with modern boosted PI-based HAART.
### Table 3.1. Percentages of nucleoside reverse-transcriptase inhibitor (NRTI) pairs and third drugs in first highly active antiretroviral therapy, stratified by year of initiation

<table>
<thead>
<tr>
<th>Years</th>
<th>nRTI Pair</th>
<th>N</th>
<th>% of Time Period</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1996-1998</td>
<td>Lamivudine/Stavudine</td>
<td>425</td>
<td>49.9</td>
<td>18.1</td>
</tr>
<tr>
<td></td>
<td>Lamivudine/Zidovudine</td>
<td>322</td>
<td>37.8</td>
<td>13.7</td>
</tr>
<tr>
<td></td>
<td>Didanosine/Stavudine</td>
<td>48</td>
<td>5.6</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>56</td>
<td>6.6</td>
<td>2.4</td>
</tr>
<tr>
<td>1999-2001</td>
<td>Lamivudine/Stavudine</td>
<td>450</td>
<td>55.6</td>
<td>19.2</td>
</tr>
<tr>
<td></td>
<td>Lamivudine/Zidovudine</td>
<td>203</td>
<td>25.1</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>Lamivudine/Didanosine</td>
<td>96</td>
<td>11.9</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>Didanosine/Stavudine</td>
<td>42</td>
<td>5.2</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>19</td>
<td>2.4</td>
<td>0.8</td>
</tr>
<tr>
<td>2002-2004</td>
<td>Lamivudine/Zidovudine</td>
<td>241</td>
<td>35.0</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>Lamivudine/Stavudine</td>
<td>208</td>
<td>30.2</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>Lamivudine/Didanosine</td>
<td>108</td>
<td>15.7</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>Lamivudine/Tenofovir</td>
<td>71</td>
<td>10.3</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>61</td>
<td>8.9</td>
<td>2.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Years</th>
<th>Third Drug</th>
<th>N</th>
<th>% of Time Period</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1996-1998</td>
<td>Indinavir</td>
<td>597</td>
<td>70.2</td>
<td>25.4</td>
</tr>
<tr>
<td></td>
<td>Nevirapine</td>
<td>118</td>
<td>13.9</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>Saquinavir</td>
<td>69</td>
<td>8.1</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>67</td>
<td>7.9</td>
<td>2.9</td>
</tr>
<tr>
<td>1999-2001</td>
<td>Nevirapine</td>
<td>387</td>
<td>47.8</td>
<td>16.5</td>
</tr>
<tr>
<td></td>
<td>Nelfinavir</td>
<td>165</td>
<td>20.4</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Indinavir/r</td>
<td>75</td>
<td>9.3</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>Indinavir</td>
<td>62</td>
<td>7.7</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Lopinavir/r</td>
<td>38</td>
<td>4.7</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>83</td>
<td>10.3</td>
<td>3.5</td>
</tr>
<tr>
<td>2002-2004</td>
<td>Nevirapine</td>
<td>224</td>
<td>32.5</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>Lopinavir/r</td>
<td>206</td>
<td>29.9</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>Efavirenz</td>
<td>83</td>
<td>12.1</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Atazanavir/r</td>
<td>75</td>
<td>10.9</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>Nelfinavir</td>
<td>27</td>
<td>3.9</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>74</td>
<td>10.7</td>
<td>3.2</td>
</tr>
</tbody>
</table>

**Note:** The element “/r” indicates boosting with ritonavir.
### Table 3.2. Baseline characteristics of patients initiating triple-combination antiretroviral therapy

<table>
<thead>
<tr>
<th>Variable</th>
<th>Resistance</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No (N=1700)</td>
<td>Yes (N=650)</td>
</tr>
<tr>
<td><strong>Sex, no. (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>310 (18.2)</td>
<td>122 (18.8)</td>
</tr>
<tr>
<td>Male</td>
<td>1390 (81.8)</td>
<td>528 (81.2)</td>
</tr>
<tr>
<td><strong>CD4+ cell count (cells/mm&lt;sup&gt;3&lt;/sup&gt;), no. (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;200</td>
<td>856 (50.4)</td>
<td>354 (54.5)</td>
</tr>
<tr>
<td>200-350</td>
<td>451 (26.5)</td>
<td>151 (23.2)</td>
</tr>
<tr>
<td>≥350</td>
<td>393 (23.1)</td>
<td>145 (22.3)</td>
</tr>
<tr>
<td><strong>Plasma HIV-1 RNA level (log&lt;sub&gt;10&lt;/sub&gt; copies/mL), no. (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4</td>
<td>153 (9)</td>
<td>30 (4.6)</td>
</tr>
<tr>
<td>4-4.99</td>
<td>619 (36.4)</td>
<td>188 (28.9)</td>
</tr>
<tr>
<td>≥5</td>
<td>928 (54.6)</td>
<td>432 (66.5)</td>
</tr>
<tr>
<td><strong>AIDS diagnosis, no. (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1440 (84.7)</td>
<td>549 (84.5)</td>
</tr>
<tr>
<td>Yes</td>
<td>260 (15.3)</td>
<td>101 (15.5)</td>
</tr>
<tr>
<td><strong>Injection drug use, no. (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1306 (76.8)</td>
<td>402 (61.8)</td>
</tr>
<tr>
<td>Yes</td>
<td>394 (23.2)</td>
<td>248 (38.2)</td>
</tr>
<tr>
<td><strong>Regimen, no. (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI-single</td>
<td>640 (37.6)</td>
<td>351 (54)</td>
</tr>
<tr>
<td>PI-boosted</td>
<td>416 (24.5)</td>
<td>59 (9.1)</td>
</tr>
<tr>
<td>NNRTI</td>
<td>644 (37.9)</td>
<td>240 (36.9)</td>
</tr>
<tr>
<td><strong>Adherence level, no. (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0% - &lt;40%</td>
<td>256 (15.1)</td>
<td>81 (12.5)</td>
</tr>
<tr>
<td>40% - &lt;80%</td>
<td>246 (14.5)</td>
<td>187 (28.8)</td>
</tr>
<tr>
<td>80% - &lt;95%</td>
<td>188 (11.1)</td>
<td>120 (18.5)</td>
</tr>
<tr>
<td>≥95%</td>
<td>1010 (59.4)</td>
<td>262 (40.3)</td>
</tr>
<tr>
<td><strong>Year of first therapy, no. (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1996-1998</td>
<td>542 (31.9)</td>
<td>309 (47.5)</td>
</tr>
<tr>
<td>1999-2001</td>
<td>574 (33.8)</td>
<td>236 (36.3)</td>
</tr>
<tr>
<td>2002-2004</td>
<td>584 (34.4)</td>
<td>105 (16.2)</td>
</tr>
<tr>
<td><strong>Age, years</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>39.3</td>
<td>37.2</td>
</tr>
<tr>
<td>Interquartile range</td>
<td>33.5 - 46.6</td>
<td>32.2 - 43.3</td>
</tr>
</tbody>
</table>
Table 3.3. Association between baseline variables and emergence of any drug resistance in participants first prescribed triple-combination antiretroviral therapy

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unadjusted OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.00 (—)</td>
<td>1.00 (—)</td>
</tr>
<tr>
<td>Male</td>
<td>0.97 (0.77, 1.22)</td>
<td>—</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.97 (0.96, 0.98)</td>
<td>0.98 (0.97, 0.99)</td>
</tr>
<tr>
<td><strong>CD4+ cell count (cells/mm³)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;200</td>
<td>1.00 (—)</td>
<td>1.00 (—)</td>
</tr>
<tr>
<td>200 – 350</td>
<td>0.81 (0.65, 1.01)</td>
<td>0.67 (0.53, 0.86)</td>
</tr>
<tr>
<td>≥350</td>
<td>0.89 (0.71, 1.12)</td>
<td>0.61 (0.47, 0.79)</td>
</tr>
<tr>
<td><strong>Plasma HIV-1 RNA level (log₁₀ copies/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥5</td>
<td>1.00 (—)</td>
<td>1.00 (—)</td>
</tr>
<tr>
<td>4-4.99</td>
<td>0.65 (0.54, 0.80)</td>
<td>0.59 (0.48, 0.74)</td>
</tr>
<tr>
<td>&lt;4</td>
<td>0.42 (0.28, 0.63)</td>
<td>0.39 (0.25, 0.59)</td>
</tr>
<tr>
<td><strong>AIDS diagnosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.00 (—)</td>
<td>1.00 (—)</td>
</tr>
<tr>
<td>Yes</td>
<td>1.02 (0.79, 1.31)</td>
<td>—</td>
</tr>
<tr>
<td><strong>Injection drug use</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.00 (—)</td>
<td>1.00 (—)</td>
</tr>
<tr>
<td>Yes</td>
<td>2.05 (1.68, 2.48)</td>
<td>1.71 (1.39, 2.11)</td>
</tr>
<tr>
<td><strong>Regimen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI-single</td>
<td>1.00 (—)</td>
<td>1.00 (—)</td>
</tr>
<tr>
<td>PI-boosted</td>
<td>0.26 (0.19, 0.35)</td>
<td>0.42 (0.28, 0.62)</td>
</tr>
<tr>
<td>NNRTI</td>
<td>0.68 (0.56, 0.83)</td>
<td>1.09 (0.84, 1.42)</td>
</tr>
<tr>
<td><strong>Adherence level</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥95%</td>
<td>1.00 (—)</td>
<td>1.00 (—)</td>
</tr>
<tr>
<td>80% - &lt;95%</td>
<td>2.46 (1.89, 3.21)</td>
<td>2.30 (1.74, 3.05)</td>
</tr>
<tr>
<td>40% - &lt;80%</td>
<td>2.93 (2.32, 3.70)</td>
<td>2.48 (1.93, 3.19)</td>
</tr>
<tr>
<td>0% - &lt;40%</td>
<td>1.22 (0.92, 1.62)</td>
<td>0.98 (0.73, 1.34)</td>
</tr>
<tr>
<td><strong>Year of first therapy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1996-1998</td>
<td>1.00 (—)</td>
<td>1.00 (—)</td>
</tr>
<tr>
<td>1999-2001</td>
<td>0.72 (0.59, 0.89)</td>
<td>0.83 (0.64, 1.08)</td>
</tr>
<tr>
<td>2002-2004</td>
<td>0.32 (0.25, 0.41)</td>
<td>0.43 (0.30, 0.61)</td>
</tr>
</tbody>
</table>
Figure 3.1. Estimated probability of drug resistance, by several baseline characteristics: adherence rate (0% to <40%, 40% to <80%, 80% to <95%, or ≥95%) (a), adherence and CD4 cell count (<200, 200–350, or ≥350 cells/mm³) (b), year of first therapy (1996–1998, 1999–2001, or 2002–2004) (c), and adherence and plasma HIV-1 RNA load (<5 or ≥5 log₁₀ copies/mL) (d).

Data are median values; bars show interquartile ranges.
Figure 3.2. Estimated probability of drug resistance, by first regimen (boosted protease inhibitor [PI], nonnucleoside reverse-transcriptase inhibitor [NNRTI], or nonboosted PI), adherence rate (0% to <40%, 40% to <80%, 80% to <95%, or ≥95%), and plasma HIV-1 RNA load (<5 or ≥5 log₁₀ copies/mL).

Data are median values; bars show interquartile ranges.
3.5. ACKNOWLEDGMENTS

We thank Fern Ragnier, Elizabeth Ferris, Nada Gataric, Kelly Hsu, Myrna Reginaldo, Marnie Gidman, and Peter Vann for their research and administrative assistance. We also thank the British Columbia Centre for Excellence in HIV/AIDS research laboratory staff for the collection of genotype data.
3.6. REFERENCES


CHAPTER 4

SILENT MUTATIONS ARE SELECTED IN HIV-1 REVERSE TRANSCRIPTASE AND AFFECT ENZYMATIC EFFICIENCY

4.1. INTRODUCTION

The HIV-1 reverse transcriptase facilitates the conversion of the viral single stranded RNA genome into double stranded DNA. Reverse transcriptase is a multifunctional enzyme and exhibits RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and ribonuclease H activities. Because of its essential role in HIV-1 replication, reverse transcriptase is a primary target for drug development [1]. Currently, two therapeutic classes of reverse transcriptase inhibitors (RTIs) – the nucleoside or nucleotide RTIs (NRTIs) and nonnucleoside RTIs (NNRTIs) [1] – are routinely used to treat HIV infection. However, the long-term efficacy of combination therapies that contain RTIs is limited by the selection of missense mutations, which confer amino acid substitutions in the reverse transcriptase that lead to HIV drug resistance [2].

Amino acid substitutions in reverse transcriptase associated with NRTI and NNRTI resistance have been generally well defined and well characterized [3]. For example, NRTI-associated resistance mutations can be broadly categorized into two groups, depending on their phenotypic mechanism of resistance. The K65R, K70E, L74V, Q151M, and M184V mutations primarily increase the selectivity of reverse transcriptase for the incorporation of a natural deoxynucleotide triphosphate (dNTP)

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1 A version of this chapter has been published. Harrigan PR, Sheen CW, Gill VS, Wynhoven B, Hudson E, Lima VD, Lecocq P, Aguirre R, Poon AF, Sluis-Cremer N. Silent mutations are selected in HIV-1 reverse transcriptase and affect enzymatic efficiency. AIDS 2008;22:2501-8.
substrate over an NRTI-triphosphate [4]. By contrast, the mutations M41L, D67N, K70R, L210W, T215F/Y, and K219Q/E – typically referred to as thymidine analogue mutations (TAMs) – augment the ability of HIV-1 reverse transcriptase to excise the chain-terminating NRTI-monophosphate from a prematurely terminated DNA chain [4].

In contrast to missense mutations, silent mutations do not result in a change to the primary amino acid sequence of the enzyme. As a result, they are typically treated as evolutionarily neutral and are largely overlooked in HIV genotypic analyses. However, in some cases, silent mutations have been shown to alter protein translational kinetics, which in turn may affect splicing, or transcriptional control, ultimately affecting subsequent protein expression and activity [5,6]. In this study, we investigated whether HIV-1 coselects for silent mutations in the HIV-1 reverse transcriptase gene under selective antiviral drug pressure.

4.2. METHODS

Prevalence of mutations

The prevalence of all reverse transcriptase mutations with respect to a wild-type reference sequence (HIV HXB2; GenBank Accession #K03455) was determined in 20,017 archived plasma samples. Using a list of key drug mutations modified from the International AIDS Society, USA (IAS) [3], we examined the prevalence of all mutations in samples stratified as either ‘wild-type’ (i.e. not having IAS ‘key resistance mutations’) or ‘nonwild-type’ (Fig. 4.1a and b, respectively). The mutations we examined by drug category were lamivudine (184I/V); any other nucleoside reverse transcriptase (NRTIs) (41L, 62V, 65R, 67N, 69D, or insertion, 70R, 74V, 75I, 151M, 210W, 215F/Y, or 219E/Q); any reverse transcriptase NNRTIs (100I, 103N, 106A/M, 108I, 181C/I,
188C/H/L, 190A/S, P225H, M230L, or 236L); and any protease inhibitors (30N, 33F, 46I/L, 48V, 50L/V, 54V/L/M, 82A/F/S/T, 84V, or 90M). To show amino acid changes, we compared the difference in the prevalence of amino acid substitutions between ‘wild-type’ and ‘nonwild-type’ samples (b minus a) (Fig. 4.1c).

**Clinical correlates of silent mutation selection**

In order to establish the clinical correlates of these silent mutations, data for silent AAG (or mixtures) at reverse transcriptase codons 65 and/or 66 were pooled from all individuals with available genotypes in members of the previously well characterized HOMER (‘HAART Observational Medical Evaluation and Research’) cohort [7,8] infected with subtype B HIV-1 (n = 2542). Patients were at least 18 years of age and antiretroviral therapy-naive when they started HAART and initiated treatment between 1 August 1996 and 30 November 2005. All participants had a CD4 cell count and plasma viral load (pVL) measurement within 6 months of their first antiretroviral start date. Study data from eligible participants were extracted from the British Columbia Centre for Excellence in HIV/AIDS' monitoring and evaluation system. Associations between baseline characteristics (sex, AIDS diagnosis, history of injection drug use, year of first therapy, adherence strata, CD4 cell count, and pVL) and exposure to specific NRTI drugs during therapy (lamivudine, abacavir, zidovudine, stavudine, zalcitabine, didanosine, and tenofovir) were tested using the $\chi^2$ method, with Bonferroni corrections made for multiple comparisons. We also wished to assess the clinical impact of K65/66K silent mutations on pVL during therapy. Significance of the differences in pre and post K65/66K pVL was determined using the Wilcoxon signed-rank test.
**Steady-state kinetic assays**

D67N/K70R/T215F/K219Q (TAM67) and TAM67/K65R HIV-1 reverse transcriptase were generated by site-directed mutagenesis of wild-type HIV-1LAI reverse transcriptase [9,10]. Recombinant reverse transcriptases were overexpressed and purified to homogeneity as described previously [11,12]. Reverse transcriptase concentration was determined spectrophotometrically at 280 nm, using an extinction coefficient (ε280) of 260 450 M^-1 cm^-1 and the active site concentration was calculated from presteady-state burst experiments [13]. All experiments were performed using corrective active site concentrations.

DNA and RNA oligomers were synthesized by Integrated DNA technologies (Coralville, Iowa, USA). The RNA templates spanned codons 62–77 of the HIV-1 reverse transcriptase gene and included different combinations of mutations at codons 65 (AAA, AGA, AAG), 66 (AAA, AAG), 67 (GAC, AAC), and 70 (AAA, AGA). A complementary 20 nucleotide DNA primer (5’-TGAAATCTCTAATTTTCTCC-3’)) was used to prime reverse transcriptase-mediated DNA synthesis reactions. The primer was 5’-radiolabeled with [γ-32P]-ATP (GE Healthcare, Piscataway, New Jersey, USA) and annealed to the respective RNA templates, as described previously [9,10].

Steady-state DNA polymerization assays were carried out in 50 mmol/l Tris–HCl pH 7.8 (37°C), 50 mmol/l KCl, 10 mmol/l MgCl2 containing 200 nmol/l of T/P, and 5 µmol/l dNTP. Reactions were initiated by the addition of 50 nmol/l wild-type or mutant (TAM67 or TAM67/K65R) HIV-1 reverse transcriptase. Aliquots were removed at designated times (1, 2.5, 3.5, 5.5, 7.5, 10, and 20 min) and mixed with an equal volume of sample loading buffer [98% deionized formamide, 10 mmol/l
ethylenediaminetetraacetic acid (EDTA), and 1 mg/ml each of bromophenol blue and xylene cyanol]. Samples were denatured at 85°C for 5 min, products were separated on a 7 mol/l urea–14% polyacrylamide gel, and detected using a Bio-Rad GS525 Molecular Imager (Bio-Rad Laboratories, Hercules, California, USA).

RNA structural determinations

Preliminary modeling of the minimum free energy secondary structure of isolated HIV-1 reverse transcriptase RNA sequences, spanning codons 55–75, was conducted using the Vienna RNA Secondary Structure Package (http://www.tbi.univie.ac.at/RNA/). Input sequences included wild-type and several variants containing nonsynonymous mutations at codons 65, 67, and 70, as well as silent mutations at codons 65 and 66.

4.3. RESULTS

Silent mutations in HIV-1 reverse transcriptase are associated with treatment experience

Figure 4.1a shows the prevalence of all mutations with respect to HXB2 at the individual reverse transcriptase codons for samples that were classified as ‘wild-type’, whereas Fig. 4.1b shows mutation prevalences for ‘nonwild-type’ samples. The difference in prevalence between ‘wild-type’ and ‘nonwild-type’ samples for mutations that conferred amino acid changes is shown in Fig. 4.1c. The prevalence of silent mutations, relative to HXB2 clade B consensus (Fig. 4.1d), revealed that only two silent mutations at codons K65 and/or K66 in reverse transcriptase were strongly associated with treatment experience and both were highly significant after correction for multiple comparisons. The prevalence of silent mutations at codons K65 and/or K66 in treatment-experienced patients increased by 3.0 and 9.8%, respectively, relative to the naive
population. This degree of selection actually exceeds the degree of selection of most of the known IAS drug resistance mutations. Silent AAA to AAG (or mixtures) mutations at reverse transcriptase codons 65 and/or 66 were observed in 812 samples from 351 patients and 2129 samples from 829 patients, respectively. Similar selection of silent mutations at codons 65 and 66 was observed in the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu; data not shown). We also investigated the prevalence of all mutations with respect to HXB2 at individual reverse transcriptase codons from first available pretherapy genotypes, as well as the prevalence of mutations at the last available on-therapy genotypes, to ensure that only a single post-treatment sample per patient was examined. The results were similar, showing an increase in the prevalence of silent mutations at codons 65K and/or 66K between last available on-therapy and first available pretherapy sample (data not shown).

**Thymidine analogue mutations are strongly associated with silent mutations**

Pooled data from available HIV-1 subtype B reverse transcriptase sequences in the British Columbia Centre for Excellence in HIV/AIDS database were used to identify the resistance mutations that are most commonly associated with the presence of 65K and/or 66K silent mutations (Table 4.1). In subtype B samples, there was a very strong correlation between the silent mutations and TAMs. In particular, at position 67 of the reverse transcriptase, 7% of wild-type D67 samples had the 65K and/or 66K silent mutations, compared with over 80% of samples with the D67N substitution ($P << 0.001$). These associations were confirmed in two independent resistance datasets (the Stanford HIV Drug Resistance Database and Virco; data not shown).
Clinical correlates of silent mutation selection

Although the TAMs are considered to arise on exposure to the thymidine analogues (stavudine or, particularly, zidovudine), exposure to zidovudine was not associated with the selection of the silent mutations. However, exposure to the other NRTIs (abacavir, stavudine, didanosine, or tenofovir) during therapy was associated with the selection of silent mutations at position 65 and 66 (Table 4.2) in a dose-dependent manner [analysis of variance (ANOVA) F-test $P = 0.01$] (Fig. 4.2). Note that the amino acid substitution K65R has been implicated in resistance to many of these agents and is itself antagonistic to TAMs. The mean pVL for individuals before the first positive test for K65/66K silent mutations was 3.89 log$_{10}$ and 4.01 log$_{10}$ at the first positive test (Wilcoxon signed-rank test $P = 0.0064$). Although statistically significant, this is not likely to be a clinically significant difference. Male sex, no history of injection drug use, and low baseline CD4 cell count were each associated with the presence of the K65/66K mutations (Table 4.2). However, these associations may not be causal, but could be correlated with silent mutations because of the association of these variables with the development of extensive resistance in general.

RNA structural determinations

Preliminary investigations modeling the secondary structure of isolated wild-type HIV-1 RNA templates and several templates with various combinations of codon changes at positions 65, 66, 67, and 70 showed most of the silent changes occurred in a loop of the secondary RNA structure, and therefore, it would not be expected to result in significant structural changes between secondary structures.
Silent mutations compensate for replication block of D67N/K70R

To address a possible mechanism by which the silent mutations at codons 65 and 66 in the HIV-1 reverse transcriptase gene might affect virus replication, we assessed the ability of recombinant HIV-1 reverse transcriptase that was wild-type or contained the TAMs D67N, K70R, T215F, and K219Q (TAM67 RT) to copy RNA templates that spanned codons 62–77 in reverse transcriptase and included different combinations of mutations at codons 65 (AAA, AGA, AAG), 66 (AAA, AAG), 67 (GAC, AAC), and 70 (AAA, AGA). Under steady-state kinetic conditions, both the wild-type and TAM67 reverse transcriptases exhibited a stronger tendency to pause/dissociate at codons 65 and 66 on RNA templates containing the D67N/K70R mutations (template RH1) in comparison to the wild-type sequence (Fig. 4.3a, c). However, when the K65 or K66 AAA to AAG mutations were added to the background of the D67N and K70R mutational changes (templates RH3 and RH2, respectively), these pausing and/or dissociation events were largely alleviated (Fig. 4.3a, c). Interestingly, for both reverse transcriptase enzymes, the introduction of the K65R mutation (AAA to AGA) into the RNA template (template RH4) also alleviated the replication block resulting from the 67 and 70 mutational changes (Fig. 4.3b, c). Together, these results suggest that mutations at codons 65 and 66 in the RNA template compensate for a replication block triggered by the D67N/K70R mutations.

As described above, silent mutations at codons 65 and 66 in the reverse transcriptase gene are highly associated with TAMs, and in particular D67N. However, antagonism between missense mutations at K65 (e.g. K65R) and TAMs has previously been reported at the genomic, viral, and enzyme level in subtype B reverse transcriptase,
indicating that the virus cannot readily accommodate both TAMs and K65R in reverse transcriptase [10,14,15]. Accordingly, we were also interested in the ability of reverse transcriptase enzymes containing TAMs and K65R (i.e. TAM67/K65R RT) to copy RNA templates that contained codon changes at positions 65, 66, 67, and 70 (Fig. 4.3d). In comparison with wild-type and TAM67 HIV-1 reverse transcriptase, the TAM67/K65R enzyme exhibited a decreased tendency to pause at codons 66 and 67 on the RH1 template. Furthermore, the silent AAA to AAG change at codon 65 in combination with the D67N and K70R (template RH3) had minimal effect of the overall polymerization pattern of the enzyme. However, on templates that included the K65R mutation (RH4), TAM67/K65R reverse transcriptase exhibited a very strong tendency to pause at codons 65, 66, and 67. Interestingly, the introduction of silent mutation into templates with 67N/70R/K65R mutations does not alleviate the block experienced by reverse transcriptase with K65R+TAMs (Fig. 4.3d; RH5). Furthermore, the addition of the K66 AAA to AAG silent mutation to this template (RH5) did not compensate for this increased pausing. In fact, the enzyme exhibited a very distributive mode of polymerization; by contrast, both the wild-type and TAM67 reverse transcriptase could replicate very efficiently on this template (data not shown). Taken together, these data show that antagonism between reverse transcriptase codon 65 and TAMs may also extend to the RNA level.

4.4. DISCUSSION

Our results reveal the surprising finding that silent mutations at K65 and/or K66 of the HIV-1 reverse transcriptase are strongly selected for during antiretroviral therapy. These silent mutations are particularly observed in association with mutations at HIV-1
reverse transcriptase codon 67. Selection was associated with exposure to the NRTIs abacavir, didanosine, stavudine, and/or tenofovir. Enzymatic data suggest that the silent mutations may play a role in the efficiency of viral DNA synthesis and also highlight a previously unobserved aspect of antagonism between K65 mutations and TAMs at the level of reverse transcription. Subsequently, there have been two independent follow-up studies that showed similar results [16,17].

Other studies have focused on the predisposition of virus with silent mutations in the evolution of HIV drug resistance, particularly with respect to non-B subtypes, but none have tied the actual evolution of silent mutations to drug exposure. Loemba et al. [18] identified several silent mutations at codons 62, 65, 106, 138, and 161 associated with antiretroviral resistance in subtype C. Additionally, Turner et al. [19] described a G48G silent mutation within the protease region of subtype C subtypes, as well as a T215T silent mutation present in subtype A and A/E subtypes. However, to our knowledge no silent mutations have been demonstrated to be selected for by antiretroviral drug pressure, and indeed, this may represent the first observation of the selection of a synonymous mutation in response to a known selective pressure occurring in a natural setting.

Using data from the Los Alamos HIV sequence database, we noted that consensus codons 64, 65, and 66 in wild-type subtype C HIV-1 differ from other subtypes in this region. Whereas subtype C has AAA, AAG, and AAG at codons 64, 65, and 66, respectively, subtypes B–F have AAG, AAA, and AAA at codons 64, 65, and 66, respectively. There have been recent suggestions that silent mutations in the subtype C reverse transcriptase gene were responsible for the rapid selection of the K65R mutation
Another unique feature of subtype C virus is that the K65R mutation that develops in subtype C is AGG, whereas in subtype B it is almost exclusively AGA (data not shown). Although our results are analogous, they are likely not directly relevant to subtype C HIV-1.

An important limitation of our preliminary investigations of secondary RNA structure was that they were conducted on isolated RNA sequences and we cannot make any inferences about the effects on a RNA sequence that is undergoing reverse transcription. In addition, we have only examined the effect of the silent mutations on RNA-dependent DNA polymerization. As HIV-1 reverse transcriptase is a multifunctional enzyme, it is also possible that silent changes in the RNA template affect the efficiency of the DNA-dependent DNA polymerization and/or ribonuclease H activity of HIV-1 reverse transcriptase, but these were not investigated.

The data show that mutations at codons 65 and/or 66 are coselected with TAMs, and therefore, provide an interesting and potentially clinically relevant example of situations in which silent amino acid changes may not be evolutionarily neutral. These data also provide the first evidence for an RNA-level mechanism of direct relevance to drug resistance. Further investigations should be conducted to more accurately identify the mechanism by which these silent mutations are affecting viral evolution, along with other studies to try and identify any further examples of silent mutation selection in response to therapy across the HIV genome.
Table 4.1. HIV RT resistance mutations most commonly associated with the presence of 65K and/or 66K silent mutations

<table>
<thead>
<tr>
<th>RT Codon</th>
<th>Number With Silent Mutation (%)</th>
<th>Number Without Silent Mutation (%)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>67</td>
<td>1755 (69.6)</td>
<td>767 (30.4)</td>
<td>2.6 x 10^{-153}</td>
</tr>
<tr>
<td>219</td>
<td>1182 (64.6)</td>
<td>649 (35.5)</td>
<td>5.5 x 10^{-86}</td>
</tr>
<tr>
<td>70</td>
<td>921 (51.4)</td>
<td>871 (48.6)</td>
<td>9.4 x 10^{-48}</td>
</tr>
<tr>
<td>215</td>
<td>1691 (42.5)</td>
<td>2291 (57.5)</td>
<td>2.0 x 10^{-45}</td>
</tr>
<tr>
<td>41</td>
<td>1408 (43.2)</td>
<td>1851 (56.8)</td>
<td>3.0 x 10^{-35}</td>
</tr>
<tr>
<td>65</td>
<td>33 (15.1)</td>
<td>185 (84.9)</td>
<td>1.8 x 10^{-29}</td>
</tr>
<tr>
<td>184</td>
<td>1973 (33.2)</td>
<td>3969 (66.8)</td>
<td>1.8 x 10^{-27}</td>
</tr>
<tr>
<td>210</td>
<td>1054 (45.5)</td>
<td>1262 (54.5)</td>
<td>5.3 x 10^{-26}</td>
</tr>
</tbody>
</table>

**Note:** Data were pooled from available clade B RT sequences in the BC Centre for Excellence database. Data are sorted by p-value for illustration.
Table 4.2. Association between baseline characteristics and drug exposures with K65/66K for members of the HOMER cohort

<table>
<thead>
<tr>
<th>Variable</th>
<th>Subcategory</th>
<th>Presence of 65K and/or 66K silent Mutation (N [%])</th>
<th>No</th>
<th>Yes</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>1531 (80.1)</td>
<td>548 (87.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>381 (19.9)</td>
<td>82 (13.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIDS Diagnosis</td>
<td>Yes</td>
<td>264 (13.8)</td>
<td>103 (16.4)</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>1648 (86.2)</td>
<td>527 (83.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of Injection Drug Use</td>
<td>Yes</td>
<td>693 (36.2)</td>
<td>186 (29.5)</td>
<td>0.0021</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>1219 (63.8)</td>
<td>444 (70.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year of First Therapy</td>
<td>1996 - 1999</td>
<td>1104 (57.7)</td>
<td>367 (58.3)</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1999 - 2003</td>
<td>616 (32.2)</td>
<td>196 (31.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2003 - 2005</td>
<td>192 (10.0)</td>
<td>67 (10.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adherence</td>
<td>0% - &lt;40%</td>
<td>355 (18.7)</td>
<td>104 (16.6)</td>
<td>0.066</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40% - &lt;80%</td>
<td>459 (24.2)</td>
<td>127 (20.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>80% - &lt;95%</td>
<td>281 (14.8)</td>
<td>104 (16.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥95%</td>
<td>804 (42.3)</td>
<td>292 (46.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Variable</td>
<td>Subcategory</td>
<td>Presence of 65K and/or 66K silent Mutation (N [%])</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------------</td>
<td>-------------</td>
<td>--------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>Yes</td>
<td>p-Value</td>
<td></td>
</tr>
<tr>
<td>CD4+ Cell Count (cells/mm³)</td>
<td>&lt;200</td>
<td>766</td>
<td>295</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 - &lt;350</td>
<td>528</td>
<td>155</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥350</td>
<td>597</td>
<td>176</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma Viral Load (log₁₀)</td>
<td>&lt;4</td>
<td>131</td>
<td>43</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 - &lt;5</td>
<td>627</td>
<td>195</td>
<td></td>
<td></td>
</tr>
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<td></td>
<td>≥5</td>
<td>904</td>
<td>279</td>
<td></td>
<td></td>
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<tr>
<td>Antiretroviral Drug Exposure</td>
<td>Lamivudine</td>
<td>1801</td>
<td>599</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abacavir</td>
<td>308</td>
<td>157</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zidovudine</td>
<td>1091</td>
<td>359</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stavudine</td>
<td>1210</td>
<td>444</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zalcitabine</td>
<td>67</td>
<td>31</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Didanosine</td>
<td>742</td>
<td>286</td>
<td>0.0035</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tenofovir</td>
<td>271</td>
<td>130</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** Participants initiated treatment between August 1, 1996 and November 30, 2005.
Figure 4.1. Prevalence of mutations at individual reverse transcriptase codons before and after therapy

(a) Prevalence of all mutations at individual reverse transcriptase codons, with respect to HXB2, from all samples stratified as ‘wild-type’ [i.e. having no International AIDS Society (IAS) ‘key resistance mutations’]. (b) Prevalence of all mutations at individual reverse transcriptase codons, with respect to HXB2, from all samples stratified as ‘nonwild-type’ (i.e. having at least one IAS ‘key resistance mutation’). (c) Difference in prevalence between ‘wild-type’ and ‘nonwild-type’ sequences, at individual reverse transcriptase codons, for all mutations that conferred amino acid changes (b minus a). (d) Difference in prevalence between ‘wild-type’ and ‘nonwild-type’ sequences, at individual reverse transcriptase codons, for silent mutations only (b minus a). Codons 65 and 66 are highlighted by the large arrow. RT, reverse transcriptase.
Figure 4.2. Percentage of patients with a 65K and/or 66K silent mutation as a function of the number of nucleoside or nucleotide reverse transcriptase inhibitors exposed to during treatment (abacavir, stavudine, didanosine, or tenofovir)

The total number of patients for each exposure, including those without K65/66K mutations, is shown below each grouping. Analysis of variance (ANOVA) F-test; \( P = 0.01 \). NRTI, nucleoside or nucleotide reverse transcriptase inhibitor.
Figure 4.3. DNA synthesis reactions carried out by wild-type, TAM67, and TAM67/K65R HIV-1 reverse transcriptase on RNA templates with substitutions at codons 70 (AAA to AGA), 67 (GAC to AAC), 66 (AAA to AAG), and/or 65 (AAA to AAG or AGA)

(a) DNA synthesis reactions carried out on the wild-type template as well as RH1, RH3, and RH2. (b) DNA synthesis reactions carried out on the RH1, RH3, and RH4 templates. (c) DNA synthesis reactions carried out by TAM67 HIV-1 reverse transcriptase on the wild-type, RH1, RH3, and RH4 templates. (d) DNA synthesis reactions carried out by TAM67/K65R HIV-1 reverse transcriptase on the wild-type, RH1, RH3, RH4, and RH5 templates. Reactions were carried out and analyzed as described in ‘Methods’. K65K* and K66K* signify the AAA to AAG silent mutations at codons 65 and 66. Times each aliquot ran for, from left to right, are 1, 2.5, 3.5, 5.5, 7.5, 10, and 20 min, respectively. WT, wild-type.
4.5. ACKNOWLEDGMENTS

The present study was conceived and designed by P.R.H. and N.S.C. Data acquisition was performed by V.S.G., E.H., B.W. (genotypic data), as well as C.W.S. (steady-state kinetic assays). Data analysis was conducted by V.D.L., B.W., P.L., A.F.P., and R.A. Initial drafting of the article was conducted by V.S.G., E.H., and N.S.C. All authors contributed to the interpretations of data and critical revision of the article.

The present work was supported by the Canadian Institutes of Health Research (CIHR) through a fellowship for V.D.L., by GlaxoSmithKline/CIHR through a Chair in Clinical Virology for P.R.H., and by grants from the University of Pittsburgh Clinical and Translational Science Institute (CTSI RR024153) to N.S.C.

A portion of this work was presented at the XVI International HIV Drug Resistance Workshop, 12–16 June 2007, Needham's Point, Barbados.
4.6. REFERENCES


17. Gonzalez LMF, Pinto AFN, Martins AN, Ahuiar RS, Afonso AO, Brindeiro RM, et al. Silent mutations at reverse transcriptase codons 65 and 66 in B and C strains found in Brazil are very strongly associated with treatment and these signatures can impact on zidovudine mutation acquisition in vitro [Abstract]. Antivir Ther 2008;13 Suppl 3:A63.


CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

5.1. INTRODUCTION

Chapters 2-4 of this thesis each address issues of importance to the understanding of HIV antiretroviral drug resistance. This chapter summarizes the important findings from each analysis and proposes future research to build upon the work carried out in this thesis. Each chapter will be discussed independently, with a final section for general comments.

5.2. CHAPTER 2 – POPULATION INCIDENCE OF ANTIRETROVIRAL RESISTANCE

Despite increases in annual exposure to antiretrovirals there has been a drastic decrease in the incidence of new cases of HIV-1 drug resistance, which has been concomitant with a steady increase in the proportion of treated patients achieving virological suppression. Furthermore, for all drug classes and years of initiation of antiretrovirals, the incidence of resistance per person-months of therapy appears to decline with increasing duration of therapy in our relatively well-monitored patient population.

An important extension of these analyses would be to monitor the incidence of HIV-1 drug resistance in resource-poor settings, particularly those where the standard of care is vastly different from the resource-rich setting. Currently, the World Health Organization recommends a preferred first-line regimen for adults and adolescents in resource-limited settings of the NRTIs zidovudine or tenofovir combined with either of lamivudine or emtricitabine and either of the NNRTIs efavirenz or nevirapine [1]. As an alternative to the preferred regimen, a triple-NRTI regimen is recommended in order to preserve PI-based...
therapies for second-line treatment [1]. These standards are in stark contrast to those recommended for resource-rich settings, where the options for first-line therapy and alternatives are much greater [2, 3]. While treatment in resource-rich settings is largely tailored for individuals, with extensive and complex laboratory monitoring available for the average individual, the WHO strategy for resource-limited countries steers clinicians away from making individualized decisions on treatment regimens and makes the selection of antiretroviral regimens a matter of national policy that is guided by the recommendations of the WHO [1]. Importantly, because of limited access to viral load monitoring in poor nations, the WHO recommends clinical progression criteria and, if possible, CD4 counts as alternative measures to define treatment failure and the need for a regimen change [1]. Changes in CD4 counts and clinical progression are reliable indicators of immunological and clinical failure, respectively, but lack sensitivity to identify individuals with virological failure [4]. If patients are experiencing increases in viral load due to an outbreak of resistant virus that occurs ahead of measurable CD4 decreases or clinical disease, the continued use of those antiretroviral drugs may exacerbate further resistance development, and that could reduce the effectiveness of second-line regimens [5].

In regard to the monitoring of antiretroviral resistance, the WHO has assembled of global network of over 50 institutions called WHO HIVResNET [6]. WHO HIVResNet is charged with performing analyses and making recommendations on strategies to limit the development of antiretroviral resistance in resource-limited settings. Currently, WHO HIVResNet is initiating the monitoring of antiretroviral resistance in resource-poor nations by targeting selected sentinel sites for pilot investigations [6]. Countries are recommended to identify 9-30 sites which are representative of their treatment population, and surveys of
resistance are to be conducted in 3-10 of those sites annually for 3 years, with the cycle restarting in the year 1 sites in the fourth year. Each site will follow a cohort of 99-129 patients beginning first-line therapy. This monitoring will be used as a guide to plan more detailed studies within each country. While any efforts made to monitor antiretroviral resistance in resource-limited settings are laudable, the current population-based monitoring efforts of WHO HIVResNet are undeniably poor in comparison to resistance monitoring in resource-rich settings.

The implications of subtype diversity as well as large-scale NNRTI use on treatment-related outcomes, in particular the development of resistance, have not been fully and clearly characterized. The increased use of WHO-recommended regimens in resource-poor settings can be expected to contribute to a vastly different picture for the incidence of drug resistance in the developing world. With the strong preference for NNRTI-based regimens, the selective pressure from therapy will be of a different nature in the developing world than in the West. Furthermore, the virus the pressure is acting on will also be different than that in the resource-rich setting. While HIV-1 subtype B virus is the predominant strain in the Western world, it accounts for only 10% of the global prevalence of HIV strains [7]. In regards to the achievement of viral load suppression and immune response, current antiretroviral medications have been shown to be effective against non-subtype B viruses for patients undergoing treatment in the resource-rich setting [8]. However, there have been some demonstrated differences between subtypes for the development of resistance (for a review see [9]). The clinical implications of these differences, if any, are not yet clear. One potential issue is a demonstrated ease of selection, in biochemical studies and certain treatment settings, for the K65R mutation in subtype C HIV-1 (which accounts for almost
50% of the global subtype prevalence) versus subtype B virus [7, 10-14]. The development of K65R is correlated with exposure to tenofovir, one of the WHO’s recommended first-line NRTIs. Should the development of K65R in resource-poor settings become problematic, studies designed to discern the prevalence of resistance may only identify its emergence as a problem after it becomes a well-established issue. Studies designed to monitor the incidence of resistance will provide better indicators of the emergence of K65R and other resistance-related mutations, and these studies will be useful in guiding changes to protocols before resistance-related mutations can become larger issues.

5.3. CHAPTER 3 – BOOSTED PI REGIMENS RESILIENT TO THE DEVELOPMENT OF RESISTANCE

Chapter 3 demonstrates the superiority of modern boosted protease inhibitor-based regimens, relative to NNRTI-based and unboosted PI-based regimens, for the development of drug resistance at the populational level. Overall, individuals initiating their first HAART with a boosted PI regimen had a 2.4-fold lower odds ratio of developing resistance, at all adherence levels, than for other regimens. Furthermore, the data demonstrate a temporal decrease in the likelihood of developing resistance with calendar year of therapy initiation.

Interestingly, the simple methodology of logistic regression was sufficient to show the simultaneous effects of adherence, pVL, and antiretroviral regimen on development of drug resistance. The methods applied here can be used to assess the adjusted risk of developing resistance among potential future first-line regimens that incorporate newer antiretroviral drugs. Continued investigations of the risk of developing resistance for newer therapeutic agents and at different time-points in treatment (e.g. after a change in antiretrovirals) may also be undertaken in order to better inform drug choices for second-line
and salvage antiretroviral regimens. Furthermore, the finding that a history of injection drug use was a significant risk factor for the development of resistance warrants further exploration, including whether unaccounted confounders distinguish injection drug users in a significant way from other HIV-infected individuals and explain the increased risk for resistance acquisition. It would be particularly interesting to explore whether the mode of HIV transmission (i.e., sexual versus injection drug use) plays a mechanistic role in the risk of developing antiretroviral resistance. Further knowledge of whether or not the mode of transmission plays a mechanistic role in the development of resistance would further basic knowledge of HIV evolution in the context of treatment and could have many potential downstream implications, including affecting clinical outcomes by leading to adjustments in treatment based on the mode of transmission.

5.4. CHAPTER 4 – SELECTED SILENT MUTATIONS IN HIV-1 REVERSE TRANSCRIPTASE

The selection of non-synonymous mutations that confer resistance to HIV antiretroviral drugs has been well-established [15]. However, prior to the undertaking of this analysis there was no evidence of the selection of silent mutations due to antiretroviral therapy and their contribution to the development of antiretroviral resistance. Our results reveal the surprising finding that silent mutations at K65 and/or K66 of the HIV-1 subtype B reverse transcriptase gene are strongly selected for during antiretroviral therapy. There was a strong correlation between the silent mutations and thymidine analog mutations, particularly D67N. The selection of these silent mutations was associated with exposure to the NRTIs in a dose-dependent manner. Data obtained from biochemical assays suggest the silent mutations may play a role in the efficiency of viral DNA synthesis and also highlight a
previously unobserved aspect of antagonism between K65 mutations and thymidine analog mutations (TAMs) at the level of reverse transcription.

There are several candidates for future investigations that can build upon this work. Similar studies which take a broader look at the entire HIV-1 genome may be able to identify other non-synonymous and silent mutations that are associated with antiretroviral therapy and may find other unexpected evidence of drug selection. Interestingly, recent cell culture experiments with NRTI drug selection showed increased development of K65R in a subtype B virus that contained tandem silent subtype C nucleotide polymorphisms at reverse transcriptase positions 64 and 65 versus the wild-type subtype B strain [11]. This study provided further evidence of the importance of silent mutations in drug resistance, and future investigations of HIV-1 subtypes other than B may identify other subtype-specific selected silent mutations that can act as signature polymorphisms for antiretroviral resistance.

Longitudinal analyses may be undertaken to assess the timing of the selection of the silent mutations relative to known resistance mutations, especially D67N. These analyses would help determine if the silent mutations precede certain mutations or are acting as compensatory mutations for fitness defects created by the selection of non-synonymous mutations.

Within this study, an attempt was made to model the secondary RNA structure of isolated HIV-1 reverse transcriptase RNA sequences spanning codons 55 to 75 using software from the Vienna RNA Secondary Structure Package. These preliminary investigations showed most of the silent changes occurred in a loop of the secondary RNA structure, and therefore would not be expected to result in significant changes between secondary structures. However, this preliminary analysis was limited by the fact that it relied
on computed rule-based modeling of isolated reverse transcriptase RNA structures of an arbitrary length. Recently, the architecture and secondary structure of the HIV-1 RNA genome for a laboratory strain of virus, called NL4-3, was determined via an *in vitro* technique called high-throughput selective 2’-hydroxyl acylation analyzed by primer extension (SHAPE) analysis [16]. Application of the SHAPE analysis results may allow for a more in-depth and accurate investigation of the secondary structure of HIV-1 reverse transcriptase than the preliminary modeling exercises undertaken in our original analysis.

5.5. FINAL COMMENTS

The use of combinations of antiretroviral medication in the form of HAART has resulted in marked improvements in clinical outcomes for patients infected with human immunodeficiency virus type 1 (HIV-1) [17, 18]. However, despite the success of HAART, the development of HIV antiretroviral resistance remains a significant obstacle to its effectiveness [19, 20]. Therefore, it is important to monitor the development of antiretroviral resistance in “real world” settings. Chapter 2 explored the biochemical mechanisms of resistance development and found previously unidentified silent mutations may play a role in the evolution of resistance. Chapter 2 used population-level data for the province of British Columbia to determine the incidence of antiretroviral resistance over time, and was able to demonstrate drastic decreases in the development of new resistance since the advent of antiretroviral therapy. Chapter 3 used data from a cohort of antiretroviral-naïve individuals to demonstrate the effects of several factors on the risk of developing resistance. Chapters 2 and 3 provide evidence that modern HAART, especially boosted PI-based regimens, can be highly effective in guarding against the development of resistance at the population level. Chapter 4 provides an example of how gaps remain in the basic understanding of the
mechanisms driving the evolution of resistance, despite extensive study of antiretroviral resistance.

Analyses of antiretroviral resistance will benefit from improvements in technology, such as the development of modern “ultra-deep” pyrosequencing techniques, which offer the ability to detect resistance mutations at much lower levels than were previously possible with older Sanger-based sequencing [21-23]. Also, the use of SHAPE analyses and other techniques that characterize the RNA genome of HIV will allow for new investigations of the mechanisms of HIV drug resistance at the RNA level [16]. The data shown in this thesis is encouraging, especially for a resource-rich setting such as British Columbia. The thesis provides novel insights into the mechanisms of drug resistance that may serve to improve strategies to impede its development. Furthermore, it shows the benefit of improved antiretroviral regimens to the decreased development of resistance over time. Future studies should build upon these findings in the resource-rich setting as well as resource-poor countries.
5.6. REFERENCES


APPENDIX A

ETHICS CERTIFICATE OF EXPEDITED APPROVAL: ANNUAL RENEWAL

PRINCIPAL INVESTIGATOR: Robert S. Hogg
DEPARTMENT: PHGRI
UBC-PHC REB NUMBER: H05-50123

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:
- Providence Health Care
- St. Paul's Hospital
Other locations where the research will be conducted:
N/A

CO-INVESTIGATOR(S):
- David Moore
- P. Richard Harrigan
- Julio S.G. Montaner

SPONSORING AGENCIES:
- Unfunded Research

PROJECT TITLE:
- BC HIV/AIDS Drug Treatment Program

EXPIRY DATE OF THIS APPROVAL: May 26, 2010

APPROVAL DATE: May 26, 2009

CERTIFICATION:
1. The membership of the UBC-PHC REB complies with the membership requirements for research ethics boards defined in Part C Division S of the Food and Drug Regulations of Canada.
2. The UBC-PHC REB carries out its functions in a manner fully consistent with Good Clinical Practices.
3. The UBC-PHC REB has reviewed and approved the research project named on this Certificate of Approval including any associated consent form and taken the action noted above. This research project is to be conducted by the principal investigator named above at the specified research site(s). This review of the UBC-PHC REB have been documented in writing.

The UBC-PHC Research Ethics Board Chair or Associate Chair, has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal.

Approval of the UBC-PHC Research Ethics Board Chair or Associate Chair, verified by the signature of one of the following:

- Dr. Kuo-Hsing Kuo, Chair
- Dr. J. Kernahan, Associate Chair
- Dr. I. Feldman, Associate Chair