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Clinical Implications of Minority HIV-1 Resistant Variants

Directors:

Daniel R. Kuritzkes
Bonaventura Clotet i Sala

Doctoral Thesis
Departament de Medicina
Universitat Autònoma de Barcelona
2009

DEPARTAMENT DE MEDICINA
UNIVERSITAT AUTÒNOMA DE BARCELONA

DOCTORAL THESIS, 2009

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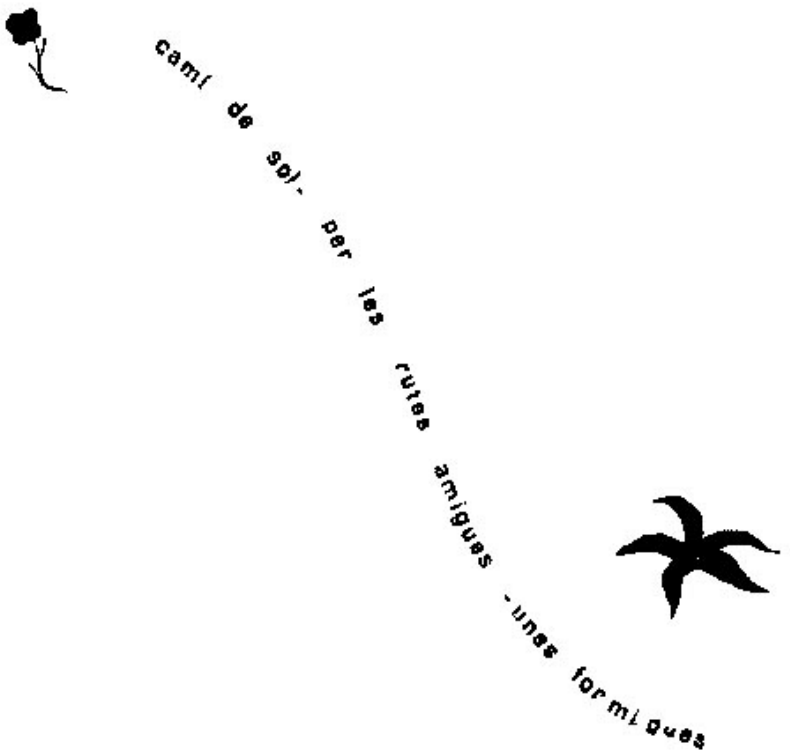
Thesis Directors:

Daniel R. Kuritzkes, MD
Harvard Medical School, Boston, Massachusetts, USA.

Bonaventura Clotet i Sala, MD, PhD
Facultat de Medicina
Universitat Autònoma de Barcelona, Barcelona, Catalonia, Spain.

Als meus pares, els meus mestres



Joan Salvat-Papasseït
Les formigues - *L'irradiador del port i les gavines.*

"(...)I took several specimens of an Octopus, which possessed a most marvellous power of changing its colours; equalling any chamaelion, & evidently accommodating the changes to the colour of the ground which it passed over, yellowish green, dark brown & red were the prevailing colours: this fact appears to be new, as far as I can find out. (...)"

Letter from Charles Darwin to John Stevens Henslow
Cape Verde, 16 June 1832

Abbreviations code

3TC	Lamivudine
ABC	Abacavir
APV	Amprenavir
ART	Antiretroviral therapy
ASPCR	Allele-specific polymerase chain reaction
ATV/r	Ritonavir-boosted atazanavir
d4T	Stavudine
ddI	Didanosine
DRV/r	Ritonavir-boosted darunavir
EFV	Efavirenz
ENF	Enfuvirtide
ETV	Etravirine
FAPV/r	Ritonavir-boosted fosamprenavir
gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus
IDV	Indinavir
IN	Integrase
InSTI	Integrase strand-transfer inhibitor
LPV/r	Ritonavir-boosted lopinavir
NFV	Nelfinavir
NNRTI	Non-nucleoside analogue reverse transcriptase inhibitor
NRTI	Nucleoside analogue reverse transcriptase inhibitor
NVP	Nevirapine
PASS	Parallel allele-specific sequencing
PI	Protease inhibitor
PR	Protease
RT	Reverse transcriptase
RTV	Ritonavir
SGS	Single genome sequencing
SQV/r	Ritonavir-boosted saquinavir
TAM	Thymidine analogue-associated resistance mutations
TDF	Tenofovir disoproxil fumarate
TPV/r	Ritonavir-boosted tipranavir
UDS	Ultradeep sequencing
ZDV	Zidovudine

Contents

Preface

Since the dawn of our species, humans have lived in constant interaction with microbial agents capable of causing disease. Retroviruses are not an exception. The human DNA contains approximately 80,000 proviruses or their remnants, comprising 6-8% of the genome, whereas it "only" harbors about 30,000 genes. Therefore, "there are more proviruses in us than there is us in us."[1]

Endogenous proviruses are widespread in nature, and have been found in most vertebrate and invertebrate species studied, indicative of the barrage of retroviruses to which all species have been subjected throughout their evolutionary history. Ancient proviruses entered the germ line before the species originated; they are found in most vertebrates and are located at the same genomic position in all members of the same species. Conversely, recent proviruses entered the germ line after speciation; they may not be fixed in the species and may still be capable of yielding infectious virus. Both ancient and recent endogenous proviruses closely resemble the retroviruses existing today. Endogenous proviruses can sometimes block infection of the host by related viruses; in other cases, they can induce disease in non-human hosts and maybe in humans as well.

Besides their present role in promoting or preventing disease, the presence of human endogenous retroviral elements (HERVs) indicates

[1] *Coffin J. Evolution of Retroviruses: Fossils in our DNA. Proc Am Philos Soc. 2004;148:264-80.*

that humans co-evolved with retroviruses ever since our species emerged –just like our ancestors did before us. Moreover, the presence of HERVs in our genome indicates that such co-evolution led toward a stable state in which viruses could infect and spread from one individual to another without causing disease severe enough to hinder this transmission or to reduce the pool of available hosts.

Stable interactions between retroviruses and host tend to be highly specific of the host's genetic environment. Transmission of the virus to a new species followed by spread within that species is associated with considerable morbidity and mortality, leading to selection of variant hosts that resist infection and/or disease.

Although the debate about the origin of HIV remains open, most scientists agree that approximately 100 years ago, a close ancestor of HIV highly related to current Simian Immunodeficiency Viruses (SIVs), was transferred from monkeys to humans in the region comprised between the Niger and Congo rivers in West Africa. SIVs have been found in more than 35 African primate species. Each SIV is highly species-specific, suggesting that SIVs co-evolved with each primate species since ancient times. Likely, the M, N and O groups of HIV-1 were introduced into humans by at least three separate cross-species transmissions of SIVcpz from chimpanzees, particularly *Pan troglotydes troglotydes* living in the West Central region of Africa. Recently, SIV infection was noted in gorillas. The SIVgor virus closely resembles the HIV-1 group 0–like viruses, suggesting that the group 0 HIV-1 could, indeed, have originated from gorillas. SIVs in monkeys originating in West Africa, particularly sooty mangabeys (*Cercocebus atys*) (SIVsmm), likely originated HIV-2.

SIV-related viruses have clear pathogenic potential in humans and macaques. However, SIV infections in their natural hosts are nonpathogenic –despite being characterized by relatively high viral loads in peripheral blood and tissues. Natural defense mechanisms like the APOBECG and TRIM-5-alfa proteins play a major role in restricting retroviral infections, but often fail to protect against retroviruses from different species. Surely, other natural defense

mechanisms against retroviral infections will be elucidated in the coming years.

All these findings converge towards the idea that humans were not prepared for the irruption of HIV, a virus that thrives by infecting precisely the immune cells in charge of eliminating viral infections and subverting the immune system to its own replicative advantage.

Unfortunately, this means that the fight against HIV will probably last much more than any of us would like to imagine. As our species overcame all previous epidemics, there is no doubt that, someday, we will overcome HIV/AIDS, too. The challenges posed by HIV to our species, however, are unprecedented and entirely different from those presented by other infectious agents before. Even if we can mitigate the effects of HIV infection quite effectively with antiretroviral drugs, we cannot eradicate HIV and, given the current knowledge, there is no realistic indication that an effective vaccine will be generated anytime soon.

HIV combines five characteristics previously unseen in any other human pathogen simultaneously:

(a) It infects and destroys most regulator and effector immune cells, particularly those residing in the gut-associated lymphoid tissue (GALT) within few days after primary infection and before any effective immune response can be mounted against it;

(b) Once immune responses are generated, it subverts the immune system by inducing immune activation and utilizing its milieu toward its own replicative advantage; in addition, HIV is prone to rapid antigen variation and uses host's autologous glycoproteins to mask epitopes that could elicit neutralizing antibodies;

(c) Like other retroviruses, HIV irreversibly integrates its genetic material into the host's genome, including cells with very low turnover or entering latency, thus establishing latent cellular viral reservoirs that cannot be cleared by current antiretrovirals and from which it can emerge when needed;

(d) HIV infection is compartmentalized in anatomical sanctuaries with distinct replication kinetics and different levels of antiretroviral drug penetration; and
(e) Finally, HIV has a quasispecies distribution that allows rapid fitness adaptation to varying environments;

HIV's huge ability to diversify within the infected host and across human populations is one of its most salient features. As a reference, the diversity of viral variants infecting a single individual in any given moment is much higher than the variability of all influenza viruses generated around the globe every year. The random generation of viral variants with immune and drug escape mutations even before the virus is challenged by immune responses or drugs, is a fundamental survival strategy that allows HIV to rapidly adapt to changing environments and overcome the adverse pressure of both immune system and pharmacologic treatment. Indeed, viral evolution further accelerates in the presence of active replication under the selective pressure of therapy or immune responses.

In order to treat HIV infection properly, we need to be aware that we are not confronting a single agent, but a swarm of genetically related variants infecting each patient. Some of them predominate because they have a fitness advantage in that particular environment, being easily detectable by standard populating sequencing techniques. Others remain at very low frequency in the viral quasispecies, but are ready to emerge as soon as the environmental conditions change to confer them a fitness advantage. Therefore, it is paramount to understand the determinants of such diversity and to develop tools than allow us to study the quasispecies structure and dynamics with more detail.

In the following pages, we will discuss the relevance of minority variants harboring resistance mutations in the clinical management of HIV infection. The first chapter will present a systematic evaluation of allele-specific polymerase chain reaction (ASPCR), as a tool to detect low-frequency viral variants harboring resistance mutations in the reverse transcriptase (M184V, M184I) and protease (D30N)-coding regions of *pol*, as well as in *env* (V38A). In the second chapter, we will

use this technique alongside others to characterize with detail the decay dynamics of M184V mutants in subjects infected with multidrug-resistant HIV-1 who interrupt treatment with reverse transcriptase inhibitors and continue protease inhibitors. This study will show that ASPCR can be used to estimate the fitness of particular allelic variants *in vivo* and help improve our understanding of quasispecies dynamics in the presence and absence of therapy. The third chapter will show how detection of low-frequency mutants can be applied to the surveillance of primary antiretroviral resistance, increasing the prevalence of resistance mutations by 2 to 3-fold relative to using bulk sequencing of plasma viruses. In the fourth chapter we will show that antiretroviral naïve HIV-1-infected pregnant women frequently select resistance mutations to drugs with low-genetic barrier during pregnancy-limited antiretroviral therapy; again, the frequency of resistance mutations will increase more than two-fold using the ASPCR assay. These findings have important clinical implications, given that women selecting resistance mutations during pregnancy-limited antiretroviral therapy may be more likely to fail first-line therapy. The fifth chapter will show that pre-existing minority variants harboring resistance to non-nucleoside reverse transcriptase inhibitors more than triple the risk of virological failure to first-line efavirenz-based antiretroviral therapy, even in drug-adherent subjects. Then, we will discuss our findings in the context of other studies and provide a general overview of their implications. We will finish this thesis by presenting the main conclusions derived from our work and by outlining future research questions that need to be pursued to reach a better understanding of the clinical implications of minority HIV-1 variants.

In summary, this work demonstrates that minority HIV-1 resistant variants, which are often missed by standard viral population sequencing assays, do modify antiretroviral therapy outcomes and therefore are of major clinical importance.

Introduction

CLASSIFICATION

Human Immunodeficiency Virus (HIV) is a member of the genus *Lentivirus* in the *Retroviridae* family (Table 1). As a retrovirus, its RNA genome is transcribed into DNA within the cell using the viral enzyme reverse transcriptase (RT).

Table 1. Classification of Retroviruses

Genus	Example	Virion morphology[a]	Genome
Avian sarcoma and leukosis viral group	Rous sarcoma virus	central, spherical core "C particles"	simple
Mammalian B-type viral group	Mouse mammary tumor virus	eccentric, spherical core "B particles"	simple
Murine leukemia-related viral group	Moloney murine leukemia virus	central, spherical core "C particles"	simple
Human T-cell leukemia–bovine leukemia viral	Human T-cell leukemia virus	central, spherical core	complex
D-type viral group	Mason-Pfizer monkey virus	cylindrical core "D particles"	simple
Lentiviruses	Human immunodeficiency virus	cone-shaped core	complex
Spumaviruses	Human foamy virus	central, spherical core	complex

[a] Distinctive features seen in transmission electron micrographs.
Source: *Retroviruses. 1997. John M. Coffin, Stephen H. Hugues and Harold E. Varmus (Editors). Cold Spring Harbor Laboratory Press.*

All lentiviruses have characteristics in common. (Table 2) Clinically, lentiviral infections have a long incubation period; they frequently induce immune deficiency, involve the hematopoietic and central nervous system, and can be associated with arthritis and autoimmunity. Biologically, lentiviral infections are highly host-specific; lentiviruses are exogenous, non-oncogenic agents with a cone-shaped nucleocapsid that exert cytopathic effects. Lentiviral infections are usually associated with accumulation of unintegrated circular and linear viral cDNA in infected cells, and are able to achieve latent or persistent cell infection. From a molecular perspective, lentiviruses have large genomes (≥9 Kb) with a truncated *gag* gene that enables processing for several Gag proteins. Lentiviral genomes are highly polymorphic, particularly in the envelope region, and include a novel central open reading frame that separates the *pol* and *env* genes. Finally, all lentiviruses have a highly glycosylated envelope.

Table 2. Lentiviruses

Virus	Host Infected	Primary cell type infected	Major clinical disorder
Equine infectious anemia virus	Horse	Macrophages	Cyclical infection in the first year, autoimmune hemolytic anemia, encephalopathy
Visna/maedi virus	Sheep	Macrophages	Encephalopathy / pneumonitis
Caprine arthritis-encephalitis virus	Goat	Macrophages	Immune deficiency, arthritis, encephalopathy
Bovine immunodeficiency virus	Cow	Macrophages	Lymphadenopathy, lymphocytosis, central nervous system disease
Feline immunodeficiency virus	Cat	T lymphocytes	Immune deficiency, encephalopathy
Simian immunodeficiency virus	Primate	T lymphocytes	Immune deficiency, encephalopathy
Human immunodeficiency virus	Human	T lymphocytes	Immune deficiency, encephalopathy and enteropathy

Source: *Levy, Jay A. HIV and the Pathogenesis of AIDS –3rd Edition. 2008. ASM Press, American Society for Microbiology (Editors) & HIV Sequence Compendium 2008.*

HIV STRUCTURE

The HIV virion is about 100 to 120 nm in diameter. (Figure 1, Table 3) Infectious viruses contain the envelope and three structural Gag proteins: matrix (MA, p17), capsid (CA, p24) and nucleocapsid (NC, p7). MA forms the inner shell in the particle below the viral membrane; CA forms a conical-shaped core that encloses the viral genomic RNA, and NC interacts with the viral RNA inside the capsid. These viral proteins are generated by the viral protease (PR) processing of the HIV p55 Gag precursor polyprotein. Inside the Gag capsid are two identical RNA strands. The viral RNA-dependent DNA polymerase, RT (p66, p51), and the NC proteins (p9, and p6) are closely associated to the genetic material. The inner portion of the viral membrane is surrounded by a myristoylated p17 core protein (MA) that is part of the viral structure and is possibly needed for directing HIV assembly and incorporation of the Env proteins into mature virions. The Vif and Nef proteins are closely associated with the core. Approximately, 7 to 20 Vif molecules exist per virion. The Vpr protein (Vpx in HIV-2) is also within the virion but likely outside the core.

Tsibris et al. provided an excellent review of the envelope structure and transformations during viral entry.[1] The envelope proteins derive from gp160, which is cleaved by cellular enzymes to gp120 (SU) and gp41 (TM) in the Golgi apparatus. Gp41 is anchored to the viral membrane by its C-terminal region, whereas the central and N-terminal regions are expressed outside of the virion. The central region of the viral TM protein binds noncovalently to the gp120 protein, primarily at two hydrophobic regions in the amino and carboxyl termini of gp120 with a stoichiometry of one molecule of gp120 to one molecule of gp41. Three of these units aggregate on the membrane surface to form the gp120/gp41 heterotrimer.[2-4] By the time the virus is released from a cell, only 7 to 14 Env spikes appear to be present on the virion surface. The association of gp120 with gp41 in the trimer traps gp41 in a conformationally metastable state, the energy from which can later be exploited to accelerate the rate of fusion.[5] Gp120 contains the binding site(s) for the cellular receptor(s) and the major antibody –neutralizing domains.

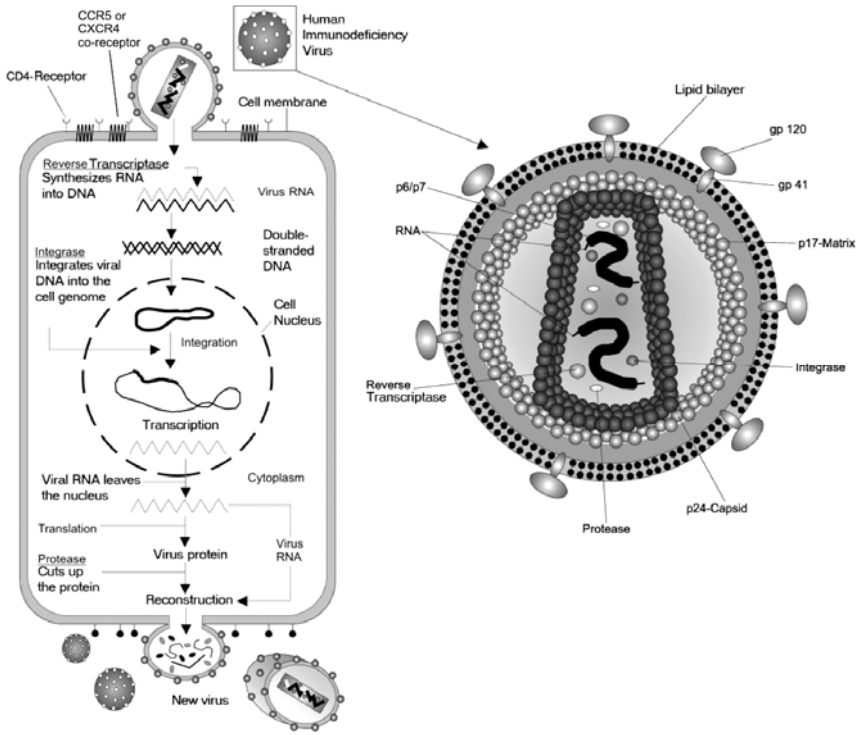


FIGURE 1. SCHEMATIC REPRESENTATION OF THE HIV-1 STRUCTURE (RIGHT) AND CELL CYCLE (LEFT).
Source: *Apadpted from Wikipedia Commons.Original available at: http://en.wikipedia.org/w/index.php?title=Image%3AHiv_gross.png.*

Several host cellular proteins can be found within the virus, such as certain cytoskeletal proteins (e.g. actin, ezrin, emerin and moesin).[6-10] Emerin seems to be essential for the interaction between viral cDNA with chromatin and subsequent integration of the provirus.[7] In addition, the heat shock protein 70 (hsp70) is incorporated in the membrane of primate lentiviral virions, including HIV-1 cores,[10] and seemingly helps to maintain the core's structural integrity.

As with other retroviruses, specific lipid domains from the host cell membrane are selectively incorporated to the viral membrane during budding. In addition to the HIV envelope spikes, HIV particles carry in

their membranes numerous host cell-derived glycoproteins and an array of serum proteins nonspecifically attached to the virion surface.[11] Many of the original functional spikes have shed their gp120 subunits and may display a conformationally irrelevant postfusion gp41. The remaining intact spikes are highly glycosylated, flexible on the surface and may differ by up to 10% of amino acids between different HIV virions within an individual at a particular time point, thus interfering with the affinity maturation of antibodies.[11]

Table 3. HIV proteins and their functions

Proteins[a]	Designation[b] and size (kDA)	Function
Gag	p24	Capsid (CA), structural protein. The genomic region encoding the capsid proteins (group specific antigens). The precursor is the p55 myristylated protein protein, which is processed to p17 (MAtrix), p24 (CApsid), p7 (NucleoCapsid), and p6 proteins, by the viral protease. Gag associates with the plasma membrane where the virus assembly takes place. The 55 kDa Gag precursor is called assemblin to indicate its role in viral assembly.
	p17	Matrix (MA) protein, myristylated
	p7	Nucleocapsid (NC) protein; helps in reverse transcription
	p6	Role in budding (L domain)
Polymerase (pol)	p66, p51	Reverse transcriptase (RT): RNAseH –inside core. The genomic region encoding the viral enzymes protease, reverse transcriptase, RNAse, and integrase. These enzymes are produced as a Gag-Pol precursor polyprotein, which is processed by the viral protease; the Gag-Pol precursor is produced by ribosome frameshifting near the 30end of gag.
Protease (PR)	p10	Posttranslational processing of viral proteins
Integrase (IN)	p32	Viral cDNA integration
Envelope (env)	gp120	Envelope surface (SU) protein. Viral glycoproteins produced as a precursor (gp160) which is processed to give a noncovalent complex of the external glycoprotein gp120 and the transmembrane glycoprotein gp41. The mature gp120-gp41 proteins are bound by non-covalent interactions and are associated as a trimer on the cell surface. A substantial amount

		of gp120 can be found released in the medium. gp120 contains the binding site for the CD4 receptor, and the seven transmembrane domain chemokine receptors that serve as co-receptors for HIV- 1.
	gp41	Envelope transmembrane (TM) protein
Tat	p14	Transactivator of HIV gene expression. One of two essential viral regulatory factors (Tat and Rev) for HIV gene expression. Two forms are known, Tat-1 exon (minor form) of 72 amino acids and Tat-2 exon (major form) of 86 amino acids. Low levels of both proteins are found in persistently infected cells. Tat has been localized primarily in the nucleolus/ nucleus by immunofluorescence. It acts by binding to the TAR RNA element and activating transcription initiation and elongation from the LTR promoter, preventing the 50LTR AATAAA polyadenylation signal from causing premature termination of transcription and polyadenylation. It is the first eukaryotic transcription factor known to interact with RNA rather than DNA and may have similarities with prokaryotic anti-termination factors. Extracellular Tat can be found and can be taken up by cells in culture.
Rev	p19	Regulation of viral mRNA expression. The second necessary regulatory factor for HIV expression. A 19 kDa phosphoprotein, localized primarily in the nucleolus/nucleus, Rev acts by binding to RRE and promoting the nuclear export, stabilization and utilization of the unspliced viral mRNAs containing RRE. Rev is considered the most functionally conserved regulatory protein of lentiviruses. Rev cycles rapidly between the nucleus and the cytoplasm.
Nef	p27	Pleiotropic, can increase or decrease virus replication. A multifunctional 27-kDa myristylated protein produced by an ORF located at the 30end of the primate lentiviruses. Other forms of Nef are known, including nonmyristylated variants. Nef is predominantly cytoplasmic and associated with the plasma membrane via the myristyl residue linked to the conserved second amino acid (Gly). Nef has also been identified in the nucleus and found associated with the cytoskeleton in some experiments. One of the first HIV proteins to be produced in infected cells, it is the most immunogenic of the accessory proteins. The nef

		genes of HIV and SIV are dispensable in vitro, but are essential for efficient viral spread and disease progression in vivo. Nef is necessary for the maintenance of high virus loads and for the development of AIDS in macaques, and viruses with defective Nef have been detected in some HIV-1 infected long term survivors. Nef downregulates CD4, the primary viral receptor, and MHC class I molecules, and these functions map to different parts of the protein. Nef interacts with components of host cell signal transduction and clathrin-dependent protein sorting pathways. It increases viral infectivity. Nef contains PxxP motifs that bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of HIV but not for the downregulation of CD4.
Vif	p23	Increases virus infectivity and cell-to-cell transmisión; helps in proviral DNA síntesis and/or in virion assembly. Viral infectivity factor, a basic protein of typically 23 kDa. Promotes the infectivity but not the production of viral particles. In the absence of Vif the produced viral particles are defective, while the cell-to-cell transmission of virus is not affected significantly. Found in almost all lentiviruses, Vif is a cytoplasmic protein, existing in both a soluble cytosolic form and a membrane-associated form. The latter form of Vif is a peripheral membrane protein that is tightly associated with the cytoplasmic side of cellular membranes. In 2003, it was discovered that Vif prevents the action of the cellular APOBEC-3G protein which deaminates DNA:RNA heteroduplexes in the cytoplasm.
Vpr	p15	Helps in virus replication; transactivation. Vpr (viral protein R) is a 96-amino acid (14 kDa) protein, which is incorporated into the virion. It interacts with the p6 Gag part of the Pr55 Gag precursor. Vpr detected in the cell is localized to the nucleus. Proposed functions for Vpr include the targeting the nuclear import of preintegration complexes, cell growth arrest, transactivation of cellular genes, and induction of cellular differentiation. In HIV-2, SIV-SMM, SIVRCM, SIV-MND-2 and SIV-DRL the Vpx gene is apparently the result of a Vpr gene duplication event, possibly by recombination.
Vpu[cd]	p16	Helps in virus release; disrupts gp160:CD4 complexes. Vpu (viral protein U) is unique to HIV-1, SIVcpz (the closest SIV relative of HIV-

		1), SIV-GSN, SIV-MUS, SIVMON and SIV-DEN. There is no similar gene in HIV-2, SIV-SMM or other SIVs. Vpu is a 16 kDa (81-amino acid) type I integral membrane protein with at least two different biological functions: (a) degradation of CD4 in the endoplasmic reticulum, and (b) enhancement of virion release from the plasma membrane of HIV-1-infected cells. Env and Vpu are expressed from a bicistronic mRNA. Vpu probably possesses an N-terminal hydrophobic membrane anchor and a hydrophilic moiety. It is phosphorylated by casein kinase II at positions Ser52 and Ser56. Vpu is involved in Env maturation and is not found in the virion. Vpu has been found to increase susceptibility of HIV-1 infected cells to Fas killing.
Vpx[c]	p15	Helps in entry and infectivity. A virion protein of 12 kDa found in HIV-2, SIV-SMM, SIV-RCM, SIV-MND-2 and SIV-DRL and not in HIV-1 or other SIVs. This accessory gene is a homolog of HIV-1 vpr, and viruses with Vpx carry both vpr and vpx. Vpx function in relation to Vpr is not fully elucidated; both are incorporated into virions at levels comparable to Gag proteins through interactions with Gag p6. Vpx is necessary for efficient replication of SIV-SMM in PBMCs. Progression to AIDS and death in SIV-infected animals can occur in the absence of Vpr or Vpx. Double mutant virus lacking both vpr and vpx was attenuated, whereas the single mutants were not, suggesting a redundancy in the function of Vpr and Vpx related to virus pathogenicity.
Tev[c]	p26	Tat/Rev activities

[a] See figure 2 for location of the viral genes on the HIV genome. [b] Numbers in designations are sizes, in kilodaltons. [c] Not found to be associated with the virion, [d] Only present with HIV-1.[c] Only enclosed by HIV-2. May be a duplication of Vpr
Sources: *Levy, Jay A. HIV and the Pathogenesis of AIDS –3d Edition. 2008. ASM Press, American Society for Microbiology (Editors) & HIV Sequence Compendium 2008. Carla Kuiken, Thomas Leitner, Brian Foley, Beatrice Hahn, Preston Marx, Francince McCutchan, Steven Wolinsky, and Bette Korber editors. 2008. Publisher: Los Alamos National Laboratory, Theoretical Biology and Biophysics, Los Alamos, New Mexico. LA-UR 08-03719.*

GENOMIC ORGANIZATION

HIV's genome is about 10kB long with different open reading frames (ORFs) coding for several viral proteins. Figure 2 summarizes the processing of viral proteins from HIV-1 genome. The HIV genomic structural elements are shown in Table 4. HIV-1 proteins translated from 10 different viral transcripts are further processed by cellular and viral proteases. Sixteen viral proteins are made from 46 translated ORFs. They form the virion structure, direct viral enzymatic activities, and serve regulatory and accessory activities. Regulatory proteins are translated first, and modulate the following synthesis of viral structural proteins.

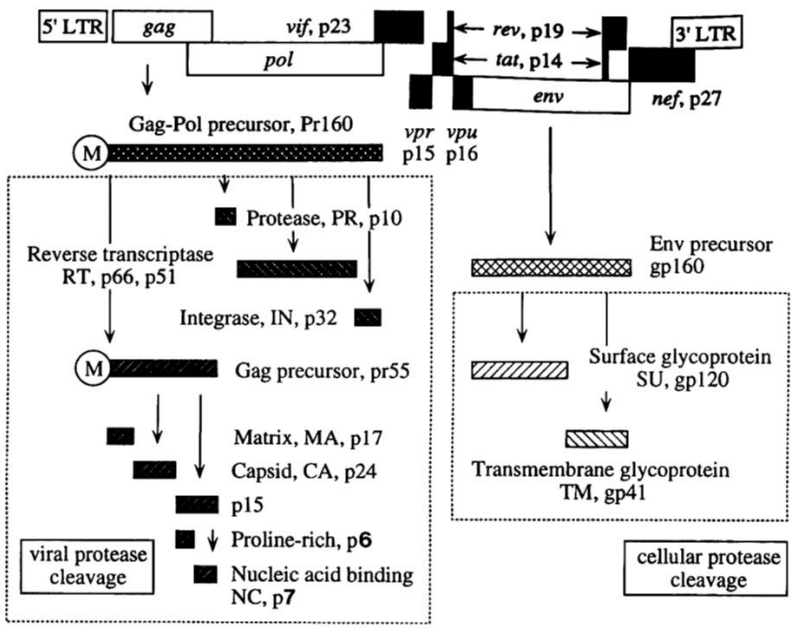


FIGURE 2. PROCESSING OF VIRAL PROTEINS. The Gag-Pol precursor of 160 kDa is processed by the viral aspartyl protease into seven proteins, which include four Gag proteins (MA, p17; CA, p24; late domain, p7; and NC, p9), protease (P, p10), reverse transcriptase/RNAse (RT, p66, p51), and integrase (IN, p32). The Env precursor (gp160) is processesed by a cellular protease into the surface glycoprotein (SU, gp120) and the transmembrane glycoprotein (TM, gp41). Viral regulatory and accessory proteins, which include Tat (p14), Tev (p20), Rev (p19), Nef (p27), Vif (p23), Vpr (p15), and Vpu (p16), are not processed. M, myristoylated. Source: *Levy, Jay A. HIV and the Pathogenesis of AIDS –3rd Edition. 2008. ASM Press, American Society for Microbiology (Editors)*

Table 4. HIV genomic structural elements

Designation	Name	Function
LTR	Long terminal repeat	DNA sequence flanking the genome of integrated proviruses. It contains important regulatory regions, especially those for transcription initiation and polyadenylation.
TAR	Target sequence for viral transactivation	Binding site for Tat protein and for cellular proteins; consists of approximately the first 45 nucleotides of the viral mRNAs in HIV-1 (or the first 100 nucleotides in HIV-2 and SIV.) TAR RNA forms a hairpin stem-loop structure with a side bulge; the bulge is necessary for Tat binding and function.
RRE	Rev responsive element	RNA element encoded within the env region of HIV-1. It consists of approximately 200 nucleotides (positions 7327 to 7530 from the start of transcription in HIV-1, spanning the border of gp120 and gp41). The RRE is necessary for Rev function; it contains a high affinity site for Rev; in all, approximately seven binding sites for Rev exist within the RRE RNA. Other lentiviruses (HIV- 2, SIV, visna, CAEV) have similar RRE elements in similar locations within env, while HTLVs have an analogous RNA element (RXRE) serving the same purpose within their LTR; RRE is the binding site for Rev protein, while RXRE is the binding site for Rex protein. RRE (and RXRE) form complex secondary structures, necessary for specific protein binding.
PE	Psi elements	a set of 4 stem-loop structures preceding and overlapping the Gag start codon which are the sites recognized by the cysteine histidine box, a conserved motif with the canonical sequence CysX2CysX4HisX4Cys, present in the Gag p7 MC protein. The Psi Elements are present in unspliced genomic transcripts but absent from spliced viral mRNAs.
SLIP	SLIP	A TTTTTT slippery site, followed by a stem-loop structure, is responsible for regulating the -1 ribosomal frameshift out of the Gag reading frame into the Pol reading frame.
CRS	Cis-acting repressive sequences	Sequences postulated to inhibit structural protein expression in the absence of Rev. One such site was mapped within the pol region of HIV-1. The exact function has not been defined; splice sites have been postulated to act as CRS sequences
INS	Inhibitory/ Instability	RNA sequences found within the structural genes of HIV-1 and of other complex retroviruses.

RNA sequences	Multiple INS elements exist within the genome and can act independently; one of the best characterized elements spans nucleotides 414 to 631 in the gag region of HIV-1. The INS elements have been defined by functional assays as elements that inhibit expression posttranscriptionally. Mutation of the RNA elements was shown to lead to INS inactivation and up regulation of gene expression.

Source: HIV Sequence Compendium 2008. Carla Kuiken, Thomas Leitner, Brian Foley, Beatrice Hahn, Preston Marx, Francince McCutchan, Steven Wolinsky, and Bette Korber editors. 2008. Publisher: Los Alamos National Laboratory, Theoretical Biology and Biophysics, Los Alamos, New Mexico. LA-UR 08-03719.

CELL CYCLE

VIRAL ENTRY

Entry of HIV-1 into target cells proceeds by the fusion of viral and cellular membranes. This event involves viral and cellular protein interactions that lead to conformational changes in critical protein structures. The mechanism of HIV-1 entry shares a number of features in common with other enveloped viruses. The HIV-1 SU and TM subunits of the envelope glycoprotein mediate viral binding to and fusion with host target cells.

As summarized by Tsibris et al.,[1] the first step in membrane fusion is binding of gp120 to its primary receptor on the cell surface, CD4. Although CD4-independent entry can occur in vitro, all primary HIV-1 isolates require CD4 for viral entry.[12] The CD4 binding site is not fully formed in unliganded gp120 but is stabilized and fixed by the approach of CD4.[13] Binding to CD4 typically is followed by binding to either the CCR5 or CXCR4 coreceptor, which is required for fusion to proceed.[14-19] Coreceptor recognition is defined by several structural elements of gp120 that include the first and second hypervariable regions (V1-V2), the bridging sheet (an antiparallel, four-stranded beta sheet that connects the inner and outer domains of gp120), and most importantly, the V3 loop.[20-23] The V1-V2 stem influences coreceptor usage through its amino acid composition as well as by the degree of N-linked

glycosylation.[24] Little structural variation of the bridging sheet is found in human and primate lentiviruses, suggesting that this structure serves as a common determinant for recognition of either coreceptor. The V3 loop, by contrast, is highly variable and is the principal determinant of coreceptor specificity.[15, 24-26]

According to current models of HIV-1 entry, sequential binding of gp120 to CD4 and the CCR5 or CXCR4 coreceptor leads to the release of gp41 from its metastable conformation. The hydrophobic N-terminus, or fusion domain, of the gp41 ectodomain is thereby freed to insert into the target cell membrane.[5, 27, 28] Two trimeric coiled-coil structures in gp41, comprising heptad repeats 1 and 2 (HR-1 and HR-2, respectively), rearrange in an antiparallel orientation to form a six-helix bundle that leads to the approximation of the two membranes and eventual fusion.[5]

REVERSE TRANSCRIPTION

Reverse transcription begins when the viral particle enters the cytoplasm of a target cell. The viral RNA genome enters the cytoplasm as part of a nucleoprotein reverse transcription complex (RTC). Although this RTC remains to be fully characterized, it includes the MA and CA structural proteins and the accessory Vpr protein, together with RT and IN. Several studies have shown that the CA protein dissociates from this RTC soon during uncoating.

The process of reverse transcription generates, in the cytoplasm, a linear DNA duplex via an intricate series of steps. This DNA is colinear with its RNA template, but it contains terminal duplications known as the long terminal repeats (LTRs) that are not present in viral RNA (Fig. 3). The synthesis of full-length viral DNA in the RTC produces the pre-integration complex (PIC) that will be responsible for integrating the viral DNA into cromosomic human DNA.

Retroviral DNA synthesis is absolutely dependent on the two distinct enzymatic activities of RT: a DNA polymerase that can use either RNA or DNA as a template, and a nuclease, termed ribonuclease H (RNase H), that is specific for the RNA strand of RNA:DNA duplexes. Although a role for other proteins cannot be

ruled out, and it is likely that certain viral proteins (e.g., nucleocapsid, NC) increase the efficiency of reverse transcription, all of the enzymatic functions required to complete the series of steps involved in the generation of viral DNA can be attributed to either the DNA polymerase or the RNaseH of RT.

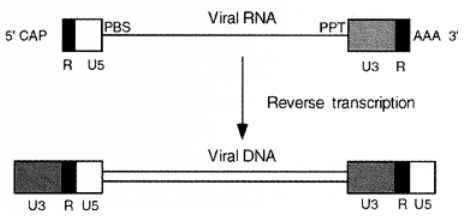


FIGURE 3. REVERSE TRANSCRIPTION OF THE VIRAL RNA GENOME GENERATES A LINEAR DNA DUPLEX. The positions of the R, U5, and U3 regions, the polypurine tract (PPT), and the primer-binding site (PBS) are indicated. Reverse transcription creates duplications of the U5 and U3 regions such that the DNA product is longer than the RNA at both ends. This is the origin of the two long terminal repeats (LTRs) (each consisting of U3/R/U5 regions) that are characteristic of the DNA form of the viral genome. Source: *Retroviruses. 1997. John M. Coffin, Stephen H. Hugues and Harold E. Varmus (Editors). Cold Spring Harbor Laboratory Press.*

Extant models for reverse transcription propose that two specialized template switches known as strand-transfer reactions or "jumps" are required to generate the LTRs. Coffin[29] has summarized the process of retroviral DNA synthesis in the following steps (Figure 4):

1. Minus-strand DNA synthesis is initiated using the 3'end of a partially unwound transfer RNA (tRNA). This tRNA anneals to the primer-binding site (PBS) in genomic RNA and acts as a primer. Minus-strand DNA synthesis proceeds until the 5'end of genomic RNA is reached. This generates a DNA intermediate of discrete length termed *minus-strand strong-stop DNA* (–sssDNA). Since the binding site for the tRNA primer is near the 5' end of viral RNA, –sssDNA is relatively short, on the order of 100–150 bases

2. Following RNase-H-mediated degradation of the RNA strand of the RNA:–sssDNA duplex, the first strand transfer causes –sssDNA to be annealed to the 3'end of a viral genomic RNA. This transfer is mediated by identical sequences known as the repeated (R) sequences, which are present at the 5' and 3'ends of the RNA genome. The 3'end of –sssDNA was copied from the R sequences at the 5'end of the viral genome and, therefore, contains sequences complementary to R. After the RNA template has been removed, –sssDNA can anneal to the R sequences at the 3'end of the RNA genome. The annealing reaction appears to be facilitated by the NC.
3. Once the –sssDNA has been transferred to the 3'R segment on viral RNA, minus-strand DNA synthesis resumes, accompanied by RNaseH digestion of the template strand. This degradation is not complete, however.
4. The RNA genome contains a short polypurine tract (PPT) that is relatively resistant to RNase H degradation. A defined RNA segment derived from the PPT primes plus-strand DNA synthesis. Plus-strand synthesis is halted after a portion of the primer tRNA is reverse-transcribed, yielding a DNA called *plus-strand strong-stop DNA* (+sssDNA).
5. RNase H removes the primer tRNA, exposing sequences in +sssDNA that are complementary to sequences at or near the 3'end of plus-strand DNA.
6. Annealing of the complementary PBS segments in +sssDNA and minus-strand DNA constitutes the second strand transfer.
7. Plus- and minus-strand syntheses are then completed, with the plus and minus strands of DNA each serving as a template for the other strand.

Recent analyses using single-molecule fluorescence resonance energy transfer (FRET) have demonstrated that RT has a remarkable ability to slide and rapidly shuttle between the opposite termini of long nucleic acid duplexes flipping from RNAseH-competent binding mode into the polymerase-competent binding mode when needed.[30]

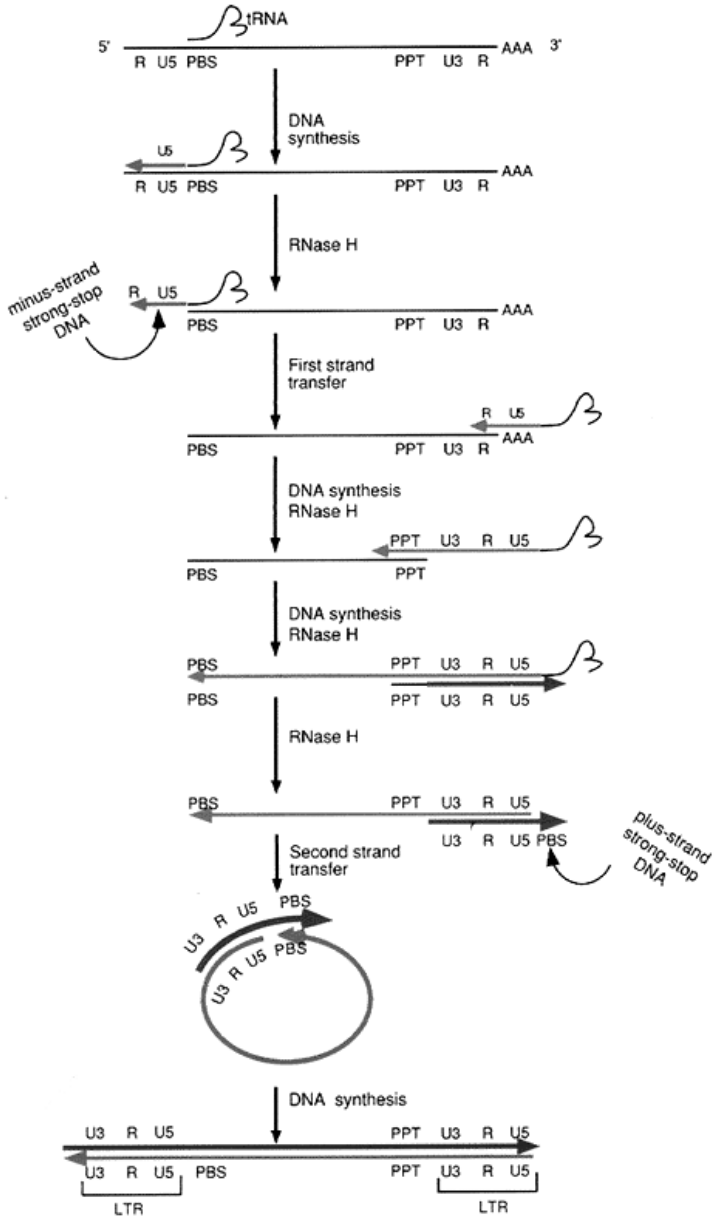


FIGURE 4. PROCESS OF REVERSE TRANSCRIPTION OF THE RETROVIRAL GENOME. (thin black line) RNA; (light grey) minus-strand DNAs; (dark grey) plus-strand DNA. See text for a description of this process. Source: *Retroviruses. 1997. John M. Coffin, Stephen H. Hugues and Harold E. Varmus (Editors). Cold Spring Harbor Laboratory Press.*

INTEGRATION

The integration process encompasses all the events between completion of viral DNA synthesis and initiation of the expression of the newly integrated provirus. As was mentioned earlier, synthesis of full-length viral DNA in the reverse transcription complex leads to the formation of the preintegration complex (PIC). This PIC carries the newly synthesized viral DNA from the cytoplasm to the cell nucleus, and mediates its integration.

The PIC is formed by the HIV-1 proteins MA, NC, Vpr, RT and IN; to which a number of cytopasmic proteins are incorporated, including the Barrier-to-Autointegration factor (BAF) and the lens-epithelium-derived growth factor (LEGDF/p75).[30] Whereas other retroviruses enter the nucleous during mitosis, HIV-1 has the ability to integrate in both dividing and non-dividing cells while the nucleus is intact. Because the PIC is a large complex (50nm in diameter) at least as big as ribosomes, it cannot enter the cell nucleus by passive diffusion.

Current models of nuclear trafficking suggest that the PIC reaches the nuclear envelope by active transport along microtubules that bind nucleocapsid proteins, toward microtubule-organizing centers that lie next to the nuclear pores. Viral determinants of nuclear import are the MA, Vpr and IN proteins. MA, Vpr, and probably the central polypurine tract of viral DNA have kariophillic residues that probably interact with importins and nucleoporins that determine nuclear import. Recent studies indicate that HIV-1 IN lacks a transferable nuclear localization signal and that the kariophillic property of IN is conferred by LEDGF/p17, a transcriptional regulator that associated with HIV-1 IN and protects it from proteosomal degradation. LEDGF/p17 does have an N-terminal nuclear localization signal and may contribute to nuclear accumulation of IN and its stable tethering to chromatin. LEDGF/p17 is possibly more important in regulating the integration efficiency and/or integration-site selection than in nuclear translocation of viral DNA. In addition to LEDGF/p17, other proteins like emerin or BAF have been implicated in anchoring PICs to chromatin. [30]

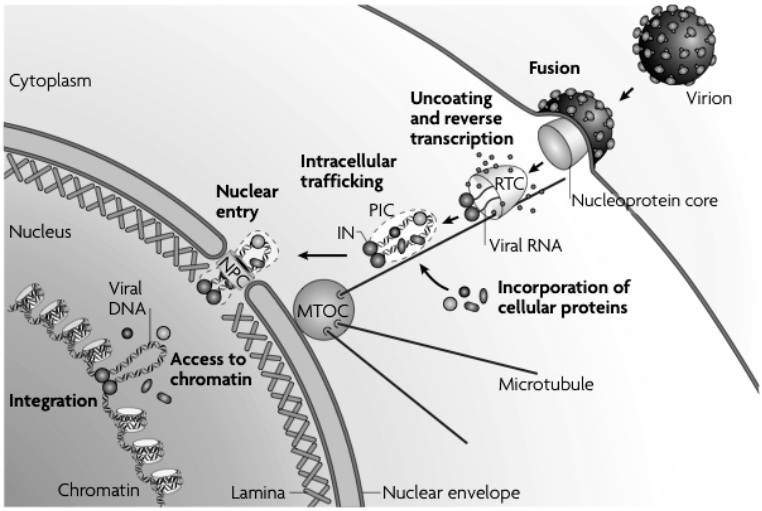


FIGURE 5. FROM CELL ENTRY TO DNA INTEGRATION. The virus enters the target cell by fusion between the cellular and viral membranes, and delivers the nucleoprotein core containing the genomic RNA into the cytoplasm. Uncoating of the viral core forms the reverse transcription complex (RTC) in which reverse transcription occurs. The resulting viral DNA remains associated with viral and cellular proteins in the pre-integration complex (PIC). The PIC probably reaches the nuclear envelope by active transport along microtubules. HIV-1 PICs can cross the intact nuclear envelope, presumably through the nuclear pore complex (NPC). After entry into the nucleus, the PIC gains access to chromatin and viral DNA is integrated by the viral integrase protein (IN). MTOC, microtubule-organizing centre. Source: *Suzuki Y and Craigie R. The Road to Chromatin – Nuclear Entry of retroviruses. Nat Rev Microbiol 2007; 5 (3): 187-96.*

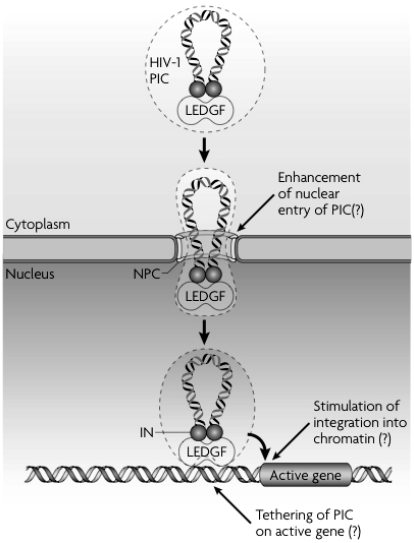


FIGURE 6. LEDGF/P75 AND NUCLEAR ENTRY OF PICS. Several roles for lens-epithelium-derived growth factor (LEDGF/p75) have been proposed for human immunodeficiency virus 1 (HIV-1) DNA integration. LEDGF/p75 might regulate HIV-1 replication through the tethering of integrase protein (IN) and chromatin. NPC, nuclear pore complex; PIC, pre integration complex. Source: *Suzuki Y and Craigie R. The Road to Chromatin – Nuclear Entry of retroviruses. Nat Rev Microbiol 2007; 5 (3): 187-96.*

Once the PIC reaches human chromatin, the following steps ensue (Figure 7):[29]

1. The viral DNA molecule at the completion of its synthesis is a blunt-ended linear molecule whose termini, corresponding to the boundaries of the long terminal repeats, are specified by the primers for plus- and minus-strand DNA synthesis.
2. Soon after completion of viral DNA synthesis, usually while still in the cytoplasm, IN cleaves the 3'termini of the viral DNA, eliminating the terminal two (or, rarely, three) bases from each 3'end. The resulting recessed 3'-OH groups provide the sites of attachment of the provirus to host DNA and thus ultimately define the ends of the integrated provirus.
3. Upon entry into the nucleus, the preintegration complex encounters the host DNA. Although specific target sequences are not required for integration, the host genome is not uniformly used as a target. Highly bent DNA sites, such as are those found at specific positions in nucleosomes, are strongly preferred. Host-cell DNA-binding proteins may occlude potential target sites, preventing their use. In some cases, cellular proteins that bind to host DNA may be recognized by the viral integration machinery, directing integration to specific sites. Ongoing cellular DNA synthesis or transcription of the target DNA sequences are not required.
4. Binding of host DNA by the integrase-viral DNA complex is followed by a concerted, integrase-catalyzed reaction in which the 3'-OH groups at the viral DNA ends are used to attack phosphodiester bonds on opposite strands of the target DNA, at positions staggered by four to six bases in the 5' direction, and therefore on the same face of the double helix, separated by the major groove. In this direct transesterification reaction, the energy of the broken phosphodiester bonds in the target DNA is used for formation of new bonds joining the viral 3'ends to the target DNA.

5. DNA synthesis, perhaps guided by viral proteins or carried out by the viral reverse transcriptase, extends from the host DNA 3'-OH groups that flank the host-viral DNA junctions, filling in the gaps that flank the viral DNA and displacing the usually mismatched viral 5'ends. Following a ligation step, proviral integration is complete.
6. The mechanism by which the preintegration complex is quickly disassembled once integration is completed is not well known.

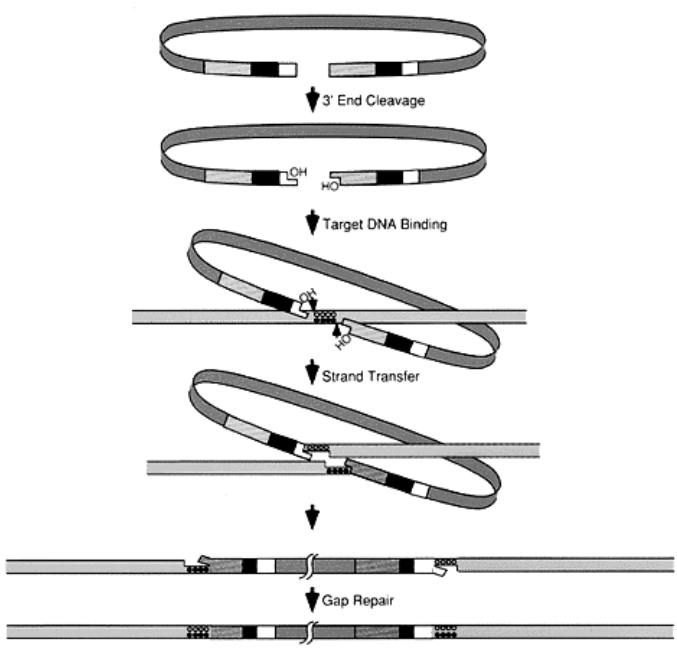


FIGURE 7. SCHEMATIC OUTLINE OF THE PRINCIPAL STEPS IN RETROVIRAL DNA INTEGRATION. Source: *Retroviruses. 1997. John M. Coffin, Stephen H. Hugues and Harold E. Varmus (Editors). Cold Spring Harbor Laboratory Press.*

TRANSCRIPTION

Once integrated in the human genome, the cell does not differentiate between the provirus and autologous genomic sequences. Therefore, the machinery involved in the expression of the provirus is the same as with any other autologous gene. Indeed, the viral DNA

contains recognition sequences that interact with components of the machinery the cell uses to express its own genes. Moreover, as a complex retroviruses, HIV encodes accessory proteins that regulate the timing and level of expression of its own genes.

Retroviral transcription initiates at the U3-R boundary (also called the "cap" site), whereas the majority of the important binding and regulatory sequences are within U3.[31-33] These sites resemble sites found in normal cellular genes and include both basal elements (such as the TATA box) as well as enhancer sequences. The enhancer elements are often in very complex combinations. For example, the HIV LTR contains enhancers that bind to the NF-kB, a transcription factor that is expressed in active form only in activated cells.[31-33] This binding site silences proviruses that are not in active state. If CD4 cells are infected just as they are entering a quiescent state and as NF-kB is being down-regulated, these cells may become latent reservoirs of HIV that could be activated later by immune stimulus. Unlike initiation, termination of RNA synthesis is imprecise and frequently continues into flanking DNA until the polyadenilation machinery cleaves the RNA.[29]

Retroviral transcription is mediated by the host-cell RNA polymerase II, which synthesizes cellular mRNAs and some small nuclear RNAs (snRNAs). The full-length viral transcript, which is packaged as the viral genome, contains a unique copy of all of the information encoded in the proviral DNA, plus a short direct repeat at each end termed R.

RNA PROCESSING

The newly synthesized RNA has to be modified in a number of ways before it is suitable for use as genome or mRNA. First, like almost all cell mRNA, a 200 nucleotide poly (A) sequence is added at the 3' R-U5 border. This end is now recognized by the poly (A) polymerase. Like cellular mRNAs, retroviral RNAs have the standard signal for cleavage and poly (A) addition, AAUAAA, about 24 bases upstream of the poly (A) site.[34, 35]

The other important modification is splicing to create genomic mRNAs for *env*, as well as for the other genes. Splicing is also affected

by the cellular enzymatic machinery. Only a small fraction of the retroviral RNA molecules can be spliced before transport to the cytoplasm because the genome itself cannot be spliced, or all information in "intron" sequences would be lost, including *gag* and *pol*.[36] Rev favors HIV binding to constitutive transport elements, which transport unspliced RNA to the cytoplasm. In the absence of Rev most retroviral RNA would be spliced in the nucleous.

Therefore, HIV transcription proceeds in two phases. Early in infection, no Tat or Rev proteins are present. RNA synthesis is at very low levels, and all transcripts are fully spliced and no genomes or mRNA for virion proteins are made. Only the set of "early" proteins – particularly Tat, Rev, and Nef- are synthesized. As Tat protein increases in concentration, expression of early proteins increases. Finally, increasing amounts of Rev cause a shift in expression to late proteins at the expense of early gene expression. After this point, Rev serves as a negative feedback regulator, maintaining the balance between expression of the two types of genes. The feedback regulation permits a phased expression but also allows the virus to express a much more complex set of genes than possible with simple retroviruses. [29]

PROTEIN SYNTHESIS

Retroviral protein synthesis takes place at two sites in the cell: *env* is translated on membrane-bound polyribosomes, giving rise to a primary product that spans the membrane of the endoplasmic reticulum. The remaining proteins are synthesized on free polyribosomes.[37] Rybososomes bind to the capping group at the 5' end of the mRNA and move along until they find the first methionine codon, at which point translation begins, following the *one mRNA-one protein* rule.

As an exception, *Gag*, *pro* and *pol* are translated coordinately from the same initiation codon, but maintaining the proper balance of the gene products: because of their structural role, Gag proteins need to be present at about 20 times the amount in virions as the enzymes Pro and Pol. For this purpose, all retroviruses contain a run of U residues just upstream a stem-loop structure near the *gag* terminator. Ribosomes encountering the stem-loop often slip into de the pro-pol frame. Given

that this occurs about 5% of the time, the primary translation products Pr65 Gag and Pr160 Gag-Pro-Pol are generated in a 20:1 ratio.[37]

Another exception to the *one mRNA-one protein* rule is that, despite *vpu* and *env* genes are located in the same mRNA, ribosomes often skip the *vpu* starting codon allowing frequent initiation at *env*.[29] This mechanism allows the coordinated expression of both genes and places newly synthesized Vpu at the inner surface of the endoplasmic reticulum, where it is needed for CD4 removal.

The signal peptide at the N-terminus of *env* directs traslation and migration through the endoplasmic reticulum to the Golgi apparatus where it is extensively glycosylated and cleaved by a cellular enzyme into the functional subunits SU and TM before reaching the cell surface.

VIRAL BUDDING AND MATURATION

Viral budding proceeds by simultaneous association of RNA, Gag and Gag-Pro-Pol proteins, and membrane. However, only some portions of Gag are required for the assembly of virion-like particles. Due to a specific interaction between MA and the cytoplasmic tail of SU, Env proteins become associated with the surface of the budding particle. Other non-specific cellular proteins are also incorporated on the surface of HIV and SIV, virions including MHC proteins.[29] Finally, virions are released from the infected cell. Of note, p6 mutants are unable to leave the cell and remain attached by a short stalk, probably because of problems in the bud closure. To prevent cell-surface CD4 from binding to freshly budded viruses (which could stop the release of virions), Vpu removes newly synthesized CD4 from the endoplasmic reticulum, Nef removes and degrades cell-surface CD4,[38, 39] and Env binds to itself and sequesters CD4.

Free virions are initially immature and unable to infect new cells until the protease is activated and cleaves the Gag-Pro-Pol precursors. Unlike cellular proteases, HIV-1 PR is active only in a dimeric state because the monomer encoded by Pro contains only half of an active site. Likely, self-assembly of Gag-Pro-Pol precursor permits formation of few active dimers. These PR dimers cleave the Pro peptide rapidly

out of the remaining precursors, leading to a chain reaction and acceleration of the processing of the whole structure. This process leads to condensation of the core into its characteristic cone shape, and ends up with the development of fully infective viruses. [29]

ANTIRETROVIRAL THERAPY

Antiretroviral therapy is designed to arrest viral replication by interfering with critical steps of the virus cell cycle. Most antiretroviral drugs inhibit HIV proteins essential for virus cell entry,[5] reverse transcription,[40] integration[41] and maturation.[42, 43] The new small molecule CCR5 or CXCR4 antagonists cause allosteric inhibition of these human chemokine receptors.[1] In few infectious diseases treatment has evolved so much in so little time. Today, the therapeutic arsenal of HIV infection comprises 22 antiretroviral drugs from 7 different drug classes,[44, 45] with many more in the pipeline.

Table 5. Antiretroviral drugs in 2008

Drug class	Drug name	Trade Mark
Reverse Transcriptase Inhibitors (RTIs)		
Nucleoside analogues (NRTIs)	Abacavir (ABC)	Ziagen
	Zidovudine (AZT)	Retrovir
	Stavudine (d4T)	Zerit
	Emtricitabine (FTC)	Emtriva
	Didanosine (ddI)	Videx
	Lamivudine (3TC)	Epivir
Nucleotide analogues (NtRTI)	Tenofovir (TDF)	Viread
Non-nucleoside analogues (NNRTIs)	Efavirenz (EFV)	Sustiva
	Nevirapine (NVP)	Viramune
	Etravirine (ETV)*	Intellence
Protease Inhibitors (PIs)	Atazanavir (ATV)	Reyataz
	Darunavir (DRV)	Prezista
	Fosamprenavir (FAPV)	Telzir
	Indinavir (IDV)	Crixivan
	Lopinavir (LPV)	Kaletra
	Ritonavir (RTV)	Norvir

	Saquinavir (SQV)	Invirase
	Tipranavir (TPV)	Aptivus
Fusion inhibitors	Enfuvirtide (ENF, T-20)	Fuzeon
CCR5 antagonists	Maraviroc (MRC)	Celsentri
Integrase Inhibitors		
Strand-transfer inhibitors (INSTI)	Raltegravir (RAL)	Isentress
	Elvitegravir (ELV)*	Unknown
Co-formulated pills		
NRTI	ZDV / 3TC	Combivir
	ZDV / 3TC / ABC	Trizivir
	ABC / 3TC	Kivexa
	TDF / FTC	Truvada
NRTI + NNRTI	TDF / FTC /EFV	Atripla
PI	LPV / rtv	Kaletra Meltrex

**Drugs not formally approved by the end of 2008 but close to approval.*

PRINCIPLES OF ANTIRETROVIRAL THERAPY

The primary goals of antiretroviral therapy are to reduce HIV-related morbidity, prolong survival, improve quality of life, restore and preserve immunologic function and prevent HIV transmission.[44, 45] These goals can be achieved by maximally and durably suppressing viral replication.

Combined antiretroviral therapy tackles HIV's adaptative mechanisms by taking advantage of two aspects: first, that the pre-existence of viral variants resistant to 3 drugs is unlikely in treatment-naïve subjects[46] and, second, that the rate of viral evolution is highly dependent on the viral replication rate.[46] By combining a minimum of three antiretroviral drugs, antiretroviral therapy is able to suppress viral replication to undetectable levels, increase CD4+ T-cell counts, and improve survival and quality of life. Although viral replication cannot be completely halted, antiretroviral therapy can suppress viremia below 50 copies/mL during several years.[47] Importantly, while HIV-1 RNA levels remain undetectable, the risk of HIV transmission is extremely low - but not zero.[48]

When maximal initial suppression is not achieved or is lost, changing to a new regimen with at least two active drugs is required. If this is not possible in a clinically and immunologically stable patient, an interval of persisting viremia may be acceptable while waiting for arrival of potent new therapies.[49]

In order to achieve treatment goals, clinicians must select carefully the initial combination regimen taking into account efficacy, pill burden, potential side effects, comorbidities, interactions with other required medications, and results of pretreatment genotypic drug resistance testing. Conditions that promote adherence should be maximized prior to initiating antiretroviral therapy.[44, 45]

MECHANISMS OF ACTION OF ANTIRETROVIRAL DRUGS

Nucleoside and nucleotide analogues

Nucleoside and nucleotide analogues are chain terminators. After phosphorylation by cellular kinases, NRTIs are incorporated by reverse transcriptase into the nascent chain of viral DNA. Because they lack a 3' hydroxyl group, no additional nucleotides can be appended, and the synthesis of viral DNA is arrested. Nucleotide analogues are already phosphorylated, so intracellular phosphorilation is not required.

Non-Nucleoside analogues

Non-nucleoside reverse-transcriptase inhibitors are small molecules with strong affinity for a hydrophobic pocket located near the catalytic domain of reverse transcriptase. Inhibitor binding affects the flexibility of the enzyme, thereby blocking its ability to synthesize DNA.

Protease Inhibitors

The HIV protease cleaves large polyprotein precursors at specific sites, releasing the structural protein[50]s and enzymes necessary for the assembly of infectious viral particles. In the absence of a functional protease, viral particles are produced, but they are immature and non-infectious. The protease of HIV is a symmetrically assembled homodimer with a central, symmetric, substrate-binding cavity.[51] Protease inhibitors mimic the structure of the natural viral substrates,

competing with them for attachment in the enzyme's active site, thus inhibiting the catalytic activity of the PR in a highly selective manner.[51]

Integrase Inhibitors

Raltegravir and Elvitegravir are DNA strand transfer inhibitors that block the joining of the processed viral DNA ends into the host chromosome. Strand transfer inhibitors likely interact with the Mg^{2+} cofactor present in the integrase active site, resulting in a functional sequestration of such critical metal cofactor.[52] Antiretroviral drugs inhibiting HIV integration by other mechanisms are still in early development.

Fusion Inhibitors

Enfuvirtide is a 36-mer synthetic oligopeptide whose sequence corresponds to that of the HR-2 region of the HIV-1 envelope gp41 subunit. Binding of enfuvirtide to the trimeric HR-1 complex prevents the association of HR-1 with HR-2, thereby inhibiting fusion and blocking virus entry.

Small-Molecule CCR5 Antagonists

CCR5 antagonists are small-molecule allosteric inhibitors of the human CCR5 chemokine receptor, a receptor that can be found on several host defense cells. CCR5 antagonist binding to the CCR5 receptor is thought to alter the conformational state of the CCR5 receptor, thereby inhibiting the binding of gp120 to CCR5 by an allosteric mechanism.[1]

ANTIRETROVIRAL DRUG RESISTANCE

Antiretroviral resistance is the need of increasing concentrations of antiretrovirals to suppress viral replication compared with non-resistant virus; thus, resistance is a continuum.

In vivo, antiretroviral resistance is a function of viral susceptibility and the drug levels achieved in the target cells, where viral replication occurs. Higher drug levels can suppress partially resistant viruses. Viral

susceptibility is expressed as the drug concentration that is able to inhibit virus growth *in vitro* to 50% (50% inhibitory concentration, IC_{50}) or 90% (IC_{90}), relative to a wildtype reference virus.[53]

A critical factor for understanding HIV resistance is that HIV has a quasispecies distribution.[54] Soon after infection with a relatively homogeneous viral population, viral replication ensues at an extraordinary rate: 10^{9-12} new virions are generated every day. Because HIV's RT lacks proofreading ability, 10^{-3} to 10^{-4} mutations (one or two per genome) are spontaneously generated per replication cycle.[46, 50] Given HIV's high replication rate, any single mutant and some dual mutants could be generated per day.[46] Most mutations are deleterious and drive mutant viruses to extinction. Others, have neutral or beneficial effects on HIV's replicative capacity and remain incorporated in the quasispecies.[54]

Variants in the virus quasispecies may have different fitness in different environments.[55] The variant with better ability to replicate in the absence of therapy, the WT variant, predominates before therapy initiation. Mutants with a fitness advantage in the presence of therapy remain at very low levels in the absence of treatment. However, they can outcompete the WT within days after therapy initiation if viral replication is not rapidly averted. Secondary mutations often accumulate in the presence of continued viremia; they compensate the potential fitness losses derived from primary resistance mutations and increase cross-class resistance.

The likelihood of developing antiretroviral resistance depends on the relative potency of the antiretroviral regimen and the degree of ongoing replication in the presence of therapy.[46, 51, 54, 56-59] (Figure 8) A regimen with small antiviral potency creates a minimal selective pressure to the virus and leads to slow resistance evolution, even if replication persists. A more potent regimen that is unable to suppress viral replication leads to an increased selective pressure over the virus, which rapidly accumulates resistance. Finally, a highly potent regimen that decreases viral replication to minimal levels is associated with slow resistance accumulation, despite the potent selective pressure exerted to the virus.

In addition, each antiretroviral therapeutic class has a unique adherence-resistance relationship. (Figure 9) As conceptualized by Bangsberg et al.,[60] NNRTI-treated individuals rarely develop resistance at high levels of adherence due to the virological effectiveness of these regimens. NNRTI resistance develops rapidly at moderate to low levels of resistance due to the low 'fitness' costs associated with single mutations. Single PI-treated individuals may develop resistance at high levels of adherence because residual viral replication is often seen in such patients. PI resistance is uncommon at low levels of adherence because of the significant fitness costs associated with these mutations. Resistance to a ritonavir-boosted PI is only possible in a narrow range of adherence where there is sufficient drug around to select for mutations that reduce fitness while still allowing residual viral replication.

Attempts to block viral replication through intensified antiretroviral regimens including more than three antiretrovirals have not demonstrated higher antiviral efficacy than standard 3-drug regimens.[61, 62] Residual viral production persists in plasma, body compartments with limited antiretroviral penetration (CNS, testes, kidney, etc), and cellular reservoirs of low turnover or latently HIV-infected cells that can reinitiate viral production when needed. [63-69]

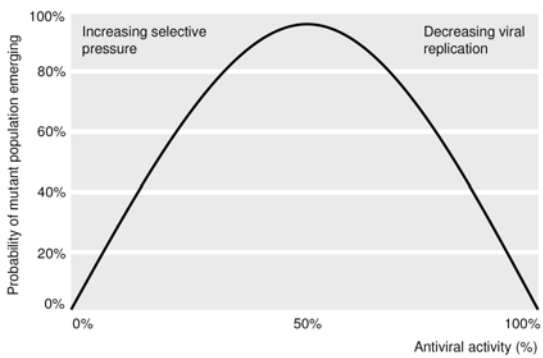


FIGURE 8. RELATION BETWEEN ANTIVIRAL DRUG ACTIVITY AND EMERGENCE OF RESISTANCE. *Source: Pillay D and Zambon M. Education and debate: Antiviral drug resistance. BMJ 1998; 317: 660-662*

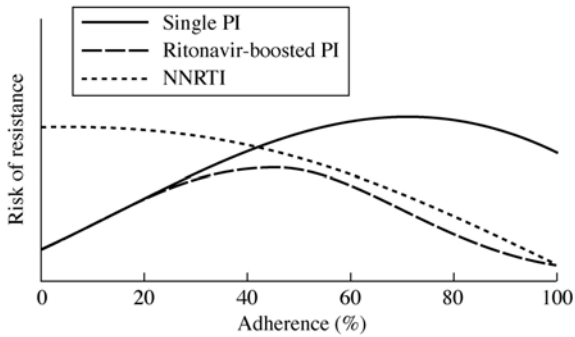


FIGURE 9. RELATIONSHIP BETWEEN MEDICATION ADHERENCE AND THE RISK OF DEVELOPING PI OR NNRTI DRUG RESISTANCE. Resistance to single PI therapy occurs most frequently at moderate to high levels of adherence, resistance to NNRTI therapy occurs at low to moderate levels of adherence, and resistance to ritonavir-boosted PI therapy is most likely to occur at middle ranges of adherence. Data in this figure are conceptual and based on trends observed in a number of recent studies PI, protease inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor. Source: *Bangsberg DR, Moss AR, and Deeks SG. Paradoxes of adherence and drug resistance to HIV antiretroviral therapy. J Antimicrob Chemother. 53, 696-699*

Given the molecular structure similarities within compounds of the same antiretroviral family and their interaction with similar target sites, the emergence of resistance to one drug will often extend to the other drugs of the same family with variable degrees. On the other hand, some mutations conferring high-level resistance to one agent may increase viral susceptibility to another compound, resulting in a so-called "hypersusceptible" virus to the other agent. In addition, many resistance-conferring mutations decrease replication capacity in comparison with the WT virus. The clinical correlates of replication capacity measurements, however, remain unclear.

FURTHER BENEFITS OF ANTIGENIC VARIATION

In addition to enabling rapid evolution of HIV resistance, HIV's huge antigenic variation capacity confers other important general advantages to the virus:

(a) *Extension of the time of infection within the host.* Antigen variation allows immune escape and continuation of vigorous infection. Extended infection time benefits the virus by increasing the chances for transmission to new hosts.

(b) *Re-infection of hosts with immune memory from previous infections.* Antigenic variants that differ from a host's previous infections escape that host's memory response. The distribution of the memory profiles between hosts determines the success of each antigenic variant.

(c) *Variation in surface antigens may allow pathogens to attach with variable success to cellular receptors of different host genotypes.* Particular antigenic variants attack some host genotypes better than others.

(d) *Variable surface antigens permit enhanced pathogen fitness by allowing colonization of different host tissues.* Some HIV variants have preferential tropism for tissues with lower replication kinetics or reduced drug penetration. Similarly, R5 to X4 tropism switches frequently occur in subjects with advanced HIV infection, allowing infection of T lymphocytes, which is associated with accelerated clinical progression.

(e) *Some variants exert antigenic interference with the immune response to others.* For example, a host may first encounter a particular antigenic type and then become infected by or develop a cross-reacting variant through antigenic mutation. The second variant may stimulate a host memory response to the first variant rather than a new, specific response to the second variant, in a phenomenon known as *original antigenic sin.* In other cases, two coexisting variants may interact with the immune system so that one or both variants benefit from the protection created by the presence of the other, through *altered peptide ligand antagonism* or other mechanisms.

CLINICAL IMPLICATIONS OF ANTIRETROVIRAL RESISTANCE

The most obvious clinical consequence of antiretroviral resistance is loss of treatment efficacy. In general, resistance-associated treatment

failure will lead to prescription of more complex, less tolerable regimens. Consecutive treatment lines are associated with progressively reduced duration of antiviral efficacy. Each failure is associated with further resistance accumulation; some patients will eventually develop viruses resistant to all drug classes.

Moreover, the emergence of antiretroviral resistance among patients starting first-line HAART is associated with a nearly 2-fold increased risk for death.[70, 71] Interestingly, emergence of resistance to NNRTIs seems to be associated with a greater risk of subsequent death (3-fold increase) than emergence of resistance to any other class of drug.

To delay the evolution of antiretroviral drug resistance, it is essential to suppress viral replication profoundly and durably, and manage viral replication rebounds aggressively.

MECHANISMS OF ANTIRETROVIRAL DRUG RESISTANCE

Most resistance mutations are simple aminoacid substitutions in the proteins targeted by antiretroviral drugs but some include insertions of one or more aminoacids. In general, resistance mutations alter the 3-dimensional structure and biochemical properties of viral proteins or co-receptors, reducing the activity of drugs through a variety of mechanisms.

NUCLEOSIDE AND NUCLEOTIDE ANALOGUES

Resistance to nucleoside and nucleotide analogues can be summarized in four different pathways: the M184V/I mutation; the thymidine-analogue (TAM) 1 and 2 pathways; the K65R and L74V pathway, and the multinucleoside resistance pathway, which includes the Q151M and/or 69 insertion complexes.

Mutations M184V, K65R and the Q151M complex promote resistance by selectively impairing the ability of reverse transcriptase to incorporate an analogue into DNA. (Figure 10) Conversely, TAMs induce removal of the nucleoside analogue from the 3' end of the terminated DNA chain. This process involves an ATP- or pyrophosphate-mediated attack to the phosphodiester bond linking the

nucleoside analogue to the DNA chain.[72, 73] Entry of ATP and pyrophosphate, a by-product of DNA polymerization, is facilitated by the structure of a reverse transcriptase expressing TAMs.[50] However, such entry is significantly decreased in the presence of the M184V mutation, what explains the difficulty for TAMs to emerge in the presence of M184V.

The M184V/I mutation

Mutations M184V (ATG→GTG) and M184I (ATG→ATA) in the conserved YMDD motif of the HIV-1 reverse transcriptase (RT) induce high-level lamivudine and emtricitabine resistance [40, 74-77] by altering the interaction between the enzyme's active site, the primer/template duplex and the incoming deoxynucleoside triphosphates. [78] Lamivudine resistance develops very early when this drug is given in monotherapy [75, 79] or in combination with zidovudine or stavudine.[57, 80, 81] The M184I mutation is the first to emerge, being detectable as early as 7 days after the initiation of treatment,[75] because of the mutational bias in the RT toward G→A replacements.[82] Under the continuous presence of drug, viruses harboring the M184V mutation emerge because they are more fit than wildtype and M184I mutants. [83] M184V variants can be detected as early as 3 weeks after starting lamivudine therapy and become fixed in virtually all subjects after 12 weeks [75]. Likewise, M184V is the first and most likely resistance-related mutation to arise in subjects with active replication under lamivudine-including 3-drug ART[84-86] and in those undergoing structured treatment interruptions (STIs).[87, 88] M184V or M184I mutations confer >1000-fold resistance to both lamivudine and emtricitabine.[40, 74-77] Mutations M184V/I also decrease the susceptibility to abacavir and didanosine. M184V hypersensitizes HIV-1 to zidovudine and tenofovir, and exerts synergy with these drugs by altering RT's processivity among multiple of other effects.[89-94] Importantly, M184V impairs the replication capacity of mutant viruses in the presence of lamivudine, relative to wildtype viruses in the absence of therapy.

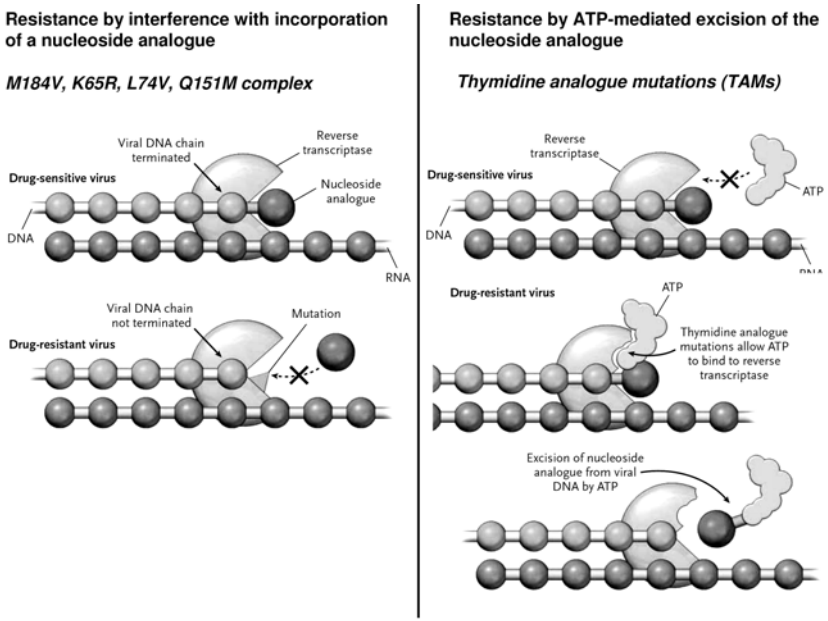


FIGURE 10. THE TWO PRINCIPAL MECHANISMS OF RESISTANCE OF HIV TO NUCLEOSIDE ANALOGUES. In the left panel, the incorporation of a nucleoside analogue into drug-sensitive viruses results in the termination of the viral DNA chain. Mutations in drug-resistant viruses prevent the incorporation of the nucleoside analogue into the growing viral DNA chain. In the right panel, ATP in drug-sensitive viruses does not have access to a reverse transcriptase that has formed a complex with a nucleoside analogue. Mutations that cause resistance to nucleoside analogues, referred to as thymidine analogue mutations, allow ATP to bind reverse transcriptase near the 3' end of viral DNA terminated by the incorporation of a nucleoside analogue. ATP then excises the analogue from viral DNA, allowing reverse transcription to proceed normally. Source: *Clavel F and Hance A. Medical Progress: HIV Drug Resistance N Engl J Med 2004;350:1023-35.*

The TAM pathways

Thymidine-analogue resistance mutations (TAMs, at positions 41, 67, 70, 210, 215, and 219),[95, 96] confer resistance to ZDV and d4T, and partially contribute to ABC and ddI resistance. [97, 98] There are two TAM pathways, TAM1 (41L, 210W, 215Y) and TAM2 (67N, 70R and 219E/Q), which tend to be mutually excluding, particularly during early TAM accumulation. The TAM1 pattern probably exerts stronger clinical cross-resistance to tenofovir than TAM2 in subtype B viruses.[99] In general, significant decreases in NRTI susceptibility require the stepwise accumulation of several resistance mutations.[55, 80, 95, 100-107]

Evolution along the TAM pathways leads to increasing resistance levels and extension of cross-resistance to more NRTIs. (Figure 11)

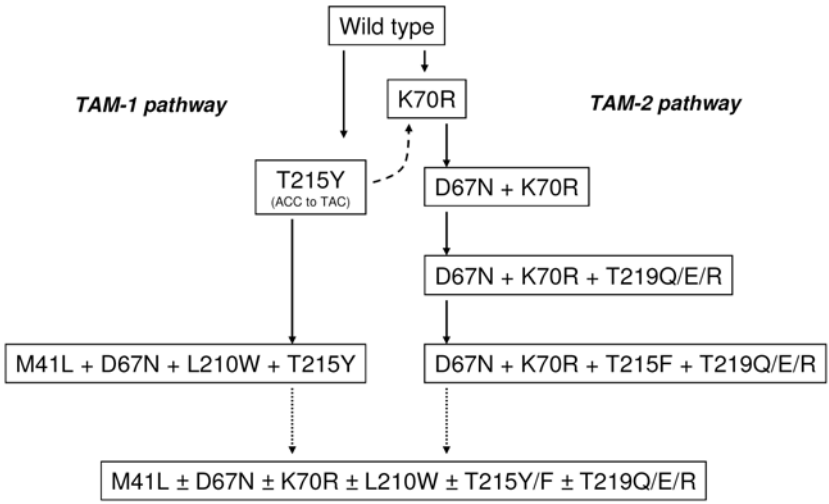


FIGURE 11. ORDERED ACCUMULATION OF THYMIDINE ANALOGUE RESISTANCE MUTATIONS. The single point mutation at codon 70 (K–>R) emerges first but confers relatively low-level ZDV resistance. Subsequently, the T215Y mutation emerges in different viral genomes and these viruses overgrow the K70R mutants. Because the T215Y substitution requires a double-nucleotide mutation (ACC to TAC), it would be expected to occur at a much lower frequency than that of the K70R point mutation, thus explaining its later emergence. Continued replication of partially resistant T215Y mutants in the setting of nonsuppressive regimens eventually results in the accumulation of the M41L, L210W, and D67N mutations. Alternatively, the D67N mutation arises in viruses already carrying a K70R mutation, with eventual accumulation of K219Q, K219E, or K219R substitution and the T215F substitution (which, like T215Y, requires a double mutation [ACC to TTC]). The poor replication kinetics and lower fitness of T215F compared to T215Y along with the modest advantage over the wild-type virus in the presence of ZDV most likely explain why this mutation rarely occurs as an initial ZDV resistance mutation or in association with M41L. If replication under thymidine analogues ensues, mixed TAM 1/2 patterns can emerge. Source: *Hu Z, Giguel F, Hatano H, Reid P, Lu J, and Kuritzkes DR. Fitness Comparison of Thymidine Analog Resistance Pathways in Human Immunodeficiency Virus Type 1. J Virol. 2006; 80(14): 7020–7027.*

Interestingly, although TAMs induce resistance to most NRTIs *in vivo*, viruses with TAMs have much smaller *in vitro* susceptibility decreases to abacavir, tenofovir or didanosine than to zidovudine.

Indeed, susceptibility to didanosine as measured in tissue cell culture is often below the level of significance. This discrepancy between *in vivo* and *in vitro* data may be explained, on one hand because zidovudine is a larger molecule that could more easily be excised. On the other hand, ddI, ABC and TDF can escape excision by forming excision refractory closed complexes, which is facilitated by high concentrations of dNTPs. Because the tumor-derived cells used in HIV phenotypic assay systems contain high levels of dNTPs, it is possible that resistance in cells with lower dNTP pools (such as the natural target cells of HIV *in vivo*) may be significantly higher.

The K65R and L74V mutations

This pathway affects susceptibility to tenofovir (TDF) and abacavir (ABC), and includes the K65R and L74V mutations plus several accessory mutations (T69N/S/A, V75T), situated in a loop between the β2 and β3 strands in the "fingers" region of the RT. [108]

Mutation K65R is the signature mutation for TDF, although it also confers intermediate levels of resistance to ddI, ABC, 3TC, FTC, and low-level resistance to d4T. [108-117] K65R hypersensitizes HIV-1 to ZDV and does not develop in patients receiving ZDV-containing regimens. [112]

L74V occurs frequently during ddI and ABC monotherapy .[118]L74V is ddI's signature mutation, being sufficient to cause failure in patients receiving ddI monotherapy. ABC failure, in contrast, requires the accumulation of additional mutations.[119] As well, L74V hypersensitizes HIV-1 to ZDV and, less clearly, to d4T and TDF.

Mutations at position 69 are the most frequent substitutions in HIV-1 genotypes other than TAMs and M184V, and have been shown to contribute to resistance to every NRTI. V75T confers resistance to d4T, ddI and ddC, and generally occurs in the context of multinucleoside resistance.

The Q151M and 69 insertion complexes

A fourth, infrequent pathway includes multinucleoside resistance driven by the Q151M mutation and/or 69 insertions. [97, 98, 120, 121]The Q151M complex is seen in less than 5% of all HIV strains with

resistance to nucleoside analogues. It is most often selected for in the course of the failure of regimens containing stavudine and didanosine, but can also be seen in subjects with prolonged ZDV exposure. Q151M is a two base-pair change in a conserved RT region that is close to the first nucleotide of the single-stranded nucleotide template. Q151M alone causes intermediate levels of resistance to ZDV, ddI, d4T, and ABC, but not to TDF. It is nearly always followed by mutations at positions 62, 75, 77, and 116. Such secondary mutations increase resistance and the activity of RT. Isolates with V75I, F77L, F116Y, and Q151M have high-level resistance to ZDV, ddI, d4T, and ABC, and low-level resistance to 3TC and TDF. Insertions in position 69 occur only in 2% of heavily pre-treated patients and confer high-level resistance to TDF and other NRTIs.

NON-NUCLEOSIDE ANALOGUES

Resistance patterns are different for first- and second-generation NNRTIs.

First generation NNRTIs: nevirapine and efavirenz

First-generation NNRTIs are characterized by having a low genetic barrier to attain resistance.[97, 98] Mutations selected for after nevirapine or efavirenz failure are all located in the drug-binding pocket.[122-128] While most NNRTI resistance mutations affect residues that are directly involved in inhibitor binding, a few have been found to act indirectly, by changing the position or the orientation of the aminoacids involved with direct contact with the inhibitor. NNRTI resistance mutations can be divided into three separate clusters:

- Cluster 1: L100I, K103N, V106A and V108I
- Cluster 2: Y181C, Y188L/C/H , and G190S/A
- Cluster 3:P225H, M230L, and P236L.

The first two clusters represent two opposite sides of the NNRTI binding pocket of RT in the main subunit p66; the third cluster is carried by the second RT subunit, p51.

Because of subtle differences in the interaction between various NNRTIs and the hydrophobic pocket,[123] however, the mutations that emerge most frequently tend to be drug-dependent. Nevirapine resistance is often associated with the Y181C mutation, but other mutations, such as Y188C, K103N, G190A, and V106A, also occur. Given that Y181C increases HIV-1 susceptibility to ZDV[128], Y181C mutants are selected against during nevirapine failure in the presence of ZDV, leading to the emergence of variants containing the K103N mutation. Initial resistance to efavirenz is generally characterized by the K103N mutation, but the Y188L mutation is also seen.

All the abovementioned mutations generate cross-resistance to EFV and NVP. The K103N and Y188L mutations generate high-level phenotypic resistance to both drugs *in vitro*, whereas Y181C causes high-level resistance to NVP but only a two-fold decrease in phenotypic susceptibility to EFV *in vitro*. Nevertheless, clinical cross-resistance to EFV is evident, since subjects failing NVP with an Y181C virus and no K103N detectable are also more likely to fail EFV therapy than those with a WT virus. Sometimes, presence of minority K103N mutants can partially explain the excess risk of virological failure.[129] Y181C mutants could also pre-exist in many subjects as minority variants. Previous reports noted the emergence of NNRTI resistant viruses (mostly Y181C mutants) within one week after the initiation of NVP when given alone or together with zidovudine.[128, 130, 131] It was calculated that minority Y181C mutants were present before therapy at a frequency of 7 and 133 per 10,000 copies of plasma HIV-1 RNA in two subjects in whom this mutation emerged during NVP therapy.[131]

In opposition to PI or NRTI-resistance mutations, NNRTI resistance mutations have a minimal impact on HIV's replication capacity,[125, 126, 132] so NNRTI maintenance is of little utility to impair viral virulence.

These data imply that sequential treatment with EFV and NVP or maintenance of any of these drugs in the presence of detectable viremia is not only futile, but could hamper the virological outcomes of a future treatment with etravirine through the accumulation of further NNRTI resistance mutations. A recent EuroSIDA Study found that

maintenance of NVP or EFV therapy in viremic subjects was associated with a rate of accumulation of NNRTI mutations of 1 every 2.2 years. Etravirine-specific mutations, however, accumulated more slowly (1 every 4 years), leading to an 8% mean reduction in etravirine activity per year. As expected, viruses with higher susceptibility to NNRTIs accumulated NNRTI resistance mutations faster.[133]

The long plasma half-life of NVP and EFV ensures sufficient drug levels during continued treatment. This characteristic, however, becomes problematic when NNRTI treatment needs to be interrupted. The slow decay of NVP or EFV plasma levels in comparison with that of the accompanying NRTIs or PIs often leads to periods of virtual monotherapy characterized by suboptimal NNRTI levels in the presence of active replication, a situation that fuels resistance evolution.

Second generation NNRTIs: etravirine

Etravirine (ETV) is a second generation NNRTI that retains antiviral activity against HIV-1 carrying the K103N mutation. Its potency appears to be related to etravirine's flexibility as a molecule. Etravirine is a diarylpyrimidine, a type of organic molecule with some conformational isomerism that can bind the enzyme reverse transcriptase in multiple conformations, allowing for a more robust interaction between etravirine and the enzyme, even in the presence of mutations.

Mutation Y181C confers a mild decrease in susceptibility to ETV; the accumulation of other resistance-associated mutations (V90I, A98G, L100I, K101E/H/P, V106I, E138A, V179D/F/T, Y181C/I/V, G190S/A and M230L) is required to render HIV-1 highly resistant to this drug. The relative weight of the 17 known etravirine resistance mutations according to clinical outcome (Table 6) and phenotypic susceptibility (Table 7) correlates has been reported.[134-136]

Table 6. Weighted classification of etravirine resistance mutations by clinical outcome in the DUET Trials (Tibotec-Jannsen®).

Weight	Mutations
3	Y181I/V
2.5	K101P, L100I, Y181C, M230L
1.5	E138A, V106I, G190S, V179F*
1	V90I, V179D/T, K101E/H, A98G, G190A

**Always detected together with Y181C*

Interpretation:

Weighted mutation score	Response rates in DUET trials
0-2	74% (highest response)
2.5-3.5	52% (intermediate response)
= or > 4	38% (reduced response)*

**comparable to the control arm*

Table 7. Weighted classification of etravirine resistance mutations by phenotypic susceptibility (Monogram Biosciences®)

Weight	Mutations
4	L100I, K101P, Y181C/I/V
3	E138A/G, V179E, G190Q, M230L, K238N
2	K101E, V106A, E138K, V179L, Y188L
1	V90I, K101H, V106M, E138Q, V179D/F/M, Y181F, V189I, G190E/T, H221Y, P225H, K238T

**Mutations not retained: A98G, V106I, G190A/S.*
Interpretation: A weighted score of 4 is equivalent with the lower clinical cutoff for etravirine (2.9) that defines reduced susceptibility. Mutations with a WF of 4 (L100I, K101P, and Y181C/I/V) are sufficient to confer reduced susceptibility to ETV on their own.

In terms of interpretation of the relative weights of each mutation, the main differences between the two scores are that Tibotec-Jannsen® gives the highest weight to mutations Y181I/V and considers mutations A98G, V106I, G190A/S as clinically relevant. Also, mutation V179F is given a 1.5 weight although it was always found in combination with Y181C. In comparison, the score by Monogram Biosciences® considers mutations L100I, K101P, Y181C/I/V to be the most important and does not retain mutations A98G, V106I, G190A/S as being phenotypically relevant.

The lack of upper cut-off in the Monogram Biosciences®, score makes it difficult to ascertain residual antiviral activity of etravirine in

the presence of multidrug resistant HIV. Finally, as with other NNRTIs, TAMs and M184V seem to increase the susceptibility to etravirine *in vitro*.[137]

In summary, etravirine may remain effective after early virological failure to EFV or NVP-including regimens. Other diarylpyrimidine-analogues like rilpivirine are currently being developed as potential anti-HIV agents.

MUTATIONS IN THE CONNECTION AND RNASE H DOMAINS OF REVERSE TRANSCRIPTASE

It is still unclear if mutations in the RT's connection or RNAseH domains affect the virological outcomes of therapy. These domains are not routinely investigated by commercial genotypic resistance assays; mutations in these regions usually remain undetected and are not incorporated in genotypic resistance scores or other interpretation systems.

Mutations in the connection and RNase H domains of the RT are virtually absent in treatment naïve individuals, but can be coselected on the same genome as TAMs, significantly increasing zidovudine resistance when combined with TAMs.[138-140] They also increase, although to a much lesser extent, cross-resistance to lamivudine, abacavir, and tenofovir but do not affect susceptibility to stavudine or didanosine.[141] Three mutations (N348I, T369I, and E399G/D) in the reverse transcriptase C-terminus are associated with the increased resistance to zidovudine and to NNRTIs. Mutation N348I decreases susceptibility to nevirapine 7.4-fold and efavirenz 2.5-fold and enhances NNRTI resistance in the context of K103N. This mutation is more likely to be selected with zidovudine and nevirapine treatment and may appear early after treatment failure, even preceding TAMs.[142]

In isolation, T369I reduced susceptibility to efavirenz and zidovudine 3 and 2.3-fold.[140] In combination with K103R/V179D T369I, N348I and E399D cause 13-, 6, and 2.6-fold increased resistance, respectively, to efavirenz. The double mutant T369I/N348I causes fold-change of 11, 60 and 7 to efavirenz, nevirapine and zidovudine, respectively. Viruses containing T369I or N348I show

reduced RTexpression, and those containing T369I, N348I or E399D show impaired processing of p55gag polyproteins.[140]

Mutation E399G increases efavirenz resistance 3.6-fold and reduces viral replication capacity when associated with other mutations in the NNRTI binding pocket (L100I, V106I, V179D, and F227C). Mutations in the C-domain could modulate NNRTI resistance by affecting dimerization of p66/p51 heterodimers [143] or slowing the RNAseH processivity, thus allowing more time for primer excision to occur.

The clinical utility of detecting mutations in the connection or RNAseH domains will depend on (a) whether these mutations are early markers of the future TAMs accumulation, and (b) the extent to which they reduce susceptibility to NNRTIs, particularly etravirine.

PROTEASE INHIBITORS

The substrate-binding domain of the protease is a symmetrical cavity delimited on both sides by the two subunits of the dimer.[50] The "floor" of the cavity contains two aspartic residues at position 25 that are essential for the proteolytic cleavage reaction. The "roof" of the cavity is formed by two mobile flaps, which open when the cavity is empty and close when it becomes occupied by its natural substrate or an inhibitor.[50] The overall shape and chemical properties of the cavity, as well as the mobility of the flap are important for proper substrate recognition, binding and cleavage. [51, 144, 145]

Protease inhibitors have been designed to fit the cavity very tightly, based on the precise knowledge of its 3-D structure.[145] Therefore, substitutions of aminoacids in direct contact with the inhibitor or of distant aminoacids that modify the overall shape of the cavity, will both be able to disrupt fitting of the PI within the cavity and induce resistance.[50, 51, 144, 145]

Most PI resistance mutations appear to enlarge the size of the substrate-binding cavity. Usually, PIs occupy more space in the substrate-binding cavity that the natural protease substrates. Such natural substrates require sequential and ordered cleavage, which implies less tight interactions with the protease and less efficient

cleavage from this enzyme. Therefore, the development of PI resistance only impairs Gag and Gag-Pol cleavage slightly, and synthesis of mature virions can continue.

Mutations in Gag or Gag-Pol can also induce PI resistance, particularly in the presence of resistance mutations in protease.[53] The primary function of Gag cleavage site mutations, however, is to partially compensate for the loss of cleavage by the protease at these sites.

Each PI is associated with its own "major" or signature mutations; persistent viral replication under PI therapy leads to the accumulation of additional major and minor mutations that increase cross-resistance to other PIs and compensate for fitness reductions derived from major mutations.[53, 146] Each individual PI mutation tends to have a small impact of virus susceptibility *in vitro;* some "secondary" mutations may cause larger decreases in susceptibility than major PI resistance mutations.[50]

Protease inhibitor-naïve subjects who experience virologic failure of a ritonavir-boosted PI regimen often lack PI resistance mutations and have less NRTI resistance mutations than with non-boosted PIs. Subjects with virological failure to boosted-PIs should be switched to new antiretroviral regimens including darunavir or tipranavir, raltegravir, etravirine or maraviroc, even in the lack of evident resistance mutations to the failing PI regimen. [41-43, 147-151]

Saquinavir resistance

Saquinavir's signature mutations are G48V and L90M. Each mutation results in a 3- to 10-fold decrease in susceptibility; viruses with both mutations have more than 100-fold decreased susceptibility.[152-154] L90M mutation is the most frequent saquinavir mutation observed *in vivo.* The G48V mutation appears *in vitro,* but it is rarely observed *in vivo,* being more frequent in subjects that received high-dose non-boosted saquinavir in the past.[155] *In vivo*, G48V is frequently found alongside V82A mutation,[152, 153] which confers cross-PI resistance. The L90M mutation reduces susceptibility to most other PIs, particularly to nelfinavir. L90M has been found to predict lopinavir/ritonavir failure and is associated with reduced virological response to ritonavir-bosted atazanavir therapy in PI experienced patients.[156] As with other PIs,

saquinavir resistance mutations are much less frequent when this drug is given alongside ritonavir boosting.[146] Saquinavir secondary mutations include L10I/R/V, L24I, I54V, I62V, A71V/T, G73S, V77I, V82A/F/T/S and/or I84V. Conversely, mutation V82T is associated with increased susceptibility to saquinavir *in vitro*.[157]

Indinavir resistance

Indinavir is a potent but highly toxic PI rarely used nowadays because of its poor tolerability. Major mutations include M46I, V82A/F/T, and I84V.[59] Amino acid substitutions at positions 10, 20, 24, 54, 63, 64, 71, and 90 in PR are also associated with resistance *in vivo*. The number of mutations predicts the degree of resistance. Mutations M46L/I and V82A are the first to occur during indinavir failure, followed by I54V or A71V/T.[158] Indinavir-associated mutations induce broad cross-resistance to ritonavir, amprenavir, lopinavir/ritonavir, and tipranavir being major mutations for these PIs; they also decrease susceptibility to saquinavir, nelfinavir, and atazanavir. The accumulation of a minimum of 4 substitutions in protease (M46I, L63P, V82T, and I84V) confers resistance to most PIs.[59]

Ritonavir resistance

Today, ritonavir is only used as a pharmacokinetic enhancer because of its capacity to potently inhibit different cytochrome P450 isoenzymes. The I84V substitution is the major determinant of ritonavir resistance. The addition of the V82F mutation significantly increases resistance to ritonavir but impairs the replication capacity of the double mutants. Additional M46I, L63P, and A71V mutations improve replication capacity. Importantly, patients with rebound viremia while on ritonavir typically accumulate resistance mutations in the following: V82F → M36I, I54V, A71V →I84V, L90M.[46, 56]

In vitro, ritonavir-resistant strains including either the single mutations I84V or V82F or the combination of the 4 mutations M46I, A71V, V82F, and I84V displayed similar levels of resistance to ritonavir and indinavir, but only a slight reduction in susceptibility to saquinavir.[58, 59] However, the triple mutants including M46I, L63P, and I84A have an

80-fold increased IC_{50} for ritonavir, saquinavir, and indinavir, and a 125-fold increased IC_{50} for nelfinavir.

Nelfinavir resistance

On the June 6th, 2007 both the Medicines and Healthcare products Regulatory Agency and the European Medicines Agency (EMEA) released an alert requesting the recall of any of the drug in circulation because of fears that batches of the therapy may have been contaminated with potentially cancer-causing chemicals. Nelfinavir is thus no longer used to treat HIV infection. Research is ongoing to try to reposition this drug as an anti-cancer agent.

For subjects who took nelfinavir in the past, it is interesting to remember that nelfinavir-resistant viruses often preserve sensitivity to other PIs. The D30N mutation is the first to appear during nelfinavir failure in patients infected with subtype B HIV; L90M is the first mutation in non-subtype B HIV-1 infected subjects. The D30N mutation confers high-level nelfinavir resistance *in vitro* (>30-fold increase in IC_{50}), but also impairs replication capacity[159] to the extent of constraining the accumulation of additional mutations. Occasionally, substitutions at positions 36, 46, 71, 88 can be found alongside D30N. The D30N mutation does not reduce susceptibility to other PIs. Indeed, mutation N88S can cause hypersusceptibility to amprenavir in vitro.[160, 161]

The L90M mutation is the signature mutation in non-subtype B HIV-1, but can also emerge in subtype B viruses in the face of persistent viremia under nelfinavir exposure. L90M is often associated with other minor mutations that confer cross-resistance to other PIs, especially to saquinavir, ritonavir, indinavir, tipranavir, and lopinavir/ritonavir, but also to amprenavir and atazanavir.

Amprenavir and fosamprenavir resistance

Amprenavir has been replaced by its calcium phosphate ester prodrug, fosamprenavir, which has a much more favorable pharmacokinetic profile, reduced pill burden, and no food interactions. However, fosamprenavir is quickly hydrolyzed in the gut to produce the

active compound amprenavir and virtually no fosamprenavir reaches the bloodstream. Therefore, the resistance profile of both drugs is superimposable. Ritonavir boosting of fosamprenavir, however, decreases the frequency of of resistance mutations.[146]

Patients with early virologic failure to amprenavir often lack genotypic or phenotypic evidence of cross-resistance to other PIs *in vitro*, although low-level cross-resistance to ritonavir and lopinavir is sometimes noted.[162] On the other hand, many patients with virologic failure to other PIs may remain susceptible to amprenavir. [163]

The signature resistance mutation for amprenavir is I50V,[164] which is rare after virological failure to most PIs. It appears early after amprenavir failure and, as a single mutation, confers 2- to 3-fold decreased susceptibility to amprenavir. However, I50V confers low fitness, so acquisition of high-level resistance to amprenavir sometimes requires additional substitutions in Gag.[165-167] Of note, I50V is associated with the greatest reduction in darunavir susceptibility but increases viral susceptibility to tipranavir.[147]

The second amprenavir signature mutation is I84V, although it is less common than I50V, I54L/M, or the V32I + I47V combination.[168] Mutation I84V is associated with reduced susceptibility to lopinavir, indinavir, nelfinavir, ritonavir, saquinavir, and tipranavir.[169, 170] The addition of V82F to I84V significantly increases resistance to ritonavir, indinavir, nelfinavir, and amprenavir.[171]

Mutations I54L/M and/or the combination V32I + I47V are often seen in patients failing ritonavir-boosted amprenavir, but they usually appear in patients with further experience to PIs.[172] I54L/M and V32I + I47V reduce the virologic response to ritonavir-amprenavir[173] and confer cross-resistance to other PIs.

In addition to the four main pathways of amprenavir resistance, namely I50V, I54L/M, V32I + I47V, and I84V, multiple accessory mutations are also associated with resistance to amprenavir *in vivo*, including substitutions at residues 10, 32, 46, 47, 54, 73, and 90.[168] Significant resistance to amprenavir can be predicted when one of the following combinations of mutations is encountered

1. I84V alone;

2. I50V plus one or two mutations of the following group: L10F/I/R/V, V32I, M46I/L, I54L/V, V82I/F, or L90M; and
3. three or more mutations of the above listed group (L10F/I/R/V, V32I, M46I/L, I54L/V, V82I/F, or L90M).

Finally, mutations K20T and N88S[157, 174] and the atazanavir-related mutation I50L[175] have been associated with hypersusceptibility to amprenavir and other PIs, particularly when other resistant mutations are present.

The specific mutational pathways observed at first failure of unboosted fosamprenavir b.i.d. are either the I54L/M mutation or the V32I plus I47V mutations. The I50V mutation can emerge if fosamprenavir is continued in the presence of ongoing viral replication. Other major fosamprenavir mutations include I54M, L76V and I84V[176-180] The accessory mutations L33F and to a lesser extent V11I and L89V are associated with decreased fosamprenavir susceptibility.[180-182] Finally, mutations M46I/L, I54V/A/T/S, V82A/T/F/S, and L90M rarely occur during fosamprenavir failure, but are common with other PIs and can confer low-to-intermediate cross-resistance to this drug. N88S increases fosamprenavir susceptibility *in vitro* and *in vivo.* [183, 184]

Lopinavir/ritonavir resistance

Resistance to lopinavir/ritonavir can develop through two different pathways:[185, 186] One resembles the indinavir pathway, with mutations M46I/L, I54V/T/A/S, and V82A/T/F/S.[169] The second is similar to the amprenavir resistance profile and includes mutations V32I, I47V/A, I50V, I54L/M and L76V.[180, 187-189]

Mutations at position 82 reduce susceptibility approximately 2-fold. Mutations at positions 54 + 82 reduce susceptibility approximately 10-fold. The combination of mutations at positions 46, 54, and 82 together with accessory mutations at positions 10 and 20, reduces LPV susceptibility >50-fold and reduce the virologic response to LPV/r salvage therapy.[169, 187, 190, 191]

I47V and less commonly I47A are selected during LPV/r salvage therapy.[180, 189] I47A is a rare mutation which occurs in combination with V32I in which case it reduces LPV susceptibility >50-fold.[192-194] L76V is selected by and is associated with decreased LPV susceptibility.[187, 188]

The accesory mutations L24I and F53L have been associated with reduced LPV susceptibility.[169] L33F is selected during LPV salvage therapy and associated with reduced LPV susceptibility and virological response.[195]

Mutations I84V and L90M acquired during virological failure to other PIs, each reduce LPV susceptibility approximately 2-fold and can contribute to higher levels of resistance when associated with other LPV-resistance mutations. Both mutations appear to reduce susceptibility to LPV less than that of other PIs except darunavir. G48V is not selected by LPV but has been associated with decreased virological response in a large multivariate analysis.[195] G48M and I84A/C are rare mutations associated with high-levels of resistance to multiple PIs including LPV.[185]

Atazanavir resistance

Atazanavir is a semisymmetrical azapeptide PI that has shown equal potency to efavirenz in antiretroviral-naive patients and, when boosted with ritonavir, is as efficacious as lopinavir/ritonavir in antiretroviral naïve patients.[43] When used without ritonavir as the initial PI, atazanavir selects for the I50L mutation; this signature mutation for atazanavir differs from the I50V mutation seen with amprenavir. I50L occurs less frequently in patients receiving atazanavir/ritonavir or in previously PI-treated patients receiving atazanavir.[196, 197] The I50L mutation produces a significant reduction in susceptibility to atazanavir but has been associated with increased susceptibility to other PIs. In initial PI failure, atazanavir may select for the A71V mutation together with I50L, which increases atazanavir resistance but diminishes viral fitness. When used in PI-experienced patients or when combined with saquinavir, atazanavir can select for the I84V mutation and, less frequently, for the I54L mutation. Whereas the I50L mutation does not confer cross-resistance to other PIs, the I84V mutation confers broad PI cross-

resistance. Moreover, the I54L is a major darunavir resistance mutation. In vitro, atazanavir selects for V32I, M46I, I84V, and N88S. Classical PI mutations, such as V82A and L90M, have been shown to confer cross-resistance to atazanavir, particularly when present in combination with each other or with other known PI resistance mutations (V32I, G48V/M, F53L, I54V/L/M/T/A, G73S/C/T/A and I84V/A/C). However, as with many PI-based regimens, in patients who are naive to PIs, the most common resistance profile observed with early atazanavir failure is wild-type virus.

Tipranavir Resistance

Tipranavir is a sulfonamide-containing dihydropyridone. It is metabolized by the cytochrome P450 system and, while ritonavir coadministration increases its exposure, tipranavir in turn markedly increases ritonavir metabolism, necessitating use of ritonavir 200 mg twice daily when boosting tipranavir in PI-experienced patients. The major tipranavir-associated mutations are V32I, I47V, I54V/A/M, V82L/T and I84V. Mutations V82L/T, and I84V have been the most common major PI-resistance mutations to emerge during tipranavir/ritonavir salvage therapy.[198] Whereas V82L occurs in viruses that were wildtype at baseline, mutation V82T develops mostly in viruses with the V82A substitution at baseline.[199] Mutations V82L/T are associated with the greatest decreases in tipranavir susceptibility *in vitro*. In addition, I47V, I54A/V/S/M, I84V have also been associated with reduced TPV susceptibility.[199, 200] The accessory mutations L33F/I are among the most commonly occurring substitutions to emerge during tipranavir/ritonavir treatment and are associated with decreased virologic response when combined with other major mutations. E35G, K43T, Q58E, T74P, N83D, and L89V are nonpolymorphic mutations that have been associated with decreased virologic response to tipranavir/ritonavir in multivariate analyses.[198, 200]

Importantly, mutations L24I, I50V, I50L, I54L, and L76V are associated with increased tipranavir susceptibility. This effect is particularly large for I50V/L and I54L.

Different algorithms have been developed for tipranavir. The most comprehensive and better performing one was developed by independent investigators together with its manufacturer using the data from the RESIST trials[201] to predict virologic response (Table 8). A few existing TPV score mutations, most of which are uncommon in patients who have not used TPV, had the greatest weights (47V, 54A/M/V, 58E, 74P, 82L/T, 83D) while the others were considered minor or not important in terms of accurately predicting virologic response. The score also included mutations associated with increased susceptibility to tipranavir (24I, 50L/V, 54L, 76V). These mutations remained in the final score with large negative weights. Tested on an independent dataset against other commonly used scores, the new weighted score compared favourably, showing a better prediction than the unweighted score.

Table 8. Weight classification of tipranavir mutations

Class	Classification according to weight	Mutations (weight of each mutation)
Increased response	<0	24I (-2), 50L/V (-4), 54L (-7), 76V (-2)
Minor mutations	1–2	10V (+1), 36I (+2), 43T (+2), 46L (+1), 84V (+2)
Major mutations	>2	47V (+6), 54A/M/V (+3), 58E (+5), 74P (+6), 82L/T (+5), 83D (+4)

Interpretation:

Weighted mutation score	Interpretation based on RESIST trials
< or = 3	Susceptible
> 3 and <= 10	Partially Susceptible
> 10	Resistant

Darunavir resistance

The major darunavir-associated resistance mutations are V11I, V32I, L33F, I47V, I50V, I54L/M, G73S, L76V, I84V, and L89V. In an analysis of pooled week 24 data from POWER 1, 2, and 3 studies using darunavir/ritonavir 600/100 mg bid (N = 458), baseline darunavir

fold change in IC_{50} was a strong predictor of virological response at week 24.[147] Preliminary phenotypic clinical cut-offs of 10 and 40 were established. Protease mutations associated with diminished darunavir/r virological response were V11I, V32I, L33F, I47V, I50V, I54L/M, G73S, L76V, I84V, and L89V (darunavir RAMs). These mutations occurred in the presence of a high number of other PI resistance mutations. About 60% with 0, 45% with 1-2, and <=20% with >=3 darunavir RAMs had RNA <50 copies/ml at wk 24. A diminished response to darunavir/ritonavir was also observed in the presence of >or=14 PI resistance mutations other than the darunavir RAMs. The fold-change decrease in darunavir susceptibility is summarized in Table 9. Mutations developing during darunavir/r virological failure were V32I, L33F, I47V, I54L, and L89V. In phenotypic studies, I50V, I54M, L76V, and I84V reduced susceptibility to the greatest extent. V32I emerged in 30% of failures according to prescribing information. Of note, mutation I50V is associated with the largest susceptibility loss to darunavir in vitro but it hypersensitizes the virus to tipranavir. Other mutations that increase susceptibility to tipranavir are D30N, 41K, G48V, I50L, 53L/W/Y, I54L, H69K, 12S, 61K, N88S, and 76V.

In a larger follow-up study, which also included the DUET studies, all of the mutations with the exception of G73S were confirmed and T74P was added as a predictor of decreased virological.[147] In an independent study, V32I, L33F, and I47VA were found to be associated with decreased virological response to DRV/r salvage therapy.[202] In addition to the seven major PI-resistance mutations, mutations V11I, L33F, G73S/T/C and L89V were associated with a decreased virological response to DRV/r in the POWER and DUET studies.[147]

Finally, although mutation V82F has not been reported to develop in viruses from patients receiving DRV/r, it has had a major effect on DRV/r susceptibility in multivariate analyses of the mutations present in DRV/r resistant virus isolates.[203]

Table 9. Weight classification of darunavir mutations based on fold-change in IC_{50} in vitro

Fold-change	Mutations
>4	50V
3-4	54M, 76V, 84V
2-3	32I, 33F, 47V, 74P*
<2	11I, 54L, 89V

** in some studies, mutation 74P is associated with a fold-change comparable to that of I50V. Interpretation: Diminished response to darunavir when 3 or more of these mutations were present at baseline.*

Combined effect of etravirine and darunavir resistance mutations

Because darunavir is often given together with etravirine as salvage therapy, it is important to estimate the response rates to these two drugs in the presence of combined resistance mutation patterns. In a pooled subgroup analysis of Week 24 data from DUET-1 and DUET-2 trials,[204] favorable combined, etravirine and darunavir resistance profiles predicted higher virologic response in multiclass-experienced patients receiving etravirine plus darunavir/ritonavir together with optimized NRTIs but without *de novo* enfuvirtide. The highest response rates were seen in patients with etravirine FC ≤ 3 and darunavir FC ≤ 40. There were high response rates in patients with no etravirine RAMs and ≤ 3 darunavir RAMs and in patients with a total of ≤ 3 combined etravirine plus darunavir RAMs. In addition, there were also high responses rates in patients with weighted etravirine genotypic score ≤ 2 with ≤ 3 darunavir RAMs, and those with weighted etravirine genotypic score ≤ 3.5 with ≤ 1 darunavir RAMs.[205]

INTEGRASE INHIBITORS

Raltegravir (formerly MK-518) is an hydroxypyrimidinone carboxamide derivative of the diketobutanoic acid family approved by the FDA and the EMEA for treating, in combination with other antiretroviral drugs, treatment-experienced HIV-1 infected adult patients. Studies are on their way to assess the role of raltegravir in first-line therapy. By the time of finishing this review, elvitegravir (GS-9137)

was still in Phase II of development, although Phase III trials were expected to begin shortly. It is likely that elvitegravir will be positioned similarly to raltegravir in antiretroviral therapy schedules. Cumulative data, however, indicate that there is extensive cross-resistance between the two drugs.

The need for ritonavir boosting of elvitegravir (raltegravir is eliminated through glucuronidation, so does not require ritonavir boosting) may also imply differential toxicity patterns that may affect their relative positioning in HIV therapy.

Integration is a multistep process that occurs in discrete biochemical stages: (i) assembly of a stable complex with specific DNA sequences at the end of the HIV-1 long terminal repeat (LTR) regions, (ii) endonucleolytic processing of the viral DNA to remove the terminal dinucleotide from each 3' end, and (iii) strand transfer in which the viral DNA 3' ends are covalently linked to the cellular (target) DNA. Each of the catalytic reactions (3' processing and strand transfer) is metal-dependent and requires integrase to be appropriately assembled on a specific viral DNA donor substrate. A DDE motif in the integrase catalytic site coordinates the interaction of the enzyme with two magnesium ions. The acidic moiety of raltegravir and elvitegravir is essential for inhibition and confers distinct metal-dependent properties on the inhibitor.[206] Binding requires divalent metal and resistance is metal dependent with active site mutants displaying resistance only when the enzymes are evaluated in the context of Mg(2+). The mechanism of action of these inhibitors is therefore likely a consequence of the interaction between the acid moiety and metal ion(s) in the integrase active site, resulting in a functional sequestration of the critical metal cofactor(s). [52]

Resistance to raltegravir (RAL) evolves through at least three separate pathways defined by the signature mutations Q148H/K/R, N155H and Y143C/R.[207] These mutations impair viral replication to different extents.

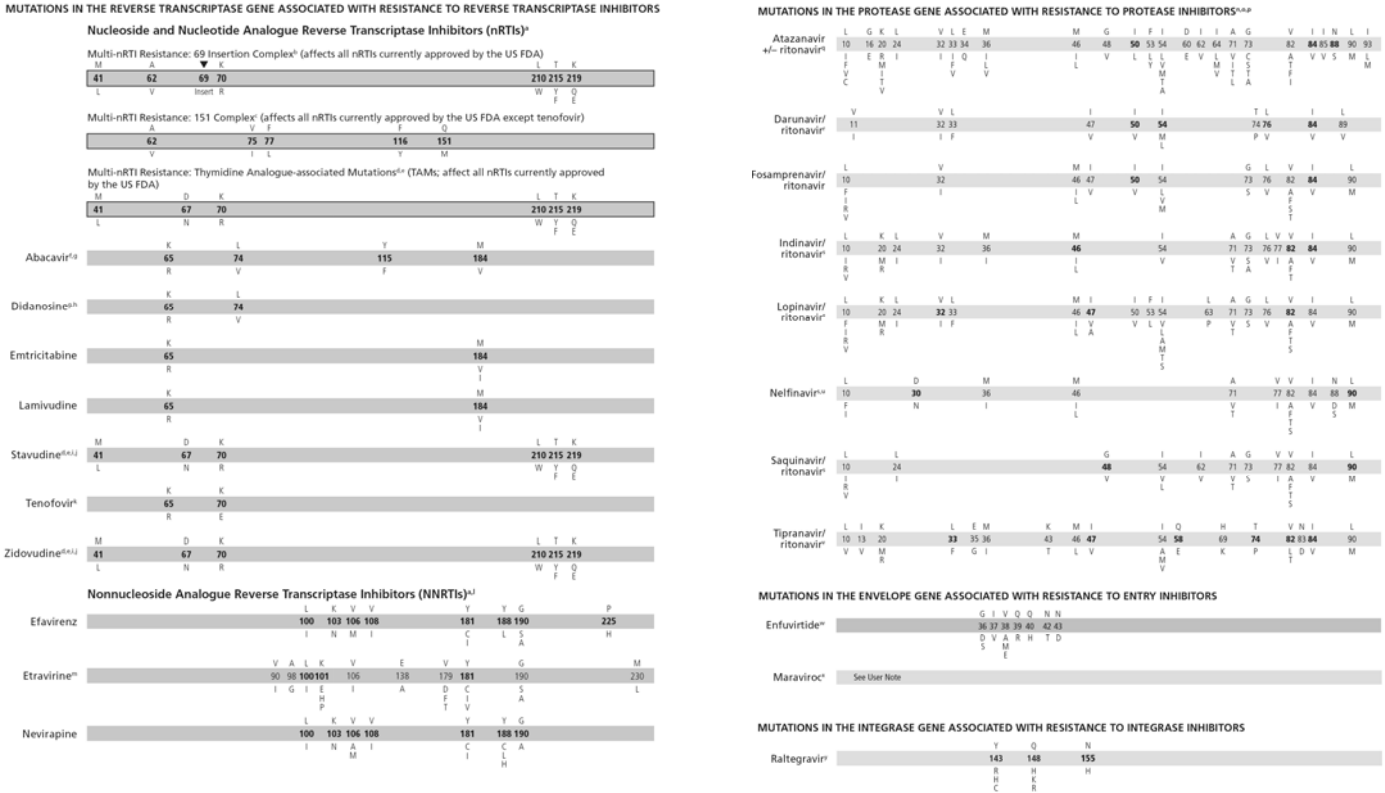


FIGURE 12. MUTATIONS IN HIV-1 THAT AFFECT SUSCEPTIBILITY TO ANTIRETROVIRAL DRUGS, BY HIV GENE TARGET. The letter above each position is the wild-type amino acid and the letter(s) below each position indicate the substitution(s) that are associated with drug resistance. Source: IAS-USA. December 2008 Update. Top HIV Med 2008;16(5):138-145

Mutations in residue 148 and N155H confer 10- to 25-fold, respectively, decreased susceptibility to raltegravir. Mutation Q148H is the one associated with greatest reductions in replication capacity. Accessory or secondary mutations accumulate to compensate for replication capacity losses (G140S in the presence of Q148H), or to further decrease viral susceptibility to raltegravir. Mutations L74M, E92Q, T97A, E138K, V151I, G163G/R, and D232D/N may also be present in raltegravir virologic failures. These secondary mutations by themselves contribute only in a modest way to resistance to raltegravir. However, when combined with either of the primary mutations N155H or Q148R/H/K, they lead to substantial further decreases in raltegravir susceptibility. The continued accumulation of resistance mutations in the absence of major fitness variations *in vivo*, suggests that raltegravir retains residual antiviral efficacy on viruses with major resistance mutations.

Mutations frequently encountered in the N155H pathway include E92Q, V151I, T97A, G163K and L64M. The most frequent secondary mutations in the Q148K/R/H pathways are G140A/S and E138K. Mutation G140S restores the fitness cost of Q148H. This combination of mutations is associated with the greatest susceptibility loss to RAL. The Y143C/R pathway is being characterized.

Development of resistance to raltegravir *in vivo* seems to be an ordered, stepwise phenomenon. Mutation N155H seems to emerge early in subjects failing raltegravir. In the presence of continued viral replication in the presence of raltegravir, this mutation is then replaced by Q148H, the fitness cost of which is compensated by the accumulation of G140S. Studies are undergoing to understand the mechanisms of these *in vivo* findings.

Mutations Q148H/K/R and N155H also confer resistance to elvitegravir (ELV). The E92Q mutation is probably a signature mutation for ELV, although it can also develop under RAL treatment. Recently, the L68V/I mutation was reported to increase resistance to both RAL and ELV in the presence of the other resistance mutations. The most frequent mutations found after elvitegravir failure in a phase IIb trial[208] were: E92Q, E138K, Q148R/K/H and N155H (each

observed in 39% of subjects); S147G (32%); and T66I/A/K (18%). Viruses of subjects experiencing virologic failure in this study showed a mean increase in IC50 value of greater than 151-fold for elvitegravir and greater than 28-fold for raltegravir compared with control, further confirming the extent of cross-resistance between these two compounds.

FUSION INHIBITORS

Enfuvirtide (T-20) is the only fusion inhibitor currently available in clinical practice. The clinical use of this drug is limited to deep salvage therapy, when no other treatment options are available, because it requires twice daily subcutaneous administration that is almost invariably associated with local adverse reactions at the puncture sites.

Development of enfuvirtide resistance is associated with changes in a conserved amino acid triad (GIV) at positions 36–38 and in aminoacids 39-45 in the HR1 region of gp41.[209, 210] Regions associated with co-receptor specificity and susceptibility to enfuvirtide, such as the V3 loop of gp120 do not play a primary role in the development of clinical resistance to enfuvirtide.[211]

A variety of different mutations has been noted in the amino acid region 36–45 (Table 10), each conferring a distinct level of resistance or susceptibility to enfuvirtide in a defined molecular background. Enfuvirtide is a drug with low genetic barrier to attain resistance. Single amino acid substitutions in this region are the most common and cause variable degrees of susceptibility loss. Serial mutations, where the reversion of the primary mutation coincides with the generation of the second, are also known. Single substitutions exhibit a 5- to 10-fold reduction in susceptibility to enfuvirtide.[209] Double amino acid substitutions have been associated with the highest levels of resistance.[209] In addition, considerable differences in enfuvirtide IC50 have been observed between primary isolates bearing the same pattern of mutations in gp41 as well as differences between viruses isolated from patients enrolled in Phase II clinical trials and site-directed mutants bearing the same mutations (e.g. G36S/L44M). This suggests that other viral factors (e.g. the V3 loop, or the HR2 region) may modulate the

sensitivity of the gp41 36–45 amino acid core region. [210-212] Mutations in the HR2 region (N126K and S138A), can also contribute to enfuvirtide resistance.[213]

Table 10. Enfuvirtide susceptibility of HIV-1 mutants carrying single and double substitutions in gp41 amino acids 36–45[211]

Substitution(s)[a]	Enfuvirtide EC50 (mg/L)	Enfuvirtide fold change[b]
NL4-3Gc	0.012	
G36D	0.091	8
G36S	0.088	7
V38A	0.188	16
Q40H	0.256	21
N42T	0.045	4
N42E	0.015	1
N42S	0.006	1
N43D	0.210	18
N43S	0.067	6
N43K	0.063	5
L44M	0.021	2
L45M	0.017	1
G36S + L44M	0.181	15
N42T + N43K	0.388	32
N42T + N43S	0.727	61
V38A + N42D	1.685	140
V38A + N42T	1.782	149
V38E + N42S	6.156	513

a Relative to a consensus wild-type sequence of GIVQQQNNLL (NL4-3G).

b Relative to NL4-3G.

c NL4-3 altered to match the consensus sequence at amino acid position 36 (aspartic acid replaced by glycine).

Source: Michael L. Greenberg and Nick Cammack. Resistance to enfuvirtide, the first HIV fusion inhibitor. Journal of Antimicrobial Chemotherapy 2004 54(2):333-340; doi:10.1093/jac/dkh330

Because amino acids 36–45 in the HR1 region of gp41 are highly conserved, mutations in this region are invariably associated with a fitness cost to the virus.[214] In the absence of drug, wild-type virus was able to replicate with faster kinetics than viruses bearing mutations. Within amino acids 36–38 of HR1 a relative order of GIV > DIV > DTV > DIM > SIM was found for the replication kinetics of mutant clones in the absence of drug. This relative order of fitness was reversed

in the presence of enfuvirtide.[214] Viruses with double amino acid substitutions were less fit than those with single substitutions.

One consequence of the development of enfuvirtide-resistant mutants with attendant reduced fitness is that following discontinuation of enfuvirtide, wild-type virus has been found to outgrow resistant virus, restoring replicative capacity associated with normal drug susceptibility.[215]

In a study assessing the proportion of plasma virus carrying the V38A mutation in gp41 by allele-specific PCR in serial samples collected from 3 subjects who interrupted enfuvirtide, we demonstrated *in vivo* fitness differences for mutant versus wild type ranging from -25% to -65% in the absence of drug. The V38A mutant virus reemerged rapidly (within 1-2 weeks) during a subsequent enfuvirtide pulse, confirming that enfuvirtide resistance remains archived in the viral quasispecies and is ready to emerge very soon after re-exposure.

CCR5 ANTAGONISTS

The dominant pathway to virological failure to CCR5 antagonist therapy *in vivo* is not the development of resistance mutations in envelope, but a shift from CCR5 to CXCR4 use. Such shifts have been seen in 55% of subjects experiencing virological failure in the MOTIVATE-1 and 2 trials[216, 217] (maraviroc in treatment-experienced subjects), 35% of individuals in the ACTG A5211 trial[218] (vicriviroc in treatment-experienced patients) and in 31% of subjects in the MERIT study[219] (maraviroc as first-line therapy in antiretroviral-naïve individuals). Different analyses suggest that X4 viruses emerging during virological failure to CCR5 antagonists originate from pre-existing minority viral populations. [220]

Most maraviroc or vicriviroc-resistant viruses described to date, both *in vivo* and *in vitro*, contained mutations in the V3-loop stem. Maraviroc-resistant HIV-1 variants were generated by serial passage in vitro.[221] Two mutations in the V3 loop, T316A and V323I, were associated with maraviroc resistance; a third V3 loop mutation, A319S, was not consistently observed. The effect of such resistance mutations, however, is dependent on the remaining envelope context. The same

mutations that confer CCR5 antagonist resistance in one subject may not affect viral susceptibility in a different envelope background. Thus, unlike resistance to reverse transcriptase and protease inhibitors, resistance to small-molecule CCR5 antagonists may not result in the selection of stereotypical mutations.[1] Rather, the selected mutations may lead to env-specific structural changes that allow gp120 to adapt to an inhibitor-bound conformation of CCR5. The effect of such mutations is context-dependent

The multiple conformations of CCR5 that exist in vivo, as for any allosteric protein, may also contribute to the variety of CCR5-inhibitor resistance mutations observed to date. Small-molecule antagonists may bind to several different conformations of CCR5. The emergence of a variety of seemingly unrelated mutations could, in the context of a particular envelope molecule, give rise to common structural changes that improve the efficiency of HIV-1 fusion and viral entry into target cells.

Resistant viruses acquire the ability to recognize receptor conformations stabilized by maraviroc and therefore can no longer be inhibited even at high compound concentrations. Phenotypically, this is evidenced by decreases in the percent maximal inhibition in resistant viruses relative to control, instead of right shifts in the IC_{50} curves.[222] Plateaus in dose–response curves are obtained with viruses that recognize both compound-free and compound-bound forms of the receptor.[222] The association between emergence of clinical resistance to maraviroc and plateaus in maximum inhibition or shifts in dose response is currently under investigation.

It was also seen that CCR5 antagonist resistance mutations confer a fitness cost to the virus in the absence of drug.[223] The extent of of cross-resistance within the class is not yet known.

The phase IIb study of the CCR5 antagonist vicriviroc (ACTG A5211) studied 29 subjects with virologic failure from a and identified one individual with HIV-1 subtype C who developed vicriviroc resistance.[223] Studies with chimeric envelopes demonstrated that changes within the V3 loop were sufficient to confer vicriviroc resistance. Resistant virus showed vicriviroc-enhanced replication, cross-resistance

to another CCR5 antagonist, TAK779, and increased sensitivity to aminooxypentane-RANTES and the CCR5 monoclonal antibody HGS004. Pretreatment V3 loop sequences reemerged following vicriviroc discontinuation, implying a fitness cost of vicriviroc resistance mutations to the virus.[223]

ANTIRETROVIRAL RESISTANCE SURVEILLANCE

Antiretroviral drug-resistant HIV can be transmitted from person-to-person.[224, 225] In addition, most subjects experiencing virological failure develop antiretroviral-resistant viruses.[53] Periodic surveillance of HIV drug resistance is essential from a public health perspective. Because different surveys in different areas may show different results, specific countries and regions require their own surveillance systems to monitor transmitted HIV drug resistance.

PRIMARY RESISTANCE

Variants with primary resistance can be transmitted from person-to-person through contact with blood or blood products, sexual intercourse or from mother-to-child.[224, 225] Interestingly, only a few variants present in the "donor" viral population are actually transmitted during primary HIV infection, even if transmission occurs through direct blood-to-blood contact. Moreover, resistant viruses are transmitted less efficiently than wildtype, [226] although multidrug-resistant variants can also be transmitted.[224, 227-229] This suggests a transmission bottleneck, the nature of which remains poorly understood. Because resistant variants are often transmitted alone, the viral population in the recipient subject is almost exclusively conformed by resistant viruses, which remain predominant until wild-type revertants are spontaneously generated through back-mutation.[230]

Mathematical models suggest that, in the absence of adverse drug pressure and lack of competing viral populations with better fitness, fixation of new mutations is a slow process.[231] A recent study characterized HIV drug resistance and replication capacity in

longitudinal plasma samples from 14 recently HIV-infected patients with transmitted drug-resistant virus who were followed for a median of 2.1 years after the estimated date of infection without receiving antiretroviral therapy. They found that the median time to loss of detectable drug resistance using population-based assays ranged from 4.1 years to longer than the lifetime of the individual. The transmission of drug-resistant virus was not associated with virus with reduced replication capacity.[230]

The prevalence of primary resistance is a consequence of:

a) the prevalence of acquired (secondary) HIV resistance in the general population;
b) the fraction of subjects on antiretroviral therapy who remain viremic;[48]
c) the genetic barrier to attain resistance of the drug regimens given to that population;
d) the existence of HIV transmission "hotspots", or clusters of individuals frequently engaging in high-risk transmission practices,[230] particularly if these cluster include subjects infected with resistant viruses who remain viremic.
e) To some extent, the fitness cost associated with particular resistance mutations, which influences the detectability of such mutations in plasma (and thus contributes to bias the relative prevalence of some mutants vs. others in different drug environments).

The prevalence of primary resistance in a population varies as effective antiretroviral therapy is progressively introduced in that population: When there is no treatment, there is no detectable resistance, at least by bulk sequencing. Suboptimal therapy and suboptimal therapeutic monitoring lead to increased rates of secondary resistance in the treated population; these are followed by increased rates of primary resistance. As antiretroviral therapy becomes more potent and includes drugs with higher genetic barrier to attain resistance, more people remain aviremic and harbor less resistant

viruses at the time of treatment failure. At this point, primary resistance may begin to decrease.

The prevalence of primary resistance in well-resourced countries varies in different studies, ranging 7.7%-19.2% for any drug, 5.5%-12.4% for NRTIs, 1.9%-8.1% for NNRTIs and 2.7%-6.6% for PIs. A large increase in overall primary resistance, from 13.2% for the period 1995–1998 to 24.1% for the period 2003–2004, was reported in New York, New York, and the rate of transmitted multidrug resistance increased from 2.6% to 9.8% over the same period.[232] High rates of primary resistance were found in the UK in 2003: 19.2% for any drug, 12.4% for NRTIs, 8.1% for NNRTIs, and 6.6% for PIs. High-level resistance was found in 9.3% of subjects. In contrast, a representative 10-year transmission surveillance study (1996–2005), conducted by the Swiss HIV Cohort Study, showed considerably lower rates: 7.7% for any drug, 5.5% for NRTIs, 1.9% for NNRTIs, and 2.7% for PIs. Dual– or triple–drug class resistance was observed in only 2% of patients.[233] The rate of transmission, including the transmission of multidrug-resistant virus, remained stable over a 10-year period, with the exception of NNRTI-resistant virus transmission, which—as has been reported by other groups—increased in 2005. [227, 232-234]

The large pan-European SPREAD program[235] recently showed that the prevalence of viruses with drug-resistance mutations in Western Europe was 9.1% between years 2002 and 2003. Seventy-one percent of viruses harbored only a single amino acid substitution with limited effect on predicted drug susceptibility. Mutations associated with resistance to NRTIs were observed most frequently [57/1050 (5.4%)], followed by mutations related to PIs [32/1050 (3.0%)] and mutations related to NNRTIs [27/1050 (2.6%)]. Transmission of extensively drug resistant viruses was rare, although of evident clinical relevance.

In a large cohort of 3542 ART-naïve HIV-infected patients from 36 US states and District of Columbia enrolling into clinical trials between 2001 and 2007,[236] the prevalence of primary resistance evolved increased from 2001 to 2007, mainly at the expense of the prevalence of primary NNRTI resistance (Table 11)

Integrase strand-transfer inhibitors (InSTIs) have been recently incorporated into clinical practice. Surveillance of resistance mutations in the integrase-coding region of *pol*, which are not routinely investigated by commercial genotypic assays, needs to be implemented urgently.

Table 11. Prevalence of antiretroviral resistance mutations in US ART-naïve HIV-infected patients, 2001- 2007[236]

	2001		2007	
	IAS	Stanford	IAS	Stanford
Major resistance mutations	9%	5%	20%	13%
NRTI	3%	4%	4%	5%
NNRTI	6%	2%	15%	8%
PI	2%	2%	3%	3%
Dual class	2%	2%	2%	2%
Triple class	<1%	<1%	<1%	<1%

IAS refers to the International AIDS Association-USA; Stanford refers to the Stanford HIV Drug Resistance Database.

To date, rates of primary resistance have remained low in developing countries. However, the high frequency of secondary resistance in several resource-limited settings, suggests that the prevalence of primary antiretroviral resistance could increase in the coming years in countries where antiretrovirals are being rolled-out.

In a study conducted in Zambia, where subtype C predominates, primary drug resistance mutations were observed in 5% of patients starting first-line ART.[237] Among treatment-naive patients in western India (mostly with subtype C), drug resistance mutations were documented in 10% of those tested. Mutations identified included a protease major mutation (V82A) as well as reverse-transcriptase mutations, such as D67N and M184V.[238] Among treatment-naive patients in southern Brazil, the presence of primary drug resistance was seen in approximately 5% of patients. The most frequent mutations were those associated with NNRTI resistance, particularly the K103N mutation, which was observed in 4.3% of patients.[239] In other countries

like Mali (year 2006)[240] and Tanzania (year 2005)[241], however, the prevalence of primary resistance remains below 5%.

Several primary resistance surveillance studies including some that will be presented in this thesis, have demonstrated that assays able to detect minority HIV-1 variants increase detection of primary resistance mutations at least 2 to 3-fold relative to standard population-based sequencing.[242, 243] Moreover, as we shall discuss later, there is increasing evidence that baseline detection of minor variants with mutations conferring resistance to drugs with low genetic barrier (e.g. K103N, Y181C and NNRTIs) increases the risk of virological failure 3- to 6-fold.[244, 245] We will argue that more sensitive resistance testing assays need to be incorporated in primary resistance surveillance.

ACQUIRED OR SECONDARY RESISTANCE

Most subjects failing ART harbor viruses with resistance mutations. This can abate the efficacy of second-line and salvage regimens. In statistically rigorous analyses, the prevalence of drug resistance in therapy-exposed subjects was estimated to be 50-60% in 1999, and decreased to 39%-53% in 2006, likely due to an improved efficacy of therapy and, perhaps, a better understating of the importance of optimal adherence. [246] The prevalence of triple-drug-resistant virus remained stable at 5%. Fortunately, extensive virological failure of the three veteran classes of drugs occurs slowly in routine clinical practice.

Less information exists about the prevalence of acquired resistance in developing countries. In a recent evaluation by the South African Resistance Cohort Study,[247] the overall prevalence of resistance mutations in individuals failing their first HAART regimen in KwaZulu Natal was higher than 80%. Of 115 individuals recruited in the study, 62 (54%) were receiving d4T+3TC+NNRTI; 43 (38%) were treated with ZDV+3TC+NNRTI; 5 (4%) were receiving 2 NRTI+LPV/rtv, and 5 (4%) were being treated with other ARV combinations. The prevalence of dual-class resistance (essentially due to the detection of the M184V mutation plus at least one NNRTI resistance mutation) was higher than 60%. The most common mutation detected at the time of virological failure was M184V/I (64.3%); K103N was present in 51.3% and

V106M in 19.1%. Thymidine analogue resistance mutations were found in 32% of subjects, with a predominance of the TAM-2 over the TAM-1 pattern (TAM-2: 19%; TAM-1: 7%; both TAM-1 and 2: 6%). The K65R mutation was only found in 3 subjects (2.6%) and each K70E and L74V mutations were detected in only 2 subjects (1.7%).

Similarly, genotypic resistance testing in 98 HIV-1-infected patients from Thailand who experienced treatment failure with their first antiretroviral regimen (a fixed-dose combination of stavudine, lamivudine, and nevirapine) during 2003-2005 showed a prevalence of at least one major NRTI or NNRTI mutation of 95% and 92%, respectively. M184V was observed in 89% of patients. TAMs, K65R, and Q151M were observed in 37%, 6%, and 8% of patients, respectively.[248]

STANDARDIZED RESISTANCE TESTING

The International AIDS Society-USA (IAS-USA) updated its resistance testing guidelines[53] in July 2008 to include the many recent additions to the HIV armamentarium and increasing knowledge and understanding of HIV drug resistance. These changes include: a) the incorporation of new drug classes like fusion inhibitors, CCR5 antagonists and integrase strand-transfer inhibitors; b) the increased complexity of ART, with the incorporation of new NNRTIs (etravirine) and PIs (e.g. darunavir-ritonavir) retaining activity against resistant virus; c) the need to address the biochemical correlates and clinical significance of antiretroviral drug resistance in non-B HIV-1 strains, as ART programs grow in resource limited settings, d) the development of more sensitive techniques to detect drug resistance in minority variants, and e) the need to define a clinical role for viral tropism and replication capacity testing. All these factors play key roles in the prevention and treatment of drug resistant virus.

The Department of Health and Human Services (DHHS) Panel on Antiretroviral Guidelines for Adults and Adolescents, also included recommendations for antiretroviral drug resistance testing in the last

update of the *Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents* in November 23rd, 2008.[249] These guidelines show high concordance with the latest IAS-USA guidelines regarding the clinical indication of resistance testing.

CLINICAL INDICATIONS OF RESISTANCE TESTING

The goal of resistance testing is to provide information to assist in the selection of the antiretroviral regimen(s) more likely achieve and maintain viral suppression. All guidelines agree that HIV drug resistance testing should be performed when HIV-infected persons enter into clinical care, whether or not they will be treated immediately[53, 249] (Tables 12 and 13). The goal of this strategy is to maximize the chances of detecting transmitted resistance. In those individuals in whom treatment is deferred, resistance testing should be repeated before therapy initiation. In addition, genotypic resistance testing is recommended for all pregnant women prior to initiation of therapy and for those entering pregnancy with detectable HIV RNA levels while on therapy. In HIV-infected individuals receiving antiretroviral therapy, resistance testing should be performed in the presence of virological failure. To ensure adequate performance of resistance testing, HIV-1 RNA levels should be at least 1000 copies/mL at the time of testing, although guidelines agree that resistance testing could be also attempted in individuals with HIV-1 RNA levels between 500 and 1000 copies/mL. In this last group of patients, however, the chances of amplifying HIV-1 sequences are markedly lower. The guidelines also suggest that drug resistance testing might also be helpful when managing suboptimal viral load reduction. [53, 249] This is less clear, however, because the addition of, or switch to, new antiretroviral drugs could be very helpful to achieve viral suppression in this situation regardless resistance testing results.

Importantly, given that drug resistance mutations wane after treatment interruption, drug resistance testing in the setting of virologic failure should be performed while the patient is taking his/her antiretroviral drugs, or within 4 weeks after discontinuing therapy.

Table 12. Summary of clinical situations in which resistance testing is recommended (IAS-USA, July 2008). [53]

Clinical setting	Comments
Before initiation of therapy	
Primary (acute and early) infection	Resistance testing is recommended. Initial therapy may be altered based on resistance test results.
First evaluation of chronic HIV-1 infection	Resistance testing is recommended, including for patients for whom therapy is delayed, because plasma wild-type isolates may replace drug-resistant virus with time in the absence of treatment.
Treatment initiation for chronic HIV-1 infection	Resistance testing is recommended because of a rising prevalence of baseline HIV-1 drug resistance in untreated patients with chronic infection, unless preexisting data or stored samples for testing are available.
In antiretroviral-treated patients	
Treatment failure	Resistance testing is recommended. The decision to change therapy should integrate treatment history, new and prior resistance results (if available), and evaluation of adherence and possible drug interactions.
In specific settings	
Pregnancy[a]	Resistance testing is recommended before initiation of therapy to effectively treat the mother and prevent mother-to-child transmission.
Other considerations and general recommendations	Postexposure prophylaxis should consider treatment history and resistance data from the source, when available; A sudden increase in HIV-1 plasma RNA may reflect superinfection, possibly with drug-resistant virus; Plasma samples to be tested for drug resistance should contain at least 500 HIV-1 RNA copies/mL to ensure successful PCR amplification required for all sequencing approaches; It is preferable that the blood sample for resistance testing be obtained while the patient is receiving the failing regimen, if possible; Resistance testing should be performed by laboratories that have appropriate operator training, certification, and periodic proficiency assurance;

	Genotypic and phenotypic test results should be interpreted by individuals knowledgeable in antiretroviral therapy and drug resistance patterns; Inhibitory quotient testing is not recommended for clinical decision-making.

[a]If resistance test results are available from before the pregnancy, clinical judgment should guide whether retesting for resistance is necessary. Source: Hirsch et al. Antiretroviral Drug Resistance Testing in Adult HIV-1 Infection: 2008 Recommendations of an International AIDS Society-USA Panel. Clin. Infect Dis. 2008;47:266–85

Table 13. Recommendations of the (DHHS) Panel on Antiretroviral Guidelines for Adults and Adolescents, Nov 3rd, 2008.[249]

Clinical Setting/Recommendation	Rationale
Drug-resistance assay recommended	
In acute HIV infection: Drug resistance testing is recommended, regardless of whether treatment will be initiated immediately (AIII). A genotypic assay is generally preferred (AIII).	If treatment is to be initiated, drug resistance testing will determine whether drug-resistant virus was transmitted and will help in the design of initial or changed (if therapy was initiated prior to test results) regimens.
If therapy is deferred, repeat resistance testing should be considered at the time of ART initiation (CIII).	If treatment is deferred, testing still should be performed because of the potentially greater likelihood that transmitted resistance-associated mutations will be detected earlier in the course of HIV infection; results of testing may be important when treatment is eventually initiated. Repeat testing at the time ART is initiated should be considered because of the possibility that the patient may have acquired drug-resistant virus.
In chronic HIV infection: Drug resistance testing is recommended at the time of entry into HIV care, regardless of whether therapy will be initiated (AIII). A genotypic assay is generally preferred (AIII).	Transmitted HIV with baseline resistance to at least one drug may be seen in 6%–16% of patients, and suboptimal virologic responses may be seen in patients with baseline resistant mutations.
If therapy is deferred, repeat resistance testing should be considered at the time ART is initiated (CIII).	Repeat testing at the time ART is initiated should be considered because of the possibility that the patient may have acquired drug-resistant virus.

With virologic failure during combination antiretroviral therapy with HIV RNA levels >1,000 copies/mL (AII). In persons with >500 but <1,000 copies/mL, testing may be unsuccessful but should still be considered (BII).	Testing can help determine the role of resistance in drug failure and thus maximize the number of active drugs in the new regimen, if indicated. Drug resistance testing should be performed while the patient is taking his/her antiretroviral drugs or immediately (i.e., within 4 weeks) after discontinuing therapy.
With suboptimal suppression of viral load after antiretroviral therapy initiation (AIII).	Testing can help determine the role of resistance and thus maximize the number of active drugs in the new regimen, if indicated.
In HIV-Infected Pregnant Women: Genotypic resistance testing is recommended for all pregnant women prior to initiation of therapy (AIII) and for those entering pregnancy with detectable HIV RNA levels while on therapy (AII).	The goals of antiretroviral therapy in HIV-infected pregnant women are to achieve maximal viral suppression for treatment of maternal HIV infection as well as for prevention of perinatal HIV transmission. Genotypic resistance testing will assist the clinician in selecting the optimal regimen for the patient.
Drug resistance assay not usually recommended	
After discontinuation (>4 weeks) of drugs (BIII).	Drug resistance mutations might become minor species in the absence of selective drug pressure, and available assays might not detect minor drug-resistant species. If testing is performed in this setting, the detection of drug resistance may be of value, but its absence does not rule out the presence of minor drug- resistant species.
When plasma viral load <500 copies/mL (AIII).	Resistance assays cannot be consistently performed because of low HIV RNA levels.

Strength of Recommendation: A: Strong recommendation for the statement; B: Moderate recommendation for the statement; C: Optional recommendation. Quality of Evidence for Recommendation: I: One or more randomized trials with clinical outcomes and/or validated laboratory endpoints; II: One or more well designed, nonrandomized trials or observational cohort studies with long-term clinical outcomes; III: Expert opinion. Source: Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services. November 3, 2008; 1-139. Available at http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf. (Accessed Nov 26th, 2008)

Because the CCR5 antagonist maraviroc was incorporated into clinical practice during 2008, resistance testing guidelines address for the first time the clinical indications of tropism testing.

Both the IAS-USA and DHHS guidelines agree that co-receptor tropism assays should be performed whenever the use of a CCR5 inhibitor is being considered. [53, 249] In addition, co-receptor tropism testing might be considered for patients who exhibit virologic failure on maraviroc (or any other CCR5 antagonist) because virological failure to CCR5 antagonists is frequently associated with a CCR5 to CXCR4 tropism switch. The practical applications of detecting such tropism change, however, are unclear at this moment. On one hand, there is only one CCR5 antagonist available for clinical use. Even if other CCR5 antagonists became available, it is unclear whether exposure to a new CCR5 antagonist in subjects with previous CCR5 antagonist failure would be an effective strategy, even if no tropism switch were detected after the first virological failure. Finally, it is unknown whether the rates of virological suppression with a salvage regimen not including a CCR5 antagonist might be different according to the viral tropism at the previous treatment failure.

Given that that the emergence of X4 viruses is associated with accelerated progression towards AIDS or death,[250] co-receptor tropism testing has been proposed as a tool to guide initiation of antiretroviral therapy or to establish prognosis. However, these indications are not supported by current guidelines because studies addressing these questions specifically are lacking. It is also unclear whether upfront co-receptor testing in subjects initiating first-line antiretroviral therapy would useful in case a CCR5 antagonist would be required later on (e.g. in case of toxicity). Viral tropism shifts could potentially occur, even if subjects with sustained viremia suppression.

Resistance Testing Assays

Genotypic and phenotypic assays are used to assess viral strains and select treatment strategies. The so-called "Virtual Phenotype" is essentially a genotype interpretation rule based on interrogating a query sequence against a large database of stored genotype-phenotype pairs.

The advantages and disadvantages of genotypic and phenotypic assays are summarized in Table 14.

Table 14. Advantages and limitations of genotypic and phenotypic resistance tests

	Genotyping	Phenotyping
Advantages	More rapid	Direct measure of susceptibility
	Less expensive	Delivers direct cross-resistance data
	Simple technology, available in regular hospital laboratories	Assesses the net effect of all mutations contained in the viral insert tested
	Allows detection of emerging mutations before onset of resistant phenotype	Straightforward interpretation
	Detects allele mixtures	Potential to measure viral tropism and replication capacity in parallel
Limitations	Indirect measure	Longer turn-around time
	Does not assess the effect of unknown/not described resistance mutations	Restricted availability due to complexity and cost
	Unable to detect mutation linkage	Requires establishment of clinically significant cut-offs
		Lack of standardization of cut-off values between tests
		Assesses the phenotype of only one (usually the most predominant) variant of the viral swarm
	Not useful for samples with HIV-1 RNA levels <500 – 1000 copies/mL	
	Limited sensitivity for the detection of minority variants in the viral population	
	Detection of non-B subtypes may be limited for some tests	
	Results require expert interpretation	

Genotypic Assays

Genotypic resistance assays amplify regions of the HIV genome where resistance mutations frequently occur, followed by population-based Sanger sequencing of the amplified products. Genotypic assays can be performed "in-house" or using commercial kits. "In-house" population sequencing is usually cheaper and more flexible; it allows sequencing of any region of the genome in both HIV-1 and 2 and in different subtypes. However, "in-house" sequencing is not standardized and is susceptible to inter-subject variability. Initiatives to standardize "in-house" sequencing methods across different laboratories are in place in Europe.

To ensure clinical validation, approval by regulatory agencies, decrease inter-assay and inter-subject variability and increase overall throughput, commercial genotyping assays are developed as "closed" systems that target pre-defined, specific regions of the HIV-1 genome. (Table 15); primers and specific PCR reactions are typically unknown to the user. These assays are usually preferred for clinical trials where antiretroviral resistance is a variable of interest, because they are clinically validated, consistent across laboratories, and fulfill the standards for performance characteristics and all other quality control and assurance requirements established by the US Congress *Clinical Laboratory Improvement Act* (CLIA) and the European Regulatory agencies. As a limitation, most commercial genotyping tests were designed to detect HIV-1 subtype B viruses. Sequencing of non-B subtypes can be difficult with these assays, and is generally impossible with HIV-1 from the O or N groups and with HIV-2. They are also more expensive than "in-house" methods, being unaffordable at their current cost for resistance surveillance in resource-limited settings.

Table 15. Comparison of commercial genotypic and phenotypic resistance assays

	Region amplified (aminoacids)	Minimum VL (c/mL) required	Comments
GENOTYPIC RESISTANCE ASSAYS			
GeneSeq™ HIV (Monogram Biosciences)	PR (1-99) RT (1-305)	500	CLIA-certified
TruGene™ HIV Genotyping Kit (Siemens	PR (1-99) RT (1-247)	1000	FDA-cleared CLIA-certified Resistance interpretation is based upon interpretation by an international expert panel
ViroSeq™ HIV-1 Genotyping System (Abbot Molecular)	PR (1-99) RT (1-335)	2000	FDA-cleared (16 and 96 capillary systems) CLIA-certified Hands-free gel electrophoresis & sequencing
PHENOTYPIC RESISTANCE ASSAYS			
Antivirogram ® Phenotype (Virco BVBA)	PR (1-99) + p7/p1/p6 gag cleavage sites RT (1-400)	1000	Not FDA-cleared CLIA-certified Works with all group M subtypes Includes possibly relevant mutations in the connection domain of RT relevant for NRTI and NNRTI susceptibility Includes gag cleavage sites relevant for PI susceptibility
PhenoScript™ (Viralliance)	PR (1-99) + p2/p7/p1/p6 gag cleavage sites RT (1-400) ENV (gp160)	500	Separate amplifications and transfections for PR, RT and ENV Includes gag cleavage sites relevant for PI susceptibility Works with all group M subtypes
PhenoSense™ HIV (Monogram Biosciences)	PR (1-99) + p7/p1/p6 gag cleavage sites RT (1-305)	500	FDA-cleared CLIA-certified Continuous amplicon Includes gag cleavage sites relevant for PI susceptibility Works with all M group subtypes

			Provides estimates of replication capacity (RC) relative to a wild-type control
PhenoSense™ Entry (Monogram Biosciences)	ENV (gp160)	500	Not FDA cleared CLIA-certified Research Use Only (RUO) Assesses resistance to enfuvirtide (T-20) and CCR5 antagonists The susceptibility cutoff for enfuvirtide is at the 99th percentile of the distribution of 220 enfuvirtide phenotypes in a naive reference population (baseline isolates from the TORO1 and TORO2 clinical trials). Susceptibility to CCR5 antagonists reported as percent maximal inhibition
PhenoSense™ Integrase (Monogram Biosciences)	RT (C-terminal) IN (1-288)	500	First commercial assay to assess phenotypic susceptibility to IN inhibitors Not FDA cleared CLIA-certified Research Use Only (RUO) Provides estimates of replication capacity (RC) relative to a wild-type control

One variant of genotypic testing available a few years ago was line probe assay kit or LiPA, a differential hybridization assay developed by Innogenetics® and later commercialized by Bayer Diagnostics® as the "Versant® HIV-1 RT or PR resistance Assay". The assay included LiPA strips that contained discrete lines of probes specific for particular codons in the RT and PR coding regions. The initial amplifications were carried out using biotinylated primers to produce a labeled PCR product that was then incubated with the LiPA strips. Hybridisation to LiPA probes was detected using colorimetric methods. This assay had the advantage of being more sensitive to detect individual mutations than the other genotypic assays. However, it had a comparable cost to other methods and could only detect a limited set of mutations,

requiring periodic technical updates as more drugs were being developed and more mutations and more complex mutational patterns were being described. The "Versant® HIV-1 RT or PR resistance Assay" stopped being manufactured when Siemens® bought Bayer Diagnostics®.

Phenotypic resistance tests

Phenotypic assays measure the ability of a virus to grow in different concentrations of antiretroviral drugs. Initially, viral phenotyping tests attempted to produce large virus stocks directly from the patient's blood by growing them in specific cell lines. This proved to be a slow, expensive, highly variable and labor-consuming procedure that was only available to few laboratories. Moreover, virus growth during multiple infection cycles made the virus to evolve away from the one that had been sampled originally.[251]

All current phenotyping technologies include slight variations of essentially the same procedure (Table 16): the generation of a recombinant virus by PCR-amplifying the HIV genomic region of interest from patient's plasma, and inserting it into the backbone of a laboratory clone of HIV from which this region of the genome has been removed, either by cloning or by *in vitro* recombination.[252] Large quantities of replication-competent recombinant virus are thus produced and transfected into susceptible cells. Replication of the recombinant virus at different drug concentrations is monitored by expression of a reporter gene and is compared with replication of a reference HIV strain. The drug concentration that inhibits 50% of viral replication (i.e., the median inhibitory concentration [IC] 50) is calculated, and the ratio of the IC50 of test and reference viruses is reported as the fold increase in IC50 (i.e., fold resistance).

Table 16. Principles of the commercial phenotypic assays

Assay	Characteristics
Antivirogram® (Virco BVBA)	Recombinant viruses containing the patient insert are transfected into MT4 cells. Cell cultures are monitored for the appearance of cytopathic effect (CPE). Infectivity is determined by the viral CPE assay by using a 50% endpoint method (50% cell culture infectious dose). HIV-1 drug susceptibility is determined by -3(4,5-dimethylthiazol-2-yl)-2,5-dyphenyltetrazolium bromide (MTT) (MT4-MTT)-based CPE protection assay. MT4 cells are infected with 50% cell culture infective doses of recombinant viruses in the presence of five-fold dilutions of the different antiretroviral drugs. In general, the wildtype HXB2 viruses are tested in parallel with clinical samples for each assay. Fold-resistance values are calculated by dividing the mean IC50 of the patient-derived recombinant virus by the mean IC50 of the wildtype control.
PhenoSense™ (Monogram Biosciences)	The genes of interested are amplified from HIV sequence pools and incorporated into an indicator gene viral vector (IGVV) by conventional cloning methods using ApaI and PinAI restriction sites to construct a resistance test vector (RTV). Host cells are co-transfected with RTV DNA and a plasmid that expresses the envelope protein of amphotropic murine leukemia virus (MLV). Following transfection, virus particles are harvested and used to infect fresh target cells. The completion of a single cycle of viral replication results in the production of luciferase. Serial dilutions of PIs are added at the transfection step and RT inhibitors at the infection step. For the measurement of susceptibility to entru inhibitors (EI), indicator cells expressing CCR5 or CXCR4 co-receptors are treated with serial dilutions of drugs and infected with recombinant viruses harvested from the producer cellDrug susceptibility is measured by comparing the luciferase activity in the presence and absence of drugs. Susceptible viruses result in decreased levels of luciferase activity in the presence of drugs, whereas viruses with reduced susceptibility produce comparable levels to the wildtype control.
PhenoScript™ (Viralliance)	The Phenoscript is based on a single cycle of in vitro replication and measures viral capacity of replication in the presence of drugs. Plasma is obtained from the patient's blood sample viral RNA is extracted and three regions – gag-protease (GP), reverse-transcriptase (RT) and envelope (ENV) – are separately amplified to test PIs, RTIs and EIs respectively. Each PCR product is then separately co-transfected into producer cells along with the corresponding PHENOSCRIPT™ plasmid. For the PI and RTI assays, the single cycle of infection is ensured by the deletion of the envelope encoding region of the HIV plasmid. The envelope of the recombinant virus is provided by the G protein of the Vesicular Stomitis virus (VSV-G protein), for which the genetic information is carried on a separate plasmid. Serial dilutions of

PIs are added at the transfection step and RT inhibitors at the infection step. For the measurement of susceptibility to EIs, indicator cells expressing CCR5 or CXCR4 co-receptors are treated with serial dilutions of drugs and infected with recombinant viruses harvested from the producer cell. The reporter cells used contain a LacZ gene under control of the HIV LTR. Once cells are infected, b-galacosidase is produced, the amount of which is detected using a CPRG based colorimetric assay and measured by Optical density.

In the PhenoSense™ and Phenoscript™ assays, data are analyzed by plotting percent inhibition of luciferase activity or beta-galactosidase production, respectively, versus $\log_{10}$ concentration of drug. The drug susceptibility curve is used to calculate the concentration of drug required to inhibit viral replication by 50% (IC_{50}) or 90% (IC_{90}). The IC_{50} of the patient's virus is compared to the IC_{50} of a drug-sensitive reference virus control to calculate fold change in susceptibility. Reduced drug susceptibility is indicated by a shift in the patient inhibition curve toward higher drug concentrations (to the right). In the case of CCR5 antagonist susceptibility, reduced susceptibility is indicated by a reduction in the percent maximal inhibition (PMI) ("plateau" effect) relative to the wildtype control. This reduction in PMI is characteristic of the development of resistance to all non-competitive inhibitors including allosteric inhibitors (Figure 13) The Antivirogram® assay[252] reports the ability of the infectious recombinant virus to grow in CD4 cells in various concentrations of the antiretroviral drugs under investigation, from which the virus IC_{50} is derived.

Automated, recombinant phenotypic assays are commercially available with results available in 2–3 weeks. Viral phenotypic susceptibility can also be measured using "in-house" methods, which are usually restricted to few specialized laboratories.

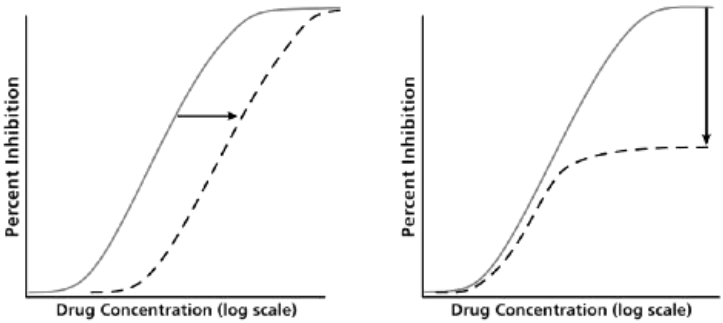


FIGURE 13. INHIBITION CURVES OF COMPETITIVE AND NON-COMPETITIVE INHIBITORS. The left panel shows a typical inhibition curve of a susceptible virus (solid line) with a typical competitive inhibitor (e.g., a protease inhibitor). The IC50 value of the resistant virus (dotted line) is shifted to the right (arrow). The right panel shows an example of a noncompetitive inhibitor (e.g., a chemokine receptor 5 antagonist). The susceptible virus (solid line) shows a typical inhibition curve, but in this case, the resistant virus (dotted line) reaches a plateau. The maximum achievable percent inhibition is shifted downward (arrow), but the curve does not shift to the right; hence, the IC50 value remains unchanged. Source: *Hirsch et al. Antiretroviral Drug Resistance Testing in Adult HIV-1 Infection: 2008 Recommendations of an International AIDS Society-USA Panel. Clin. Infect Dis. 2008;47:266–85*

Some commercial phenotypic assays report the virus replication capacity (RC). This indicates the relative ability of recombinant viruses containing patient-derived inserts (PR and RT, or C-terminal RT plus IN) to replicate in the absence of drug in comparison with the wildtype control. Overall, the clinical utility of RC measurements is uncertain. No study has clearly demonstrated that accounting for the capacity of a virus to replicate modifies therapy outcomes in a clinically meaningful manner.[53, 249] Moreover, the overall RC of a virus *in vivo* is not only determined by the genomic regions studied, but also by epistatic effects of other viral genes and complex interactions with the other viral variants in the quasispecies and between the quasispecies as a whole and the particular environment in which viruses replicate. Current guidelines for resistance testing in HIV-infected subjects do not recommend using RC measurements for guiding antiretroviral therapy choices.[53, 249]

Virtual phenotype

The "virtual phenotype" is an alternative approach for interpreting genotypic drug resistance information. The virtual phenotype (Virco®TYPE HIV-1, Virco BVBA; Geno2Pheno, http://www.geno2pheno.org) correlates genotypic data obtained from the plasma HIV-1 RNA of a candidate gene with a large database of paired phenotypes and genotypes.[253-256] Such linkage assigns calculated fold-changes in IC_{50} to query genotypes. Virtual and actual phenotypes show good correlation for most drugs. However, superiority of virtual phenotype over genotype alone could not be demonstrated in predicting clinical response to salvage regimens.[255, 257, 258] The main limitation of virtual phenotype is that its predictive power depends on the number of matched datasets available. Thus, variation is frequently higher in smaller datasets as well as for newer drugs or complex resistant patterns. Moreover, matches are based on preselected codons, not on the entire nucleotide sequence, and most genotype-phenotype pairs in the database are were obtained on subtype B viruses. This warrants caution when inferring phenotypes from non-B subtype genotypic data using virtual phenotype.

VIRAL TROPISM ASSAYS

The incorporation of CCR5 antagonists into clinical practice has renewed the interest in viral tropism assays. Co-receptor tropism determination is mandatory before initiating CCR5 antagonist therapy. Most subjects failing CCR5 antagonist therapy show rebounds of X4-using HIV-1 at the time of virological failure.[216-219] Switches in co-receptor use from CCR5 to CXCR4 have been associated with accelerated CD4+ count decay and an increased risk of AIDS-defining diseases and death.[250] Several studies have found that most X4-using viruses emerging at the time of CCR5 antagonist failure were already present before therapy as minority species and went undetected by standard tropism assays.[220] Therefore, the presence of low levels of X4 virus is a challenge to all assay methods, resulting in reduced sensitivity in clinical, patient-derived samples when compared to clonally derived samples.

Methods to determine viral tropism include recombinant phenotypic tests, such as the Monogram Trofile™ assay, as well as genotype-based predictors, heteroduplex tracking assays, and flow cytometry based methods. Currently, the best evidence supports the use of phenotypic methods, although other methods of screening for HIV co-receptor usage prior to the administration of CCR5 antagonists may reduce costs and increase turnaround time over phenotypic methods.

Phenotypic tropism assays

Phenotypic tropism assays require the amplification of *env* sequences from plasma HIV-1 RNA and the construction of viral pseudotypes or infectious recombinant viruses that express the patient-derived *env* sequences along with a reporter gene.[259] These pseudotyped viruses or viral recombinants are then inoculated onto cells that express CD4 along with CCR5 or CXCR4. Coreceptor tropism is determined by measuring the abilities of these pseudovirus populations to efficiently infect CD4+/U87 cells expressing either the CXCR4 or CCR5 coreceptor. Viruses exclusively and efficiently infecting CXCR4+/CD4+/U87 cells are designated X4-tropic. Conversely, viruses exclusively and efficiently infecting CCR5+/CD4+/U87 cells are designated R5-tropic. Viruses capable of infecting CXCR4+/CD4+/U87 and CCR5+/CD4+/U87 cells are designated dual/mixed-tropic

HIV-1 isolates that use CCR5 exclusively are termed R5 viruses, those that use only CXCR4 are termed X4 viruses, and those that use both are termed R5/X4, or dual-tropic viruses.[1, 53, 249] Because these assays do not distinguish between the presence of truly dual-tropic viruses and a mixture of R5 and X4 viruses, samples that can infect both CCR5- and CXCR4-expressing cells are often termed dual-mixed viruses.[53]

Tropism testing generally requires a plasma sample with an HIV-1 level of ≥1000 copies/mL. The assay used in most clinical trials of CCR5 antagonists in the Trofile™ assay (Monogram Biosciences). This is the only clinically validated assay to identify tropism and is considered the current gold standard. Most clinical CCR5 antagonist clinical assays were performed using the older version of this assay, that was able to detect CXCR4-using viruses when they constituted at least 5%-10% of

the virus population.[259] Recent technical improvements allow the new version of Trofile™ (also known as the Enhanced Sensitivity [ES]-Trofile™ assay) to detect down to 0.3% CXCR4-using or dual-mixed virus.[260] The enhanced sensitivity of the Trofile™ assay has important implications for the clinical management of HIV-infected subjects. A reanalysis of baseline plasma specimens of the vicriviroc ACTG A5211 study[218] and the maraviroc MOTIVATE 1 and 2[216, 217] and MERIT[219] trials showed that roughly 10%, 8% and 15% subjects that had been classified harboring R5-using HIV-1, respectively, actually had X4 or dual-mixed viruses.[261] The higher sensitivity of the ES-Trofile™ assay narrows the number of patients to which CCR5 antagonists can be prescribed but improves the outcome of CCR5 antagonist therapy. Using the first version of the Trofile™ assay to evaluate viral tropism, the MERIT trial found maraviroc to be inferior to efavirenz, both with an AZT/3TC backbone, as initial therapy for drug-naïve individuals.[219] A subsequent reanalysis of the MERIT trial showed that if participants had been screened for X4-using viruses with the ES-Trofile™ assay, more subjects harboring X4 viruses would have been excluded, but both arms would likely have reached comparable virological outcomes.[261] The main limitation of the Trofile™ assay is its cost (between $750 and $1000 per sample) and the need to ship patient samples to a central laboratory in San Francisco, USA, what slows the turn-around time. Also, the current Trofile™ assay does not allow tropism determinations in subjects with undetectable viremia who might require switching to a CCR5 antagonist for toxicity of tolerability reasons.

An alternative "in-house" phenotypic method to determine co-receptor tropism is co-cultivation of patient-derived peripheral blood mononuclear cells (PBMCs) with MT2 cells.[262] The MT2 cells are human HTLV-1-transformed lymphoblasts isolated from cord blood from subjects with Human T-cell leukemia and cocultured with cells from patients with adult T-cell leukemia. The MT2 cells are CD4+ and have the CXCR4 co-receptor but lack CCR5, being susceptible to infection by X4 HIV-1. Infection with HIV is cytopathogenic on MT2 cells, being detected by the induction of syncytia. Direct co-cultivation of IL-2 and phytohaemagglutinin (PHA)-stimulated patient-derived

PBMCs with MT2 cells is considered the most sensitive non-commercial phenotypic method to detect X4 viruses. Overall, direct comparisons have shown equivalent results between this method and the earlier version of the Trofile™ assay; comparisons with the enhanced sensitivity Trofile™ are under way. The main limitation of the MT2 assays is that infectious viruses may be difficult to obtain from stored frozen PBMCs and, possibly, may be more difficult in subjects with prolonged undetectable viremia. Conversely, this technology is cheaper than commercial phenotypic assays and can be performed in most HIV laboratories with a relatively high throughput. Because the source of virus is PBMCs instead of plasma, MT2 assays can be performed in subjects with undetectable viremia.

Genotypic tropism assays

The main genetic determinant of co-receptor tropism in the HIV envelope is the V3-loop region in gp120. However, changes in this region alone are not always necessary or sufficient to confer a particular phenotype in viruses expressing engineered gp120 proteins, since changes in other regions of gp120, particularly in V1/V2[263-267] and C4,[268] have been shown to influence phenotype either alone or in conjunction with V3. In addition, isolates with identical V3 sequences can have dissimilar patterns of coreceptor usage, cell tropism, or replication capacity.[21, 265, 266, 269] Typically, specific changes in these other regions do not have consistent effects in a wide range of sequence contexts.

Genotypic approaches to determining co-receptor use are thus based on amplifying the envelope gene and sequencing the V3 loop and, sometimes, additional regions like V2. Different algorithms and interpretation rules that attempt to infer a co-receptor tropism phenotype from the genotypic information provided are available online. The most commonly used measures for predicting CXCR4 are the bioinformatic algorithms Geno2Pheno (www.geno2pheno.org),[270] hosted by the *Max Planck Institut Informatik*, Germany; the Position-Specific Scoring Matrix (PSSM) (http://indra.mullins.microbiol.washington.edu/pssm/),[271] hosted by the Microbiology Department of the University of Washington, Seattle, US.

Whereas the Geno2Pheno algorithm is being updated regularly, the PSSM web site was last updated on April 27, 2006 (web site accessed Nov 27th 2008).

Other interpretation approaches include the presence of positively charged amino acids at positions 11 and 25, often referred to as the "11/25 rule," and the total charge of V3 loop amino acid residues of +5 or greater.[272, 273] In addition to the PSSM,[271] other bioinformatic approaches include neural networks,[274] or machine-learning techniques.[275] The heteroduplex tracking assay has also been used to detect the presence of CXCR4-using virus.[276] In this assay, the electrophoretic mobility of PCR-amplified *env* genes is assayed after hybridization to V3-coding sequences from viruses with phenotypically defined coreceptor use.

When verified against phenotypic assays, genotypic approaches showed excellent specificity but poor sensitivity for detecting the presence of dual-mixed or CXCR4 viruses in clinical samples.[270]

The main limitation of genotypic methods to assess coreceptor tropism is related to the following factors:

(a) The heterogeneity of HIV *env* in plasma virus populations makes it difficult to obtain coherent sequence data with population-based sequencing approaches.

(b) Not all determinants of viral tropism reside in the V3 loop. The V1, V2, C4 regions might sometimes confer different coreceptor use than expected from the V3-loop sequence.

(c) Viral population sequencing of *env* regions does not account for minority X4-using viral variants. This is an important limitation since, as we will discuss later, such minority variants, even if present at very low levels in the viral population (0.008%), can be the ones that emerge during virological failure to CCR5 antagonist therapy.

(d) Some bioinformatics algorithms for phenotype prediction like the Geno2Pheno were originally constructed using clonal sequence data matched with phenotypes. Therefore, they tend to perform better when clonal instead than consensus sequences are interrogated.

(e) Limited information is available for inferring tropism for non-subtype B viruses. The PSSM algorithm allows predictions for subtype C (although based on relatively few genotype-phenotype pairs), but warns against using it with subtypes other than B or C.

Due to the abovementioned limitations and lack of clinical validation, current guidelines for antiretroviral resistance testing do not recommend using genotypic approaches for identifying patients who may be suitable candidates for CCR5 antagonist therapy.[53] A number of studies are ongoing, particularly in Europe, to try to validate genotypic tropism testing for clinical use. Another question that needs to be answered is whether the sensitivity of genotypic tropism methods to detect X4-using viruses can be increased by using PBMC-associated DNA instead of plasma RNA as the source of genetic information. Because PBMC-associated DNA acts like a "repository" of genetic information of current and past viral variants, PBMCs-associated DNA may "store" genetic information of natural oscillations in coreceptor use over time, which may enable identifying subjects at higher risk of virological failure of CCR5 antagonist treatment with more sensitivity and specificity than with plasma RNA. This method would also allow evaluating coreceptor tropism in subjects with undetectable viremia. We shall discuss later on the possibility of using massively parallel sequencing of V3-loop sequences to predict viral tropism in minority variants.

TESTING FOR MINORITY RESISTANT VARIANTS

Assays able to detect, quantify or characterize minority HIV-1 variants include: standard cloning and sequencing of multiple clones, single-genome sequencing,[277] allele-specific PCR (ASPCR),[243, 278, 279] parallel allele-specific sequencing (PASS),[280] LigAmp,[138] the phenotypic analysis using *S. cerevisiae* TyHRT,[281, 282 281, 283] and massively-parallel sequencing in microfabricated PicoTiterPlates™ –also known as ultra-deep

sequencing- (454 Life Sciences/Roche). [284, 285] New high-throughput parallel sequencing systems are being developed by Illumina (SOLEXA™) and Applied Biosystems (SOLiD™), but they have not been used to detect minority HIV variants so far.

Standard cloning and sequencing of multiple clones

In standard cloning, a DNA region of interest is PCR-amplified and inserted into a vector containing an antibiotic resistance gene, and colour selection markers which provide blue/white screening (⟨-factor complementation) on X-gal medium. To preserve the original population variability and avoid founder effects, the source DNA is usually obtained by pooling the product of parallel PCRs, or by amplifying the source DNA after end-point dilutions. The recombinant construct is then transfected into bacterial cells which are plated into solid media containing an antibiotic that restricts growth to bacteria transformed with the recombinant construct. Recombinant DNA is purified from colony forming units containing a single clone each and sequenced. The sensitivity of this assay depends directly on the number of colonies analyzed. Although this is an easy and relatively cheap procedure available in most laboratories that allows the assessment of mutations linked in individual genomes, its capacity to detect minority variants is very limited. According to the Poisson distribution, in order to detect a variant present in 10% of the viral population with 95% confidence, one should analyze at least 30 CFUs. If the target sequence was present in 1% of the viral population, 300 CFUs should be analyzed to achieve the same levels of confidence. In practice, this in not viable for studies including several patient samples. Therefore, with the usual cloning and sequencing of 30-40 CFUs, one should not expect to detect viral variants present in less than 10% of the viral population.

Table 17. Principal techniques to detect minority HIV drug-resistant variants.[a]

	Standard Cloning	Single Genome Sequencing (SGS)	Allele-specific PCR (ASPCR)	Parallel Allele-Specific Sequencing (PASS)	LigAmp	Ultra deep sequencing (UDS)*
Principle	Analysis of single CFUs with indiviual clones	Massive sequencing of single genome molecules	Differential amplification of mutants vs WT in real-time PCR	Single-base allele sequencing of polonies fixed to an acrylamide surface	Template-dependent ligation of 2 primers and quantification with Q-PCR	Massively parallel microfluidic solid-surface sequencing of single molecules
Sensitivity	> 10%	2%	0.003 - 0.4%	0.1%	0.1%	0.5 – 1%
# mutations	multiple	Multiple	1	1 per round (up to 22 rounds)	1	300-400 bp
Linked mutations	Yes	Yes	No	Yes	No	Yes
Labor Intensity	↑↑↑	↑↑↑↑↑	↑↑	↑↑↑	↑↑	↑
Cost	↑	↑↑↑↑	↑↑	↑↑↑	↑↑	↑↑↑↑↑↑↑
Best	Experience, PPV	Enables linkage of mutations	S, PPV, NPV, Affordable	S, enables linkage of mutations	Same as ASPCR, increased specificity	Linkage, Accuracy, S, NPV, Rapidity of results
Worst	S, NPV	Cost, time and labor consuming	Only 1 allele 7 reaction, Sp, affected by polymorphisms	Cost, Labor intensity	Same as ASPCR	Requires strong bioinformatics support, Sp

[a] PCR: polymerase chain reaction; CFU: colony-forming units; WT: wildtype; bp: base pairs; S: Sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; Q-PCR: Quantitative PCR

* 454 sequencing, 454 Life Sciences/Roche

Single-genome sequencing

This technique was developed by Palmer and colleagues,[277] based on earlier limiting-dilution assays.[286-289] Single-genome sequencing allows more refined analyses of HIV-1 populations by obtaining DNA sequences derived from many single viral genomes in a plasma sample.

Viral RNA is extracted from 250 and 1,800 µl of plasma containing a minimum of 1,000 copies of HIV-1 RNA, after centrifugation at 16,000 x g for 1 h at 4°C. The entire viral RNA extraction is used for cDNA synthesis using random primers. To obtain PCR products derived from single cDNA molecules, the resulting cDNA is serially diluted 1:3 to a maximum dilution of 1:2,187. Ten separate real-time PCR amplifications are performed for each cDNA dilution. According to Poisson's distribution, the cDNA dilution yielding PCR product in 3 out of the 10 real-time PCRs contains 1 copy of cDNA per positive PCR about 80% of the time. Thus, 70 nested PCRs are then set up using the cDNA dilution yielding approximately 30% positive reactions. Positive nested PCRs are identified by agarose gel electrophoresis and sequenced by direct dideoxyterminator sequencing. DNA sequences derived from 20 to 40 single genomes are typically analyzed per sample, although the number of genomes obtained can be increased simply by analyzing more genomes.

Single genome sequencing detects minority variants that are present in at least 2% of the viral population. Although it is time and labor-consuming, SGS is perfectly suited for assessing the linkage of several mutations in individual genomes, which can be of major importance in heavily pre-treated patients and in NNRTI-experienced candidates to receive etravirine.

Allele-specific PCR[243, 278, 279]

Based on a modification of the amplification refractory mutation system (ARMS)[290], the allele-specific PCR performs separate real-time PCR amplifications of viruses containing, respectively, mutant and wildtype alleles of a codon. Alternatively, one can amplify the mutant allele and, separately, all variants using a non-specific primer that is identical to the mutant-specific oligonucleotide, but has a 3' end

finishing just one basepair before the target base. (Figure 14) We chose this second method because it confers several technical advantages and improves consistency (discussed in Chapter 1). To increase the specificity for the mutant allele, the mutant-specific primer usually contains one intentional base mismatch at the -1 to -1 position of the 3'end. Both the mutant-specific and the non-specific (or wild-specific) PCR reactions are run in separate wells, always in duplicate and simultaneously with standards. The copy number of mutant and all (or wild-type) variants is calculated, and the proportion of mutants is determined with the formula:

% of mutants = (copy number of mutants / total copy number) * 100

If a wildtype-specific primer is used instead of a non-specific primer, the calculation would be:

% of mutants = (copy number of mutants / copy number of WT) * 100

In blinded comparisons, ASPCR was the most sensitive method to detect minority variants.[291] It is also the cheapest, fastest and less labor-consuming method. The main limitation is that only one allele can be interrogated per experiment, thereby missing the impact of other mutations that could coexist with the target mutation; it is unsuitable for mutation linkage analysis. Also, polymorphisms at primer sites, particularly if those near the 3' end, can affect the relative amplification efficiencies of the two reactions, leading to an underestimation of mutant proportions.[292] This problem is shared also by other PCR-based techniques like parallel allele-specific sequencing (PASS), but is theoretically cirvumvented by 454 sequencing and LigAmp. Finally, due to the typical loss of linearity in the measurement of nominal proportions below 0.1% to 1%, it may be more difficult to establish clinically relevant thresholds for minority variants with ASPCR than with other techniques. As we shall show later, ASPCR remains an important and affordable research tool to investigate the clinical role of minority variants.

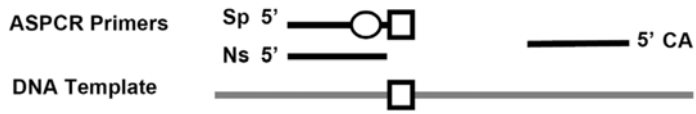


FIGURE 14. OVERVIEW OF ASPCR PRIMER DESIGN. The mutant-specific primer (Sp) incorporates the target mutant sequence (square) in its 3'-end plus an intentional mismatch (circle) in the –1 to –3 position to increase the specificity of the mutant-specific amplification reaction (i.e. constraint the amplification of wild-type variants). The non-specific primer (NS) is identical to the Sp primer, except that ends right before the target codon and does not incorporate any intentional mismatch. Both specific and non-specific amplification reactions are performed in separate wells using a common antiparallel (CA) primer. Amplified DNA can be quantified in real-time using SYBR+™Green, a TaqMan™ Probe, molecular beacons or scorpions. Source: *Paredes R, Marconi VC, Campbell TB, Kuritzkes DR. Systematic evaluation of allele-specific real-time PCR for the detection of minor HIV-1 variants with pol and env resistance mutations. J Virol Methods 2007;146(1-2):136-46.*

Parallel allele-specific sequencing (PASS),[280]

This assay simultaneously analyzes a large number of viral genomes by applying the polony technique[293, 294]A *pol* gene fragment containing sites of all major resistance mutations in reverse transcriptase and protease is amplified using acrydited primers. Because one acrydited primer becomes immobilized by covalently incorporating into polyacrylamide gels during the polymerizaton, the PCR products accumulate around individual DNA templates and form distinct spots (polonies) at the amplification sites (Figure 15). After amplification, the solid-phase negative DNA strands hybridize to complementary sequencing primers whose 3' end is juxtaposed to the site where a single-base mutation confers resistance. After single-base extension of this primer in the presence of nucleotides labeled with different fluorophores, imaging with a microarray scanner can be used to distinguish wild type and mutant populations.

This technology is more efficient, faster and more sensitive for detection of minor resistance populations than clonal sequencing. The PASS assay permits a detailed linkage analysis of multiple mutations,

allowing study of the impact of different combinations of mutations existing as minor and major viral populations. In addition, because viral cDNA molecules are directly embedded into polyacrylamide gel, and PCR amplification is carried out at a single-molecule level, artifact sequences that are generated through recombination or resampling during conventional PCR are eliminated. This technology, however, is not more sensitive than allele-specific PCR; it is also affected by polymorphisms in primer sites, requires a few days to analyze all primary mutations, and is more expensive and labor consuming and enables lower throughput than ASPCR.

LigAmp[281, 282]

The 'LigAmp' stratregy involves a ligation step followed by an amplification-detection step. (Figure 16) LigAmp can be used to detect and directly quantify mutant DNA alone or to simultaneously detect and quantify mutant and wild-type DNA by using a pair of mutant and wild-type upstream oligonucleotides. The LigAmp assay is designed to convert single-base differences into more distinctive molecules that can more easily detected and quantified.

First, two oligonucleotides are hybridized to a DNA template and ligated to one another. Each primer contains a region specific to the target gene and an M13 tail. The M13 tails permit amplification of the ligated product in a subsequent universal quantitative realtime PCR (Q-PCR) detection reaction. The upstream oligonucleotide also contains a region of unique foreign DNA (e.g. lacZ DNA, red) that serves as the binding region for a probe in the Q-PCR reaction. The upstream primer is designed to match either the mutant or wild-type sequence at the 3' end. When an upstream mutant primer is used, the 3' end of the primer to match the mutant template perfectly. The same oligonucleotide should mispair at the 3' end when hybridized to a wild-type template, preventing ligation.

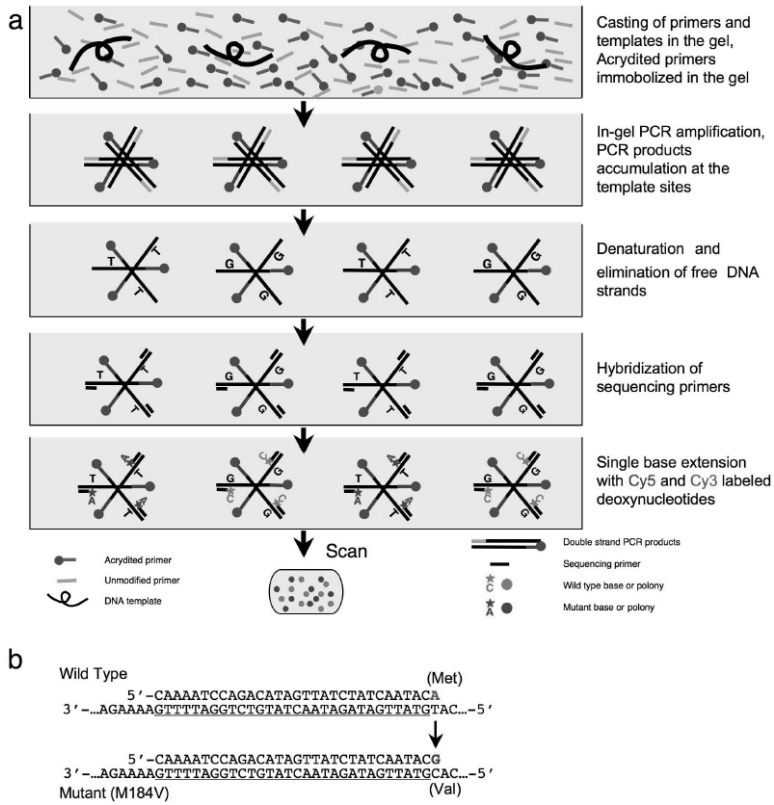


FIGURE 15. SCHEMATIC PRESENTATION OF THE PARALLEL ALLELE-SPECIFIC SEQUENCING (PASS) ASSAY. Panel A. Unmodified 5' primers, acrydited 3' primers, DNA templates and PCR reagents were imbedded in the 6% acrylamide gel. Because the acrydited primers are covalently coupled with the polyacrylamide gel during polymerization and become immobilized, the amplified PCR products accumulate around the DNA templates and form individual spots (polonies) at the amplification sites. After the double strand DNA is denatured, the free strand DNA is washed off while the DNA strands extended from the acrydited primers are kept in the gel. The sequencing primers are then annealed to the single strand templates and extended with fluorescence-labeled wildtype or resistance bases. Gels are then scanned on a microarray scanner to acquire images. Panel B. The 3' end of the primer is juxtaposed to the drug-resistance mutation position. When the primers are extended with fluorescence-labeled nucleotides, the base-identity at the mutation site can be determined by the fluorophore incorporated. The template sequence is shown at the bottom with the M184V primer sequence underlined. The primer sequence is shown at the top. The incorporated wildtype base is labeled withCy3. The incorporated mutation base is labeled with Cy5.

Source: Cai F, Chen H, Hicks C, Bartlett J, Zhu J, and Gao F. Detection of minor drug-resistant populations by parallel allele-specific sequencing. Nature Methods-4, 123-125 (2007). Supplementary Figure 1.

Then, the ligated DNA is amplified using M13 primers and detected it in a Q-PCR reaction. This step is independent of the specific gene or mutation targeted in the ligation step. A universal probe (such as LacZ) containing a fluorophore and quencher can be used for the Q-PCR amplicons. Because both the M13 forward primer and the lacZ probe have the same polarity as the upstream ligation oligonucleotide, the lacZ probe cannot bind to the ligation oligonucleotide. Binding of the probe requires ligation of the two oligonucleotides and subsequent polymerization of the complementary DNA strand in the Q-PCR step. If no ligation occurs in the first step because of mispairing, there is no template for amplification in the Q-PCR step.

The specificity of LigAmp relies on the differentiating power of a DNA ligase to ligate the upstream and downstream oligonucleotides only when both hybridize to the template with no mismatches at the adjacent terminal nucleotides.

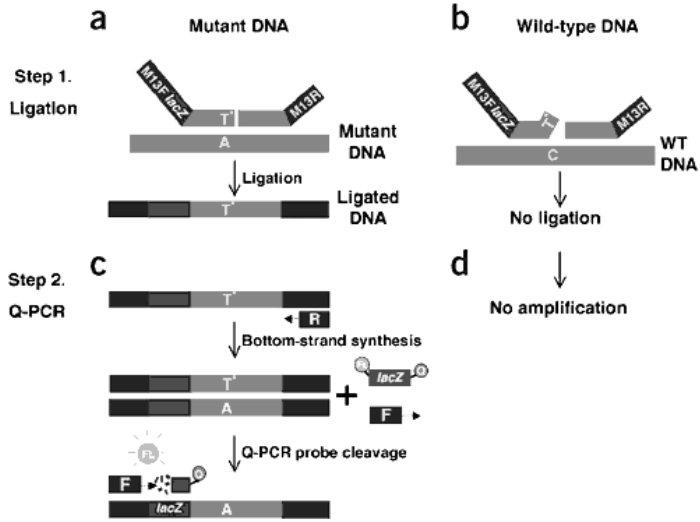


FIGURE 16. OVERVIEW OF THE LIGAMP ASSAY. (a–d) The assay includes two steps: (a,b, step 1) template-dependent ligation of two oligonucleotides and (c,d, step 2) detection and quantification with Q-PCR. F, Forward M13 primer; R, reverse M13 primer; FL, fluorophore; Q, quencher. The asterisk indicates the terminal thymidine base on the upstream mutant oligonucleotide. *Source: Shi C, Eshleman SH, Jones D, Fukushima N, Hua L, Parker AR, Yeo CJ, Hruban RH, Goggins MG and Eshleman JR. LigAmp for sensitive detection of single-nucleotide differences. Nature Methods 2004, 1 (2): 141 - 147*

The TyHRT System

This is a phenotypic assay based on hybrid elements derived from the *Saccharomyces cerevisiae* Ty1 retrotransposon in which reverse transcriptase is provided by HIV-1 RT (TyHRT).[295]

In the TyHRT system, amplified HIV-1 pol region DNA from test samples is cotransformed into yeast with a plasmid containing a TyHRT element with a deletion in the RT region. HIV-1 RT DNA is introduced into the TyHRT element by homologous recombination. Each isolate carries a unique RT domain, and the library of isolates is representative of the RT domains present in the original viral sample. The TyHRT elements carry the reverse-transcription indicator gene his3AI. Expression and reverse transcription of the TyHRT element by the recombined HIV-1 RT results in conversion of the his3AI gene into a functional HIS3 gene. RT activity is detected as the ability to give rise to cells that are able to grow on medium lacking histidine. Selection in the presence of NNRTIs measures the NNRTI susceptibility of individual RT clones. Analysis of the RT activity and NNRTI susceptibility of the isolates present in large libraries of clones makes it possible to detect NNRTI-resistant RT variants that are present at low frequencies.

Ultra-deep pyrosequencing. [284, 285]

Ultra-deep pyrosequencing (UDS, 454 sequencing, 454 Life Sciences / Roche Diagnostics) is an emulsion-based parallel pyrosequencing technique that provides significantly greater throughput and lower cost per sequenced base pair than Sanger sequencing. (Figure 17) Originally designed for high throughput sequencing or mammal and bacterial genomes, this technique is particularly well-suited for an in-depth analysis of a population of heterogeneous genomes like those of retroviruses.[284] Emulsion-droplets are used to separate individual DNA templates and amplification and sequencing are performed within the each droplet. Sequences are thus generated from a large number of independent genomes, what yields a quantitative readout of the genomic diversity. At present, UDS allows the parallel sequencing of more than 300 000-400 000 independent HIV-1 clones at once. If

lower coverage is needed, multiple specimens can be tested in a single plate using nucleotide barcoding (i.e., a sample-specific 4-base code in the 5' end of the amplification primers). Currently, the length of sequencing reads per clone is between 300 to 400 contiguous base pairs. This allows studying the genetic linkage of clinically relevant resistance mutation clusters, like resistance mutations surrounding the NNRTI-binding pocket, the integrase catalytic site, most mutations in protease, and the complete V3 loop sequence of gp120. Ultra-deep sequencing is also well suited for studying immune escape variants and host immune responses like immunoglobulin or T-cell receptor rearrangements during the adaptative immune response.

Following reverse transcription and amplification of the region of interest of the HIV-1 genome using primers with fusion adapters in the 5' end, each DNA molecule is immobilized onto a 28 μm DNA capture bead. The bead-bound amplicons are emulsified with amplification reagents in a water-in-oil mixture resulting in microreactors containing just one bead with one unique DNA molecule. Each unique DNA molecule is amplified in an emulsion PCR within its own microreactor, which in principle excludes competing or contaminating sequences. Amplification of the entire fragment collection is done in parallel; for each fragment, this results in a copy number of several million per bead. Subsequently, the emulsion PCR is broken while the amplified fragments remain bound to their specific beads. The clonally amplified fragments are enriched and loaded onto a PicoTiterPlate™ device for sequencing. The diameter of the PicoTiterPlate™ wells is 44μM, which allows for only one bead per well. After addition of sequencing enzymes, individual nucleotides are flown in a fixed order across the hundreds of thousands of wells containing one bead each. Addition of one (or more) nucleotide(s) complementary to the template strand results in a chemiluminescent signal recorded by a CCD camera. The light signals are processed and transmitted to a computer which generates a report with the DNA sequence for each clones. Each cycle is completed in less than 10 hours. The accuracy per base is higher 99.5% (> 99.99% if a consensus sequence is used as a referenceof

reference) and the cost per base is less than half than by Sanger sequencing.

This technique, however, has several limitations. First, it is an open sequencing system that requires extensive bioinformatic support in order to fully analyse the massive amount of genetic information generated. Second, the lower limit of detection of low-abundant resistant variants remains to be defined for this technology, although it seems to be close to 0.5%. Third, the clinically-relevant lower limit of detection of minority drug resistant variants remains unknown. Drug resistant variants present at frequencies significantly lower than the apparent lower detection theshold of 454 sequencing can emerge *in vivo* under selective drug pressure. On the contrary, subjects harboring minority resistant variants can achieve prolonged virological suppression on triple therapy. Fourth, it is unclear whether new genotypic drug resistance interpretation systems will need to be developed for this technique. Five, the accuracy per sequenced base pair is lower than with Sanger sequencing or with the SOLEXA™ (Illumina®)or SOLiD™ (Applied Biosystems®) high-throughput massively parallel sequencing systems. Six, accuracy is significantly impaired in homopolymeric regions, particularly in those with more than 5-6 consecutive identical bases. This requires of specific base-calling protocols to be established in order to analyze the output sequence data properly. Finally, the cost of this technology remains high, although it is decreasing. Techniques like primer barcoding increase the efficiency of reagents and hardware use.

New high-throughput massively parallel sequencing systems: SOLEXA™ and SOLiD™

The SOLEXA™ and SOLiD™ systems developed by Illumina® and Applied Biosystems®, respectively, are at the forefront of the many other high thropughput sequencing technologies currently in the pipeline (Table XX). Both assays have higher accuracy per base than 454 sequencing and achieve a much better treatment of homopolymeric regions. These technologies are starting to be applied to detect minority HIV variants, but data has not been produced yet.

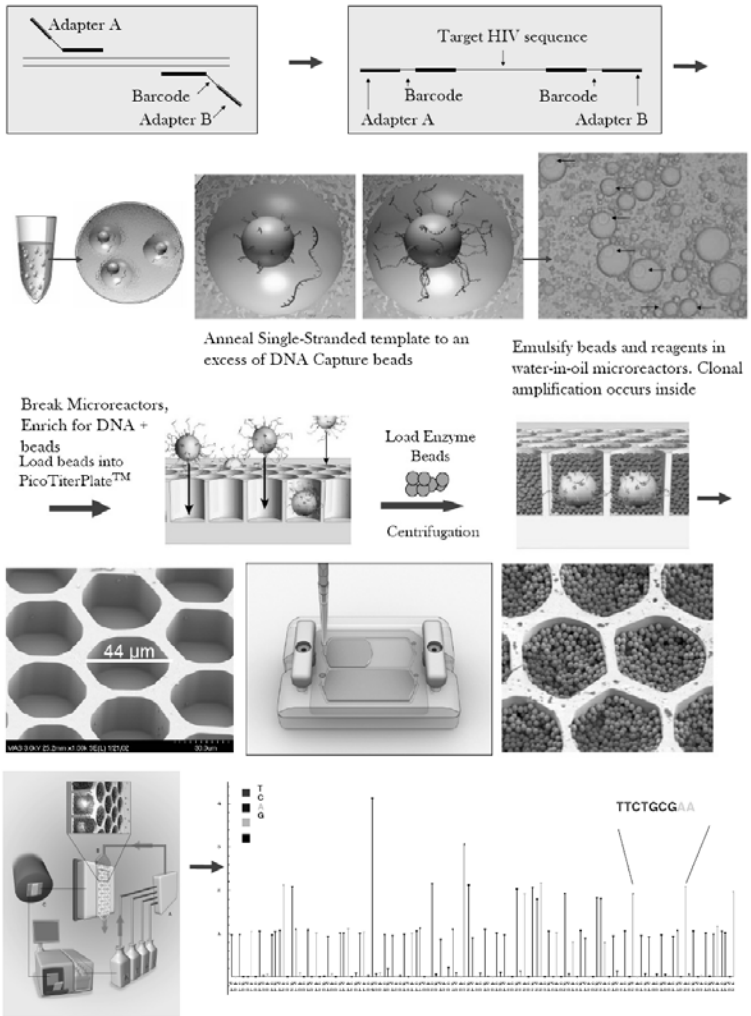


FIGURE 17. SCHEMATIC PROCEDURE FOR 454 SEQUENCING IN HIV. The target sequence is amplified with primers tagged with a sample-specific identification 4 basepair barcode plus an adapter A and B, respectively, that will anneal to 28µM beads. An water/oil emulsion is prepared so each droplet (microreactor) contains 1 bead and 1 amplicon; each amplicon is then clonally amplified by emulsion PCR within the droplet. The emulsion is disrupted and 28µM beads are loaded into 44 µM wells in PicoTiterPlates, so, theoretically, there is only one bead per well. Sequencing enzimes including luciferase and sulfurilase are loaded into each wells. Pyrosequencing proceeds by series of flows of known nucleotides. When a nucleotide hibridizes with its complementary a pyrofosfate is released and this results in the release of a chemiluminescent signal recorded by a CCD camera and integrated by a computer. Vertical bars in the sequencing flowchart represent number of bases. Source: *454 Sequencing website: www.454.com*.

The sensitivity threshold to detect point mutations is unknown, but it could be at least at low as that of 454 sequencing. The main limitation relative to 454 sequencing is that, at present, read lengths are 35 basepairs or shorter, what limits the ability to assess several resistance mutations in single amplicons. These technologies and many others in the pipeline, however, are rapidly evolving.

Table 18. Other high-throughput ultrasensitive sequencing techniques with potential application to detection of minority HIV-1 variants

	SOLiD™ (Applied Biosystems®)	SOLEXA™ (Illumina®)
Principle	Massively parallel sequencing of clonally amplified DNA fragments linked to magnetic beads, attached to a solid phase → Sequencing by sequential ligation with dye-labeled probes.	Massively parallel sequencing of DNA fragments incorporating adapters, that are attached to a solid phase → Bridge amplification on solid phase → Sequencing-by-synthesis using labelled reversible terminators
Read Length	35 bp	35 bp
Accuracy	99.94% (99.999% consensus accuracy @ 15X coverage)	99.99% @ 3X coverage
Sensitivity	Uncertain for HIV (likely <1%)	Uncertain for HIV (likely <1%)
Advantages over 454/Roche ultradeep sequencing	Bidirectional sequencing of the same amplicon feasible Less cumulative and sequential errors Better treatment of homopolymeric zones	Accurate homopolymer sequencing High accuracy
Disadvantages for HIV	Short sequence reads, no linkage	Short sequence reads, no linkage

SUMMARY

Despite the outstanding therapeutic advances achieved during the past 25 years, the cure of HIV infection remains out of reach. In part, this is because HIV is a highly complex virus with a vast capacity to diversify and escape from immune and drug pressure. The quasispecies distribution of HIV enables fast dynamic adaptation to varying environments. Resistant HIV can be transmitted from person-to-person. Moreover, the high turnover and production of genetic variants

ensure that every possible variant containing one resistance mutation and many variants with two resistance mutantions can be spontaneously generated in treatment-naïve individuals. In general, exposure to suboptimal therapy will force these mutants to rapidly evolve toward higher susceptibility losses, cross-class resistance and improved fitness through the accumulation of additional mutations in their genome. Standardized antiretroviral resistance tests only detect variants that are present in at least 15%-20% of the virus population, thereby underestimating the frequency of resistant mutants. New ultrasensitive resistance assays are being developed to detect minority viral variants carrying antiretroviral resistance mutations. Such assays will also allow a better understanding of the quasispecies structure and dynamics in different environments. Hereafter, we will describe the development and characteristics of an allele-specific PCR assay to detect drug resistance mutants with high sensitivity. Then, we will show its utility to assess mutant kinetics and viral fitness *in vivo*. We will continue by discussing the applicability of ASPCR assay to surveillance of antiretroviral resistance. Finally, we will analyze the effect of pre-existing minority NNRTI-resistant viruses on the virologic outcomes of first-line NNRTI therapy in antiretroviral naïve subjects.

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Hypotheses

1. Minority drug-resistant variants increase the risk of virological failure to initial antiretroviral therapy.

2. Allele-specific PCR is a sensitive and reliable method for detecting low-abundant resistant variants carrying single mutations.

3. Allele-specific PCR is a suitable method for assessing kinetics of particular mutants and their relative fitness *in vivo.*

4. Minority variant assays can improve surveillance of primary and secondary antiretroviral drug resistance.

5. More sensitive resistance assays could improve the clinical management of HIV-infected subjects.

Chapter 1

Systematic Evaluation of Allele-Specific Real Time PCR for the Detection of Minor HIV-1 Variants with *pol* and *env* Resistance Mutations

Roger Paredes[1,2], Vincent C. Marconi[1,3], Thomas B. Campbell[4], Daniel R. Kuritzkes[1]

[1]Section of Retroviral Therapeutics, Brigham and Women's Hospital and Division of AIDS, Harvard Medical School, Boston, MA, USA. [2]Fundacions "irsiCaixa" i Lluita contra la SIDA, Hospital Universitari Germans Trias i Pujol, Universitat Autonòma de Barcelona, Badalona, Catalonia, Spain. [3]Wilford Hall USAF Medical Center, San Antonio, TX, USA. [4]Division of Infectious Diseases, Department of Medicine, University of Colorado Health Sciences Center, Denver, CO, USA.

J Virol Methods 2007;146(1-2):136-46.

ABSTRACT

Allele-specific PCR (ASPCR) is a highly sensitive, and reproducible method for the study of minor HIV-1 variants harboring resistance mutations and is significantly less labor-intensive and time-consuming than other techniques used for similar purposes. Furthermore, ASPCR has multiple applications in HIV research: it provides earlier and more sensitive detection of evolving resistance mutations, a more accurate assessment of transmitted drug-resistant mutants and a better evaluation of resistance selection after post-exposure or mother-to-child-transmission prophylaxis programs. This article outlines the principles of ASPCR and illustrates technical challenges in the design and application of ASPCR protocols by describing ASPCR assays developed for detecting resistance mutations in the protease (PR)- and reverse transcriptase (RT)–coding regions of pol and env. The assays achieved sensitivities of <1% for the D30N mutation in HIV-1 PR, M184V and I mutations in RT, and V38A in gp41. This method can be easily adapted to the quantitative detection of other mutations in HIV-1 or other viruses by introducing minor modifications to the methods described. In addition, ASPCR can be used to assess the dynamics of mutant populations in the viral quasispecies in response to changing selection pressures, allowing inferences on viral fitness in vivo through mathematical modeling.

INTRODUCTION

Accurate detection of resistance mutations is important for the clinical management of HIV-infected persons and has major public health implications. [1, 2] Antiretroviral drug resistance is associated with worse virological, immunological and clinical outcomes. [3-7] Conversely, use of resistance information for the design of treatment combinations significantly improves such outcomes.[8-14]

As with other RNA viruses,[15-17] the HIV population in an infected person constitutes a *quasispecies* [18, 19]. Standard genotypic tests only detect resistance mutations present in more than 20% of the viral population,[20-22] likely underestimating the prevalence of drug resistance mutations at any given time point. Genotypic resistance tests performed by reference laboratories generally rely on population-based sequencing and report the consensus sequence at each nucleotide position. Although these tests can detect the presence of mixed populations, they provide only a rough estimate of the relative proportions of wild-type and mutant species in the population. Mathematical models estimate significant delays between the emergence of resistance and its detection partly because of the low sensitivity of current genotyping methods [23]. More sensitive techniques to assess drug resistance in minor variants have been developed in the recent years, including single-genome sequencing, [24] allele-specific PCR (ASPCR), [25-27] hybridization assays, [28, 29] phenotypic analysis using *S. cerevisiae* [28, 30] and massively-parallel sequencing in microfabricated PicoTiterPlates [31, 32].

The first use of allele-specific nested PCR (ASPCR) to detect resistance mutations in HIV-1 was reported in 1991. [33] The more recent application of real-time PCR technology to ASPCR has increased the sensitivity of this technique several-fold and permitted quantification of the PCR products. [25, 26]

Here, an ASPCR protocol to detect resistance mutations in HIV-1 *pol* and in the gp41-coding region of *env* with high sensitivity, accuracy and reproducibility is described. The theoretical reasoning that supports this method is discussed and some practical guidance is offered to researchers interested in applying this technique to the detection of these and other mutations in HIV-1 and other viruses.

Table 1. Oligonucleotide sequences of RT-PCR, ASPCR and TaqMan Probes[a]

		Primer Name	Length (bp)	Tm (°C)	Sequence (5' – 3)	Nucleotide Position (HXB2)
M184V ASPCR Assay						
RT-PCR		OOPF	22	64	GAAGCAGGAGCCGATAGACAAG	2211-2232
		OOR2	23	63	TTTTCTGCCAGTTCTAGCTCTGC	3466-3444
ASPCR	(NS)	VN	21	52	GACATAGTTATCTATCAATAC	3078-3098
	(Sp)	V4	22	55	GACATAGTTATCTATCAAT***A***C**G***	3078-3099
	(R)	ASR2	20	56	GGCTGTACTGTCCATTTATC	3277-3258
M184I ASPCR Assay						
RT-PCR		OOPF	22	64	GAAGCAGGAGCCGATAGACAAG	2211-2232
		OOR2	23	63	TTTTCTGCCAGTTCTAGCTCTGC	3466-3444
ASPCR	(NS)	IN	23	54	GACATAGTTATCTATCAATACAT	3078-3100
	(Sp)	I5	24	57	GACATAGTTATCTATCAATACA***A*****	3078-3101
	(R)	ASR2	20	56	GGCTGTACTGTCCATTTATC	3277-3258
D30N ASPCR Assay						
RT-PCR		OOPF	22	64	GAAGCAGGAGCCGATAGACAAG	2211-2232
		OOR2	23	63	TTTTCTGCCAGTTCTAGCTCTGC	3466-3444
ASPCR	(NS)	DN	21	55	CTATTAGATACAGGAGCAGAT	2319-2339
	(Sp)	DS4	22	55	CTATTAGATACAGGAGCA***A***ATA*	2319-2340
	(R)	DR2	20	56	CTGGCTTTAATTTTACTGGTAC	2592-2571
V38A ASPCR Assay						
RT-PCR		GP41OF	24	62	GAGGGACAATTGGAGAAGTGAATT	7649-7672
		GP41OR	24	62	GTGAATATCCCTGCCTAACTCTAT	8364-8341
ASPCR	(NS)	IFN8	21	53	GACAATTATTGTCTGGTATAG	7849-7869
	(Sp)	IFS3C2	22	58	GACAATTATTGTCTGGTAT***C***G**C***	7849-7870
	(R)	IR4	18	56	AATCCCCAGGAGCTGTTG	8009-7992
TaqMan Probe		V38Probe	27	68	(6-FAM)-TCCTTTAGGTATCTTTCCACAGCCAGG-(TAMRA)(phosphate)	7990-7964

[a]The target mutation is shown in boldface, underlined and next to an asterisk. Intentional mismatches in mutant-specific primers are shown in boldface italics and underlined. ASPCR means allele-specific polymerase chain reaction. RT-PCR means one-step reverse transcription and PCR amplification. NS means non-specific primer. Sp means specific primer. R means reverse (antiparallel) primer.

MATERIALS AND METHODS

PRIMERS AND PROBES

The design of all PCR primers and probes (Table 1) was based on the HIV-1_{Hxb2} *pol* and *env* sequences.

CONSTRUCTION OF STANDARDS

Plasmids carrying wild-type HIV-1 *pol* and *env* were constructed by cloning the relevant segments of HIV-1 NL4-3 into a pGEM® T-Easy vector (pGEM® T-Easy Vector System, Promega Corporation, Madison, WI, USA) to create pPOL-W and pENV-W, respectively. For pPOL-W, the insert corresponded to a 1256-base pair amplicon obtained with primers OOPF and OOR2 (Table 1); for pENV-W, the insert corresponded to a 715-base pair amplicon derived from the gp41-coding region of env, obtained with primers GP41OF and GP41OR.

The following mutations were introduced individually by site-directed mutagenesis (Quick Change® XL Site Directed Mutagenesis Kit, Stratagene, La Jolla, USA) into pPOL-W to generate three different single-mutant plasmids (Figure 1): M184V (ATG → GTG, [pPOL-184V]), M184I (ATG → ATA, [pPOL-184I]) and D30N (GAT → AAT, [pPOL-30N]). Likewise, a plasmid (pENV-38A) carrying the V38A (GTG → GCG) mutation was obtained by introducing this mutation into pENV-W. Presence of the appropriate mutations was confirmed by sequencing the resulting plasmids.

PCR products were purified (QIAquick® PCR Purification Kit, QIAGEN Sciences, Maryland, USA) and quantified by spectrophotometry. Equivalence between starting amounts of wild type and mutant plasmid DNA was verified by real-time PCR. Serial 10-fold dilutions of each amplicon were prepared ranging from 10^6 to 10 copies per reaction. Standards were always run in duplicate at the same time as test samples. Given that the non-selective amplification did not depend on the nucleotide composition of the target codon, only mutant amplicons were used to generate both specific and non-specific standards curves for clinical specimen analysis. This ensured identical starting DNA copies for both standard reactions and enabled parallel, comparable curves. Wild type amplicons were used to prepare

mixtures with mutant amplicons in order to test the properties of the technique.

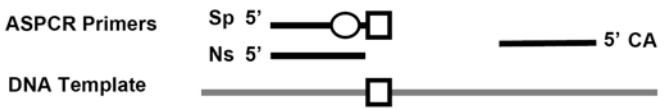

FIGURE 1. OVERVIEW OF ASPCR PRIMER DESIGN. The mutant-specific primer (Sp) incorporates the target mutant sequence (square) in its 3'-end plus an intentional mismatch (circle) in the –1 to –3 position to increase the specificity of the mutant-specific amplification reaction (i.e. constraint the amplification of wild-type variants). The non-specific primer (NS) is identical to the Sp primer, except that ends right before the target codon and does not incorporate any intentional mismatch. Both specific and non-specific amplification reactions are performed in separate wells using a common antiparallel (CA) primer. Amplified DNA can be quantified in real-time using SYBR+™Green, a TaqMan™ Probe, molecular beacons or scorpions.

HIV-1 RNA EXTRACTION FROM PLASMA

Plasma samples were obtained from subjects participating in clinical trials of antiretroviral therapy approved by the respective human subjects research committees, and were stored at -70°C. HIV-1 RNA was extracted from EDTA-anticoagulated plasma using the QIAamp® Viral RNA MiniKit (QIAGEN Sciences, Maryland, USA) according to the manufacturer's instructions. Part of each RNA sample was used for cDNA synthesis immediately after extraction, and the remainder was stored at – 80°C.

REVERSE TRANSCRIPTION AND FIRST-ROUND DNA AMPLIFICATION

The extracted RNA was transcribed to cDNA and amplified by PCR in a one-step process (Superscript III One-step RT-PCR with Platinum Taq Kit, Invitrogen™, Carlsbad, CA, USA) following the manufacturer's instructions. *Pol* and *env* fragments were amplified separately. Cycling conditions for *pol*-derived DNA included an initial cDNA synthesis step at 55°C during 25 min, followed by a denaturation step at 94°C during 2 min, 25 cycles of PCR amplification (94°C during

40 sec, 60°C during 40 sec, 68°C during 1 min and 20 sec), and a final 5 min extension step at 68°C. The PCR mix contained 25 µL of 2X Reaction Mix (including 0.4 mmol/L of each dNTP and 3.2 mmol/L of $MgCl_2$), 0.2 mmol/L of each primer OOPF and OOR2, 15 µL of extracted RNA as template and nuclease-free H_2O to a final volume of 50 µL. The same PCR mix was used for the *env*-derived DNA but with primers GP41OF and GP41OR. Thermal cycling conditions for *env*-derived DNA included 55°C for 25 min, followed by a denaturation step at 94°C for 2 min, 20 cycles of PCR amplification (94°C for 15 sec, 50°C for 30 sec, 68°C for 1 min), and a final 5 min extension step at 68°C. The PCR products were purified using the QIAquick® PCR Purification Kit (QIAquick® PCR Purification Kit, QIAGEN Sciences, Maryland, USA).

QUANTIFICATION OF VIRAL POPULATIONS USING REAL-TIME PCR.

Different fluorescence reporter molecules were used for each protocol, SYBRgreen for *pol*-derived samples and a TaqMan probe for *env*-derived samples, in order to illustrate different approaches to ASPCR design. To quantify the proportion of mutant sequences contained within each specimen, 5 µL of RT-PCR product were added to the real-time PCR together with selective or nonselective primers. Conditions for nonselective amplification of *env*-derived samples were 1× TaqMan® PCR Master Mix™ (Applied Biosystems, Warrington, UK), 1 µM of each primer IFN8 and IR4 (Figure 1, Table 1), 500 nM of V38A TaqMan® Probe, and deionized water to a final volume of 50 µL. Conditions for selective amplification of the V38A mutant sequence were identical except that primer IFN8 was replaced by IFS3C2. Conditions for nonselective amplification of *pol*-derived samples were 1× SYBR® green PCR Master Mix™ (Applied Biosystems, Warrington, UK), 900 nM of each non-specific and reverse primers, and deionized water to a final volume of 50 µL. Conditions for selective amplification of *pol* mutant sequences were identical except that the specific primer replaced the non-specific primer. Non-specific primers for the D30N, M184V and M184I ASPCR protocols

were primers DN, VN and IN, respectively (Figure 1, Table 1). Specific primers for the D30N, M184V and M184I ASPCR reactions were, respectively, primers DS4, V4, and I5. Reverse primers for D30N and M184V/I ASPCR experiments were, respectively, primers DR2 and ASR2.

Each sample was evaluated by real-time PCR in an ABI 7000 Sequence Detection System thermocycler (Perkin Elmer Applied Biosystems, Foster City, CA, USA), using the following cycling parameters: 50°C for 2 min to activate the AmpErase® UNG included in the mastermix which prevents PCR product carryover, 95°C for 10 min to activate the AmpliTaq Gold® DNA Polymerase, followed by 50 cycles at 95°C for 15s and 50°C for 1 min. Amplicons prepared from the relevant plasmids using primers OOPF and OOR2, or GP41OF and GP41OR served as standards. The number of cycles required to reach threshold fluorescence (C_t) was determined and the quantity of sequences initially present calculated by interpolation onto the standard curve.

The three different ASPCR protocols (D30N, M184V/I and V38A) were performed separately, each with a corresponding set of standards. Nonselective and selective amplifications were always performed in parallel. All reactions were performed in duplicate, and the mean of the two values was used for calculation. The percentage of viral sequences containing each mutation was calculated as follows: % mutant sequences = [(quantity of mutant sequences in the sample)/(quantity of total viral sequences in the sample)] × 100.

RESULTS

STANDARD CURVES AND AMPLIFICATION EFFICIENCY

For each set of specific and non-specific primers, C_t was linearly correlated with input DNA copy number over the range of 10^1 – 10^6 copies (Figure 2). The specific (Sp) and non-specific (NS) amplification efficiencies (defined as: $E=10^{(-1/slope)}$) were comparable within each ASPCR set. Correlation coefficients (r^2) were higher than 99.6 % for all primer pairs on their respective target standards.

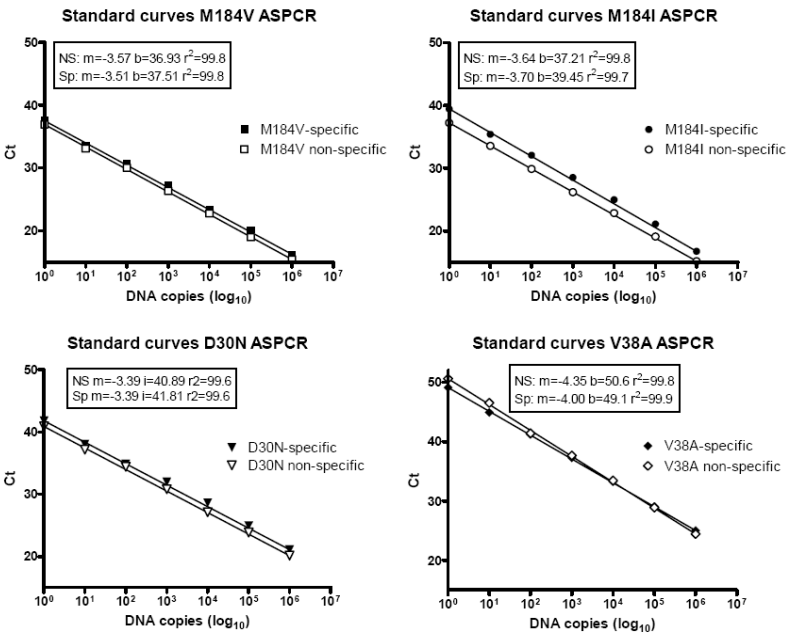

FIGURE 2. MUTANT-SPECIFIC (SP, SOLID SYMBOLS) AND NON-SPECIFIC (NS, OPEN SYMBOLS) STANDARD CURVES OF M184V, M184I, D30N AND V38A-ALLELE-SPECIFIC REAL-TIME PCR ASSAYS. Specific and NS regression lines, defined as: y= slope*x + b, where b is the y-intercept, were derived from each standard dilution set. In clinical samples, Sp and NS input copy numbers were determined using this formula by interpolation of the Cts (x) into the corresponding standard curve. Correlation coefficients (r^2) were higher than 99.6% in all cases.

ALLELIC DISCRIMINATION

Allelic discrimination of mutant-specific primers was tested by determining the difference in C_t values (ΔC_t) when identical amounts of mutant and wild-type DNA were amplified with the corresponding mutant-specific primer set (Figure 3, Panels A-D). Each experiment was conducted in triplicate; data shown represent the mean ± SD.

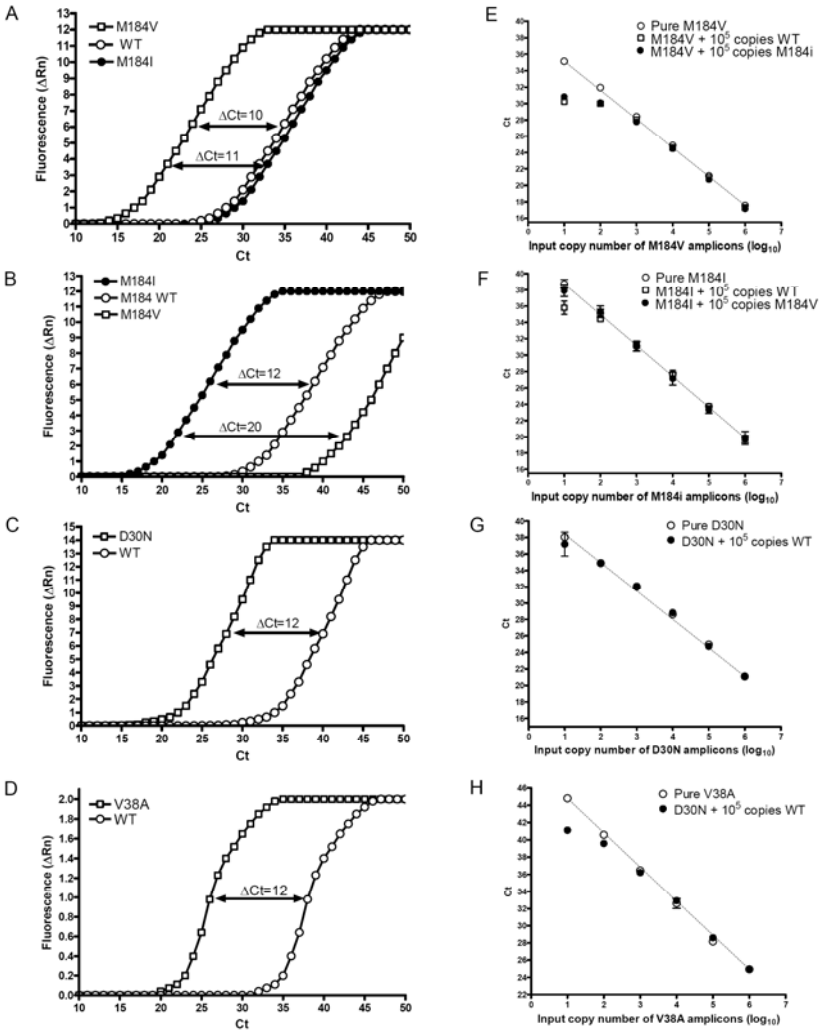

FIGURE 3 . ALLELIC DISCRIMINATION OF M184V, M184I, D30N AND V38A ASPCR ASSAYS. Panels A-D: Identical amounts of mutant and wild-type DNA were amplified with the corresponding mutant-specific primer set [i.e. M184V (Panel A), M184I (Panel B), D30N (Panel C) and V38A (Panel D)]. ΔC_t was ≥10 in all 4 assays. Panels E-H: The addition of 10^5 copies of a non-complementary allele (WT in all assays and M184I and M184V in M184V and M184I ASPCR assays, respectively) to each 10-fold dilution of the mutant standard did not significantly alter the standard curve until the mutant DNA was present at ≤10^3 copies. This illustrates that the discriminatory ability remained unaltered until the standard containing the target mutation was ≤0.1% of the total standard copies [e.g. (10^3 copies of M184V) / (10^3 copies of M184V + 10^5 copies of M184I) x 100]. Values in panels E-H correspond to the mean ± SD of triplicate measurements.

The fold-decrease in amplification efficiency (AE) was derived from ΔC_t. For the M184V-specific primer, ΔC_t between the amplification of M184V and wild type or M184I was 10 (>700-fold decrease in AE for WT) and 11 (>750-fold decrease in AE for M184I), respectively. For the M184I-specific primer, the ΔC_t between M184I and wild type was 12 (>2100-fold decrease in AE for M184V), whereas the ΔC_t between M184I and M184V was 20 (>200,000-fold decrease in AE for WT). Similarly, the ΔC_t for D30N versus WT was 12 (>5400-fold decrease in AE for WT); the ΔC_t for V38A versus WT was also 12 (>4,000-fold decrease in AE for WT).

In a second experiment (Figure 3, Panels E-H), the discriminatory ability of each assay was tested in mixing experiments by adding 10^5 copies of wild-type DNA to serial dilutions (10^6-10^1 copies) of mutant DNA. The threshold cycle was compared to PCRs performed without the addition of wild-type DNA. In each case, C_t was linearly correlated to input copy number over a range of mutant/wild-type ratios until the mutant DNA was present at 0.1% or less of total DNA copies.

SENSITIVITY

The sensitivity of each ASPCR assay was defined as the mean plus 3 standard deviations (SD) above the copy number determined on wild-type template with mutant-selective primer in 12 to 15 independent determinations. The sensitivity of the ASPCR assays was 0.4% for M184V, 0.04 % for M184I, 0.1% for D30N and 0.8% for V38A.

ACCURACY

Serial 10-fold dilutions of mutant amplicons were prepared in a background of WT amplicons and measured with the corresponding ASPCR assay (Figure 4). Measurements were accurate down to 1% (M184V), 0.1% (M184I), 0.1% (D30N), and 1% (V38A) in the various ASPCR assays, respectively. Measurements between these points and the limit of detection of each assay (e.g. between 1% and 0.4% in the M184V assay) tended to slightly overestimate the actual proportion of mutant variants.

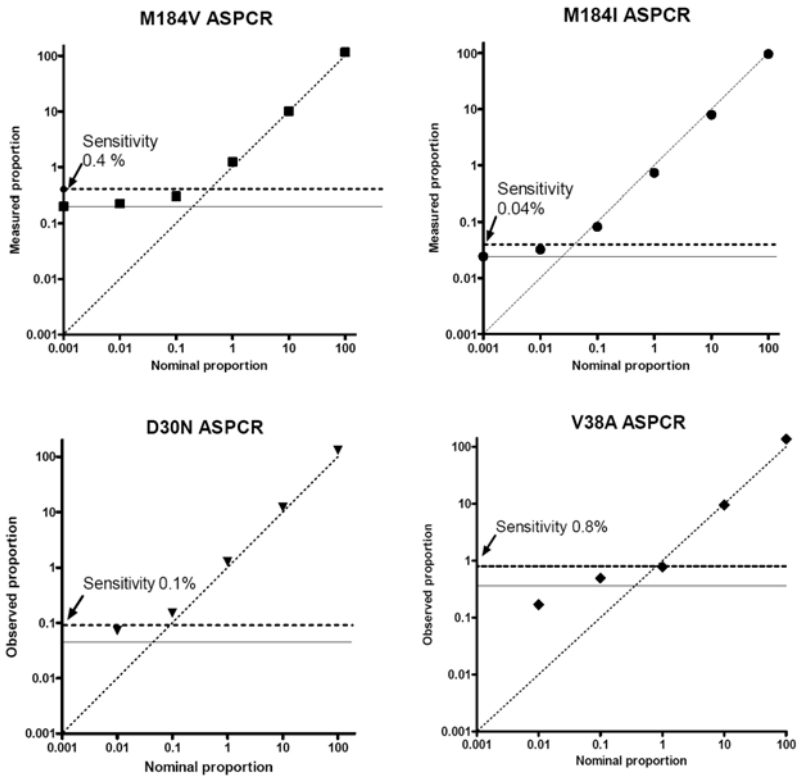

FIGURE 4: SENSITIVITY AND ACCURACY OF M184V, M184I, D30N AND V38A ASPCR ASSAYS. The sensitivity of each assay was calculated as the mean plus 3 standard deviations of 12 to 15 replicate measurements of wild-type amplicons (negative controls) obtained from recombinant viral constructs. Accuracy was evaluated by measuring serial dilutions of mutant amplicons in a background of wild-type amplicons. The nominal proportion of mutants ranged from 100% to 0.01% or 0.001% mutants. The proportion of mutants was measured as: 100×(DNA copy number obtained by mutant-specific amplification)/(DNA copy number obtained by non-specific amplification). Horizontal solid line: mean proportion of 12 to 15 replicate measurements of wild-type amplicons. Horizontal dashed line: Limit of detection (attributed sensitivity) of the assay. Diagonal dashed line: theoretical perfect match between nominal and observed proportions.

REPRODUCIBILITY

The coefficients of variation (CV) for quantifying samples with proportions of mutant DNA ranging from 100% to 1% of the population were measured in order to assess the reproducibility of the assay. Intra-assay variation was determined by triplicate determinations of each mixture containing a given proportion of mutant and wild-type

sequences. The CVs were below 0.40 for each proportion analyzed, being lower than 0.20 for the nominal mutant proportions of 100% and 10%. Inter-assay variation was assessed by testing three different aliquots of each prepared mixture in three independent assays performed on different days. Results of different runs revealed CVs of around 0.20 for nominal mutant proportions of 100%, and around 0.60 for nominal proportions of 1% (Table 2)

Table 2: Intra-assay and inter-assay coefficients of variation (CVs)

	CV Intra-assay				CV Inter-assay			
Nominal mutant Proportion	M184V	M184I	D30N	V38A	M184V	M184I	D30N	V38A
100 %	0.14	0.01	0.19	0.06	0.21	0.23	0.14	0.13
10 %	0.11	0.08	0.14	0.17	0.13	0.06	0.40	0.49
1 %	0.18	0.09	0.39	0.21	0.60	0.11	0.64	0.50

CLINICAL NEGATIVE CONTROLS

Stored plasma specimens from 6 different HIV-1-infected patients obtained in 1991 were tested for the presence of M184V/I and D30N mutations. Because neither lamivudine nor nelfinavir were approved for use at that time, the subjects studied should not have been exposed to these drugs. Population-based sequencing of these specimens demonstrated a wild type allele at both codons. Allele-specific PCR yielded M184V and D30N proportions below the limit of detection in all 6 specimens (data not shown). Of note, three of six specimens showed proportions of the M184I mutant that were slightly above the limit of detection (0.05% to 0.07%), suggesting that M184I mutants may have been present at very low levels in the virus populations of these subjects before any lamivudine exposure.

Plasma samples from 6 additional patients were tested as negative controls for V38A in *env.* These were six antiretroviral therapy-experienced but enfuvirtide-naïve patients with multidrug-resistant HIV-1 infection, but no evidence of enfuvirtide resistance by standard sequencing at the time of specimen sampling. In all six cases, the proportion of V38A mutations by ASPCR was below the limit of detection of the assay (data not shown).

MUTANT DYNAMICS IN CLINICAL SAMPLES

The ability of ASPCR to characterize the dynamics of mutants in vivo was investigated by measuring the proportion of M184V/I variants in serial plasma specimens from 4 subjects with multidrug resistant HIV-1 (MDR HIV) who selectively interrupted lamivudine therapy. [34] Allele-specific PCR measurements were compared to genotypic data obtained at each time point by standard techniques (Figure 5).

Allele-specific PCR measurements were concordant with the results of standard genotyping. As expected, M184V variants were predominant in patients 1, 2 and 3 before lamivudine interruption. In contrast, the proportion of M184V mutants was only slightly higher than 20% in Patient 4. Pre-interruption levels of M184V variants remained stable during 6 to 8 weeks and decayed thereafter. Resumption of lamivudine therapy was associated with a rapid rebound of M184V variants. Samples with 10%-20% mutant sequences by ASPCR were consistently reported as mixtures of 184V and 184M by standard genotyping, whereas samples with less than 10% mutant were consistently negative.

Resumption of lamivudine treatment was consistently associated with the transient detection of M184I variants at very low levels (≤ 0.1%). Such transient increases were associated with parallel increases in the proportion of M184V variants in patients 1, 3 and 4 but not in patient 2. As well, M184I increments were associated with increases in HIV-1 RNA load in patients 2 and 3, but with HIV-1 RNA decreases in patients 1 and 4.

EFFECT OF POLYMORPHISMS AT PRIMER SITES ON THE ACCURACY OF ASPCR

To investigate the influence of genetic polymorphisms at primer sites on the accuracy of ASPCR measurements, 12 clonal pol sequences were obtained per patient (HXB2 positions: 2218-3457) before lamivudine interruption. (Table 3) As expected, all clones exhibited an I→A mismatch in the –2 position relative to the 3' end of the M184V-specific primer. The M184V mutation was present in all but one of 12 clones (92%) from the baseline sample of Patient 3.

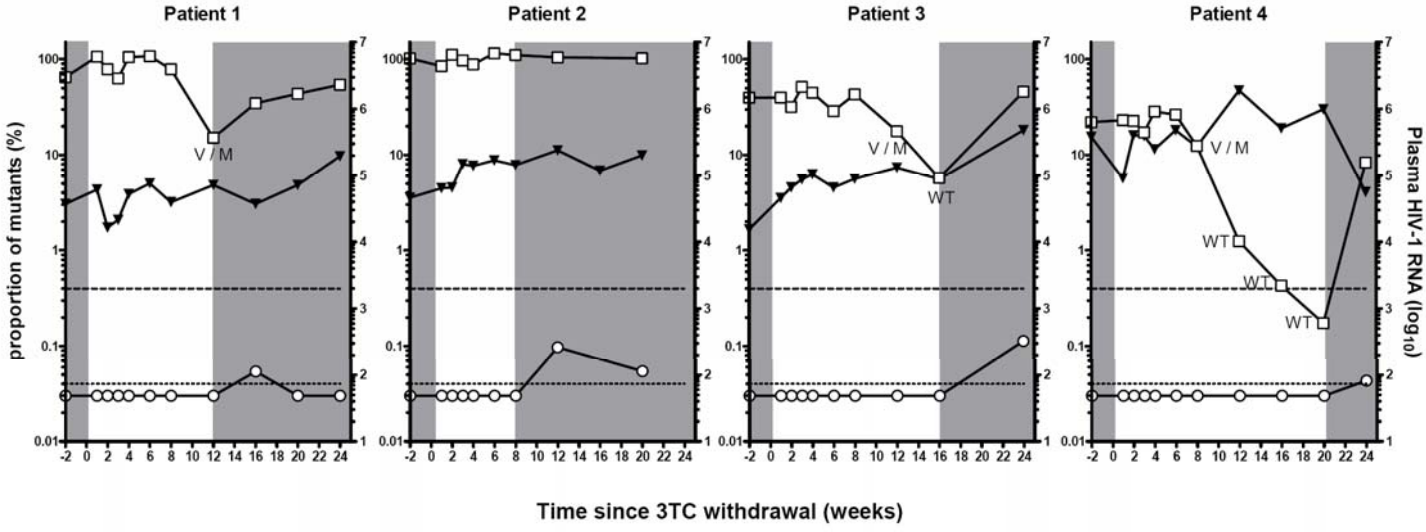

FIGURE 5: M184V AND/OR M184I DECAY AND SUBSEQUENT RE-SELECTION AMONG SUBJECTS WITH MDR HIV INFECTION UNDERGOING A TRANSIENT INTERRUPTION OF LAMIVUDINE THERAPY: ASSESSMENT BY ALLELE-SPECIFIC PCR (ASPCR) AND STANDARD GENOTYPE (SG). Four individuals infected with MDR HIV including the M184V mutation while on highly active antiretroviral therapy, transiently interrupted lamivudine therapy and continued the remaining antiretrovirals. The proportion of viral variants with the M184V or M184I mutations was investigated in serial samples before, during and after the lamivudine interruption period. Vertical shaded areas indicate periods "on" lamivudine; non-shaded areas indicate periods "off" lamivudine. Open squares: proportion of variants with the M184V mutation. Open circles: proportion of variants with the M184I mutation. Triangles: plasma HIV-1 RNA levels. Horizontal dashed line (----): Threshold of detection for M184V variants (0.4%). Horizontal dotted line (.....):Threshold of detection for M184I variants (0.04%). Standard genotypic data indicating the consensus sequence at codon 184 of the reverse transcriptase are annotated next to the corresponding M184V ASPCR results. No annotation indicates detection of M184V; "V / M" indicates the presence of M184V and WT variants; "WT" indicates detection of a wild-type sequence by standard genotype.

The existence of 1 or 2 polymorphisms near the 5' end of the discriminative primer set did not have a major impact on ASPCR measurements in Patients 1, 2 and 3. However, presence of an A→G mismatch at the –4 position relative to the 3' end of the M184V-specific primer was associated with an underestimation of the proportion of M184V variants by ASPCR in Patient 4 (Table 3, Figure 6). The incorporation of the A→ G mismatch in both specific (V4) and non-specific (NS) primers increased the accuracy of the pre-interruption values and subsequent measurements proportionally (Figure 6). Importantly, this mismatch also had to be incorporated into the standards. Otherwise, the amplification of standards lacking the mismatch using primers with the mismatch resulted in a decrease in the specific amplification of standards (not shown) and, therefore, an overestimation of the proportion of mutants.

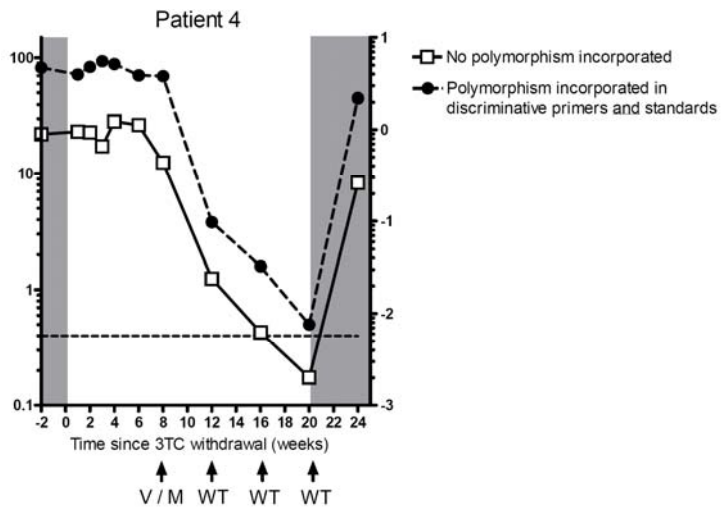

FIGURE 6: EFFECT OF PRE-EXISTING POLYMORPHISMS ON THE ACCURACY OF ASPCR MEASUREMENTS 'IN VIVO'. As shown in Table 4, 12/12 HIV-1 pol clones in patient 4 contained a pre-existing A→G polymorphism in the –4 position, relative to the 3'-end of the M184V-specific primer (V4). Vertical shaded areas indicate periods "on" lamivudine; non-shaded areas indicate periods "off" lamivudine. Open squares: proportion of variants with the M184V mutation. Horizontal dashed line (----): Threshold of detection for M184V variants (0.4%). Standard genotypic data indicating the consensus sequence at codon 184 of the reverse transcriptase are annotated next to the corresponding week. No annotation indicates detection of M184V; "V / M" indicates the presence of M184V and WT variants; "WT" indicates detection of a wild-type sequence by standard genotype.

Table 3. Clonal sequences at primer sites [a]

Sequences at discriminative forward primers site (5' – 3')		Sequences at common reverse primer site (5' – 3')
Primer V4 [b] (M184V-specific)	GACATAGTTATCTATCAAT*I*C**G***	Primer ASR2 (common reverse primer) GGCTGTACTGTCCATTTATC
Primer VN [c] (non-specific)	..................A.	
Laboratory reference strains		
HXB2	..................A.A	....
pNL4.3	........C.........A.A	....
Patient 1 clones		
10/12	......A.C.........A..	....
1/12	......A.C.........A..	.C.................
1/12	......A.C.........A..	..................T
Patient 2 clones		
2/12	......A.C.........A..	..T...............
2/12	..................A..	..T...............
2/12	..................A..	....
2/12	......A...........A..	....
1/12	......A...........A..	................G..
1/12	........C.........A..	....
1/12	.....G............A..	....
1/12	......A...........A..	..T...............
Patient 3 clones		
9/12	..GC..............A..	....
2/12	..GC..............A..	..T...............
1/12	...C..............A.A	....
Patient 4 clones		
12/12	................G.A..	....

[a] Amplicons encompassing the 2218-3457 positions relative to the HXB2 laboratory reference strain, were obtained from patient plasma specimens, PCR-purified (QIAquick® PCR Purification Kit, QIAGEN Sciences, Maryland, USA), and cloned into a pGEM® T-Easy Vector (pGEM® T-Easy Vector System, Promega Corporation, Madison, WI, USA), as directed by the manufacturer. Twelve clonal pol sequences were obtained per patient before lamivudine interruption. Here, clonal sequences at the site of the discriminative primer set (left) and at the site of the common reverse primer (right) are presented. Respectively, the M184V-specific primer (V4) sequence and the ASR2 primer sequence are used as the reference sequence for patients' clones.

[b] In the M184V-specific primer (V4), the target mutation is shown in boldface, underlined and next to an asterisk, while the intentional A→**I** mismatch in the -2 position of the 3' end is shown in boldface, italics and underlined.

[c] The non-specific (VN) primer is one base pair shorter than the M184V-specific primer (V4) and does not incorporate an intentional mismatch.

DISCUSSION

The study of minor viral variants in HIV-1 infection is relevant to understanding the mechanisms of viral persistence, escape from pharmacologic and immunologic pressure, and co-receptor usage. Detection of drug-resistant minority variants may help predict virological failure in patients with HIV-1 that appears to be wild-type by standard sequencing methods. Among the several techniques available to study minor viral variants, [24-27, 31, 32] ASPCR is one of the most sensitive, accurate and reproducible, being less expensive and time-consuming than single-genome sequencing or clonal sequence analysis. [22] Due to its high throughput, this technique can also be used as a way to rapidly screen very large pooled populations.

This article presents a systematic evaluation of ASPCR assays targeted to four important drug resistance mutations in RT (M184V and I), PR (D30N), and env (V38A in gp41). Each assay could detect the presence of mutant species with a limit of detection of <1%, but assays for certain mutant codons were more sensitive than others. Sensitivity was directly proportional to the discrimination ability of the primers, which was, in turn, strongly influenced by the particular base sequence of the codon being interrogated. For example, the M184I-specific primer was better able to discriminate M184I (ATA) from WT (ATG) and M184V (GTG) than was the M184V-specific primer.

A number of factors, however, were important to enable adequate performance of the assay, both in laboratory constructs and in clinical specimens. To ensure equal amplification efficiencies, both the specific and non-specific primers should anneal to the same DNA region. ASPCR assays using Sp and NS primers that annealed to different sites yielded inaccurate results, often overestimating the proportion of mutants (not shown). In this study, the NS primer was identical to the Sp except that it ended one base pair before the target locus and did not incorporate intentional mismatches. The antiparallel primer was common to both primer pairs. To maximize the discrimination ability of the assay, it was also important to use relatively short primers. Because the cycling conditions were the same for all four ASPCR sets,

several assays could be run at the same time, increasing overall efficiency.

Based on a modification of the amplification refractory mutation system (ARMS) [35], the Sp primer in this assay contained the target nucleotide substitution at its 3' end plus an intentional nucleotide mismatch at positions –1 to –3 from the 3' end to increase its specificity for the target mutation. [26, 35] When several different nucleotide substitutions within a codon can give rise to the same mutation, it may be necessary to use degenerate Sp primers (e.g. K103N in RT). [36] Annealing temperature (Ta) adjustments including primer dissociation curves were also important to attain optimal discrimination and lack of non-specific amplification. In general, the Ta should be as high as possible for each given primer set without compromising the amplification efficacy of the real-time PCR reactions. The optimal Ta could be determined empirically by repeating the experiments shown in Figure 3, panels A-D, in different thermal conditions.

The ASPCR assays described had good reproducibility. Because inter-assay CVs were higher than intra-assay CVs, serial specimens from an individual patient should be run in a single batch. As expected, CVs were greatest for samples with a low nominal proportion of mutant species. However, the magnitude of variation was small relative to the proportion of mutant species detected. For example, the intra-assay CV of the M184V ASPCR when the mutant species constituted 1% of the population was 0.18, which corresponded to a 99 % confidence interval of [0.5% - 2%]. [37].

In reconstruction experiments, the four ASPCR assays were highly accurate in quantifying the proportion of mutant species over the range 100%-1%, but overestimated the proportion of mutant species at proportions below 1%. The total input DNA copy number did not affect the accuracy of measurements provided that the C_T values fell within the linear range of amplification for the respective primer pairs (10^{6-7}-10^{1}). However, a minimum input of 10^{4} DNA copies in the real-time PCR step was required to ensure a sensitivity of at least 0.1% (10^{1} mutants / 10^{4} total variants = 0.1%).

Table 4. Minimum HIV-1 RNA load needed to detect minor variants at a frequency (λ) of 0.1%, 1%, and 10%, assuming that the RNA is extracted from 1 mL of plasma.[a]

Variant Frequency (λ)	Number of RNA molecules to be tested, according to the Poisson distribution (P>99%)	Fraction of elution volume used for cDNA synthesis (f_e)	Minimum HIV-1 RNA load (copies/mL)
0.1%	5000	0.25	29 762
		0.5	14 881
		1	7 440
1%	500	0.25	2 976
		0.5	1 488
		1	744
10%	50	0.25	298
		0.5	148
		1	74

[a]Calculations were derived from the formula: $pVL = N_{RNA(\lambda)} / (V \times f_e \times E_{RNAX} \times E_{cDNA})$, where pVL is the plasma HIV-1 RNA copy number; $N_{RNA(\lambda)}$ is the number of RNA copies that need to be tested according to the Poisson distribution to detect at least 1 variant with a probability > 99% if this variant is present at a frequency λ; V is the volume of plasma used for the RNA extraction in milliliters; f_e is the fraction of the RNA elution volume used for cDNA synthesis; E_{RNAX} is the efficiency of the RNA extraction process ; and E_{cDNA} is the efficiency of the cDNA synthesis. HIV-1 RNA values were estimated using the following assumptions: V=1 mL, E_{RNAX}=0.96 and E_{cDNA}= 0.7, based on http://www1.qiagen.com/literature/qiagennews/0398/983hiv1.pdf and http://omrf.ouhsc.edu/~frank/CDNA.html. Importantly, E_{RNAX} and particularly E_{cDNA} are subject to significant variation in different conditions, and may need to be determined empirically. Therefore, HIV-1 RNA values would need to be adjusted if different plasma volumes were used for the RNA extraction or if E_{RNAX} or E_{cDNA} were different. Note that the volume of plasma required for RNA extraction in order to detect a variant with frequency λ can be easily calculated as: $V = N_{RNA(\lambda)} / (pVL \times f_e \times E_{RNAX} \times E_{cDNA})$.

As with any method for assessing minor HIV-1 variants, true assay sensitivity depends on the number of RNA molecules in the original sample. According to the Poisson distribution, to detect at least 1 mutant variant present in 0.1% of the total population with a 99% probability, at least 5000 variants need to be tested. Therefore, the template RNA for the RT-PCR step had to include at least 5000 RNA molecules to permit a sensitivity of 0.1%, regardless any other characteristic of the ASPCR assay. The RNA copy in the assay depends

on plasma HIV-1 RNA concentration, the volume of plasma used and the efficiency of the RNA extraction process (Table 4). The efficiency of the reverse transcriptase step also must be taken into account. Large volumes of plasma are required for the RNA extraction in specimens with low viral loads in order to preserve the ability of any assay to detect minor variants (Table 4).

Another limitation of ASPCR is that polymorphisms that occur in the primer or probe binding sites can significantly impair the accuracy of ASPCR measurements. In accordance with prior data, [38] whereas polymorphisms near the 5' end of the discriminative primer set had little effect on the accuracy of proportions, those occurring near the 3' end could result in the underestimation of the proportion of mutant variants. The incorporation of polymorphisms detected by prior population-based sequencing into the discriminatory primer set has been suggested to overcome this problem. [38] Moreover, relevant polymorphisms also had to be incorporated into the standards (not shown). Otherwise, the presence of an additional mismatch between the discriminative primer set (with the polymorphism) and the standard sequence (without the polymorphism) preferentially constrained the amplification of the mutant-specific standard curve, relative to the non-specific standard curve. In some situations, substituting SYBR™green or molecular beacons for TaqMan™ probes may also help to overcome problems caused by sequence polymorphisms.

In summary, ASPCR is a valuable technique for studying point mutations in viral genomes. The high sensitivity, accuracy and reproducibility of this technique make it a particularly useful tool for resistance surveillance. Other possible applications include studying the kinetics of selection and decay of point resistance mutations, *in vivo* turnover of cell populations and viral fitness estimations [39, 40]. The approach described herein easily be adapted to study other mutations in the HIV-1 genome or in other viruses, in plasma, peripheral blood mononuclear cells [36], and other tissues. Allele-specific PCR will remain highly relevant in elucidating the importance of minor variants to the clinical outcomes of patients infected with drug-resistant HIV.

ACKNOWLEDGEMENTS

This work was supported by the following sources: Harvard University Center for AIDS Research (P30 AI60354), a Virology Specialty Laboratory subcontract from the AIDS Clinical Trials Group (U01 AI 38858 and AI68636), and K24 RR16482 (to D.R.K.). R.P. is a recipient of the "La Caixa" Fellowship Grant for Post-Graduate Studies, Caixa d'Estalvis i Pensions de Barcelona, Catalonia, Spain.

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Chapter 2

In Vivo Fitness Cost of the M184V Mutation in Multidrug-Resistant HIV-1 in the Absence of Lamivudine

Roger Paredes,[1,2] Manish Sagar,[1] Vincent C. Marconi,[1,3] Rebecca Hoh,[4] Jeffrey N. Martin,[4] Neil T. Parkin,[5] Christos J. Petropoulos, [5] Steven G. Deeks,[4] and Daniel R. Kuritzkes[1]

[1]Section of Retroviral Therapeutics, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA. [2]Fundacions irsiCaixa i Lluita contra la SIDA, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, Catalonia, Spain. [3]Infectious Disease Service, Brooke Army Medical Center, San Antonio Military Medical Center, San Antonio, TX, USA. [4]University of California San Francisco and San Francisco General Hospital, San Francisco, CA, USA. [5]Monogram Biosciences, Inc., South San Francisco, CA, USA.

J Virol 2009; 83(4) (Epub ahead of print)

ABSTRACT

Lamivudine therapy selects for the M184V mutation. Although this mutation reduces the replicative capacity of human immunodeficiency virus in vitro, its impact on viral fitness in vivo has not been well defined. We used quantitative allele-specific PCR to precisely calculate the fitness differences between the mutated M184V virus and one that had reverted to the wild type in a cohort of patients by selectively interrupting reverse transcriptase inhibitor therapy, and we found that the M184V variants were consistently 4 to 8% less fit than the wild type in the absence of drug. After a lag phase of variable duration, wild-type variants emerged due to continued evolution of pol and back mutation rather than through emergence of an archived wild-type variant.

Despite recent advances, the management of multidrug-resistant (MDR) HIV-1 remains a major clinical problem. One strategy to avoid immune deterioration while minimizing toxicity in viremic patients infected with MDR HIV-1 is to prevent the emergence of wild type (WT) HIV-1 by continuing selected drugs in a failing antiretroviral regimen. Studies show that reverse transcriptase inhibitors (RTI) continue to exert anti-viral activity in the presence of resistance mutations.(8, 21) In particular, continuation of lamivudine (3TC) or emtricitabine (FTC) in the presence of the M184V mutation may provide clinical benefit. (3, 4) We previously showed that M184V is lost a median of 20 weeks following interruption of 3TC together with other RTIs while continuing protease inhibitors (PIs) in viremic subjects with MDR HIV-1.(8) In this study, we performed a detailed analysis of the decay of the M184V-carrying mutants in these individuals.

Subjects were antiretroviral treatment-experienced HIV-1-infected patients enrolled in an ongoing prospective cohort study. (8) This particular substudy focused on 5 adherent, highly treatment-experienced, viremic subjects with HIV-1 resistant to antiretroviral drugs from at least two classes who interrupted 3TC together with other RTIs but remained on PIs. A sixth participant (subject 3158) enrolled in the parent study while receiving 3TC, stavudine (d4T), and nelfinavir (NFV) and selectively interrupted NFV. At week 52, this patient discontinued 3TC and d4T therapy and subsequently remained off all antiretroviral therapy. Subjects were followed weekly for the first 4 weeks, every 2 weeks for the next 8 weeks and every 4 weeks thereafter for at least 48 weeks or until treatment was modified. Participants provided written, informed consent for participation in these studies, which were approved by the University of California, San Francisco Committee on Human Research and the Partners HealthCare Systems Institutional Review Board.

At the time of PTI, the median plasma HIV-1 RNA level was 3.65 log copies/mL, and the median CD4+ count was 336 cells/mm^3. Further details of this cohort have been reported. (8)

Table 1. Mutations in HIV-1 reverse transcriptase at codons associated with drug resistance in subjects interrupting reverse transcriptase inhibitors, as assessed by population sequencing of plasma viruses.

Subject or isolate	Treatment interrupted	Treatment continued	Week post PTI [a]	Codon [b]												
				41	62	67	69	70	103	118	181	184	190	210	215	219
Clade B consensus				M	A	D	T	K	K	V	Y	M	G	L	T	K
3005	ZDV/3TC	IDV/RTV	0	L	.	N	D	.	.	.	.	V	.	W	Y	.
			34	M/L	.	N	D	.	.	V/I	.	M/V	.	L/W	Y	.
			36	M	.	N	D	.	.	V/I	.	M/V	.	L/W	Y	.
			48	.	.	N	D	.	.	V/I	.	.	.	L/W	D/N/Y	.
3040	D4T/3TC	LPV/RTV	0	.	.	N	T/A	R	.	.	Y/C	V	.	.	T/S	Q
			12	.	.	N	T	R	.	.	.	V	.	.	.	Q
			16	.	.	N	.	R	.	.	.	V	.	.	.	Q
			22	.	.	N	.	R	.	.	.	M/V	.	.	.	Q
			24	.	.	N	.	R	.	.	.	M/V	.	.	.	Q
			36	.	.	N	.	R	.	.	.	.	.	.	.	Q
3057	D4T/3TC	IDV	0	L	V	.	T/A	K/R	.	.	.	V	.	.	.	K/Q
			7	L	V	.	.	.	.	.	.	V	.	.	.	.
			36	L	V	.	.	.	.	.	.	.	.	.	.	.
			48	L	V	.	.	.	.	.	.	.	.	.	.	.
3151	DDI/D4T/3TC/EFV	APV/RTV	0	L	.	N	D	R	.	I	C	V	S	W	F	Q
			16	L	.	N	D	R	.	I	C	V	S	L/W	F	Q
3167	ZDV/3TC	NFV	0	.	.	.	.	.	.	.	.	V	.	.	.	.
			4	.	.	.	.	.	.	.	.	V	.	.	.	.
			8	.	.	.	.	.	.	.	.	V	.	.	.	.
			12	.	.	.	.	.	.	.	.	M/V	.	.	.	.
3158	D4T/3TC	None	0	.	.	N	.	R	.	.	.	V	.	.	.	Q
			5	.	.	N	.	R	.	.	.	V	.	.	.	Q
			12	.	.	N	.	R	.	.	.	M/V	.	.	.	Q
			16	.	.	N	.	R	.	.	.	M/V	.	.	.	Q

[a]PTI: partial treatment interruption. [b]Amino acid residues are indicated by single-letter abbreviations. HIV-1 clade B consensus sequence is shown for comparison. Dots indicate no change from consensus sequence. Antiretroviral use: zidovudine (ZDV), stavudine (d4T), lamivudine (3TC), didanosine (ddI), efavirenz (EFV), indinavir (IDV), ritonavir (RTV), nelfinavir (NFV), saquinavir (SQV), amprenavir (APV).

Population sequencing of plasma viruses and phenotypic antiretroviral susceptibility tests (GeneSeq® and Phenosense HIV®; Monogram Biosciences™, South San Francisco, CA) obtained before the RTI interruption and at multiple timepoints thereafter showed a slow decay of NRTI resistance. Mutations decayed gradually at a rate roughly proportional to their associated fitness cost, as estimated *in vitro* in the absence of drug.(6) (Table 1). The thymidine analogue mutations (TAMs) waned more slowly than M184V. Changes in phenotypic susceptibility to 3TC and other NRTI paralleled the genotypic changes (not shown). As expected, all subjects maintained viruses with high-level PI resistance throughout the study period (not shown).

An allele-specific PCR (ASPCR) assay that detects M184V variants present in at least 0.4% of the quasispecies population (18) was used to quantify more precisely the proportion of viruses carrying the M184V mutation over time, and to estimate the fitness of M184V-containing viruses *in vivo*. By ASPCR, M184V mutants were present as the predominant viral species at baseline in all subjects. (Figure 1) In subjects 3167 and 3151, there was no significant change in the proportion of M184V-containing virus through 12 and 16 weeks of follow-up, respectively (not shown), consistent with the population sequencing results. In subjects 3005, 3040, 3057 and 3158, M184V variants exhibited a biphasic decay: after an initial lag phase of variable duration, M184V variants decreased exponentially (Figure 1). The lag phase lasted 20 weeks in subject 3057, 24 weeks in subject 3040 and 32 weeks in subject 3005, but was much shorter (12 weeks) in subject 3158, who was receiving only d4T and 3TC and therefore interrupted all antiretroviral drugs in his regimen. Despite the variable lag time, once decay began the duration and slopes of the exponential decay phase were similar in all subjects, lasting approximately 12 weeks, although minor M184V variants remained detectable for up to 48 weeks after 3TC interruption in subject 3005.

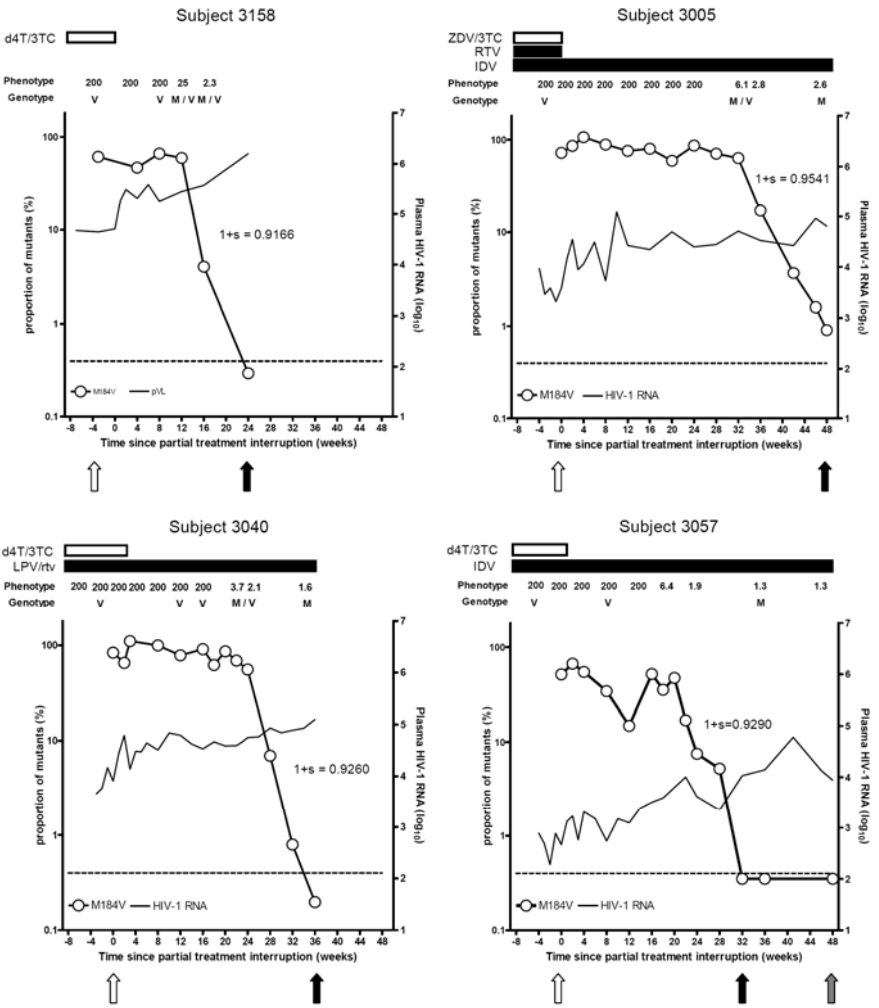

FIGURE 1. DYNAMICS OF M184V VARIANTS AFTER THE INTERRUPTION OF REVERSE TRANSCRIPTASE INHIBITORS. Only subjects achieving complete M184V mutant decay are shown. Open circles, proportion of M184V mutants by allele-specific PCR testing (in logarithmic scale). For the ASPCR assay, viral RNA was extracted from 500 μL of plasma. Continuous line, HIV-1 RNA levels (in log10 copies/mL). Horizontal dashed line, sensitivity threshold to detect M184V variants (0.4%). Horizontal bars in the superior part of each graph, duration of antiretroviral treatment. Phenotype refers to fold-change in lamivudine susceptibility. Genotype refers to the codon 184 allele detected by population-based sequencing: methionine (M), valine (V) or a mixture of variants with methionine and variants with valine in codon 184 (M/V). Arrows show the timepoints when T0 (white), T1 (black) and T2 (grey) clonal sequences were obtained. The fitness (1+S) values of M184V viruses relative to 184M variants, are shown next to the exponential M184V virus decay phase.

Relative fitness was calculated in the four subjects with M184V reversion using the average method, which incorporated multiple ASPCR measurements during the exponential M184V mutant decay phase, the HIV-1 RNA level at each timepoint and the death rate of infected cells (1, 15) (we used a death rate [δ]= 0.5, as this value approximates the mean of many independent estimates (2)). The relative fitness disadvantage of M184V ranged from 4.6% to 8.3% in these multidrug-resistant viruses (Figure 1).

To further characterize the viral variants emerging after NRTI discontinuation, clonal HIV-1 *pol* sequences were obtained from subjects 3040, 3057, 3005 and 3158 at the time of RTI interruption (T0), and at the first time point at which M184V became undetectable by ASPCR (T1). An additional time point (T2) was available for subject 3057, 16 weeks after T1. A mean of 24 clones [range 21-29] (Table 2) were analyzed at each time point. All sequences obtained at T0 carried the M184V mutation, whereas all sequences obtained at T1 and T2 were WT at this codon (Table 2). In addition to the changes in the frequency of TAMs observed by population sequencing, clonal analysis revealed a decrease in the frequency of K70R variants in subjects 3040 and 3158, and evidence of back mutation at codon 215 from the mutant TAC (Tyr) to the partial revertants GAC (Asp) and AAC (Asn) in subject 3005.

Phylogenetic trees reconstructed in PAUP v4.0b (HKY model) and MEGA v3.1 (Tamura-Nei model), (13) using both maximum likelihood and neighbor-joining (NJ) approaches plus 1,000 NJ bootstrapping replicates showed that in subjects 3057, 3005 and 3158, sequences from T0 and T1 were intermingled and shared a most-recent common ancestor (MRCA) (Figure 2), as expected from back-mutated sequences. Interestingly, in these 3 subjects there was an increase in sequence diversity in both the protease and RT genes, suggesting ongoing exploration of sequence space by the virus population (Table 2). Conversely, viral sequences from subject 3040 at T0 and T1 clustered separately with different MRCAs. In addition, in this subject sequence diversity increased in the protease gene but decreased in the RT gene (Table 2). The T1 sequences were more closely related to the

M184V variants at T0 (genetic distance=0.019) than to a pre-3TC consensus sequence (genetic distance=0.034) obtained in 1995, providing further support for the inference that the 184M variant arose from back-mutation as opposed to re-emergence of archived viruses. Analyses of the rate of non-synonymous and synonymous substitutions for samples from all patients and timepoints (MEGA software v3.1 (13)) indicated that purifying selection (i.e., reduction of alleles with a deleterious effect on the phenotype) was the main mechanism for M184V reversion in all subjects (Table 2).

This study showed that the M184V mutation disappeared quickly after a variable lag phase that lasted as long as 32 weeks. Differences between individual subjects in time to M184V disappearance appeared related to the length of the initial lag phase rather than differences in the rate of exponential decay. The estimates of the relative fitness of MDR HIV-1 carrying the M184V mutation were in close agreement with previous data, indicating a fitness cost of the M184V mutation of approximately 10% relative to WT. (14)

In contrast to observations when all drugs are discontinued, (9) we did not observe evidence for the escape of a pre-existing MDR-184M variant, nor we did find evidence of emergence of viruses with a WT RT and a mutant PR through recombination of actively replicating and archived variants. (16) Several findings suggested that 184M viruses emerged through continuous evolution of *pol* and back-mutation. (12) First, NRTI mutations decayed in serial clonal and population-based sequences in an ordered, stepwise fashion at a rate roughly proportional to their associated fitness cost, as estimated *in vitro* in the absence of drug.(6) Second, there was clonal evidence for back mutation through partial revertants at codon 215 [GAC (Asp) and AAC (Asn) derived from the mutant TAC (Tyr) in subject 3005]. Third, the MDR-M184V and MDR-184M variants shared a MRCA in three out of four subjects and, even in the fourth subject (3040), emerging MDR-184M variants were more closely related to on-treatment MDR-M184V mutants than to a pre-lamivudine WT sequence.

Table 2. Clonal analysis.[a]

Subject or isolate[b]	Week no.	Total no. of clones	No. of clones	Codon									Sequence Diversity (d ± SE)[c]			Ka/Ks ratio[d]		
				41	62	67	69	70	184	210	215	219	PR+RT	PR	RT	PR+RT	PR	RT
Clade B consensus				M	A	D	T	K	M	L	T	K						
3005	0	24	24	L	.	N	D	.	V	W	Y	.	0.0052 ± 0.0014	0.0010 ± 0.0007	0.0067 ± 0.0017	0.20	0.00	0.22
	48	29	16	.	.	N	D	.	.	.	Y	.	0.0111 ± 0.0016*	0.0045 ± 0.0011*	0.0135 ± 0.0021*	0.19	0.22	0.18
			4	.	.	N	D	.	.	.	N	.						
			4	.	.	N	D	.	.	W	N	.						
			3	.	.	N	D	.	.	W	D	.						
			1	L	.	N	D	.	.	R	D	.						
			1	.	.	N	D	.	.	.	D	.						
Overall													0.0112 ± 0.0017	0.0047 ± 0.0017	0.0118 ±0.0028	0.29	0.44	0.25
3040	0	22	22	.	.	N	.	R	V	.	.	Q	0.0065 ± 0.0016	0.0073 ± 0.0032	0.0063 ± 0.0016	0.08	0.01	0.11
	36	27	15	.	.	N	.	R	.	.	.	Q	0.0056 ± 0.0016	0.0078 ± 0.0034	0.0048 ± 0.0015	0.40	0.78	0.21
			12	.	.	N	.	.	.	.	.	Q						
Overall													0.0149 ± 0.0024	0.0172 ± 0.0054	0.0128 ±0.0028	0.26	0.27	0.16
3057	0	22	22	L	V	.	.	.	V	.	.	.	0.0061 ± 0.0014	0.0061 ± 0.0027	0.0061 ± 0.0015	0.50	NA†	0.40
	32	21	20	L	V	.	.	.	.	.	.	.	0.0119 ± 0.0021*	0.0100 ± 0.0039	0.0126 ± 0.0024*	0.20	0.17	0.19
			1	L	V	G	.	.	.	.	.	.						
	48	21	21	L	V	.	.	.	.	.	.	.	0.0104 ± 0.0019	0.0095 ± 0.0038	0.0107 ± 0.0020	0.27	0.46	0.27
Overall													0.0123 ± 0.0013	0.0103 ± 0.0029	0.0129 ±0.0023	0.32	0.44	0.28
3158	0	22	22	.	.	N	.	R	V	.	.	Q	0.0061 ± 0.0010	0.0082 ± 0.0023	0.0053 ± 0.0011	0.14	0.28	0.10
	24	24	15	.	.	N	.	R	.	.	.	Q	0.0089 ± 0.0010	0.0127 ± 0.0031	0.0076 ± 0.0015	0.16	0.37	0.11
			8	.	.	N	.	.	.	.	.	Q						
			1	.	.	N	.	S	.	.	.	Q						
Overall		46											0.0087± 0.0013	0.0113 ± 0.0029	0.0073 ±0.0013	0.24	0.36	0.15

[a] For each plasma sample, the products of five separate RT-PCRs were pooled and purified. Five separate nested PCR reactions were then performed with each pooled RT-PCR product using primers OOPF2 and OOR3 (18) and cloned within the TOPO-TA 2.1 vector (Invitrogen Corporation, Carlsbad, CA, USA). [b] Subject 3005 interrupted treatment with zidovudine/lamivudine and continued indinavir/ritonavir; subject 3040 interrupted treatment with stavudine/lamivudine and continued lopinavir/ritonavir; subject 3057 interrupted treatment with stavudine/lamivudine and continued indinavir; Subject 3158 interrupted treatment with stavudine/lamivudine and did not continue any other treatment. Amino acid residues are indicated by single-letter abbreviations. HIV-1 clade B consensus sequence is shown for comparison. Dots indicate no change from consensus B sequence. [c] Clonal sequence diversity and the non-synonymous/synonymous nucleotide substitution (Ka/Ks) ratio are shown per each subject in whom M184V viruses reverted back to 184M. Data are presented by week of clonal sequence sampling, as well as considering all patient clones together ("overall").Distances were estimated with MEGA v3.1, using a Tamura-Nei model for nucleotide substitution, assuming a gamma distribution with an alpha=0.5. Standard Errors (SE) were estimated using 1000 bootstrap replicates. * $p<0.05$, 2-tailed P value (one sample t-test), compared with baseline. [d] The Ka/Ks ratio was also estimated with MEGA v3.1, using the Nei-Gojobori method and a Jukes-Cantor model for nucleotide substitution. †NA: calculation not available because dS=0.

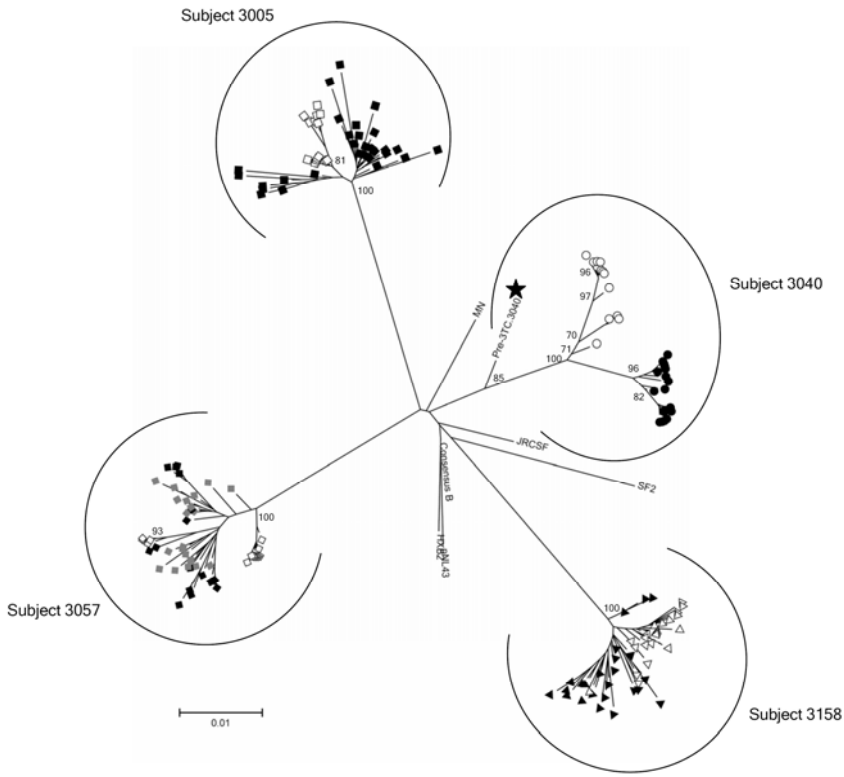

FIGURE 2. CONTINUED EVOLUTION OF POL (AND BACK-MUTATION) AS A MAJOR MECHANISM OF MDR-184M VARIANT EMERGENCE AFTER REVERSE TRANSCRIPTASE INHIBITOR INTERRUPTION. This is an unrooted neighbor-joining phylogenetic tree generated with MEGA 3.1. Data was derived from a multiple sequence alignment including non-identical clonal pol sequences from all subjects plus the laboratory and patient-derived HIV-1 reference sequences. We assumed a Tamura-Nei (TN93) model of nucleotide evolution including transitions and transversions and a gamma-distributed variability rate among sites with an alpha value=0.8. The node reliability was assessed using 1000 bootstrap replications. Percentual bootstrap values >70% are presented. Additional analyses using different models of nucleotide evolution, maximum-likelihood tree reconstruction approaches, separate alignments per each subject or separate analyses of the protease and the revere transcriptase-coding regions of pol, yielded identical results. In subjects 3005, 3057 and 3158, MDR-184M variants emerging after treatment interruption [black (T1) and grey (T2) symbols] and baseline MDR-M184V viruses (T0, white symbols) did not have different most recent common ancestors (MRCAs). In subject 3040, 184M viruses emerging after treatment interruption (black circles) derived from a significantly different MRCA than baseline M184V variants. However, a wild type consensus sequence obtained before the initiation of lamivudine (black star) was more closely related to the baseline M184V MRCA (genetic distance ± standard error, d=0.0162 ± 0.0038) than to the 184M MRCA (d= 0.0336 ± 0.0057).

The opposing selective pressures exerted on different coding regions of *pol* by interrupting RTI therapy and continuing PI treatment likely favored the loss of RT mutations that conferred a fitness cost in the absence of RTI therapy, while favoring the persistence of PI resistance mutations. Given the high mutation rate of HIV-1 (5, 11), however, 184M revertants should have been generated frequently in the setting of ongoing virus replication. Thus, it appears that the probability of the 184V revertants becoming fixed in the quasispecies was low. This finding could be explained by: a) the existence of lower than expected levels of viral replication (i.e., limited effective population size despite relatively high viral loads); b) mutations or recombination occurring outside the PR and RT modifying the overall fitness of the 184M revertants (7) ; and c) the continued competition of MDR-M184V viruses (not actively inhibited by treatment) with the MDR-184M variants after treatment interruption. (17) In addition, the MDR-184M population presentat the time of RTI interruption might have been quite small in these subjects who had initiated 3TC prior to or together with PI therapy, since all PI-resistant mutants would be linked to M184V and few if any MDR variants with a WT 184 codon would exist in the quasispecies.

Other factors such as defective CTL responses in subjects with advanced HIV disease (10, 19, 20) and the presence of other RTI resistance mutations could also have modulated the fitness cost of the 184V mutation and influenced the rate of reversion (6).

In conclusion, withdrawal of RTI therapy and continuation of PI treatment was associated with slow decay of the M184V mutation in MDR HIV-1-infected subjects. Time to back-mutation appeared to be the rate-limiting step in replacement of 184V by 184M. The challenge for the virus of generating variants with a wild-type RT while maintaining PI resistance likely contributed to the observed delay. Wild type RT variants eventually emerged due to continued evolution of *pol* and back mutation in the context of negative selection.

ACKNOWLEDGEMENTS

This work was supported in part by the following U.S. Public Health Service grants from the National Institutes of Health: R01 AI42567, R01 AI052745, R37 AI055357, K24 RR16482, a Virology Support Laboratory contract from the Adult ACTG (U01 AI068636), the Harvard Medical School Center for AIDS Research Virology Core (P30 AI060354) to D.R.K; and the UCSF/Gladstone CFAR (P30 MH59037), and the UCSF Clinical and Translational Science Institute (UL1 RR024131-01) to S.G.D; Roger Paredes received the "La Caixa" Fellowship Grant for Post-Graduate Studies, Caixa d'Estalvis i Pensions de Barcelona, "La Caixa", Barcelona, Catalonia, Spain.

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Chapter 3

High Prevalence of Primary Lamivudine and Nelfinavir Resistance in HIV-1 Infected Pregnant Women in the US, 1998-2004

Roger Paredes,[1,2] Irene Cheng,[3]
Daniel R. Kuritzkes,[1] Ruth E. Tuomala,[4]
& the Women and Infants Transmission Study Group

[1]Section of Retroviral Therapeutics, Brigham and Women's Hospital and Division of AIDS, Harvard Medical School, Boston, MA, USA. [2]Fundacions irsiCaixa i Lluita contra la SIDA, Universitat Autònoma de Barcelona, Catalonia, Spain. [3]Clinical Trials & Surveys Corp., Baltimore, MD, USA. [4]Division of Maternal-Fetal Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA.

AIDS 2007; 21(15):2103-6.

ABSTRACT

Using a highly sensitive allele-specific PCR assay we detected the M184V mutation for lamivudine (3TC) resistance in plasma from 9.4% of HIV-1-infected pregnant women enrolled in the Women and Infant Transmission Study between 1998 and 2004. The prevalence of nelfinavir resistance (D30N) was 6.3%. These results suggest a high prevalence of primary lamivudine and nelfinavir resistance among HIV-1-infected pregnant women in the US, and support routine genotypic resistance testing before initiating mother-to-child-transmission prophylaxis.

Antiretroviral (ARV) drug resistance can reduce the efficacy of mother-to-child transmission (MTCT) prophylaxis programs and limit future ARV treatment options for mother and child. Primary ARV resistance may limit the suppression of viral replication during pregnancy-limited antiretroviral therapy [1] and facilitate further resistance evolution. [2] Resistant variants can be transmitted from mother to child, before, during or after delivery. [3]

Zidovudine, lamivudine and nelfinavir are the most frequently prescribed antiretrovirals for MTCT prophylaxis in resource-rich settings. Phenotypic resistance to zidovudine requires the gradual accumulation of resistance mutations. [4] In contrast, single mutations in the reverse transcriptase (M184I/V) and protease (D30N) coding regions of *pol,* respectively, confer high-level resistance to lamivudine and nelfinavir. [5, 6] Routine genotypic resistance tests, based on viral population sequencing, may not detect resistant variants present in less than 20% of the viral quasispecies. Allele-specific PCR (ASPCR) could increase the accuracy and sensitivity of primary resistance surveillance in HIV-1-infected pregnant women.

We assessed the prevalence of primary resistance to lamivudine and nelfinavir in HIV-1-infected pregnant women enrolled in the Women and Infants Transmission Study (WITS) using population-based sequencing (PBS) of plasma virus as well as ASPCR to detect the D30N and M184V mutations. In April 2005, 1323 women who enrolled in WITS between June 1, 1998 and December 31, 2004, were evaluated for eligibility for this study. Study participants were HIV-1-infected pregnant women who initiated zidovudine and lamivudine therapy or zidovudine, lamivudine, and nelfinavir/nevirapine therapy during pregnancy, had never received antiretroviral therapy or had been treated for less than 15 days upon plasma specimen collection, and had detectable HIV RNA viral load ≥ 500 copies/ml. Of the 1323 enrolled women, 654 were ART naive prior to pregnancy. One hundred and thirty-four received one of the targeted regimens during pregnancy and had a study visit within ≤ 14 days of starting medications. Of these women, 94 had a viral load of ≥ 500 copies at that visit and 89 had adequate repository sample volume to participate in this study. Blinded

plasma specimens were collected between June 1998 and March 2004 and were analyzed in 2006 in a single laboratory.

Viral RNA was extracted from plasma (QIAamp Viral RNA kit, QIAGEN Inc., Valencia, CA, USA), reverse-transcribed and PCR-amplified during 30 cycles (Superscript III OneStep RT/PCR, Invitrogen Corp., Carlsbad, CA, USA) using primers OOPF ($_{HXB2}$:2211-2232) [5'-GAAGCAGGAGCCGATAGACAAG-3'] and OOR2 ($_{HXB2}$:3466-3444) [5'-TTTTCTGCCAGTTCTAGCTCTGC-3']. The resulting PCR product was used as the starting template for a 30-cycle nested PCR amplification (High Fidelity Platinum Taq, Invitrogen Corp., Carlsbad, CA, USA) using primers OOPF2 ($_{HXB2}$:2218-2241) [5'-GAGCCGATAGACAAGGAACTGTAT-3'] and OOR3 ($_{HXB2}$:3457-3432) [5'-AGTTCTAGCTCTGCTTCTTCAGTTAG-3']. The nested PCR product was purified and sequenced (3730XL DNA Analyzer, Applied Biosystems, Foster City, CA, USA). Resistance mutations and polymorphisms were defined according to the International AIDS-Society-USA Panel (Fall 2006 Update). [7] Standard phylogenetic analyses ruled out sequence contamination.

Mutations D30N and M184V were detected by ASPCR using $\approx 10^6$ copies of PCR product as the starting template. In brief, a separate quantification of "mutant" versus "all" viral variants was performed by real-time PCR. Mutant-specific primers ([$_{HXB2}$:2319-2340] 5'-CTATTAGATACAGGAGCAAATA-3' for D30N-ASPCR and [$_{HXB2}$:3078-3098] 5´- GACATAGTTATCTATCAATICG-3' for M184V-ASPCR) included the target codon in its 3' end and an intentional mismatch at positions –3 and –2, respectively, relative to the 3' end. Non-specific primers ([$_{HXB2}$:2319-2339] 5'-CTATTAGATACAGGAGCAGAT-3' for D30N-ASPCR and [$_{HXB2}$:3078-3098] 5-GACATAGTTATCTATCAATAC-3' for M184V-ASPCR) were similar to the former, but ended just before the target base pair and did not include intentional mismatches. The antiparallel primer ([$_{HXB2}$:2592-2571] 5'-CTGGCTTTAATTTTACTGGTAC-3' for D30N-ASPCR and [$_{HXB2}$:3277-3258] 5'-GGCTGTACTGTCCATTTATC-3' for M184V-ASPCR) was common for each pair of mutant-specific and non-specific reactions.

Real time PCR reactions were performed in an ABI7000 thermocycler during 50 cycles using SYBR®green as reporter. All reactions were performed in separate wells. Results reported the mean (± SD) proportion of duplicate measurements of the rate of mutants relative to the total quasispecies. The sensitivity threshold for detecting D30N and M184V mutations was, respectively, 0.1% and 0.4%.

Eighty-nine women were included into the study. (Analysis I, Table 1) Resistance data were available from 64 women (72%). The D30N mutation was detected in 4/64 specimens (6.3%) by ASPCR. The proportion of D30N variants in the positive specimens was in the 0.2% - 31.5% range. Population-based sequencing detected the D30N mutation in two specimens (3.1%) as a mixture with the wildtype allele. By PBS, D30N was not associated with other PI resistance mutations.

The M184V mutation was detected in 6/64 (9.4%) specimens by ASPCR. The range of proportions of M184V variants in positive specimens was 0.8%-130%. Population-based sequencing confirmed the presence of M184V mutation in 4 specimens (6.2% of all specimens), being found as a mixture with the wildtype allele in 2 of them. The M184V mutation was not associated with other RT mutations in any of these specimens.

The K103N mutation was detected by PBS in one subject (1.6%) one day after starting treatment with zidovudine / lamivudine. The frequency of natural polymorphisms (Table 1) was similar to that in publicly available drug resistance databases.

The overall prevalence of resistance mutations and polymorphisms did not differ when the analysis was restricted to plasma specimens obtained before any exposure to antiretrovirals (n=45, Analysis II, Table 1), except for exclusion of the single specimen with the K103N mutation and one specimen with the D30N mutation.

Table 1. Patient characteristics and prevalence of primary resistance mutations and polymorphisms in the protease and reverse transcriptase-coding regions of *pol*: analysis by population-based sequencing and allele-specific polymerase chain reaction.[a]

	Analysis 1 (< 15 days of ARV therapy)	Analysis 2 (ARV naive)
Baseline characteristics		
Number of women (n)	89	61
Age (average years, range)	28.3 (16.8-42.7)	28.1 (17.9-42.7)
Time from HIV Dx (median years, range)	0.13 (0-15.7)	0.16 (0-15.7)
CDC AIDS Category (n, %)	A: 69 (77.6%) B: 18 (20.2%) C: 2 (2.2%)	A: 47 (77.0%) B: 13 (21.3%) C: 1 (1.6%)
Previous intravenous drug use (n, %)	5 (5.6%)	2 (3.3%)
CD4+ T-cell count (median, $_{25\text{-}75}$IQR)	439 (312-583) cells/mm^3	446 (312-607) cells/mm^3
Plasma HIV-1 RNA (median, $_{25\text{-}75}$IQR)	6,196 (1353-17,262) copies/mL	11,851(1,604-20,097) copies/mL
Samples with informative resistance data	64 (72%)	45 (74%)
Primary mutations or polymorphisms		
Protease, Population-Based Sequencing (n, %)		
L10V	6 (9.4%)	5 (11.1%)
I13V	9 (14.1%)	6 (13.3%)
K20R/M	1 (1.6%)	1 (2.2%)
D30N	2 (3.1%)	1 (2.2%)
L33I/V	2 (3.1%)	1 (2.2%)
M36I	10 (15.6%)	7 (15.6%)
D60E	4 (6.3%)	4 (8.9%)
I62V	11 (17.2%)	7 (15.6%)
L63P	29 (45.3%)	20 (44.4%)
A71V	3 (4.7%)	3 (6.7%)
V77I	10 (15.6%)	7 (15.6%)
I93L	8 (12.5%)	5 (11.1%)
Reverse Transcriptase, Population-Based Sequencing (n, %)		
K103N	1 (1.6%)	0 (0%)
M184V	4 (6.2%)	4 (8.8%)
D30N and M184V-allele-specific PCR (n, %)		
D30N	4 (6.3%)	3 (6.7%)
M184V	6 (9.4%)	6 (13.3%)

[a]Analysis 1 included specimens drawn before 15 days of dual or trio ARV therapy (not inclusive). Analysis 2 included specimens drawn before any antiretroviral therapy. ARV, Antiretroviral; CDC, Centers for Disease Control; PCR, polymerase chain reaction.

This study suggests a high prevalence of primary lamivudine and nelfinavir resistance in HIV-1 infected pregnant women in the United States during 1998-2004. Despite the small sample size of the study, our data are consistent with prior studies indicating an increase in the prevalence of primary NRTI, NNRTI and PI resistance during the same time period. [8] The high prevalence of primary lamivudine and nelfinavir resistance likely reflects an increase in transmitted drug resistant variants among the general HIV-1-infected population during the study period. The relatively short time span between the diagnosis of HIV-1 infection and resistance testing in our study may have facilitated the detection of transmitted resistant variants. Injection drug use was infrequent in our cohort and was not significantly associated with an increased risk of resistance (not shown). Detection of the K103N mutation in one subject one day after starting zidovudine / lamivudine therapy suggests that this mutation was present before the initiation of therapy, and that further K103N mutations could have been detected using ASPCR.

Allele-specific PCR is a more sensitive method to detect individual resistance mutations than PBS, being a useful tool for surveillance of particularly relevant resistance mutations. The frequency of D30N and M184V mutations increased 2 to 3-fold and 1.5-fold, respectively, when ASPCR tests were utilized. Further research is warranted to establish the clinical significance of detecting very low levels of resistant variants. One retrospective study found that the presence of minor variants containing mutations K103N, Y181C and/or M184V in antiretroviral naïve subjects was associated with a higher likelihood of subsequent virologic failure. [9]

Our findings support routine genotypic resistance testing before initiating MTCT prophylaxis in the US. They also confirm the utility of ASPCR to detect single mutations conferring high-level resistance to key drug components of MTCT regimens, and support using triple-drug MTCT regimens to maximize the efficacy of this strategy and reduce the likeliness of resistance evolution. Further assessments of primary resistance in larger populations of HIV-1-infected pregnant women should be undertaken to confirm and extend our results.

Additional analyses to assess the development of resistance in HIV-infected pregnant women during pregnancy-limited antiretroviral treatment and virologic response to subsequent antiretroviral regimens are planned.

ACKNOWLEDGEMENTS

This work was supported in part by the following U.S. Public Health Service grants from the National Institutes of Health: R01 AI42567, K24 RR16482, a Virology Support Laboratory contract from the Adult ACTG (U01 AI-38858), and the Harvard Medical School Center for AIDS Research Virology Core (P30 AI60354). Roger Paredes is a recipient of the "La Caixa" Fellowship Grant for Post-Graduate Studies, Caixa d'Estalvis i Pensions de Barcelona, Catalonia, Spain. The Women and Infants Transmission Study Principal investigators, study coordinators, program officers and funding include: Clemente Diaz, Edna Pacheco-Acosta (University of Puerto Rico, San Juan, PR; U01 AI 034858); Ruth Tuomala, Ellen Cooper, Donna Mesthene (Boston/Worcester Site, Boston, MA; 9U01 DA 015054); Phil LaRussa, Alice Higgins (Columbia Presbyterian Hospital, New York, NY; U01 DA 015053); Sheldon Landesman, Herman Mendez, Ava Dennie (State University of New York, Brooklyn, NY; U01 HD 036117); Kenneth Rich, Delmyra Turpin (University of Illinois at Chicago, Chicago, IL; U01 AI 034841); William Shearer, Norma Cooper (Baylor College of Medicine, Houston, TX; U01 HD 041983); Joana Rosario (National Institute of Allergy and Infectious Diseases, Bethesda, MD); Kevin Ryan, (National Institute of Child Health and Human Development, Bethesda, MD); Vincent Smeriglio, Katherine Davenny (National Institute on Drug Abuse, Bethesda, MD); and Bruce Thompson (Clinical Trials & Surveys Corp., Baltimore, MD, N01 AI 085339). The scientific leadership core include: Kenneth Rich (PI), Delmyra Turpin (Study Coordinator) (1 U01 AI 050274-01). Additional support was provided by local Clinical Research Centers as follows: Baylor College of Medicine, Houston,TX; NIH GCRC RR000188; Columbia University, New York, NY; NIH GCRC RR000645.

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Chapter 4

Antiretroviral Drug Resistance during Pregnancy-Limited Antiretroviral Therapy in the US

Roger Paredes,[1,2] Irene Cheng,[3]
Daniel R. Kuritzkes,[1] Ruth E. Tuomala,[4]
& the Women and Infants Transmission Study Group

[1]Section of Retroviral Therapeutics, Brigham and Women's Hospital and Division of AIDS, Harvard Medical School, Boston, MA, USA. [2]Fundacions irsiCaixa i Lluita contra la SIDA, Universitat Autònoma de Barcelona, Catalonia, Spain. [3]Clinical Trials & Surveys Corp., Baltimore, MD, USA. [4]Division of Maternal-Fetal Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA.

AIDS 2009; (in press)

ABSTRACT

BACKGROUND: Pregnancy-limited antiretroviral therapy (PLAT) may select for antiretroviral drug resistance mutations (DRM) in mothers.

METHODS: We evaluated antiretroviral-naïve, HIV-1-infected pregnant women enrolled in the WITS cohort who received PLAT between 1998 and 2005, and had 2- or 6-month postpartum plasma samples available with HIV-1 RNA levels (VL) >500 copies/mL. Postpartum rates of DRM were assessed blindly using bulk sequencing (BSQ) and allele-specific PCR (ASPCR) of the M184V, K103N and D30N mutations. Factors associated with emergence of DRM were investigated.

RESULTS: 146 women were included in the study. All women received zidovudine + lamivudine during pregnancy; 76% also received nelfinavir and 8.2% nevirapine. Resistance data were available from 114 women. Postpartum rates of single-, dual-, and triple-class resistance were, respectively, 43.0%, 6.1% and 0% by BSQ, and 63.2%, 10.5% and 1.7% by ASPCR. In women receiving dual or triple PLAT, respectively, postpartum M184V/I rates were 65.0% (95.0% by ASPCR) and 28.7% (51.6% by ASPCR), respectively ($p<0.01$). Postpartum NNRTI resistance rates among women receiving nevirapine were 25% for K103N (37.5% by ASPCR) and 12.5% for Y188C. PI resistance rates in women receiving nelfinavir were: 1.1% for D30N (1.1% by ASPCR) and 1.1% for L90M. Dual versus triple PLAT and prolonged zidovudine exposure were independently associated with emergence of M184V. Nevirapine use and length of zidovudine plus lamivudine exposure were associated with emergence of K103N.

CONCLUSIONS: PLAT is associated with frequent selection of resistance to drugs with low-genetic barrier. Routine postpartum genotypic resistance testing may be useful to guide future treatment decisions in mothers.

INTRODUCTION

Programs to prevent mother-to-child HIV-1 transmission (MTCT) have averted millions of new HIV-1 infections worldwide. Present rates of vertical transmission in women with sustained access to triple-drug antiretroviral therapy (ART), perinatal zidovudine treatment, elective C-sections, and alternatives to breast-feeding are extremely low.[1] Even a simplified approach based on the intrapartum and neonatal administration of a single dose of nevirapine with or without zidovudine, lamivudine or tenofovir has reduced MTCT in resource-poor areas. [2, 3] In spite of these successes, emergence of antiretroviral drug resistance during MTCT prophylaxis may hinder the long-term efficacy of MTCT preventive programs as well as severely constrain future treatment options for both mother and child.[3-9]

A suitable way of investigating how often antiretroviral MTCT prophylaxis selects for drug-resistant HIV-1 in mothers is to assess the postpartum rates of resistant viruses in antiretroviral-naïve pregnant women who receive pregnancy-limited antiretroviral therapy (PLAT). Selection of resistant viruses during PLAT, even as minority variants, could impair the virological outcome when women subsequently initiate long-term ART,[10, 11] particularly if treatment is started within 6-12 months after delivery.[12, 13]

This study sought to investigate post-partum drug resistance in ARV-naive women who received PLAT in the US between 1998 and 2005, using population-based sequencing of plasma virus and allele-specific PCR testing for the M184V, K103N and D30N mutations. We also examined factors associated with an increased risk for selecting resistant viruses during pregnancy.

METHODS

STUDY DESIGN

This was a substudy utilizing specimens obtained from participants in the Women and Infants Transmission Study (WITS). The WITS is a multi-site observational study designed to examine the impact of HIV infection on HIV infected women and their infants. WITS sites are

located in Brooklyn, NY; New York City, NY; Boston and Worcester MA; Houston, TX; Chicago, IL; and Puerto Rico.

SUBJECTS

Women enrolled in WITS between June 1, 1998 and December 31, 2004, were evaluated for eligibility for this study. Study participants included HIV-1-infected pregnant women who had never received antiretroviral therapy before pregnancy, initiated PLAT consisting of zidovudine plus lamivudine either solely or in addition to either nevirapine or nelfinavir, stopped therapy postpartum, and had specimens available from 2- or 6-month postpartum visits during which the presence plasma virus by RNA-PCR testing had previously been documented. Of 1323 women, 146 were included in this study. A single laboratory, blinded as to PLAT received by participants, analyzed all specimens. Specimens collected within the first 14 days of antiretroviral therapy were also analyzed post-hoc, when available. However as most PLAT initiation occurred prior to WITS enrollment, these specimens were lacking for the majority.

DETECTION OF RESISTANCE MUTATIONS

Population sequencing of plasma viruses. HIV-1 RNA was extracted from 500 μL EDTA-anticoagulated plasma using the QIAamp® Viral RNA MiniKit (QIAGEN Sciences, Maryland, USA) after centrifugation at 24000*g* for 1 hour at 4°C. Part of each RNA sample was used for cDNA synthesis immediately after extraction, and the remainder was stored at –80°C.

The extracted RNA was transcribed to cDNA and amplified by PCR in a one-step process (Superscript III One-step RT-PCR with Platinum Taq Kit, Invitrogen™, Carlsbad, CA, USA) following the manufacturer's instructions. Cycling conditions included an initial cDNA synthesis step at 55°C for 25 min followed by a denaturation step at 94°C for 2 min; 30 cycles of PCR amplification (94°C fof 40 sec, 60°C for 40 sec, 68°C for 1 min and 20 sec); and a final 5 min extension step at 68°C. The PCR mix contained 25 μL of 2X Reaction Mix (including 0.4 mmol/L of each dNTP and 3.2 mmol/L of $MgCl_2$),

0.2 mmol/L of each primer OOPF ($_{HXB2}$2211-2232) [5'-GAAGCAGGAGCCGATAGACAAG-3'] and OOR2 ($_{HXB2}$3466-3444) [5'-TTTTCTGCCAGTTCTAGCTCTGC-3'], 15 µL of extracted RNA as template and nuclease-free H_2O to a final volume of 50 µL.

The resulting PCR product was purified using the QIAquick® PCR Purification Kit (QIAquick® PCR Purification Kit, QIAGEN Sciences, Maryland, USA) and used as the starting template for a 30-cycle nested PCR amplification (High Fidelity Platinum Taq, Invitrogen Corp., Carlsbad, CA, USA) using primers OOPF2 ($_{HXB2}$2218-2241) [5'-GAGCCGATAGACAAGGAACTGTAT-3'] and OOR3 ($_{HXB2}$3457-3432) [5'-AGTTCTAGCTCTGCTTCTTCAGTTAG-3'].

The nested PCR product was purified and sequenced (3730XL DNA Analyzer, Applied Biosystems, Foster City, CA, USA). Resistance mutations and polymorphisms were defined according to the International AIDS-Society-USA Panel (Spring 2008 Update). [14] Standard phylogenetic analyses ruled out sequence contamination.

Allele-specific PCR. To quantify the proportion of mutant sequences contained within each specimen, 5 µL of RT-PCR product were added to the real-time PCR together with selective or nonselective primers. When the initial DNA copy number was lower than 10^6, the nested DNA product was used. Conditions for nonselective amplification of *pol*-derived samples were 1× SYBR® green PCR Master Mix™ (Applied Biosystems, Warrington, UK), 900 nM of each non-specific and reverse primers, and deionized water to a final volume of 50 µL. Conditions for selective amplification of *pol* mutant sequences were identical except that the specific primer replaced the non-specific primer.

Mutant-specific primers ([$_{HXB2}$2319-2340] 5'-CTATTAGATACAGGAGCAAATA-3' for D30N; [$_{HXB2}$3078-3098] 5´- GACATAGTTATCTATCAATICG-3' for M184V; [$_{HXB2}$2884-2858] 5'-CCCACATCCAGTACTGTTACTGATTGG-3' for the K103N AAC allele; and [$_{HXB2}$2884-2858] 5'-CCACATCCAGTACTGTTACTGATTCA-3' for the K103N AAT allele) included the target codon in its 3' end and an intentional

mismatch at positions –3 or –2, relative to the 3' end. Non-specific primers ([$_{HXB2}$2319-2339] 5'-CTATTAGATACAGGAGCAGAT-3' for D30N; [$_{HXB2}$3078-3098] 5-GACATAGTTATCTATCAATAC-3' for M184V; and [$_{HXB2}$2884-2859] 5'-CCCACATCCAGTACTGTTACTGATTT-3 for both AAC and AAT K103N alleles) were similar to the former, but ended just before the target base pair and did not include intentional mismatches. The antiparallel primer ([$_{HXB2}$2592-2571] 5'-CTGGCTTTAATTTTACTGGTAC-3' for D30N; [$_{HXB2}$3277-3258] 5'-GGCTGTACTGTCCATTTATC-3' for M184V; and [$_{HXB2}$2757-2785] 5'-AAATGGAGAAAATTAGTAGATTTCAGAGA-3' for both AAC and AAT K103N alleles) was common for each pair of mutant-specific and non-specific reactions.

Each sample was evaluated by real-time PCR in an ABI 7000 Sequence Detection System thermocycler (Perkin Elmer Applied Biosystems, Foster City, CA, USA). The cycling parameters for the D30N and M184V ASPCR assays were: 50°C for 2 min, 95°C for 10 min, followed by 50 cycles at 95°C for 15s and 50°C for 1 min. The cycling parameters for the K103N ASPCR assays were identical to the former except that the annealing temperature was 60ºC. Amplicons prepared from the relevant plasmids using primers OOPF and OOR2, served as standards. The number of cycles required to reach threshold fluorescence (C_t) was determined and the quantity of sequences initially present calculated by interpolation onto the standard curve.

The different ASPCR assays were performed separately. Within each ASPCR assay, nonselective and selective amplifications were always performed in parallel. All reactions were performed in duplicate, and the mean of the two values was used for calculation. The percentage of viral sequences containing each mutation was calculated as follows: % mutant sequences = [(quantity of mutant sequences in the sample)/(quantity of total viral sequences in the sample)] × 100.

In addition to the sensitivity threshold for each ASPCR assay, we calculated a specific detection threshold per each sample, defined as the minimum proportion of variants that could be detected based on the specimen's HIV-1 RNA level (pVL), the volume of plasma used in the

RNA extraction (V), the fraction of the RNA elution volume used for cDNA synthesis (fe), and the assumed efficiencies of the RNA extraction (E_{RNAX}) and cDNA synthesis (E_{cDNA}). The sample specific detection threshold was calculated as 1/ N_{RNA}, where N_{RNA} was the number of viral RNA copies that were effectively sampled after RNA extraction and reverse transcription. NRNA was calculated as: N_{RNA}= pVL × V × fe × E_{RNAX}× E_{cDNA}. We assumed an E_{RNAX} of 0.96 and an E_{cDNA} of 0.7.[15, 16] Allele-specific PCR values between the ASPCR assay sensitivity threshold and the sample-specific detection threshold were considered undetectable.

STATISTICAL ANALYSIS

Subjects' characteristics and postpartum rates of resistance mutations were described using standard descriptive methods.

Variables associated with the emergence of M184V, K103N and D30N mutations were investigated using Chi-square, Fisher's exact test or F-test, as needed. Odds of developing M184V or K103N were evaluated using the Generalized Estimating Equations model (SAS procedure GENMOD). Alpha was set at 0.05 for determining statistical significance in all univariate and multivariate analyses. All analyses were intention-to-treat.

RESULTS

PERFORMANCE OF THE ASPCR ASSAYS

Using the mean plus 3 standard deviations of 20 negative control repeats (wild-type laboratory viral constructs), the detection threshold of the ASPCR was calculated at: 0.1%, 0.4% and 0.003% for the D30N, M184V and K103N ASPCR assays, respectively. Delta Cts between mutant and wild-type DNA equivalents were > 10 cycles for the D30N and M184V assays and > 17 cycles for the K103N assays. Proportion measurements were linear down to at least 1% for the D30N and M184V assays and to 0.01% for the K103N ASPCR assays.[11, 16]

SUBJECT'S CHARACTERISTICS

Of the 146 women, mean age was 27 year, most were African-American or Hispanic, the majority were CDC disease category A, and almost 30% had used hard drugs (crack, cocaine, heroin, methadone or any intravenous drug) and/or had a known history of alcohol use prior to delivery (Table 1). The first available CD4+ count during pregnancy was ≥ 200 cells/mm^3 in more than 90% of women. The median time of blood sample collection was 2.2 months postpartum (IQR: 1.9-4.6 months), with 76% of samples collected at the 2-month postpartum visit [median 2.0 months (IQR: 1.8-2.5 months)]; the remaining 24% were collected at the 6-month postpartum visit [median 6.2 months (IQR: 5.9 – 6.9 months)]. Median postpartum CD4+ counts were 575 cells/mm^3 (IQR: 397-767), and median postpartum HIV-1 RNA levels were 4780 copies/mL (IQR: 1352-18121 copies/mL). Nearly 48% of women maintained HIV-1 RNA levels <400 copies/mL at all timepoints during PLAT after their enrollment in WITS; 27% had all HIV-1 RNA levels >400 copies/mL during PLAT, and the remaining 23% alternated viremic with aviremic periods during PLAT. All women initiated PLAT including zidovudine + lamivudine; 64% also started nelfinavir and 7% nevirapine. Some women switched between dual- and triple-PLAT during pregnancy. Of the 146 women, 18 (12%) switched from dual- to triple-PLAT (1 added nevirapine and 17 added nelfinavir). Another 2 women switched between triple-PLAT with nevirapine and triple-PLAT with nelfinavir. The duration of exposure to zidovudine, lamivudine, nelfinavir and nevirapine are shown in Table 1.

POSTPARTUM RATES OF ANTIRETROVIRAL RESISTANCE

Post-partum resistance data were available from 114 women (78%). The characteristics of this subset of women did not differ from those of the main cohort. Pre-treatment resistance data was available from 25 of these 114 women (22%).

Overall, 49 women (43.0%) had at least 1 resistance mutation detected by the population-based sequencing analysis postpartum; 7 women (6.1%) had dual-class resistance after delivery. Three (2.6%) had resistance to NRTIs and PIs, 3 (2.6%) had resistance to NRTIs and

NNRTIs and 1 (0.9%) had resistance to NNRTIs and PIs. When including the results from the ASPCR analysis for D30N, M184V and K103N mutations, 72 women (63.2%) had at least 1 resistance mutation detected postpartum, 12 women (10.5%) had dual-class resistance, and 2 (1.7%) women had triple-class resistance after delivery. Of the 12 women who had dual-class resistance, 5 (4.4%) had resistance to NRTIs and PIs, and 7 (6.1%) had resistance to NRTIs and NNRTIs.

NRTI resistance. Using bulk sequencing of plasma viruses, the M184V/I mutation was detected postpartum in 65.0% of women receiving dual PLAT compared to 28.7% of women treated with 3 drugs (p=0.001). (Figure 1) Using ASPCR, this mutation was detected in 95.0% of women who had received dual PLAT and in 51.6% of those having received triple-drug PLAT. (p=0.001). Using ASPCR, the M184V mutation was detected postpartum in 2/5 (40%) women with M184V present by bulk sequencing at baseline and in 15/20 (75%) women who did not have this mutation before treatment.

Mutations associated with resistance to nucleoside analogues (NAMs) were also more frequent among women exposed to dual-drug PLAT (M41L, 5.0%; D67N, 5.0%; K70R, 10.0; and T215Y, 5.0%) than in those treated with 3 drugs (M41L, 1.1%; D67N, 1.1%; K70R, 1.1%; L210F, 1.1%; K219Q, 1.1%). Such differences were, at most, marginally significant.

NNRTI resistance. Using bulk sequencing of plasma viruses, the postpartum rates of NNRTI resistance among the eight women receiving nevirapine were:25% for K103N (2 cases), and 12.5% for Y188C (1 case). Using ASPCR, the K103N mutation was detected in three out of eight women exposed to nevirapine (37.5%), including the two cases detected through bulk sequencing, and in 8 out of 106 women not exposed to nevirapine (7.5%) (p=0.029). Using ASPCR, the K103N mutation was detected postpartum in 1 woman harboring this mutation before treatment and in 2/24 (8.3%) women who did not have this mutation before starting PLAT.

Table 1. Subject characteristics

Number of subjects evaluated	146
Age (Mean ± SD)	26.7 ± 5.2
Ethnicity	
White	4 (2.7)
Black	84 (57.5)
Hispanic	52 (35.6)
Other/unknown	6 (4.1)
CDC category, n (%)	
A	124 (84.9)
B	19 (13.0)
C	3 (2.1)
Ever used hard drug before delivery, n (%)	
Yes	40 (27.4)
No	105 (71.9)
Unknown	1(0.7)
Alcoholism, n (%)	
Yes	42 (28.8)
No	101 (69.2)
Unknown	3 (2.0)
First available CD4+ cell count during pregnancy, n (%)	
<200 cells/mm^3	9 (6.2)
200-350 cells/mm^3	33 (22.6)
>200 cells/mm^3	100 (68.5)
Unknown	4 (2.7)
Postpartum CD4+ cell counts (median, Q1-Q3)	
Absolute (cells/mm^3)	575 (397-767)
Percent (%)	29 (22-34)
HIV-1 RNA during PLAT, n (%)	
Always > 400 copies/mL	40 (27.4)
Mixed <400 and > 400 copies/mL	33 (22.6)
Always < 400 copies/mL	70 (47.9)
Unknown	3 (2.1)
Postpartum HIV-1 RNA copies (median, Q1-Q3) (copies/mL)	4780 (1352- 18121)
Timing of blood simple collection (months since delivery, median, (Q1-Q3))	
Overall, n=146	2.2 (1.9-4.6)
Samples collected at the 2-month postpartum visit, n=111	2.0 (2.5-1.8)
Samples collected at the 6-month postpartum visit, n=35	6.2 (5.9-6.9)
Samples tested, n (%)	146 (100%)
Initial PLAT, n (%)[b]	
AZT-3TC	43 (29.4)
AZT-3TC-Nelfinavir	93 (63.7)
AZT-3TC-Nevirapine	10 (6.8)
Length of exposure to AZT (days, up to delivery) (mean ± SD) [range]	122.5 ± 61.3 [2 , 309]
Length of exposure to AZT-3TC (days, up to delivery) (mean ± SD) [range]	118.2 ± 60.0 [1 , 270]
Nelfinavir exposure during PLAT, n (%)	
Yes	111 (76.0)
No	35 (24.0)
Length of exposure to Nelfinavir[c] (days, up to delivery) (mean ± SD) [range]	109.9 ± 58.9 [1 , 270]
Nevirapine exposure during PLAT, n (%)	
Yes	12 (8.2)
No	134 (91.8)
Length of exposure to Nevirapine[d] (days, up to delivery) (mean ± SD) [range]	68.1 ± 61.3 [1 , 177]

[a] PLAT, pregnancy-limited antiretroviral therapy; AZT, zidovudine; 3TC, lamivudine. [b] Refers to initial treatment; some subjects changed from dual-PLAT to triple-PLAT, and vice-versa. [c] For 111 exposed subjects. [d] For 12 exposed subjects

PI resistance. Using bulk sequencing, each of the D30N and L90M mutations were detected in one out of 87 women receiving nelfinavir during PLAT (1.1% for each mutation). The ASPCR testing confirmed the detection of the D30N mutation in one out of 87 women exposed to nelfinavir (1.1%) and allowed detection of this mutation in two women out of 27 (7.4%) that did not receive nelfinavir during PLAT. One D30N mutation detected at baseline was not found post-partum.

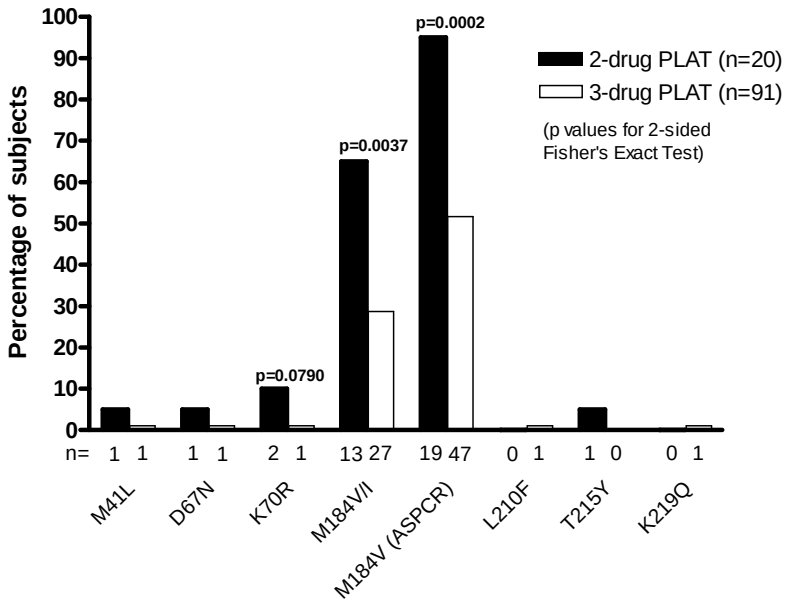

FIGURE 1. POST-PARTUM RATES OF NUCLEOSIDE ANALOGUE RESISTANCE MUTATIONS IN WOMEN TREATED WITH TWO- AND THREE-DRUG PREGNANCY-LIMITED ANTIRETROVIRAL THERAPY (PLAT)

FACTORS ASSOCIATED WITH EMERGENCE OF POST-PARTUM ANTIRETROVIRAL RESISTANCE

In the univariate analysis (Table 2), postpartum detection of the M184V mutation was more likely in women who had all HIV-1 RNA levels > 400 copies/mL during PLAT (OR= 2.65, 95%CI=1.02-6.87, p=0.05) relative to those who remaining aviremic through delivery, and in those with longer exposure to zidovudine (OR=1.25, 95%CI=1.01-1.54, p=0.04, per each additional month). The M184V mutation,

conversely, was less likely to be observed in women who received triple-drug PLAT including either nelfinavir or nevirapine (OR=0.06, 95%CI=0.007-0.44, p=0.006), relative to dual therapy only; in those treated with triple-drug PLAT including nelfinavir (OR=0.06, 95% CI=0.008-0.46, p=0.007) or nevirapine (OR=0.04, 95% CI=0.003-0.48, p=0.01), relative to dual therapy alone; and in those treated with triple-drug PLAT including nelfinavir, relative to any other regimen (OR=0.25, 95%CI=1.01-1.54, p=0.04). Univariate factors associated with postpartum detection of K103N were nevirapine exposure (OR=7.35, 95%CI=1.48-36.5, p=0.01), and longer exposure to zidovudine (OR=1.40, 95%CI=1.02-1.91, p=0.04) or to the combination of zidovudine and lamivudine (OR=1.42, 95%CI=1.04-1.94, p=0.03).

In the multivariate analysis, however, the only variables significantly associated with emergence of M184V were exposure to dual versus triple-drug PLAT (OR=19.64, 95%CI=2.47-156.25, p<0.01), and duration of zidovudine exposure (OR =1.29, 95%CI=1.03-1.63, p=0.03, per additional month). Variables associated with emergence of K103N were nevirapine use (OR=9.75, 95%CI=1.62-58.84, p=0.01) and length of zidovudine + lamivudine exposure (OR =1.46, 95%CI=1.05- 2.02, p=0.02, per additional month).

Of note, other factors such as presence of resistance mutations before starting PLAT, the first available CD4+ count during pregnancy, use of hard drugs before delivery, alcoholism before delivery, ethnicity, or age at delivery, were not associated with the postpartum detection of the M184V or K103N mutations.

Table 2. Factors associated with emergence of the M184V and the K103N mutation during pregnancy-limited antiretroviral therapy.

Characteristic*	M184V						K103N					
	Univariate Model			Multivariate Model			Univariate Model			Multivariate model		
	HR	CI	p-value	HR	CI	p-value	HR	CI	p-value	HR	CI	p-value
HIV RNA viral load during PLAT												
All VLs >400 vs. all VLs < 400	2.65	1.02-6.87	0.05	-	-	-	-	-	-	-	-	-
ART exposure during pregnancy												
Triple therapy (with NFV or NVP) vs. Dual therapy only	0.06	0.007-0.44	0.006	19.64	2.47-156.25	<0.001	-	-	-	-	-	-
Triple therapy with NFV vs. Dual therapy only	0.06	0.008-0.46	0.007	-	-	-	-	-	-	-	-	-
Triple therapy with NVP vs. Dual therapy only	0.04	0.003-0.48	0.01	-	-	-	-	-	-	-	-	-
NFV vs. No NFV	0.25	0.09-0.72	0.01	-	-	-	-	-	-	-	-	-
NVP vs. No NVP	-	-	-	-	-	-	7.35	1.48-36.50	0.01	9.75	1.62-58.84	0.01
Length of AZT/3TC combination (per additional month)	-	-	-	-	-	-	1.42	1.04-1.94	0.03	1.46	1.05-2.02	0.02
Length of AZT exposure (per additional month)	1.25	1.01-1.54	0.04	1.29	1.03-1.63	0.03	1.40	1.02-1.91	0.04	-	-	-

* Only characteristics with statistically significant association with postpartum detection of M184V or K103N mutants are shown. Factors not significantly associated with postpartum M184V or K103N mutations were: presence of resistance mutations before starting PLAT, the first available CD4+ count during pregnancy, use of hard drugs before delivery, alcoholism before delivery, ethnicity or age at delivery.

DISCUSSION

This study showed that pregnancy-limited antiretroviral therapy (PLAT) was associated with postpartum detection of antiretroviral resistance mutations in a high proportion of previously antiretroviral-naïve mothers. The main determinants of resistance selection during pregnancy were the characteristics of the antiretroviral regimen chosen to prevent MTCT.

Virtually all women receiving dual therapy developed the M184V mutation. This mutation confers high-level resistance to lamivudine and emtricitabine[17, 18] and is associated with an increased risk of virological failure of treatment combinations including these drugs.[12] Thymidine analogue resistance mutations (TAMs) were detected in few women but were also more frequent in those receiving dual therapy. A high proportion of women receiving triple-drug therapy also selected resistance mutations during pregnancy. Based on allele-specific PCR testing, 50% of women treated with three drugs developed the M184V mutation. Moreover, although few women received nevirapine in this study, almost 40% of these women had non-nucleoside analogue (NNRTI) resistance mutations detected at the postpartum visit. On the contrary, emergence of PI resistance was rare in women treated with nelfinavir.

Use of dual therapy and duration of zidovudine exposure, which reflects the overall duration of antiretroviral therapy, were the only two variables that were independently associated with an increased risk of M184V detection after delivery. Similarly, the post-partum detection of the K103N mutation was independently associated with exposure to nevirapine and duration of exposure to zidovudine and lamivudine. These findings strongly argue against using dual therapy to prevent MTCT whenever triple therapy is available.

The fact that resistance mutations were so frequently detected among women receiving triple therapy contrasts with previous estimates from Latin America and Caribbean countries,[19] and suggests that PLAT was less effective than expected at continuously suppressing HIV-1 RNA levels. In the univariate risk factor analysis, women who had all HIV-1 RNA levels above 400 copies/mL during the study were 2.7

times more likely to have the M184V mutation detected postpartum than those remaining aviremic through delivery. Similarly, subjects receiving nelfinavir were 4 times less likely to develop postpartum M184V than those not receiving this drug. These findings suggest that women treated with drugs with high genetic barrier to attain resistance are less likely to develop the M184V mutation. None of these variables, however, remained independently associated with risk of postpartum resistance in the multivariate analyses.

The pre-existence of primary resistance mutations could also have explained the high frequency of postpartum resistance found in this study. The prevalence of primary resistance increased during the last decade in pregnant women in the US.[20] Using allele-specific PCR in women enrolled in WITS with similar characteristics to those included in this study, we previously reported a 9.4% prevalence of primary lamivudine and emtricitabine resistance, and a 6.3% prevalence of nelfinavir resistance between 1998 and 2004.[21] As most women included in this analysis started antiretroviral therapy before WITS enrollment, pre-treatment resistance data was only available from one third of women. Based on this limited number of subjects, we did not observe an association between pre-existing resistance and the postpartum selection of M184V or K103N.

We did not evaluate treatment adherence; therefore we cannot rule out an association between suboptimal adherence and the observed rates of postpartum resistance. Ethnicity and hard drug or alcohol consumption have been previously associated with lower adherence to antiretroviral therapy and worse virological outcomes,[22-24] however, these factors were not associated with postpartum resistance in this study.

Finally, altered drug pharmacokinetics due to physiological changes occurring in women during pregnancy could have favored the existence of suboptimal drug levels during pregnancy or prolonged drug elimination in the postpartum period. Exposure to most PIs, including nelfinavir, is reduced in HIV-1-infected women during pregnancy due to increased intestinal and/or hepatic CYP3A activity. [25-29] Pregnant women, as well, have increased nevirapine clearance and lower plasma concentrations than non-pregnant women, although plasma levels are

largely influenced by body weight [30]. Chaix et al[31] found that emergence of nevirapine-resistance strongly correlated with higher median nevirapine plasma concentration. Prolonged nevirapine elimination after delivery in subjects with higher plasma levels could have allowed viral replication in the presence of suboptimal nevirapine levels after delivery.

In concordance with previous studies,[11, 21, 32] allele-specific PCR increased the frequency of detection of key resistance mutations relative to bulk sequencing of plasma viruses. Several studies have shown that pre-treatment detection of minority NNRTI-resistant variants more than triples the risk of virological failure to subsequent NNRTI-based therapy.[11, 33] Therefore, the results of this study have important clinical implications for women receiving PLAT during pregnancy. It is well established that women selecting lamivudine, emtricitabine or NNRTI-resistant mutants during PLAT are at a higher risk of failing subsequent NNRTI-based antiretroviral therapy, particularly if treatment is started within 6 to 12 months after delivery.[12, 13]

In order to reduce resistance selection during pregnancy, dual antiretroviral MTCT prophylaxis should be avoided. Our data support the use of triple-drug therapy as the preferred approach to MTCT prevention in order to preserve future treatment options for mothers. When possible, antiretroviral regimens to prevent MTCT should include drugs with high genetic barrier. Nelfinavir-based therapy is no longer a preferred regimen for MTCT,[34] but the findings of this study likely apply to other PIs. In women treated with nevirapine-based regimens the optimal timing of nevirapine interruption and length of continuation of other concomitant agents merits further inquiry to avoid active viral replication in the presence of suboptimal nevirapine levels. All efforts should be undertaken to ensure optimal adherence to antiretroviral therapy during pregnancy. Lastly, given that resistance mutations selected during pregnancy will wane after PLAT interruption, performing postpartum genotypic resistance testing within 1 to 2 months after delivery would be highly informative for designing future treatment regimens for women exposed to PLAT and may be useful in guiding the choice of antiretroviral regimen postpartum.

ACKNOWLEDGEMENTS

This work was supported in part by the following U.S. Public Health Service grants from the National Institutes of Health: K24 RR16482, a Virology Support Laboratory contract from the Adult ACTG (U01 AI-38858), and the Harvard Medical School Center for AIDS Research Virology Core (P30 AI60354). Roger Paredes is a recipient of the "La Caixa" Fellowship Grant for Post-Graduate Studies, Caixa d'Estalvis i Pensions de Barcelona, Catalonia, Spain. The Women and Infants Transmission Study Principal investigators, study coordinators, program officers and funding include: Clemente Diaz, Edna Pacheco-Acosta (University of Puerto Rico, San Juan, PR; U01 AI 034858); Ruth Tuomala, Ellen Cooper, Donna Mesthene (Boston/Worcester Site, Boston, MA; 9U01 DA 015054); Phil LaRussa, Alice Higgins (Columbia Presbyterian Hospital, New York, NY; U01 DA 015053); Sheldon Landesman, Herman Mendez, Ava Dennie (State University of New York, Brooklyn, NY; U01 HD 036117); Kenneth Rich, Delmyra Turpin (University of Illinois at Chicago, Chicago, IL; U01 AI 034841); William Shearer, Norma Cooper (Baylor College of Medicine, Houston, TX; U01 HD 041983); Joana Rosario (National Institute of Allergy and Infectious Diseases, Bethesda, MD); Kevin Ryan, (National Institute of Child Health and Human Development, Bethesda, MD); Vincent Smeriglio, Katherine Davenny (National Institute on Drug Abuse, Bethesda, MD); and Bruce Thompson (Clinical Trials & Surveys Corp., Baltimore, MD, N01 AI 085339). The scientific leadership core include: Kenneth Rich (PI), Delmyra Turpin (Study Coordinator) (1 U01 AI 050274-01). Additional support was provided by local Clinical Research Centers as follows: Baylor College of Medicine, Houston,TX; NIH GCRC RR000188; Columbia University, New York, NY; NIH GCRC RR000645.

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Chapter 5

Pre-existing Minority Drug-Resistant HIV-1 Variants and Risk of Antiretroviral Treatment Failure

Roger Paredes,[1,2] Christina M. Lalama,[3] Heather J. Ribaudo,[3] Bruce Schackman,[4] Cecilia Shikuma,[5] Francoise Giguel,[1] Victoria A. Johnson,[6,7] Susan A. Fiscus,[8] Richard T. D'Aquila,[9] Roy M. Gulick,[4] and Daniel R. Kuritzkes,[1] for the AIDS Clinical Trials Group (ACTG) 5095 Study Team.

[1]Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA; [2]Fundacions irsiCaixa & Lluita contra la SIDA, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, Catalonia, Spain; [3]Harvard School of Public Health, Boston, MA, USA; [4]Weill Medical College, Cornell University, New York City, NY, USA; [5]University of Hawaii, Honolulu, HI, USA; [6]Birmingham Veterans Affairs Medical Center, Birmingham, AL,USA; [7]University of Alabama at Birmingham School of Medicine, Birmingham, AL,USA; [8]University of North Carolina, Chapel Hill, NC,USA; [9]Vanderbilt University, Nashville, TN, USA

Journal Infectious Diseases 2009; (in press)

ABSTRACT

BACKGROUND: The clinical relevance of detecting low-abundance drug-resistant HIV-1 variants is uncertain.

METHODS: To determine the effect of pre-existing minority non-nucleoside reverse transcriptase inhibitor (NNRTI)-resistant variants on the risk of virologic failure (VF), we reanalyzed a case-cohort substudy of efavirenz recipients in ACTG A5095. Minority K103N or Y181C populations were determined by allele-specific PCR (ASPCR) in subjects without NNRTI resistance by population sequencing. Weighted Cox proportional hazards models adjusted for recent adherence estimated the relative risk of VF in the presence of pre-existing NNRTI-resistant minority variants.

RESULTS: The evaluable case-cohort sample included a 195 subjects from the randomly selected subcohort (51 with VF, 144 without failure [NF]), plus 127 of the remaining subjects with VF. Pre-existing presence of minority K103N or Y181C mutations, or both, was detected in 8 (4.4%), 54 (29.5%) and 11 (6%), respectively, of 183 evaluable subjects in the random subcohort. Detection of pre-existing minority Y181C mutants was associated with an increased risk of VF in the setting of recent adherence (HR=3.45, CI=1.90, 6.26), but not in non-adherent subjects (HR=1.39, CI=0.58, 3.29).

CONCLUSIONS: In adherent patients, pre-existing minority Y181C mutants more than tripled the risk of VF of first-line efavirenz-based ART.

CLINICAL TRIALS REGISTRATION: clinicaltrials.gov Identifier: NCT00013520

INTRODUCTION

Antiretroviral treatment guidelines recommend using the non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz or a ritonavir-boosted protease inhibitor (PI), plus a fixed-dose combination of nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs) for initial anti-HIV therapy[1, 2].The efficacy of NNRTI-based regimens, however, is threatened by the increasing prevalence of drug-resistant HIV-1 among newly infected or newly diagnosed persons[3,4]. We previously showed that presence of pre-existing NNRTI-resistant mutants detected by population sequencing was associated with a 2.3-fold increased risk of virologic failure to first-line efavirenz-based ART.[5]

Antiretroviral drug resistance testing is recommended in HIV-1-infected subjects before starting antiretroviral therapy (ART) to guide the selection of appropriate first-line regimens [2, 3]. Studies show that antiretroviral drug resistance testing is cost-effective [6], and improves the virologic, immunologic and clinical outcomes of ART [5, 7-10]. Current genotypic resistance assays, however, do not detect resistant viruses present in less than 15-20% of the viral population [11, 12]. New assays such as allele-specific real-time PCR (ASPCR) enable detection of low-abundance mutants with greater sensitivity [13-20]. Through preferential amplification of different allelic variants in real-time PCR conditions, ASPCR consistently detects mutants present in less than 0.1% of the virus population [14].

Relative to population sequencing of plasma viruses, ASPCR testing increases the detection of particular antiretroviral drug-resistance resistance mutations by 1.5- to 3-fold in different clinical settings [14, 15, 21, 22]. Whether drug-resistant mutants present at such low levels are associated with an increased risk of virologic failure of ART remains unresolved. We sought to address this question using ASPCR to detect selected NNRTI resistance mutations in pre-treatment plasma specimens from subjects in a case-cohort study from the efavirenz arms of AIDS Clinical Trials Group (ACTG) protocol A5095[23, 24], a randomized trial of initial ART.

METHODS

STUDY PARTICIPANTS

The ACTG A5095 study (clinicaltrials.gov identifier: NCT00013520) was a randomized, controlled trial that compared the efficacy of efavirenz plus a fixed-dose combination of two or three nucleoside reverse transcriptase inhibitors (NRTIs) with that of a fixed-dose triple-nucleoside regimen in previously untreated HIV-1-infected subjects with plasma HIV-1 RNA level of 400 copies/mL or greater (Amplicor or UltraSensitive HIV-1 Monitor Assay version 1.0; Roche Molecular Systems, Branchburg, NJ)[23, 24]. For subjects meeting the criteria for virologic failure (2 consecutive measurements of HIV-1 RNA level ≥200 copies/mL, with the first measurement at least 16 weeks after study entry), population sequencing (TruGene; Siemens, Norwood, MA) of plasma viruses was performed at the time of first virologic failure and at baseline from stored samples.

STUDY DESIGN

To determine the prevalence of NNRTI resistance and its impact on treatment outcome in the efavirenz-containing arms of this trial a case-cohort study was performed [5, 25]. The case-cohort sample consisted of a random sample (subcohort) stratified by and drawn from the efavirenz-containing arms of A5095, plus the additional cases (virologic failures) that were not selected to be in the subcohort. The current report presents further analyses of the existing case-cohort study[5].

To evaluate the prevalence of pre-treatment minority K103N and Y181C variants and their association with subsequent virologic outcome, blinded pre-treatment plasma samples in the case-cohort study with no NNRTI resistance detected by population sequencing were reanalyzed using ASPCR. The primary outcome measure for the case-cohort study was the occurrence of virologic failure; the primary variable of interest was presence or absence of minority K103N and/or Y181C variants in the pre-treatment samples. Minority variants were defined as variants detected by ASPCR but not by population sequencing. Data on recent adherence were captured as part of A5095 while the subject was on randomized treatment at weeks 4, 12, and 24

and then every 24 weeks using a self-administered adherence questionnaire. Given that non-adherence was associated with an increased risk of virologic failure in the main A5095 study [23], as-treated analyses in the current study were adjusted for recent self-reported adherence, defined as not missing any doses over the past 4 days [26].

DETECTION OF K103N AND Y181C MUTANTS USING ALLELE-SPECIFIC PCR.

Viral RNA was extracted from one milliliter of plasma (QIAamp Viral RNA Mini Kit, Valencia, CA) after centrifugation at 24000 x g for 1 hour at 4ºC. One-step reverse transcription of viral RNA and cDNA PCR amplification was performed using primers OOPF ($_{HXB2}$2211-2232) 5'-GAAGCAGGAGCCGATAGACAAG-3', and OOR2 ($_{HXB2}$ 3466-3444) 5'-TTTTCTGCCAGTTCTAGCTCTGC-3'. To ensure that 10^6-10^7 copies of total starting template DNA were introduced in the real-time PCR, a nested PCR step using primers OOPF2 ($_{HXB2}$ 2221-2241) 5'-GAGCCGATAGACAAGGAACTGTAT-3' and OOR3 ($_{HXB2}$ 3454-3432) 5'-AGTTCTAGCTCTGCTTCTTCTGTTAG-3' was performed. The PCR product was then quantified and diluted. Primers used for the ASPCR reactions were adapted to subtype B HIV-1 from a previous publication by Palmer et al [19]. Primers for the K103N mutation (AAC and AAT alleles) included a common upstream primer F-TC ($_{HXB2}$2757-2785) 5'-AAATGGAGAAAATTAGTAGATTTCAGAGA-3', a common non-specific downstream primer K-NS ($_{HXB2}$2884-2859) 5'-CCCACATCCAGTACTGTTACTGATTT-3, a K103N AAC allele-specific primer KC ($_{HXB2}$2884-2858) 5'-CCCACATCCAGTACTGTTACTGATTGG-3', and a K103N AAT allele-specific primer KT ($_{HXB2}$2884-2858) 5'-CCACATCCAGTACTGTTACTGATTCA-3'. Primers for the Y181C mutation included an upstream primer YF ($_{HXB2}$2965-2993) 5'-CACCAGGGATTAGATATCAGTACAATGTG-3', a non-specific downstream primer YN ($_{HXB2}$3117-3090) 5'-CTACATACAAATCATCCATGTATTGA-3, and an Y181C allele-specific primer YS ($_{HXB2}$3117-3092) 5'-

CTACATACAAATCATCCATGTATTGCC -3'. All mutant-specific primers incorporated an intentional mismatch at position -1 relative to the 3'-end to increase the discrimination of the target allele from other alleles including wild-type. PCR reactions proceeded as previously published [14, 19]. Clinical specimens were analyzed in the same batch with serially diluted standards (range, 10^2 to 10^7 standard DNA copies). The percentage of HIV-1 sequences containing each mutation was calculated as: percent mutated sequences = 100 × (quantity of mutant sequences)/(quantity of total HIV-1 sequences).

In addition to the sensitivity threshold for each ASPCR assay, we calculated a specific detection threshold for each sample, defined as the minimum proportion of variants that could be detected based on the subject's plasma HIV-1 RNA level (pVL), the volume of plasma used in the RNA extraction (V), the fraction of the RNA elution volume used for cDNA synthesis (fe), and the assumed efficiencies of the RNA extraction (E_{RNAX}) and cDNA synthesis (E_{cDNA}). The sample specific detection threshold was calculated as $1/ N_{RNA}$ where N_{RNA} was the number of viral RNA copies that were effectively sampled after RNA extraction and reverse transcription. N_{RNA} was calculated as: N_{RNA}= pVL × V × fe × E_{RNAX}× E_{cDNA}. We assumed an E_{RNAX} of 0.96 and an E_{cDNA} of 0.7 [14, 27]. Allele-specific PCR values between the ASPCR assay sensitivity threshold and the sample-specific detection threshold were considered undetectable.

STATISTICAL METHODS

Based on the random subcohort, the prevalence of baseline minority K103N and/or Y181C mutants was estimated; the prevalence of each minority variant was compared between virologic failures and non-failures using the Fisher's exact test. Using an exact test for homogeneity of odds ratios, the prevalence of Y181C mutants was compared between virologic failures and non-failures across the following subgroups: subjects with or without the K103N mutation, 4-drug or 3-drug EFV-based treatment, and screening HIV-1 RNA level. Summary statistics of the demographics of subjects in the random subcohort by pre-existing minority K103N and/or Y181C mutants, population resistance, or no NNRTI resistance are described, as well

as for additional subjects with virologic failure. Weighted Cox proportional hazards models [28] were used to estimate the risk of virologic failure in the presence and absence of minority K103N and/or Y181C mutants at baseline among subjects without NNRTI resistance mutations by population sequencing. Unadjusted intent-to-treat and as-treated analyses showed similar results. Further as-treated analyses were adjusted for recent self-reported adherence; the presence of an interaction between recent self-reported adherence and baseline NNRTI resistance was examined. Such an interaction would imply a different impact of the presence of NNRTI resistance mutations at baseline dependent on recent adherence. In all analyses, subjects without ASPCR results for either codon 103 or 181 were counted as missing unless otherwise specified. All P-values and confidence intervals presented are nominal, unadjusted for multiple comparisons.

RESULTS

CASE-COHORT SAMPLE

Of the 220 randomly sampled subjects, 57 (26%) were cases (virologic failures) and 163 (74%) were controls (non-failures) (Figure 1). Eleven controls had less than 16 weeks of follow-up and therefore were not evaluable for the protocol-defined criteria for virologic failure; these subjects were excluded from analyses of virologic failure and were not assayed by ASPCR. Reasons for premature study discontinuation included loss to follow-up (5), unable to get to clinic (4), toxicity (1) and clinical event (1). Of the remaining 209 subjects from the random subcohort, 12 had NNRTI resistance by population sequencing, 3 had missing samples, and the remainder were assayed for the presence of minority variants using ASPCR. Eleven of these subjects had incomplete ASPCR results (no amplification): 7 subjects had no results for both K103N and Y181C; 2 had no K103N result; 2 had no Y181C result. A viral genotype could not be obtained by population sequencing for one (non-failure) subject from the randomly selected subcohort, but results were obtained by ASPCR. The evaluable random cohort sample therefore included 195 subjects with at least 16 weeks of follow-up of whom 12 were considered NNRTI resistant by

population sequencing and 183 had complete ASPCR results. Of the additional 136 subjects with virologic failure who were not randomly chosen to be in the subcohort, 7 had NNRTI resistance by population sequencing, 4 had missing samples, and the remainder were assayed by ASPCR. Two subjects had no results (no amplification) for both K103N and Y181C, 2 had no K103N result, and 1 had no Y181C result. Therefore, 127 additional failures were added to the random subcohort (7 NNRTI resistant by population sequencing and 120 with complete ASPCR results). Overall, the total case-cohort sample included 322 subjects (178 failures and 144 non-failures).

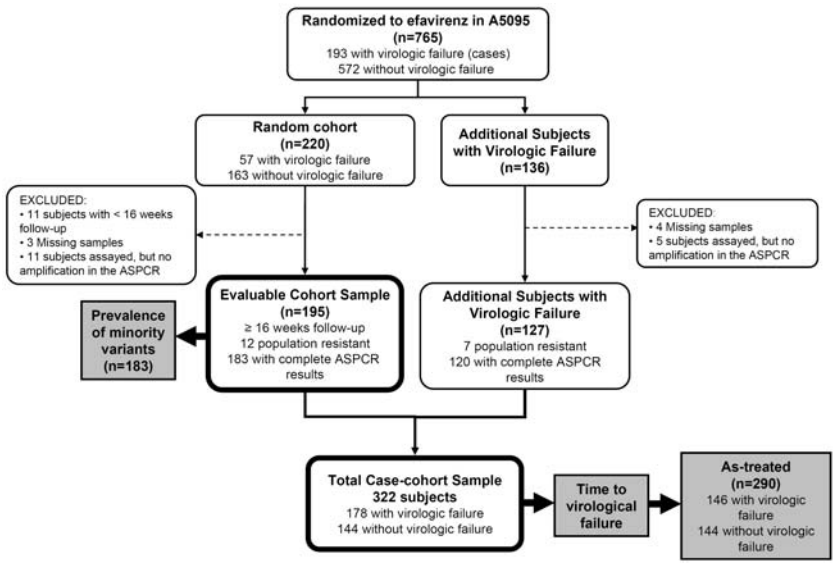

FIGURE 1. CASE-COHORT DESIGN AND SUBJECT DISPOSITION. Someone was counted as missing if he/she had no result for one or both minority variant.

Table 1: Baseline characteristics of the case-cohort sample

		Random Subcohort						Additional virologic failures (n=127)
		No NNRTI resistance[c] (n=110)	Resistance by ASPCR			NNRTI Resistance by population sequencing[d] (n=12)	All of Random Subcohort (n=195)	
			K103N only (n=8)	Y181C only (n=54)	Both K103N+Y181C (n=11)			
Screening HIV-1 RNA	< 100,000	67 (61%)	2 (25%)	25 (46%)	7 (64%)	8 (67%)	109 (56%)	66 (52%)
	>= 100,000	43 (39%)	6 (75%)	29 (54%)	4 (36%)	4 (33%)	86 (44%)	61 (48%)
Sex	Male	82 (75%)	8 (100%)	47 (87%)	9 (82%)	11 (92%)	157 (81%)	105 (83%)
	Female	28 (25%)	0 (0%)	7 (13%)	2 (18%)	1 (8%)	38 (19%)	22 (17%)
Age (yrs)	Mean (SD)	36 (9)	36 (8)	40 (11)	37 (10)	37 (7)	37 (10)	37 (9)
Race/ethnicity	White Non-Hispanic	48 (44%)	4 (50%)	23 (43%)	4 (36%)	8 (67%)	87 (45%)	46 (36%)
	Black Non-Hispanic	36 (33%)	2 (25%)	23 (43%)	4 (36%)	4 (33%)	69 (35%)	55 (43%)
	Hispanic (Regardless of Race)	24 (22%)	2 (25%)	7 (13%)	3 (27%)	0 (0%)	36 (18%)	23 (18%)
	Asian, Pacific Islander	1 (1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1%)	2 (2%)
	American Indian, Alaskan Native	1 (1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1%)	1 (1%)
	Subject does not know or other	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0 (0%)	1 (1%)	0 (0%)
IV drug history	Never	95 (86%)	6 (75%)	46 (85%)	8 (73%)	7 (58%)	162 (83%)	112 (88%)
	Currently	1 (1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1%)	
	Previously	14 (13%)	2 (25%)	8 (15%)	3 (27%)	5 (42%)	32 (16%)	15 (12%)
HIV-1 RNA (log_{10} cp/mL)	Mean (SD)	4.85 (0.71)	5.08 (0.93)	4.94 (0.65)	5.32 (0.47)	4.65 (0.46)	4.90 (0.69)	4.94 (0.71)
CD4 Count (cells/mm^3)	Median (Q1, Q3)	204 (99, 322)	74 (17, 334)	199 (44, 330)	201 (46, 287)	283 (208, 443)	206 (65, 324)	203 (75, 356)

a The case-cohort sample consisted of a random sample (Random subcohort) stratified by and drawn from the efavirenz-containing arms of the ACTG A5095 study, plus the additional cases (Additional virologic failures) that were not selected to be in the subcohort. The baseline characteristics of subjects included in the random subcohort are shown for the following groups: subjects without NNRTI resistance mutations detected by either population sequencing or ASPCR; those without NNRTI resistance detected by population sequencing but in whom ASPCR detected only K103N mutants (K103N only), only Y181C mutants (Y181C only) or both K103N and Y181C mutants (both K103N + Y181C); subjects in whom NNRTI resistance mutations were detected by population sequencing of plasma viruses, and for all subjects included in the random subcohort (All of Random Subcohort). Of note, subjects with NNRTI resistance mutations detected by population sequencing were not retested using ASPCR.

b The table does not include subjects with less than 16 weeks of follow-up (n=11) or with no ASPCR results (n=23).

c NNRTI, non-nucleoside reverse transcriptase inhibitor; ASPCR, allele-specific polymerase chain reaction

d Population sequencing of plasma viruses

ALLELE-SPECIFIC PCR

The detection threshold of the ASPCR was defined as more than 3 standard deviations above the mean of 20 repeated assays using the wild-type RT from pNL4-3 as a control target. Detection thresholds were: K103N (AAC) = 0.003%, K103N (AAT) = 0.001% and Y181C = 0.03%. The difference in real-time PCR threshold cycle values (ΔCt) between mutant and wild-type DNA equivalents was always > 17 cycles. Proportion measurements were linear down to at least 0.1% in all cases.

BASELINE CHARACTERISTICS

As previously reported[5], the prevalence of pre-treatment NNRTI resistance by population sequencing in the randomly sampled subcohort was 5%, which included 6 subjects with K103N alone, 2 with K103N together with a second NNRTI (other than Y181C), 0 with Y181C, and 1 with both K103N and Y181C by population sequencing at baseline. Of the 183 subjects assayed for the presence of pre-existing low-abundance K103N and/or Y181C mutants using ASPCR, variants carrying the K103N or Y181C mutations, or both were detected in 8 (4.4%), 54 (29.5%) and 11 (6%) subjects, respectively. Table 1 summarizes baseline demographics of the random subcohort by presence or absence of pre-existing minority NNRTI resistance mutations, and for the additional subjects with virologic failure.

LEVELS OF LOW-ABUNDANCE DRUG-RESISTANT MUTANTS AT BASELINE.

Among subjects in the random subcohort in whom minority NNRTI-resistant variants were detected, the median (interquartile range) levels of mutants were: K103N, AAC allele= 0.012% (0.008%-0.116%); K103N, AAT allele= 0.013% (0.005%-0.053%); and Y181C=0.060% (0.048%-0.089%). Of note, the levels of Y181C mutants detected in individual samples were all below 1% (Figure 2); levels of the K103N mutant alleles were similarly low (data not shown). Because we tested only those subjects with wild-type virus by standard genotypic resistance tests, these results suggest that presence of NNRTI

mutations at levels greater than 1% of the virus population had been efficiently identified by population sequencing.

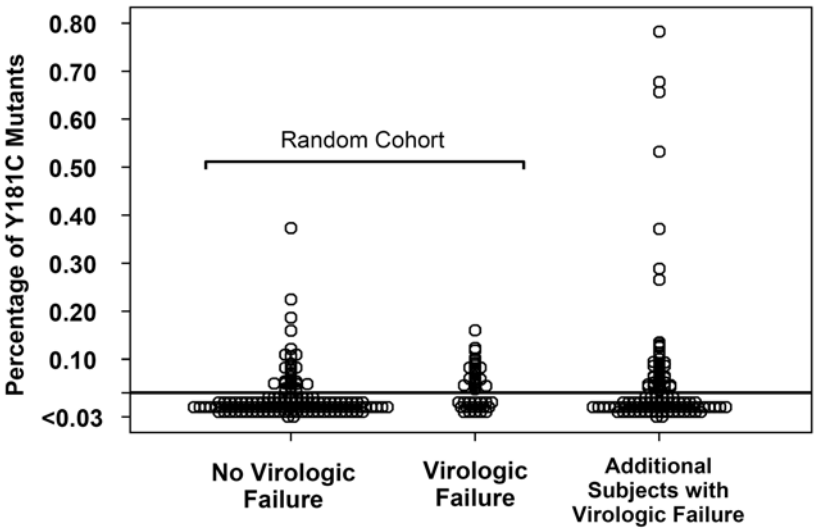

FIGURE 2. LEVELS OF MINORITY Y181C MUTANTS IN THE VIRUS POPULATION. The horizontal line represents the sensitivity threshold of the allele-specific PCR assay for detecting Y181C mutants (0.03%). Circles represent the levels at which Y181C were found among each plasma sample. Each circle corresponds to one subject.

LOW-ABUNDANCE K103N AND/OR Y181C MUTANTS AND VIROLOGIC FAILURE

Pre-existing low-abundance K103N mutants were detected less often than Y181C variants and were as frequent in virologic failures as in non-failures among subjects in the random subcohort with complete K103N ASPCR data and ≥ 16 weeks of follow-up (Figure 3). Of the 185 subjects in the random subcohort with complete Y181C ASPCR data and ≥ 16 weeks of follow-up, 58% of virologic failures compared to 29% of non-failures had low-abundance Y181C mutants at baseline (P=0.001). The relative prevalence of Y181C mutants in subjects with virologic failure compared to subjects without virologic failure was similar across subgroups defined by presence or absence of the K103N mutation, assignment to the 4-drug or 3-drug arm, and screening HIV-1 RNA level (Figure 3).

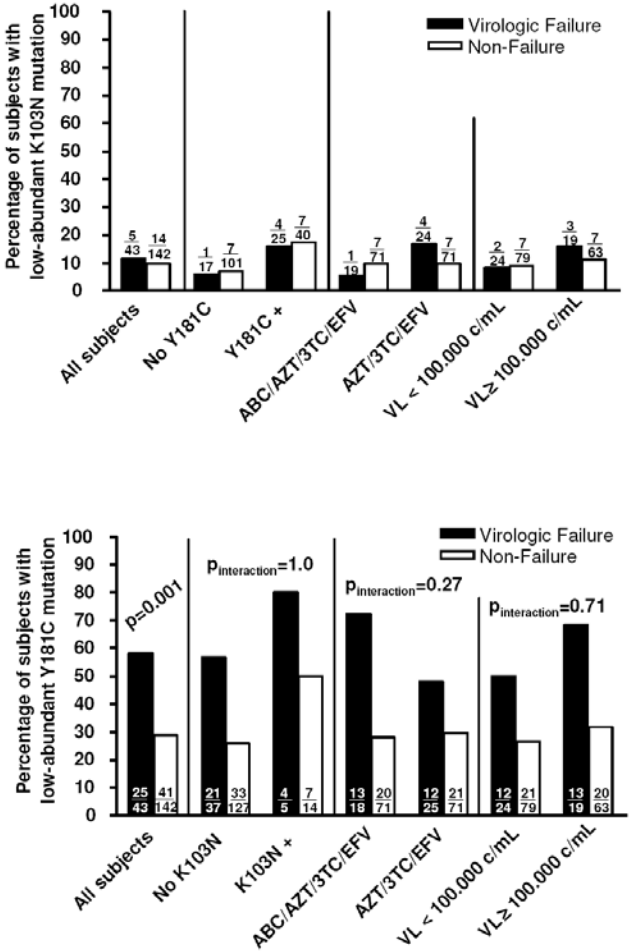

Figure 3. Prevalence of baseline minority K103N and Y181C mutants according to virologic outcome. Figures show the proportion of failures and non failures with low-abundant K103N (upper figure) and Y181C (lower figure) mutations detected at baseline among those without NNRTI resistance by population sequencing in the random subcohort. Analyses were done overall; for subjects with the Y181C mutation detected by ASPCR (Y181C+) or not (No Y181C); for subjects with the K103N mutation detected by ASPCR (K103N+) or not (No K103N); for those receiving abacavir (ABC), zidovudine (AZT), lamivudine (3TC) and efavirenz (EFV) or those receiving zidovudine (AZT), lamivudine (3TC) and efavirenz (EFV); and for subjects with screening HIV-1 RNA levels (VL) greater or lower than 100,000 copies/mL. The prevalence of low-abundant K103N mutants among failures and non-failures was not significantly different overall (p-value not shown). P-value for difference in prevalence of low-abundant Y181C mutants among failures and non-failures overall is shown. Pinteraction values are based on exact test for homogeneity of odds ratios across subgroups. P-values are nominal and unadjusted for multiple comparisons.

In exploratory analyses we were unable to define a threshold level of Y181C mutants that distinguished failures and non-failures with high sensitivity and specificity (not shown). A post-hoc analysis of the baseline characteristics of subjects with low-abundanceY181C mutants in the randomly sampled subcohort showed no differences between virologic failures and non-failures regarding screening HIV-1 RNA levels, CD4+ T-cell counts, or race/ethnicity (not shown).

An as-treated weighted Cox proportional hazards model adjusted for recent adherence (Table 2) showed a significantly increased risk of virologic failure for subjects with an NNRTI-resistant virus by population sequencing compared with those with wild-type virus by population sequencing and ASPCR (hazard ratio [HR]= 4.00, 95% confidence interval [CI]= 1.72, 9.09). Among subjects with wild-type HIV-1 by population sequencing, detection of low-abundance Y181C mutants by ASPCR was associated with an increased risk of virologic failure (HR=2.54, 95% CI= 1.53, 4.20). A significant association with the detection of minority K103N mutants and an increased risk of virologic failure was not detected (P=0.22), but the direction of the effect was similar (HR=1.58, 95% CI=0.76, 3.28). As seen in the study overall [23], subjects with recent non-adherence also had an increased risk of virologic failure compared with adherent subjects (HR=2.30, 95% CI=1.40, 3.78).

Table 2. Weighted Cox proportional hazards model for virologic failure adjusted for recent adherence – Main effects model

	Hazard Ratio for virologic failure [95% CI]
Resistant (Population sequencing) vs. sensitive (Population sequencing & ASPCR)	4.00 [1.72, 9.09]
Presence of minority K103N by ASPCR vs. absence (given sensitive by population sequencing)	1.58 [0.76, 3.28]
Presence of minority Y181C by ASPCR vs. absence (given sensitive by population sequencing)	2.54 [1.53, 4.20]
Recent adherence: non-adherent vs. adherent	2.30 [1.40, 3.78]

Further modeling suggested an interaction between baseline presence of low-abundance Y181C mutants and recent adherence

(P=0.08), showing that in the presence of recent non-adherence, the effect of minority Y181C was diminished (adjusting for the presence of K103N) (Figure 4). Among adherent subjects, the presence of minority Y181C by ASPCR had an increased risk of virologic failure compared to those that were sensitive by both population sequencing and ASPCR (HR=3.45, 95% CI= 1.90, 6.26); among non-adherent subjects, the presence of minority Y181C did not show a significantly increased risk of virologic failure (HR=1.39, 95% CI= 0.58, 3.29). Similar results were obtained when repeating this analysis using the presence of any minority variant (either K103N or Y181C) (data not shown).

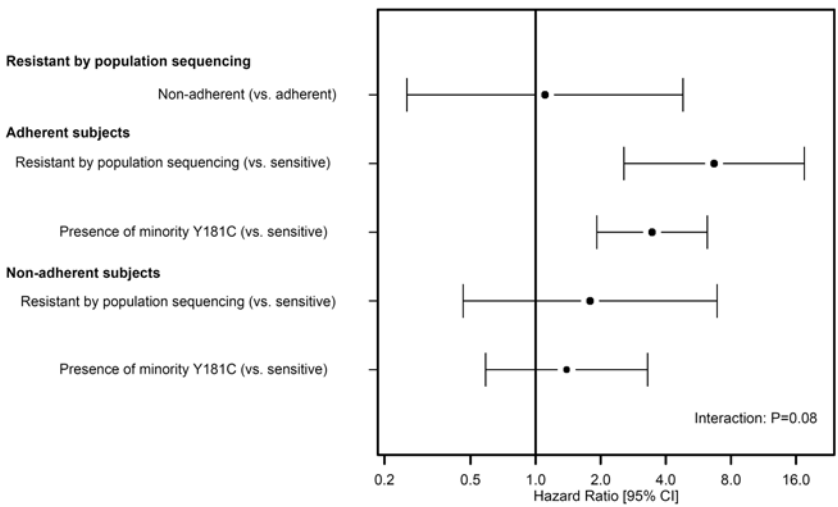

FIGURE 4. EFFECT OF Y181C IN THE PRESENCE OF RECENT ADHERENCE (ADJUSTED FOR PRESENCE OF MINORITY OF K103N BY ASPCR). As-treated weighted Cox proportional hazards model of time to virologic failure. Population sequencing (HIV-1 TruGeneTM assay, version 10 (Bayer HealthCare Diagnostics, Berkeley, CA)); Adherent, adequate recent self-reported adherence to therapy; Sensitive, no evidence of non-nucleoside reverse transcriptase inhibitors (NNRTI) resistance mutations by population sequencing and by allele-specific PCR (ASPCR); 95% CI, 95% confidence interval of the hazard ratio.

NNRTI RESISTANCE MUTATIONS AT VIROLOGIC FAILURE.

Sixty-five subjects with low-abundance Y181C mutants at baseline experienced virologic failure and had a viral genotype (by population sequencing) available at the time of virologic failure. No resistance

mutations were detected in 27 (41.5%), K103N was detected in 25 (38.5%), Y181C in 5 (7.7%) and K101E in 4 (6.2%); 2 of these 4 [3.1%] also had the K103N mutation (Table 3).

Table 3. Resistance mutations in HIV reverse transcriptase identified by population sequencing at the time of virologic failure

Resistance mutations	Number of subjects
None[a]	27
K103N	11
M184V,K103N	6
Y181C	3
M184V	2
D67N,K70R,M184V,K101E,G190A	1
D67N,M184V,K103N,P225H	1
K101E	1
K101Q,K103N	1
K103N,P225H	1
K65R,M184V,K103N,P225H	1
L100I,K101E,K103N	1
M184I,K103N	1
M184V,K101E,K103N	1
M184V,K103N,M230L	1
M184V,K219Q	1
M184V,V179D	1
V118I	1
Y181C,V108I	1
Y181C,G190S	1
Y188C,G190S	1

[a] Absence of resistance mutations by population sequencing.

DISCUSSION

Detection of pre-existing minority Y181C mutants encoding NNRTI resistance was associated with a more than 3-fold increased risk of virological failure to initial ART with efavirenz-based regimens in ART-naive HIV-1-infected subjects. The increased risk persisted across subjects with diverse baseline characteristics, including those with plasma HIV-1 RNA levels greater than or less than 100,000 copies/mL;

the risk magnitude was considerable and clinically relevant. The impact of the presence of low-abundance Y181C mutants on the risk of virologic failure was diminished among non-adherent subjects. These findings confirm the importance of pre-existing resistant viruses present as minority members of the viral quasispecies in determining the virologic outcome of ART, particularly in the case of drugs with a low genetic barrier to resistance. They also underscore the clinical need for improving the sensitivity of genotypic drug resistance assays.

Minority Y181C and K103N mutants were detected by ASPCR in nearly 40% of subjects with wildtype virus by standard genotypic testing. This prevalence represented an almost 9-fold increase in the detection of primary NNRTI resistance when the results of ASPCR plus population sequencing (44%) were compared to population sequencing alone (5%). The high prevalence of Y181C mutants was consistent with the rapid emergence of NNRTI-resistant viruses (mostly Y181C mutants) after the initiation of nevirapine when given alone or together with zidovudine [29-31].

In the current study, we did not detect an association with the presence of low-abundance K103N mutants and increased risk of virologic failure. This observation contrasts with previous studies, including our own finding in the same study population of a significantly increased risk of virologic failure when K103N was detected by population sequencing. However, this discrepancy may be attributable to the relatively small number of subjects with low-abundance K103N mutants identified by ASPCR.

It is noteworthy that all of the mutants identified by ASPCR in our study were present at levels below 1%. Although these low levels could represent underestimation due to polymorphisms at the primer binding sites in the target sequences, it would be surprising if this were the case in every subject tested. A more likely explanation is that Y181C and K103N mutants present at higher levels had already been identified by population sequencing, since samples from those subjects were not retested by ASPCR. This interpretation is consistent with data generated by ultradeep pyrosequencing [22], which found that NNRTI-resistant mutants were either present at relatively high levels (and thus

detectable by population sequencing) or at low levels (generally below 1%-5%). These findings suggest that ultrasensitive resistance assays should have sufficient sensitivity to detect variants present at less than 1%-5% of the plasma virus population.

Our study extends the findings of two earlier studies. A retrospective case-control analysis from the U.S. Centers for Diseases Control and Prevention (CDC) applied a modified ASPCR technique to baseline samples drawn from two clinical trials of efavirenz-containing first-line regimens [32]. Presence of minority mutations at RT codons 103, 181 or 184 was associated with an 11-fold increased odds of virological failure, but these mutations were detected in only a small number of subjects (7/95 with virologic failure and 2/221 with virologic suppression). The contribution of each individual mutation to the risk of virologic failure could not be assessed.

Analysis of baseline resistance by ultradeep pyrosequencing in the Flexible Initial Retrovirus Suppressive Therapies (FIRST) study, which compared initial ART strategies including an NNRTI, PI or both [33] found that pre-existing minority NNRTI-resistant variants more than tripled the hazard of virological failure in ART-naive subjects starting NNRTI-based therapy [22]. Similarly, all 4 subjects in the PI arm in whom PI-resistant minority variants were detected experienced virologic failure, but the numbers were too small to show a statistically significant increase in the risk of virologic failure.

The clinical application of ASPCR or any other resistance assay requires a precise refinement of thresholds that identify subjects at greatest risk of virologic failure. We were unable to define a threshold level of mutants that distinguished between subjects with virologic failure and subjects without virologic failure with high sensitivity and specificity. Although subjects with minority Y181C variants were at greater risk of virologic failure, 70% of these subjects nevertheless achieved long-term viral suppression on their initial efavirenz-based regimen. In post-hoc exploratory analyses, we were unable to identify factors that explained this difference. The high sensitivity of ASPCR may capture natural fluctuations within the quasispecies over time that are not necessarily clinically significant. Conversely, the other two

studies addressing the clinical relevance of minority variants used higher thresholds for detecting minor variants. Because the modified ASPCR method used in the CDC study [32] was designed to detect mutant viruses above the natural quasispecies frequency of each mutation, the actual threshold for detecting the K103N and Y181C mutants was 0.9% and 1.0%, respectively, which is at least two orders of magnitude higher than with our approach. Similarly, due to the error rate of pyrosequencing, the cut-off for detecting minority variants in the FIRST study [22] was established at 1%. Whereas the CDC and the FIRST studies could have missed clinically relevant minority mutants, a number of minority mutants detected in our study did not contribute to virologic failure during the study period. Determining the optimum threshold to maximize sensitivity and specificity requires analysis of a larger number of samples than available in studies performed to date.

Although the presence of pre-existing low-abundance Y181C mutants was associated with a greater risk of virologic failure, other EFV resistance mutations were more commonly found at the time of virologic failure. Similar results were obtained in the FIRST study [22]. It is possible that presence of the Y181C mutants was a marker for presence of other, undetected NNRTI mutants that emerged under efavirenz selection. Alternatively, the low-level EFV resistance conferred by Y181C could have allowed ongoing virus replication that led, in turn, to the later accumulation of other NNRTI resistance mutations such as K103N or G190S. Persistence of Y181C might have been selected against by the coadministration of zidovudine, since Y181C increases HIV-1 susceptibility to that drug [31].

In conclusion, low-abundance NNRTI-resistant variants significantly increased the risk of virologic failure to initial antiretroviral therapy with efavirenz. More sensitive resistance assays could improve the clinical management of HIV-infected subjects. The clinical application of such assays, however, will require further technical developments, a better understanding of the role of low-abundance resistant variants in different clinical scenarios and, refinement of assay thresholds that identify patients at greatest risk of virologic failure.

AUTHOR INFORMATION

CORRESPONDING AUTHOR: Daniel R. Kuritzkes, M.D., Section of Retroviral Therapeutics, Brigham and Women's Hospital, 65 Landsdowne St, Rm 449, Cambridge, MA 02139 (dkuritzkes@partners.org)

AUTHOR CONTRIBUTIONS: Dr. Paredes, Dr. Ribaudo, Ms. Lalama, Dr. Gulick and Dr. Kuritzkes had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

STUDY CONCEPT AND DESIGN: Paredes, Ribaudo, Shikuma, Gulick, Kuritzkes.

ACQUISITION OF DATA: Paredes, Ribaudo, Shikuma, Lalama, Schackman, Meyer, Giguel, Johnson, Fiscus, D'Aquila, Gulick, Kuritzkes.

ANALYSIS AND INTERPRETATION OF DATA: Paredes, Ribaudo, Lalama, Gulick, Kuritzkes.

DRAFTING OF THE MANUSCRIPT: Paredes, Ribaudo, Lalama, Gulick, Kuritzkes.

CRITICAL REVISION OF THE MANUSCRIPT FOR IMPORTANT INTELLECTUAL CONTENT: Paredes, Ribaudo, Shikuma, Lalama, Schackman, Johnson, Fiscus, D'Aquila, Gulick, Kuritzkes.

STATISTICAL ANALYSIS: Ribaudo, Lalama.

STUDY SUPERVISION: Paredes, Ribaudo, Shackman, Shikuma, Gulick, and Kuritzkes.

FINANCIAL DISCLOSURES:

Dr. Paredes reports having received research grants (awarded to the irsiCaixa Foundation) from Boehringer-Ingelheim, Monogram, Pfizer, and Merck; and received speaker honoraria from Siemens Medical Solutions. Dr. Shikuma reports having had affiliations with or financial involvement with Boehringer-Ingelheim, Bristol-Myers Squibb, Gilead, and GlaxoSmithKline. Dr. Johnson reports serving as a consultant to and/or having received grant support from Bayer, Bristol-Myers Squibb and GlaxoSmithKline. Dr. Fiscus reports having received speaker honoraria from Gen-Probe and Abbott Molecular and receiving kits

from Gen-Probe, Abbott Molecular, and Perkin-Elmer.Dr. D'Aquila reports having received grant support from Bristol-Myers Squibb and being a consultant to Boehringer-Ingleheim and GlaxoSmithKline.Dr. Gulick reports having received research grants (awarded to Cornell University) from Merck, Panacos, Pfizer, Schering and Tibotec; served as an ad hoc consultant to Bristol-Myers Squibb, Gilead, GlaxoSmithKline, Merck, Monogram, Pathway, Pfizer, Progenics, Schering, Tibotec, and Virostatics; and serving as DSMB Chair for Koronis. Dr. Kuritzkes reports having served as a consultant for and received speaker's fees and/or research support from Boehringer-Ingelheim, Bristol-Myers Squibb, and GlaxoSmithKline; and served as a consultant for and received research support from Bayer and Siemens. Ms. Lalama, Dr. Ribaudo and Dr. Shackman had no financial disclosures to report. None of the authors or clinical/laboratory site personnel received direct compensation from any of the pharmaceutical or diagnostic company collaborators for their participation in this study. All authors and clinical/laboratory site personnel received partial support from the National Institute of Allergy and Infectious Diseases (NIAID) through the AIDS Clinical Trials Group cooperative agreement. The pharmaceutical representatives to the protocol team were compensated as employees by the respective pharmaceutical cosponsors.

FUNDING/SUPPORT: This work was supported by NIH grants AI38858 and AI068636 (AIDS Clinical Trials Group Central Grant), AI069419, AI051966, AI069472, AI069452, RR00047; subcontracts from grants AI38858 and AI06836 with the Virology Support Laboratories at Massachusetts General Hospital, the University of Alabama, the University of North Carolina, and Vanderbilt University; the Birmingham Veterans Affairs Medical Center; and the Harvard University and University of Alabama at Birmingham and the University of North Carolina at Chapel Hill Centers for AIDS Research (AI060354, AI027767, and AI50410). Dr. Paredes was awarded the "La Caixa" Grant for Post-Graduate Studies, Caixa de Pensions de Barcelona, "La Caixa", Spain. Bristol-Myers Squibb and

GlaxoSmithKline provided drug for this study as well as financial support for plasma HIV-1 RNA determinations.

ROLE OF THE SPONSORS: This study was designed and conducted by investigators of the ACTG. Pharmaceutical company representatives to the protocol team had an opportunity to comment on the study design, but all final design decisions were made by the investigators. The NIAID provided final approval of the study prior to implementation. Conduct of the study was entirely the responsibility of the investigators, with regulatory oversight by the NIAID. Data collection, management, and interpretation were entirely the responsibility of the ACTG investigators. All members of the protocol team, including pharmaceutical company representatives and the NIAID, had an opportunity to comment on interpretation of the data, but final decisions regarding data interpretation were the prerogative of the ACTG investigators. The manuscript was prepared by a writing team comprising Drs Paredes, Ribaudo, Lalama, Gulick, and Kuritzkes and circulated to coauthors for review, comment, and approval. Once all of the authors had granted approval, the manuscript was circulated to protocol team members, including the pharmaceutical company representatives and the NIAID, for review and comment. The manuscript also received internal review by the ACTG scientific leadership and by the ACTG Statistics and Data Analysis Center prior to submission. Final responsibility for approval of the manuscript rests with the authors.

STATISTICAL ANALYSIS: For this National Institutes of Health–sponsored study of the ACTG, the statistical analysis was conducted by Heather Ribaudo, PhD, and Christina Lalama, MS, of the Statistical and Data Analysis Center at Harvard School of Public Health, Boston, Mass.

PREVIOUS PRESENTATION: Presented in part at the 15th Conference on Retroviruses and Opportunistic Infections, Boston, MA, 2008, Oral Abstract 83.

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Discussion

This doctoral thesis shows that pre-existing minority drug-resistant HIV-1 variants impair the virological outcomes of first-line antiretroviral therapy with non-nucleoside reverse transcriptase inhibitors. Our findings likely apply to other drugs with low genetic barrier to attain resistance, although this hypothesis should be directly addressed in specific studies. Given that minority resistant variants are systematically overlooked by standard drug resistance tests, more sensitive drug resistance assays must be incorporated to the clinical management of HIV-1-infected subjects. The clinical application of such assays, however, will require the definition of a threshold level of mutants that predicts treatment outcomes with high sensitivity and specificity, an objective that remains elusive.

TECHNICAL ASPECTS OF MINORITY VARIANT ASSAYS

Minority viral variants are rare events that follow a Poisson distribution;[1] all methods aiming to detect rare viral variants must account for this factor. Regardless the theoretical sensitivity of the resistance assay used, any analysis aiming to detect minority variants must ensure that enough viral variants are sampled to detect such rare variants with reasonable probability. Table 1 shows the number of variants that need to be assessed in order to detect at least one minority virus with various Poisson probabilities. As expected, the chances of finding such rare event depend on its frequency in the viral population.

For example, in order to detect one variant present in 1% of the viral population with a 95% Poisson probability, one needs to assess, at least, 300 sequences. If this variant was present in 0.1% of the population, 3000 sequences would need to be analyzed to attain 95% confidence. Indeed, if a higher degree of confidence was sought, more variants would need to be sampled. These figures clearly show that standard cloning procedures analyzing about 30 clones cannot detect variants present in less than 10% of the viral population. Ensuring an adequate sampling of the viral population is, therefore, the first essential step toward detection of minority viral variants.

Table 1. Number of sequences needed to be tested in order to detect at least 1 mutant with a given Poisson probability (p[x≥1]), if such mutation is present in 10%, 1%, 0.1% or 0.01% of the virus population.

Poisson	Frequency of the allelic variant in the population			
P (x ≥ 1)	10%	1%	0.1%	0.01%
9.52	1	10	100	1000
32.97	4	40	400	4000
39.35	5	50	500	5000
45.12	6	60	600	6000
50.34	7	70	700	7000
55.07	8	80	800	8000
59.3	9	90	900	9000
63.21	10	100	1000	10000
86.47	20	200	2000	20000
95.02	30	300	3000	30000
98.17	40	400	4000	40000
99.33	50	500	5000	50000
99.75	60	600	6000	60000
99.91	70	700	7000	70000
99.97	80	800	8000	80000
99.99	90	900	9000	90000
100	100	1000	10000	100000

The next question is how to guarantee that variant sampling is sufficient. As we show in Chapter 1, factors affecting viral sampling include the sample HIV-1 RNA levels, the volume of plasma from

which viral RNA is extracted, the fraction of eluted RNA used for reverse transcription and, finally, the efficiencies of RNA extraction and reverse transcription. The latter two factors can be calculated empirically, but this is a laborious and not always accurate procedure. For practical reasons, we decided to assume previously reported efficiencies for these two processes, i.e. an RNA extraction efficiency of 96% (http://www1.qiagen.com/literature/qiagennews/0398/983hiv1.pdf) and a reverse transcription efficiency of 70% (http://omrf.ouhsc.edu/~frank/CDNA.html). Although, such efficiencies may often be lower in reality, these assumptions provide a working framework that helps to set up specific criteria to rule out false positive results.

The sample HIV-1 RNA levels and the volume of plasma from which viral RNA is extracted are critical factors for ensuring adequate sampling that sometimes are overlooked in studies assessing minority variants. As we show in Chapter 1, high viral loads and high plasma volumes are required to guarantee the detection of minority viral variants. Unfortunately, a number of studies assessing minority variants perform ASPCR or other ultrasensitive resistance tests directly from viral RNA extraction products used for conventional genotypic resistance assays. Such genotypic assays usually require viral RNA to be extracted from 140 to 200 microliters of plasma. Whereas this volume is adequate for detecting the predominant viral variant, it is clearly insufficient to identify rare constituents of the viral quasispecies. For example, if a variant is present in 0.1% of the quasispecies, extraction of viral RNA from 200 microliters of plasma would allow detecting such variant only if the HIV-1 RNA levels were 100,000 copies/mL or higher. It is not surprising that studies using small plasma volumes for RNA extraction detect few minority variants, if any.

Another important consequence of the previous considerations is that low-abundant variants cannot be detected in subjects with low viral loads. It is easy to understand that one cannot find a variant present in 0.1% of the virus population (1 in 1000 viruses) from a sample with a viral load 100 copies/mL if viral RNA is extracted from 1 mL of plasma. We calculate in Chapter 1 the plasma volume from which viral

RNA should be extracted to enable the detection of a minority variant present at a percentage P in the viral population with 99% Poisson probability. The same equation can be used to assess the minimum HIV-1 RNA load needed to detect minor variants at a frequency of 0.1%, 1%, and 10%, assuming that the RNA is extracted from 1 mL of plasma.

An important contribution of our work relative to other studies is that we used these calculations to determine a sample-specific threshold of mutants that could be detected with 99% Poisson probability, according to the HIV-1 RNA extraction volume, the sample HIV-1 RNA levels and the assumed RNA extraction and reverse transcription efficiencies. In the clinical studies presented in this thesis, viral variants had to be detected at a frequency above both the theoretical ASPCR threshold and the sample-specific threshold to be considered detectable. This likely helped reduce false positive rates and strengthened clinical and virological correlations.

Another potential problem affecting all PCR-based minority variant assays is the existence of "founder effects" by which the viral sampling process can bias the population structure. Potentially, PCR reactions following cDNA synthesis could also modify the original quasispecies distribution. To avoid these problems, it is recommended to run RT-PCR and nested-PCR reactions in triplicate and pool PCR products, fine-tune the PCR conditions, and run negative and positive controls in parallel with patient-derived samples. The use of primers tagged with non-HIV sequences during RT-PCR to which nested PCR primers could anneal, could further reduce the chances of introducing population bias during nested-PCR steps.

Once DNA is ready for allele-specific real-time amplification, it is critical to ensure that the input DNA copies are high enough to be reliably quantified in real-time conditions. With all real-time PCR assays, amplification of samples with less than 100 DNA input copies becomes affected by random amplification events. Amplification of samples with less than 10 input DNA copies is usually achieved in less than 50% of the cases. Therefore, in order to quantify a variant that is present in 1 out of 1,000 viruses, one must ensure that the DNA input

of such minority variant is at least 100 copies; as a result, the total DNA input must be 100,000 copies or larger. We decided to establish a standard curve ranging 10^7 to 10^2 copies per reaction to ensure that real-time amplification proceeded in the most reliable conditions.

A major limitation of the ASPCR assay and of all other PCR-based minority variant methods is that polymorphisms in primer sites can affect the relative amplification efficiencies, thereby biasing the calculated proportions. Because we used a non-specific primer to amplify all sequences and a mutant-specific primer to selectively amplify mutants, and because both primers annealed in the same DNA regions, the occurrence of polymorphisms in primer sites tended to produce underestimations of the proportion of mutants. In assays using one specific primer for the mutant allele and another for the wild-type allele, proportions can either be over or underestimated. We show that inclusion of such polymorphisms in both primers and standards, corrects for such underestimation.

One final technical aspect that merits discussion is that, in our hands, it was essential that both discriminatory primer sets annealed to the same DNA positions (except for the extra base pair in the 3' end of the mutant-specific primer) in order to ensure the accuracy of measured proportions. One variant of the ASPCR assay used by the US Center for Disease Control and Prevention[2] uses standard viral load quantification to assess the total copy number, and allele-specific primers to assess the copy number of the different alleles. Although this modified assay has shown to produce clinically relevant data, it cannot calculate the proportion of mutants in blinded samples accurately.

VIRAL PATHOGENESIS STUDIES: MUTANT KINETICS AND VIRAL FITNESS

Once the performance of our ASPCR assay was ensured, we applied this assay to *in vivo* fitness studies. Viral fitness describes the ability of one virus (or viral variant) to generate progeny relative to another. There are multiple methods to assess relative fitness, both *ex vivo* and *in vivo*. *In vivo* fitness assays compare the relative proportions of the two variants over time in a specific clinical context, and use

different mathematical methods to assess the relative capacity of each variant to survive by generating progeny over time. Due to the technical aspects mentioned above, ASPCR is one of the most sensitive and accurate methods to quantify the proportion of different alleles over time.

In Chapter 2, we used this method to characterize with great detail the dynamics of M184V mutant decay over time in subjects infected with multidrug-resistant HIV who interrupted treatment with reverse transcriptase inhibitors and remained on protease inhibitors. Two technical aspects outstand from this study: first, that ASPCR measurements we highly concordant with bulk sequencing and phenotypic estimations; second, that the ASPCR was able to detect mutant viruses in the exponential decay phase with high accuracy, while the other methods did only detect wildtype. The precision of ASPCR in detecting mutant decay allowed us to: (a) characterize the exponential mutant decay phase with greater resolution, thereby improving the relative fitness calculations, and (b) describe two clearly differentiated phases after treatment interruption. We observed that lag phase following treatment interruption while mutant proportions remained relatively constant. This lag phase was of variable duration and its extent seemed to be the main determinant of the time to mutant decay. The initial lag phase was followed by an exponential decay phase that had a similar slope in all subjects. Because direct competition between virus variants is though to occur during the exponential decay phase, we used multiple proportion measurements obtained during this stage to calculate the relative fitness of M184V mutants versus 184M viruses. Further analyses showed that viruses likely decayed because of continued reverse transcriptase evolution and back mutation, rather than by the emergence of a pre-existing viral variant. We observed that negative selection or elimination of the less fit variants in the absence of therapy was the main selective mechanism driving mutant decay.

In a similar study by Marconi et al.,[3] the *in vivo* fitness cost of enfuvirtide resistance was determined by analyzing dynamic shifts in the HIV-1 quasispecies under changing drug selective pressure. The study analyzed three subjects on failing enfuvirtide-based regimens who

interrupted enfuvirtide while maintaining stable background regimens, and subsequently received this drug during 4 weeks as "pulse intensification." Using ASPCR to measure the proportion of plasma virus carrying the V38A mutation in gp41, this study demonstrated fitness differences for mutant versus wild type ranging from -2.5% to -6.5%, thereby providing *in vivo* evidence for the reduced fitness of enfuvirtide-resistant HIV-1. Interestingly, whereas the V38A mutants decayed slowly, they rapidly reemerged during the enfuvirtide pulse, and decayed slowly again after the second enfuvirtide interruption. This shows that (a) V38A mutants had remained incorporated in the virus quasispecies and were ready to emerge immediately after re-exposure to enfuvirtide, and (b) the viral fitness advantage of V38A mutants in the presence of drug was far larger than their fitness disadvantage in the absence of treatment.

Allele-specific PCR thus provides further insights into the pathogenesis of HIV and produces novel research questions. Future studies should assess whether the biphasic mutant decay dynamics observed in our study are universal or, instead, are only characteristic of certain mutants. This question is relevant because viral fitness calculations may vary depending on the number and type of allelic proportion measurements included. Two-timepoint *in vivo* fitness calculations would produce erroneous results by including the initial lag phase, where no direct competition is expected. On the other hand, our data suggest that the slope of exponential decay is constant for different subjects. It might be interesting to ascertain in larger cohorts whether the exponential mutant decay slope is a mutation-dependent constant, while environmental influences preferentially impact the duration of the initial lag phase. Finally, mathematical modeling could help explain the events occurring during the initial lag-phase, which seems to be the main determinant of the velocity at which mutants are lost *in vivo*. Time to back mutation seemed to be the rate-limiting step for mutant decay in this study, but reasons for delayed mutant decay may be different in other clinical contexts.

SURVEILLANCE OF ANTIRETROVIRAL DRUG RESISTANCE

Given that ASPCR is a more sensitive method to detect single mutations than population sequencing, we decided to apply this technology to antiretroviral resistance surveillance in well-defined clinical cohorts. For this purpose, we evaluated the prevalence of primary (Chapter 3) and postpartum (Chapter 4) antiretroviral resistance in previously antiretroviral-naïve, HIV-1-infected pregnant women enrolled in the *Women and Infants Transmission Study* (WITS). The WITS is a multi-center, longitudinal, natural history study of pregnant women with HIV infection and their offspring. This prospective cohort study is designed to answer questions such as likelihood of HIV transmission, the effect of perinatally acquired HIV infection on the infant, and the most effective means for early diagnosis of HIV infection in the infant. Current research also seeks to determine risk factors for ante-partum vs. intra-partum transmission and the most useful predictors for HIV disease progression in postpartum.

The studies presented in Chapters 3 and 4 show that resistance tests based on viral population sequencing underestimate the prevalence of variants harboring resistance mutations to lamivudine and emtricitabine (M184V), nelfinavir (D30N) and nevirapine and efavirenz (K103N). Using ASPCR, these mutations could be detected in 1.5 to 3-fold more subjects than with population sequencing alone. Several studies, including the one presented in Chapter 5, show that minority drug resistant variants impair the virological outcomes of antiretroviral therapy.[2, 4, 5] Therefore, incorporating ultrasensitive resistance assays to surveillance of resistance mutations may provide clinically relevant information for the management of HIV-1-infected patients.

Chapter 3 describes a high prevalence of M184V and D30N mutations in antiretroviral-naïve, HIV-1 infected pregnant women in the US between 1998 and 2004. These findings are concordant with the increased transmission of resistant variants during this period in the US.[6-9] They also support routine genotypic resistance testing before initiating mother-to-child transmission prophylaxis (PMTCT) and using triple-drug PMTCT regimens to maximize the efficacy of this strategy and reduce the likeliness of resistance evolution. The utility of ASPCR

for primary resistance surveillance was also shown by Metzer et al.[10] in a study assessing the prevalence of mutations L90M, K103N and M184V in 49 recent seroconverters by ASPCR and bulk sequencing. Drug-resistant variants were detected in 20.4% of subjects. The L90M, K103N and M184V were found, respectively, in one (2%), five (10.2%) and six out of 49 (12.2%) patients. In five of the 10 individuals with detectable drug-resistant virus, the detected population represented a minor viral variant that had been missed by bulk sequencing. Therefore, assays that are more sensitive detect more resistance mutations; this enables a better understanding of the extent, mechanisms and consequences of transmitted resistance. Moreover, a careful evaluation of the level of mutants detected in antiretroviral naïve, recently HIV-1-infected subjects with and without transmitted resistance, may be highly useful to establish the threshold level of minority variants that differentiates transmitted resistance from spontaneous mutant generation.

In Chapter 4, we analyzed the prevalence of resistance post-partum in antiretroviral-naïve HIV-1-infected pregnant women that received pregnancy-limited antiretroviral therapy to prevent vertical transmission. From a methodological perspective, this chapter shows how ASPCR can also be applied to assess the prevalence of secondary –or acquired- antiretroviral resistance. As with studies in antiretroviral naïve patients, this study found that 2- to 3-fold more resistance mutations could be detected by using ASPCR in comparison with population sequencing of plasma viruses. In addition, this study provided highly relevant data for the clinical management of HIV-infected pregnant women.

We observed that pregnancy-limited antiretroviral therapy was associated with postpartum detection of antiretroviral resistance mutations in a high proportion of previously antiretroviral-naïve mothers. Virtually all women receiving dual therapy developed the M184V mutation, which confers high-level resistance to lamivudine and emtricitabine[11, 12] and is associated with an increased risk of virological failure of treatment combinations including these drugs.[13] Thymidine analogue resistance mutations were also more frequent in those receiving dual therapy. The most striking finding, however, was that up

to 50% of women treated with three antiretroviral drugs also developed the M184V mutation, as assessed by ASPCR. Moreover, NNRTI resistance mutations were detected in 40% of women receiving nevirapine during pregnancy-limited therapy. This high prevalence of postpartum NNRTI resistance should be interpreted with caution because of the number of women surveyed was small and pre-treatment resistance data was available from few women. However, it suggests that a remarkable proportion of women receiving nevirapine during pregnancy may develop viruses resistant to at least 2 of the drugs included in the initial regimen most frequently prescribed nowadays, i.e. co-formulated tenofovir / emtricitabine / efavirenz. It is well established that women selecting lamivudine, emtricitabine or NNRTI-resistant mutants during pregnancy-associated treatment are at a higher risk of failing subsequent NNRTI-based antiretroviral therapy, particularly if treatment is started within 6 to 12 months after delivery.[5, 13] One encouraging finding of our study was that detection of PI resistance was rare in women treated with nelfinavir. Although nelfinavir-based therapy is no longer a preferred regimen for PMTCT,[14] the findings of this study likely apply to other protease inhibitors.

The abovementioned findings suggest that, in order to reduce resistance selection during pregnancy, dual antiretroviral MTCT prophylaxis should be avoided. In addition, when possible, PMTCT regimens should include drugs with high genetic barrier. Finally, given that resistance mutations will wane after treatment interruption and might be undetectable in subsequent genotypic tests in the absence of therapy, postpartum genotypic resistance testing within 1-2 months after stopping treatment could be the most effective way of identifying resistance mutations selected during pregnancy-limited therapy. Postpartum genotypic resistance testing would, thus, be useful in guiding the choice of future antiretroviral regimens in women receiving pregnancy-limited antiretroviral therapy.

CLINICAL OUTCOMES IN THE PRESENCE OF MINORITY DRUG-RESISTANT VARIANTS

The last and most important finding of this thesis was that pre-existing minority NNRTI-resistant viruses more than tripled the risk of virological failure to initial NNRTI-based therapy in ART-naive HIV-1-infected subjects with adequate recent adherence. As expected, the risk of virological failure of non-adherent subjects was not different between those with or without pre-existing minority NNRTI-resistant variants. These findings confirm that pre-existing minority resistant viruses impair the virological outcome of antiretroviral therapy, and underscore the need for improving the sensitivity of current drug resistance assays.

The study presented in Chapter 5 was a case-cohort substudy of the AIDS Clinical Trials Group (ACTG) 5095 Study. The ACTG comprises the largest network of expert clinical and translational investigators and therapeutic clinical trials units in the world. The ACTG 5095 Study demonstrated the virological inferiority of 3-NRTI regimens in comparison with regimens including efavirenz plus two or three NRTIs. As has been explained previously by Kuritzkes et al.,[15] "a case-cohort study design uses a sub-sampling technique in survival data for estimating the relative risk of disease in a cohort study without collecting data from the entire cohort. This design is an efficient and economical way to study risk factors for infrequent disease in a large cohort. It involves the collection of covariate data from all disease cases observed in the entire cohort, and from the members of a random subcohort. In addition to ascertaining the relative risk of disease in relation to the risk factors of interest, the random subcohort also offers the ability to assess the prevalence of risk factors that may be too costly to evaluate on the entire cohort."

As with previous analyses, our study showed that ASPCR increased the detection of resistance mutations several-fold. Whereas the increased prevalence of K103N mutants was commensurate with previous observations using ASPCR or ultradeep sequencing,[10, 16, 17] there was a surprisingly high prevalence of Y181C mutants that, nevertheless, had been suggested in previous studies.[18-20] The finding that nearly 40% of subjects with wildtype virus by standard genotypic testing had

minority NNRTI-resistant variants, mostly Y181C represented an almost nine-fold increase in the overall prevalence of primary NNRTI resistance relative to population sequencing alone. The high prevalence of minority Y181C mutants might also help explain the worse virological outcomes of nevirapine relative to efavirenz in subjects with prior exposure to suboptimal NRTI regimens.

A highly relevant finding for the continued development of ultrasensitive resistance assays was that minority mutants were present at levels below 1%. This observation is consistent with data generated by ultradeep sequencing,[17] which found that most NNRTI-resistant mutants were either present at relatively high levels (and thus detectable by population sequencing) or at very low levels, generally below 1%-5%. Therefore, ultrasensitive resistance assays should have sufficient sensitivity to detect variants present at less than 1%-5% of the plasma virus population to be clinically useful.

At least two other studies show similar results to ours. The first study is a retrospective case-control analysis from the U.S. Centers for Diseases Control and Prevention (CDC) that detected minority variants carrying the M184V, K103N or Y181C mutations in 7/95 (7%) subjects with virological failure to first-line regimens including efavirenz, versus 2/221 subjects (0.9%) with virological suppression through the first 48 weeks of therapy.[2] Such differences were statistically significant in a logistic regression model. However, only four subjects with virological failure and one individual with persistent viral suppression had minority K103N mutants detected before treatment, and only one in each group had preexisting minority Y181C mutants. Therefore, the contribution of each individual mutation to the risk of virological failure could not be assessed.

The CDC study used a modified ASPCR technique and defined clinical cutoffs for each mutant using pre-treatment stored samples. The aim was to avoid detecting mutants generated by spontaneous errors during reverse transcription, assuming that only transmitted and not spontaneously generated mutants are clinically relevant. This strategy has two potential problems, in our opinion.

On one hand, there is no evidence that minority resistant mutants originated from transmission events may be present at different levels in the virus population than mutants generated spontaneously. Resistant viruses are often transmitted without simultaneous transmission of wildtype variants; thereby, transmitted resistant mutants gradually loose resistance mutations through back mutation. Given the absence of competition with wildtype, mutant reversion is an extremely slow process and transmitted mutants remain detectable by population sequencing many years after transmission.[21] However, once the appropriate revertant is generated, it outcompetes the transmitted mutant at a rate proportional to its relative fitness advantage in the absence of therapy. The logical consequence of such competition is that the resistant mutant is brought to minimum levels in a relatively fast phase transition. It remains to be demonstrated whether such mutant remains incorporated in the virus quasispecies at a higher level than if it had been spontaneously generated.

The second conceptual problem of the CDC study is the assumption that spontaneously generated mutants do not have the capacity to impair the virological outcomes of antiretroviral therapy. From an evolutionary perspective, the only reason for the virus to generate such mutants is, precisely, to escape from adverse environmental pressure, including that of antiretroviral drugs.

Another study concordant with our findings is a reanalysis of baseline resistance of the CPCRA 058 FIRST Study.[22] In this study, antiretroviral-naïve HIV-1-infcted subjects were randomly assigned to receive initial antiretroviral therapy including 2 nucleoside analogues plus an NNRTI (nevirapine or efavirenz), a PI, or one NNRTI plus one PI. Interestingly, the parent trial failed to demonstrate an association between pre-existing antiretroviral resistance by bulk sequencing and treatment outcomes. The reanalysis looking for minority variants showed that pre-existing low-abundant NNRTI-resistant variants detected by ultradeep pyrosequencing more than tripled the hazard of virological failure in ART-naive subjects starting NNRTI-based therapy.[17] By contrast, detection of minority PI-resistant mutants was not associated with an increased risk of virological failure

in the PI arm[17], suggesting that minority variants might not reduce the efficacy of regimens with high genetic barrier.

Before ultrasensitive resistance tests can be routinely applied in clinical practice, however, thresholds that identify patients at greatest risk of virologic failure need to be identified. Despite being at higher risk for virological failure, still 70% of subjects with minority Y181C variants in our study achieved long-term viral suppression on their initial efavirenz-based regimen, and there were no significant differences in levels of minority variants between responders and non-responders. The CDC study used a method that does not allow the precise measurement of mutant proportions in patient samples. Using external controls, however, they reported a sensitivity threshold to detect the K103N and Y181C mutants of 0.9% and 1.0%. The cut-off for detecting minority variants in the FIRST study[17] was established at 1%. As we discuss in Chapter 5, the CDC and the FIRST studies likely missed clinically relevant minority mutants whereas a several low-abundance mutants detected in our study did not contribute to virological failure. Determining the optimum threshold to maximize sensitivity and specificity is the main research venture ahead to enable the clinical application of these assays. However, this will probably require analysis of a larger number of samples than available in studies performed to date.

In conclusion, Chapter 5 shows that minority NNRTI-resistant variants significantly increase the risk of virological failure to initial antiretroviral therapy with efavirenz. These findings have been included in the 2008 update of the IAS-USA resistance testing guidelines.[23] The same observations could apply to other treatment combinations including drugs with low-genetic barrier, although specific studies should address this question. Minority variants might be less important for first-line regimens including drugs with high genetic barrier like ritonavir-boosted PIs. It is uncertain if they could be more relevant to guide the design of salvage therapy regimens in including ritonavir-boosted PIs, or in the event of transmitted variants with resistance mutations in protease.

Whereas more sensitive resistance assays could improve the clinical management of HIV-infected subjects, the clinical application of such assays, will require further technical developments, a better understanding of the role of minority resistant variants in different clinical scenarios and, refinement of assay thresholds that identify patients at greatest risk of virologic failure.

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Conclusions

1. In the setting of adherence, pre-existing minority Y181C mutants more than triple the risk of virological failure of first-line antiretroviral therapy with non-nucleoside reverse transcriptase inhibitors.

2. Allele-specific PCR is a sensitive and reliable method for detecting low-abundant resistant variants carrying single mutations and allows precise estimations of the kinetics of particular mutants and their relative fitness *in vivo.*

3. In vivo, M184V variants are consistently 4 to 8% less fit than the wild type in the absence of drug. After a lag phase of variable duration, wild-type variants emerge due to continued evolution of *pol* and back mutation rather than through emergence of an archived wild-type variant.

4. Minority variant assays improve surveillance of primary and secondary antiretroviral drug resistance.

5. There is a high prevalence of primary lamivudine and nelfinavir resistance among HIV-1-infected pregnant women in the US.

6. Pregnancy-limited antiretroviral therapy is associated with frequent selection of resistance to drugs with low-genetic barrier. Routine postpartum genotypic resistance testing may be useful to guide future treatment decisions in mothers.

7. More sensitive resistance assays could improve the clinical management of HIV-infected subjects.

8. The clinical application of such assays, however, requires further technical developments, a better understanding of the role of low-abundance resistant variants in different clinical scenarios and, refinement of assay thresholds that identify patients at greatest risk of virologic failure.

Future Research Questions

Based on the findings of this thesis, the following research questions are suggested:

1. What is the threshold level of minority mutants that identifies patients at greatest risk of virologic failure with high sensitivity and specificity?

2. In case this threshold exists, which are its determinants? Is it mutant-specific? Is it technique-specific? Do CD4+ counts or other HIV-associated biological parameters modify this threshold?

3. Do HIV-specific immune responses influence the threshold level of mutants that predicts virological failure?

4. Do HIV-1 variants harboring resistance to integrase inhibitors, fusion inhibitors and CCR5 antagonists exist in antiretroviral naïve subjects?

5. In case they pre-exist, do they also impair the virological outcome of regimens including integrase inhibitors, fusion inhibitors or CCR5 antagonists?

6. What is the level of minority resistant variants generated by spontaneous reverse transcription errors?

7. Is this level significantly different from that of transmitted variants, once the latter are outcompeted by wildtype revertants?

8. Does the level of minority variants decrease over time in the absence of selective pressure? At what rate?

9. Do minority variants ever become extinct?

10. Do minority resistant variants affect the outcomes of drugs with high genetic barrier like ritonavir-boosted inhibitors? In which clinical contexts?

11. Do minority X4 viruses pre-exist in all subjects?

12. Is the level of preexisting minority X4 viruses different in various stages of HIV infection? Why?

13. Are minority X4 viruses ever transmitted? At what rate? Under which circumstances?

14. Are pre-existing minority X4 viruses associated with worse virological outcomes of CCR5 antagonist therapy?

15. How many different single mutants are spontaneously generated? How many dual mutants? Are there any triple mutants being generated spontaneously?

16. Which are the clinical correlates of preexisting viruses with several mutations linked on single genomes?

17. What ultrasensitive resistance test is most useful for the clinical management of HIV-infected patients?

18. Is it cost-effective to detect minority resistant HIV-1 variants for the clinical management of HIV-1-infected subjects?

19. Do all mutant viruses decay in two phases in the absence of treatment?

20. What biological events characterize the lag phase in mutant decay observed after treatment interruptions?

21. What is the best mathematical approach to calculate viral fitness *in vivo* to account for the different viral decay phases and factors contributing to viral decay?

22. Which are the most conserved HIV genomic regions in the quasispecies?

Addendum I

Published Manuscripts in Portable Document Format (PDF)

CHAPTER 1

Roger Paredes, Vincent C. Marconi, Thomas B. Campbell, Daniel R. Kuritzkes. **Systematic Evaluation of Allele-Specific Real Time PCR for the Detection of Minor HIV-1 Variants with pol and env Resistance Mutations.** J Virol Methods 2007;146(1-2):136-46

CHAPTER 2

Roger Paredes, Manish Sagar, Vincent C. Marconi, Rebecca Hoh, Jeffrey N. Martin, Neil T. Parkin, Christos J. Petropoulos, Steven G. Deeks, and Daniel R. Kuritzkes. **In Vivo Fitness Cost of the M184V Mutation in Multidrug-Resistant HIV-1 in the Absence of Lamivudine.** J Virol 2009; 83(4). [Epub ahead of print]

CHAPTER 3

Roger Paredes, Irene Cheng, Daniel R. Kuritzkes, Ruth E. Tuomala, & the Women and Infants Transmission Study Group **High Prevalence of Primary Lamivudine and Nelfinavir Resistance in HIV-1 Infected Pregnant Women in the US, 1998-2004.** AIDS 2007; 21(15):2103-6.

Available online at www.sciencedirect.com
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Journal of Virological Methods 146 (2007) 136–146
Journal of Virological Methods
www.elsevier.com/locate/jviromet

Systematic evaluation of allele-specific real-time PCR for the detection of minor HIV-1 variants with *pol* and *env* resistance mutations

Roger Paredes[a,b], Vincent C. Marconi[a,c], Thomas B. Campbell[d], Daniel R. Kuritzkes[a,*]

[a] *Section of Retroviral Therapeutics, Brigham and Women's Hospital and Division of AIDS, Harvard Medical School, Boston, MA, USA*
[b] *Fundacions "irsiCaixa" i Lluita contra la SIDA, Hospital Universitari Germans Trias i Pujol, Universitat Autonòma de Barcelona, Badalona, Catalonia, Spain*
[c] *Wilford Hall USAF Medical Center, San Antonio, TX, USA*
[d] *Division of Infectious Diseases, Department of Medicine, University of Colorado Health Sciences Center, Denver, CO, USA*

Received 12 March 2007; received in revised form 13 June 2007; accepted 20 June 2007
Available online 26 July 2007

Abstract

Allele-specific PCR (ASPCR) is a highly sensitive, and reproducible method for the study of minor HIV-1 variants harboring resistance mutations and is significantly less labor-intensive and time-consuming than other techniques used for similar purposes. Furthermore, ASPCR has multiple applications in HIV research: it provides earlier and more sensitive detection of evolving resistance mutations, a more accurate assessment of transmitted drug-resistant mutants and a better evaluation of resistance selection after post-exposure or mother-to-child-transmission prophylaxis programs. This article outlines the principles of ASPCR and illustrates technical challenges in the design and application of ASPCR protocols by describing ASPCR assays developed for detecting resistance mutations in the protease (PR)- and reverse transcriptase (RT)-coding regions of *pol* and *env*. The assays achieved sensitivities of <1% for the D30N mutation in HIV-1 PR, M184V and I mutations in RT, and V38A in gp41. This method can be easily adapted to the quantitative detection of other mutations in HIV-1 or other viruses by introducing minor modifications to the methods described. In addition, ASPCR can be used to assess the dynamics of mutant populations in the viral quasispecies in response to changing selection pressures, allowing inferences on viral fitness in vivo through mathematical modeling.

Keywords: HIV-1; Antiretroviral drug resistance; Allele-specific PCR; Real-time PCR; Minority variants; Quasispecies

1. Introduction

Accurate detection of resistance mutations is important for the clinical management of HIV-infected persons and has important public health implications (Johnson et al., 2005; Wensing et al., 2005). Antiretroviral drug resistance is associated with worse virological, immunological and clinical outcomes (Boucher et al., 1992; Harrigan et al., 1999; Japour et al., 1995; Middleton et al., 2001; Ross et al., 2001). Conversely, use of resistance information for the design of treatment combinations significantly improves such outcomes (Baxter et al., 2000; Cingolani et al., 2002; Cohen et al., 2002; Durant et al., 1999; Haubrich et al., 2005; Meynard et al., 2002; Tural et al., 2002).

As with other RNA viruses (Cristina, 2005; Gonzalez-Lopez et al., 2005; Jerzak et al., 2005) the HIV population in an infected person constitutes a quasispecies (Coffin, 1995; Domingo and Holland, 1997). Standard genotypic tests only detect resistance mutations present in more than 20% of the viral population (Brun-Vezinet et al., 2004; Grant et al., 2003; Halvas et al., 2006) likely underestimating the prevalence of drug resistance mutations at any given time point. Genotypic resistance tests performed by reference laboratories generally rely on population-based sequencing and report the consensus sequence at each nucleotide position. Although these tests can detect the presence of mixed populations, they provide only a rough estimate of the relative proportions of wild-type and mutant species in the population. Mathematical models estimate significant delays between the emergence of resistance and its detection partly because of the low sensitivity of current genotyping methods (D'Amato et al., 1998). More sensitive techniques to assess drug resistance in minor variants have been developed in the

* Corresponding author at: Section Retroviral Therapeutics, Brigham and Women's Hospital, 65 Landsdowne Street, Room 449, Cambridge, MA, USA. Tel.: +1 617 768 8371; fax: +1 617 768 8738.
E-mail address: dkuritzkes@partners.org (D.R. Kuritzkes).

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doi:10.1016/j.jviromet.2007.06.012

recent years, including single-genome sequencing (Palmer et al., 2005), allele-specific PCR (ASPCR) (Bergroth et al., 2005; Metzner et al., 2003, 2005), hybridization assays (Flys et al., 2005; Shi et al., 2004), phenotypic analysis using *S. cerevisiae* (Nissley et al., 2005; Shi et al., 2004) and massively parallel sequencing in microfabricated PicoTiterPlates (Leamon et al., 2003; Margulies et al., 2005).

The first use of allele-specific nested PCR (ASPCR) to detect resistance mutations in HIV-1 was reported in 1991 (Larder et al., 1991). The more recent application of real-time PCR technology to ASPCR has increased the sensitivity of this technique several-fold and permitted quantification of the PCR products (Metzner et al., 2003, 2005).

Here, an ASPCR protocol to detect resistance mutations in HIV-1 *pol* and in the gp41-coding region of *env* with high sensitivity, accuracy and reproducibility is described. The theoretical reasoning that supports this method is discussed and some practical guidance is offered to researchers interested in applying this technique to the detection of these and other mutations in HIV-1 and other viruses.

2. Materials and methods

2.1. Primers and probes

The design of all PCR primers and probes (Table 1) was based on the HIV-1_{Hxb2} *pol* and *env* sequences.

2.2. Construction of standards

Plasmids carrying wild-type HIV-1 *pol* and *env* were constructed by cloning the relevant segments of HIV-1 NL4-3 into a pGEM® T-Easy vector (pGEM® T-Easy Vector System, Promega Corporation, Madison, WI, USA) to create pPOL-W and pENV-W, respectively. For pPOL-W, the insert corresponded to a 1256 bp amplicon obtained with primers OOPF and OOR2 (Table 1); for pENV-W, the insert corresponded to

Table 1
Oligonucleotide sequences of RT-PCR, ASPCR and TaqMan probes[a]

	Primer name	Length (bp)	T_m (°C)	Sequence (5′–3)	Nucleotide position (HXB2)
M184V ASPCR assay					
RT-PCR	OOPF	22	64	GAAGCAGGAGCCGATAGACAAG	2211–2232
	OOR2	23	63	TTTTCTGCCAGTTCTAGCTCTGC	3466–3444
ASPCR					
NS	VN	21	52	GACATAGTTATCTATCAATAC	3078–3098
Sp	V4	22	55	GACATAGTTATCTATCAAT***T*****CG***	3078–3099
R	ASR2	20	56	GGCTGTACTGTCCATTTATC	3277–3258
M184I ASPCR assay					
RT-PCR	OOPF	22	64	GAAGCAGGAGCCGATAGACAAG	2211–2232
	OOR2	23	63	TTTTCTGCCAGTTCTAGCTCTGC	3466–3444
ASPCR					
NS	IN	23	54	GACATAGTTATCTATCAATACAT	3078–3100
Sp	15	24	57	GACATAGTTATCTATCAATACA***I*****A***	3078–3101
R	ASR2	20	56	GGCTGTACTGTCCATTTATC	3277–3258
D30N ASPCR assay					
RT-PCR	OOPF	22	64	GAAGCAGGAGCCGATAGACAAG	2211–2232
	OOR2	23	63	TTTTCTGCCAGTTCTAGCTCTGC	3466–3444
ASPCR					
(NS)	DN	21	55	CTATTAGATACAGGAGCAGAT	2319–2339
(Sp)	DS4	22	55	CTATTAGATACAGGAGCA***A*****AT*****A***	2319–2340
(R)	DR2	20	56	CTGGCTTTAATTTTACTGGTAC	2592–2571
V38A ASPCR assay					
RT-PCR	GP41OF	24	62	GAGGGACAATTGGAGAAGTGAATT	7649–7672
	GP41OR	24	62	GTGAATATCCCTGCCTAACTCTAT	8364–8341
ASPCR					
NS	IFN8	21	53	GACAATTATTGTCTGGTATAG	7849–7869
Sp	IFS3C2	22	58	GACAATTATTGTCTGGTAT***C*****G*****C***	7849–7870
R	IR4	18	56	AATCCCCAGGAGCTGTTG	8009–7992
TaqMan probe	V38Probe	27	68	(6-FAM)-TCCTTTAGGTATCTTTCCACAGCCAGG-(TAMRA) (phosphate)	7990–7964

[a] The target mutation is shown in boldface, underlined and next to an asterisk. Intentional mismatches in mutant-specific primers are shown in boldface italics and underlined. ASPCR means allele-specific polymerase chain reaction. RT-PCR means one-step reverse transcription and PCR amplification. NS means non-specific primer. Sp means specific primer. R means reverse (antiparallel) primer.

Fig. 1. Overview of ASPCR primer design. The mutant-specific primer (Sp) incorporates the target mutant sequence (square) in its 3′-end plus an intentional mismatch (circle) in the −1 to −3 positions to increase the specificity of the mutant-specific amplification reaction (i.e. constrain the amplification of wild-type variants). The non-specific primer (NS) is identical to the Sp primer, except that it ends right before the target codon and does not incorporate any intentional mismatch. Both specific and non-specific amplification reactions are performed in separate wells using a common antiparallel (CA) primer. Amplified DNA can be quantified in real-time using SYBR+™Green, a TaqMan™ probe, molecular beacons or scorpions.

a 715 bp amplicon derived from the gp41-coding region of *env*, obtained with primers GP41OF and GP41OR.

The following mutations were introduced individually by site-directed mutagenesis (Quick Change® XL Site Directed Mutagenesis Kit, Stratagene, La Jolla, USA) into pPOL-W to generate three different single-mutant plasmids (Fig. 1): M184V (ATG → GTG, [pPOL-184V]), M184I (ATG → ATA, [pPOL-184I]) and D30N (GAT → AAT, [pPOL-30N]). Likewise, a plasmid (pENV-38A) carrying the V38A (GTG → GCG) mutation was obtained by introducing this mutation into pENV-W. Presence of the appropriate mutations was confirmed by sequencing the resulting plasmids.

PCR products were purified (QIAquick® PCR Purification Kit, Qiagen Sciences, Maryland, USA) and quantified by spectrophotometry. Equivalence between starting amounts of wild-type and mutant plasmid DNA was verified by real-time PCR. Serial 10-fold dilutions of each amplicon were prepared ranging from 10^6 to 10 copies per reaction. Standards were always run in duplicate at the same time as test samples. Given that the nonselective amplification did not depend on the nucleotide composition of the target codon, only mutant amplicons were used to generate both specific and non-specific standard curves for clinical specimen analysis. This ensured identical starting DNA copies for both standard reactions and enabled parallel, comparable curves. Wild-type amplicons were used to prepare mixtures with mutant amplicons in order to test the properties of the technique.

2.3. HIV-1 RNA extraction from plasma

Plasma samples were obtained from subjects participating in clinical trials of antiretroviral therapy approved by the respective human subjects research committees, and were stored at −70 °C. HIV-1 RNA was extracted from EDTA-anticoagulated plasma using the QIAamp® Viral RNA MiniKit (Qiagen Sciences, Maryland, USA) according to the manufacturer's instructions. Part of each RNA sample was used for cDNA synthesis immediately after extraction, and the remainder was stored at −80 °C.

2.4. Reverse transcription and first-round DNA amplification

The extracted RNA was transcribed to cDNA and amplified by PCR in a one-step process (Superscript III one-step RT-PCR with Platinum Taq Kit, Invitrogen™, Carlsbad, CA, USA) following the manufacturer's instructions. *Pol* and *env* fragments were amplified separately. Cycling conditions for *pol*-derived DNA included an initial cDNA synthesis step at 55 °C during 25 min, followed by a denaturation step at 94 °C during 2 min, 25 cycles of PCR amplification (94 °C during 40 s, 60 °C during 40 s, 68 °C during 1 min and 20 s), and a final 5 min extension step at 68 °C. The PCR mix contained 25 μL of 2× Reaction Mix (including 0.4 mmol/L of each dNTP and 3.2 mmol/L of $MgCl_2$), 0.2 μmol/L of each primer OOPF and OOR2, 15 μL of extracted RNA as template and nuclease-free H_2O to a final volume of 50 μL. The same PCR mix was used for the *env*-derived DNA but with primers GP41OF and GP41OR. Thermal cycling conditions for *env*-derived DNA included 55 °C for 25 min, followed by a denaturation step at 94 °C for 2 min, 20 cycles of PCR amplification (94 °C for 15 s, 50 °C for 30 s, 68 °C for 1 min), and a final 5 min extension step at 68 °C. The PCR products were purified using the QIAquick® PCR Purification Kit (QIAquick® PCR Purification Kit, Qiagen Sciences, Maryland, USA).

2.5. Quantification of viral populations using real-time PCR

Different fluorescence reporter molecules were used for each protocol, SYBRgreen for *pol*-derived samples and a TaqMan probe for *env*-derived samples, in order to illustrate different approaches to ASPCR design. To quantify the proportion of mutant sequences contained within each specimen, 5 μL of RT-PCR product were added to the real-time PCR together with selective or nonselective primers. Conditions for nonselective amplification of *env*-derived samples were 1× TaqMan® PCR Master Mix™ (Applied Biosystems, Warrington, UK), 1 μM of each primer IFN8 and IR4 (Fig. 1, Table 1), 500 nM of V38A TaqMan® probe, and deionized water to a final volume of 50 μL. Conditions for selective amplification of the V38A mutant sequence were identical except that primer IFN8 was replaced by IFS3C2. Conditions for nonselective amplification of *pol*-derived samples were 1× SYBR® green PCR Master Mix™ (Applied Biosystems, Warrington, UK), 900 nM of each non-specific and reverse primers, and deionized water to a final volume of 50 μL. Conditions for selective amplification of *pol* mutant sequences were identical except that the specific primer replaced the non-specific primer. Non-specific primers for the D30N, M184V and M184I ASPCR protocols were primers DN, VN and IN, respectively (Fig. 1, Table 1). Specific primers for the D30N, M184V and M184I ASPCR reactions were, respectively, primers DS4, V4, and I5. Reverse primers for D30N and M184V/I ASPCR experiments were, respectively, primers DR2 and ASR2.

Each sample was evaluated by real-time PCR in an ABI 7000 Sequence Detection System thermocycler (Perkin-Elmer Applied Biosystems, Foster City, CA, USA), using the following cycling parameters: 50 °C for 2 min to activate the AmpErase® UNG included in the mastermix, which prevents PCR product carryover, 95 °C for 10 min to activate the AmpliTaq Gold®

DNA Polymerase, followed by 50 cycles at 95 °C for 15 s and 50 °C for 1 min. Amplicons prepared from the relevant plasmids using primers OOPF and OOR2, or GP41OF and GP41OR served as standards. The number of cycles required to reach threshold fluorescence (C_t) was determined and the quantity of sequences initially present was calculated by interpolation onto the standard curve.

The three different ASPCR protocols (D30N, M184V/I and V38A) were performed separately, each with a corresponding set of standards. Nonselective and selective amplifications were always performed in parallel. All reactions were performed in duplicate, and the mean of the two values was used for calculation. The percentage of viral sequences containing each mutation was calculated as follows: % mutant sequences = [(quantity of mutant sequences in the sample)/(quantity of total viral sequences in the sample)] × 100.

3. Results

3.1. Standard curves and amplification efficiency

For each set of specific and non-specific primers, C_t was linearly correlated with input DNA copy number over the range of 10^1 to 10^6 copies (Fig. 2). The specific (Sp) and non-specific (NS) amplification efficiencies (defined as: $E = 10^{(-1/\text{slope})}$) were comparable within each ASPCR set. Correlation coefficients (r^2) were higher than 99.6% for all primer pairs on their respective target standards.

3.2. Allelic discrimination

Allelic discrimination of mutant-specific primers was tested by determining the difference in C_t values (ΔC_t) when identical amounts of mutant and wild-type DNA were amplified with the corresponding mutant-specific primer set (Fig. 3, Panels A–D). Each experiment was conducted in triplicate; data shown represent the mean ± S.D. The fold decrease in amplification efficiency (AE) was derived from ΔC_t. For the M184V-specific primer, ΔC_t between the amplification of M184V and wild-type or M184I was 10 (>700-fold decrease in AE for WT) and 11 (>750-fold decrease in AE for M184I), respectively. For the M184I-specific primer, the ΔC_t between M184I and wild-type was 12 (>2100-fold decrease in AE for M184V), whereas the ΔC_t between M184I and M184V was 20 (>200,000-fold decrease in AE for WT). Similarly, the ΔC_t for D30N versus WT was 12 (>5400-fold decrease in AE for WT); the ΔC_t for V38A versus WT was also 12 (>4000-fold decrease in AE for WT).

In a second experiment (Fig. 3, Panels E–H), the discriminatory ability of each assay was tested in mixing experiments by adding 10^5 copies of wild-type DNA to serial dilutions (10^6 to 10^1 copies) of mutant DNA. The threshold cycle was compared to PCRs performed without the addition of wild-type DNA. In each case, C_t was linearly correlated to input copy number over a range of mutant/wild-type ratios until the mutant DNA was present at 0.1% or less of total DNA copies.

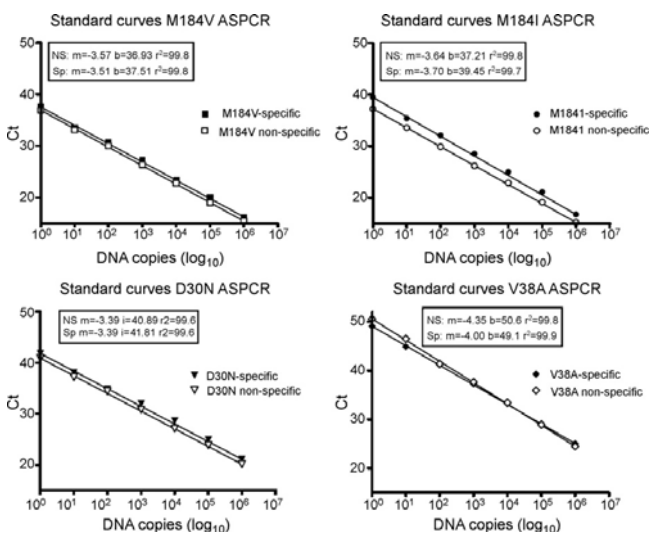

Fig. 2. Mutant-specific (Sp, solid symbols) and non-specific (NS, open symbols) standard curves of M184V, M184I, D30N and V38A-allele-specific real-time PCR assays. Specific and NS regression lines, defined as: $y = \text{slope}\, x + b$, where b is the y-intercept, derived from each standard dilution set. In clinical samples, Sp and NS input copy numbers were determined using this formula by interpolation of the Cts (x) into the corresponding standard curve. Correlation coefficients (r^2) were higher than 99.6% in all cases.

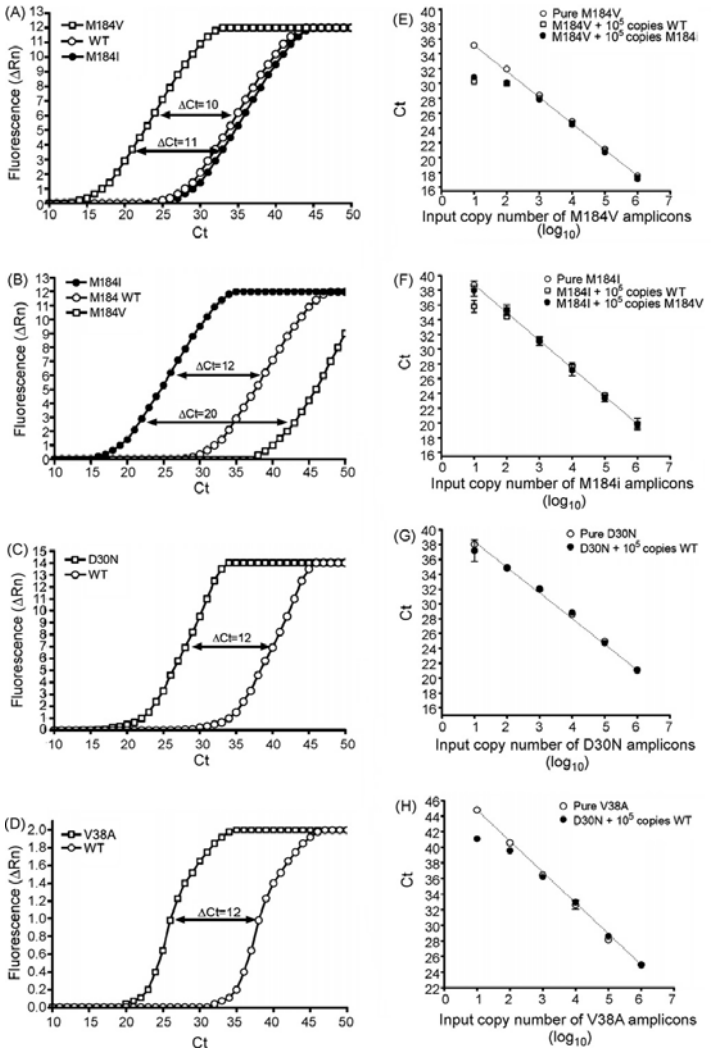

Fig. 3. Allelic discrimination of M184V, M184I, D30N and V38A ASPCR assays. Panels A–D: identical amounts of mutant and wild-type DNA were amplified with the corresponding mutant-specific primer set [i.e. M184V (Panel A), M184I (Panel B), D30N (Panel C) and V38A (Panel D)]. ΔC_t was $\geq$10 in all four assays. Panels E–H: the addition of 10^5 copies of a non-complementary allele (WT in all assays and M184I and M184V in M184V and M184I ASPCR assays, respectively) to each 10-fold dilution of the mutant standard did not significantly alter the standard curve until the mutant DNA was present at $\leq 10^3$ copies. This illustrates that the discriminatory ability remained unaltered until the standard containing the target mutation was $\leq$0.1% of the total standard copies [e.g. (10^3 copies of M184V)/(10^3 copies of M184V + 10^5 copies of M184I) × 100]. Values in panels E–H correspond to the mean ± S.D. of triplicate measurements.

3.3. Sensitivity

The sensitivity of each ASPCR assay was defined as the mean plus 3 standard deviations (S.D.) above the copy number determined on wild-type template with mutant-selective primer in 12–15 independent determinations. The sensitivity of the ASPCR assays was 0.4% for M184V, 0.04% for M184I, 0.1% for D30N and 0.8% for V38A.

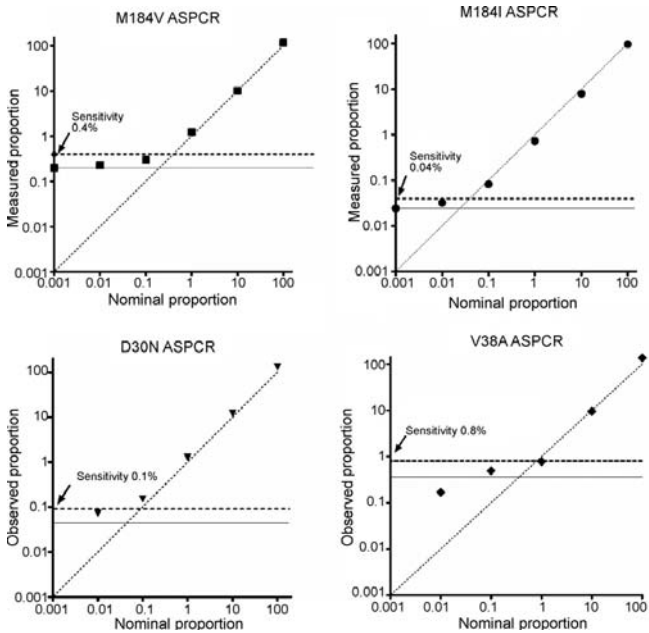

Fig. 4. Sensitivity and accuracy of M184V, M184I, D30N and V38A ASPCR assays. The sensitivity of each assay was calculated as the mean plus 3 standard deviations of 12–15 replicate measurements of wild-type amplicons (negative controls) obtained from recombinant viral constructs. Accuracy was evaluated by measuring serial dilutions of mutant amplicons in a background of wild-type amplicons. The nominal proportion of mutants ranged from 100% to 0.01% or 0.001% mutants. The proportion of mutants was measured as: 100 × (DNA copy number obtained by mutant-specific amplification)/(DNA copy number obtained by non-specific amplification). Horizontal solid line, mean proportion of 12–15 replicate measurements of wild-type amplicons; horizontal dashed line, limit of detection (attributed sensitivity) of the assay and diagonal dashed line, theoretical perfect match between nominal and observed proportions.

3.4. Accuracy

Serial 10-fold dilutions of mutant amplicons were prepared in a background of WT amplicons and measured with the corresponding ASPCR assay (Fig. 4). Measurements were accurate down to 1% (M184V), 0.1% (M184I), 0.1% (D30N), and 1% (V38A) in the various ASPCR assays, respectively. Measurements between these points and the limit of detection of each assay (e.g. between 1% and 0.4% in the M184V assay) tended to slightly overestimate the actual proportion of mutant variants.

3.5. Reproducibility

The coefficients of variation (CV) for quantifying samples with proportions of mutant DNA ranging from 100% to 1% of the population were measured in order to assess the reproducibility of the assay. Intra-assay variation was determined by triplicate determinations of each mixture containing a given proportion of mutant and wild-type sequences. The CVs were below 0.40 for each proportion analyzed, being lower than 0.20 for the nominal mutant proportions of 100% and 10%. Inter-assay variation was assessed by testing three different aliquots of each prepared mixture in three independent assays performed on different days. Results of different runs revealed CVs of around 0.20 for nominal mutant proportions of 100%, and around 0.60 for nominal proportions of 1% (Table 2).

3.6. Clinical negative controls

Stored plasma specimens from six different HIV-1-infected patients obtained in 1991 were tested for the presence of M184V/I and D30N mutations. Because neither lamivudine nor nelfinavir were approved for use at that time, the subjects studied should not have been exposed to these drugs. Population-based sequencing of these specimens demonstrated a wild-type allele at both codons. Allele-specific PCR yielded M184V and D30N proportions below the limit of detection in all six specimens (data not shown). Of note, three of six specimens showed proportions of the M184I mutant that were slightly above the limit of detection (0.05–0.07%), suggesting that M184I mutants may have been present at very low levels

Table 2
Intra-assay and inter-assay coefficients of variation (CVs)

Nominal mutant proportion (%)	CV intra-assay				CV inter-assay			
	M184V	M184I	D30N	V38A	M184V	M184I	D30N	V38A
100	0.14	0.01	0.19	0.06	0.21	0.23	0.14	0.13
10	0.11	0.08	0.14	0.17	0.13	0.06	0.40	0.49
1	0.18	0.09	0.39	0.21	0.60	0.11	0.64	0.50

in the virus populations of these subjects before any lamivudine exposure.

Plasma samples from six additional patients were tested as negative controls for V38A in *env*. These were six antiretroviral therapy-experienced but enfuvirtide-naïve patients with multidrug-resistant HIV-1 infection, but no evidence of enfuvirtide resistance by standard sequencing at the time of specimen sampling. In all six cases, the proportion of V38A mutations by ASPCR was below the limit of detection of the assay (data not shown).

3.7. Mutant dynamics in clinical samples

The ability of ASPCR to characterize the dynamics of mutants in vivo was investigated by measuring the proportion of M184V/I variants in serial plasma specimens from four subjects with multidrug-resistant HIV-1 (MDR HIV) who selectively interrupted lamivudine therapy (Campbell et al., 2005). Allele-specific PCR measurements were compared to genotypic data obtained at each time point by standard techniques (Fig. 5).

Allele-specific PCR measurements were concordant with the results of standard genotyping. As expected, M184V variants were predominant in patients 1–3 before lamivudine interruption. In contrast, the proportion of M184V mutants was only slightly higher than 20% in patient 4. Pre-interruption levels of M184V variants remained stable during 6–8 weeks and decayed thereafter. Resumption of lamivudine therapy was associated with a rapid rebound of M184V variants. Samples with 10–20% mutant sequences by ASPCR were consistently reported as mixtures of 184V and 184M by standard genotyping, whereas samples with less than 10% mutant were consistently negative.

Resumption of lamivudine treatment was consistently associated with the transient detection of M184I variants at very low levels ($\leq$0.1%). Such transient increases were associated with parallel increases in the proportion of M184V variants in patients 1, 3 and 4 but not in patient 2. As well, M184I increments were associated with increases in HIV-1 RNA load in patients 2 and 3, but with HIV-1 RNA decreases in patients 1 and 4.

3.8. Effect of polymorphisms at primer sites on the accuracy of ASPCR

To investigate the influence of genetic polymorphisms at primer sites on the accuracy of ASPCR measurements, 12 clonal *pol* sequences were obtained per patient (HXB2 posi-

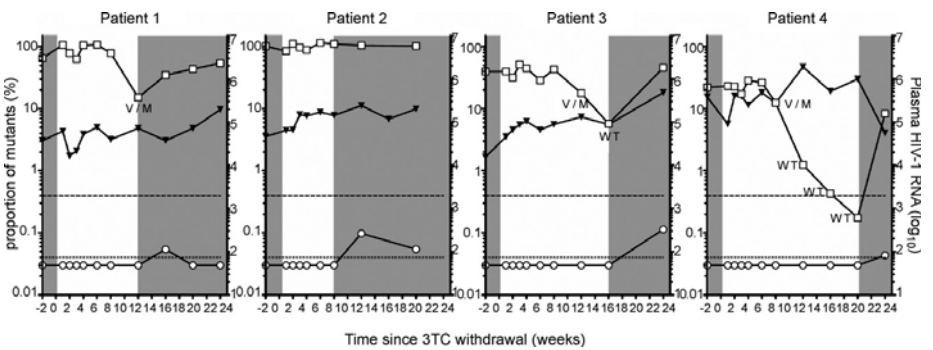

Fig. 5. M184V and/or M184I decay and subsequent re-selection among subjects with MDR HIV infection undergoing a transient interruption of lamivudine therapy: assessment by allele-specific PCR (ASPCR) and standard genotype (SG). Four individuals infected with MDR HIV including the M184V mutation while on highly active antiretroviral therapy, transiently interrupted lamivudine therapy and continued the remaining antiretrovirals. The proportion of viral variants with the M184V or M184I mutations was investigated in serial samples before, during and after the lamivudine interruption period. Vertical shaded areas indicate periods "on" lamivudine; non-shaded areas indicate periods "off" lamivudine. Open squares: proportion of variants with the M184V mutation. Open circles: proportion of variants with the M184I mutation. Triangles: plasma HIV-1 RNA levels. Horizontal dashed line (– – –) threshold of detection for M184V variants (0.4%). Horizontal dotted line (· · ·) threshold of detection for M184I variants (0.04%). Standard genotypic data indicating the consensus sequence at codon 184 of the reverse transcriptase are annotated next to the corresponding M184V ASPCR results. No annotation indicates detection of M184V; "V/M" indicates the presence of M184V and WT variants; "WT" indicates detection of a wild-type sequence by standard genotype.

Table 3
Clonal sequences at primer sites[a]

Sequences at discriminative forward primers site (5' – 3')		Sequences at common reverse primer site (5' – 3')	
Primer V4 [b] (M184V-specific)	GACATAGTTATCTATCAATI<u>C</u><u>G</u>*	Primer ASR2 (common reverse primer)	GGCTGTACTGTCCATTTATC
Primer VN [c] (non-specific)	..................A.		
Laboratory reference strains			
HXB2	..................A.A		....
pNL4.3	........C..........A.A		....
Patient 1 clones			
10/12	......A.C..........A..		....
1/12	......A.C..........A..		.C....................
1/12	......A.C..........A..		...................T
Patient 2 clones			
2/12	......A.C..........A..		..T...................
2/12	..................A..		..T...................
2/12	..................A..		....
2/12	......A............A..		....
1/12	......A............A..		.................G..
1/12	........C..........A..		....
1/12	.....G.............A..		....
1/12	......A............A..		..T...................
Patient 3 clones			
9/12	..GC...............A..		....
2/12	..GC...............A..		..T...................
1/12	...C...............A.A		....
Patient 4 clones			
12/12	................G.A..		....

[a] Amplicons encompassing the 2218–3457 positions relative to the HXB2 laboratory reference strain, were obtained from patient plasma specimens, PCR-purified (QIAquick® PCR Purification Kit, Qiagen Sciences, Maryland, USA), and cloned into a pGEM® T-Easy Vector (pGEM® T-Easy Vector System, Promega Corporation, Madison, WI, USA), as directed by the manufacturer. Twelve clonal *pol* sequences were obtained per patient before lamivudine interruption. Here, clonal sequences at the site of the discriminative primer set (left) and at the site of the common reverse primer (right) are presented. Respectively, the M184V-specific primer (V4) sequence and the ASR2 primer sequence are used as the reference sequence for patients' clones.

[b] In the M184V-specific primer (V4), the target mutation is shown in boldface, underlined and next to an asterisk, while the intentional A → I mismatch in the −2 position of the 3′-end is shown in boldface, italics and underlined.

[c] The non-specific (VN) primer is one base pair shorter than the M184V-specific primer (V4) and does not incorporate an intentional mismatch.

tions: 2218–3457) before lamivudine interruption (Table 4). As expected, all clones exhibited an I → A mismatch in the −2 position relative to the 3′-end of the M184V-specific primer. The M184V mutation was present in all but one of 12 clones (92%) from the baseline sample of patient 3. The existence of one or two polymorphisms near the 5′-end of the discriminative primer set did not have a major impact on ASPCR measurements in patients 1–3. However, presence of an A→G mismatch at the −4 position relative to the 3′-end of the M184V-specific primer was associated with an underestimation of the proportion of M184V variants by ASPCR in patient 4 (Table 3, Fig. 6). The incorporation of the A → G mismatch in both specific (V4) and non-specific (NS) primers increased the accuracy of the pre-interruption values and subsequent measurements proportionally (Fig. 6). Importantly, this mismatch also had to be incorporated into the standards. Otherwise, the amplification of standards lacking the mismatch using primers with the mismatch resulted in a decrease in the specific amplification of standards (not shown) and, therefore, an overestimation of the proportion of mutants.

4. Discussion

The study of minor viral variants in HIV-1 infection is relevant to understanding the mechanisms of viral persistence, escape from pharmacologic and immunologic pressure, and co-receptor usage. Detection of drug-resistant minority variants may help predict virological failure in patients with HIV-1 that appears to be wild-type by standard sequencing methods. Among the several techniques available to study minor viral variants (Bergroth et al., 2005; Leamon et al., 2003; Margulies et al., 2005; Metzner et al., 2003, 2005; Palmer et al., 2005), ASPCR is one of the most sensitive, accurate and reproducible, being less expensive and time-consuming than single-genome sequencing or clonal sequence analysis (Halvas et al., 2006). Due to its high throughput, this technique can also be used as a way to rapidly screen very large pooled populations.

This article presents a systematic evaluation of ASPCR assays targeted to four important drug resistance mutations in RT (M184V and I), PR (D30N), and *env* (V38A in gp41). Each assay could detect the presence of mutant species with a limit

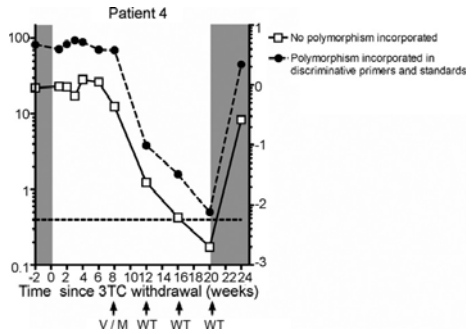

Fig. 6. Effect of pre-existing polymorphisms on the accuracy of ASPCR measurements 'in vivo'. As shown in Table 4, 12/12 HIV-1 *pol* clones in patient 4 contained a pre-existing A → G polymorphism in the −4 position, relative to the 3′-end of the M184V-specific primer (V4). Vertical shaded areas indicate periods "on" lamivudine; non-shaded areas indicate periods "off" lamivudine. Open squares: proportion of variants with the M184V mutation. Horizontal dashed line (– – –): threshold of detection for M184V variants (0.4%). Standard genotypic data indicating the consensus sequence at codon 184 of the reverse transcriptase are annotated next to the corresponding week. No annotation indicates detection of M184V; "V/M" indicates the presence of M184V and WT variants; "WT" indicates detection of a wild-type sequence by standard genotype.

of detection of <1%, but assays for certain mutant codons were more sensitive than others. Sensitivity was directly proportional to the discrimination ability of the primers, which was, in turn, strongly influenced by the particular base sequence of the codon being interrogated. For example, the M184I-specific primer was better able to discriminate M184I (AT**A**) from WT (AT**G**) and M184V (**G**TG) than was the M184V-specific primer.

A number of factors, however, were important to enable adequate performance of the assay, both in laboratory constructs and in clinical specimens. To ensure equal amplification efficiencies, both the specific and non-specific primers should anneal to the same DNA region. ASPCR assays using Sp and NS primers that annealed to different sites yielded inaccurate results, often overestimating the proportion of mutants (not shown). In this study, the NS primer was identical to the Sp except that it ended one base pair before the target locus and did not incorporate intentional mismatches. The antiparallel primer was common to both primer pairs. To maximize the discrimination ability of the assay, it was also important to use relatively short primers. Because the cycling conditions were the same for all four ASPCR sets, several assays could be run at the same time, increasing overall efficiency.

Based on a modification of the amplification refractory mutation system (ARMS) (Newton et al., 1989), the Sp primer in this assay contained the target nucleotide substitution at its 3′-end plus an intentional nucleotide mismatch at positions −1 to −3 from the 3′-end to increase its specificity for the target mutation (Metzner et al., 2003; Newton et al., 1989). When several different nucleotide substitutions within a codon can give rise to the same mutation, it may be necessary to use degenerate Sp primers (e.g. K103N in RT) (Loubser et al., 2006). Annealing temperature (T_a) adjustments including primer dissociation curves were also important to attain optimal discrimination and lack of non-specific amplification. In general, the Ta should be as high as possible for each given primer set without compromising the amplification efficacy of the real-time PCR reactions. The optimal T_a could be determined empirically by repeating the experiments shown in Fig. 3 (panels A–D), in different thermal conditions.

The ASPCR assays described had good reproducibility. Because inter-assay CVs were higher than intra-assay CVs, serial specimens from an individual patient should be run in a single batch. As expected, CVs were greatest for samples with a low nominal proportion of mutant species. However, the mag-

Table 4
Minimum HIV-1 RNA load needed to detect minor variants at a frequency (λ) of 0.1%, 1%, and 10%, assuming that the RNA is extracted from 1 mL of plasma[a]

Variant frequency, λ	No. of RNA molecules to be tested, according to the Poisson distribution ($P > 99\%$)	Fraction of elution volume used for cDNA synthesis, f_e	Minimum HIV-1 RNA load (copies/mL)
0.1%	5000	0.25	29,762
		0.5	14,881
		1	7,440
1%	500	0.25	2,976
		0.5	1,488
		1	744
10%	50	0.25	298
		0.5	148
		1	74

HIV-1 RNA values were estimated using the following assumptions: $V = 1$ mL, $E_{RNAX} = 0.96$ and $E_{cDNA} = 0.7$, based on http://www1.qiagen.com/literature/qiagennews/0398/983hiv1.pdf and http://omrf.ouhsc.edu/~frank/CDNA.html. Importantly, E_{RNAX} and particularly E_{cDNA} are subject to significant variation in different conditions, and may need to be determined empirically. Therefore, HIV-1 RNA values would need to be adjusted if different plasma volumes were used for the RNA extraction or if E_{RNAX} or E_{cDNA} were different. Note that the volume of plasma required for RNA extraction in order to detect a variant with frequency λ can be easily calculated as $V = N_{RNA(\lambda)}/(\text{pVL} f_e E_{RNAX} E_{cDNA})$.

[a] Calculations were derived from the formula: $\text{pVL} = N_{RNA(\lambda)}/(V f_e E_{RNAX} E_{cDNA})$, where pVL is the plasma HIV-1 RNA copy number, $N_{RNA(\lambda)}$ the number of RNA copies that need to be tested according to the Poisson distribution to detect at least one variant with a probability >99% if this variant is present at a frequency λ, V the volume of plasma used for the RNA extraction in millilitres, f_e the fraction of the RNA elution volume used for cDNA synthesis, E_{RNAX} the efficiency of the RNA extraction process and E_{cDNA} is the efficiency of the cDNA synthesis.

nitude of variation was small relative to the proportion of mutant species detected. For example, the intra-assay CV of the M184V ASPCR when the mutant species constituted 1% of the population was 0.18, which corresponded to a 99% confidence interval of [0.5–2%] (Reed et al., 2002).

In reconstruction experiments, the four ASPCR assays were highly accurate in quantifying the proportion of mutant species over the range of 100–1%, but overestimated the proportion of mutant species at proportions below 1%. The total input DNA copy number did not affect the accuracy of measurements provided that the C_t values fell within the linear range of amplification for the respective primer pairs ($10^{6–7}$ to 10^1). However, a minimum input of 10^4 DNA copies in the real-time PCR step was required to ensure a sensitivity of at least 0.1% (10^1 mutants/10^4 total variants = 0.1%).

As with any method for assessing minor HIV-1 variants, true assay sensitivity depends on the number of RNA molecules in the original sample. According to the Poisson distribution, to detect at least one mutant variant present in 0.1% of the total population with a 99% probability, at least 5000 variants need to be tested. Therefore, the template RNA for the RT-PCR step had to include at least 5000 RNA molecules to permit a sensitivity of 0.1%, regardless any other characteristic of the ASPCR assay. The RNA copy in the assay depends on plasma HIV-1 RNA concentration, the volume of plasma used and the efficiency of the RNA extraction process (Table 4). The efficiency of the reverse transcriptase step also must be taken into account. Large volumes of plasma are required for the RNA extraction in specimens with low viral loads in order to preserve the ability of any assay to detect minor variants (Table 4).

Another limitation of ASPCR is that polymorphisms that occur in the primer or probe binding sites can significantly impair the accuracy of ASPCR measurements. In accordance with prior data (Palmer et al., 2006), whereas polymorphisms near the 5′-end of the discriminative primer set had little effect on the accuracy of proportions, those occurring near the 3′-end could result in the underestimation of the proportion of mutant variants. The incorporation of polymorphisms detected by prior population-based sequencing into the discriminatory primer set has been suggested to overcome this problem (Palmer et al., 2006). Moreover, relevant polymorphisms also had to be incorporated into the standards (not shown). Otherwise, the presence of an additional mismatch between the discriminative primer set (with the polymorphism) and the standard sequence (without the polymorphism) preferentially constrained the amplification of the mutant-specific standard curve, relative to the non-specific standard curve. In some situations, substituting SYBR™ green or molecular beacons for TaqMan™ probes may also help to overcome problems caused by sequence polymorphisms.

In summary, ASPCR is a valuable technique for studying point mutations in viral genomes. The high sensitivity, accuracy and reproducibility of this technique make it a particularly useful tool for resistance surveillance. Other possible applications include studying the kinetics of selection and decay of point resistance mutations, in vivo turnover of cell populations and viral fitness estimations (Marconi et al., 2006; Paredes et al., 2006). The approach described herein can easily be adapted to study other mutations in the HIV-1 genome or in other viruses, in plasma, peripheral blood mononuclear cells (Loubser et al., 2006), and other tissues. Allele-specific PCR will remain highly relevant in elucidating the importance of minor variants to the clinical outcomes of patients infected with drug-resistant HIV.

Acknowledgements

This work was supported by the following sources: Harvard University Center for AIDS Research (P30 AI60354), a Virology Specialty Laboratory subcontract from the AIDS Clinical Trials Group (U01 AI 38858 and AI68636), and K24 RR16482 (to D.R.K.). R.P. is a recipient of the “La Caixa” Fellowship Grant for Post-Graduate Studies, Caixa d’Estalvis i Pensions de Barcelona, Catalonia, Spain.

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JVI Accepts, published online ahead of print on 19 November 2008
J. Virol. doi:10.1128/JVI.02154-08

TITLE PAGE

In Vivo Fitness Cost of the M184V Mutation in Multidrug-Resistant HIV-1 in the Absence of Lamivudine

Roger Paredes,[1,2] Manish Sagar,[1] Vincent C. Marconi,[1,3] Rebecca Hoh,[4] Jeffrey N. Martin,[4] Neil T. Parkin,[5] Christos J. Petropoulos,[5] Steven G. Deeks,[4] Daniel R. Kuritzkes[1]

[1]Section of Retroviral Therapeutics, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA. [2]Fundacions *irsiCaixa* i Lluita contra la SIDA, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, Catalonia, Spain. [3]Infectious Disease Service, Brooke Army Medical Center, San Antonio Military Medical Center, San Antonio, TX, USA. [4]University of California San Francisco and San Francisco General Hospital, San Francisco, CA, USA. [5]Monogram Biosciences, Inc., South San Francisco, CA, USA.

Word count: Abstract: 100 (max 100), Text: 1660 (max 1600)

Corresponding Author:

Daniel R. Kuritzkes, M.D.
Section of Retroviral Therapeutics
Brigham and Women's Hospital
65 Landsdowne St, Rm 449
Cambridge, MA 02139
Tel: 617 768 8371
Fax: 617 768 8738
email: dkuritzkes@partners.org

This work was presented in part at the 13th (Abstract 628) and 14th (Abstract 588) Conferences on Retroviruses and Opportunistic Infections, held in Denver, CO, 2006 and Los Angeles, CA, 2007, respectively.

Keywords: HIV-1; allele-specific PCR; viral fitness; resistance; partial treatment interruption; quasispecies; antiretroviral therapy; salvage therapy;

NOTE

Lamivudine therapy selects for the M184V mutation. Although this mutation reduces the replicative capacity of HIV in vitro, its impact on viral fitness in vivo has not been well defined. We used quantitative allele-specific PCR to precisely calculate the fitness differences between M184V and M184M in a cohort of patients selectively interrupting reverse transcriptase inhibitors, and found that the M184V variants were consistently 4-8% less fit than wild-type in absence of drug. After a lag phase of variable duration, wild-type variants emerged due to continued evolution of pol and back mutation rather than through emergence of an archived wild-type variant.

Despite recent advances, the management of multidrug-resistant (MDR) HIV-1 remains a major clinical problem. One strategy to avoid immune deterioration while minimizing toxicity in viremic patients infected with MDR HIV-1 is to prevent the emergence of wild type (WT) HIV-1 by continuing selected drugs in a failing antiretroviral regimen. Studies show that reverse transcriptase inhibitors (RTI) continue to exert anti-viral activity in the presence of resistance mutations.(8, 21) In particular, continuation of lamivudine (3TC) or emtricitabine (FTC) in the presence of the M184V mutation may provide clinical benefit. (3, 4) We previously showed that M184V is lost a median of 20 weeks following interruption of 3TC together with other RTIs while continuing protease inhibitors (PIs) in viremic subjects with MDR HIV-1.(8) In this study, we performed a detailed analysis of the decay of the M184V-carrying mutants in these individuals.

Subjects were antiretroviral treatment-experienced HIV-1-infected patients enrolled in an ongoing prospective cohort study. (8) This particular substudy focused on 5 adherent, highly treatment-

experienced, viremic subjects with HIV-1 resistant to antiretroviral drugs from at least two classes who interrupted 3TC together with other RTIs but remained on PIs. A sixth participant (subject 3158) enrolled in the parent study while receiving 3TC, stavudine (d4T), and nelfinavir (NFV) and selectively interrupted NFV. At week 52, this patient discontinued 3TC and d4T therapy and subsequently remained off all antiretroviral therapy. Subjects were followed weekly for the first 4 weeks, every 2 weeks for the next 8 weeks and every 4 weeks thereafter for at least 48 weeks or until treatment was modified. Participants provided written, informed consent for participation in these studies, which were approved by the University of California, San Francisco Committee on Human Research and the Partners HealthCare Systems Institutional Review Board.

At the time of PTI, the median plasma HIV-1 RNA level was 3.65 log copies/mL, and the median CD4+ count was 336 cells/mm^3. Further details of this cohort have been reported. (8)

Population sequencing of plasma viruses and phenotypic antiretroviral susceptibility tests (GeneSeq® and Phenosense HIV®; Monogram Biosciences™, South San Francisco, CA) obtained before the RTI interruption and at multiple timepoints thereafter showed a slow decay of NRTI resistance. Mutations decayed gradually at a rate roughly proportional to their associated fitness cost, as estimated *in vitro* in the absence of drug.(6) (Table 1). The thymidine analogue mutations (TAMs) waned more slowly than M184V. Changes in phenotypic susceptibility to 3TC and other NRTI paralleled the genotypic changes (not shown). As expected, all subjects maintained viruses with high-level PI resistance throughout the study period (not shown).

An allele-specific PCR (ASPCR) assay that detects M184V variants present in at least 0.4% of the quasispecies population (18) was used to quantify more precisely the proportion of viruses carrying the M184V mutation over time, and to estimate the fitness of M184V-containing viruses *in vivo*. By ASPCR, M184V mutants were present as the predominant viral species at baseline in all subjects. (Figure 1) In subjects 3167 and 3151, there was no significant change in the proportion of M184V-containing virus through 12 and 16 weeks of follow-up, respectively (not shown), consistent with the population

sequencing results. In subjects 3005, 3040, 3057 and 3158, M184V variants exhibited a biphasic decay: after an initial lag phase of variable duration, M184V variants decreased exponentially (Figure 1). The lag phase lasted 20 weeks in subject 3057, 24 weeks in subject 3040 and 32 weeks in subject 3005, but was much shorter (12 weeks) in subject 3158, who was receiving only d4T and 3TC and therefore interrupted all antiretroviral drugs in his regimen. Despite the variable lag time, once decay began the duration and slopes of the exponential decay phase were similar in all subjects, lasting approximately 12 weeks, although minor M184V variants remained detectable for up to 48 weeks after 3TC interruption in subject 3005.

Relative fitness was calculated in the four subjects with M184V reversion using the average method, which incorporated multiple ASPCR measurements during the exponential M184V mutant decay phase, the HIV-1 RNA level at each timepoint and the death rate of infected cells (1, 15) (we used a death rate [δ]= 0.5, as this value approximates the mean of many independent estimates (2)). The relative fitness disadvantage of M184V ranged from 4.6% to 8.3% in these multidrug-resistant viruses (Figure 1).

To further characterize the viral variants emerging after NRTI discontinuation, clonal HIV-1 *pol* sequences were obtained from subjects 3040, 3057, 3005 and 3158 at the time of RTI interruption (T0), and at the first time point at which M184V became undetectable by ASPCR (T1). An additional time point (T2) was available for subject 3057, 16 weeks after T1. A mean of 24 clones [range 21-29] (Table 2) were analyzed at each time point. All sequences obtained at T0 carried the M184V mutation, whereas all sequences obtained at T1 and T2 were WT at this codon (Table 2). In addition to the changes in the frequency of TAMs observed by population sequencing, clonal analysis revealed a decrease in the frequency of K70R variants in subjects 3040 and 3158, and evidence of back mutation at codon 215 from the mutant TAC (Tyr) to the partial revertants GAC (Asp) and AAC (Asn) in subject 3005.

Phylogenetic trees reconstructed in PAUP v4.0b (HKY model) and MEGA v3.1 (Tamura-Nei model), (13) using both maximum likelihood and neighbor-joining (NJ) approaches plus 1,000 NJ bootstrapping replicates showed that in subjects 3057, 3005 and 3158, sequences from T0 and T1 were intermingled and

shared a most-recent common ancestor (MRCA) (Figure 2), as expected from back-mutated sequences. Interestingly, in these 3 subjects there was an increase in sequence diversity in both the protease and RT genes, suggesting ongoing exploration of sequence space by the virus population (Table 2). Conversely, viral sequences from subject 3040 at T0 and T1 clustered separately with different MRCAs. In addition, in this subject sequence diversity increased in the protease gene but decreased in the RT gene (Table 2). The T1 sequences were more closely related to the M184V variants at T0 (genetic distance=0.019) than to a pre-3TC consensus sequence (genetic distance=0.034) obtained in 1995, providing further support for the inference that the 184M variant arose from back-mutation as opposed to re-emergence of archived viruses. Analyses of the rate of non-synonymous and synonymous substitutions for samples from all patients and timepoints (MEGA software v3.1 (13)) indicated that purifying selection (i.e., reduction of alleles with a deleterious effect on the phenotype) was the main mechanism for M184V reversion in all subjects (Table 2).

This study showed that the M184V mutation disappeared quickly after a variable lag phase that lasted as long as 32 weeks. Differences between individual subjects in time to M184V disappearance appeared related to the length of the initial lag phase rather than differences in the rate of exponential decay. The estimates of the relative fitness of MDR HIV-1 carrying the M184V mutation were in close agreement with previous data, indicating a fitness cost of the M184V mutation of approximately 10% relative to WT. (14)

In contrast to observations when all drugs are discontinued, (9) we did not observe evidence for the escape of a pre-existing MDR-184M variant, nor we did find evidence of emergence of viruses with a WT RT and a mutant PR through recombination of actively replicating and archived variants. (16) Several findings suggested that 184M viruses emerged through continuous evolution of *pol* and back-mutation. (12) First, NRTI mutations decayed in serial clonal and population-based sequences in an ordered, stepwise fashion at a rate roughly proportional to their associated fitness cost, as estimated *in vitro* in the absence of drug.(6) Second, there was clonal evidence for back mutation through partial revertants at

codon 215 [GAC (Asp) and AAC (Asn) derived from the mutant TAC (Tyr) in subject 3005]. Third, the MDR-M184V and MDR-184M variants shared a MRCA in three out of four subjects and, even in the fourth subject (3040), emerging MDR-184M variants were more closely related to on-treatment MDR-M184V mutants than to a pre-lamivudine WT sequence.

The opposing selective pressures exerted on different coding regions of *pol* by interrupting RTI therapy and continuing PI treatment likely favored the loss of RT mutations that conferred a fitness cost in the absence of RTI therapy, while favoring the persistence of PI resistance mutations. Given the high mutation rate of HIV-1 (5, 11), however, 184M revertants should have been generated frequently in the setting of ongoing virus replication. Thus, it appears that the probability of the 184V revertants becoming fixed in the quasispecies was low. This finding could be explained by: a) the existence of lower than expected levels of viral replication (i.e., limited effective population size despite relatively high viral loads); b) mutations or recombination occurring outside the PR and RT modifying the overall fitness of the 184M revertants (7) ; and c) the continued competition of MDR-M184V viruses (not actively inhibited by treatment) with the MDR-184M variants after treatment interruption. (17) In addition, the MDR-184M population presentat the time of RTI interruption might have been quite small in these subjects who had initiated 3TC prior to or together with PI therapy, since all PI-resistant mutants would be linked to M184V and few if any MDR variants with a WT 184 codon would exist in the quasispecies.

Other factors such as defective CTL responses in subjects with advanced HIV disease (10, 19, 20) and the presence of other RTI resistance mutations could also have modulated the fitness cost of the 184V mutation and influenced the rate of reversion (6).

In conclusion, withdrawal of RTI therapy and continuation of PI treatment was associated with slow decay of the M184V mutation in MDR HIV-1-infected subjects. Time to back-mutation appeared to be the rate-limiting step in replacement of 184V by 184M. The challenge for the virus of generating variants with a wild-type RT while maintaining PI resistance likely contributed to the observed delay. Wild type

RT variants eventually emerged due to continued evolution of *pol* and back mutation in the context of negative selection.

Acknowledgements

This work was supported in part by the following U.S. Public Health Service grants from the National Institutes of Health: R01 AI42567, R01 AI052745, R37 AI055357, K24 RR16482, a Virology Support Laboratory contract from the Adult ACTG (U01 AI068636), the Harvard Medical School Center for AIDS Research Virology Core (P30 AI060354) to D.R.K; and the UCSF/Gladstone CFAR (P30 MH59037), and the UCSF Clinical and Translational Science Institute (UL1 RR024131-01) to S.G.D; Roger Paredes received the "La Caixa" Fellowship Grant for Post-Graduate Studies, Caixa d'Estalvis i Pensions de Barcelona, "La Caixa", Barcelona, Catalonia, Spain.

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FIGURE LEGENDS

Figure 1. Dynamics of M184V variants after the interruption of revere transcriptase inhibitors. Only subjects achieving complete M184V mutant decay are shown. Open circles, proportion of M184V mutants by allele-specific PCR testing (in logarithmic scale). For the ASPCR assay, viral RNA was extracted from 500 mL of plasma. Continuous line, HIV-1 RNA levels (in log10 copies/mL). Horizontal dashed line, sensitivity threshold to detect M184V variants (0.4%). Horizontal bars in the superior part of each graph, duration of antiretroviral treatment. Phenotype refers to fold-change in lamivudine susceptibility. Genotype refers to the codon 184 allele detected by population-based sequencing: methionine (M), valine (V) or a mixture of variants with methionine and variants with valine in codon 184 (M/V). Arrows show the timepoints when T0 (white), T1 (black) and T2 (grey) clonal sequences were obtained. The fitness (1+S) values of M184V viruses relative to 184M variants, are shown next to the exponential M184V virus decay phase.

Figure 2. Continued evolution of pol (and back-mutation) as a major mechanism of MDR-184M variant emergence after reverse transcriptase inhibitor interruption. This is an unrooted neighbor-joining phylogenetic tree generated with MEGA 3.1. Data was derived from a multiple sequence alignment including non-identical clonal pol sequences from all subjects plus the laboratory and patient-derived HIV-1 reference sequences. We assumed a Tamura-Nei (TN93) model of nucleotide evolution including transitions and transversions and a gamma-distributed variability rate among sites with an alpha value=0.8. The node reliability was assessed using 1000 bootstrap replications. Percentual bootstrap values >70% are presented. Additional analyses using different models of nucleotide evolution, maximum-likelihood tree reconstruction approaches, separate alignments per each subject or separate analyses of the protease and the revere transcriptase-coding regions of pol, yielded identical results. In subjects 3005, 3057 and 3158, MDR-184M variants emerging after treatment interruption [black (T1) and grey (T2) symbols] and baseline MDR-M184V viruses (T0, white symbols) did not have different most recent common ancestors (MRCAs). In subject 3040, 184M viruses emerging after treatment interruption (black circles) derived from a significantly different MRCA than baseline M184V variants. However, a wild type consensus sequence obtained before the initiation of lamivudine (black star) was more closely related to the baseline M184V MRCA (genetic distance ± standard error, d=0.0162 ± 0.0038) than to the 184M MRCA (d= 0.0336 ± 0.0057).

TABLES AND FIGURES

Table 1. Mutations in HIV-1 reverse transcriptase at codons associated with drug resistance in subjects interrupting reverse transcriptase inhibitors, as assessed by population sequencing of plasma viruses.[a]

Subject or isolate	Treatment interrupted	Treatment continued	Week post PTI	Codon												
				41	62	67	69	70	103	118	181	184	190	210	215	219
Clade B consensus				M	A	D	T	K	K	V	Y	M	G	L	T	K
3005	ZDV/3TC	IDV/RTV	0	L	.	N	D	.	.	.	.	V	.	W	Y	.
			34	M/L	.	N	D	.	.	V/I	.	M/V	.	L/W	Y	.
			36	M	.	N	D	.	.	V/I	.	M/V	.	L/W	Y	.
			48	.	.	N	D	.	.	V/I	.	.	.	L/W	D/N/Y	.
3040	D4T/3TC	LPV/RTV	0	.	.	N	T/A	R	.	.	Y/C	V	.	.	T/S	Q
			12	.	.	N	T	R	.	.	.	V	.	.	.	Q
			16	.	.	N	.	R	.	.	.	V	.	.	.	Q
			22	.	.	N	.	R	.	.	.	M/V	.	.	.	Q
			24	.	.	N	.	R	.	.	.	M/V	.	.	.	Q
			36	.	.	N	.	R	.	.	.	.	.	.	.	Q
3057	D4T/3TC	IDV	0	L	V	.	T/A	K/R	.	.	.	V	.	.	.	K/Q
			7	L	V	.	.	.	.	.	.	V	.	.	.	.
			36	L	V	.	.	.	.	.	.	.	.	.	.	.
			48	L	V	.	.	.	.	.	.	.	.	.	.	.
3151	DDI/D4T/3TC/EFV	APV/RTV	0	L	.	N	D	R	.	I	C	V	S	W	F	Q
			16	L	.	N	D	R	.	I	C	V	S	L/W	F	Q
3167	ZDV/3TC	NFV	0	.	.	.	.	.	.	.	.	V	.	.	.	.
			4	.	.	.	.	.	.	.	.	V	.	.	.	.
			8	.	.	.	.	.	.	.	.	V	.	.	.	.
			12	.	.	.	.	.	.	.	.	M/V	.	.	.	.
3158	D4T/3TC	None	0	.	.	N	.	R	.	.	.	V	.	.	.	Q
			5	.	.	N	.	R	.	.	.	V	.	.	.	Q
			12	.	.	N	.	R	.	.	.	M/V	.	.	.	Q
			16	.	.	N	.	R	.	.	.	M/V	.	.	.	Q

[a] *Amino acid residues are indicated by single-letter abbreviations. HIV-1 clade B consensus sequence is shown for comparison. Dots indicate no change from consensus sequence. Antiretroviral use: zidovudine (ZDV), stavudine (d4T), lamivudine (3TC), didanosine (ddI), efavirenz (EFV), indinavir (IDV), ritonavir (RTV), nelfinavir (NFV), saquinavir (SQV), amprenavir (APV). PTI: partial treatment interruption.*

Table 2. Clonal analysis.[a]

Subject or isolate[b]	Week	# of clones (total)	# of clones	Codon									Sequence Diversity (d ± SE)[c]			Ka/Ks ratio[d]		
				41	62	67	69	70	184	210	215	219	PR+RT	PR	RT	PR+RT	PR	RT
Clade B consensus				M	A	D	T	K	M	L	T	K						
3005	0	24	16	.	.	N	D	.	.	.	Y	.	0.0052 ± 0.0014	0.0010 ± 0.0007	0.0067 ± 0.0017	0.20	0.00	0.22
	48	29	4	.	.	N	D	.	.	.	N	.	0.0111 ± 0.0016*	0.0045 ± 0.0011*	0.0135 ± 0.0021*	0.19	0.22	0.18
			4	.	.	N	D	.	.	W	N	.						
			3	.	.	N	D	.	.	W	D	.						
			1	L	.	N	D	.	.	R	D	.						
			1	.	.	N	D	.	.	.	D	.						
	Overall												0.0112 ± 0.0017	0.0047 ± 0.0017	0.0118 ±0.0028	0.29	0.44	0.25
3040	0	22	22	.	.	N	.	R	V	.	.	Q	0.0065 ± 0.0016	0.0073 ± 0.0032	0.0063 ± 0.0016	0.08	0.01	0.11
	36	27	15	.	.	N	.	R	.	.	.	Q	0.0056 ± 0.0016	0.0078 ± 0.0034	0.0048 ± 0.0015	0.40	0.78	0.21
			12	.	.	N	.	.	.	.	.	Q						
	Overall												0.0149 ± 0.0024	0.0172 ± 0.0054	0.0128 ±0.0028	0.26	0.27	0.16
3057	0	22	22	L	V	.	.	.	V	.	.	.	0.0061 ± 0.0014	0.0061 ± 0.0027	0.0061 ± 0.0015	0.50	NA[†]	0.40
	32	21	20	L	V	.	.	.	.	.	.	.	0.0119 ± 0.0021*	0.0100 ± 0.0039	0.0126 ± 0.0024*	0.20	0.17	0.19
			1	L	V	G	.	.	.	.	.	.						
	48	21	21	L	V	.	.	.	.	.	.	.	0.0104 ± 0.0019	0.0095 ± 0.0038	0.0107 ± 0.0020	0.27	0.46	0.27
	Overall												0.0123 ± 0.0013	0.0103 ± 0.0029	0.0129 ±0.0023	0.32	0.44	0.28
3158	0	22	22	.	.	N	.	R	V	.	.	Q	0.0061 ± 0.0010	0.0082 ± 0.0023	0.0053 ± 0.0011	0.14	0.28	0.10

24	24	15	.	.	N	.	R	.	.	.	Q	0.0089 ± 0.0010	0.0127 ± 0.0031	0.0076 ± 0.0015	0.16	0.37	0.11
		8	.	.	N	.	.	.	.	.	Q						
		1	.	.	N	.	S	.	.	.	Q						
Overall	46											0.0087± 0.0013	0.0113 ± 0.0029	0.0073 ±0.0013	0.24	0.36	0.15

[a] *For each plasma sample, the products of five separate RT-PCRs were pooled and purified. Five separate nested PCR reactions were then performed with each pooled RT-PCR product using primers OOPF2 and OOR3 (18) and cloned within the TOPO-TA 2.1 vector (Invitrogen Corporation, Carlsbad, CA, USA).*

[b] *Subject 3005 interrupted treatment with zidovudine/lamivudine and continued indinavir/ritonavir; subject 3040 interrupted treatment with stavudine/lamivudine and continued lopinavir/ritonavir; subject 3057 interrupted treatment with stavudine/lamivudine and continued indinavir; Subject 3158 interrupted treatment with stavudine/lamivudine and did not continue any other treatment. Amino acid residues are indicated by single-letter abbreviations. HIV-1 clade B consensus sequence is shown for comparison. Dots indicate no change from consensus B sequence.*

[c] *Clonal sequence diversity and the non-synonymous/synonymous nucleotide substitution (Ka/Ks) ratio are shown per each subject in whom M184V viruses reverted back to 184M. Data are presented by week of clonal sequence sampling, as well as considering all patient clones together ("overall").Distances were estimated with MEGA v3.1, using a Tamura-Nei model for nucleotide substitution, assuming a gamma distribution with an alpha=0.5. Standard Errors (SE) were estimated using 1000 bootstrap replicates.* * *p<0.05, 2-tailed P value (one sample t-test), compared with baseline.*

[d] *The Ka/Ks ratio was also estimated with MEGA v3.1, using the Nei-Gojobori method and a Jukes-Cantor model for nucleotide substitution.* † *NA: calculation not available because dS=0.*

Figure 1. Dynamics of M184V variants after the interruption of revere transcriptase inhibitors.

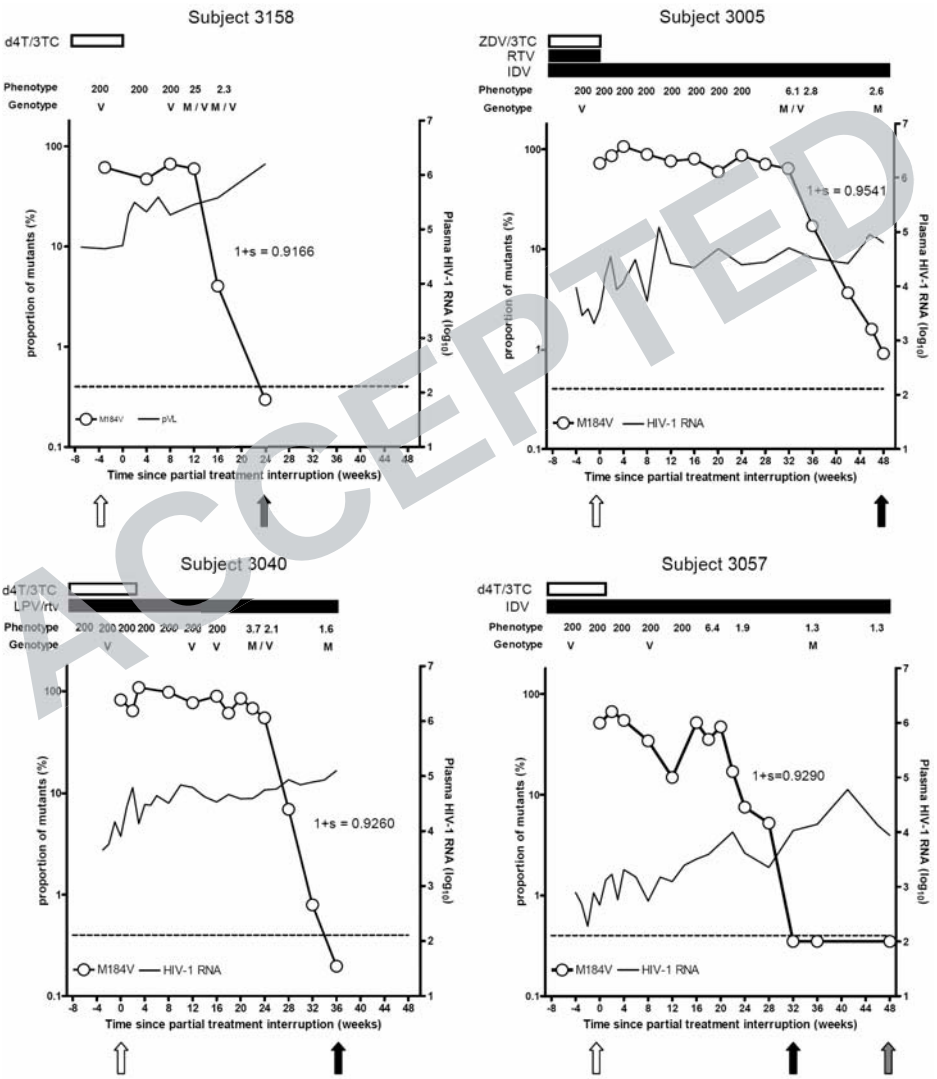
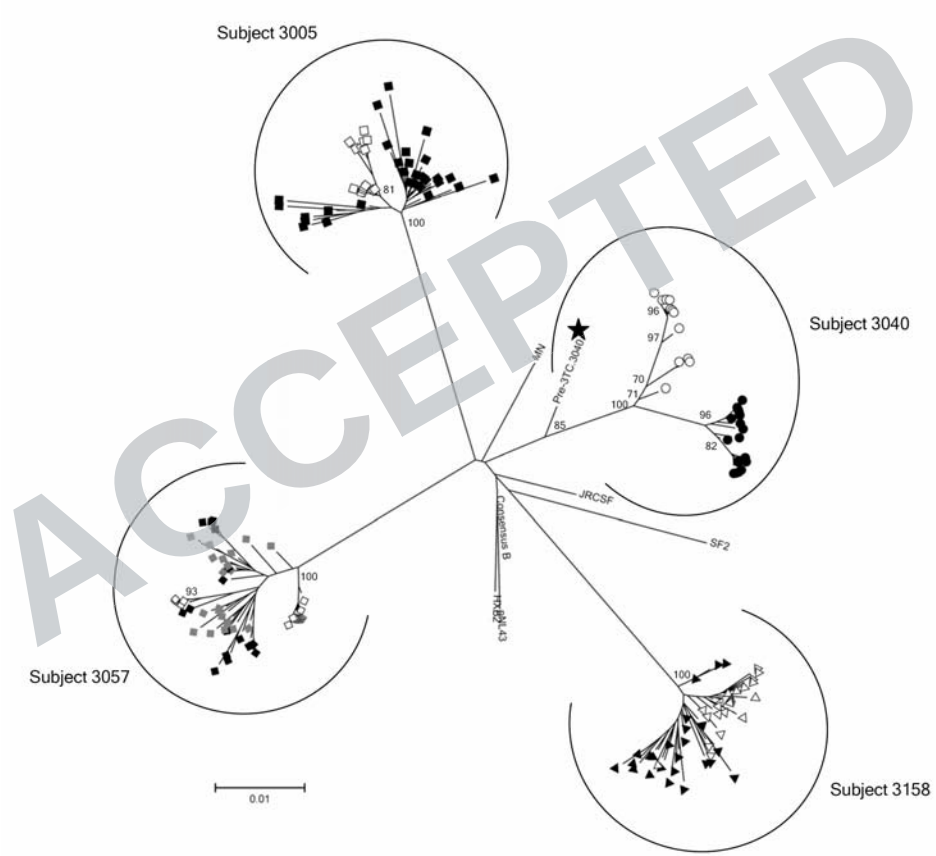


Figure 2. Continued evolution of *pol* (and back-mutation) as a major mechanism of MDR-184M variant emergence after reverse transcriptase inhibitor interruption.



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High prevalence of primary lamivudine and nelfinavir resistance in HIV-1-infected pregnant women in the United States, 1998–2004

Roger Paredes[a,b], Irene Cheng[c], Daniel R. Kuritzkes[a] and Ruth E. Tuomala[d]

Using a highly sensitive allele-specific polymerase chain reaction assay we detected the M184V mutation for lamivudine resistance in plasma from 9.4% of HIV-1-infected pregnant women enrolled in the Women and Infant Transmission Study between 1998 and 2004. The prevalence of nelfinavir resistance (D30N) was 6.3%. These results suggest a high prevalence of primary lamivudine and nelfinavir resistance among HIV-1-infected pregnant women in the United States, and support routine genotypic resistance testing before initiating mother-to-child-transmission prophylaxis.

Antiretroviral drug resistance can reduce the efficacy of mother-to-child transmission (MTCT) prophylaxis programmes and limit future antiretroviral treatment options for mother and child. Primary antiretroviral resistance may limit the suppression of viral replication during pregnancy-limited antiretroviral therapy (ART) [1] and facilitate further resistance evolution [2]. Resistant variants can be transmitted from mother to child, before, during, or after delivery [3].

Zidovudine, lamivudine and nelfinavir are the most frequently prescribed antiretroviral agents for MTCT prophylaxis in resource-rich settings. Phenotypic resistance to zidovudine requires the gradual accumulation of resistance mutations [4]. In contrast, single mutations in the reverse transcriptase (M184I/V) and protease (D30N) coding regions of *pol*, respectively, confer high-level resistance to lamivudine and nelfinavir [5,6]. Compared with routine viral population sequencing-based genotypic tests, allele-specific polymerase chain reaction (PCR) could increase the accuracy and sensitivity of primary resistance surveillance in HIV-1-infected pregnant women.

We assessed the prevalence of primary resistance to lamivudine and nelfinavir in HIV-1-infected pregnant women enrolled in the Women and Infants Transmission Study using population-based sequencing of plasma virus as well as allele-specific PCR to detect the D30N and M184V mutations. In April 2005, 1323 women who enrolled in the Women and Infants Transmission Study between 1 June 1998 and 31 December 2004 were evaluated for eligibility for this study. Study participants were HIV-1-infected pregnant women who initiated zidovudine and lamivudine therapy or zidovudine, lamivudine, and nelfinavir/nevirapine therapy during pregnancy, had never received antiretroviral therapy or had been treated for less than 15 days upon plasma specimen collection, and had a detectable HIV-RNA viral load of 500 copies/ml or greater. Of the 1323 enrolled women, 654 were ART naive before pregnancy. A total of 134 received one of the targeted regimens during pregnancy and had a study visit within 14 days of starting medications. Of these women, 94 had a viral load of 500 copies or greater at that visit and 89 had an adequate repository sample volume to participate in the study. Blinded plasma specimens were collected between June 1998 and March 2004 and were analysed in 2006 in a single laboratory.

Viral RNA was extracted from plasma (QIAamp viral RNA kit; Qiagen Inc., Valencia, California, USA), reverse-transcribed and PCR-amplified during 30 cycles (Superscript III OneStep RT/PCR; Invitrogen Corp., Carlsbad, California, USA) using primers OOPF ($_{HXB2:2211}$-2232) [5′–GAAGCAGGAGCCGATAGACAAG–3′] and OOR2 ($_{HXB2:3466}$-3444) [5′–TTTTCTGCCAGTTCTAGCTCTGC–3′]. The resulting PCR product was used as the starting template for a 30-cycle nested PCR amplification (High Fidelity Platinum Taq; Invitrogen Corp.) using primers OOPF2 ($_{HXB2:2218}$-2241) [5′–GAGCCGATAGACAAGGAACTGTAT–3′] and OOR3 ($_{HXB2:3457}$-3432) [5′–AGTTCTAGCTCTGCTTCTTCAGTTAG–3′]. The nested PCR product was

purified and sequenced (3730XL DNA analyser; Applied Biosystems, Foster City, California, USA). Resistance mutations and polymorphisms were defined according to the International AIDS Society – USA Panel (Fall 2006 update) [7]. Standard phylogenetic analyses ruled out sequence contamination.

Mutations D30N and M184V were detected by allele-specific PCR using approximately 10^6 copies of PCR product as the starting template as reported elsewhere [8]. Results reported the mean (±SD) proportion of duplicate measurements of the rate of mutants relative to the total quasispecies. The sensitivity threshold for detecting D30N and M184V mutations was, respectively, 0.1 and 0.4%.

Eighty-nine women were included in the study (analysis I, Table 1). Resistance data were available from 64 women (72%). The D30N mutation was detected in four out of 64 specimens (6.3%) by allele-specific PCR. The proportion of D30N variants in the positive specimens was in the 0.2–31.5% range. Population-based sequencing detected the D30N mutation in two specimens (3.1%) as a mixture with the wild-type allele. By population-based sequencing, D30N was not associated with other protease inhibitor resistance mutations.

The M184V mutation was detected in six out of 64 (9.4%) specimens by allele-specific PCR. The range of proportions of M184V variants in positive specimens was 0.8–130%. Population-based sequencing confirmed the presence of the M184V mutation in four specimens (6.2% of all specimens), being found as a mixture with the wild-type allele in two of them. The M184V mutation was not associated with other reverse transcriptase mutations in any of these specimens.

The K103N mutation was detected by population-based sequencing in one subject (1.6%) one day after starting treatment with zidovudine/lamivudine. The frequency of natural polymorphisms (Table 1) was similar to that in publicly available drug resistance databases.

The overall prevalence of resistance mutations and polymorphisms did not differ when the analysis was restricted to plasma specimens obtained before any exposure to antiretroviral drugs ($n = 45$, analysis II, Table 1), except for the exclusion of the single specimen with the K103N mutation and one specimen with the D30N mutation.

Table 1. Patient characteristics and prevalence of primary resistance mutations and polymorphisms in the protease and reverse transcriptase coding regions of *pol*; analysis by population-based sequencing and allele-specific polymerase chain reaction.[a]

	Analysis 1 (< 15 days ART)	Analysis 2 (ART naive)
Baseline characteristics		
Number of women (*n*)	89	61
Age (average years, range)	28.3 (16.8–42.7)	28.1 (17.9–42.7)
Time from HIV diagnosis (median years, range)	0.13 (0–15.7)	0.16 (0–15.7)
CDC AIDS category (*n*, %)	A: 69 (77.6%)	A: 47 (77.1%)
	B: 18 (20.2%)	B: 13 (21.3%)
	C: 2 (2.2%)	C: 1 (1.6%)
Previous intravenous drug use (*n*, %)	5 (5.6%)	2 (3.3%)
CD4 T-cell count (median, $_{25\text{-}75}$IQR)	439 (312–583) cells/μl	446 (312–607) cells/μl
Plasma HIV-1 RNA (median, $_{25\text{-}75}$IQR)	6196 (1353–17 262) copies/ml	11 851 (1604–20 097) copies/ml
Samples with informative resistance data	64 (72%)	45 (74%)
Primary mutations or polymorphisms		
Protease, population-based sequencing (*n*, %)		
L10V	6 (9.4%)	5 (11.1%)
I13V	9 (14.1%)	6 (13.3%)
K20R/M	1 (1.6%)	1 (2.2%)
D30N	2 (3.1%)	1 (2.2%)
L33I/V	2 (3.1%)	1 (2.2%)
M36I	10 (15.6%)	7 (15.6%)
D60E	4 (6.3%)	4 (8.9%)
I62V	11 (17.2%)	7 (15.6%)
L63P	29 (45.3%)	20 (44.4%)
A71V	3 (4.7%)	3 (6.7%)
V77I	10 (15.6%)	7 (15.6%)
I93L	8 (12.5%)	5 (11.1%)
Reverse transcriptase, population-based sequencing (*n*, %)		
K103N	1 (1.6%)	0 (0%)
M184V	4 (6.3%)	4 (8.9%)
D30N and M184V allele-specific PCR (*n*, %)		
D30N	4 (6.3%)	3 (6.7%)
M184V	6 (9.4%)	6 (13.3%)

ART, Antiretroviral therapy; CDC, Centers for Disease Control and Prevention; IQR, interquartile range; PCR, polymerase chain reaction.
[a]Analysis 1 included specimens drawn before 15 days of dual or trio antiretroviral therapy (not inclusive). Analysis 2 included specimens drawn before any antiretroviral therapy.

This study suggests a high prevalence of primary lamivudine and nelfinavir resistance in HIV-1-infected pregnant women in the United States during 1998–2004. Despite the small sample size of the study, our data are consistent with earlier studies indicating an increase in the prevalence of primary nucleoside reverse transcriptase inhibitor, non-nucleoside reverse transcriptase inhibitor and protease inhibitor resistance during the same time period [9]. Our data probably reflect an increase in transmitted drug-resistant variants among the general HIV-1-infected population during the study period. The relatively short timespan between the diagnosis of HIV-1 infection and resistance testing in our study may have facilitated the detection of transmitted resistant variants. Injection drug use was infrequent in our cohort, and was not significantly associated with an increased risk of resistance (not shown). Detection of the K103N mutation in one subject one day after starting zidovudine/lamivudine therapy suggests that this mutation was present before the initiation of therapy, and that further K103N mutations could have been detected using allele-specific PCR.

Allele-specific PCR is a more sensitive method of detecting individual resistance mutations than population-based sequencing, being a useful tool for the surveillance of particularly relevant resistance mutations. The frequency of the D30N and M184V mutations increased two to threefold and 1.5-fold, respectively, when allele-specific PCR tests were utilized. Further research is warranted to establish the clinical significance of detecting very low levels of resistant variants. One retrospective study found that the presence of minor variants containing mutations K103N, Y181C and M184V in antiretroviral-naive subjects was associated with a higher likelihood of subsequent virological failure [10].

Our findings support routine genotypic resistance testing before initiating MTCT prophylaxis in the United States. They also confirm the utility of allele-specific PCR to detect single mutations conferring high-level resistance to key drug components of MTCT regimens, and support using triple-drug MTCT regimens to maximize the efficacy of this strategy and reduce the likeliness of the evolution of resistance. Further assessments of primary resistance in larger populations of HIV-1-infected pregnant women should be undertaken to confirm and extend our results. Additional analyses to assess the development of resistance in HIV-infected pregnant women during pregnancy-limited ART and virological response to subsequent antiretroviral regimens are planned.

[a]Section of Retroviral Therapeutics, Brigham and Women's Hospital and Division of AIDS, Harvard Medical School, Boston, Massachusetts, USA; [b]Fundacions irsi Caixa i Lluita contra la SIDA, Universitat Autònoma de Barcelona, Catalonia, Spain; [c]Clinical Trials and Surveys Corp., Baltimore, Maryland, USA; and [d]Division of Maternal–Fetal Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA.

This work was partly supported by the following US Public Health Service grants from the National Institutes of Health: R01 AI42567, K24 RR16482, a Virology Support Laboratory contract from the Adult ACTG (U01 AI-38858), and the Harvard Medical School Center for AIDS Research Virology Core (P30 AI60354). R.P. is a recipient of the 'La Caixa' Fellowship Grant for Post-Graduate Studies, Caixa d'Estalvis i Pensions de Barcelona, Catalonia, Spain. The Women and Infants Transmission Study principal investigators, study coordinators, programme officers and funding include: Clemente Diaz, Edna Pacheco-Acosta (University of Puerto Rico, San Juan, PR; U01 AI 034858); Ruth Tuomala, Ellen Cooper, Donna Mesthene (Boston/Worcester Site, Boston, MA; 9U01 DA 015054); Phil LaRussa, Alice Higgins (Columbia Presbyterian Hospital, New York, NY; U01 DA 015053); Sheldon Landesman, Herman Mendez, Ava Dennie (State University of New York, Brooklyn, NY; U01 HD 036117); Kenneth Rich, Delmyra Turpin (University of Illinois, Chicago, IL; U01 AI 034841); William Shearer, Norma Cooper (Baylor College of Medicine, Houston, TX; U01 HD 041983); Joana Rosario (National Institute of Allergy and Infectious Diseases, Bethesda, MD); Kevin Ryan (National Institute of Child Health and Human Development, Bethesda, MD); Vincent Smeriglio, Katherine Davenny (National Institute on Drug Abuse, Bethesda, MD); and Bruce Thompson (Clinical Trials and Surveys Corporation, Baltimore, MD; N01 AI 085339). The scientific leadership core included: Kenneth Rich (principal investigator), Delmyra Turpin (study coordinator) 1 U01 AI 050274-01. Additional support was provided by local clinical research centers as follows: Baylor College of Medicine, Houston, TX; NIH GCRC RR000188; Columbia University, New York, NY; NIH GCRC RR000645.

Received: 29 March 2007; revised: 11 June 2007; accepted: 20 June 2007.

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The intestinal mucosa as a reservoir of HIV-1 infection after successful HAART

Liliana Belmonte[a], Martin Olmos[c], Ana Fanin[c], Cecilia Parodi[a], Patricia Baré[a], Hugo Concetti[d], Héctor Pérez[b], Maria Marta E. de Bracco[a] and Pedro Cahn[b]

The presence of HIV-1 RNA in distal duodenal mucosa was evaluated in 44 HIV-1-positive patients. HIV-1 RNA was detected in gut tissue in antiretroviral-naive patients with high plasma viral loads, as well as in patients on HAART with plasma viral loads below the limit of detection and in patients on HAART with virological failure. The intestinal mucosa seems to serve as a reservoir poorly influenced by levels of plasma viral load or HAART.

With the use of HAART, it is now possible to reduce plasma HIV-1 RNA to undetectable levels and increase CD4 T-cell counts in peripheral blood in the majority of adherent patients [1,2]. T lymphocytes in peripheral blood represent only 2–5% of the total of lymphocytes in the body, whereas the majority of lymphocytes are located in lymphoid tissues. Gut-associated lymphoid tissue (GALT) represents the largest lymphoid organ. It is extremely permissive to HIV-1 infection and supportive of HIV-1 replication, providing a persistent viral reservoir [3–5]. Several studies have demonstrated that replication-competent virus persists in blood cells and lymphoid tissue after at least 2 years of HAART, despite the complete suppression of HIV-1 RNA in blood [6–8]. The presence of HIV-1 in intestinal mucosa has been demonstrated, but there are relatively few reports of the effects of HAART in these tissues [9–11].

In this study we examined the presence of HIV-1 RNA in paraffin-embedded distal duodenal mucosa biopsies from 44 HIV-positive patients by in-situ hybridization. Twenty-five out of 44 patients had been on HAART for more than 4 years. The characteristics and clinical data of the participants in the study were: 30 men, 14 women; average CD4 T-cell count 265 ± 222 cells/μl (mean ± SD); mean age 38.2 ± 7 years; mode of transmission men who have sex with men, 18 cases; heterosexual transmission, 19; intravenous drug users, six; transfusion, one. As controls, distal duodenal mucosa biopsies from 10 HIV-1-seronegative individuals were also analysed.

The duodenal mucosa biopsies were obtained for diagnostic purpose and the remaining tissue was embedded in paraffin and used for the in-situ hybridization analysis later. This protocol was reviewed and approved by the Ethics Committee of the Academia Nacional de Medicina and Juan A. Fernandez Hospital.

The HIV-RNA concentration in plasma was determined using the Amplicor HIV monitor quantitative assay (Roche Diagnostic Systems, Branchburg, New Jersey, USA). CD4 lymphocyte counts were determined by flow cytometry (Becton-Dickinson, San Jose, California, USA).

Twenty-five out of the 44 HIV-1-positive patients had HIV-1 RNA detectable in plasma (plasma viral load $> 100\,000$ copies/ml). The remaining 19 patients had undetectable HIV-1 RNA (< 50 copies/ml).

In-situ hybridization using biotin-labeled probes and tyramide signal amplification (DakoCytomation, Gen Point; Dako Denmark A/S, Denmark) was performed to allow the qualitative detection of HIV-1 RNA in duodenal mucosa sections from patients with chronic HIV infection. Two probes were used: Gag/5′ genome 5′GGA TGT ACT CTA TCC CAT TCT GCA GCT TCC TCA 3′; Rev probe 5′TCC TGC CAT AGG AGA TGC/CAG TCG CCG CCC CTC 3′. The signal was developed by adding the chromogenic indicator dye diaminobencidine, which is oxidized by the peroxidase enzymes. The specificity of the in-situ hybridization was demonstrated by the lack of signals in the duodenal biopsies from HIV-1-seronegative patients or when the probe was omitted from the hybridization mixture. The slides were counterstained with haematoxilin. Visualization of in-situ hybridization signals was carried out using an Olympus microscope equipped with 20×, 40×, 60× and 100× objectives (Fig. 1a).

Positive HIV-1 hybridization signals were observed in the duodenal biopsies from 20 of the 44 HIV-1 patients (45.5%). Ten of the 20 HIV-1-positive duodenal biopsies corresponded to patients who had plasma viral loads below 50 copies/ml and 10 to patients who had plasma viral loads greater than 100 000 copies/ml ($P = 0.4$ ns, chi-square test).

Addendum II

Publications Related with this Thesis under Journal Review as of January 2009

CHAPTER 4

Roger Paredes, Irene Cheng, Daniel R. Kuritzkes, Ruth E. Tuomala, & the Women and Infants Transmission Study Group. **Antiretroviral Drug Resistance during Pregnancy-Limited Antiretroviral Therapy in the US.** AIDS.
(Submitted, Under Review)

CHAPTER 5

Roger Paredes, Christina M. Lalama, Heather J. Ribaudo, Bruce Schackman, Cecilia Shikuma, Francoise Giguel, Victoria A. Johnson, Susan A. Fiscus, Richard T. D'Aquila, Roy M. Gulick, and Daniel R. Kuritzkes, for the AIDS Clinical Trials 5095 Study Team. **Pre-existing Minority Drug-Resistant HIV-1 Variants and Risk of Antiretroviral Treatment Failure.** Journal of Infectious Diseases.
(Submitted, Under Review)

Addendum III
Other Publications by the Author

PUBMED INDEXED MANUSCRIPTS

1. Athe M. N. Tsibris,* **Roger Paredes**,* Amy Chadburn, Zhaohui Su, Timothy J. Henrich, Amy Krambrink, Michael D. Hughes, Judith A. Aberg, Judith S. Currier, Karen Tashima, Catherine Godfrey, Wayne Greaves, Charles Flexner, Paul R. Skolnik, Timothy J. Wilkin, Roy M. Gulick, and Daniel R. Kuritzkes. Lymphoma Diagnosis and Plasma Epstein-Barr Load during Vicriviroc Therapy: Results of the AIDS Clinical Trials Group 5211. Clin Infect Dis 2009; 48: (in press). (*both authors contributed equally)

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Acknowledgements

Thanks, first, to my family: to my grandparents Manuel, Joan, Anna and Pilar, my parents Antoni and Pilar, my sister Kina and my brother Jordi. To my wife Judith and my kids Ernest and Martí, the best thing ever! To Víctor and Maria Rosa and the Serna Mont-Ros Family. To my friends Àlex, Guillermo, Pere, Marc, David and Isma. To Jose and Ivan. To Jordi, Ana, Mayte, Héctor, Mireia, David, Miriam, Iban, Sergio, Anna, Nayra, Bea, and all my resident fellows. To Joan Ramon and Montero. To "*el tito*" Dani. To Sara and Jordi. To David and Jordi. To Vince and Claudia. To Athe. To Françoise and Kim. To Nina and Nori. To Zixin and Manish. To Luis and Fae. To my teachers. To my team, Rocío, Christian, Mattia and Paco. To Lourdes and Albert. To everyone at irsiCaixa and Lluita contra la SIDA. To the coauthors of all my papers. To all the funding agencies cited throughout this thesis. To the "La Caixa" Grant for Postgraduate Studies, for funding my research fellowship. To Dan, my mentor at Harvard, for giving me the opportunity to work in a fantastic scientific environment and provide me with wonderful projects. To Ventura, my lifetime mentor, the only person I know for whom everything seems to be possible, a good friend, and one of the most extraordinary physicians and human beings I know.


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