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Improved Prediction of Salvage Antiretroviral Therapy Outcomes Using Ultrasensitive HIV-1 Drug Resistance Testing

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Background. The clinical relevance of ultrasensitive human immunodeficiency virus type 1 (HIV-1) genotypic resistance testing in antiretroviral treatment (ART)-experienced individuals remains unknown.

Methods. This was a retrospective, multicentre, cohort study in ART-experienced, HIV-1-infected adults who initiated salvage ART including, at least 1 ritonavir-boosted protease inhibitor, raltegravir or etravirine. Presalvage ART Sanger and 454 sequencing of plasma HIV-1 were used to generate separate genotypic sensitivity scores (GSS) using the HIVdb, ANRS, and REGA algorithms. Virological failure (VF) was defined as 2 consecutive HIV-1 RNA levels ≥200 copies/mL at least 12 weeks after salvage ART initiation, whereas subjects remained on the same ART. The ability of Sanger and 454-GSS to predict VF was assessed by receiver operating characteristic (ROC) curves and survival analyses.

Results. The study included 132 evaluable subjects; 28 (21%) developed VF. Using HIVdb, 454 predicted VF better than Sanger sequencing in the ROC curve analysis (area under the curve: 0.69 vs 0.60, Delong test $P = .029$). Time to VF was shorter for subjects with 454-GSS < 3 vs 454-GSS ≥ 3 (Log-rank $P = .003$) but not significantly different between Sanger-GSS < 3 and ≥3. Factors independently associated with increased risk of VF in multivariate Cox regression were a 454-GSS < 3 (HR = 4.6, 95 CI, [1.5, 14.0], $P = .007$), and the number of previous antiretrovirals received (HR = 1.2 per additional drug, 95 CI, [1.1, 1.3], $P = .001$). Equivalent findings were obtained with the ANRS and REGA algorithms.

Conclusions. Ultrasensitive HIV-1 genotyping improves GSS-based predictions of virological outcomes of salvage ART relative to Sanger sequencing. This may improve the clinical management of ART-experienced subjects living with HIV-1.

Clinical Trials Registration. NCT01346878.

Keywords. HIV-1; antiretroviral drug resistance; deep sequencing; salvage antiretroviral therapy; genotypic susceptibility score.

Choosing the right antiretroviral therapy (ART) is one of the most cost-effective actions in medicine [1, 2]. Effective ART provides enormous benefits in health status, survival, and quality of life to people living with human immunodeficiency virus type 1 (HIV-1) [3, 4]. It also delivers important benefits to our society, including virtual elimination of onward HIV-1 transmission in ideal conditions of treatment access and adherence [5, 6]. Antiretroviral drug resistance testing provides key information to clinicians to select the optimal ART for each patient [7]. Surveillance of transmitted

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and acquired antiretroviral drug resistance is essential for public health policies to control the HIV/AIDS pandemic [8–12].

During more than a decade, genotypic HIV-1 drug resistance testing has relied on inferring antiretroviral susceptibility from HIV-1 sequences obtained by Sanger sequencing of plasma viruses. This approach is effective, fast, affordable, and easy to standardize and implement in routine diagnostic laboratories. However, Sanger sequencing only provides a consensus sequence reflecting the most abundant viral variants (>15%–20%) in the HIV-1 population for each patient. Thereby, clinicians miss potentially relevant information on low-frequency drug-resistant (LFDR) HIV when making their treatment choices [13]. Accounting for such additional information could improve ART design and therefore provide further clinical benefits to people living with HIV.

Antiretroviral-experienced subjects with multidrug-resistant HIV-1 are our most difficult-to-treat patients and could highly benefit from more precise resistance evaluations. Failure to suppress viral replication with subsequent salvage ART might result in exhaustion of treatment options and increased mortality [14]. Although data analysing the impact of LFDR HIV and minority CXCR4 viruses on first-line antiretroviral therapy (ART) outcomes has accumulated in recent years [15–26], the clinical relevance of ultrasensitive genotyping in treatment-experienced individuals remains largely unexplored.

METHODS

Study Design

This was a retrospective, multicentre, cohort study developed in 4 Spanish HIV tertiary care academic hospitals. The study was registered at Clinicaltrials.gov under the ID: NCT01346878.

Subjects

Study participants were ART-experienced, HIV-1-infected adults who initiated salvage ART including, at least 1 ritonavir-boosted protease inhibitor (PI/r), raltegravir (RAL), or etravirine (ETR). Subjects had to have 1 mL of plasma available for genotypic resistance testing with HIV-1 RNA levels ≥5000 copies/mL within 48 weeks before treatment change (baseline).

Data Collection

The demographic and clinical characteristics of study participants, including HIV-1 RNA and CD4+ T-cell counts were obtained from the clinical records of each participating centre and collected in a standardized, curated, and study-dedicated database (TherapyEdge-DeepCheck-HIV, ABL S.A., Luxembourg) centralised at the irsiCaixa AIDS Research Institute. Virological failure was defined as 2 consecutive HIV-1 RNA levels ≥200 copies/mL at least 12 weeks after salvage ART initiation and while subjects were still on the same ART. Due to its retrospective nature, no formal adherence evaluation was available to this study.

Sanger HIV-1 Drug Resistance Testing

The protease (PR) and reverse transcriptase (RT) genes were evaluated in all participants with either the TRUGENE HIV-1 Genotyping Assay (Siemens Healthcare, Spain) or the ViroSeq HIV-1 Genotyping System (Abbott Molecular, Spain). The integrase (IN) gene was sequenced in subjects initiating RAL using an in-house HIV-1 sequencing method (Supplementary methods).

454 Sequencing

Ultrasensitive HIV-1 genotyping was performed using 2 overlapping amplicons for PR, 5 for the RT and 4 for the IN, which was only sequenced in subjects initiating salvage ART including RAL. 454 sequencing was performed at the irsiCaixa AIDS Research Institute using a 454 GS FLX equipment with Titanium chemistry. According to sequencing strand-dependant error patterns and negative control testing results, only those variants showing frequency values on forward and reverse reads within a 1 log ratio and an overall frequency >0.5% were used for downstream analysis (Supplementary methods).

Genotypic Sensitivity Scores

Separate genotypic sensitivity scores were generated for Sanger (Sanger-GSS) and 454 (454-GSS) information using HIVdb (v6.3.1), ANRS (v2012.09), and REGA (v9.1.0) algorithms, by interfacing with the Stanford HIV-1 Web Service (Sierra, beta v1.0.1). All algorithms were interpreted using a Sensitive/Intermediate/Resistance (S/I/R) scale, corresponding to 1/0.5/0 scores, except for the REGA PI/r S/I/R scores, which were 1.5/0.75/0, respectively. A score of 1 was added per each maraviroc (MRV) and enfuvirtide (T-20) included in the regimen, making the conservative assumption that subjects initiating these drugs had a virus fully susceptible to them. When IN sequences were available, they were used for GSS calculations; otherwise, a score of 1 was added if the subject initiated RAL.

Statistical Analysis

The characteristics of the study population were described overall and according to virological outcomes. Differences in baseline characteristics between subjects with and without virological failure were evaluated for statistical significance using the χ^2 or Fisher exact test for categorical variables and the Mann Whitney rank sum test for continuous variables, as needed. The effect of 454 over Sanger sequencing on GSS categories was described using bubble plots where the diameter of each bubble was proportional to the number of subjects included in each combined category. The overall ability of Sanger and 454-GSS to predict virological failure was compared using by receiver operating characteristic (ROC) curves. Pairwise

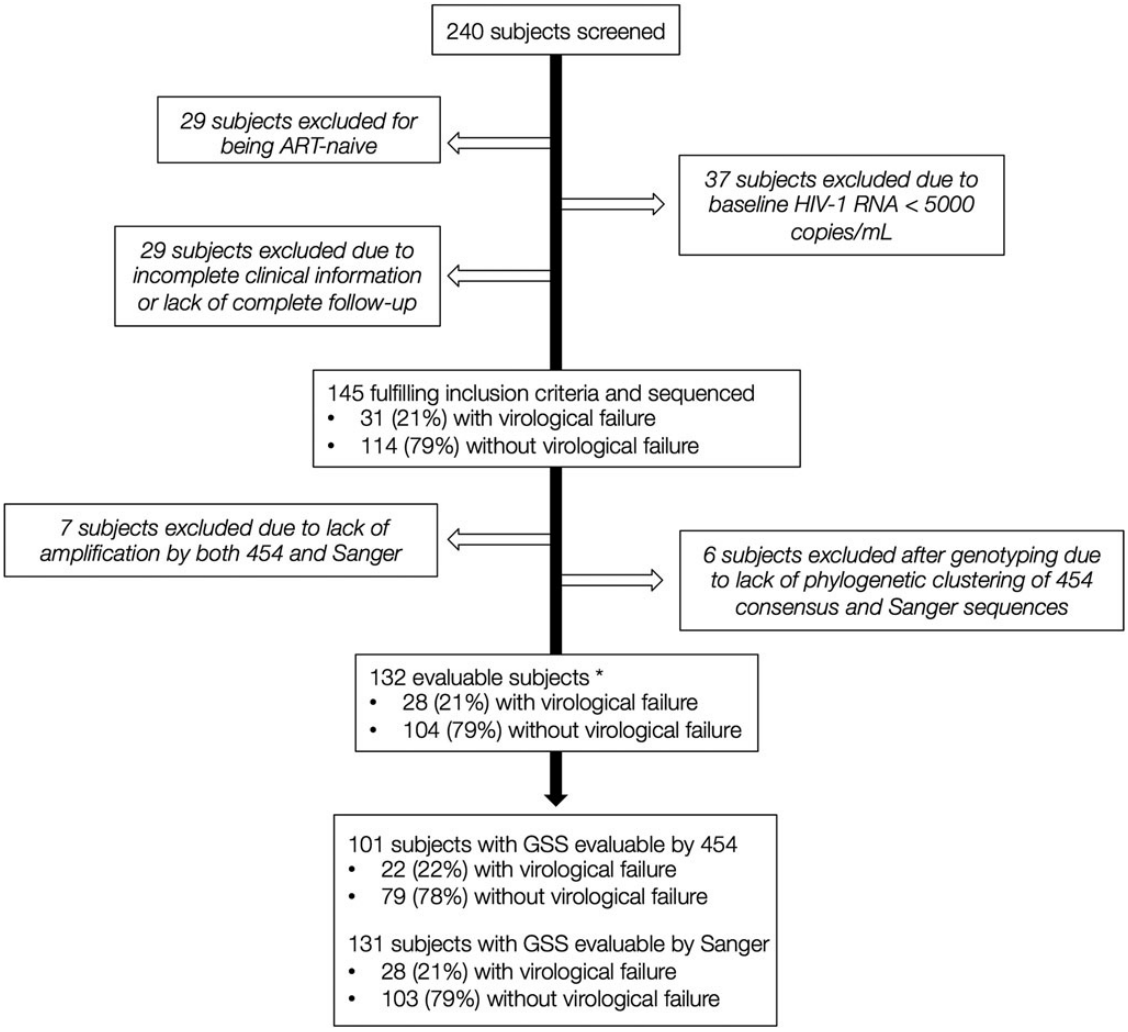

Figure 1. Subject disposition. This diagram summarizes the reasons for inclusion/exclusion of subjects in the study and the number of subjects evaluable by the 454 and Sanger sequencing. Subjects were considered evaluable if they fulfilled all inclusion criteria and had a genotypic test that provided complete information in, at least, protease and reverse transcriptase to construct genotypic sensitivity score. *There were 31 subjects with Sanger but no 454 data and 1 subject with 454 but no Sanger data; 100 subjects had both 454 and Sanger data. Abbreviations: GSS, genotypic sensitivity score; HIV-1, human immunodeficiency virus type 1. Abbreviation: ART, antiretroviral therapy.

differences in area under the curve between 454 and Sanger GSS estimations were assessed for statistical significance using the method of Delong, Delong, and Clarke-Pearson [27]. Kaplan Meier curves were then used to describe time to virological failure according to 454 and Sanger GSS categories <3 and ≥3, using HIVdb, ANRS, and REGA algorithms. Statistically significant differences were evaluated using the Log-Rank test. Observations were right-censored if subjects changed the ART regimen received at the time of GSS calculation or if follow-up was interrupted. Finally, univariate and multivariate Cox proportional hazards models were constructed to evaluate baseline parameters associated with virological failure. The multivariate Cox model was built using covariates achieving a P-value < .05 in the univariate analysis. Statistical analyses were performed using SigmaPlot v12.5 (Systat Software, Inc.) and R software.

Sequence Datasets

Raw 454 sequences were deposited in the NCBI Sequence Read Archive (BioProject PRJNA243019).

RESULTS

Subjects' Selection

The original screening of the participating sites' databases identified 240 individuals potentially fulfilling the inclusion criteria

(Figure 1). Twenty-nine individuals were excluded because they were ART-naïve; 37 had baseline HIV-1 RNA levels <5000 copies/mL and 29 additional subjects lacked complete clinical information or follow-up. The remaining 145 subjects fulfilled the inclusion criteria and were sequenced. In 7 subjects, HIV-1 could not be amplified by either sequencing method; 6 additional individuals were excluded due to lack of phylogenetic clustering of Sanger and 454 consensus sequences. This left 132 evaluable subjects, 28 (21%) experiencing virological failure. A GSS could be constructed in 131 individuals using Sanger sequencing and in 101 using 454 sequencing; 100 subjects had both Sanger and 454 GSS estimates available.

Baseline Characteristics

The evaluable subjects were mostly men (72%), of white ethnicity (97%), intravenous drug users (50%), with the previous CDC AIDS C category (71%), prolonged ART experience (median 13.8 years and 13 previous ARV drugs), and low nadir $CD4^+$ T-cell counts (median 83 cells/mm^3; Table 1). The median calendar year of the treatment change (TC) episode was 2008. At the time of TC, subjects were 43 years old, had 232 $CD4^+$ cells/mm^3, and 36 904 plasma HIV-1 RNA copies/mL (all median values). The salvage ART regimen started included, at least, ritonavir-boosted darunavir (DRV/r) and/or raltegravir (RAL) in approximately half of subjects, PI/r other than DRV/r in 33%, etravirine (ETR) in 25%, and enfuvirtide (T-20) and/or maraviroc (MVC) in approximately 15% of subjects (see Supplementary Results Table R1 for detailed information on salvage treatments started). The median follow-up after TC was 13.6 months, and the median time between genotypic testing and TC was 1.5 months. The abovementioned characteristics were well balanced between virological outcome groups, except for marginally significant increases in number of ARV drugs exposed to (15 vs 12, $P = .078$), time between genotypic testing and TC (3.1 vs 1.4 months, $P = .080$), and recruitment from center 02 (36% vs14%, $P = .057$) among subjects developing virological failure.

GSS Distribution by Sanger and 454 Sequencing

Median GSS values were 3.0 by Sanger and 454 sequencing, except for the 454-GSS estimation using the HIVdb algorithm, which was 2.5 (Table 1). Median 454-GSS values were significantly lower and the proportion of subjects with a 454-GSS < 3 significantly higher in subjects developing virological failure than in nonfailures with any of the 3 algorithms (Table 1); such differences were not found when GSS calculations were based on Sanger sequencing. In the analysis of 454 vs Sanger GSS categories (Figure 2), 69%–73% of subjects retained the same GSS value with both sequencing methods; the most frequent category was GSS = 3 for HIVdb and ANRS, and GSS = 3.5 for REGA. In total, 454 sequencing produced a smaller GSS estimate than Sanger sequencing in 23%–25% of subjects, but led to higher GSS estimates in 4%–7% of individuals. Reasons for the latter observation were further explored (Supplementary results, Table R2).

Ability of Sanger and 454 Sequencing GSS Estimates to Predict Virological Failure

GSS estimates calculated with 454 sequencing achieved higher diagnostic accuracy than those calculated with Sanger sequencing regardless of the algorithm used (Figure 3). Pairwise differences in the area under the curve in the receiver operating characteristic curves between 454 and Sanger sequencing were: 0.69 vs 0.60 ($P = .029$) for HIVdb; 0.72 vs 0.60 ($P = .005$) for ANRS, and 0.67 vs 0.60 ($P = .008$) for REGA.

Survival Analysis of Time to Virological Failure

Overall, 28/132 subjects (21%) developed protocol defined virological failure, which mostly occurred during the first year after TC (Figure 4 for HIVdb; Supplementary Results Figure R1 for ANRS and REGA). Time to virological failure was significantly shorter for subjects with a 454-GSS < 3 than in those with a 454-GSS ≥ 3 (Log-rank *P*-values: .003 for HIVdb, .004 for ANRS and < .001 for REGA). Conversely, time to virological failure was not significantly different between Sanger GSS estimates <3 or ≥3. In a sensitivity analysis using combined Sanger and 454-GSS categories (Supplementary Results Figure R2), categories including a 454-GSS < 3 were associated with shorter time to virological failure regardless of their Sanger-GSS. However, this analysis was underpowered to address statistical significance.

Cox Regression Models of Risk of Virological Failure

Baseline factors associated with virological failure in the univariate Cox analysis (Table 2) were having a 454-GSS < 3 by the HIVdb (HR = 4.6, 95 CI, [1.5; 13.6], $P = .006$), the ANRS (HR = 3.5, 95 confidence interval [CI], [1.4; 8.7], $P = .006$) or the REGA (HR = 5.2, 95 CI, [1.9; 14.1], $P = .001$) algorithms; having been recruited at center 02 (HR = 2.4, 95 CI, [1.0; 5.5], $P = .044$), having been exposed to more ARV drugs at the time of TC (HR = 1.1 per each additional drug, 95 CI, [1.0, 1.2], $P = .007$), performing the TC at an earlier calendar year (HR = 0.9 per each additional calendar year, 95 CI, [.7, 1.0], $P = .052$), having more time between the genotypic test and treatment initiation (HR = 1.1 per each additional month, 95 CI, [1.0, 1.2], $P = .067$), and having >100 000 HIV-1 RNA copies/mL (HR = 2.0, 95 CI, [.9; 4.4], $P = .073$).

The only factors that remained independently associated with an increased risk of virological failure in the multivariate Cox regression model (Table 3) were a 454-GSS (HIVdb) < 3 vs 454-GSS (HIVdb) ≥3 (HR = 4.6, 95 CI, [1.5, 14.0], $P = .007$), and the number of previous antiretroviral drugs received (HR = 1.2 per additional drug, 95 CI, [1.1, 1.3], $P = .001$).

Table 1. Subject Characteristics

	Overall (n = 132)*	No Virologic Failure (n = 104)	Virologic Failure (n = 28)	*P* Value
Gender, n (%)				
Female	37 (28.0%)	30 (28.8%)	7 (25.0%)	.869
Age at TC, years, median (IQR)	43 (38, 47)	42 (38, 46)	43 (38, 48)	.711
Calendar year at TC, median (IQR)	2008 (2006, 2008)	2008 (2006, 2008)	2007 (2006, 2008)	.610
Mode of infection, n (%)				.360
IVDU	66 (50.0%)	45 (43.3%)	11 (39.3%)	
MSM	34 (25.7%)	28 (26.9%)	6 (21.4%)	
HTS	19 (14.4%)	16 (15.4%)	3 (10.7%)	
Other/Unknown	23 (17.4%)	15 (14.4%)	8 (28.6%)	
Centre, n (%)				.057
01	59 (44.7%)	47 (45.2%)	12 (42.9%)	
02	25 (19.0%)	15 (14.4%)	10 (35.7%)	
03	18 (13.6%)	16 (15.4%)	2 (7.1%)	
04	30 (22.7%)	26 (25.0%)	4 (14.3%)	
Previous AIDS diagnosis, n (%)	105 (79.5%)	82 (78.9%)	23 (82.1%)	.905
Previous CDC Category C, n (%) *	85 (71.4%)	64 (69.6%)	21 (77.8%)	.556
Nadir $CD4^+$ T cells (cells/mm^3)*				
Median (IQR)	83 (29, 191)	86 (29, 191)	69 (22, 173)	.476
<100, n (%)	71 (54.2%)	54 (52.4%)	17 (60.7%)	.571
$CD4^+$ T cells at TC (cells/mm^3)*				
Median (IQR)	232 (104, 398)	225 (108, 366)	255 (94, 477)	.429
<200, n (%)	56 (42.7%)	44 (42.7%)	12 (42.9%)	.840
HIV-1 RNA at TC (copies/mL)				
Median (IQR)	36 904 (16 955, 97 400)	34 540 (13 250, 90 000)	46 049 (23 149, 159 970)	.108
≥1 00 000, n (%)	31 (23.5%)	21 (20.2%)	10 (35.7%)	
Follow-up years before TC, median (IQR)	13.8 (8.9, 15.0)	13.3 (8.6, 15.0)	13.5 (11.5, 14.5)	.757
Months of follow-up after TC, median (IQR)	13.5 (6.0, 22.6)	12.3 (5.9, 23.1)	19.0 (7.3, 22.4)	.306
Months between the genotypic test and TC, median (IQR)	1.5 (0.0, 4.6)	1.4 (0.0, 4.3)	3.1 (0.3, 6.3)	.080
No. of ARV drugs before TC	13 (9, 17)	12 (9, 17)	15 (10, 19)	.078
Drugs included in the salvage regimen, n (%)				
Maraviroc	19 (14.4%)	17 (16.3%)	2 (7.1%)	.353
Enfuvirtide	20 (15.1%)	16 (15.4%)	4 (14.2%)	.878
Raltegravir	62 (47.0%)	50 (48.1%)	12 (42.9%)	.781
Etravirine	33 (25.0%)	27 (26.0%)	6 (21.4%)	.806
Darunavir/r	70 (53.0%)	58 (55.8%)	12 (43.0%)	.316
PI/r other than DRV/r	43 (32.6%)	32 (30.8%)	11 (39%)	.531
Sanger-GSS (HIVdb)*				
Median (IQR)	3.0 (2.0–3.0)	3.0 (2.0, 3.0)	2.5 (1.5, 3.0)	.088
GSS < 3, n (%)	65 (49.6%)	49 (47.6%)	16 (57.1%)	.493
Sanger-GSS (ANRS)*				
Median (IQR)	3.0 (2.5, 3.0)	3.0 (2.5, 3.0)	2.75 (2.0, 3.0)	.095
GSS < 3, n (%)	49 (37.4%)	35 (34.0%)	14 (50%)	.182
Sanger-GSS (REGA)*				
Median (IQR)	3.0 (2.0, 3.5)	3.25 (2.5, 3.5)	3.0 (2.0, 3.5)	.113
GSS < 3, n (%)	47 (35.9%)	34 (33.0%)	13 (46.4%)	.275
454-GSS (HIVdb)*				
Median (IQR)	2.5 (1.5–3.0)	3.0 (2.0, 3.0)	1.75 (1.375, 2.5)	.001
GSS < 3, n (%)	53 (52.5%)	35 (44.3%)	18 (81.8%)	.004

Table 1 continued.

	Overall (n = 132)*	No Virologic Failure (n = 104)	Virologic Failure (n = 28)	*P* Value
454-GSS (ANRS)*				
Median (IQR)	3.0 (2.0, 3.0)	3 (2.5, 3.0)	2.0 (1.87, 3.0)	.004
GSS < 3, n (%)	44 (43.6%)	29 (36.7%)	15 (68.2%)	.017
454-GSS (REGA)*				
Median (IQR)	3.0 (2.0, 3.5)	3.0 (2.5, 3.5)	2.25 (1.70, 2.75)	.006
GSS < 3, n (%)	47 (46.5%)	30 (38.0%)	17 (77.3%)	.002

P-values were obtained with the χ^2 or Fisher tests for categorical data, and the Mann-Whitney rank sum test for continuous data). The total number of subjects was 132, except for the following variables (*): previous CDC category C (n = 119); Nadir CD4$^+$ T cells and CD4$^+$ T cells at TC (n = 131); all Sanger-GSS categories (n = 131, 28 with and 103 without virological failure), and all 454-GSS categories (n = 101, 22 with and 79 without virological failure).

Abbreviations: ARV, antiretroviral; CDC, centers for disease control and prevention; HIV, human immunodeficiency virus; HTS, heterosexual; IQR, 25%–75% interquartile range; IVDU, intravenous drug use; MSM, men who have sex with men; PI/r, ritonavir-boosted protease inhibitor; Sanger-GSS, genotypic susceptibility score calculated from Sanger sequencing data; 454-GSS, genotypic susceptibility score calculated from 454 sequencing data; TC, treatment change.

Equivalent findings were obtained with the ANRS and REGA algorithms (Supplementary Results Table 3).

DISCUSSION

This study found that genotypic sensitivity scores based on ultrasensitive HIV-1 genotyping discriminated salvage ART outcomes better than those relying on Sanger sequencing. Our findings were consistent across resistance interpretation algorithms in ROC curve and survival analyses, including multivariate Cox regression, where having <3 active drugs in the salvage regimen by 454 sequencing was a strong independent predictor of subsequent virological failure. Ultrasensitive genotyping reduced the GSS estimate achieved by Sanger sequencing in approximately one quarter of subjects, suggesting that at least one quarter of treatment-experienced subjects requiring salvage ART might benefit from a genotyping approach with increased sensitivity. Our observations thus suggest that ultrasensitive HIV-1 genotyping might indeed be helpful to optimizing salvage ART in subjects infected with multidrug-resistant HIV-1.

To our knowledge, this is the first cohort study addressing the clinical value of ultrasensitive genotyping in ART-experienced subjects. Strict selection criteria and several rounds of database curation were put in place to minimize biases and confounders

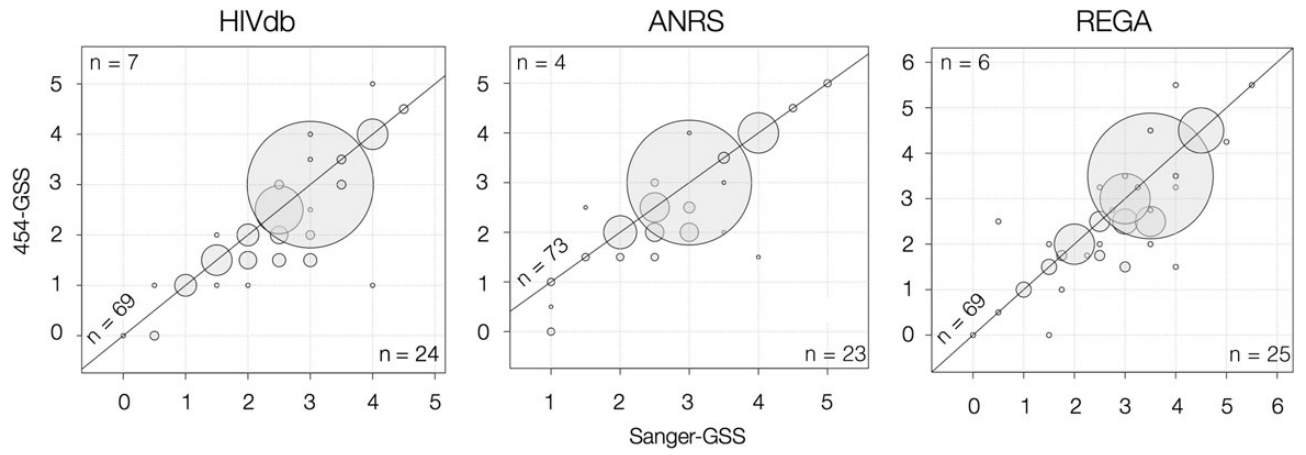

Figure 2. Genotypic sensitivity scores (GSS) by 454 vs Sanger sequencing. Bubble plots of the combined Sanger and 454 sequencing GSS categories for the 100 subjects with both genotypic data types available. Separate graphs are shown for each HIVdb (v6.3.1), ANRS (v2012.09), and REGA (v9.1.0) algorithms. The diameter of each bubble is proportional to the number of subjects included in the corresponding combined GSS category. In bubbles centred on the diagonal line (overall number of subjects shown on top of the diagonal line), Sanger and 454 sequencing produced the same GSS estimates. In bubbles below the diagonal line (overall number of subjects shown on the lower-right corner), 454 sequencing produced a smaller GSS estimate than Sanger sequencing. In bubbles above the diagonal line (overall no. of subjects shown on the upper-left corner), 454 sequencing produced a larger GSS estimate than Sanger sequencing.

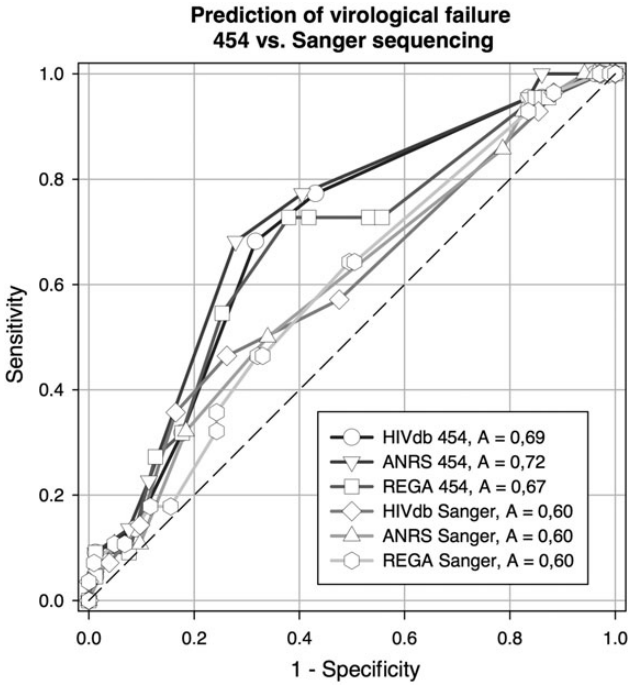

Figure 3. Improved prediction of virological failure with 454 relative to Sanger sequencing. Receiver operating characteristic curves of the ability of HIVdb, ANRS, and REGA algorithms to predict virological failure when genotypic sensitivity scores are calculated using 454 sequencing (HIVdb 454, ANRS 454, REGA 454 groups) or Sanger sequencing (HIVdb Sanger, ANRS Sanger, REGA Sanger groups). The legend shows the area under the curve values (*A*) for each category. Pairwise differences in area under the curve between 454 and Sanger categories were statistically significant using the method of Delong, Delong, and Clarke-Pearson [27], ie, $P = .029$ for HIVdb; $P = .005$ for ANRS, and $P = .008$ for REGA.

as much as possible. Rather than evaluating discrete sets of mutations, we used GSS to assess the overall virus susceptibility to salvage ART. Multivariate Cox regression models attempted to control for the effect of the most common potential confounders, such as HIV-1 RNA levels of $CD4^+$ T-cell counts. We also excluded all subjects with any suspicion of nonadherence by clinical records. However, no formal adherence data were available in this study due to its retrospective nature. Previous studies in antiretroviral-naive subjects showed that both the presence of LFDR HIV-1 and suboptimal adherence were independent risk factors for virological failure to first-line NNRTI ART but also potentiated each other's effects on virologic failure [28]. It may be easier to detect LFDR HIV-1 in non-fully adherent individuals due to lower degrees of fixation of mutants in the viral population [29]; in practice, this could also help clinicians make better-informed ART choices in non-fully adherent subjects.

Our findings are thus encouraging and set a basis for future confirmatory prospective evaluations but should be interpreted with caution. Importantly, our study highlights a number of issues that should be addressed by future research.

One circular problem with interpreting ultrasensitive drug resistance data is that, on one hand, all genotypic interpretation algorithms, including those used in our study, have been developed and validated using Sanger sequencing data, but, on the other hand, next-generation sequencing (NGS) data are needed to develop new algorithms or adapt the existing ones to address minority variant information. It is unknown, for example, how variant frequency data should be treated in these algorithms, whether mutations should be weighted according to their frequency in the virus population, or how such weights should be established. Answering these questions would require much larger data sets with, possibly, thousands of treatment change episodes.

After balancing the advantages and limitations of the different possible approaches to mutational data interpretation, we deliberately chose using the HIVdb, ANRS, and REGA algorithms "as they are," and treat LFDR variants as binary variables (presence vs absence) disregarding their frequency in the population. Our assumption was that LFDR variants detected at HIV-1 RNA levels ≥5000 copies/mL represented a clinically meaningful amount of viruses. Such assumption is partially supported by previous studies in ART-naive subjects, which showed that even the presence of 10 copies/mL of mutant virus had a significant impact on clinical outcomes to first-line NNRTI ART. In contrast with studies in naive individuals, we did not use mutational load information to evaluate outcomes because, in our population, subjects had multiple mutations in different genes as well as an extensive treatment history, and no theoretical framework is available yet to properly analyse that information. Nevertheless, our simplified approach was able to improve Sanger-based GSS predictions, suggesting that (a) a reductionist approach to ultrasensitive HIV-1 genotyping may already provide benefits to patients, and (b) such strategy can potentially be improved, albeit through nontrivial methodological approaches.

The small sample size of our study only allowed us to test GSS categories above and below 3. Although this cutoff is meaningful from a clinical perspective—all HIV management guidelines recommend treating patients with at least 3 active drugs—the study was not powered enough to assess GSS strata with increased granularity. Similarly, we were not able to assess the effect of specific antiretroviral drugs other than accounting for their presence in the regimen in the Cox analysis. Also, historical genotypes, which have been shown to correlate with 454 data in previous studies [30], were not available to us in a consistent manner to enable a direct comparison of their diagnostic performance with that of ultrasensitive HIV-1 genotyping. Although recruitment of virological failures differed by center, this was not an independent predictor of virological failure in the multivariate Cox analysis.

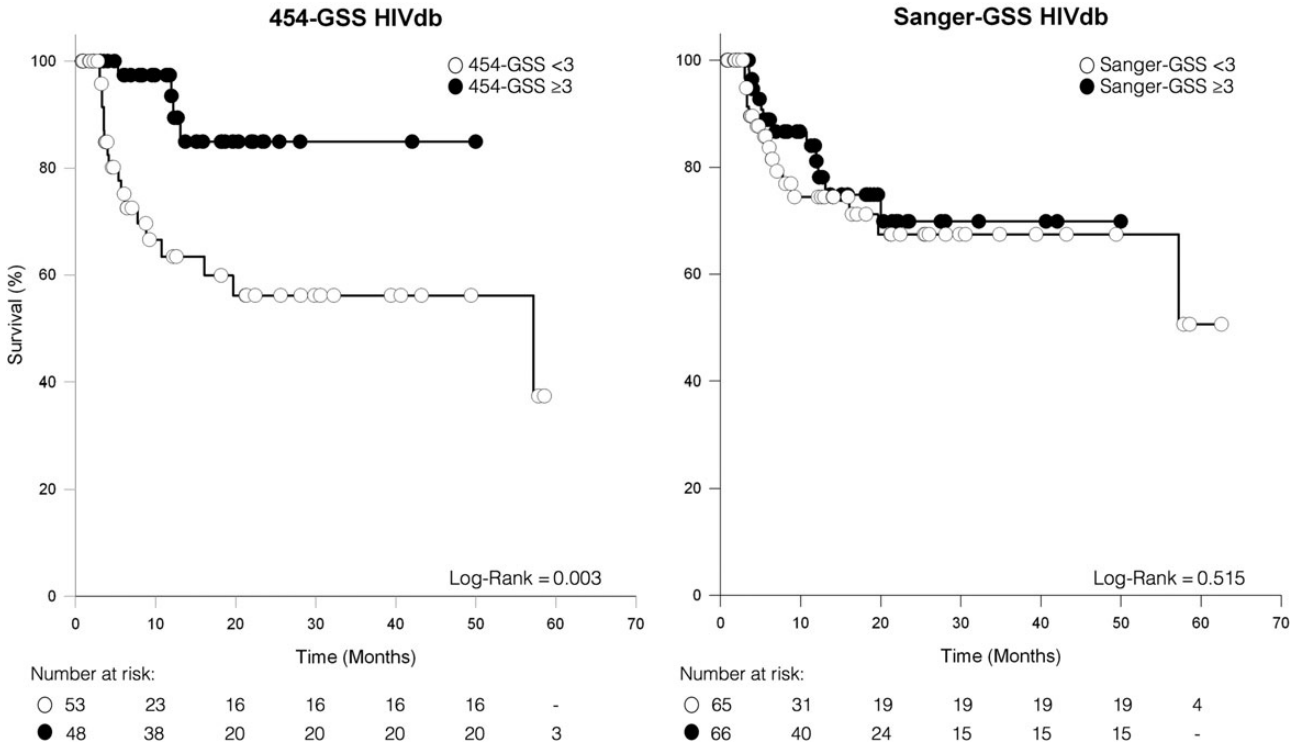

Figure 4. Kaplan-Meier curves of time to virological failure, HIVdb algorithm. In the 454-GSS panel, 454 sequencing data were used to calculate the GSS using the HIVdb algorithm (v6.3.1); in the Sanger-GSS panel, Sanger sequencing of HIV populations was used to calculate the GSS using the HIVdb algorithm (v6.3.1). Symbols show censored events. Similar results were obtained when the same analyses were performed using the ANRS (v2012.09) and REGA (v9.1.0) algorithms (Supplementary results). Abbreviations: GSS, genotypic sensitivity score; HIV, human immunodeficiency virus.

Previous studies have shown that ultrasensitive HIV-1 genotyping is useful for treatment-naive subjects initiating first-line NNRTI therapy, women exposed to pMTCT programs including single-dose NVP who require NNRTI ART, and individuals requiring treatment with small-molecule CCR5 antagonists at any stage of the disease [15–25]. Our study suggests that deep HIV-1 sequencing is also useful to tailor salvage ART in treatment-experienced subjects. Our observations likely hold for different NGS platforms, which have consistently shown technical equivalence to 454 sequencing in HIV-1 genotyping [31]. Of note, due to our stringent quality criteria for GSS calculation, we could not calculate 454-GSS in almost one quarter of subjects.

Subjects included in this study were highly ART-experienced. We could expect similar findings for salvage ART in earlier stages of the disease, because clinicians would have more antiretroviral options to choose from. Previous studies [29, 32], for example, showed that LFDR HIV-1 information improved GSS evaluations to drugs like etravirine, which might have an impact on which treatment combinations are chosen for second or third-line ART. Finally, HIV-1 genotyping was performed on subjects with HIV-1 RNA levels >5000 copies/mL, which reduces sampling biases and false-positive mutations. If virological failure occurred at lower HIV-1 RNA levels, NGS would, at least, provide technical noninferiority to Sanger sequencing [33], although the chance of detecting low-frequency variants would logically decrease if less RNA molecules were sampled.

The technical performance of ultrasensitive HIV-1 genotyping is only one factor determining its applicability in the clinical setting. Cost, workload, and technical complexity of NGS platforms are rapidly decreasing, as throughput, scalability, and automation of workflows and sequence interpretations improve [13]. The field is evolving rapidly, and even diagnostic companies currently supporting Sanger sequencing are adapting routine HIV-1 genotyping to NGS. Continuous decreases in sequencing costs coupled with adequate logistics, including centralised testing of large amounts of samples, might turn HIV-1 genotyping into a more affordable tool for HIV-1 resistance surveillance and clinical management in the coming years, even for resource-limited settings.

In conclusion, our study shows that ultrasensitive HIV-1 genotyping consistently improves GSS-based predictions of virological outcomes of salvage ART relative to Sanger sequencing. Ultrasensitive HIV-1 genotyping could become a useful tool to

Table 2. Univariate Cox Regression Model of Risk of Virological Failure

	HR	95% CI	*P* Value
454-GSS (HIVdb)			
≥3	1	. . .	. . .
<3	4.6	(1.5, 13.6)	.006
454-GSS (ANRS)			
≥3	1	. . .	. . .
<3	3.5	(1.4, 8.7)	.006
454-GSS (REGA)			
≥3	1	. . .	. . .
<3	5.2	(1.9, 14.1)	.001
Sanger-GSS (HIVdb)			
≥3	1	. . .	. . .
<3	1.3	(.6, 2.7)	.517
Sanger-GSS (ANRS)			
≥3	1	. . .	. . .
<3	1.8	(.8, 3.7)	.135
Sanger-GSS (REGA)			
≥3	1	. . .	. . .
<3	1.6	(.8, 3.5)	.190
Age at TC			
Per each additional year	1.0	(1.0, 1.0)	.746
Gender			
Male	1	. . .	. . .
Female	0.9	(.4, 2.1)	.809
Transmission route			
IVDU	1	. . .	. . .
MSM	0.8	(.3, 2.1)	.609
HTS	0.8	(.2, 2.8)	.694
Other	1.6	(.7, 4.1)	.294
Calendar Year at TC,			
Per each additional year	0.9	(.7, 1.0)	.052
Time between genotypic test and TC			
Per each additional month	1.1	(1.0, 1.2)	.067
Time since HIV diagnosis			
Per each additional year	1.1	(1.0, 1.2)	.124
# previous ARV drugs			
Per each additional drug	1.1	(1.0, 1.2)	.007
Centre			
01	1	. . .	. . .
02	2.4	(1.0, 5.5)	.044
03	0.5	(.1, 2.1)	.315
04	0.7	(.2, 2.1)	.478
CDC 1993 AIDS Category			
C	1	. . .	. . .
A	0.4	(.1, 1.5)	.169
B	1.0	(.4, 3.1)	.924
Nadir $CD4^+$ T-cell counts (cells/mm^3)			
<100	1	. . .	. . .
≥100	0.6	(.3, 1.3)	.213
$CD4^+$ T-cell counts at TC (cells/mm^3)			
<200	1	. . .	. . .
≥200	1.0	(.5, 2.1)	.985
HIV-1 RNA at TC (copies/mL)			
<100 000	1	. . .	. . .
≥100 000	2.0	(.9, 4.4)	.073
Maraviroc in the salvage ART[a]			
No	1	. . .	. . .
Yes	0.5	(.1, 2.0)	.304
Enfurvirtide in the salvage ART[a]			
No	1	. . .	. . .
Yes	1.6	(.5, 5.0)	.363
Raltegravir in the salvage ART[a]			
No	1	. . .	. . .
Yes	0.9	(.4, 1.9)	.734
Etravirine in the salvage ART[a]			
No	1	. . .	. . .
Yes	0.8	(.3, 2.1)	.714
Darunavir/r in the salvage ART[a]			
No	1	. . .	. . .
Yes	0.7	(.3, 1.4)	.317
PI/r other than Darunavir in the salvage ART[a]			
No	1	. . .	. . .
Yes	1.2	(.5, 2.6)	.689

Table 2 continued.

Abbreviations: ART, antiretroviral therapy; ARV, antiretroviral; CDC, centers for disease control and prevention; CI, confidence interval; HIV-1, human immunodeficiency virus type 1; HR, hazard ratio; HTS, heterosexual; IVDU, intravenous drug use; MSM, men who have sex with men; PI/r, ritonavir-boosted protease inhibitor; Sanger-GSS, genotypic sensitivity score calculated from Sanger sequencing data; 454-GSS, genotypic sensitivity score calculated from 454 sequencing data; TC, treatment change.

[a] The salvage ART regimen started contains, at least, this drug.

Table 3. Multivariate Cox Regression Model of Risk of Virological Failure Using the HIVdb Algorithm

	HR	95% CI	*P* Value
454-GSS (HIVdb)			
≥3	1	. . .	. . .
<3	4.6	(1.5, 14.0)	.007
Centre			
01	1	. . .	. . .
02	2.7	(.9, 7.0)	.064
03	2.3	(.4, 12.9)	.333
04	0.9	(.2, 3.2)	.837
No. of previous ARV drugs			
Per each additional drug	1.2	(1.1, 1.3)	.001

Abbreviations: ARV, antiretroviral; CI, confidence interval; HR, hazard ratio; 454-GSS, genotypic sensitivity score calculated from 454 sequencing data.

improve the clinical management of treatment-experienced individuals living with HIV-1.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflict of interest. B. C. has been a consultant on advisory boards or participated in speakers' bureaus or conducted clinical trials with Boehringer-Ingelheim, Abbott, GlaxoSmithKline, Gilead, Janssen, Merck, Shionogi, and ViiV. R. P. has received consulting fees from Pfizer and grant support from Pfizer, Roche Diagnostics, Siemens, Merck, and Boehringer-Ingelheim. F. G. has been a consultant on advisory boards or participated in speakers' bureaus with Boehringer-Ingelheim, Abbott, Gilead, Merck, and ViiV. R. D. has been a consultant on advisory boards or participated in speakers' bureaus with Roche, Abbott, Janssen, ViiV, and BMS. D. D. has been a consultant on advisory boards or participated in speakers' bureaus with Boehringer-Ingelheim, Abbott, Gilead, Merck, Janssen, and ViiV. F. P. has been a consultant on advisory boards or participated in speakers' bureaus or conducted clinical trials with Abbott, GlaxoSmith Kline, Gilead, Janssen, Merck, and ViiV. M. A. T. works for Roche Diagnostics, SL, which commercializes the 454 sequencing technology. D. G. is an employee and owns shares in ABL SA. C. S. is the CEO, cofounder, and shareholder of ABL, which owns TherapyEdge and DeepChek software systems. All other authors report no potential conflicts.

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