## ORIGINAL ARTICLE

# Genetic evolution of HIV in patients remaining on a stable HAART regimen despite insufficient viral suppression

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**Abstract**

Our objective was to investigate whether steadily increasing resistance levels are inevitable in the course of a failing but unchanged Highly Active Antiretroviral Therapy (HAART) regimen. Patients having an unchanged HAART regimen and a good CD4 response (100 cells/µl above nadir) despite consistent HIV-RNA levels above 200 copies/ml were included in the study. The study period spanned at least 12 months and included 47 plasma samples from 17 patients that were sequenced and analysed with respect to evolutionary changes. At inclusion, the median CD4 count was 300 cells/ml (inter-quartile range (IQR): 231–380) and the median HIV-RNA was 2000 copies/ml (IQR: 1301–6090). Reverse transcription inhibitor (RTI) mutations increased 0.5 mutations per y (STD = 0.8 mutations per y), while major protease inhibitor (PI) resistance mutations increased at a rate of 0.2 mutations per y (STD = 0.8 mutations per y) and minor PI resistance mutations increased at a rate of 0.3 mutations per y (STD = 0.7 mutations per y). The rate at which RTI mutations accumulated decreased during the study period ($p = 0.035$). Interestingly, the rate of mutation accumulation was not associated with HIV-RNA level. The majority of patients kept accumulating new resistance mutations. However, 3 out of 17 patients with viral failure were caught in an apparent mutational deadlock, thus the development of additional resistance during a failing HAART is not inevitable. We hypothesize that certain patterns of mutations can cause a mutational deadlock where the evolutionary benefit of further resistance mutation is limited if the patient is kept on a stable HAART regimen.

## Introduction

Human immunodeficiency virus (HIV) is able to develop resistance towards all currently used antiretroviral agents, i.e. the nucleoside analogue reverse transcriptase inhibitors (NRTI), non-nucleoside analogue reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI) and the fusion inhibitors. Resistance develops because of error prone reverse transcription of HIV-RNA causing the emergence of a diverse viral population followed by natural selection of virus able to replicate in the presence of 1 or more antiviral drugs. Because of the high mutation rate of HIV, resistance is almost guaranteed to emerge in patients on single drug therapy. HAART combines 3 or more drugs in an attempt to counteract the development of resistance [1–4] in 2 ways:

first, viral replication is suppressed to a level where very few new mutants are generated, and secondly the simultaneous presence of multiple drugs requires the virus to acquire several different resistance associated mutations more or less simultaneously. In a substantial proportion of patients, HAART suppresses replication to a level that does not allow the evolution of drug resistance over a time frame of y [5]. In an attempt to regain viral suppression and prevent the development of increased resistance among patients receiving HAART with insufficient viral suppression, these are often switched to new drug regimens according to a genotypic antiretroviral test [6]. However, the number of drugs available as well as cross-resistance limits this approach, and in many situations it is unlikely that renewed viral control can be achieved.

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Patients often remain healthy and retain an immunological response to HAART despite insufficient replication suppression, i.e. having an increased and stable CD4 count compared to the CD4 count prior to the instigation of therapy [7]. Patients with immunological success despite viral failure are said to have a discordant response towards HAART. Deeks et al. [8] found that discontinuation of PI-based HAART in a cohort of discordant responders with multi-drug resistant HIV and stable CD4 counts despite detectable HIV-RNA, resulted in increasing HIV-RNA. It is believed that acquisition of additional resistance conferring mutations during a stable failing HAART regimen will eventually optimize viral fitness and cause a deteriorating CD4 count. As yet it is unclear which biological mechanism causes the discordant immune response; however, several studies have shown that HIV carrying resistance mutations is less fit than wild-type HIV [9–17].

We explore whether steadily increasing resistance levels are inevitable in the course of a failing but unchanged HAART regimen. To test this hypothesis and to improve the understanding of the genetic evolution of HIV under a constant selection pressure, we examined 17 patients with a discordant response. Thus, they all had benefit from the treatment with good CD4 response despite measurable HIV-RNA on an unchanged HAART regimen. Only patients with a good CD4 response of 100 cells/μl above nadir for the entire study period were included to ensure that the treatment had an immunological effect, thus continuously inducing a selection pressure on viral evolution.

## Material and methods

### Subjects

All patients were diagnosed with HIV-1 and followed at the Department of Infectious Diseases, Hvidovre University Hospital, Copenhagen, Denmark. HIV infected patients are followed at the outpatient clinic every 3 months for a routine check-up at which time several biochemical tests, including measurements of CD4 count and HIV-RNA, are performed. In addition, a plasma sample is collected and stored. Discordant responders were selected for HIV-RNA sequencing retrospectively using the following inclusion criteria:

1. All patients initiated HAART at least 18 months before study inclusion. HAART was defined as the combination of at least 3 antiretroviral drugs.

2. Patients remained on the same HAART regimen for at least 12 months without any changes. This HAART regimen was defined as 'current treatment'.
3. HIV-RNA values were above 200 copies/ml for the entire study period.
4. An increase in CD4 count of at least 100 cells/μl on current treatment for the entire study period when compared to nadir CD4 count.

Clinicians working in the outpatient clinic identified 23 individuals as discordant responders according to the above inclusion criteria; thus the true prevalence of discordant patients in our cohort is not known. Careful examination of patient records showed that 4 of these patients did not fulfil the inclusion criteria for a full y; in 1 case samples spanning 1 y were not available, and in 1 case it was not possible to achieve sequencing results at time-points spanning 1 y. In total 17 patients were included: 14 of these had 3 samples available, in 1 case the sequencing failed, 3 of the patients only had 2 samples available. In total 13 patients had a genotypic resistance test performed at 3 time-points while the remaining 4 patients had a genotypic test performed at 2 time-points. The interval between first and last sample determined the study period for each patient. Blood samples were obtained from November 1997 through September 2001. The included patients were kept on a stable therapy for a number of reasons: no other treatment options were available, side-effects were an issue, some patients were not interested in having any treatment changes whereas other patients were kept on a stable treatment regimen because of a durable CD4 response despite ongoing viral replication. In some patients no reason was given. From patient records, data on demographics, antiretroviral treatment, CD4 count, and HIV-RNA values were collected in a standardized form. Forms and samples were coded before analysis. The Medical Ethics Committee of Copenhagen County approved the study.

### CD4 count and HIV-RNA

CD4+ T-cell counts were measured by FACS analysis using standard procedures. HIV-RNA was determined using the Roche Amplicor HIV-1 assay (Roche Molecular Systems, Inc., Branchburg, N, USA) according to the manufacturer's instructions. The detection threshold was 20 copies/ml [18].

*HIV-sequencing*

Sequencing was carried out on bulk PCR products using the Viroseq™ HIV-1 genotyping system version 2 (Applied Biosystems, CA, USA) according to the manufacturer's instructions. The sequenced region has a length of 1269 nucleotides and corresponds to the entire protease (PR) gene (codon 1–99) followed by the first 324 codons of the reverse transcriptase (RT) gene. All sequences have been submitted to GenBank [19]. Resistance associated mutations in the PR and RT gene were identified using the resistance analysis module of the Stanford HIV Reverse Transcriptase and Protease Sequence Database [20].

*Correlation of HIV-RNA and CD4 against time*

In order to investigate whether any patients displayed consistent changes in HIV-RNA or CD4 counts during the study period, we computed the Kendall rank correlation coefficient between time and HIV-RNA/CD4 count. If there is a consistent rise or fall in either measure during the investigated time span, this will be seen as a significant correlation between the measure and time. The Bonferroni correction for multiple testing was applied.

*Construction of phylogenetic tree*

A phylogenetic tree was constructed using the neighbour joining method, based on a multiple alignment of all population sequences from all time-points and all patients. Specifically, gapped positions were excluded and there was no correction for multiple substitutions as population sequences are not real sequences; using model-based methods to correct for unseen events is somewhat problematic).

*Population sequence mutation rates*

For every patient the 'population sequence mutation rate' (expressed as nucleotide substitutions per site per y) was calculated between each pair of consecutive time-points. One rate was computed for the 4 patients having population sequences from 2 time-points, whereas 2 rates were calculated for the 13 patients having population sequences from 3 time- points. The mutation rate was calculated by dividing the number of differing nucleotide sites by the length of the sequence and by the time elapsed. Two sites were only scored as identical if they contained exactly the same International Union of Pure and Applied Chemistry (IUPAC) nucleotide symbol. For instance, if the symbol Y (pyrimidine, i.e. C or T) was present at a given position in 1 sequence, while the symbol C was present at the corresponding position in another sequence, it was counted as 1 change. It is important to realize that since we are comparing population sequences this is not an actual mutation rate (the calculation of which would have required sequences from individual clones), but rather a way of quantifying how fast the average of an entire population of HIV clones in 1 patient evolves over time.

Table I. Cohort characteristics ($n = 17$).

| | |
|---|---|
| Median Age | 35 (IQR: 34–47) |
| Gender (female/male) | 2/15 |
| Median plasma HIV-1 RNA at start of study period | 2000 copies/ml (IQR 1301–6090) |
| Median CD4 cell count at start of study period | 300 cells/μl (IQR 231–380) |
| Median CD4 cell count at nadir | 64 cells/μl (IQR: 26–159). |
| Median increase in CD4 cell count above nadir at initiation of study period | 223 cells/μl (IQR 160–294) |
| Median time from HIV diagnosis to initiation of first antiretroviral therapy | 444 days (IQR: 34–2380) |
| Patients were started on these antiretroviral regimens | |
| Monotherapy (zidovudine) | 9 |
| Dual therapy | 7 |
| Triple therapy | 1 |
| Median time from first treatment to implementation of HAART | 490 days (IQR: 341–1008) |
| Median time from first treatment to current treatment | 927 days (IQR: 589–1461) |
| Median time from initiation of HAART to current treament | 440 days (IQR: 0–762) |
| Median time from initiation of current treatment to first samle | 159 days (IQR: 59–411) |
| Median length of study period | 526 days (IQR: 476– 945) |
| Median number of resistance associated mutations at start of study period | 9 (IQR: 9–13) |
| Number of patients recieving these current antiretroviral regimens | |
| 2 NRTI+1 PI | 7 |
| 2 NRTI+1 PI+1NNRTI | 5 |
| 2 NRTI+2 PI | 3 |
| 3 NRTI+1 PI | 1 |
| 3 NRTI+1 NNRTI | 1 |

*Rate of change of resistance associated mutations*

At every time-point a list of known PI or reverse transcription inhibitor (RTI) resistance associated mutations in the population sequence was constructed, and at each pair of consecutive time-points, we computed a 'resistance associated mutation rate' by taking the absolute number of resistance associated mutations at the first time-point minus the absolute number of such mutations at the last time-point, finally dividing this difference by the time elapsed (in y). This rate is measured in mutations per y and indicates whether a patient has experienced a rise or fall in the total number of resistance associated mutations and how fast this change occurred. One patient (No.13, see Table II) did not receive any PIs, and mutation data from the PR gene from this patient were not included in the analysis; however, mutation data from the RT gene were included in the analysis.

*Pair-wise correlation of mutations in the RT and PR genes*

Fisher's exact test was used to investigate whether the presence of 1 resistance-associated mutation was correlated with the presence (or absence) of other resistance-associated mutations. We arbitrarily used the last time-point sequence from each patient (bulk sequences at consecutive time-point from 1 patient will have a common history, and are therefore not independent observations of pairs of mutations), and the presence or absence of resistance-associated mutations was analysed for all possible pairs of codon sites at which mutations may lead to resistance. If the presence of 1 mutation is independent of the presence of a second mutation, then the test will not be significant, otherwise it will. Specifically, we used Fisher's exact test as implemented in the R-package for statistical analysis (R Development Core Team, 2005). The Bonferroni correction for multiple testing was applied (in total 703 comparisons were performed). Since this lowers the $p$-value considerably, comparisons at a $p$-value of 0.05 were also performed. If a relationship is found at this $p$-value it must be confirmed in another study and be theoretically meaningful to be validated.

**Results**

*Cohort characteristics*

Clinical and biological characteristics of the 17 patients are outlined in Table I. Data on HIV-RNA levels prior to any treatment or off-treatment were only available on 2 patients; for these 2 patients 'viral set-point' was higher than during the study period (data not shown).

One patient did not receive any PI; all other patients received at least 1 PI.

*Changes in HIV-RNA and CD4 counts during the study period*

For none of the patients was any significant directional change in CD4 over time observed (investigated by correlating CD4 count and time; data not shown). Similarly, no significant directional change in HIV-RNA over time was observed (data not shown).

*Population mutations*

All patients were infected with clade B HIV-1. A phylogenetic tree constructed from all available sequence data, showed that the population sequences behaved in a reasonable manner: sequences occurred in clusters corresponding to the individual patients, and branch order was furthermore consistent with the actual temporal order of sampling (Figure 1). The average population mutation rate for all nucleotide changes over the entire sequenced region (1269 nucleotides) was 0.014 substitutions site-1 y-1 (STD = 0.011).

*Genotypic resistance*

The development in HIV-RNA, CD4 count, and genotypic resistance is listed in Table I. 10 patients developed new PI mutations (59%), 7 patients developed new NRTI mutations (41%) and 5 patients developed new NNRTI mutations (29%). Of the 16 patients receiving PIs 6 did not develop new PI mutations; of the 17 patients receiving NRTIs 10 did not develop new NRTI mutations, and of the 6 patients receiving NNRTIs 1 did not develop any new NNRTI mutations. All new mutations could be explained, by previous ('re-emergence of lost mutations') or present ('novel mutations') treatment, although attention must be drawn to patient 14 who developed the K101Q mutation while never having received any NNRTIs (K101Q is known to occur in untreated individuals [20]). Interestingly, patient 1 did not have any changes in genotypic resistance during 434 d of follow-up and patient numbers 2 and 8 did not develop any new mutations, while some mutations no longer selected for were lost. Patient 11 only acquired new minor PI-mutations and was stable concerning the remaining resistance associated mutations. The other patients continued to accumulate new mutations. 13 patients all on a 3TC-based HAART expressed the M184V mutation during the entire period of follow-up, while 2 patients no longer on a 3TC-based HAART lost the M184V.

Table II. The table lists the antiretroviral treatment regimen, time-points, CD4 counts and plasma viral load (pVL) as well as resistance associated mutations for each of the patients. For 12 patients a genotypic test was only performed at 2 time-points.

| | Treatment | Time (d) | CD4 (cells/ul) | VL (Copies/ml) | Specific PR mutations | | Specific RT mutations | | | |
| | | | | | Major mutations | Minor mutations | 3TC | TAMs | Other NRTI mutations | NNRTI mutations |
|---|---|---|---|---|---|---|---|---|---|---|
| Pt1 | d4T, 3TC, Ritonavir, Saquinavir | 0 | 616 | 9080 | I54V, I84V, L90M | L10I, L63P, A71V | M184V | M41L, T215FY | | |
| | | 304 | 687 | 3620 | I54V, I84V, L90M, | L10I, L63P, A71V | M184V | M41L, T215FY | | |
| | | 434 | 679 | 17500 | I54V, I84V, L90M, | L10I, L63P, A71V | M184V | M41L, T215FY | | |
| Pt2 | d4T, 3TC, Indinavir | 0 | 442 | 6090 | M46LM, G48V, I54V, V82A, L90M | L10I, L63A, A71T, V77I, I93L | M184V | D67N, K70R, T215F, K219Q | V118I | |
| | | 245 | 479 | 8650 | M46LM, G48V, I54V, V82A, L90M | L10I, L63AP, A71T, V77I, I93L | M184V | D67N, K70R, T215F, K219Q | V118I | |
| | | 637 | 532 | 5480 | G48V, I54V, V82T, L90M | L10I, L63P, A71T, V77I, I93L | M184V | D67N, K70R, T215F, K219Q | V118I | |
| Pt3 | AZT, ddI, Saquinavir | 0 | 364 | 1710 | M46L, G48V, L90M | A71V, V77I, I93L | | M41L, L210W, T215Y | | |
| | | 229 | 367 | 3880 | M46L, G48V, L90M | A71V, V77I, I93L | | M41L, L210W, T215Y | V118I | |
| | | 522 | 374 | 2000 | G48V, V82A, L90M | L63P, A71V, V77I, I93L | | M41L, L210W, T215Y | V118I | |
| Pt5 | d4T, ddI, Nelfinavir, NVP | 0 | 238 | 300 | G73S, I84V, L90M | L10I, L63P, A71V, V77I, I93L | M184V | M41L, D67N, L210W, T215Y, K219KN | E44D, V118IMV | Y318Y |
| | | 672 | 272 | 2230 | G73S, I84V, L90M | L10I, L63P, A71V, V77I, I93L | | M41L, D67N, L210W, T215Y, K219KN | V118IMV | A98G, K103N, Y181C |
| | | 1148 | 348 | 389 | M46I, G73S, I84V, L90M | L10I, L63P, A71V, V77I, I93L | | M41L, D67N, L210W, T215Y, K219KN | V118IV | A98G, K103N, Y181C |
| Pt6 | d4T, 3TC, Nelfinavir | 0 | 180 | 38517 | M46IM, G73ST, I84V, L90M | L10I, L63P, A71V, V77I, I93L | M184V | M41L, D67N, K70R, L210W, T215Y | E44D, V75M, V118I | A98G |
| | | 293 | 229 | 117000 | M46I, G73ST, I84V, L90M | L10I, L63P, A71V, V77I, I93L | M184V | M41L, D67N, L210W, T215Y, K219KR | E44D, L74IL, V75M, V118I | A98G, V108I |

Table II (*Continued*)

| | Treatment | Time (d) | CD4 (cells/ul) | VL (Copies/ml) | Specific PR mutations | | Specific RT mutations | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Major mutations | Minor mutations | 3TC | TAMs | Other NRTI mutations | NNRTI mutations |
| | | 476 | 179 | 73100 | M46I, G73ST, I84V, L90M | L10I, L63P, A71V, V77I, I93L | M184V | M41L, D67N, L210W, T215Y, K219R | E44D, L74I, V75M, V118I | A98G, V108I |
| Pt7 | d4T, 3TC, Nelfinavir, NVP | 0 | 247 | 70000 | V32I, M46I, V82T | L63P, A71V | M184V | M41L, D67N, T215Y | | |
| | | 373 | 448 | 4595 | V32I, M46I, V82T | L63P, A71V | M184V | M41L, D67N, T215Y | | Y188L |
| | | 1058 | 599 | 3910 | V32I, M46I, V82T, L90M | L63P, A71V | M184V | M41L, T215Y | | Y188L |
| Pt 8 | d4T, 3TC, ABC, Ritonavir | 0 | 300 | 1780 | M46I, F53FL, I54V, V82A, I84V, L90M | L10I, L63P, A71AV | M184MV | M41L, D67N, L210W, T215Y, K219KR | E44D | K103N |
| | | 412 | 431 | 11200 | M46I, I54V, V82T, I84V | L10I, l63P | M184MV | M41L, D67N, L210W, T215Y, K219KR | E44D | K103N |
| Pt9 | 3TC, ABC, EFV, Nelfinavir | 0 | 231 | 2777 | G48A, I54V, V82A, L90M | L10I, M36I, L63P, A71I, I93L | M184V | M41L, T215Y | | K103N |
| | | 95 | 221 | 6740 | I54V, V82A, L90M | L10I, M36I, L63P, A71I, I93L | M184V | M41L, T215Y | L74V | L100I, L103N |
| | | 474 | 222 | 9250 | I54V, V82A, L90M | L10I, M36I, L63P, A71I, I93L | M184V | M41L, T215Y | L74V | L100I, L103N |
| Pt10 | d4T, ABC, Indinavir, NVP | 0 | 182 | 2000 | I54V, V82A, L90M | L10I, M36I,L63P, I93L | | D67N, K70R, K219Q | L74IL | Y181C |
| | | 504 | 179 | 18100 | M46IM, I54V, V82A, L90M | L10I, M36I, L63P, I93L | | D67N, K70R, K219Q | | K103N, Y181C |
| Pt11 | d4T, 3TC, Nelfinavir | 0 | 357 | 5318 | L24I, M46L, I54V, V82A, I84V | L10I, L33V, L63P, A71V | M184V | M41L, D67N, L210W, T215Y | | |
| | | 483 | 503 | 4740 | L24I, M46L, I54V, V82A, I84V | L10I, L33V, L63P, A71V | M184V | M41L, D67N, L210W, T215Y | | |
| | | 875 | 401 | 4110 | L24I, M46L, I54V, V82A, I84V | L10I, K20KM, L33ILV, L63P, A71V | M184V | M41L, D67N, L210W, T215Y | | |

Table II (*Continued*)

| | Treatment | Time (d) | CD4 (cells/ul) | VL (Copies/ml) | Specific PR mutations | | Specific RT mutations | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Major mutations | Minor mutations | 3TC | TAMs | Other NRTI mutations | NNRTI mutations |
| Pt12 | AZT, ddI, Indinavir, Ritonavir | 0 | 380 | 2400 | M46I, V82L, L90M | L63P,A71T, V77I | M184V | M41L, L210W, T215Y | | |
| | | 526 | 358 | 12500 | M46I, G73A, I84V, L90M, | L10I, L63P, A71T, V77I | | M41L, L210W, T215Y | T69RT, V118IV | |
| Pt13 | d4T, ABC, 3TC, NVP | 0 | 182 | 14900 | | L63P | M184V | M41L, D67DN, L210W, T215Y, K219KN | E44D, V118I | |
| | | 149 | 190 | 11200 | | L63P | M184V | M41L, D67DN, L210W, T215Y, K219N | E44D, V118I | A98G, K103KN, Y181CY |
| | | 520 | 258 | 3670 | | L63P | M184V | M41L, D67N, L210W, T215Y, K219N | E44D, L74IL, V118I | A98G, K103KN, Y181CY |
| Pt14 | d4T, 3TC, Indinavir | 0 | 756 | 235 | V82A | L63P | M184V | D67N, K70R, T215Y, K219Q | | |
| | | 444 | 400 | 3230 | V32I, K46L, V82A | L63P, A71V | M184V | D67N, K70KR, T215CY, K219Q | | |
| | | 945 | 559 | 1970 | V32I, K46L, V82A, L90M | L10FL, L63P, A71V | M184V | D67N, T215CY, K219Q | | K101Q |
| Pt16 | 3TC, d4T, NVP, Nelfinavir. | 0 | 204 | 346 | M46LM, L90M | L63P, I93L | M184V | D67N | | K103N |
| | | 547 | 402 | 1520 | M46IM, L90M | L63P, I93L | M184V | D67N, K70R, K219E | | K103N |
| | | 987 | 338 | 566 | M46IM, L90M | L63P, I93L | M184V | D67N, K70R, K219E | | K103N |
| Pt17 | AZT, 3TC, Indinavir | 0 | 249 | 2000 | | L63P | M184V | M41L, L210W, T215Y | | |
| | | 518 | 179 | 8245 | M46L, I54V, V82A, L90M | L10I, L63P, A71V | M184V | M41L, L210W, T215Y | | |
| | | 940 | 210 | 4120 | L24IL, M46L, I54V, V82A, L90LM | L10I, M36LM, L63P, A71V | M184V | M41L, E44DE, L210W, T215Y | | |
| Pt18 | 3TC, AZT, Saquinavir, Ritonavir | 0 | 600 | 1301 | L90M | L10I, A71T, V77I, I93L | M184V | M41L, L210W, T215Y | | |

Table II (*Continued*)

| Treatment | Time (d) | CD4 (cells/ul) | VL (Copies/ml) | Specific PR mutations | | Specific RT mutations | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Major mutations | Minor mutations | 3TC | TAMs | Other NRTI mutations | NNRTI mutations |
| | 526 | 593 | 2420 | I84V, L90M | L10I, A71I, V77I, L93L | M184V | M41L, L210W, T215Y | | |
| | 1094 | 626 | 1620 | I54L, I84V, L90M | L10I, A71I, I93L | M184V | M41L, L210W, T215Y | | |
| Pt19  3TC, AZT, Nelfinavir | 0 | 312 | 785 | D30N, N88D | L63P, V77I, | M184V | D67N, K70R, K219Q | | |
| | 385 | 362 | 601 | D30N, N88D | M36I, L63P, A71V, V77I | M184V | D67N, K70R, K219Q | | |

The development of resistance was analysed by calculating the change in the total number of resistance-associated mutations per y for each gene. During the entire study period, the 17 investigated patients displayed an average increase of 0.5 RTI resistance-associated mutations per y (STD = 0.8 mutations per y), while major PI resistance-associated mutations in the gene increased at an average rate of 0.2 mutations per y (STD = 0.8 mutations per y). Minor PI mutations changed 0.3 mutations per y (STD = 0.7 mutations per y).

Using data from the 13 patients with 3 time-points, we further calculated the rate of mutation between time-points 1 and 2 as well as between 2 and 3 separately. RTI resistance associated mutations increased at an average rate of 1.8 mutations per y (STD = 2.7 mutations per y) in the first part of the study period, whereas the average rate of mutation for the second part of the study period was 0.1 mutations per y (STD = 0.4 mutations per y). The mutation rate in the second part of the study period was significantly lower ($p = 0.035$) than the mutation rate in the first part of the study period. The number of major PI mutations increased at 0.2 mutations per y (STD = 1.5 mutations/y) in the first part of the study period and 0.1 mutations per y (STD = 0.7 mutations/y) in the second part of the study, not significantly different ($p = 0.58$). The number of minor PI mutations increased at 0.2 mutations per y (STD = 0.5 mutations/y) in the first part of the study period and 0.3 mutations per y (STD = 0.5 mutations/y) in the second part of the study, not significantly different ($p = 0.25$).

### Pair-wise correlation of resistance mutations

Using Fisher's test for comparison, we also investigated whether any pair of mutations in the RT and PR genes was correlated in the sense that the presence of 1 mutation could predict the presence or absence of a mutation at a second site. No significant pair-wise linkage was observed. We did not test whether more complex patterns of mutations were associated. If the Bonferroni correction for multiple comparisons (in total 703 comparisons are made) is not applied and a significance of 0.05 is used, a possible association between 12 mutations was suggested (Table III).

### No association between mutation and HIV-RNA

Neither the rate at which the number of resistance associated mutations changed, nor the overall rate of nucleotide change was correlated to the HIV-RNA level, when analysed across all patients. For the PR and RT sequence the Pearson linear correlation ($r$)
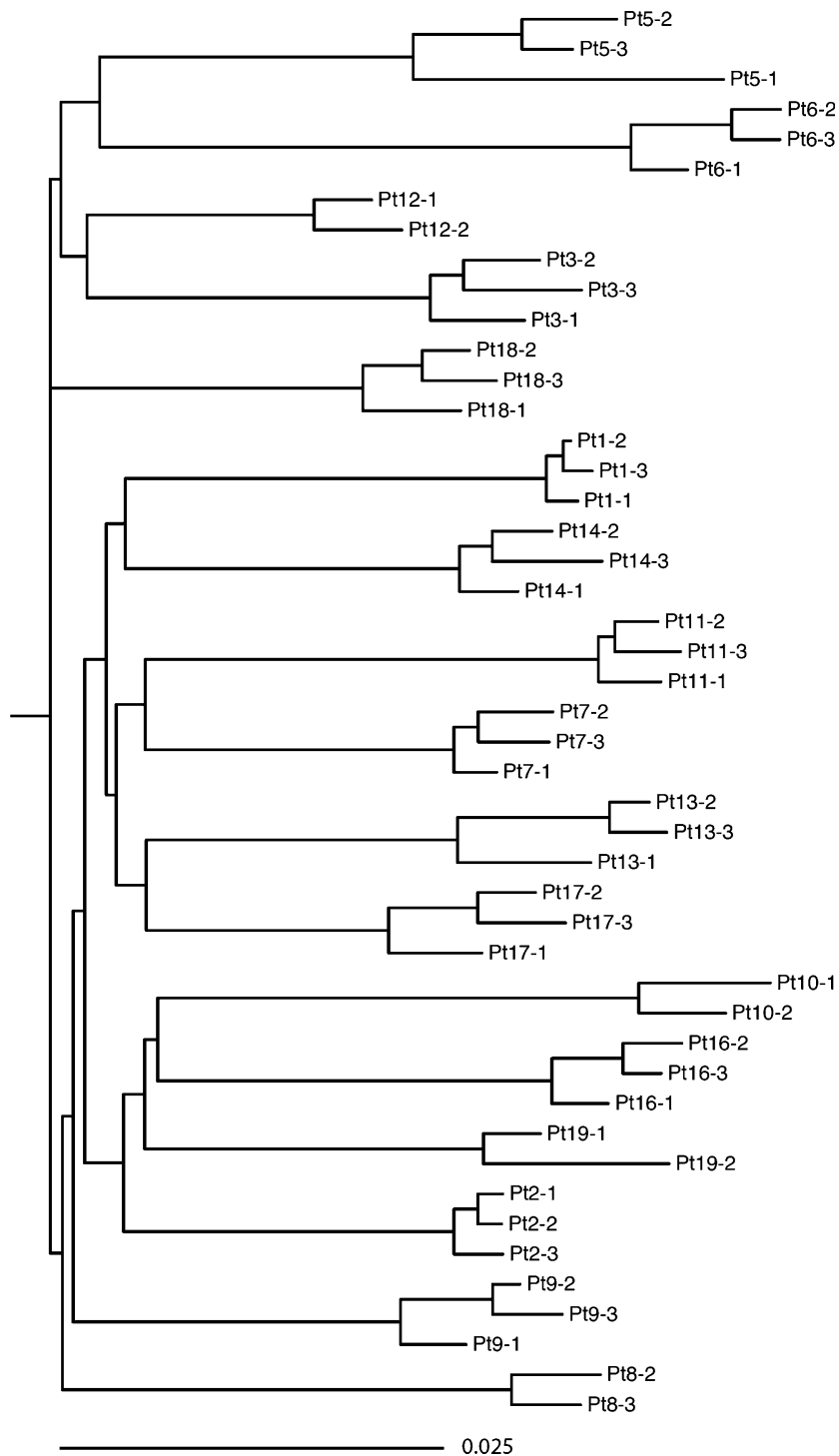
Figure 1. A phylogenetic tree was constructed from all available sequence data. Id-number 10_1 denotes: patient 10, 1st sample. The phylogenetic tree shows that consensus sequences behaved in a reasonable manner: sequences occurred in clusters corresponding to the individual patients from which they were obtained, and branch order was consistent with the actual temporal order of sampling.

between population mutation rate and HIV-RNA level was −0.09 and 0.03, respectively.

## Discussion

From this study concerning the genetic evolution of HIV in patients receiving a stable HAART regimen

with good CD4 response despite ongoing HIV-RNA replication, several tentative conclusions can be drawn.

HIV continued to accumulate new mutations when looking at all sites in the sequenced RT and PI gene. The average mutation rate (silent and non-silent mutations) at all nucleotide sites was

Table III. Possible pair-wise linkage of resistance mutations.

| Position 1 | Position 2 | P-value |
| --- | --- | --- |
| RT219 | RT67 | 0.0004 |
| PR77 | RT118 | 0.005 |
| RT210 | RT41 | 0.009 |
| RT215 | RT41 | 0.01 |
| PR10 | PR54 | 0.02 |
| PR73 | PR77 | 0.03 |
| PR73 | RT118 | 0.03 |
| RT103 | RT181 | 0.03 |
| RT118 | RT98 | 0.03 |
| PR84 | RT210 | 0.04 |
| PR10 | PR84 | 0.04 |
| PR84 | RT41 | 0.04 |

0.014 substitutions site-1 y-1; this mutation rate is very similar to previously published estimates on treatment naïve patients [21–23]. The level of HIV-RNA did not influence the mutation rate. The analysis with regard to resistance mutations showed that 3 out of 17 patients did not develop new mutations during the study period, while 1 patient only acquired minor PI resistance mutations. The remaining 13 patients continued to accumulate resistance mutations. This finding is in agreement with a recent study showing that 75% of patients kept on a stable HAART regimen despite viral loads above 400 copies/ml for more than 2 months developed new drug resistance mutations [24]. Also, our study elucidated several aspects of HIV evolution during a discordant immune response in the face of a constant selection pressure. During the study period, the average rate of amino acid change associated with RTI resistance was 0.5 mutations per y, whereas the average rate of amino acid change associated with PI resistance was 0.4 mutations per y. The rate of mutation associated with RTI resistance decreased during the study period. The change in mutation rate associated with PI resistance was not significant.

The data presented suggest that during viral failure a mutational deadlock can occasionally arise, though more frequently the acquisition of additional resistance mutations will continue to occur though at a declining rate. This is what could be expected; an adaptive population such as a HIV quasispecies will move towards its optimum after the environment has changed and once reached it will stay there until the environment changes again, thus in the face of a constant unchanged HAART it is possible for a mutational deadlock to arise. Furthermore, the quasispecies will probably acquire the most beneficial mutations first and then less beneficial ones will follow at a declining rate as the selection pressure gradually becomes weaker.

It has been shown that in the presence of a selection pressure induced by HAART the single most important factor driving the development of drug resistance is viral replication [25,26]. Interestingly, neither the rate of amino acid change associated with drug resistance nor the rate of mutation at all sites was correlated to the HIV-RNA level, i.e. the mutation rates in patients with high viral loads was not higher than the mutation rates in patients with low viral loads. This implies that a mechanism other than viral replication alone may drive mutation and that viral replication in the face of a constant drug selection pressure is not always sufficient to cause further resistance mutation.

Since treatment effect on CD4 count was an inclusion criterion, a residual effect of treatment could explain the effects observed in this study; however, the extent of such a residual effect on viral replication is not possible to estimate since viral set-point is not available. Several studies have shown the effect of HAART even during viral failure. In a study by Cozzi-Lepri et al. [27] investigating patients kept on a stable HAART despite insufficient viral suppression, it was found that viral load over the ensuing 12-month period increased at a relatively slow rate. Similarly, the Plato Collaboration [28] recently showed that treatment regimens which maintain the viral load below 10,000 copies/ml or at least $1.5 \log_{10}$ copies below off-treatment value, do not seem to be associated with an appreciable CD4 cell count decline.

A reduced fitness of resistant HIV could also explain the discordant response observed in this study. Fitness represents the competitive advantage for propagation of HIV within the patient; fitness is interrelated but not absolutely equivalent to replication capacity [29]. Within the HIV quasispecies present in a patient the variant with the highest fitness will be the dominant. High fitness in vivo combines high replication capacity and best capability of evading selective pressures such as host immune response [30,31], target cell availability [32], tropism [33] and antiretroviral treatment. Thus, in the presence of antiretroviral drugs appearance of resistance mutations will cause increased fitness. Several assays measure fitness by determining replication capacity in vitro, but fail to estimate the cumulative selective pressures within the patients [34]. In this study no in vitro measurement of viral fitness was conducted, but the surrogate marker viral load was available. Despite ongoing viral replication and a constant selection pressure induced by treatment during the study period no increase in viral load was observed. This does not completely exclude

the possibility of a steadily improving viral fitness not reflected in an increasing viral load. However, increased viral fitness would be expected to cause a declining CD4 count. Furthermore, selectively reduced fitness of PI resistant virus in human thymus cells [35] and changes in the HIV-specific cellular immune response towards resistant HIV could explain the discordant outcome [36,37].

The 3TC associated M184V substitution has been suggested to confer benefit and increased susceptibility to drugs other than 3TC [38] and the presence of the M184V mutation could explain the discordant response. However, the COLATE study showed no clinical benefit from continuing 3TC to maintain the M184V mutation in patients switching their HAART regimen after viral failure [39]. In our study 13 out of 17 patients expressed M184V during the entire period of follow-up, and 2 patients lost the M184V mutation during follow-up. Since only 13 patients expressed M184V and 2 of these patients lost this mutation while retaining a discordant immune response, it is unlikely that increased susceptibility to other drugs than 3TC induced by the M184V substitution could cause the observed discordant immune response.

In conclusion, the rate of mutation associated with RTI resistance decreased during the study period. Furthermore, no correlation between HIV-RNA and mutation was observed. Finally, 3 out of 17 patients with a discordant immune response kept on a stable HAART despite viral failure were caught in an apparent mutational deadlock. We hypothesize that the selective pressure induced by an unchanged HAART in certain cases drives the virus onto a 'local hill top in the fitness landscape within the patient', and that, even though higher peaks may exist, the virus has trouble accessing these since it would require several simultaneous mutations to cross the valleys separating these peaks. It is unclear whether the observed situation in certain circumstances is stable and caused by an evolutionary mechanism, or if other mutations than those known to be associated with resistance may eventually make the virus comfortable on another fitness top, or if genetic recombination may enable the virus to access the next fitness top without having to go down the fitness valley. It is also possible that the observed effect is caused by a residual effect of treatment, by reduced fitness of resistant virus, or that the chance of accumulating new resistance mutations simply declines when very many mutations are already present. Thus further studies – preferably comparing a strategy of continued versus changing selection pressure – are warranted.

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