PATRÍCIA MUNERATO

A RESISTÊNCIA AOS ANTI-RETROVIRAIS E A DIVERSIDADE GENÉTICA DO HIV-1 NO BRASIL

Tese apresentada à Universidade Federal de São Paulo – Escola Paulista de Medicina, para obtenção do Título de Doutor em Ciências.

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Orientador: Prof. Dr. Ricardo Sobhie Diaz

Co-Orientador: Prof. Dr Luiz Mário Ramos Janini

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Antiretrovirals resistance and HIV-1 genotypic diversity in Brazil.

1. antiretroviral resistance 2.resistance mutation 3.resistance pathway 4. phenotypic resistance 5.genotypic diversity

Dedicatória

A minha mãe, ***Luiza Zerlin Munerato****, que pacientemente esperou 34 anos até que eu entendesse o significado completo do seu papel em minha vida.*

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“- Você poderia me dizer, por favor, qual caminho eu devo seguir?- perguntou Alice

- Isso depende muito de onde você deseja chegar." - disse o Gato.

- Para mim tanto faz .....contanto que eu chegue em algum lugar...- respondeu Alice.

- Então, pouco importa o caminho que você tome, certamente você chegará lá se continuar andando bastante..." - respondeu o Gato."

Alice no País das Maravilhas

Lewis Carroll

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Sumário

1. Introdução

O Vírus da Imunodeficiência Humana Tipo 1 (HIV-1) é um exemplo clássico dos retrovírus e, como tal, sofre mutações numa velocidade alarmante. Substituições nucleotídicas, tais como, insersões, deleções e recombinações são comuns durante o curso da infecção por este agente.[1] Neste contexto de geração de diversidade, o HIV-1 está classificado em três grupos distintos e altamente divergentes: M (major), O (outlier) e N (new), os quais podem representar três introduções separadas do Vírus da Imunodeficiência Símia (SIV) aos seres humanos. A grande maioria das variantes genéticas do HIV-1 está inseridas no grupo M, o qual está subdividido em 9 subtipos (A, B, C, D, F, G, H, J e K) e inúmeras Formas Recombinantes Circulantes (CRFs) que têm sido descritas num número cada vez maior de publicações.[2,3,4] As CRFs são fruto da ocorrência de eventos de recombinação entre dois subtipos distintos e contribuem para o processo dinâmico de expansão e heterogeneidade da epidemia.

Atualmente, os 33,2 milhões de indivíduos infectados pelo HIV estão espalhados pelos 5 continentes, porém a distribuição de subtipos não é homogênea (http://www.unaids.org/en/KnowledgeCentre/HIVData). A África, berço da pandemia, abriga todos os subtipos do HIV-1, tendo o C como predominante. Porém, na América do Norte e Europa Ocidental o vírus do subtipo B circula quase que com exclusividade, embora outras variantes venham sendo descritas nos últimos anos.[5,6,7,8,9,10,11,12] No Brasil, a diversidade é um pouco maior, onde cerca de 85% dos vírus são B e 25% subtipo F em locais como São Paulo e Rio de Janeiro, com uma proporção significativa de recombinantes B/F. No sul do país há uma alta prevalência do subtipo C, que chega a 50% no estado do Rio Grande do Sul, enquanto a prevalência do subtipo F aumenta em direção ao norte do país.[13]

Recentemente, as CRFs 28 e 29, recombinantes entre as clades B e F, foram caracterizadas e descritas no estado de São Paulo, corroborando o fato da ocorrência de eventos de recombinação onde mais de um subtipo co-circula.[14]

Avaliar os diferentes subtipos e CRFs do HIV-1 é de importância crucial para o entendimento da tendência da pandemia. Mais do que isso, a diversidade do HIV-1 pode afetar testes diagnósticos, principalmente aqueles baseados em manipulação dos ácidos nucléicos.[15,16,17] O mesmo pode acontecer com a eficácia dos anti-retrovirais atuais, que foram inicialmente testados em pacientes infectados pelo vírus B, podendo ter desempenho diferente frente aos vírus não-B, além destes últimos poderem apresentar padrões de resistência e vias mutacionais distintas.

A maioria dos testes de susceptibilidade a anti-retrovirais foram também desenhados e desenvolvidos principalmente para o subtipop B, haja vista que este é o subtipo mais prevalente nos países desenvolvidos, onde concentram-se os grandes centros de pesquisa e desenvolvimento farmacêuticos. No entanto, com o aumento global de subtipos não-B, surge a necessidade de investigar o desempenho dos ensaios de resistência aos medicamentos em relação a outros subtipos que não o B. Estes testes se tornaram uma ferramenta importante no acompanhamento de pacientes infectados pelo HIV sob terapia anti-retroviral.[18] Tanto métodos genotípicos (genotipagem) quanto fenotípicos (fenotipagem) parecem ser igualmente úteis na determinação da susceptibilidade aos anti-retrovirais.[19] Enquanto a genotipagem identifica as posições das mutações e/ou dos polimorfismos no genoma do HIV associados à resistência aos anti-retrovirais, a fenotipagem examina a susceptibilidade relativa do vírus em questão às diferentes concentrações de medicamentos *in vitro*.

Não existem dados conclusivos até o momento, se mutações ou vias mutacionais específicas de resistência são selecionadas em subtipos não-B.

Da mesma forma, não se sabe com certeza se os polimorfismos presentes nos demais subtipos influenciam a susceptibilidade às drogas. Polimorfismos que ocorrem naturalmente no gene *pol*, o qual codifica enzimas que são alvos de medicamentos anti HIV (protease, transcriptase reversa (TR) e integrase), resultam da alta taxa de replicação viral, que atinge 10^9 a 10^{10} virions por dia, e da baixa fidelidade da enzima TR, que possui taxa de erro de 3×10^{-5} por base por ciclo replicativo. [20]

Tais mudanças genéticas são freqüentemente relatadas em indivíduos virgens de tratamento infectados por subtipos não-B. [21,22,23,24,25,26,27,28] Algumas destas mudanças, tais como K20I, M36I e V82A, estão associadas à resistência aos inibidores da protease (IPs) e são consideradas mutações secundárias ou acessórias, ou seja, emergem naturalmente e, via de regra, recuperam o *fitness* perdido pelo aparecimento de mutações principais, além de poderem propiciar perda modesta de susceptibilidade aos anti-retrovirais.[29] De modo geral, a alteração genética M36I na protease pode ser considerada um marcador genético de subtipos não-B do grupo M,[29] e a substituição *K20I* aparece quase exclusivamente em seqüências da protease pertencentes ao subtipo G.[29] Outro polimorfismo da protease, o *L89M*, comumente presente nos vírus clade F, já foi associado à perda de susceptibilidade ao Nelfinavir.[30] Por outro lado, o polimorfismo *L93I*, presente no subtipo C, foi descrito por causar hipersusceptibilidade deste vírus ao Lopinavir.[31] A *L93I* também está presente nos vírus do subtipo F, porém seu afeito nesta clade não foi demonstrado.

É interessante notar, com relação às vias mutacionais para a resistência aos anti-retrovirais, que quando existe a pressão seletiva do inibidor de protease Nelfinavir sobre os vírus dos subtipos não-B, a mutação que emerge quase exclusivamente é a *L90M*, sendo muito rara a via pela *D30N*.[32] A razão para existência de uma via preferencial divergente entre o B e os não-B parece ser a presença do poliformfismo *L89M* nestes últimos, a qual representa uma barreira para seleção da *D30N*.[33] Essa é uma das evidências

de que as vias mutacionais têm relação com a estrutura do vírus em questão e não ocorrem de maneira puramente aleatória. O entendimento sobre as vias mutacionais seguidas por cada subtipo viral tem relevância no sentido de que algumas delas podem implicar em resistência cruzada e, conseqüentemente, impedir o sucesso de determinados tratamentos.

Outro exemplo de padrão de resistência diferenciado entre os subtipos é em relação aos Inibidores não-nucleosídeos da Transcriptase Reversa (ITRNN), onde a seleção da mutação *V106M* pelo vírus do subtipo C possibilita a rápida resistência a esta classe de medicamentos.[34] Normalmente no subtipo B, a mutação de resistência relacionada a este códon é a *V106A* (substituição de GTG por GCA) ou *V106I* (GTG por ATT, ATC ou ATA), que não emerge com facilidade. A mutação *V106M* (substituição de GTG por ATG) ocorre rapidamente nos vírus do subtipo C, levando a alto nível de resistência aos ITRNN.[35]

Alguns estudos retrospectivos sobre a relevância clínica destes polimorfismos basais mostraram que indivíduos infectados por vírus da clade F apresentaram comprometimento na resposta aos anti-retrovirais quando comparados a indivíduos infectados pela clade B.[36] Sugerem ainda, que a presença de grande número de substituições em posições associadas à resistência pode, de alguma maneira, colocar em risco o sucesso do tratamento. [35,37,38,39] Por outro lado, outros estudos sustentam a idéia de que a resposta aos anti-retrovirais parece ser independente do subtipo.[40]

De acordo com o cenário exposto acima, fica implícita a importância de se realizar mais estudos sobre a diversidade genética e sua correlação com a resposta aos anti-retrovirais e os padrões de resistência oriundos do tratamento.

Para o Brasil, onde a epidemia é composta por três subtipos (B, C e F) e seus recombinantes e que foi o primeiro país a ter um programa governamental gratuito para controle da Síndrome da Imunodeficiência Humana Adquirida (AIDS), atendendo atualmente mais de 180 mil pessoas,

seria importantíssimo estudos a partir de amostras brasileiras representando a diversidade genética local que pudessem jogar luz acerca de padrões de resistência a resposta aos anti-retrovirais.

2. Objetivos

As propostas dos dois trabalhos apresentados a seguir foram:

1. Delinear o perfil mutacional de resistência aos anti-retrovirais para os subtipos de HIV-1 que compõem a epidemia nacional.

2. Analisar a resposta fenotípica aos antiretrovirais de vírus circulantes no Brasil frente a um teste de fenotipagem.

Profiling HIV-1 antiretroviral drug resistance mutations by subtype in a Brazilian population

Running head: HIV-1 resistance mutations in Brazil

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Abstract

Objectives: In Brazil, where three distinct HIV-1 subtypes (B, F, and C) co-circulate, a significant portion of the HIV-infected population has been exposed to antiretroviral drugs. This study analyzes the antiretroviral resistance profiles of HIV-1-infected individuals failing antiretroviral therapy.

Methods: We analyzed the genotypic resistance profiles of 2,474 patients presenting virologic failure to antiretroviral therapy in the city of São Paulo, Brazil.

Results: We detected high levels of resistance to a variety of antiretroviral classes: resistance mutations to protease inhibitors and nucleoside reverse transcriptase inhibitors were less common in subtype C viruses, whereas non-nucleoside reverse transcriptase inhibitor resistance mutations were less common in subtype F viruses. The thymidine analog mutation pathway known as pathway 1 was more prevalent in subtype B viruses than in subtype C viruses, whereas pathway 2 was more prevalent in subtype C viruses. Selected resistance mutations varied according to subtype for all three classes of antiretrovirals. We describe two pathways of non-nucleoside reverse transcriptase inhibitor resistance (to nevirapine and efavirenz). Although cross-resistance to etravirine should occur more frequently among individuals failing nevirapine treatment, the prevalence of cross-resistance to etravirine, darunavir, and tipranavir was found to be low. We found that increases in the number of resistance mutations will be related to increases in the viral load.

Conclusions: Special attention should be given to resistance profiles in non-B subtype viruses. The accumulation of knowledge regarding such profiles in the developing world is desirable.

Keywords: drug resistance, multiple, viral/genetics, anti-retroviral agents, HIV-1/drug effects, anti-HIV agents/therapeutic use

Introduction

Understanding the genetic basis for antiretroviral resistance might be particularly important for predicting cross-resistance to new antiretrovirals within a given population. In developing countries such as Brazil, which provides HIV-infected patients with full and free access to all U.S. Food and Drug Administration-approved antiretrovirals, the issue of antiretroviral resistance could be especially critical. Given the sequential use of antiretrovirals in many patients in Brazil and the extensive use of unboosted protease inhibitors (PIs) in the recent past, it is assumed that a high proportion of patients experience virologic failure. One study showed that the time to virologic failure after the first treatment regimen is only 9 months in Brazil [1]. The rate of virologic failure after an initial 48 weeks of successful viral suppression is 82.5% [2], and it can be expected that extensive resistance will emerge in this population. Another repercussion of antiretroviral failure with secondary resistance is the high rate of transmission of antiretroviral-resistant HIV, which can be as high as 36% among recently infected individuals in some areas of Brazil [3]. The HIV-1 epidemic in Brazil is concentrated around the city of São Paulo, which harbors more than 20% of all AIDS cases in the country (www.aids.gov.br). In this region, as in many other areas of Brazil, subtype F and subtype C viruses, as well as B/F recombinant viruses, co-circulate with subtype B viruses.

There are many unresolved issues regarding HIV-1 behavior in relation to its genetic diversity. It is conceivable that the genetic diversity of the virus impairs immune responses to candidate vaccines, causes false-negative results in diagnostic/monitoring

laboratory tests (especially those involving nucleic acids) [4, 5], and alters disease progression [6, 7]. Most of the resistance mutation profiles described to date has been in subtype B HIV-1 viruses. However, in non-B subtypes subjected to the selective pressure exerted by antiretroviral therapy, specific resistance mutations or pathways can be selected. For instance, some thymidine analog mutations (TAMs), as well as some PI resistance mutations, are more frequently selected in subtype B viruses than in non-B subtypes [8, 9]. Emerging *in vitro* data suggest that some natural polymorphisms in the *pol* gene interfere with antiretroviral efficacy. For instance, in subtype C viruses, the *V106M* mutation is related to lower activity of non-nucleoside reverse transcriptase inhibitors (NNRTIs) [10], and the *I93L* mutation increases susceptibility to lopiinavir [11]. In addition, subtype C viruses, due to a silent polymorphism, also have a lower genetic barrier to tenofovir resistance [12]. In the present study, we sought to analyze the secondary resistance among HIV-1-infected individuals failing antiretroviral therapy in the city of São Paulo, Brazil in a cohort of patients for whom information related to previous antiretroviral exposure was available, with a special emphasis on how well subtype correlates with resistance and with resistance pathway.

Patients and methods

Population

Samples from 2,474 patients were collected between 2002 and 2006. Samples were processed at the Federal University of São Paulo, in São Paulo, Brazil. All participating patients gave written informed consent.

Viral load and number of mutations

The number of resistance mutations was calculated as the sum of all nucleoside reverse transcriptase inhibitor (NRTI), NNRTI, and major PI resistance mutations (minor PI resistance mutations were excluded). Means, standard deviations, and medians were calculated for viral load in each group of patients. Analysis of variance was performed in order to identify statistically significant differences.

The same analyses were performed using the proportion test based on binominal distribution to determine the number of patients in each group presenting a viral load ≥ 100,000 copies/mL. All groups were compared to an antiretroviral-naïve group of 194 individuals, in which 0.19% of the patients had a viral load ≥ 100,000 copies/mL. Values of $P \leq 0.05$ were considered statistically significant.

Genotypic resistance analysis

All samples were sequenced using ViroSeq™ v2.0 (Celera Diagnostics, Alameda, California, USA). Resistance mutations were identified according to the IAS mutations list [13]. Statistical analyses were performed using Minitab Release 14 Statistical Software.

The prevalence of mutations in subtype B viruses was compared with that observed for subtype F and C viruses using chi-square tests and tests of proportion based on a binomial distribution.

HIV-1 subtype analysis

The nucleotide sequences of *pol* gene were subtyped using BLAST analysis (http://hivdb6.stanford.edu).

Results

Patient characteristics

2,474 consecutive patients were enrolled in this study. All were being treated with antiretroviral drugs, and all were experiencing virologic failure: for the first time (17%); for the second time (33%); or for the third or subsequent time (50%). The mean viral load was 123,845 copies/mL.

Sample characteristics

Only 1.2% of strains presented no antiretroviral drug resistance mutations. The subtyping analysis showed that 78.3% (1,938) of the viruses belonged to subtype B, 4.3% (106) belonged to subtype C, and 8.6% (214) belonged to subtype F. In addition, 0.5% (12) were B/C recombinants, and 8.2% (204) were B/F recombinants. According to the analysis of individual fragments, the prevalence of protease and reverse transcriptase subtype B was, respectively, 79.6% and 85.8%, compared with 4.3% and 4.8% for subtype C, and 16.1% and 9.5% for subtype F.

Genotypic resistance patterns

Mutations were identified in the following proportions: 54.3% (major PI resistance mutations); 89.7% (NRTI resistance mutations); and 62.5% (NNRTI resistance

mutations). The prevalence of antiretroviral resistance by drug class and by HIV-1 subtype is presented in Table 1. Overall, 21% of individuals presented resistance to one antiretroviral class, 44.8% presented resistance to two classes, and 34% presented resistance to all three classes. As can be seen, there was a differential acquisition of resistance mutations by subtype, which was not explained by antiretroviral exposure (data not shown). Considering only major PI resistance mutations, the prevalence of PI resistance was lower in subtype C than in the other subtypes. Additionally, subtype C viruses accumulated fewer NRTI resistance mutations than did subtype B or subtype F viruses. Subtype F viruses presented significantly fewer NNRTI resistance mutations than did subtype B viruses.

Analysis of PI resistance mutations

Of the major PI resistance mutations, *L90M*, *M46I/L*, *N88D/S*, and *D30N* were the most frequently observed, whereas *G48V* and *I50V/L* were the rarest (Figure 1A). Individuals failing nelfinavir as the first PI were evaluated. Among those infected with subtype B (n = 168) the following resistance pathways were identified: *D30N* in 52.5%; *L90M* in 38.3%; and the *D30N-L90M* combination in 9.6%. Among those infected with subtype F (n = 48), 30.4% presented the *D30N* pathway, and 47.8% presented the *L90M* pathway. Using end-point PCR, we generated 10 clones from 5 individuals that presented the *D30N-L90M* combination and observed that the *D30N* or *L90M* mutations never co-existed in the same viral clone (data not shown). At the time of plasma sample collection from this group of

patients, there had been no exposure to tipranavir or darunavir. Of the samples evaluated, 2.21% harbored three or more darunavir resistance mutations: *V11I* (1.18%); *V32I* (3.51%); *L33F* (3.55%); *I47V* (2.75%); *I50V* (1.07%); *I54M* (0.61%); *G73S* (5.57.8%); *L76V* (2.10%); *I84V* (7.44%); and *L89V* (1.49%). In addition, 22.15% of samples harbored 3 or more tipranavir resistance mutations: *L10V* (12.21%); *I13V* (30.46%); *K20M/R* (10.50%); *L33F* (6.49%); *E35G* (1.34%); *M36I* (49.43%); *K43T* (4.31%); *M46L* (5.73%); *I47V* (2.75%); *I54A/M/V* (2.60%); *Q58E* (5.31%); *H69K* (7.86%); *T74P* (2.21%); *V82L/T* (3.09%); *N83D* (0.84%); and *I84V* (7.52%). Of the individuals evaluated, 0.38% harbored viruses with three or more mutations associated with resistance to tipranavir and darunavir, 18.9% harbored viruses with three or more mutations associated with resistance to tipranavir, but not to darunavir, and 1.8% harbored viruses with three or more mutations associated with resistance to darunavir, but not to tipranavir.

Although exposure to different PIs or to the number of previous PIs did not vary among the different subtypes, there were differences in the prevalence of primary and secondary PI resistance mutations. In subtype B viruses, resistance mutations at codons 33, 34, 58, 63, 73, 71, 77, and 84 were more common than in subtype F or C viruses, whereas those at codons 20, 36, and 89 were less common. In subtype F and C viruses, resistance mutations at codons 20, 36, and 89 were more common. In subtype F viruses, resistance mutations were more common at codons 10, 35, 48, 74, 57, and 82, whereas they were less common at codons 47 and 93. In subtype C viruses, the frequency of resistance mutations was higher at codon 93 and lower at codons 10, 30, 43, 46, and 74 ($P < 0.05$ for all).

Analysis of NRTI resistance mutations

The NRTI resistance mutation *M184V/I* was present in 66.3% of patients (Figure 1B), whereas 3.5% harbored viruses with multiple NRTI resistance mutations related to the *Q151M* complex and to the codon 69 insertion. The *K65R* mutation was found in only 1% of patients. The NRTI resistance selected by the TAMs *M41L*, *D67N*, *K70R*, *L210W*, *T215Y/F*, and *K219Q/E* was 70%. Of the individuals evaluated, 13.31% presented one TAM, 22.35% presented two TAMs, 17.09% presented three TAMs, 12.13% presented four TAMs, 2.48% presented five TAMs and 1.37% presented six TAMs. Interestingly the TAM pathway 1, which includes mutations at codons 41, 210, and 215, was most prevalent among subtype B viruses, whereas pathway 2, which includes mutations at codon 67, 70, and 219, was most prevalent among subtype C viruses (Figure 2). In subtype F viruses, pathway 1 and pathway 2 were equally distributed.

In subtype B viruses, resistance mutations other than TAMs were most often observed at codons 75 and 151; in subtype C viruses, such mutations were least common at codons 44 and 118 ($P < 0.05$ for all).

Analysis of NNRTI resistance mutations

The prevalence of individual NNRTI resistance mutations is presented in Figure 1C. An interesting new pathway of NNRTI resistance was detected in this study. Individuals failing efavirenz presented *K103N* as a key selected mutation, whereas those failing nevirapine presented the *Y181C* mutation (Figure 3A). In addition, mutations *L100I* and

P225H were strongly associated with the *K103N* mutation, whereas mutations *K101E* and *G190A* were strongly associated with the *Y181C* mutation ($P < 0.000$ for all). Therefore, we were able to describe two pathways of NNRTI resistance: one in which failing nevirapine is most often related to mutations *Y181C*, *K101E*, and *G190A*; and one in which failing efavirenz is most often related to mutations *K103N*, *L100I*, and *P225H* (Figure 3B). Potential cross-resistance to etravirine was evaluated, and 1.06% of the individuals presented three or more resistance mutations to this new antiretroviral drug: *A98G* (9.00%); *L100I* (5.40%); *K101P/E* (7.20%); *V106I* (5.30%); *V179E/F/I* (9.84%); *Y181C/I/V* (9.36%); *G190A/S* (15.4%); and *M230L* (0.90%).

Resistance mutations at codon 106 were most prevalent in subtype C viruses, resistance mutations at codon 190 were less prevalent in subtype F viruses, and resistance mutations at codon 225 were less prevalent in subtype B viruses ($P < 0.05$ for all).

Viral load and number of resistance mutations

Figure 4 shows the mean viral load according to the number of resistance mutations accumulated during antiretroviral treatment. The results for all groups were compared with those obtained for the treatment-naïve group, which consisted of 194 patients with recent HIV-1 infection, as determined using the serologic testing algorithm for recent HIV seroconversion [14]. Viral loads analyzed for each individual patient in this group of naïve patients were chosen after the viral load set point (6 months after inclusion in the cohort). Initially, viral load decreased in parallel with the increase in the number of resistance

mutations, after which it increased as the number of resistance mutations peaked and stabilized (Figure 4).

The variance test results did not show statistical significance for the mean viral load among groups with different numbers of resistance mutations. Therefore, the proportion test based on binomial distribution was used in order to analyze the groups stratified according to the number of mutations in terms of the distribution of patients with viral loads greater than 100,000 copies/mL. When all resistance mutations were analyzed, the proportion of individuals with viral loads greater than 100,000 copies/mL was significantly higher in the groups with eight or more resistance mutations than in the treatment-naïve group. There was also a higher proportion of individuals with viral loads greater than 100,000 copies/mL in the groups with at least one NNRTI resistance mutation than in the treatment-naïve group.

Discussion

This study enabled us to profile genotypic resistance to antiretrovirals in a large group of patients failing antiretroviral therapy and for whom information related to previous treatment was available. It can be seen that the majority of individuals failing antiretroviral treatment in this region of Brazil, where genotyping was requested by the attending physician, had extensive antiretroviral exposure, and 50% were failing their third or subsequent antiretroviral regimen. This extensive antiretroviral exposure and failure, consequences of, among other factors, long term treatment with suboptimal antiretroviral schemes, leads to extensive antiretroviral resistance. Of these patients, 34% harbored resistance to all three classes of antiretrovirals, a scenario that has been associated with a high risk of mortality [15].

In the present study, resistance to NRTIs revealed the extensive exposure to thymidine analogs and lamivudine. Rare resistance mutations related to didanosine (codon 74), and stavudine (codon 75) were also prevalent in our patient sample, with a prevalence of approximately 10%. We observed a differential prevalence of resistance mutations among HIV-1 subtypes without any difference in the previous antiretroviral exposure. Subtype B viruses presented more NRTI resistance mutations than did subtype C or F viruses. Another recent report also showed that, among infected individuals failing antiretroviral therapy, those infected with subtype C viruses present less accumulation of resistance mutations to NRTIs than do those infected with subtype B viruses [16]. The reasonable explanation for this differential acquisition of antiretroviral drug resistance mutations is

related to the current ignorance regarding the genotypic correlates of viral resistance among HIV-1 subtypes other than subtype B. Alternatively, and perhaps less likely, resistance to NRTIs in these non-B subtypes could be related to resistance mutations to other as yet uninspected HIV genomic regions, such as RNaseH [17, 18]. A similar explanation could be given for the fact that NRTI resistance mutations were more frequently acquired in subtype B viruses than in subtype F viruses. We found less acquisition of protease mutations in subtype C viruses than in subtype B or F viruses. Indeed, previous studies have also produced the same results comparing subtype C and B only [16]. However, it must be borne in mind that some natural polymorphisms, such as the *L89M* substitution present in subtype F viruses, might be sufficient to decrease the susceptibility to a number of PIs [19], perhaps underscoring the importance of using boosted PIs for the treatment of subtype F-infected individuals [20].

Identifying pathways of antiretroviral resistance is considered important for predicting cross-resistance to antiretrovirals. The term antiretroviral resistance pathway refers to the group of resistance mutations selected *in vivo* or *in vitro* by a given antiretroviral agent. One likely explanation for these resistance pathways is the genetic structure of the virus. This insight comes from the observation that subtype B viruses exposed *in vivo* or *in vitro* to nelfinavir will typically select the *D30N* mutation, whereas non-B viruses will select the *L90M* mutation [21]. Supposedly, the *D30N* mutation rarely co-exists with the *L90M* mutation. In confirmation of this, we found that, in the few cases in which these two resistance mutations occurred concomitantly, they arose in different genomes. We also found that the *L90M* mutation was present in subtype B-infected individuals at a higher

proportion than that previously reported, in 47.9% of our patients, including the viruses containing the *D30N-L90M* combination. This indicates that cross-resistance to nelfinavir was higher than expected in this population, since cross-resistance to other PIs is lower among *D30N*-containing viruses. In addition and in contrast of what has been generally speculated, we found *D30N* mutations in 30.4% of the subtype F-infected individuals in our sample.

Another important pathway for resistance described more recently refers to the TAM profile of resistance mutations. The initial selection of TAMs has been shown to occur either within pathway 1, which includes resistance mutations at codons 41, 210, and 215, or within pathway 2, which includes resistance mutations at codons 67, 70, and 219. The practical implication of this is cross-resistance to tenofovir, where resistance mutations at codon 41 or codon 210 in a total of three TAMs will result in a marked decrease in the activity of this adenosine analog [22]. Although it has been demonstrated that pathway 1 and pathway 2 present an approximately equal risk of initiating resistance [23, 24], we found that both of these pathways correlated strongly with the viral subtype. Clearly, the prevalence of acquired resistance mutations related to pathway 1 would be higher among subtype B-infected individuals, whereas that of those related to pathway 2 would be higher among subtype C-infected individuals. In this sense, cross-resistance to Tenofovir would be more prevalent in subtype B-infected individuals than in subtype C-infected individuals. Therefore, although the results of *in vitro* studies have suggested that the genetic barrier to tenofovir resistance is lower in subtype C viruses due to rapid selection of the *K65R* mutation [12], we can speculate that salvage therapy with tenofovir is more

effective among subtype C-infected individuals, especially in view of the possibility that *K65R* will not emerge in the presence of TAMs, since TAMs and *K65R* rarely co-exist [25, 26]. It is of note that, in the present study, subtype F viruses were intermediate between subtype B and C viruses in terms of the pathway 1- and pathway 2-selected TAMs, suggesting that, in subtype F viruses, the chance of pathway 1 TAMs being selected is approximately the same as that of pathway 2 TAMs being selected.

In the present study, we also detected two distinct pathways to NNRTI resistance, one related to the selective pressure of efavirenz and other to that of nevirapine. The most common mutation related to efavirenz resistance is the *K103N* mutation, whereas the most common mutation related to nevirapine resistance is the *Y181C* mutation. It is noteworthy that mutations *K101E* and *G190A* were associated with the *Y181C* mutation and with nevirapine exposure, whereas mutations *L100I* and *P225H* were associated with the *K103N* mutation and with efavirenz exposure. All three mutations associated with nevirapine exposure are related to etravirine cross-resistance, whereas, among those associated with efavirenz exposure, only the *L100I* mutation is related to etravirine cross-resistance. Therefore, nevirapine failure might impair future salvage therapy with etravirine, which was designed to be active against NNRTI-resistant HIV strains. Nevertheless, cross-resistance to etravirine appears to be low (1.06% of samples presented three or more related mutations), and etravirine might be a good option for salvage therapy in this population (those failing other NNRTIs). Similarly, darunavir and tipranavir might also be good options for salvage therapy, since cross-resistance to both seems to be equally low in this population. Since strains resistant to darunavir will

sometimes remain sensitive to tipranavir and vice versa, the availability of both drugs will be important in such cases.

A well-defined paradigm related to the accumulation of antiretroviral drug resistance mutations is the concomitant decrease in the replicative capacity of a virus, which will ultimately decrease viral fitness. Some experts even suggest that the presence of a resistant virus will lead to a more modest decrease in CD4 T-cell counts [27], as well as to less pronounced cell activation [28], thereby potentially delaying disease progression. It has been established that the viral load can be a surrogate marker of viral fitness [29]. On the basis of this assumption, we attempted to draw a correlation between the number of resistance mutations accumulated and the viral load in order to infer the impact of that the accumulation of resistance mutations has on viral fitness. We found that, as the number of resistance mutations increases, there is in fact a drop in the mean viral load. However, after the number of resistance mutations reaches a certain threshold, the viral load begins to increase in direct proportion to the number of resistance mutations. We therefore speculate that the replicative capacity of the virus is restored after a virus has accumulated a large number of resistance mutations, and that this explains the more rapid disease progression seen in individuals presenting more extensive resistance [15]. Another possible explanation for viral load increasing in parallel with the number of resistance mutations is that, since all of our patients were receiving antiretroviral therapy, there was less residual activity. Nevertheless, it is undeniable that the strains containing a high number of resistance mutations and producing high viral loads also present high fitness. Since the probability of accumulating antiretroviral drug resistance mutations over time is

directly proportional to the magnitude of the residual viral replication in cases of treatment failure [30], it would be reasonable to pursue a viral load that would be as low as possible, even in patients harboring resistant viruses, in order to minimize the chances of accumulating additional resistance mutations.

In conclusion, it is becoming clear that special attention should be given to the resistance profile selected in non-B subtypes, and further studies along this line are warranted.

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Figure Legends

Fig. 1. Prevalence of codons with antiretroviral resistance mutations according to HIV-1 subtype (B, F and C). Panel A shows the major PI resistance mutations, Panel B shows the NRTI resistance mutations, and Panel C shows the NNRTI resistance mutations.

Fig. 2. Prevalence of TAMs by subtype. Pathway 1 TAMs are represented by reverse transcriptase codons 41 and 210, and pathway 2 TAMs are represented by codons 67 and 219. Statistical analyses were performed using chi-square tests, and *P* values are shown for each subtype comparison.

Fig. 3. Analysis of 798 patients failing NNRTIs as the most recent treatment regimen. Panel A shows the prevalence of NRTI resistance mutations in patients treated with nevirapine (NVP) or efavirenz (EFV). Panel B shows the association between mutations related to *K103N*, which was the mutation most often selected by EFV, and *Y181C*, which was the mutation most often selected by NVP.

Fig. 4. Mean viral load according to the number of antiretroviral drug resistance mutations: Number of NRTI, NNRTI, and PI resistance mutations (panel A); Number of NRTI and PI resistance mutations (panel B); and Number of NNRTI resistance mutations (panel C).

Table 1. Prevalence of resistance to different classes of antiretrovirals by HIV-1 subtype.

Antiretroviral class	Subtype	Resistance mutations, n	%		Subtypes compared	*P*
PI*	B	1076	54.6	*P* = 0.00	B vs. F	NS
	F	226	57.0		B vs. C	0.00
	C	41	39.0		F vs. C	0.00
NRTI	B	1914	90.1	*P* = 0.00	B vs. F	NS
	F	212	90.5		B vs. C	0.00
	C	94	79.6		F vs. C	0.01
NNRTI	B	1347	63.5	*P* = 0.01	B vs. F	0.00
	F	126	53.8		B vs. C	NS
	C	73	61.8		F vs. C	NS

*PI mutations included in the analysis were *L23I*, *L24I*, *D30N*, *V32I*, *V33F*, *M46I/L*, *I47V/A*, *G48V/M*, *I50L/V*, *F53F/V*, *I54V/T/A/L/M*, *G73S/T*, *L76L/V*, *V82A/F/T/S*, *I84V/A/C*, *N88T/S* and *L90M*. PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor.

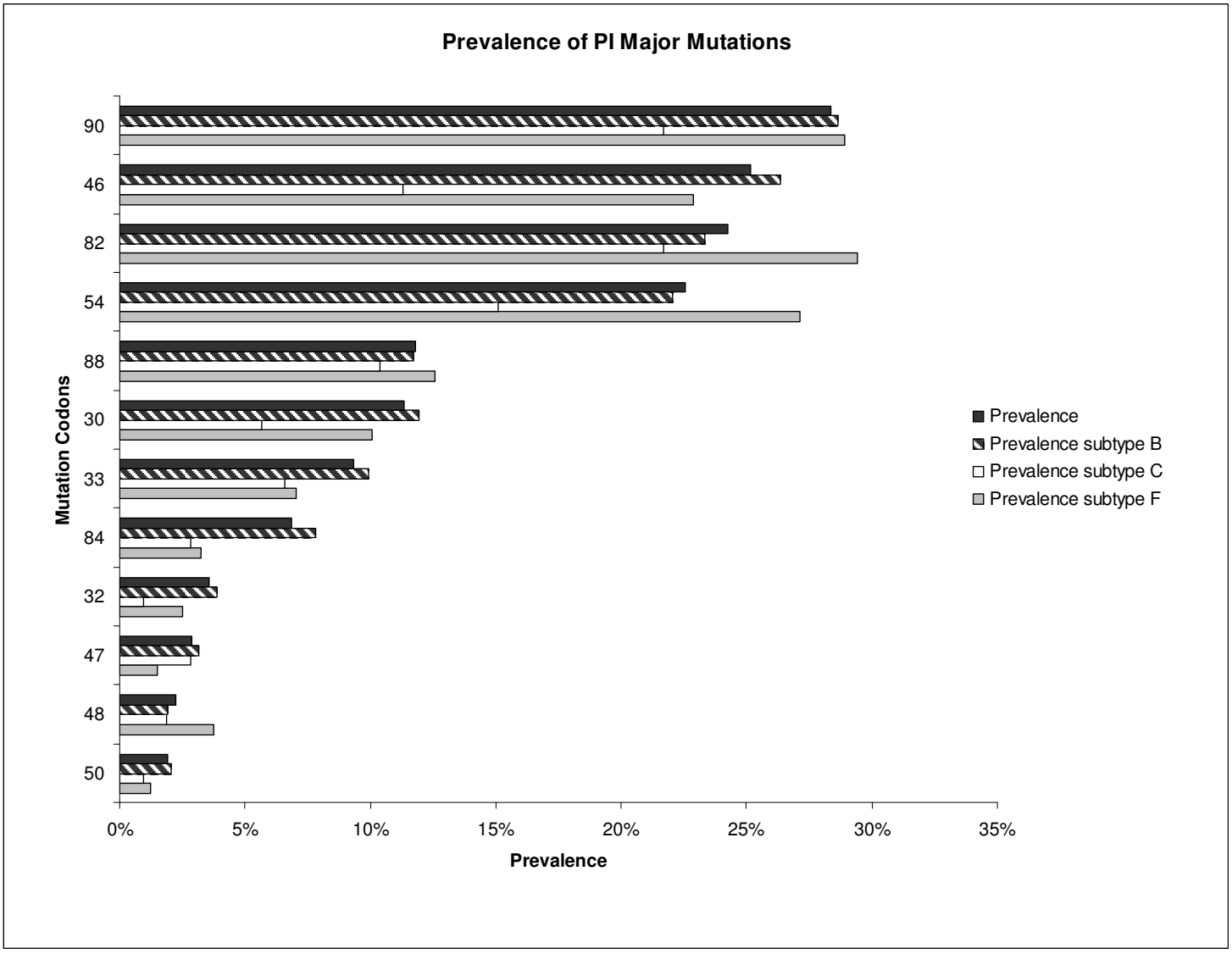


Figure 1, panel A.

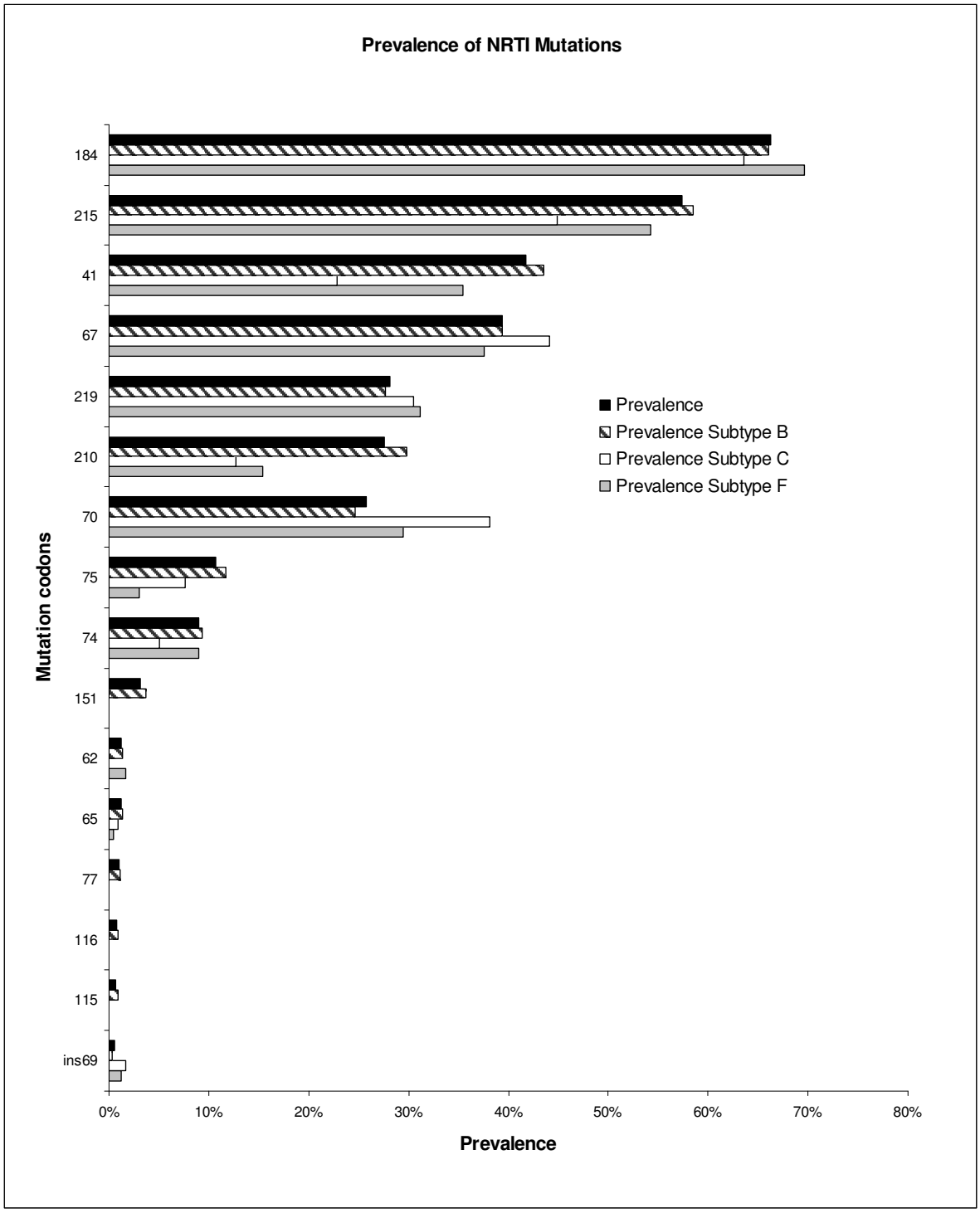


Figure 1, panel B.

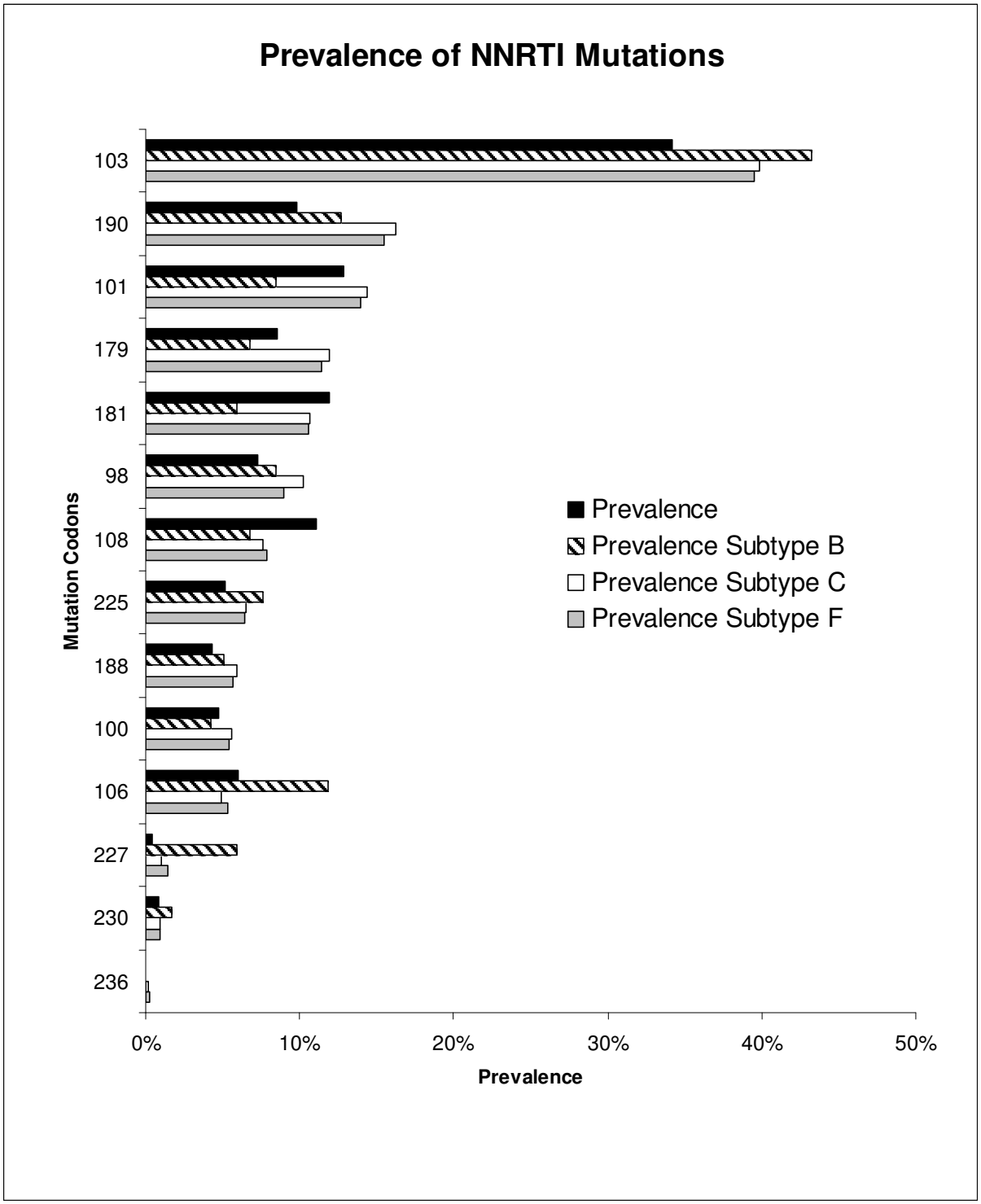
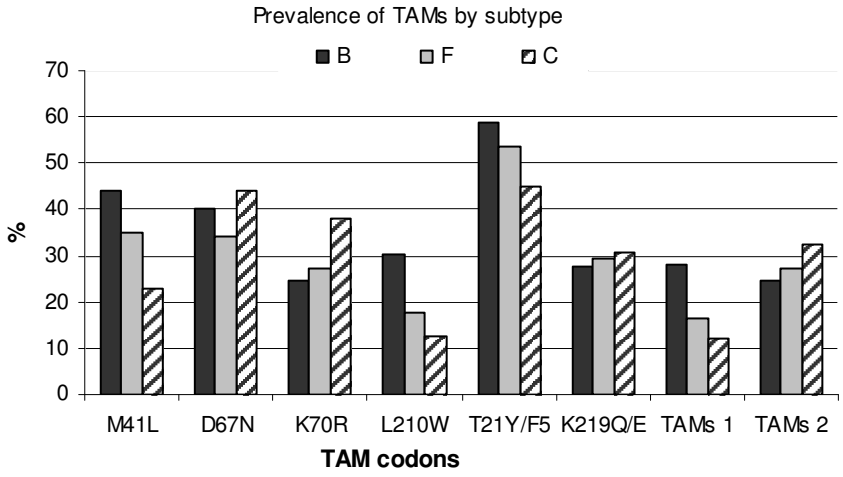


Figure 1, panel C.

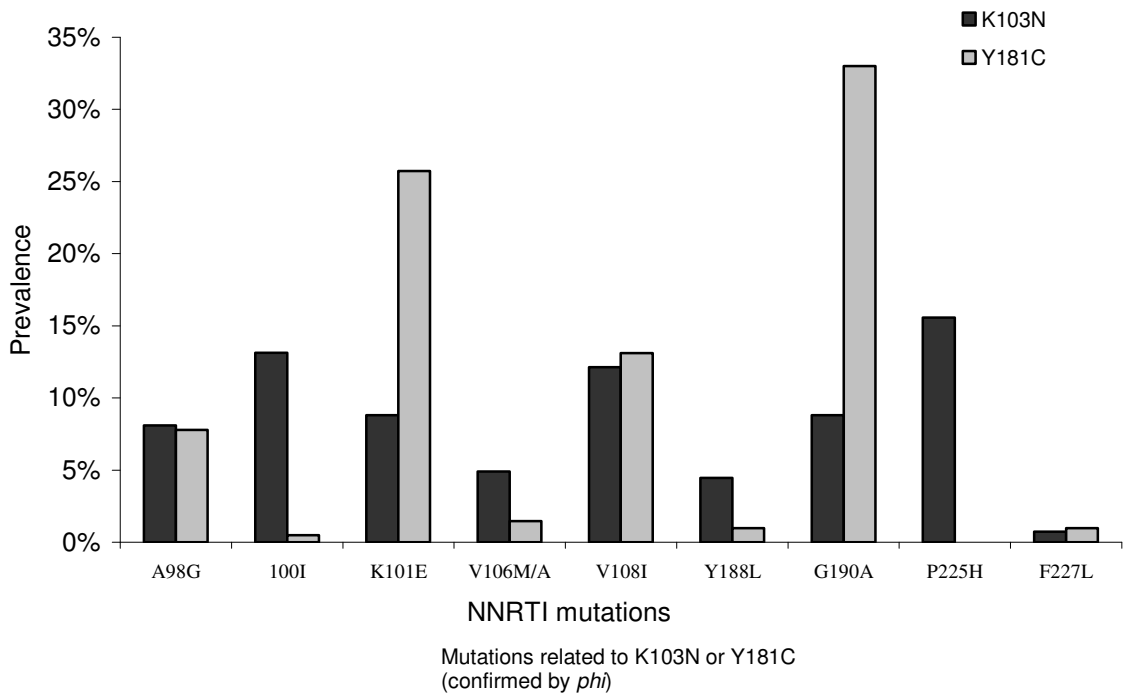
Fig. 2.



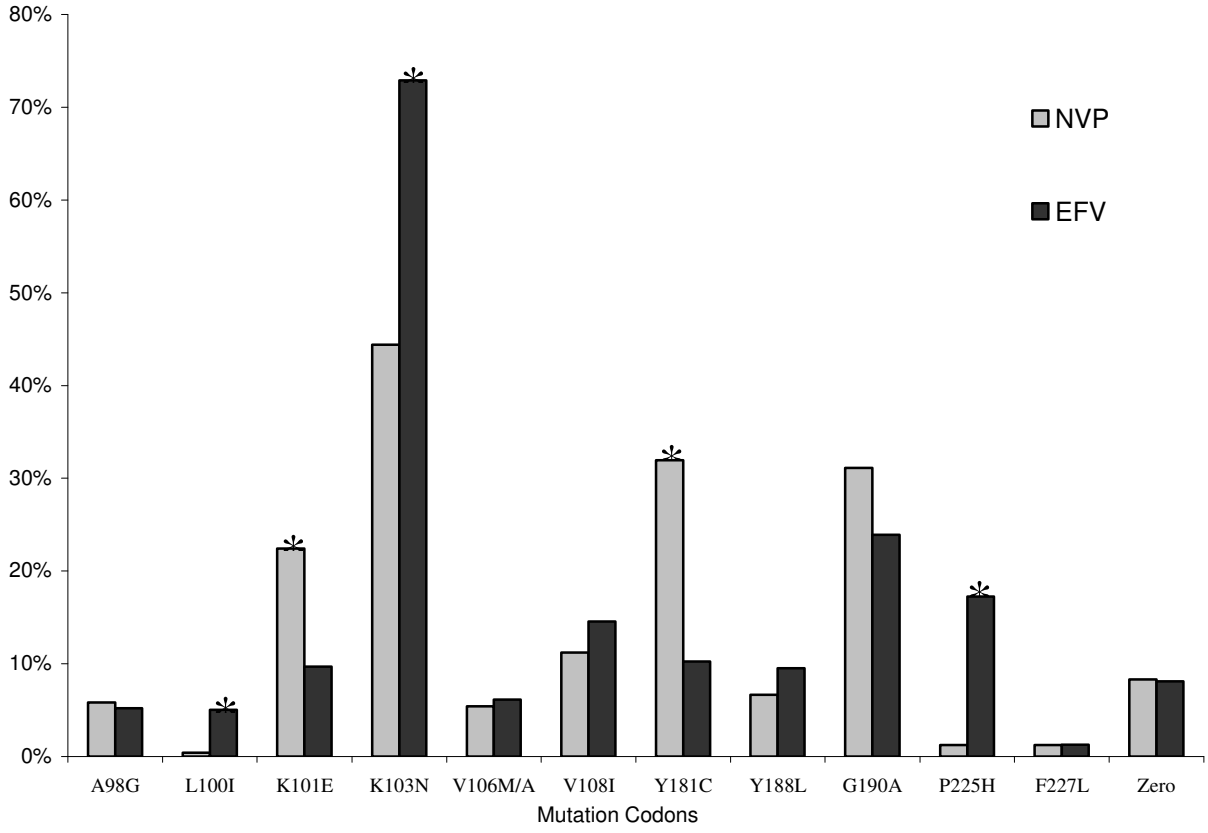
Mutation	B vs. F	B vs. C	F vs. C
M41L	0.001	0.000	0.013
D67N	0.036	0.387	0.055
K70R	0.278	0.001	0.026
L210W	0.000	0.000	0.215
T215Y/F	0.063	0.003	0.095
K219Q/E	0.572	0.529	0.795
M41L/L210W	0.000	0.000	0.321
D67N/K219Q/E	0.573	0.015	0.104

Figure 3. Panels A and B.

(A)



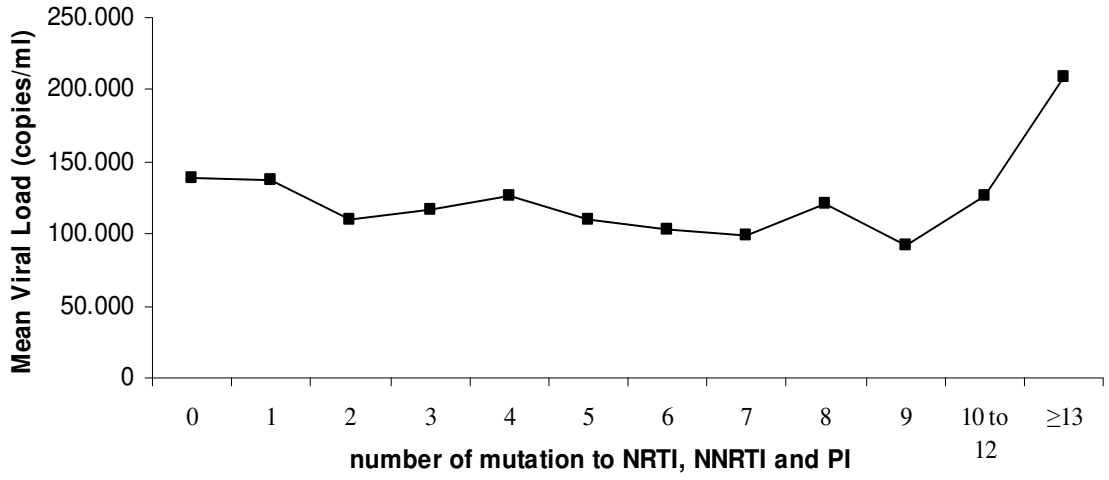
(B)



Codons selected by nevirapine or efavirenz

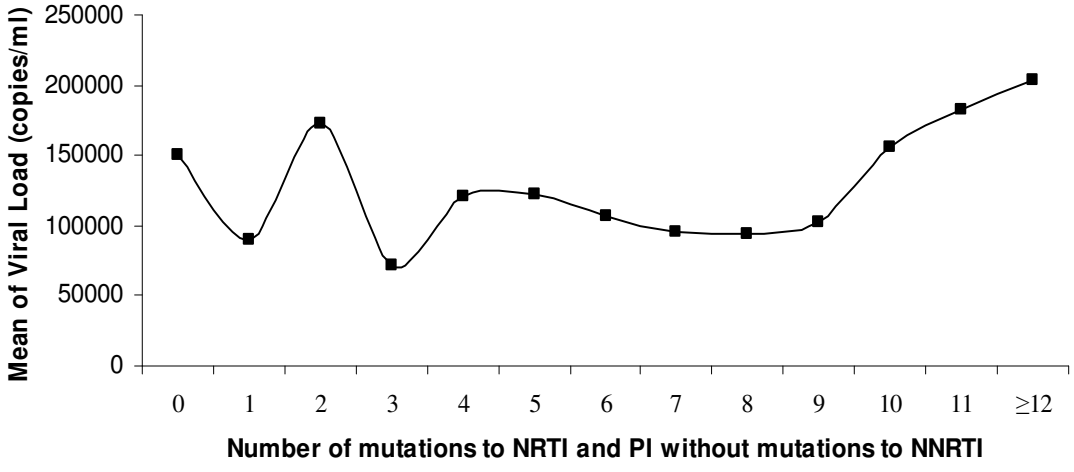
* = p<0.01

Figure 4, Panes A, B, and C.



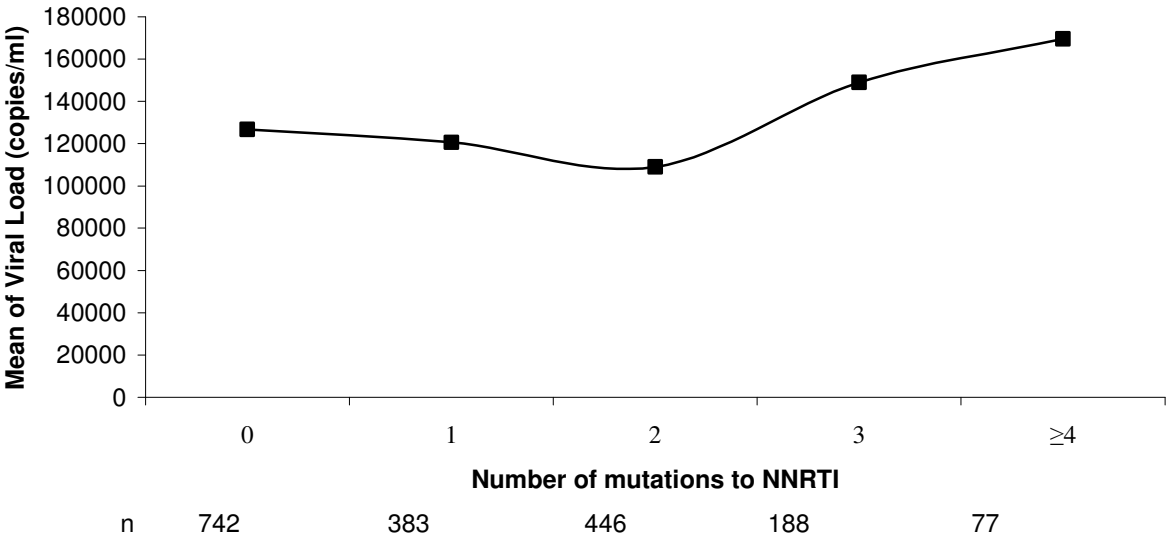
n	109	92	118	146	175	166	173	190	143	124	247	153

(A)



n	155	195	153	158	166	211	167	144	126	100	76	68	117

(B)


Mean of Viral Load (copies/ml)
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
0
1
2
3
≥4
Number of mutations to NNRTI
n 742 383 446 188 77

(C)

Decreased Phenotypic Susceptibility to Reverse Transcriptase Inhibitors in the Absence of Known Resistance Mutations in Clades C, F, and B/F Recombinant Antiretroviral Naïve HIV-1 Strains

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Running title: phenotypic susceptibility in non-B HIV-1

Abstract

The impact of clade-specific polymorphisms on HIV antiretroviral susceptibility is not completely understood. To evaluate the antiretroviral phenotypic susceptibility of wild-type HIV-1 strains circulating in Brazil, samples from 32 antiretroviral-naïve individuals infected with non-B subtypes C (n = 16), F (n = 9), or B/F (n = 7) where RT is B and protease is F, were phenotyped. Reduced susceptibility to protease inhibitors (PIs) was observed in three F and one C isolates. None of these strains presented any known resistance mutation correlated to PI. BR43 was the only strain containing the Y59H substitution, and presented Saquinavir resistance. The fold change in susceptibility to one or more PIs was above the cut-off value in 3.1% of clade F isolate and 1.0% of C isolates. Phenotypic resistance to at least one nucleoside reverse transcriptase inhibitor (NRTI) was found for one B/F isolate, three C, and three F isolates. The fold change in susceptibility to NRTIs was above the cut-off value in 6.2% of clade C isolates, compared with 4.8% for clade F and 2.0% for clade B. The fold change in non-NRTI (NNRTI) susceptibility was above the cut-off in 21.9% of C isolates, whereas none of F isolates presented such susceptibility. Only two of these sixteen C samples presented a known resistance mutation. Surprisingly, the NNRTI susceptibility fold change was above the cut-off value in 21.4% of Brazilian B/F recombinants, presenting clade B reverse transcriptase. In HIV-1 clades F and C, PI and NRTI susceptibility is apparently preserved, whereas NNRTI susceptibility should be better investigated in clade C and B/F recombinants.

Introduction

Drug resistance testing has become an important tool in the management of HIV-infected individuals undergoing antiretroviral therapy.[1] Genotyping and phenotyping methods appear to be equally useful for determining the susceptibility of HIV-1 to antiretroviral drugs.[2] Genotyping identifies mutations in the HIV genome associated with antiretroviral resistance, whereas phenotyping examines the relative susceptibility of viruses to different antiretroviral concentrations *in vitro*. Drug susceptibility tests have been designed for subtype B strains and are performed mainly on those strains. However, the increasing global prevalence of non-B subtypes creates the need to determine the performance of commercial drug resistance assays testing HIV-1 subtypes other than B, since it is conceivable that the genetic diversity of HIV-1 influences the susceptibility to antiretroviral drugs. Furthermore, it is not well known whether specific mutations or pathways are differently selected by antiretrovirals among HIV-1 clades. Some retrospective results suggest that individuals infected with clade F strains respond more poorly to antiretroviral therapy than do clade B-infected individuals.[3] It has also been suggested that the *L89M* polymorphism, which is highly prevalent in the clade F protease, plays a role in the antiretroviral response, since this polymorphism decreases the susceptibility of viruses to most protease inhibitors (PIs).[4] In contrast, it has been also speculated that the *I93L* polymorphism increases the susceptibility of clade C strains to lopinavir.[5] Although the *I93L* polymorphism is also common in clade F viruses, its effect in that subtype has not

been determined. Moreover, a study conducted with subtypes B and C-infected patients in southern Brazil has shown that the latter acquires less drug resistance mutations under treatment exposure, particularly to nucleoside analogues and protease inhibitors. In Brazil, HIV-1 clades B, C, and F co-circulate, B/F recombinants accounting for a significant proportion.[6] In the present study, we evaluated the mutation profile and *in vitro* antiretroviral response in strains isolated from antiretroviral-naïve Brazilian patients infected with subtypes C, F, or B/F. In order to assess the natural susceptibility of the C and F HIV subtypes to PIs, as well as to nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs), we evaluated the phenotypic susceptibility and genotypic correlates of resistance in antiretroviral-naïve strains.

Materials and Methods

Isolates from 32 antiretroviral-naïve Brazilian patients were evaluated: 16 were subtype C, 9 were subtype F1, and 7 were B/F recombinant strains, all of which carried a F1 protease and a B reverse transcriptase. Viruses from plasma samples were genotyped at the Federal Universities of Sao Paulo and Rio de Janeiro, Brazil, using the ViroSeq® System (Celera Diagnostics, Alameda, CA, USA). Sequences were analyzed using an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA). All genotyping was performed based on an analysis of a portion of the *pol* gene sequence profile, spanning reverse

transcriptase and protease regions. Samples identified as recombinants were analyzed using the SimPlot program, version 3.5.1 [7] and confirmed by bootscanning analysis. The basic principle of bootscanning is that mosaicism is suggested when one observes high levels of phylogenetic relatedness between a query sequence and more than one subtype reference sequence in different genomic regions.[8] Sample sets were subjected to antiretroviral phenotypic analysis using the Antivirogram™ Assay (Virco, Mechelen, Belgium). This phenotyping assay uses HIV-1 genomes generated by recombination between PCR amplified *pol* products derived from patient viruses and a subtype B proviral clone with deletion of protease and reverse transcriptase regions. Recombinant viral production and detection methods were conducted as previously described.[9,10] For clade C, clade F and B/F recombinant isolates obtained from treatment-naïve subjects, the mean, median, and interquartile range of fold change values for each drug, as determined using the Antivirogram™ assay, were compared to the same parameters for clade B isolates in the Virco database. The mutation patterns associated with reduced susceptibility to one or more drugs in clade C, clade F, and B/F isolates were evaluated and compared to genotypic correlates of drug resistance described for clade B isolates.

Results

Sample identification, protease/reverse transcriptase subtypes, polymorphisms/mutations at codons associated to resistance, and fold change in susceptibility to PIs, NRTIs and NNRTIs, as well as the mean fold change by subtype, are shown in Tables 1A and 1B.

As in any diagnostic test that produces a quantitative result, the Antivirogram™ HIV-1 fold change values are interpreted using cut-off values that separate viral strains that are considered to be highly susceptible to antiretrovirals from those that are considered to present low antiretroviral susceptibility. Biological cut-off values are derived from *in vitro* experiments and mark the upper limit of natural variation in phenotypic susceptibility among wild-type viruses, which includes 97.5% of strains after two standard deviations to the right of the normal distribution.[11] Since these biological cut-off values were typically calculated using clade B strains, we analyzed the percentage of viruses from clades C, F and Brazilian B/F recombinant that remained above the biological cut-off value. For PI susceptibility, 1.0% of the isolates from clade C and 3.1% from clade F presented fold change values above the upper limit of the biological cut-off values. For NRTI susceptibility, 6.2% of fold change values were above the biological cut-off for clade C isolates, compared with 4.8% for clade F and 2.0% for B/F isolates. The phenotyping results for NNRTIs susceptibility showed 21.9% of fold change values above the biological cut-off value in clade C isolates. None of clade F

isolates showed decreased susceptibility to NNRTIs. Most importantly, clade C samples did not present any known mutation to correlate with NRTI resistance, and only two samples presented mutation that correlates with NNRTI resistance, A98G and K103N (samples BR76 and BR87, respectively, Table 1B). Surprisingly, 21.4% of the Brazilian clade B/F isolates presented fold change that was above the biological cut-off value for NNRTI susceptibility.

All but four viral strains were fully susceptible to all six PIs tested (Table 1A). Three strains belonging to subtype F (BR38, BR43, and BR44) presented reduced susceptibility to PIs. The first strain, BR38, presented a fold change of 1.8 for saquinavir and carried the following protease polymorphisms/mutations: V3I, I15V, E35D, M36I, S37N, R41K, R57K, D60E, Q61E, L63P, K70T, and V77I. The BR43 isolate also presented decreased susceptibility to saquinavir, with a fold change of 2.1, and carried the protease polymorphisms/mutations V3I, L10V, I13V, I15V, G16E, K20M, E35D, M36I, S37N, R41K, R57K, Y59H, Q61N, I72T, and L89M. The BR44 isolate presented a fold change of 2.3 for lopinavir, containing the amino acid substitutions V3I, I15V, G17E, K20R, E35D, M36I, S37N, R57K, Q61N, L63T, E65D, and I72V. Only one clade C isolate (BR86) presented a low level of resistance, with a 0.1 fold change above the biological cut-off value. This isolate contained the following amino acid substitutions: V3I, I15V, M36I, S37K/N, R41N, L63I/P/S/T, H69K, V82I/V, L89M, and I93L. As can be seen in Table 1A, these four viruses presented fold changes discreetly above the biological cut-off values for each respective drug[11]. Since other strains

displayed the same mutation profile without reduced PI susceptibility, direct involvement of those amino acid changes with drug resistance could not be speculated. One exception was the mutation Y59H, which was only detected in the BR43 isolate, which showed a loss of susceptibility to saquinavir. All twenty-eight of the remaining isolates (13 of subtype F and 15 of subtype C) were fully susceptible to all PIs tested. The mean fold change in antiretroviral susceptibility per subtype sample set was nearly 1.0, which is comparable to that found for subtype B wild-type strains.

Amino acid substitutions that were considered accessory mutations and correlated with selective pressure from antiretroviral drugs among subtype B strains, including substitutions at codons 10, 20, 36, 63, 77, and 82, were detected in the protease region of non-B strains isolated from antiretroviral-naïve individuals. Changes were found at various rates: L10I (12.5% in subtype F and 6.2% in subtype C), L10V (18.7% in subtype F and 6.2% in subtype C), K20R (43.7% in subtype F and 12.5% in subtype C), M36I (87.5% in subtype F and 81.2 in subtype C), M36T (12.5% in subtype C), L63P (12.5% in subtype F and subtype C), L63A/G/H/I/L/Q/S/T/V (31.2% in subtype F and 43.7% in subtype C), V77I (18.7% in subtype F) and V82I (6.2% in subtype F and 31.2% in subtype C). The L89M polymorphism did not have any impact on PI susceptibility among clade F strains, which presented fold change less than or equal to 1.0. Nor did the I90L polymorphism have any impact on PI susceptibility among clade C strains, for which the mean fold change in susceptibility to lopinavir was 1.0. Although

previous studies have shown that the L89M polymorphism has an impact on saquinavir, nelfinavir, ritonavir, amprenavir and lopinavir susceptibility,[4,12] we found no data to support a consistent change in PI susceptibility related to this polymorphism. It has also previously been reported that susceptibility to lopinavir increases in the presence of the I93L substitution in clade C strains, and that the poor response to PI therapy is empowered by I93L.[5,13] In contrast, we found I93L in all subtype C strains analyzed, and the mean fold change value for clade C samples set was very close to 1.0 (0.8) and the susceptibility to other PI was not compromised. The remaining polymorphisms are of uncertainly significance. As previously described,[14] 100% of the clade F isolates carried V3I, and R57K; 81.2% carried S37N, R41K, and Q61N; 62.5% carried E35D; 56.2% carried I15V; and 50% carried L89M. For clade C, 100% of the isolates carried S37K, R41N, H69K, I89M, and I93L; 87.5% carried I15V; and 31.2% carried G16A/E. It is of note that, whereas subtype F isolates presented S37N, R41K, and R57K polymorphisms, subtype C isolates presented S37K, R41N, and H69K polymorphisms in all samples. When different substitutions at amino acid positions known to be related to PI resistance in clade B, such as K20I, M36V/L, and L63S/A/T/F, were taken into account, all but two subtype F strains (87.5%) carried two to four mutations. Nearly 62% of subtype C strains carried one to three of those mutations.

We also analyzed the susceptibility of clades C and F to reverse transcriptase inhibitors. Seven NRTIs and two NNRTIs were tested for Brazilian isolates: 7 from

subtype B/F reverse transcriptase, 16 from subtype C and 9 from subtype F reverse transcriptase (Table 1B). Decreased susceptibility to at least one of the reverse transcriptase inhibitors tested was observed in 10 viral strains: 2 clade B/F (BR36 and BR39), 3 clade F (BR43, BR44 and BR46) and 6 clade C (BR76, BR82, BR86, BR87, BR94, and BR99; Table 1B). Since clades C and F carried many reverse transcriptase polymorphisms, Table 1B shows only amino acid substitutions known to be related to drug resistance. In the analysis of clade F samples set, the BR43 presented decreased susceptibility to ddI with 0.1 fold change above the cut-off value, BR44 and BR46 presented decreased susceptibility to zidovudine with 0.2 and 0.5 fold changes above the cut-off values, respectively. These three clade F samples presented loss of susceptibility without any amino acid substitutions previously described to be related to antiretroviral resistance. Into clade C samples set, the BR76 sample presented A98G and a 20.7 fold change in susceptibility to nevirapine, compared with 18.7 and 61.6 fold changes in susceptibility to efavirenz and nevirapine, respectively, presented by BR87 sample, which was found to carry K103N. The BR86 strain presented decreased susceptibility to abacavir, zidovudine and efavirenz, with only one NRTI resistance-related codon previously described, although with a different amino acid substitution: E40D. In contrast, BR43 from clade F presented the same amino acid change, but no decreased susceptibility was observed. The other four subtype C strains that presented decreased susceptibility to NNRTIs did not show any known resistance mutations. The mean fold change values for

clade F samples were comparable to those found for clade B in all reverse transcriptase inhibitors tested. For the clade C sample set, the mean fold change values were also near 1.0, except those for susceptibility to efavirenz and nevirapine, which were 2.6 and 7.1, respectively.

Discussion

Despite the fact that genetic polymorphisms at positions associated with PI resistance were found at a higher rate in the two non-B subtypes isolated from antiretroviral-naïve subjects, no phenotypic decrease in PI susceptibility was identified in this set of samples. In contrast, some level of decrease in the susceptibility to NRTIs and NNRTIs was found among strains belonging to these non-B HIV-1 viruses. Strikingly, only two of the twelve samples revealed known resistance mutations related to reverse transcriptase inhibitors, suggesting that the genotypic correlates of decreased susceptibility among clade C, clade F and BF recombinants are yet obscure. One recent study demonstrated that the rates of resistance-related mutation acquisition among clade C strains might be inferior to those of clade B strains[15]. One reasonable explanation for the fact that the prevalence of mutations is lower among clade C-infected individuals than among clade B-infected individuals is again the fact that the correlates of genotypic resistance in HIV-1 clade C might be unknown.

Another hypothetical explanation for the lack of mutations known to confer resistance to reverse transcriptase inhibitors in the presence of a low level of resistance is that genetic mutations outside the reverse transcriptase catalytic region could be influencing susceptibility to certain reverse transcriptase inhibitors. In fact, there is mounting data that mutations in the RT connection and RNase H regions lead to either NRTI or NNRTI resistance. [16,17] Resistance

mutations outside the reverse transcriptase region might explain the lack of resistance mutations found, since B/F recombinant strains cluster with clade B in the reverse transcriptase region, although one sample showed resistance to NRTIs and two samples presented resistance to NNRTIs. Sequencing outside the protease and reverse transcriptase catalytic regions was not performed, and we are therefore unable to assign the outside regions to any particular HIV-1 subtype.

Although all clade C strains tested here presented the I93L polymorphism in the protease region, we were unable to confirm previous results suggesting that this natural polymorphism causes *in vitro* hypersusceptibility to lopinavir [5]. In fact, the I93L substitution has been related to lower *in vivo* susceptibility to indinavir and nelfinavir among clade B-infected individuals [18]. Likewise, we were unable to confirm that the L89M present in clade F strains would compromise the natural susceptibility to PIs, as previously described [4]. However, it can be argued that the high number of clade-related polymorphism found in non-B strains contributes to decreasing the genetic barrier to PI resistance, even in the absence of baseline resistance to PIs, as described in the present study. It has been reported that the polymorphisms found at positions 10 and 36 are the strongest predictors of virologic failure, appearing in nearly 40% of antiretroviral-naïve subjects in whom PI-based therapies failed.[19] It is of note that the M36I mutation was present in 87.5% of the F and 75.0% of C subtype proteases in our study, and that the phenotypic assays did not recognize any significant loss in PI susceptibility.

Although previously antiretroviral-naïve individuals treated with boosted PIs do not typically present PI mutations or virologic resistance, [20,21,22,23] one recent study revealed genetic progression in five patients treated with boosted lopinavir monotherapy, two infected with a clade B strain and three infected with CRF02_AG strains [24]. Although no phenotypic resistance was confirmed in these two clade B strains, it has been confirmed in two CRF02_AG strains with the emergence of mutation L76V, as well as other mutations. It can be speculated that, in those cases, the high number of polymorphisms present in non-B strains allows genetic evolution resulting in selective pressure of PIs.

Another point of great interest is the relationship between the genetic diversity of HIV and response to treatment. An unrecognized level of risk of antiretroviral failure might be related to a high number of natural polymorphisms present in non-B strains, and, although the results of some studies indicate that the level of response is lower among patients infected with non-B HIV, [3] other studies have shown the opposite [25].

We recognize that the small size of the sample analyzed here is a major limitation of this study, and that further studies are needed in order to confirm our results. Nevertheless, we believe that, until better correlates of genotypic resistance become available, phenotypic tests for non-B clades should be more widely accessible.

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TABLE 1A. Results of phenotypic susceptibility to protease inhibitors (PIs) using Antivirogram™ Assay and protease genotypic profile from HIV-1 Brazilian samples from clades F and C.

		FC in PI susceptibility vs. wild-type																			
		APV	AZT	IDV	LPV	NFV	SQV														
		*Virco biological cut-off value**																			
Sample ID	Protease Subtype	(1.8)	(2.0)	(2.1)	(1.6)	(2.3)	(1.7)	Protease polymorphisms/mutations													
BR29	F	1.1	0.7	0.5	1.6	1.4	0.8	V3I	R41K	R57K	Q61N	K70R	L89M								
BR35	F	1	1	0.7	0.6	0.8	0.4	V3I	L10I	I13V	L33V	E35D	M36I	S37N	R41K	R57K	D60E	Q61N	G68E	L89M	
BR36	F	0.7	1.1	1	1.6	0.7	1.5	V3I	G17D	K20R	E35D	M36I	S37N	R41K	K43R	R57K	Q61N	I72R			
BR37	F	0.8	1.1	1	0.6	0.9	0.5	V3I	T12I	I13V	M36I	S37N	R41K	R57K	Q61H/N	L89M					
BR38	F	1.4	0.9	0.7	1.2	1.7	**1.8**	V3I	I15V	E35D	M36I	S37N	R41K	R57K	D60E	Q61E	L63P	K70T	V77I		
BR39	F	0.3	0.2	1.4	0.7	0.6	0.3	V3I	T12K	I15V	L19I	E35D	M36I	S37N	R41K	R57K	Q61N	E65D	I72T		
BR40	F	0.6	0.5	0.9	0.9	1	1	V3I	L10V	I13V	G17E	K20R	M36I	S37E	R57K	Q61N	L63A/V	K70R			
BR31	F	0.9	1	0.8	0.6	0.8	0.5	V3I	I13I/V	I15I/L	M36I	S37N	R41K	R57K	Q61N	H69Y	L89M				
BR43	F	1.2	1.3	0.7	1.1	1.2	**2.1**	V3I	L10V	I13V	I15V	G16E	K20M	E35D	M36I	S37N	R41K	R57K	Y59H	Q61N	I72R	L89M
BR44	F	0.6	0.4	0.6	**2.3**	1.4	0.6	V3I	I15V	G17E	K20R	E35D	M36I	S37N	R57K	Q61N	L63T	E65D	I72V		
BR46	F	1.1	1.5	0.5	1.3	1.4	0.9	V3I	I15V	K20R	E35D	M36I	S37N	P39Q	R41K	R57K	D60E	Q61D	L63P/S	L89M	
BR48	F	0.3	0.1	0.2	0.7	1.1	0.2	V3I	L10V	I15V	S37E	R57K	Q61N	L63A	I64L	I72V	V77I	I93L			
BR50	F	1.3	0.7	0.7	1.1	0.5	0.5	V3I	L10I	T12S	I13V	K14R	G16E	K20R	E35D	M36I	S37N	R41K	R57K	Q61N	L63V	I72T
BR51	F	0.3	0.6	1.5	0.9	1	0.9	V3I	I15V	E35D	M36I	S37N	P39Q	R41K	R57K	Q61N	V77I	L89M			
BR52	F	0.3	0.4	0.3	0.5	0.3	0.4	V3I	I15V	K20R	M36I/L	S37K	R41N	H69K	V82I	L89M	I93L				
BR53	F	0.4	0.3	0.2	0.7	0.8	0.3	V3I	I15V	E35D	M36I	S37N	R41K	R57K	Q61N	L63S	E65D	I72T			
Mean FC		**0.8**	**0.7**	**0.7**	**1**	**0.9**	**0.8**														
BR76	C	0.2	0.5	0.3	0.7	0.4	0.5	V3I	I15V	G16A	M36I	S37K	R41N	H69K	L89M	I93L					
BR78	C	0.2	0.6	0.3	0.6	0.4	0.5	V3I	I15V	G16E	M36I	S37K	R41N	H69K	L89M	I93L					
BR79	C	0.6	0.8	0.5	0.9	1.4	0.5	V3I	I15V	M36I	S37K	R41N	L63T	H69K	L89M	I93L					
BR80	C	0.7	0.8	0.4	0.6	0.4	0.9	V3I	I15V	M36I	S37K	R41N	L63T	H69K	V82I	L89M	I93L				
BR81	C	0.2	0.1	0.2	0.7	0.7	0	V3I	I15V	M36I	S37K	R41N	L63G/V	H69K	L89M	I93L					
BR82	C	0.7	0.6	0.2	1.2	1	0.6	V3I	G16E	M36I	S37K	R41N	L63H/L/Q/V	I64L	H69K	L89M	I93L				
BR83	C	0.5	0.6	0.8	1	1.1	0.9	V3I	I15V	L19I	M36I	S37K	R41N	M46V	H69K	V82I	L89M	I93L			
BR84	C	0.4	0.9	0.8	0.7	0.5	0.7	V3I	T12P	I15V	M36T	S37K	R41N	L63T	H69K	L89M	I93L				
BR86	C	1.3	1.1	0.8	1.2	1.6	**1.8**	V3I	I15V	M36I	S37K/N	R41N	L63I/P/S/T	H69K	V82I/V	L89M	I93L				
BR87	C	0.9	1	1.8	0.6	0.5	1.4	V3I	I15V	L19I	S37K	R41I	D60E	H69K	L89M	I93L					
BR88	C	0.3	0.3	0.2	0.8	1.3	0.8	V3I	T12N	I15V	M36I	S37N	R41K	H69K	L89M	I93L					
BR89	C	0.5	0.6	0.6	0.7	1.1	0.7	V3I	L10I/V	I13V	K14R	M'36I/T	S37K	R41N	L63P	H69K	L89M	I93L			
BR91	C	0.8	1.1	0.3	1	1.3	0.5	V3I	I15V	G16E	K20R	E35D	M36I	S37K	P39S	R41N/S	H69K	L89M	I93L		
BR92	C	0.2	0.4	0.2	0.3	0.2	0.3	V3I	T12K	K14R	I15V	M36I	S37K	R41N	H69K	V82I	L89M	I93L			
BR94	C	0.6	0.4	0.3	0.6	0.6	0.5	V3I	I15V	S37K	R41N	L63V	H69K	V82I	L89M	I93L					
BR99	C	0.8	1.2	1.7	1.4	1.2	0.7	V3I	K14R	I15V	G16E	K20R	M36I	S37K	P39S	R41N	H69K	L89M	I93L		
Mean FC		**0.6**	**0.7**	**0.6**	**0.8**	**0.9**	**0.7**														

FC, fold change; PI, protease inhibitor; APV, amprenavir; AZT, zidovudine; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; SQV, saquinavir.

*Values refer to the second generation Virco biological cut-off values.[11]

TABLE 1B. Results of phenotypic susceptibility to reverse transcriptase inhibitors (NRTIs and NNRTIs) using Antivirogram™ Assay and reverse transcriptase related resistance codons from HIV-1 Brazilian samples.

		FC susceptibility vs. wild-type									
		NRTIs							NNRTIs		RT resistance mutation codons
		*Virco biological cut-off value**							*Virco biological cut-off value**		
Sample ID	RT Subtype	ABC (2.2)	ddI (2.2)	FTC (3.5)	3TC (2.4)	d4T (2.3)	TDF (2.1)	AZT (2.7)	EFV (3.4)	NVP (5.5)	
BR29	B/F	0.7	0.5	0.4	0.8	0.4	1	2.7	1.6	0.7	
BR35	B/F	0.5	0.7	2	0.7	0.6	0.4	1.2	0.9	2.8	
BR36	B/F	0.5	1.1	1.2	0.7	1.6	1	0.7	**4.1**	**7.2**	
BR37	B/F	1.1	1	0.7	1.1	1.1	1	1.2	2.6	1.2	
BR38	B/F	0.7	1.9	0.9	1.4	1.8	1.7	0.7	1	1.1	
BR39	B/F	0.8	2.1	3.6	0.6	0.4	0.7	0.7	**3.8**	1.6	
BR40	B/F	0.5	0.6	1.1	1.1	0.8	0.4	1.9	0.2	0.9	
Mean FC		**0.7**	**1.1**	**1.4**	**0.9**	**1.0**	**0.9**	**1.3**	**2.0**	**2.2**	
BR31	F	0.8	0.9	0.7	0.5	1.3	0.8	1.2	1.5	1.7	
BR43	F	0.2	**2.3**	0.4	1.1	1.5	1	0.6	0.7	1.1	**E40D**
BR44	F	1.7	0.7	1.3	0.6	1.5	0.6	**2.9**	1.2	0.8	
BR46	F	0.8	1.1	1	0.7	2.2	0.5	**3.2**	0.7	1.8	
BR48	F	0.2	0.2	0.1	0.4	0.2	0.6	0.2	0.5	0.5	**I50V**
BR50	F	1	0.7	1.4	0.9	1.3	1.4	1.2	1.1	0.9	
BR51	F	1.6	0.3	0.5	0.6	0.3	0.3	0.3	0.2	0.7	**G333D**
BR52	F	0.3	0.5	0.9	1.1	0.7	0.4	1.8	0.9	0.2	
BR53	F	0.5	0.2	0.3	0.6	0.2	0.5	0.4	0.2	0.2	
Mean FC		**0.7**	**0.9**	**1.1**	**0.8**	**1.0**	**0.8**	**1.3**	**1.4**	**1.5**	
BR76	C	0.4	0.5	0.7	1	0.3	1.4	0.8	1.9	**20.7**	**A98G**
BR78	C	0.4	0.9	1	0.9	0.4	0.9	0.6	0.2	1.2	
BR79	C	0.4	0.8	1.7	1.2	0.5	1.4	0.5	2.2	0.8	
BR80	C	0.6	0.4	3.3	0.6	0.4	0.8	1.9	2.1	4.7	**A98S**
BR81	C	0.2	0.2	0.1	0.7	0.2	0.6	0.2	0.5	0.5	
BR82	C	0.6	1.8	1.5	1.6	**2.6**	1	1.1	2	**6.3**	
BR83	C	0.5	1.2	1.3	0.8	0.6	1.4	0.9	2.2	0.9	
BR84	C	0.4	1.3	0.5	1.6	0.3	0.8	0.6	2.3	0.4	
BR86	C	**2.7**		1.4	2	2	1.5	**3.4**	**3.9**	0.8	**E40D**
BR87	C	1.9	1.8	2.2	1.1	2.1	1.4	2.6	**18.7**	**61.6**	**K103N**
BR88	C	0.2	0.3	0.3	0.7	0.3	1.6	0.5	0.5	0.7	
BR89	C	0.8	0.5	1.4	0.8	0.4	0.6	0.9	0.6	0.7	
BR91	C	1.3	1	0.9	1.2	1	0.5	1.2	0.9	0.5	
BR92	C	0.3	0.3	0.6	0.8	0.3	0.2	0.7	0.5	0.3	
BR94	C	0.5	0.7	0.1	0.6	0.6	0.8	0.4	0.5	**6.1**	
BR99	C	1.2	**2.4**	1.1	**2.6**	**4**	**2.4**	2.3	3	**7.2**	
Mean FC		**0.8**	**0.9**	**1.1**	**1.1**	**1.0**	**1.1**	**1.2**	**2.6**	**7.1**	

FC, fold change; NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors; RT, reverse transcriptase; ABC, abacavir; ddI, didanosine; FTC, emtricitabine; 3TC, lamivudine; d4T, stavudine; TDF, tenofovir; AZT, zidovudine; EFV, efavirenz; NVP, nevirapine.

*Values refer to the second generation Virco biological cut-off values.[11]

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3. Conclusões

1. Altos níveis de resistência anti-retroviral, incluindo resistência a múltiplas classes, foram encontradas na população analisada neste estudo. Mutações de resistência relacionadas aos inibidores da protease e inibidores da transcriptase reversa análogos aos nucleosídeos foram menos frequentes no subtipo C, enquanto as mutações relacionadas aos inibidores não-nucleosídeos da transcriptase reversa foram menos frequentes no F.

2. Os virus do subtipo B seguem, preferencialmente, a via 1 no acúmulo de mutações aos análogos de timidina (TAM1), enquanto os virus C seguem mais frequentemente a via 2 (TAM2). Os virus do subtipo F ocupam uma posição intermediária quando comparados aos subtipos B e C.

3. A seleção de mutações de resistência varia de acordo com os subtipos do HIV-1 analisados para as três classes de anti-retrovirais (IP, ITRN, ITRNN).

4. Duas vias mutacionais de resistência foram descritas para os inibidores não nucleosídeos da transcriptase reversa nevirapina e efavirenz. A substituição de aminoácido K103N é mais frequentemente selecionada em esquemas utilizando efavirenz, enquanto Y181C é mais selecionada pela nevirapina. Uma alta correlação positiva foi encontrada entre as mutações K103N, L100I, P225H, caracterizando assim a via mutacional relacionada ao efavirenz. Por outro lado,

Y181C esteve fortemente associada com K101E e G190A, caracterizando a via da nevirapina.

5. De acordo com as vias mutacionais de resistência descritas, pode-se antecipar que resistência cruzada à etravirina ocorrerá mais frequentemente entre os indivíduos sob falha terapêutica da nevirapina.

6. Usando valores de carga viral plasmática como um marcador substitutivo de fitness viral, pode-se demonstrar uma tendência de restabelecimento do fitness após um determinado número de mutações de resistência acumuladas.

7. A susceptibilidade aos inibidores de protease e análogos aos nucleosídeos parece estar preservada nos virus dos subtipos F e C. Porém, a susceptibilidade aos inibidores não nucleosídeos da TR merece ser mais bem investigada para os subtipos C e recombinantes B/F brasileiros.

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