

Phenotype or virtual phenotype for choosing antiretroviral therapy after failure: a prospective, randomized study

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Background: Resistance testing is useful in the management of virological failure patients, although the best method to be used in clinical practice has not been determined.

Methods: A prospective, randomized, double-blind, multicentre, controlled clinical trial was performed to compare the usefulness of drug resistance testing with a recombinant viral phenotype method or with a virtual phenotype, a genotyping interpretation system. Planned 300 HIV-infected adults failing their current antiretroviral therapy (HIV RNA >1000 copies/ml) were centrally randomized 1:1 to resistance testing with a recombinant viral phenotype method or with a virtual phenotype, after stratifying according to previous drug exposure (one or two versus three drug classes). Percent of patients with HIV RNA suppression (% <400 copies/ml) after 24 weeks was the primary outcome variable. Median HIV RNA concentration and change from baseline in HIV RNA concentration were also used to compare effectiveness. An extended analysis was performed at week 48.

Results: Of the 300 patients enrolled, a total of 276 patients could be analysed; 139 patients were randomized to the phenotype group and 137 patients were randomized to the virtual phenotype group. After 24 weeks of follow-up, 46.8 and 56.2% of patients had HIV RNA <400 copies/ml ($P=0.1$) in the phenotype and virtual phenotype, respectively. Mean decrease from baseline in viral load was 1.0 and 1.3 log copies/ml in the phenotype and virtual phenotype groups, respectively ($P=0.017$). In a multivariate linear regression analysis, after adjusting for baseline HIV RNA and adherence to treatment, the virtual phenotype was associated with a greater mean decrease in plasma HIV RNA ($P=0.0063$). The results observed at week 48 were similar.

Conclusions: Virtual phenotype is at least as effective as phenotype when used to select an optimized treatment for patients who have failed one or more antiretroviral regimens.

Introduction

The treatment options for patients infected with HIV-1 have increased importantly over the last several years [1]. Nonetheless, a considerable proportion of patients fail treatment despite the availability of numerous effective drugs. Viral, immunological and treatment-related factors have been identified as reasons for this failure [2-4]. Adherence and baseline resistance of HIV to one or more of the chosen drugs or the emergence over time of resistance mutations have been identified as principal reasons [5-9].

Several studies have demonstrated the utility of resistance testing in the long-term management of the

HIV-infected patient [10,11]. Recent recommendations by different expert panels have consistently recommended testing for drug sensitivity in certain patient populations [5,12,13]. Drug sensitivity can be evaluated using the HIV genotype for key sequences within the reverse transcriptase or the protease gene of HIV or by direct determination of the drug sensitivity phenotype [5]. Genotype assays provide information about viral mutations that may result in changes in viral susceptibility to particular drugs or classes of drugs. Phenotype assays directly quantitate the level of susceptibility of a patient's virus sample to specific

drugs *in vitro*. Each of the two methods has clear advantages and inconveniences. Determination of a drug sensitivity phenotype provides information that seems relatively straightforward in interpretation, while the interpretation of a genotype assay is more complex. However, phenotyping is a more labour-intensive and expensive method.

Virtual phenotyping is an interpretation of genotypic data based on a database linking protease and reverse transcriptase gene sequences from more than 20 000 viral strains with the corresponding observed phenotype. An intelligent neural network transforms the genotypic information into the most likely associated phenotype [14]. A high degree of correlation with phenotype has been demonstrated, and has proved it to be accurate predictors of virological response [14,15]. There are no reports on the clinical utility of virtual phenotype compared to other resistance testing methods.

In this prospective, randomized clinical trial we compared the usefulness of phenotyping, using standard recombinant methods, to the usefulness of virtual phenotyping in selecting an effective follow-up anti-retroviral regimen in patients who were failing their current treatment.

Materials and methods

Study population and clinical setting

Patients were enrolled from five outpatient HIV/AIDS clinics in Spain of five different hospitals (Ramón y Cajal, La Princesa, La Paz, Alcalá de Henares, from Madrid and Virgen de las Nieves, from Cuenca) in Spain. A local institutional review board at each centre approved the study and written informed consent was obtained for each patient participating in the study.

Adult patients (>18 years) failing their prescribed antiretroviral regimen were eligible for the study. Patients must have received their current regimen for at least 3 months. Failure was defined as a plasma HIV RNA concentration >1000 copies/ml measured on at least two consecutive occasions. Subjects were excluded if they were naive to antiretroviral therapy, were suspected to be poor adherent to their treatment or had been off antiretroviral therapy for greater than 21 days.

Randomization and patient management

Patients were randomized centrally (1:1) to be tested for resistance using either a recombinant viral phenotype or a virtual phenotype. Patients were stratified prior to randomization according to previous antiretroviral treatment with one or two drug classes [nucleoside reverse transcriptase inhibitor (NRTI), protease inhibitor (PI) or non-NRTI (NNRTI)] versus three drug classes.

Patients, clinicians and statisticians were blinded to randomization. A novel resistance testing report was used in this study, in order to assure the blinded nature of the test results. This report was generated in the central coordinator laboratory. Susceptibility was reported as sensitive, intermediate and resistant, for all commercially available drugs. The original reports (Antivirogram[®], genotype and virtual phenotype, either VircoGen I or II[®]) were not sent to clinicians. Therefore, the exact IC₅₀ and the genotype were not available for prescribing the new combination in the phenotype and virtual phenotype arms, respectively.

Occasionally, no result could be obtained for a patient using the resistance testing method as randomized. If no result could be obtained after two attempts, the patient was tested using the alternative method. According to intent-to-treat rules, however, the patient was included in the primary analysis according to his original randomization.

Determination of HIV RNA concentrations were performed locally using either an ultrasensitive branched-DNA assay (Quantiplex, V 3.0, Bayer, Emeryville, Calif., USA) or RT-PCR (Amplicor HIV monitor standard version, Roche Diagnostic Systems, Branchburg, NJ, USA).

Antiretroviral treatment available included all approved drugs by EMEA (the European agency for the evaluation of medical products). Lopinavir/ritonavir was available on expanded access, but no resistance information was provided in the test results for this drug.

Viral load assessment, immunological and clinical evaluations were performed at 12, 24 and 48 weeks.

Laboratory resistance testing methods

Phenotype

Phenotypic resistance testing was performed by Virco laboratories, using the recombinant virus assay described by Hertogs *et al.* [5] with minor modifications (Antivirogram[®], Virco, Mechelen, Belgium). The results of the analysis were expressed for each drug as the fold-increase in IC₅₀ relative to a reference wild-type virus isolate (HIVIII_B/LAI). Sensitive was defined as <fourfold increase), intermediate as a four- to 10-fold-increase and resistant as >10-fold increase.

Virtual phenotype

The laboratory of Infectious Diseases, Ramon y Cajal Hospital, determined the HIV genotype using reverse transcription PCR of a plasma-derived viral RNA, extracted using Qiagen columns. Standard dideoxiterminators were used (ABI377 sequencer) according to the manufacturers' instructions. The PCR product encompassed the entire protease gene and the first 250

amino acids of the reverse transcriptase gene [5]. Results of the consensus sequence analysis were transmitted to Virco, where it was evaluated with proprietary software (VircoGen I or II[®], Virco, Ireland) that predicts the drug susceptibility phenotype via interrogation of a large database of HIV-1 clinical isolates with previously determined viral genotypes and drug susceptibility test results. For each drug, key mutations in the patient's viral genotype are identified and summarized as a mutational profile, which details the presence (and absence) of mutations affecting drug susceptibility. The number of isolates in the database with the same mutational profile and the mean fold change in IC₅₀ for these previously tested isolates is reported. As with the phenotype, sensitive was defined as <fourfold increase, intermediate as a four- to 10-fold-increase and resistant as >10-fold-increase in the versions of the virtual phenotype used in this study.

Results of the resistance test were sent directly to the Infectious Diseases Laboratory, Ramon y Cajal Hospital, and the unique, blinded report was generated for the clinician by the laboratory coordinator.

The central virology laboratory used in this study was accredited by an international quality control program (ENVA). Additionally, Virco validated the resistance testing procedures used for this study.

Statistical methods

A sample of 300 patients was estimated to be necessary to demonstrate equivalence between both methodologies, a two-sided χ^2 was performed, assuming α and β errors of 0.05 and 0.10, respectively, for detecting a relevant difference of 20% between the two resistance methods, with an estimated dropout rate of 10%. Furthermore these two strata (stratum-1 patients exposed to two or less drug classes and stratum-2 patients exposed to three drug classes) were taken into account before the randomization. The main outcome measure was a sustained antiviral effect measured as the proportion of subjects at 24 weeks with serum concentrations of HIV RNA <400 copies/ml. Median HIV RNA concentration and median change from baseline at 24 weeks were also compared between the randomization groups, using Gehan's modification of Wilcoxon's test to allow for censoring [6].

Demographics, antiretroviral treatment history and adherence to the previous regimen were evaluated at baseline. Adherence was evaluated by patient self-reports and from the hospital pharmacy dispensation records and categorized as >90% of prescribed doses or <90% of prescribed doses.

An intent-to-treat analysis was performed, in which all patients were considered according to the original randomisation. Patients for whom no resistance test result could be obtained using the test method as

randomized and who were then tested according to the alternative method were, nonetheless included in this analysis as randomized. Only patients lost to follow-up before starting treatment and patients who died before receiving the test result were excluded from this analysis.

A secondary, as-treated analysis was performed in which only patients who were tested according to their randomization were included. Patients without a result using the method as randomized were excluded from this analysis.

A by-stratum analysis was also performed according to the prior history of exposure to antiretroviral drugs. In this analysis, patients with a treatment history that included antiretrovirals from one or two of the three classes of these drugs were analysed separately from the patients who had prior exposure to all three classes of antiretrovirals.

Patients without a viral load result at 24 or 48 weeks, or who had changed treatment due to failure were considered virological failures. However, patients who switched from their newly prescribed regimen for other reasons, such as toxicity, intolerance or convenience were included in the analysis according to the actual HIV RNA concentration determined at 24 or 48 weeks.

Linear model accounting for censored measurement were used to multivariate analysis [17], using SAS V8.2.

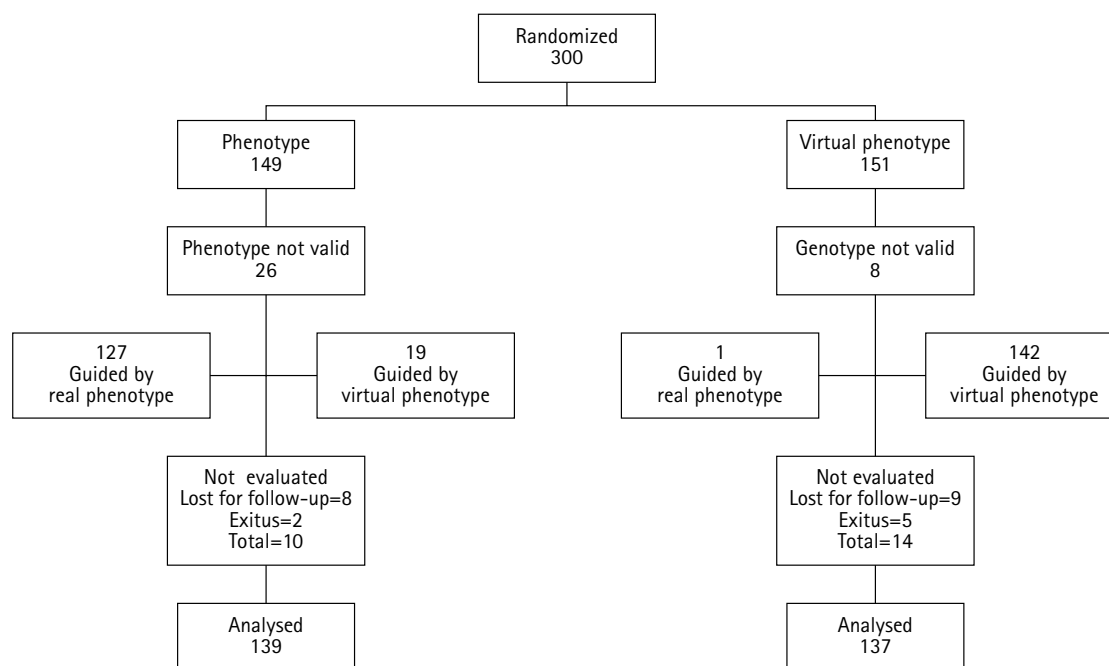
Results

Population distribution and baseline demographics

Between February 2000 and February 2001, 300 patients were enrolled in the study; 149 patients were assigned to resistance testing using a phenotype method, and 151 patients were assigned to resistance testing using a virtual phenotype. Ten patients from the phenotype group and 14 patients from the virtual phenotype group were excluded from the 24-week analysis as lost-to-follow-up or dead. As a result, there were 139 subjects in the phenotype randomization group and 137 subjects in the virtual phenotype group (Figure 1).

A valid test result could not be obtained at entry for 26 of the patients randomized to the phenotype group. These subjects were then tested using the virtual phenotype. In contrast, a result was not obtained for only eight patients randomized to the virtual phenotype arm of the study. These patients were included in the intent-to-treat analysis according to their randomization. A valid result was obtained with the virtual phenotype method for 19 of the 26 patients originally randomized to the phenotype group for whom a valid result could not be obtained. A phenotype was alternatively obtained for only one patient of the eight who could not be successfully genotyped.

Figure 1. Flow chart of patients after randomization



No differences in baseline characteristics were apparent between arms (Table 1).

Baseline drug resistance information and prescribed antiretroviral treatment

No statistically significant differences were detected in baseline antiretroviral regimens (Figure 2). There was a more frequent use of protease inhibitors boosted with ritonavir in patients randomized to virtual phenotype than on patients in the phenotype group (87 vs 78%), whereas more protease inhibitor-sparing regimens were used in the phenotype arm (42 vs 35%).

Baseline resistance to NRTIs, PIs and NNRTIs, was found in 85.5, 64.1 and 57.6% of the patients, respectively. More HIV isolates from subjects in the phenotype group were shown to be sensitive to zidovudine, didanosine, abacavir and ritonavir than in the virtual phenotype group (Figure 3).

Antiviral response

The percentage of patients with an adherence higher than 90% was similar in the two groups (86 and 89% in the phenotype and virtual phenotype groups, respectively). The median decrease in viral load observed after 24 weeks of treatment with the newly prescribed regimen was 1.0 and 1.3 log copies/ml in the phenotype and the virtual phenotype groups, respectively ($P=0.017$).

In the intention to treat analysis, the percentage of patients with HIV RNA <400 copies/ml at 24 weeks

was 46.8 and 56.2% ($P=0.1$) for the phenotype and virtual phenotype groups, respectively. The difference in median viral loads was maintained at weeks 36 ($P=0.052$) and 48 ($P=0.031$) (Figure 4).

In a multivariate linear regression analysis (accounting for censoring measures) after adjusting for baseline HIV RNA concentration and adherence to treatment, the difference still favoured the virtual phenotype arm of the study [0.47; 95% CI: 0.13–0.82, $P=0.0063$].

A separate analysis of each stratum was performed. In patients with a history of treatment with only one or two of the classes of antiretrovirals, a trend towards a better virological response were observed in median HIV RNA concentration (2.4 vs 2.27 log copies/ml) and in the median decrease from baseline (1.15 vs 1.35 log copies/ml, $P=0.15$) in HIV RNA concentration, at 24 weeks, between the phenotype and virtual phenotype arms of the study. Furthermore, when the analysis was restricted to patients who had received treatment with drugs from all three classes of antiretrovirals, a greater benefit was seen in patients randomized to the virtual phenotype arm (0.81 vs 1.3, $P=0.02$); however, no statistically significant differences were found between both stratum in terms of viral load decrease.

An as-treated analysis was also performed, excluding patients for whom a result could not be obtained by the method to which they were originally randomized (113 and 129 for the phenotype and

virtual phenotype groups, respectively). The results of the as-treated analysis were no different from the results of the intent-to-treat analysis (data not shown).

No difference was detected between the groups in CD4 cell number, CD4 cell count increased to a median 407 cells/mm³ in the phenotype group compared to a median 391 cells/mm³ in the virtual phenotype group.

An extended analysis was performed after 48 weeks of follow-up. A total of 129 patients in the phenotype group and 127 patients in the virtual phenotype group could be analysed. HIV RNA below 400 copies/ml was reached in 39% and 51% of the phenotype and virtual phenotype groups, respectively ($P=0.06$). A trend towards a higher viral load drop was observed in the virtual phenotype arm at 48 weeks (1.3 vs 1.03 log₁₀ copies/ml) $P=0.163$. No differences were observed between groups in the number of HIV-associated clinical events (8.7 and 7.8% in the virtual and the phenotype groups, respectively) or deaths (0.8 and 2.3%, respectively).

Discussion

Testing for viral resistance to available treatment regimens is critical in the long-term clinical management of the HIV-infected patient [9,10]. This paradigm becomes all the more true as the number of drugs available for the treatment of HIV increases, the numbers of patients who have received multiple treatments for their disease increases, and the risk of transmission of drug-resistant strains of HIV increases. All of these elements work together to make the treatment decisions progressively complex. Consequently, resistance testing is widely accepted as valuable even though our experience with the testing methods available and interpretation of the results is still developing.

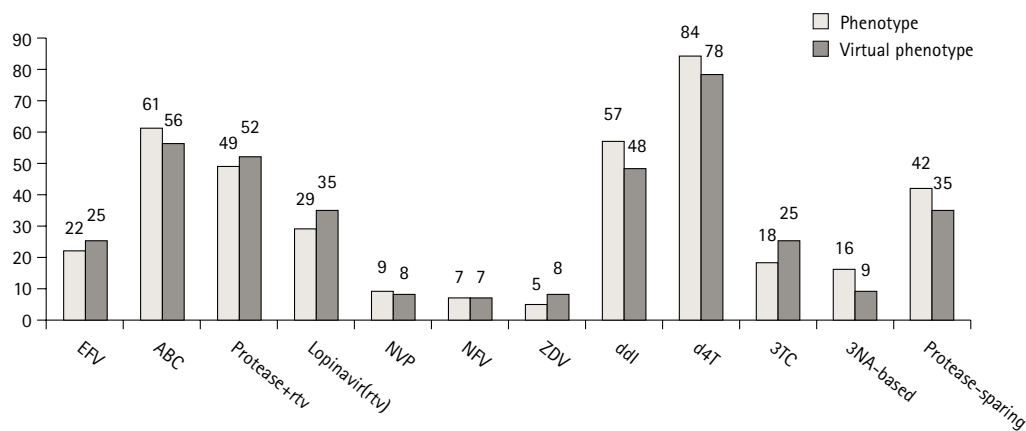
The results of this study indicate that virtual phenotype was at least as useful as phenotype in selecting an effective new treatment regimen for patients who were failing their current therapy. Preliminary results of another study comparing phenotype and virtual phenotype have shown a similar efficacy, of the regimen chosen by the two methods, to reduce viral load [16]. The virological benefit of the virtual phenotype was detected clearly in subjects who had previously received treatment with drugs from all three classes of antiretrovirals, but differences were not found between both groups. Similar results have been obtained in another study evaluating long-term efficacy of resistance testing [17]. The virtual phenotype arm had a better virological outcome in terms of HIV RNA decrease; the more complex mutational profile of the viral population may have been better interpreted by this methodology, which is based on genotyping [11].

Table 1. Baseline characteristics by randomization arm

	Phenotype	Virtual phenotype
<i>n</i> =276	139	137
Male sex [<i>n</i> (%)]	101 (73)	107 (78)
Route of HIV acquisition IVDA [<i>n</i> (%)]	76 (55)	71(52)
Age median [IQR]	37 [34–41]	38 [31–45]
Prior AIDS diagnosis [<i>n</i> (%)]	55 (41)	53 (39)
HIV RNA log ₁₀ copies/ml (median [IQR])	3.95 [3.4–4.6]	4.0 [3.4–4.7]
CD4 cells/μl (Median [IQR])	332 [162–482]	276 [169–476]
Adherence >90% of doses prescribed with previous regimen [<i>n</i> (%)]	99 (71%)	101 (74%)
Prior length of antiretroviral therapy, Months, median [IQR]	63 [42–82]	60.5 [43–84]
NRTI (276)	62 [42–82]	59.5 [43–84]
PI (248)	38 [29–45]	40 [27–46]
NNRTI (87)	15 [8–21]	12 [6–19]
Number of drug classes prior to resistance testing		
1 [<i>n</i> (%)]	7 (5)	5 (3.6)
2	53 (38)	49 (36)
3	79 (57)	83 (61)
Pre-test antiretroviral washout (%)	4	4
Days, median [IQR]	22.5 [7–30]	24 [3–30]
Post-test antiretroviral washout (%)	22	20
Days, median [IQR]	91.5 [67–182]	87 [45–121]
PI washout prior to resistance test (<i>n</i>)	48	37
Days, median [IQR]	161 [76–265]	260 [119–345]
Delay from test to switch antiretroviral therapy (<i>n</i>) days, median [IQR]	73.5 [47–104]	75 [53–103]
Attending clinicians test fidelity (%) median [IQR]	100 [83–100]	100 [80–100]
Number of active drugs prescribed median [IQR]	3 [3–3]	3 [2–3]

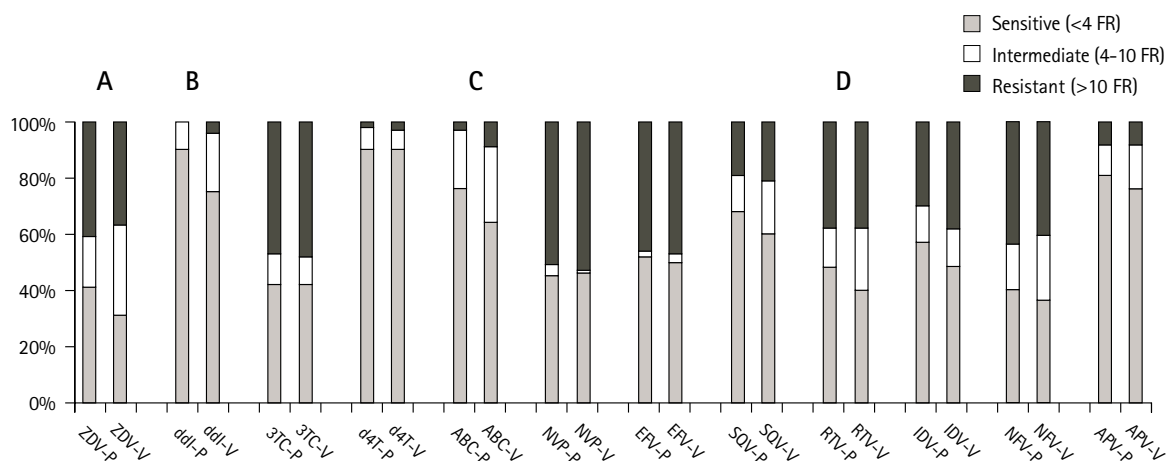
IQR, interquartile range.

Figure 2. Baseline treatment and strategies prescribed according to randomization arm



EFV, efavirenz; ABC, abacavir; Protease+rtv, a protease inhibitor boosted with ritonavir; NVP, nevirapine; NFV, nelfinavir; ZDV, zidovudine; ddl, didanosine; d4T, stavudine; 3TC, lamivudine; 3NA-based, three-nucleoside analogue-based regimen; protease sparing, protease-sparing regimen.

Figure 3. Baseline resistance pattern according to randomization arm



P, phenotype; V, virtual phenotype. (A) Phenotype 41% sensitive, virtual phenotype 31% ($P=0.038$); (B) phenotype 90% sensitive, virtual phenotype 75% ($P=0.005$); (C) phenotype 76% sensitive, virtual phenotype 64% ($P=0.037$); (D) phenotype 48% sensitive, virtual phenotype 40% ($P=0.048$).

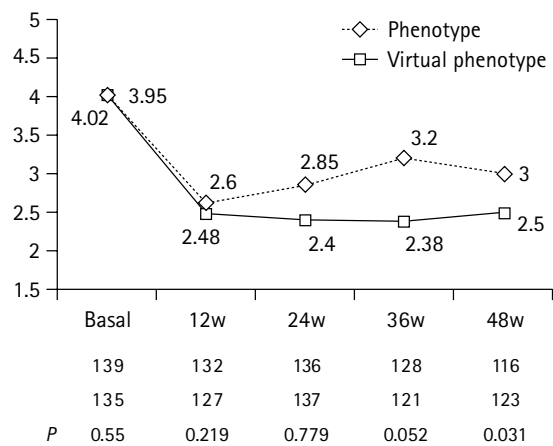
While the reasons for this outcome are unclear, it could be hypothesized that the complex interaction of multiple drug resistance mutations is not adequately reflected in the IC_{50} for these drugs and the average measure, which results of virtual phenotype reflects more accurately the viral strain susceptibility.

The percentage of patients in this study with a viral response to the prescribed treatment regimen was similar to that observed in previous studies with similar design, regardless of resistance testing method. However, the evidence for the usefulness of resistance testing from prospective clinical trials is variable. The results from four earlier studies, VIRADAPT, GART, Havana and NARVAL [10,11,18,19] indicated that treatment decisions based on interpretation of HIV genotype are more

effective than treatment decisions based on consensus recommendations and patient history. On the other hand, three studies, VIRA3001, CCTG 575 and NARVAL [19–21], failed to detect a widely applicable benefit of treatment decisions based on the drug resistance HIV phenotype. The results of these latter studies may be confounded by inadequate understanding of the clinical significance of an increased IC_{50} as well as the limited numbers of active drugs that remain to be administered to heavily pre-treated patients with highly cross-resistant strains of virus.

A result was obtained with the virtual phenotype for 95% of patients while a result was obtained for only 82% of subjects randomized to phenotyping. This result probably reflects the technical ease associated with genotyping HIV compared to the complexity of

Figure 4. Decrease in median HIV RNA concentration (\log_{10} copies/ml) throughout 48 weeks



the methods required to establish a phenotype. This reliability is a clear advantage favouring the use of a virtual phenotype.

Resistance testing based on the viral genotype requires a method for adequately interpreting the complex data that are acquired. Interpretation of the changes detected in the reverse transcriptase and protease genes of HIV and translating these changes into an effective new treatment regimen is beyond the expertise of many clinicians who have had to turn to expert advice for a recommendation for new treatments based on this complex information. However, as the number of antiretroviral drugs increases along with our understanding of the mutational patterns that emerge during long-term treatment with combinations of these drugs, such interpretation will likely be beyond any one expert or panel of experts [22].

Rule-based genotype interpretation is an alternative to the expert panel. Such interpretations, based on paradigms derived from the scientific literature, however, are limited by rapid changes in this field and the difficulty of establishing a robust paradigm, some discordance have been identified between methods, and they have not been designed and validated to predict virological response [23].

A virtual phenotype combines the characteristics of genotyping and phenotyping. The clinician must only provide the HIV genotype for the patient in question and receives, in return, the average of historical phenotypes associated with similar protease and reverse transcriptase mutations at critical sequences. IC₅₀ information obtained from a virtual phenotype is essentially the average from all phenotypes within the database associated with the submitted genotype. Relevant clinical cut-offs should be identified for a better performance of this interpretation methodology.

Conclusions

A high reliability combined with the accuracy in defining an active new regimen reported in this study makes virtual phenotyping a highly valuable clinical tool for the long-term management of HIV-infected persons. Studies are needed that compare the performance of virtual phenotype with other genotyping interpretation systems.

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