

Clinically relevant interpretation of genotype for resistance to abacavir

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Objective: To develop a stepwise methodology for the development and validation of clinically relevant genotypic score for resistance to antiretroviral drugs and to apply this approach to the genotypic resistance to abacavir.

Methods: All patients having received abacavir during the Narval trial were included in this study. The impact of each nucleoside analogue resistance mutation on the virologic response to abacavir was studied in a univariate analysis. Mutations with a *P* value < 0.20 and those selected by abacavir were retained. According to the number of mutations three levels of resistance were defined. A multivariate analysis accounting for confounding variables assessed whether the genotypic score was an independent predictor of the response. The robustness of the score was analysed using the bootstrap resampling method.

Results: In the 175 patients exposed to abacavir, the strongest association between the decrease in viral load and the number of mutations was observed with a set of six mutations at codons 41, 67, 210, 215, 74 and 184 of the reverse transcriptase gene. In patients with fewer than four mutations (no evidence of resistance) the median decrease in viral load was $-1.64 \log_{10}$ copies/ml while it was $-0.69 \log_{10}$ and $-0.19 \log_{10}$ in those with four (possible resistance) and five or six (resistance) mutations respectively. In the multivariate analysis this score was an independent predictor of the response. The bootstrap analysis showed the robustness of the score.

Conclusions: We developed a new strategy for the analysis of correlation between genotype profile at baseline and virologic response. © 2003 Lippincott Williams & Wilkins

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Introduction

Abacavir is a 2'-deoxyguanosine analogue reverse transcriptase (RT) inhibitor used in antiretroviral combination therapy for the treatment of HIV infected patients. The *in vitro* and *in vivo* patterns associated with resistance to abacavir have been described in several studies.

Sequential *in vitro* passages of a laboratory strain in the presence of increasing concentrations of abacavir selected for mutations K65R, L74V, Y115F and M184V. A combination of at least three mutations was required to produce a decrease in phenotypic susceptibility with an increase in the 50% inhibitory concentration (IC₅₀) of more than eightfold [1]. *In vitro* cross-resistance to abacavir, conferred by mutations selected by the nucleoside reverse transcriptase inhibitors (NRTI), has been shown to arise with M184V plus at least three nucleoside associated mutations (NAM) [1]. The NAM are the six RT mutations, selected by zidovudine and stavudine, including M41L, D67N, K70R, L210W, T215Y/F and K219Q/E. High phenotypic resistance to abacavir is also associated with two multi-NRTI resistance profiles: the Q151M mutation complex and the 69 insertion complex [2,3].

The most common mutations detected in therapy-naïve patients during abacavir monotherapy were M184V and L74V. The mutations K65R and Y115F were detected at very low frequency [4,5]. Very few therapy-naïve patients receiving abacavir in combination with zidovudine and lamivudine had detectable RT mutations other than the M184V at the time of virological failure [6]. The cross-resistance to abacavir resulting from prior NRTI therapy was studied by a meta-analysis of four trials where abacavir was added as a single agent to the background therapy [7,8]. A set of 15 RT mutations was considered in the analysis: the six NAM, the four mutations selected *in vitro* and *in vivo* by abacavir, the mutations T69D/N and V75MSAT and the substitutions corresponding to the multi-drug resistance profiles (F116Y, Q151M and the 69SS insertion). The antiviral activity of abacavir was similar when baseline virus was wild-type or had one, two or three mutations, including M184V. Thirty-five percent of the patients with four mutations or more still achieved a virological reduction, defined in this study as a decrease in viral load of at least 0.5 log₁₀ copies/ml or a viral load < 400 copies/ml at week 12.

The Narval trial was a multicenter trial, conducted in 1999 and 2000, comparing the virological response in patients failing a protease inhibitor containing regimen and randomized into three arms with treatment choice at screening based on phenotype results, on genotype results, or on standard of care [9]. Using this dataset we developed a methodology for constructing genotypic

algorithms and defined a clinically relevant genotype interpretation for resistance to abacavir.

Patients and methods

Study population

In the Narval trial, 541 patients were enrolled and 190 were randomized to phenotype, 192 to genotype and 159 to standard-of-care arms. In this specific study we analysed the virological response in only those patients who were exposed to abacavir during the trial.

Laboratory methods

Plasma HIV-1 RNA assay

Plasma HIV-1 RNA was measured in a single laboratory at day 0 and week 12 by the Amplicor HIV-1 Monitor kit (Cobas 1.5 test, Roche Diagnostic Systems, Meylan, France) with a detection limit of 200 copies/ml.

Genotyping

Plasma HIV-1 RNA was genotyped using the TrueGene HIV-1 kit (Visible Genetics, Toronto, Ontario, Canada). Genotyping was performed at baseline in all patients but only the results for patients enrolled in the genotype arm were made available to the physician. The genotype was interpreted according to the French ANRS algorithm available in 1998 [9]. This algorithm classified the viral isolates of the patients into one of the three following categories: evidence of resistance, possible resistance or no evidence of resistance. As regards the reports of the genotypic results for abacavir, the presence of the mutation T215Y/F associated with M41L and M184V/I, as well as the two multi-drug resistance profiles: Q151M or 69SS insertion, were considered as constituting resistance to this drug. Possible resistance to abacavir corresponded to one of the following profiles: at least three NAM including the T215Y/F, or presence of one of the mutations: M184V/I, K65R, L74V or Y115F.

Statistical analyses

The end-point for the analysis was the change in plasma HIV-1 RNA (log₁₀ copies/ml) between day 0 and week 12. First we evaluated the algorithm which was used for abacavir during the trial by comparing responses for each of the resistance categories. We then analysed the impact of the presence of each mutation associated with resistance to NRTI [10] on response, by comparing the change in plasma HIV-1 RNA in patients with and without the mutation using a non-parametric Mann-Whitney test. Mutations that were present in at least 10% of patients and for which *P* was < 0.20 in the above univariate analysis were retained for further analysis. Mutations known to be selected during use of abacavir (K65R, L74V, Y115F, M184V)

when they were present in more than 10% of patients were also retained and used in the subsequent analysis. We calculated the average response for patients harbouring viruses with an incremental number of mutations (0, 1, 2, etc.) among those retained at the previous stage and studied the association between this number of mutations and the change in plasma HIV-1 RNA using nonparametric Kruskal–Wallis test. Several combinations of mutations were tested and that with the smallest *P* value was retained for further analyses. Taking into account the results observed, three levels of resistance were defined depending on the number of mutations, representing the genotypic score: resistance, possible resistance and no evidence of resistance.

To assess whether or not the genotypic score was an independent predictor of response, a linear multivariate regression was used, accounting for those variables that were predictive of response in the overall multivariate analysis of the Narval trial [11]. Eight variables were included in the analysis: prescription of efavirenz in non-nucleoside reverse transcriptase inhibitor (NNRTI)-naïve patients, prescription of lamivudine, prescription of abacavir in patients who were abacavir naïve, prescription of nelfinavir, baseline plasma HIV-1 RNA, the randomization arm, presence of three or more major protease inhibitor (PI) mutations, previous exposure to PI for more than 30 months. In addition prescription of abacavir as the only NRTI was also accounted for.

Finally we used the bootstrap resampling method to assess the robustness of the score obtained. This approach was initially suggested for cross-validation of the Cox regression model [12]. Univariate and multivariate analyses of the score were performed on 100 samples drawn from the initial 175 patients by sampling with replacement. We report the number of times the score had a *P* value < 0.05 and < 0.10 in the univariate and the multivariate analyses. For the univariate analyses we also report the mean changes in viral load in the three resistance groups, while for the multivariate analyses we report the mean value of the regression parameter and its standard deviation.

Results

Patients baseline characteristics

Of the 541 patients enrolled in Narval, 180 were prescribed abacavir at baseline. Of these 175 had available baseline genotype and viral load measurements at baseline and week 12. Among these 175 patients, 70 were on the phenotype arm, 30 on the genotype arm and 75 on the standard-of-care arm. The baseline characteristics of these 175 patients are presented in Table 1. Of

Table 1. Baseline characteristics of the 175 patients exposed to abacavir.

CDC stage C (%)	44
Mean plasma HIV-1 RNA [\log_{10} copies/ml (SD)]	4.4 (0.9)
Mean CD4 cell count [$\times 10^6/l$ (SD)]	290 (202)
Mean duration (months) of exposure to:	
Antiretroviral agents (SD)	64 (26)
Protease inhibitors (SD)	30 (7)
Median number of previous:	
Antiretroviral agents (range)	7 (3–13)
Nucleoside analogues (range)	4 (2–6)
Protease inhibitors (range)	2 (1–5)
Nucleoside analog exposure [number of patients (%)]	
Zidovudine	171 (98)
Stavudine	159 (91)
Didanosine	150 (86)
Lamivudine	167 (95)
Zalcitabine	83 (47)
Abacavir	21 (12)

the 175 patients, 114 (65%) had three or more NAM at baseline and the median number of major PI mutations was 2 (range, 0–5).

In these patients the nucleoside analogues combined with abacavir were very varied: 43 patients received abacavir as the only nucleoside analogue, 73 and 59 one and two other nucleoside analogues respectively. Of the 175 patients, 120 (69%) had never been exposed to NNRTI and 101 were prescribed this class of drug at baseline, mainly efavirenz (76 patients). The most frequent PI co-prescribed with abacavir was amprenavir, in 65 patients (37%), mainly not boosted by ritonavir.

Virological response to abacavir

The mean decrease in viral load between baseline and week 12 in the patients exposed to abacavir was $1.3 \pm 1.3 \log_{10}$ copies/ml (mean \pm SD).

Using the original Narval genotype interpretation the mean reduction in HIV-1 plasma RNA was $1.4 \pm 1.4 \log_{10}$ copies/ml in the 25 patients with isolates considered susceptible to abacavir while it was 1.4 ± 1.3 and $1.0 \pm 1.3 \log_{10}$ copies/ml in those with isolates scored as possibly resistant (*n* = 79) and resistant (*n* = 71) respectively (*P* = 0.07).

Impact of the NRTI mutations on the virological response

Table 2 shows the univariate analysis of the virological response according to the presence of mutated or wild-type codons at specific sites of the RT gene associated with resistance to NRTI. Six mutations were present in at least 10% of the patients and associated with a reduced virological response to abacavir (*P* < 0.2): M41L, E44D, D67N, V118I, L210W and T215Y/F. Among the NAM the mutations K70R and K219Q/E

Table 2. Mean decrease in HIV-1 plasma RNA according to mutations at specific codons in the reverse transcriptase gene at baseline.^a

Codon	WT (n): decrease in viral load (log ₁₀ copies/ml ± SD)	Mutated (n): decrease in viral load (log ₁₀ copies/ml ± SD)	P
M41L	(58) 1.4 ± 1.3	(117) 1.2 ± 1.3	0.159
E44D	(155) 1.3 ± 1.3	(20) 0.7 ± 1.1	0.028
A62V	(168) 1.3 ± 1.3	(7) 1.2 ± 1.4	0.861
D67N	(85) 1.4 ± 1.4	(90) 1.1 ± 1.2	0.115
T69 (D/N/S)	(142) 1.3 ± 1.3	(33) 1.1 ± 1.3	0.532
K70R	(121) 1.2 ± 1.3	(54) 1.4 ± 1.3	0.394
L74V	(155) 1.3 ± 1.3	(20) 1.0 ± 1.3	0.334
V75T	(165) 1.3 ± 1.3	(10) 0.9 ± 1.1	0.378
F116Y	(171) 1.3 ± 1.3	(4) 0.1 ± 0.6	0.068
V118I	(123) 1.3 ± 1.3	(52) 1.1 ± 1.4	0.116
Q151M	(169) 1.3 ± 1.3	(6) 0.6 ± 1.2	0.151
M184V/I	(69) 1.3 ± 1.3	(106) 1.2 ± 1.3	0.647
L210W	(107) 1.4 ± 1.3	(68) 1.0 ± 1.3	0.016
T215Y/F	1.5 ± 1.4	(135) 1.2 ± 1.3	0.091
T215Y		(109) 1.2 ± 1.3	
T215F		(26) 1.2 ± 1.5	
K219Q/E	(122) 1.2 ± 1.3	(53) 1.3 ± 1.3	0.928
K219Q		(35) 1.5 ± 1.3	
K219E		(18) 0.8 ± 1.3	

^aThe K65R, F77L and Y115F mutations were present in only two patients and the insertion 69SS in only one; no statistical comparisons were performed.

had no impact on the virological response. Among the mutations selected by abacavir, the substitutions L74V and M184V/I were not associated with decreased viral load response and the mutations K65R and Y115F were present in very few patients.

Description of the resistance mutation profiles

Table 3 shows the most frequent combinations of NAM (present in at least 3 % of cases) for 120 of the 175 patients (69%). The T215Y mutation was observed in 109 patients (81%) and the T215F in 26 (19%). The substitution Y or F at codon 215 was not associated with similar profiles of NAM. The mutation L210W, that most strongly associated ($P = 0.016$) with a poor virological response was always, except in three patients, present with T215Y. Similarly the mutation M41L was associated mainly with T215Y and less frequently with T215F. The substitution D67N was combined equally with T215Y or T215F. In contrast the mutations K70R and K219Q were associated with each other and with T215F.

The mutation E44D was always associated with the T215Y mutation. The mutation V118I was more frequently combined with T215Y than with the wild-type 215 codon ($P = 0.0156$). There was no significant association between E44D and/or V118I substitutions and the M184V/I mutation.

Abacavir genotypic score

From the univariate analysis we retained four NAM at codons 41, 67, 210, and 215 and substitutions at codons 44 and 118 which were also associated with reduced response to abacavir. The mutations M184V/I and L74V selected *in vitro* and *in vivo* by abacavir and present in at least 10% of the Narval population were also considered. Table 4 shows the P values of the Kruskal–Wallis analysis of the mean decrease in viral load according to the number of substitutions for eight different sets of mutations.

The strongest association between the decrease in viral load response and the number of mutations was ob-

Table 3. Combinations of nucleoside associated mutations (NAM) detected in at least 3% of patients (n = 120, 69% of the 175 patients). Shaded blocks represent the patients with mutated codons

M41L	D67N	K70R	L210W	T215Y	T215F	K219Q	Number of patients
							11 WT* for NAM
							32 (13 with 44D, 21 with 118I)
							27 (2 with 44D, 2 with 118I)
							22 (3 with 44D, 8 with 118I)
							6 (1 with 118I)
							6 (3 with 118I)
							6 (2 with 118I)
							10 (1 with 118I)

*WT = wild type

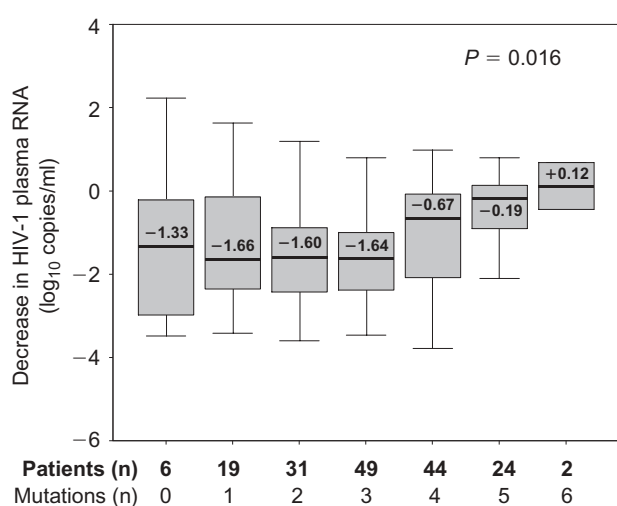
Table 4. Combinations of mutations: *P* value of the univariate analysis (Kruskal–Wallis) assessing the mean decrease in viral load according to the number of mutations in each different set.

Combinations of mutations	<i>P</i>
1: 4 NAM—M41L + D67N + L210W + T215Y/F	0.046
2: 4 NAM ^a + E44D + V118I	0.084
3: 4 NAM ^a + E44D	0.041
4: 4 NAM ^a + V118I	0.081
5: 4 NAM ^a + E44D + V118I + M184V/I + L74V	0.060
6: 4 NAM ^a + V118I + M184V/I + L74V	0.031
7: 4 NAM ^a + E44D + M184V/I + L74V	0.026
8: 4 NAM ^a + M184V/I + L74V	0.016

^aM41L + D67N + L210W + T215Y/F.

served when using combination 8 which included the four NAM, the M184V/I and the L74V mutations ($P = 0.016$). The introduction of M184V/I and L74V in the set of mutations led to a better discrimination of the response as compared with combination 1 which included only the four NAM. Accounting for V118I (combinations 2, 4, 5 and 6) and to a lesser extent for E44D (combinations 3 and 7) decreased the strength of the association between the number of mutations and the reduction in virological response to abacavir.

Fig. 1 shows the median decrease in viral load according to the number of mutations from combination 8. The viral load decrease was significantly lower when the number of mutations increased. To build the resistance score we grouped the number of mutations for which the viral load reduction was similar. When zero to three mutations were present at baseline the median reduction in plasma HIV-1 RNA was $1.6 \log_{10}$ copies/ml whereas it was 0.7 and $0.2 \log_{10}$ copies/ml

**Fig. 1. Median decrease in HIV plasma RNA according to the number of mutations when considering the combination of M41L + D67N + L210W + T215Y/F + M184V/I + L74V.**

in patients with four or more mutations, respectively (Fig. 2; $P = 0.001$). We therefore defined viral isolates as ‘not resistant’, ‘possibly resistant and resistant when they possessed fewer than four mutations, four mutations and more than four mutations, respectively.

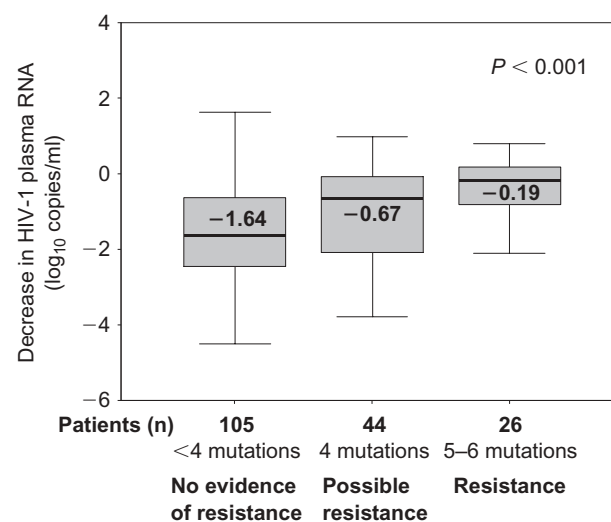
Multivariate and bootstrap analyses

Except for previous duration of exposure to PI, other variables that were predictive of response in the overall multivariate analysis of the Narval trial, were also associated with virological response in the subset of patients exposed to abacavir considered in this study (univariate analysis with $P < 0.2$).

In the 43 patients who received abacavir as the only NRTI the mean reduction in HIV-1 plasma RNA was $1.4 \pm 1.3 \log_{10}$ copies/ml as compared with $1.2 \pm 1.3 \log_{10}$ copies/ml in those having received abacavir combined with other NRTI ($P = 0.07$). This variable was included in the multivariate analysis.

In the multivariate analysis, taking into account all the predictive variables of the Narval trial and the prescription of abacavir as the only NRTI, the abacavir genotypic score was an independent predictor of the virological response at week 12 ($P = 0.005$). Only the prescription of efavirenz in patients naive to NNRTI also remained an independent predictor of the response.

In the 100 bootstrap samples, the *P* value for the univariate analysis was significant (< 0.05) in 93 cases and < 0.10 in 97 cases. For the multivariate analyses the results were slightly less robust as the corresponding

**Fig. 2. Abacavir genotypic score according to the number of mutations among: M41L + D67N + L210W + T215Y/F + M184V/I + L74V.**

figures were 73 and 81 cases respectively. The estimated mean decrease (\pm standard error) in viral load of the 100 bootstrap samples was -1.50 ± 0.14 , -1.11 ± 0.20 , and -0.49 ± 0.20 in the group classified as no evidence of resistance, possible resistance and resistance, respectively. The mean β parameter of the score in the multivariate regression analysis was estimated as 0.357 and its standard deviation as 0.146 ($P = 0.014$).

Discussion

In this study we propose a stepwise methodology for the development and validation of clinically relevant genotypic resistance scores for antiretroviral drugs. Here we applied the method to abacavir, in the context of the Narval trial. Six mutations were found to constitute a resistance score associated with a reduced virological response to abacavir: four of the six NAM at codons 41, 67, 210 and 215 and the mutations L74V and M184V/I. The genotypic score classifies the isolates as no evidence of resistance (0–3), possible resistance (4) or resistance (5–6) according to the number of mutations. Patients with isolates scored as possibly resistant still achieved a virologic reduction of 1 \log_{10} copies/ml in 45% of cases compared with 75% and 23% of cases in those with isolates classified as not resistant and resistant, respectively.

Appropriate interpretation of the results of drug resistance testing is a challenging problem for both phenotype and genotype assays [13]. Genotype interpretation is usually based first on the *in vitro* correlation studies with phenotype. However recent data showed difficulty in determining reliable phenotypic cut-offs for some drugs such as stavudine, didanosine and amprenavir [14,15]. Correlation studies analysing the virological response in treatment-experienced patients according to the genotypic profile at baseline should provide the most relevant information for establishing algorithms [16].

To build up a resistance genotypic score from clinical studies for one individual drug, two types of patient populations might be used. In an ideal situation all the patients included in the study would have received the same antiretroviral drug treatment with the exception of the drug to be studied. These circumstances will no longer or rarely occur as, in the trials evaluating the antiviral effect of a new antiretroviral drug in experienced patients, the choice of the other drugs is usually optimized according to the results of resistance assays. In most cases patients are prescribed a wide variety of different drugs besides the one being analysed. In experienced patients with high inter-subject response variability (in our sample the standard deviation was

estimated as 1.3 \log_{10} copies/ml) a sample size of 125 subjects is needed to detect a difference of 0.5 \log_{10} copies/ml when the mutation is present in 20% of the patients. When the mutation is present in only 10% of the population the required sample size is 220 patients. Moreover, the definition of clinically relevant criteria for resistance genotypes requires multivariate analyses accounting for all the confounding factors. Finally, the results must be validated; one approach for validation is to define the genotypic score in a patient population and then to validate it in another set of patients. Here we propose a methodology using the re-sampling bootstrap method in the validation step, allowing use of the same patient population to define the genotypic score and then evaluate its robustness. The score was robust as demonstrated by the results of the bootstrap analysis.

In our study the patients were highly experienced with a high prevalence of NRTI mutations at baseline: 77% had the T215Y/F substitution and 61% the M184V/I mutation. Nevertheless the mutations K65R and I15Y/F were underrepresented in this population and could not be accounted for in the genotypic score although they could have an impact on the response to abacavir. Similarly the Q151M complex and the 69SS insertion were present in too few patients to assess the response. However they confer high and unequivocal phenotypic resistance to abacavir and isolates with such mutations should be considered as resistant.

Among the six NAM we found that the K70R and K219Q/E mutations were not associated with a reduced virological response to abacavir. This is consistent with a recent study evaluating algorithms for the prediction of abacavir phenotypic resistance from genotype profile in clinical samples, which showed that inclusion of the mutation K70R did not improve the algorithms [17]. Contrary to our study where the D67N is retained in the genotypic score Walter *et al.* showed that the D67N substitution did not improve phenotypic prediction. Our analysis demonstrates that both the number and the type of NAM are important for the prediction of abacavir resistance. A similar conclusion emerged from the studies analysing the virological response to Tenofovir DF according to the NAM at baseline [18].

Interestingly, regarding the type of NAM, two different NAM patterns were observed in this study population with the association of M41L, L210W with T215Y or of K70R and K219Q with or without T215F. The D67N substitution was equally combined with both patterns. The second NAM pattern is much less frequent than the first. These associations between NAM have been reported previously in a large database of NRTI-experienced patients where a significant association between mutations at codons 41 and 210

was observed while the combination of mutations at codons 70 and 210 was never observed [19]. More recently the two distinct patterns of NAM in association with 215Y or 215F have been described in the analyses of antiviral response to Tenofovir DF therapy in antiretroviral experienced patients [18,20].

The E44D and V118I mutations have been described as being selected in the NAM background [17,21]. In our study both mutations were associated with T215Y/F, irrespective of the presence of M184V/I. Although identified in the univariate analysis of correlation with response, we did not retain these two mutations in the genotypic score as their introduction in the set of mutations reduced the strength of the association between the number of mutations and response. In site-directed mutagenesis experiments it has been shown that, in the context of clones already containing the substitutions at codons 41, 210 and 215, the addition of E44D and V118I increased phenotypic resistance to abacavir only slightly [17]. More recently it has been shown on a panel of site-directed mutants that in the background of 41L/67N/210W/215Y, E44D and V118I contribute to moderate abacavir resistance [22].

The single 184V or 74V variants displayed a three- to fivefold reduction in phenotypic susceptibility. The double mutant has a nine- to 10-fold increase in IC₅₀ compared with the wild type virus [1]. While the mutations M184V/I and L74V were not associated with a reduced response to abacavir in the univariate analysis, their introduction in the set of mutations improved the genotypic score.

We have developed a new approach for correlation study between genotype at baseline and virological response to an antiretroviral drug. This strategy is particularly suitable for studies involving patients receiving a wide variety of different drugs besides the molecule being analysed.

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