Received: 3 July 2023 | Accepted: 12 September 2023

DOI: 10.1111/hiv.13556

ORIGINAL ARTICLE

Rate of response to initial antiretroviral therapy according to level of pre-existing HIV-1 drug resistance detected by next-generation sequencing in the strategic timing of antiretroviral treatment (START) study

Alessandro Cozzi-Lepri[1] | **David Dunn**[1] | **Anna Tostevin**[1] | **Rasmus L. Marvig**[2] | **Marc Bennedbæk**[3] | **Shweta Sharma**[4] | **Michael J. Kozal**[5] | **Mark Gompels**[6] | **Angie N. Pinto**[7] | **Jens Lundgren**[8] | **John D. Baxter**[9] | **for the INSIGHT START Study Group**

[1]Institute for Global Health, UCL, London, UK

[2]Center for Genomic Medicine, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark

[3]Virus Research and Development Laboratory, Virus and Microbiological Special Diagnostics, Statens Serum Institute, Copenhagen, Denmark

[4]Division of Biostatistics, School of Public Health, University of Minnesota, Minneapolis, Minnesota, USA

[5]Stanford University, Palo Alto, California, USA

[6]North Bristol NHS Trust, Bristol, UK

[7]The Kirby Institute, University of New South Wales, Sydney, New South Wales, Australia

[8]Copenhagen HIV Programme, Rigs Hospitalet, University of Copenhagen, Copenhagen, Denmark

[9]Cooper Medical School of Rowan University and Cooper University Health Care, Camden, New Jersey, USA

Correspondence
Alessandro Cozzi-Lepri, Institute for Global Health, UCL, London, UK.
Email: a.cozzi-lepri@ucl.ac.uk

Funding information
National Institute of Allergy and Infectious Diseases; National Institutes of Health, Grant/Award Numbers: RO1HL096453, UM1-AI120197, UM1-AI068641

Abstract

Objectives: The main objective of this analysis was to evaluate the impact of pre-existing drug resistance by next-generation sequencing (NGS) on the risk of treatment failure (TF) of first-line regimens in participants enrolled in the START study.

Methods: Stored plasma from participants with entry HIV RNA >1000 copies/mL were analysed using NGS (llumina MiSeq). Pre-existing drug resistance was defined using the mutations considered by the Stanford HIV Drug Resistance Database (HIVDB v8.6) to calculate the genotypic susceptibility score (GSS, estimating the number of active drugs) for the first-line regimen at the detection threshold windows of >20%, >5%, and >2% of the viral population. Survival analysis was conducted to evaluate the association between the GSS and risk of TF (viral load >200 copies/mL plus treatment change).

Results: Baseline NGS data were available for 1380 antiretroviral therapy (ART)-naïve participants enrolled over 2009–2013. First-line ART included a non-nucleoside reverse transcriptase inhibitor (NNRTI) in 976 (71%), a boosted

protease inhibitor in 297 (22%), or an integrase strand transfer inhibitor in 107 (8%). The proportions of participants with GSS <3 were 7% for >20%, 10% for >5%, and 17% for the >2% thresholds, respectively. The adjusted hazard ratio of TF associated with a GSS of 0–2.75 versus 3 in the subset of participants with mutations detected at the >2% threshold was 1.66 (95% confidence interval 1.01–2.74; $p = 0.05$) and 2.32 (95% confidence interval 1.32–4.09; $p = 0.003$) after restricting the analysis to participants who started an NNRTI-based regimen.

Conclusions: Up to 17% of participants initiated ART with a GSS <3 on the basis of NGS data. Minority variants were predictive of TF, especially for participants starting NNRTI-based regimens.

KEYWORDS

antiretroviral therapy, HIV drug resistance, human immunodeficiency virus (HIV), next generation sequencing

INTRODUCTION

HIV-1 drug resistance genotyping is recommended to help guide the selection of antiretroviral therapy (ART) and to prevent virological failure (VF). Although drug-resistant mutations (DRMs) are typically detected by Sanger sequencing if present in 15–25% of the total viral population [1–3], more sensitive techniques [4–8] with an increased sensitivity range have been developed.

Minority variants have been shown to be associated with the risk of ART failing, but the strength of the associations varies across drug classes (strongest for non-nucleoside reverse transcriptase inhibitors [NNRTIs] and C-C chemokine receptor type 5 antagonists). In contrast, the evidence is only moderate for nucleoside reverse transcriptase inhibitors (NRTIs) and the integrase strand transfer inhibitor (INSTI) raltegravir and is very low for the protease inhibitors (PIs) and the INSTIs elvitegravir and dolutegravir [9–13].

A pooled analysis of data from 10 studies showed that people in whom minority variants were detected at baseline had a 2-fold higher risk of VF than those without these variants [14]. Of note, the increased risk of VF appeared to be mainly driven by NNRTI-resistant minority variants and was independent of medication adherence. These results were confirmed in several subsequent studies conducted in other settings [15–21]. However, a more recent qualitative review that identified 25 studies investigating the association between detection of minority variants and risk of VF of an NNRTI-based first-line regimen reported conflicting findings [22].

In light of these newer findings, the aim of this analysis was to re-evaluate the impact of minority variants using next-generation sequencing (NGS), including the role of NNRTI-associated resistance, on the risk of failure of first-line regimens initiated by the target population enrolled in the START trial.

METHODS

Study population and sequencing

The START trial, conducted by the International Network for Strategic Initiatives in Global HIV Trials, enrolled ART-naïve participants with HIV between April 2009 and December 2013. The study design and data collection plan for START has previously been reported [23, 24]. A plasma sample, taken within 60 days before enrolment was obtained from all participants who provided consent for stored specimens.

Methods for sample preparation, amplification of viral RNA and NGS, identification of DRM by means of VirVarSeq, and determination of HIV subtype have been described elsewhere [25, 26].

Sequence reads (FASTQ files) were analyzed with VirVarSeq version 20140929, which calls variants at the codon level [27]. From the output, we extracted amino acid frequencies in the *pol* gene from amino acid position 1–935 where positions 1–99 encode protease protein, positions 100–659 encode reverse transcriptase protein, and positions 660–935 partially encode integrase protein (our amplicon did not cover positions 936–947) [25].

Definition of pre-existing drug resistance and phenotypic drug susceptibility

In this analysis, we considered all mutations used by the Stanford HIV Drug Resistance Database (HIVDB) HIVdb

algorithm v8.6 to provide genotypic test result interpretation. Of note, the Stanford HIVdb algorithm considers a much wider range of mutations than those considered by the World Health Organization (WHO) 2009 surveillance list used in previous analyses of these datasets [27–29]. The Stanford HIVdb interpretation was used to calculate a genotypic susceptibility score (GSS) for the drugs included in participants' first-line regimens as follows [30]: each drug included in the initial regimen was given an individual numerical score of 1 if the interpretation was 'no resistance' (fully active drug), 0.75 if potential low level, 0.5 if low level, 0.25 if intermediate, and 0 if high level of resistance (zero activity of the drug). The GSS for a regimen was the sum of the individual scores for the drugs included in the regimen and estimated the total number of drugs predicted to be active. For a triple combination regimen, GSS varies between 0 and 3. Detail regarding our choice for sequencing depth and thresholds for calling DRMs are reported in the Appendix.

Statistical analysis

Characteristics of participants were described and compared across GSS strata, grouped as 0–2.75 versus 3 (i.e., partially vs. fully active regimen) at the >20% threshold. The compared the distribution of categorical variables using a chi-squared test and of continuous variables using the Mann–Whitney U test. The breakdown of the exact first-line treatment was also shown overall and by GSS strata. We also described the distribution of the HIVDB interpretation scores separately by antiretroviral drug and according to threshold window used.

The primary endpoint of this analysis was treatment failure (TF), a composite outcome defined at the time of two possible failure events: (1) the time of experiencing a single plasma HIV RNA >200 copies/mL after >6 months of therapy initiation if this event was followed by a change of one or more drugs (including discontinuation) of the regimen received at time of the elevated HIV RNA value or (2) the time of pure confirmed VF, defined at the time of the first of two consecutive HIV-RNA viral loads >200 copies/mL. For both components of the primary endpoint, survival time accrued from the date of therapy initiation until the date of the failure event or the date of the last available HIV RNA measure, whichever occurred first. All available viral loads were used, and the main assumption behind definition (1) is that therapy changes occurring after having observed a viral load >200 copies/mL were with the intent of re-suppressing the viral load.

Standard unweighted Kaplan–Meier and Cox regression model analysis were performed. Suboptimal GSS was defined as having fewer than three active drugs. The association between GSS (fitted as a binary covariate 0–2.75 vs. 3 at the various thresholds) and the risk of experiencing the endpoints was evaluated in univariable analyses and after controlling for a number of variables chosen from the following initial list: age, sex, geographical region, mode of HIV transmission, hepatitis co-infection status, baseline CD4 count and HIV RNA, trial arm, non-adherence, HIV subtype, year of starting ART, and type of ART started. We used a direct acyclic graph to describe our assumptions regarding the underlying causal structure of the data. On the basis of these assumptions, only baseline HIV RNA, geographical region, intervention arm of START (immediate vs. deferred ART), and year of starting ART were deemed as common causes of both the exposure of interest and the outcome and so were included in the multivariable models as potential confounders (Supplementary Figure S1).

A first sensitivity analysis was performed after restricting participants to those with Sanger GSS ≥2 (the subset in which NGS is likely to provide additional benefit, $n = 1349$, Table S1B). A second sensitivity analysis was performed using an alternative endpoint of TF, which, for the component (1), only counted the discontinuations of ART following failure as events. Reasons for discontinuing drugs as coded by the treating physicians were summarized. Linear regression analysis controlling for extra-sample error distribution by means of cross validation was also used to identify mutations at the 2–20% level associated with the week 4 decline in HIV RNA. We also calculated the hazard ratios (HRs) of TF associated with these identified mutations. More details for the linear regression analysis are shown in the Supplementary material.

A two-sided test of <0.05 was considered statistically significant. All statistical analyses were performed using SAS software, version 9.4 (Carey NC, USA).

RESULTS

Overall, of the 1819 participants with NGS data at the ≥200 read depth, 1380 (76%) who started ART with three or more drugs, either in the immediate or the deferred arm, and had virological follow-up of ≥1 month were included in this analysis. Table 1 shows the main characteristics of the study population, a selection of factors potentially associated with the risk of VF to HIV treatment, stratified by GSS groups using the 20% detection threshold. Of note, 96 of 1380 participants (7%) started a regimen predicted by GSS to include fewer than three active drugs at this threshold. The two GSS groups

TABLE 1 Characteristics of participants according to Stanford University Drug Resistance Database (HIVDB) genotypic susceptibility score (>20% threshold).

Characteristics	0 < GSS[#] ≤2.75 (*N* = 96)	GSS[#] = 3 (*N* = 1284)	*p*-value[$]	Total (*N* = 1380)
Sex			0.992	
Female	15 (15.6)	201 (15.7)		216 (15.7)
Race			0.258	
Asian	7 (7.3)	90 (7.0)		97 (7.0)
Black	10 (10.4)	246 (19.2)		256 (18.6)
White	57 (59.4)	724 (56.4)		781 (56.6)
Hispanic	18 (18.8)	199 (15.5)		217 (15.7)
Other	4 (4.2)	36 (2.8)		40 (2.9)
Mode of HIV transmission			0.553	
Heterosexual contacts	19 (19.8)	327 (25.5)		346 (25.1)
PWID	1 (1.0)	19 (1.5)		20 (1.4)
MSM	73 (76.0)	886 (69.0)		959 (69.5)
Other/unknown	3 (3.1)	52 (4.0)		55 (4.0)
Region of enrollment			<0.001	
Africa	3 (3.1)	136 (10.6)		139 (10.1)
Asia	7 (7.3)	78 (6.1)		85 (6.2)
Australia	5 (5.2)	40 (3.1)		45 (3.3)
Europe	29 (30.2)	567 (44.2)		596 (43.2)
South America	46 (47.9)	309 (24.1)		355 (25.7)
USA	6 (6.3)	154 (12.0)		160 (11.6)
Age, years	35 (28–47)	35 (28–43)	0.884	35 (28–44)
CD4 count, cells/mm^3	654 (588–730)	632 (575–721)	0.177	633 (575–721)
HIV RNA, $\log_{10}$ copies/mL	4.45 (4.00–4.79)	4.48 (4.01–4.86)	0.630	4.47 (4.01–4.86)
CD4/CD8 ratio	0.6 (0.5–0.9)	0.6 (0.4–0.8)	0.208	0.6 (0.4–0.8)
HBsAg, *n* (%)	2 (2.1)	34 (2.7)	0.720	36 (2.7)
HCVAb, *n* (%)	1 (1.1)	36 (2.9)	0.299	37 (2.7)
BMI, kg/m^2	23.8 (22.0–26.6)	24 (22–27)	0.645	24 (22–27)
Calendar year of starting ART	2012 (2011–2013)	2012 (2011–2013)	0.55	2012 (2011–2013)
Months from HIV diagnosis to enrolment	8 (3–22)	11 (4–31)	0.015	10 (4–30)
Months from GRT to start of ART	12 (2–28)	12 (3–30)	0.286	12 (3–29)
Co-morbidities				
Cardiovascular disease	2 (2.1)	8 (0.6)	0.104	10 (0.7)
Diabetes	2 (2.1)	37 (2.9)	0.649	39 (2.8)
Dyslipidaemia	2 (2.1)	53 (4.1)	0.323	55 (4.0)
Hypertension	7 (7.3)	125 (9.7)	0.433	132 (9.6)

Note: Data are presented as n (%) or median (interquartile range) unless otherwise indicated.

Abbreviations: ART, antiretroviral therapy; BMI, body mass index; GRT, genotypic resistance test; GSS, genotypic susceptibility score; HBsAg, hepatitis B surface antigen; HCVAb, hepatitis C virus antibodies; MSM, men who have sex with men; PWID, people who inject drugs.

[$]Chi-squared or Mann–Whitney U as appropriate.

[#]HIVDB v8.6 with >20% threshold.

appeared to be balanced in terms of demographics and immune-virological factors. The only marked differences were in region of enrolment, where South America was over-represented in the group with a GSS of 0–2.75. In contrast, participants in Africa and the USA appeared to be more likely to have a GSS = 3 (chi-squared $p < 0.001$). In addition, the median time from HIV diagnosis for participants in the GSS of 0–2.75 group was on average

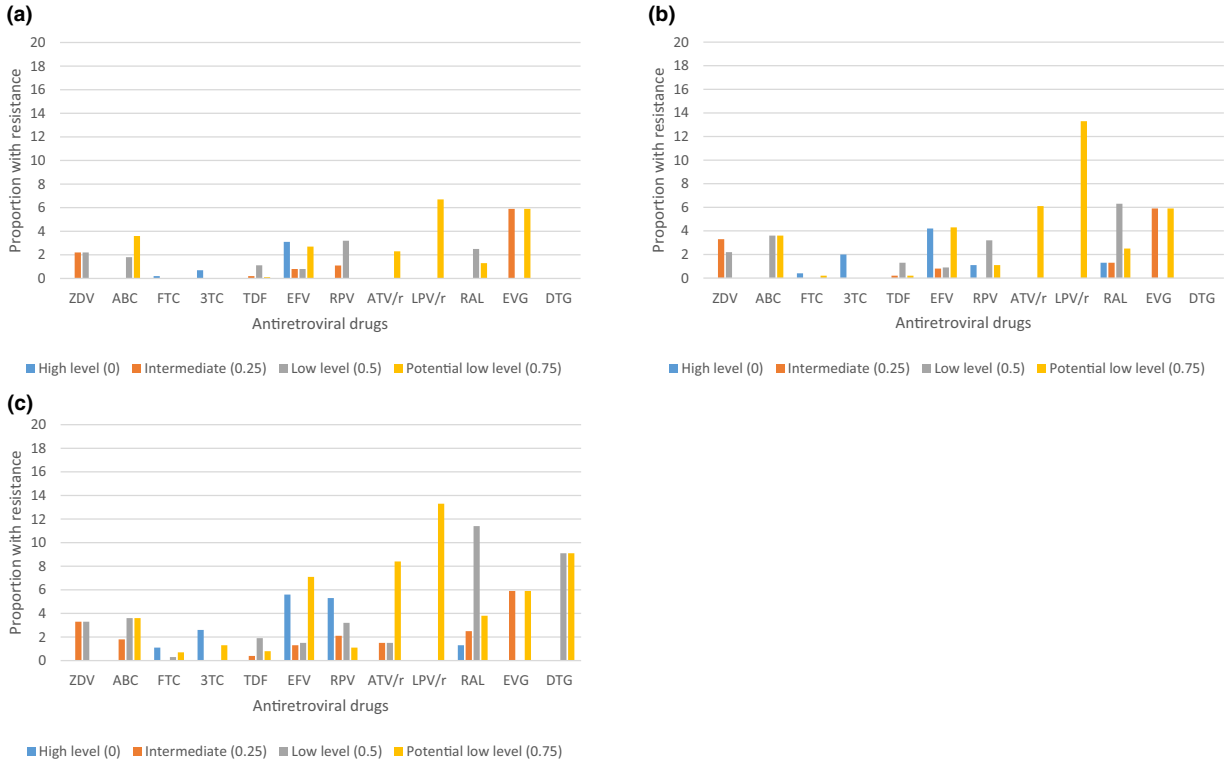

FIGURE 1 HIVDB interpreted drug activity <1 according to anchor drug started in first-line regimen by (a) >20%, (b) >5%, and (c) 2% threshold windows. NB only interpretations for the drugs included in the first-line regimen are shown. ABC, abacavir; ATV, atazanavir; DTG, dolutegravir; EVG, elvitegravir; EFV, efavirenz; FTC, emtricitabine; HIVDB, Stanford University HIV Drug Resistance Database; LPV, lopinavir; RAL, raltegravir; RPV, rilpivirine; TDF, tenofovir; 3TC, lamivudine; ZDV, zidovudine.

3 months shorter than that of the GSS = 3 group ($p = 0.02$, Table 1). Of note, participants' characteristics were similar when other window thresholds were used to define the GSS groups (see Table S1A for the >2% threshold for example). The proportion of participants with a GSS of 0–2.75 using this threshold was increased at 17.0% (235/1380).

Supplementary Figure S2 shows the breakdown of antiretroviral regimens; the large majority of the population initiated a triple-drug regimen that included tenofovir/emtricitabine (FTC) ($n = 80$ [83% of those with GSS = 0–2.75] and $n = 1148$ [89% of those with GSS = 3]) in combination with efavirenz (77% of the GSS = 0–2.75 group vs. 63% of the GSS = 3 group).

Figure 1a–c shows the breakdown of the Stanford predictions for drug components of the initial regimen of participants, stratified by the threshold window used to define resistance. Using the 20% Sanger threshold, participants' viruses showed high-level resistance to efavirenz (3%) and lamivudine (3TC) or FTC (0.7 and 0.2%, respectively) while there appeared to be intermediate resistance to elvitegravir (6%) but not to the other INSTIs. Interestingly, as seen in previous analyses of this same dataset, by lowering the threshold for resistance detection to >2%, a higher percentage of participants appeared to have a virus with high-level resistance to raltegravir (1.5%) and 1–3% retaining high-level resistance to 3TC/FTC and 6% to rilpivirine and efavirenz. Because a large proportion of mutations were detected at very low levels, more resistance (at any level) was detected at the >2% threshold (Figure 1c) than at the >5% threshold (Figure 1b).

At the >2% threshold, T215 revertant ($n = 47$, 3.4%), M41L ($n = 31$, 2.3%), and K219QENR ($n = 28$, 2.0%) were the most prevalent NRTI mutations. Mutations D67NGE and K70RE were detected in 1.2% of participants ($n = 17$ and $n = 16$, respectively). Notably, the K65R mutation was not observed in any sample, even at this low threshold. M184V and M184I were detected in seven (0.5%) and 11 (0.8%) participants, respectively. The most common NNRTI DRMs were K103NS ($n = 47$, 3.4%), G190ASE ($n = 39$, 2.8%), and E138K ($n = 22$, 1.6%). The prevalence of PI PDR was strongly influenced by the M46IL mutation, which was observed in 6.2% of participants ($n = 86$). The D30N mutation was detected in 28 (2.0%) and the L90M in six (0.4%). Other commonly detected mutations in the PI region were F53LY, I54VLMATS, and V82ATFSCML (in ~0.6% of

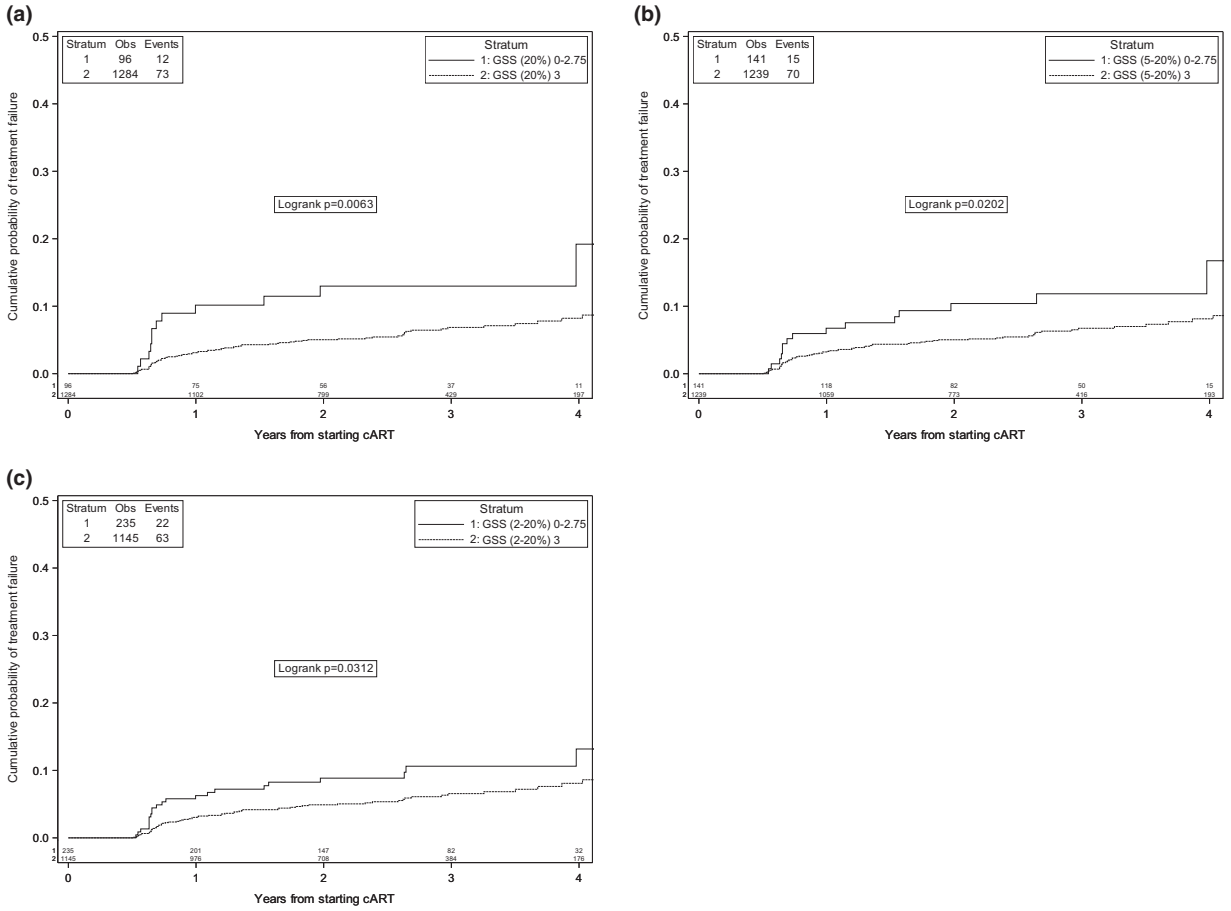

FIGURE 2 Kaplan–Meier estimates of the time to treatment failure according to Stanford University HIV Drug Resistance Database genotypic susceptibility score (GSS) categories and (a) >20%, (b) >5%, and (c) >2% threshold windows. Outcome: confirmed viral load >200 copies/mL or single viral load >200 copies/mL followed by antiretroviral therapy change. ART, antiretroviral therapy; cART, combined ART; Obs, observations; VL, viral load.

participants). The most common individual INSTI DRMs were T66AIK ($n = 6$, 0.4%), G140ACRS ($n = 8$, 0.6%), Y143CHR ($n = 7$, 0.5%), and Q148HKR ($n = 7$, 0.5%), all with a prevalence of <1%.

Overall, 85 participants met our definition of primary endpoint of TF. Using the 20% threshold, by 2 years from starting ART, the estimated proportion of participants who experienced TF were 13.0% (95% confidence interval [CI] 5.8–20.2) in the GSS 0–2.75 group versus 5.0% (95% CI 3.8–6.3) in the GSS = 3 group (log-rank $p = 0.006$, Figure 2a). Importantly, ∼10% in the GSS 0–2.75 group (95% CI 3.9–16.5%) had already experienced TF after 12 months of starting therapy.

Interestingly, in the unweighted survival analysis, performed separately using the three threshold windows, the strongest evidence against the null hypothesis was for the Sanger threshold (>20%) as compared with the minority variants thresholds (Figure 2a–c). For the >2% threshold, the Kaplan–Meier estimates of TF by 2.5 years were 7.1% (95% CI 3.2–10.9%) in the GSS 0–2.75 group versus 5.0% (95% CI 3.5–6.5) in the GSS = 3 group (log-rank $p = 0.03$, Figure 2c).

The results obtained in the unadjusted analysis were confirmed after controlling for the set of identified potential confounders (Supplementary Figure S1, Table 2). In particular, we estimated a >2-fold difference in risk of TF by GSS group using the Sanger window threshold, although an effect as small as a 16% increase in risk as well as a 4-fold increase in risk were all values compatible with the data (Table 2A). Of note, participants with a GSS in the 0–2.75 group were also at increased risk of TF when using the >2% window threshold for resistance, although the effect size was much smaller (adjusted for the confounders described in the direct acyclic graph [Figure S1], HR 1.66; 95% CI 1.01–2.71, $p = 0.04$). Among the 27 ART discontinuations, the reasons for the change were as follows: 12 (44%) for failure (high HIV RNA $n = 9$, low CD4 count $n = 1$ and detection of resistance $n = 2$, one of which also had an elevated HIV RNA), nine (33%) for intolerance/toxicity, four (15%) for

TABLE 2 Hazard ratio from fitting an unweighted Cox regression model with time-fixed covariates at entry.

	Unadjusted HR (95% CI)	*p*-value	Adjusted* HR (95% CI)	*p*-value
Panel (A): Unadjusted and adjusted HR of treatment failure endpoint[&]				
GSS (>20% window)				
3	1		1	
0–2.75	2.29 (1.24–4.21)	0.008	2.18 (1.16–4.09)	0.015
GSS (>5% window)				
3	1		1	
0–2.75	1.91 (1.10–3.34)	0.022	1.74 (0.99–3.07)	0.056
GSS (5–20% window[£])				
3	1		1	
0–2.75	1.72 (0.95–3.10)	0.074	1.56 (0.85–2.84)	0.150
GSS (>2% window)				
3	1		1	
0–2.75	1.69 (1.04–2.75)	0.033	1.66 (1.01–2.74)	0.046
GSS (2–20% window[£])				
3	1		1	
0–2.75	1.57 (0.95–2.60)	0.079	1.54 (0.93–2.56)	0.096
Panel (B): Subsets of participants who started a NNRTI-based regimen				
GSS (>20% window)				
3	1		1	
0–2.75	3.12 (1.61–6.05)	<0.001	2.93 (1.47–5.84)	0.002
GSS (>5% window)				
3	1		1	
0–2.75	2.58 (1.38–4.81)	0.003	2.25 (1.19–4.25)	0.012
GSS (5–20% window[£])				
3	1		1	
0–2.75	2.27 (1.17–4.42)	0.016	1.99 (1.01–3.90)	0.046
GSS (>2% window)				
3	1		1	
0–2.75	2.35 (1.35–4.12)	0.003	2.32 (1.32–4.09)	0.003
GSS (2–20% window[£])				
3	1		1	
0–2.75	2.16 (1.21–3.85)	0.009	2.15 (1.20–3.86)	0.011

Abbreviations: ART, antiretroviral therapy; CI, confidence interval; GSS, genotypic susceptibility score; HR, hazard ratio; NNRTI, non-nucleoside reverse transcriptase inhibitor;

*Adjusted for geographical region, baseline HIV RNA, intervention arm of START (immediate vs. differed) and year of starting ART.

[&]Pure confirmed virological failure or a single viral load >200 copies/mL followed by an ART change.

[£]Restricting to those with GSS[$] ≥2; to exclude those who could be detected as participants initiating a suboptimal regimen by Sanger sequence alone.

[$]HIVDB v8.6 with >20% threshold.

simplification (i.e., stop before switching to a simpler regimen), and the remaining two (0.7%) for other/unknown reasons. Results were similar when we used the alternative endpoint of TF, which only counted the discontinuations due to failure as events ($n = 77$ total events, Table S2).

Interestingly, in the main adjusted analysis, results were compatible with the null hypothesis of no difference when using the intermediate >5% window threshold. Of note, all these associations were much stronger and were all significant ($p < 0.05$) when we restricted the analysis to participants who started a regimen with an NNRTI as

the anchor drug (Table 2B, Table S3). The results of the sensitivity analysis after restricting to participants with windows of 2–20% and 5–20% were consistent with those of the main analysis (Table 2A,B). Supplementary Figure S3 and some supplemental text show the results of the analysis investigating the association between individual mutations and HIV RNA decline.

DISCUSSION

One key finding of this analysis is that PDR detected in >20% of the virus population was a confirmed determinant of failure of first-line ART. Of note, participants in START were treatment naïve because this was an entry criterion for the trial. Therefore, the most likely mechanism for study participants to have detectable HIV genotypic resistance at study entry would be due to infection with a resistant strain. Importantly, the results also confirm that PDR detected at >2% was also predictive of TF in our study population. We estimated a 66% increase in risk of TF in those with suboptimal versus optimal GSS but an increase risk as small as 1% was also compatible with the data, as indicated by the confidence limits. Importantly, the association was particularly strong when restricting to the participants who started first-line therapy with NNRTI-based regimens (>2-fold increase in risk). The results were also confirmed in several sensitivity analyses.

Of interest, the proportion that started a regimen predicted by the Stanford GSS to have suboptimal activity was low at 6.7% (96/138) when using the routine detection threshold of >20%. This could be due to the lack of routine testing in a particular region of recruitment in the trial, to the use of different systems to interpret the results, or to the time between the date of the stored sample and that of ART initiation. Indeed, our data support the fact that people who started suboptimal therapy were enriched in South America as compared with in Europe or the USA. The GSS 0–2.75 group also appeared to have had their HIV infection diagnosed more recently than those in the GSS = 3 group. In contrast, the median time from the date of stored sample and ART initiation was 12 months because of the inclusion of participants in both study treatment arms, with no difference by GSS groups.

The analysis also confirms the results presented in our previous work, showing that a large proportion of the PDR mutations were detected at a level between 2% and 5% [24]. This appeared to be particularly marked for INSTI-associated mutations but to a lesser extent for NNRTI-associated mutations, namely rilpivirine. When the association between individual mutations and week 4 change in viral load was investigated, INSTI-associated mutations N155H and G140ACRS detected at the 2–20% threshold appeared to be those more strongly associated with a slower decline (association was not significant, but the prevalence of these mutations was very low, affecting the power of the analysis).

In a systematic review of 25 studies examining the impact of minority variants on initial ART, only 11 (44%) showed an association between the detection of pre-existing minority variants and risk of VF of NNRTI-based first-line regimens [13, 14, 22, 31–34]. However, the review included heterogeneous studies with respect to target population, definition of the exposure, exact regimen initiated, and definition of the outcome, and a quantitative meta-analysis has not been performed. Also, little was done to rank the studies according to quality of the study design and statistical methods employed (i.e. how confounding was handled, the presence of other systematic biases such as selection and observer bias). For these reasons, it is difficult to compare results, and it appears to be misleading to present the crude percentage of studies in favour or against an association. Interestingly, the majority of studies showing no association were more recent analyses, conducted in the resource-limited settings in people receiving rilpivirine-based regimens [35–39]. Furthermore, the authors of this review, surprisingly, eventually concluded that minority variants have been shown to be clinically relevant, especially before the initiation of a first-line NNRTI-based regimen [22]. Our data support this conclusion.

One unmet need only partially addressed by this analysis is whether the data support a specific threshold for minority variants (i.e., 2% vs. 5% of virus populations) to be used in routine care.

Our findings seem to slightly favour the >2% threshold, although much larger studies are needed that stratify by specific target populations, mutations, and treatment started to be able to give recommendations for one threshold over another.

A number of limitations should be discussed. One key limitation is that most participants initiated regimens that are no longer routinely started in the first line. Unfortunately, although one of the goals was to assess the role of minority variants to predict response to INSTI-based regimens, our analysis was underpowered to answer this question. Nevertheless, results are relevant for countries in which NNRTI-based regimens are still a prevalent option for first-line therapy. WHO antiretroviral guidelines recommend the use of efavirenz as an alternative option in first-line ART regimens [40], which is still implemented in low- and medium-income counties if levels of pretreatment drug resistance to NNRTIs are <10%. Of course, all our results are valid

under the set of strong, mainly untestable, assumptions, including no unmeasured confounding, which we cannot rule out. Of note, by decreasing the threshold of the detection to 2%, the number of unusual mutations increased, which may lead to the detection of spurious associations because of misclassification of the exposure. Nevertheless, the NGS data were carefully reviewed by stipulating a minimum read depth of 200 across the HIV regions spanning all relevant mutations within each gene to minimize chance detection of unusual mutations at low levels. Finally, genotypic resistance tests were not conducted at time of failure. These additional data could have been useful to verify the extent of outgrowth of the minority variants detected at baseline and provide additional evidence towards a putative causative effect of minority variants as a key determinant of TF. Also, it would have given an estimate of the potential impact of accumulated resistance on the response to second-line regimens.

In conclusion, this analysis confirms an association between detectable minority variants and risk of failure of first-line ART. Importantly, given prior conflicting results regarding the impact of NNRTI minority variants, the data confirmed a strong association when the analysis was restricted to the subset of participants who initiated a NNRTI-based regimen. Further studies are needed to address other relevant unanswered questions, such as the role of minority variant INSTI-associated mutations and whether the ability of minority variants to predict TF might vary by mutational load.

AUTHOR CONTRIBUTIONS

Alessandro Cozzi-Lepri drafted the first version of the manuscript with support from John D. Baxter, David Dunn, Anna Tostevin, and Shweta Sharma. Alessandro Cozzi-Lepri, David Dunn, and Anna Tostevin were primarily responsible for performing the statistical analyses. All authors reviewed the study data and provided comments and approval prior to manuscript submission.

ACKNOWLEDGEMENTS

The authors thank the study participants and clinical staff of the START trial (see Initiation of antiretroviral therapy in early asymptomatic individuals. *New England Journal of Medicine* 2015;373:794-807 for a complete list of START investigators [23]).

FUNDING INFORMATION

The study was supported in part by the National Institute of Allergy and Infectious Diseases; National Institutes of Health (grants UM1-AI068641, UM1-AI120197); National Heart, Lung, and Blood Institute (NHLBI; grant RO1HL096453); National Institutes of Health Clinical Center; National Cancer Institute; NHLBI; Eunice Kennedy Shriver National Institute of Child Health and Human Development; National Institute of Mental Health; National Institute of Neurological Disorders and Stroke; National Institute of Arthritis and Musculoskeletal and Skin Diseases; Agence Nationale de Recherches sur le SIDA et les Hepatites Virales (France); National Health and Medical Research Council (Australia); National Research Foundation (Denmark); Bundes ministerium fur Bildung und Forschung (Germany); European AIDS Treatment Network; Medical Research Council (UK); National Institute for Health Research; National Health Service (UK); and the University of Minnesota. Antiretroviral drugs were donated to the central drug repository by AbbVie, Bristol-Myers Squibb, Gilead Sciences, GlaxoSmithKline/ViiV Healthcare, Janssen Scientific Affairs, and Merck.

CONFLICT OF INTEREST STATEMENT

JDB serves as a consultant for Quest Diagnostics. All other authors declare no conflicts of interest.

ORCID

Alessandro Cozzi-Lepri https://orcid.org/0000-0002-6339-919X
Mark Gompels https://orcid.org/0000-0002-8006-325X
Angie N. Pinto https://orcid.org/0000-0003-3583-9490

REFERENCES

1. Stella-Ascariz N, Arribas JR, Paredes R, Li JZ. The role of HIV-1 drug-resistant minority variants in treatment failure. *J Infect Dis*. 2017;216(suppl_9):S847-S850. doi:10.1093/infdis/jix430
2. Johnson JA, Li JF, Wei X, et al. Minority HIV-1 drug resistance mutations are present in antiretroviral treatment-naïve populations and associate with reduced treatment efficacy. *PLoS Med*. 2008;5(7):e158. doi:10.1371/journal.pmed.0050158
3. Li JZ, Kuritzkes DR. Clinical implications of HIV-1 minority variants. *Clin Infect Dis*. 2013;56(11):1667-1674. doi:10.1093/cid/cit125
4. Paredes R, Marconi VC, Campbell TB, Kuritzkes DR. Systematic evaluation of allele-specific real-time PCR for the detection of minor HIV-1 variants with pol and env resistance mutations. *J Virol Methods*. 2007;146:136-146.
5. Ellis GM, Vlaskin TA, Koth A, et al. Simultaneous and sensitive detection of human immunodeficiency virus type 1 (HIV) drug resistant genotypes by multiplex oligonucleotide ligation assay. *J Virol Methods*. 2013;192:39-43.
6. Jakobsen MR, Tolstrup M, Søgaard OS, et al. Transmission of HIV-1 drug-resistant variants: prevalence and effect on treatment outcome. *Clin Infect Dis*. 2010;50:566-573.
7. Li JZ, Chapman B, Charlebois P, et al. ACTG A5262 study team. Comparison of Illumina and 454 deep sequencing in participants failing raltegravir-based antiretroviral therapy. *PloS One*. 2014;9:e90485.
8. Gibson RM, Meyer AM, Winner D, et al. Sensitive deep-sequencing-based HIV-1 genotyping assay to simultaneously

determine susceptibility to protease, reverse transcriptase, integrase, and maturation inhibitors, as well as HIV-1 coreceptor tropism. *Antimicrob Agents Chemother*. 2014;58:2167-2185.
9. 2022 Update of the Drug Resistance Mutations in HIV-1 Topics in Antiviral Medicine. Volume 30 Issue 4 October/November 2022.
10. Tang MW, Shafer RW. HIV-1 Antiretroviral resistance scientific principles and clinical applications. *Drugs*. 2012;72:1-25.
11. Gibson RM, Weber J, Winner D, Miller MD, Quiñones-Mateu ME. Contribution of human immunodeficiency virus type 1 minority variants to reduced drug susceptibility in patients on an integrase strand transfer inhibitor-based therapy. *PloS One*. 2014 Aug 11;9(8):e104512. doi:10.1371/journal.pone.0104512
12. Fonager J, Larsson JT, Hussing C, Neess Engsig F, Nielsen C, Fischer TK. Identification of minority resistance mutations in the HIV-1 integrase coding region using next generation sequencing. *J Clin Virol*. 2015;73:95-100.
13. Li JZ, Paredes R, Ribaudo HJ, et al. Low-frequency HIV-1 drug resistance mutations and risk of NNRTI-based antiretroviral treatment failure: a systematic review and pooled analysis. *JAMA*. 2011;305:1327-1335.
14. Cozzi-Lepri A, Noguera-Julian M, Di Giallonardo F, et al. CHAIN minority HIV-1 variants working group. Low-frequency drug-resistant HIV-1 and risk of virological failure to first-line NNRTI-based ART: a multicohort European case-control study using centralized ultrasensitive 454 pyrosequencing. *J Antimicrob Chemother*. 2015;70:930-940.
15. Halvas EK, Wiegand A, Boltz VF, et al. Low frequency nonnucleoside reverse-transcriptase inhibitor-resistant variants contribute to failure of efavirenz-containing regimens in treatment-experienced patients. *J Infect Dis*. 2010;201:672-680.
16. Casadellà M, Manzardo C, Noguera-Julian M, et al. ADVANZ and ADVANZ-3 investigators. Clinical value of ultradeep HIV-1 genotyping and tropism testing in late presenters with advanced disease. *Aids*. 2015;29:1493-1504.
17. Casadellà M, Noguera-Julian M, Sunpath H, et al. Treatment options after virological failure of first-line tenofovir-based regimens in South Africa: an analysis by deep sequencing. *Aids*. 2016;30:1137-1140.
18. Charpentier C, Lee GQ, Rodriguez C, et al. Highly frequent HIV-1 minority resistant variants at baseline of the ANRS 139 TRIO trial had a limited impact on virological response. *J Antimicrob Chemother*. 2015;70:2090-2096.
19. Kyeyune F, Gibson RM, Nankya I, et al. Low-frequency drug resistance in HIVinfected Ugandans on antiretroviral treatment is associated with regimen failure. *Antimicrob Agents Chemother*. 2016;60:3380-3397.
20. Mohamed S, Penaranda G, Gonzalez D, et al. Comparison of ultra-deep versus sanger sequencing detection of minority mutations on the HIV-1 drug resistance interpretations after virological failure. *Aids*. 2014;28:1315-1324.
21. Nishizawa M, Matsuda M, Hattori J, et al. Longitudinal detection and persistence of minority drug-resistant populations and their effect on salvage therapy. *PloS One*. 2015;10:1-13.
22. Mbunkah HA, Bertagnolio S, Hamers RL, et al. Low-abundance drug-resistant HIV-1 variants in antiretroviral drug-naive individuals: a systematic review of detection methods, prevalence, and clinical impact. *J Infect Dis*. 2020;221(10):1584-1597.
23. Group ISS, Lundgren JD, Babiker AG, et al. Initiation of antiretroviral therapy in early asymptomatic HIV infection. *N Engl J Med*. 2015;373:795-807.
24. Baxter JD, Dunn D, Tostevin A, et al. Transmitted HIV-1 drug resistance in a large international cohort using next-generation sequencing: results from the strategic timing of antiretroviral treatment (START) study. *HIV Med*. 2020;22:360-371.
25. Bennedbæk M, Zhukova A, Tang M-HE, et al. For the INSIGHT START Study Group, phylogenetic analysis of HIV-1 shows frequent cross-country transmission and local population expansions. *Virus Evolution*. 2021;7(2):1-10.
26. Verbist B, Thys K, Reumers J, et al. VirVarSeq: a low-frequency virus variant detection pipeline for Illumina sequencing using adaptive base-calling accuracy filtering. *Bioinformatics (Oxford, England)*. 2015;31:94-101.
27. Baxter J, Dunn D, White E, et al. Global HIV-1 transmitted drug resistance in the INSIGHT S trategic T iming of a nti R etroviral T reatment (START) trial. *HIV Med*. 2015;16:77-87.
28. Bennett DE, Camacho RJ, Otelea D, et al. Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. *PloS One*. 2009;4:e4724.
29. Database SUHDR. 2016 Surveillance Drug Resistance Mutation (SDRM) Worksheet:INIs.
30. Liu TF, Shafer RW. Web resources for HIV type 1 genotypic-resistance test interpretation. *Clin Infect Dis*. 2006;42:1608-1618.
31. Inzaule SC, Hamers RL, Noguera-Julian M, et al. PanAfrican studies to evaluate resistance. Clinically relevant thresholds for ultrasensitive HIV drug resistance testing: a multicountry nested case-control study. *Lancet HIV*. 2018;5:e638-e646.
32. Geretti AM, Fox ZV, Booth CL, et al. Low-frequency K103N strengthens the impact of transmitted drug resistance on virologic responses to first-line efavirenz or nevirapinebased highly active antiretroviral therapy. *J Acquir Immune Defic Syndr*. 2009;52:569-573.
33. Metzner KJ, Giulieri SG, Knoepfel SA, et al. Minority quasispecies of drug-resistant HIV-1 that lead to early therapy failure in treatment-naive and -adherent patients. *Clin Infect Dis*. 2009 Jan 15;48(2):239-247.
34. Paredes R, Lalama CM, Ribaudo HJ, et al. Pre-existing minority drug-resistant HIV-1 variants, adherence, and risk of antiretroviral treatment failure. *J Infect Dis*. 2010;201(5):662-671. doi:10.1086/650543
35. Metzner KJ, Scherrer AU, von Wyl V, et al. Swiss HIV cohort study. Limited clinical benefit of minority K103N and Y181C-variant detection in addition to routine genotypic resistance testing in antiretroviral therapy-naive patients. *Aids*. 2014;28:2231-2239.
36. Raymond S, Nicot F, Pallier C, et al. French National Agency for Research on AIDS and Viral Hepatitis (ANRS) AC11 resistance study group. Impact of human immunodeficiency virus type 1 minority variants on the virus response to a rilpivirine-based first-line regimen. *Clin Infect Dis*. 2018;66:1588-1594.
37. Su B, Zheng X, Liu Y, et al. Detection of pretreatment minority HIV-1 reverse transcriptase inhibitor-resistant variants by ultra-deep sequencing has a limited impact on virological outcomes. *J Antimicrob Chemother*. 2019;74:1408-1416.
38. Van Eygen V, Thys K, Van Hove C, et al. Deep sequencing analysis of HIV-1 reverse transcriptase at baseline and time of

failure in patients receiving rilpivirine in the phase III studies ECHO and THRIVE. *J Med Virol*. 2016;88:798-806.
39. Zoufaly A, Jochum J, Hammerl R, et al. Virological failure after 1 year of first-line ART is not associated with HIV minority drug resistance in rural Cameroon. *J Antimicrob Chemother*. 2015;70:922-925.
40. https://www.who.int/publications/i/item/9789240031593

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Cozzi-Lepri A, Dunn D, Tostevin A, et al. Rate of response to initial antiretroviral therapy according to level of pre-existing HIV-1 drug resistance detected by next-generation sequencing in the strategic timing of antiretroviral treatment (START) study. *HIV Med*. 2023;1-11. doi:10.1111/hiv.13556