

Development of Drug Resistance Mutations in Patients on Highly Active Antiretroviral Therapy: Does Competitive Advantage Drive Evolution

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Abstract

Most physicians that treat individuals with HIV-1 disease are able to successfully suppress viral replication with the pharmacologic armamentarium available today. For the majority of patients this results in immune reconstitution and improved quality of life. However, a large fraction of these patients have transient elevations in their viral burden and even persistence of low-level viremia. In fact, many individuals whose viral load is suppressed to < 50 c/ml have evidence of low-level viral replication.

The impact of low-level viremia and persistent viral replication is an area of significant study and interest owing to the potential for the development of drug resistance mutations. Here the fundamental question is whether and perhaps what factors provide a venue for the development of resistant virus. The concern is clearly the eventual progression of disease with the exhaustion of treatment options.

The purpose of this review is to evaluate the current literature regarding the effect of low-level viremia on the development of drug resistance mutations. Herein, we discuss the impact of different levels of viral suppression on the development of mutations. In addition, we look at the role that resistance and fitness play in determining the survival of a breakthrough mutation within the background of drug. (AIDS Reviews 2007;9:68-74)

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Introduction

The advances in HIV antiretroviral treatment have had an extraordinary impact on disease progression for infected individuals on combination therapy. Over the past decade, deaths due to HIV infection have diminished when individuals take medication in a reliable and adherent manner, resulting in an improved quality of life and maintenance of immunologic function for extended peri-

ods of time. In fact, the pharmacokinetics of the newer agents have been more forgiving in terms of adherence when compared to the early days of combination therapy when individuals were burdened with high pill number and high dosing frequency throughout the day.

But, as HIV has developed into a chronic disease, lapses in adherence or other factors that reduce drug concentrations provide a window of opportunity for the virus to mutate to maintain a survival advantage. It is this persistence of virus layered upon an increasing prevalence of HIV-infected individuals that drives an increase in AIDS in countries with combination therapy.

For individuals on no therapy, viral evolution is a dynamic situation that can be described in large part by deterministic theory¹. As time progresses from the point of infection the virus evolves, generating progeny that differ from the founder population and therefore some distance from the point of origin in terms of divergence and diversity. Early after infection this divergence from the

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original virus increases linearly at a rate of about 1% per year and eventually levels off in late disease². It is important to recognize that the virus swarm in the host is therefore not uniform and perhaps of an increased complexity based on the fact that evolution in one anatomical area may be driven by the local environment.

When an individual is treated with antiretroviral therapy, the goal is to stop viral replication. Although combination therapy has been successful in lowering viral loads below levels of clinical detection, sensitive laboratory techniques have routinely demonstrated evidence of persistent viral replication. Moreover, transient elevations in viral load occur during combination antiretroviral therapy in a majority of individuals. To further complicate matters, some patients have episodes of low but detectable viral burdens. The fact that viral replication has been difficult to stop begs the question regarding whether and at what rate does viral evolution occur, impacting on the ultimate development of breakthrough drug resistance mutations. Addressing this question is not easy because viral reservoirs are heterogeneous in factors that affect viral replication. As such, evolution of virus for one individual with low viral burden may be different than evolution for another individual with the same plasma viral burden. As the viral load decreases on therapy, viral evolution moves from one described by deterministic theory to one described by local constraints. Because of ongoing viral replication in various anatomical areas, local viral replication may determine individual patterns of evolution. Early studies have shown that low viral burdens do not necessarily translate into low viral loads in lymph nodes³. Indeed, indirect measurements of viral activity such as PET scanning using fluorodeoxyglucose to label metabolically active cell populations demonstrate an increased metabolic activity in lymph nodes in infected individuals with non-detectable plasma viral replication⁴. Furthermore, earlier⁵ and more recent⁴ studies evaluating distribution of fluorodeoxyglucose activity revealed a correlation of viral load with standardized uptake. This activity is reflected in other studies that look at lymphocyte turnover after pulse-chase labeling experiments using D-glucose⁶. In these experiments, effective antiretroviral therapy diminishes CD4 and CD8 cell proliferation and increase survival, but not to levels of those seen for control subjects, suggesting the persistence of viral factors that drive host immunity. Cell activation, another indirect marker of response to virus, continues to remain elevated for many infected individuals on suppressive therapy^{7,8}. The scatter in the levels of activation parameters from patient to patient on therapy may reflect heterogeneity of response and persistence of virus^{7,8}. More

direct measurements of viral DNA in lymph node tissue demonstrate that individuals on dual nucleoside reverse transcriptase inhibitor (NRTI) therapy have significant levels of viral DNA in lymph nodes when the plasma viral load is suppressed^{9,10}. Even with nonnucleoside reverse transcriptase inhibitor (NNRTI) or protease inhibitor (PI) containing regimens, viral DNA can be frequently found in tissue of suppressed individuals⁹⁻¹². Whether viral DNA in extravascular compartments translates into replication-competent virus that impacts on treatment or viral evolution is an area of discussion. In one study⁹, data was presented that looked at treatment failure for patients with suppressed viral burdens, and compared those individuals with no measurable tonsillar DNA to those with measurable tonsillar DNA. Over time, those with measurable tonsillar viral DNA failed therapy at a greater rate than those patients with non-detectable tonsillar viral DNA. Evidence that viral evolution occurs in individuals with suppressed viral burdens can be seen in an earlier study in which virally suppressed patients (20-200 c/ml) were followed over time for the development of drug resistance mutations¹³. In that study, phylogenetic tree analysis for one of the patients on a triple NRTI regimen demonstrated the development of an M184V mutation during suppression¹³. In this case, lamivudine was added later in the course of therapy, enhancing the possibility that this mutation followed the addition and was not likely transmitted¹³. Although this patient was not on combination antiretroviral therapy as we use it today, the point to be made from the current discussion is that viral DNA can exist in anatomical sites that have ongoing replication, even when the plasma viral load is not detectable, and may evolve in some cases. With this as a historical starting point, we revisit the question of the impact of viral evolution with low and suppressed plasma viral burdens for those individuals on combination antiretroviral therapy.

Impact of plasma viral load on the development of drug resistance mutations

Although guidelines reflect expert consensus on when to modify treatment and targets for goals of therapy, numerous confounding variables exist for the practicing physician. Finite treatment choices as well as patient-driven variables frequently dictate intervention behavior. The fundamental tenet held is that the lower the viral burden the lower the rate of viral evolution and therefore breakthrough resistance.

Numerous studies have looked at viral evolution when viral loads are persistently elevated (> 400 c/ml). One

study demonstrated that plasma viral burdens on the order of 1000 c/ml had lymph node viral loads indistinguishable from untreated individuals³. Although limited in size, such a finding suggests plasma viral loads that are low may have areas of active replication that ultimately drive the emergence of drug resistance mutations. This possibility has been substantiated in a number of studies that evaluated the emergence of new viral mutations at low persistent plasma viral loads under unchanged combination antiretroviral therapy¹⁴⁻¹⁷. In these studies, the median log viral load was circa 3.5 and the median CD4 counts were 200-300/mm. Most of these patients were highly experienced to treatment and had baseline mutations. In all studies, the addition of new drug resistance mutations as well as non-drug resistance mutations was common over a period of one year. Although variable from study to study, an incidence rate of 1.0 mutation per person-year for those with low persistent viral burdens is within the confidence limits of the studies and almost 100-fold greater than the incident rate for viral evolution in naive HIV-infected individuals^{2,18}. It should be noted that this incidence rate is determined by numerous factors such as drug experience, but certainly gives the clinician some idea regarding the rapidity that mutations can occur at these viral burdens, setting an upper limit on the development of mutations for patients with lower plasma viral burdens. In one study the acquisition of mutations was biphasic, with the highest risk occurring for those individuals with log HIV-RNA between 3 to 4¹⁵, which is consistent with the lymph node data, suggesting that active viral replication is ongoing at these plasma viral loads³. A consistent finding in all the studies was that as the number of mutations accumulates in the viral genome, there is a decrease in the rate at which new mutations are added. Whether the decrease in addition of mutations is a consequence of a decrease in viral fitness is possible. Some studies have addressed this question and found that for patients with suboptimal viral suppression and the concomitant development of drug resistance mutations, there is frequently a drop in the virus replication capacity (a rough indirect measure of fitness)^{19,20}. That cost does not necessarily translate into full control or stability of the viral burden¹⁹. As we will discuss later, the addition of new mutations does not always negatively affect fitness since compensatory mutations can improve viral survival.

The goals of antiretroviral therapy are to diminish the viral burdens to below levels of detection (< 50 c/ml). This has been difficult to sustain since it is common for individuals to have transient and sometimes persistent low-level elevations in their plasma viral burdens²¹⁻²⁴.

In a study by Nettles, et al.²⁴, virally suppressed patients on HAART underwent sampling of viral loads every two to three days for a period of three to four months. Nine of 10 patients had transient viral load elevations (blips) and none developed new mutations. These data suggest that the vast majority of individuals on HAART undergo transient elevations in plasma viral load during their course of therapy. These studies lead one naturally to ask, what is the impact of low (50-400 c/ml) persistent viral burdens or transient viral blips on the development of drug resistance mutations?

This is a difficult question to address since the development of new mutations is driven by local considerations. Perhaps the accumulation of these mutations is similar to those described by evolutionists such as George Simpson in the development of rapid evolutionary changes that occur in paleontology and even at the molecular level²⁵. The impact of local effects may be seen both directly as well as indirectly (described previously). In infected individuals with suppressed viral burdens, evaluation of lymph node viral DNA demonstrates significant measurable virus in the tissue⁹⁻¹². One study showed that individuals with measurable tonsillar viral DNA failed their antiretroviral therapy at a greater rate than those with non-detectable viral burden. These findings are consistent with data evaluating T-cell activation and the potential role it plays in immunologic recovery and progression^{7,8}. Perhaps as telling are the earlier data evaluating the development of drug resistance mutations for individuals on dual NRTI therapy. One study evaluated data from three studies (INCA, AVANTI-2, AVANTI-3) for viral rebound and asked whether these episodes of rebound were determinative of therapeutic failure in virally suppressed individuals²⁶. In this retrospective study, at 52 weeks, individuals on triple therapy had less failure than those on dual NRTI therapy. One study evaluated viral evolution for individuals with viral loads suppressed in the range of 20-200 c/ml. Those on NRTI therapy demonstrated *env* divergence and the addition of new mutations in the RT *pol* sequences¹³. These data strongly suggest that the plasma viral burden although important does not necessarily reveal the full degree of viral activity in compartments not easily accessed.

Treatment options have improved since these earlier studies. Combination therapy has impacted positively on both survival and durability of viral load suppression. The newer agents and combinations provide higher drug concentrations for longer times with adjunctive benefits of increased penetration into various sites. As such, these improvements are expected to impact on viral replication and therefore viral evolution. For indi-

viduals on HAART, viral burdens between 50-1000 c/ml are sufficient to allow viral evolution with the development of drug resistance mutations^{3,27,28}. Recently, clonal genotyping was used to evaluate the HIV-1 *pol* gene sequence from plasma specimens from 21 patients with persistent viral burdens between 50-400 c/ml for at least three months²⁷. Drug resistance mutations were found in nine of 21 patients, and three of 21 could definitively be attributed to the new regimen. Using the three mutations for the 21 patients over the duration of the low-level viremia for the group, a calculated incidence of developing a drug resistance mutation was 0.16 per patient year. This is almost 10-fold less than that found for the development of drug resistance mutations for individuals with viral loads from 1000-10,000 c/ml.

The data on whether transient blips in the viral load portend failure or the development of drug resistance mutations is somewhat mixed. As indicated above, intense sampling of viral burden demonstrates that a large proportion of individuals have viral load blips over time²⁴ but do not develop drug resistance mutations. An earlier study²⁹ used phylogenetic tree analysis to evaluate pairwise distances from the most recent common ancestor (MRCA) for the HIV-1 *env* region and was able to demonstrate viral evolution. They found that evolutionary distance correlated with the area under the curve for viral decay and transient viral blips after initiation of therapy, suggesting that viral replication and evolution in suppressed patients (<50 c/ml) was occurring. Comparatively, a study in the pediatric/adolescent population evaluated the effect of viral load blips on viral evolution³⁰. They found that genetic divergence from the MRCA in plasma and PBMC virus could be demonstrated in the *pol* encoding RT and *env* encoding C2-V5 regions for three of 11 patients. In two of these patients viral blips were relatively frequent (32-42%) and they developed drug resistance mutations over time. From these data one can calculate the incidence of drug resistance mutations to be 0.044 per patient year. By using these drug resistance mutations per year described above at various viral burdens and placing those with blips below persistent viremia, a graph can be constructed as a function of plasma viral load, understanding that this is simply a visualization of data extracted from various sources described herein (Fig. 1). The dashed curve is an exponential decay curve, suggesting that the mutational rate is correlated with the viral burden. This picture provides a framework to discuss why some groups may find mutations and others do not, since when the mutation rate is low the number of patient years that need to be followed to find a mutation is in the order of decades in this model.

As the plasma viral burden is further suppressed so that transient elevations in viral load are lowered or abrogated, the mutation rate drops as well. Numerous groups have demonstrated this fact^{29,31,32}. In the work by Bailey, et al.³² a phylogenetic tree analysis of multiple viral clonal sequences from plasma and cells were derived over time for a series of patients. In no cases were the researchers able to demonstrate the development of new drug resistance mutations. They did find, however, that there were predominant clonal species in the plasma, representing > 50% of the independent plasma sequences derived from an individual patient. Interestingly, these predominant plasma clones were underrepresented in the cellular pool of integrated virus. Why there are these predominant plasma clones and where they originate is not known at this time.

Role for resistance and fitness in the development of drug resistance mutations

So, it seems that the development of productive drug resistance mutations are small and decrease as the viral load is tightly controlled with combination antiretroviral therapy. Then, how do these drug resistance mutations originate and take hold? We discussed the fact that the viral load is not a good indicator of viral replication in the face of dual NRTI therapy or, perhaps more importantly, with viral load blips given sufficient time on therapy. As such, it is clear that there will be times where anatomical islands of viral replication are ongoing. So, in these microcosms of viral opportunity, what determines whether a resistance mutation will survive and grow? It is important that the virus be able to replicate effectively and have targets to infect to sustain growth. In most cases, many different viral quasispecies³³ exist and therefore will potentially have the opportunity to recombine³⁴. As such, the fact that the mutation rate is high may not be as telling as the variability found in the viral genomic structure that leads to the production of new viral species through a high incidence of recombination events³⁴. This mutation opportunity through recombination further provides a potential to escape from bottlenecks. In contrast, however, there is substantive evidence that the extent of viral diversity is restricted. In the envelop region, for example, a maximum viral diversity of around 8% has been found, whereas in the protease region diversity is limited to approximately 1%^{34,35}. In addition, modifications that occur in one genomic location may impact on other regions³⁶. The diversity in the viral genome as well as host and local factors provide a fitness landscape that is complex. Because of

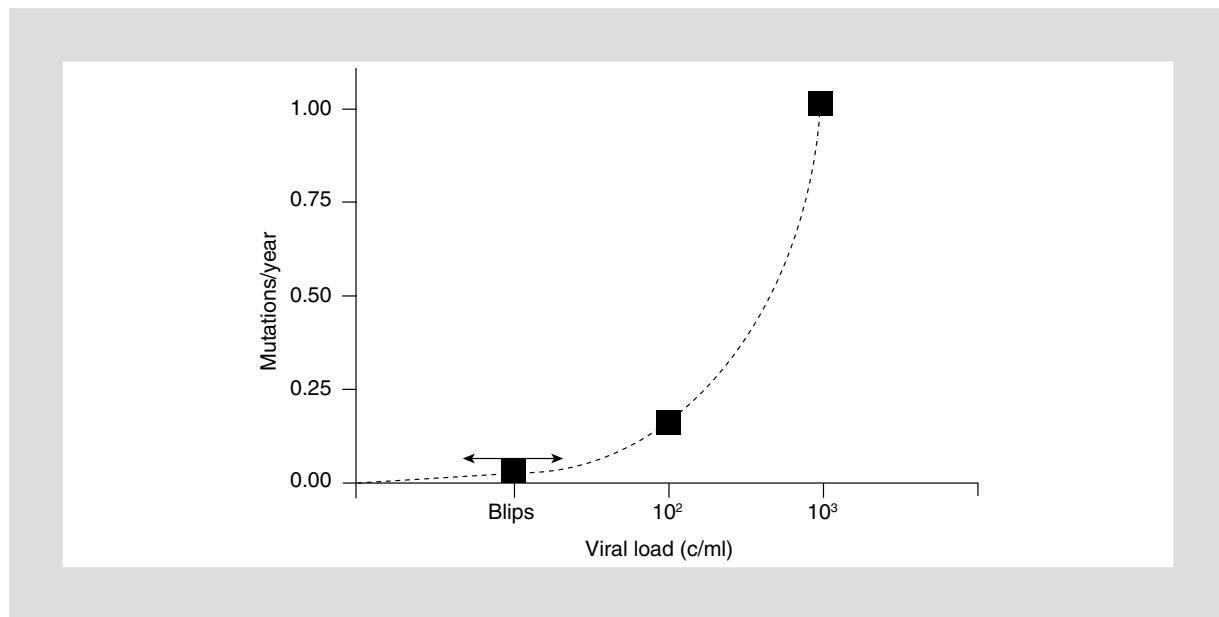


Figure 1. Incidence of drug resistance mutations as a function of plasma viral burden. The incidence of drug resistance mutations is shown as a function of plasma viral load. Points on the graph represent a compilation of data from different studies as described in the text. The dashed line is an exponential decay curve that is displayed for demonstrative purposes. The arrows on the point designating viral blips are to demonstrate that this point is not fixed and may vary significantly in either direction.

this, the dominant viral species may not be the one that exists on a local fitness maximum determined solely by viral considerations since other contributing factors impact on fitness as well. This has been demonstrated in a number of recent studies^{32,37}.

Within all the local interactions and multiplicity of variables it does seem that there exist driving forces that determine the sequence and development of drug resistance mutations. This is most easily appreciated when one looks at the fact that viral evolution in terms of database analysis is describable by modeling^{38,39} and therefore demonstrates evidence of potential predictability. Two factors considered by numerous researchers as important are resistance and viral fitness. Although these two parameters are not exclusionary, resistance is thought of as the inability to suppress viral replication under drug pressure suppressive of a wild-type virus as generally displayed in phenotypic assays. Fitness is a broader term and reflects on the ability of the virus to reproductively grow in a particular environment. This is frequently measured using competitive assays between two viral species to determine which virus dominates and under what conditions. Other studies of a biochemical nature can be used to evaluate particular steps in the replication process and how they could affect growth.

One important question is whether the interplay between resistance and fitness is able to describe viral evolution. The K103N mutation is the most common

mutation for those individuals failing efavirenz therapy⁴⁰, but does not confer the greatest resistance when compared to other mutations such as the G190S mutation⁴¹. One explanation for this may be given by looking at competition experiments that demonstrate that there are no concentrations of efavirenz at which G190S confers a greater fitness than the K103N mutation⁴². Therefore, it may be this fitness advantage of the K103N mutation that explains its prevalence and persistence as the dominant efavirenz breakthrough mutation, even in light of its effect on resistance. Similarly, the K103N mutation is found with double mutations, but the most common ones are not the most resistant⁴¹. Unlike the single mutations of K103N and G190S, the fitness between the dual K103N+L100I and K103N+V108I is dependent on the efavirenz concentration⁴³. In this case, the L100I dual mutants were more fit than the V108I mutants under efavirenz drug pressure but not in the absence of efavirenz. Considering recent adherence data⁴⁴ showing that good viral load suppression occurs with 50-100% adherence to NNRTI therapy, failure may result from low drug levels driving the system away from L100I containing mutants.

Looking at the development of PI mutations yields similar information. Development of the I50V mutation for amprenavir-treated individuals seemed to be strongly linked to either the L449F or P453L mutations⁴⁵. By itself, the I50V mutation produced a poorly

fit virus, whereas with the addition of the mutations in the region of the *gag* cleavage sites, fitness was improved and further enhanced by addition of the M46I mutation. The interpretation of these data was that the viral escape from amprenavir occurred secondary to viral adaptation in both the protease and Gag regions, resulting in a decrease in active site binding of the drug and an increase in fitness. Earlier studies on resistance and fitness have demonstrated the complexity involving these two variables^{46,47}. Mammano, et al.⁴⁷ looked at the effect of different drug resistance mutations on resistance and infectivity (as a measure of fitness) in the absence and presence of different PI concentrations. In this work it was clear that similar levels of resistance to a PI occur with different drug resistance mutations (e.g. ritonavir, virus containing 46I-82A or 71V-82A) whereas the infectivity was vastly different for each viral species. Additionally, when infectivity was viewed as a function of PI drug concentration the curves were generally biphasic, demonstrating the importance and complexity for when drug resistance mutations develop in the background of drug.

The effect of drug concentration on fitness is also seen on drugs that constitute the backbone of HAART regimens. Thymidine analog mutations (TAM) D67N and K219Q do not confer resistance, but they can affect the development of resistance to zidovudine. In the presence of zidovudine, these mutations provide a significant competitive advantage for growth when compared to wild-type⁴⁸, demonstrating that modification of fitness under drug pressure can impact on the development of drug resistance mutations.

The role of fitness in modifying the development of drug resistance mutations may be seen in the impact of certain mutations which by themselves do not affect this parameter. The L63P mutation is a common polymorphism in wild-type virus⁴⁹. In the presence of a D30N or L90M mutation, an L63P containing virus results in improved fitness, and a potential explanation for the frequency with which D30N or L90M may be found with L63P⁵⁰. This also demonstrates that polymorphisms in naive individuals that do not contain drug resistance mutations could impact in an *a priori* manner on the development of drug resistance mutations.

Conclusions

In summary, the incidence of productive drug resistance mutations diminishes in a manner that seems exponentially dependent on plasma viral load for those on HAART. The differences found from study to study in

terms of viral load transient elevations may be attributed to factors such as time on therapy, sampling times, and the number of elevations. Indeed, mitigating factors such as the number of drug resistance mutations, wild-type polymorphisms and genomically distant mutations that provide a background unto which new proximal drug resistance mutations occur, impact evolution as well. The host haplotype can impact on viral immunity and selective pressures for the development of viral diversity as well as drug metabolism for those on antiretroviral therapy⁵¹⁻⁵³. How these additional variables participate in the evolution of drug resistance mutations will, no doubt, become part of the growing picture as technology and database utilization continue to expand.

Even with the layered complexity described above, variables such as resistance and fitness provide some foundation for discussing which drug resistance mutations break through under drug pressure. Although most studies use these variables to explain findings for the development of resistance, they cannot be reliably predicted. This said, as our ability to control variables such as drug concentration improves, it will become easier to discern whether there are fundamental parameters dominating the development of drug resistance mutations.

It has been remarkable when viewing the advances over the past decade to see how the marriage of experiment with information technology and computing science has advanced our knowledge. One can expect this only to improve over the coming years.

References

1. Rouzine I, Rodrigo A, Coffin J. Transition between stochastic evolution and deterministic evolution in the presence of selection: general theory and application to virology. *Microbiol Mol Biol Rev* 2001;65:151-85.
2. Shankarappa R, Margolick J, Gange S, et al. Consistent viral evolutionary changes associated with the progression of HIV-1 infection. *J Virol* 1999;73:10489-502.
3. Gunthard H, Wong J, Ignacio C, et al. HIV replication and genotypic resistance in blood and lymph nodes after a year of potent antiretroviral therapy. *J Virol* 1998;72:2422-8.
4. Brust D, Polis M, Davey R, et al. Fluorodeoxyglucose imaging in healthy subjects with HIV infection: impact of disease stage and therapy on pattern of nodal activation. *AIDS* 2006;20:985-93.
5. Iyengar S, Chin B, Margolick J, Sabundayo B, Schwartz D. Anatomical loci of HIV-associated immune activation and association with viremia. *Lancet* 2003;362:945-50.
6. Mohri H, Perelson A, Tung K, et al. Increased turnover of T lymphocytes in HIV-1 infection and its reduction by antiretroviral therapy. *J Exp Med* 2001;194:1277-87.
7. Benito J, Lopez M, Lozano S, Martinez P, Gonzalez-Lahoz J, Soriano V. CD38 expression on CD8 T lymphocytes as a marker of residual virus replication in chronically HIV-infected patients receiving antiretroviral therapy. *AIDS Res Hum Retroviruses* 2004;20:227-33.
8. Cohen Stuart J, Hazenberg M, Hamann D, et al. The dominant source of CD4+ and CD8+ T-cell activation in HIV infection is antigenic stimulation. *J Acquir Immune Defic Syndr* 2000 ;25:203-11.

9. Martinez E, Arnedo M, Giner V, et al. Lymphoid tissue viral burden and duration of viral suppression in plasma. *AIDS* 2001;15:1477-82.
10. Ruiz L, van Lunzen J, Arno A, et al. Protease inhibitor-containing regimens compared with nucleoside analogs alone in the suppression of persistent HIV-1 replication in lymphoid tissue. *AIDS* 1999;13:F1-8.
11. Lafeuillade A, Chollet L, Hittinger G, Profizi N, Costes O, Poggi C. Residual HIV-1 RNA in lymphoid tissue of patients with sustained plasma RNA of < 200 copies/ml. *J Infect Dis* 1998;177:235-8.
12. Dybul M, Chun T, Ward D, et al. Evaluation of lymph node virus burden in HIV-infected patients receiving efavirenz-based protease inhibitor-sparing HAART. *J Infect Dis* 2000;181:1273-9.
13. Martinez M, Cabana M, Ibanez A, Clotet B, Arno A, Ruiz L. HIV-1 genetic evolution in patients with prolonged suppression of plasma viremia. *Virology* 1999;256:180-7.
14. Kantor R, Shafer R, Follansbee S, et al. Evolution of resistance to drugs in HIV-1-infected patients failing antiretroviral therapy. *AIDS* 2004;18:1503-11.
15. Napravnik S, Edwards D, Stewart P, Stalzer B, Matteson E, Eron J. HIV-1 drug resistance evolution among patients on potent combination antiretroviral therapy with detectable viremia. *J Acquir Immune Defic Syndr* 2005;40:34-40.
16. Kristiansen T, Pedersen A, Eugen-Olsen J, Katzenstein T, Lundgren J. Genetic evolution of HIV in patients remaining on a stable HAART regimen despite insufficient viral suppression. *Scand J Infect Dis* 2005;37:890-901.
17. Hatano H, Hunt P, Weidler J, et al. Rate of viral evolution and risk of losing future drug options in heavily pretreated, HIV-infected patients who continue to receive a stable, partially suppressive treatment regimen. *Clin Infect Dis* 2006;43:1329-36.
18. Fu Y. Estimating mutation rate and generation time from longitudinal samples of DNA sequences. *Mol Biol Evol* 2001;18:620-6.
19. Barbour J, Wrin T, Grant R, et al. Evolution of phenotypic drug susceptibility and viral replication capacity during long-term virologic failure of protease inhibitor therapy in HIV-infected adults. *J Virol* 2002;76:11104-12.
20. Nicastrì E, Sarmati L, d'Ettoire G, et al. Replication capacity, biological phenotype, and drug resistance of HIV strains isolated from patients failing antiretroviral therapy. *J Med Virol* 2003;69:1-6.
21. Greub G, Cozzi-Lepri A, Ledergerber B, et al. Intermittent and sustained low-level HIV viral rebound in patients receiving potent antiretroviral therapy. *AIDS* 2002;16:1967-9.
22. Moore A, Youle M, Lipman M, et al. Raised viral load in patients with viral suppression on HAART: transient increase or treatment failure? *AIDS* 2002;16:615-8.
23. Di Mascio M, Markowitz M, Louie M, et al. Viral blip dynamics during HAART. *J Virol* 2003;77:12165-72.
24. Nettles R, Kieffer T, Kwon P, et al. Intermittent HIV-1 viremia (Blips) and drug resistance in patients receiving HAART. *JAMA* 2005;293:817-29.
25. Simpson G. *Tempo and Mode in Evolution*. New York: Columbia Univ Press 1944.
26. Raboud J, Rae S, Woods R, Harris M, Montaner J. INCAS and AVANTI Study Groups. Consecutive rebounds in plasma viral load are associated with virologic failure at 52 weeks among HIV-infected patients. *AIDS* 2002;16:1627-32.
27. Nettles R, Kieffer T, Simmons R, et al. Genotypic resistance in HIV-1-infected patients with persistently detectable low-level viremia while receiving HAART. *Clin Infect Dis* 2004;39:1030-7.
28. Sungkanuparph S, Groger R, Overton E, Fraser V, Powderly W. Persistent low-level viremia and virologic failure in HIV-1-infected patients treated with HAART. *HIV Med* 2006;7:437-41.
29. Gunthard H, Frost S, Leigh-Brown A, et al. Evolution of envelope sequences of HIV-1 in cellular reservoirs in the setting of potent antiviral therapy. *J Virol* 1999;73:9404-12.
30. Tobin N, Learn G, Holte S, et al. Evidence that low-level viremia during effective HAART result from two processes: expression of archival virus and replication of virus. *J Virol* 2005;79:9625-34.
31. Frenkel L, Wang Y, Learn G, et al. Multiple viral genetic analyses detect low-level HIV-1 replication during effective HAART. *J Virol* 2003;77:5721-30.
32. Bailey J, Sedaghat A, Kieffer T, et al. Residual HIV-1 viremia in some patients on antiretroviral therapy is dominated by a small number of invariant clones rarely found in circulating CD4+ T-cells. *J Virol* 2006;80:6441-57.
33. Briones C, de Vicente A, Molina-Paris C, Domingo E. Minority memory genomes can influence the evolution of HIV-1 quasispecies *in vivo*. *Gene* 2006;384:129-38.
34. Charpentier C, Nora T, Tenaillon O, Clavel F, Hance AJ. Extensive recombination among HIV-1 quasispecies makes an important contribution to viral diversity in individual patients. *J Virol* 2006;80:2472-82.
35. Wolinsky S, Korber B, Neumann A, et al. Adaptive evolution of HIV-1 during the natural course of infection. *Science* 1996;272:537-42.
36. Delwart E, Pan H, Neumann A, Markowitz M. Rapid, transient changes at the env locus of plasma HIV-1 populations during the emergence of protease inhibitor resistance. *J Virol* 1998;72:2416-21.
37. Fernandez G, Clotet B, Martinez M. Fitness landscape of HIV-1 protease quasispecies. *J Virol* 2007;81:2485-96.
38. Foulkes A, De Gruttola V. Characterizing the progression of viral mutations over time. *J Am Stat Ass* 2003;98:859-67.
39. Beerwinkler N, Daumer M, Sing T, et al. Estimating HIV evolutionary pathways and the genetic barrier to drug resistance. *J Infect Dis* 2005;191:1953-60.
40. Bachelier L, Anton E, Kudish P, et al. HIV-1 mutations selected in patients failing efavirenz combination therapy. *Antimicrob Agents Chemother* 2000;44:2475-84.
41. Bachelier L, Jeffrey S, Hanna G, et al. Genotypic correlates of phenotypic resistance to efavirenz in virus isolates from patients failing NNRTI therapy. *J Virol* 2001;75:4999-5008.
42. Wang J, Dykes C, Domaoal R, Koval C, Bambara R, Demeter L. The HIV-1 reverse transcriptase mutants G190S and G190A, which confer resistance to NNRTI, demonstrate reductions in RNaseH activity and DNA synthesis from tRNA(Lys, 3) that correlate with reductions in replication efficiency. *Virology* 2006;348:462-74.
43. Koval C, Dykes C, Wang J, Demeter L. Relative replication fitness of efavirenz-resistant mutants of HIV-1: correlation with frequency during clinical therapy and evidence of compensation for the reduced fitness of K103N + L100I by the nucleoside resistance mutation L74V. *Virology* 2006;353:184-92.
44. Bangsberg D. Less than 95% adherence to NNRTI therapy can lead to viral suppression. *Clin Infect Dis* 2006;43:939-41.
45. Maguire M, Guinea R, Griffin P, et al. Changes in HIV-1 Gag at positions L449 and P453 are linked to I50V protease mutants *in vivo* and cause reduction of sensitivity to amprenavir and improved viral fitness *in vitro*. *J Virol* 2002;76:7398-406.
46. Borman A, Paulous S, Clavel F. Resistance of HIV-1 to protease inhibitors: selection of resistance mutations in the presence and absence of the drug. *J Gen Virol* 1996;77:419-26.
47. Mammano F, Trouplin V, Zennou V, Clavel F. Retracing the evolutionary pathways of HIV-1 resistance to protease inhibitors: virus fitness in the absence and in the presence of drug. *J Virol* 2000;74:8524-31.
48. Garcia-Lerma J, MacInnes H, Bennett D, Weinstock H, Heneine W. Transmitted HIV-1 carrying the D67N or K219Q/E mutation evolves rapidly to zidovudine resistance *in vitro* and shows a high replicative fitness in the presence of zidovudine. *J Virol* 2004;78:7545-52.
49. Lech W, Wang G, Yang Y, et al. *In vivo* sequence diversity of the protease of HIV-1: presence of protease inhibitor-resistant variants in untreated subjects. *J Virol* 1996;70:2038-43.
50. Quiros-Roldan E, Moretti F, Torti C, Tirelli V, Casari S, Carosi G. HIV-1 genotype resistance pattern and evolution in patients failing nelfinavir-containing regimens. *J Clin Lab Anal* 2005;19:26-9.
51. Karlsson A, Deeks S, Barbour J, et al. Dual pressure from antiretroviral therapy and cell-mediated immune response on the HIV-1 protease gene. *J Virol* 2003;77:6743-52.
52. Schmitt M, Harrer E, Goldwisch A, et al. Specific recognition of lamivudine-resistant HIV-1 by cytotoxic T lymphocytes. *AIDS* 2000;14:653-8.
53. Wang J, Sonnerborg A, Rane A, et al. Identification of a novel specific CYP2B6 allele in Africans causing impaired metabolism of the HIV drug efavirenz. *Pharmacogenet Genomics* 2006;16:191-8.