Mutational Bias and Emergence of Drug Resistance in the Human Immunodeficiency Virus Type 1

Matthew McCallum

Master of Science

Microbiology and Immunology

McGill University, Montréal

845 Sherbrooke Street West, Montréal, QC H3A 0G4

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Abstract

E138K, a G→A mutation in the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT), is preferentially selected by etravirine (ETR) and rilpivirine (RPV) over other substitutions at position E138 that offer greater drug resistance. We hypothesized that there was a mutational bias for the E138K substitution and designed an allele-specific PCR to monitor the emergence of E138A/G/K/Q/R/V during ETR or RPV selection experiments. E138K, as well as E138G, consistently emerged first during selection experiments, followed by E138A, E138Q and E138R. Surprisingly, E138K was identified as a minority in 23% of drug-naïve subtype B patients, and was not further enriched in patients with the M184I substitution. The high prevalence of E138K minority species could reflect a low fitness cost of E138K; however, E138K was one of the least fit substitutions at codon E138, even after taking into account the dNTP pools of the cells used in competition experiments. Ultra-deep sequencing analysis revealed other minority species in a pattern consistent with the mutational bias of HIV-1 RT. These results confirm the mutational bias of HIV-1 in patients and highlight the importance of G→A mutations in HIV-1 drug resistance evolution.

This G→A bias reflects enriched adenosine in HIV-1 codons, a feature that is mysteriously targeted by the anti-HIV-1 restriction factor, Schlafen family protein 11 (SLFN11). Our *in silico* modeling of SLFN11 suggested putative structure-function relationships and a relation to Ski2-family RNA helicases.

Résumé

E138K, une mutation G → A dans l'immunodéficience humaine de type virus humain 1 (VIH-1) et dans la transcriptase inverse (TI), est de préférence choisie par l'étravirine (ETR) et rilpivirine (RPV) plutôt que d'autres substitutions à la position E138 qui offrent une plus grande résistance. Nous avons supposé qu'il y avait un biais mutationnel pour la substitution E138K et conçu une PCR allèle spécifique pour surveiller l'émergence de E138A/G/K/Q/R/V lors d'expériences de sélection avec ETR ou RPV. E138K, ainsi que E138G, constamment apparue au cours d'expériences de sélection, suivi d'E138A, E138Q et E138R. Étonnamment, E138K a été identifiée comme une infime minorité dans 23% des cas de sous-type B de patients naïfs aux médicaments, et n'a pas augmenté chez les patients atteints de la substitution M184I. La prévalence élevée des espèces minoritaires de E138K pourrait refléter un faible coût de remise en forme de E138K, mais E138K était l'un des substitutions moins performants au niveau du codon E138, même après avoir pris en compte la concentration de dNTP dans cellules utilisées dans des expériences de compétition. Une analyse de séquençage en profondeur a révélé d'autres espèces minoritaires dans un modèle cohérent avec le biais mutationnel du VIH-1 TI. Ces résultats soulignent l'importance de G → A mutations du VIH-1 dans l'évolution de la résistance aux médicaments.

Ce G → A biais a enrichi l'adénosine dans le codons VIH-1, une fonctionnalité qui est mystérieusement ciblé par le facteur anti-VIH-1 restriction, Schlafen protéine de la famille 11 (SLFN11). Notre modélisation *in silico* de SLFN11 suggère des relations structure/fonction présumée et une relation à l'hélicase ARN de famille Ski2.

Dedication

This work is dedicated to individuals infected with HIV,
whose lives depend on antiretroviral drugs.

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Preface

All results presented are original scholarship; however, much of this work has recently been published (McCallum, M., Oliveira, M., Ibanescu, R. I., Kramer, V. G., Moisi, D., Asahchop, E. L., Brenner, B. G., Harrigan, R., Xu, H. T., and Wainberg, M. A. (2013) Basis for the Early and Preferential Selection of the E138K Substitution in HIV-1 Reverse Transcriptase with Etravirine. Antimicrob. Agents Chemother. Epub ahead of print). Excerpts from this publication are presented here with permission of Antimicrobial Agents and Chemotherapy.

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List of Abbreviations

Human Immunodeficiency Virus (HIV), Acquired Immune Deficiency Syndrome (AIDS), Simian Immunodeficiency Virus (SIV), primer binding site (PBS), transactivation responsive (TAR), schlafen family protein 11 (SLFN11), SAM domain and HD domain-containing protein 1 (SAMHD1), uracil-DNA glycosylase 2 (UNG2), interferon-inducible transmembrane protein family (IFITM), apolipoprotein B mRNA-editing enzyme-catalytic polypeptide 3 (APOBEC3), APOBEC3A-H (A3A-H), tripartite motif containing 5 alpha (Trim5α), superfamily 1 and 2 (SF1 and SF2), ATPases associated with diverse cellular activities (AAA+), highly active antiretroviral therapy (HAART), nucleoside/nucleotide reverse transcriptase inhibitor (NRTI), non-NRTI (NNRTI), protease inhibitor (PI), Didanosine (ddI), Zalcitabine (ddC), Stavudine (d4T), Lamivudine (3TC), Delavirdine (DLV), Efavirenz (EFV), nevirapine (NVP), TSAO, Raltegravir (RAL), Elvitegravir (EVG), diarylpyrimidine (DAPY), Etravirine (ETR), Rilpivirine (RPV), tenofovir (TDF), Emtricitabine (FTC), allele-specific polymerase chain reaction (AS-PCR), hydroxyurea (HU), deoxynucleoside (dN), primary HIV infection (PHI), root-mean-square deviation (RMSD), deoxycytidine (dC), and tetrahydrouridine (THU).

1 INTRODUCTION

We have entered a new era in the struggle against the human immunodeficiency virus (HIV), the causative agent of the Acquired Immunodeficiency Syndrome (AIDS). Biomedical advances have changed HIV infection from a death sentence into a manageable chronic disease, and additional innovations have revealed effective techniques to prevent infection. Most of these advances reflect innovations in antiretroviral drugs that inhibit viral replication. Unfortunately, HIV inevitably evolves resistance against antiretroviral drugs in patients, and these patients must be given alternative antiretrovirals that are not susceptible to such resistance. Understanding the mechanisms of HIV drug resistance is essential to wisely choosing primary therapy, salvage therapy, and antiretrovirals designed to prevent infection. After almost 30 years of clinical antiretroviral drug use, elucidating these mechanisms is still a necessity as novel drugs continue to be introduced into clinical practice.

1.1 HIV Epidemiology

Independent cross-species transmission events of the simian immunodeficiency virus (SIV) into humans resulted in several lineages of HIV. It is thought that HIV-1 groups M and N arose from separate zoonotic infections of chimpanzee SIV, HIV-1 groups O and P arose from separate zoonotic infections of gorilla SIV, and HIV-2 from a zoonotic infection of sooty mangabey SIV (1-5). Group M accounts for almost all HIV infections, and can be subdivided into subtypes A-K, as well as circulating recombinant forms of these subtypes. During the early 1980s, HIV-1 Group M Subtype B was first identified in gay men,

intravenous drug users, Haitians, and hemophiliacs in major cities of North America and Western Europe. To this day, subtype B infections in gay men account for most infections in North America and Western and Central Europe (6-8). Conversely, Subtype C infections in heterosexual populations account for the majority of infections globally, with an overwhelming prevalence in sub-Saharan Africa (8, 9).

Early studies found that HIV-1 can be transmitted via bodily fluid exchange during sexual activity, needle sharing, blood transfusions, breast-feeding, and during pregnancy (10-16). Typically within a decade of infection, HIV-1 infection causes a massive depletion in CD4 T-cells that leads to AIDS, which is characterized by rare cancers, weight loss, and various opportunistic infections that ultimately result in death (10, 17-19). It is estimated that over 35 million people have died of AIDS, and more than 34 million people are currently infected with HIV-1 (9).

1.2 The Viral Lifecycle

1.2.1 Entry

HIV-1 infects $CD4^+$ cells, such as CD4 T-cells, macrophages, and dendritic cells. Entry of HIV-1 into these cells is a two-step process: the viral envelope first binds the CD4 receptor, and then the viral envelope binds to either CXCR4 or CCR5 co-receptors (20-23). This sequential binding triggers a conformational change allowing fusion of the viral envelope with the host-cell plasma membrane (23).

1.2.2 Reverse Transcription

Upon entry into the cytoplasm, reverse transcription of the sense single stranded RNA genome into double stranded DNA begins (24). RT catalyzes the RNA dependent DNA polymerase-mediated conversion of the RNA genome into negative sense DNA, and then RT uses its DNA dependent DNA polymerase activity to generate double stranded viral DNA (25). The reverse transcription process involves strand-transfer events, whereby RT switches templates during polymerization (26, 27). This capacity enables the two RNA copies carried in each HIV-1 virion to recombine up to 30 times during reverse transcription (28). RT also has poor substrate specificity, and no 3'→5' exonuclease proof-reading activity, making the reverse transcription process notoriously error prone (29-31). The efficiency of reverse transcription is dependent on the host-cell dNTP pools, as demonstrated by the inefficiency of reverse transcription in cells with low dNTP pools, like resting T-cells, macrophage, and dendritic cells, compared with cells with high dNTP pools, like activated T-cells (32-34).

1.2.3 Integration

Various viral and host proteins form a pre-integration complex that facilitates the nuclear localization of the viral DNA genome (35-38). The viral integrase then catalyzes the covalent integration of the viral DNA genome into the host genome (39, 40). The integrated viral DNA is referred to as a provirus.

1.2.4 Viral Transcription

Following integration, the viral promoter in the 5' LTR may bind multiple transcription factors if they are available, including nuclear factor kappa-light-chain-enhancer of activated B cells and Specificity Protein 1 trigging viral

genome transcription by host RNA polymerase II (41, 42). However, only a short leader sequence termed the transactivation responsive (TAR) element is transcribed (43). The viral protein, Tat, binds the TAR element, as well as to positive transcription elongation factor b, TATA-binding protein, and several other transcription factors, promoting the initiation and elongation of viral transcription past the TAR element (44-48). This generates full length viral transcripts in the nucleus that are subsequently spliced multiple times by the host-splicosome (49).

1.2.5 Viral Translation

Similar to host mRNA, these heavily spliced viral transcripts are exported to the cytoplasm where they are translated to Tat, Nef, and Rev viral proteins (49). In a positive feedback loop, Tat amplifies viral transcription (47). Rev binds the Rev response element of incompletely spliced viral transcripts exporting them to the cytoplasm prior to further splicing (50). In this way, Rev permits the expression of additional viral proteins Gag, Gag-Pol, Env, Vif, Vpu, and Vpr. Gag assembles on the inside of the plasma membrane promoting viral budding and actively recruiting Gag-Pol and viral RNA into the budding virion, as well as Env on the cell surface (51-53). Following budding, in a process termed virion-maturation, the protease in Gag-Pol cleaves Gag and Gag-Pol into capsid, matrix, nucleocapsid, spacer 1, spacer 2, p6, integrase, p51, p66, and protease (54). p51 is an alternatively cleaved version of p66 that lacks RNAseH; p51 and p66 form the heterodimeric viral RT; p66 is catalytically active while p51 is not (55, 56). Together, two full-length viral RNA transcripts and these viral proteins form a functional virus. However, host-cells are not defenseless against viral infection

and have evolved proteins that restrict viral replication, i.e. restriction factors. Likewise, the viral proteins, Vif, Vpu, and Vpr are actively involved with countering these restriction factors.

1.3 HIV-1 Restriction Factors

When human tissues are infected by a virus, molecular pattern recognition receptors in infected cells sense conversed viral patterns, and trigger the secretion of the antiviral and antitumor cytokines known as interferons (57, 58). In an autocrine and paracrine fashion, interferons activate certain cells of the immune system, bias the adaptive immune response towards antiviral functions, and up-regulate antiviral restriction factors (59). Numerous potential HIV-1 restriction factors have been identified, though only a few have been characterized (60, 61).

1.3.1 Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide 3 (APOBEC3) Family Enzymes

Seven APOBEC3 family enzymes encoded by the human genome have been found to have anti-HIV-1 activity: APOBEC3A (A3A), APOBEC3B (A3B), APOBEC3C (A3C), APOBEC3DE (A3DE), APOBEC3F (A3F), APOBEC3G (A3G), and APOBEC3H (A3H) (62-66). These proteins restrict HIV-1 replication by deaminating numerous deoxycytidine nucleotides to deoxyuracil on negative sense DNA during reverse transcription (66-71). Ultimately, this causes the positive sense viral RNA genome to have extensive guanosine to adenonsine G→A mutations, termed hypermutation. Hypermutation can restrict viral replication by introducing premature stop codons or deleterious mutations in viral genes (66, 68, 72). Some APOBEC3 enzymes also restrict viral replication by

directly interfering with reverse transcription in a deaminase-independent mechanism (73-75).

HIV-1 encodes the Vif protein, which targets most APOBEC3 family proteins for proteosomal degradation (76-79). To function effectively, APOBEC3 enzymes must be incorporated into budding virions; hence, Vif protects progeny virions from the antiviral activities of APOBEC3 enzymes (80). Accordingly, HIV-1 expressing functional Vif replicates proficiently in human T-cells despite constitutive expression of APOBEC3 enzymes (81). Nonetheless, Vif is saturable, and sometimes there is APOBEC3 activity despite Vif expression (82, 83). Likewise, hypermutated sequences are consistently identified in 6-40% of the proviral genomes in patients (84-88).

APOBEC3 enzymes have dinucleotide substrate specificity with A3G preferentially mutating GG→AG, A3DE preferentially mutating GT→AT, and the other APOBEC3 enzymes preferentially mutating GA→AA (64, 71, 77, 89-94). Mutations introduced at other stages of the viral lifecycle, i.e. reverse transcription, do not have such specificity (95). Hence, quantifying and comparing G→A mutations in a dinucleotide specific context provides a straightforward method of identifying hypermutated viral sequences (Hypermut 2.0; http://www.hiv.lanl.gov). With overlapping substrate specificities and expression patterns, the relative contribution of each family member to HIV-1 hypermutation and restriction in patients is unclear, though A3G and A3F are thought to play a more significant role than the others (94).

1.3.2 Uracil-DNA glycosylase 2 (UNG2)

UNG2 restricts the integration of HIV-1 DNA that has accumulated large amounts of deoxyuracil nucleosides (96). HIV-1 DNA can accumulate deoxyuracil nucleosides via dUTP misincorporation, as well as by APOBEC3 activity. UNG2 begins the base-excision repair pathway that intermediately causes a single stranded break; accumulation of multiple deoxyuracil-excision repair initiations may cause lethal double stranded breaks (96, 97). Likewise, the viral protein, Vpr, targets UNG2 for proteosomal degradation (98). The relation between this mode of restriction and APOBEC3 mediated deamination is unclear, though it is known that UNG2 is not required for APOBEC3 mediated viral restriction (99-101).

1.3.3 Tetherin

Tetherin is a membrane protein that tethers budding virions to the surface of infected cells (102, 103). The viral protein, Vpu, antagonizes Tetherin by targeting it for proteosomal degradation (104).

1.3.4 Tripartite motif containing 5 (Trim5α)

By an unclear mechanism, Trim5α binds retroviral capsid causing premature uncoating, blocking reverse transcription and nuclear import (105, 106). Human Trim5α cannot bind HIV-1 capsid, and does not restrict HIV-1; though the Trim5α of some old world monkeys potently inhibit HIV-1 replication (107).

1.3.5 SAM domain and HD domain-containing protein 1 (SAMHD1)

SAMHD1 is an interferon inducible dNTPase that lowers cellular dNTP pools in monocytes, dendritic cells, and macrophages, reducing the efficiency of reverse transcription and infection (108-110). There is currently no known HIV-1 encoded SAMHD1 antagonist.

1.3.6 Interferon-inducible transmembrane protein family (IFITM)

The mechanism of IFITM restriction is currently unclear, though it is thought that some IFITM proteins restrict HIV-1 entry (111). Like SAMHD1, there is no known viral antagonist to IFITM.

1.3.7 Schlafen family member 11 (SLFN11)

SLFN11 is a newly discovered interferon inducible HIV-1 restriction factor, and a member of the largely uncharacterized SLFN family of proteins (112). SLFN family proteins are important for orthopoxvirus virulence and T-cell quiescence, development, and cell-cycle arrest (113-117). It was recently shown that SLFN11 restricts HIV-1 replication by inhibiting the synthesis of viral proteins that use codons rarely used in human genes (112). The codon use in HIV-1 is divergent from most human genes due to the heavy adenosine bias of the HIV-1 genome (112, 118). Intriguingly, it was also recently shown that SLFN11 expression in cancer cells is causally associated with the effectiveness of DNA-damaging anti-cancer drugs (119, 120).

The molecular mechanism of SLFN family proteins, including SLFN11, is currently unclear. Sequence alignment searches reveal no characterized relatives.

Detailed sequence analysis only reveals two putative ATPase-associated-with-diverse-cellular-activities (AAA+) domains (121). This functionally diverse family of proteins is actively involved with conformational changes in proteins, DNA, or RNA (122). One study suggested that murine SLFN family members have motifs with weak homology to DEAD-box motifs common to Superfamily 1 and 2 (SF1 and SF2) DNA/RNA helicases (114). Consistent with this premise, SLFN11 was found to bind tRNA *in vitro* (112). If tRNA binding is relevant *in vivo*, the subsequent sequestering, processing, or degradation of tRNAs important to viral replication could explain the codon-dependent antiviral function of SLFN11. Unfortunately, this hypothetical mechanism cannot adequately justify the relation of SLFN11 to cancer therapeutics. Thus, the putative molecular function of SLFN11 remains a mystery.

1.4 Antiretroviral Therapy

1.4.1 Early nucleoside reverse transcriptase inhibitors (NRTIs)

Despite the diversity of host restriction factors, HIV-1 replicates proficiently in humans. Likewise, for the health of infected individuals, HIV-1 replication must be challenged by biomedical intervention. Zidovudine (AZT), a nucleoside reverse transcriptase (RT) inhibitor (NRTIs), was first reported to decrease mortality in patients with AIDS in the late 1980s (123). Shortly thereafter, additional NRTIs, Didanosine (ddI), Zalcitabine (ddC), Stavudine (d4T), and Lamivudine (3TC), were approved for clinical use (124-129). NRTIs are substrate analogues that are mistaken by RT for normal nucleotides and incorporated into viral DNA during reverse transcription (130). NRTIs lack a

3'OH group, and RT lacks 3'→5' exonuclease proof-reading activity; therefore, NRTI incorporation during reverse transcription causes lethal chain termination (29, 130).

The efficacy of early NRTI treatment was transient. The rapid and error prone nature of viral replication and high viral titers allowed HIV-1 to quickly diversify and acquire drug resistance mutations that limited the effectiveness of early NRTIs (131). For example, in response to 3TC treatment, patients rapidly developed the M184I or M184V mutations within the RT active site; M184I usually emerged first, and then was replaced by M184V (132, 133). These mutations in RT sterically hinder incoming deoxynucleotides, especially 3TC (134). As a result, RT with the M184I/V substitution adequately discriminates against 3TC at the cost of having a higher reverse transcription fidelity, lower affinity for deoxynucleotides, and decreased enzyme processivity (135, 136). This results in viruses with the M184I/V substitution having decreased replicative fitness (137-140). HIV-1 replicative fitness is defined as the capacity to adapt and reproduce within a given environment (141, 142). Decreased viral fitness is correlated with decreased viral pathogenicity and is also thought to decrease the likelihood of transmission (143, 144). As a result of substitutions like M184I/V, early NRTI use had limited therapeutic benefit.

1.4.2 Drug combinations and protease inhibitors

When the protease inhibitors (PIs) were approved for clinical use in the mid and late 1990s, they were used in combination with two NRTIs, usually AZT with 3TC (145, 146). This multi-drug combination therapy was termed highly active

antiretroviral therapy (HAART). HIV-1 had to develop many drug resistance mutations prior to clinical HAART failure; likewise, HAART suppressed viral replication better than ever before with dramatic decreases in morbidity and mortality (147-150).

1.4.3 Early non-nucleoside reverse transcriptase inhibitors (NNRTIs)

Shortly after the approval of the first PIs, the first NNRTIs were approved for combination drug therapy: Delavirdine (DLV), Efavirenz (EFV), and Nevirapine (NVP) (151-153). All of these early NNRTIs developed similar drug resistance mutations, including K103N, Y181C, and G190A, and treatment failure to one NNRTI usually prevented alternative NNRTI use (154). Likewise, an alternative family of NNRTIs with a distinct drug resistance profile was formulated: TSAO-derivatives (155, 156). However, TSAO-derivatives were never approved for clinical use as it was found that the E138K substitutions in RT quickly emerged leading to very high-level TSAO resistance (157, 158).

1.4.4 Issues with early HAART

Despite the successes of early HAART, treatment required taking many different pills, several times a day, with specific food requirements, as well as a long list of severe side effects (159-163). Likewise, adherence to these drugs was low, and this permitted viral replication and drug resistance in treated patients (164-166). Multi-drug resistant HIV-1 variants emerged and were transmitted within populations, threatening the success of HAART (167, 168). With poor adherence, limited treatment alternatives, the inevitability of developing drug

resistance, and unclear long-term side effects of antiretroviral therapy, HIV-1 infected individuals were recommended to be placed on therapy just before or after development of AIDS (169, 170). Newer drugs and improved treatment regimens were a necessity.

1.4.5 Newer drugs and drug targets

Likewise, new antiretroviral drugs were formulated, some with alternative viral targets. Entry inhibitors, Miraviroc and Enfuvirtide, as well as integrase inhibitors, Raltegravir (RAL) and Elvitegravir (EVG), have been approved for clinical use (171, 172). In addition, two diarylpyrimidine (DAPY) based NNRTIs, Etravirine (ETR) and Rilpivirine (RPV), were approved for clinical use (173-175). Like TSAO-compounds, DAPY compounds are effective against HIV-1 resistant to earlier NNRTIs and also select for the E138K substitution (176). Newer NRTIs with greatly improved pharmacokinetics were also approved: Tenofovir (TDF) and Emtricitabine (FTC) (177-179). Of note, FTC shares many structural properties with 3TC; likewise, both drugs strongly select for the M184I/V substitution (180).

1.4.6 Current HAART formulations and policies

In addition to newer drugs, improved drug co-formulations have made a dramatic impact on the practicality of HAART regimens. For example, there are now three all-in-one co-formulations that are approved to be taken just once a day: EFV/FTC/TDF, RPV/FTC/TDF, and EVG/FTC/TDF/Cobicistat (181-186).

FTC together with TDF is a common backbone in contemporary HAART regimens (187).

Improved treatment regimens of contemporary HAART, such as once-a-day co-formulations, have minimized poor adherence and reduced the likelihood of drug resistance (188, 189). Furthermore, the long-term side-effects of HAART appear minimal compared with the benefits (190). Indeed, there is a growing consensus that HAART should be initiated as soon after infection as possible (191, 192). This is seconded by the finding that persons treated with HAART are much less likely to transmit the virus (193). Uninfected persons taking TDF/FTC as pre-exposure prophylaxis (PrEP) are also less likely to be infected with the virus (194-196). With the approval of new drugs and drug-combinations for use in more populations than ever before, new and poorly characterized drug resistance mutations must be studied to gauge how best to use these drugs for the long-term benefit of current HAART.

1.5 Anomalous selection of E138K by ETR and RPV

1.5.1 ECHO and THRIVE Clinical Trials

The recent ECHO and THRIVE clinical trials were designed to assess the effectiveness of RPV/FTC/TDF compared with EFV/FTC/TDF in treatment naïve patients (197-199). In the EFV arm of the trial, K103N and M184V were the most frequently selected NNRTI and NRTI resistance mutations, respectively. This was expected as the K103N substitution is the most commonly selected EFV resistance mutation, and the M184V substitution is the most commonly selected FTC resistance mutation (200-202). In the RPV arm of the trial, the E138K and the M184I substitutions were the most frequently selected NNRTI and NRTI

resistance mutations, respectively (197-199). This was unexpected as the M184V substitution is identified much more often at treatment failure than M184I as it has greater viral fitness than the M184I substitution (137). This was also unexpected because several other substitutions offer similar or greater RPV resistance than E138K, including other substitutions at codon E138 (203-205).

1.5.2 Mutual compensation with M184I

It was discovered that the E138K/M184I mutational combination offers greater drug resistance than the E138K/M184V combination (206, 207), which explains why the M184I substitution was selected more frequently than the M184V substitution. It was also found that E138K and M184I have a mutually compensatory effect on HIV-1 RT kinetics (206, 208), which explains why E138K was preferentially selected instead of alternative RPV resistance mutations. It was later shown that other substitutions at codon E138, like E138Q and E138R, also display a mutually compensatory relationship with M184I (205). Interestingly, even in the absence of FTC or M184I/V, E138K is still preferentially selected by RPV or ETR in cell culture (209, 210). Thus, the reason for the preferential selection of E138K may be independent of FTC or M184I/V. The preferential selection of E138K is complicated by the finding that E138K offers less ETR and RPV resistance than E138Q and E138R and a similar level of resistance to E138A and E138G (205, 211, 212).

1.5.3 E138 substitution relative fitness

A simple explanation for the preferential selection of E138K may be that E138K results in higher viral fitness than other E138 substitutions. Indeed, a

previous study showed that when E138K was passaged with E138A, E138Q, or E138G, E138K eventually dominated E138A and E138Q but not E138G; the relative fitness of E138R was not assessed (213). However, this result is not consistent with the fact that E138A is fit enough to be a polymorphism in drug-naïve patients, but E138K is not found in drug-naïve patients (214, 215), suggesting that E138K is less fit than E138A. Thus, the relative fitness of E138K should be re-evaluated with a contemporary viral fitness assay. Serial passaging of viruses in cell culture for extended periods until one virus dominates may have been susceptible to bottleneck effects and genetic drift that could obscure viral fitness determination. Contemporary methods of determining viral fitness rely on infecting cell cultures with multiple viruses and measuring viral outgrowth (216-218). This method of determining viral fitness is highly reproducible and can show quantitative differences in viral fitness.

1.5.4 dNTP pool size and E138 substitutions

The relative fitness of E138 substitutions should be evaluated with regard to the dNTP pool size of the cells that are infected. It has been shown that substitutions at codon E138 have dNTP dependent effects on RT kinetics *in vitro*, and the cell types infected by HIV-1 have a wide range of dNTP pool sizes (34, 136, 208, 212). Likewise, it would be very informative to perform E138 fitness experiments under a range of dNTP pool sizes.

1.5.5 E138K and the HIV-1 mutational bias

If drug resistance cannot explain the preferential selection of E138K, and it is found that E138K is not one of the most fit substitutions at codon E138, another explanation for the preferential selection of E138K may be that E138K emerges first, prior to other substitutions and gains preeminence via this mechanism. This is plausible since E138K is a G→A mutation, and there is mutational bias toward G→A mutations in both cell-free RT assays and in single cycle HIV-1 replication experiments performed in cell lines (30, 31, 219).

Host factors like APOBEC3 family members may also favor the premier emergence of the E138K substitution. Indeed, E138K is a GA→AA mutation, which as previously mentioned is a potential target of several APOBEC3 enzymes. Indeed, it has been shown that E138K frequently appears in the proviral reservoir of drug-naïve patients, which suggests that APOBEC3 enzymes may play a major role in the emergence of E138K (220). This suggests that it might be possible to identify small E138K minority species genetically linked to hypermutated viruses in plasma RNA. However, hypermutated viral sequences are rarely or never identified in plasma RNA, and it has been noted elsewhere that the data only weakly support the ability of APOBEC3 enzymes to facilitate viral diversity and drug resistance emergence (84, 221-224). It has even been demonstrated that A3G mediated GG→AG hypermutation is practically too deleterious to permit viral reemergence from proviral reservoirs (221). On the other hand, in contrast to A3G activity, A3F and A3C neutralization are not required for viral propagation (225, 226), which suggests that these APOBEC3 family members may cause only sub-lethal hypermutation that could facilitate

drug resistance emergence. This may be because A3F and A3C primarily induce GA→AA mutations that do not have the ability to create stop codons. Likewise, it is worth investigating the relation of APOBEC3 activity to the emergence of GA→AA drug resistance mutations, like E138K.

1.6 Hypothesis and Objectives

We hypothesized that the mutational bias of HIV-1 was responsible for the preferential selection of E138K. Furthermore, we hypothesized that E138K emergence could be associated with APOBEC3 enzyme activity.

To assess these hypotheses, we developed a sensitive, inexpensive, and quantitative allele-specific PCR (AS-PCR) method for detecting E138A, E138G, E138K, E138Q, E138V, and E138R minority species (227-231). AS-PCR is one of the most sensitive methods of detecting minority variants, and it is also the simplest, fastest, and least expensive method (227-229, 232). It relies on the observation that Taq polymerase amplifies DNA less efficiently if the 3' ultimate position in a primer is mismatched (230). This allows differential amplification of genetic mutants, such that DNA with a particular mutation amplifies far better than DNA without a particular mutation. This property can be harnessed by standard qPCR techniques to quantify the proportion of mutant DNA in a sample (232-234). Of note, the inclusion of a deliberate mismatch in the penultimate position of an AS-primer dramatically increases allele-specificity (231). Developing an accessible AS-PCR method for detecting these mutations is clinically and diagnostically relevant, since pre-existing minority species can be an indicator of future virologic failure in otherwise adherent patients (235).

Furthermore, since these primers preferentially amplified DNA with specified mutations, simply sequencing the amplicon of AS-PCR reactions revealed mutations linked to the identified minority species (236). We used this AS-PCR technique, as well as ultra-deep sequencing (UDS), to search for evidence of APOBEC3 activity in plasma RNA as well as to gauge the mutational bias of HIV-1 in patients. In addition, to work on E138 substitutions, we also gained insight into SLFN11 structure-function by creating an *in silico* model of SLFN11, and cloned putative domains identified in this model.

2 METHODS

2.1 Viral isolates, cells, drugs, and plasmids

Viral isolates 5326, 5331, 8336, 8116, and BG-05 were obtained from drug-naïve patients during acute infection (<6 months) with informed consent at our clinics in Montreal, Canada. Cord blood mononuclear cells (CBMCs) were obtained through the Department of Obstetrics, Jewish General Hospital, Montreal, Canada. Through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, the pNL-4.3 vector (which encodes the full genome of HIV-1 subtype B) was obtained from Dr. Malcolm Martin (237), and ETR and RPV were obtained from Janssen Pharmaceuticals, Inc. NL-4.3 virus was generated by HEK293T cell transfection (Lipofectamine® 2000, Life Technologies) with pNL-4.3 as per manufacturer instructions. Clinical isolates or NL-4.3 virus were used in selection experiments with ETR or RPV as previously described (238); selection experiments were performed by Maureen Oliveira and

Eugene Asahchop in CBMCs. ETR selection experiments with viral isolates 5326, 5331, and BG-05 were performed previously (239).

2.2 Viral RNA extraction and RT-PCR

HIV-1 RNA was purified from EDTA-anticoagulated plasma or from cell culture supernatants (QIAamp® Viral RNA MiniKit, Qiagen Sciences, Maryland), according to the instructions of the manufacturer. Purified RNA (20µl) was reverse transcribed and PCR amplified in a single step (SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase, Invitrogen, Burlington, ON) using the manufacturer's instructions, with cDNA synthesis performed at 53°C for 30 minutes and the PCR annealing temperature set at 55°C for 30 seconds. 20 units of RNaseOUT (Invitrogen, Burlington, ON) was used in each reaction. The primers used for reverse transcription and first round of PCR were RT_sense (CCTGAAAATCCATACAATAC) and RT_Antisense (TATTGACAAACTCCCACTC). RT-PCR was also performed using a clinical protocol (Virco BVBA, Mechelen, Belgium). The PCR products were purified (QIAquick® PCR Purification Kit, Qiagen Sciences, Maryland) and quantified by spectrophotometry (Nanodrop 1000, Thermo Scientific).

2.3 Quantifying E138 minority species by AS-PCR

The sequences of all primers were based on the 2010 Los Alamos HIV sequence compendium of HIV-1 subtype B *pol* (240). All AS-PCR reaction conditions were the same: 300nM of sense primer, 300nM of antisense primer, 2µM SYTO9, 2% DMSO, and 1×Platinum® Quantitative PCR SuperMix-UDG

(Invitrogen, Burlington, ON) in a volume of 10µl in a transparent tube (0.1ml Strip Tubes and Caps, Qiagen Sciences, Maryland, USA). Samples were evaluated by qPCR in a Rotor-Gene^{TM} 6000 Real-Time Thermocycler (Corbett Research Pty Ltd, Australia) using the following parameters: 50°C for 2 minutes (for UDG), then 94°C for 2 minutes, and then 50 cycles of 94°C denaturation for 15 seconds, 50°C annealing for 15 seconds, and 72°C elongation for 60 seconds. The E138A, E138G, E138K, E138Q, E138R, and E138V substitutions were introduced into pNL4-3 RT by site-directed mutagenesis (Quick Change XL kit, Stratagene, La Jolla, CA). After transfecting and harvesting of viral mutants, viral RNA was extracted and RT-PCR was performed to generate AS-PCR standards. Assessments of sensitivity and running of samples were performed as reported elsewhere (241). Briefly, standard curves of E138 mutants from 10^8 to 10^4 amplicons/µl were used to quantify the total and mutant DNA concentrations in samples, with the appropriate primers. The total DNA concentration in most AS-PCR reaction mixtures was 10^7-10^8 amplicons/µl. The linearity of the AS-PCR assay was confirmed by measuring DNA with the indicated mutations at codon E138 serially diluted in wildtype DNA. Adding three standard deviations to the mean measurement recorded for wildtype DNA set the sensitivity of our assay.

2.4 Study Populations

The study included analysis of 37 M184I containing samples in our database for the presence of E138 substitutions by bulk sequencing. 18 of these samples (from 18 different patients) were analyzed by AS-PCR for E138 substitutions. The median viral load in these patients was 7000; consequently, the

median theoretical sensitivity for E138 minority identification was approximately 1% (233). As a *post-hoc* control, we analyzed 18 patients that were wildtype at codon M184. To make these controls more meaningful, we matched each M184I patient sample with an M184 patient sample of nearly an identical sequence; this was done by building a large phylogenetic tree of all full length *pol* sequences in our database via the Neighbor-TreeMaker (233), using the Kimura 2-Parameter Method. Consequently, the average sequence identity between pairs was 97%. The use of NNRTIs was similar between M184I and M184 patients: two M184I patients and two M184 patients were treated with NVP, and two M184I and two M184 patients were treated with EFV. No patients were treated with ETR or RPV. Each sample pair was analyzed and compared at the same sensitivity, which was determined by the theoretical sensitivity set by sample with the lower viral load, as well as the empirical AS-PCR sensitivity.

The study also included evaluation of two datasets of drug-naïve populations prior to the approval of ETR or RPV for clinical use. The first was an HIV-1 *pol* sequence dataset from the Quebec drug resistance genotyping program between January 2001 and December 2007 ($n_{\text{subtype B}}$= 1223, $n_{\text{subtype C}}$=135). The second was a subtype B HIV-1 *pol* sequence dataset from the Montreal Primary HIV Infection (PHI) Cohort Study (n = 335). Individuals in this study were recruited from all major Montreal HIV-1 clinics (<6 months after serconversion). Patients provided informed consent for blood collection and resistance testing. We evaluated the prevalence of E138 resistance mutations by bulk sequencing in both datasets. In addition, we randomly identified 22 drug-naïve subtype B

patients during primary infection obtained before January 2008 who were assessed for minority species by AS-PCR. We ensured that only patients with a viral load >150,000 were chosen to achieve at least 0.05% sensitivity as described elsewhere (242). Similarly, we randomly identified 9 drug-naïve subtype C patient samples obtained before January 2008 with a viral load >100,000 (instead of >150,000, due to limited sample size) to achieve at least 0.08% sensitivity.

2.5 Ultra-deep sequencing (UDS)

Ultra-deep sequencing was performed on a Roche 454 deep sequencing apparatus in the laboratory of Dr. Richard Harrigan. To remove UDS errors, and also in an attempt to increase sensitivity, we used the PrimerID method (233). Automated UDS programs designed to analyze raw sequence data invariably caused unrecoverable misalignments that reduced UDS accuracy (243). Furthermore, these programs disrupted the PrimerID sequence tag needed to ensure accurate population sampling and remove PCR errors. Indeed, there are no publically accessible programs for analysis of PrimerID tagged HIV-1 sequences. Therefore, to analyze these data and maintain the integrity of our sequences, we opted to perform alignments and analysis through multiple programs. We aligned all sequences in Clustal Omega (244), and these were corrected by hand in MEGA5 (245, 246). Consensuses of sequences with the same PrimerID tag were made (Consensus Maker v2.0.0, http://www.hiv.lanl.gov). This yielded on average 1088 complete and unique sequences per patient from codons 105 to 194. We considered minority species to be present when the majority of at least 3 sequences had the same mutation with the same PrimerID tag. With an average of

2 reads per sequence, this method alone could not identify many small minority species. Therefore, we also relied on statistical tests to identify small minority species. PrimerID consensus sequences were opened in Microsoft Excel (2010 Microsoft Corporation), where the frequency of minority species at each position was calculated. We only considered mutations present when the mutational frequency was significantly greater (Fisher Exact Test, $p<0.05$) than the frequency of this mutation in >5000 sequence reads that should not have possessed this mutation but occasionally did due to PCR induced substitutions (i.e. sequences that had the same PrimerID tag). This protocol was followed for each individual nucleotide position regarding the mutation type, and permitted the detection of mutations below 1% in proportion. Data were analyzed using Prism 5 (GraphPad Software, Inc.). Phylogenetic analysis was performed in MEGA5 (247); after the gamma parameter was estimated, NJ maximum composite likelihood trees were created and tested using the interior branch test (1000 bootstrap replications).

2.6 Linkage analysis by AS-PCR and DNA sequencing

The method used to determine genetic linkage following AS-PCR is similar to a method published elsewhere (247). After AS-PCR, 20µl of the PCR reaction mixture were immediately purified (QIAquick® PCR Purification Kit, Qiagen Sciences, Maryland, USA) and sequenced with 125nM of the 138_Antisense primer. We compared the sequences and chromatograms of amplicons generated with the 138K_AA AS-primer with those generated with the 138_Total non-allele-specific primer to reveal mutations specifically linked to E138K.

2.7 Competition Experiments

Competition experiments were performed with 65 000 MT-2 cells in 2ml RPMI medium supplemented with 10% fetal bovine serum, 1% penicillin, 1% streptomycin, and 1% L-glutamine. Virus was normalized based on multiplicity of infection (MOI), and cells were infected with a total MOI of 0.05. 1ml of supernatant was removed and frozen at -80°C each day for future AS-PCR analysis, and replaced with 1ml of fresh medium. 1.5mM hydroxyurea (HU; Sigma) was used in some studies because this was the highest concentration that could be used on MT-2 cells before toxic effects were noted. 0.65mM deoxynucleosides (dN; Sigma) was used in some studies because this was the lowest concentration that partially rescued MT-2 cells subjected to excess (50mM) HU. MT-2 cells were preincubated for 24 hours with HU or dN prior to infection. Relative fitness was calculated by least squares (236).

2.8 *In silico* SLFN11 Modeling, Cloning, and Purification

SLFN11 was modeled in the I-TASSER server without providing restraints or templates (248). This model was then energy minimized in the Swiss-PDB Viewer using default parameters (249-251). The DaliLite alignment server was then used to identify proteins with structural similarly (252). Based on the domains identified within SLFN11, we designed primers to clone fragments of SLFN11 into the pET-19b vector. The pET-19b vector (Novogen) was chosen as a protein expression vector as it contains an enterokinase cleavage site for the removal an N-terminal His-tag, which may be critical for functional and structural studies. The enterokinase cleavage site ‘DDDDR’ is several fold more efficiently

cleaved than the pET-19b 'DDDDK' enterokinase cleavage site (253). Thus, the pET19b 'DDDDK' cleavage site was modified to 'DDDDR' by site-directed mutagenesis and confirmed by sequencing. *E. coli* BL21(DE3)pLysS was transformed with the pET19b vector harboring SLFN11, and was grown at 37°C with vigorous shaking (300rpm) in 500ml Luria Broth supplemented with 100 µg/ml ampicillian, 170 µg/ml chloramphenicol, and 0.5% glucose. When the culture reached OD_{600nm}=0.9, 2mM IPTG was added to the media and the temperature was reduced to room temperature for 7 hours. Cells were pelleted and frozen at -80°C until purification. For purification, cells were thawed on ice, and resuspended in binding buffer (20mM Tris-HCl pH 8.0, 100mM NaCl, 200mM KCl, and 30mM Imidazole) with 1g/l lysozyme. The cells were sonicated 3 times for 30 seconds at 70% intensity, with one-minute pauses in between. Insoluble fractions were pelleted at 20,000×g for 1 hour at 4°C. The supernatant was filtered using a 0.2µM filter, then incubated with 3ml of new Ni-NTA Agarose beads (Qiagen) for 1 hour at 4°C. The beads were then loaded into columns and washed with 10ml of Wash Buffer (20mM Tris-HCl pH 8.0, 50mM NaCl, 100mM KCl, and 30mM Imidazole). SLFN11 was eluted with step-wise increasing concentrations of elution buffer (20mM Tris-HCl pH 8.0, 50mM NaCl, 100mM KCl, and 1M Imidazole) in Wash Buffer.

3 RESULTS

3.1 Design of an AS-PCR assay for E138A, E138G, E138K, E138Q, E138R, and E138V

The design of this AS-PCR assay offered a unique challenge in that it had to be specific for two nucleotides to adequately distinguish between six different substitutions. Therefore, the 3' ultimate and penultimate positions of the AS-primer were designed to overlap with the first two nucleotides of the E138 codon. Intentional mismatches were placed on two invariant adenine nucleotides of residue N137 (254-256) to increase sensitivity. Of note, approximately half of subtype-B and subtype-C viruses should not have any mutations in the AS-primer-binding site (241). After selecting the optimal primers (Table 1), sensitivity of our assay was determined by diluting DNA with the indicated substitutions at position E138 in wild-type DNA (Figure 1A-F and Table 2). To demonstrate that the AS-PCR assay was not detecting the incorrect substitutions, DNA with the indicated substitution at codon E138 was also diluted in other common E138 substitutions (Figure 1G-AJ and Table 2).

Table 1: Summary of the AS-PCR oligonucleotide primers used

Position$_{HXB2}$	**Name**	**Sequence***
2942→2960	138_Total	CATACCTAGTATAAACAAT
2942→2962	138A_GC	CATACCTAGTATAAAC**TT**T**GC**
	138G_GG	CATACCTAGTATAAAC**TC**T**GG**
	138K_AA	CATACCTAGTATAAAC**TG**T**AA**
	138Q_CA	CATACCTAGTATAAAC**TC**T**CA**
	138R_CG	CATACCTAGTATAAAC**TC**T**AG**
	138V_GT	CATACCTAGTATAAAC**TC**T**GT**
	138E_GA	CATACCTAGTATAAAC**CT**T**GA**
3212←3230	138_Antisense	GAATGGAGGTTCTTTCTGA

*Sequences are provided 5' to 3'. Bolded nucleotides were intentionally mismatched; bolded and underlined nucleotides base-pair with the specified mutant codon.

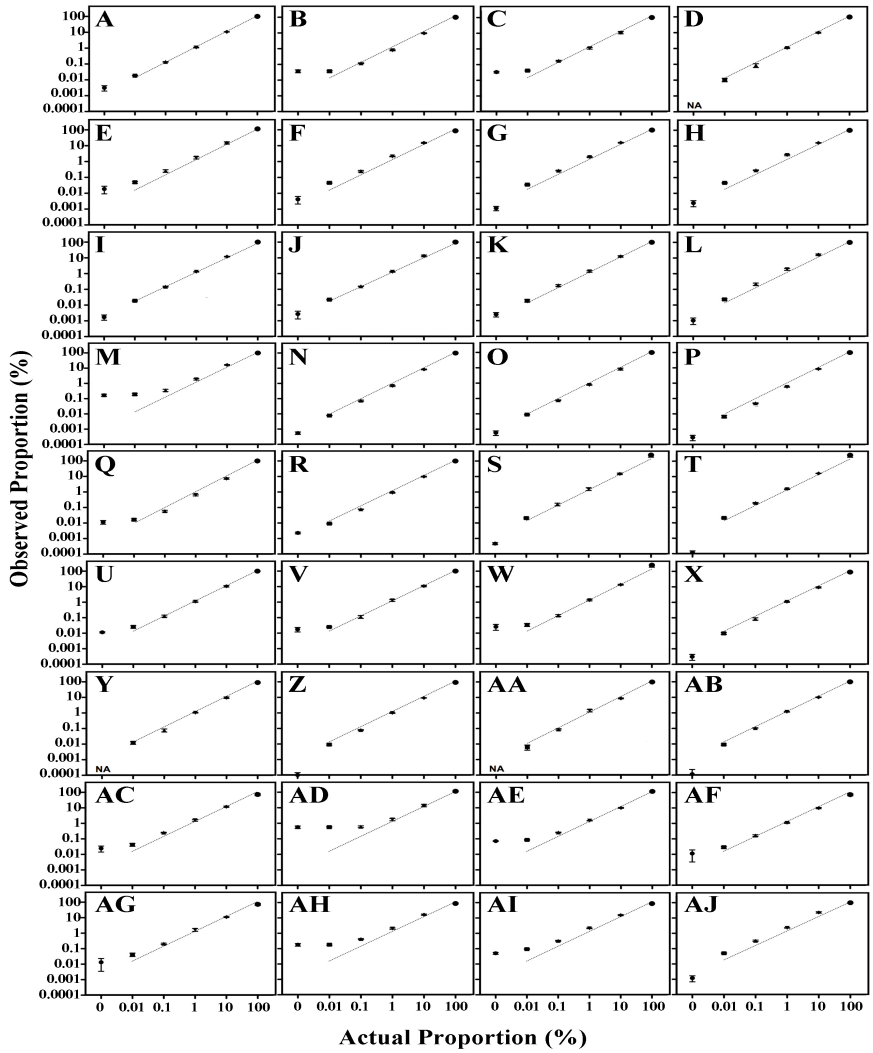


Figure 1: PCR amplicons of E138 substitutions diluted in other common E138 substitutions were measured by AS-PCR

The dotted line shows equivalence between actual proportion and observed proportion. NA, the PCR reaction with the AS-primer failed to amplify. **A,** E138A serially diluted in E138. **B,** E138G serially diluted in E138. **C,** E138K serially diluted in E138. **D,** E138Q serially diluted in E138. **E,** E138R serially diluted in E138. **F,** E138V serially diluted in E138. **G,** E138V serially diluted in E138Q. **H,** E138V serially diluted in E138R. **I,** E138A serially diluted in E138G. **J,** E138A serially diluted in E138K. **K,** E138A serially diluted in E138Q. **L,** E138A serially diluted in E138R. **M,** E138A serially diluted in E138V. **N,** E138G serially diluted in E138A. **O,** E138G serially diluted in E138K. **P,** E138G serially diluted in E138Q. **Q,** E138G serially diluted in E138R. **R,** E138G serially diluted in E138V. **S,** E138K serially diluted in E138A. **T,** E138K serially diluted in E138G. **U,** E138K serially diluted in E138Q. **V,** E138K serially diluted in E138R. **W,** E138K serially diluted in E138V. **X,** E138Q serially diluted in E138A. **Y,** E138Q serially diluted in E138G. **Z,** E138Q serially diluted in E138K. **AA,** E138Q serially diluted in E138R. **AB,** E138Q serially diluted in E138V. **AC,** E138R serially diluted in E138A. **AD,** E138R serially diluted in E138G. **AE,** E138R serially diluted in E138K. **AF,** E138R serially diluted in E138Q. **AG,** E138R serially diluted in E138V. **AH,** E138V serially diluted in E138A. **AI,** E138V serially diluted in E138G. **AJ,** E138V serially diluted in E138K.

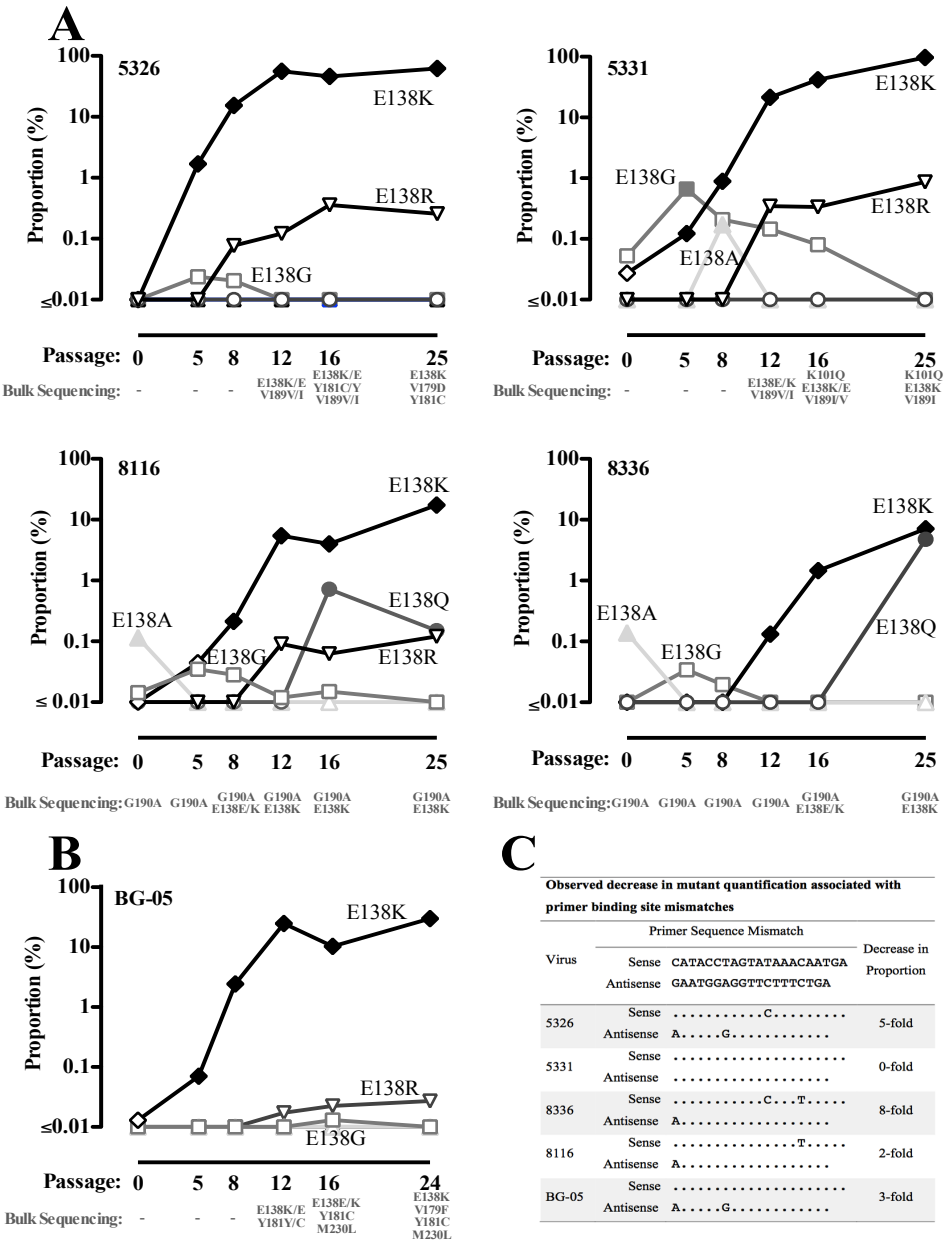
Table 2: Summary of empirically determined AS-PCR sensitivities

		Substitutions used to Serially Dilute the Substitutions Assessed by AS-PCR						
		E138	E138A	E138G	E138K	E138Q	E138R	E138V
Substitutions Assessed by AS-PCR	E138A	0.01	—	0.01	0.01	0.01	0.01	0.4
	E138G	0.3	0.01	—	0.01	0.01	0.03	0.01
	E138K	0.06	0.01	0.01	—	0.02	0.06	0.1
	E138Q	0.01	0.01	0.01	0.01	—	0.01	0.01
	E138R	0.09	0.1	1	1	0.07	—	0.09
	E138V	0.4	0.4	0.3	0.01	0.01	0.01	—

3.2 E138K emerges first during ETR selection experiments

To validate the assay and gain insights into the evolution of substitutions at codon E138, we analyzed cell culture ETR selection experiments that used patient derived subtype B and C viruses. Four viruses had mutations in primer-binding sites, and such mutations are known to impact the observed proportion and sensitivity of AS-PCR methods (241, 257). By comparing the observed proportion of points at which E138K was measurable by bulk sequencing, we validated the assay and estimated the approximate fold decrease in the observed proportion of viruses with mutations in the primer binding sites (Figure 2).

Analysis of these ETR selections by AS-PCR also revealed the dynamic process of E138 substitution selection (Figure 2A and B). E138K consistently emerged first for each particular experiment. The higher limit of detection for E138G combined with primer binding site mismatches prevented the detection of E138G above the technical limit of detection in most of these selections; nonetheless, the trend is clear that E138G also emerged very early above background measurements. Using similar logic, it would appear that E138R consistently emerged below the limit of detection a few weeks after E138K. However, our extensive efforts to clarify how these AS-PCR assays act when the detected substitution is diluted in substitutions other than wildtype revealed that



C

Observed decrease in mutant quantification associated with primer binding site mismatches

Virus	Primer Sequence Mismatch		Decrease in Proportion
	Sense	CATACCTAGTATAAACAATGA	
	Antisense	GAATGGAGGTTCTTTCTGA	
5326	Sense	..........C.........	5-fold
	Antisense	A.....G..........	
5331	Sense	.....	0-fold
	Antisense	.....	
8336	Sense	..........C...T.....	8-fold
	Antisense	A.....	
8116	Sense	.....T.....	2-fold
	Antisense	A.....	
BG-05	Sense	.....	3-fold
	Antisense	A.....G..........	

Figure 2: Allele-specific PCR analysis of E138 substitutions selected in viral isolates from acute HIV-1 infections harboring wildtype and G190A viral quasispecies under etravirine pressure

Raw data from the AS-PCR analysis of E138A/G/K/Q/R/V are presented. Substitutions measurable by bulk sequencing are noted. Open symbols indicate proportions of mutant viruses at levels below AS-PCR sensitivity. Closed symbols show proportions of mutants above the levels of AS-PCR sensitivity. **A,** Etravirine selection experiments performed in cord blood mononuclear cells (CBMCs) using viral samples taken from the plasma of HIV-1 subtype B infected, drug-naïve patients. **B,** Etravirine selection experiments performed in CBMCs with viral samples taken from the plasma of HIV-1 subtype C infected, drug-naïve patient. **C,** The approximate decrease in observed proportion caused by primer binding site mismatches, determined by comparing the observed proportion of E138K at points when E138K was measurable by both AS-PCR and bulk sequencing. Sequences are provided from 5' to 3'.

this is an artifact caused by the increased background for E138R AS-PCR in E138K containing DNA (refer to Figure 1AE), which was confirmed by UDS. Hence, even though E138R conferred the highest level of resistance to ETR (233, 258), there is no evidence that this substitution appeared during these experiments. Of note, the presence of G190A, a common first generation NNRTI resistance mutation (203-205), did not appear to influence the evolution of E138 minority species. It is also worth noting that the Y181C substitution emerged alongside E138K twice; simultaneously, there was a transient drop in the proportion of E138K and a substitution at codon V179 was subsequently selected.

The consistently early selection of E138K and E138G indicated that these substitutions have a low genetic barrier for emergence. The order of appearance of the E138 substitutions correlated with the mutational bias of HIV-1 (259): E138K and E138G (G→A and A→G, respectively) preceded E138A and E138Q (A→C and G→C, respectively), and E138R (G→A + A→G) never appeared.

3.3 M184I delays the selection of E138K by ETR and RPV

Given the aforementioned mutual fitness compensation between M184I and E138K, we suspected that E138K might be selected faster in viruses that had the M184I substitution. Therefore, using AS-PCR we analyzed selection experiments performed with ETR and RPV in wildtype and M184I containing clonal NL-4.3 viruses. In wildtype viruses under ETR or RPV pressure we noted a similar selection dynamic to previous experiments: E138K and E138G minorities appeared very early (Figure 3). Interestingly, E138R was eventually selected very late in one ETR selection experiment. When the M184I substitution was introduced into the NL-4.3 virus, the emergence of an E138K minority

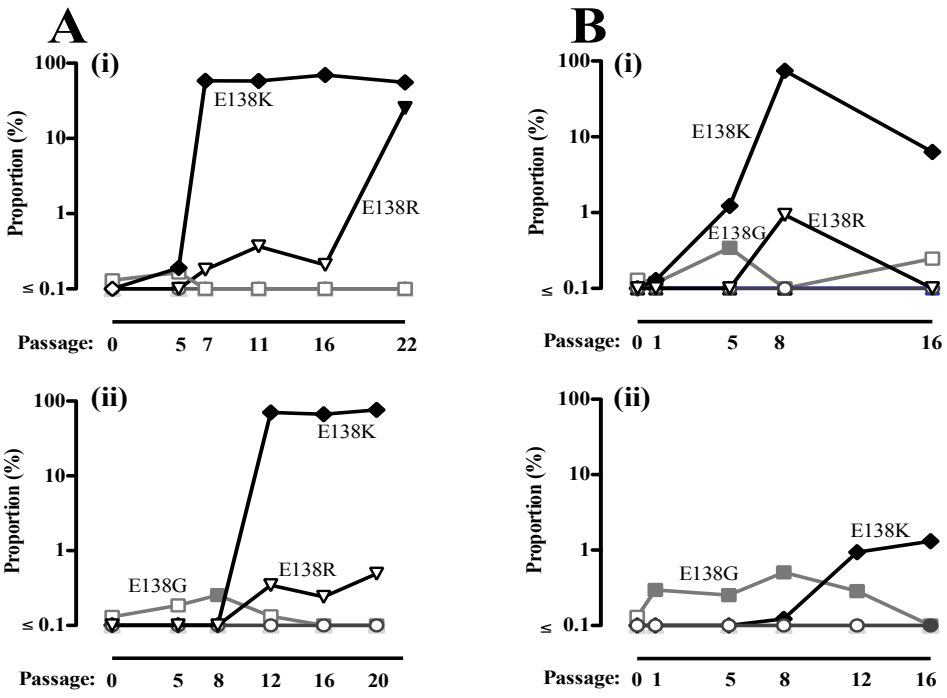


Figure 3: Allele-specific PCR analysis of E138 substitutions selected by etravirine or rilpivirine from NL-4.3 clonal viruses with and without the M184I substitution.

Raw data from the AS-PCR analysis of E138A/G/K/Q/R/V are presented. Open symbols indicate proportions of mutated viruses at levels below AS-PCR sensitivity. Closed symbols show proportions of mutants above the levels of AS-PCR sensitivity. **A,** Etravirine selection experiments performed in cord blood mononuclear cells (CBMCs) using **(i)** wildtype NL-4.3 virus and **(ii)** using NL-4.3 virus with the M184I substitution introduced by site-directed mutagenesis. **B,** Rilpivirine selection experiments performed in CBMCs using **(i)** wildtype NL-4.3 virus and **(ii)** using NL-4.3 virus with the M184I substitution introduced by site-directed mutagenesis.

species in response to ETR and RPV was delayed by approximately 7 and 8 weeks, respectively. Since E138K and M184I display mutual fitness compensation, this result was surprising. On the other hand, M184I increases the mutational fidelity of HIV-1 RT, and thus this delay in selection may reflect that it took more replicative cycles to mutate the E138K substitution.

3.4 E138 substitutions are not enriched in patients with M184I

Even if the M184I substitution delayed the emergence of the E138K substitution in cell culture, the mutual fitness compensation between some E138 substitutions and the M184I substitution should favor the long-term positive selection of E138 substitutions in M184I containing viral populations. For this

reason, we assessed whether any patients with the M184I substitution in our database also had substitutions at the E138 codon.

None of the 37 M184I containing samples in our database had any E138 substitution measurable by bulk sequencing. M184I is a transient substitution: while it is selected by 3TC or FTC, M184I will be replaced by M184V with continued 3TC or FTC use (137). In the absence of NRTIs, M184I quickly reverts to wildtype (210). It may be that due to the transient nature of M184I, there is not enough time for E138K to reach a proportion measurable by bulk sequencing. In this case, emerging E138K minority species, not detectable by bulk sequencing, may be enriched in M184I harboring patients. Therefore, we screened patients with the M184I substitution by AS-PCR to determine if indeed this substitution is present as a minority species; we screened only 18 different patients due to sample availability. We identified 11% (2/18) of patients with E138K and 11% (2/18) of patients with E138G (Figure 4). As a control, we analyzed 18 patients wildtype at codon M184; these patients were controlled for viral *pol* sequence, viral load, and NNRTI use. The identification of E138K and E138G minority species was similar between the patients with M184I and M184, while M184 harbored somewhat more E138A, E138Q, and E138R minority species (Figure 4). Interestingly, some of the patients identified to have E138 minority species were not taking any antiretroviral drugs at the time of sample acquisition. The significance of these findings is unclear given the small sample sizes between groups; nonetheless, it appears that M184I may not harbor substantially more E138 minority species than wildtype virus in patients.

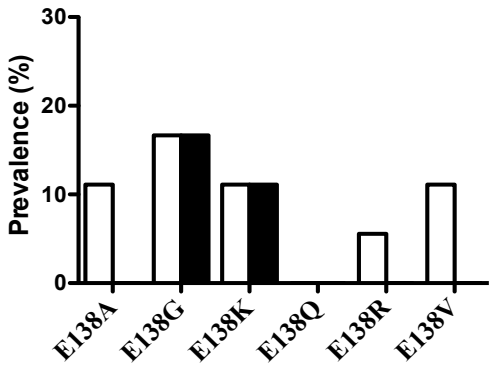


Figure 4: Comparative prevalence (%) of E138 minority species evaluated by AS-PCR in patients with and without the M184I substitution.

Open bars, E138 minority species prevalence in patients with M184, n_{M184}=18. Closed bars, E138 minority species prevalence in patients with the M184I substitution, n_{M184I}=18. Analyses were controlled by sequence, limit of detection, and NNRTI use in a pairwise manner between M184 and M184I harboring patients.

3.5 K103R emerges in E138K containing virus passaged without drugs

Given the fitness compensation between E138K and M184I, it is unclear why M184I would not spontaneously acquire E138K, and raises the question whether E138K would acquire M184I in the absences of NRTI pressure. We passaged virus with the E138K substitution in the absence of drug pressure acknowledging that under these conditions E138K may eventually revert back to wildtype. In three out of three passaging experiments, E138K did not revert to wildtype after 18 weeks. Intriguingly, by bulk sequencing we discovered that the K103R substitution had emerged at week 12 in two out of the three passaging experiments. The M184I substitution did not emerge, though we have yet to assess this with techniques more sensitive than bulk sequencing.

3.6 Prevalence of substitutions at position E138 prior to the availability of ETR and RPV

The identification of some E138 minority species in patients not on therapy suggested these minority species could represent remnants of transmitted drug resistance substitutions, since many of these samples were taken after the approval of ETR and RPV. To rule out this possibility, we next evaluated drug-naïve patients by bulk sequencing, AS-PCR, and UDS, for the historic prevalence

of E138 substitutions at times prior to the clinical availability of ETR and RPV. By bulk sequencing, we evaluated the viral sequences from 1558 subtype-B and 135 subtype-C infected patients (Table 3). Only E138A, E138G, and E138V were identified in drug-naïve patients by bulk sequencing.

Table 3: Prevalence of resistance substitutions at codon 138 prior to the approval of etravirine or rilpivirine by bulk sequencing in antiretroviral naïve or treated populations

Subtype	Population	Unique Patients	Substitution Prevalence (%)					
			138A	138G	138K	138Q	138R	138V
B	Untreated	741	2	0.1	0	0	0	0
	Treated	817	2	0.5	0.5	0.2	0	0
C	Untreated	89	4	0	0	0	0	1
	Treated	46	9	2	2	2	0	0

We then used our AS-PCR to evaluate E138 minority species in some of these drug-naïve patients that had a high viral load (>150,000), to increase the sensitivity of the assay. Due to these conditions, as well as sample availability, we were limited to analyzing only 22 patients. We found that E138K was the most prevalent minority species, found surprisingly in 23% (5/22) of these patients (Figure 5A). E138G and E138A were also found at a high prevalence of 18% (4/22) and 9% (2/22), respectively, while E138Q, E138R, and E138V were never detected.

We confirmed these results by UDS, although one patient, identified by AS-PCR as possessing E138K, was not analyzed due to lack of plasma availability. Identifying mutations at levels below the 1% proportion by UDS was achieved by statistically evaluating each substitution on a site and mutation-type specific basis. We were able to confirm the high prevalence of E138K at 19% (4/21) as well as E138A at 10% (2/21) (Figure 5B). The high prevalence of

E138G substitutions could not be confirmed due to a high UDS background for this specific mutation. In addition, a patient with an E138V minority was also identified.

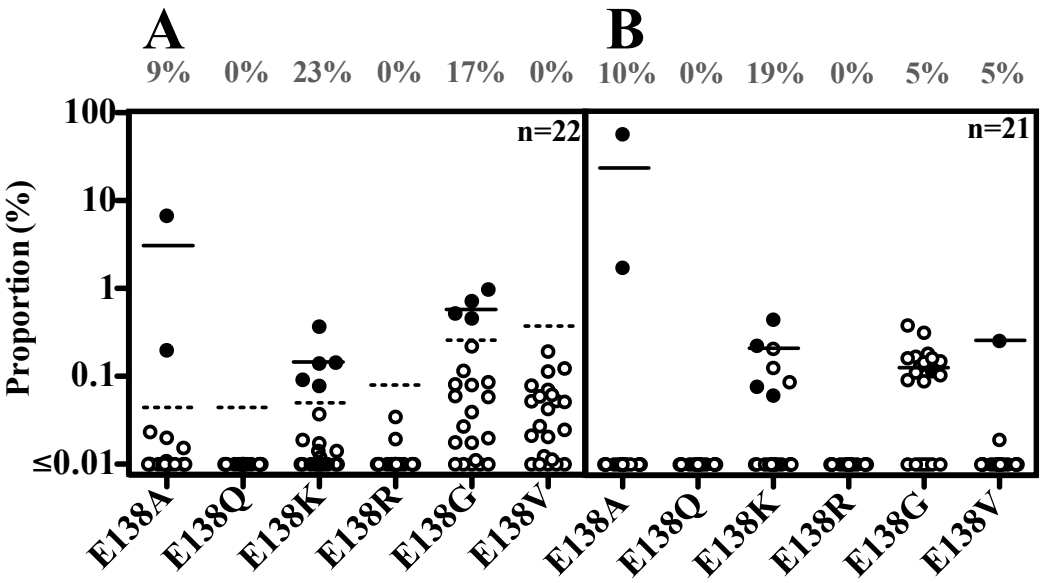


Figure 5: Prevalence of minority E138 species in drug-naïve subtype B patients prior to 2008.

A, Prevalence of minority E138 species in 22 patients measured by AS-PCR, as confirmed in two or three independent experiments. The dotted line represents the AS-PCR limit of detection for a specified substitution, taking into account a minimum limit of detection for patients based on viral load. **B,** Prevalence of minority E138 species in the same patients as in B above, except one patient due to plasma availability, was also followed by 454 ultra-deep sequencing. Open circles indicate values below the sensitivity of the assay. Closed circles represent values above the sensitivity of the assay; for UDS this meant a level significantly higher than that of background PCR mutations ($p<0.05$, Fisher Exact Test). Solid line shows the mean proportion of minority species detected at levels above the sensitivity of the assay. Grey numbers represent the prevalence of corresponding minority species.

By AS-PCR, we also evaluated 9 subtype C drug-naïve persons from whom samples were available before the approval of ETR or RPV (Figure 6). More samples would have been analyzed; however, there was very low sample availability of untreated subtype C patients with a very high viral load. In these patients, we found the prevalence E138K to be 22% (2/9), while E138A, E138G, E138Q, E138R, and E138V were never detected. This prevalence has not yet

been confirmed by UDS as only four of these plasma samples were available in sufficient quantities for such analysis.

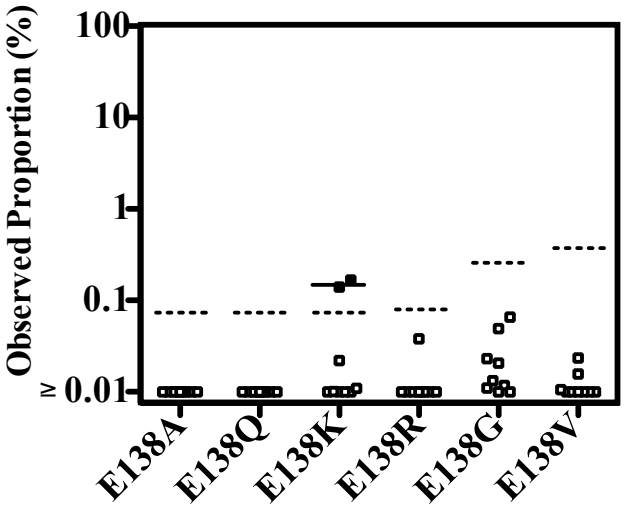


Figure 6: Prevalence of minority E138 species in 9 in subtype C patients prior to 2008 measured by AS-PCR.

Results were confirmed in two or three independent experiments. The dotted line represents the AS-PCR limit of detection for a specified substitution, taking into account a minimum limit of detection for patients based on viral load. Open squares indicate values below the sensitivity of the assay. Closed squares represent values above the sensitivity of the assay.

3.7 E138K is one of the least fit substitutions at codon E138

The early selection and high prevalence of E138K minority species could be rationalized if E138K had a low fitness cost relative to other substitution at codon E138. As previously mentioned, competition experiments with an E138K-containing virus have been performed previously with paradoxical results that suggest the E138K substitution is more fit than the E138A polymorphism (213). Given that these competition experiments were long-term passaging experiments, and we found here that E138K rapidly acquires the K103R substitution during long-term passaging experiments, we sought to confirm this finding with shorter-term experiments. We performed competition experiments using viruses that contained E138A, E138G, E138K, E138Q, and E138R. We capitalized on the

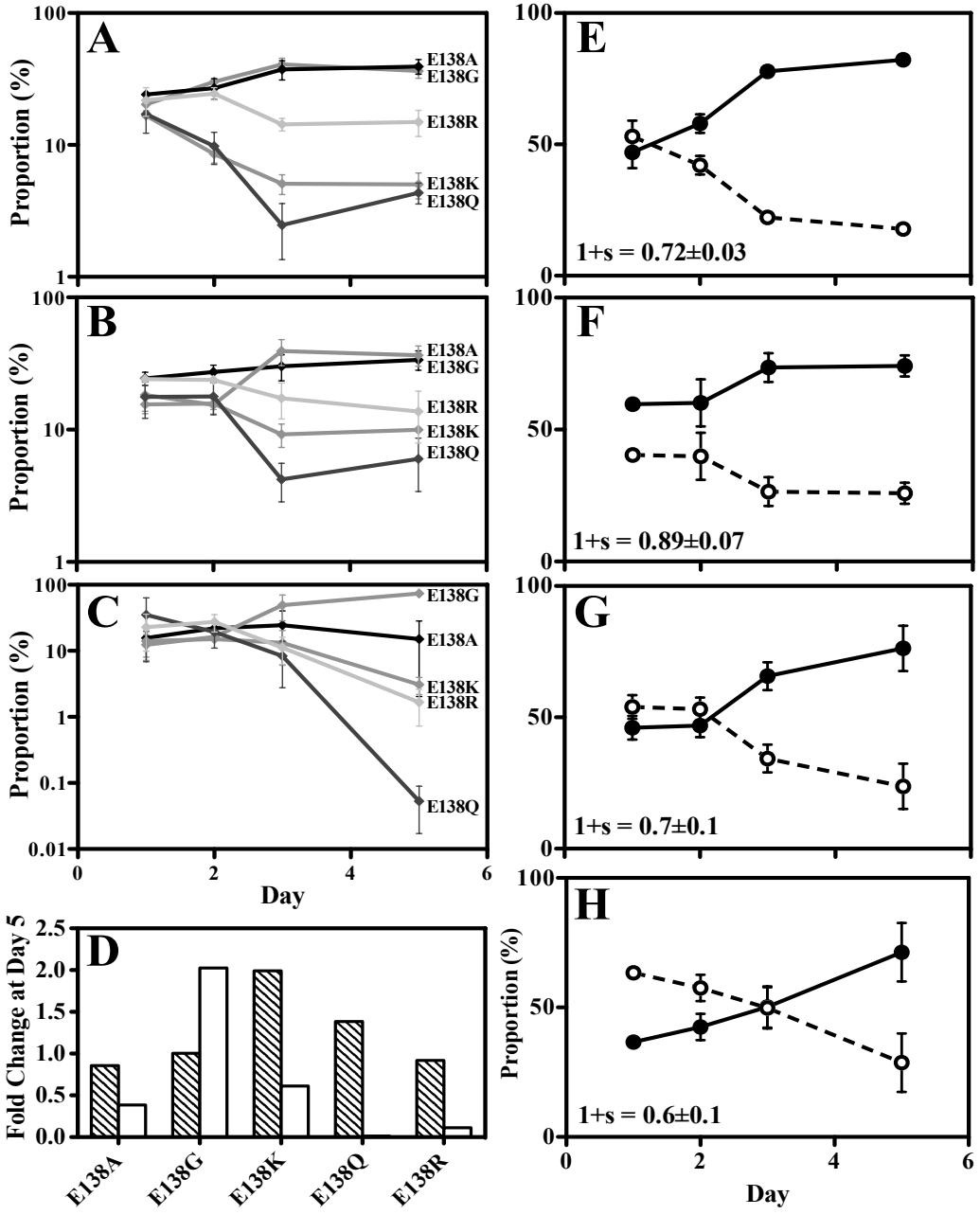


Figure 7: Competition experiments with substitutions at codon E138 in MT-2 cells monitored by AS-PCR.

The data are representative of the mean ± SEM of three independent experiments. **A-D,** Results of competition experiments using E138A, E138G, E138K, E138Q, and E138R. **A,** Competition experiments performed without altering the dNTP concentration. **B,** 1.5mM hydroxyurea (HU) was added to the media. **C,** 0.65mM of deoxynucleosides (dN) were added to the media. **D,** Fold change in the proportions of individual substitutions at the end of the competition experiment (Crosshatched bars) fold-change with the addition of HU. (Empty bars) fold-change with addition of deoxynucleosides. **E-H,** Results of competition experiments using E138K virus and wildtype. (Solid line) Wildtype. (Dashed line) E138K. (s+1) Relative viral fitness with the standard error of the mean noted. **E,** Competition experiment performed without altering the dNTP concentration. **F,** 1.5mM hydroxyurea (HU) was added to the media. **G,** 0.65mM of deoxynucleosides (dN) was added to the media. **H,** Both 1.5mM HU and 0.65mM dN were added to the media; note that this combination caused a minor decrease in MT-2 cell replication.

ability of the AS-PCR method to distinguish among these substitutions by competing all viruses together in an internally controlled experiment (Figure 7A). Consistent with the majority-species prevalence of E138A in drug-naïve patients, we found that E138A and E138G were the most fit substitutions, followed by E138R; the least fit substitutions were E138K and E138Q.

3.8 dNTP pool size affects E138 substitution relative fitness.

We have previously shown that some E138 substitutions have a dNTP concentration-dependent effect on RT kinetics *in vitro* (260). We assessed whether modified dNTP concentrations *in vivo* may permit E138K to become the most fit substitution at codon E138 (Figure 4B-H). Using hydroxyurea (HU), an inhibitor of ribonucleotide reductase, to lower the dNTP concentrations of MT-2 cells resulted in a modest increase in the fitness of viruses containing E138K and E138Q relative to E138A, E138G, and E138R (Figure 7B and D). Conversely, using deoxynucleosides (dNs) to exogenously raise the dNTP concentration of the cells resulted in E138G gaining a strong fitness advantage over the other substitutions at codon E138 (Figure 7C and D). Under the conditions tested, E138K was consistently one of the least fit substitutions at codon E138. E138K virus was also competed against wildtype virus under similar conditions (Figure 7E-H); E138K also had modestly improved relative fitness with the addition of HU, but was nonetheless consistently less fit than wildtype virus.

3.9 Small minority species correlates to HIV-1 mutational bias

Considerations of fitness and drug-resistance cannot explain the preferential selection of E138K. On the other hand, the order of E138 minority

species emergence under drug pressure of E138 minority species is consistent with the HIV-1 mutational bias, suggesting that the mutational bias is responsible for the preferential selection of E138K. Likewise, the mutational rate and bias of HIV-1 may also be responsible for the high prevalence of small E138K minority species. If this were the case, then the small E138 minority species identified previously may be one of many examples of codons with a prevalence of minority species consistent with the HIV-1 mutational bias. Indeed, further UDS analysis on the 21 drug-naïve patients previously mentioned revealed that the pattern of small minority species was consistent with that of HIV-1 mutational bias during single cycle replication experiments (Figure 8A) (31). The prevalence in patients of any particular G→A mutation was approximately 15%, usually as a tiny (<1%) minority. This indicates that the high prevalence of E138K as a small minority is not specific to E138K, and can be explained by the fact that it is a G→A mutation.

We also estimated the mean substitution bias by phylogenetic means in these drug-naïve patients (Figure 8B). This technique provided a direct measure of relative substitution bias by compensating for mutations that were present incidentally in viral linages compared with mutations that repeatedly arose. However, with this technique, it was not possible to remove PCR errors. Therefore, the mean substitution matrix provided the average of HIV-1 errors and some PCR errors. Nonetheless, the substitution matrix estimate of the HIV-1 mutational bias in these patients was comparable with the small minority species identified in these patients, and both were distinct from the UDS PCR error

mutational bias (Figure 8C). As expected, the UDS PCR error mutational bias favoring T→C and A→G mutations closely matched that of the Taq polymerase mutational bias calculated elsewhere (31).

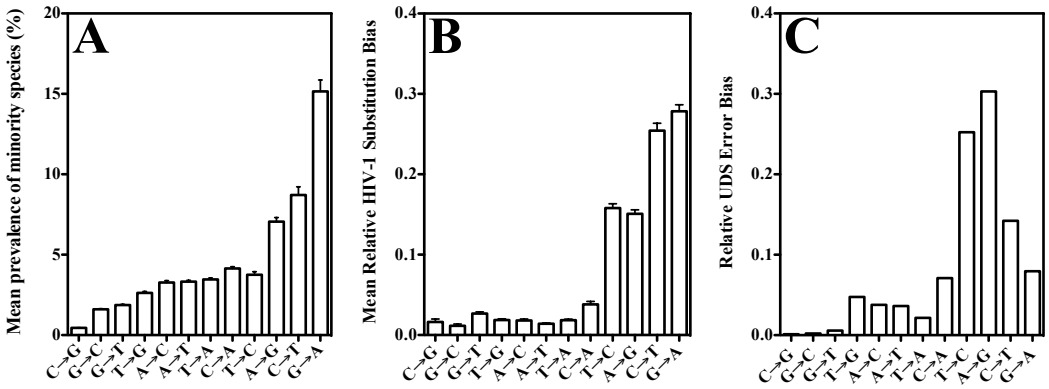


Figure 8: Ultra-deep sequencing revealed prevalence of minority species per available nucleotide site in HIV pol (HXB2 2862→3131) in 21 drug-naïve patients.

A, Mean prevalence of minority species (<5% proportion) stratified on the basis of mutational type. Prevalence of minority species per patient was calculated and then averaged together. Error bars represent standard deviation (SD). **B,** Mean relative substitution bias (±SD) calculated using the Estimate Substitution Matrix function available through the MEGA5 program with the general time reversible model. **C,** Relative mutational bias of UDS errors; data represent the relative frequency of mutations in 5000 sequence reads that should not have possessed mutations but occasionally did due to UDS errors (i.e. sequences that had the same PrimerID tag).

Despite the fact that *in vitro* RT preferentially introduces G→A mutations during positive and negative sense DNA polymerization, positive sense G→A mutations were significantly higher than positive sense C→T mutations here. This indicated that RT exhibits a slightly different mutational bias for RNA- and DNA-dependent DNA polymerization as suggested previously (261), or that a host-factor was responsible for additional positive sense G→A mutations sufficient to impact the mutational bias.

3.10 Determining genetic linkage by sequencing AS-PCR amplicons

Frequent APOBEC3-mediated G→A hypermutation may functionally lower the genetic barrier for emergence of E138K. To determine whether E138K

minority species appeared linked to APOBEC3-hypermutated viruses, we adapted a sensitive, reliable, and rapid AS-PCR method for assessment of mutational linkage (Figure 9A) (31). To validate this method, we serially diluted E138K/G190 containing virus into E138/G190A containing virus, and analyzed the dilutions. We were able to detect the linkage of E138K to G190 with 0.1 to 0.01% sensitivity (Figure 9B-M). Importantly, the reverse transcription and amplification steps prior to and during the AS-PCR did not interfere with the linkage of E138K to G190.

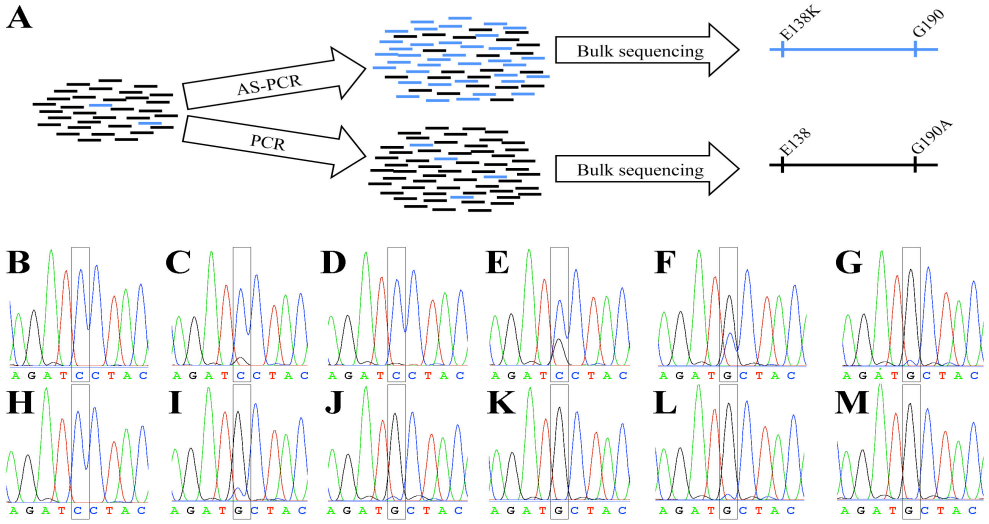


Figure 9: Sequencing the amplicon of AS-PCR reactions with the antisense primer to reveal genetically linked mutations.

A, Schematic representation of the method; horizontal bars represent HIV-1 DNA and genetically linked mutations. **B-M,** Viruses containing G190 and the E138K substitution were serially diluted in virus with E138 and the G190A substitution; after RT-PCR and AS-PCR, the PCR amplicon was sequenced with the 138_Antisense primer. **B-G,** AS-PCR was performed with the 138K_AA AS-primer preceding sequencing. **H-M,** AS-PCR was performed with the 138_Total non-allele-specific primer preceding sequencing. The viral dilution series with E138K virus diluted in G190A virus was as follows: **B & H,** 100% E138K; **C & I,** 10% E138K; **D & J,** 1% E138K; **E & K,** 0.1% E138K; **F & L,** 0.01% E138K, **G & M,** 0% E138K. Note that the G190 anticodon is TCC and the G190A anticodon is TGC.

3.11 Genetic linkage of E138K in Subtype B

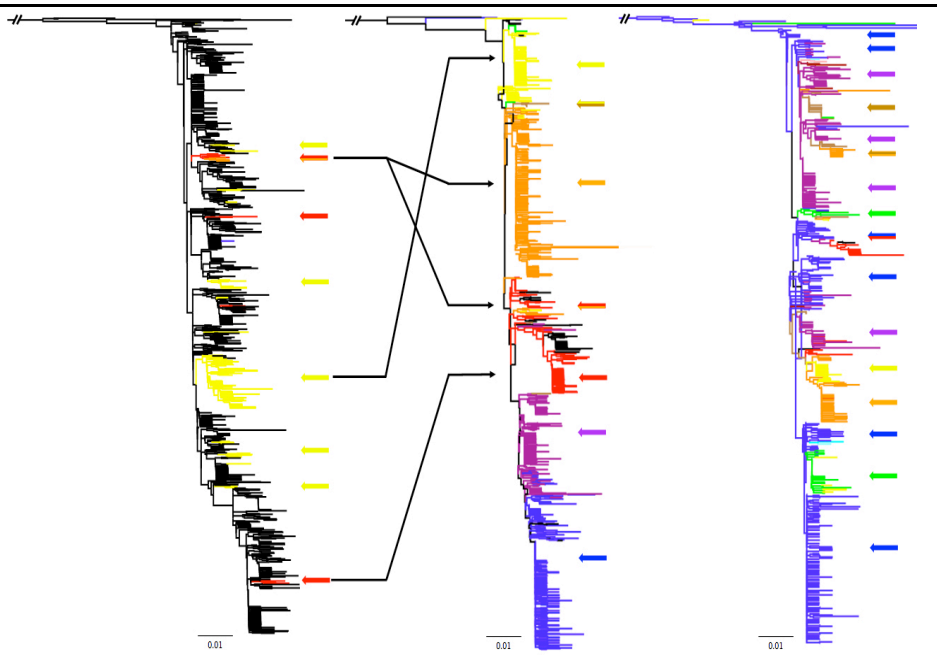
Using this method, we assess whether E138K arose on hypermutated viruses during ETR cell culture selection experiments (Table 4). There was no

indication that E138K emerged on hypermutated viruses in these selection experiments. The genetic linkage of E138K in isolate 5326 followed the most complex mutation selection pattern, and was thus ideal for further confirmation of this AS-PCR technique by UDS. We analyzed isolate 5326 passage 8, 12, and 16 by UDS and confirmed the accuracy of the AS-PCR method of detecting mutation linkage (Figure 10).

Table 4: Sequencing of the E138K AS-PCR from etravirine selection experiments

Isolate	Passage	E138K Proportion (%)[a]	Not Allele-Specific	E138K-Linked
5326	8	1	None	E138K
	12	30	E138E/K, Y181Y/C, V189V/I	E138K, V189I
	16	20	E138E/K, Y181C	E138K, Y181C
	25	100	E138K, V179D, Y181C	E138K, V179D, Y181C
5331	5	0.1	None	E138K
	8	0.9	None	E138K
	12	20	E138E/K, V189V/I	E138K, V189V/I
	16	40	E138K/E, V189I/V	E138K, V189I/V
	25	100	E138K, V189I	E138K, V189I
8116	12	1	G190A	E138K, G190A
	16	14	G190A	E138K, G190A
	25	100	E138Q/K, G190A	E138K, G190A
8336	5	3	G190A	G190A
	8	30	E138E/K, G190A	E138K, G190A
	12	100	E138K, G190A	E138K, G190A
	16	80	E138K, G190A	E138K, G190A
	25	100	E138K, G190A	E138K, G190A
BG-05	5	0.2	None	E138K
	8	8	None	E138K
	12	80	E138K/E, Y181Y/C	E138K
	16	30	E138E/K, Y181C	E138K, Y181C
	24	100	E138K, Y181C	E138K, Y181C

[a]Proportion corrected based on fold decrease in observed proportion due to primer mismatches



		Passage 8	Passage 12	Passage 16
G→A	**V106I**	0.1	1.0	4.0
	E138K	1.9	54.6	37.7
	V189I	12.7	55.0	21.2
	G190E	0.1	0.5	0.1
A→G	**E138G**	0.3	0.5	0.2
	Y181C	0.2	43.4	81.4
C→A	**E138Q**	0.0	0.0	0.8
T→A	**V179D**	0.0	0.0	6.1
A→C	**E138A**	0.0	0.0	0.0
G→T	**V179F**	0.0	0.0	0.0
G→C	**V179L**	0.0	0.0	0.0
G→C	**G190A**	0.0	0.0	0.0

Figure 10: 454 Ultra-deep sequencing analysis of ETR selection with patient isolate 5326.

Top, NJ Maximum Composite Likelihood phylogenetic trees of selection experiment with 1000 bootstrap replications, rooted to SIV_{cpz}. Black, Wildtype viral sequences. Red, Viral sequences with E138K. Yellow, Viral sequences with V189I. Blue, Viral sequences with Y181C. Orange, Viral sequences with E138K and V189I. Purple, Viral sequences with E138K and Y181C. Green, Viral sequences with V189I and Y181C. Brown, Viral sequences with E138K, Y181C, and V189I. Arrows with color, phylogenetically distinct subpopulations of mutations (bootstrap confidence > 99%). Black arrows, passage 8 mutant subpopulations that clustered with passage 12 mutant subpopulations after all sequences from passage 8 and passage 12 were combined in a single NJ Maximum Composite Likelihood tree (not shown). **Bottom,** Proportions (%) of single-nucleotide ETR resistance mutations in the selection experiment sorted by mutation type.

UDS of the 5326 selection experiment also revealed that, consistent with the impact of mutational bias on the selection of E138 substitutions, the emergence of other minority species agreed with the HIV-1 mutational bias: the G→A mutations V106I, E138K, and V189I emerged first, closely followed by the A→G mutation Y181C, followed by C→A and T→A mutations (Figure 10). Strikingly, phylogenetic analysis of this UDS data identified the G→A mutations, E138K and V189I, in several phylogenetically distinct subpopulations at passage 8. Creating a single phylogenetic tree with the sequences from passage 8 and passage 12 identified multiple E138K subpopulations from passage 8 that clustered with passage 12 E138K subpopulations (Figure 10, black arrows) indicating that multiple passage 8 E138K viruses were viable and contributed to the emerging ETR resistance. A similar pattern was seen with passage 8 and 12 V189I subpopulations. Currently, it is not clear whether these phylogenetically distinct passage 8 subpopulations represent the same mutations emerging multiple times in a single selection experiment or recombination events that could not be controlled for. Nonetheless, the fact that V106I, E138K and V189I, all G→A mutations, could emerge independently by passage 8 demonstrates that multiple E138K minority species could also emerge independently by this time point.

We also analyzed the genetic linkage of the E138K minority species we previously identified in drug-naïve subtype B infected patients. There was no indication that E138K emerged on hypermutated viruses in the drug-naïve subtype B patients identified with E138K minority species (Table 5). UDS

Table 5: Mutations specifically enriched with E138K by sequencing of allele-specific PCR amplicon from five drug-naïve subtype B infected patients

Patient	Measured Proportion of E138K (%)	Mutations*			Corresponding E138K-Linked Substitutions
		$Position_{HxB2}$	Total	E138K-Linked	
B(4)	0.4	2972	G/A	G	Silent
		2993	G	G/A	Silent
		3050	C	C/T	Silent
		3125	C	C/T	Silent
B(15)	0.1	2972	G/A	A	Silent
B(18)	0.08	3026	A/C	A	Silent
		3046	A/G	A	K/R166K
		3118	G/C	G	G/A190G
		3161	G/A	A	Silent
B(21)	0.09	3002	G/A	A	Silent
B(22)	0.1	3035	C/T	C	Silent

*Only mutations between position 2963 to ~3160—the region amplified during AS-PCR—were screened. All mutations linked to E138K are not reported; simply the mutations that were different between amplicons produced with non-allele specific primer 138_Total and the AS-primer E138K_AA have been reported. A slash indicates that the mutation is in a mixture with the species on the left of the slash in greater proportion that that on the right. Also, arrows highlight G→A mutations.

analysis did not reveal any linkage of E138K to hypermutated viruses; indeed, there were no hypermutated viruses at all (Hypermut 2.0 Program, p-value <0.05). It is possible that most reactivated APOBEC3-hypermutated viruses fail to replicate sufficiently to be detectable by UDS. In this case, hypermutation followed by early recombination could enrich G→A minority species in specific dinucleotide contexts. While this would provide indirect evidence of APOBEC3 activity, no significant enrichment of G→A mutations was observed (Figure 11). This questions the role of APOBEC3 enzymes regarding the overall HIV-1 mutational bias for G→A mutations in subtype B, such as E138K.

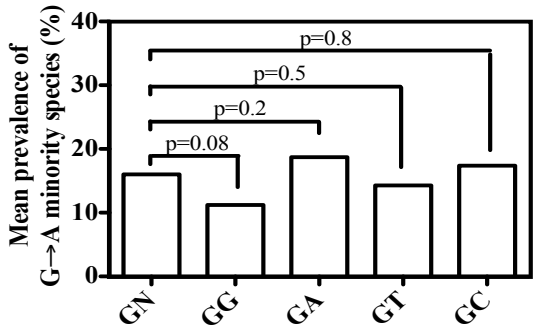


Figure 11: Prevalence of G→A minority species detected by ultra-deep sequencing in 21 drug-naïve patients stratified on the basis of dinucleotide context.

Frequencies were compared by Fisher Exact Test with p-values noted.

3.12 Genetic linkage of E138K in Subtype C

We also assessed the subtype C ETR selection experiment and patients with the E138K minority species for evidence of hypermutation. While the subtype C virus during ETR selection showed no evidence of hypermutation, surprisingly the two subtype C E138K containing minority species did show some evidence of hypermutation (Table 6). In sample C1, E138K minority species were linked to thirteen G→A mutations, eleven of which were GA→AA

Table 6: Mutations specifically enriched with E138K by sequencing of allele-specific PCR from two subtype C patients with small E138K minority species

Patient	Measured Proportion of E138K (%)	Mutations*				Corresponding E138K-Linked Substitutions
		$Position_{HxB2}$	Total		E138K-Linked	
C(1)	0.2	3041	G	→	A	M164I
		3065	G	→	A	Silent
		3098	T/C		C	Silent
		3102	G	→	A	D185N
		3105	G	→	A	D186N
		3110	G	→	A	Silent
		3118	G	→	A	G190E
		3123	G	→	A	D192N
		3129	G	→	A	E194K
		3135	G	→	A	G196K
		3136	G	→	A	G196K
		3159	G	→	A	E204K
		3166	G	→	A	R206K
		3168	G	→	A	E207K
C(9)	0.1	3032	G/A		G	Silent
		3047	A/G		G	Silent
		3065	G/A		G	Silent
		3066	G	→	A	E173K
		3069	C/A		C	Q/K174Q
		3098	C/T		C	Silent
		3123	G	→	A	D192N
		3125	C/T		T	Silent
		3146	G	→	A	Silent
		3165	G	→	A	E206K

*Only mutations between position 2963 to ~3160—the region amplified during AS-PCR—were screened. All mutations linked to E138K are not reported; simply the mutations that were different between amplicons produced with non-allele specific primer 138_Total and the AS-primer E138K_AA have been reported. A slash indicates that the mutation is in a mixture with the species on the left of the slash in greater proportion that that on the right. Also, arrows highlight G→A mutations.

mutations. Sample C9 was less convincingly hypermutated as E138K minority species were linked to four G→A mutations, only two of which were GA→AA mutations. Due to limited sample size, further investigation must be done prior to suggesting that E138K has a greater propensity to arise on hypermutated viruses in subtype C. More importantly, these data demonstrate that hypermutated minority species can be identified in the plasma if they were actually present, which validates the negative findings in subtype B.

3.13 *In silico* modeling of SLFN11

Similar to the HIV-1 mutational bias, SLFN11 may have a tremendous capacity to globally influence the pattern of drug resistance emergence by differentially restricting particular codons. Gauging the extent of this possibility is hindered by the lack of a known molecular mechanism of SLFN11. In the absence of any characterized SLFN11 relatives identified by sequence conservation, identifying the structure of SLFN11 may provide clues as to its specific function.

We modeled SLFN11 in I-TASSER, which is currently the premier application for automated protein structure prediction (205). The generated SLFN11 model showed substantial structural similarity to the Brr2 helicase, with an excellent root-mean-square deviation (RMSD) value of 1.8Å (Figure 12A). The next most structurally similar proteins were Hel308 and Mtr4. Brr2, Hel308, and Mtr4 are Ski2-like helicases; a family of proteins that can carry out tasks ranging from DNA unwinding important for DNA repair to RNA unwinding

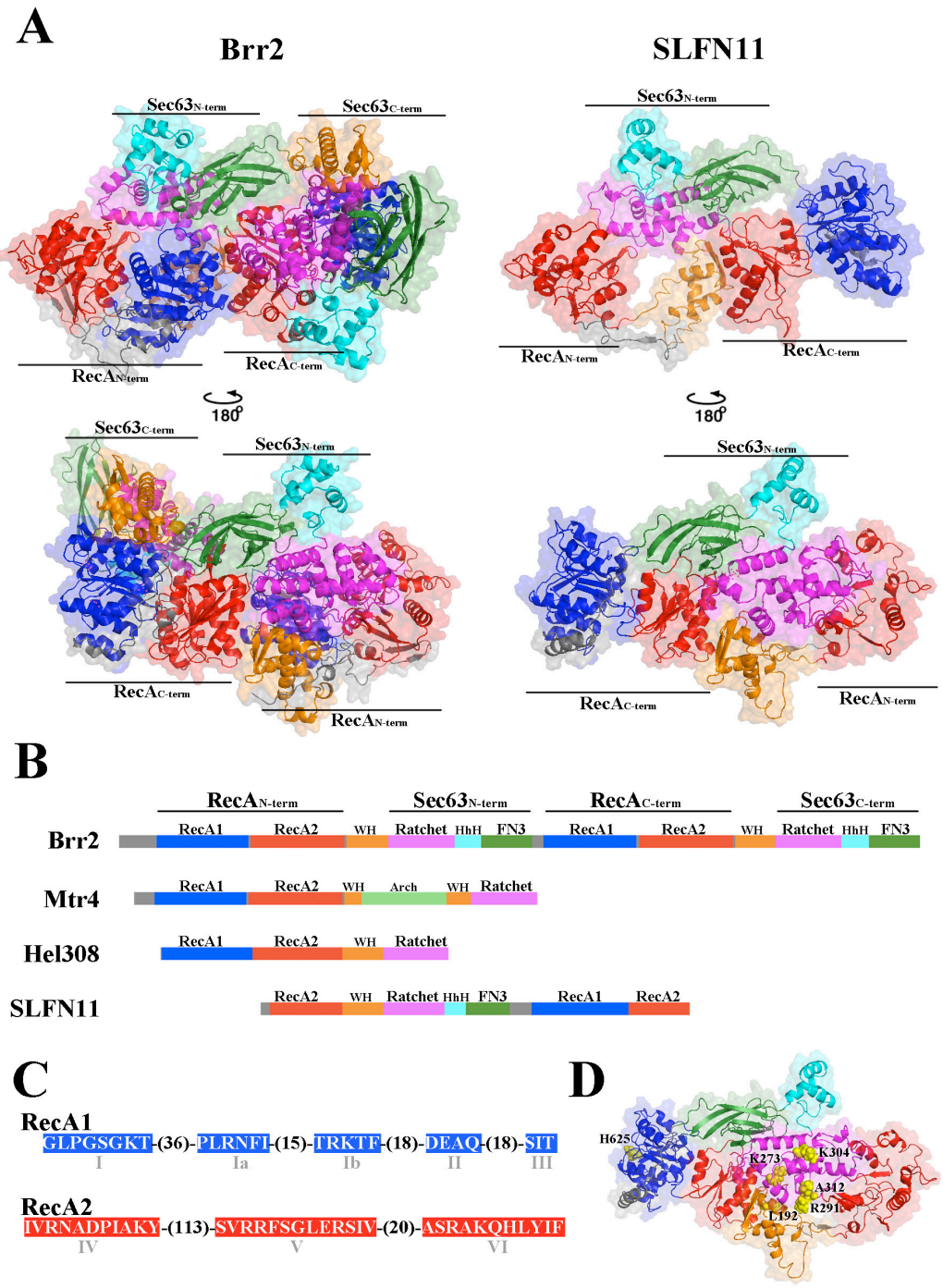


Figure 12: *In silico* modeling of SLFN11. Domains based on Brr2 (PDB 4F91) domain assignments.

Blue, RecA1 domain. Red, RecA2 domain. Orange, Winged-helix (WH) domain. Magenta, Ratchet domain. Light blue, helix-hairpin-helix (HhH) domain. Green, fibronectin type III (FN3) domain. **A,** Crystal structure of Brr2 solved elsewhere (262) and our SLFN11 model built using I-TASSER automated protein modeling. **B,** Domain arrangement of three Ski2-like helicases (263) and SLFN11. **C,** DEAD-box helicase motifs identified within the C-terminal RecA portion of SLFN11. **D,** Human SLFN11 with amino acids determined elsewhere (264) to be under positive selection compared with SLFN11 in several other species.

important for RNA degradation or splicing (250). By hydrolyzing ATP, the RecA1 and RecA2 domains of the RecA enzymatic core of Ski2-like helicases power helicase movement, while the Ratchet domain clamps substrate RNA or DNA onto the RecA portion. Both the RecA core and the Ratchet domain are thought to be important for substrate recognition and helicase directionality (263).

Like the Brr2 helicase, our SLFN11 model has two putative RecA cores. The N-terminal RecA core lacks the RecA1 domain while the C-terminal domain has both RecA1 and RecA2 domains, which suggests that only the C-terminal RecA core might be catalytically active. This is peculiar given that the functional RecA core is N-terminal to the functional Ratchet domain in other Ski2-like helicases (Figure 12B). This raises the question whether SLFN11 is a functional helicase and the role of the C-terminal RecA domain. Nonetheless, we identified several conserved DEAD-box helicase motifs important for catalysis, also identified in murine SLFN family members (114), in the C-terminal RecA portion: motif I, Ia, Ib, II, and III in RecA1, and motif IV, V and VI in RecA2 (Figure 12C). In DEAD-box helicases with solved structures, motifs I, Ia, Ib, II, and III are located on a separate and adjacent domain from motifs IV, V, and VI (264), which is consistent with the motif organization within our SLFN11 model. The identified motifs, the conserved and potentially functional residues in these motifs, and the distribution of these motifs within RecA1 and RecA2 suggest that the C-terminal RecA has the potential to be a functional enzyme. On the other hand, the C-terminal location of RecA is unusual and suggests novel structure-

function relationships, urging the need for empirical structural analysis of SLFN11.

Whether or not SLFN11 is a functional helicase, the Ratchet domain and the RecA1 domain may be important to its molecular function. A previous study identified sites of positive selection within the SLFN11 proteins of several animals (264); in human SLFN11 these residues are L192, K273, R291, K304, A312, and H625. Highlighting these residues in our SLFN11 model revealed a series of surface-exposed residues in the Ratchet domain, as well as a single residue in the C-terminal RecA1 domain (Figure 12D). This suggests that at least the Ratchet domain and RecA1 are important for the function of SLFN11, consistent with the importance of these domains for Ski2-like helicase function.

Table 7: SLFN11 primer sequences and combinations used in this study

A

Primer Number	Primer Name	Primer Sequence* 5' to 3'
P1	**F_pET-19b_DDDDR**	CCATATCGACGACGACGAC*CGT*CATATGCTCGAGGATCCGG
P2	**R_pET-19b_DDDDR**	CCGGATCCTCGAGCATATG*ACG*GTCGTCGTCGTCGATATGG
P3	**Sense.hSLFN11.aa1**	*CTACTGTT***CATATG**GAGGCAAATCAGTGC
P4	**Sense.hSLFN11.aa175**	*CTACTGTT***CATATG**GAGCTCCCTAACTCGGAT
P5	**Sense.hSLFN11.aa441**	*CATCAGTT***CATATG**TCTAGAAGTTGGGCTGTGGAC
P6	**Sense.hSLFN11.aa546**	*CTACTGTT***CATATG**GAAGCCCTGCTGCAGTCC
P7	**Antisense.hSLFN11.aa175**	*CTTATGTT***GCTCAGC***CTACTA*TTGGTATACACCCTTGTGAA
P8	**Antisense.hSLFN11.aa441**	*CTTATGTT***GCTCAGC***CTACTA*GAAGATCAAAATTCCCCGAAA
P9	**Antisense.hSLFN11.aa546**	*CTTATGTT***GCTCAGC***CTACTA*GTGCTGGGTGCCTGCAAG
P10	**Antisense.hSLFN11.aa901**	*ATCATGTA***GCTCAGC***CTA*CTAATGGCCACCCCACGGA

B

Fragment Number	Primer Combinations	Domains in Fragment	Size (kb)	Translated Size (kDa)
F1	P3+P7	RecA2	0.6	26
F2	P3+P8	RecA2-WH-Ratchet-HhH	1.4	57
F3	P3+P9	RecA2-WH-Ratchet-HhH-FN3	1.7	68
F4	P3+P10	Full SLFN11	2.8	109
F5	P4+P8	WH-Ratchet-HhH	0.9	37
F6	P4+P9	WH-Ratchet-HhH-FN3	1.2	48
F7	P4+P10	WH-Ratchet-HhH-FN3-RecA1-RecA2	2.2	89
F8	P5+P9	FN3	0.4	17
F9	P5+P10	FN3-RecA1-RecA2	1.4	58
F10	P6+P10	RecA1-RecA2	1.1	47

*Bolded sequences highlight restriction sites; underlined sequences represent start or stop codons; italicized sequences represent introduced nucleotides that do not basepair with the original template.

3.14 Progress towards cloning and expressing SLFN11

Previously it has been shown that an N-terminal portion of SLFN11 (amino acid 1-579, corresponding to the deletion of the C-terminal RecA domain) is soluble, can be purified from *E. coli* by N-term His-tagged Nickel affinity purification, and can bind tRNA (112). With putative domains mapped by *in silico* modeling, we designed primers to clone 10 fragments of SLFN11 into the pET-19b vector (Table 7). Thus far, cloning seven SLFN11 fragments into this modified pET-19b vector appears to have been successful (Table 8), though six fragments are still pending DNA sequencing confirmation.

Table 8: Summary of SLFN11 fragment cloning progress

Cloning Step*	Insert**							
	F1	F3	F4	F6	F7	F10	F9	H_2O
CFUs from Cloning	46	>200	>200	~100	~150	~100	32	25
Plasmids with Inserts	2/15	9/10	6/10	10/10	4/10	7/10	2/10	0/5
Cloning confirmed by DNA sequencing	—	—	—	—	—	—	2/2	—

*CFU=colony forming units. The number of plasmids with the correct inserts was confirmed by PCR amplification using the appropriate primers. Long dash indicates sequencing has not yet been completed.
**Refer to table 8 for description of fragments.

The C-terminal F9 fragment has been sequenced, and the expression conditions of this fragment in *E. coli* BL21(pLys) were optimized. As with the expression of the N-terminal portion of SLFN11, this fragment could be purified to >90% purity in a single step by Nickel Affinity chromatography (Figure 13). With various fragments of SLFN11 cloned and a successful system for purification, functional and structural studies on SLFN11 at the molecular level may be possible.

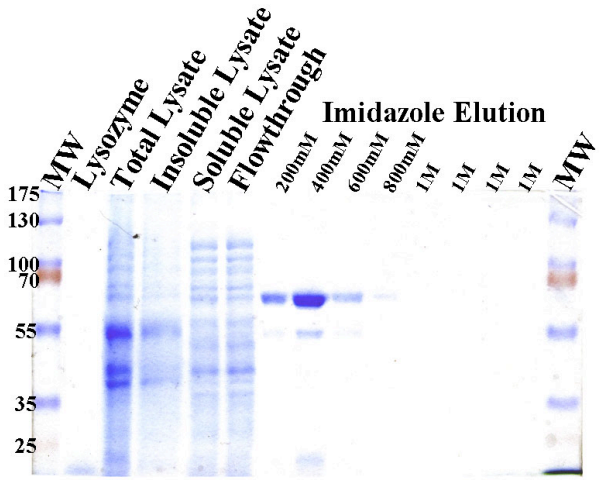


Figure 13: Polyacrylamide gel electrophoresis analysis of nickel affinity purification of SLFN11 F9 fragment.

E. coli lysate from 500ml of culture was loaded onto 2ml of Ni-NTA agarose beads, and eluded with a discontinuous gradient of imidazole. 1ml of each imidazole concentration was added to the beads and the flowthrough was collected. MW, Molecular weight (kDa) Fermentas PageRuler prestained protein ladder.

4 DISCUSSION

Here we present evidence to indicate that the HIV-1 mutational bias is a major player in the evolution of ETR and RPV drug resistance. The order of mutational emergence following ETR and RPV treatment, as well as the prevalence of very small minority species indicate that the mutational bias of HIV-1 is responsible for the preferential selection and high prevalence of E138K. In contrast, neither viral fitness nor drug-resistance can explain the preferential selection and high prevalence of E138K.

The mutational bias of HIV-1 is also critical to the over-representation of the adenine nucleotide in HIV-1 codons, which is targeted by SLFN11 inhibiting viral replication by an unknown mechanism. By performing preliminary *in silico* structural studies we can now suggest potential functions for SLFN11 that can be tested *in vitro* and *in vivo*. Our modeling argues that SLFN11 might act as an RNA helicase: the closest structural relative of SLFN11 was the Brr2 RNA helicase, and we identified RNA helicase motifs in the C-terminal RecA portion. This assignment is in agreement with the finding that SLFN11 binds tRNA (112).

On the other hand, it has been shown that SLFN11 is still capable of binding tRNA and effecting an antiviral response without its C-terminal RecA portion (112). Since this is the only putatively functional motor in SLFN11, the antiviral activity of SLFN11 may not be enzymatic. At any rate, the determinants of substrate-specificity and potential enzymatic activity are important avenues of SLFN11 function that should be investigated in the future. The identification of a putative molecular function and functional motifs, as well as cloning putative domains, thoroughly lays down the groundwork for future structure-function studies.

Investigating the evolution of E138 substitutions under ETR drug pressure has revealed the early selection of E138K over other E138 substitutions. Since each of E138K, E138G, and E138A confer ETR resistance and appeared at times that E138 still dominated the quasispecies, one might assume that their order of appearance is proportional to their genetic barrier for emergence: E138K ≤ E138G < E138A. E138Q and E138R were selected only after E138K first became a majority species. For E138Q, this suggests E138K followed by K138Q (G→A→C), may have a higher likelihood for occurrence than the single E138Q (G→C) substitution, consistent with the mutational bias of HIV-1 that favors G→A or A→C mutations over G→C mutations (31). Likewise, for E138R, this indicates that E138K followed by K138R (G→A, A→G) has a higher likelihood for occurrence than the single E138R (GA→AG) substitution. Since E138Q and E138R offer greater ETR and RPV resistance than E138K (205), this analysis implies that E138K may increase the propensity for more drug-resistant

substitutions at codon E138 to emerge. In addition, by emerging in multiple subpopulations, E138K may also have preserved some of the original viral diversity lost during drug selection and increased the likelihood of emerging with additional drug-resistance mutations. In other words, E138K is a 'gateway mutation': permitting and facilitating the acquisition of more dangerous drug-resistance mutations.

The fact that E138K and E138G were both selected early and were frequently identified in drug-naïve patients helps to explain why both were the most common E138 substitutions in treatment failure during clinical trials with RPV, despite the fact that E138G only confers low-level RPV resistance (31). A different study indicated that E138K was selected over E138G by TSAO derivatives primarily because of the mutational bias of HIV-1. Using exogenously provided deoxycytidine (dC) and tetrahydrouridine (THU) to artificially increase the concentration of dCTPs in cells lead to the selection of E138G instead of E138K by TSAO derivatives (204, 265). This was interpreted to be the result of an augmented dCTP/dTTP ratio that skewed the RT mutational bias. However, we show here that E138G often emerges alongside E138K, and that high dNTP concentrations provide E138G with a strong fitness advantage. In the future, we hope to test whether dC and THU augments the fitness of E138G over E138K as opposed to altering the mutational bias of HIV-1.

While the early emergence of E138G along with E138K is consistent with the HIV-1 mutational bias, it is not consistent with the notion that APOBEC3 enzymes substantially lower the genetic barrier to emergence of E138K.

Furthermore, the best explanation for the delayed emergence of E138K in M184I harboring viruses is that M184I increased RT fidelity; this explanation implies that APOBEC3 does not have a major impact on E138K emergence. In addition, we could find no evidence of APOBEC3 activity in the plasma of subtype B patients. Likewise, it seems superfluous to suggest that this restriction factor plays a role in the preferential emergence of E138K in light of ample evidence that the HIV-1 mutational bias favors the emergence G→A drug resistance mutations.

That said, the evidence presented here regarding APOBEC3 is observational and must be tested experimentally. For example, in the future we hope to perform ETR and RPV selection experiments with cell lines that do and do not express APOBEC3 enzymes with viruses that do and do not express Vif. Such cell lines are available and are derived from the CEM T-cell line: the CEM A3.01 cell line expresses APOBEC3 enzymes and is semi-restrictive to HIV-1 replication, while the CEM-SS cell line does not express APOBEC3 enzymes (266, 267). Monitoring E138K by AS-PCR in such selection experiments should clearly demonstrate the impact of APOBEC3 enzymes on the emergence of this substitution. Given the identification of hypermutated E138K minority species in subtype C, as well as recent studies from our laboratory demonstrating reduced anti-APOBEC3 activity of subtype C Vif (268), these experiments should be performed in a subtype-specific manner. In addition, even if APOBEC3 activity is superfluous compared to the mutational rate and bias of HIV-1 for single mutations, APOBEC3 activity may be important for the earlier emergence of

double G→A drug-resistance mutations. For instance, it has been suggested that APOBEC3 may contribute to the combined emergence of E138K and M184I (220). Therefore, we hope to simultaneously select for E138K and M184I with RPV and FTC in cell lines that do and do not express APOBEC3 enzymes under the aforementioned conditions. These experiments should clearly demonstrate the role of APOBEC enzymes to single and double drug resistance mutation emergence in a subtype specific manner. Since the M184I substitution may delay the emergence of E138K, APOBEC3 mediated co-emergence of E138K and M184I could substantially decrease genetic barrier for this mutational combination with important clinical implications.

Aside from M184I, we also noted another indication of drug-resistance mutation interactions with E138K by monitoring ETR selections by AS-PCR. We noted twice when Y181C was selected after E138K by ETR, the proportion of E138K transiently decreased. We have subsequently shown that Y181C has greater ETR resistance than Y181C and E138K, and Y181C and E138K has greater ETR resistance than E138K alone (269). This explains how virus harboring the E138K mutation could acquire the Y181C mutation, and then subsequently begin to lose the E138K mutation. However, in these experiments E138K was not totally lost with Y181C acquisition, but instead recovered with the acquisition of a mutation at V179. Likewise, we are currently investigating if V179 substitutions increase the drug resistance or fitness of E138K/Y181C harboring viruses.

Here we used viral competition experiments to compare the fitness of mutations at codon E138 under different dNTP conditions. In contrast to a previously published work (213), we demonstrated that E138K is less fit than E138A. Consistent with this difference in fitness, we confirmed that E138A is a common polymorphism in HIV-1 while E138K is not. Likewise, under the various dNTP concentrations tested E138K was still less fit than E138A or WT. Since we did not directly measure the dNTP pools, the *in vivo* relevance of the absolute fitness measurements with modulated dNTP levels is less clear. Therefore, we also hope to assess the fitness of the E138 mutants in primary human cells. Nonetheless, here we present the first study to show that an RT mutation has improved viral fitness with reduced dNTP levels. This result has interesting applications to gene therapy; use of the E138K substitution in lentiviral vectors may modestly increase the specificity of these vectors for low dNTP containing cells, such as microglial cells (270).

Monitoring multiple drug resistance substitutions at the E138 codon during these selection experiments would not have been possible without the development of an AS-PCR method that distinguished among six substitutions at a single codon. This was accomplished by building on archetypal AS-PCR primers, which are specific for one nucleotide at the 3' ultimate position, to be specific for two nucleotides at the 3' ultimate position (271). In addition, we adapted a previously published technique to study linked mutations following AS-PCR (230, 231) in order to identify E138K mutational combinations with high sensitivity. These assays have diagnostic applications, given that the

E138K/M184I combination was identified in the majority of virologic failures in the ECHO and THRIVE trials.

That said, the prevalence identified here of E138K minority species in drug-naïve patients is unexpectedly high questioning the accuracy of this PCR-based method. However, this finding does not likely represent an artifact of PCR, since we specifically controlled for this during the AS-PCR optimization as well as during UDS analysis. Likewise, the prevalence of minority species revealed by UDS was consistent with HIV-1 mutational bias, and was very distinct from that of Taq polymerase mutational bias (265). Furthermore, this is not the first study to note very small and unfit minority species in patients who are not under drug pressure (261). Nonetheless, our findings serve as a warning to cautiously interpret the discovery of minority drug resistance mutations. The presence of E138A minority species at the start of some of the ETR selection experiments shows that not every detectable minority species will be positively selected under drug pressure, since most circulating viruses are not infectious (233, 272, 273).

On the other hand, the frequent identification of minority species appears to reflect a mutational bias that could favor the selection of a particular substitution. For example, we previously identified a genetic template bias for the selection of K65R in subtype C compared with subtype B (274, 275). Likewise, 9% of drug-naïve subtype C infected patients harbor tiny K65R minority species, while this is true for only 2% of subtype B infected patients (276). Consequently, a relatively high number of subtype C patients have failed first-line therapy with the K65R substitution (272). The fact that G→A mutations were the most

frequent minority species detected here, combined with the fact that G→A mutations are the most common type of HIV-1 drug resistance mutation, further underlines the critical role that mutational bias plays in the evolution of clinically relevant HIV-1 drug-resistance.

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