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Publication Date

2013

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Association of Changes in Viral Fitness and Genotype at Position
184 in Reverse Transcriptase with Viral Load and CD4 Count in
Persons Recently Infected with Drug-resistant Human
Immunodeficiency Virus Type I

by

Thomas P. Young III, MS, NP, PhD(c)

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Nursing

in the

GRADUATE DIVISION

Acknowledgements

In 2005, at a motivational speaker program, the speaker discussed one's "vocation" versus one's "mission" in life and challenged the audience to ask ourselves what our work really meant to us. I have committed 30 years to nursing care for persons living with HIV. It was shortly after hearing this talk that I decided to pursue doctoral studies as a natural extension of my own mission in life.

My doctoral studies have developed and advanced my skills in research theory and methods. Coupled with over 25 years of practical experience, this has improved my ability pursue not just "good science but relevant science." As I observe the evolution of evidenced-based research in treating disease, I have an increasing appreciation for the impact of basic science research in drug development, diagnostic advances, and disease management. I have witnessed how these advances impact and improve patient care outcomes. As the result of my work in providing education to nurses, nurse practitioners, physicians, physician assistants, pharmacists, health educators, social workers, and patients, I have developed a keen interest in making science "translational and trans-disciplinary."

It has been through this work that I've established a network of remarkable colleagues and mentors. I owe a debt of gratitude to the generosity shared by these doctors, nurses, researchers, professors, and other professionals, as well as to the many kind friends and my family for having helped me develop my professional and academic career. I could never begin to list everyone here and if I've excluded anyone it in no way reflects my lack of appreciation for our work together or the support they have shared with me. I'd like to acknowledge the following people who have played a significant role in my achievements and career:

Colleagues and Mentors

Holly Batterman, MD

Yvette Cuca, PhD

Guy de la Rosa, MD

Milton Estes, MD

Michael Fath, PhD

Jeffrey Fessel, MD

Signe Fransen, MS

Stephen Follansbee, MD

Brian Goodroad, RN, DNP

John Hackett Jr., PhD

W. David Hardy, MD

Mark Holodniy, MD

John Miller, RN, MS

J.B. Molaghan, NP

Ellen Paxinos, PhD

Christos Petropoulos, PhD

Clarissa Ramstead, MS, NP

Juergen Rockstroh, MD, PhD

Stephan Rowniak, NP, PhD

John Stansell, MD

S. Stringari-Murray, MS, NP

Roger Trinh, MD

Ming Li Tsang, MD

Jackie Tulsy, MD

Friends and Family

Tama Adelman

Steve Abedon

Kristin and Tim Bennett

Graham A. Bigelow

Susan Campbell

Keith Cunningham

Carmen Dett

Lee Dixon

Dick and Pauline Kaku

Annette and Will Kelty

Julia and Paul Khait

Debra Golden

LaRee and Matt Maguire

Elizabeth McDermott

Paul McDermott

Nicole Mezo

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The Options Project Team

To my dissertation committee members who have truly mentored me through the research process, a special note of thanks. Their multidisciplinary expertise, standard of academic excellence, and extraordinary generosity have been exceptional and I will be forever grateful. I hope to one day follow their example and share with others in the same way they have with me.

I also owe an unconventional note of appreciation for my three fantastic pets, Patrick, Casey, and Sean, who remind me on a daily basis of life's humanity and to take time for play! To Mark S Karmendy, the timing of our meeting is indescribably remarkable. Your intellect and wisdom are inspiring. You've been generous in time and spirit and I honestly can't imagine making it to the finish line without the support and love you have shared. I'll be forever grateful. Above all, I owe a debt of gratitude to the many patients who continue to share their amazing life stories with me and allow me the privilege to care for them.

Abstract

As the human immunodeficiency virus (HIV-1) epidemic enters its fourth decade, we continue to advance our understanding of viral transmission, pathogenesis, epidemiology, prevention, and antiretroviral (ARV) treatment. Although this expanding knowledge has helped us manage and decrease morbidity and mortality for persons living with HIV-1 infection, patients on therapy must deal with long-term challenges and complications of therapy. One of these complications is the development of drug resistance. The presence of HIV-1 drug resistance can affect a person's response to treatment and lead to therapy failure. Once resistance-associated mutations are acquired it is permanently integrated into the host's viral genome and its clinical impact will wax and wane depending on selective drug pressure that is present. This is why HIV drug resistance is often considered to be an irreversible complication of ARV therapy.

The first ARV regimen provides the best chance of success in achieving HIV-1 RNA suppression and immune recovery. Historically, each subsequent regimen has had a decreasing chance of success because these regimens possessed challenges with regard to patient tolerability and adherence. The introduction of more potent therapies, improved tolerability profiles, and less toxic regimens have increased the chance of success with second- and third-line regimens. This however does not negate the impact that drug resistance has on a patient's chance for successful therapy. Failing a regimen is not inconsequential and if the underlying reasons for failure are not addressed, subsequent regimen failures may leave patients with limited treatment options. In order to maximize ARV therapy response, treatment guidelines recommend utilization of HIV drug resistance testing prior to initiation for treatment-naïve patients, for patients failing ARV therapy, and for women prior to starting prenatal treatment regimens. Developing expertise in how to interpret resistance test results is crucial in a provider's repertoire of clinical management skills. As resistance testing technologies evolve, it is increasingly critical to stay abreast of advances in the science and translational application of these assays into clinical practice.

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List of Acronyms

3TC: lamivudine	HS: hypersusceptibility
AIDS: autoimmune deficiency disorder syndrome	IAS: International AIDS Society
ARI: AIDS Research Institute	INI: integrase strand transfer inhibitor
ARMS: allele-specific primers	LLV: low level viremia
ARV: antiretroviral	MUT: mutant
ART: antiretroviral therapy	NRTI: nucleoside reverse transcriptase inhibitor
ASPCR: allele-specific polymerase chain reaction	NNRTI: nonnucleoside reverse transcriptase inhibitor
CDC: Center for Disease Control	PBMC: peripheral blood mononuclear cells
CLIA: C laboratory	PCR: polymerase chain reaction
CLPI: combined amplification and sequencing reaction	PI: protease inhibitor
DHHS: Department of Health and Human Services	<i>pr</i>: protease
DNA: deoxyribonucleic acid	PT: phenotype
EIA: enzyme-linked immunosorbent assay	qMVA: quantitative minor variant assay
GT: genotype	RAM: resistance-associated mutation
EI: entry inhibitor	RC: replication capacity
FDA: Food and Drug Administration	RNA: ribonucleic acid
FTC: emtricitabine	<i>rt</i>: reverse transcriptase
HCSUS: HIV Cost and Services Utilization Study	SNP: single nucleotide polymorphism
HIV-1: human immunodeficiency virus type-1	SOP: standard operating procedure
HOPS: HIV Outpatient Study	US: United States
	UDS: ultra-deep sequencing
	VL: viral load
	WT: wild-type

I. The Study Problem

a. Introduction to the Problem

As the human immunodeficiency virus (HIV-1) epidemic enters its fourth decade, we continue to advance our understanding of viral transmission, pathogenesis, epidemiology, prevention, and antiretroviral (ARV) treatment. To date, 34 ARV drugs (27 individual agents and 7 fixed dose combinations) have been approved by the FDA (Food and Drug Administration) for the treatment of HIV-1 infection. Additionally, one drug combination tablet is now approved for use in pre-exposure prophylaxis of HIV-1 infection (Centers for Disease & Prevention, 2012; Hankins & Dybul, 2012). The United States (US) Department of Health and Human Services (DHHS) Guidelines state that with use of these drugs in first-line treatment, patients can expect a 70%-80% virologic response rate, defined as achieving a viral load (VL) below the limits of detection (less than 50 copies/ml) at 48 weeks (DHHS, 2013). The first ARV regimen provides the best chance of success in achieving viral-load suppression and immune recovery.

Although these advances have decreased morbidity and mortality for persons living with HIV-1 infection, patients on therapy must deal with long-term challenges and complications of therapy (Palella et al., 2009; Palella et al., 1998). One of these complications is the development of drug resistance. There are several factors that can affect the evolution of resistance; these include the genetic barrier of the ARV regimen, viral fitness and replication capacity, mutation background (both primary and secondary mutations effects), viral load, and other host genetic factors that are often unmeasured in routine clinical care (Liovat et al., 2012; R. Wang, Bosch, Benson, & Lederman, 2012). Mutations in viral target genes conferring drug resistance can affect the response to treatment and lead to virologic, immunologic, and clinical failure. Once resistant viruses have been selected, their proviral DNA becomes permanently integrated into the host cellular genome and can serve as a reservoir for outgrowth of resistant virus as a result of changes in drug selective pressure (Parisi et al., 2006; Shafer, Kantor, &

Gonzales, 2000; Van de Perre, 2006). For this reason HIV drug resistance is often considered to be an irreversible complication of ARV therapy that affects the patient as well as anyone to whom they transmit the resistant virus.

b. Statement of the Problem

Most resistance-associated mutations (RAMs) impart some level of decreased viral fitness, which is defined as the ability of a virus to replicate in a given environment. Pathogenicity and virulence are defined as the ability of an organism to cause disease or induce harm to its host (Barbour & Grant, 2005; Cong, Heneine, & Garcia-Lerma, 2007; Skowron et al., 2009). While measurement of viral fitness is complex and time-consuming, one component of viral fitness is replication capacity. Replication capacity (RC) is defined as the ability of the virus to replicate under ideal conditions in vitro, under no selective drug pressure, and without host immune factors (Barbour & Grant, 2004, 2005; Van de Perre, 2006). RC can be measured as a component of other resistance testing assays and is considered a surrogate marker of viral fitness (Bates, Wrin, Huang, Petropoulos, & Hellmann, 2003; Quinones-Mateu et al., 2000). Therefore, in some clinical situations, replication capacity can be followed over time with drug resistance as a marker of virological and immunological response to treatment (Daar et al., 2005; De Luca et al., 2007; Goetz et al., 2010; Grant RM, 2001). For the purposes of this work, the term replication capacity (RC) will be used synonymously with viral fitness.

The degree by which fitness is affected is partially influenced by specific drug resistance-associated mutations and the accumulation of mutations. It is also a relative measure and subject to variations based on when it is measured in relation time of infection and treatment. It is also measured against a wild-type reference strain in vitro (Wrin, 2001). Some mutations have a greater impact than others on fitness and the clinical implications of such mutations may influence how ARVs are selected and utilized (L. Chen & Lee, 2006; Gotte, 2012; Paredes et al., 2009).

Fitness mutations do occur across all classes of ARV drugs as in the case of the protease inhibitor, Nelfinavir, and the D30N mutation, and for the K65R mutation associated with the nucleotide inhibitor tenofovir (Viread) (Deval et al., 2004; Martinez-Picado, Savara, Sutton, & D'Aquila, 1999). There are also data suggesting that fusion inhibitor resistance-associated mutations for enfuvirtide (Fuzeon) can cause reductions in fitness (Lu, Sista, Giguél, Greenberg, & Kuritzkes, 2004). Persistence of these fitness-impairing mutations depends on continued selective pressure of the respective ARV agents as well as effects of several other factors. These may include host cellular response to infection, adaptive and innate immune response, co-infections, HIV-1 viral load and other dynamics yet undefined (McMichael & Rowland-Jones, 2001; Quinones-Mateu et al., 2000; Quinones-Mateu, Moore-Dudley, Jegede, Weber, & E, 2008). Accumulation of additional mutations will eventually compensate for losses and gains in RC changes (Barbour et al., 2002; Nijhuis et al., 1999). For example, the M184V mutation usually persists between 4-24 weeks following interruption of therapy or upon initial detection in newly infected persons with HIV-1. The K103N mutation can persist for up to 3 years after discontinuation of therapy or after initial detection in recent infection (Jain et al., 2011; Li et al., 2011; Little et al., 2008; Wainberg, Moisi, Oliveira, Toni, & Brenner, 2011).

Detection of these mutations would likely have an impact on the selection of ARV therapy or the decision to continue therapy in a failing regimen. For this reason assay sensitivity may have an important role in detecting these mutations at the time of HIV diagnosis or at therapy failure. In the case of the newly infected person, the start of ARV therapy may not coincide with seroconversion or the person's initial resistance test (Buckton, Harris, Pillay, & Cane, 2011; Jakobsen et al., 2010; Kuritzkes et al., 2008; Li & Kuritzkes, 2013). Additionally, host immune responses such as CD4+ t-cell impact, HLA (human leukocyte antigen) phenotypes, and other adaptive immune interactions may play a role in whether replication capacity kinetics have a contributing effect on clinical response to therapy or discontinuation of therapy (Bonhoeffer, Coffin, & Nowak, 1997; Miura et al., 2010).

Viral fitness and replication capacity have been pivotal points of discussion in the pathogenesis of HIV, drug resistance–associated mutations for fitness, and the impact these mutations have on host immune response, in acute and chronic infection. In understanding a key mutation affecting fitness, the M184V is important. The transmission of this mutation and its impact on a person’s immune system both have clinical implications when managing care along the continuum of HIV disease (Bonhoeffer et al., 1997; Lyles et al., 2000; Miura et al., 2010; Quinones-Mateu et al., 2008; Trkola et al., 2003). Understanding the technologies of resistance testing is one of the first steps in a clinician’s path to gaining knowledge that is critical to delivering expert care to persons infected with HIV-1.

The two primary methods used in clinical practice for determination of resistance to ARV drugs are genotyping (GT) and phenotyping (PT). Genotype tests detect resistance-associated mutations in target genes of HIV-1, specifically *pol* (Clavel & Hance, 2004; Grant et al., 2003; Kuritzkes, Grant, et al., 2003; Shafer, 2002). Phenotype tests directly measure the susceptibility of recombinant viruses to varying concentrations of ARV drugs in cell culture (Hertogs et al., 1998; Petropoulos et al., 2000). In addition, as noted previously, PT assays can also be used to measure replication capacity as a surrogate marker for viral fitness (Barbour, Hecht, Wrin, Segal, et al., 2004; Barbour et al., 2002).

The clinical utility of genotype and phenotype assays have been debated and compared in clinical trials (Wegner et al., 2004; Zolopa, 2003; Zolopa et al., 2005). Both resistance tests are complex and require a degree of expert interpretation. In addition, both are relatively insensitive to the presence of low-abundance resistant virus populations, since they use population (bulk) polymerase chain reaction (PCR) products amplified from the target viral genes (Alcorn & Faruki, 2000; D’Aquila, 2000; Shafer, 2002; Tang & Shafer, 2012). Standard sequencing assays that use bulk PCR products are usually able to detect mutations represented at levels between 15%-20% in the total viral population and can vary by assay and

mutation (Moser et al., 2005; Schuurman, Brambilla, de Groot, Huang, Land, Bremer, Benders, & Boucher, 2002; Schuurman et al., 1999).

In recent years, assays with increased sensitivity have become available and can detect mutations at levels well below that of standard population sequencing genotyping. For example, allele-specific PCR (ASPCR) assays are able to measure proportions of low-abundance variants to levels below 1% (Buckton, Harris, et al., 2011; Church et al., 2008; Li & Kuritzkes, 2013; Paredes, Marconi, Campbell, & Kuritzkes, 2007). ASPCR uses PCR target amplification with homogeneous real-time fluorescent detection for the discrimination of known single-base polymorphisms. This approach offers a higher degree of sensitivity and reproducibility enabling the quantification of resistant virus present at very low levels with sensitivities in the range of 0.003%-0.1%.

ASPCR sensitivity can be further increased by using “nested PCR,” whereby highly conserved non-discriminatory primers are used to pre-amplify a larger region of interest, and ASPCR primers are then used to further interrogate very specific, short regions of this enriched sample to identify and quantify single nucleotide changes of interest (Buckton, Harris, et al., 2011; Church et al., 2008; Li & Kuritzkes, 2013; Li et al., 2011; Newton et al., 1989; Paredes et al., 2007; Walker & Rapley, 2005). It is this short length of amplified nucleic acids and the fact that ASPCR interrogates a single nucleotide position that does not normally allow for the assessment of linkage between mutations. Furthermore, some amino acid substitutions can be encoded by more than one codon, which requires that the ASPCR primers be designed to detect all possible codons, e.g., AAC or AAT for 103N, further increasing the already labor-intensiveness and cost requirements of this assay (Ugozzoli & Wallace, 1992).

Sensitive assays such as ASPCR and qMVA, which is defined as a type of assay that uses ASPCR, have not yet been validated for patient management, and are available for research use only. Additionally, deep sequencing technologies are novel approaches being used to detect and analyze minor variants (Shafer, 2009). It is possible that these new assays

and the next generation of sequencing technologies will offer an improvement in guidance regarding initial ARV treatment selection and clinical outcome, compared to standard sequencing assays. However, this will require establishing clinically relevant cut-offs, which can be influenced by several factors including drug selection, background viral fitness, RC, and other host immune factors.

Research studies designed to address this question have reported conflicting or inconsistent results, especially regarding the proportion of resistant virus that is predictive of treatment failure (Jain et al., 2011; J. A. Johnson et al., 2008; Kuritzkes et al., 2008; Paredes et al., 2010). One reason for these inconsistencies may be related to uncertainty about the optimal output measurement of low-abundance resistant variant assays, as described by Goodman and colleagues (2011). These authors reported a stronger association between clinical outcome and absolute viral load of the resistant subpopulation, compared to the relative percentage of resistant mutants in the population. It remains a consistent finding that there is a lack of validated minor variant assays available for patient management. This will continue to limit a clinician's ability to test patients for drug resistance at low levels, of which there is still an undefined consensus on clinical impact (Gianella & Richman, 2010; Goodman et al., 2011; J. A. Johnson et al., 2008; Li & Kuritzkes, 2013; Shafer, 2009; Simen et al., 2009).

c. Purpose of the Study

The purpose of this study is to better understand the relative usefulness of various assays that measure drug resistance and to provide insight into clinically relevant cut-offs for detection of drug resistant variants. The specific aims are (a) to review the literature on drug resistance and its evolution into clinical practice; (b) to review the current and evolving technologies for HIV drug resistance testing with a focus on clinical utility of these assays; and (c) to examine the association of changes in viral fitness and genotype at position 184 in reverse transcriptase with viral load and CD4 count in persons recently infected with drug-resistant human immunodeficiency virus type 1. Having a greater understanding of the most

useful assays and appropriate clinical cut-offs can help clinicians determine how best to initiate ARV therapy in recently infected persons with HIV-1, thereby improving long-term health outcomes.

d. Need for the Study

This study explores utilizing a sensitive ASPCR quantitative minor variant assay (qMVA) assay that can target a specific mutation that confers resistance to particular drugs. In this study, the M184V mutation is the focal mutation of exploration. It is the signature mutation for the NRTIs lamivudine (3TC) and emtricitabine (FTC), and is important because both drugs are major components of initial preferred regimens on the DHHS, International AIDS Society (IAS), and World Health Organization (WHO) treatment guidelines for persons initiating ARV therapy (DHHS, 2013; Hirnschall, Harries, Easterbrook, Doherty, & Ball, 2013; Thompson et al., 2012). Additionally, the M184V mutation is one of the most frequently observed resistance-associated mutations in viruses present following failure of 3TC- or FTC-containing regimens (Charpentier et al., 2013; Gallego et al., 2001; Maguire et al., 2000; Marcelin et al., 2012; Miller et al., 2012), and it is prevalent in viruses from patients with transmitted drug resistance (Bennett et al., 2009; Boden et al., 1999; Little et al., 2002; Pillay, 2004; Ross et al., 2007; Wheeler et al., 2010). The clinical impact of the M184V mutation is associated with significant reduction in viral fitness and may be partially responsible for preservation of immunologic function even after virologic failure (Daar et al., 2005; Deeks et al., 2001; Nijhuis, Deeks, & Boucher, 2001; Vaidya et al., 2010).

Considering its impact on viral fitness, it is not surprising that M184V has often been observed to be one of the first mutations reverting to wild-type following transmission of 3TC/FTC-resistant virus in untreated, newly infected patients (Frost, Nijhuis, Schuurman, Boucher, & Brown, 2000; Jain et al., 2011; Little et al., 2008; Wainberg, Hsu, Gu, Borkow, & Parniak, 1996; Wainberg et al., 2011). Finally, given the significance of this mutation in patients failing ARV therapy and those recently infected with transmitted drug resistance, understanding the evolution and reversion kinetics of this mutation may help to expand the clinician's

understanding of how to treat newly infected persons with HIV-1 infection and the M184V mutation.

Previous studies utilizing population sequencing to assess the impact of transmitted drug resistant variants in newly infected persons with HIV-1 infection may underestimate the impact that these mutations may have on initial response to therapy. Additionally, although TDR mutation can wane below the detection threshold of population sequencing assays over time and vary depending on what mutations are being measured, the M184V mutation can be relatively rapid in its reversion. Further studies are needed to investigate what other host immune factors might be influencing the kinetics of such rapid reversion including host and viral fitness factors. Determining whether minor variant assays will have clinically diagnostic relevance in early infection can add to the foundation of knowledge as it relates to utilizing new assays in transmitted drug resistance (Jain et al., 2011; Little et al., 2008).

II. Literature Review and Conceptual Framework

a. Overview of Relevant Research Directly Related to the Problem

In order to examine the gaps in our knowledge about ARV resistance, an in-depth literature review was conducted. This review of HIV-1 drug resistance in the United States focused on the history and extent of the problem, technologies for identifying resistance, and the clinical utility of drug resistance testing. A PubMed query utilizing the search terms “HIV drug resistance”, “HIV drug resistance testing technologies,” and “HIV drug resistance epidemiology” resulted in 14, 283; and 2,793 citations, respectively. Combined, these search terms totaled 17,218 citations. Advanced search limits for “HIV drug resistance” included English language only, clinical and research focused HIV/AIDS related journals, and types of articles including clinical trials, editorial, letters, meta-analysis, practice guidelines, randomized controlled trials, and review articles that provided a current and critical evaluation of a current topic in HIV drug resistance and/or study critique. Application of these limits narrowed the number of citations to 5,321. Selection of the literature was based on the evolution of knowledge over time regarding their contribution to treatment guidelines; research published by key opinion leaders and major research institutions in the field; relevance to the current topic of HIV drug resistance; unanswered questions related to drug resistance technologies; and utility and application of drug resistance testing in clinical practice. The temporal relatedness of data and studies was also considered so that an overview of shifts in treatment paradigms could be incorporated into the review. Two hundred and thirty eight citations met these criteria and included three sets of clinical guidelines (US Department of Health and Human Services, International AIDS Society—USA, and World Health Organization) and reference text books. The distribution of literature selected for review is as follows: 51% of papers were published between 2007 and 2013, 32% between 2002 and 2006, and 17% between 1989 and 2001. The literature review focuses on the following three significant topical areas: (1) epidemiology, (2) drug resistance technologies, and (3) clinical utility of resistance testing.

i. Epidemiology of HIV Drug Resistance

Epidemiology surveillance reports in the United States estimate that there are over one million persons living with HIV/AIDS, and that as individuals' access healthcare and initiate treatment, there will be an increasing risk of patients failing therapy and developing resistance (Centers for Disease & Prevention, 2012; Das et al., 2010; Schreibman & Friedland, 2003). Additionally, it is estimated that approximately 21% of HIV-infected persons are unaware of their infection, further increasing the risk of forward transmission (Centers for Disease & Prevention, 2012; H. I. Hall et al., 2008; Prejean et al., 2011). Recent studies have presented estimates that provide a landscape of drug resistance patterns in the US (Boden et al., 1999; Brennan et al., 2009; Grant et al., 2002; Little et al., 2002; Rahim, Fredrick, da Silva, Bernstein, & King, 2009; Weinstock et al., 2004; Wheeler et al., 2010). There is also a substantial amount of data on the global prevalence of HIV drug resistance and the impact it has on clinical outcomes. However, there are significant limitations on access to resistance testing technologies in developing countries that limit the accuracy of reporting hence, underestimating true prevalence of the problem (Bennett, 2006; Booth & Geretti, 2007; De Luca et al., 2013; Gupta, Hill, Sawyer, & Pillay, 2008; Pillay, 2004; Programme, 2008; Rahim et al., 2009; Stadel & Richman, 2013).

In the HIV Cost and Services Utilization Study (HCSUS), investigators reported that up to 76% of HIV-1 plasma RNA samples from patients in care and on treatment across the US have evidence of resistance to at least one NRTI, NNRTI, or PI; 48% of these patients demonstrated dual resistance and 13% had triple-class resistance. Resistance was measured for the 15 approved ARV drugs at the time utilizing the PhenoSense Assay (Monogram Biosciences [formerly, ViroLogic, Inc.], South San Francisco, CA) (Richman et al., 2004). Since the initial HCSUS report, two additional classes of ARV agents have been introduced: entry inhibitors and integrase inhibitors (Grinsztejn et al., 2007; Markowitz et al., 2009; Reynes et al., 2007). Epidemiological understanding regarding the development of resistance to these agents and other new drugs approved by the FDA continues to evolve. Monitoring the evolution of new

resistance data is crucial as it has impact on the clinical utility of resistance testing assays (Marcelin, Ceccherini-Silberstein, Perno, & Calvez, 2009).

In the HCSUS study there were increased rates of NNRTI resistance compared to other classes; this has also been described by investigators at the CDC and in recent studies looking at adolescents and newly infected persons (Varghese et al., 2009; Viani et al., 2006). This observation is significant because drugs in the NNRTI class are on the list of preferred first-line ARV regimens in the treatment guidelines of the DHHS, International AIDS Society (IAS), and the World Health Organization (WHO) (Bennett, Myatt, Bertagnolio, Sutherland, & Gilks, 2008; DHHS, 2013; Thompson et al., 2012). Incidence and prevalence patterns of drug resistance—associated mutations have correlated with the introduction and use of new agents along the continuum of drug development (D'Aquila et al., 2002; V. A. Johnson et al., 2008; V. A. Johnson et al., 2011; Wainberg, Mesplede, & Raffi, 2013); (V. A. Johnson et al., 2010; Jordan, Bennett, Bertagnolio, Gilks, & Sutherland, 2008). There is a dearth of research assessing resistance to newer classes of ARV drugs and although the amount of data is increasing, it is important to stay attuned to patterns of resistance to new agents as they are incorporated into clinical practice.

The investigators of the French Aquitaine Group, a large cohort of prospectively enrolled HIV-1 infected participants, presented data in 2007 on the first documented case of transmitted HIV-1 infection with entry inhibitor resistance. This case reported a tropism shift and corresponding decline of CD4+ t-cell from 419 to 184 cells/ μ l in an ARV treatment-naïve patient. The authors describe the dual-tropic to R5 shift and subsequent clinical response occurring in combination with multidrug-resistant (PI, NRTI, and NNRTI) resistance. The evolution and transmission of quadruple-class resistance and the potential for quintuple-class resistance with the introduction of integrase inhibitors will require careful monitoring. Although multidrug-resistant viruses tend to have impaired replication capacity, making transmission of such mutations more difficult, when transmission of multiclass drug-resistant virus does occur, there

are significant clinical implications for the options for ARV therapy combinations. While continued drug development is important for ongoing treatment strategies particularly in novel targets, ongoing attention should be focused on understanding drug resistance mechanisms.

Baseline HIV drug resistance testing is particularly important in areas where there is an increased prevalence of drug resistance in the HIV infected community and where there is evidence for ARV class-specific patterns of resistance. For the US and other developed countries, access to drug resistance testing is more readily available. However, in the case of novel or new classes of ARV drugs, there is no data on drug resistance-associated mutations in the community because use of these new agents is restricted to the confines of clinical trials. Furthermore, there is no consensus on what guidelines to follow. Thus, no evidence to support baseline resistance for the newest class of ARV exists for integrase strand transfer inhibitors (INI). Young and colleagues recently presented data on the first documented case of transmitted drug resistance to integrase inhibitors in a subject enrolled on a clinical trial for treatment-naïve patients (2011). This was followed by another report describing an HIV-infected patient with pretreatment resistance to raltegravir (still an investigational/ new ARV agent at the time). Unlike the subject described by Young and colleagues, this subject was infected with a subtype CRF_AG virus (Boyd et al., 2011). The risk of resistance to a recently approved drug was thought to be negligible and therefore the initial resistance went undetected. This underscores the utility of baseline resistance testing prior to initiation of ARV treatment as delineated in the treatment guidelines. It also highlights the critical need to understand how companion diagnostics such as HIV drug resistance testing continue to play a role in HIV drug development. This is particularly important as new therapies are introduced into clinical care and practice (Daar, 2007; Kozal, 2009; Marcelin et al., 2009; Volberding, 2009).

Genetic Diversity

Drug and technology development has historically been driven by North America and Western Europe, resulting in a subtype B-centric approach. When monitoring for drug

resistance it is important to have accurate and reliable technologies. The demand therefore is a global issue as access to care and treatment continues to expand (Kumarasamy & Krishnan, 2013; Mee, Fielding, Charalambous, Churchyard, & Grant, 2008). Increasing awareness of HIV's genetic diversity on drug development and clinical response to therapy requires that more attention be paid to the development of technologies. Awareness of viral genetic differences between the common HIV-1 groups and their impact on the performance characteristics on resistance testing assays can have clinical implications. It is therefore important to understand the background of HIV subtypes, circulating recombinant forms (CRFs) and unique recombinant forms (URFs), and the implications on resistance testing.

There are three classes of HIV-1 based on the diversity of the viral envelope: M (major), O (outlying), and N (new). The M group, which includes more than 90% of HIV infections globally, is sub-classified into nine major subtypes including A–D, F–H, J, and K, as well as several recombinant forms (Kuiken, Korber, & Shafer, 2003; Taylor & Hammer, 2008). With an estimated viral production of 10^{10} virions per day and a reverse transcriptase enzyme that lacks proofreading capacity, millions of viral variants with single-point mutations are produced within an infected person in a single day. This level of polymorphism has led to a rapid proliferation of viral subtypes. Genetic variation within an HIV subtype can range from 15% to 20%, whereas variation between subtypes may be as much as 35% (Hemelaar, Gouws, Ghys, & Osmanov, 2006; Taylor & Hammer, 2008). Although Group M, subtype B accounts for approximately 11% of the global pandemic, it is the most studied as it dominates in North America and Western Europe as well as the Caribbean, Latin America, Central Europe (Hemelaar et al., 2006; Rahim et al., 2009).

The underlying genetic diversity or polymorphism of HIV-1 may pose complicating challenges. PCR and sequencing reactions depend on the hybridization of synthetic oligonucleotide primers to specific viral nucleotide sequences. Mismatches between the viral and primer sequence can result in the failure of a PCR reaction. In light of this extreme genetic

diversity, designing PCR primers that will robustly hybridize to all HIV viral genotypes is extremely challenging. To ensure reproducibility and generate sequence alignment in the face of genetic mismatch, assay developers employ a strategy of slightly overlapping, redundant sequencing primers. If point mutations result in the failure of a sequencing primer to hybridize to its viral target, useful sequence will be generated by an overlapping primer. These technical enhancements help to ensure accurate and consistent detection of mutations with a high degree of precision and reproducibility. For the purposes of this study, the minor variant assay utilized was developed with these concerns in mind via the addition of a “CURE” step. The qMVA assay normalizes areas of sequence heterogeneity adjacent to the single nucleotide polymorphism considering resistance and is described in detail in the methods section.

As a result of rapid drug development in the United States and Western Europe, diagnostic manufacturers have focused on the predominant virus found in the developed world (Group M, subtype B) as the template for the design of PCR primers used in commercial HIV resistance testing (RT) assays (Hackett, 2012). As global access to HIV treatment expands so will the need to understand how the variability in HIV-1 genetic diversity will impact drug resistance technologies, viral-load technologies, and drug development. Ultimately all of these factors affect how we understand and address issues related to global problems of HIV drug resistance (Hackett, 2012; H. H. Lin et al., 2006; N. T. Parkin & Schapiro, 2004).

In an era of jet travel, the world has become a global village and the map of the HIV pandemic continues to evolve rapidly. Recent studies have shown that HIV, non-B subtypes and circulating recombinant forms (CRFs) are becoming more prevalent in North America and that all groups, subtypes, and major CRFs are increasingly well represented in Western Europe (Hirigoyen & Cartwright, 2005; H. H. Lin et al., 2006; Rahim et al., 2009; Wheeler et al., 2010). Diagnostic manufacturers of HIV RT assays need to closely monitor the evolution of the pandemic. They may need to adjust their primer sequences to keep pace with ever-changing

viral evolution if they are to have effective assays where HIV Group M, subtype B is not the predominant viral genotype (Hackett, 2012; Taylor & Hammer, 2008).

ii. Drug Resistance Technologies

Primer on Technology

Resistance testing provides predictions or measures of drug susceptibility for HIV. In many cases, resistance can be correlated to the presence of known mutations in the protease or reverse transcriptase regions of the HIV genome of an individual patient's virus. The codons are identified by numeric position on the protein coding region preceded by the wild-type (usually consensus B subtype) amino acid (AA), and followed by the mutant amino acid (Lodish, 2003; Singer & Berg, 1991). For example, the signature mutation for resistance to lamivudine and emtricitabine (structurally related nucleoside reverse transcriptase inhibitor [NRTI] ARV agents) is a substitution of valine for methionine at position 184 in reverse transcriptase (*rt*). At position 184 in *rt*, methionine is the wild-type AA, hence M184 is the wild-type (WT) codon and the mutation is referred to as the M184V with valine being mutant (de Mendoza, Gallego, & Soriano, 2002). This is the most commonly observed AA change at this codon in virus from patients on lamivudine- or emtricitabine-containing ARV regimen. Based on the algorithm and rules of what is known about clinical response to therapy, it is predicted that viruses with the M184V mutation will generally be resistant to lamivudine and emtricitabine (Epivir or Emtriva), respectively (V. A. Johnson et al., 2011).

Sometimes a population of viral species can contain mixtures; specifically, the virus population contains both wild-type and mutant viral strains. For example, "M184M/V" indicates that viruses with a methionine and valine at position 184 are both detected. This could occur if the test was performed at a time when the viral population is in transition from wild-type to mutant (or vice versa). Interpretation of genotype and phenotype test results when mixtures are present can be challenging; this will be discussed below (de Mendoza et al., 2002; Shafer et al., 2000; Shafer & Schapiro, 2008).

There are different mutation types and understanding what impact these have can help with the interpretation of resistance tests results. Primary mutations are those known to be associated with reductions in susceptibility to specific ARV drugs. Secondary mutations are those that alone do not cause resistance to a drug but are selected along with primary mutations. Secondary mutations may compensate for loss of replication capacity or fitness of viruses that have become resistant. Polymorphisms occur naturally in the absence of drug pressure but can affect ARV susceptibility if they accumulate in combination and at key sites in the genome, thus some polymorphisms may also be characterized as secondary mutations. These polymorphic mutations can also be passed on in the case of transmitted drug resistance (Clavel & Hance, 2004; Shafer, 2002; Shafer & Schapiro, 2008).

Nucleotide sequence changes resulting in amino acid substitutions will eventually lead to mutations that affect reductions in drug susceptibility. The number of these mutations will affect clinical response to therapy and is referred to as the genetic barrier.(Gotte, 2012; Tang & Shafer, 2012). Additionally, each ARV has an associated genetic barrier to resistance, which is comprised of primary and secondary mutations that vary in number for each drug. Each drug can have more than one pathway to resistance with different genetic barriers. Resistance to a drug with a high genetic barrier requires more mutations to effect resistance, and resistance to a drug with a low genetic barrier requires fewer mutations to confer resistance to the drug (Beerenwinkel et al., 2013; Bongiovanni et al., 2003; Kuritzkes, Boyle, Gallant, Squires, & Zolopa, 2003; Luber, 2005; van de Vijver et al., 2006). It is also important consider that drug concentrations can affect the genetic barrier in that effective concentrations must be achieved in order to prevent drug-resistant variants from overcoming selective drug pressure. Ineffective drug levels may be the result of suboptimal dosing, poor adherence, or lowered drug levels due to pharmacokinetics of drug interactions in a person's concomitant medications. Assessment of and understanding dose response curves and pharmacokinetics are becoming increasingly

important when discussing the genetic barrier of individual drugs and ARV regimens (la Porte, 2008; Shen, Rabi, & Siliciano, 2009; Siliciano, 2011).

HIV drug resistance testing has progressed significantly since the first case of drug resistance was described by Larder and colleagues (1989). It is important to understand that ARV therapy does not cause mutations. Resistance mutations already exist and drugs inhibit the growth of the sensitive wild-type strains, allowing the resistant mutants to predominate. Initially, genotype assays were the only resistance tests available and as phenotype resistance tests were developed, both assays were incorporated into clinical practice. Drug resistance testing informs clinicians about which drugs will most likely not be effective, thereby enabling the construct of an individualized, tailored, potent ARV regimen. The initial studies were instrumental in changing HIV treatment guidelines that now recommend resistance testing as part of routine clinical care for patients with HIV infection. Although these early studies varied in the type of tests utilized, patient populations (treatment-naïve or experienced), and duration of observation, the overall findings collectively supported the use of HIV resistance testing in guiding clinical decision making for selection of ARV regimens along the treatment continuum (Badri, Adeyemi, Max, Zagorski, & Barker, 2003; Baxter et al., 2000; Clevenbergh et al., 2000; Cohen et al., 2002; V. A. Johnson et al., 2011). Resistance testing is technically sophisticated and the results require expert interpretation to ensure maximum clinical utility when making decisions and constructing ARV regimens. In order to understand the difficulties faced with resistance testing, it is important to understand the basics of such testing.

Two principal methodologies for assessment of drug resistance to ARV have been previously noted, genotyping (GT) and phenotyping (PT). GT tests detect mutations in target proteins within the genes of HIV-1 that are associated with drug resistance, specifically, protease, reverse transcriptase, integrase, and envelope regions of the genome. PT tests measure the susceptibility of a patient's virus to ARV drugs in cell culture. PT assays measure the ability of a patient's virus to replicate in the presence of varying drug

concentrations compared to a wild-type laboratory reference strain. Measurements are calculated using the inhibitory concentration of varying drug concentrations for both the person's virus and the reference strain, and calculated difference is expressed as a fold-change. The wider the fold-change of the patient compared to the reference strain, the more resistance the virus.

Differences exist between GT and PT assays with respect to cost and how quickly laboratory results can be obtained (de Mendoza et al., 2002; Hertogs et al., 1998; Kuritzkes, 2011; Kuritzkes, Boyle, et al., 2003; Petropoulos et al., 2000; Richman, 2006; Shafer, 2002; Shafer et al., 2000; Tang & Shafer, 2012).

HIV-1 Genotype Testing

Genotypic assays detect the presence or absence of mutations that are associated with HIV drug resistance. Regions of the HIV genome that are targets of the ARV drugs are amplified by reverse transcription-polymerase chain reaction (RT-PCR) and sequences using the Sanger sequencing technique, based on chain termination chemistry and gel electrophoresis. This technology sequentially determines the base composition of nucleic acids in the gene sequence of interest. Chain termination chemistry involves the incorporation of labeled dideoxynucleotide-tri-phosphates (ddNTPs) at the end of a strand of deoxyribonucleic acid (DNA) during the extension phase of DNA synthesis. The incorporation of these labeled deoxynucleotide-tri-phosphates (dNTPs) ends the extension of the strand. The resulting fragments of differing lengths are then separated using electrophoresis and analyzed using autoradiography or fluorescence depending on the label used in the ddNTPs. The results are then read from an autoradiogram or chromatogram and interpretations are made based on the reading of nucleotide sequences and their corresponding amino acids (Hartl & Jones, 2005; Lodish, 2003).

Genotype assays are able to detect the predominant viral populations in a person's plasma or other fluids if the sample comprises approximately 15%-20% of the total viral population. This threshold varies depending upon the assay being utilized. Some limitations of

GT assays are the inability to detect minority species at levels of less than 15%, the qualitative algorithm for interpretation of quantitative results and the dependence on the previous knowledge regarding which mutations cause resistance to a drug. This is not always known for new drugs, novel mutations, or newer drug classes. Alternative technologies (discussed below) have lower limits of detection for low-level, minority-resistant variants, varying from 0.1 to 5% (Cai et al., 2007; Gianella & Richman, 2010; Paredes et al., 2007; Shafer, 2009). However, as with many new technologies, there are unanswered questions regarding clinical utility and application of findings. There have been small studies reporting that ultra-sensitive RT assays are able to detect resistant variants that have been missed by standard genotype assays up to 40% of the time (Charpentier et al., 2004; J. A. Johnson et al., 2008; Nissley et al., 2005; Palmer et al., 2005). These studies have been retrospective in nature and have yet to answer the questions of whether utilizing ultra-sensitive resistance testing assays improves ARV drug selection and treatment outcomes. Additionally, as “ultra-sensitive” tests have only been available in research laboratories, the limits of detection have not been established or standardized. Furthermore, these assays are labor intensive and can be costly, and the timeline for commercial availability is not clear (Gianella & Richman, 2010; Li & Kuritzkes, 2013).

As mentioned previously, one limitation of genotype assays is the algorithms that guide clinicians in decision making. These algorithms are best derived from clinical response data, most often clinical trial data, which are then used to create rules-based computer programs that are used in bioinformatics systems along with drug resistance testing assays. These programs and algorithms are often simple and straightforward, as in the case of ARVs with single-point mutations that are known to confer high-level resistance. An example of this would be M184V for 3TC and FTC, the D30N mutation for nelfinavir (Viracept), an un-boosted protease inhibitor (PI), or the K103N mutation for nevirapine (Viramune) and efavirenz (Sustiva), nonnucleoside reverse transcriptase inhibitors (NNRTI). K103N is an example of a mutation that is associated

with class resistance for the first generation of NNRTIs, whereby one mutation causes cross-resistance to all of the NNRTIs. This type of class mutation is problematic because it limits future treatment options, making drug selection a challenge for patients in advanced lines of therapy (Beerenwinkel et al., 2003; de Mendoza et al., 2002; Hirsch et al., 1998; Hirsch et al., 2008; Kuritzkes, 2004; Kuritzkes, Boyle, et al., 2003; Schmidt, Walter, Zeitler, & Korn, 2002). For the second generation of NNRTIs, such as etravirine (Intelence) and rilpivirine (Edurant), there are additional mutations and combination of mutations required to cause resistance and these drugs. These new agents were specifically developed to overcome resistance conferred by the signature mutations associated with resistance to the first generations NNRTIs (Gulick, 2010; Hirsch et al., 2008; Jayaweera & Dilanchian, 2012).

Keeping GT interpretation algorithms up-to-date as new drugs become available is a challenge. New data relating mutation patterns to clinical response or drug susceptibility are generated by multiple sources, leading to lack of standardization in the resulting algorithm. Laboratories that have expertise in running HIV resistance tests are often in the best position to develop good algorithms. Strong algorithms are those with updated clinically relevant data, expert panel review, timely updates, and other quality control measures in place. Quality control and standardization (discussed below) for resistance-testing algorithms and rules-based interpretations are needed (Bennett, 2006; Bennett, Myatt, et al., 2008; Schuurman, Brambilla, de Groot, Huang, Land, Bremer, Benders, & Boucher, 2002; Schuurman et al., 1999; Shafer, Rhee, & Bennett, 2008).

As patients advance in HIV disease and fail regimens, GT resistance patterns become increasingly complex, making patient management more difficult. These complexities and unique shifts in resistance effects make expert knowledge extremely important in interpreting resistance test results from the multidrug treatment failure patient (de Mendoza et al., 2002; Eron et al., 2011; R. Haubrich & Demeter, 2001; R. H. Haubrich et al., 2005; Katzenstein, 2003; Katzenstein et al., 2003; N. Parkin et al., 2002; Wegner et al., 2004; Zolopa, 2003). As the field

of resistance testing and its interpretation becomes broader and more complex, increasing expertise is required and additional testing technologies are sometimes complimentary.

HIV Phenotype Testing

Phenotype assays are a direct, quantitative measure of the virus' ability to replicate in the presence of drug at increasing concentrations. PT assays require that a portion of the patient's virus sequence, typically HIV *pr* and *rt*, be isolated and recombined with a laboratory strain virus, resulting in a recombinant virus vector that includes patient-specific sequences. The vectors are then cultured and tested in the presence of varying ARV concentrations against a laboratory reference strain of HIV. The measurement of resistance varies by the assay and recombination technique, but all assays report a quantitative measure of susceptibility (newer assays can also measure integrase [*in*] resistance and entry [*er*] inhibitor resistance and will be discussed later).

Most PT assays measure the IC₅₀ (inhibitory concentration of 50%, which means the concentration of an inhibitor that is required for 50% inhibition of HIV replication) of each drug against the virus in vitro. Typically, results are reported as fold-change (FC) in IC₅₀, as compared with a drug-susceptible control strain, often WT virus noted above. A FC susceptibility curve is generated for each drug being tested. If the FC of a patient's virus is higher than the reference virus, then the patient's virus is considered to have a reduced susceptibility to that drug. The magnitude of the FC determines how likely the person's virus is to respond to that drug in a regimen; there are currently two commercially available assays available to clinicians, the PhenoSense Assay (Monogram Biosciences, South San Francisco, CA, USA) and the Antivirogram (Virco, Mechelen, Belgium) (Hertogs et al., 1998; Petropoulos et al., 2000; K. Wang, Samudrala, & Mittler, 2004).

The clinically relevant FC where one would expect a drug to have statistically significant reductions in susceptibility is referred to as a cut-off. There are three types of cut-offs; assay variability cut-offs, biologic cut-offs, and clinical cut-offs. Assay cut-offs, also referred to as

technical cut-offs or reproducibility cut-offs, are based on assay sensitivity. The current range on commercially available PT assays range from 2-fold to 4-fold change and are assay-specific (Eshleman et al., 2007; Hertogs et al., 1998; Petropoulos et al., 2000; Schmidt et al., 2002). This narrow range can detect relatively small changes in susceptibility that may or may not be clinically relevant. This is why clinical cut-offs are generally considered to be of increased benefit when utilizing the phenotype assay. Biologic cut-offs refer to the natural variability of wild-type susceptibility to ARV drugs, and have been defined as the FC in IC₅₀ below which 99% of wild-type samples fall in the distribution for the PhenoSense assay (Hertogs et al., 1998; Petropoulos et al., 2000). In the absence of clinical response data, clinicians cannot assume that all viruses within or outside of the natural distribution will respond equally well to a specific drug. Clinical history and other patient-related factors must be intercalated into the clinical decision process when managing ARV therapy using resistance testing data in complex cases.

Therefore, for reasons aforementioned, the most useful measure for clinicians is the clinical cut-off. These data are obtained from clinical trials and thus provide actual treatment response information. Clinical cut-offs are determined by comparing baseline PT susceptibility before and after the administration of a new or additional ARV drug to a failing regimen. Changes in susceptibility and corresponding changes in VL are compared to assess clinical response over a designated, observed time period in a clinical trial (e.g., 24-weeks, 48-weeks). This allows an assessment of the impact that the addition of the new drug contributes to the regimen as well as changes in susceptibility curves, thus establishing clinically relevant responses, thus establishing a clinical cut-off (Harrigan, Miller, McKenna, Brumme, & Larder, 2002; Kempf et al., 2001). These clinical cut-offs therefore reflect the FC above which there is a statistically significant decreased likelihood of a clinical response. FC values below the clinical cut-off predict the greatest chance of a response to a particular ARV drug, and FC values above the clinical cut-off predict no response or an attenuated response to a particular agent. It is important to understand that the concept of a cut-off is based on a continuum. There is no

absolute point at which a patient is guaranteed a response or no response. One key feature of HIV drug resistance testing is that results can provide clinicians with information on targeted, individualized therapy.

Regardless of which cut-off is being used and interpreted, it is important to remember that these values are distinct for each assay and are not interchangeable. Clinical cut-offs must be established for individual assays and vary between each ARV drug; there is also a high and low cut-off established for each individual agent. Further, the results of PT assays provide information on the average plasma viral population at the time of sampling and while the patient is on therapy. Like genotypes, a major limitation of the phenotype assays is that they can assess only one drug at a time; they do not provide a measure of ARV drug combinations. Additionally, the clinical cut-offs have not been established for all ARV drugs, and like genotype assays, phenotype assays only detect resistance in the majority of the viral population and may not detect minority drug-resistant variants (DeGruttola et al., 2000; Hirsch et al., 1998; Katzenstein, 2003; N. T. Parkin et al., 2004).

Combination Testing

Although genotype and phenotype technologies have inherent differences, they provide complementary information, particularly given the complex nature of ARV resistance in highly-treated patients who may harbor virus that is cross-resistant to multiple ARV drugs. It is not uncommon for providers to utilize combination testing in complex clinical cases (Fehr et al., 2011; Sarmati et al., 2002; Zolopa, 2003). GT results may also indicate that the virus is sensitive to one or more drugs, while PT may detect a reduction in susceptibility (resistance). This is referred to as GT/PT discordance (N. Parkin et al., 2002; Wegner et al., 2004). Patients who have been heavily treated with multiple ARV regimens are particularly prone to discordant results because they can develop multiple, complex patterns of mutations over their course of treatment. As patients progress through sequential lines of ARV therapy, it is important to remember to seek expert guidance (Call et al., 2001; Harrigan & Larder, 2002; R. H. Haubrich et

al., 2005; Katzenstein, 2003; Perez-Elias et al., 2000). It's also practical to keep in mind that historically, many regimens were the result of sequential mono-therapy and done without the benefit of resistance-guided drug switches, therefore, it's critical to correlate resistance test results with ARV treatment history.

Experts and guidelines suggest that in highly treatment-experienced patients, combination resistance testing may enhance clinical utility in constructing new ARV regimens for patients who are failing advanced lines of therapy. The mechanisms for complex mutation patterns and reasons for combination testing are expanded upon below (DHHS, 2013; N. Parkin et al., 2002; Zolopa, 2003; Zolopa et al., 2005).

Replication Capacity/Viral Fitness Assays

Viral fitness is a complex evolutionary term used to describe replication capacity in a defined environment. As previously noted, there are resistance associated mutations that can have effects on viral fitness and there have been varied results on clinical impact of fitness measures and outcome. When discussing performance characteristics of assays for in-vitro and in-vivo measures of viral fitness, it is important to remember what these definitions entail and what assays are being utilized (Barbour & Grant, 2004, 2005). This is particularly true because replication capacity assays are used as surrogate markers for viral fitness and these terms are sometimes used interchangeably.

Due to the high replication dynamics of HIV-1, it provides an ideal model to test both fitness and evolution theories. In order to test fitness of a viral species, in-vitro studies would include quantifying replication kinetics in a cell-based culture of two or more viral isolates from the same viral strains, observing viral evolution over time. In-vitro fitness data are often obtained in well controlled environments that include well-defined and validated cell lines, standardized reference strains, calculations of input ratios, and varied timing for analysis of progeny populations. Well controlled studies are essential to ensure accurate and reliable data because in-vitro variables and viral strain factors interplay in culture-based media and

conditions. Conversely, differentiating changes over time as done with in-vivo studies are often more challenging. Host immune variables and responses can affect measures as can antiviral impairing agents, and the development of fitness impairing mutations and target cell responses (CD4+ t-cells). These variables are what make in-vivo studies more challenging but more representative of true clinical response (Quinones-Mateu et al., 2000; Wargo & Kurath, 2012).

The complex nature of true fitness assays has presented limitations for the clinical care and management of patients. Recombinant drug-resistant testing technologies such as those used by HIV phenotyping allow for the incorporation of replication capacity (RC) testing. RC, being a component of viral fitness, can be measured as a part of some phenotype resistance tests assays and have been described elsewhere (Bates et al., 2003; Campbell, Schneider, Wrin, Petropoulos, & Connick, 2003). There have been studies that correlate specific HIV-1 drug resistance-associated mutations with impaired or increased RC. A number of studies have examined the clinical utility of RC as a predictor of virologic and immunologic failure. These studies have been inconclusive on clinical utility of this assay as a consistent marker of clinical outcome, most likely because they have analyzed different patient populations along different trajectories of care (Barbour et al., 2002; Campbell et al., 2003; Deeks et al., 2001; Trkola et al., 2003).

Other Technologies

Additional technologies for resistance testing exist beyond genotype, phenotype, and replication capacity assays including, for example, tropism testing. The chemokine receptors CCR5 and CXCR4 are necessary for viral entry into the target cells in HIV-1 infection. Tropism refers to the type of co-receptor(s) on the cell surface that a particular HIV-1 virus or variant uses to enter the cell. R5-tropic variants use the CCR5 co-receptor and X4-tropic variants use the CXCR4 co-receptor; there are also dual-tropic variants that can use both co-receptors. There are three drug classes of entry inhibitors: attachment inhibitors, co-receptor antagonist, and fusion inhibitors. Attachment inhibitors block initial binding of cell surface proteins to the

CD4+ t-cell receptor. Co-receptor antagonist blocks entry by interfering with attachment to one of two co-receptor receptors, namely CCR5 or CXCR4. Fusion inhibitors prevent membrane penetration by a core viral particle and ultimate viral entry (Tilton & Doms, 2010; Whitcomb et al., 2007).

Tropism-testing technologies now include both phenotypic and genotypic assays that can detect and assess evolution of tropism and associated resistance-associated mutations with co-receptor tropism. Assessment and response to ARV therapy for drugs in this class are important because tropism shifts or evolution of co-receptor usage from R5 to X4 has been shown to have significant impact on the pathogenesis and clinical outcome. X4 variants are associated with increased virulence and acceleration of HIV disease. Detection of these tropism shifts is essential when a patient is on entry inhibitor therapy as there can be clinical implications; the change in tropism can be signaling ARV failure requiring a change in therapy (Daar et al., 2005; N. H. Lin & Kuritzkes, 2009; Miller & Hazuda, 2004; Skowron et al., 2009). Several studies have demonstrated that HIV-1 drug-resistant variants present at low abundance that are not detectable by standard genotype assays can impair clinical response to therapy (Daar et al., 2007).

A variety of technologies have been developed that allow characterization of HIV drug-resistant variants at lower limits of detection compared to standard “bulk sequencing” techniques, which has inherently poor resolution of mixed populations. Two of these methods, clonal analysis and single-genome amplification, have relied upon traditional capillary based sequencing. Although these methods are more sensitive and allow for phasing of variants, they are labor and cost intensive. Recently, “ultra-deep sequencing” (UDS) applications have been developed on several next generation sequencing (NGS) sequencing platforms (Buckton, Harris, et al., 2011; Buckton, Prabhu, et al., 2011; Church et al., 2008; Dudley et al., 2012; J. A. Johnson et al., 2007; Palmer et al., 2005; Paredes et al., 2007; Shafer, 2009)..

Quality Control Measures: Technologies

The performance characteristics of diagnostic tests are important for clinicians to understand because results are translated into clinical decision making regarding ARV therapy. Therefore, demonstrating that resistance testing assays perform consistently under various laboratory conditions reassures providers and patients that test results are reliable and valid. There are several factors that affect assay performance, such as reagent and supply lots, supplies and suppliers of instrumentation, and systems manufacturing. Local shipping and storage conditions as well as draw-site specimen handling and processing can also affect tests results. Additionally, technical skills of the analysts, much like provider expertise, can also affect outcomes. In the US, assay validation studies are required by Clinical Laboratory Improvement Amendments (CLIA) regulations. The approval of resistance testing assays by the Food and Drug Administration (FDA) or CLIA is a topic clinicians should be familiar with because not all tests are CLIA approved. Some resistance tests are FDA approved and others are not. FDA- or CLIA-approved assays undergo rigorous validation with established operating and performance characteristics for a given purpose. These studies ensure that assays are clinically effective as part of the current treatment algorithm and that the results reported are accurate and robust. FDA approval enables the diagnostic manufacturer to market and distribute these reagents and devices as a test with an intended and commercially labeled use established during clinical trials leading up to its approval (U.S. Food and Drug Administration, 2013; Westgard & Barry, 2008).

In instances where FDA-approved diagnostic assays are not available, laboratories can develop and self-validate in-house "laboratory-developed tests" (LDT) in accordance with regulations of the CLIA of 1988. Regulations governing the validation and approval of LDTs are currently under review by the FDA and initial guidance implies that all such tests will require a submission and approval from a regulatory body prior to use in a patient care (U.S. Food and Drug Administration, 2013).

Reliability of resistance test data is dependent on quality control measures of the reference laboratory performing the assays. There have been proficiency panel studies comparing laboratory consistency in accurately identifying mutations and making predictions of resistance. Significant performance variations have been seen across laboratories for blinded samples regarding detection capacity for mutant virus even for samples expressing pure mutant genotypes. Performance metrics were significantly worse for samples with mutant or mixed genotypes. Challenges with over- or under-calling resistance can have clinical consequences if clinicians are not familiar with their labs' proficiency or quality assurance procedures (Galli, Satta, Wynhoven, O'Shaughnessy, & Harrigan, 2003; Sayer et al., 2003; Schuurman, Brambilla, de Groot, Huang, Land, Bremer, Benders, & Boucher, 2002; Schuurman et al., 1999). There is also a lack of standardized algorithms for GT interpretation, and the clinically relevant cut-points for PT assays have also not been established for all drugs. Additionally, there are limitations for both GT and PT assays with newer ARV agents, as current GT algorithms may not account for the new mutation patterns that may arise, and the corresponding PT cut-offs may have not yet been established (discussed elsewhere). Quality control data and processes should be in place and continue to be monitored for impact on clinical care and outcome.

iii. Clinical Utility of Drug Resistance Testing

There are two different forms of HIV drug resistance: transmitted drug resistance (TDR) which is observed in individuals who have never received ARV therapy and acquired drug resistance (ADR) which is observed in individuals who have been on ARV therapy and experience virologic failure. (Bangsberg, Kroetz, & Deeks, 2007; Bansi et al., 2010; F. M. Hecht et al., 1998; Richman, 2006; Shafer et al., 2000). Acquired drug resistance differs from transmitted drug resistance in several important ways and can have different implications for clinical management of patients. Importantly, TDR occurs when an individual is infected with a virus that is already resistant to an ARV (Grant et al., 2002; R. Haubrich & Demeter, 2001; Kantor et al., 2004; Kuritzkes, Boyle, et al., 2003; Phillips et al., 2005; Simen et al., 2009; von

Wyl et al., 2007), while ADR occurs in an individual initially susceptible to an ARV but subsequently develops resistance after starting ARV therapy. TDR is of clinical concern because it can impact the selection of a person's initial ARV regimen as well as subsequent regimens.

Studies have shown that pre-existing mutations at the time of infection affect treatment outcomes (Buckton, Harris, et al., 2011; Grant et al., 2002; Li et al., 2011; Little et al., 2002; Paquet et al., 2011; Poon et al., 2011). Although a newly infected patient may be naïve to ARV therapy, the source patient's transmitted resistant virus has left molecular footprints of drug resistance, complicating the newly infected person's initial ARV therapy selection. Transmitted drug resistance is a significant concern especially in geographic areas where ARV treatment has been widely implemented for extended periods of time. One major concern about TDR is the inability to detect drug resistance associated mutations prior to treatment beginning resulting in a potentially sub-optimal regimen being utilized. In the case of TDR, reversion of resistance mutations back to wild-type can occur at varying rates and is dependent on the mutation that is transmitted (Barbour, Hecht, Wrin, Liegler, et al., 2004; Little et al., 2008). A genetic reversion is a mutation that reverts to its wild-type or an intermediary. Since drug resistance mutations may wane over time, early testing is key and the sensitivity of the drug resistance testing assay is also important. There is conflicting data on the differential benefits of utilizing standard population based sequencing genotypes versus newer sensitive assays that are being used in this study such as the qMVA or ASPCR. Some studies have demonstrated a clinical benefit of utilizing a minor variant assay while others have not. These clinical benefits have varied from increased detection in baseline mutations to sustained virologic suppression and improved immunologic responses (Goodman et al., 2011; J. A. Johnson et al., 2008; Li & Kuritzkes, 2013; Li et al., 2011). One variable that impacts whether TDR is detected is the time at which the resistance testing is done. The earlier a virus is tested for resistance following infection, the higher the likelihood of detecting any transmitted drug associated mutations (Little et al., 2008).

The second type of HIV drug resistance, acquired drug resistance (ADR), occurs in chronically infected, ARV-treated patients in whom a diverse population of viruses, including drug-sensitive wild-type virus, has had ample opportunity to integrate into the latent reservoir. Therefore, upon drug discontinuation following virologic failure with drug resistance, it is likely that the wild-type drug sensitive virus which has higher fitness will again become the predominant variant, since no drug pressure is present. This is in contrast to the genetic reversion of the drug resistance mutations which occurs with TDR. For example, in an individual infected with TDR who does not begin ARV therapy, virus may revert back to wild-type via back-mutation rather than the selection of archived wild-type virus, since no archived drug-sensitive virus is present. This process is generally slower and may occur at different rates depending on the actual sequence change. If the virus can restore fitness by reverting the mutation to a variant different from the wild-type (WT), this may be preferred if it involves fewer nucleotide changes e.g. T215 revertant mutations (Mitsuya et al., 2008). The impact these revertant mutations have on resistance testing is discussed elsewhere.

There are different mechanisms by which a virus can become resistant to an ARV and these mechanisms are specific to the ARV drug and class (Clavel & Hance, 2004). For NRTIs, resistance may develop via one of two different mechanisms: (1) impairment of analogue incorporation or (2) removal of an analogue from the terminated DNA chain (also known as chain termination). The NNRTI and PI class of ARV have a similar mechanism of acquiring resistance and usually entail substrate binding interference either by blocking of substrates or by conformational changes in the catalytic core of the viral genome created by resistance associated mutations. For entry inhibitors, the drug resistance associated mutations affect conformational changes on the cell surfaces in the *env* region of the HIV-1 genome. Drug resistance is a direct consequence of sub-therapeutic drug levels, which can be caused by many different factors, thereby allowing for drug resistant mutants to escape selective drug pressure of a person's specific regimen. This is based on the assumption that the person was

infected with a quasi-species containing a compendium of viruses harboring multiple variants, primed for eventual selective pressure opportunities (Clavel & Hance, 2004; de Mendoza et al., 2002; Gianella & Richman, 2010; Richman, 2006). Thus, strategies to improve adherence are critical in order to decrease a person's risk of developing drug resistance; it is a complex topic as there are many reasons for suboptimal adherence. Ideal rates of adherence are dependent upon the combination of ARV drugs selected and the genetic barrier (previously described) that these regimens provide (Bangsberg et al., 2003; Bangsberg et al., 2007)

Regardless of what mechanism HIV-1 drug resistance occurs, the current guidelines recommend the use of drug resistance testing in the clinical management of persons on antiretroviral (ARV) therapy or considering treatment. Although a significant proportion of patients are able to achieve success on treatment as demonstrated by reductions in HIV-1 viral load (VL) to undetectable levels, increases in CD4 cells, and decreased morbidity and mortality, there is still a significant cohort of patients who experience treatment failure (DHHS, 2013) and are at risk for development of ARV drug resistance. Following is an overview of HIV-1 drug resistance technology and its role in clinical management of persons on ARV therapy.

Treatment failure is defined as the inability of anti-HIV drugs to control HIV infection and it can be categorized into three different types: virologic failure, immunologic failure, and clinical failure. Virologic failure often occurs first and is characterized by the inability to suppress HIV ribonucleic acid (RNA) VL to below the limit of detection on standard laboratory assays. Virologic failure can also be defined when HIV VL is initially suppressed but increases or rebounds to detectable levels on two or more consecutive measures. These consecutive measures are important to help distinguish isolated VL blips from true signals of virologic failure (Cohen et al., 2002; Havlir et al., 2001). Immunologic failure is a consequence of the immune system not responding to ARV therapy and is characterized by a lack of an increase in CD4+ t-cells or a further decrease in CD4+ t-cells. Clinical failure is characterized by HIV disease progression despite a patient's being on ARV therapy and can be the result of many factors.

Reasons for failure include drug potency, inadequate drug levels due to suboptimal adherence or drug-drug interactions that cause sub-therapeutic drug levels, or the development of drug resistance (Kuritzkes, Boyle, et al., 2003; Quinones-Mateu et al., 2008). Regardless of the reasons for ARV therapy failure, continued ARV therapy in the presence of virology failure allows for the accumulation of drug resistance mutations (Goetz et al., 2006; Kantor et al., 2004; Kuritzkes, 2004; N. T. Parkin et al., 2000; Resch, Parkin, Watkins, Harris, & Swanstrom, 2005).

The use of resistance testing can guide clinicians in the identification of ARV agents that are likely not to be effective for the HIV that a particular person harbors, thus making it possible to individualize and construct a person's medication regimen. In 1989 the first reports of HIV drug resistance to zidovudine, a NRTI and the first drug approved by the FDA for the treatment of HIV-1 infection, were published (Larder et al., 1989; Larder & Kemp, 1989). Since then, drug resistance has been reported for all ARV agents currently used as part of highly active antiretroviral therapy (HAART) (Clavel & Hance, 2004; de Mendoza et al., 2002; Kuritzkes, 2011; Shafer & Schapiro, 2008; Tang & Shafer, 2012).

Second generation ARV agents have been developed in part to overcome resistance to the first generation of HIV-1 drugs, and also to improve adverse effect profiles and enable more convenient dosing schedules (once or twice daily versus three or four times daily). Fixed dose combinations of two or more agents have also decreased the pill count burden. Patients failing therapy are treated with these improved ARV agents in their second-line regimens, and although they can expect treatment success, there are still other issues to contend with. ARV drug costs remain high for newer drugs and tolerability problems, although improved, are not completely resolved. Deep into "salvage" therapy, often described as third-line therapy and beyond, viruses are often multiclass resistant. The increased complexity of a person's HIV-1 drug resistance profile often requires combination resistance testing and expert interpretation in order to construct an effective salvage regimen (N. Parkin et al., 2002; Zolopa et al., 2005).

Recently, data from the HIV Outpatient Study (HOPS) were evaluated to establish the impact that HIV drug resistance testing had on survival (Palella et al., 2009). In this prospectively enrolled observational cohort of patients, utilization of resistance testing in the management of ARV therapy was independently associated with improved survival among ARV-experienced patients. Although the investigators were unable to fully address the impact of resistance testing in naïve patients due to small numbers in this subgroup, they noted that benefits trended toward favoring the utility of resistance testing in this group. There are a number of other studies that support the utility of resistance testing for treatment-naïve and newly diagnosed patients as reviewed earlier (Boden et al., 1999; Dunn, Coughlin, & Cane, 2011; R. Haubrich & Demeter, 2001; Marcelin et al., 2009; Pillay, 2004; Weinstock et al., 2004).

While the benefits of resistance testing are well accepted, understanding the limits of resistance tests is also important as there are several factors that can complicate interpretation of the results. A person can be infected with multiple strains of HIV-1 (referred to as quasi-species) at the time of infection. It is therefore important to remember that currently available genotype technology is limited to detection of resistant variants representing at least 15%-20% of the total viral population. As stated above, new technologies offer improved sensitivity for detection of minority drug-resistant variants, and the clinical utility of these new tests is still being developed and tested. Quality assurance measures were mentioned previously; however, it is important to remember that previously mentioned improvement programs must also take into account assay specificity. The mutation discrimination capacity of an assay is vital to accurately and reliably report on all point mutations detected. Regardless of the sensitivity for the assay being utilized, the internal and external validation of these tests is critical as drug therapy and patient well-being are dependent upon accurate laboratory results.

Reproducibility studies, in which population and clonal analysis have been done with samples of equal mixtures of viral populations at a given amino acid position, can lead to mixed signals on corresponding electropherograms. This is a challenge for interpretation as there is a

degree of subjectivity in interpretation that can lead to discordant results due to under- or over-calling of HIV drug resistance (Shafer et al., 2001). This was reported in the ENVA-1 and ENVA-2 studies, where laboratories with less expertise reported variable results versus laboratories with more experience (Schuurman, Brambilla, de Groot, Huang, Land, Bremer, Benders, Boucher, et al., 2002; Schuurman et al., 1999). The discordant results were more problematic with samples containing mixtures and highlight the need for ongoing expertise for clinicians and laboratory personnel (Korn, Reil, Walter, & Schmidt, 2003; Sayer et al., 2003; Schuurman, Brambilla, de Groot, Huang, Land, Bremer, Benders, & Boucher, 2002; Schuurman et al., 1999).

Another concern with sensitivity of mutation detection is an algorithm's ability to recognize transmitted strains of resistant mutations. In the absence of treatment, some transmitted mutations become revertant or intermediary, as in the case of the M215F/Y to M215C/D/N/S. Some of these revertant mutants can persist for extended periods and have significant clinical implications for ARV drug selection. It is critical that genotypes be in alignment with standardized, accepted algorithms to recognize these revertant mutations as genetic footprints of previous primary resistance-associated mutations (Baraboutis, Papastamopoulos, Georgiou, & Skoutelis, 2007; Bennett, 2006; Bennett, Bertagnolio, Sutherland, & Gilks, 2008; Bennett et al., 2009; Bennett, Myatt, et al., 2008; Garcia-Lerma, Nidtha, Blumoff, Weinstock, & Heneine, 2001; Lanier, 2002; Mitsuya et al., 2008; Riva, 2002).

Following on the concept of revertant mutations, archived resistance-associated mutations can also be a challenge in sanctuary sites other than plasma. Recent studies done in detection of resistance-associated mutations in viral reservoirs other than plasma have demonstrated that resistance in other compartments can exist independent of viral species in plasma (Pao et al., 2004; Parisi et al., 2006). Studies done in cerebral spinal fluid (CSF), lymph nodes, gut-associated lymphoid tissue (GALT), vaginal secretions and semen, demonstrated that these fluids can harbor HIV-1 drug-resistant variants and be its distinct reservoir for drug

resistance–associated mutations (J. L. Adams et al., 2013; Bergroth, Ekici, Gisslen, Hagberg, & Sonnerborg, 2009; Best et al., 2009; Dumond et al., 2012; Ghosn et al., 2004; Hightower et al., 2009; Martin et al., 2006; Patterson et al., 2013; Yeh et al., 2009). Penetration of ARV drugs into these physiologic reservoirs is being studied and data on multi-compartment HIV-1 drug resistance is expanding. RT technologies have been adapted to measure drug resistance in fluids other than plasma and will continue to enhance our understanding of this phenomenon (Alcorn & Faruki, 2000; D'Aquila, 2000; Letendre et al., 2008; Yeh et al., 2009).

Complex HIV drug resistance mutation patterns detected by GT and the corresponding PT can complicate interpretation of resistance testing (Fehr et al., 2011; R. Haubrich & Demeter, 2001; Zolopa, 2003). These complex patterns can occur within any class of ARV drug and are due to the many ways in which resistance mutations can confer “cross-resistance” from one ARV agent to another (Bachelier et al., 2001; Coakley, Gillis, & Hammer, 2000; Jorgensen et al., 2000; N. T. Parkin, Chappey, & Petropoulos, 2003). Additionally, clusters of mutations within the same drug class can result in “class resistance,” as in the case of the K103N, which leads to pan-resistance to all NNRTIs in the first generation of drugs in this class (J. Adams, Patel, Mankaryous, Tadros, & Miller, 2010; Bachelier et al., 2001; Deeks, 2001; Melikian et al., 2013). Unlike the NNRTI drug class, NRTIs and PIs usually require an accumulation of mutations to confer resistance to individual agents in their class, previously described as the genetic barrier. However, there are multidrug resistance–associated mutations that can confer pan-resistance in the NRTI class, as demonstrated in vivo and in vitro by the Q151M (Loveday, 2001; Miller et al., 2012; Miller, Margot, Hertogs, Larder, & Miller, 2001; Palmer, Shafer, & Merigan, 1999).

There are also other mutations within the same class that do cause resistance to one drug but increase susceptibility or re-sensitizes an otherwise resistant virus to another drug (previously noted). Re-sensitizing effects can sometimes result in discrepant results between

the GT (resistant) and PT (sensitive). This is demonstrated in the case of the M184V and zidovudine resistance. Although a signature mutation for lamivudine or emtricitabine, the mutation has been shown to phenotypically cause reversion shifts in susceptibility curves for zidovudine (Gotte, Arion, Parniak, & Wainberg, 2000; Larder, Kemp, & Harrigan, 1995; Ross et al., 2004).

Further evidence of the complexities of genotype/phenotype discordance is the phenomenon described as hypersusceptibility (HS) (R. H. Haubrich et al., 2002; Shulman et al., 2001; Whitcomb et al., 2002). HIV drug resistance mutations conferring resistance to one class of ARV drugs can increase sensitivity to drugs in another ARV class; this has been described for NNRTI hypersensitivity (Delgado & Shulman, 2005). This is contrary to PI hypersensitivity and the N88S mutation, which is not related to resistance in other ARV drug classes (Ziermann et al., 2000). The exact mechanism of HS is not understood, but it has primarily been described in heavily treated patients with significant mutations in the NRTI class but who are naïve to drugs in the NNRTI class and who present as hypersusceptible on phenotype. There is some data to suggest that HS may enhance clinical response to therapy for NNRTI drugs, but careful interpretation of results and previous treatment history should be considered when utilizing HS results in sequencing of ARV therapy (Delgado & Shulman, 2005; Shulman et al., 2001; Whitcomb et al., 2002).

Another challenge associated with interpretation of GT and PT is the impact of mixtures. Mixtures of WT and mutant (resistant) viral strains can occur for a number of reasons. The impact of mixtures affects both assays in different ways and can result in GT/PT discordance. The detection of mixtures is limited by the sensitivity of the assays being utilized. Other causes of mixtures can be changes in selective pressure due to variations in drug levels that allow for the low-level percolation of drug-resistant variants “mixed” with wild-type clones that are both detected by drug resistance tests (De Wolf et al., 2011; Fantin et al., 2013; Woods et al., 2012).

Mixtures are expressed and sometimes interpreted differently for genotypes and phenotypes. An example of this would be for the M184V mutation, a genotype can detect mutation when present in the range of 20 percent of the viral population but to observe a phenotype shift of 2 fold-change in susceptibility is not observed until the mutation is present in at least 50% of the total population and when present in 60% of the viral pool, the shift increases to 3.5 fold (Underwood et al., 2009). The converse proportion is true of the G190A mutation, it can cause a FC shift in susceptibility on phenotype when present in five percent of the viral population but not detected on genotype until between 15%-20% of the total species (Hellmann N., 1994). These extreme examples of mixtures highlights why the interpretation requires consultation with an expert in order to formulate an optimal treatment strategy for the patient (N. Parkin et al., 2002; Sarmati et al., 2002; Zolopa, 2003) and why quality control measures are important as previously discussed.

Irrespective of whether a person with HIV-1 infection acquires drug resistance mutations through sub-optimal drug levels or becomes infected with transmitted drug resistance mutations, there is potential impact on clinical outcome and the underlying reasons must be addressed (Booth et al., 2007; Jakobsen et al., 2010; Louvel et al., 2008; Metzner et al., 2005; Parikh & Mellors, 2012; Peuchant et al., 2008; Poon et al., 2011; Varghese et al., 2009; Wheeler et al., 2010). One key interchange between acquired and transmitted drug resistance is that the relationship is very interdependent; they are the cause and consequence of each other. There is a forward and revolving cycle of events that occur. Transmitted drug resistance occurs because acquired drug resistance exists, at the time of infection and while on ARV therapy, respectively. The latter can also possibly due to a super-infection event, although this concept is still debated by some (Piantadosi, Chohan, Chohan, McClelland, & Overbaugh, 2007; Smith et al., 2005). So though the reasons for drug resistance may be different, they are very much dependent on the underlying challenges related to behavior, namely adherence to ARV therapy,

safe sex practices and harm reduction activities around drug use and other risk factors (Bangsberg et al., 2007; Bansi et al., 2010; Kozal, 2009).

Therefore addressing strategies and interventions that can reach for higher rates of adherence and harm reduction practices that will help to stop transmission events and the development of resistance are important components of HIV prevention and ARV therapy. Data consistently demonstrate that increased rate of adherence to ARV therapy significantly improves clinical outcome (Bangsberg & Deeks, 2002; Bangsberg et al., 2007).

Patterson et al (2000) estimates that an adherence rate of 95% is required to achieve success on un-boosted PI-based therapy. This high rate of adherence is required because un-boosted PI therapy usually requires a single-point mutation for the drug to become fully resistant, hence it is a low-genetic barrier drug. For a twice daily, un-boosted PI regimen, this would mean a patient could miss 0.7 doses of medications per week, less than a single dose of drug per week. For the NRTI component of the same regimen taken daily, they could miss only 0.35 of a single dose for the week. This high degree of adherence would be a challenge for many people even under the best of circumstances. Low levels of adherence in very unforgiving regimens containing ARV drugs with low genetic barriers and missing a little as one dose can equal failure over time (Bangsberg & Deeks, 2002; Bangsberg et al., 2007). Newer drugs that include boosted-protease inhibitors (BPI), and fixed dose combinations of ARV agents have improved tolerability profiles, and have contributed to convenience of dose schedules and subsequent better adherence strategies (Bangsberg & Deeks, 2002; Paterson et al., 2000). Boosted PIs, because of their enhanced pharmacokinetic profile that allows for higher and prolonged drug concentrations, often require that several mutations be accumulated before drug resistance is acquired; hence, a high-genetic barrier drug (la Porte, 2008; Luber, 2005; Winston et al., 2006).

b. Conceptual and Theoretical Framework

In order to conceptually address our evolving understanding of HIV drug resistance and genetic barriers, we can look back to early models of other viruses. Herrmann and Hermann described drug resistance as a viral phenomenon versus a host mechanism (1977). They postulated that protein changes within viruses would allow for functional changes that could subsequently permit selective advantages for resistant clones to emerge and predominate.

Even before the publication of the Hermann and Hermann theory, Oxford and colleagues had demonstrated resistance to the first drug approved for the treatment of influenza type-A (Oxford, Logan, & Potter, 1970). Shortly after this initial report of influenza resistance to first-line treatment, resistance to rimantadine, a second agent to treat influenza, was reported, demonstrating that mutants evolve and adapt quickly over time. This further supported the theory of adaptive and selective pressure that continues to be the foundation of work today for evolutionary biologist (C. B. Hall et al., 1987; Webster, Kawaoka, Bean, Beard, & Brugh, 1985). The evolution of influenza has continued to plague the scientific community with deleterious clinical effects and outcomes, and has often been compared to the HIV epidemic (Webster et al., 1985).

Interestingly, the work done with influenza has also led to other work with antiviral drug development, most notably the herpes simplex virus (HSV) and varicella zoster virus (VZV). It has been through the expanding knowledge of drug resistance mechanisms in many disease states that has driven science and technology along across multiple therapeutic areas. Isolation of gene targets such as thymidine kinase and DNA polymerase for HSV and VZV respectively has led to development of other antiviral discoveries and progress. These isolated targets have accelerated drug development strategies, expanded treatment options, and improved clinical outcome. Unfortunately, this is also where drug resistance can develop and cause problems with regard to treatment failure (Coen, 1996; Gilbert, Bestman-Smith, & Boivin, 2002).

Parallels can be drawn from the work done with influenza and other viral disease states. HIV-1 lacks proofreading capacity in its replication enzymes, specifically reverse transcriptase so although the virus can replicate rapidly it is prone to high rates of replication errors. Thus, in untreated or wild-type states, the proliferation of viruses can result in naturally occurring transcription errors resulting in mutations or polymorphisms. These polymorphic mutations occur randomly, rarely impact susceptibility alone and can be detected by resistance testing assays (J. Coffin & Swanstrom, 2013; Drake & Holland, 1999). Their presence and impact should be understood.

Understanding these viral dynamics is important because the development of mutations requires an additional step to fully characterize the impact a mutation and combination of mutations will have on clinical outcome. In order to quantify the characterization of a mutation, site-directed mutants are often generated to confirm phenotypic responses under various conditions in-vitro and in-vivo. Additionally, clinical samples with single-point mutations or combinations of mutations are often used for correlation assays (comparing genotype and phenotype responses) to gain a better understanding of these characterizations (Clavel & Hance, 2004; de Mendoza et al., 2002). It is essential to gain a clear understanding of these mutations in order to develop predictive algorithms for calling and identifying resistance (Shafer, 2002; Tang & Shafer, 2012).

It is through these early models and concepts that evolutionary biology theories have led to technology developments we use today (R. A. Fisher, 1930). It is also the early understanding of selective pressure that has added to our expanding knowledge of viral fitness and its impact on drug resistance. The concept of evolution is often attributed to Darwin and in fact, in one of his early writings he states, “natural selection can act only by taking advantage of slight successive variations; she can never take a leap, but must advance by the shortest and slowest steps” (Darwin, 1859). The works on the true nature of inheritance followed much later

by Mendel; however, Darwin laid the foundations of our understanding for the interplay between environment and adaptation (Maynard Smith, 1982).

In the case of transmitted drug resistance, undetected resistance-associated mutations have the opportunity to adapt and evolve. In theory, these micro-mutational advantages provide for optimal adaptation and evolution of resistance. As described by Fisher and Mendel, host immune factors and variability, rapid viral dynamics and turn over offer an ideal epigenetic scenario for proliferation and accumulation of compensatory mutations. One mutation leads to impairment of replication capacity that leads to mutations that compensate for loss of fitness; this leads to alternative pathways selection and to survival of the fittest clone to emerge. Acute HIV infection and the evolution kinetics of transmitted drug resistance allows for replication capacity and fitness to impact immune response. It's this immune response that triggers the host micro-environmental changes that cascades by a decline in CD4+ t-cells, or clinical failure, resulting in manifestations of symptoms or AIDS-defining events (J. M. Coffin, 1996; Swanstrom & Coffin, 2012).

Relative to any species or theory, evolutionary change is slow and gradual, but in the case of HIV-1, with high viral-replication dynamics, the expression of genetic error and drift is comparatively rapid. These changes results in mutations that can be affected by treatment with ARV therapy, adherence to ARV therapy, and failure on ARV therapy. There may be several other factors that play a role in how these resistance mutations are controlled. Host immune factors, co-infection variables (e.g., hepatitis C virus, syphilis), initial ARV drug regimen, drug adherence, drug-drug interactions that can affect ARV levels, and sanctuary site penetration of ARVs that may also be affecting how drug mutations are contained or restricted from proliferation. There are many unanswered questions.

Evolutionary geneticists often debate how to bridge gaps between theory and data. Clinicians debate how to integrate theory into clinical practice. Incorporating a theoretical understanding of HIV-1 drug resistance to individual effects and clinical utility of drug resistance

assays is important because it provides a foundation for translating science into practice (Orr, 2010).

Fitness is an intrinsic characteristic of all organisms and in the virologic sense, it becomes important to understand what is in the construct of transmitted drug-resistant quasi-species at the time of transmission (Bates et al., 2003). Studies with viruses other than HIV-1 and bacteria have demonstrated that genetic changes occurring early in adaptation impact fitness greater than later-occurring changes. It is also noted that parallel changes also occur indicating that the organism is always looking for alternative pathways for fitness compensation or escape. Genetic adaptation is always looking for a new phenotype, and Fisher's work also described a correlation between specific mutations and phenotypic impact. A parallel could be made to a genotypic sensitivity score (GSS) or phenotypic sensitivity (PSS) used in HIV drug resistance. Both conceptually aim to quantify the degree of resistance on a continuum based on accumulation of mutations for the GSS or shifting inhibitory concentration or fold-change in resistance against a laboratory reference strain for the PSS (De Luca et al., 2003; DeGruttola et al., 2000; Ross et al., 2001; Winters et al., 2009). Regardless of which assay is being used, both conceptually aim to quantify the predicted phenotypic response of HIV-1 in a treatment failing regimen or predict response for a naïve patient starting therapy.

If the theories of early works are correct as expressed by Darwin and Fisher, then the evolution of the M184V mutation for recently infected persons with HIV-1 is relevant to the theory that early detection of minor variants in patients with TDR is clinically important. Moreover, understanding the reversion kinetics of this mutation and the impact that it has on viral fitness are relevant if these contribute to the evolution of additional mutations and impact the replication capacity of a person's HIV infection. Evolutionary theory informs this study by posing that early genetic changes influence evolutionary pathways of viral changes which may suggest the importance of being able to detect minor variants early and as close to the point of transmission as possible. Our ability to monitor evolutionary changes early in therapy failure will

inform clinicians to make management decisions that will prevent the development of resistance-associated mutations by modifying therapy sooner. Further, early intervention can prevent the accumulation of additional mutations thus avoiding fitness changes that may have an impact in providing the organism with an alternative route to escape. It's this viral or pathogen escape that can lead to evolution of increased virulence and disease progression (Drake & Holland, 1999; Holland, de la Torre, Clarke, & Duarte, 1991; McMichael & Rowland-Jones, 2001).

However, these evolutionary theorists could not evolve in isolation. As noted in a historical review of evolutionary theory by Orr, the author describes evolutionary geneticist's conundrum of having an expanding foundation of knowledge built of models of mathematical theories and phenotypic evolution (2005). These theories and technologies are coupled with significant amounts of data from microbial experimental banks. In such microbial work, environmental adaptation is a key element to the observed evolutionary changes. The parallel environmental adaptation in HIV-1 infection could be considered adherence. Changes in therapeutic drug levels are the driving force in titrating inhibitory concentrations that lower or raise the genetic barriers. As previously noted these genetic barriers are ARV drug and class specific and have varying degrees of impact of viral fitness. Microbial studies have noted that genetic changes occurring earlier in evolution frequently have a larger fitness impact than those that ensue later in transition. The studies also support the notion that parallel evolution or compensatory pathways also occur commonly (Orr, 2005, 2010). Parallel to the work presented in this study, early detection of the HIV-1 *rt* drug resistance associated mutation, M184V and its impact on fitness in newly infected persons with HIV-1 is of key interest. A significant amount of data has been collected and knowledge has expanded because of advances in technology. As a result of these advances, theories have evolved.

c. Assumptions

Based on the above review, we propose that the following assumptions are relevant to this work. First, HIV drug resistance testing has progressed significantly since the first case of HIV drug resistance was reported (Larder & Kemp, 1989). Initially, genotype assays were the only resistance tests available. As phenotype resistance tests were developed, both assays were incorporated into clinical practice. The initial studies were instrumental in changing HIV treatment guidelines, which now recommend HIV drug resistance testing as part of routine clinical care for patients with HIV infection. As drug resistance technologies evolve, we assume that there will be improved performance characteristics of these assays. These improvements should include lower sensitivity to increase the detection capacity of HIV-1 drug resistance mutations.

Second, although these early studies varied in the assays utilized, patient populations, and duration of observation, findings supported the use of HIV drug resistance testing in guiding clinical decisions for selection of ARV regimens along the treatment continuum (Badri et al., 2003; Baxter et al., 2000; Clevenbergh et al., 2000; Cohen et al., 2002; V. A. Johnson et al., 2011). Newer drug resistance assays will also be added to the current recommendations for the clinical management of persons recently infected with HIV-1. It is assumed that the detection of low-abundance resistance variants could be clinically relevant and that their detection would improve surveillance of antiretroviral drug resistance (Bennett et al., 2009).

The third assumption is that viral fitness is of clinical importance. Although there have been inconsistent findings in research to date, there have been some compelling data to demonstrate that it correlates with changes in plasma HIV-1 RNA levels and CD4+ t-cell counts in treatment interruption and failure (Deeks, 2006; Deeks et al., 2001). Fitness measurements will provide additional predictive value in the clinical management of newly infected persons with HIV-1.

Finally, it is assumed that more sensitive resistance testing assays could improve the clinical management of persons infected with HIV-1 because minority variants increase the risk of failure to initial ARV therapy in persons with transmitted drug resistance mutations. Minor variant assays are suitable methods for assessing kinetics of particular mutants and their relative fitness in vivo (Barbour, Hecht, Wrin, Segal, et al., 2004; Frost et al., 2000; Wrin, 2001).

d. Research Questions and Hypotheses

As described in the review of the literature, there is a substantial gap in our understanding about the clinical utility of minor variant resistance testing assays. Based on this research gap, the proposed theoretical framework, and the assumptions described above, the *primary* research question of the study presented here is this: Is there an association between virological and immunological parameters and the presence of M184V containing variants at various proportions over time in recently infected patients with transmitted drug resistance before they initiate antiretroviral therapy?

Specifically, this study tested whether the reversion to drug-sensitive, more-fit virus (for examples, as determined by RC or relative fitness in vivo) was associated with lower or more rapid loss of CD4 count or higher viral load.

Secondary study objectives included (1) examining the difference between resistance testing methodologies and potential impact of M184V mutation detection by AS-PCR compared to standard genotyping in clinical practice, (2) examining the comparison and correlation of replication capacity and *s*-values as measures of fitness, and (3) identifying the clinical relevance of these technologies on the care and treatment of people with HIV.

e. Definition of Terms

Allele-specific PCR (ASPCR): An application of the PCR that permits direct detection of any point mutation in a target genome sequence analyzing the PCR products in an ethidium bromide-stained agarose or polyacrylamide gel. ASPCR works because an oligonucleotide primer that forms a 3 mismatch with the DNA template will be refractory to primer extension.

Therefore, oligonucleotide primers specific for all known alleles can be synthesized and used to detect the alleles in DNAs of unknown genotype. This technique is used in molecular diagnostic techniques involving the diagnosis of genetic and infectious diseases.

Drug resistance: The reduction in effectiveness of a drug such as an anti-viral, anti-infective, or anti-neoplastic in curing a disease or condition. More commonly, the term is used in the context of resistance that pathogens have "acquired," or have been transmitted. When an organism is resistant to more than one drug, it is said to be multidrug resistant. The development of antibiotic resistance in particular stems from the drugs targeting only specific bacterial proteins. Because the drug *is* so specific, any mutation in these proteins will interfere with or negate its destructive effect, resulting in antibiotic resistance.

Genotype: The genetic makeup of a cell, an organism, or an individual, usually with reference to a specific characteristic under consideration.

Phenotype: The composite of an organism's observable characteristics or traits, such as its morphology, development, biochemical or physiological properties, and products of behavior. Phenotypes generally result from expression of an organism's genes as well as other epigenetic influences of environmental factors and the interactions between the two.

Polymorph: When two or more clearly different phenotypes exist in the same population of a species.

Population sequencing: The process of determining differences in the genotype of an individual using biological assays and comparing it to another individual's sequence or a reference sequence. It reveals the alleles an individual has inherited from his or her parents. Traditionally, genotyping is the use of DNA sequences to define biological populations utilizing molecular tools. Current methods include RFLPI of genomic DNA, random amplified polymorphic detection of genomic DNA, PCR, DNA sequencing, ASPCR and DNA microarrays.

Replication capacity: A component of fitness; fitness is generally accepted to refer to the ability of an organism to replicate in a defined environment and thus is used to describe the viral

replication potential in the absence of the drug. Although viral fitness and replication capacity are related in some ways, it is important to recognize that viral fitness is not the same as viral replication capacity.

Reversion: Genetic reversion is a mutation that occurs when a mutant gene is reverted to wild-type. This reversion is located in the same place of the first mutation, which reverses the modification made previously, aka, “revertant.”. Revertant mutations are “genetic footprints” of previous mutant or ‘resistance’ as in the case of HIV resistance associated mutations and the T215C/D/E/L/S.

S value co-efficient: The formula equation utilized in population genetics, the selection co-efficient is a measure of the relative fitness of a phenotype. Usually denoted by the letter s , it compares the fitness of a phenotype to another favored phenotype, and is the proportional amount that the considered phenotype is *less* fit as measured by fertile progeny. $s=0$ then is selectively neutral compared to the favored phenotype, while $s=1$ indicates complete lethality.

Tropism: A biological phenomenon indicating growth or turning movement of a biological organism, usually a plant, in response to an environmental stimulus. In tropisms, this response is dependent on the direction of the stimulus (as opposed to nastic movements, which are non-directional responses). Viruses and other pathogens also affect what is called "host tropism" or "cell tropism," in which case tropism refers to the way in which different viruses/pathogens have evolved to preferentially target specific host species, or specific cell types within those species.

Viral fitness: In a given environment, different viral mutants compete with each other for limited (microenvironment) resources with which to replicate. Viral fitness is defined as the ability of a variant to contribute to successive generations. Viral fitness of a drug-resistant mutant, as measured in cell culture, may correlate with its relative frequency in replicating isolates.

Wild-type (WT): Refers to the phenotype of the typical form of a species as it occurs in nature. Originally, the wild-type was conceptualized as a product of the standard, normal allele

at a locus, in contrast to that produced by a non-standard, mutant allele. It is now appreciated that most or all gene loci exist in a variety of allelic forms, which vary in frequency throughout the geographic range of a species, and that a uniform wild-type does not exist. In general, however, the most prevalent allele, the one with the highest gene frequency, is the one deemed as wild-type.

III. Methodology

a. Research Design

This is an observational, retrospective, proof-of-concept sub-study of persons with transmitted drug resistance who were recently (<6 months) infected with HIV-1. Participants are enrolled into The Options Project at the University of California, San Francisco. This cohort has previously been described (F. Hecht, 1996).

b. Description of Research Setting

Data were collected at The Options Project Research Unit at San Francisco General Hospital Medical Center at the Positive Health Program (PHP), Ward 84/86. The PHP is a large, urban clinic in San Francisco, CA, providing multidisciplinary services and care to persons living with HIV in San Francisco (<http://hiv.ucsf.edu/care/>).

c. Sample

A total of 820 participants have been enrolled (1758 screened) to The Options Project (nine participants were selected for this sub-study and are detailed below). The study spans 17 years and full details of the cohort's demographics and other risk factor data has been updated and described by Hecht and colleagues (2002). Informed consent was obtained for all laboratory study-related testing (including future testing) and utilization of clinical data. Participants were seen nine times during the first year after seroconversion, and every 3-6 months thereafter. Extensive biologic and behavioral evaluations were conducted at each visit, including plasma HIV RNA levels, CD4+ and CD8+ t-cell enumeration, PBMC, plasma, serum, and saliva storage. Through interviews, data were collected on substance use, medication adherence levels, and barriers to adherence and sexual behaviors. More specialized laboratory data were also collected regularly, including resistance testing genotyping and immune-phenotyping for activation markers and for additional investigational studies as outlined in the informed consent.

i. Human Subjects Assurance

The UCSF Committee on Human Research reviewed and approved this study (IRB Number 10-00301; Reference No. 065712; Approval Date 05/02/13). All participants signed The Options Project approved informed consent form. The informed consent included an explanation of study procedures, and consent to collect and retrieve additional laboratory and other medically relevant data from the participants' medical records. All data were de-identified to remove any personal identifying information. Additionally, their Options Study ID numbers were converted to ordinal three-digit numbers (e.g., 001, 002, 003...009) for the purposes of publications and presentations.

All study staff members were extensively trained and have many years of experience in providing clinical services and psychosocial support to persons with or at risk for acute HIV infection, such as the participants in this study. All staff received direct oversight from either the Principal Investigator or the Project Director, and all new cases were reviewed every two weeks with the Principal Investigator to ensure adequacy of services provided and to ensure that the welfare of the participant was adequately protected (e.g., appropriate referrals were made for indicated services that we do not provide as part of study participation). Funding for this project is extensive, with multi-year NIH Program Project funding as well as multiple secondary funding sources. Study procedures were conducted at SFGH, with trauma center capabilities in the event of participant injury. Psychological support was available to study participants after study participation by calling the study hotline phone number.

ii. Nature and Size of Sample

The Options Project is a longitudinal observational cohort study of adults enrolled at the time of acute or early HIV-1 infection and followed throughout the course of HIV disease. The study presented here is an analysis of nine untreated Options Project participants who were newly diagnosed with HIV-1 infection, and with evidence of transmitted HIV-1 drug resistance,

including the M184V mutation. The demographics of the nine participants including age, gender, and risk factors for HIV-1 infection are in **Table 1**.

iii. Criteria for Sample Selection

Upon screening for enrollment to The Options Project, participants' recent infection with HIV-1 was confirmed utilizing a 'detuned' (less-sensitive) HIV antibody test. Negative results of a detuned EIA in conjunction with a positive routine antibody test provide evidence of infection within the preceding six months (Highleyman, 1999; McFarland et al., 1999; Parekh et al., 2002).

All participants enrolled into The Options Project were also screened for baseline drug resistance mutations utilizing population sequencing genotype assays (TRUGENE HIV-1 Genotype Kit). Nine participants with the transmitted drug resistance mutation at M184V were identified. These nine participants were followed longitudinally by additional population sequencing genotyping and qMVA assays until the initiation of ARV therapy. In addition to the M184V mutation, other baseline mutations identified by population sequencing are also included in the participant's demographic information (**Table 1**).

d. Data Collection Methods

i. Techniques

The primary laboratory assays of interest were the population sequence resistance test TRUGENE HIV-1 Genotype Kit (Siemens) and the ASPCR minor variant assay (qMVA) adapted and performed at UCSF's AIDS Research Institute (ARI), Laboratory of Clinical Virology. Participants with M184V were further monitored longitudinally by population sequencing and a ultra-sensitive, quantitative ASPCR assay (described below) until initiation of ARV therapy and following termination of therapy. Matched time point samples were utilized for population sequencing and ASPCR qMVA where possible along all designated study intervals. The time to reach background levels of M184V was compared utilizing both population sequencing and ASPCR qMVA.

Additional plasma and serum samples collected for immune studies (CD4+ t-cells and other Options Project assays previously mentioned and outlined on The Options Project Schedule of Evaluations and Procedures, **Appendix A**) were run at a commercial, clinical reference laboratory (Quest Diagnostics) contracted by the UCSF Options Project. All samples were collected in compliance within protocol-designated time points and where necessary due to participants' missed visits or other unforeseen circumstances; adjusted time points were noted and managed in data analysis.

The selection co-efficient (*s* co-efficient or *s*-value) is a measure of relative fitness of an expressed phenotype. It compares the proportional fitness of one phenotype to another and one PT is considered less fit as measured by fertile progeny or generational measurement (often calculated). Selection coefficient methods are novel analytical methods using concentrations of virus adjusting for estimates in relative fitness from exponential replication and deterioration or decline. The measurement is projected from the slope of the drug resistant proportion (r_t) that is evolving over time relative to the drug sensitive (S_t) ratios. The selection co-efficient calculations for this model is adapted from Holland and colleagues and used extensively in works with HIV and other viruses (1991; Resch et al., 2005).

Another definition is used in population genetics states that “the selection co-efficient is a measure of the relative fitness of a phenotype. Usually denoted by the letter *s*, it compares the fitness of a phenotype to another favored phenotype, and is the proportional amount that the considered phenotype is *less* fit as measured by fertile progeny. $s = 0$ then is selectively neutral compared to the favored phenotype, while $s = 1$ indicates complete lethality. For example, if the favored phenotype produces 100 fertile progeny, and only 90 are produced by the phenotype selected against then $s = 0.1$. An alternative way of expressing this is to describe the fitness of the favored phenotype as 1.0 and that of the phenotype selected against as 0.9 (1.0–0.9 or 1–*s*). The terminology is used in the same way to refer to the selective differences between genotypes to which it extends in a natural fashion.” Despite the common use of the symbol *s* to

describe a selective advantage *against* a phenotype, in some contexts the letter *s* is used to describe a selective *advantage* instead. One can, for example, speak of "a new mutation that improves fitness by $s = 0.001$ " (Carroll, 1997; Ridley, 2004).

For this study, the difference in fitness between two competing strains at time t was computed by use of the following function:

$$S_1 = \ln \left[\left(\frac{r_t/s_t}{r_{t-1}/s_{t-1}} \right) \right]$$

where r_t/r_{t-1} and s_t/s_{t-1} give the growth rates for drug-resistant and drug susceptible strains respectively. Therefore, s can be interpreted as the natural logarithm of the quotient of the growth rates of the competing strains. S is negative if ARV resistance reduces viral fitness, and s is positive if resistance increases viral fitness relative to that of the drug-susceptible competitor strain. Relative viral fitness at time of t (fit) was calculated as $fit_1 = 1 + S_1$. So then $fit(t)$ is equal to 1 if there is no difference in fitness between the competing strains, $fit(t)$ is less than 1 if ARV resistance reduces viral fitness and $fit(t)$ is greater than 1 if resistance increases viral fitness relative to that of the drug-susceptible competitor strain.

The relative fitness of two variants was estimated as described by Frost and colleagues (2000) and provides a more relative example as it assessed fitness in resistant HIV-1. In brief, the logarithm of the ratio of the abundance of two variants ($\ln[\text{variant 1}/\text{variant 2}]$) is plotted against time in generations. The slope of this line is an estimate of the selective advantage of variant 1, (s). The fold-change of the ratio per generation (e^{slope}) is referred to as relative fitness. We utilized 2.3 days per generation which was extrapolated from a series of studies cited by Resch and colleagues. The generation factor appears to be variable from 1-2.1 +/- 0.4 days with minimal impact on relative fitness estimates numbers (2005).

Relative viral fitness was calculated as described by Frost and Resch respectively (2000; 2005). The rate of change in relative proportion of M184V versus wild-type was calculated over the period between the time when the wild-type 184M was first detected by

qMVA (M184V <99.5%) and the last time at which the mutant M184V was still detectable (>0.5%). The number of generations was estimated by dividing the number of days between these 2 time points by 2.3 days per generation (Resch et al., 2005). The difference in natural logarithm of the 184V:184M percentage ratio at the beginning and end of this reversion time window was divided by the number of generations to calculate the s coefficient. Relative fitness was then calculated as e^s . Relative fitness is less than 1 if 184V reduces viral fitness relative to that of the drug-susceptible 184M wild-type virus.

ii. Instruments

The HIV RNA viral load tests were run using various assays through the course of the study due to the reference laboratory changes. At each assay change, internal validation protocols were run and quality control checks were met with standardized metrics per the manufacturer's recommendations.

The study was initiated with the Versant bDNA assay (Bayer HIV-1 RNA Assay V 3.0, Chiron 1st Generation bDNA; Chiron 2.0, Chiron 3.0 respectively). The lower limit of quantification as labeled is 75 copies/ml. The study transitioned to the Roche AMPLICOR MONITOR v1.5 with a lower limit of quantification of 50 copies/ml followed by the current assay in use the Abbott RealTime HIV-1 with a lower limit of quantification of 40 copies/ml. The major proportion of viral load results are with bDNA but in analysis, PCR was utilized if bDNA was at the lower threshold and there was a PCR measure lower than the bDNA threshold. Where bDNA was missing and there was a PCR measure available, we imputed the missing bDNA values based on co-efficient from a linear regression model that used all available data when both assay types were done, with PCR results predicting bDNA results (Elbeik et al., 2000).

The ASPCR procedure used in this study, referred to as the quantitative minor variant assay (qMVA), was run on the StepOnePlus real-time PCR™ system (Applied Biosystems, Inc., Foster City CA), described below.

iii. Description

The AS-PCR procedure and protocol has been validated for use in research studies and not for the management of HIV-1 infected individuals (Westgard & Barry, 2008). This test quantifies the relative proportion of antiretroviral drug susceptible (WT) and drug resistant (MUT) HIV-1 RNA when present as a mixture in blood plasma specimens from HIV-1 infected individuals. The drug resistance mutations interrogated by the assay are in HIV-1 reverse transcriptase (RT) and include K65R, K70E, M184V and M184I. For the purposes of this study, the mutation of interest is the M184V.

Oligonucleotide amplicons generated from the TRUGENE HIV-1 Genotyping kit (Siemens Healthcare Diagnostics, Inc. Tarrytown NY) are used as starting material. Amplicons are a 1.3 kb region spanning all of HIV-1 protease and the majority of reverse transcriptase (*rt*). Standard Operating Procedures (SOP) can be obtained by referring to ARI-UCSF LCV SOP #P0001 and #P0002:

- 1) Minor variant drug resistant populations are quantified by an allele-specific PCR (AS-PCR) amplification strategy using real-time PCR detection of SYBR Green, preceded by a “cure” PCR step to minimize the destabilizing effect from sequence heterogeneity in the primer binding regions.
- 2) The % minor variant of an unknown sample at a given drug resistance site is quantified by direct extrapolation against a standard curve using “delta-Ct” (Δ -Ct) measurements with paired MUT and WT allele-specific PCR (AS-PCR) amplifications.
- 3) Multiple primer sets each consisting of 1) a “cure” primer pair and 2) an allele-specific and universal primer pair was validated for cross-clade amplification and minor variant quantification. Primer sets are selected for each sample based on 1) proximity to the drug resistance site and 2) and closest match to the population sequence for that particular specimen.

- 4) Quality Assurance guidelines have been established for results reporting and are described in the current version ARI-UCSF LCV SOP-R #:P0008. These include but are not limited to the following:
- a) The range of Ct values for the standard curve, external plate controls (1% and 10%) and unknowns should be within the range of 10 and 32 Ct. If criteria are not met, assay must be repeated using freshly prepared standard curve, external controls and unknowns.
 - b) The standard curve R² value must be equal to or above the designated values specific for each primer set. Values were determined by calculating the mean R² value from 12 consecutive standard curves for a given primer set, minus 3SD.
 - c) The CV of triplicate Ct measurements must be ≤2.0%. If any standard curve point, control or sample CV is between 1.0 and 3.0%, the value is considered invalid. If >3.0%, the obvious outlier may be removed and %CV recalculated based on remaining duplicate specimens. The sample (standard curve, control or unknown) is considered valid if the recalculated value is ≤2.0%. This is allowable no more than 2 times per run (96-well plate with single standard curve) and no more than once each in the standard curve and external plate controls. If this occurs 3x per plate, the run is considered invalid and must be repeated.

Assay Exclusions:

- 1. Site-specific primer mismatches to population sequence, all resistance sites.
 - a. Cure or universal (downstream) primer mismatches at 3' positions 0, -1, or -2.

Primer Sets:

There are currently 8 primer sets consisting of the cure, allele-specific (ARMS) primers (mutant and wild-type), and the cognate universal primer. For the purposes of this study only 2 primer sets were utilized for the M184V subtype B viruses in these subjects.

- a. The primer sets were performed, but not included in this document; primer name, HXB2 reference location, and the 5' → 3' oligonucleotide sequence.

iv. Reliability and Validity

Assay Linear Range (reportable): the assay linear range was determined for each primer set by repeat measurements of a 7-point standard curve consisting of a varying mixture of MUT and WT DNA template for the specified drug resistance site (0.033%, 0.1%, 0.3%, 0.9%, 2.7%, 8.1% and 24.3%). The upper limit of the standard curve was determined as representing the lower limit of detection of a sequence variant using population sequencing under clinical protocols. The lower limit of the assay linear range was determined for each resistance site by visual assessment of the standard curve points, determining the lowest % MUT that consistently falls on a straight line of the standard curve. The assay dynamic range for each primer set was performed but not included in this document.

Lower limit % MUT for reporting drug resistance (biologic cutoff, BCO): HIV-1 drug resistance mutations are generated and exist within an individual in the absence of drug selection due to the relatively high mutation rate of HIV-1 reverse transcriptase. The level may be distinct for each drug resistance mutation, reflecting the mutation rate and viral replication fitness cost, and should be empirically determined in order to independently evaluate the presence of drug resistance mutations generated by selection. The background level of drug resistance was measured by directly assaying clinical specimens and viral isolate panels from 12 each subtype B, isolated from ARV-untreated individuals infected prior to the distribution of any compound selecting for mutations at *rt* 184. The absence of clinical drug resistance at the *rt* sites of interest was confirmed by population sequencing. The ranges in % minor variant drug resistance determined by the qMVA using the appropriate primer sets were performed but not included in this document. In some cases, multiple primer sets for a particular drug resistance site (e.g., "M184V" and "M184V v2 DIA") were created and validated in order to best fit natural sequence heterogeneity. A reasonable and consistent biological cutoff value was determined for each primer set by adding 3SD to the highest value determined in the panel. Values $\leq 0.50\%$ are assigned 0.50% as their biological cutoff. Those primer sets measuring $>0.5\%$ are assigned

the highest value of all panel samples +3SD (e.g., 184Vv2 D1A biologic cutoff >0.6%). Note: results above determined cutoff values represent % drug resistance variants above background levels measured from untreated HIV-1 positive individuals, and do not necessarily infer clinical significance.

Assay reproducibility (variance) measurements: assay reproducibility was measured by iterative measurements of approximate 1% and 10% standards constructed by mixing WT and MUT DNA templates at known concentrations determined by real-time PCR. The DNA sequence of each set of standards was constructed to match its cognate primer set. Twenty independent measurements of both 1% and 10% standards were made, five each replicates measured against four independent standard curves. Similar replicate assays were performed for each primer set, but not included in this document. For the 1% standards, laboratory %CV ranged between 8% and 33% including all primer sets. For the 10% standards, laboratory %CV ranged between 9% and 21% including all primer sets. Within-run and between-run variance calculations reference (Westgard & Barry, 2008):

One characteristic that differentiates the qMVA from other point mutation assays is that the ASPCR primers are based on consensus sequence, with potential target primer mismatch neutralized by the cure PCR step preceding the ASPCR. Nucleotide sequences from population genotype data were used to assign correct cure primer. The current limit of detection for variants containing M184I (M/methionine; I/isoleucine) or V (valine) is 0.3% (biological cut-off 0.5%) of the total population. Linkage of M184I or V with other mutations was not assessed.

HIV-1 drug resistance genotyping from blood plasma samples was performed by the ARI-UCSF Laboratory of Clinical Virology (LCV) using the TRUGENE HIV-1 RNA genotyping kit (Siemens Medical Solutions Diagnostics), an FDA-approved integrated assay coupled with rules-based interpretation of drug resistance mutations. The assay is based on dye-primer, cycle sequencing of the entire protease and the majority of the reverse transcriptase reading frames, and has several advantages over other existing drug resistance tests. Published

performance characteristics include a high degree of accuracy for nucleotide base identification (97%) and for codon identification at 54 sites associated with drug resistance (97.6 %) (Grant et al., 2003; Kuritzkes, Grant, et al., 2003; Tong et al., 2005). The combined amplification and sequencing reaction (CLIP) allows successful genotyping of specimens with as low as 100 RNA copies/ml of clade B and non-clade B virus. The dye primer sequencing chemistry allows reliable interpretation of viral mixtures down to 20% minority species; detection of mutant HIV-1 present in 50% of the virus population is seen with 98.9% accuracy. The fingerprinting module in the analysis software allows detection of sample or amplified product contamination by comparing recently determined sequences with sequences that have been derived in the laboratory previously. This feature is critical as a quality control measure.

All other laboratory assays were run in compliance with manufacturers' recommendations. Package insert protocols were followed in accordance with strict quality control measures in a CLIA certified laboratory (San Francisco General Hospital, Medical Center; the Department of Public Health, San Francisco; and ARI-UCSF Laboratory of Clinical Virology and Quest Diagnostics). Current CLIA certificates for each laboratory are on file as required by the CFR 21 and the CHR at the University of California, San Francisco.

e. Procedures

Demographic and clinical data were extrapolated from The Options Project's study files and supporting clinical source documents. Data were stored and managed utilizing Access Database Microsoft (Version 2010). Inconsistencies, discrepancies, or quality checks are confirmed by comparing primary care source documentation against the subject's de-identified Options study file.

Plasma samples were collected at scheduled intervals and stored at -80°C. Other biomarker samples, fluids, psycho-social data, and interviews were also collected. Some interactions are also recorded. A full schedule and explanation of procedures are outlined in The Options Project, Schedule of Evaluations and Procedures (**Appendix A**).

f. Data Analysis

The relationship between the decline in the M184V mutation and HIV-1 RNA viral load was assessed using a linear mixed model. Viral load was \log_{10} transformed for all analyses. The percent effect of change in %M184V was modeled as the difference between the %M184V and the within-cluster average %M184V, with the individual as the cluster (Neuhaus & Kalbfleisch, 1998). To avoid problems of wrong way causation, the values were lagged such that the values of the predictor arise from the measurement immediately prior to the time when the outcome was measured. Random effects for intercept and time allowed for individual differences in viral load slope and initial level. A similar modeling approach was used to assess the relationship between changes in the M184V population over time and CD4+ T cell count. CD4+ count was log transformed in the models for consistency in units of measure and linearity. The time to reach background levels (0.5%) of M184V was estimated by linear regression of % MUT during logarithmic decline using Prism GraphPad software. Analyses were done in Stata version 12.0 (Stata Corp., College Station, TX).

IV. Results

a. Summary of Results

This chapter presents a summary of the results of this study, based on each research question.

Question 1: What is the review of the literature on drug resistance and its evolution into clinical practice? The literature currently supports that resistance testing can assist clinicians and patient to:

- Avoid unnecessary switch to second-line ARV regimens prematurely, because early detection of resistance is clinically important to avoid accumulation of additional mutations, further compromising future treatment options;
- Consider thoughtfully on whether progressive regimens are truly indicated as they are often more complex and can be more costly; also leading to disease progression and clinical events; and
- Understand that prolonged periods of virologic failure and viremia can potentiate transmission events and transmission of drug resistance variants.

Question 2: What are the current and evolving technologies for HIV drug resistance testing technologies for HIV drug resistance testing with a focus on clinical utility of these assays? Two key points are:

- Given the continual diversification and global redistribution of HIV subtypes, drug resistance testing technologies are being developed to accommodate multiple strains. The need to select primer and probe sequences from highly conserved regions of the HIV genome that can build assays to reliably detect and monitor all HIV infections is becoming an essential reality.
- Newer assays with lower limits of detection and improved algorithms are necessary to keep pace with the expanding clinical needs of the patients and clinicians. Along with this is a

need to make the science translational; meaning, that the utility of the assay must be clinically meaningful and treatment decisions must be able to be rendered from the results.

Question 3: What is the association of changes in viral fitness and genotype at position 184 in reverse transcriptase with viral load and CD4 count in persons recently infected with drug-resistant human immunodeficiency virus type 1?

In this cohort of nine HIV-infected participants, with an average of 8.87 weeks of infection at study entry (**see Table 2**) who also exhibited evidence of transmitted drug resistance, the aim was to answer the following question: was there an association between virological and immunological parameters and the presence of M184V containing variants at various proportions over time before the subjects initiated antiretroviral therapy? Specifically, this study tested whether the reversion to drug-sensitive, more-fit virus (as determined by *s* co-efficient calculations or relative fitness in vivo) was associated with lower or more rapid loss of CD4 count or higher viral load.

The replication kinetics relative to immunologic and virologic responses were measured with relative difference in fitness being represented as value approached 1 and was calculated against a value of 0 to indicate no difference. As association was observed between viral fitness and viral load at the end of reversion. In a linear regression model using one time point per participant, there was no difference in viral load at the start of reversion, for each 0.1 unit increase in *s*-coefficient there was a -0.61 unit change in viral load \log_{10} copies/ml (95% CI: -2.24 to +1.02, $p=0.40$). At the end of reversion, evidence of an association was observed, for each 0.1 unit of increase in *s* co-efficient there was a decrease in viral load of 1.37 \log_{10} copies/ml (95%CI: 2.40 to 0.35 \log_{10} copies/ml; $p=0.017$). Adjusting for the duration of infection, a model of *s* co-efficient and viral load still showed a relationship: for each 0.1 unit increase in *s* co-efficient a 1.48 \log_{10} copies/ml decrease in viral load (95%CI: 2.89 to 0.07 unit decrease, $p=0.043$) was observed.

In contrast, there was no evidence for an association between viral load at the start of reversion and the s -coefficient ($\beta = 0.41$, 95%CI: -0.86, 1.69, $p=0.46$). Participant 007 excluded because his initial minor variant data sample was missing and therefore reversion kinetics could not be calculated.

Regarding the immunologic response, the linear regression models failed to show statistical significance at both the start (pre) and end (post) of reversion related to CD4 count changes with a $p=0.28$ and 0.35 , respectively, although both pre and post reversion CD4 cell increases were 186 cells. The directional change hypothesized was a more rapid or increased loss of viral load or CD4+ t-cells, therefore the null can neither be accepted nor rejected because (1) the viral load response included both a statistically significant and non-significant value when adjusted for duration of infection (the hypotheses as stated were inclusive for both virologic and immunologic response), and (2) the definition for rapid and 'increased' loss was not clearly defined and therefore subject to interpretation.

The secondary objectives included (1) examining the difference between resistance testing methodologies and potential impact of M184V mutation detection by AS-PCR compared to standard genotyping in clinical practice. A difference of approximately 6 months was observed in the detection capacity of the M184V mutation, favoring the quantitative minor variant assay (qMVA) over the population sequencing (**see Table 5**). Since improved detection of minor variants was a goal of this study and stated as such in the hypothesis, this end point was met. Whether it translated into clinical benefit is to be discussed. The clinical implications for this finding is that in newly infected persons with acute or recently acquired HIV infection, the window of opportunity for detection of HIV transmitted drug resistance mutations is relatively narrow as it has been corroborated by others (F. M. Hecht et al., 2002; Little et al., 2008). Early detection of acute HIV infection is important, but equally important is the need to establish early primary care with a HIV specialist who can provide expert guidance in the baseline diagnostics required to guide newly infected persons with fastidiousness along the treatment continuum

Differences in the detection capacity of the qMVA versus population sequencing genotype were observed but this did not translate into any clinical advantages as measured by HIV-1 RNA or CD4 + t-cells. It was noted that the slope of HIV-1 RNA decline and M184V reversion did occur early in the trajectory post-acute infection and were consistent across all participants over time. Reversion occurred quickly and the average time of infection at the start of reversion ranged from 11 to 27 weeks. Decline in HIV-1 RNA levels were more rapid compared to the fluctuant CD4+ t-cells responses that were observed. These variable responses were most likely attributable to host immune influences and individual variability. As such, immune influences on response were not measured in this study but have been described in previous works and are noted above. The clinical utility of the minor variant assay was not significant, unfortunately.

Participant 009

In this study nine participants were analyzed, but only eight were included in the final analysis. Participant 009, female, was not included as this person was on ARV therapy until approximately reversion week eight. Her data was initially included in the early analysis because this participant was the only female in this small study group. Results included a distinct linear relationship between the reversion kinetics of the M184V mutation and HIV-1 RNA viral load and CD4+ t-cell responses which were distinct in comparison to the others. To illustrate this, arbitrarily we restricted the graph to 8 weeks before the start of reversion (the length of time she was on therapy) to 181 weeks after the initial reversion point (which provided 2 data points at the lowest point on her reversion curve). The start of reversion was defined as the first time when %MUT was <99.5%, which was 99.4% for this participant, and which occurred for the first year that reversion had not started. The second time point has %MUT >99.5% but then the third was 37.5%. This time point observation did not meet the qMVA defined criteria for the lower limit of quantification and the biologic cut-off and was therefore excluded from analysis at this juncture. It is hypothesized that the participant's initial treatment that included a

lamivudine based regimen, transformed the evolution of her M184V mutation thereby affecting not only the kinetics of the mutation but also resulting shifts in fitness.

Reversion

The start of reversion was defined as the first specimen with minor variant assay values of %V<99.5%, which was the upper limit of detection for the assay. Complete reversion was identified as the first specimen where the minor variant assay had a value of %V<0.5%, which was the lower limit of detection for the assay. The total time to reversion was the difference between the two time points (from the start of reversion to when it was first complete). The mean infection duration at start of reversion was 15.21 weeks (range 3.1 to 27 weeks) with a median of 14.6 weeks (95% CI, -0.48, 55.24, p=.0537). The mean infection duration when reversion was complete was 60.4 weeks (range 30.3 to 155.1 weeks) with a median of 49.3 weeks (95% CI, 31.77, 89.03, p=.0013). The mean total weeks to reversion was 45.19 with a median of 34.9 (95% CI, 13.66, 76.72, p=.0108). **Table 3** outlines full reversion dynamics and **Figure 1** is a complement of the table that shows the full reversion kinetics of the minor variant assay for all nine participants with accompanying HIV-1 RNA and CD4 results from time point 0 through week 60.

Infection weeks were adjusted for Participant's 001 and 009 to remove time on ARV that occurred before mutation detection and when viral replication would be expected to be asymptomatic. Participant 009 had low level viremia while on ARV therapy, raising suspicion on evolution of resistance and fitness kinetic changes. The adjusted weeks were defined as the weeks since the estimated infection date minus the number of weeks on ARVs. The unadjusted times for participant 001 were 42.3 weeks and 73.0 weeks, respectively. Removing Participant 009 from analysis of reversion adjusts the p-value to <0.0001 across all previous measures for infection duration at start of reversion, completion of reversion and total weeks to reversion.

Furthermore, one caveat for Participant 007 was that the earliest minor variant assay result was 50%V (all other participants had initial values >80%V). It is likely that the "total weeks

to reversion” for Participant 007 reflects only the second half of the reversion process. This is consistent with the fact that the time to reversion measured for this person was shorter than that observed for other participants. Because of this, the value listed for infection duration at the start of reversion more likely represents some number of weeks after the true start of reversion, and the total time to reversion is underestimated. Of note this participant’s first viral load was unavailable due to an unaccounted for laboratory specimen processing error. The participant’s primary source of care was able to provide background documentation of HIV-1 RNA results but unable to provide plasma sample for minor variant assay determinations of M184V minor variant detection. There was no recalculation of statistics for this participant.

Relationship between % Mutant and Viral Load

The first model analyzed changes in the prior M184V (defined as difference between measures and average value) associated with current viral load (M184V from time point before viral load time point). This allowed for assessment of effect from one time point to the next, the model demonstrated that for every 10% drop in %V (MUT), there is a 0.01 unit increase in Log_{10} viral load or a $10^{(10 \cdot \text{coef})} = 0.969$ -fold change in viral load, which translates to a $(100 \cdot (10^{(10 \cdot \text{coef})} - 1)) = 3.1\%$ increase in viral load with every 10% decrease in %V. This was not statistically significant with $p = 0.38$. No improvement in fit was observed when adjusting for average %V and this adjusted model is similar to the first model, showing no statistically significant relationship between %V and VL differences (a 3.0% increase in viral load, or a 0.01 unit increase in Log_{10} viral load, for every 10% decrease in %V; $p = 0.39$). The co-efficient for average %V suggests that at the start of reversion, for every 10% increase in average %V, there is a 0.24 unit decrease in log_{10} viral load, $p = 0.37$.

When time is included in the model, defined as the relationship between the M184V and viral load response separate from change in viral load over time, neither term was statistically significant. However, when %V is in the model, an inverse relationship between time and viral load developed; for every 10% point decrease in %V, the model demonstrated an 8.0%

increase in viral load copies/ml (or a 0.04 unit change in \log_{10} viral load), $p=0.17$. Controlling for %V, for every 10 weeks that pass, a 16.4% decrease in viral load copies/ml (or a 0.08 unit change in \log_{10} viral load), was observed, $p=0.28$.

A random intercept model which allows for individual differences in viral load slopes and intercepts did not improve fit with statistical significance. The model is similar to the previous model above; for every 10% point decrease in %V, the model shows a 7.3% increase in VL copies/ml (or a 0.03 unit change in \log_{10} viral load, $p=0.20$. Again controlling for %V, for every 10 weeks that pass, a 15.0% decrease in VL copies/ml (or a 0.07 unit change in \log_{10} viral load), $p=0.35$.

Finally, a model was analyzed to assess for viral load against time, solely evaluating the relationship for any modifying effects of the M184V and viral load separate from change in viral load over time. This model exhibited a 14.6% increase in VL copies/ml for every 10 weeks that pass (vs. decrease over time when %V is in the model). The interaction was not statistically significant ($p=0.31$).

Participant 001 did not initiate ARV therapy after reversion was completed. Participant 002 only completed 2 weeks of ARV therapy prior to reversion being completed and never resumed therapy after that time point while on study. A parallel set of models was run restricting observations to exclude HIV-1 RNA (viral load) values lower than <1000copies/ml. This was due to possible measurement error with the minor variant assay and detection limits of the M184V mutation with low copy numbers. Participants 002 and 005 lost three time point measures with additional analysis excluding LLV because of sensitivity limitations associated with qMVA and low copy detection of the minor variants. These samples and the lower limits of quantification did not meet internal quality control standards and were therefore not included in the query. Reanalysis of this data set with low level viremia data points removed did not change statistical significance of findings.

Immune Response/CD4 Change(s)

Immune response was measured with CD4+ t-cells at regular intervals and the first model in the analysis similar to the viral load analysis looked at immune response in mixed model method. The first model examined CD4 response against the average %V (MUT); for every 10% decrease in %V, we see a 2.4% decrease in CD4 count ($= 100 * (\exp(10 * \text{coef}) - 1)$); $p=0.001$. Subsequent models explore whether this relationship is discrete from the change in CD4 count over time.

The next analysis included time and change in percent mutant virus, demonstrating that for every 10% decrease in %V we see a 2.4% decrease in CD4 count, $p=0.001$. Adjusting for differences in average %V, for every 10% difference in average %V, an 8.3% difference in CD4 count (across individuals); $p=0.57$.

In similar modeling as for viral load response incorporating a random slope and intercept, including time and change in % mutant virus, the interaction between %V and CD4 count was no longer statistically significant (and the relationship between %V and CD4 count becomes attenuated). The models show a 1.3% decrease in CD4 count for every 10% decrease in %V ($p=0.25$), and a 3.1% decline in CD4 count for every 10 weeks that pass ($p=0.24$).

Incorporating a random slope, random intercept, only factoring time against CD4 count change, a 0.7% decrease in CD4 count for every 10% decrease in %V ($p=0.46$), and a 5.1% decrease in CD4 count for every 10 weeks that pass were observed ($p=0.099$). The CD4 slope alone (change over time in model with only time) shows a 6.4% drop in CD4 count every 10 weeks ($p=0.01$).

Overall, immune responses were varied and probably reflective of the individual proportion of viremia and modified by interaction of fitness changes for each participant over time. The graphs of each participant provide a visual picture of the individual variability (see

Figures 2-10). Only absolute CD4+ t-cells were assessed; other CD4 parameters and immune markers were collected but not analyzed for the purposes of this study.

Fitness Measures

The role of impaired fitness of drug-resistant mutations such as the M184V in maintaining partial virologic suppression during reversion was observed by correlating relative fitness with changes in plasma HIV RNA levels. The selection co-efficient was estimated from the slope of the relationship between drug resistant and drug sensitive ratios over time. Measurements of the selection co-efficient (s) kinetics for the M184V resistance-associated mutation versus HIV-1 wild-type are presented on **Table 4**. Participant 007 was not included in analysis for calculation of s co-efficient because of the delayed initial time point in their first assessment of minor variant detection (previously noted). However, this participant was included for other analyses because he had TDR with the M184V mutation and other time point measures were available and were of valuable contribution regarding other assessments and variables.

The mean time of infection in weeks at time 0 for calculations of the s co-efficient was 19.8 weeks (range 11.7 to 42.3). The minimum and maximum time between s co-efficient calculation measures were 18.3 and 64.4 weeks, respectively. The slope variant that (representing variant) ranged from -0.386 to -0.032 , with a mean of -0.1544 ($p=0.0013$, 95% CI -0.2479 , -0.0609). The mean relative fitness of variant 1 (the start of reversion to variant 2) or the plateau was 0.8615 (range $0.680 - 0.978$), $p<0.0001$ (95% CI 0.7857 , 0.9372) both logarithm to the base e measures. This value is representative of the relative proportion that is changing over time, fitness is relative in relation to variant 1; variant 1 is always less fit than wild-type. Measurements across all time points and participants demonstrate comparable estimates of mutant proportions, except Participant 007.

In a linear regression model using one time point per participant, there was no difference in viral load at the start of reversion (see **Figure 11**), for each 0.1 unit increase in s -coefficient

there was a -0.61 unit change in viral load \log_{10} copies/ml at the end of reversion (95% CI: -2.24 to +1.02, $p=0.40$). At the end of reversion, evidence of an association between viral fitness and viral load is observed. For each 0.1 unit of increase in s co-efficient being correlated with a decrease in viral load of 1.37 \log_{10} copies/ml (95%CI: 2.40 to 0.35 \log_{10} copies/ml; $p=0.017$). Adjusting for the duration of infection, a model of s co-efficient and viral load still showed a relationship: for each 0.1 unit increase in s co-efficient a 1.48 \log_{10} copies/ml decrease in viral load (95%CI: 2.89 to 0.07 unit decrease, $p=0.043$) was observed.

In contrast to the viral load results, neither linear regression models of CD4 count at the start nor end of reversion had statistically significant results (see **Figure 12**). For each 0.1 unit increase in s co-efficient, there was a 186 cells/mm increase in CD4 count at the start of reversion ($\beta = -129$, 95%CI: -201 to +572; $p=0.28$). At the end of reversion there was also an increase of 186 CD4+ t-cells for every 0.1 unit increase of s co-efficient ($\beta = -140$, 95%CI: -264 to +637, $p=0.35$).

Population Sequencing Versus qMVA

Differences in detection capacity between resistance testing methods were calculated and compared to demonstrate the time from estimated infection date to the time when M184V reversion was detected by population sequencing or the quantitative Minor Variant Assay (qMVA). Difference between reversion detection by qMVA versus reversion detection by population sequencing showed that qMVA detected the M184V mutation an average (mean) of 3.9 months longer (SD=1.9 months) than population sequencing, with a range of 2.0 to 7.4 months ($p=0.0003$ by paired t test).

These times and differences were analyzed and plotted in months. Time for person 1 (= Participant 001) is ARV-adjusted time (we removed the months on ARV from the total time because this participant was virologically suppressed on ARV therapy for several months after the minor variant was detected and before reversion was found to have occurred). All other times are unadjusted (i.e., the raw data were used for patients 2–8).

b. Other Findings

To study the effect of M184V on treatment response, we looked at virologic and immunologic response to treatment among Options patients who had the mutation and who went on ART for at least 24 weeks. The first group included six subjects with M184V that was found to have completely reverted to wild type by qMVA before ART initiation, and who went on to have at least 24 weeks of ART use. The comparison group were all remaining Options participants with M184V identified by genotype within three weeks of ARV initiation (most were genotyped on their ARV start date), who were thus known to have the mutant genotype during treatment (and who also had at least 24-weeks of treatment). Using a linear mixed model with random effects for subject and left-censoring for undetectable viral loads , participants who had M184V during ARV had an average of 3.04 \log_{10} (copies/ml) higher viral loads 24-weeks after ARV initiation compared with people whose M184V had reverted to wild type before initiation of ARV therapy (95% confidence interval: -0.10 to 6.19; $p=0.057$)(Thiebaut & Jacqmin-Gadda, 2004).

In adjusting for pre-treatment \log_{10} HIV-1 RNA viral load, participants with M184V while on treatment had an average of 3.35 \log_{10} (copies/ml) higher viral load after 24 weeks of treatment compared with participants whose virus had reverted to wild-type before ARV initiation (95% confidence interval: 0.31 to 6.39; $p=0.034$). Viral load was \log_{10} transformed in all models.

The original analysis plan did not include intent to assess treatment response; however, several subjects did initiate ARV therapy. We were interested in seeing whether treatment outcomes were different in subjects starting therapy before reversion was complete versus persons who initiated treatment after reversion was completed. This was important because reversion response can be a differentiating factor between early treatment interventions versus persons who defer treatment. Given the push for early treatment in acute HIV infection it is more relevant than ever to know whether there are different outcomes for those patients who

start therapy before reversion is complete versus those who delay therapy. To examine the effect of M184V on treatment response, viral load on treatment was compared starting 24-weeks after ARV initiation between two groups. The selection of the 24-week time point was made to account for an adequate response time after the initiation of ARV therapy in treatment, naïve persons (DHHS, 2013).

V. Discussion

a. Interpretation of Findings

While the study did not answer all of the questions or support all hypotheses proposed, it did provide valuable information and support background on the questions related to transmitted drug resistance in newly infected persons, provided support for future studies. This study is unique because it looks at a small cohort of subjects recently infected with TDR focusing on fitness measurements in viruses from TDR without archived wild-type. Previously published work has presented similar measurements from acquired resistance where rebound viremia is presumably from archived wild-type. This is a unique opportunity to show different fitness values in acute and recent infection.

There is more homogenous viral population since persons infected with drug resistant virus do not harbor wild-type variants at the transmission event. Although at the time of infection, the person is exposed to numerous strains of virus; there is a virologic “bottleneck” prior to a true quasi-species” being established. Loss of detectable drug resistant variants requires replacement by a wild-type virus rather than the re-emergence of a pre-existing WT such as that in a chronically infected person where a more heterogeneous population of viruses exists. Chronically infected persons have had time for clonal expansion of their wild-type quasi-species to proliferate and for resistance mutations to “back mutate” or to be replaced.

Based on this unique perspective, utilizing resistance testing early in primary HIV infection remains important; the guidelines have established this as a standard of practice and the current data and research are moving the field towards a new generation of assays that will lower the sensitivity of assays and increase detection capacity. This small study is important because it continues to explore the question of what is the clinically relevant cut-off that needs to be established for the new generation of drug resistant assays. The use of minority variant assays is an evolving field and the technology must continue to grow and make changes to advance the understanding of drug resistance, drug development and HIV pathogenesis.

Second, the M184V mutation is one of the most common mutations in the evolution of resistance and in the transmission of drug resistance. In treatment experienced patients on failing regimens, there has been data to demonstrate that even in the presence of this mutation and in failing regimens that patients failing therapy have maintained immunologic benefit by remaining on a lamivudine or emtricitabine based regimen (Campbell et al., 2005). Furthermore, because lamivudine and emtricitabine remain on treatment guidelines as components of first-line recommended ARV regimens; this key, primary NRTI mutation is still important. Ongoing understanding of the kinetics and mechanisms of this drug resistance mutation remains critical. Should we continue to use these agents when detected in persons with TDR and the M184V mutation? In this study, of the eight participants who started ARV therapy in the course of the observation period, seven participants' initiated treatment with 3TC- or FTC- containing regimens. In primary HIV-1 infection is there benefit of initiating therapy with 3TC-, or FTC-based regimen regardless of transmitted or baseline resistance?

Third, it is generally accepted that fitness impairment of drug resistance mutations has interplay with a host's immune response and with the pathogenesis of HIV-1 infection. Though the M184V mutation is known to confer a fitness cost to HIV-1 and impair replication capacity, there is no consistent body of work that clearly demonstrates how to use fitness assays as a surrogate marker of immune or virologic predictors. Work in this area must continue and drug resistance technologies are very much an integral part of this science and research.

Finally, viral fitness can be utilized as a surrogate marker for virologic and immunologic responses that were consistent with previous data. In the presence of drug resistance mutations, e.g., the M184V, there are fitness changes as shifts in the mutation wane, fitness increases and there is a corresponding increase virologic response and resultant decline in immunologic function. The s co-efficient is reflective of a corresponding levels of in-vivo fitness and the shifts of mutant versus wild-type populations. These shifts in viral fitness can fluctuate more in acute or recent infection and are more subject to a person's host immune factors; thus,

the variability in immune response. As time progressed for participants in this study, there was an increase in corresponding viral load as fitness increased, most likely related to the return of the viral population to wild-type. The CD4+ t-cell response waned over time but increased mid-trajectory for all participants, probably reflecting some form of immune control at some point, although there is a net gain average of >100/mm cells at the start and end of reversion.

Analysis done for fitness correlations at the start and end of reversion failed to show any statistical significance changes for CD4+ t-cell response and viral load response did not show an association at the start of reversion but at the end of reversion. The limited sample sizes may have affected the ability to observe or detect a statistically significant difference. In clinical practice, selection of therapy may come into play? As previously noted, of the 8 participants in this study who initiated ARV therapy, 7 regimens included 3TC- or FTC- containing regimens. Although both of these agents are known to impair fitness, other ARV agents may or may not have fitness impacting effects. Three regimens included NRTIs plus protease inhibitors; Three participants initiated with NRTIs and NNRTI containing regimens with one of them switching to an integrase containing regimen and one switching to a protease inhibitor containing regimen. One participant initiated therapy with a triple NRTI regimen that eventually converted to an integrase containing regimen then an NNRTI regimen but a second generation NNRTI. . The last participant who initiated with a FTC-containing regimen included a protease containing regimen that rolled over to an integrase inhibitor regimen.

No data is available for the reasons for ARV switches however the switches are evidence of an important signal that makes an important point on the management of HIV drug resistance. A change of ARV agents is possible for adverse event management such as tolerability and there is no resistance management issue, however, if changes are made due to virologic failure, and whether these changes are made in tandem with drug resistance testing, these drugs should not be recycled. It is presumed that patients are resistant to these drugs if they have detectable viral loads while on therapy with these agents and should be educated to

this fact. The management of ARV therapy in relation to how viral fitness assays are utilized has been in debate and whether it can be used in acute infection or when to initiate therapy remains an unanswered question.

b. Significance

The 6-month window of detectability of the M184V mutation remains the one significant finding for the minor variant assay, but does this translate into an advantage for better treatment decisions? At face value and based on the detection of a single mutation, the M184V, it appears the answer is not completely definitive. It is unclear if additional mutations had been included by both population sequencing and minor variant assay that a difference would have been observed in clinical benefit. The limitation of a small sample size may have impacted the significance of the findings. These factors are included in the discussion on limitations.

The value of using viral fitness as a marker in relation to viral load and CD4+ t-cell response in persons with recent HIV-1 infection is ambiguous at best in this study and has limitations in this small cohort. In-vivo fitness measures are dependent on a complex cascade of factors not explored by the technique utilized in this study. Our measures were also limited by a single time point measure. Presumably these multiple host immune factors were relatively stable within individuals as reversion occurred as these participants were ARV naïve with the exception of one subject who was later removed from analysis (Participant 009).

The significance of this study is the overall richness of the data for these nine participants. There is an incredible amount of historical and longitudinal data collected on individuals who were acutely infected with transmitted drug resistant virus. While this study only looked at one mutation, there is an opportunity to ask different questions of the data and apply different statistical methods that can better inform clinicians on the utility of minor variant assays as it relates to transmitted drug resistant-associated mutations. The contributions of these participants have not been lost as the observations made and gained are valuable tools and answers to drive further questions in exploring not just good science but relevant science. It's

taking the data from the bench to the bedside and working towards interpreting these data into other opportunities across other disciplines.

The challenge with drug resistance both, in acquired and transmitted, is the heavy vigilance and consistent education needed by patients on adherence counseling and prevention (J. D. Fisher et al., 2004). It is the combined elements of prevention, education, and adherence that can lead to the bio-behavioral fundamentals needed to drive change at the bedside and in the communities among persons who struggle to live with HIV infection and need to gain motivation to adhere to medication regimens and participate in harm-reduction strategies to promote health (Bandura, 2004; Barford, Hecht, Rubow, & Gerstoft, 2006). The development of motivation takes time and can often involve persons recently infected reflecting on events leading up to their infection and needing time to think about life changing events as it relates to their own experience and how willing and able they can change behaviors and not have a repeat occurrence of infection.

There is a wealth of education required for the newly diagnosed person with HIV infection and whether knowing they have transmitted drug resistance can impact their behavior varies for individuals. A person's response around safer sex practices, other harm reduction strategies if they are injection drug users or if it affects their adherence on newly started ARV regimens will vary on an individual's previous knowledge in this area coupled with their experience in the healthcare system. Promoting a positive experience where persons with HIV infection are not stigmatized is important to ensure that access to care is achieved (Bandura, 2004; Bansi et al., 2010). The impact of an acute diagnosis of HIV seroconversion can have on a person is often devastating, when coupled with the knowledge that their seroconversion includes transmitted drug resistance, which often worsens the impact of the news. While a newly infected person may be naïve to ARV treatment, the strain of virus is not and therefore, their first regimen of therapy will not be their "true initial" therapy that "their virus" receives. This

intensity of information is often difficult for newly infected persons to comprehend and assimilate and requires intensive education for newly infected persons.

c. Limitations

The benefits utilizing a qMVA versus standard resistance testing may be limited to certain drug classes or to certain patient types; for example, only to those persons who are newly infected and who enter care early enough to receive an HIV drug resistance test.

Additionally, the small sample size, limited study demographics of all Caucasian males and who were men who have sex with men would have limits on generalizability. Perhaps the performance characteristics of the qMVA (ASPCR) and the limitations related to analyzing only the impact of the M184V mutation became too narrow of an approach. There were other transmitted drug resistance mutation data collected with the qMVA at the time of enrollment for these nine participants, but due to the small sample size and small numbers of mutations collected, the decision was made to focus on the M184V for reasons already detailed. A future study that includes the cumulative effect of multiple mutations may add to additional insights into the use of minor variant assays and improve its clinical utility. The utilization of ultra-deep sequencing technologies can provide both drug resistance testing and minor variant assessment, and studies are evolving into the use of these next generation assays and platforms (C. Wang, Mitsuya, Gharizadeh, Ronaghi, & Shafer, 2007).

Moreover, for this small group of participants, there was the random manner in which ARV treatment was initiated and that was controlled for by the participant's primary care provider. Although this is reflective of real-world scenarios, in a controlled clinical trial, one could conceivably have more opportunity to monitor for medication adherence, provide necessary support and education on therapy, and provide other enhanced study-related support inherent to a study environment.

Finally, although there were data collected on other minority variant mutations for these nine participants, only the M184V mutation was analyzed for the reasons discussed in the

statement of the problem. One reason that some studies have been inconsistent on establishing the clinical utility of minor variant assays is that they have often observed or analyzed only one mutation at a time. Some reasons center on convenience of populations, drugs of interests, and limits of technology. Five of nine participants had evidence of dual-class drug resistance, one had triple-class drug resistance, and the remaining three had evidence only of the M184V mutation at baseline. All sequence data was run through the Stanford HIV Drug Resistance database and met the criteria and definition for transmitted surveillance drug resistance mutations (Shafer et al., 2008). This is a key element of defining transmitted drug resistance: the uniformity in which mutations are defined. (Bennett, Bertagnolio, et al., 2008; Bennett et al., 2009; Bennett, Myatt, et al., 2008). Since response to ARV therapy is based on the combination of ARV drugs, it is reasonable to take into account the cumulative effect of all mutations observed at baseline in the context of TDR and minority variant detection.

d. Implication for Nursing

Nurses have a historic role in the care of persons with HIV infection and AIDS. This has included not only direct care but also a pivotal role in prevention and treatment. The role of the nurse has focused heavily on patient education and includes knowledge of HIV treatment and prevention strategies for patients. Long-term success for patients on ARV therapy depends on this knowledge of their regimens and the prevention of HIV drug resistance.

Expert knowledge and understanding of the mechanisms of drug resistance have far reaching implications for nursing practice and patient care. Implications include (1) the influence of drug resistance on selection of ARV drug therapy, (2) epidemiologic impact on the transmission of drug resistance among newly infected persons, and (3) the ongoing problem of acquired drug resistance for person on ARV therapy. Additionally, the discussion on prevention of drug resistance is an excellent opportunity for nurses to incorporate education on adherence strategies to ARV therapy. Adherence strategies are complex and require ongoing support as ARV therapy is a lifelong commitment. Constructing ARV therapy must take into consideration

many aspects of a person's needs including their baseline and historical HIV drug resistance profile, ARV treatment history, concomitant medical history and individual lifestyle and activities. Acknowledging a person's multiple needs can help construct individual care a plan ensuring each person is individually cared for and can be maximally primed for success on therapy.

The problem of HIV drug resistance is not limited to developed countries where treatment is more readily available; as developing countries' and "the worlds" access to ARV treatment expands, so will the challenges of failing regimens and subsequent drug resistance. HIV drug resistance has been described above as an irreversible complication of HIV treatment. Nursing continues to play a pivotal role in advocating and promoting successful treatment of HIV disease. Expanding nursing's capacity to understand the mechanisms of drug resistance will enhance our ability to promote the kind of education to persons infected with HIV/AIDS with the goal of preventing acquisition of drug resistance. It is important to translate our own knowledge into "patient-speak" to support persons living with HIV/AIDS in understanding the importance of adherence to their individualized ARV therapy regimens.

Translation of science into "patient-speak" requires that nursing integrate additional knowledge into their repertoire of bedside skills and care. Most nursing curricula at the undergraduate and graduate level do not currently require upper division basic science training or course work. Is it time to initiate and require as pre-requisites for admission, advance science training into nursing programs? Has nursing as a discipline in its mission to study human response to illness in holistic terms undervalued basic science knowledge and not kept pace with biomedical advances (Drew, 1988)?

Across many disease states, there is an increasing demand for response guided therapies to be monitored by companion diagnostics. This is true of many oncology driven therapies (Hodgson, Whittaker, Herath, Amakye, & Clack, 2009; Taube, 2009). Biomarkers and other molecular diagnostic tools are increasingly ordered as part of primary care management. For HIV patients, healthcare providers' routinely utilize HLA B5701 testing to prevent abacavir

(Ziagen) hypersensitivity reactions (Ma, Lee, & Kuo, 2010; Watson et al., 2009). Additionally, QuantiFERON-TB Gold and T-SPOT TB assays are now utilized clinically to monitor for mycobacterium tuberculosis infections as a more reliable methodology for detection of latent TB infections for immune compromised persons with HIV-1 infection who cannot mount an anergic response to routine PPD skin testing (Gray, Reves, Johnson, & Belknap, 2012; Santin, Munoz, & Rigau, 2012; Sultan et al., 2013). Both these technologies, like HIV-1 RNA viral load assays and HIV-1 drug resistance testing are molecular based technologies. We are well past the introduction phase of these molecular biological assays in the care and management of HIV disease. If nurses are expected to keep pace in a dynamic world of advancing technologies, it is time to start with the foundational learning and upgrade the entry requirements into the field.

Additionally, increasing understanding of the technologies for detecting and monitoring drug resistance will further allow nurses to work collaboratively across disciplines to provide state-of-the-art, evidence-based care to patients. Evidence has consistently demonstrated that collaborative models for healthcare are associated with positive clinical outcomes. Nursing continues to be a key touchstone to many aspects of care as nurses translate many elements of what transpire across the continuum of care (W. T. Chen et al., 2010; Gallant et al., 2011; Sherer et al., 2002).

In developing models of education for patients, do nurses include discussions on what resistance testing means to the patient's long-term outcome? In a study presented by the Community Programs for Clinical Research on AIDS (CPCRA), the primary grade 4 events in a cohort of over 2000 persons on ARV included liver toxicity, neutropenia, anemia, cardiovascular disease, pancreatitis, renal event, and psychiatric symptomatology. All but neutropenia were statistically significant and required discontinuation of therapy or changes in regimen. The cohort of persons was 53% and 47% treatment naïve and experienced respectively. There was a 6% rate of HBV co-infection, 17.9% HCV co-infection and <1% B and C co-infection. This early study highlights the complexity that nurses deal with in the education and management of

persons with HIV and on ARV therapy. In this study, grade 4 adverse events in the era of ARV therapy are described as important as AIDS defining events (Reisler, Han, Burman, Tedaldi, & Neaton, 2003). Although this study included older regimens that were often more cumbersome to take and had more tolerability challenges, newer regimens are not without side effects. New ARV agents remain a challenge regardless of improvements made in pill burden and side effect profiles.

Patient's fears are a major consideration when discussing adherence to potentially challenging regimens. Another study that specifically addressed predictors of adherence examined reasons for missing doses of ARV medications. The three more noted reasons included being "too busy or forgetting," "being away from home," or "having a change in their daily routine." These were followed by feeling "depressed," "taking a break or holiday from their regimen," or "having run out of their meds." Being able to incorporate the ARV regimen into their daily routine appears to be an important factor for many individuals and breaking routine requires that patients develop strategies to accommodate variations in their activities like back up doses in their gym bags or their brief cases when revealing their HIV status is not a threat. Being "outed" as HIV-positive was also a source of missing doses and developing strategies to manage medication doses was also important so once daily regimens are very effective in this scenario assuming there are no pre-existing resistance to the ARV drugs in the regimen (Gifford et al., 2000).

Nurses provide a great deal of support and strategic planning to persons facing these many types of issues and concerns. Incorporating these types of strategies coupled with the information from the patient's resistance test results can help providers to tailor the ARV regimen whether it is a daily regimen that is food dependent or non-food dependent. That may be dependent on being taken with a proton pump inhibitor 2-hours before or after their ARV dose or not; being taken without their calcium supplement or with; there are often many drug-drug interactions that make ARV regimen selection very complex. Ensuring proper dosing

schedules and strategies will ensure proper drug levels are maintained and therefore can prevent the development of drug resistance and the cycle it creates of acquisition and transmission (Gotte, 2012; Lubber, 2005).

e. Future Research

This study confirmed that standard population sequencing is able to detect a proportion of transmitted drug resistance-associated mutation, namely the M184V for a range of 2.0—7.4 months. Additionally, the minor variant assay was able to extend the detection capacity of the M184V variant for an average of 3.9 months over standard population sequencing. This extended time period of detection may have clinical benefits in early infection. If the clinician sees this mutation when making early treatment decisions, will decisions be made differently? In clinical practice, providers often see only one resistance test, the one done at the initial evaluation. This may or may not be done near the point of transmission. Rarely is a second resistance test done in clinical practice, outside the scope of a clinical trial. The question arises, should clinicians consider repeat testing and if so should a minor variant assay be utilized? The data extrapolated from this study would suggest that if early as time progresses, changing assays should be considered, particularly in early infection. As noted above, for those participants starting therapy, all but one were initiated on a lamivudine/emtricitabine-based regimen in spite of having the population sequencing data; would they have made a different decision had they seen the minor variant assay information? Understanding the value and clinical benefit of minority variant assays remains an unanswered question. More studies are needed to determine which assay provides the most consistent and relative clinical utility, and more importantly, establish the relative clinical cut-offs for detection of minor variants.

Additionally, the ideal timing of the assay's advantage, obtained nearest the point of seroconversion, also needs to be established. Are serial assays required to monitor for kinetics and reversion of minor variants to decide on the optimal timing of intervention? This study failed to establish a signal to answer this question.

Like several previous studies, despite the presence of minority variants, participants were able to achieve non-detectable HIV-1 RNA levels despite harboring the signature mutation of a key component in their initial ARV regimen. Some postulate it is a matter of proportion of drug-resistant mutants in the total quasi-species; this would be consistent with Darwinian Theory of Evolution and the survival of the fittest models previously discussed. The concept of relative proportion may also be related to the specific ARV drug and corresponding genetic barrier it is associated with. This may be why some regimens have demonstrated less clinical efficacy with higher HIV-1 RNA levels (Kozal et al., 2012; Molina et al., 2013). Studies may be necessary to evaluate if the clinical cut-offs would be to be ARV- and mutation-specific. It will be a new challenge for drug developers to come up with an individualized drug and mutation inhibitory quotient.

While the correlation between impaired replication capacity and HIV-1 drug resistance-associate mutations are well described, both wild-type and drug resistant strains of HIV have wide ranges of fitness, and it remains a relative measure. Therefore, the full utility of viral fitness assays as a true surrogate marker for disease progression remains unanswered and undefined. However, in this study there is evidence that the M184V mutation does affect fitness and did exhibit some statistical correlates with reversion kinetics. The sample size was relatively small and measures were used against only one mutation with limited time points and intervals assessed.

Additionally, as laboratory advances continue to progress with the next generation of sequencing technologies, there will no doubt be assay development in the area of viral fitness and replication-capacity dynamics. There may be ways to consolidate technologies. There are studies demonstrating that ultra-deep pyrosequencing assays can be utilized to evaluate sequence data that identify markers of acute-infection, transmitted drug resistance, and minority drug resistance variants (Andersson et al., 2013; Zheng et al., 2013).

To what degree these new technologies will impact clinical care and outcome remains to be seen. Nursing is in a key position to collaborate with its basic science research colleagues, laboratory collaborators, and physician partners; it's through these cross-disciplinary relationships that specialties expand and foundations of knowledge grow. It is essential that we translate science from the bench to the bedside.

VI. Table 1: Subject Demographics and Population Genotype

Subject ID	Gender	Age (years)	Risk Factor	List of baseline SDR mutations Stanford HIVDB
001	M	56.9	MSM	M46I, N88D, L90M D67N, M184V, T215Y, K219Q
Other NRTI/NNRTI mutations				I37X, D67N, K104R, D123E, I135L, M184V, I195L, T200A, Q207E, T215Y, K219Q
Protease mutations				T12K, L19V, E35D, M36I, N37D, M46I, L63P, A71T, V82I, N88D, L90M, I93L
002	M	37.6	MSM	M41L, M184V, T215Y
Other NRTI/NNRTI mutations				I37X, M41L, K103N, K122E, I135V, I142V, E169D, D177E, I178L, V179A, M184V, R211K, T215Y, K238T
Protease mutations				I15V, L19I, N37D, L63V, T74A, V77I
003	M	34.4	MSM	M41L, M184V, L210W, T215Y M46L, I54V, V82A, L90M
Other NRTI/NNRTI mutations				I37X, M41L, K43Q, K103N, V118I, I135T, E138Q, Q174R, M184V, L210W, R211K, T215Y
Protease mutations				L10I, T12P, L19I, K20R, E35D, M36I, N37D, M46L, I54V, R57K, Q61R, I62V, L63P, A71V, T74S, V82A, L90M, I93L, C95F
004	M	41.5	MSM	M184V
Other NRTI/NNRTI mutations				I37X, K122E, I135T, M184V, R211K
Protease mutations				I15V, M36L, N37A, D60E, Q61E, I62V, L63P
005	M	47.8	MSM	K70R, M184V L24I, M46I, I54V, V82A, N88D
Other NRTI/NNRTI mutations				I37X, S48T, D123E, I142V, D177E, M184V, T200A, R211K, V241L
Protease mutations				L10V, N37S, L63T, V77I, I93L
006	M	49.0	MSM	D67N, K70R, M184V, K219Q K103N
Other NRTI/NNRTI mutations				I37X, V60I, D67N, K70R, K103N, I135T, Q174H, M184V, G196E, T200I, I202V, Q207K, R211G, K219Q, K238N
Protease mutations				T12P, K14R, I62V, L63P, I93L
007	M	61.4	MSM	F77L, Y115F, F116Y, Q151M, M184V I54V, G73S, V82A, L90M
Other NRTI/NNRTI mutations				I37X, K46Q, K49R, A62V, V75I, F77L, V108I, Y115F, F116Y, D123E, Q151M, D177E, M184MV, Q207N, R211K
Protease mutations				L10I, I15V, L19I, K20R, E35D, M36I, N37E, R41K, I54V, Q58E, D60N, I62V, L63P, K70R, A71V, G73S, V82A, L90M, I93L
008	M	24.5	MSM	M184V
Other NRTI/NNRTI mutations				I37X, T39A, R83K, K104Q, D123E, M184V, T200A, Q207A, R211K
Protease mutations				L10I, I15V, L19I, K20R, E35D, M36I, N37E, R41K, I54V, Q58E, D60N, I62V, L63P, K70R, A71V, G73S, V82A, L90M, I93L
009	F	41.6	Hetero	M184V
Other NRTI/NNRTI mutations				I37X, S48T, D123E, I142V, D177E, M184V, T200A, R211K, V241L
Protease mutations				L10V, N37S, L63T, V77I, I93L

* All participants were Caucasian

VI. Table 2: Duration of Infection

Participant	Infection Duration at Study Entry (weeks)	Infection Duration at First M184V Genotype¹ (weeks)
001	3.4	3.4
002	10.3	27.0
003	3.4	14.7
004	10.3	20.0
005	3.4	3.4
006	10.3	12.3
007	14.6	18.6
008	10.3	11.6
009	12.1	12.1

First M184V genotype was done by population sequencing for all patients; qMVA quantification of the minor variant was also performed on the first specimen for patient numbers 002, 004, 007, and 008.

VI. Table 3: Reversion Dynamic Calculations (relation to duration of infection in weeks)

Participant	Infection duration at start of reversion	Infection duration when reversion is complete	Total weeks to reversion
001 ²	17.2	47.9	30.7
002	27.0	73.3	46.3
003	11.7	46.6	34.9
004	20.0	41.0	21.0
005	14.6	49.7	35.1
006	14.4	50.4	36.0
007 ³	14.3	30.3	16.0
008	14.6	49.3	34.7
009 ¹	3.1	155.1	152.0

Duration of HIV infection at times associated with M184V mutation, and time to reversion (all times in weeks). The start of reversion was defined as the first specimen with minor variant assay values of %V<99.5%, which was the upper limit of detection for the assay. Complete reversion was identified as the first specimen where the aMVA valuer reached %V<0.5%, or was at the lower limit of detection for the assay. The total time to reversion was the difference between the two times (from the start of reversion to when it was first complete). ^{1,2} Infection weeks were adjusted for Participants 009 and 002 to remove time on ARV (that occurred before mutation detection and when viral replication would be expected to be asymptotic, although subject 37 had low level viremia while on ARV). The adjusted weeks were defined as the weeks since the estimated infection date minus the number of weeks on ARV. The unadjusted times for 009 were as follows: 182 weeks of infection at the start of reversion, and 334 weeks of infection when reversion was complete. The unadjusted times for 002 were 42.3 weeks and 73.0 weeks, respectively). ³ The earliest minor variant assay result for Participant 007 was 50%V (all other participants had initial values >80%V). It is likely that the “total weeks to reversion” this subject reflects only the second half of the reversion process.

VI. Table 4: S Co-Efficient Calculations

Study ID	time 0 (weeks) start of reversion	time 1 (weeks) end of reversion	time (weeks)	Generations (2.3.days)	%V time 0	ln(%V0)	%V time 1	ln(%V1)	delt ln(%)	Slope Variant 1 (WT)	S (relative fitness variant 1 [WT] in relation to Variant 2 [M184V])
009	182	334	152	462.61	99.4	5.110	0.47	-5.355	-10.465	-0.023	0.978
001	42.3	60	17.7	53.87	95.33	3.016	5.61	-2.823	-5.839	-0.108	0.897
002	27	64.4	37.4	113.83	89.3	2.122	18.66	-1.472	-3.594	-0.032	0.969
003	11.7	37.7	26	79.13	97.95	3.867	0.76	-4.872	-8.739	-0.110	0.895
004	20	31.3	11.3	34.39	82.7	1.565	8.33	-2.398	-3.963	-0.115	0.891
005	14.6	33.6	19	57.83	86.4	1.849	4.1	-3.152	-5.001	-0.086	0.917
006	14.4	31.3	16.9	51.43	93.03	2.591	0.59	-5.127	-7.718	-0.150	0.861
007	14.3	18.3	4	12.17	50	0.000	0.9	-4.701	-4.701	-0.386	0.680
008	14.6	26.3	11.7	35.61	99.38	5.077	2.4	-3.705	-8.782	-0.247	0.781

The mean time of infection in weeks at time 0 for calculations of the s co-efficient was 19.8 weeks (range 11.7 to 42.3). The minimum and maximum time between s co-efficient calculation measures were 18.3 and 64.4 weeks, respectively. The slope variant that (representing WT) ranged from -0.386 to -0.032, with a mean of -0.1544 ($p=0.0013$, 95% CI -0.2479, -0.0609). The mean relative fitness of WT (the start of reversion to M184V) or the plateau was 0.8615 (range 0.680 – 0.978), $p<0.0001$ (95% CI 0.7857, 0.9372) both logarithm to the base e measures. This value is representative of the relative proportion that is changing over time, fitness is relative in relation to variant 1; variant 1 is always less fit than wild-type. Measurements across all time points and participants demonstrate comparable estimates of mutant proportions, except Participant 007.

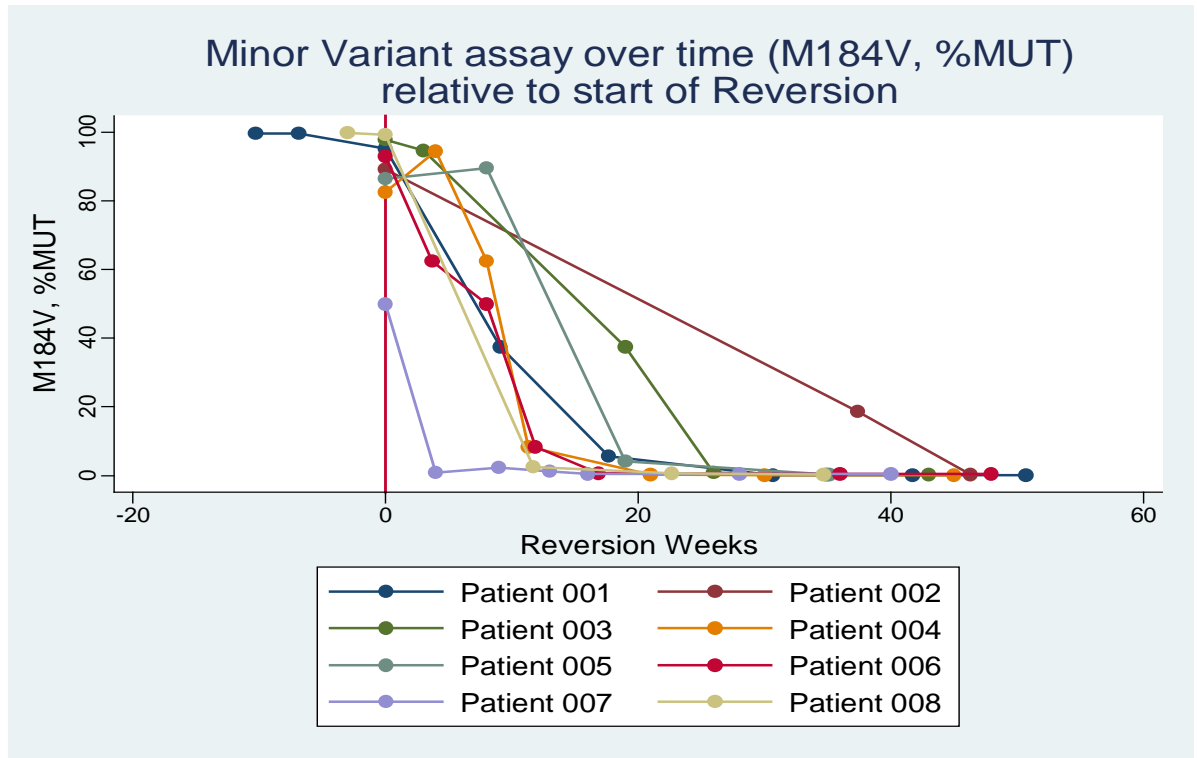
VI. Table 5: Differential Detection Capacity

Population Sequencing vs. Quantitative Minor Variant Assay (qMVA)

Participant	Infection Duration at Reversion (months)	
	AS-PCR (minor variant assay)	Population Sequencing
001	11.0	6.0
002	16.9	14.8
003	10.7	8.7
004	9.4	6.4
005	11.4	7.7
006	11.6	4.2
007	8.0	5.2
008	11.3	6.0
009	35.7	35.7

Difference between reversion detection by qMVA versus reversion detection by population sequencing showed that qMVA detected the M184V mutation an average (mean) of 3.9 months longer (SD=1.9 months) than population sequencing, with a range of 2.0 to 7.4 months ($p=0.0003$ by paired t test).

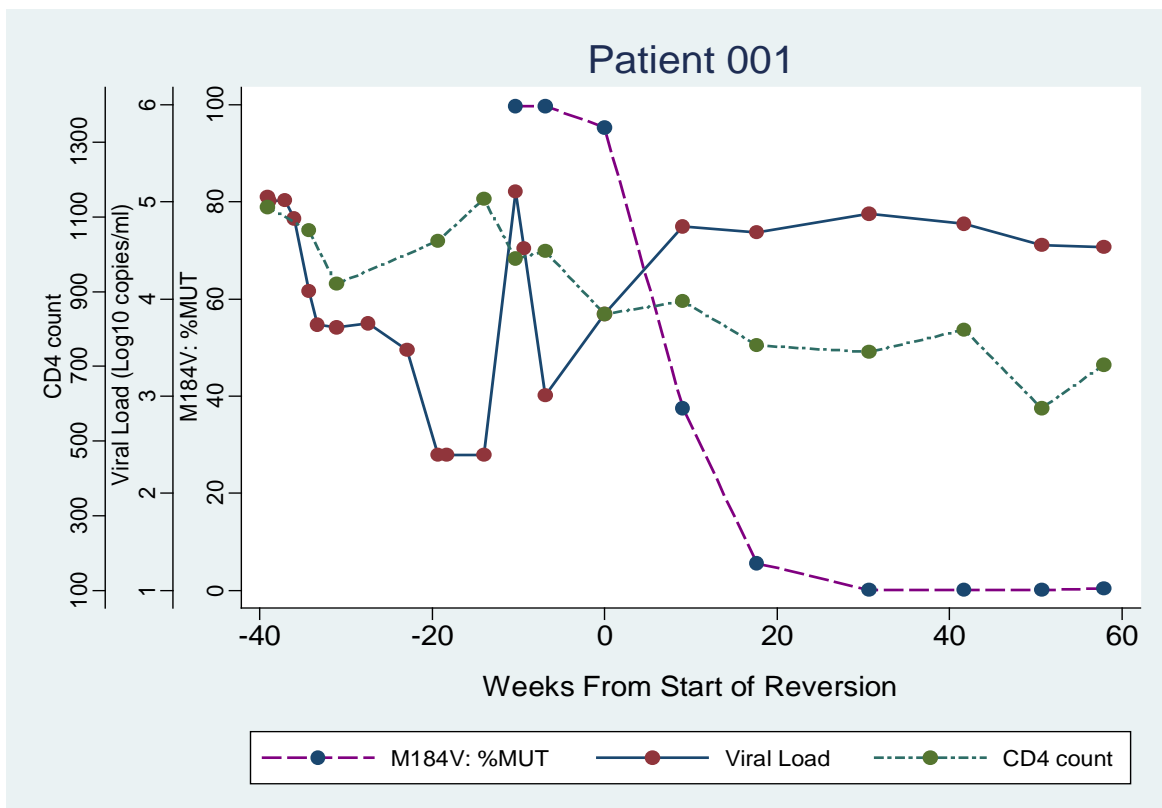
VII. Figure 1: Reversion Kinetics Quantitative Minor Variant Assay
(% Mutant M184V from Start of Reversion to Plateau)



The start of reversion is defined as the first qMVA value of % V < 99.5% = week 0.
 End point analysis truncated at week 60 for all participants (extended data available for all subjects).

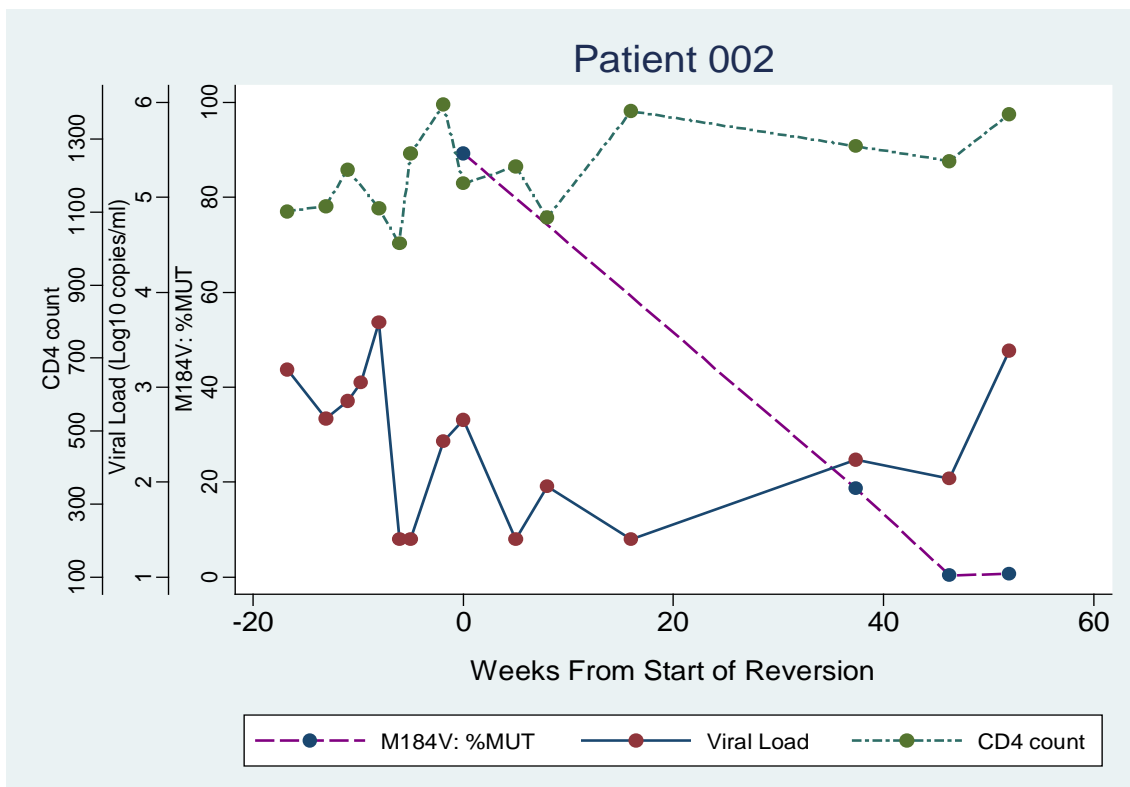
VII. Figure.2: Reversion Kinetics (HIV-1 RNA, CD4, % Mutant M184V)

Participant 001



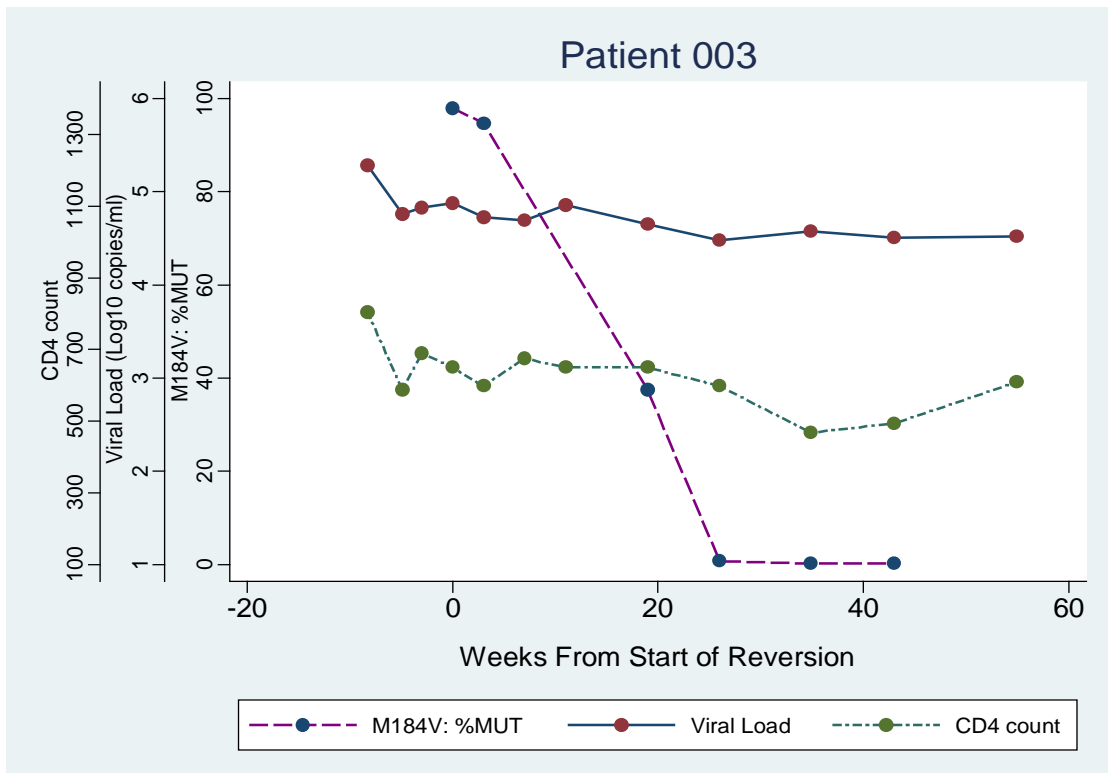
VII. Figure 3: Reversion Kinetics (HIV-1 RNA, CD4, % Mutant M184V)

Participant 002



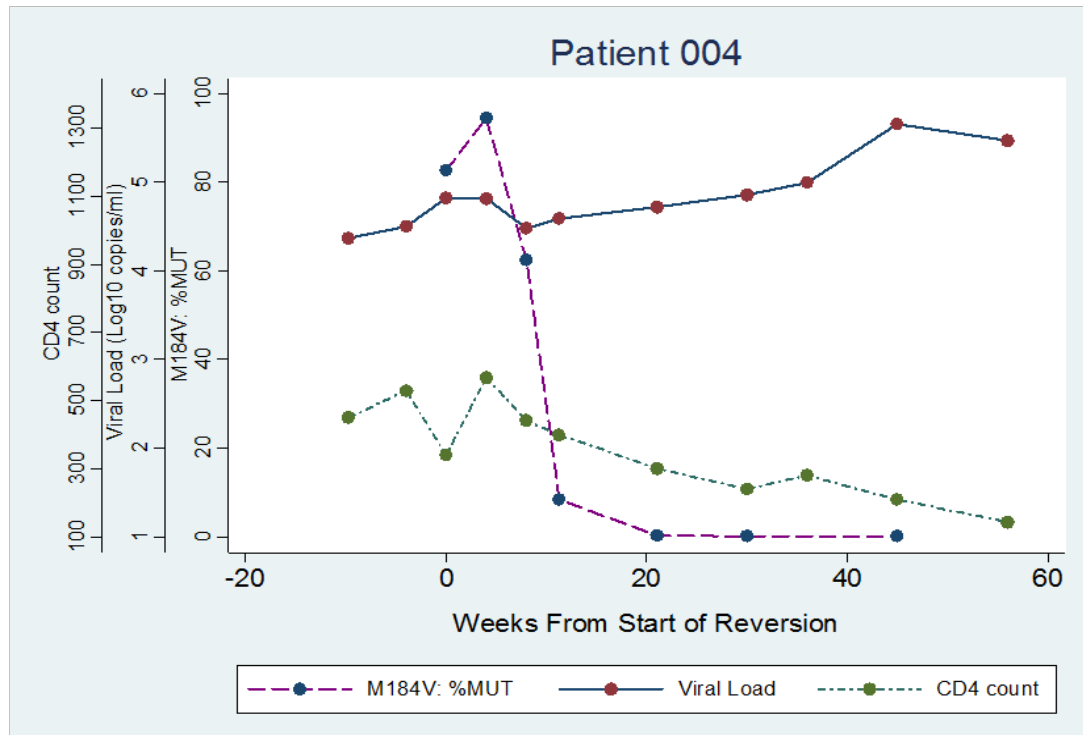
VII. Figure 4: Reversion Kinetics (HIV-1 RNA, CD4, % Mutant M184V)

Participant 003



VII. Figure 5: Reversion Kinetics (HIV-1 RNA, CD4, % Mutant M184V)

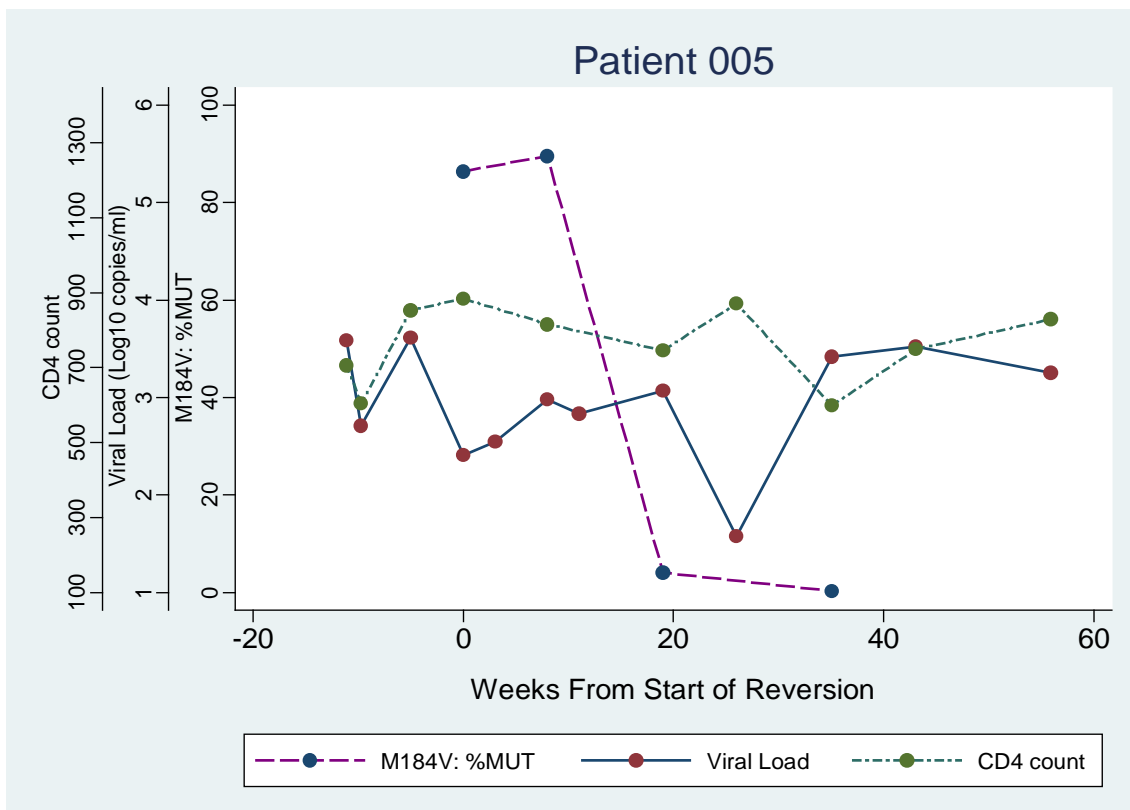
Participant 004



Initiated ARV at 19.5 months (on a regimen to which M184V converts resistance), however, changed regimens several times over a few weeks and started initial regimen not impacting M184V at 20.3 months. The apparent drop in VL just after 75 months is possibly an artifact of the assays—the lab switched from bDNA (LOD=75 copies/ml) to PCR (LOD=40 copies/ml); for these analyses, (0.5*LOD) (log10 transformed) was used for VL values at the lower limit of detection.

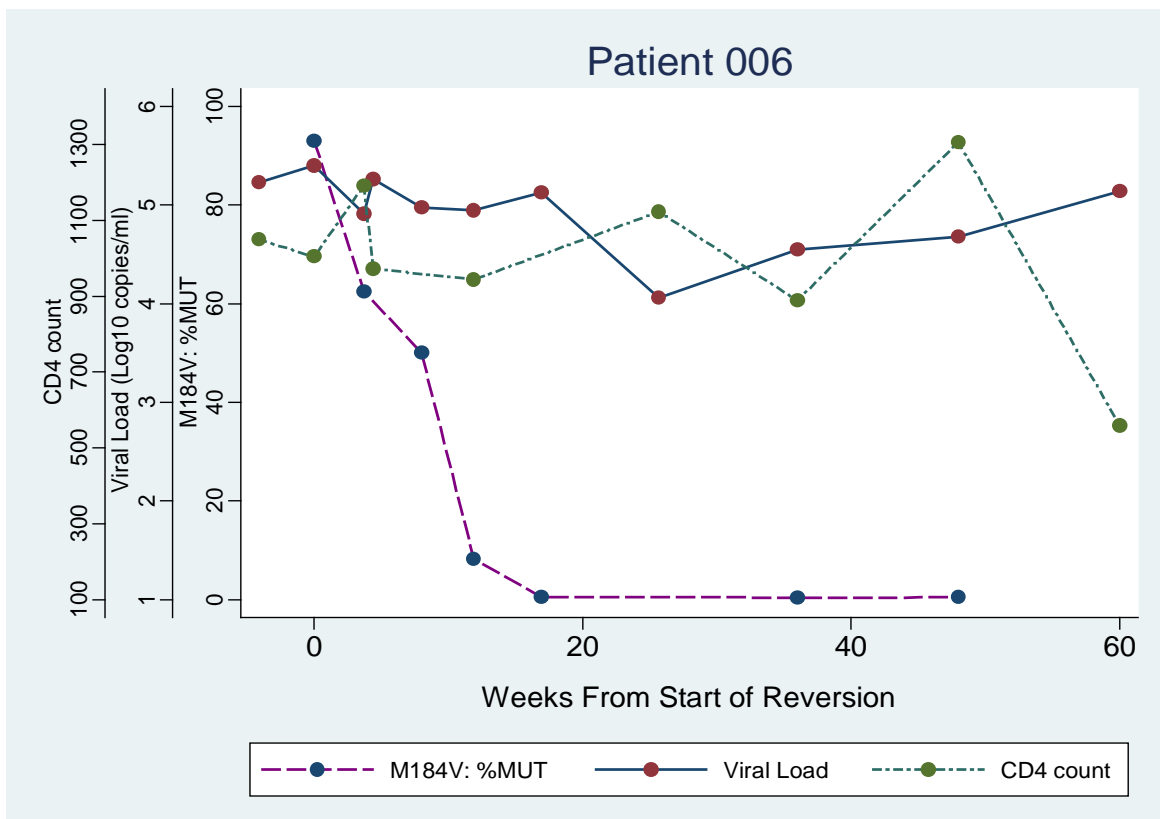
VII. Figure 6: Reversion Kinetics (HIV-1 RNA, CD4, % Mutant M184V)

Participant 005



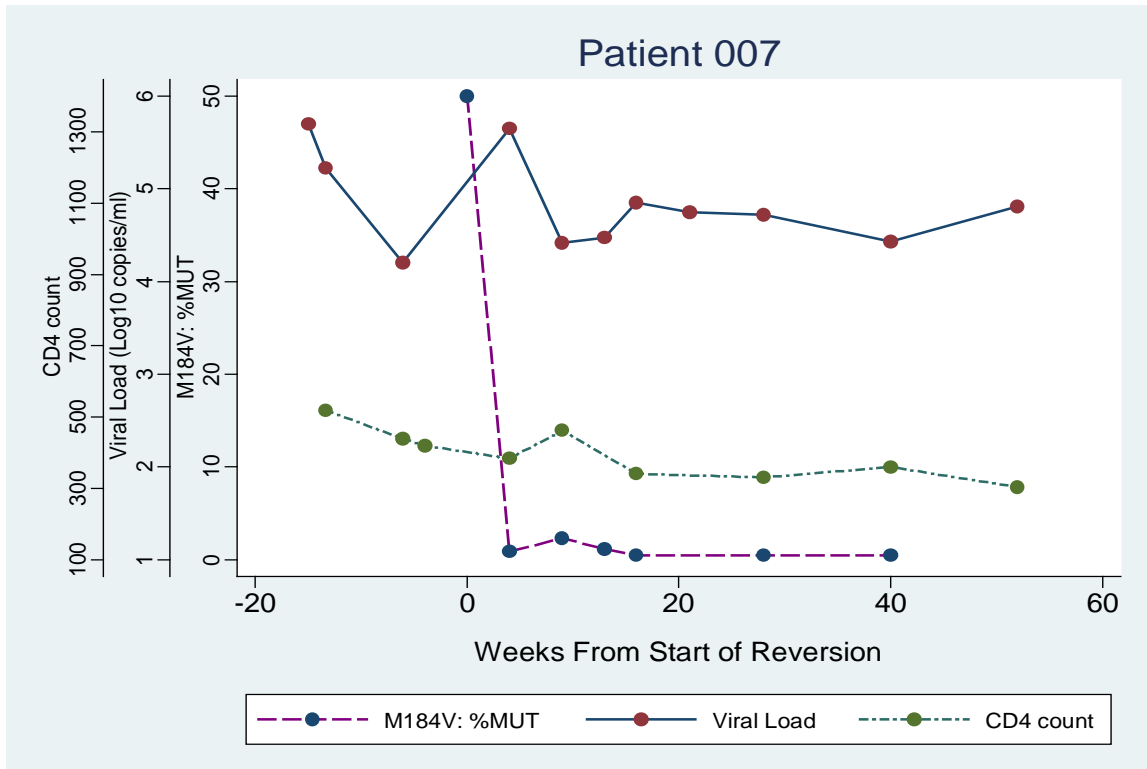
VII. Figure 7: Reversion Kinetics (HIV-1 RNA, CD4, % Mutant M184V)

Participant 006



VII. Figure 8: Reversion Kinetics (HIV-1 RNA, CD4, % Mutant M184V)

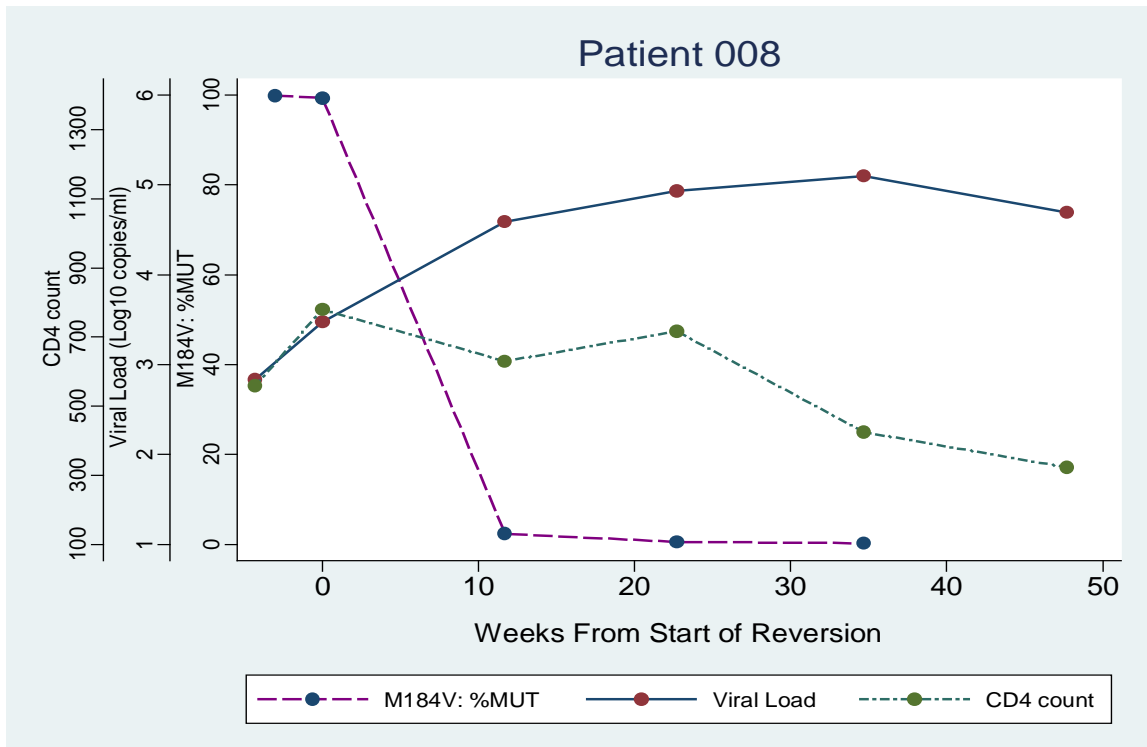
Participant 007



This is the only participant whose ARV regimen did not include medications to which M184V confers resistance. Note that this graph includes an updated infection date and additional lab data immediately following seroconversion that were recently recovered from the primary care provider. Missing initial minor variant data and not included in final s co-efficient analysis.

VII. Figure 9: Reversion Kinetics (HIV-1 RNA, CD4, % Mutant M184V)

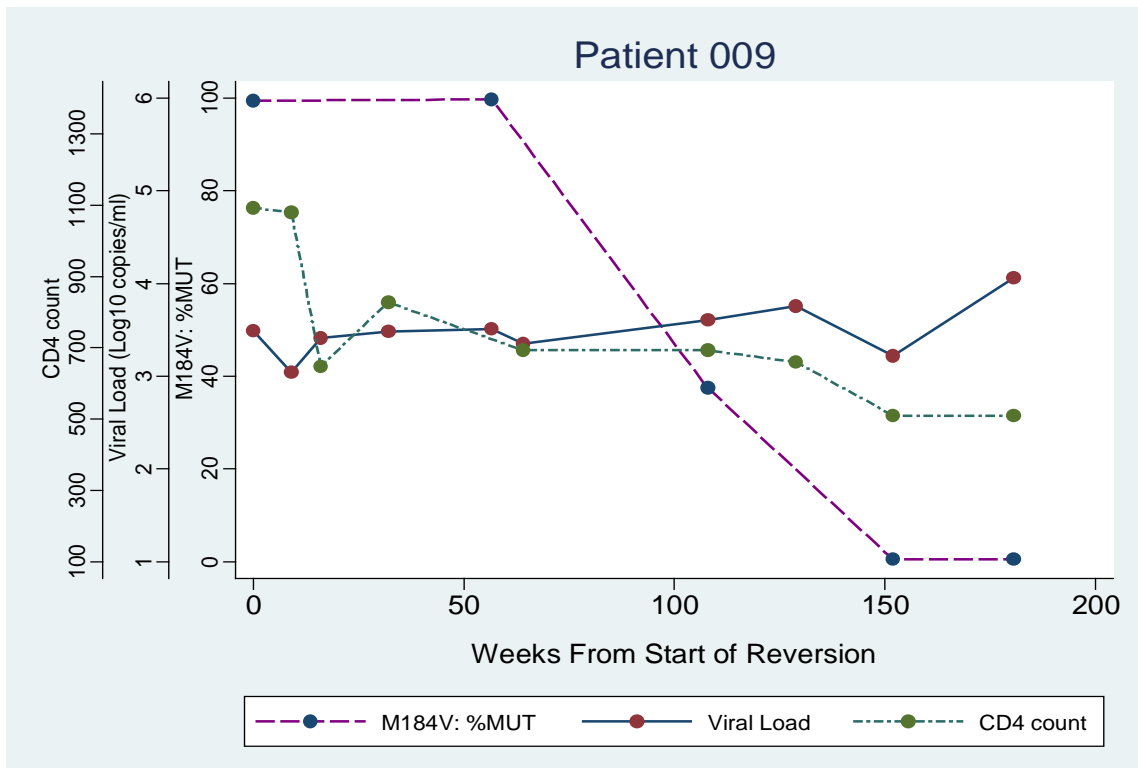
Participant 008



Participant has a large gap between VL measures around the start of ARV. Due to this gap between measures, the VL slope around ARV initiation in the graph below may mis-represent the true change in VL over time in the immediate post-ARV-initiation period. It was a more reasonable assumption to draw a horizontal line from the last pre-ARV VL to the start of ARV, and then draw the line down from that point to the first post-ARV initiation VL.

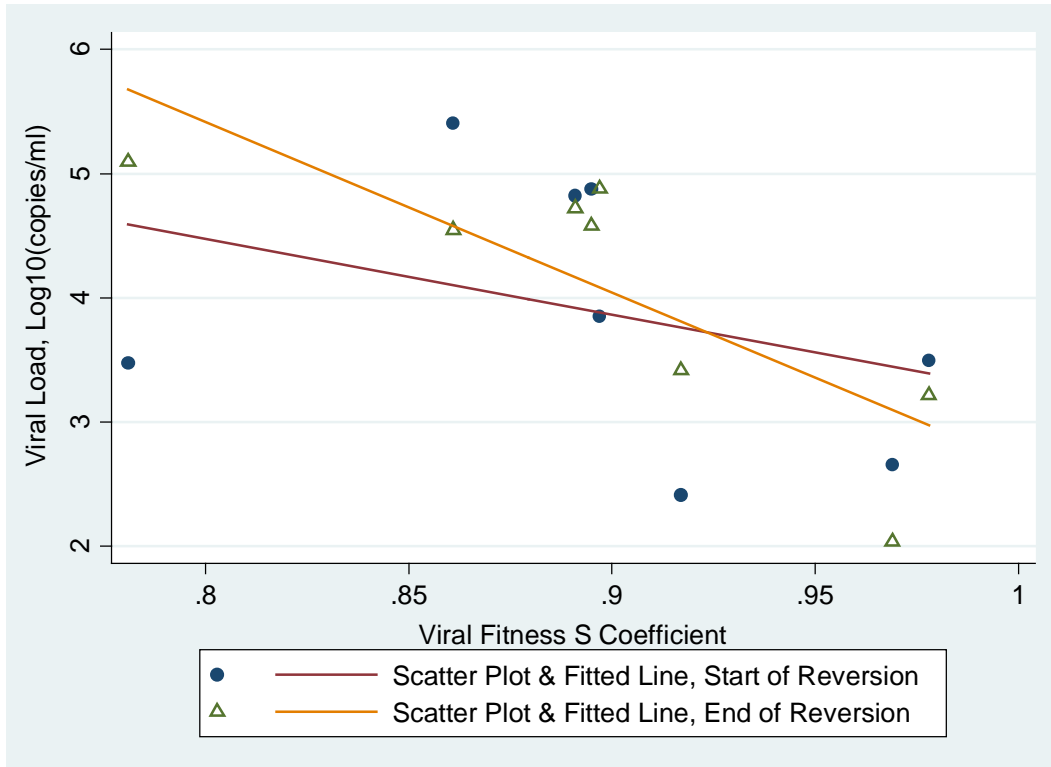
VII. Figure 10: Reversion Kinetics (HIV-1 RNA, CD4, % Mutant M184V)

Participant 009



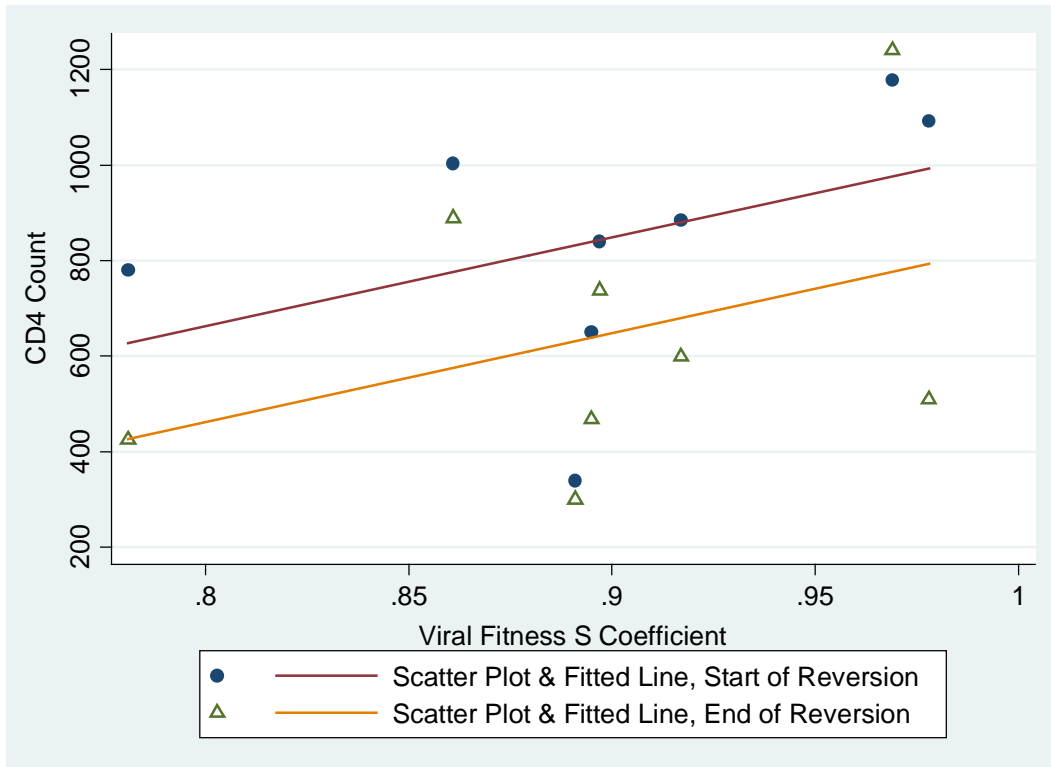
Because of prolonged treatment without adequate viral suppression before M184V reversion was detected, this individual is not included in modeling analyses.

VII. Figure 11: S Co-efficient and Viral Load at the Start and End of Reversion



In a linear regression model using one time point per participant, there was no difference in viral load at the START of reversion, for each 0.1 unit increase in *s*-coefficient there was a -0.61 unit change in viral load log₁₀ copies/ml (95% CI: -2.24 to +1.02, *p*=0.40). At the END of reversion, evidence of an association was observed, for each 0.1 unit of increase in *s* co-efficient there was a DECREASE in viral load of 1.37 log₁₀ copies/ml (95%CI: 2.40 to 0.35 log₁₀ copies/ml; *p*=0.017). Adjusting for the duration of infection, a model of *s* co-efficient and viral load still showed a relationship: for each 0.1 unit increase in *s* co-efficient a 1.48 log₁₀ copies/ml DECREASE in viral load (95%CI: 2.89 to 0.07 unit decrease, *p*=0.043) was observed. *N*=8; Participant 007 excluded (initial data point missing); Participant 009 excluded from final virologic and immunologic analysis but included in fitness analysis for observational purposes.

VII. Figure 12: S Co-efficient and CD4+ t-cell at the Start and End of Reversion



In contrast to viral load results, linear regression models of CD4+ t-cell counts at both the start and end of reversion did not have statistically significant results. For each 0.1 unit increase in s-coefficient, there was a 186 cell decrease in CD4+ t-cell counts at the start of reversion ($\beta = -129$, 95%CI: -201, +572; $p=0.28$). The results were similar with CD4+ t-cells increase of 186 cells at the end of reversion ($\beta = -140$, 95%CI: -264, +637; $p=0.35$). N=8; Participant 007 excluded (initial data point missing); Participant 009 excluded from final virologic and immunologic analysis but included in fitness analysis for observational purposes).

VIII. Appendix 1: The OPTIONS Project–Schedule of Evaluations and Procedures

Visit	Screen ²	Result ¹¹	Entry ³							Wk48 - Wk240	Beyond Wk240 ¹⁴
Cohort Study Week ¹¹	-4	-2	0	4	8	12	16	24	36	48, q12wk ¹³	q 24 wk
Visit Window, weeks (w) ¹			+30d ¹⁵	±2w	±2w	±2w	±2w	±4w	±4w	±6w	
Administrative											
Informed consent	X										
Eligibility checklist	X										
Demographic information	X										
Medical records consent	X										
Patient feedback form ¹⁷			X					X		q24wks	X
Locator information ¹⁸	X		X	X	X	X	X	X	X	X	X
Reimbursement	X	X	X	X	X	X	X	X	X	X	X
Clinical											
ARS assessment	X										
Medical history, exam ⁴			X					X		q24wks	X
STD testing	X ²⁵	[X]	[X]	[X]	[X]	[X]	[X]	VDRL	[X]	VDRL q24wk	[X]
Rx and AE review			X	X	X	X	X	X	X	X	X
Psychosocial											
Pre/post-test counseling	X	X									
Psychosocial assessment	X										
Risk behavior survey ²³	X		X ¹⁹			X		X	X	X	X ²¹
Partner referral review ²	X	X	X	X	X	[X]	[X]	[X]	[X]	[X]	[X]
Risk reduction counseling, condoms	X	X	X	X	X	X	X	X	X	X	X
Phlebotomy											
HIV-1 testing ^{5,12}	X		[X]	[X]	[X]	[X]				[X] ¹²	
Hemoglobin ⁶	X	[X]	[X]	X	X	X	[X]	X	[X]	X	X
CBC with CD4+ cell count ⁷	X		X	X	X	X	X	X	X	X	X
HIV-1 RNA viral load ⁷	X	X	X	X	X	X	X	X	X	X	X
Genotyping/resistance ⁸		X		[X]	[X]	[X]	[X]	[X]	[X]	[X]	[X]
LFT/chemistries ¹⁶	X			X						q48wks	
PT/PTT	X										
Hepatitis screening			X								
Dried Blood Spots	X		X	X	X	X	X	X	X	X	
PBMC, plasma (mL)	90	60/90 ²⁴	100	60	60	90	60	90	60	60/90 ²²	60
Min Blood Volume (mL)	146	88	124	94	80	110	80	117	80	109	103
Max Blood Volume (mL)¹⁰	175	175	175	175	200	200	200	200	200	200	200
Other biosampling											
Genital and oral fluids ⁹	X		X								
Hair (substudy only)		X									
Urine (kidney function)	X					X					
Other tests for specific aims	[X]	[X]	[X]	[X]	[X]	[X]	[X]	[X]	[X]	[X]	[X]

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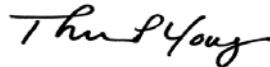
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