AN INVESTIGATION OF HUMAN AND VIRAL GENETIC FACTORS
INFLUENCING HIV DISEASE PROGRESSION AND RESPONSE TO TREATMENT
IN THE ERA OF HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART)

by

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ABSTRACT

Objective: Host and viral genetic factors contribute significantly to the natural course of untreated HIV infection; however, the impact of these genetic factors on response to modern antiretroviral therapies remains poorly understood. The primary objective of this thesis is to establish the impact of specific host and viral genetic factors on HIV disease progression and treatment response in the era of Highly Active Antiretroviral Therapy (HAART).

Methods: Host genetic factors selected for investigation include polymorphisms in chemokine receptor genes known to play a role in HIV disease progression during untreated infection (CCR5 and CX3CR1), or genes involved in drug metabolism (MDR-1). Viral genetic factors selected for investigation include polymorphisms in HIV genes known to influence untreated disease progression (HIV envelope), or mutations in the viral genome known or suspected to confer resistance to antiretroviral agents. The impact of host and viral genetic factors on short and longer-term clinical outcomes were assessed using retrospective, population-based cohort and case-control analyses of HIV-infected individuals enrolled in the HIV/AIDS Drug Treatment Program in British Columbia, Canada.

Results: Naturally-occurring polymorphisms in human chemokine receptor genes CCR5 and CX3CR1, as well as genes involved in drug metabolism (MDR-1) appear to be associated with differential short and longer-term responses to antiretroviral therapy; however, the relative contribution of individual human genetic polymorphisms to therapy response is likely to be small. In contrast, genetic variation in the HIV envelope appears to significantly affect both short and longer-term clinical responses to HAART, supporting the use of HIV envelope genotypes as determinants of therapy response. An investigation of the impact of antiretroviral resistance mutations on long-term clinical outcomes, in particular, mortality,
suggested that drug resistance, although associated with poor short-term therapy efficacy, is not yet a major factor contributing to mortality among HIV-infected persons in British Columbia, at least on a population basis.

**Conclusion:** Results support the potential incorporation of HIV envelope genotyping as a monitoring tool for the clinical management of HIV-infected individuals. The impact of human genetic variation on therapy response, however, is likely to be mediated by the contributions of polymorphisms in many different genes; a more comprehensive approach may therefore be necessary before incorporating host genotypes into routine HIV clinical management.
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CO-AUTHORSHIP STATEMENT

Each of chapters 3-8 consists of a manuscript that is published in an international, peer-reviewed journal. The candidate is either the lead author (chapters 3, 4, 6, 7, 8) or second author (chapter 5) on all of these manuscripts.

This statement is to certify that the candidate was the major contributor to study identification and design, design of laboratory methods, collection and/or supervision of laboratory data collection, and data analysis. The candidate also wrote the manuscripts and, in the cases of chapters 7 and 8, also co-wrote the successful grant applications.

Primary co-authors of these manuscripts include the thesis supervisor (Dr. Richard Harrigan, thesis committee members (Dr. Julio Montaner, Dr. Robert Hogg, Dr. Michael O'Shaughnessy), statistical analysts (Jerome Asselin, Keith Chan, Chanson Brumme, Benita Yip), authors who provided data access and assistance with data analysis (Brian Wynhoven, Chanson Brumme), Laboratory Technical Staff (Winnie Dong, Theresa Mo, Bethany Henrick) and External Collaborators.
CHAPTER 1

GENERAL INTRODUCTION AND THESIS OBJECTIVES

1.1 Human Immunodeficiency Virus infection: worldwide impact

Human Immunodeficiency Virus Type 1 (HIV-1), the virus that causes Acquired Immunodeficiency Syndrome (AIDS), has emerged as a major worldwide pandemic since its identification as a novel pathogenic retrovirus in the early 1980s [1,2,3]. At the end of 2004, 39.4 million people worldwide (37.2 million adults and 2.2 million children under 15 years of age) were estimated to be living with HIV/AIDS, with Sub-Saharan Africa bearing the major burden of these infections [4]. During the year 2004, it was estimated that a total of 3.1 million individuals died of HIV/AIDS (2.6 million adults and 510,000 children) [4]. To date the total cumulative HIV/AIDS-associated death toll worldwide is estimated to exceed 25 million [4,5]. Currently, an estimated 1.6 million adults and children are living with HIV/AIDS in North America [4]. The most recent estimates indicate that approximately 56,000 Canadians were living with HIV/AIDS at the end of 2002, approximately 30% of whom were unaware of their diagnosis [6].

1.2 Human Immunodeficiency Virus infection: pathogenesis

HIV-1 is a pathogenic retrovirus that specifically targets and destroys the CD4+ subset of T-lymphocytes of the human immune system [7,8]. If left untreated, a progressive deterioration of immune function occurs, leaving the infected individual susceptible to a wide range of opportunistic bacterial, viral, parasitic and/or fungal infections eventually leading to AIDS and death. Currently, the case definition for AIDS, as classified by the U.S. Centres for Disease Control and World Health Organisation, is defined as the presence of an AIDS-defining illness and by a CD4 T-lymphocyte count of less than 200 cells/mm³ [9].
1.3 Brief Overview of HIV life cycle and genomic organization.

The HIV-1 genome is comprised of two single strands of positive (+)-sense RNA, each approximately 9700 base-pairs long, which encode the major structural and regulatory proteins needed for viral replication [10]. HIV-1 shares a common genomic structure and organization with all known replication-competent retroviruses: from the 5' to 3' ends of the genome are encoded the gag (group-specific antigen), pol (polymerase) and env (envelope) regions, encoding proteins needed for retroviral replication [for reviews, see 11,12]. The Gag region encodes a polyprotein precursor (Pr55$^{\text{Gag}}$) which is cleaved by the HIV protease to release the major structural components of the virion, namely the viral matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7) and another small protein p6$^{\text{Gag}}$. The HIV pol region encodes the major viral enzymes required for the replication, integration and processing of the HIV genome and gene products, including the HIV protease, reverse transcriptase, RNaseH, and integrase enzymes. The pol genes are initially expressed as a large gag-pol polyprotein precursor (Pr160$^{\text{Gag-Pol}}$), generated by a ribosomal frameshift event during translation of the viral mRNA message; individual proteins are later cleaved and released by the HIV protease. The env region encodes the envelope glycoproteins gp120 and gp41, which form the surface (SU) and transmembrane (TM) components of the HIV envelope, respectively. Env genes are also initially produced as a polyprotein precursor (gp160), which is cleaved into gp41 and gp120 by a cellular protease [13]. In addition, the HIV genome encodes two regulatory proteins, tat and rev, as well as a number of accessory proteins, vif, vpr, vpu and nef, all of which are necessary for viral replication in vivo.

HIV begins its replication cycle by binding to a target cell through an interaction between HIV gp120 (envelope) and the CD4 receptor on the surface of the target cell.
CD4 alone, however, is not sufficient for successful viral infection of the host cell. In the mid-1990s, the human chemokine receptors CXCR4 [15] and CCR5 [16,17,18] were identified as necessary co-receptors, required in addition to CD4, for efficient entry of HIV into target cells. The CXCR4 receptor is expressed primarily on activated CD4+ T-lymphocytes, while the CCR5 receptor is expressed primarily on naïve and memory CD4+ T-lymphocytes as well as macrophages; thus, the major cellular targets for HIV infection are CD4+T-lymphocytes and macrophages. In rare cases, other minor co-receptors have also been shown to mediate entry of HIV-1 into target cells, at least in vitro [19,20,21]. In addition, evidence suggests that HIV may, to a lesser extent, also be able to infect CD8+ T-lymphocytes [22,23,24], although the relevance of this to the pathogenesis of HIV remains unclear.

The binding of HIV gp120 to CD4 on the surface of the host cell induces a conformational change in the HIV envelope which exposes the co-receptor binding site on gp120 [for a review on co-receptors and viral entry, see 25]. The subsequent binding of gp120 to the co-receptor then triggers the virus-cell membrane fusion event, in which the transmembrane portion of the HIV envelope (gp41) changes conformation to catalyze the insertion of the “fusion peptide” (located at the N-terminus of gp41) into the host cell membrane. Following virus-cell fusion, the virus core particle (representing the remaining viral structure once the lipid bilayer and envelope proteins are removed) enters the host cell and a poorly-understood “uncoating” process occurs whereby the viral genetic material is released into the host cell cytoplasm [11]. The HIV reverse transcriptase then converts the single-stranded RNA genome into double-stranded DNA (dsDNA) and the HIV integrase, in complex with a number of other HIV and host proteins, transports the HIV dsDNA into the
nucleus, where it catalyzes the insertion of the dsDNA into the host cell genome. Once integrated, the HIV DNA is referred to as a “provirus”, and serves as a template for the synthesis of viral RNAs. HIV RNA expression from the provirus proceeds from the 5’ Long-Terminal Repeat (LTR), a regulatory region located at the 5’ end of the genome. The first viral genes to be transcribed are those encoding the regulatory proteins Tat and Rev. Once synthesized, Tat and Rev regulate expression of the remainder of viral RNAs, which include full-length genomic RNAs (for inclusion in new retroviral particles as well as serving as templates for Gag/Pol proteins) as well as singly and multiply-spliced viral mRNAs that serve as templates for the synthesis of the remainder of the viral structural, enzymatic and accessory proteins [26].

The assembly of new retroviral particles takes place at the host cell plasma membrane, and is largely mediated by the structural proteins encoded within HIV gag [11]. As mentioned earlier, the gag (structural) and pol (enzymatic) proteins are expressed as Pr55Gag and Pr160Gag-Pol polyproteins, respectively, which are targeted to the host cell plasma membrane by the MA (matrix) domain of gag. The gag proteins are also responsible for binding and encapsulating the HIV genomic RNA and subsequently promoting the assembly of progeny virions at the host cell membrane, although the details of this process remain incompletely understood. The final steps of the viral life cycle are mediated in part by specific retroviral sequences termed “late (L) domains”. In HIV, the p6Gag protein, located at the 3’ end of Gag, is the L domain responsible for mediating the release of new virus particles from the plasma membrane [27]. After the release of immature progeny virions, a final “maturation” step occurs, in which the HIV protease cleaves and releases the individual structural and enzymatic proteins contained in the gag and gag/pol polyproteins. The
structural rearrangement of viral components thus results in the formation of mature infectious virus particles.

1.4 Brief History of Antiretroviral Treatment

Prior to the availability of antiretroviral therapy, the median time from infection to AIDS diagnosis ranged from approximately 5 to 11 years, depending on age at seroconversion [28], and the median survival time following AIDS diagnosis was approximately 9.5 to 12 months [29,30]. The introduction of zidovudine (AZT), a Nucleoside Reverse Transcriptase Inhibitor (NRTI), as the first antiretroviral agent in the late 1980s led to improvements in the prognosis of HIV-infected individuals, at least in the short term [31,32,33]. Shortly thereafter, the introduction of additional NRTIs didanosine (ddI) and zalcitabine (ddC) led to the use of dual-combination therapies that resulted in improved clinical responses and survival when compared to AZT monotherapy [34,35,36,37,38]. It became quickly evident, however, that mono and dual combination therapies had only a relatively short period of effectiveness, in part due to the rapid selection of drug-resistant HIV variants [39,40,41]. It was not until the development of additional classes of antiretroviral agents and the universal recommendation of triple combination therapy (also known as “Highly Active Antiretroviral Therapy”, or “HAART”) in the mid-1990s, that dramatic and sustained reductions in HIV-related morbidity and mortality were observed on a population basis [42,43,44,45], resulting in a complete change in outlook for individuals infected with HIV.

Currently, there are at least 19 antiretroviral agents approved by the U.S. Food and Drug Administration (FDA) and Health Canada for treatment of HIV infection, which may be classified into four broad antiretroviral classes based on the protein target of the
antiretroviral agent and the mechanism of inhibition. The first available class of antiretroviral agents, Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs), are nucleoside analogues which inhibit HIV reverse transcription by terminating synthesis of the elongating HIV DNA. To date there are at least 8 NRTIs currently approved by the U.S. FDA/Health Canada for treatment of HIV infection, including zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir, tenofovir, emtricitabine, and combinations thereof. The second class of antiretroviral agents, the Protease Inhibitors (Pis), target the viral maturation step mediated by the HIV protease enzyme. Pis were licensed for use beginning in the mid-1990s, and include ritonavir, nelfinavir, indinavir, amprenavir, saquinavir, lopinavir, and atazanavir. The third class of antiretroviral agents, the Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), also target the HIV reverse transcriptase, through allosteric inhibition of the enzyme itself. NNRTIs currently licensed for treatment of HIV infection include nevirapine, delavirdine and efavirenz. The majority of current first-line antiretroviral combination therapies include a backbone of two NRTIs in combination with either a PI or an NNRTI [46]. Finally, the fourth class of antiretroviral agents, the “fusion inhibitors”, inhibit the virus-cell fusion event mediated by HIV gp41. Enfuvirtide (T20) is currently the only licensed example of this class [47,48].

Antiretroviral agents currently in development include coreceptor antagonists (aimed at inhibiting the interaction between HIV gp120 and coreceptors CCR5 and/or CXCR4) [49], integrase inhibitors (inhibiting the integration of the HIV provirus into the host cell genome) [50], RNAseH inhibitors (inhibiting the degradation of the HIV RNA genome in the transient RNA-DNA complex that occurs during reverse transcription) [51] as well as others [52].

1.5 Surrogate markers for monitoring of HIV disease progression
In the initial clinical trials evaluating the efficacy of novel antiretroviral therapies, clinical outcomes were measured primarily in terms of the reduction in the burden of opportunistic infections (or the duration of AIDS-free survival), or using death as an endpoint. The evaluation of long-term treatment outcomes, however, was replaced in later years by the use of surrogate markers, allowing more rapid monitoring and evaluation of novel antiretroviral agents in the context of clinical trials as well in the clinical management of HIV-infected individuals [53,54]. The first available surrogate marker for HIV disease progression was the CD4 count [55], which quantifies the number of circulating CD4+ T-lymphocytes as an indirect measure of immunologic function. It was not until the mid-1990s, however, that a virologic marker of HIV disease progression was made available in the form of the reverse-transcriptase polymerase-chain-reaction (RT-PCR)-based HIV plasma viral load (pVL) assay [56]. At the present time, additional HIV viral load assays are also available, including the nucleic acid sequence-based amplification (NASBA) [57] as well as a signal amplification technology termed "branched-chain DNA" (bDNA) [58]. The plasma viral load assay, which quantifies the amount of HIV RNA in the peripheral blood, was shown to be a significant independent predictor of progression to AIDS and death in the context of untreated infection [59,60]. Furthermore, reductions in HIV plasma viral load as a direct consequence of the administration of combination antiretroviral therapy were found to be highly predictive of improved clinical prognosis, response to treatment, as well as a decreased risk of progression to AIDS and death [61,62,63,64]. A recent analysis of antiretroviral therapy outcomes from >12000 subjects in prospective cohort studies concluded that antiretroviral treatment response could be best predicted by the changes in
CD4 cell count and HIV plasma viral load observed after 6 months of treatment, regardless of pre-treatment values [65,66].

Just as CD4 counts and HIV plasma viral loads were useful and accurate surrogate markers of HIV disease progression in untreated infection, they remain the primary tools today in the clinical management of HIV-infected individuals treated with combination antiretroviral therapies [46,67]. Current treatment guidelines recommend the use of CD4 and, to a lesser extent, pVL to guide decisions regarding the optimal time to initiate or change antiretroviral therapy [46,67,68].

1.6 Identification of host and viral genetic factors which influence HIV disease progression in untreated infection

The natural history of HIV disease progression varies widely in infected individuals, ranging from persons who remain HIV seronegative despite multiple high-risk exposures [69,70], to persons who progress to AIDS and death within a relatively short period (months to a few years) following infection [71]. It is now appreciated that a wide spectrum of host and viral factors contribute significantly to the natural course of HIV disease progression.

a) Host genetic factors

Probably the best-studied example of human genetic variation playing a direct role in HIV infection and pathogenesis is a naturally-occurring 32-base pair deletion in the gene encoding the human CCR5 chemokine receptor, one of the major co-receptors for HIV entry into target cells [72,73]. The 32-base pair deletion (CCR5Δ32) causes a reading-frame shift that generates a premature stop codon in the CCR5 mRNA, resulting in a truncated, nonfunctional protein [74]. Individuals homozygous for this mutation (CCR5Δ32/Δ32, a rare genotype that occurs at a prevalence of <1% in Caucasian populations), do not express
CCR5 on the surface of their cells and therefore are relatively "resistant" to infection with HIV [72,73,75]. Individuals heterozygous for this mutation (CCR5wt/Δ32, a genotype that occurs at a prevalence of ~15% in Caucasian populations) express vastly reduced levels of CCR5 on the surface of their cells [76], and although they may be infected with HIV, exhibit a significantly delayed disease progression when compared to CCR5 wild-type individuals [72,73]. Variation in genes encoding other human chemokine receptors [77,78], chemokine receptor ligands [79,80], and genes involved in the control and modulation of the human immune response [81] are also now known, or suspected, to play a significant role in the body's response to HIV infection and the subsequent rate of disease progression in the context of untreated infection.

b) Viral genetic factors

Even before the HIV structure, genomic organization and replication cycle was fully understood, it was known that HIV variants differed in their capacity to induce disease progression [82]. One notable example is an observational clinical study from the late 1980s describing rates of HIV disease progression in haemophiliacs transfused with HIV-infected blood prior to the availability of a test to screen blood donors [83]. It was observed that recipients infected with HIV from donors who had progressed rapidly to AIDS (within 4 years) were significantly more likely to also experience a rapid disease progression, while persons infected with HIV from "slow progressors" also displayed a slower disease progression [83], strongly suggesting that viral genetic factors were important determinants of the rate of HIV disease progression.

It is now known that not only does HIV have an extensive capacity for mutation and adaptation, but also that sequence variation in the HIV genome contributes significantly to
the natural course of HIV disease. Due to the lack of 3'-5' proofreading ability of the HIV reverse transcriptase, nucleotides are misincorporated, inserted and/or deleted during the conversion of the HIV RNA genome to DNA with an average frequency of one “mistake” per RNA-to-DNA genomic conversion [84]. This frequency, when combined with a total \textit{in vivo} production of up to $10^9$ virions per day in untreated infection [84], provides HIV with an extraordinary capability for mutational diversity and adaptation. On an individual basis, genetic variation in many regions of the HIV genome, most notably \textit{env} [85,86], as well as genes encoding the various HIV accessory proteins [87,88,89] is known or suspected to play a significant role in the clinical course of untreated HIV infection. On a population basis, the extreme worldwide diversity of HIV-1 is best illustrated by the existence of multiple viral classes: groups M (major), O (outlying) and N (new) [90]. Group M, which accounts for the vast majority of HIV-1 infections worldwide, may be further subdivided into multiple subtypes, or clades (A-D, F-H and K), as well as numerous circulating recombinant forms (CRFs), a fact which has important implications for the development of future treatments and/or vaccines for HIV, as well as for resistance to antiretroviral agents [90,91].

\textit{1.7 Relevance of host and viral genetic factors in the era of HAART}

The majority of HIV-infected individuals, at least in the developed world, now have potential access to combination antiretroviral treatment. It is therefore of importance to re-evaluate the relative contributions of known host and viral genetic determinants of untreated HIV disease progression to clinical outcomes the current era of modern antiretroviral therapies. As HAART is such a potent and effective tool in the treatment of HIV infection, it is not known what residual effects, if any, host and/or viral genetic factors may contribute above and beyond the effects of antiretroviral treatment. Population-based studies conducted
on HIV-infected, treated populations are therefore necessary to evaluate the influence of specific host and viral genetic variants on short and longer-term responses to therapy.

The current era of HAART has also resulted in the need to identify factors which may more directly affect antiretroviral therapy response. The field of pharmacogenetics, or the study of how host genetic factors influence the uptake, metabolism, bioavailability, clearance, clinical response, and adverse reactions to medications, is now a major focus of HIV research. Considering the complexity of current combination therapies, as well as the lifelong nature of antiretroviral treatment, it is of importance that a greater understanding of interindividual differences in medication responses be achieved. Examples of human genes implicated in differential responses to antiretroviral therapy include a naturally-occurring polymorphism in a gene encoding a membrane multidrug efflux transporter whose substrates include HIV protease inhibitors [92,93], as well as naturally-occurring genetic variation in the HLA region, involved in the recognition of foreign antigen by cytotoxic (CD8+) T-lymphocytes, where a specific HLA-B genetic variant (HLA-B*5701) is believed to cause a severe and potentially life-threatening hypersensitivity reaction to the NRTI abacavir [94,95].

The relevance of viral genetic factors in the era of antiretroviral therapy is most clearly demonstrated by the emergence of drug-resistant HIV as a major barrier to antiretroviral treatment efficacy [96,97,98,99,100]. In fact, current guidelines for the clinical management and treatment of HIV-infected individuals recommend the routine use of HIV antiretroviral resistance genotyping assays to help guide decisions regarding therapy [101,102]. As discussed in later chapters, it is highly likely that variation in the genes encoding current and future drug targets, as well as in other areas of the HIV genome [103],
will play a significant role in determining an individual’s response to current and future antiretroviral therapies.

Finally, although plasma viral load, CD4 counts and HIV genotypic drug resistance tests currently are, and will remain important tools in the clinical monitoring of HIV disease and therapy outcomes, it is important to continue to investigate and evaluate additional markers, including genetic markers, which may independently influence therapy response. Again, the relevance of novel genetic markers to antiretroviral therapy response, as well as the potential clinical utility of these genetic markers to the management of HIV infection should be evaluated in large, observational, population-based studies of HIV-infected, treated populations, over substantial follow-up times.

Although the "correction" of human genetic variation using techniques such as gene therapy remain a very distant goal, the incorporation of relevant genetic screening assays into routine clinical practise will likely be a realistic and feasible option for clinical management of HIV infected individuals in the future. Data obtained through longitudinal, population-based studies of individuals receiving antiretroviral treatment will help elucidate the individual and combined contributions of host and/or viral genetic factors to therapy outcomes, and hopefully will contribute to resolving still outstanding questions regarding the optimal time to initiate antiretroviral therapy [68] and/or which therapies may be most appropriate under different circumstances.

As outlined in detail below, the main objective of this thesis is to evaluate the clinical relevance of host and viral genetic variation in the era of HAART in a population setting, in order to investigate the potential utility of host and viral genetic factors as prognostic indicators of response modern antiretroviral therapies.
1.8 Thesis objectives and organization

This thesis is divided into nine chapters. This first chapter provides a general introduction to HIV pathogenesis and treatment, as well as outlines the relevance and objectives of the thesis research. Chapter two is a detailed literature review outlining the impact of host and viral genetic variation on HIV infection and disease progression, and addresses the relevance of these factors in context of the modern era of combination antiretroviral therapy. Chapters three through eight address the main thesis objectives as outlined below. Chapter nine provides a detailed discussion and summary of the research results, and comments on the implications of the thesis work.

The general aim of this thesis is to evaluate the relevance of selected host and viral genetic factors to HIV disease in the modern era of combination antiretroviral therapy in a population setting. The host and viral genetic factors selected for investigation are all factors which have been shown to influence HIV infection and/or disease progression in untreated infection, or have been implicated as playing a direct role in the metabolism and/or bioavailability of specific antiretrovirals. Chapters 3, 4, 6, 7, and 8 are population-based studies conducted using retrospective, longitudinal analysis of cohorts of HIV-infected, antiretroviral naïve individuals initiating their first therapy in order to investigate the relative contributions of host chemokine receptor polymorphisms (chapters 3, 4), polymorphisms potentially affecting drug bioavailability (chapter 4), as well as viral genetic factors implicated in HIV pathogenesis (chapters 6, 7, 8) to short and long-term clinical responses to antiretroviral therapy. The specific objectives of these investigations are 1. to determine the prevalence of specific human and viral genetic factors in a cross-sectional analysis of the cohort population at baseline (pre-therapy); 2. to investigate sociodemographic and clinical
correlates of these genetic factors in the context of untreated infection; 3. to use longitudinally-collected plasma viral load and CD4 count data in order to establish the clinical impact of these specific genotypes on initial virologic (plasma viral load) and immunologic (CD4 count) responses to antiretroviral therapy; and 4. to evaluate the long-term impact of these genotypes on response to HAART using mortality data. Chapter 5 employs a retrospective case-control approach and is designed to evaluate the relative contribution of viral genetic variation in the form of HIV antiretroviral resistance to mortality in the HAART era. The thesis will therefore address both the short and longer-term implications of host and viral genetic factors in the modern era of antiretroviral therapy.

1.9 Brief overview of data sources, and evolution of our population-based cohorts

As will be discussed in detail in the following chapters, the population-based cohorts of HIV-infected, antiretroviral-treated individuals studied as a part of this thesis are drawn from the B.C. Centre for Excellence in HIV/AIDS Drug Treatment Program (DTP), based at St. Paul's Hospital in Vancouver, Canada. Briefly, antiretroviral medications in British Columbia are available free of charge to HIV-infected individuals and are centrally distributed through the DTP, which remains the only free source of antiretroviral medication in the province. The B.C. Centre for Excellence in HIV/AIDS Drug Treatment Program captures ~99% of HIV-infected individuals in British Columbia receiving antiretroviral therapy [104]. Individuals are automatically entered into the DTP when they are first prescribed any antiretroviral agent. At DTP entry and with each subsequent physician visit, the participant’s complete history (if any) of antiretroviral use, CD4 count, plasma viral load, and disease stage, as well as sociodemographic information, are recorded [105]. As a part of
the current HIV/AIDS treatment guidelines, HIV-infected patients are monitored at approximately three-month intervals with respect to CD4 counts and HIV plasma viral loads [105]. In fact, the DTP represents the largest HIV treatment-based, Canadian cohort developed to date, and one of the longest followed and best-studied cohorts in the world with the capacity to integrate epidemiological, clinical and laboratory data. The B.C. Centre for Excellence in HIV/AIDS Drug Treatment Program is therefore uniquely positioned to conduct population-based studies that reflect the virologic and clinical status of large numbers of HIV-infected individuals being treated with antiretroviral agents, and to undertake studies investigating the impact of human and viral genetic variation on antiretroviral therapy outcomes, such as described here.

As the DTP represents an open cohort in which patients are continuously being enrolled, and follow-up data continuously being collected, there has been substantial evolution of cohort composition over the course of time, reflecting changes in patient sociodemographics, treatment guidelines and other aspects of clinical practise. Also by definition, there is a ongoing accumulation of follow-up data as individuals continue to be monitored through the DTP. At the time the thesis research was initiated, research focused on a population-based cohort of 461 individuals, of which ~80% were treated with HAART (chapters 4 and 6), while later on, a substantially larger cohort comprising >1000 individuals, all of whom initiated HAART, was created (chapters, 3, 7 and 8). In addition, as time progressed, it was possible to extend follow-up times and include evaluations of long-term outcomes such as mortality (chapters 3, 7 and 8). Throughout this thesis, therefore, there are some variations in cohort composition, follow-up times and available genotype data, which reflect the fact that with each study we wished to include as much longitudinal outcome data...
as possible. Please note that this in some cases, slight inter-chapter differences in reported
N, p-values, odds ratios etc. may be observed, due to maturation of the cohorts and inclusion
of as much follow-up data as was available at the time. In addition, for reasons of style and
composition, please note that the thesis chapters are not presented in the order in which they
were undertaken.

This thesis is prepared according to a manuscript-based format. Chapter two is a
literature review summarizing the current knowledge surrounding the relevance of host and
viral genetic factors in the era of HAART. Chapters three through eight each represent stand­
alone manuscripts, which have all been published in international, peer-reviewed journals.
Journals in which the individual thesis chapters have been published include Journal of
Infectious Disease, AIDS and Antiviral Therapy. The candidate is either the lead author
(chapters 3, 4, 6, 7, 8) or second author (chapter 5) on all manuscripts. The final chapter
(chapter 9) discusses and summarizes the overall study results, and comments upon the
implications and relevance of these findings in the context of the current state of knowledge.
References are found at the end of each chapter.
1.10 REFERENCES


CHAPTER 2
LITERATURE REVIEW:

Impact of human and viral genetic variation on HIV disease progression and response to therapy in the era of Highly Active Antiretroviral Therapy (HAART)

2.1 Introduction

The natural course of Human Immunodeficiency Virus Type 1 (HIV-1) disease progression varies widely among individuals [for a review, see 1]. Not all people exposed to HIV-1 become infected, and of those who do, the time course of untreated disease varies greatly. Although the median time from infection to AIDS diagnosis ranges from approximately 5 to 11 years, depending on age at seroconversion [2], there exist individuals who progress to AIDS in as little as one year from infection ("fast progressors"), and others who remain asymptomatic with normal CD4 counts and low plasma viral loads for 20 years or more ("slow progressors"). Historically, studies of multiply-exposed yet uninfected individuals helped to identify factors which determine "natural" protection from HIV infection [3,4,5,6], while studies comparing characteristics of fast and slow progressors led to the identification of host and viral factors which influence the natural course of HIV disease progression [7,8,9,10,11,12]. A wide spectrum of host and viral genetic factors contribute to the extensive variability in the natural course of HIV-1 disease [7,8,9,10,11]. This review will describe some of the most important findings in this area, and will comment upon the relevance of these findings in context of the modern era of Highly Active Antiretroviral Therapy (HAART) [13].
2.2 Human Genetic Factors: Chemokine receptor polymorphisms

In the mid-1990s, the identification of the human chemokine receptors CXCR4 [14] and CCR5 [15,16,17] as co-receptors for HIV entry into CD4+ cells led to the discovery that genetic variation among chemokine receptor genes could directly influence susceptibility to infection with HIV, as well as the subsequent rate of disease progression. To date, the best-studied example of the contribution of chemokine receptor variation to HIV disease is the CCR5Δ32, a naturally-occurring 32 base-pair deletion in the gene encoding CCR5 [6]. Individuals homozygous for CCR5Δ32 do not express CCR5 and are thus naturally "resistant" to HIV infection [6,22,23], although rare exceptions have been documented where CCR5Δ32/Δ32 individuals have been infected with CXCR4-using strains [18,19]. Individuals heterozygous for the CCR5Δ32 have been shown to express CCR5 at significantly reduced levels on the cell-surface [20], and although these individuals are equally susceptible to HIV infection as CCR5 wild-type individuals, they appear to progress to AIDS and death at a significantly slower rate after infection than those without this mutation [21,22,23,24,25,26]. Polymorphisms in the promoter region of CCR5, presumably affecting expression of the receptor, have also been shown to influence HIV disease progression [27,28].

In addition, several naturally-occurring polymorphisms in minor HIV co-receptors have been reported, including a V62I mutation in the CCR2 gene, which has been shown to confer a protective effect with respect to progression to AIDS [29,30], although not in all studies [31]. The fact that the V62I mutation is in complete linkage disequilibrium with the aforementioned CCR5 promoter polymorphisms [30] may explain this association. More recently, two common single nucleotide polymorphisms in the human CX3CR1 receptor, a
minor co-receptor for HIV-1 [32] have been identified [33]. HIV-1 infected individuals homozygous for the CX3CR1 249I and 280M amino acid substitutions were shown to progress to AIDS and death more rapidly than those with other haplotypes [33], although these results were not confirmed in an independent study (which examined the polymorphism at codon 280 only) [34].

To date no polymorphisms relevant to HIV transmission or pathogenesis have been reported in CXCR4, the major co-receptor for T-cell-tropic, syncytium-inducing strains of HIV, although a polymorphism has been reported in the gene encoding its natural chemokine ligand, stromal-derived factor (SDF)-1 [35]. SDF-1 may act as a natural competitive inhibitor for HIV-1 binding to CXCR4-expressing cells. The SDF-1 3’α mutation, which lies in an untranslated region of the gene, may upregulate the expression of SDF-1 and thus potentially increase its ability to act as a competitive inhibitor [8]. However, the clinical relevance of the SDF-1 3’α mutation has been controversial and reports have ranged from a protective effect [35] to no effect [29] to a detrimental effect [36] of this mutation on progression to AIDS in untreated HIV infection.

2.3 Mutations in genes that modulate the immune response

Variation in the genes modulating the immune response also influence the course of HIV disease. There is now ample evidence that specific alleles of the Human Leukocyte Antigen (HLA) system, located on chromosome 6 and representing one of the most genetically diverse regions in the human genome, play a significant role in the immune control of HIV [10,37,38]. Their function is to allow the immune system to recognize and combat a wide range of pathogens, including HIV, through mediation of the cytotoxic (CD8+) T-cell response [10,37,38]. It is been suggested that HLA genetic variation
contributes to diversity of antigen recognition on both an individual as well as a population level [39]. Evidence strongly suggests that individuals homozygous at any of the HLA loci are at higher risk of more rapid HIV disease progression [40,41]. This is most likely because HLA homozygosity may limit the immune system’s ability to respond to a diverse range of peptide antigens, thus allowing HIV to more easily adapt to avoid the host immune response [41]. In addition, several specific HLA alleles, most notably at the HLA-B locus, influence the natural course of HIV disease, including HLA-B57, HLA-B27 and HLA-B35, among others [42,43,44,45,46,47,48]. In addition, HLA genetic variation directly influences viral genetic diversity: at the population level, the predominant or “consensus” HIV sequence reflects viral genetic adaptation to the most common HLA types in the infected population [49]. Finally, a combined analysis of both HLA type and corresponding viral sequence predicted pre-treatment plasma viral load [49], indicating that the HLA region is a major contributor to the natural course of HIV infection.

2.4 Relevance of chemokine and immunogenetic factors in the era of HAART

Although host genetic variation plays a significant role in untreated HIV infection, the relevance of these factors in the context of HAART remains to be determined. Studies investigating whether the CCR5Δ32 mutation confers any additional protective effect during treatment with HAART have produced conflicting results, with some studies reporting an independent protective effect [50,51,52,53], and others report no significant effect [54,55]. A recent study from our laboratory investigating the effect of CCR5Δ32 on initial virologic and immunologic outcomes as well as survival in >1000 HIV-infected individuals initiating HAART reported a small protective effect of CCR5Δ32 on survival, although this was not statistically significant [56]. Similarly a study from our laboratory has identified the
CX3CR1 249I mutation as being associated with a poorer immunologic response to HAART, although the mechanism for this remains undetermined [57]. A small number of studies investigating other chemokine receptor and/or ligand polymorphisms in the context of HAART have reported variable outcomes [58,59]. Overall, studies to date appear to indicate that host chemokine genetic factors may indeed contribute to disease progression and treatment response in the context of HAART; although in some cases the relative contribution may be small. Perhaps of greater interest is emerging evidence that human chemokine genotypes may act as a selective pressure mediating genetic evolution of the virus, and thus indirectly contribute to disease progression and/or response to current and future antiretroviral agents [60].

2.5 Pharmacogenetics in the era of HAART

The modern era of antiretroviral therapy has opened up an entirely new area of research in the field of HIV/AIDS: pharmacogenetics, or the study of how polymorphisms in the human genome affect the uptake, metabolism, bioavailability, clearance, clinical response as well as adverse reactions to medications.

An example of a well-studied human gene in the field of pharmacogenetics is the Multi-Drug-Resistance (MDR)-1 gene, implicated in the development of resistance to a variety of medications [61,62], including antiretrovirals. The MDR-1 gene encodes the P-glycoprotein (P-gp) membrane efflux transporter, which is expressed on a variety of cell types including CD4+ lymphocytes [63], and possesses a broad substrate specificity [64,65,66,67] which includes HIV protease inhibitors [68, 69]. A single C-to-T nucleotide polymorphism (C3435T) in exon 26 of MDR-1 is significantly correlated with decreased intestinal expression levels and activity of P-glycoprotein [70]. MDR-1 expression levels are
correlated with the resistance of cancer cells to chemotherapeutic agents [61,62]. Indeed it has been shown that overexpression of multidrug transporters significantly reduces the accumulation of PIs in human lymphocytes [72], and recently that intracellular nelfinavir concentrations in lymphoblastoid cell lines expressing the homozygous 3435T/T genotype were significantly higher than those with the 3435C/C genotype [71]. It is possible, therefore, that differential expression of P-gp could affect the intracellular concentration of PIs and thus influence their efficacy [63,72], at least in some populations [73]. Indeed, data have confirmed a correlation between initial CD4 response, nelfinavir and efavirenz levels, and MDR-1 polymorphisms in adherent patients [74]. A study conducted in our laboratory investigating the effects of the MDR-1 C3435T polymorphism on initial clinical response to first combination antiretroviral therapy reported a trend to earlier virologic failure (defined as a rebound in plasma HIV RNA to >500 copies/ml) in subjects with the MDR-1 3435C/C genotype [57], consistent with previous reports suggesting a potential effect of the MDR-1 polymorphism on antiretroviral treatment efficacy [72]. In a small study subset, however, our study was unable to confirm that this earlier virologic failure might be attributed to the selection of resistance mutations to protease inhibitors [57], although this remains to be confirmed in other datasets. Somewhat in contrast, a recent study reported a significant trend to improved virologic response to nelfinavir-containing regimens in children with the heterozygous 3435 C/T genotype [75]. With respect to immunologic response to antiretroviral treatment, some studies have reported a significantly improved CD4 response in individuals with the 3435T/T genotype after initiating therapy [74], although other studies have reported no effect [57,71]. Overall, the clinical importance of MDR-1 genotyping for
antiretroviral therapy remains to be determined [71], but the search for polymorphisms in human genes that affect antiretroviral therapy response has only just begun [76].

The contribution of HLA genetic polymorphisms in the context of HAART has only begun to be investigated, as significant challenges are associated with teasing out the individual contributions of each specific HLA allele on an individual's response to antiretroviral treatment. A rather important, and clinically significant contribution of HLA genetic variation to treatment response, however, is demonstrated by the HLA-B*57 allele, which has been shown to mediate up to 10-times increased risk of a very dramatic, and potentially life-threatening hypersensitivity reaction to the nucleoside reverse transcriptase inhibitor (NRTI) abacavir [77,78]. Due to the clinical implications of this reaction, small studies have begun to evaluate the clinical utility of “patch-testing” for abacavir hypersensitivity, (in which a small amount of the drug is placed on the skin to evaluate whether a contact reaction occurs) [79], and it is likely that clinical genetic screening programs for HLA-B*57 will soon follow [80,81]. It remains to be determined whether HLA allelic variation, or other human genetic variation for that matter, mediates hypersensitivity or intolerance to other antiretroviral agents.

2.6 Viral Genetic Factors: resistance to current and future antiretroviral agents

The complete HIV genome sequence was published in 1985 [82]. Technical advances made in DNA sequencing technologies, most notably in the last decade, have greatly facilitated the large-scale sequence analysis of HIV genomes from diverse populations, and have resulted in an appreciation of the extreme plasticity and continuing diversification and evolution of this retrovirus [83]. Over the last two decades, variation in nearly every region of the HIV genome, whether coding or noncoding, has been shown to
influence virulence, infectivity and/or pathogenesis of HIV both in vitro and in vivo. The significance of viral genetic diversity to HIV pathogenesis remains one of the most pertinent questions in HIV research in the current era of HAART.

Possibly the best example of viral genetic factors relevant in the HAART era is the emergence of antiretroviral-resistant HIV. Although combination antiretroviral therapies have significantly reduced HIV-related morbidity and mortality in the last decade [84,85], it is now recognised that drug-resistant HIV variants are a major cause of treatment failure and therefore a barrier to long-term antiretroviral efficacy in both antiretroviral-naïve and experienced patients [86,87,88,89]. In fact, it is estimated that virologic failure occurs in up to 20% of previously antiretroviral-naïve individuals [90] and up to 30-50% of previously-treated individuals within two years of initiation of therapy [90,91]. In addition, a survey of drug resistance in the United States undertaken in the late 1990s estimated that 63% of patients receiving HIV care had plasma viremia of > 500 copies/ml, 76% of whom harbored HIV resistant to one or more antiretroviral agents [92]. A survey of antiretroviral resistance among treated individuals in England with plasma viral loads >2000 copies/ml reported resistance prevalences of 69%, 88% and 55%, respectively, over the years 1998-2000 [93].

Because of the implications of HIV drug resistance on the long-term efficacy of HAART, it is important to identify factors which may influence the emergence of drug resistance in clinical practise. The identification of these factors may yield interventions to minimize or at least delay the development of resistance. Our laboratory recently undertook a large-scale, retrospective, population-based analysis of >1000 HIV-infected, previously antiretroviral-naïve individuals initiating their first triple combination antiretroviral therapy between the dates of August 1996-September 1999 [94]. In this population, the incidence of drug-resistance
mutations was documented over a 2.5-year period following initiation of therapy, and risk factors for the development of drug resistance mutations were identified. Over the first 30 months after initiation of treatment, 25% of the study population developed antiretroviral resistance mutations, and the strongest determinants of development of resistance were high baseline viral load and substantial but incomplete levels of adherence [94]. These results are in agreement with a study of antiretroviral resistance in the United States, which reported that higher plasma HIV viral load and low CD4 cell count were significant predictors of the selection of drug resistance mutations [92]. In the US study [92], previous antiretroviral experience and advanced HIV disease were also associated with the presence of drug resistance.

Although resistance to multiple antiretroviral classes has indeed emerged as a major challenge in the treatment of HIV-infected individuals [86,87,88,89], the long-term effects of multidrug resistance remain to be determined. As HIV therapy is a lifelong commitment, a realistic consequence of multidrug resistance is the exhaustion of available treatment options through repeated failure of antiretroviral regimens. Although the ten years that have elapsed since the introduction of HAART are not likely to provide sufficient time to conclusively address this issue, a recent study of HIV-infected individuals in British Columbia investigating the potential impact of antiretroviral resistance on mortality suggests that multidrug resistance is not yet a strong net contributor to HIV-related deaths in this province, at least on a population basis [95]. Whether this continues to be the case in the future remains to be determined. However, the development of novel antiretroviral agents will continually expand HIV treatment options, even for individuals who have developed resistance to the currently available antiretroviral agents.
2.7 *HIV envelope and co-receptor phenotype*

The HIV envelope, encoding the gp41 (transmembrane) and gp120 (surface) proteins, is believed to be the most genetically variable region in the viral genome and thus contributes significantly to HIV pathogenesis. The HIV envelope's capacity for mutation, for example, facilitates the virus' continual evasion of the humoral (antibody-mediated) immune response [for reviews, see 96,97], possibly contributing to HIV disease progression. In addition, genetic variation in the HIV envelope also defines both HIV biological phenotype as well as target cell tropism as determined by coreceptor usage. As already outlined, HIV primarily uses host chemokine co-receptors CCR5 and CXCR4, in combination with CD4, in order to attach, fuse and gain entry into host cells [14,15,16]. The ability of HIV to use one co-receptor over another lies in the genetics of the HIV envelope [98,99].

Coreceptor usage appears to play an important role in HIV pathogenesis. In general, most HIV variants isolated from newly-infected individuals utilize CCR5 in combination with CD4 to gain entry to host cells [100]. These CCR5-using HIV strains (termed "R5" variants [101]) predominantly infect activated CD4\(^+\) T-lymphocytes as well as macrophages. While R5 variants are generally detectable over the entire course of HIV infection [102], variants able to utilize CXCR4 ("X4" variants [101]) emerge in approximately 40-50% of infected persons over the course of disease [103], a phenomenon referred to as R5-to-X4 "phenotype switch" [104]. These X4 variants predominantly target naïve and resting CD4\(^+\) T-lymphocytes and display different biological properties than their R5 counterparts, including increased replication rate, pathogenicity and syncytium-inducing capacity in MT-2 T-cell culture [102,105]. In addition, dual-tropic variants capable of using both CXCR4 and CCR5 may also arise over the course of disease [106].
Although the factors mediating the R5-to-X4 "phenotype switch" are incompletely understood, the consequences of this switch may be quite severe with respect to clinical prognosis. The emergence of CXCR4-using variants is associated with a rapid decline in CD4+ T-lymphocyte counts, accelerated disease progression and reduced survival time in untreated individuals [107,108,109,110], as well as poorer response to treatment in the pre-HAART era [111,112,113,114,115,116]. The mechanisms whereby X4 viruses contribute to accelerated disease progression and poorer prognosis, however, remain incompletely understood. In fact, the association may not be directly causative; it is not known whether X4 variants are inherently more pathogenic and are directly responsible for rapid disease progression, or whether CXCR4-using variants may emerge as a consequence of progressive immune dysfunction [117]. Regardless of the direction of causation, the association of X4 variants with poorer prognosis and inferior therapy response is an issue immediately relevant to the clinical management of HIV infection, and remains so in the era of HAART.

Currently, commercial assays exist to experimentally determine HIV coreceptor phenotype in clinical isolates; however these assays are time-consuming and costly and therefore not generally used in clinical practise. However, HIV envelope genotyping, targeted at env regions responsible for coreceptor determination, is much less resource-intensive and may prove to be of clinical utility. Briefly, the capacity of the virus to switch to CXCR4 usage lies at least partially in a change in two key amino acids in the third hypervariable region, known as the V3 loop, of HIV gp120 [98,99], although genetic variation at other gp120 sites also play a role [118]. In general, X4 variants display positively charged amino acids (namely lysine or arginine) at codons 11 and/or 25 of V3, while R5 variants do not [119,120]. V3 amino acid analysis has been used to distinguish X4
from R5 isolates in an oversimplified approach termed the "11/25" rule [121]. Of the available methods for sequence-based predictions of viral phenotype, the 11/25 rule has proven to be the most reliable [120], although it is by no means perfect. Studies of cloned isolates report specificities of >85% for the prediction of HIV phenotype from V3 loop genotype [119,122], however studies employing direct ('bulk') PCR and sequencing approaches report much lower sensitivities and specificities for prediction of HIV coreceptor usage [60].

2.8 Relevance of HIV envelope variation in the era of HAART

Whether HIV envelope genotyping and/or HIV coreceptor phenotyping may be relevant as independent predictors of HIV clinical prognosis and therapy response in the current era of HAART remains to be determined. Two recent studies from our laboratory have attempted to address this issue [60,123]. By analyzing clinical outcomes from a population-based cohort of >1000 HIV-infected, antiretroviral-naïve individuals initiating first HAART, our group attempted to establish the impact of baseline (pre-therapy) V3 genotype and baseline HIV coreceptor usage on initial clinical response and survival following initiation of HAART [60,123]. Consistent with studies reporting an association of X4 HIV and disease progression in untreated infection, we observed a dramatic correlation between the presence of X4-using variants and poorer clinical prognosis at the time of HAART initiation: subjects harboring X4-using variants had significantly lower CD4 counts and higher plasma viral loads than subjects harboring exclusively R5 variants [60]. In addition we observed that subjects harboring X4 variants at baseline had significantly poorer survival following initiation of HAART than subjects harboring exclusively R5 variants [60], an observation that is not surprising considering that pre-treatment pVL and especially CD4
count remain the strongest predictors of response to HAART [124,125]. After adjustment
for pre-treatment CD4 and viral load, however, the presence of X4 variants did not remain an
independent predictor of survival following initiation of HAART [60], suggesting that a
costly co-receptor determination assay may not provide any additional information above and
beyond pre-treatment CD4 and viral load, at least in terms of response to HAART [60]. The
potential utility of coreceptor phenotyping in other contexts, for example treatment with
novel antiretroviral agents such as coreceptor inhibitors [126], remains to be determined.

Of interest, a different result was observed when baseline V3 genotypes were
evaluated with respect to HAART response [123]. In contrast to the coreceptor phenotypes,
V3 genotypes were not associated with pre-treatment clinical parameters, but were
independently predictive of clinical outcomes following initiation of HAART [123]. The
presence of positively-charged amino acids at codons 11 and/or 25 of the HIV V3 loop (the
"11/25" genotype) was independently predictive of poorer CD4 response as well as mortality
in the 5 years following therapy initiation, suggesting that an HIV envelope genotype may
provide useful supplementary information, in addition to CD4 count and viral load, in the
clinical management of HIV-infected individuals [123].

At present, the epidemiology of HIV coreceptor usage and envelope sequence
variation is of particular relevance in light of the development of HIV co-receptor
antagonists, a new class of antiretroviral agents designed to prevent HIV entry into target
cells through blockade of CCR5 and/or CXCR4 coreceptors [117,127]. In anticipation of
the approval of this new class of antiretrovirals, there is increasing interest in determining the
prevalence, risk factors and effect on outcomes associated with coreceptor usage in
population-based studies of both therapy naïve and experienced patients [60,126]. HIV
coreceptor usage and envelope variation will therefore continue to be of relevance in the HAART era and beyond.

2.9 Relevance of variation in other regions of the HIV genome in the era of HAART

HIV genetic variation in regions other than those described here have also been implicated in playing a potential role in HIV disease progression, response to therapy and/or the development of antiretroviral resistance, either directly or indirectly. The HIV-1 nef gene, for example, located at the 3' end of the HIV-1 genome, encodes a 25 kDa N-myristilated protein which is a major determinant of HIV pathogenesis in untreated infection [128]. Correlations between infection with nef-deleted or nef-defective HIV strains and dramatically decreased rates of disease progression have been reported [129,130,131], and sequence variations in nef have been shown to correlate with plasma viral load (pVL), CD4 counts and disease stage [132]. More recently, sequence variation in the accessory gene vpr has also been associated with HIV disease progression in untreated infection [133,134]. Finally, amino acid insertion mutations in HIV p6\textsuperscript{Gag} have been associated with increased viral infectivity in vitro, and may also be associated with resistance to NRTIs [135], and thus influence response to therapy [136]. The relevance of these HIV genetic differences in the HAART era remains to be determined.

In conclusion, there is abundant evidence to suggest that host and viral genetic factors will continue to be of relevance in the current era of antiretroviral treatment. As more information is made available on the clinical implications of both host and viral genetic variation in the current treatment era, and innovations in DNA sequencing technologies continue to be made [137,138], it is likely that the future of HIV clinical management will feature the incorporation of novel host and viral genotyping assays.
2.10 REFERENCES


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CHAPTER 3

HUMAN GENETIC VARIATION: IMPLICATIONS OF THE CCR5Δ32 MUTATION ON RESPONSE TO HAART

3.1 INTRODUCTION

The CCR5 chemokine receptor is a key cell-surface co-receptor for HIV-1 [1,2]. A naturally-occurring 32-base pair deletion in CCR5 confers protective effects with respect to HIV infection and disease progression in untreated individuals [3,4], and in some studies, improved response to antiretroviral therapy [5,6,7] although this remains controversial [8,9,10]. Our laboratory previously investigated the effect of the CCR5Δ32 genotype on initial virologic and immunologic response to first antiretroviral treatment in a cohort of 436 subjects initiating dual (20%) and triple (80%) therapy [9]. We observed no significant influence of CCR5Δ32 genotype on initial virologic or immunologic response over a two-year follow-up [9].

Here we investigate the influence of CCR5Δ32 on first virologic and immunologic response to triple therapy in a cohort of >1000 antiretroviral naive adults initiating their first triple therapy in British Columbia, Canada, over a median >5-year follow-up period. In addition we investigate the association between CCR5Δ32 and survival following initiation of treatment, which was not possible in the previous study [9] due to shorter duration.

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1 This chapter has been published as:
3.2 MATERIALS AND METHODS

*The B.C. Centre for Excellence in HIV/AIDS Drug Treatment Program*

In the province of British Columbia, Canada, antiretrovirals are distributed free of charge to HIV-infected individuals through a centralized drug treatment program based at the B.C. Centre for Excellence in HIV/AIDS (the Centre). Antiretrovirals are prescribed according to specific guidelines set by the B.C. Therapeutic Guidelines Committee, which are revised regularly and are in accordance with international guidelines [11]. Patients who enroll in the program may provide informed consent and participate in a survey that collects sociodemographic data. Routine clinical monitoring of patients takes place at approximately 3-month intervals at which time plasma HIV viral load testing (Roche Amplicor Monitor Assay) and CD4 counts are performed. These data are stored in the Centre's Drug Treatment Program database.

*The HOMER cohort*

The HAART Observational Medical Evaluation and Research (HOMER) cohort includes all HIV-positive, antiretroviral naïve adults who started triple antiretroviral therapy (consisting of two nucleoside reverse transcriptase inhibitors [nRTIs] and either a protease inhibitor [PI] or a non-nucleoside reverse transcriptase inhibitor [NNRTI]) through the B.C. Drug Treatment Program between August 1996 and September 1999 (N=1188). This cohort has been the focus of a number of population-based studies and has been described in detail previously [11,12]. In the present study, HOMER cohort subjects were followed over a median of 5.8 years following therapy initiation.
**CCR5 genotyping**

PCR primers flanking CCR5Δ32 were used to amplify this region from nucleic acid material extracted from a single blood or plasma sample from each subject, as described in [9]. Amplified products were visualised by agarose gel electrophoresis and CCR5Δ32 genotypes confirmed by automated DNA sequencing on an ABI 3700 DNA sequencer. Note that there is some overlap between the current and previous [9] cohorts (N=322, 27.0%).

**Statistical Analysis**

Associations between CCR5Δ32 and baseline sociodemographic and clinical parameters (age, gender, prior AIDS diagnosis, history of injection drug use, pVL and CD4 count) were determined using the Chi-Squared test (binary variables) or the Wilcoxon Rank-sum test (continuous variables). Associations between CCR5Δ32 and treatment outcomes were assessed using Kaplan-Meier survival analysis and Cox proportional hazards regression. The primary endpoint investigated was time to non-accidental death (occurring on or before September 30, 2004, the date of last linkage with the British Columbia Vital Statistics Agency). Deaths were classified according to the International Classification of Diseases, Tenth revision (ICD-10). Additional surrogate clinical endpoints included time to first virologic suppression (time to the first of 2 consecutive pVL <400 copies/ml from date of therapy initiation), time to subsequent virologic rebound (time elapsed between pVL suppression and to the first of two pVL ≥ 400 copies/ml), and time to CD4 decline (time to the first of two CD4 counts below baseline), consistent with definitions in [9]. Event-free subjects were censored on the draw date of the last pVL or CD4 sample up to and including September 30, 2004. Subjects were followed for a median of 5.8 years following therapy.
initiation. Ethical approval was granted by the institutional Research Ethics Board (REB). Individual consent from subjects was not obtained as samples were analyzed in an anonymized manner consistent with the current REB guidelines.
3.3 RESULTS

*Association of CCR5Δ32 with baseline parameters*

CCR5Δ32 genotyping was successful for 1174 of 1188 subjects (98.8%). The heterozygous CCR5wt/Δ32 genotype was observed at a prevalence of 14.6% (n=171 of 1174). No CCR5Δ32/Δ32 individuals were observed. At baseline, subjects with and without the CCR5Δ32 mutation were comparable with respect to sociodemographic and clinical parameters including gender (overall cohort 84% male), history of injection drug use (30%), and proportion previously diagnosed with an AIDS-defining illness (13%). However, CCR5wt/Δ32 subjects were on average slightly older at the time of therapy initiation when compared to CCR5wt/wt subjects (median 38 vs. 37 years p=0.04), had significantly higher baseline CD4 counts when compared to CCR5wt/wt subjects (CD4 median 320 cells/mm$^3$; Interquartile range [IQR 140-470] vs. 270 cells/mm$^3$ [IQR 130-420], p=0.04) and slightly lower baseline plasma viral loads when compared CCR5wt/wt subjects (pVL median 110,000 copies HIV RNA/ml [IQR 35,000-240,000] vs. 130,000 copies HIV RNA/ml [IQR 43,000-320,000], p=0.06).

*Association of CCR5Δ32 with shorter-term clinical surrogate outcomes*

In multivariate analyses adjusting for baseline parameters as well as time spent on antiretrovirals in the first year of therapy (estimated by dividing the number of months of prescriptions dispensed by months of follow-up in the first year, a surrogate of adherence [13]), we observed a significant association between the CCR5wt/Δ32 genotype and a shorter time to initial virologic suppression <400 copies HIV RNA/ml (multivariate HR [95% CI] 1.20 [1.01-1.44], p=0.044). The percentages of CCR5wt/wt and CCR5Δ32/wt subjects who achieved initial virologic suppression were 76% and 84%, respectively (p=0.02, chi-squared
Among subjects who achieved virologic suppression, the median time to suppression was 10.8 months for the CCR5 wt/wt group vs. 9.6 months for the CCR5Δ32/wt group.

However, no significant difference was observed between CCR5wt/Δ32 and CCR5wt/wt genotype groups and subsequent time to first virologic rebound ≥400 copies HIV RNA/ml after initial suppression (multivariate p>0.1). Similarly, no significant association was observed between CCR5wt/Δ32 and CCR5wt/wt genotype groups and immunologic HAART outcomes, as measured by the time to the first decline of CD4 count below baseline (multivariate p>0.1).

Association of CCR5Δ32 with survival following initiation of triple therapy

A total of 179 non-accidental deaths were recorded over study follow-up; 16 (9.4%) among CCR5wt/Δ32 and 163 (16.3%) among CCR5wt/wt subjects. Of the non-accidental deaths among CCR5wt/Δ32 and CCR5wt/wt subjects, 75% and 72%, respectively, were coded as directly HIV and/or AIDS-related. In univariate analyses we observed improved survival in the (median) >5-year period following therapy initiation among CCR5wt/Δ32 subjects (univariate Hazard Ratio [HR] 0.56, 95% Confidence Interval [CI] 0.34-0.94, p=0.03) (Figure and Table). This trend remained significant after adjustment for baseline age, CD4, pVL, and presence of AIDS-defining illnesses (p=0.03, data not shown).

However, in a multivariate model additionally controlling for time spent on antiretrovirals in the first year of therapy [13], a surrogate of adherence, the association between CCR5wt/Δ32 and improved survival did not remain statistically significant (multivariate HR [95% CI] 0.64 [0.38- 1.07], p= 0.09) (Table). In addition an analysis controlling for adherence over the entire course of study follow-up yielded comparable results (data not shown). Finally, a
survival analysis taking into consideration *death from all causes* produced results entirely consistent with those of the original analysis (data not shown).
3.4 DISCUSSION AND CONCLUSION

Studies investigating whether the CCR5Δ32 mutation confers any protective effect during treatment with HAART have reported somewhat conflicting results. Some studies have reported a significant association between the heterozygous CCR5wt/Δ32 genotype and an increased likelihood of plasma virus suppression after treatment with HAART [6,7,14], improved short-term CD4 responses [6,15], as well as improved 6-and 12-month virologic responses to HAART in subjects with advanced disease [5]. In contrast, other studies have reported no significant correlation between CCR5Δ32 genotype and response to treatment [8,9,10]. In most cases, however, these studies were limited to the evaluation of the effect of CCR5Δ32 on shorter-term (<2-year) clinical outcomes.

The present study evaluated the effect of the CCR5Δ32 mutation on survival and treatment outcomes in a cohort of >1000 antiretroviral naive individuals initiating their first HAART, over a median >5-year follow-up. After adjustment for baseline sociodemographic and clinical factors known to affect therapy response, individuals heterozygous for the CCR5Δ32 deletion experienced significantly more rapid initial suppression of plasma HIV RNA below 400 copies/ml. However consistent with our previous findings [9], no significant association between CCR5Δ32 and the subsequent duration of initial virologic response was observed. Similarly we observed no association between CCR5Δ32 and initial immunologic response, as measured by the time to a decline of CD4 count to below baseline.

Over the longer-term (median >5-year) follow-up, a trend to improved survival in CCR5wt/Δ32 subjects was observed; however, the magnitude of difference was relatively small and most importantly, the association did not remain significant after controlling for adherence. Among the parameters investigated, the strongest predictors of survival over the
study follow-up period were age at therapy initiation, baseline CD4 count and adherence (see Table).

As it has been firmly established that the CCR5wt/Δ32 allele does indeed confer a protective effect with respect to HIV disease progression in the context of untreated infection [3,4,16,17], the survival advantage observed among CCR5wt/Δ32 subjects in this study may possibly be explained by a "natural history effect" in this cohort, which is minimized after controlling for adherence. These results emphasize the need to include data on adherence in studies evaluating the effects of potential prognostic markers on treatment response, and may partially explain the contrasting reports on the relevance of CCR5Δ32 to treatment outcomes thus far.

These results indicate that CCR5Δ32 is likely not a clinically significant predictor of longer-term clinical responses or survival in the context of HAART. Results therefore suggest that CCR5Δ32 is likely not relevant from a clinical management perspective and results do not support the utility of the CCR5Δ32 genotype as an independent clinical prognostic marker for response to HAART. However, the potential relevance of CCR5Δ32 genotypes evaluated in combination with other host genetic factors [18,19] as well as the relevance of CCR5Δ32 genotypes in context of future antiretroviral agents, (including co-receptor antagonists) to therapeutic outcomes, remains to be determined.
Table 3.1: Influence of CCR5Δ32 genotype on time to non-accidental death after initiation of HAART (median >5-year follow-up)

<table>
<thead>
<tr>
<th>Risk Factor</th>
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<th></th>
<th>Multivariate</th>
<th></th>
</tr>
</thead>
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<td>Hazard Ratio</td>
<td>95% CI</td>
<td>p</td>
<td>Hazard Ratio</td>
</tr>
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<td>0.34-0.94</td>
<td>0.03</td>
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<td>age (per 10 year increment)</td>
<td>1.46</td>
<td>1.26-1.69</td>
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<td>1.50</td>
</tr>
<tr>
<td>baseline HIV RNA (per log_{10} increment)</td>
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<td>1.29-2.16</td>
<td>&lt;0.0001</td>
<td>1.37</td>
</tr>
<tr>
<td>baseline CD4 cell count (per 100 cell decrement)</td>
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<td>1.14-1.34</td>
<td>&lt;0.0001</td>
<td>1.19</td>
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<td>1.17-2.43</td>
<td>0.005</td>
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<td>History of injection drug use</td>
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<td>0.79-1.47</td>
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<td>-</td>
</tr>
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<td>PI vs. NNRTI-containing initial regimen</td>
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<td>0.98-2.00</td>
<td>0.09</td>
<td>-</td>
</tr>
<tr>
<td>95% adherent (yes vs. no)*</td>
<td>0.36</td>
<td>0.28-0.52</td>
<td>&lt;0.0001</td>
<td>0.31</td>
</tr>
</tbody>
</table>

* Adherence was estimated by determining the proportion of time spent on antiretrovirals in the first year of therapy. This parameter was calculated by dividing the number of months of antiretroviral prescriptions dispensed by the number of months of follow-up in the first year [13]
Figure 3.1: Kaplan-Meier analysis of survival following initiation of HAART, by CCR5 genotype

CCR5wt/Δ32 subjects are represented by circle (o) symbols while CCR5wt/wt individuals are represented by cross (+) symbols. Symbols indicate time points at which events occur (in this case, only nonaccidental deaths are considered events. Note that accidental deaths are not considered events and subjects censored on date of death). In this Kaplan-Meier analysis, a significant association between CCR5wt/Δ32 genotype and improved survival after initiating HAART was observed (p<0.05). However it is important to note that this association did not remain statistically significant after controlling for the effects of adherence (see Table 3.1).
3.5 REFERENCES


CHAPTER 4

HUMAN GENETIC VARIATION: IMPLICATIONS OF SINGLE NUCLEOTIDE POLYMORPHISMS IN CX3CR1 AND MDR-1 GENES ON INITIAL RESPONSE TO ANTIRETROVIRAL THERAPY

4.1 INTRODUCTION

Many recent studies have focused on the influence of naturally occurring polymorphisms within the human genome on HIV-1 infection and clinical disease progression. Probably the most notable polymorphism lies within the gene encoding the human chemokine receptor CCR5, a key cell-surface co-receptor for R5 strains of HIV-1 [1-3]. A homozygous 32-base pair deletion in the coding region of CCR5 (CCR5Δ32/Δ32) has been associated with a protective effect with respect to HIV-1 infection [4,5], while the heterozygous genotype (CCR5wt/Δ32) has been associated with slower HIV-1 disease progression [4, 6-8] in untreated individuals.

CX3CR1, a leukocyte chemotactic and adhesion receptor for the human chemokine fractalkine, has also been defined as a minor co-receptor for HIV-1 [9, 10]. Two common single nucleotide polymorphisms (SNPs) within the human CX3CR1 gene have recently been identified: HIV-1 infected patients homozygous for the CX3CR1 249I and 280M amino acid substitutions (a G-to-A nucleotide substitution encoding a V-to-I amino acid substitution at position 249, and a C-to-T nucleotide substitution encoding a T-to-M amino acid substitution at position 280) were shown to progress to AIDS and death more rapidly than those with other haplotypes [11], although these results were not confirmed in an independent study.

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1 This chapter has been published as: Brumme ZL, Dong WW, Chan KJ, Hogg RS, Montaner JS, O'Shaughnessy MV, Harrigan PR. "Influence of polymorphisms within the CX3CR1 and MDR-1 genes on initial antiretroviral therapy response." AIDS. 2003;17:201-8.
(which examined the polymorphism at codon 280 only) [12]. Although the influence of CCR5 polymorphisms on response to antiretroviral therapy has been examined [13-15], to date, no published studies appear to have examined whether polymorphisms in the CX3CR1 gene are associated with altered response to antiretroviral therapy.

SNPs in other genes may also affect response to antiretroviral therapy more directly. The human multidrug-resistance (MDR)-1 gene encodes the ATP-dependent P-glycoprotein (P-gp) efflux membrane transporter which possesses a broad substrate specificity [17, 18] and therefore the ability to export a wide range of hydrophobic molecules from cells [19, 20]. The HIV-1 protease inhibitors (PIs) are substrates for the P-gp drug efflux pump in vitro [21-23], a finding which could potentially affect the bioavailability of HIV-1 protease inhibitors and limit the therapeutic efficacy of PIs in P-gp expressing cells [24], including CD4+ cells [25]. A single C-to-T nucleotide polymorphism in exon 26 (C3435T) of the MDR-1 gene is significantly correlated with decreased intestinal expression levels and activity of P-glycoprotein [26]. In other disease models, mainly cancer, the level of MDR-1 expression is correlated with the resistance of tumour cells against chemotherapeutic agents [27, 28]. It has also been found that overexpression of multidrug transporters significantly reduces the accumulation of PIs in human lymphocytes [24], and recently shown that individuals homozygous for the 3435C allele exhibited increased efflux of the P-gp substrate rhodamine 123 from CD56+ natural killer cells [29]. Based on these findings and evidence of racial differences in 3435C allele distribution, it was hypothesized that differential P-gp expression could affect the utility of PIs in African populations [30]. Finally, data have confirmed a correlation between initial CD4 response, nelfinavir and efavirenz levels, and MDR-1 polymorphisms in adherent patients [31].
To address whether previously identified SNPs in CX3CR1 or MDR-1 affect response to antiretroviral therapy in an unselected patient population, we retrospectively determined the prevalence of the CX3CR1 249I and 280M amino acid substitutions, and the MDR-1 C3435T polymorphisms in a group of 461 antiretroviral naïve individuals initiating their first HIV therapy in British Columbia, Canada, between June 1996 and August 1998, and assessed the potential effects of these natural human polymorphisms on the initial virological and immunological response to antiretroviral therapy.
4.2 MATERIALS AND METHODS

Study Design and Patients

In the province of British Columbia, Canada, antiretroviral drugs are distributed free of charge to HIV-infected individuals through a centralised drug treatment program based at the British Columbia Centre for Excellence in HIV/AIDS (the B.C. Centre). Briefly, B.C. treatment guidelines recommend that plasma viral load testing be performed at baseline, again at 4 weeks after initiating or changing therapy, and approximately every 3 months thereafter [see reference 32 for more details of policies and treatment guidelines]. The median time between plasma viral load measurements for subjects included in this study was three months (Interquartile Range 2-4 months). Less than 5% of sequential viral load measurements had an elapsed time of ≥ 8 months. All antiretroviral naïve individuals first seeking treatment at the B.C. Centre between June 1, 1996 and August 31, 1998, were eligible for analysis in this retrospective study (N=479). Of these, 461 (96.2%) had a pre-therapy plasma sample available for testing. Greater than 80% of the antiretroviral naïve patients were prescribed triple therapy including two nucleoside analogues and one protease inhibitor, consistent with treatment guidelines existing at the time. Adherence to medication was evaluated by analyzing patients' prescription records and determining the frequency of prescriptions filled on time during the first year of therapy [33, 34], with "adherence" being defined here as filling 100% of prescriptions on schedule.

Ethical Considerations

Ethical approval for this study was obtained from the local ethics board at St. Paul's Hospital, Vancouver. All patient samples were assigned new anonymous code identifiers
prior to genotypic analyses. Laboratory analyses were performed using the coded identifiers only.

**Plasma Viral Load and CD4 Cell Count**

CD4 cell counts were measured by standard techniques. Plasma HIV-1 RNA levels (viral loads) were determined using the Roche Amplicor Monitor assay (Roche Diagnostics, Laval, Quebec, Canada) using either the standard method or the ultrasensitive adaptation. The ultrasensitive adaptation to this assay was not routinely available over the time period of this study; for this reason a constant cut-off of 500 HIV RNA copies/ml was used throughout the analysis.

**Determination of CX3CR1, MDR-1, CCR5 Δ32 polymorphisms, and HIV-RNA Genotyping for drug resistance.**

Plasma nucleic acids were extracted and precipitated with guanidine thiocyanate and isopropanol followed by two ethanol washes. The region of CX3CR1 containing the polymorphisms of interest was amplified in a single round of PCR, using the oligonucleotide primers CX3CR1 F1 (5’ CTGAATCAGTGACAGAAAACTTT 3’) and CX3CR1 R1 (5’ GTAGACACAAGGCTTTGGGATTC 3’), to generate a 1.1 Kb product. In the case of the MDR-1 polymorphism in exon 26, one round of PCR using oligonucleotide primers MDR-1 F1 (5’ CAATTATGACCTTGGTGTTAGGTTAA 3’) and MDR-1 R1 (5’ AGATGCTTGTATACAGGTAAGG 3’) was performed, to generate a 0.5 Kb product. PCR products were then sequenced directly in both the 5’ and 3’ directions using these primers and the BigDye dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and resolved on an ABI 3700 automated sequencer. CCR5Δ32 status [13] and HIV-1 drug-resistance genotyping for the protease gene [35] were performed as previously described.
Statistical Analysis

Time to virological success was defined as the time to achieve a plasma viral load (pVL) \( \leq 500 \) copies HIV-1 RNA/ml from baseline. Time to virological failure was defined as the time elapsed between virological success and the second of two consecutive pVL measurements > 500 copies/ml. If a person never achieved virological success, they were excluded from the analysis of time to virological failure. If a patient reached therapeutic success and did not subsequently fail then they were censored at the time of their last viral load date (occurring prior to December 1, 2000). Time to immunological failure was defined as the time to the second of two successive CD4 cell counts below baseline. The effects of the CX3CR1, MDR-1, and combined CX3CR1 and CCR5 polymorphisms on time to virological and immunological success and/or failure were analyzed by Kaplan-Meier methods. Group differences were assessed using the log-rank test. Cox proportional hazard regression was used to model the effect of the CX3CR1 and MDR-1 polymorphisms on time to virological and immunological failure while adjusting for age, gender, baseline pVL, baseline CD4 cell count, adherence, type of antiretroviral therapy at initiation (triple therapy with 2 NRTIs and a PI vs. dual therapy), and "time to virological success" in the virological failure model only. Finally, Fisher's exact test was used to assess the potential correlation between MDR-1 genotype and the development of HIV-1 resistance to protease inhibitors in the first two years of therapy (an analysis restricted to those patients initiating therapy including a protease inhibitor).

All of the 461 patients in the study were included in the "time-to-virological success" analyses. In order to be included in the "time-to-virological failure" analysis, patients had to register at least one post-baseline HIV pVL \( \leq 500 \) copies/ml; this included 374 of 461
individuals (81.1%). 382 of 461 individuals (82.9%) had at least one post-baseline CD4 count, and were therefore included in the time-to-immunological-failure analysis.
4.3 RESULTS

Baseline characteristics of the 461 HIV-1 infected individuals examined for CX3CR1 249I and 280M amino acid substitutions and the MDR-1 C3435T polymorphisms are presented in Table 1. There were no significant differences with respect to age, gender, baseline plasma viral load, CD4 count, AIDS defining-illness or history of injection drug use between the 479 patients eligible for the study and the 461 (96.2%) patients for whom plasma samples were available for testing. However, patients in the study group (N=461) were more likely to have been prescribed triple therapy (p=0.03). There were no statistically significant differences observed between any baseline parameter and presence or absence of SNPs (p>0.1, data not shown). The median follow up time after starting therapy was 40 months.

PCR products and CX3CR1 genotypes were obtained for a total of 451 of 461 individuals (97.8%). At CX3CR1 codon 249, 282 individuals (62.5%) were homozygous G/G (encoding amino acid 249V), 54 individuals (12.0%) were homozygous A/A (encoding amino acid 249I), and the remaining 115 (25.5%) were heterozygous. At CX3CR1 codon 280, 339 (75.2%) were homozygous C/C (encoding amino acid 280T), 27 (6.0%) were homozygous T/T (encoding amino acid 280M), and the remaining 85 (18.8%) were C/T heterozygous. The CX3CR1 249G allele (encoding amino acid 249V) was in complete linkage disequilibrium with the 280C allele (encoding amino acid 280T) [11]. PCR products and MDR-1 genotypes were obtained for 455 of 461 individuals (98.7%). A total of 137 (30.1%) individuals were 3435 C/C, 165 (36.3%) were 3435 T/T, and the remaining 153 (33.6%) were 3435 C/T. As described elsewhere, patients were also genotyped for the CCR5Δ32 [13]. The allele and haplotype frequencies for all polymorphisms in the CX3CR1, MDR-1 and CCR5 genes are summarized in Table 2. There was no genetic linkage detected.
between any of the polymorphic groups (with the exception of the linkage observed within the CX3CR1 gene).

Kaplan-Meier analysis of the influence of MDR1 genotype on time elapsed to virological failure (defined as the time elapsed to the second of two successive pVL>500 copies/ml from initial success) is shown in figure 1. The proportion of individuals not achieving virological success was between 18-20% for all three groups. There was a trend to earlier virological failure in the homozygous 3435C/C group which did not attain statistical significance (p=0.07). No effect of the 3435 C/T nucleotide polymorphism in the MDR-1 gene on immunological failure, defined as the time elapsed to the second of two consecutive CD4 counts below baseline, was detected (p=0.46, data not shown). When only the homozygous groups (3435C/C and 3435T/T) were considered in the analysis, the correlation between 3435C/C and earlier virological failure reached statistical significance (p=0.046), but no effect was detected on immunological failure (p=0.97). In a secondary Kaplan-Meier analysis, there was no detectable influence of MDR-1 genotype on the time elapsed to virological success (time to HIV pVL ≤ 500 copies/ml) (p=0.371, data not shown).

In addition, we wished to determine whether the polymorphism in MDR-1 could be correlated with the development of resistance to HIV protease inhibitors [24]. A search of our HIV-1 genotype database identified a subset of 124 (of 461) individuals (26.9%) who had at least one HIV-1 antiretroviral resistance genotype requested by a physician performed during the course of study follow-up, at least 6 months after initiation of therapy. 13 of 124 patients (10.5%) displayed decreased susceptibility to one or more protease inhibitors in at least one post-therapy genotype using a "virtual" phenotype analysis [35] where "decreased susceptibility" was defined as a > 3-, 3.5-, 4-, 2.5-, 2-, or 2.5-fold decrease in susceptibility to
indinavir, ritonavir, nelfinavir, saquinavir, amprenavir and lopinavir, respectively [36]. Samples for which genotypes were available revealed no correlation between the MDR-1 polymorphism and development of resistance to protease inhibitors using the Fisher's exact test, in an analysis restricted to those patients initiating therapy including a protease inhibitor (n=83, p=0.99). PI-resistance was evenly distributed between the MDR-1 3435 C/C, T/T, and C/T genotype groups.

For the Kaplan-Meier analyses of the influence of the CX₃CR1 genotype on time elapsed to virological and immunological failure, patients were classified into categories based on their amino acid polymorphisms at codons 249 and 280. The first analysis included homozygous amino acid combinations only (CX₃CR1 genotypes 249I 280T, 249I 280M and 249V 280T). The proportion of individuals censored in the time to virological failure analysis was similar for all three groups. No significant influence was observed between the 3 groups with respect to time elapsed to virological failure (p=0.99, data not shown), however, a tendency to earlier immunological failure was observed in the two groups containing the 249I polymorphism which achieved statistical significance (p=0.02, figure 2). When heterozygous allele combinations were included in the analysis, the effect on virological outcome remained not statistically significant (p=0.92, data not shown), and a trend for earlier time to immunological failure for the two groups containing the homozygous 249I polymorphism remained (p=0.07, data not shown). Kaplan-Meier survival curves for the heterozygous allele groups fell near those of the homozygous 249V 280T group. Finally, when both homozygous and heterozygous allele combinations at codons 249 (I, I/V, V) and 280 (T, T/M, M) were analyzed independently (N=451), no effects of variation at codons 249 or 280 on virological outcome could be demonstrated (p=0.78 and p=0.52, respectively).
Similarly, no effect of variation at codon 280 alone [12] on immunological outcome could be demonstrated (p=0.167). However, a statistically significant trend for earlier immunological failure in the homozygous 249I group was observed (p=0.036, data not shown). In a secondary analysis, no influence of CX3CR1 genotype on the time elapsed to virological success was observed (p=0.89, data not shown).

No significant association between the CX3CR1 249I or 249V genotypes and CCR5Δ32 status was observed (N=446, p>0.1, data not shown). No effect of the combined CX3CR1 and CCR5 genotypes on time to virological success or virological or immunological failure was observed (p>0.6, data not shown).

Cox proportional hazard regression was used to model the effect of the CX3CR1 and MDR-1 polymorphisms on time to virological and immunological failure while adjusting for age, gender, baseline pVL, baseline CD4 cell count, adherence, type of antiretroviral therapy at initiation, and "time to virological success" in the virological failure model only (table 3). The results were consistent with the previous analyses. The MDR-1 virological analysis reached statistical significance (p=0.04) (risk ratios [and 95% confidence intervals] were 0.67 [0.45-0.99] for the MDR-1 3435 T/T and 0.71 [0.47-1.06] for the MDR-1 3435 C/T (Y) genotypes), and results of the CX3CR1 immunological analysis also remained significant (p=0.05) (risk ratios were 1.92 [1.00-3.69] for the CX3CR1 249I 280T and 1.46 [0.80-2.70] for the 249I 240M genotypes). Of interest, the only baseline variable that was consistently protective in the multivariate analyses was "100% adherence" (p=0.0001, Table 3), which was evaluated using prescription refill records [33,34], with "adherence" being defined as filling 100% of prescriptions on schedule.
In order to control for non-adherence, a secondary subset analysis was performed on only those individuals who were most likely highly adherent to therapy based on the above definition (240 of 461 subjects, or 52.1%). The adherence subset analyses were consistent with previous results (p>0.05 for CX3CR1 and MDR-1 virological failure and MDR-1 immunological failure, and p <0.05 for CX3CR1 immunological failure).
4.4 DISCUSSION AND CONCLUSIONS

This study investigated the effects of single nucleotide polymorphisms (SNPs) within the human CX3CR1 and MDR-1 genes on time to virological and immunological treatment failure in a large cohort of antiretroviral naive patients initiating therapy. We report a small but statistically significant correlation between the CX3CR1 2491 polymorphism and slightly earlier immunological failure; and a trend to earlier virological failure in the MDR-1 3435C/C genotype group.

The role of MDR-1 gene polymorphisms and P-glycoprotein expression in HIV pathogenesis remains largely unresolved. It has been suggested that overexpression of multidrug transporters may accelerate the development of HIV-1 resistance to protease inhibitors [24], and that the MDR-1 3435C/C genotype may be a factor restricting access of HIV-1 protease inhibitors to target cells expressing P-gp [30]. The MDR-1 3435C/C genotype has a much higher frequency among individuals of African origin (between 60-80%) [30] than in individuals of Caucasian or Asian origin (> 30%) [26, 30, 37]. Although complete ethnic background data is not available for our cohort (individuals are not obligated to answer any questions pertaining to ethnic origin), partial data indicates that the cohort is mainly comprised of persons of caucasian and aboriginal descent. Based on this partial ethnic data, the frequency of the homozygous MDR-1 3435C/C genotype (30.1%) was consistent with the frequency previously reported for Caucasian and Asian populations [26, 30, 37]. In support of previous reports suggesting a potential effect of the MDR-1 polymorphism on the effectiveness of HIV-1 protease inhibitors [24], a strong trend to earlier virological failure in the MDR-1 3435C/C genotype group was observed. However, there was no detectable effect of the MDR-1 polymorphism on initial immunological response to
antiretroviral therapy using our *a priori* definition, or, in a limited subset, HIV-1 drug resistance development. These observations extend the findings from a recent study of 123 Caucasian, drug-naïve patients initiating antiretroviral therapy, which reported a beneficial effect of the MDR-1 3435T/T genotype on short-term CD4 cell count increases [31]. Differences in virological response could not be detected over the 6-month follow-up period [31], compared to the 40 months of follow-up reported here.

The CX3CR1 haplotype frequencies determined in this study are consistent with those previously reported [11]: 249V 280T was the most common, with a haplotype frequency of 0.75 (compared to 0.678-0.730 in French cohorts), followed by 249I 280M, at 0.15 (France 0.126-0.198), and finally 249I 280T, at 0.1 (France 0.123-0.142). Similarly, we observed complete linkage disequilibrium within CX3CR1 SNPs, and no discernible genetic linkage between the CX3CR1 and CCR5 polymorphisms [11]. Thus, despite the fact that the CX3CR1 and CCR5 genes are found in close proximity on chromosome 3 [11], any effects of the CX3CR1 polymorphisms on disease progression and therapy outcome are not due to linkage to the CCR5Δ32 allele. CX3CR1 249I 280M was previously identified as a recessive HIV genetic risk factor in untreated individuals, with 280M (but not necessarily 249I) independently associated with faster progression to clinical AIDS and more rapid CD4 decline [11]. However, McDermott *et al.* examined CX3CR1 280M alone and could not confirm these results [12]. Although not directly addressed here, the discrepancy between the two studies may be explained by our observation that the polymorphism at codon 249, and not codon 280, may be largely responsible for the differences observed in response to therapy. The homozygous 249I polymorphism was associated with reduced immunological response alone and in combination with variation at codon 280, while independent variation
at codon 280 was not significant in our analyses. Variation in CX3CR1 genotype at either codon 249 or 280 however, did not influence virological response in any of our analyses.

A limitation of this study relates to the frequency of viral load and CD4 tests performed on the study group. Although the majority of patients had tests performed at baseline, one month, and every three months thereafter (the median time between viral load tests in this study was 3 months), a minority of patients may have had viral load tests performed more or less often. It is possible therefore, that the therapy follow-up data may be biased towards longer times to success and/or failure, due to the possibility that some study subjects may have large gaps between viral load or CD4 tests. Another possible limitation of this study relates to the potential confounding effect provided by incomplete adherence. We addressed this using a previously validated definition based on prescription refills [33,34]. Although we cannot rule out the possibility of incomplete adherence, it is unlikely that this would be unbalanced between SNP groups. Other potential limitations of this study include its retrospective nature, the fact that the cohort, although large, represents only a geographically limited population, and the fact that the resistance subset for the MDR-1 analysis is non-random, and therefore potentially subject to bias. Finally, the biological mechanism of the effects of the CX3CR1 and MDR-1 polymorphisms is unclear. In support of previous studies identifying the CX3CR1 249I 280M genotype as a genetic risk factor in AIDS progression in untreated individuals [11], and linking the MDR-1 3435 C/C genotype to reduced CD4 cell responses [31], we have observed a correlation between the CX3CR1 249I polymorphism and the MDR-1 3435C/C genotype and slightly earlier immunological and virological failure, respectively. The magnitude of these effects is relatively minor despite the fact that they remain statistically significant after adjusting for multiple factors.
The actual utility of determining these genotypes in routine clinical practice is therefore not clear.
Table 4.1: Baseline characteristics of the study population, prior to the initiation of antiretroviral therapy (N=461).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>median</th>
<th>IQR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sample size</td>
<td>461</td>
<td>-</td>
</tr>
<tr>
<td>age (years)</td>
<td>36</td>
<td>31-42</td>
</tr>
<tr>
<td>Gender (%)</td>
<td>391 Male (84.8%)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>70 Female (15.2%)</td>
<td>-</td>
</tr>
<tr>
<td>plasma viral load (x10^3 copies/ml)</td>
<td>89</td>
<td>30-280</td>
</tr>
<tr>
<td>CD4 count (x10^6 cells/l)</td>
<td>310</td>
<td>140-460</td>
</tr>
<tr>
<td>Baseline AIDS diagnosis</td>
<td>49 (10.6%)</td>
<td>-</td>
</tr>
<tr>
<td>% Triple Therapy</td>
<td>385 (83.5%)</td>
<td>-</td>
</tr>
</tbody>
</table>

The distribution of baseline parameters was similar for all the individual CX3CR1 and MDR-1 genotype groups (p>0.1). (IQR = interquartile range)
Table 4.2: Allele and haplotype frequencies for the CX3CR1, MDR-1 and CCR5 polymorphisms.

### CX3CR1

<table>
<thead>
<tr>
<th>Genotypes at codon 249</th>
<th>codon 280</th>
<th># of patients (N = 451)</th>
<th>% of patients</th>
<th>Independent allele frequencies</th>
<th>haplotype frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/A (249I)</td>
<td>C/C (280T)</td>
<td>21</td>
<td>4.7</td>
<td>249A=0.25</td>
<td>249I 280T = 0.1</td>
</tr>
<tr>
<td>A/A (249I)</td>
<td>T/T (280M)</td>
<td>27</td>
<td>6.0</td>
<td>249G=0.75</td>
<td>249I 280M = 0.15</td>
</tr>
<tr>
<td>A/A (249I)</td>
<td>C/T (280T/M)</td>
<td>6</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G (249V)</td>
<td>C/C (280T)</td>
<td>282</td>
<td>62.5</td>
<td>280C=0.85</td>
<td>249V 280T = 0.75</td>
</tr>
<tr>
<td>G/A (249V/I)</td>
<td>C/C (280T)</td>
<td>36</td>
<td>8.0</td>
<td>280T=0.15</td>
<td></td>
</tr>
<tr>
<td>G/A (249V/I)</td>
<td>C/T (280T/M)</td>
<td>79</td>
<td>17.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### MDR-1

<table>
<thead>
<tr>
<th>Genotypes at nucleotide 3435</th>
<th># of patients (N = 455)</th>
<th>% of patients</th>
<th>allele frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>3435 C/C</td>
<td>137</td>
<td>30.1</td>
<td>3435C=0.47</td>
</tr>
<tr>
<td>3435 T/T</td>
<td>165</td>
<td>36.3</td>
<td>3435T=0.53</td>
</tr>
<tr>
<td>3435 C/T</td>
<td>153</td>
<td>33.6</td>
<td></td>
</tr>
</tbody>
</table>

### CCR5

<table>
<thead>
<tr>
<th>CCR5 Genotype</th>
<th># of patients (N = 461)</th>
<th>% of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR5 wt/wt</td>
<td>392</td>
<td>85.0</td>
</tr>
<tr>
<td>CCR5 Δ32/wt</td>
<td>68</td>
<td>14.8</td>
</tr>
<tr>
<td>CCR5 Δ32/Δ32</td>
<td>1</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Table 4.3: Multivariate Cox Model showing effect of baseline parameters, including MDR-1 and CX3CR1 polymorphisms, on time to Virological and Immunological Failure.

<table>
<thead>
<tr>
<th>Baseline Variable</th>
<th>Time to virological failure</th>
<th>Time to immunological failure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Risk ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td>MDR-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.91</td>
<td>0.83-0.99</td>
</tr>
<tr>
<td>Male sex</td>
<td>0.77</td>
<td>0.48-1.22</td>
</tr>
<tr>
<td>plasma viral load</td>
<td>1.14</td>
<td>0.91-1.43</td>
</tr>
<tr>
<td>CD4 count</td>
<td>1.09</td>
<td>1.02-1.15</td>
</tr>
<tr>
<td>100% adherence</td>
<td>0.84</td>
<td>0.79-0.90</td>
</tr>
<tr>
<td>Triple Therapy</td>
<td>0.39</td>
<td>0.26-0.59</td>
</tr>
<tr>
<td>MDR-1 3435T/T</td>
<td>0.67</td>
<td>0.45-0.98</td>
</tr>
<tr>
<td>MDR-1 3435C/T (Y)</td>
<td>0.71</td>
<td>0.47-1.06</td>
</tr>
<tr>
<td>time to virological success</td>
<td>1.00</td>
<td>0.97-1.03</td>
</tr>
<tr>
<td>CX3CR1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.90</td>
<td>0.80-1.01</td>
</tr>
<tr>
<td>Male sex</td>
<td>0.96</td>
<td>0.54-1.71</td>
</tr>
<tr>
<td>plasma viral load</td>
<td>1.36</td>
<td>1.03-1.81</td>
</tr>
<tr>
<td>CD4 count</td>
<td>1.09</td>
<td>0.99-1.19</td>
</tr>
<tr>
<td>100% adherence</td>
<td>0.84</td>
<td>0.78-0.90</td>
</tr>
<tr>
<td>Triple Therapy</td>
<td>0.37</td>
<td>0.22-0.64</td>
</tr>
<tr>
<td>CX3CR1 249I/280T</td>
<td>0.96</td>
<td>0.39-2.33</td>
</tr>
<tr>
<td>CX3CR1 249I/280M</td>
<td>1.13</td>
<td>0.59-2.20</td>
</tr>
<tr>
<td>time to virological success</td>
<td>1.00</td>
<td>0.96-1.03</td>
</tr>
</tbody>
</table>

CI, confidence interval
Figure 4.1: Kaplan-Meier plot comparing the influence of nucleotide variation at position 3435 of the human P-glycoprotein (MDR-1) gene on time to virological failure.

The number of patients in each genotype group (3435 C/C, C/T, and T/T) still experiencing virological success at 24 months was (55, 72 and 74). (p=0.07)
Figure 4.2: Kaplan-Meier plot comparing the influence of amino acid variation at codons 249 and 280 of the human CX3CR1 receptor gene on time to immunological failure.

For clarity, only those genotype groups involving homozygous amino acid combinations are shown. The number of patients in each genotype group (CX3CR1 249I 280T, 249I 280M, and 249V 280T) still experiencing immunological success at 24 months was (9, 10, 133). (p=0.02)
4.5 REFERENCES


CHAPTER 5

HIV GENETIC VARIATION: IMPLICATIONS OF ANTIRETROVIRAL RESISTANCE TO LONG-TERM HAART OUTCOMES

5.1 INTRODUCTION

Morbidity and mortality associated with Human Immunodeficiency Virus (HIV) infection and Acquired Immunodeficiency Syndrome (AIDS) have declined significantly in North America and Western Europe as a result of the introduction of combination antiretroviral therapies [1-6]. Despite these advances in HIV treatment, HIV-related deaths continue to occur across the developed world [1-4, 7, 8].

Drug-resistant HIV variants are often cited as a major barrier to long-term antiretroviral efficacy and a major cause of treatment failure [9-16]. However, it remains to be conclusively determined what proportion of recent HIV-related deaths among those with access to antiretroviral therapy are due to diminished treatment options as a consequence of multi-drug resistance and what proportion may be due to other factors.

Using data from a centralized HIV/AIDS Drug Treatment Program in British Columbia, Canada, the prevalence of antiretroviral resistance among HIV-infected persons who died between July 1997 and December 2001 was contrasted to the prevalence of resistance among living individuals experiencing virologic therapy failure over the same study period. Our objective was to determine the degree to which antiretroviral resistance may contribute to mortality among HIV-infected individuals in the era of Highly Active Antiretroviral Therapy (HAART).

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1 This chapter has been published as:
5.2 MATERIALS AND METHODS

British Columbia HIV/AIDS Drug Treatment Program

In the province of British Columbia, Canada, antiretroviral agents have been distributed at no cost to all eligible HIV-infected individuals through a centrally administered HIV/AIDS Drug Treatment Program (DTP) since 1986. The DTP remains the only source of free-of-charge antiretroviral therapy in the province, and pharmaceutical sales suggest that <1% of HIV-infected British Columbians purchase antiretrovirals outside the DTP [17]. The DTP database contains sociodemographic data as well as comprehensive antiretroviral prescription and treatment data for all those accessing HIV care and treatment in British Columbia. Details on patient enrolment, policies and DTP treatment guidelines have been previously published [17].

As a part of routine patient monitoring, plasma HIV-1 RNA levels (HIV viral loads) and CD4 cell counts are generally measured at baseline, after one month, and at three month intervals thereafter. A portion of the plasma sample used for HIV viral load testing is also archived. Ethical approval for this study was obtained from the research ethics board at the University of British Columbia/St. Paul's Hospital, Vancouver.

Study Sample and Variables of Interest

A total of 637 deaths were recorded amongst persons enrolled in the DTP between July 1997 and December 2001. Deaths were identified through physician reports and through linkages with the BC Division of Vital Statistics. Deaths were classified according to ICD-9 (1995-1999) and ICD-10 (2000-2001) coding. Underlying causes of death coded under ICD-9 categories 042-044 and ICD-10 categories B20- B24 identified deaths directly caused by HIV infection. July 1997 was selected as the study start date because HAART became
universally available to all HIV-positive individuals seeking treatment in British Columbia at this time. Subjects included in this study form a part of a larger group of HIV-infected individuals who have died in British Columbia between 1995 and 2001, who have recently been described in detail with respect to sociodemographic characteristics, risk factors and access to antiretrovirals [17]. Time spent on antiretroviral treatment was estimated using a previously validated method based on analyzing prescription refill data [18].

**Genotypic Resistance Testing**

Accidental deaths were assumed to be not directly related to antiretroviral resistance and these plasma samples were not genotyped. Samples with viral loads < 500 copies HIV RNA/ml were not genotyped due to an inadequate amount of plasma HIV. However, because these plasma samples originated from individuals with successful suppression of plasma HIV RNA, it is unlikely that these samples contained HIV harboring resistance mutations in sufficient quantities to compromise effectiveness of therapy.

Genotypic drug resistance testing was restricted to those individuals who were prescribed antiretrovirals and who died of a non-accidental cause (Figure 1). For each subject meeting these requirements, the last on-therapy plasma sample was genotyped for drug resistance if the plasma viral load (pVL) was ≥500 copies HIV RNA/ml. Resistance genotyping was performed as previously described [19].

Samples were considered "resistant" if they displayed one or more major resistance mutations, based on the IAS-USA list [20]: lamivudine resistance (184I/V); any other nucleos(t)ide Reverse Transcriptase Inhibitor (nRTI) resistance (41L, 62V, 65R, 67N, 69A/N/D or insertion, 70R, 74V, 75I, 151M, 210W, 215F/Y or 219E/Q); any Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) resistance (100I, 103N, 106A, 108I,
181C/I, 188C/H/L/Y, 190A/S, or 236L); or any Protease Inhibitor (PI) resistance (30N, 46I/L, 48V, 50L/V, 54L/M, 82A/F/S/T, 84V, or 90M).

**Drug resistance among living HIV-infected persons with virologic therapy failure**

In the evaluation of HIV treatment outcomes, mortality is often used as an endpoint in the definition of "therapy failure". However, surrogate markers such as HIV plasma viral load and CD4 cell count are also commonly used as definitions of "therapy failure" in clinical trials and population-based studies. In order to provide context, the prevalence of resistance in the study group (where "therapy failure" was defined as mortality) was contrasted to the prevalence of resistance in a large group of living individuals selected based on a plasma-viral load driven definition of "therapy failure". A "virologic failure" group of N=1220 subjects was therefore selected from the HIV/AIDS Drug Treatment Program database which represented all living HIV-infected individuals in British Columbia failing their therapy (as defined by a pVL ≥ 500 copies HIV RNA/ml) between the study dates of July 1997-December 2001, for whom a physician-requested HIV genotypic drug resistance test had been performed. In cases where an individual had multiple genotypes performed over the period of interest, the latest available on-therapy genotype was selected. Genotypic resistance testing and interpretation of resistance genotypes was performed as described above. Individuals included in the mortality group (N=637) and the virologic failure group (N=1220) represent nearly 40% of the total of ~4600 patients who ever received therapy in B.C. in this time period.

**Statistical Analyses**

Categorical variables were compared between groups using Pearson’s chi-squared test. Contingency tables that contained one or more expected counts of less than five were
analyzed by the Fisher’s exact test. The Cochran-Armitage test was used to assess resistance trends over time. Comparisons of continuous variables were carried out using Wilcoxon’s rank-sum test. All reported p-values are two-sided.
5.3 RESULTS

*Causes of mortality and antiretroviral therapy in the study group*

A total of 637 deaths were recorded among HIV-infected individuals enrolled in a province-wide HIV/AIDS Drug Treatment Program between July 1997 and December 2001 (Figure 1). Of these, 83 (13.0%) were attributed to accidental causes, the two most common causes (by ICD-9 or ICD-10 coding) being illicit drug overdoses (57.8%) and concussion (18.1%). The remaining accidental deaths included suicides, traffic accidents, assaults and other injuries. Although HIV disease may have played an indirect role in these deaths, accidental deaths were assumed not to be directly related to antiretroviral resistance and were not included in genotypic analyses.

The remaining 554 deaths (87.0%) were attributed to non-accidental causes. Deaths included 383 (69.1%) directly related to HIV infection (ICD-9 categories 042-044 and ICD-10 categories B20-B24), 34 (6.1%) liver disease, 25 (4.5%) various cardiac conditions, 20 (3.6%) viral and/or bacterial infections, 18 (3.2%) malignant neoplasms, 44 (7.9%) other causes, and 31 (5.6%) unknown causes.

Of the 554 non-accidental deaths, 58 (10.5%) occurred in individuals who had enrolled in the province's HIV/AIDS Drug Treatment Program, but who had never been dispensed any antiretrovirals. Plasma samples from these individuals were not genotyped, as resistance mutations would not likely have been present. The remaining 496 (89.5%) non-accidental deaths occurred in individuals with at least some exposure to antiretroviral agents.

The number of non-accidental deaths per year among treated individuals increased slightly over the period of interest (Table 1). The median time elapsed between initiation of first antiretroviral therapy and date of death in this cohort increased over the study period,
from 28 months for deaths occurring in the last half of 1997, to 47 months for deaths occurring in 2001 (p<0.001). The median time actually spent on antiretroviral therapy increased from 11 months of antiretroviral exposure for deaths occurring in the last half of 1997, to 28 months of antiretroviral exposure for deaths occurring in 2001. Similarly, the median time spent on HAART increased from 4 to 15 months over the study period. The median time elapsed between stopping therapy and death remained constant over the study period, with death occurring a median of 2 months after discontinuation of therapy [IQR 0.5-7.4 months]. Although reasons for discontinuation of therapy are unknown, it is of interest to note that the majority of individuals (287 of 496, 58%) were not being prescribed antiretrovirals at time of death.

Of the 496 non-accidental deaths among treated individuals, 99 (20.0%) occurred in individuals for whom no viral load data were available and for whom no plasma samples were available for resistance testing. The median duration between initiation of therapy and death in these individuals was 18 months [IQR 5-44 months], however, the median time actually spent on antiretrovirals was only 2 months [IQR 0-4 months]. It is therefore unlikely that broad drug resistance would have played a major role in their deaths, given their relatively short exposure to antiretroviral agents. The median CD4 count before death in this group was 90 cells/mm$^3$ [IQR 20-250].

**Genotypic Resistance Testing**

Of the 397 treated individuals for whom plasma samples were available (Table 2), 147 (37.0%) deaths occurred in individuals whose last on-therapy viral load was <500 copies HIV RNA/ml. Although it was not possible to genotype these samples, the fact that plasma HIV RNA was suppressed below 500 copies/ml suggests that drug resistance mutations were
not present in quantities sufficient to compromise therapy. Of interest, 60 (41%) of these 147 individuals discontinued antiretrovirals for unknown reasons shortly after this viral load measurement, and were therefore not receiving any antiretrovirals at time of death. The remaining 250 (63.0%) deaths occurred in individuals whose last on-therapy viral load was ≥500 copies/ml; drug resistance genotyping was successful in 232 of these 250 samples (92.8%).

The majority of the last on-therapy plasma samples (212 of 379, 55.9%) represented individuals with either successful suppression of plasma HIV RNA <500 copies/ml (N=147) or contained HIV harboring no major antiretroviral resistance mutations (N=65). The proportion of isolates with and without resistance-associated mutations remained relatively stable over the course of the study period (Figure 2A). A total of 167 samples (44.1%) harbored HIV with at least one major resistance mutation (Figure 2A), an observation strongly driven by resistance to nRTIs (142 samples, 37.4%), specifically, resistance to lamivudine arising from the M184I/V mutation (101 samples, 26.6%). A total of 83 (21.9%) and 53 (13.9%) samples had mutations conferring resistance to NNRTIs and PIs, respectively (data not shown). Isolates harboring HIV with mutations conferring resistance to drugs from at least 2 classes and drugs from all 3 classes were observed relatively infrequently, at 23.4% and 5.8% of isolates, respectively (Figure 2A).

Individuals who started on mono or dual therapy were significantly more likely to harbor resistant HIV than individuals who received HAART exclusively (Figure 2B). Resistance to at least one class of drugs was observed in 138 of 253 individuals (54.5%) who started on mono or dual therapy, compared to 29 of 126 individuals (23.0%) who received HAART exclusively (p<0.001). Similarly, resistance to at least two drug classes was
observed in 77 individuals (30.4%) who started on mono or dual therapy, compared to 12 of 126 individuals (9.5%) who received HAART exclusively (p<0.001). Of interest, *not a single individual* who received HAART as their first therapy harbored HIV with resistance to all 3 antiretroviral classes. All individuals with 3-class resistance had initially received mono or dual therapy before receiving HAART (22 of 210, 10.4%) (p<0.001).

Individuals with confirmed resistance mutations had a significantly longer median duration of therapy (32 months [IQR 16-52]) compared with individuals confirmed to have no resistance mutations (15 months [IQR 7-62]) (p<0.001). There were no other significant differences between these two groups (Table 2).

**Antiretroviral Resistance in individuals living with HIV and experiencing virologic therapy failure**

To put these observations into perspective, a virologic failure group was selected which included all *living* DTP participants with a pVL> 500 copies HIV RNA/ml over the same study period, for whom a physician-requested genotypic resistance test had been performed (N=1220). We observed a dramatically higher prevalence of antiretroviral resistance in the virologic failure group than in the mortality group. Of the N=1220 individuals in the virologic failure group, 924 (75.7%) harbored HIV with resistance to at least one drug class, compared to only 167 of 379 (44.1%) in the mortality group (p<0.0001). Similarly the prevalence of resistance to ≥2- and 3 drug classes was also significantly higher in the virologic failure group (42.3% and 11.1% of isolates, respectively) than in the mortality group (23.4% and 5.8% of isolates, respectively) (p<0.001). Resistant isolates in the mortality and virologic failure groups were similar with regards to the number of resistance mutations detected. Isolates from the virologic failure group with single-class
resistance had a median of 2 major resistance mutations, compared to a median of only one major resistance mutation in single-class resistant isolates from the mortality group. The number of resistance-associated mutations in isolates with 2 and 3-class antiretroviral resistance was the same for both groups (median 4 and 8 mutations, respectively).

In contrast to the relatively stable prevalence of antiretroviral resistance in the mortality group (Figure 2A), we observed a dramatic increase in the proportion of isolates in the virologic failure group with resistance to ≥2 classes of drugs over the study period (Figure 3). This was primarily due to an increase in NNRTI resistance among this group: in 1997, only 5.9% of isolates displayed NNRTI resistance, a figure that increased to 38% by 2001 (data not shown).

Reliability of using a single plasma sample for Resistance Testing

For the mortality group, resistance data were obtained from the last on-therapy plasma sample prior to death. It is possible, however, that resistance-associated mutations may have been present in earlier samples. In an attempt to determine the reliability of using this single sample, we analyzed retrospective data to assess the presence of resistance in earlier samples, if available. Earlier genotype data were available for approximately half of patients. Despite this nonrandom sample, patterns of previous antiretroviral resistance were largely consistent with results from the last on-therapy genotype, with resistance to two or three drug-classes observed at some point in the individual's history in only 7% of those that were classified as "not resistant" and 12% of those with pVL<500 copies/ml at the last on-therapy genotype.
5.4 DISCUSSION AND CONCLUSIONS

In order to determine the degree to which antiretroviral resistance may contribute to mortality among HIV-infected individuals in a province where antiretroviral medications and HIV care are provided at no cost through a government-sponsored program, the prevalence of antiretroviral resistance in two groups was investigated. The two groups were selected based on two independent definitions of antiretroviral therapy failure: a group comprising all HIV-infected individuals who had died over the period 1997-2001 (where "therapy failure" was defined as mortality) and a group comprising all living individuals for whom a resistance genotype had been requested over the same study period (where "therapy failure" was defined as a pVL ≥ 500 copies HIV RNA/ml). Specifically, we wished to determine whether mortality in this population was primarily due to an exhaustion of available treatment options arising as a direct result of multi-drug resistance.

The majority (56%) of individuals in the mortality group who received antiretroviral therapy harbored HIV with either no major resistance mutations or had plasma viral load suppression <500 copies HIV RNA/ml, suggesting that drug resistance was not sufficient to compromise therapy. Less than 25% of the mortality group harbored HIV with mutations conferring resistance to antiretrovirals from two or more drug classes, while only a minority (~5%) harbored HIV with mutations conferring resistance to drugs from all three classes. Of interest, triple-class resistance was not observed in any individual who received HAART exclusively. In the larger population, regardless of therapy access, we estimate that resistance to all three classes was present in <4% of the 554 individuals who died of non-accidental causes.
These results clearly suggest that exhaustion of available and effective treatment options, arising as a direct result of multi-drug resistance, was not a driving factor in the vast majority of deaths in this cohort. These results are somewhat in contrast to those from a recent study of 29 HIV-related deaths, that reported mutations conferring resistance to both HIV reverse transcriptase and protease inhibitors in 10 (34%) of the isolates [21].

The relatively low level of antiretroviral resistance observed in the mortality group contrasts to the high level of resistance observed in the virologic failure group. Consistent with the increased use of triple combination therapies over the period of interest, rates of two- and three-class resistance increased dramatically in the virologic failure group, while levels of multidrug resistance remained relatively stable in the mortality group over the same period.

These results represent an interesting paradox: HIV drug resistance, and especially multi-class resistance, is common and increasingly prevalent over time in individuals experiencing virologic therapy failure who remain alive, but remains relatively uncommon in those who have died. Although the inverse association between antiretroviral resistance and mortality may seem counter-intuitive, the duration of antiretroviral therapy in the mortality group suggests that the low level of drug resistance was mainly driven by insufficient antiretroviral exposure. Among individuals who died, the median time elapsed between initiation of therapy and death was over three years; however the time actually spent on antiretrovirals was only 19 months. Given the large number of treatment possibilities today, 19 months is not long enough to have attempted and failed all possible therapy combinations. The discrepancy between these times indicates that in many individuals, antiretroviral therapies were used only intermittently or were discontinued. In fact, the majority of the
study population (including ~40% of individuals whose last on-therapy viral load was <500 copies/ml) were not receiving any antiretrovirals at the time of death, having discontinued therapy for unknown reasons a median of two months previously. In the case of 10% of the study population, there was no antiretroviral exposure at all.

Consistent with these observations are a recent study of HIV-infected individuals in British Columbia which reported a positive association between intermittent use of antiretroviral therapy and mortality [22], and a recent study of 88 HIV-related deaths occurring in 1999-2000 which reported that the majority of patients who died were not receiving HAART despite access to care [8]. In that study [8], an inability to tolerate HAART due to side effects or therapy toxicity, as well as non-adherence, were cited as the major reasons why patients were not receiving antiretrovirals at time of death. In addition, a recent study investigating barriers to antiretroviral access among 1239 HIV-infected British Columbians who died between 1995-2001, of which our cohort was a part, identified female gender, aboriginal ethnicity and low socioeconomic status as the strongest predictors of lack of therapy access, intermittent therapy use and/or discontinuation of therapy despite availability of antiretrovirals at no cost through a government-funded treatment program [17]. Although reasons for intermittent use of therapy are not known for individuals in our study, it is highly likely that side effects, illness, toxicity and other factors would have played a large role.

Finally, a large study estimating the prevalence of antiretroviral resistance in the United States reported that ~78% of HIV-infected individuals with viral loads >500 copies/ml harbored resistant HIV [23], a result which is entirely consistent with the observed prevalence of antiretroviral resistance in our virologic failure group. Most notably, these
investigators also reported a positive correlation between drug resistance and antiretroviral exposure; in this study the prevalence of drug resistance was significantly associated with greater access to treatment [23].

A limitation of this study is the fact that no viral load data or plasma samples were available for approximately 20% of deaths occurring in treated individuals. Although we cannot exclude the possibility that these individuals may have harbored drug-resistant HIV, the fact that these individuals only received a median of 2 months of therapy strongly suggests that drug resistance did not play a large role in their deaths. Additional limitations of this study include its retrospective nature, and the fact that reasons for interruption and/or discontinuation of therapy are largely unknown. Finally, it is important to note that the two different endpoints used (clinical failure as defined by mortality and virologic failure as determined by pVL≥ 500 copies/ml) results in groups which are not directly comparable. The mortality group includes all individuals who died, regardless of whether or not they received antiretrovirals, while the "virologic failure" group includes all individuals who have received therapy and maintained a pVL≥ 500 copies/ml. The total prevalence of resistance in all living HIV-infected individuals in British Columbia is likely much lower than in the "virologic failure" group presented here, as this group excludes those who consistently maintained pVL<500 copies/ml (approximately 1000 individuals), those with at least one pVL≥500 copies/ml but for whom a genotypic resistance test was not requested by their physician (~1700 individuals), those lost to follow-up (~30 individuals), and those who have never received antiretrovirals (number unknown). The data do, however, show the tremendous difference in the prevalence and trends in resistance using these two different definitions of treatment failure.
In conclusion, results of this study strongly indicate that treatment failure due to antiretroviral resistance is not a major factor influencing mortality in this cohort for whom antiretrovirals were available at no cost. For most of the individuals studied, insufficient and/or intermittent exposure to antiretrovirals, comorbidities and other factors likely played a larger role.
Table 5.1: Number of deaths and antiretroviral treatment history in this cohort, by year. The considerable discrepancy between the median time elapsed between initiation of therapy and death, and the median time actually spent on antiretrovirals suggests that for many individuals, antiretroviral use was intermittent.

<table>
<thead>
<tr>
<th>Year of Death</th>
<th>Number of Deaths</th>
<th>Median months elapsed between start of therapy and death [IQR]</th>
<th>Median months spent on any antiretrovirals [IQR]</th>
<th>Median months spent on HAART [IQR]</th>
<th>Median months between stopping therapy and death [IQR]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997 (July-Dec)</td>
<td>45</td>
<td>28 [8-54]</td>
<td>11 [4-33]</td>
<td>4 [0-8]</td>
<td>2.5 [0.6-4.4]</td>
</tr>
<tr>
<td>1998</td>
<td>90</td>
<td>32 [19-60]</td>
<td>18 [7-33]</td>
<td>6 [0-13]</td>
<td>2.8 [0.6-8.0]</td>
</tr>
<tr>
<td>1999</td>
<td>105</td>
<td>35 [17-56]</td>
<td>15 [6-34]</td>
<td>8 [3-14]</td>
<td>1.8 [0.6-8.0]</td>
</tr>
<tr>
<td>2000</td>
<td>135</td>
<td>44 [28-71]</td>
<td>20 [8-44]</td>
<td>10 [4-22]</td>
<td>2.0 [0.5-8.5]</td>
</tr>
<tr>
<td>2001</td>
<td>121</td>
<td>47 [20-68]</td>
<td>28 [10-48]</td>
<td>15 [5-33]</td>
<td>1.6 [0.5-6.0]</td>
</tr>
<tr>
<td>TOTAL OVERALL</td>
<td>496</td>
<td>40 [19-62]</td>
<td>19 [7-41]</td>
<td>8 [2-18]</td>
<td>2.0 [0.5-7.4]</td>
</tr>
</tbody>
</table>

p<0.001  p=0.002  p<0.001  p=0.8

[IQR]: Interquartile range
Table 5.2: Comparison between those who died without any antiretroviral resistance compared to those who showed any antiretroviral resistance.

<table>
<thead>
<tr>
<th></th>
<th>No antiretroviral resistance (N=65) (A)</th>
<th>Virologic suppression (N=147) (B)</th>
<th>Any antiretroviral resistance (N=167) (C)</th>
<th>p (A vs. B)</th>
<th>p (A vs. C)</th>
<th>p (B vs. C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at start of therapy</td>
<td>39.6 (9.3)</td>
<td>42.4 (10.3)</td>
<td>39.2 (9.1)</td>
<td>0.039</td>
<td>0.767</td>
<td>0.002</td>
</tr>
<tr>
<td>Age at death (std dev)</td>
<td>42.3 (8.8)</td>
<td>45.8 (10.6)</td>
<td>43.2 (8.9)</td>
<td>0.008</td>
<td>0.247</td>
<td>0.031</td>
</tr>
<tr>
<td>% Male</td>
<td>81.2%</td>
<td>87.2%</td>
<td>87.4%</td>
<td>0.263</td>
<td>0.230</td>
<td>0.944</td>
</tr>
<tr>
<td>% IDU</td>
<td>31.3%</td>
<td>30.4%</td>
<td>29.3%</td>
<td>0.903</td>
<td>0.777</td>
<td>0.837</td>
</tr>
<tr>
<td>Median CD4 count</td>
<td>30 [10-160]</td>
<td>200 [70-370]</td>
<td>50 [10-200]</td>
<td>&lt;0.001</td>
<td>0.207</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(cells/mm^3) [IQR]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median plasma viral load</td>
<td>109,000 [3185-416,000]</td>
<td>&lt;500</td>
<td>49,000 [5100-272,000]</td>
<td>&lt;0.001</td>
<td>0.250</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(copies HIV RNA/ml) [IQR]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median length of time on therapy (months)</td>
<td>15 [7-62]</td>
<td>31 [13-47]</td>
<td>32 [16-52]</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.153</td>
</tr>
<tr>
<td>Adherence [IQR]*</td>
<td>78 [33-100]</td>
<td>100 [73-100]</td>
<td>83 [58-100]</td>
<td>&lt;0.001</td>
<td>0.084</td>
<td>0.006</td>
</tr>
</tbody>
</table>

IDU: Injection Drug User
IQR: Interquartile Range
*Adherence defined as % of prescriptions refilled during first 12 months of therapy
Figure 5.1 (Next Page): Flowchart summarizing number of subjects in mortality group.

Dotted boxes contain subsets of individuals whom antiretroviral resistance was either not detected in a genotypic resistance test, or for whom antiretroviral resistance was not likely to have contributed to death. Individuals in the "no follow-up pVL" group had only a very short duration of antiretroviral exposure; drug resistance therefore was not likely a relevant factor in their deaths. The thick solid box indicates individuals for whom antiretroviral resistance was confirmed in a genotypic test.
Deaths from all causes
July 1997- December 2001
N=637

Accidental Death
N=83

Non-Accidental Death
N=554

Never received therapy
N=58

Received therapy
N=496

Last pVL<500 HIV RNA/ml
N=147

Last pVL>500 HIV RNA/ml
N=250

successfully genotyped
N=232

No Antiretroviral Resistance
N=65

Antiretroviral Resistance
N=167
Figure 5.2A: Prevalence of antiretroviral resistance in the last on-therapy plasma sample, by year of death.

Gray bars indicate isolates which contained no major resistance mutations or that represented samples from individuals with pVL <500 copies/mL. Hatched bars represent isolates with resistance to at least 1 drug class. White bars represent isolates with resistance to at least 2 drug classes, and black bars represent isolates with resistance to all three drug classes. Overall, the majority of isolates either contained no major resistance mutations or represented individuals with successful pVL suppression <500 copies/mL. Resistance to drugs from all three classes was rarely observed.
Figure 5.2B: Proportion of resistant isolates, stratified by type of therapy received.

Gray bars indicate isolates which contained no major resistance mutations or that represented samples from individuals with pVL<500 copies/mL. Hatched bars represent isolates with resistance to at least 1 drug class. White bars represent isolates with resistance to at least 2 drug classes, and black bars represent isolates with resistance to all three drug classes. Individuals who received mono or dual therapy at some point in their treatment history were significantly more likely to harbor HIV with resistance mutations than individuals who received HAART exclusively. None of the patients who received HAART exclusively had mutations conferring resistance to all three drug classes.
Figure 5.3: Prevalence of antiretroviral resistance mutations, by year, in a group of living individuals enrolled in the Province's HIV/AIDS Drug Treatment program failing their current therapy (N=1220).

Gray bars indicate isolates which contained no major resistance mutations or that represented samples from individuals failing therapy with plasma viral loads $\geq 500$ copies HIV RNA/ml. Hatched bars represent isolates with resistance to at least 1 drug class. White bars represent isolates with resistance to at least 2 drug classes, and black bars represent isolates with resistance to all three drug classes. Note the dramatic increase in the proportion of isolates with resistance to $\geq 2$ classes of drugs over the study period.
5.5 REFERENCES


CHAPTER 6

HIV GENETIC VARIATION: IMPLICATIONS OF HIV p6\textsuperscript{Gag} INSERTIONS ON RESPONSE TO HAART\textsuperscript{1}

6.1 INTRODUCTION

The emergence of antiretroviral-resistant strains of Human Immunodeficiency Virus Type-1 (HIV-1) is a major factor influencing the effectiveness of antiretroviral therapy. Historically, antiretroviral resistance has been associated with mutations in the protease and reverse transcriptase genes, whose protein products are the targets of currently available antiretroviral agents. However, genetic alterations outside these regions may also contribute to drug resistance. For example, mutations at the HIV \textit{gag} protease cleavage sites have been associated with reduced susceptibility to protease inhibitors [1,2]. More recently, insertion mutations within the HIV p6\textsuperscript{Gag} gene, located at the C-terminal end of the \textit{gag} immediately upstream of \textit{pol}, have been implicated in HIV antiretroviral resistance [3]. The p6\textsuperscript{Gag} protein, involved in the process of HIV budding from infected cells [4-7], contains a highly conserved, amino-terminal proline-rich region referred to as the L-domain or "PTAP" motif. In the late stages of the viral life cycle, the PTAP motif appears to recruit the cellular protein tumor susceptibility gene (Tsg)-101, involved in the regulation of intracellular protein trafficking, to sites of HIV assembly at the plasma membrane [8-10]. The interaction of HIV p6\textsuperscript{Gag} with Tsg-101 is believed to be required for the release of new infectious viral particles from the host cell surface [8,9], in a cell-type-dependent manner [4,5].

\textsuperscript{1} This chapter has been published as: Brumme ZL, Chan KJ, Dong WW, Wynhoven B, Mo T, Hogg RS, Montaner JS, O'Shaughnessy MV, Harrigan PR. "Prevalence and clinical implications of insertions in the HIV-1 p6Gag N-terminal region in drug-naive individuals initiating antiretroviral therapy". Antiviral Therapy, 2003; 8:91-6.
A recent study of the p6\textsuperscript{Gag} region in 296 HIV-infected individuals reported that a duplication of the proline-rich PTAP motif occurred relatively frequently in individuals treated with nucleoside analog reverse transcriptase inhibitors (NRTIs), but only rarely in drug naive individuals without associated resistance mutations, suggesting a link between the PTAP motif and resistance to NRTIs [3]. Furthermore, clones generated with the PTAP duplication exhibited increased infectivity and a competitive growth advantage in the presence of NRTIs \textit{in vitro} [3]. However, an independent study of a small cohort of treatment-experienced individuals reported only a weak correlation between insertions in the proline-rich p6\textsuperscript{Gag} motif and a lower risk of virological failure, which was not statistically significant [11].

The objective of this study was to assess the prevalence and influence of amino acid insertions within the HIV p6\textsuperscript{Gag} N-terminal proline-rich region on initial virological and immunological response to antiretroviral therapy in a large, population-based cohort of HIV-infected, drug naive individuals initiating their first antiretroviral therapy.
6.2 MATERIALS AND METHODS

Study Design and Patients

In the province of British Columbia, Canada, antiretroviral drugs are distributed free of charge to eligible HIV-infected individuals through a centralized drug treatment program based at the British Columbia Centre for Excellence in HIV/AIDS ("the B.C. Centre") [see reference 12 for details of policies and treatment guidelines]. All antiretroviral naïve individuals first seeking treatment at the B.C. Centre between June 1, 1996 and August 31, 1998, were eligible for analysis (N=479). Of these, 461 (96.2%) had a pre-therapy plasma sample available for testing. Greater than 80% of patients were prescribed triple therapy including two nucleoside analogues and one protease inhibitor, consistent with treatment guidelines at the time. Ethical approval for this study was obtained from the institutional ethics board at St. Paul's Hospital, Vancouver. Samples were assigned anonymous identifiers prior to genotypic analyses.

Plasma Viral Load and CD4 Cell Count

Plasma HIV-1 RNA levels (viral loads) were determined using the Roche Amplicor Monitor assay (Roche Diagnostics, Laval, Quebec, Canada) using either the standard method or the ultrasensitive adaptation. The ultrasensitive adaptation to this assay was not routinely available over the time period of this study; for this reason a constant cut-off of 500 HIV RNA copies/ml was used throughout the analysis. CD4 cell counts were measured by flow cytometry, followed by fluorescent monoclonal antibody analysis (Beckman Coulter, Inc., Mississauga, Ontario, Canada).
Detection of HIV p6\textsuperscript{Gag} PTAP insertions, HIV envelope V3 loop sequences, and HIV-RNA genotyping for drug resistance.

Plasma nucleic acids were extracted and precipitated with guanidine thiocyanate and isopropanol, followed by two ethanol washes. The HIV p6\textsuperscript{Gag} amino-terminal region (located in the C-terminal region of gag, directly upstream of pol) was amplified by nested RT-PCR, using forward primer 5' GCCCCTAGGAAAAAGGGCTGTGGG 3' and reverse primer 5' TTCTGTCAATGGCCATTGTTTAAC 3' in the first round, and forward primer 5' CCTAGGAAAAAGGGCTGTGGAAATGTG 3' and reverse primer 5' CCTGGCTTTAATTTTACTGGTACAG 3' in the second round, to generate a final 0.6 Kb product. Note that mutations and/or insertions in the C-terminal region of the HIV p6\textsuperscript{Gag} were not included in the analysis presented here. The region encoding the HIV V3 loop within the env gene was amplified by nested RT-PCR, using forward primer 5' CAGAAAAATTGTGGGTCACAGTCTATTATGGGGTACCT 3' and reverse primer 5' TATAATTCATTCTCTCAATTGTCC 3' in the first round, and forward primer 5' ACACATGCCTGTGACCCCAGA 3' and reverse primer 5' ATTACAGTACAAAAATTTCCCTCCAC 3' in the second round, to generate a 0.95 Kb product. PCR amplification products were sequenced directly in both the 5' and 3' directions using the BigDye dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and resolved on an ABI 3700 automated sequencer. HIV drug resistance genotyping was performed as previously described [13].

Statistical Analysis

Time to virological success was defined as the time to achieve a plasma viral load (pVL) ≤ 500 copies HIV-1 RNA/ml from baseline. Time to virological failure was defined as
the time elapsed between virological success and the second of two consecutive pVL measurements > 500 copies/ml. If a person never achieved virological success, they were excluded from the analysis of time to virological failure. If a patient reached therapeutic success and did not subsequently fail then they were censored at the time of their last viral load date (occurring prior to December 1, 2000). Time to immunological failure was defined as the time to the second of two successive CD4 cell counts below baseline. Event-free subjects were right censored as of December 1, 2000, or at the time of their last viral load or CD4 cell test.

The influence of HIV p6\textsuperscript{Gag} N-terminal insertions on time to virological success and virological and immunological failure were assessed by Kaplan-Meier survival analysis, over a median follow-up time of 40 months. Cox proportional hazard regression was used to calculate univariate and adjusted risk ratios and 95% confidence intervals (CIs). In this analysis, gender, prior diagnosis of AIDS, initiation of triple therapy, detection of HIV p6\textsuperscript{Gag} insertion, 100% adherence (evaluated using a previously validated approach based on the frequency of prescriptions filled on time during the first year of therapy, where an adherence level of 100% was defined as filling all prescriptions on schedule [14]) and history of injection drug use were tested as binary variables. Age, pVL, and CD4 cell count were treated as either ordinate or continuous variables. All tests of significance were two-sided, with a p-value of less than 0.05 indicating statistical significance.
6.3 RESULTS

Prevalence of HIV \( p_6^{Gag} \) insertions and association with baseline parameters

Of the 423 of 461 drug-naive individuals (91.8%) who were successfully genotyped, 70 (16.5%) contained a \( p_6^{Gag} \) N-terminal amino acid insertion (Table 1). N-terminal \( p_6^{Gag} \) sequences have been deposited in GenBank (Accession numbers: AF538974-AF539396). The most commonly observed insertion was "PTA" (34/70, 48.5%). Note that because different equivalent alignments are possible in some cases, the classification of insertions is somewhat arbitrary. For example, "PTA" may be designated "TAP" or "APP", depending on the alignment chosen.

Baseline demographic characteristics of the sample population were generally evenly matched between those with or without HIV \( p_6^{Gag} \) insertions (Table 2). HIV \( p_6^{Gag} \) insertions were significantly associated with a lower median baseline CD4 count (\( p=0.002 \)).

Association between HIV \( p_6^{Gag} \) insertions and HIV envelope

The majority of the study group (382 of 423 patients, or 90.3%) were additionally genotyped for their HIV envelope V3 loop sequence. Briefly, nested RT-PCR and direct DNA sequencing were used to identify basic amino acid residues at positions 11 and 25 of the V3 loop, which have been associated with an HIV syncytium-inducing (SI) phenotype [15,16]. A significant association between the presence of a \( p_6^{Gag} \) insertion and the presence of basic amino acid residues in the HIV V3 loop was observed (\( p_6^{Gag} \) insertion observed in 50 of 335 inferred non-syncytium-inducing [NSI] viruses vs. 17 of 47 inferred SI viruses, \( p=0.0003 \)). Of interest, although the presence of an HIV \( p_6^{Gag} \) insertion was significantly associated with a lower baseline CD4 count (\( p=0.002 \), Table 2), there was no association between HIV V3 sequence and baseline CD4 count (\( p=0.2 \), data not shown).
A search of the HIV-1 complete genome sequences available from the Los Alamos HIV sequence database indicated a prevalence of HIV p6\textsuperscript{Gag} insertions of approximately 10.5%, however no significant association was observed between presence of p6\textsuperscript{Gag} insertions and inferred HIV tropism in this data set (p>0.1, data not shown).

**Association between HIV p6\textsuperscript{Gag} insertion and baseline antiretroviral resistance**

The relationship between HIV p6\textsuperscript{Gag} insertions and antiretroviral resistance [3] was also investigated. Baseline genotypic resistance analysis (performed as described in [13] using the same pre-therapy plasma sample) was completed for all 423 samples. Baseline antiretroviral resistance was defined as the presence of one or more of the following key mutations: D30N, M46I/L, G48V, I50V, V82A/F/T/S, I84V, L90M in the HIV protease, or M41L, K65R, T69D, K70R, L74V, K103N, Q151M, Y181C/I, M184V/I or T215Y/F in the HIV reverse transcriptase (adapted from [17]). According to this definition, baseline resistance mutations were observed in 15 of 423 samples (3.5%): 3 M41L, 4 M184V (including one sample with both M184V and L90M), 4 M46L 1 M46I, 1 I50V, 1 T69D and 1 V82A. There was no association between the presence of HIV p6\textsuperscript{Gag} insertions and the presence of resistance-associated mutations at baseline (p=0.7, data not shown).

**Association of HIV p6\textsuperscript{Gag} insertion with initial virological and immunological treatment response**

In this study, individuals were initially therapy naïve, but generally the time since seroconversion was not known. Some individuals may have been infected with drug-resistant virus, and it is conceivable that over time, drug resistance mutations in the protease and/or RT may have reverted to wild-type, while the p6\textsuperscript{Gag} insertion did not. For this reason,
we investigated the effect of insertions in the HIV p6<sup>Gag</sup> on initial virological and immunological therapy response (Figure 1).

There was no influence of HIV p6<sup>Gag</sup> insertions on time to virological success (p=0.8, data not shown), virological failure (p=0.5), or immunological failure (p=0.7). The proportion of individuals never achieving virological success was 17.1% and 20.4% for the groups with and without p6<sup>Gag</sup> insertions, respectively (p=0.5).

Cox proportional hazard regression was used to calculate univariate and multivariate risk ratios (R.R.) and 95% confidence intervals (CIs). In multivariate analyses, time to virological and immunological failure were significantly associated with initiating triple therapy [virological R.R. 0.41; 95%CI 0.27-0.63, immunological R.R. 0.61; 95%CI 0.39-0.95] and adherence [virological R.R. 0.28; 95%CI 0.20-0.41, immunological R.R. 0.30; 95%CI 0.21-0.45]. Consistent with the Kaplan-Meier analyses, there was no influence of N-terminal HIV p6<sup>Gag</sup> insertions on time to virological failure [R.R. 1.08; 95%CI 0.70-1.67], time to immunological failure [R.R. 1.19; 95%CI 0.75-1.89] or time to virological success [RR 1.06; 95%CI 0.78-1.45] (p>0.1, data not shown).
6.4 DISCUSSION AND CONCLUSIONS

This study suggests that N-terminal insertions in HIV p6Gag are much more common in drug naive individuals than previously reported [3]. Almost 17% of this study population had p6Gag insertions, compared to a previous study that identified p6Gag insertions in 5.4% of drug naive individuals [3]. These results are consistent with a recent study that reported a p6Gag insertion prevalence of 17.6% in an antiretroviral naive population [18]. In contrast to a previous study suggesting a link between p6Gag PTAP insertions and transmission of drug-resistant HIV [3], we observed no association between p6Gag insertions and the presence of antiretroviral resistance mutations at baseline. These data are consistent with a recent study of 100 individuals examined during primary HIV infection, which reported that despite an increase in frequency of an HIV p6Gag motif duplication, which paralleled an increase in incidence of infection with drug resistant strains over the course of study follow-up, there was no direct association between p6Gag insertions and primary-resistance-associated mutations [19]. Importantly, after adjustment for baseline factors, there was no significant influence of N-terminal HIV p6Gag insertions on virological response to antiretroviral therapy, suggesting that there may be relatively little effect of these insertions on HIV drug resistance per se. Similarly, we were unable to demonstrate any correlation between HIV p6Gag insertions and immunological therapy response. It must be noted, however, that these insertions could have had a small impact on resistance that did not result in significant differences in virological and immunological response to therapy. Interestingly, a previous study of 42 highly treatment-experienced individuals reported a trend, although not statistically significant, between p6Gag insertions and improved virological response to therapy [11].
The observation that p6\textsuperscript{Gag} insertions were significantly associated with a lower baseline CD4 cell count suggests that p6\textsuperscript{Gag} insertions may indeed be associated with a poorer prognosis and/or more rapid disease progression in untreated patients. The mechanism for this association may be direct, through an increase in viral packaging efficiency in HIV containing a p6\textsuperscript{Gag} insert [3], or as a result of variable interactions of p6\textsuperscript{Gag} with host cell factors. It has been recently suggested that Tsg-101, a protein normally involved in intracellular trafficking, interacts with the HIV p6\textsuperscript{Gag} PTAP motif at a late stage in the HIV life cycle in an event essential to viral budding and egress [8-10]. Since it has been shown that mutations within the conserved p6\textsuperscript{Gag} PTAP motif inhibit both Tsg-101 binding and HIV particle release from the cell surface [9,10], it is conceivable that duplication of the PTAP motif may increase HIV infectivity by enhancing interactions with Tsg-101.

Alternatively, the mechanism for this association may be indirect, through association with other naturally occurring mutations at various sites within the HIV genome. Indeed, in this study, basic amino acid residues at key positions in the HIV V3 loop sequence, indicative of an HIV syncytium-inducing phenotype which is associated with entry into CXCR4 co-receptor-expressing cells [20], and is associated with depletion of CD4-positive cells and more rapid disease progression [15, 16, 21, 22], were 2.4-fold more likely to be observed in individuals with p6\textsuperscript{Gag} insertions, although analysis of the available HIV-1 genome sequences from the Los Alamos Sequence database did not reveal similar correlations between p6\textsuperscript{Gag} insertions and inferred viral phenotype.

It is possible that HIV p6\textsuperscript{Gag} insertions may arise naturally, as a result of the increasing diversity of HIV-1 sequences over time, in a manner analogous to the shift from the non-syncytium-inducing (NSI) to the syncytium-inducing (SI) phenotype in later stages.
of HIV disease progression. Since neither the date of seroconversion nor reasons for starting antiretroviral therapy are known for most individuals, it is conceivable that the association of HIV p6$^{Gag}$ insertions with SI virus and a poorer baseline prognosis may simply be attributed to increased time since seroconversion and/or natural disease progression and viral evolution in these individuals.

In conclusion, results from this study suggest that HIV p6$^{Gag}$ insertions may arise naturally in drug naive individuals, rather than being directly associated with resistance to antiretrovirals, and are not predictive of antiretroviral therapy response. The association with V3 loop sequences merits further investigation.
Table 6.1: Distribution of N-terminal HIV \( p_6^{Gag} \) insertions observed in study group of antiretroviral naive individuals

HIV \( p_6^{Gag} \): N-terminus - L Q S R P E P T A P P E E S F - C-terminus

<table>
<thead>
<tr>
<th>Amino Acid Insertion</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTA</td>
<td>34</td>
</tr>
<tr>
<td>PTAPPE</td>
<td>6</td>
</tr>
<tr>
<td>SRPE</td>
<td>4</td>
</tr>
<tr>
<td>LEPTAP</td>
<td>3</td>
</tr>
<tr>
<td>PTAPPA</td>
<td>3</td>
</tr>
<tr>
<td>QSRPE</td>
<td>2</td>
</tr>
<tr>
<td>PRPE</td>
<td>2</td>
</tr>
<tr>
<td>PPA</td>
<td>2</td>
</tr>
<tr>
<td>QSRLE</td>
<td>1</td>
</tr>
<tr>
<td>QSRTE</td>
<td>1</td>
</tr>
<tr>
<td>QNRPEPTAP</td>
<td>1</td>
</tr>
<tr>
<td>PRPEPTA</td>
<td>1</td>
</tr>
<tr>
<td>RPEPTA</td>
<td>1</td>
</tr>
<tr>
<td>LEPTA</td>
<td>1</td>
</tr>
<tr>
<td>APVPTAPP</td>
<td>1</td>
</tr>
<tr>
<td>PTGPEE</td>
<td>1</td>
</tr>
<tr>
<td>PTGPPE</td>
<td>1</td>
</tr>
<tr>
<td>PSA</td>
<td>1</td>
</tr>
<tr>
<td>PP</td>
<td>1</td>
</tr>
<tr>
<td>VPT</td>
<td>1</td>
</tr>
<tr>
<td>(V/A)PT</td>
<td>1</td>
</tr>
<tr>
<td>EESR</td>
<td></td>
</tr>
</tbody>
</table>

Total 70

The first 15 N-terminal amino acids in the wild-type HIV \( p_6^{Gag} \) region are indicated above the table. Insertions are listed according to their observed frequency. In some cases, insertions are somewhat arbitrarily named, depending on the choice of different equivalent alignments.
Table 6.2: Patient Characteristics at baseline

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>All patients (N=461)</th>
<th>Stratified by presence or absence of HIV p6\textsuperscript{3ag} insertions (N=423 patients genotyped successfully)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>insert (N=70)</td>
<td>no insert (N=353)</td>
</tr>
<tr>
<td>Gender: % Male</td>
<td>84.5%</td>
<td>88.6%</td>
</tr>
<tr>
<td>Median Age, years [IQR]</td>
<td>36 [31-42]</td>
<td>34 [30-41]</td>
</tr>
<tr>
<td>HIV pVL (x10\textsuperscript{3} copies/ml) [IQR]</td>
<td>89 [30-280]</td>
<td>130 [47-420]</td>
</tr>
<tr>
<td>CD4 count (x10\textsuperscript{6} cells/L) [IQR]</td>
<td>310 [140-460]</td>
<td>195 [100-370]</td>
</tr>
<tr>
<td>baseline AIDS diagnosis (%)</td>
<td>49 (10.6%)</td>
<td>9 (12.9%)</td>
</tr>
<tr>
<td>triple therapy (%)</td>
<td>385 (83.5%)</td>
<td>58 (82.9%)</td>
</tr>
<tr>
<td>100% adherent (%)</td>
<td>240 (52.1%)</td>
<td>32 (45.7%)</td>
</tr>
</tbody>
</table>

[IQR= Interquartile Range]
Figure 6.1: Kaplan-Meier survival analyses, showing the influence of insertions within the N-terminal region of the HIV p6\textsuperscript{Gag} on time to virological (a) and immunological (b) failure.

To be included in the "time to virological failure" and "time to immunological failure" analyses, patients had to register at least one post-baseline HIV pVL \(\leq 500\) copies/ml, and at least one post-baseline CD4 cell count, respectively. The univariate p-values for the virological and immunological analyses were \(p=0.5\) and \(p=0.7\), respectively. The number of patients in each group (insertions vs. no insertions) still experiencing virological and immunological success at 24 months were [28 (insertion), 148 (no insertion)] and [28 (insertion), 167 (no insertion)], respectively.
6.5 REFERENCES


22. Spijkerman I, de Wolf F, Langendam M, Schuitemaker H, Coutinho R. Emergence of syncytium-inducing human immunodeficiency virus type 1 variants coincides with a
transient increase in viral RNA level and is an independent predictor for progression to AIDS. Journal of Infectious Diseases 1998; 2:397-403.
CHAPTER 7
HIV GENETIC VARIATION: RELEVANCE OF HIV V3 ENVELOPE GENOTYPE TO HAART OUTCOMES

7.1 INTRODUCTION

The introduction of Highly Active Antiretroviral Therapy (HAART) has resulted in dramatic decreases in morbidity and mortality in HIV-infected individuals [1-3]. However, due to concerns about long-term tolerability, drug resistance, cost and other factors, the optimal time to initiate HAART remains controversial [4-6]. A dominant prognostic factor influencing HIV disease progression and mortality in antiretroviral-naïve individuals initiating HAART is the baseline CD4 cell count [5-7]. However, evidence suggests that the baseline HIV plasma viral load is a weaker independent predictor of clinical outcomes after starting HAART [5,6].

In order to continue to develop clinically relevant tools for HIV/AIDS management in the HAART era, it is important to identify additional predictors of treatment outcome. The emergence of syncytium-inducing (SI) HIV, which arises in approximately 50% of untreated, HIV subtype B-infected individuals with late-stage disease, is associated with a rapid decline in CD4 T-cells and reduced survival time [8-11]. SI phenotype has also been associated with poorer response to mono or dual therapy [12-14] and poorer short-term responses to triple therapy in clinical trials [15,16], though some studies have reported no effect [17]. It has become clear that SI phenotype change is generally associated with a change in the preferred

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1 This chapter has been published as:
virus co-receptor, from CCR5 to CXCR4 [18]. Simple nucleic acid sequence-based methods exist to predict HIV phenotype or co-receptor usage from the HIV envelope third variable loop ("V3") sequence [19-23]. In general, SI variants (and CXCR4-coreceptor-using, or X4 variants) display positively charged amino acid residues at codons 11 and/or 25 within the V3 loop, while NSI variants (and CCR5-coreceptor-using, or R5 variants) generally do not [19, 22-24]. This "11/25" rule is one of the most reliable methods for sequence-based predictions of viral phenotype [25]. Other bioinformatic approaches for predicting phenotype from sequence data have been developed, including a published neural network approach (NN) [25], and a Position Specific Scoring Matrix approach (PSSM) [26].

Unfortunately, directly determining HIV phenotype and/or co-receptor usage remains too time consuming and costly for routine clinical testing, so these measures are rarely employed outside of clinical trials. In contrast, nucleic acid sequence-based assays are more practical and widely employed in clinical monitoring of HIV [27]. The long-term impact of V3 sequence variation on plasma viral load suppression, drug resistance, immunological response and mortality after initiation of HAART is not known. We therefore conducted the present study to evaluate directly the impact of HIV V3 sequence variation on therapy outcomes in the HOMER cohort, a population-based cohort of 1191 HIV-infected individuals initiating HAART.
7.2 MATERIALS AND METHODS

British Columbia HIV/AIDS Drug Treatment Program

In the province of British Columbia, Canada, antiretrovirals are distributed free of charge to HIV-infected individuals through a centralised drug treatment program. Antiretrovirals are prescribed according to specific guidelines set by the B.C. Therapeutic Guidelines Committee, which are revised regularly and are in accordance with international guidelines [5].

Patient selection

The study population is very similar to a cohort that has been described in detail previously [5]. Briefly, all HIV-positive, antiretroviral naïve adults who started triple HAART (consisting of 2 nRTI and either a PI or an NNRTI) in B.C. between 08/1996 and 09/1999 were eligible (N=1312). Of these, 121 (9.2%) were excluded for not having baseline CD4 and plasma HIV RNA data available within 6 months prior to the start of triple HAART; the study sample was therefore made up of the remaining 1191 subjects. These 1191 subjects form an open treatment-based cohort known as the HOMER (HAART Observational Medical Evaluation and Research) cohort. Ethical approval was obtained from the institutional ethics board. Samples were assigned anonymous code identifiers before analysis.

Laboratory Methods

Plasma HIV-1 RNA loads were determined using the Roche Amplicor Monitor assay using either the standard or ultrasensitive methods. The ultrasensitive assay was not routinely available over the study period; a constant cut-off of 500 HIV RNA copies/ml was therefore used in outcome analyses. CD4 counts were measured by standard techniques.
Plasma nucleic acids from each pre-therapy sample were extracted using standard methods and a portion of the HIV-1 envelope containing the V3 loop was amplified by nested RT-PCR, using first round primers 5’

CAGAAAAATTGTGGGTCACAGTCTATTATGGGGTACCT 3’ (HIV-1 HXB2 acc# K03455, nt 6316-6353) and 5’ TATAATTCACTTCTCCAATTGTCC 3’ (nt 7675-7652), and second-round primers 5’ ACACATGCCTGTGTACCCACAGA 3’ (nt 6435-6457) and 5’

ATTACAGTAGAAAAATTCCCCCTCCAC 3’ (nt 7382-7357). If no product was amplified, an alternative second-round forward primer was used (5’

AATGTCAGCAGCAGTACAATGTACAC 3’) (nt 6945-6969). PCR products were sequenced in both 5’ and 3’ directions using the BigDye dye terminator cycle sequencing kit (Applied Biosystems) and run on an ABI 3100 or 3700 automated sequencer.

Data Analysis:

a) Alignment of V3 sequences

The majority of sequences in our study encoded 35 amino acid residues. However to maintain alignment, gaps needed to be inserted in approximately 10% of sequences. The final alignment was performed manually, based on the amino acid profile of a large number (>400) of independent V3 loops [25]. Sequences were aligned to the following motifs: (T|A)(T|A) (that is, either threonine or alanine) at each of codons 22 & 23, (G) at codon 24, and (I|V)(I|V|T) at codons 26 and 27. Any charged residue following (G)24 or preceding (I|V)(I|V|T) 26-27 was assigned to site 25. If no charged residue intervened between (G)24 and (I|V)(I|V|T) 26-27, site 25 was gapped.
b) Interpretation of V3 sequences

In the primary analysis, HIV envelope V3 loop sequences were dichotomized by the presence of positively charged residues at codons 11 and/or 25 of the V3 loop [19]. Because we were interested in evaluating the predictive value of V3 sequence (rather than in-vitro phenotypes or co-receptor usage) on therapy outcome, we elected to use the following nomenclature: HIV isolates who displayed positively charged residues at codons 11 and/or 25 of V3 were classified as having an "11/25" genotype. In addition, we employed a neural network (NN) [25] as well as a Position-Specific Scoring Matrix (PSSM) [26] to categorize V3 sequences. V3 sequence predictions inferred using NN and PSSM are indicated in lowercase letters ("si", "nsi") [24] to distinguish them from experimentally determined phenotypes.

Nucleotide mixtures are sometimes observed upon direct DNA sequencing of clinical isolates. In these cases, nucleic acid sequences from each patient were translated into amino acid (aa) sequences containing all possible aa combinations. However, the translation of HIV sequences into all possible amino acid sequence combinations could potentially have resulted in the artificial disruption of natural sequence linkage patterns and the subsequent interpretation of amino acid combinations that would not naturally exist in vivo. We applied an arbitrary requirement that if ≥25% of all possible sequence combinations from a given patient had the "11/25" genotype (or were classified as "si" by NN or PSSM), then the sample was classified as having an "11/25" genotype (or was classified as "si"). This definition affected only one, 15 or 12 samples for the "11/25", NN or PSSM approaches, respectively. Although somewhat arbitrary, this definition corresponds to the approximate 25% minimum threshold at which minority nucleotide quasi-species are detectable by RT-
PCR and sequencing methods. Samples with poor sequence data and/or that translated into >128 possible amino acid sequence combinations were not interpreted.

Clinical Endpoints

a) Mortality

The primary endpoint in this analysis was non-accidental death. Deaths that occurred during the study period were identified through physician reports and linkage with the B.C. Department of Vital Statistics. Deaths were classified according to ICD-9 and ICD-10 coding. All non-accidental deaths were considered potentially HIV-related and classified as events. Time to death was the time elapsed between the antiretroviral start date and date of non-accidental death. Accidental deaths were considered non-events and subjects censored at time of death.

b) Other endpoints

Additional outcomes in this study were viral suppression and rebound, CD4 response and development of antiretroviral resistance. Time to viral suppression was defined as the time elapsed between the antiretroviral start date and the first of at least two consecutive plasma viral loads (pVL) < 500 copies HIV-1 RNA/ml. In the primary analysis of time to viral suppression, suppression could occur before or after switching therapies, while in a secondary analysis, individuals who had not achieved viral suppression at the time of switching therapy were censored on the switch date as non-events. This was done in order to take into consideration the possibility that some individuals may have required some optimisation of therapy before achieving plasma viral suppression.

The subsequent time to plasma viral rebound was also evaluated. The time to rebound analysis was restricted to individuals who had achieved plasma viral suppression < 500
copies HIV-1 RNA/ml, and was defined as the time elapsed between initial viral suppression (the first of at least two consecutive pVL < 500 copies HIV-1 RNA/ml) and the halfway point between the last pVL < 500 copies/ml and the first of at least two consecutive pVL ≥ 500 copies/ml. If an individual registered only one pVL ≥ 500 copies/ml but then subsequently died of a non-accidental cause, this was also considered an event.

Time to CD4 decline below baseline was defined as the time elapsed between the antiretroviral start date and the first CD4 count below the baseline CD4 count.

The relationship between V3 sequence and time to development of antiretroviral resistance was investigated. For each subject, all plasma samples with pVL ≥1000 HIV RNA/ml collected in the 2.5 years following initiation of HAART were genotyped for antiretroviral resistance [28]. Samples with pVL <1000 HIV RNA/ml were not genotyped and were assumed to carry no resistance mutations. Time to resistance was defined as the time elapsed between starting therapy and the first sample containing at least one major resistance mutation, defined as any nRTI-associated mutation (184I/V, 41L, 62V, 65R, 67N, 69D or insertion, 70R, 74V, 75I, 151M, 210W, 215F/Y or 219E/Q); any NNRTI-associated mutation (100I, 103N, 106A/M, 108I, 181C/I, 188C/H/L, 190A/S, 230L or 236L) or any PI-associated mutation (30N, 46I/L, 48V, 50L/V, 54V/L/M, 82A/F/S/T, 84V, or 90M) [29].

The study end date was March 31, 2002. For mortality analysis, event-free subjects were censored at the date of last known contact with the HIV/AIDS Drug Treatment program. For outcomes involving CD4 and viral load, event-free subjects were censored on the last test date available until March 31, 2002. For antiretroviral resistance, event-free subjects were censored at the last test date available up until 2.5 years from initiation of HAART on or before March 31, 2002.
Statistical Analyses

The influence of HIV V3 sequence on clinical outcomes was assessed by Kaplan-Meier methods. Cox proportional hazards regression was used to calculate univariate and multivariate Risk Ratios (RR) and 95% confidence intervals (CIs). Baseline variables examined included plasma HIV RNA (per log increment), CD4 count (per 100 cell decrement), age (per 10 year increment), proportion of total time spent on antiretroviral therapy in the first year of follow-up (per 10% decrement) [30], type of therapy at initiation (NNRTI-containing vs. PI-containing), AIDS diagnosis (yes vs. no), history of injection drug use (yes vs. no) and gender (male vs. female). All tests of significance were 2-sided, with a p-value < 0.05 indicating statistical significance.
7.3 RESULTS

Study subjects and V3 genotypes

The effect of HIV V3 genotype on therapy response and mortality was investigated in the HOMER cohort of 1191 individuals initiating first triple HAART. Three methods were used to categorize V3 genotype data: the presence of positively charged amino acids at codons 11 and/or 25 of the V3 loop (the "11/25" genotype) [19], a Neural Network approach [25] and a Position-Specific Scoring Matrix method [26].

Baseline V3 genotypes were successfully obtained for 1085 of 1191 subjects (91.1%). In general, unsuccessfully genotyped samples had significantly lower pVL than successfully genotyped samples (p<0.001, Table 1), but were otherwise comparable in their baseline characteristics. Of the 1085 successfully genotyped samples, 118 (10.9%) had an "11/25" genotype as determined by V3 amino acid analysis. Again, baseline characteristics were generally similar for the "11/25" and non-"11/25" genotype groups, although a lower median baseline pVL was observed in the "11/25" genotype group (p=0.008, Table 1).

Influence of V3 sequence on mortality

A total of 159 deaths were observed among the 1085 successfully genotyped HOMER subjects. Of these, 32 (20.1%) were accidental and subjects censored at date of death. Among the 127 non-accidental deaths, 22 (17.3%) occurred in subjects with the "11/25" genotype, while the remaining 105 (82.7%) occurred in subjects with the "non-11/25" genotype. Crude mortality rates were 18.6% and 10.9% in the "11/25" and "non-11/25" groups, respectively.

In an analysis of the influence of V3 sequence on time to death after initiation of HAART, we observed a statistically significant association between the "11/25" genotype
and more rapid mortality (Risk Ratio 1.77, 95%CI 1.12-2.80, p=0.015) (Figure 1 and Table 2a). This association remained significant in multivariate analyses (Risk Ratio 1.70, 95% CI 1.06-2.71, p=0.027) controlling for baseline factors including age, plasma viral load, CD4 count, the proportion of time spent on antiretrovirals in the first year of follow up [30], and AIDS diagnosis (Table 2a).

*Influence of V3 genotype on viral and immunological endpoints*

In the pre-defined primary analysis of time to achieving suppression of pVL below 500 HIV RNA copies/ml, we observed no significant influence of the V3 sequence on time to viral suppression (univariate p>0.1, data not shown). Nevertheless, two lines of evidence from post-hoc data exploration suggested the possibility that viral suppression was actually somewhat more difficult to achieve and/or maintain in patients with the "11/25" genotype. There was a significant association between the "11/25" genotype and an increased time to viral suppression < 500 copies/ml *if individuals were censored at time of switching therapy* (multivariate Risk Ratio 0.63, 95% CI 0.47-0.84, p=0.002), suggesting that patients with the "11/25" genotype were more likely to require a therapy change to achieve plasma viral suppression (data not shown). Furthermore, in patients with the "11/25" genotype, the median pVL was less consistently between 50 and 500 copies HIV RNA/ml over the course of study follow-up (Figure 2a). Although the ultrasensitive pVL assay was not consistently available over the course of the study, the proportion of "11/25" and "non-11/25" samples genotyped using the ultrasensitive assay was comparable over the course of study follow-up (data not shown). In individuals who achieved viral suppression to below 500 HIV RNA copies/ml, we observed no significant association between V3 sequence and time to subsequent viral rebound (univariate p>0.1, data not shown).
The pre-defined primary analysis of time to CD4 cell decline below baseline was shorter in individuals with the "11/25" genotype (univariate p=0.009, Table 2b). This trend remained significant in multivariate analyses controlling for baseline factors and prescription refills in the first year of follow-up [30] (Risk Ratio 1.38, 95% CI 1.07-1.77, p=0.012, Table 2b). Over the course of study follow-up, the median CD4 response was consistently nearly 100 cells lower in subjects with the "11/25" genotype (Figure 2b). Of interest, one of the strongest predictors of both CD4 decline and mortality after starting HAART was the proportion of prescriptions filled in the first year of follow-up, a surrogate of adherence [30]. This association had been demonstrated in a previous study of the HOMER cohort [5], and emphasizes the importance of obtaining and taking prescribed antiretrovirals.

Influence of V3 genotype on time to antiretroviral resistance

The influence of V3 sequence on the time to the development of antiretroviral resistance was also investigated. For each study subject, all plasma samples with pVL ≥ 1000 HIV RNA/ml collected in the 2.5 years following initiation of HAART (N>2600) were genotyped. Of the 1085 individuals for which V3 genotyping was successful, 33 (3.0%) had no follow-up plasma samples available for testing, and resistance testing failed in a further 26 (2.4%); these individuals were censored at time=0. A further 306 individuals (28.2%) had follow-up pVL measurements < 1000 HIV RNA/ml; these subjects were censored at the last pVL < 1000 and assumed to carry no resistance mutations. Of the remaining subjects with follow-up pVL ≥ 1000, 450 (41.5%) displayed no resistance mutations after genotyping, and 270 (24.9%) subjects displayed at least one major resistance mutation. Of the 270 subjects with at least one resistance mutation, 238 were in the "non-11/25" genotype group (24.6% of this group) while the remaining 32 were in the "11/25" genotype group (27.1% of this
group). No association between V3 sequence and time to development of drug resistance mutations was observed (univariate \(p>0.1\), data not shown).

*Evaluation of clinical endpoints using PSSM-based and neural-network phenotype predictions*

Both Position-Specific Scoring Matrix (PSSM) [26] and neural network (NN) approaches [25] were used as alternative methods for categorizing HIV genotypes. PSSM predicted "si" virus in 108 (10.0%) of samples, close to the 10.9% with the "11/25" genotype. Concordant values (cases where "11/25" genotypes were called "si" by PSSM or where "non-11/25" genotypes were called "nsi" by PSSM) were obtained in 96% of cases. PSSM "si" predictions were associated with earlier mortality in univariate analyses (\(p<0.05\)) and a trend remained in multivariate analyses (Risk Ratio 1.53, 95% CI 0.95-2.46, \(p=0.081\), data not shown). PSSM "si" predictions were not associated with time to viral suppression below 500 copies/ml in the primary analysis, however in a secondary analysis where individuals' data were censored at time of therapy switch, PSSM "si" predictions were significantly associated with a longer time to viral suppression in both univariate and multivariate analyses (multivariate Risk Ratio 0.68, 95% CI 0.50-0.93, \(p=0.015\), data not shown). PSSM predictions were not associated with time to virologic failure, development of drug resistance, or CD4 decline below baseline (\(p>0.1\), data not shown).

The neural network (NN) predictions diverged more strongly from those of the "11/25" and PSSM methods, with approximately 18% of viral isolates being classified as "si". Concordance was relatively low between NN sequence categories and both the "11/25" genotype (87%) and the PSSM sequence categories (87%). NN sequence categories were not significantly associated with any endpoints investigated (\(p>0.1\), data not shown).
7.4 DISCUSSION AND CONCLUSIONS

We assessed the ability of three sequence interpretation methods to predict clinical outcomes from baseline HIV envelope V3 sequences in a large cohort of antiretroviral naive individuals initiating first triple HAART: "the 11/25 rule", a method based on examining amino acid variation at two critical V3 sites, as well as two bioinformatics-based approaches, a Neural Network (NN) and a Position Specific Scoring Matrix (PSSM) method. Of the three sequence interpretation methods, the "11/25 rule" was the strongest predictor of clinical parameters, including mortality, in this cohort.

The data indicate a significant correlation between the presence of positively charged amino acids at codons 11 and 25 of the HIV envelope V3 loop (the "11/25" genotype) and inferior CD4 cell response and reduced survival time following initiation of HAART. These poorer prognoses were maintained despite the fact that the time to achieve viral suppression to below 500 HIV RNA copies/ml was similar for the two groups in a primary analysis allowing for regimen changes.

Although this association is most likely driven by the association of the "11/25" genotype with SI phenotype and/or X4 co-receptor usage, it is important to stress that we were interested in the predictive value of HIV V3 loop sequence data for clinical outcomes rather than the predictive values of *in vitro* phenotype, cellular tropism or co-receptor usage. The relative ease and lower costs associated with genotyping (when compared to phenotyping assays) may make this test of practical clinical relevance in the management of HIV-infected individuals.

Presuming V3 loop sequence is indeed predictive of viral phenotype and/or co-receptor usage [19-23], these observations are consistent with previous studies linking the
emergence of SI virus to a rapid loss of CD4-positive T-cells and accelerated disease progression in untreated individuals [8-11], and a poorer response to therapy in a limited number of studies of treated individuals [12-17]. Of interest, we observed that the "11/25" genotype was not associated with an increased time to plasma viral suppression below 500 copies/ml unless individuals were censored at the time of any switch in antiretroviral therapy. These results do not necessarily indicate that individuals harboring HIV with the "11/25" genotype are less likely to suppress plasma viral load, but rather that it may require some optimization of therapy to find a combination that is effective. Importantly, even though antiretroviral therapies led to suppression of viral load to a median below 500 HIV RNA copies/ml, immunological and clinical response was still poorer in the "11/25" genotype group. In contrast, we observed no measurable effects of the "11/25" genotype on time to viral rebound (after initial suppression) or the development of drug resistance mutations, suggesting that the "11/25" genotype does not drive virological failure.

Sequence categories using the Position-Specific Scoring Matrix (PSSM) were largely consistent with sequence interpretations using the "11/25" rule, and were similarly predictive of time to viral suppression and mortality, but not CD4 response. Note that PSSM has a potential advantage over the simple dichotomization used here, as there is likely a continuum of HIV genetic sequences representing evolution to use the CXCR4 co-receptor with increasing efficiency. In contrast to the "11/25" rule, PSSM takes this into consideration by evaluating the strength of the association between V3 sequence and inferred viral phenotype. However for reasons of simplicity and consistency, we dichotomized PSSM sequence interpretations into either "si" or "nsi". It is possible, however, that sequence-based interpretation methods that take into consideration factors such as the efficiency of co-
receptor use and/or the strength of the association between genotype and inferred phenotype may prove to be more useful than simple sequence-based prediction methods in clinically relevant settings.

Rather surprisingly, HIV phenotypes predicted using the neural network (NN) differed substantially from "11/25" and PSSM predictions, and were not associated with clinical outcomes. The NN was originally trained to interpret collected datasets of viruses that were both genotyped and phenotyped (including both lab strains and primary isolates); its potential to predict outcomes from population-based sequence data from plasma samples therefore remains to be determined. In addition, the translation of nucleotide mixtures may have potentially disrupted natural patterns of sequence linkage, which could have most strongly affected the NN predictions. Since these analyses were performed, an additional approach to V3 sequence interpretation has been published, which has not yet been investigated [31].

We are unable to investigate the possibility that the "11/25" genotype was associated with different lengths of time since seroconversion or more rapid disease progression before therapy, as seroconversion dates are largely unknown in this cohort. In addition, as our observations are based on a single V3 genotype at baseline, we cannot rule out evolution of V3 sequences during study follow-up. Finally, as RT-PCR and DNA sequencing techniques were used to evaluate V3 sequences, only the most predominant species are detected; both types of virus may co-exist within any individual representing minority quasispecies.

In conclusion, results from this study indicate a significant correlation between positively charged residues at positions 11 and 25 of the HIV-1 envelope V3 loop, and both reduced CD4 response and decreased survival time following initiation of HAART. Baseline
V3 loop sequence data may therefore provide additional information regarding therapy response and could potentially be incorporated into standard clinical genotyping procedures.
Table 7.1: Patient Characteristics at baseline. [IQR]: Interquartile range

<table>
<thead>
<tr>
<th>Baseline Characteristics</th>
<th>Total study group (N=1191)</th>
<th>Stratified by success of V3 genotyping</th>
<th>Stratified by V3 genotype (N=1085 successfully genotyped samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotyping not successful (N=106)</td>
<td>Genotyping successful (N=1085)</td>
<td>p</td>
</tr>
<tr>
<td>Male Gender (%)</td>
<td>1004 (84.3%)</td>
<td>84 (79.3%)</td>
<td>920 (84.8%)</td>
</tr>
<tr>
<td>Median CD4 count (cells/mm³) [IQR]</td>
<td>280 [130-420]</td>
<td>260 [100-440]</td>
<td>280 [130-420]</td>
</tr>
<tr>
<td>Baseline AIDS diagnosis (%)</td>
<td>154 (12.9%)</td>
<td>15 (14.2%)</td>
<td>139 (12.8%)</td>
</tr>
<tr>
<td>NNRTI-containing therapy (%)</td>
<td>306 (25.7%)</td>
<td>20 (18.9%)</td>
<td>286 (26.4%)</td>
</tr>
<tr>
<td>History of injection drug use (%)</td>
<td>321 (27.0%)</td>
<td>23 (21.7%)</td>
<td>298 (27.5%)</td>
</tr>
</tbody>
</table>
Table 7.2a: Influence of baseline factors on time to death after initiation of HAART

<table>
<thead>
<tr>
<th>Baseline Risk Factor</th>
<th>Univariate</th>
<th></th>
<th></th>
<th>Multivariate</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Risk Ratio</td>
<td>95% CI</td>
<td>p</td>
<td>Risk Ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td>&quot;11/25&quot; genotype</td>
<td></td>
<td>1.77</td>
<td>1.12-2.80</td>
<td>0.015</td>
<td>1.70</td>
<td>1.06-2.71</td>
</tr>
<tr>
<td>male gender</td>
<td></td>
<td>0.94</td>
<td>0.58-1.53</td>
<td>0.808</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age (per 10 year increment)</td>
<td></td>
<td>1.45</td>
<td>1.21-1.73</td>
<td>&lt;0.001</td>
<td>1.49</td>
<td>1.24-1.78</td>
</tr>
<tr>
<td>baseline HIV RNA (per log increment)</td>
<td></td>
<td>1.78</td>
<td>1.28-2.48</td>
<td>0.001</td>
<td>1.44</td>
<td>1.03-2.00</td>
</tr>
<tr>
<td>baseline CD4 cell count (per 100 cell decrement)</td>
<td></td>
<td>1.31</td>
<td>1.19-1.45</td>
<td>&lt;0.001</td>
<td>1.31</td>
<td>1.17-1.47</td>
</tr>
<tr>
<td>% of time on antiretrovirals (per 10% decrement)</td>
<td></td>
<td>1.16</td>
<td>1.10-1.23</td>
<td>&lt;0.001</td>
<td>1.25</td>
<td>1.18-1.32</td>
</tr>
<tr>
<td>baseline AIDS diagnosis</td>
<td></td>
<td>1.70</td>
<td>1.10-2.64</td>
<td>0.017</td>
<td>1.30</td>
<td>0.81-2.11</td>
</tr>
<tr>
<td>History of injection drug use</td>
<td></td>
<td>1.14</td>
<td>0.79-1.66</td>
<td>0.481</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NNRTI vs. PI-containing therapy</td>
<td></td>
<td>0.90</td>
<td>0.58-1.40</td>
<td>0.642</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 7.2b: Influence of baseline factors on time to CD4 decline below baseline after initiation of HAART

<table>
<thead>
<tr>
<th>Baseline Risk Factor</th>
<th>Univariate</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Risk Ratio</td>
<td>95% CI</td>
<td>p</td>
<td>Risk Ratio</td>
<td>95% CI</td>
<td>p</td>
<td></td>
</tr>
<tr>
<td>&quot;11/25&quot; genotype</td>
<td>1.40</td>
<td>1.09-1.79</td>
<td>0.009</td>
<td>1.38</td>
<td>1.07-1.77</td>
<td>0.012</td>
<td></td>
</tr>
<tr>
<td>male gender</td>
<td>0.68</td>
<td>0.55-0.85</td>
<td>&lt;0.001</td>
<td>0.92</td>
<td>0.74-1.15</td>
<td>0.465</td>
<td></td>
</tr>
<tr>
<td>age (per 10 year increment)</td>
<td>0.95</td>
<td>0.87-1.05</td>
<td>0.325</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>baseline HIV RNA (per log increment)</td>
<td>0.71</td>
<td>0.62-0.80</td>
<td>&lt;0.001</td>
<td>0.99</td>
<td>0.86-1.14</td>
<td>0.877</td>
<td></td>
</tr>
<tr>
<td>baseline CD4 cell count (per 100 cell decrement)</td>
<td>0.85</td>
<td>0.83-0.87</td>
<td>&lt;0.001</td>
<td>0.87</td>
<td>0.84-0.89</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>% of time on antiretrovirals (per 10% decrement)</td>
<td>1.22</td>
<td>1.18-1.25</td>
<td>&lt;0.001</td>
<td>1.19</td>
<td>1.16-1.22</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>baseline AIDS diagnosis</td>
<td>0.38</td>
<td>0.27-0.53</td>
<td>&lt;0.001</td>
<td>0.68</td>
<td>0.48-0.96</td>
<td>0.029</td>
<td></td>
</tr>
<tr>
<td>History of injection drug use</td>
<td>1.54</td>
<td>1.30-1.83</td>
<td>&lt;0.001</td>
<td>1.21</td>
<td>1.01-1.44</td>
<td>0.042</td>
<td></td>
</tr>
<tr>
<td>NNRTI vs. PI-containing therapy</td>
<td>1.33</td>
<td>1.11-1.61</td>
<td>0.002</td>
<td>1.38</td>
<td>1.13-1.67</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>
HIV isolates were classified into two groups based on amino acid analysis of the HIV V3 loop region. Isolates with positively charged amino acids at positions 11 and/or 25 of the V3 loop (a genotype previously associated with HIV SI phenotype [19]) were classified as having an "11/25" genotype. Individual events (non-accidental deaths) are indicated by circles ("11/25" genotype) or crosses ("non-11/25" genotype). There was a statistically significant association between the "11/25" genotype at baseline and an earlier time to death after initiation of HAART (log rank p=0.015). The number of subjects remaining in the analysis is indicated below the graphs.
Figure 7.2: Longitudinal analysis of population-based HIV viral loads (panel A) and CD4 counts (panel B), stratified by HIV V3 loop genotype.

The median post-therapy plasma viral load (A) or CD4 cell count (B) after starting triple drug therapy are indicated for individuals with the "11/25" genotype (solid lines with circles) or "non-11/25" genotype (dotted lines with crosses). Error bars represent the Interquartile Range associated with each point. If an individual had more than one CD4 or viral load test performed during any given time interval, only the latest measurement was included. The gray horizontal line in Panel A indicates the 500 copies HIV RNA/ml (2.70 log) cutoff used in the primary analyses. The ultrasensitive adaptation of the plasma viral load assay was not available over the entire study period; data are presented where this assay was available.
7.5 REFERENCES


27. Hirsch MS, Brun-Vezinet F, D'Aquila RT, Hammer SM, Johnson VA, Kuritzkes DR,


CHAPTER 8

HIV GENETIC VARIATION: RELEVANCE OF HIV ENVELOPE CORECEPTOR USAGE ON RESPONSE TO HAART

8.1 INTRODUCTION

Human Immunodeficiency Virus type 1 (HIV-1) uses host cell membrane chemokine receptors in combination with CD4 in order to gain entry into host cells [1,2,3]. The most important co-receptors in HIV pathogenesis are the chemokine receptors CCR5 [1,2,4] and CXCR4 [5], although in rare cases other co-receptors have also been shown to mediate entry of HIV-1 into target cells, at least in vitro [6,7,8]. In general, most HIV-1 variants isolated from newly-infected individuals utilize CCR5 in combination with CD4 to gain entry to host cells. These R5 variants predominantly infect activated CD4⁺ T-lymphocytes as well as macrophages. While R5 variants are generally detectable over the entire course of HIV infection [9], variants able to utilize CXCR4 emerge in approximately 40-50% of infected persons over the course of disease [10]. These X4 variants predominantly target naïve and resting CD4⁺ T-lymphocytes and display different biological properties than their R5 counterparts, including increased replication rate, pathogenicity and syncytium-inducing (SI) capacity in cell culture [9,11]. In addition, dual-tropic variants capable of using both CXCR4 and CCR5 may also arise over the course of disease [12].

The factors mediating the R5-to-X4 phenotype "switch" over the natural course of HIV infection remain incompletely understood. The emergence of CXCR4-using variants is associated with a rapid decline in CD4⁺ T-lymphocyte counts, accelerated disease progression, and increased risk of disease progression and AIDS-related events [9,11].

This chapter has been published as:
progression and reduced survival time in untreated individuals [13,14,15,16], as well as poorer response to treatment in the pre-Highly Active Antiretroviral Therapy (HAART) era [17,18,19,20,21]. However, it is not known whether X4 variants are inherently more pathogenic and are directly responsible for more rapid disease progression, or whether CXCR4-using HIV variants may emerge as a consequence of progressive immune dysfunction [22]. Regardless of the direction of causation, the association of X4 HIV with poorer prognosis and inferior therapy response remains an important issue in clinical practice, and it is important that the prognostic implications of HIV co-receptor usage be re-evaluated in the HAART era.

At present, the epidemiology of R5 and X4 HIV is also of particular relevance due to the development of HIV co-receptor inhibitors. This new class of antiretroviral agents is designed to specifically inhibit HIV binding to either CCR5 and/or CXCR4, thereby preventing HIV entry into target cells. Although co-receptor inhibitors currently show promising effects in early clinical trials [22,23], it is not known on a population basis what proportion of HIV-infected individuals may potentially benefit most from antiretroviral agents targeting CCR5 and/or CXCR4.

Using the newly-developed Virologic Phenosense assay, we investigated the epidemiology of HIV co-receptor usage in the HOMER cohort in British Columbia, Canada, consisting of 1191 antiretroviral-naïve individuals initiating their first triple combination therapy between August 1996 and September 1999 [24,25]. In this cross-sectional study, we wished to characterize the prevalence of X4 HIV at therapy initiation and to identify sociodemographic, clinical and genetic risk factors associated with phenotypic CXCR4 usage. Finally, using longitudinal clinical data collected over the course of study follow-up,
we wished to establish the impact of CXCR4 co-receptor usage on clinical and virological outcomes after initiation of triple combination therapy.
8.2 MATERIALS AND METHODS

The B.C. Centre for Excellence in HIV/AIDS Drug Treatment Program

In the province of British Columbia, Canada, antiretrovirals are distributed free of charge to HIV-infected individuals through a centralized drug treatment program based at the B.C. Centre for Excellence in HIV/AIDS (the Centre). Antiretrovirals are prescribed according to specific guidelines set by the B.C. Therapeutic Guidelines Committee, which are revised regularly and are in accordance with international guidelines [24]. Patients who enroll in the program may provide informed consent and participate in a survey that collects sociodemographic data. Routine clinical monitoring of patients takes place at approximately 3-month intervals at which time plasma viral load testing (Roche Amplicor Monitor Assay) and CD4 counts are performed. These data are stored in the Centre's Drug Treatment Program database. Ethical approval for this study was obtained from the research ethics board at the University of British Columbia, St. Paul’s Hospital site.

The HOMER cohort

The HAART Observational Medical Evaluation and Research (HOMER) cohort includes all HIV-positive, antiretroviral naïve adults who started triple antiretroviral therapy (consisting of two nucleoside reverse transcriptase inhibitors [nRTIs] and either a protease inhibitor [PI] or a non-nucleoside reverse transcriptase inhibitor [NNRTI]) through the B.C. Drug Treatment Program between August 1996 and September 1999 (N=1191). This cohort has been the focus of a number of population-based studies and has been described in detail previously [24,25]. Subjects were followed for a median of 4.7 years (56 months) after initiation of antiretroviral therapy.
Determination of baseline HIV co-receptor phenotype and envelope V3 genotype

For each study subject, a single pre-therapy (baseline) plasma sample collected ≤ 6 months prior to initiation of therapy was assayed using the Virologic Phenosense Assay (Virologic, San Francisco) in order to determine HIV co-receptor usage. Viral RNA was extracted using oligo(dT) columns and HIV-1 env-specific primers were used to amplify a 2.5-kb RT-PCR product that spanned the entire gp160 open reading frame. RT-PCR products were digested, purified and ligated into an *E. Coli* expression vector, and gene libraries constructed. A replication-defective retroviral vector (pHIVluc) containing a luciferase expression cassette inserted within the *env* gene was used to co-transfect human embryonic kidney cell cultures with the sample plasmid DNA. Recombinant viruses were harvested after 48 hours and assessed for their ability to infect cells expressing CCR5 or CXCR4. The Phenosense assay classifies isolates as R5, X4 or R5/X4 (indicating dual and/or mixed-tropic virus).

The same baseline plasma samples were used to determine HIV V3 envelope sequence as described previously [25]. Isolates displaying positively-charged amino acids at codons 11 and/or 25 of the V3 loop [26], associated with an HIV syncytium-inducing (SI) phenotype [26], were classified as having an "11/25 genotype" [25].

Determination of CCR5Δ32 genotype

Blood (n=796, 66.8%) or plasma (n=395, 33.2%) samples were available from all 1191 study subjects. DNA was extracted from blood or plasma using the Qiagen DNA kit adapted for use on the Qiagen Biorobot 9604. Extracted DNA was amplified in a single round of PCR using primers flanking the Δ32 region. PCR products were visualised by
electrophoresis on a 2% agarose gel and confirmed by automated DNA sequencing in both 5' and 3' directions on an ABI 3700 DNA sequencer.

Statistical Methods

Association of baseline HIV co-receptor use with sociodemographic, clinical and genetic variables

Associations between baseline HIV co-receptor usage and dichotomous baseline parameters including gender, baseline AIDS diagnosis, history of injection drug use, human CCR5 genotype (CCR5wt/Δ32 or wt/wt) and HIV V3 genotype ("11/25" genotype or not) [25] were determined using the Chi-squared test. Associations between baseline HIV co-receptor usage and continuous variables including age, plasma viral load (pVL) and CD4 count were determined using the Wilcoxon Rank-sum test. Baseline predictors of X4 usage and associated odds ratios were calculated using univariate and multivariate logistic regression.

Association between baseline HIV co-receptor usage and response to antiretroviral therapy

The primary endpoint investigated in the outcome analyses was time to non-accidental death, defined as the time from therapy initiation to non-accidental death (occurring on or before June 30, 2003, the date of latest linkage with mortality statistics from the British Columbia Vital Statistics Agency). Deaths were classified according to ICD-10 coding. Accidental deaths were not considered events and subjects censored at date of death. Additional clinical outcomes included time to suppression of plasma HIV RNA (time from therapy initiation to first of two consecutive viral loads <500 copies/ml), time to plasma HIV RNA rebound (subsequent time to the first of two consecutive viral loads >500 copies/ml), and time to CD4 decline (time to first decline of CD4 count below baseline). For the clinical
outcome analyses, event-free subjects were censored at the collection date of the last tested sample up to and including June 30, 2003.

The influence of HIV co-receptor usage on clinical outcomes was assessed by Kaplan-Meier methods. Cox proportional hazards regression was used to calculate univariate and multivariate Hazard Ratios (HR) and 95% confidence intervals (CI). Baseline variables included in the model were gender (male vs. female [reference group]), age (per 10 year increment), AIDS diagnosis (yes vs. no), plasma HIV RNA (per log_{10} increment), CD4 count (per 100 cell decrement), proportion of total time spent on antiretroviral therapy in the first year of follow-up (per 10% increment) [27], type of therapy at initiation (PI-containing vs. NNRTI-containing), history of injection drug use (yes vs. no), CCR5 genotype (wt/A32 vs. wt/wt) and HIV V3 genotype ('11/25' vs. not) [25].

In both the logistic regression and Cox proportional hazard regression analyses, all factors significant in univariate analyses were included in multivariate analyses. All tests of significance were two-sided, with a p-value < 0.05 indicating statistical significance.
8.3 RESULTS

Results of HIV co-receptor phenotyping

The prevalence and determinants of HIV-1 co-receptor usage were investigated in the HOMER [24,25] cohort of 1191 antiretroviral naïve individuals initiating therapy. HIV co-receptor usage data were obtained for 979 of 1191 subjects (82.2%). Phenotype data were more likely to be available for male subjects (p=0.04), and for subjects with higher plasma viral loads (p<0.0001), lower CD4 counts (p=0.007) and the human CCR5 wt/wt genotype (p=0.007) (Table 1a). Of the 979 phenotyped subjects, 801 (81.8%) harbored R5 variants, 177 (18.1%) harbored R5/X4 variants, and only one (0.1%) harbored exclusively X4 variants. For the remainder of the analyses, this individual was included in the R5/X4 group.

Baseline characteristics stratified by HIV co-receptor usage in a therapy-naïve population

Comparison of baseline parameters from subjects harboring R5/X4 variants with those harboring exclusively R5 variants revealed several important differences in clinical characteristics (Table 1a). Subjects with R5/X4 variants had significantly higher HIV plasma viral loads (p=0.0006), lower CD4 counts (p<0.001), and were significantly more likely to be diagnosed with an AIDS-defining illness prior to therapy initiation (p<0.0001). The distribution of HIV co-receptor usage at clinically useful CD4 strata highlights the strong association between the detection of X4 variants and absolute CD4 count at baseline, ranging from <10% for CD4 counts above 200 cells/mm$^3$ to >50% for CD4 counts below 25 cells/mm$^3$ (Table 1b).

As expected, subjects with basic amino acids at codons 11 and/or 25 of the HIV V3 loop, (an '11/25' genotype [25,26]), were significantly more likely to harbor R5/X4 HIV (p<0.0001, Table 1a) than subjects with neutral or negatively-charged residues at these sites.
The '11/25 genotype', in this case evaluated using direct PCR and sequencing of patient-derived isolates, displayed a specificity of 93.1% and a sensitivity of 32.7% for predicting the presence of X4 variants. In addition, individuals heterozygous for the CCR5Δ32 deletion (CCR5 wt/Δ32) were also significantly more likely to harbor R5/X4 HIV variants than individuals with the CCR5 wt/wt genotype (p=0.0085) (Table 1a).

**Baseline characteristics stratified by combined CCR5 genotype and HIV phenotype data**

In order to further investigate the association between CCR5Δ32 genotype and R5/X4 HIV, we combined the human CCR5 genotype and HIV co-receptor usage data. Combined HIV phenotype and human CCR5 genotype data were available for 967 subjects (Table 2). The four categories [CCR5 wt/wt, R5 phenotype (N=697); CCR5 wt/wt, R5/X4 phenotype (N=142); CCR5 wt/Δ32, R5 phenotype (N=94); CCR5 wt/Δ32, R5/X4 phenotype (N=34)] were compared with respect to baseline sociodemographic and clinical characteristics. Individuals heterozygous for the CCR5Δ32 deletion and who harbored exclusively R5 HIV displayed the most favourable clinical profile; of the four groups, these subjects had the lowest plasma viral loads and the highest CD4 counts at baseline (Table 2). In contrast, individuals with R5/X4 HIV, regardless of CCR5 genotype, had the poorest clinical profile; these individuals had the highest plasma viral loads, the lowest CD4 counts, and were more likely to be diagnosed with an AIDS-defining illness at time of therapy initiation than subjects harboring only R5 HIV (Table 2).

In addition, we wished to investigate whether R5/X4 HIV variants from CCR5 wt/wt or CCR5 wt/Δ32 individuals might differ in their predominant HIV V3 amino acid sequence. Although, as mentioned previously, R5/X4 HIV was significantly associated with the '11/25 genotype' [25] (p<0.0001, Table 2), we observed no significant difference in the ability of the
'11/25' genotype to predict HIV co-receptor usage in either CCR5 wt/wt or CCR5 wt/Δ32 individuals (sensitivity 30.1% vs. 39.4% respectively, p=0.3, data not shown).

**Predictors of R5/X4 HIV in a therapy-naïve population**

Univariate and multivariate logistic regression were used to identify significant baseline predictors of R5/X4 HIV and to calculate univariate and multivariate odds ratios associated with these parameters (Table 3). In multivariate analyses, the strongest predictors of R5/X4 HIV were low CD4 count (Odds Ratio [OR] 1.53 per 100-cell decrement in CD4 count, p<0.0001) and the HIV V3 loop '11/25' genotype (OR 9.11, p<0.0001). Additional predictors of R5/X4 HIV were high baseline plasma viral load (OR 1.46 per log10 increment in HIV RNA, p=0.04) and the heterozygous CCR5 wt/Δ32 genotype (OR 2.48, p=0.0005) (Table 3).

**Association of baseline R5/X4 HIV with mortality and other clinical outcomes**

In univariate analyses, the presence of R5/X4 HIV at baseline was significantly associated with decreased survival after initiating antiretroviral therapy (univariate p=0.05, Table 4a and Figure 1a). However, after adjusting for baseline factors including CD4 count, age and plasma viral load, this association was no longer significant (multivariate p=0.45, Table 4a).

We observed no significant associations between baseline HIV co-receptor usage and either CD4 response after initiation of therapy (p=0.07, Figure 1b and Table 4b) or the time to achieve suppression of plasma HIV RNA <500 copies/ml (p=0.98, HR=1.00). In univariate analyses, subjects harboring R5/X4 HIV appeared to be less likely to experience virologic failure (Hazard Ratio [HR] 0.7, p=0.012) after initial suppression (Figure 1c and Table 4c). However, in multivariate analyses adjusting for baseline parameters, HIV co-
receptor usage was not significantly associated with either virologic or immunologic response after initiation of HAART (p>0.1, Tables 4b and 4c).

**Analysis of combined CCR5 genotype and HIV co-receptor phenotype to predict therapy outcome**

Combining data from both CCR5 genotype and HIV co-receptor phenotype further clarified the association between these parameters and survival following therapy initiation (Figure 1d). In these analyses, the reference group was defined as subjects with the human CCR5 wt/wt genotype harboring exclusively R5 HIV. In univariate analyses, subjects with the CCR5 wt/A32 genotype harboring exclusively R5 HIV were at decreased risk of death (HR=0.54, p=0.09) compared with the reference group. In contrast, subjects with the CCR5 wt/wt genotype harboring R5/X4 HIV were at significantly increased risk of death when compared to the reference group (HR 1.54, p=0.04). Subjects with the CCR5 wt/A32 genotype harboring R5/X4 HIV were at comparable risk of mortality with respect to the reference group (HR 1.09, p=0.8). However, in multivariate analyses adjusting for baseline parameters including age, plasma viral load and CD4 count, these associations were not significant (p>0.1, data not shown).
8.4 DISCUSSION AND CONCLUSION

We investigated the prevalence and clinical correlates of X4 HIV in a large population of antiretroviral naïve individuals initiating first HAART. Consistent with results from other cross-sectional studies [28,29,30,31] we observed a strong correlation between the presence of CXCR4-using HIV and clinical parameters including viral load, diagnosis of AIDS defining-illnesses, and most strikingly, CD4 count. In this study, CD4 counts from subjects harboring X4 variants were on average three times lower than those from subjects harboring exclusively R5 variants.

Genotypic (sequence-based) and phenotypic HIV co-receptor usage assays as predictors of therapy response

We previously undertook a study on the same cohort that identified the HIV V3 loop sequence as a significant predictor of survival and CD4 response after initiating antiretroviral therapy [25]. As expected, we observed a highly significant correlation between positively-charged amino acids at key residues of the HIV V3 loop (an 11/25 genotype), and the presence of X4 HIV at baseline [25,26]. In the present study, the 11/25 genotype predicted the presence of CXCR4-using virus with a specificity of 93% and a sensitivity of 33%. This value appears to be considerably lower than other studies employing V3 sequence-based interpretation methods [32,33], however this may be explained by the fact that our V3 genotypes were obtained using bulk PCR and sequencing of nucleic material isolated directly from clinical isolates, rather than analysis of cloned PCR products. As population-based sequencing methods detect only the predominant circulating species, the prevalence of X4 variants may be underestimated if they are present as minority variants. HIV Genotype-Phenotype correlations will be examined in detail in a further study.
However, somewhat in contrast to the previous study linking baseline V3 sequence to therapy outcome [25], the present study suggests that HIV co-receptor use, although strongly associated with baseline clinical parameters, may not be independently associated with response to treatment. HIV co-receptor use at baseline did not remain an independent predictor of therapy response after adjusting for the strong association with baseline clinical parameters including CD4 count and viral load.

**Association between CCR5Δ32 genotype and HIV co-receptor usage**

One hypothetical but potentially important consequence of the administration of CCR5 antagonists is the selection of CXCR4-using HIV variants [34]. The naturally occurring CCR5Δ32 deletion, associated with improved prognosis and slower HIV disease progression [35,36], may provide useful insights into this issue. CCR5 wt/Δ32 individuals have only one functioning CCR5 allele and express CCR5 at much reduced levels [37], and therefore if reduced cell-surface CCR5 availability indeed selects for CXCR4-using variants, one would expect to observe an increased prevalence of X4 HIV in these individuals [38]. Indeed, our data indicate that after accounting for other baseline parameters, individuals with the CCR5 wt/Δ32 genotype are nearly twice as likely to harbor R5/X4 HIV than CCR5 wt/wt individuals, although other studies have reported no correlation [39]. Although our results suggest that limited cell-surface CCR5 may lead to selective pressure favoring the selection of CXCR4-using variants, one must be cautious in drawing conclusions from cross-sectional data. In addition we must acknowledge the potential bias resulting from the possibility that CCR5 wt/Δ32 individuals harboring CXCR4-using variants may be over-represented in our cohort due to poor survival of X4-carriers with a CCR5 wt/wt genotype.
Whether this observation may be relevant to the administration of CCR5 co-receptor antagonists remains to be determined.

When taking into consideration previous data linking the CCR5Δ32 deletion with slower HIV disease progression [35,36] and the HIV X4 phenotype with faster disease progression [13,14,15,16], the possibility that individuals with the CCR5Δ32 genotype are more likely to harbor X4 HIV appears contradictory. However, an analysis of the combined CCR5 genotype and HIV co-receptor phenotype data indicate that the association between CCR5 wt/Δ32 genotype and improved baseline clinical profile as well as survival after initiation of antiretroviral therapy is observed only when the predominant circulating virus at baseline is exclusively R5 (Table 2). The protective effects of the CCR5Δ32 deletion appear to be largely lost when X4 variants are present at baseline, consistent with previous reports [39,40]. However it is interesting to note that individuals with a CCR5 wt/Δ32 genotype harboring R5/X4 variants have a higher baseline viral load than CCR5 wt/wt individuals with R5/X4 variants (Table 2), but slightly better survival following initiation of HAART (figure 1d). A potential explanation is that the CCR5 wt/Δ32 genotype may still confer a slight survival advantage in the presence of X4-containing variants, however we must caution that the CCR5 wt/Δ32 R5/X4 group is relatively small and again the potential biases resulting from cross-sectional data and potential under-representation of X4-carriers with a CCR5 wt/wt genotype in this cohort must be acknowledged.

Limitations of this study include the fact that associations between HIV co-receptor usage and clinical parameters are based on cross-sectional data from a population of therapy-naïve individuals initiating their first antiretroviral treatment. As the contemporary guidelines for when to initiate therapy were predominantly based on the presence of AIDS-
defining illnesses or CD4- and viral load thresholds, our study population may not be representative of the HIV-infected, antiretroviral naïve population in general. In addition, because of the cross-sectional nature of this study, we are unable to comment upon the incidence of X4 variants, the length of time these variants have been present, or on the evolution of HIV co-receptor usage over the natural history of infection. Similarly as seroconversion dates are not known and historical plasma samples not generally available we also are unable to investigate what proportion of our study group may have acquired X4 variants at transmission. Finally, because population-based genotyping and phenotyping approaches were utilized, we are unable to distinguish whether subjects with R5/X4 HIV harbor dual-tropic variants, a mixture of R5 and X4 variants, or both. Despite these limitations, our study is one of the first to characterize the epidemiology and clinical predictors of HIV co-receptor usage in a large therapy naïve population and present longitudinal clinical outcome data after initiation of therapy. In addition it is one of the largest studies evaluating associations between HIV V3 genotype, co-receptor phenotype, human CCR5Δ32 genotype and other clinical parameters. Strong associations between R5/X4 HIV and clinical markers of HIV disease progression including higher plasma viral load and significantly lower CD4 counts confirm the well-characterized link between X4 HIV and poorer prognosis [13,14,15,16], although the direction of causation still remains unclear. Despite the association of X4 HIV with poorer pre-therapy prognosis, however, the presence of CXCR4-using HIV at baseline did not remain an independent predictor of survival or clinical response to HAART after adjustment for baseline parameters including CD4 count and plasma viral load.
Table 8.1a: Patient Characteristics at baseline, stratified by HIV co-receptor usage.
[IQR]: Interquartile range

<table>
<thead>
<tr>
<th>Baseline Characteristics</th>
<th>Total study group (N=1191)</th>
<th>Stratified by co-receptor usage data available</th>
<th>Stratified by HIV co-receptor use (N=979 samples for which co-receptor data available)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Co-receptor data not available (N=212)</td>
<td>Co-receptor data available (N=979)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R5 (N=801)</td>
<td>R5/X4 (N=178)</td>
</tr>
<tr>
<td>Male Gender</td>
<td>1004</td>
<td>169</td>
<td>835</td>
</tr>
<tr>
<td>(%)</td>
<td>(64.3%)</td>
<td>(79.7%)</td>
<td>(85.3%)</td>
</tr>
<tr>
<td>Median Age, years [IQR]</td>
<td>37</td>
<td>36</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>[32-44]</td>
<td>[31-43]</td>
<td>[32-44]</td>
</tr>
<tr>
<td>Median HIV pVL (x10^3 copies/ml) [IQR]</td>
<td>120</td>
<td>61</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>[42-310]</td>
<td>[13-190]</td>
<td>[55-340]</td>
</tr>
<tr>
<td>Median CD4 count (cells/mm^3) [IQR]</td>
<td>280</td>
<td>330</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td>[130-420]</td>
<td>[155-455]</td>
<td>[120-420]</td>
</tr>
<tr>
<td>Baseline AIDS diagnosis (%)</td>
<td>154</td>
<td>24</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>(12.9%)</td>
<td>(11.3%)</td>
<td>(13.4%)</td>
</tr>
<tr>
<td>History of injection drug use (%)</td>
<td>321</td>
<td>67</td>
<td>268</td>
</tr>
<tr>
<td></td>
<td>(27.0%)</td>
<td>(31.6%)</td>
<td>(27.4%)</td>
</tr>
<tr>
<td>CCR5 genetics (% CCR5 wt/A32)</td>
<td>171 of 1177</td>
<td>43 of 210</td>
<td>128 of 967</td>
</tr>
<tr>
<td>Note: N=967 with CCR5 and phenotype data available</td>
<td>(14.5%)</td>
<td>(20.5%)</td>
<td>(13.2%)</td>
</tr>
<tr>
<td>V3 '11/25' genotype (% 11/25 genotype)</td>
<td>130 of 1117</td>
<td>21 of 162</td>
<td>109 of 955</td>
</tr>
<tr>
<td>Note: N=955 with V3 geno and pheno data available</td>
<td>(11.6%)</td>
<td>(13.0%)</td>
<td>(11.4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>54 of 787</td>
<td>55 of 168</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6.9%)</td>
<td>(32.7%)</td>
</tr>
</tbody>
</table>

Note: N=967 with CCR5 and phenotype data available.

Note: N=955 with V3 geno and pheno data available.
Table 8.1b: Distribution of R5/X4 HIV by baseline (pre-therapy) CD4 count strata

<table>
<thead>
<tr>
<th>Baseline co-receptor usage</th>
<th>Baseline CD4 count strata (cells/mm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;25</td>
</tr>
<tr>
<td>R5 HIV (% of CD4 stratum)</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>45.6%</td>
</tr>
<tr>
<td>R5/X4 HIV (% of CD4 stratum)</td>
<td>43</td>
</tr>
<tr>
<td>54.4%</td>
<td>31.1%</td>
</tr>
<tr>
<td>Total for each CD4 stratum</td>
<td>79</td>
</tr>
</tbody>
</table>
Table 8.2: Patient Characteristics at baseline, stratified by HIV co-receptor usage and human CCR5A32 genotype. [IQR]: Interquartile range

| Baseline Characteristics | Stratified by HIV co-receptor usage and CCR5A32 genotype (N=967) |  |  |  |  | p
|--------------------------|-----------------------------------------------------------------|---|---|---|---|------
|                          | CCR5 wt/wt (N=697) | CCR5 wt/A32 | R5 (N=142) | R5 (N=34) | (any differences) |
| Male Gender (%):         | 584 (83.8%)        | 125 (88.0%) | 85 (90.4%) | 30 (88.2%) | 0.23             |
| Median HIV pVL (x10^3 copies/ml) [IQR] | 130 [54-340] | 165 [84-400] | 78 [29-142] | 295 [86-530] | <0.0001 |
| Baseline AIDS diagnosis (%) | 80 (11.5%)       | 33 (23.2%)  | 10 (10.6%) | 7 (20.6%) | 0.001            |
| History of injection drug use (%) | 202 (29.0%)       | 30 (21.3%)  | 24 (25.5%) | 8 (23.5%) | 0.25             |
| V3 genotype (% '11/25' genotype) (N=943 with CCR5, phenotype and genotype data available) | 50 of 685 (7.3%) | 40 of 133 (30.1%) | 4 of 92 (4.4%) | 13 of 33 (39.4%) | <0.0001 |
### Table 8.3: Univariate and Multivariate predictors of the presence of X4-using HIV variants at baseline

<table>
<thead>
<tr>
<th>Baseline Risk Factor</th>
<th>Univariate</th>
<th></th>
<th></th>
<th>Multivariate</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds Ratio</td>
<td>95% CI</td>
<td>p</td>
<td>Odds Ratio</td>
<td>95% CI</td>
<td>p</td>
</tr>
<tr>
<td>Male gender</td>
<td>1.36</td>
<td>0.83-2.22</td>
<td>0.227</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age (per 10 year increment)</td>
<td>1.04</td>
<td>0.87-1.24</td>
<td>0.705</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Baseline HIV RNA (per log incr)</td>
<td>1.62</td>
<td>1.19-2.20</td>
<td>0.002</td>
<td>1.46</td>
<td>1.02-2.08</td>
<td>0.040</td>
</tr>
<tr>
<td>Baseline CD4 cell count (per 100 cell decr)</td>
<td>1.52</td>
<td>1.69-1.35</td>
<td>&lt;0.0001</td>
<td>1.53</td>
<td>1.34-1.73</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Baseline AIDS diagnosis</td>
<td>2.26</td>
<td>1.50-3.42</td>
<td>&lt;0.0001</td>
<td>1.17</td>
<td>0.72-1.91</td>
<td>0.531</td>
</tr>
<tr>
<td>History of injection drug use</td>
<td>0.70</td>
<td>0.48-1.03</td>
<td>0.072</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CCR5 wt/Δ32 genotype</td>
<td>1.78</td>
<td>1.15-2.73</td>
<td>0.009</td>
<td>2.48</td>
<td>1.48-4.15</td>
<td>0.0005</td>
</tr>
<tr>
<td>HIV V3 '11/25' genotype</td>
<td>6.61</td>
<td>4.32-10.1</td>
<td>&lt;0.0001</td>
<td>9.11</td>
<td>5.54-14.98</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Table 8.4a: Influence of baseline factors on time to non-accidental death after initiation of HAART

<table>
<thead>
<tr>
<th>Baseline Risk Factor</th>
<th>Univariate</th>
<th></th>
<th></th>
<th>Multivariate (N=967)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard Ratio</td>
<td>95% CI</td>
<td>p</td>
<td>Hazard Ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td>Presence of X4 HIV variants</td>
<td>1.45</td>
<td>1.00-2.13</td>
<td>0.053</td>
<td>1.14</td>
<td>0.76-1.70</td>
</tr>
<tr>
<td>male gender</td>
<td>0.91</td>
<td>0.60-1.38</td>
<td>0.661</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>age (per 10 year increment)</td>
<td>1.45</td>
<td>1.24-1.69</td>
<td>&lt;0.0001</td>
<td>1.48</td>
<td>1.25-1.76</td>
</tr>
<tr>
<td>baseline HIV RNA (per log increment)</td>
<td>1.67</td>
<td>1.28-2.19</td>
<td>0.0002</td>
<td>1.38</td>
<td>0.99-1.92</td>
</tr>
<tr>
<td>baseline CD4 cell count (per 100 cell decr)</td>
<td>1.27</td>
<td>1.16-1.39</td>
<td>&lt;0.0001</td>
<td>1.32</td>
<td>1.18-1.48</td>
</tr>
<tr>
<td>% of time on antiretrovirals (per 10% incr)</td>
<td>0.86</td>
<td>0.83-0.91</td>
<td>&lt;0.0001</td>
<td>0.80</td>
<td>0.76-0.85</td>
</tr>
<tr>
<td>baseline AIDS diagnosis</td>
<td>1.71</td>
<td>1.17-2.51</td>
<td>0.006</td>
<td>1.11</td>
<td>0.70-1.76</td>
</tr>
<tr>
<td>History of injection drug use</td>
<td>1.01</td>
<td>0.72-1.41</td>
<td>0.976</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PI vs. NNRTI -containing therapy</td>
<td>1.28</td>
<td>0.87-1.87</td>
<td>0.213</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CCR5 wt/A32 genotype</td>
<td>0.64</td>
<td>0.38-1.07</td>
<td>0.087</td>
<td>0.74</td>
<td>0.41-1.32</td>
</tr>
</tbody>
</table>

Table 8.4b: Influence of baseline factors on time to CD4 decline below baseline after initiation of HAART

<table>
<thead>
<tr>
<th>Baseline Risk Factor</th>
<th>Univariate</th>
<th></th>
<th></th>
<th>Multivariate (N=967)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard Ratio</td>
<td>95% CI</td>
<td>p</td>
<td>Hazard Ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td>Presence of X4 HIV variants</td>
<td>0.81</td>
<td>0.65-1.02</td>
<td>0.074</td>
<td>1.17</td>
<td>0.93-1.48</td>
</tr>
<tr>
<td>male gender</td>
<td>0.74</td>
<td>0.61-0.91</td>
<td>0.003</td>
<td>0.98</td>
<td>0.78-1.23</td>
</tr>
<tr>
<td>age (per 10 year increment)</td>
<td>0.95</td>
<td>0.87-1.03</td>
<td>0.196</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>baseline HIV RNA (per log increment)</td>
<td>0.76</td>
<td>0.68-0.84</td>
<td>&lt;0.0001</td>
<td>0.91</td>
<td>0.78-1.06</td>
</tr>
<tr>
<td>baseline CD4 cell count (per 100 cell decr)</td>
<td>0.84</td>
<td>0.83-0.86</td>
<td>&lt;0.0001</td>
<td>0.84</td>
<td>0.80-0.87</td>
</tr>
<tr>
<td>% of time on antiretrovirals (per 10% incr)</td>
<td>0.83</td>
<td>0.81-0.85</td>
<td>&lt;0.0001</td>
<td>0.86</td>
<td>0.83-0.88</td>
</tr>
<tr>
<td>baseline AIDS diagnosis</td>
<td>0.39</td>
<td>0.29-0.53</td>
<td>&lt;0.0001</td>
<td>0.64</td>
<td>0.45-0.91</td>
</tr>
<tr>
<td>History of injection drug use</td>
<td>1.63</td>
<td>1.39-1.91</td>
<td>&lt;0.0001</td>
<td>1.42</td>
<td>1.18-1.70</td>
</tr>
<tr>
<td>PI vs. NNRTI -containing therapy</td>
<td>0.80</td>
<td>0.67-0.95</td>
<td>0.009</td>
<td>0.76</td>
<td>0.62-0.93</td>
</tr>
<tr>
<td>CCR5 wt/A32 genotype</td>
<td>1.08</td>
<td>0.87-1.33</td>
<td>0.505</td>
<td>1.02</td>
<td>0.80-1.30</td>
</tr>
</tbody>
</table>
Table 8.4c: Influence of baseline factors on time to pVL failure >500 copies/ml (after initial suppression of pVL <500 copies/ml)

<table>
<thead>
<tr>
<th>Baseline Risk Factor</th>
<th>Univariate</th>
<th></th>
<th>Multivariate (N=751)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard Ratio</td>
<td>95% CI</td>
<td>p</td>
</tr>
<tr>
<td>Presence of X4 HIV variants</td>
<td>0.72</td>
<td>0.56-0.93</td>
<td>0.012</td>
</tr>
<tr>
<td>male gender</td>
<td>0.70</td>
<td>0.55-0.88</td>
<td>0.002</td>
</tr>
<tr>
<td>age (per 10 year increment)</td>
<td>0.88</td>
<td>0.80-0.97</td>
<td>0.008</td>
</tr>
<tr>
<td>baseline HIV RNA (per log increment)</td>
<td>0.98</td>
<td>0.87-1.11</td>
<td>0.775</td>
</tr>
<tr>
<td>baseline CD4 cell count (per 100 cell decr)</td>
<td>1.09</td>
<td>1.06-1.13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>% of time on antiretrovirals (per 10% incr)</td>
<td>0.88</td>
<td>0.85-0.91</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>baseline AIDS diagnosis</td>
<td>0.56</td>
<td>0.43-0.74</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>History of injection drug use</td>
<td>1.44</td>
<td>1.20-1.74</td>
<td>0.0001</td>
</tr>
<tr>
<td>PI vs. NNRTI-containing therapy</td>
<td>0.91</td>
<td>0.75-1.10</td>
<td>0.320</td>
</tr>
<tr>
<td>CCR5 wt/A32 genotype</td>
<td>0.81</td>
<td>0.64-1.03</td>
<td>0.085</td>
</tr>
</tbody>
</table>

* note that subjects who did not achieve initial suppression of pVL <500 copies HIV RNA/ml were censored at time of HAART initiation
Figure 8.1, panels A-D (Next page): Influence of HIV co-receptor usage on survival and clinical outcomes after initiation of first triple antiretroviral therapy.

Kaplan-Meier analyses of the influence of baseline HIV co-receptor usage (R5 vs. R5/X4 virus) on time to non-accidental death (panel A); time to CD4 decline below baseline (panel B); and time to virologic rebound >500 copies/ml (panel C) after initiation of first triple antiretroviral therapy, over a median follow-up time of 56 months. Individuals with R5 HIV are represented by the circles (○), while individuals with R5/X4 HIV are indicated by the plus (+) signs.

Panel D is a Kaplan-Meier analysis of the influence of combined human CCR5Δ32 genotype and HIV co-receptor usage on time to non-accidental death following initiation of therapy. Categories are as follows: human CCR5 wt/Δ32 genotype with R5 HIV (plus [+] signs), human CCR5 wt/wt genotype with R5 HIV (stars [*]), human CCR5 wt/Δ32 genotype with R5/X4 HIV (circles [○]), human CCR5 wt/wt genotype with R5/X4 HIV (triangles [Δ]).
8.5 REFERENCES


CHAPTER 9

GENERAL DISCUSSION AND CONCLUSION

9.1 Introduction

Much attention has been focused on the elucidation of the role of host and viral genetic factors in the pathogenesis of HIV/AIDS. It is now appreciated that a wide variety of host genetic factors play a significant role in the natural course of HIV disease, and influence everything from the risk of infection upon exposure [1,2,3,4], the rate of disease progression once infected [5,6,7,8], and the strength and diversity of the immune response [8,9,10]. Similarly, HIV genetic diversity has also been shown to modulate viral infectivity [11], evasion of the host immune response [12,13], disease progression [14,15], and rate of T-cell death and subsequent decline of immune function [16].

The introduction of HAART in the mid-1990s revolutionized the treatment of HIV/AIDS, at least in areas of the world where access to antiretrovirals was available. Treatment with HAART resulted in reductions in plasma viral load to undetectable levels using sensitive assays [17,18,19], increases in CD4 counts, and improvement of immune function. This resulted in dramatically decreased incidence of morbidity due to AIDS-defining illnesses and opportunistic infections, and most importantly, significantly prolonged survival [20,21,22,23]. The initial success of treatment with triple combination antiretroviral therapies was such that some postulated the eradication of HIV/AIDS after only a few years of treatment [24], although it is now clear that this was an overly optimistic prediction. Ten years later, it is evident that despite the successes seen as a result of HAART, many challenges and outstanding questions remain. For example, for reasons that are poorly understood, approximately 10% of individuals fail to respond to HAART, despite
apparently good adherence and no evidence of resistance mutations [25]. Among those who initially achieve treatment success under HAART, subsequent virologic failure rates approach 20% in previously treatment-naive individuals [25] and 30-50% in previously treated individuals [25,26]. Other individuals exhibit what is known as a “discordant” response – meaning that while improvements in CD4 counts and/or immune function are achieved, virologic suppression is not achieved [27,28,29], or vice-versa [30]. Even among those individuals who maintain successful virologic and immune control of HIV under HAART, it is now clear that antiretroviral therapy is a lifelong commitment and that treatment success must be maintained. In addition, emerging challenges and toxicities associated with long-term antiretroviral therapy include lipid abnormalities [31], mitochondrial toxicities [32], cardiovascular [33], renal [34] and hepatic [35] toxicities, antiretroviral resistance [36] as well as others [37]. Given that long-term treatment success is essential, it is of importance that we achieve a greater understanding of the factors that contribute to the success of antiretroviral treatment, including genetic factors which may influence response to HAART. The identification of factors which influence antiretroviral therapy response may be of direct relevance in improvement of the lives of individuals living with HIV/AIDS. For example, if predictors of treatment failure are defined, then individuals at risk of treatment failure could be identified and interventions designed to optimize treatment. Furthermore, the identification of host and viral genetic factors that influence therapy response may lead to the development of novel, genotype-based tools for the clinical management of HIV-infected individuals.

As described earlier, the objectives of this thesis were to investigate whether previously-identified host and viral genetic factors exert an influence on the clinical course of
HIV/AIDS in the context of HAART, as well as to investigate additional genetic markers that may be of direct relevance to HAART outcomes. This chapter summarizes the major conclusions of each of the individual research papers, and discusses these conclusions in context of new studies that have been published since the undertaking of the studies presented here. This will be followed by a discussion of the broader implications of this work, as well as the potential application of these results in modern clinical practice.

9.2 Human Genetic Variation: the chemokine coreceptors

a) CCR5 Δ32

As already discussed, a 32-base pair deletion in the human CCR5 chemokine receptor gene is known to confer protection against HIV infection (in individuals homozygous for the deletion) [38,39,40] and contribute to slower HIV disease progression (in individuals heterozygous for the deletion) [39,40]. While some studies reported significantly improved short-term virologic and/or immunologic responses to HAART among CCR5wt/Δ32 individuals [41,42,43], others reported no association between CCR5 genotype and treatment outcome [44,45]. In most cases, however, these studies were limited to the evaluation of the effect of CCR5Δ32 on short-term clinical outcomes.

In order to more conclusively investigate this issue, we undertook a large, population-based study of >1000 HIV-infected antiretroviral naïve individuals initiating first HAART, and sought to evaluate what effect, if any, the CCR5Δ32 genotype exerted on response to HAART over a longer-term follow-up [46, chapter 3]. After adjustment for baseline sociodemographic and clinical factors known to affect therapy response, individuals heterozygous for the CCR5Δ32 deletion experienced significantly more rapid initial suppression of plasma HIV RNA below 400 copies/ml, however there was no association
between CCR5 genotype and the subsequent duration of viral suppression. Similarly, we observed no association between CCR5 genotype and initial immunologic response, as measured by the time to a decline of CD4 count to below baseline. In an analysis of longer-term outcomes, we observed a weak trend, although not statistically significant, to improved survival in individuals heterozygous for the CCR5Δ32 deletion, when compared to CCR5 wild-type subjects, over a 5-year follow-up period after initiating HAART [46, chapter 3].

Although a single study can by no means be conclusive, results from this large population-based analysis strongly suggest that any protective effect conferred by CCR5Δ32 alone in the context of HAART may not be clinically significant on an individual basis. The results of this analysis alone, therefore, do not support the utility of the CCR5Δ32 genotype as an independent clinical prognostic marker for HAART response. However, discussed later, additional evidence from our group as well as others suggests that the CCR5 genotype may be of useful prognostic value when taken in context with other viral genetic markers, namely the coreceptor usage of the predominant circulating virus [47,48,49,50], or when evaluated in context with other host factors [51,52].

b) \( CX_3CR1 \)

Despite the correlations observed between CCR5Δ32 and the natural course of untreated HIV disease, it was clear that CCR5 genetics could at most account for only a small proportion of the variation in HIV disease progression observed in HIV-infected populations. A number of studies identified additional genetic polymorphisms among chemokine ligand and receptor genes relevant to HIV disease, including a study by Faure et al reporting rapid progression to AIDS in individuals homozygous for an I249/M280 amino acid haplotype in the human \( CX_3CR1 \) chemokine receptor gene, when compared to
individuals with other haplotypes [53]. Since the publication of the original study [53] (as well as the publication of our research study investigating the effects of the CX3CR1 I249/M280 variation on response to HAART [54, chapter 4]), these researchers have subsequently confirmed their findings in a larger, population-based study [55], although another group, investigating the M280 polymorphism only, reported no significant correlation between CX3CR1 M280 and severity of HIV disease [56].

The specific role that CX3CR1 plays in the course of HIV disease, as well as the specific mechanisms by which the I249/M280 amino acid haplotype contributes to more rapid disease progression remain largely unknown. Although it is known that CX3CR1 functions as a minor coreceptor for HIV entry into target cells [57], it is not known whether this is a major mechanism by which CX3CR1 exerts its influence on HIV disease progression. It is more likely that the natural immune functions of CX3CR1 (which include adhesion and migration of leukocytes [58] as well as regulation of CD8+ T-cell function [59]) play a larger role in defining the role of CX3CR1 in HIV pathogenesis. It is now known that HIV infection induces a massive increase in the expression of CX3CR1 on CD4+ and CD8+ T-cells [58, 59], and indeed that increased CX3CR1 expression as a result of HIV infection is associated with declining CD4 T-cell number and increasing plasma viral load [59]. This, combined with the finding that fractalkine, the natural ligand for CX3CR1, displays significantly reduced binding affinity to peripheral blood mononuclear cells from CX3CR1 I249/M280 homozygotes when compared to cells expressing the “wild-type” V249/T280 receptor [55], suggests that the mechanism whereby the I249/M280 polymorphisms contribute to HIV pathogenesis may be due to alterations in receptor-ligand interactions and the immune consequences thereof.
Similarly, the mechanisms whereby the CX3CR1 polymorphisms may contribute to virologic and immunologic control of HIV infection during treatment with HAART also remain poorly understood. Since the publication of our study reporting a statistically significant trend to earlier immunological failure following initiation of HAART in individuals with the CX3CR1 I249 mutation [54, chapter 4], a recent study of HIV-infected children participating in randomized clinical trials of mono and dual therapy also reported a significant independent association of CX3CR1 I249 mutation with more rapid virologic and immunologic disease progression, even after adjustment for factors such as baseline CD4 count and viral load [60]. To date, the data supporting the value of CX3CR1 polymorphisms in predicting clinical prognosis and outcomes in both untreated and treated HIV infection is largely consistent and suggests that the CX3CR1 I249 polymorphism may be a useful prognostic indicator of more rapid HIV disease progression. Additional studies are needed in order to further elucidate the function of CX3CR1 in HIV pathogenesis and evaluate the potential clinical utility of determining CX3CR1 genotypes in treated and untreated HIV-infected individuals.

9.3 Pharmacokinetics: drug metabolism and the P-gp membrane efflux transporter

As outlined in detail in chapter 4, the human multidrug resistance (MDR)-1 gene encodes the ATP-dependent membrane efflux transporter P-glycoprotein (P-gp), whose substrates include HIV protease inhibitors. A single, synonymous C4345T polymorphism has been identified in the MDR-1 gene which affects membrane expression of the P-gp transporter [61], a polymorphism which presumably will have consequences for the absorption, bioavailability and metabolism of HIV protease inhibitors, as well as potential consequences for antiretroviral treatment response. The consequences of altered P-gp
expression levels to antiretroviral concentrations and bioavailability in different body compartments are complex: differential expression levels of P-gp in the gastrointestinal tract could affect the intestinal absorption and thus bioavailability of drugs (including protease inhibitors), expression of P-gp in the liver and kidney could affect the rate at which drugs are metabolized/eliminated, expression of P-gp in the epithelial cells forming the blood-brain barrier limit entry of drugs into the brain, and expression of P-gp on lymphocytes affects intracellular drug concentrations in target cells [62]. Indeed it has been shown that individuals homozygous for 3435T display significantly reduced P-gp expression and activity in the gastrointestinal tract [61], reduced P-gp function and expression in cells of the immune system [63,64], significantly reduced plasma levels of the PI nelfinavir [64], and significantly increased intracellular concentrations of nelfinavir [65], although other studies report somewhat contrasting results [66,67].

The debate as to whether the C3435T MDR-1 polymorphisms influence response to antiretroviral treatment, however, has been much more controversial. Since Fellay et al reported a significant association between the homozygous 3435T genotype and improved CD4 response to PI-containing regimens in 2002 [64], there have been a number of conflicting studies attempting to address this issue, the majority of which have failed to observe a strong correlation between MDR-1 C3435T and treatment response. Our laboratory, in a population-based study of ~450 antiretroviral naïve individuals initiating their first HAART reported a trend, although not statistically significant, to earlier virologic failure in individuals with the MDR-1 3435C/C genotype, but no correlation between MDR-1 genotype on immunologic response [54]. Since then, numerous studies have reported no correlation between MDR-1 C3435T genotype and initial virologic and/or immunologic
response to combination therapy among antiretroviral-naïve individuals [68,69,70] as well as individuals with previous antiretroviral exposure [65]. A recent study of HIV-1 infected children reported higher plasma nelfinavir levels and improved virologic responses to HAART among subjects with the MDR-1 3435C/T genotype when compared to those with the 3435C/C genotype [71]. The small number of study subjects with the 3435T/T genotype (n=7) displayed similar pharmacokinetic and virologic response profiles to those with the 3435C/C genotype [71].

These contrasting reports are perhaps not surprising given the fact that a single nucleotide polymorphism in a single human gene, however relevant, can realistically only account for a very small portion of the inter-individual differences observed pharmacokinetic profiles and treatment responses. This is especially true in the case of a polymorphism such as the MDR-1 C3435T, a synonymous substitution that does not affect the amino acid sequence of the protein and thus most likely confers a protective effect indirectly, possibly through linkage with polymorphisms at other sites. In fact, the MDR-1 C3435T polymorphism is now known to be in linkage disequilibrium with the MDR-1 G2677T substitution, among others [72], and it is suggested that an MDR-1 haplotype analysis, taking into consideration multiple polymorphisms on a single allele, may be superior than analysis of single nucleotide polymorphisms (SNPs) in predicting pharmacokinetics [72] and treatment response.

Currently, the available evidence is not sufficient to draw a firm association between MDR-1 C3435T and treatment response, and does not support the use of MDR-1 genotyping in clinical practice. As genotyping technologies continue to improve [73], however, and more understanding of the relative contributions of SNPs in MDR-1 and other genes relevant
to the pharmacokinetics of antiretroviral agents is achieved, it is possible that MDR-1 polymorphisms may in the future be interpreted in a broader genetic context.

9.4 Relevance of HIV Genetic Variation in the era of HAART

In addition to host genetic factors, this thesis sought to additionally investigate the effects of genetic variation in the HIV genome on the same parameters and outcomes. In the two decades since the complete sequencing of the HIV genome [74], major advances have been made in the understanding of the structure and function of all nine of HIV's protein-encoding genes, as well as in the understanding of the implications of viral genetic variation to the pathogenesis of HIV. This understanding was initially largely achieved through \textit{in vitro} mutagenesis experiments where HIV proteins and/or whole viruses harboring specific amino acid changes were engineered, expressed and analyzed in order to draw conclusions about the structural and functional consequences of specific genetic changes. The relevance of these \textit{in vitro} data to HIV infection and disease progression \textit{in vivo} could then be evaluated by investigating the prevalence and clinical consequences of specific genetic changes in HIV isolates derived from individuals at different disease stages. The combination of improvements in modern genotyping technologies, and the linkage of these sequence data to large datasets of clinical outcomes, now confers the capability to directly assess the \textit{in vivo} effects of specific mutations/polymorphisms on the response to antiretroviral therapy, on a population basis.

Currently there is more evidence supporting the clinical utility of incorporating HIV sequence-based assays (as opposed to human genotypic assays) into clinical practice, especially in the context of modern antiretroviral therapies. Indeed, viral genetic data are already extensively used in the clinical management of HIV-infected individuals in the form
of genotypic and phenotypic antiretroviral resistance assays, in order to guide decisions regarding treatment [36]. Given the extensive research currently focused on the clinical consequences of HIV genetic variation and sequence evolution, it is likely that novel HIV genotypic assays will continue to be incorporated into routine clinical practice. The following section will focus on summarizing the current evidence supporting (or not supporting) the relevance and potential clinical utility of additional specific HIV genetic markers in the context of HAART.

9.5 Antiretroviral resistance testing

As has been discussed previously, the most important example of viral genetic variation directly related to antiretroviral treatment is the emergence of drug-resistant HIV. Drug-resistant HIV variants are a major cause of treatment failure and remain a formidable barrier to long-term antiretroviral efficacy [36,75,76]. In fact, it is estimated that virologic failure occurs in up to 20% of previously antiretroviral naive individuals [25] and in >30% of previously treated individuals within two years of initiation of antiretroviral therapy [25,26]. In addition, a survey of antiretroviral resistance among HIV-infected individuals in the United States estimates that greater than 75% of individuals with detectable plasma viremia during antiretroviral therapy harboured HIV with resistance to one or more antiretroviral agents [77]. However, although the impact of antiretroviral resistance (and, especially, multidrug resistance) on the efficacy of HAART and/or the availability of appropriate treatment options is indeed a major concern, it is comforting to note that multidrug resistance is not yet a significant contributor to HIV-related deaths at least in British Columbia [78,chapter 6]. In our province, the majority of deaths among HIV-infected persons appear to be largely confined to cases where antiretrovirals were not accessed or unable to be consistently taken
Although these results clearly illustrate the need for programs to improve both antiretroviral access and adherence, these results also confirm the continuing efficacy of HAART.

The management of HIV is somewhat unique in the field of medicine because it represents one of the few examples where the evolving genotype of the infectious agent is routinely characterized and incorporated in clinical decision-making. In fact, genotypic resistance tests have been shown to improve virologic response to therapy in some randomized controlled trials, and are currently recommended for use in a variety of settings including acute or recent infection, prior to the initiation of therapy, in cases of virologic failure while receiving antiretroviral therapy, and during pregnancy [36]. In light of the continual development of novel antiretroviral agents, as well as increased research into the impact of HIV genetic variation on disease progression and response to therapy, there will undoubtedly continue to be a significant role for HIV drug resistance genotyping in clinical practice. In addition, it is likely that additional HIV genotyping assays, focusing on other regions of the genome [79,80], will be incorporated into routine clinical practice as well.

9.6 HIV p6\(^{\text{Gag}}\)

The first investigation of HIV genetic parameters and response to HAART undertaken as a part of this thesis work focused on proline-containing insertion mutations within HIV p6\(^{\text{Gag}}\). Peters et al. reported that insertion mutations within the p6\(^{\text{Gag}}\) proline-rich “PTAP” motif were associated with resistance to nucleoside analog reverse transcriptase inhibitors (NRTIs) [81]. Briefly, p6\(^{\text{Gag}}\) insertions appeared to occur more frequently among individuals treated with NRTIs when compared to antiretroviral naïve individuals, and when analyzed in an \textit{in vitro} system, clones generated with a PTAP duplication exhibited increased
infectivity and a competitive growth advantage in the presence of NRTIs [81]. In order to investigate whether p6\textsuperscript{Gag} insertions were indeed associated with NRTI resistance and/or poorer response to antiretroviral therapy, we retrospectively genotyped a cohort of antiretroviral-naïve individuals for HIV p6\textsuperscript{Gag} insertions and analyzed virologic and immunologic therapy outcomes in subjects with and without p6\textsuperscript{Gag} insertions [82, chapter 5]. As discussed, we observed a relatively high prevalence of p6\textsuperscript{Gag} insertions in this antiretroviral naïve population (~17%), suggesting that p6\textsuperscript{Gag} insertions may represent naturally-occurring genetic variation among circulating HIV strains in untreated populations [82]. Furthermore we did not observe any correlation between p6\textsuperscript{Gag} PTAP insertions and treatment response, nor with the presence of baseline NRTI resistance mutations [82]. Since the publication of our study [82] the observation that p6\textsuperscript{Gag} PTAP insertions most likely represent natural polymorphisms is supported by results from Gallego \textit{et al}., who observed similar rates of PTAP insertions (~15%) among drug-naïve and pretreated patients [83], as well as Ibe \textit{et al}., who reported that p6\textsuperscript{Gag} insertions “selected” under HAART were in all cases already present prior to initiation of therapy, and therefore most likely represent naturally-occurring polymorphisms which may confer some kind of replicative and/or survival advantage in the presence of antiretrovirals [84]. With respect to the potential associations between p6\textsuperscript{Gag} insertions and response to therapy, the only other evidence available to date are contrasting studies by Kaufmann \textit{et al}., who reported a trend, although not statistically significant, between the presence of p6\textsuperscript{Gag} insertions and decreased likelihood of virologic failure [85], and Lastere \textit{et al}., who reported a trend, again not statistically significant, towards earlier virologic failure among heavily experienced patients harbouring HIV p6\textsuperscript{Gag} PTAP insertions receiving amprenavir [86].
Of interest, in our study, we also observed a significant correlation between the presence of p6$^{\text{Gag}}$ insertions, and the "11/25" HIV envelope genotype [82], which, if confirmed in other independent studies, may further help clarify the relationship between HIV p6$^{\text{Gag}}$ genetic variation and HIV clinical prognosis and treatment response. To our knowledge, however, there have not yet been any additional studies investigating this correlation. To date, therefore, there is not enough evidence to conclusively evaluate the relevance of HIV p6$^{\text{Gag}}$ insertions in the context of HAART, although the available evidence suggests these insertions are not clinically significant. The implications of these insertions for HIV drug resistance, antiretroviral treatment response, and HIV envelope sequence variation merit further investigation.

9.7 HIV envelope

The HIV envelope may be the most genetically variable region in the viral genome and is known to play a significant role HIV pathogenesis, disease progression, and evasion of the immune response. In addition, the HIV envelope is largely responsible for determining HIV co-receptor usage. Briefly, the ability of the virus to use either the CCR5 or CXCR4 coreceptors lies at least partially in the amino acid sequence of the third variable ("V3") region of the HIV envelope [87,88]. In general, clonal isolates able to use the CXCR4 coreceptor display positively-charged amino acids at codons 11 and/or 25 of V3 (an "11/25" genotype [89]), while isolates able to use the CCR5 coreceptor do not [90]. Although much remains to be elucidated with respect to coreceptor evolution over the course of HIV disease, it is known that the appearance of viruses with the capacity to use the CXCR4 receptor is associated with rapid immune decline and accelerated progression to AIDS and death [14,15,16].
Despite the clear evidence indicating an important role of HIV envelope variation and/or coreceptor usage during untreated HIV infection, the relevance of HIV envelope variation and/or coreceptor usage in the HAART era has not been conclusively evaluated. As a part of this thesis, therefore, we wished to investigate whether HIV envelope variation/coreceptor usage were independent markers of response to HAART in antiretroviral naïve individuals. This section will summarize our findings and conclusions on this subject, as well as present any new data that have been published since the completion of our studies.

a) V3 loop genotyping

A large-scale investigation of the relevance of V3 sequence variation in the context of HAART was undertaken as part of this thesis [89, chapter 7]. As V3 loop genotyping is relatively rapid and low-cost compared to currently available phenotyping assays, we wished to evaluate the potential clinical utility of the simplified HIV V3 “11/25 genotype” as an independent prognostic factor for response to HAART.

An investigation of the baseline prevalence of the HIV envelope “11/25” genotype in the HOMER cohort, a large cohort of antiretroviral-naïve individuals in British Columbia initiating HAART, revealed an “11/25 genotype” prevalence of ~10% [89]. Subjects with and without the “11/25 genotype” were comparable with respect to their baseline clinical and sociodemographic characteristics. However, despite the fact that subjects with and without the “11/25 genotype” appeared to have similar baseline clinical prognoses, the “11/25 genotype” was significantly and independently associated with more rapid immunologic decline, as well as poorer survival in the five-year period following initiation of HAART. In addition, there was evidence suggesting that the “11/25 genotype” may also negatively affect virologic response. If, in the virologic outcome analyses, individuals were censored at time
of switching therapy, a significant association between the "11/25" genotype and an increased time to viral suppression was observed. In addition, a longitudinal analysis of the median pVL over the course of study follow-up also suggested that long-term virologic control was more difficult to maintain among subjects with the "11/25" genotype [89].

Implications of HIV V3 genotype in the context of HAART

The potential utility of HIV envelope genotyping in routine clinical practice has not been extensively evaluated. To our knowledge, ours is the first study investigating the implications of V3 genotype on response to HAART in previously antiretroviral-naïve individuals. Taken together with ample evidence indicating that the presence of positively-charged amino acids at key sites in the HIV V3 loop are predictive of HIV disease progression in untreated infection [88,90], this study strongly suggests that HIV envelope genotype has the potential to provide useful clinical information, in addition to plasma viral load and CD4 count, to help guide therapy-related decisions. Based on results showing poorer immunologic response to treatment, more therapy switches and poorer survival among individuals with “11/25” HIV, it may be argued that the “11/25 genotype” could be used as an indicator to initiate HAART at the higher end of currently recommended guidelines [76]. Alternatively, or in addition, the presence of “11/25” HIV could translate into a decision to choose a more potent initial treatment regimen, or the recommendation to monitor patients’ responses to treatment more closely.

Further evidence for the potential application of HIV V3 loop genotyping in the modern treatment era is demonstrated by a recent study by Toulson et al [91] investigating the effect of various clinical parameters on the success and length of treatment interruptions among individuals with nadir CD4 counts >200 cells/mm³. In this study, the presence of
"11/25" HIV, along with other clinical markers, was significantly and independently predictive of a rapid CD4 decline during the treatment interruption, and the need to re-initiate HAART earlier when compared to the "non-11/25" genotype [91]. Results from this study indicate that V3 genotyping might be useful as an independent prognostic marker in a variety of settings relating to antiretroviral treatment.

b) HIV coreceptor phenotyping

In a related study, we also sought to evaluate whether baseline HIV coreceptor phenotype may be a useful prognostic clinical marker for therapy response in the current era of HAART [47, chapter 8]. In addition, a baseline cross-sectional characterization of the prevalence and clinical correlates of CCR5- and CXCR4-using HIV in this cohort would be of relevance in the context of HIV coreceptor inhibitors, a new class of antiretroviral agents currently in development.

A cross-sectional analysis of the baseline HIV coreceptor usage in the HOMER cohort revealed that approximately 20% of subjects harboured X4 variants at study entry, most (with the exception of one individual with exclusively X4 HIV) as a mixture of either R5 and X4 variants and/or dual-tropic variants [47]. The presence of X4-using HIV at baseline was significantly correlated with poorer clinical prognosis in this cohort: subjects harboring X4 HIV had significantly lower CD4 counts, higher viral loads and higher incidences of AIDS-defining illnesses than individuals with exclusively R5 variants [47]. These findings are consistent with results of other recent cross-sectional analyses attempting to identify clinical correlates of X4 HIV [92,93], and are also consistent with the large body of historical research linking the presence of X4/syncytium-inducing viral variants to accelerated disease progression during untreated infection [14,15,16].
Perhaps surprisingly, however, coreceptor phenotype appeared to be less useful as an independent prognostic marker than V3 genotype in the context of HAART, mainly due to the very strong correlations between baseline coreceptor phenotype and the other major clinical markers of HIV disease progression [47]. In univariate analyses, the presence of X4-using HIV prior to the initiation of therapy was indeed correlated with reduced survival following initiation of HAART, but this association did not remain significant after adjusting for other baseline clinical parameters, most notably CD4 count. These data indicate that coreceptor usage may not provide useful additional prognostic information for HAART response above and beyond the routine clinical markers already used. Results, therefore, do not support the incorporation of HIV co-receptor phenotype into clinical practice at this point in time, at least in the context of currently-available HAART regimens.

9.8 Unresolved research questions surrounding HIV envelope genotyping/phenotyping and evidence for potential future clinical utility of coreceptor assays

a) Evolution of coreceptor phenotypes during treatment with HAART

The potential evolution of coreceptor usage and/or envelope sequence under antiretroviral therapy, as well as the relevance of this evolution to the success of HAART remains to be conclusively determined. To date, this issue has been investigated in a number of longitudinal studies, most of which have focused on a small number of subjects as the cost of HIV coreceptor phenotyping remains quite high. The majority of studies to date report that antiretroviral therapy suppresses CXCR4-using strains and/or induces a shift to predominantly R5 strains in individuals with X4 or mixed R5/X4 strains at baseline [94,95,96], although one study reports that R5 outgrowth may be only a temporary phenomenon [97]. In contrast, other studies report no effect of HAART on co-receptor
phenotype [98]. Yet others report the emergence of HIV envelope sequences with the
"11/25" genotype under effective antiretroviral therapy, in subjects that predominantly
harbored "non-11/25" HIV envelope genotypes at baseline [99].

Although we have not yet undertaken a longitudinal analysis of HIV envelope
variation and/or coreceptor phenotype evolution during antiretroviral therapy, we believe that
our studies investigating HIV envelope genotypes and coreceptor phenotypes as baseline
prognostic variables [47,89] contribute to the debate on this issue. Further characterization of
the evolution of coreceptor usage under antiretroviral therapy, especially among larger
cohorts such as HOMER, will help evaluate the clinical relevance and potential clinical
utility of HIV coreceptor phenotyping in the context of modern HAART.

b) HIV envelope genotype/coreceptor phenotype correlations

As already described, the ability of the virus to use either the CCR5 or CXCR4
coreceptors lies at least partially in the amino acid sequence of the envelope V3 loop; X4
variants generally display positively charged amino acids at codons 11 and/or 25 (an "11/25"
genotype), while R5 variants do not [88,90]. The "11/25" rule, however, is by no means a
perfect predictor of coreceptor usage: previous studies of cloned isolates report sensitivities
and specificities of ~50% and ~85-95%, respectively, for the ability of the "11/25" genotype
to predict CXCR4 usage [100,101,102], while studies employing direct ('bulk') PCR and
sequencing approaches report even lower sensitivities and specificities (33% and 93%) [47].
Despite this, in our study the presence of "11/25" HIV was associated with a >9-fold
increased risk of harboring X4-using virus [47]. Although there exist other bioinformatics-
based methods to predict coreceptor usage from V3 genotype data (for example, [101,102]),
these do not appear to exceed the “11/25” rule with respect to accuracy of coreceptor prediction, at least in our data set of matched genotypes and phenotypes (unpublished data).

Given the observation that the “11/25” rule is a relatively poor predictor of coreceptor usage, but nevertheless a very strong marker of clinical response to HAART [47], many questions remain outstanding. What, indeed, is the significance of the presence of positively-charged amino acids at key sites of V3, and why are the presence of these amino acids independently predictive of therapy outcomes? One potential explanation may lie in the continual sequence changes occurring in the HIV envelope sequence during the evolution towards CXCR4 usage discussed in chapter 7: over the course of HIV disease progression “there is likely a continuum of HIV genetic sequences representing evolution to use the CXCR4 co-receptor with increasing efficiency” [89]. The 11/25 rule, in other words, may only be representative of CXCR4-using strains at a certain stage of evolution - specifically, where the continuum of V3 genotype evolution temporarily passes through a stage where positively-charged amino acids predominate at key sites. Therefore, one may hypothesize that perhaps in the early-to-middle stages of CXCR4 evolution, the “11/25 rule” may display increased capability to predict coreceptor usage, whereas in the very last stages of CXCR4 evolution (ie at very low CD4 counts) this rule may no longer be relevant.

Two observations from our research support this hypothesis. First, there were approximately double the number of HOMER cohort participants harboring X4-using variants at baseline [47], than subjects harboring “11/25” genotype HIV at baseline [89], suggesting that the coreceptor phenotype assay was capturing an additional subset of the population. Secondly, X4-using HIV was significantly associated with poorer baseline clinical parameters (including very low CD4 counts in some cases) [47], whereas “11/25
genotype" HIV was not [89], suggesting that the phenotyping assay may have been additionally capturing the subset of the population at the most advanced stage of CXCR4-coreceptor usage, the stage in which the “11/25” genotype may no longer apply. Indeed, the inclusion of this “low baseline-CD4” subset among the CXCR4 phenotype group would explain why, after multivariate analyses controlling for baseline clinical parameters, the presence of CXCR4-using HIV was no longer predictive of survival. The “11/25 genotype” therefore perhaps remains independently predictive of therapy outcomes because these genotypes may represent early evolution towards CXCR4 usage, before other markers of disease prognosis (ie CD4 and pVL) have been affected. Further studies analysing genotype/phenotype correlations, as well as combined effects of genotypes and phenotype on therapy outcomes are currently ongoing.

c) Evidence for adaptation of HIV to human genotypes: the CCR5 mutation and HIV coreceptor usage

The availability of both HIV coreceptor usage and human CCR5Δ32 genotypes for the same patient population (again, the HOMER cohort) provided the opportunity to observe the potential effects of human genetic variation on the course of HIV evolution, and to investigate the consequences that this may have with respect to HIV disease. As outlined in chapter 8 [47], we were interested in whether reduced cell-surface expression of CCR5, (and therefore, by inference, the CCR5wt/Δ32 heterozygous genotype), may potentially drive evolution towards use of CXCR4.

Indeed, our baseline cross-sectional data indicated that CCR5wt/Δ32 individuals were nearly twice as likely to harbor X4-using HIV than CCR5 wt/wt individuals, suggesting that reduced cell-surface CCR5 expression might indeed select for X4-using variants, at least in
some individuals [47], although we do acknowledge the potential biases in these observations [47]. There is also supporting evidence from other independent studies that report a loss of protection by the CCR5 Δ32 allele in cases where X4 variants are present [103,104,105,106]. Indeed, in our study, CXCR4-using HIV appeared to be associated with poor prognosis independent of CCR5 genotype [47].

The association between HIV coreceptor phenotype and human CCR5Δ32 genotype, and the consequences of this association, may help explain some of the contrasting results obtained in various studies of the independent impact of CCR5Δ32 on HIV disease progression and/or treatment response, where corresponding HIV coreceptor phenotype was not evaluated. If the Δ32 allele confers a significant protective effect only in the presence of CCR5-using strains, then CCR5Δ32 genotypes, if ever incorporated into clinical practice, should ideally be evaluated in combination with a current HIV coreceptor phenotype. Finally, the observation that limited cell-surface CCR5 may lead to selective pressure favoring the emergence of CXCR4-using variants may be relevant for the use of CCR5 coreceptor antagonists in the treatment of HIV-infected persons. However, the full implications of this have yet to be assessed.

d) Use of HIV coreceptor phenotyping in the context of future antiretroviral agents

The epidemiology of HIV coreceptor usage in both treated and untreated populations is of direct relevance in the context of future antiretroviral therapies, including coreceptor antagonists, agents aimed at inhibiting HIV entry into target cells through blockade of the CCR5 and/or CXCR4 coreceptors. On a population basis, studies investigating the prevalence of CCR5 and CXCR4-using strains among HIV-infected populations will assist in
estimating the proportion of patients that may benefit most from treatment with CCR5 antagonists, CXCR4 antagonists, or both.

HIV coreceptor phenotyping may soon be recommended on an individual basis, in order to determine whether a patient should receive antiretroviral therapy including a CCR5 antagonist. In a recent study, Moyle et al suggest that, due to concerns regarding the clinical implications of administering CCR5-antagonists to patients with mixed R5/X4 populations, patients may have to be screened for HIV coreceptor phenotype prior to treatment with a coreceptor antagonist. Because phenotyping remains a relatively time-consuming and costly process, it would be ideal if, through epidemiological characterizations of HIV coreceptor usage such as the one undertaken as a part of this thesis work, reliable predictive markers of co-receptor usage could be established. However, although the risk of harboring X4 variants indeed increases with declining CD4 count and increasing viral load, a high CD4 count and low viral load do not necessarily exclude the presence of X4 variants. Based on the available data, therefore, these laboratory markers cannot be relied upon to accurately predict HIV coreceptor usage in the clinical setting. It is likely, therefore, that HIV coreceptor usage assays may shortly be adapted for use in the clinical setting, in the context of HIV coreceptor antagonists as antiretrovirals.

9.9 Implications and Future Directions

It is slowly being appreciated that the impact of human genetic variation on HIV pathogenesis (or indeed, the pathogenesis of any disease, whether acquired or inherited) is a complex phenomenon. To date, the majority of immunogenetic and pharmacogenetic studies of the influence of human genetic variation on HIV disease progression, including the ones highlighted in this thesis work, have focused on single nucleotide polymorphisms, or at the
most, combinations of polymorphisms at a small number of sites within a single gene. Although simple genetic markers of HIV infection and disease progression have indeed been identified (as evidenced by the discovery of the CCR5 Δ32 deletion allele), it is more likely that the course of HIV disease reflects a multitude of interacting genotypes and phenotypes reflecting variation within genes scattered throughout the whole human genome [51, 107, 108]. Improvements in molecular typing technologies, computer processing and bioinformatics, as well as an increased understanding of the results of the human genome project [109] will result in increasing potential to generate, store and manipulate large sets of DNA sequence data, as well to assess the impact of multiple interacting combinations of genotypes on clinical outcomes. In the future, therefore, not only will we most likely observe the routine use of human genetic screening assays in the clinical management of infectious disease, including HIV, but we will most likely also witness the expansion of genetic testing from single nucleotide polymorphisms to complete genetic haplotypes in order to provide a more inclusive assessment of the impact of human genetics on clinical disease prognosis.

Given the extensive research focused on the clinical consequences of viral genetic variation and sequence evolution in the context of both untreated and treated infection, as well as the fact that HIV genotypic and phenotypic assays are already routinely used in the clinical management of infected individuals, it is certain that novel HIV genotypic and phenotypic assays will continue to be incorporated into clinical practice. Based on the work included in this thesis, as well as work by others, there is strong evidence to support the incorporation of HIV envelope V3 genotyping (along with already-established markers CD4 and plasma viral load) for use in helping guide decisions as to when to initiate therapy and/or
whether a therapy interruption may be safely recommended. Current data also supports a potential role for HIV coreceptor phenotyping as a clinical management tool, in the context of emerging antiretroviral therapies.

9.10 Concluding Remarks

In conclusion, the future of HIV research will undoubtedly continue to focus on the role of human and viral genetic variation on HIV disease progression as well as response to current and upcoming novel antiretroviral agents. And as the relationship between host and viral genetics continues to be explored and understood, and genetic markers relevant to the progress of HIV disease continue to be identified, it is likely that genotyping assays will adopt an ever-increasing role in the management of HIV infection.
9.11 REFERENCES


APPENDIX

Certificates of Ethical Approval: Original, Renewals and Updates