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Changes in the HIV-1 Mutational Profile Before First-line HAART in the RESINA Cohort

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ABSTRACT

Sporadic observations have shown changing patterns of transmitted drug resistance mutations (TDRMs) in HIV infection even without selection pressure by antiretroviral treatment. Repeated genotypic resistance analyses in treatment-naïve patients was performed, in order to analyze intraindividual variances of resistance patterns over time.

Between the years 2001 and 2008 two genotypic resistance tests were performed at different time-points in 49 treatment-naïve HIV-positive patients aged >18 years.

Wild-type virus was found at baseline and during follow-up in 31 patients (group A, median time between resistance tests 146 days), while resistance mutations were found either at baseline or during follow-up in 18 patients (group B, median time between resistance tests 297 days).

In group B, the pattern of resistance changed in 8 out of 18 patients over time, with 3 patients showing decreasing numbers and 5 patients showing increasing numbers of resistance mutations. The pattern of resistance mutations remained unchanged in 10 out of 18 patients.

The mutational pattern in untreated HIV infection may change over time and a single resistance analysis may underestimate the true prevalence of preserved resistance mutations. If these findings can be confirmed in a larger number of patients, it would be prudent to perform genotypic resistance testing both at baseline and prior to the start of antiretroviral treatment in order to capture a more complete picture of preserved mutations before initiating antiretroviral treatment.

INTRODUCTION

Transmitted drug resistance mutations (TDRMs) affect significantly the success of first-line antiretroviral treatment (Johnson et al., 2008, Poggensee et al., 2007, von Hentig et al., 2007, Ghosn et al., 2006, Little et al., 2002, Harzic et al., 2002, Tang and Pillay, 2004, Simen et al., 2009). TDRMs in primary HIV infection have been recognized as a relevant problem for patient management (Little et al., 2002, Yerly et al., 2007), driving the World Health Organization (WHO) to recommend continued surveillance of primary resistance through national HIV/AIDS control programs (Lazzari et al., 2004). The prevalence of primary resistance varies significantly between studies and between geographical locations, ranging from 2% to 51% (Varella et al., 2007, Diletnia et al., 2007, Han et al., 2007, Chang et al., 2008, Oette et al., 2004, Palacios et al., 2008, Sanchez et al., 2007, Horban et al., 2002). In Germany, the prevalence of primary resistance has ranged above 10% in various studies, and thus has an impact on the management of HIV infection (Sagir et al., 2007, Oette et al., 2006, Poggensee et al., 2007, Duwe et al., 2001).

The consideration of TDRMs for tailoring first-line treatment has led to improved outcomes, with similar treatment efficacy as compared to patients without primary resistance (Oette et al., 2006, Shet et al., 2006). The determination of the resistance profile in treatment-naïve patients infected by HIV has now become standard of care and is recommended in major guidelines (German-Austrian guidelines et al., 2009, Clumeck et al., 2008, Hirsch et al., 2008).

Sporadic observations have been made about changing patterns of TDRMs even without selective pressure by antiretroviral treatment. For example, higher mutation rates have been described immediately after seroconversion as compared to a further progressed time point during chronic infection (Sanchez et al., 2007). In other cases, persistence of resistant

viruses with high replicative capacity has been observed in newly infected patients over prolonged periods of time (Little et al., 2008).

A longitudinal analysis of repeated genotypic resistance testing was undertaken in a subgroup of patients from the RESINA-cohort prior to initiation of antiretroviral treatment.

The goal was to investigate the frequency and characteristics of resistance-associated mutations over time in untreated HIV-positive patients.

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MATERIALS AND METHODS

Baseline characteristics of the study population

Between the years 2001 and 2008 a longitudinal analysis of resistance mutations was performed in HIV-positive treatment-naïve patients aged >18 years. Two blood samples were analyzed at different time-points by population sequencing for resistance mutations in the protease (PR) and reverse transcriptase (RT) of the HIV-1 *pol*-gene. Only major mutations were scored as resistance-associated according to the current Stanford HIV Drug Resistance Database (November 6, 2009), (available at <http://hivdb.stanford.edu>).

Selection of patients

Written informed consent was obtained from each patient prior to inclusion into the study. According to the protocol, testing for primary resistance in the German RESINA cohort should be performed uniformly at the time when initiation of antiretroviral treatment (ART) is imminent. In some patients, however, testing for primary resistance had been performed without subsequent initiation of ART, because the responsible physician had ultimately decided to postpone the start of treatment. Review of the patient chart and interviews with the responsible physicians revealed that retesting was performed because the first analysis had been premature without the necessity to immediately start treatment. As a consequence, resistance testing was repeated in these cases immediately before the beginning of ART. In the present study, all of these longitudinal resistance analyses were studied, no patient was excluded.

Genotypic resistance testing

Viral RNA was isolated from patient plasma using QIAamp viral RNA mini-kit (Qiagen, Hilden, Germany). Reverse transcription and polymerase chain reaction were performed using OneStep RT-PCR kit (Qiagen, Hilden, Germany) with primers Res1 and Res2 (1RES: 5' GAA GAA ATG ATG ACA GCA TGT CAG GG 3' 2RES: 5' TAA TTT ATC TAC TTG TTC ATT TCC TCC AAT 3') and second round PCR was carried out with

HotStarTaq kit (Qiagen, Hilden, Germany) and the primer pair Res3 und Res4 (3RES:5' TCTT GAT AAA TTT GAT ATG TCC 3' 4RES:5' AGA CAG GCT AAT TTT TTA GGA 3').

PCR products were purified using the QIAquick spin PCR purification kit (Qiagen, Hilden, Germany) and extended using the ViroSeq HIV-1 Genotyping System sequencing module (Applied Biosystems, Foster City, CA, USA). Extension products were purified using MultiScreen purification plates (Millipore, Bedford, MA, USA) and Sephadex G-50 superfine (Amersham Biosciences, Upsala, Sweden) and were run on an ABI PRISM 3100 Avant. Sequence data were generated by using Sequencing Analysis v3.4 (Applied Biosystems, Foster City, CA, USA). The sequences were edited using the ViroSeq HIV-1 Genotyping software v2.6 (Applied Biosystems, Foster City, CA, USA).

Multidrug resistance

Multidrug resistance (MDR) was defined as concurrent major resistance mutations against the drug classes NRTIs (= nucleoside reverse transcriptase inhibitors), NNRTIs (= non-nucleoside reverse transcriptase inhibitors) and PIs (= protease inhibitors).

Phylogenetic Analysis

The Arevir database was searched for similar sequences and the resulting set of 54 sequences, together with the HxB2 reference strain and 8 unrelated sequences, were analyzed with Clustal and Mega4 (Tamura K, Dudley J, Nei M & Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24:1596-1599. (Publication PDF at <http://www.kumarlab.net/publications>). The phylogenetic tree was constructed using the neighbor-joining method. A bootstrap test with 1000 replications was used to obtain the confidence values.

Statistical analysis

In this pilot study descriptive statistics were calculated, such as frequencies of categorical outcomes, median and range of continuous variables.

Ethical approval

The present study was approved by the ethical committee of the University of Düsseldorf.

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RESULTS

Resistance mutations were analyzed in 49 HIV-positive treatment-naïve patients. Group A consisted of patients with wild-type virus at the baseline and during follow-up. Group B comprised patients with detection of resistance mutations either at the baseline or during follow-up. Median time between resistance tests in group A (n=31 patients) were 146 days (average 391 days, range 17-2002 days) and 297 days (average 302 days, range 9-2066 days) in group B (n=18 patients).

Table 1 depicts the homogeneous distribution of baseline characteristics between group A (without TDRMs) and group B (with TDRMs). No major differences between groups were noted with regard to age, gender, mode of transmission, CD4 count, viral load and HIV-subtype.

TDRMs were detected in 18 out of 49 patients (36.78%) (group B). A changing mutational profile in the major viral population was found in 8 out of these 18 patients between the two resistance analyses over time (table 2, groups B1 with increasing mutations and B3 with decreasing mutations).

Several new mutations were found in the follow-up analysis (table 2, group B1). The median time between both resistance analyses was 335 days (average 299, range 17-491). These increasing mutations were most prevalent in the RT gene (K65R, V75I, K103N, Y181C, M184I, L210W, T215I), while only one of the emerging mutations was found in the PR gene (M46I). Homologies between sequences for patients 1 to 5 (table 2) were as follows: 99.07%, 99.05%, 97.65%, 99.54% and 99.46%, the close relationship between samples can be evaluated additionally in figure 1.

In 10 out of 18 cases with TDRMs persisting mutations were found over time (table 2, group B2). The median time between both resistance analyses was 46 days (average 181, range 10-610). Single class mutations were found in 8 cases, while multidrug TDRMs were detected in 2 cases.

In 3 patients decreasing mutations were found over time (table 2, group B3). The median time between both resistance analyses was 759 days (average 713, range 458-921). Single mutations lost were the RT mutation M184V and the revertant T215S. In the third case, multiple TDRMs in the RT gene reverted to wild type in the second analysis after 759 days, including M184V, K103N, V108I and G190A. Homologies between sequences for patients 16, 17 and 18 (table 2) were 93.01%, 99.33% and 98.29%, respectively.

Transmitted MDR virus was found in 2 cases and both showed identical resistance patterns over time (table 2, patients 12 and 14). They harboured the following mutations:

Patient 12: PR: F53L, V82A, L90M, RT: K103N, Y181C, L210W, T215D

Patient 14: PR: L24I, M46I, V82A, I84V, RT: D67G, K103N, T215V, K219Q

CD4 cells decreased by a median of 91 cells / μ l between the two resistance analyses in group A (table 3). In contrast, CD4 cells in the group with detected resistance mutations (group B) remained unchanged, with a median change of 1 cell / μ l. In analogy to the more pronounced CD4 cell decrease in group A, an increase in viral load by a median of 11,798 copies /ml was observed in this group, while in group B the viral load decreased over time (-10,392 copies /ml). A subgroup analysis in group B revealed that the improved immune situation over time was found in patients with an unchanged mutational pattern (B2), while increases or decreases in mutations did not reveal this trend (B1 and B3).

DISCUSSION

The RESINA project is a prospective multicenter study on the epidemiology of primary drug resistance and treatment outcome in North Rhine-Westphalia, Germany. In this project, ongoing surveillance of transmitted resistance mutations has been conducted since 2001 (Sagir et al., 2007, Oette et al., 2006), with more than 2000 patients included to date. Primary drug resistance was determined in patients infected chronically with unknown date of infection. The present study comprised 49 treatment-naïve patients infected chronically with HIV. These patients received two resistance tests before initiation of antiretroviral therapy. TDRMs were detected in 18 patients and a changing mutational pattern was found in 8 out of 18 patients with TDRMs over time.

The TDRM rate of 36.7% (18/49) of our patients is much higher than the estimated rate of 10% in Germany (Sagir et al., 2007, Oette et al., 2006, Poggensee et al., 2007, Duwe et al., 2001). Due to the small number of patients, the number of patients with TDRM cannot be considered representative in this sub analysis of the RESINA cohort. Furthermore, due to the retrospective nature of this study it cannot be excluded that physicians performed repeated testing more readily in patients with known TDRM.

Several new mutations were found in the follow-up analysis in 5 patients (table 2).

To date, accumulation of resistance mutations in treatment-naïve patients have been described rarely. Despite persistence of mutations, no increase of mutations was observed in several other studies (Fox et al., 2006, Ghosn et al., 2006, Pao et al., 2004, Barbour et al., 2004, Cane et al., 2009). In analogy to the present results, in one case of primary infection development of 2 additional RT mutations (M184V and V75M) was noted after 5 months of infection (Chan et al., 2003).

Mutations may be acquired newly through superinfection with a second strain (Allen and Altfeld, 2003). Homologies between sequences for our patients with increasing mutations were above 97.6% in all 5 cases, and therefore the hypothesis of superinfection was

unlikely in these cases. The phylogenetic tree (figure 1) confirms the very close relationships between the respective sample pairs. An alternative explanation is the emergence of new resistance mutations through selection pressure exerted by the immune system of the host. Some mutations which confer drug resistance incidentally can also enable the virus to evade recognition by the cytotoxic T-lymphocytes (CTL) and are therefore selected even in the absence of drugs as an immune-escape mutant (Mueller et al., 2007). An increasing body of evidence suggests that a considerable proportion of transmitted HIV-1 drug resistance is not detected by conventional genotyping and that minority strains harbouring resistance mutations can have clinical consequences (Johnson et al., 2008, Metzner et al., 2009, Simen et al., 2009). The dynamics of HIV quasispecies with changing proportions of the population can result in the detection of minority strains in a repeated standard resistance testing. Furthermore, a genetic viral diversity in different sites of the body has been observed, and from these reservoirs the re-emergence of resistant viral strains appears plausible (Lambotte et al., 2004).

In 10 out of 18 cases with TDRMs persisting mutations were found over time (table 2). It must be noted that in the group with persisting TDRMs (B2) the interval between genotypic resistance analyses was shorter as compared to the groups with changes in the mutational pattern over time (B1 and B3). The frequency of mutational changes over time may therefore have been underestimated. In accordance with the present findings, Fox et al (2006) had observed persisting TDRMs in 8 out of 9 patients. Seven patients showed single-class mutations and in 2 cases multiple mutations were found. In contrast to the rapid disappearance of secondary mutations in patients with failure of ART (Deeks et al., 2001), primary mutations in treatment-naïve patients may persist for long periods of time (Chan et al., 2003). Ghosn et al (2006) also found that acquired resistant viruses establish themselves as the dominant viral population at primary infection.

A large diversity of RT mutations without PR mutations was found among the 7 cases with persisting single-class resistance patterns: 3x M41L, Y188L, 2x K219Q, T215A, T215CS, 2x T215D and P225H. In the present analysis, persistence of TDRMs in the PR gene was not observed in patients with single class mutations. This finding is supported by a previous study (Pao et al., 2004). As an explanation for this finding, Wirden et al. (2004) observed that the kinetics of the shift to wild-type amino acid residues were significantly faster for PR mutations than for mutations in the RT gene.

Some mutant viruses may possess a better replicative capacity in comparison to wild-type virus. It can therefore be hypothesized that in contrast to wild-type-virus, long-term persistence of certain mutations is advantageous for a viral population. Several of the TDRMs found have been shown previously to persist after transmission even in the absence of antiretroviral therapy, including D67N, K70R, M41L, T69N and some T215 variants,(Barbour et al., 2004, Pao et al., 2004) as well as the K103N mutation (Ghosn et al., 2006, Capetti et al., 2005, Cane et al., 2009). Interestingly, in some cases persistence of TDRMs was documented for up to 5 years (Brenner et al., 2002).

Two multi-resistant virus strains were detected, each with identical resistance patterns over time (table 2, samples 12 and 14). Long-term persistence of MDR virus over several years in the treatment-naïve setting has been observed (Pao et al., 2004, Brenner et al., 2004, Brenner et al., 2002, Delaugerre et al., 2004) and was not outgrown by a virus strain with wild-type sequence. As an explanation for this finding, it has been hypothesized that primary mutations can be transmitted in a homogeneous viral species without coexisting wild-type virus (Simon et al., 2002). In these cases, there would be no competition with a wild-type virus.

While a primary transmitted M184V mutation persisted over a 9-month observation period in one study (Barbour et al., 2004), mutations such as K65R and M184V in the RT gene are

known to cause a considerable reduction in viral fitness. They may therefore disappear more rapidly but remain within the viral archive and may be detectable using more sensitive assays than those in current routine use (Caneet al., 2005). This concurs with the present observation that neither the K65R nor the M184V mutation was observed to persist over extended periods of time (table 2). Various other TDRMs, such as Y181C, K219Q (Barbour et al., 2004, Pao et al., 2004) and D67N (Barbour et al., 2004) have been described as reverting to wild type. In addition, there is evidence for a reversion of the T215Y mutation to a fitter revertant virus, such as T215S/D/C (Fox et al., 2006, Ghosn et al., 2006, Pao et al., 2004, Barbour et al., 2004, Brenner et al., 2004, Caneet al., 2005). Two out of 3 of our patients with decreasing mutations over time (patients 17 and 18, table 2) showed high homologies between sequences. However, in patient 16 it remains unclear whether the markedly lower similarity between the sequences (93.01%) is the result of superinfection or a laboratory error (sample swapping, contamination).

It may be concluded from these data that in order to detect as many TDRMs as possible, resistance testing should be performed soon after establishing the diagnosis.

The above results demonstrate that the resistance pattern can change without selection pressure by antiretroviral drugs, but may be driven by viral dynamics of strains with different resistance patterns and the varying ability of the immune system to display and recognize the different epitopes of the various viral gene products with resistance mutations (so called “escape mutants”). Another important factor for the persistence or reversion of resistance mutations in the absence of selective drug pressure is their impact on the viral replicative capacity or “viral fitness”.

While genotypic resistance testing is now recommended uniformly in all major treatment guidelines for treatment-naive patients, the recommended timing for genotypic resistance testing varies. In Germany and Austria resistance testing is recommended prior to start of

1 treatment. In contrast, European and U.S. Guidelines both recommend testing as part of the
2
3 initial patient assessment, even if treatment is not initiated (German-Austrian guidelines et
4
5 al., 2009, Clumeck et al., 2008, Hirsch et al., 2008).
6
7

8 Without the knowledge of the individual infection chain, our findings suggest an important
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10 role for drug resistance testing both at baseline and before the start of antiretroviral
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12 treatment in naïve patients.
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18 Without antiretroviral treatment, a trend toward more pronounced progression of disease
19
20 was observed in the group without TDRMs (group A): CD4 cells decreased by a median of
21
22 91 cells / μ l between the two resistance analyses. In contrast, in the group with detected
23
24 TDRMs (group B) CD4 cells did not change significantly over time (median of 1 cell / μ l).
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26 In analogy to the more pronounced CD4 cell decrease in group A, an increase in viral load
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28 by a median of 11,798 copies /ml was observed in this group, while in group B the viral
29
30 load did not increase over time (-10,392 copies /ml). A subgroup analysis in group B
31
32 revealed that the improved immune status over time was found in patients with an
33
34 unchanged mutational pattern (B2 in table 3), while increases or decreases in mutations
35
36 tended to be associated with a stable or a deteriorating immune function.
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42 Conflicting results are found in the literature with regard to alterations of immunological
43
44 parameters. In contrast to the above observations, a faster rate of CD4 decline was
45
46 observed in TDRMs by Fox et al (2006) and other investigators found comparable rates of
47
48 CD4 decline between TDRMs and wild-type HIV (Bhaskaran et al., 2004).
49
50

51 In support of the present findings, Cane et al. (2009) showed that TDRMs remained stable
52
53 for up to two years and that this stable situation was associated with low viral loads. The
54
55 finding of low viral loads in TDRMs was first described by Salomon et al. (2000) and may
56
57 be attributable to a compromised replicative capacity of some resistant HIV strains
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59
60

(Brenner et al., 2002), hypothetically enabling the host immune system to exert greater control than has been seen in most patients infected with wild-type virus.

Several limitations of the present analysis must be acknowledged, as the small number of patients may imply inherent bias. Rates of disease progression may have been influenced by the retrospective study approach and the clinician-driven selection of patients. Furthermore, the prevalence of transmitted drug resistance may have been underestimated, as population sequencing frequently fails to detect low-frequency variants. This problem could be solved by “deep sequencing”, but these techniques are currently not established as routine methods. A prospective study in a larger, random group of patients will be indispensable to confirm the present data regarding the clinically-relevant question of changing mutational patterns and their influence on disease progression.

Conclusions

In this longitudinal analysis of primary HIV resistance mutations, a high frequency of alterations of the mutational profile in the treatment-naïve setting was found, demonstrating that the resistance pattern of the predominant viral population can change over time without selective drug pressure. Single time resistance analyses may underestimate the true extension of preserved resistance mutations. If these data can be validated in a larger group of patients, this would imply the necessity of two resistance analyses, the first soon after establishing the diagnosis and the second directly before start of antiretroviral treatment.

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TABLES

	Group A (n=31) without TDRM	Group B (n=18) with TDRM
Median age at baseline (years)	38.6	36.4
Gender	Male 24, female 7	Male 16, female 2
Mode of transmission	MSM = 16 CHP = 3 Hetero = 6 IVDU = 2 Unknown = 4	MSM = 13 CHP = 2 Hetero = 0 IVDU = 1 Unknown = 2
Median CD4 abs (rel) at baseline	340 / μ l (19%)	309 / μ l (18%)
Median viral load at baseline	56,402 c/ml	41,471 c/ml
Subtype	A = 1 B = 24 C = 2 D = 0 F = 1 G = 1 CRF 02_AG = 1 Urf = 1	A = 1 B = 16 C = 0 D = 1 F = 0 G = 0 CRF 02_AG = 0 Urf = 0

Table 1: Baseline characteristics of studied patients. (Baseline = time of first resistance testing, MSM = men who have sex with men, CHP = country of high prevalence, IVDU = intravenous drug use, c/ml = copies per millilitre, Urf = unrecognized recombinant form, TDRM = transmitted drug resistance mutations)

Time between resistance tests			Major resistance mutations			
B1: Increase						
Sample No.	subtype	(days)	NRTI	NNRTI	PI	REVERTANTS
1a	D		0	0	0	0
1b	D	259	0	0	M46I	0
2a	B		0	0	L90M	0
2b	B	394	T215I	0	L90M	0
3a	B		0	0	0	0
3b	B	335	K65R V75I M184I	Y181C	0	0
4a	B		0	0	0	0
4b	B	491	0	L210W	0	0
5a	B		0	Y181C	V82A L90M	T215C
5b	B	17	0	K103N Y181C	V82A L90M	T215C
B2: Unchanged						
6	B	393	D67N K219Q,	0	M46L	T215S
7	B	237	M41L	0	0	T215D
8	B	386	0	Y188L P225H	0	0
9	B	61	0	0	0	215D
10	B	28	M41L	0	0	0
11	B	28	K219Q	0	0	0
12	B	10	D67G K219Q	K103N	L24I M46I V82A I84V	T215V
13	B	25	0	0	0	T215CS
14	B	31	L210W	K103N Y181C	F53L V82A L90M	T215D
15	B	610	M41L	0	0	0
B3: Decrease						
16a	A		M184V	K103N V108I G190A	0	0
16b	A	759	0	0	0	0
17a	B		M184V	0	V82L	0
17b	B	458	0	0	V82L	0
18a	B		0	0		T215S
18b	B	921	0	0		0

Table 2: Patterns of major resistance mutations over time. B1 = increasing mutations (patients 1-5), B2 = unchanged mutations (patients 6-15), B3 = decreasing mutations

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(patients 16-18). (NRTI = nucleoside reverse transcriptase inhibitor, NNRTI = non-nucleoside reverse transcriptase inhibitor, PI = protease inhibitor). *a* = first resistance test, *b* = second resistance test

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	Group A (n=31) without TDRM	Group B (n=18) with TDRM
Median days between resistance analyses (range)	146 (range 17-2002)	297 (range +9 - +2066)
Median CD4 change abs. (range)	-91 cells / μ l (-744 – +101)	Total: +1 cell / μ l B1: +28 cells / μ l (+21 – +60) B2: +78 cells / μ l (-274 – +486) B3: -11 cells / μ l (+1 – +13)
Median CD4 change rel. (range)	-2.5 % (-26 – +27)	Total: +2 % B1: -1 % (-2 – +1) B2: +3.5 % (-8 – +14) B3: -7 % (-9 – +1)
Median viral load change (range)	+11,798 copies /ml (-683,026 – +229910)	Total: - 10,392 copies /ml B1: + 5,894 copies /ml (-130,000 – +8290) B2: -161,701 copies /ml (-328,399 – +1031) B3: +16,003 copies /ml (-2364 – +247,000)

Table 3: Comparison of changes in CD4 and viral load between resistance analyses.

B1 = increasing numbers of TDRMs (n=5), B2 = unchanged numbers of TDRMs (n=10),

B3 = decreasing numbers of TDRMs (n=3)

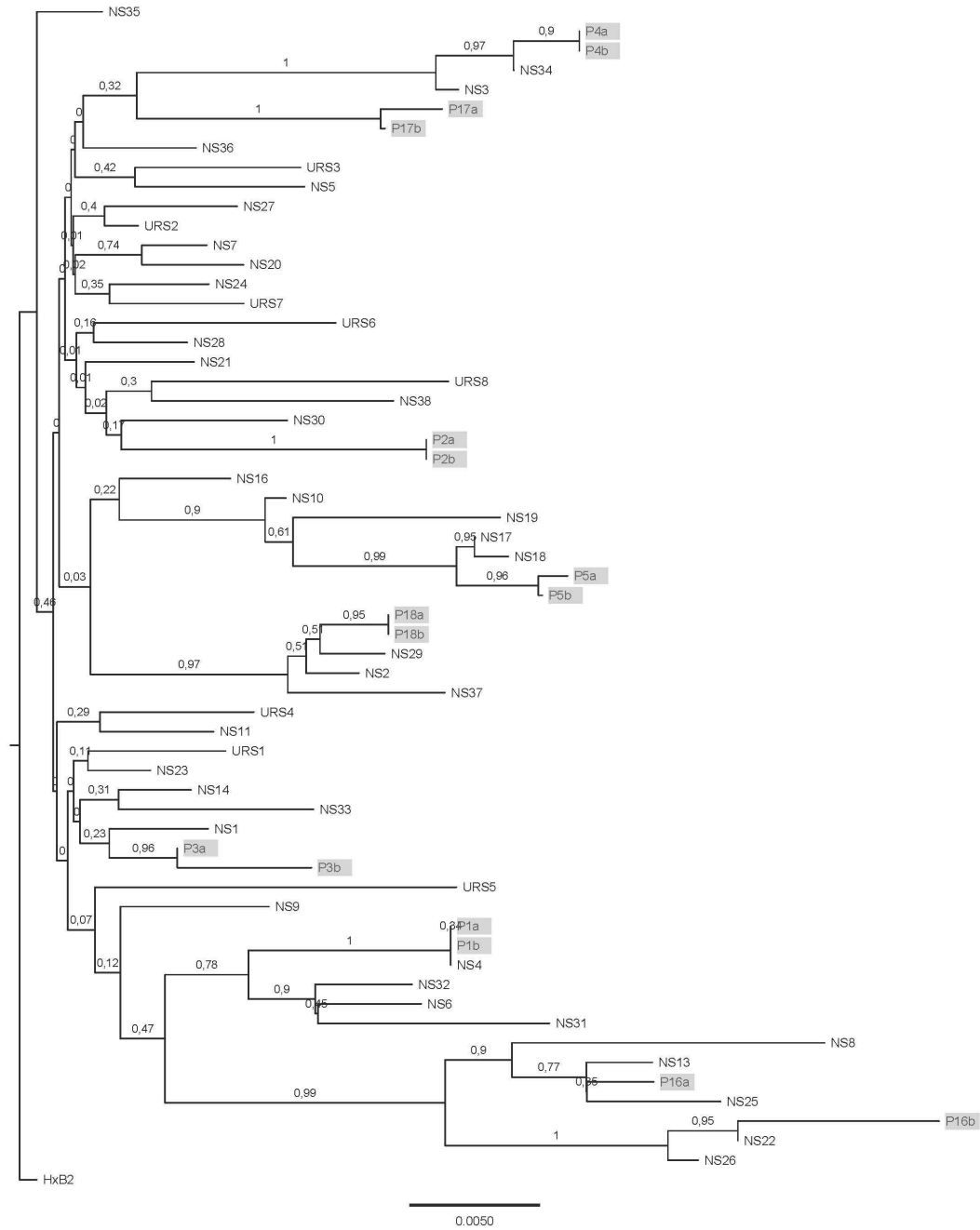


Figure 1: *Phylogenetic relationships between the HIV-1 protease/reverse transcriptase nucleotide sequences from the patients discussed (P1a – P5b and P16a – P18b), similar sequences (NS1-NS38) and unrelated sequences (URS1-URS8). The analysis was done using the neighbor-joining method and 1000 bootstrap resamplings. Except in one case (P16), the tree confirms the very close relationships between the respective sample pairs.*