HIV-1 viral load is elevated in individuals with reverse transcriptase mutation M184V/I during virological failure of first line antiretroviral therapy and is associated with compensatory mutation L74I.

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40 word summary:

Lamivudine is a cornerstone antiretroviral whose efficacy has been ascribed to high fitness cost of the lamivudine resistance mutation M184V. However, here we demonstrate elevated viral loads in the context of M184V, likely attributable to compensatory mutations such as L74I.

Abstract

Background: M184V/I cause high-level lamivudine (3TC) and emtricitabine (FTC) resistance, and increased tenofovir (TDF) susceptibility. Nonetheless, 3TC and FTC (collectively referred to as XTC) appear to retain modest activity against HIV-1 with these mutations possibly as a result of reduced replication capacity. Here we determined how M184V/I impacts virus load (VL) in patients failing therapy on a TDF/XTC plus nonnucleoside RT inhibitor (NNRTI)-containing regimen.

Methods: We compared VL in absence and presence M184V/I across studies using random effects meta-analysis. The effect of mutations on virus RT activity and infectiousness was analysed in vitro.

Results: M184I/V was present in 817 (56.5%) of 1445 individuals with VF. VL was similar in individuals with or without M184I/V (difference in $\log_{10}$VL 0.18, 95% CI 0.05-0.31). CD4 count was lower both at initiation of ART and at VF in participants who went on to develop M184V/I. L74I was present in 10.2% of persons with M184V/I but absent in persons without M184V/I ($p<0.0001$). In vitro, L74I compensated for defective replication of M184V mutated virus.

Conclusion: Virus loads were similar in persons with and without M184V/I during VF on a TDF/XTC/NNRTI-containing regimen. We therefore do not find evidence for a benefit of XTC in the context of first line failure on this combination.

Key words: antiretroviral; drug resistance; HIV; Lamivudine; fitness cost; compensatory mutation

Introduction

The global scale up of antiretroviral therapy (ART) using a public health approach with limited viral load monitoring has been accompanied by high prevalence of drug resistance to NNRTI containing regimens amongst individuals with virological failure in LMIC, [1-3] [4-6].

The cytosine analogues lamivudine (3TC) and emtricitabine (FTC), collectively referred to as XTC, are components of first and second line regimens recommended by WHO. However, high level XTC resistance can be conferred and selected by single amino acid changes at position 184 of RT in the highly conserved (Y183, M184, D185, D186) amino acid domain that includes the active (catalytic) site of the p66 polymerase subunit of RT[7]. M184V/I are the most commonly occurring drug-resistance mutations in persons with acquired resistance to first-generation NNRTI containing regimens[1-3] [4-6].

Several lines of evidence suggest that in addition to causing high-level reductions in XTC susceptibility in vitro and modestly increased TDF susceptibility, viruses with these mutations retain some in vivo susceptibility to XTC possibly because of their reduced replication capacity[8-10]. For example early studies showed that in patients receiving 3TC monotherapy, or dual therapy with AZT/3TC, VL did not return to baseline despite the development of M184V[9, 11-14]. In addition, discontinuation of lamivudine during combination ART was associated with a modest increase in VL[15-17]. By contrast the COLATE study, a randomised controlled trial conducted in Europe in the early 2000s, showed there was no effect of removal of lamivudine from a failing regimen where the endpoint was viral suppression to <200 copies/ml or viral load change of 1.4$\log_{10}$[18].

To understand the relationship between M184I/V and viral load in the era of tenofovir based cART where TAMs were not present, and also in the context of limited or no access to viral load monitoring, we therefore studied individuals failing the WHO recommended regimen first line regimen TDF/Xtc/NNRTI across a range of settings.

Methods

The study population has previously been described and is presented in Supplementary Table 1[20-41]. Patients treated with tenofovir disoproxil fumarate (TDF) plus 3TC/FTC and NVP/EFV were included where there was documented virologic failure (VF) and RT sequence data from codons 40-240 were available. VF was locally determined, and for low-middle income countries (LMIC) the threshold was 1000 copies/ml. HIV-1 RT sequences were determined by standard Sanger sequencing at individual study sites.

Mutations were defined as amino acid differences at positions 1 to 240 between each sequence and the consensus subtype B amino acid reference sequence. As some individuals may have been exposed to thymidine analogues prior to TDF-containing regimens[5], we excluded individuals with sequences containing thymidine analogue mutations (TAMs) – M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E.

Each sequence was subtyped as previously described and sequence quality control measures were taken to identify sequences with APOBEC G-to-A hypermutation[20]. Duplicate sequences were removed. All patients reported that they were ARV naïve at baseline. The primary outcome was viral load at VF, hence patients without this outcome were excluded.

Statistical analysis

We graphically compared the distribution of log10 viral loads according to presence of M184I/V mutation both within and across studies. To quantify the impact of M184I/V on viral load, we calculated mean log10 viral load in each study according to M184I/V. Differences were pooled across studies using random effects meta-analysis. Estimates of the standard error in each study were calculated by dividing the pooled estimate of the standard deviation by the square root of the number of patients with/without M184I/V in any given study. We repeated this process in subgroups of patients defined by several baseline characteristics: presence of K65R

mutation, presence of major NNRTI mutations, choice of NRTI, choice of NNRTI, categories of baseline CD4 count (< and > 200 cells/mm^{3}) and categories of baseline viral load (< and > 100,000 copies per mL). Analyses of CD4 count and treatment failure used the same methods. To assess whether M184I/V was associated with viral load at failure independently of other mutations, we performed a separate analysis in which we used a mixed linear regression model adjusting for study as a random effect and other mutations associated with increased viral load (which were identified by forward stepwise variable selection). Next, we used Fisher's exact test to identify mutations associated with M184I/V. We used two-sided p-values and Stata version 15.1 for all statistical analyses.

In vitro analyses

A patient derived *pol* sequence was identified with mutations of interest and the gag-PR-RT-IN region amplified by PCR with flanking restriction sites inserted into primers. Following cloning into an expression plasmid, site directed mutagenesis was performed to revert (i) isoleucine back to leucine at RT amino acid 74, (ii) valine back to methionine at RT amino acid 184, or both. Plasmids expressing gag-pol were co-transfected into 293T cells along with a VSV-G envelope expressing plasmid and a vector encoding luciferase expressed from an LTR promoter as previously described[42]. Supernatant containing virus was harvested 2 days later and used to infect fresh 293T cells. Luminescence as a read out of infection was read by luminometry 2 days later. Viral p24 abundance in supernatants was estimated using western blot using a p24 antibody as previously described[43].

Results

Amongst 2873 participants included in the initial group, 1445 from 32 study groups across 15 countries had an available failure viral load measurement of which M184I/V was present in 817 (56.5%) (Table 1 and supplementary Table 1). Participants were from sub-Saharan Africa (55.4%), Asia (19.2%), Europe (16.2%) and North America (9.3%). All participants were on TDF, most of them also treated with EFV (75.2%) and 3TC (64.5%), and participants harboring M184I/V mutated virus were significantly more likely to have high level tenofovir and NNRTI resistance (Table 1B). Participants harboring M184I/V were also more likely to have multiple

NNRTI mutations.

In a crude comparison of viral load at failure, patients with M184I/V present had a higher median log 10 viral load (4.7, interquartile range (IQR): 3.4-5) than patients without M184I/V (median 4.3, IQR 4.1-5.3). When restricting analyses to comparisons of patients within the same study, the estimated difference in viral load was non-significant in the vast majority of studies (Figure 1). When within-study differences were pooled across studies, there was a marginally higher viral load in patients with M184I/V present compared to absent (pooled difference in log10 viral load 0.18, 95% CI 0.05-0.31) (Figure 2). Following statistical adjustment for other mutations independently associated with increased viral load, M184I/V was no longer significantly associated with viral load at failure. However, the estimated difference and 95% confidence interval (0.09, 95% CI -0.01 to 0.20) excluded any meaningful decrease in failure viral load associated with M184I/V. There was no evidence that relationship between M184I/V and failure viral load was modified by choice of NRTI, choice of NNRTI, or drug resistance to NNRTI or tenofovir (Figure 2).

We next explored the relationship between detection of M184I/V failure and CD4 count, noting that the duration of VF was likely longer in LMIC regions. Mean baseline CD4 was significantly lower amongst patients who went on to develop M184I/V by treatment failure compared to those who did not (88 vs 180, $p<0.0001$). Similarly, at VF, presence of M184V/I was associated with lower CD4 count, though the difference was greater (Figure 3). Between baseline and treatment failure, CD4 count increased to a similar extent in patients with and without M184I/V (median increase: 79 vs 48 cells/mm^3, $p=0.55$).

We next examined NRTI mutations associated with M184V/I that might play a compensatory role for M184I/V. We looked for associations in the dataset between M184V/I and RT amino acid positions known to be associated with drug exposure. Figure 4 shows mutations with strong evidence of an association with M184I/V. Many of these mutations have previously been associated with drug resistance to tenofovir, either directly (K65R, K70E) or as compensatory mutations for K65R (A62V, S68N, F155Y). The following NNRTI mutations were also associated (A98G, L100I, K103R, V108I, Y181C, Y188L, G190A, P225H, L228R, M230L).

Of note, L74I was the only mutation to be exclusively associated with M184V/I, occurring in 83 (10.2%) of patients with M184I/V, and in none of the 628 patients in which M184I/V was absent (p for association <0.0001). L74I was observed in 11.7% of subtype C infected participants with M184I/V at VF, and in 14.4% of CRF01_AE participants with M184I/V at VF (Supplementary Table 2).

Given a previous report that L74I can restore replication to a virus with the K65R mutation without conferring drug resistance[44], we next sought to test the hypothesis that L74I could restore replication 'fitness' to a M184V mutant virus, explaining the higher than expected viral loads. Molecular characterisation of virus with the mutations M184V and L74I was undertaken. The viral isolate tested also had the NNRTI resistance mutations A98G, K103N and P225H. Site directed mutagenesis was performed to revert isoleucine back to leucine at 74 and Valine to Methionine at 184 (Figure 5A). We did not however assess the impact of M184I. We measured (i) infectivity of these viruses and (ii) reverse transcriptase efficiency in a single round replication assay (Figure 5). We found that removing the L74I mutation significantly decreased the efficiency of reverse transcription (Figure 5B, compare left bar with middle bar) whilst virus abundance was not affected as determined by western blot of viral p24 abundance in supernatants (Figure 5B bottom panel). Infectivity was also significantly decreased by reversion of the compensatory mutation (Figure 5C, compare left bar with middle bar). Mutation of M184V back to M, leaving a virus with only L74I, had no impact on reverse transcriptase efficiency and a minor effect on infectivity (Figure 5B, C compare left and right bars).

Discussion

Despite having a low genetic barrier to drug resistance, lamivudine has retained importance and a central role in both first and second line ART [45]. A complete understanding of lamivudine efficacy is therefore important, particularly given reports suggesting that lamivudine use confers viral load benefit despite high level resistance to the drug in the form of the M184V/I.

Our primary finding that viral load was similar in participants with and without M184V/I at the time of VF was robust across baseline CD4 count, baseline viral load, gender, and different NNRTI and NRTI drugs in the first line treatment regimen. We observed lower baseline and VF CD4 counts in individuals with M184V/I, though rate of change of CD4 did not differ based on M184V/I status. Lower baseline CD4 count is known to be associated with higher VF rates and a higher probability of drug resistance at VF[6, 46]. A possible explanation for this finding is that the antiviral effect of a competent immune system is important in limiting replication and emergence of resistance in tissue compartments where ARV drug penetration is suboptimal. A lower CD4 count at VF in the group with M184V/I further argues against this mutation being 'protective' or 'benign'. These data are also consistent with reports of the pathogenic potential of M184V containing viruses in both humans[47] and animal models[48].

We identified L74I as being specifically enriched in individuals with M184V and not present at all in those without M184V/I. We observed significant prevalence of L74I in subtypes C and CRF01_AE, though limited numbers of participants across subtypes limited a full understanding of subtype distribution. *In vitro* experiments demonstrated that L74I restores replication efficiency to a virus with the M184V mutation over a single round of infection, and that enhancement was due to efficiency of HIV reverse transcription in viral particles.

The emergence of L74I exclusively in patients with M184V/I suggests an *in vivo* selection advantage of L74I + M184V replication over M184V alone at least in some individuals. L74I was first reported as a mutation associated with exposure to abacavir or less commonly tenofovir[49, 50], and it appeared more common in patients with thymidine analogue mutations[50]. Correlation with M184V/I has not been made to date and in vitro experiments not performed with L74I + M184V/I containing viruses.

As L74I was observed only in around 10% of those with M184V/I, we postulate that alternative mutations, less strongly linked to M184V/I or perhaps outside the region of the *pol* gene sequenced in this study, could have similar effects as L74I in participants with M184V/I. Data from our study support the transmission potential of

M184V/I containing viruses in the context of prolonged virological failure and accumulated co-evolved mutations in RT that occurs under 'real world' conditions.

Limitations of this study include its retrospective cross-sectional design, absence of drug levels or adherence data and unknown duration of VF for participants. Our study was not designed to provide a mechanistic understanding of the relationship between M184 and fitness, rather to understand the pathogenic potential of M184V containing viruses in treated 'real world' patients. Finally. there was heterogeneity between population groups, and to account for this, analyses were conducted within study. It should also be noted that stratification by tenofovir or NNRTI resistance resulted in small numbers for sub analyses.

In summary, we show that lamivudine resistant and susceptible viruses show similar viral loads in patients failing NNRTI based ART containing lamivudine, tenofovir and NNRTI, likely in part due to viral evolution of compensatory changes that maintain replication efficiency of M184V/I containing viruses. These data reinforce the importance of effective viral load monitoring to limit HIV drug resistance and disease progression in the face of suboptimal drug pressure, particularly in low resource settings. Finally, given that we did not find benefit of lamivudine in failing first line patients, a prospective clinical trial could to determine whether there is benefit for including XTC in second-line regimens for the treatment of persons whose viruses develop M184I/V following VF on a first-line treatment regimen.

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Conflicts of interest

RG has acted as ad hoc consultant for Gilead Sciences and ViiV. CP has acted as ad hoc consulant for Gilead Sciences, Merck, Janssen, Theratechnologies and ViiV. RWS has received research funding from Janssen.

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Table 1: Baseline characteristics of participants by geographic region

Table 2: Summary of drug resistance characteristics of participants at virological failure with tenofovir + cytosine analogue + NNRTI by geographical region

Figure 1: Difference in viral load by mutations at RT position 184 in study groups with 95% confidence interval using random effects meta-analysis. Boxes represent mean with 95% CI. Estimates to the right indicate higher viral load in the presence of M184V/I, and estimates to the left lower viral load in presence of M184V/I.

Figure 2: Association of M184V/I mutation with log10 viral load across subgroups. Diamonds represent mean with 95% CI. Estimates to the right indicate higher viral load in the presence of M184V/I.

Figure 3: Differences in CD4 count during virological failure within studies by presence and absence of M184V/I. Boxes represent mean with 95% CI. Estimates to the left of centre line indicate lower CD4 count in participants with M184V/I.

Figure 4: HIV reverse transcriptase inhibitor resistance associated mutations enriched in virologically failing participants (n=1445) with M184V/I. Mutations are shown that occurred in at least 10% of individuals with M184V/ at a significance level of <0.001.

Figure 5. In vitro replication measurement of lamivudine resistant subtype C clinical isolate containing M184V and L74I and revertant mutations. A. Amino acid multiple sequence alignment of clinical isolate and revertant mutants generated by site directed mutagenesis. Numbering is relative to strain HXB2. B. In vitro reverse transcription efficiency contained in pelleted single round virus from cells producing clinical HIV isolate RT sequence and mutants. Bottom panel shows western blot of corresponding virus associated p24 in supernatants from cells. C. Single round infection of target HEK 293T cells by equal quantities of luciferase expressing VSV-G pseudotyped HIV viruses from B. Data in B and C were performed in replicate and means are presented with error bars corresponding to standard deviation. RLU: relative light units.

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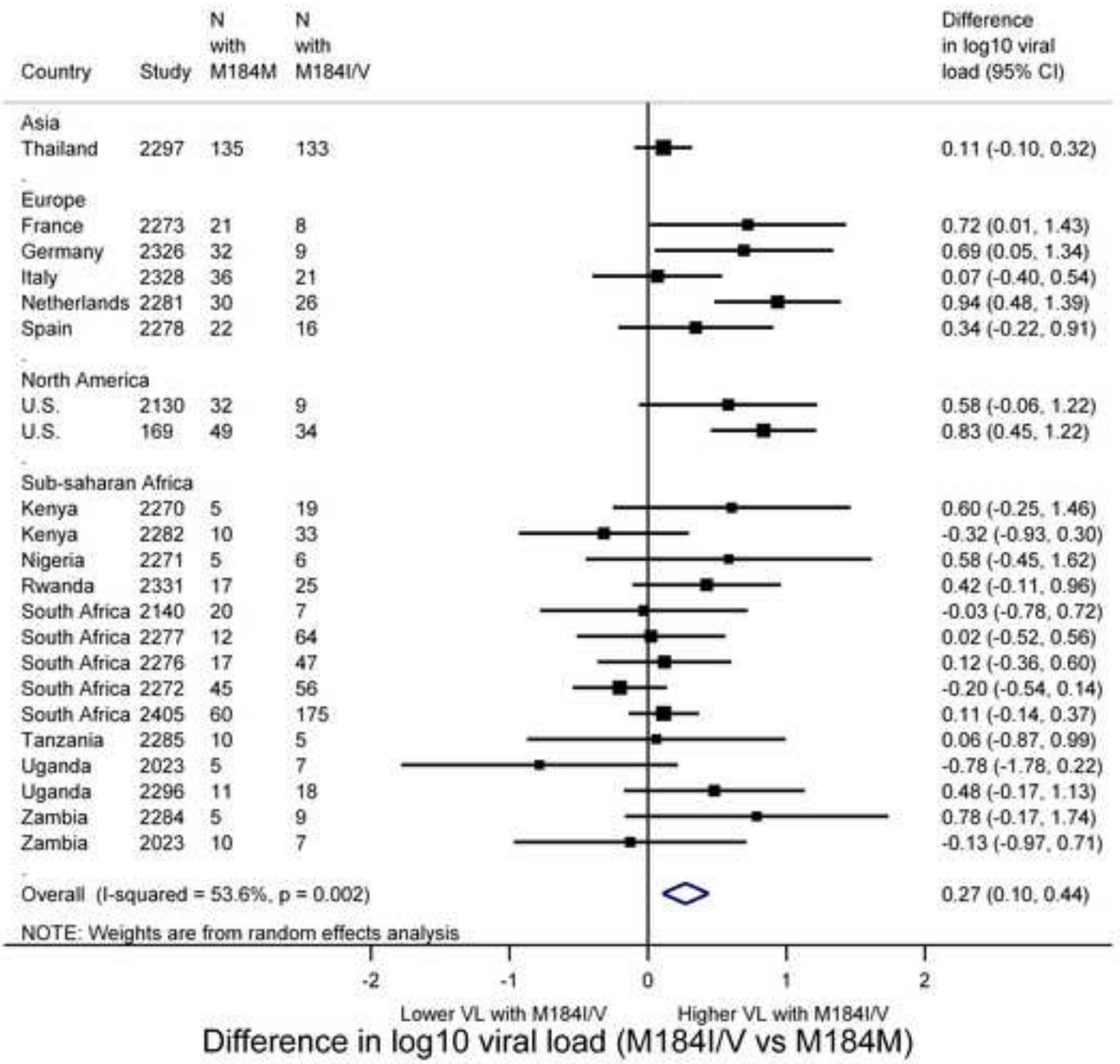
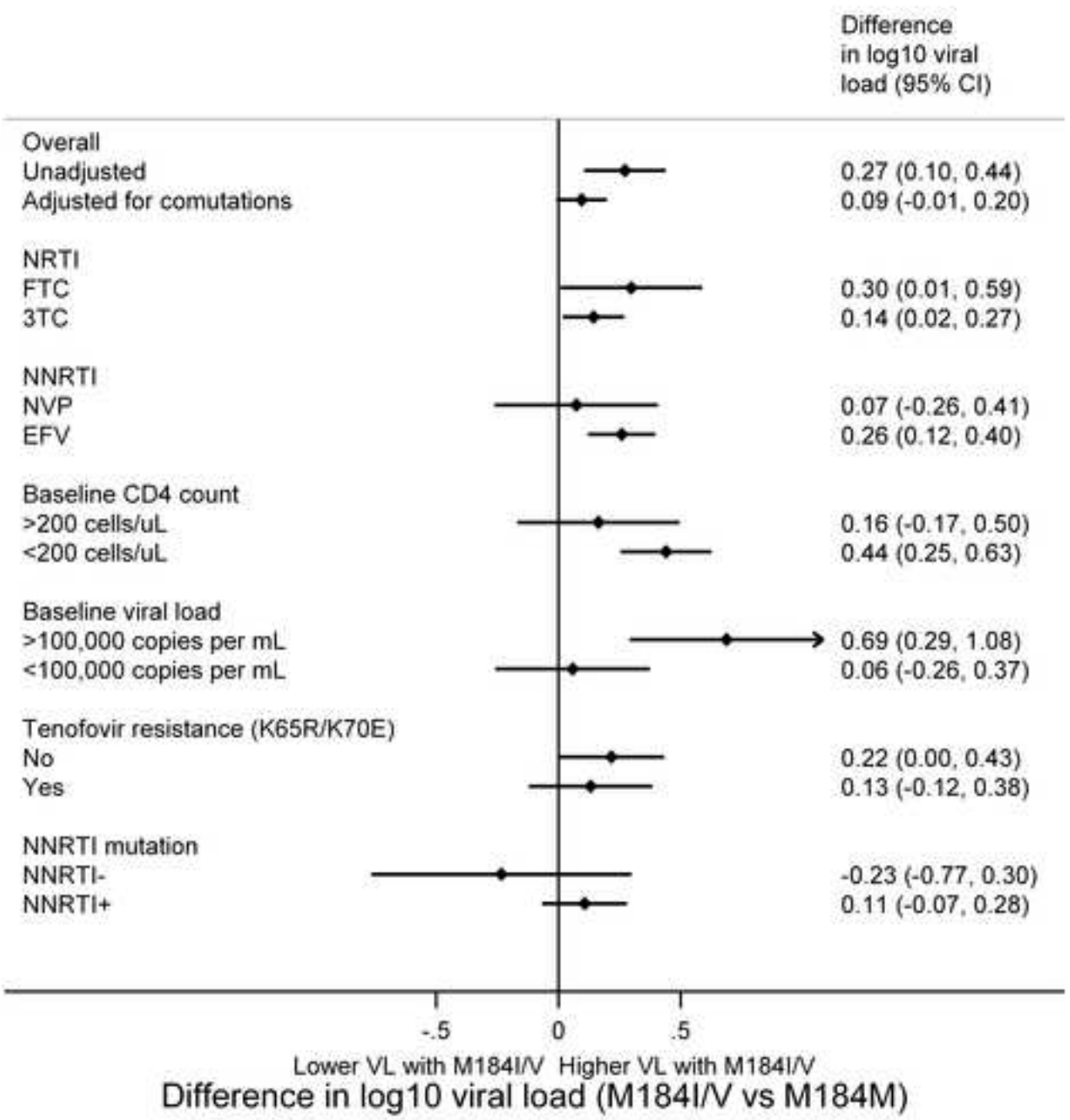
Table1

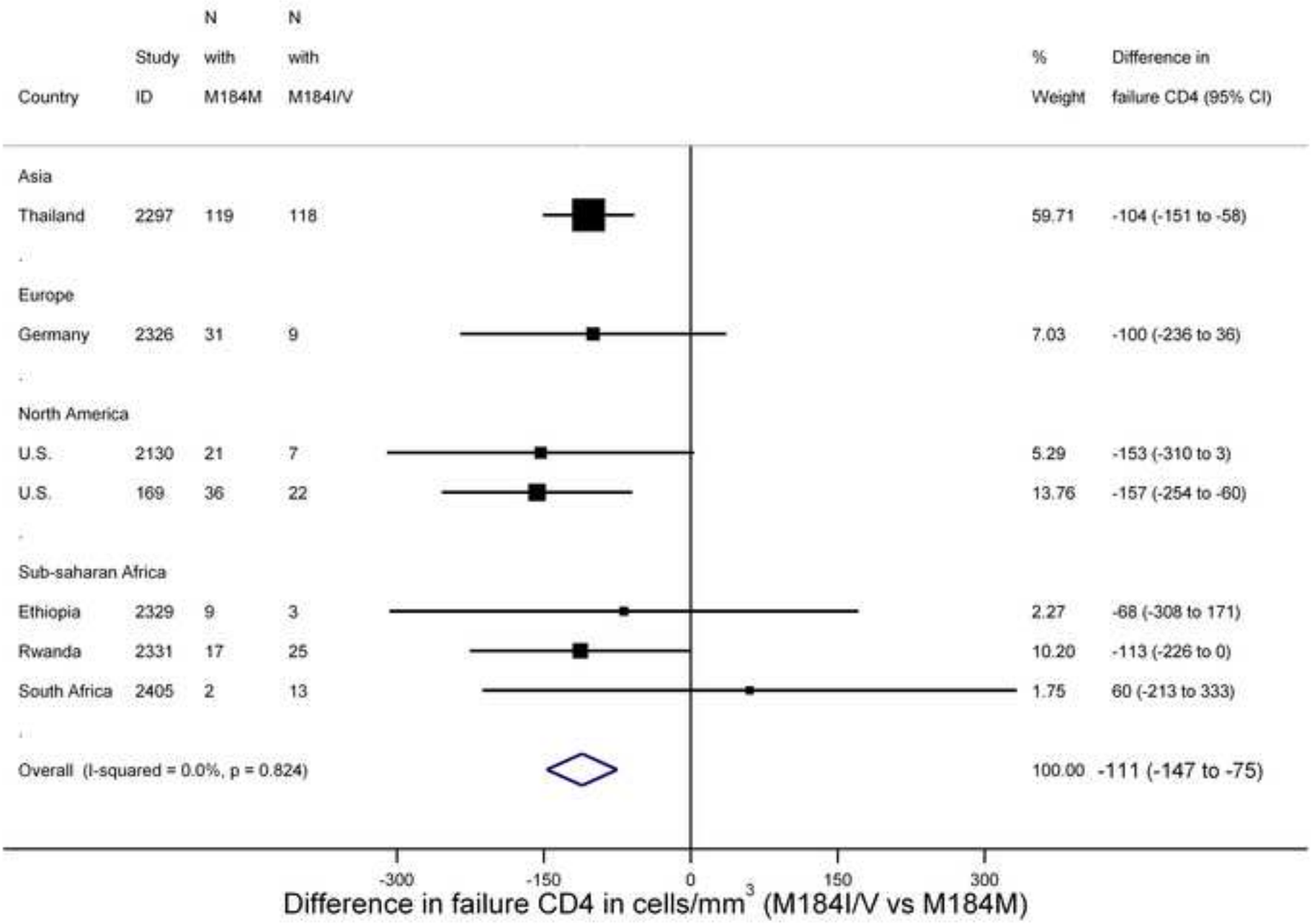
Region	M184 I/V	Patients	EFV	3TC	Baseline CD4 count		Baseline log10 viral load	
					N with data		N with data	
Overall	No	628	523 (83.3%)	350 (55.7%)	351	180.0 (82.0 to 288.0)	253	5.0 (4.5 to 5.5)
	Yes	817	564 (69.0%)	582 (71.2%)	385	88.0 (36.0 to 165.0)	187	5.2 (4.7 to 5.7)
Sub-saharan Africa	No	257	198 (77.0%)	204 (79.4%)	142	148.0 (69.0 to 264.0)	43	5.3 (4.5 to 5.7)
	Yes	543	356 (65.6%)	430 (79.2%)	270	77.0 (35.0 to 138.0)	71	5.3 (4.7 to 5.7)
Asia	No	136	112 (82.4%)	110 (80.9%)	0	-	0	-
	Yes	141	121 (85.8%)	122 (86.5%)	4	69.5 (33.5 to 159.0)	5	4.7 (4.6 to 5.9)
Europe	No	146	127 (87.0%)	25 (17.1%)	138	199.5 (84.0 to 304.0)	136	5.0 (4.6 to 5.5)
	Yes	88	53 (60.2%)	23 (26.1%)	77	157.0 (62.0 to 232.0)	76	5.1 (4.8 to 5.7)
North America	No	89	86 (96.6%)	11 (12.4%)	71	204.0 (98.0 to 351.0)	77	4.7 (4.3 to 5.3)
	Yes	45	34 (75.6%)	7 (15.6%)	34	67.5 (27.0 to 156.0)	35	5.2 (4.8 to 5.6)

Table2

Region	M184 I/V	TDF resistance, n (%)	At least one major NNRTI mutation, n (%)	Number of NNRTI mutations, mean (SD)	Failure log10 viral load	Failure CD4 count, median (IQR)	
						N with data	Median (IQR)
Overall	No	137 (21.8%)	380 (60.5%)	1.2 (1.3)	4.3 (3.4 to 5.0)	237	263.0 (121.0 to 382.0)
	Yes	539 (66.0%)	792 (96.9%)	2.9 (1.3)	4.7 (4.1 to 5.3)	211	104.0 (29.0 to 236.0)
Sub-saharan Africa	No	80 (31.1%)	175 (68.1%)	1.5 (1.4)	4.7 (3.9 to 5.2)	29	262.0 (180.0 to 360.0)
	Yes	400 (73.7%)	531 (97.8%)	2.9 (1.3)	4.8 (4.1 to 5.3)	52	137.0 (20.0 to 219.0)
Asia	No	30 (22.1%)	91 (66.9%)	1.3 (1.4)	4.8 (4.1 to 5.3)	119	188.0 (71.0 to 355.0)
	Yes	82 (58.2%)	130 (92.2%)	2.9 (1.5)	4.9 (4.2 to 5.3)	118	87.5 (29.0 to 229.0)
Europe	No	20 (13.7%)	65 (44.5%)	0.7 (1.0)	3.4 (2.7 to 4.6)	32	323.0 (238.0 to 387.0)
	Yes	38 (43.2%)	86 (97.7%)	2.6 (1.4)	4.2 (3.8 to 4.8)	12	242.5 (122.0 to 345.0)
North America	No	7 (7.9%)	49 (55.1%)	0.8 (0.9)	3.4 (2.4 to 4.3)	57	312.0 (198.0 to 476.0)
	Yes	19 (42.2%)	45 (100.0%)	2.8 (1.4)	4.2 (3.7 to 4.7)	29	173.0 (42.0 to 329.0)







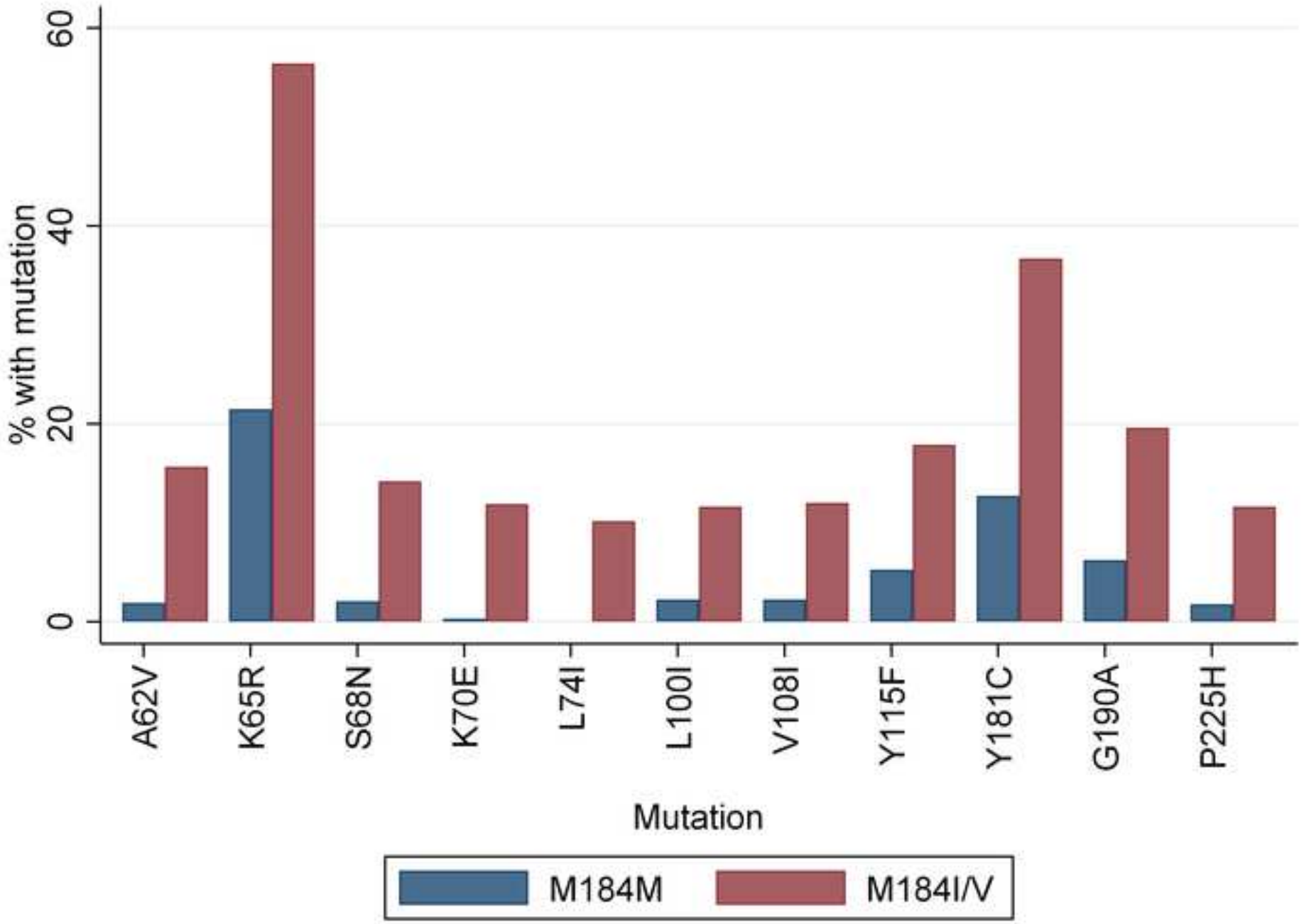
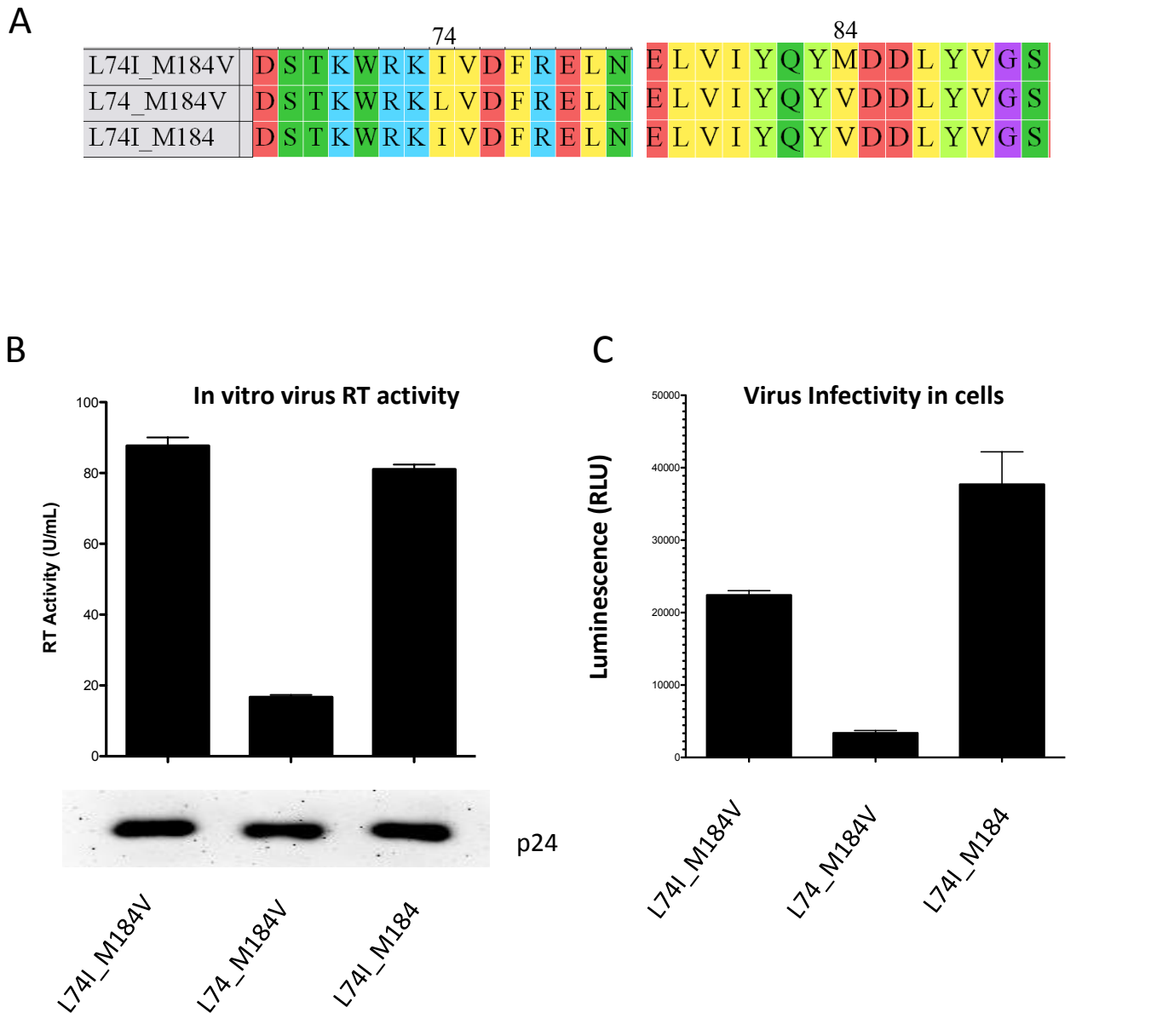


Figure 5



Supplementary Table 1. List of studies/datasets with publicly available RT sequences from individuals failing a TDF+XTC+NNRTI-containing first-line regimen

Author	PubMedID	Number of individuals	Regions
		TenoRes Studies (29 datasets, 1573 individuals)	
Sirivichayakul, S		282	Thailand
Theys, K	23183438	203	Belgium, Germany, Israel, Italy, Luxembourg, Portugal, Spain, Sweden
Stanford, A	28365230	118	U.S.
Hunt, G	28981637	115	South Africa
Goedhals, D		102	South Africa
de Oliveira, T		81	South Africa
Rokx, C	25273080	68	Netherlands
Santoro, M		65	Italy
Yang, C		56	Kenya
Schmidt, D		53	Germany
Hoffmann, CJ	23751421	50	South Africa
Sobrino-Vegas, P	21820763	40	Spain
Neogi, U	24922326	38	Sweden
Kaleebu, P	26700639	35	Uganda
Brooks, K	27231099	32	Kenya
Sunpath, H	22739389	31	South Africa
Charpentier, C		31	France
Theys, K	23027713	30	Portugal
Etiebet, MA	23079810	21	Nigeria
Kerschberger, B		21	Swaziland
Yang, C		17	Zambia
Yang, C		15	Tanzania
Shapiro, J		14	Israel
Arruda, M		13	Brazil
Ndembi, N		12	Nigeria
Yang, WL	26362944	10	Switzerland
Ugbena, R	22544206	7	Nigeria
Hamers, RL		7	Nigeria, Uganda, Zambia
Yang, C		6	Uganda
		Non-TenoRes Studies (22 datasets, 1840 individuals)	
Van Zyl, GU		466	South Africa
Steegen, K	27659733	322	South Africa
Van Zyl, GU	23840622	151	South Africa
Neogi, U	26413747	146	South Africa
Dinesha, TR	27334566	144	India
Theys, K		121	Belgium, Germany, Italy, Luxembourg, Portugal, Sweden
Lam, EP	27346600	102	Argentina, India, Israel, Malaysia, Mexico, Nigeria, South Africa, Thailand, U.K.
Skhosana, L	25659108	79	South Africa
Ndahimana, JD	27125473	68	Rwanda
Hamers, RL	22474222	47	Nigeria, South Africa, Uganda, Zambia, Zimbabwe
Mollan, K	23148287	44	U.S.
Hawkins, CA	19644383	24	Nigeria
Sigaloff, KC	21694603	21	Kenya, Nigeria, South Africa, Uganda, Zambia
Ngo-Giang-Huong, N	22132100	19	Thailand
Seu, L	25754408	19	Zambia
Jiamsakul, A	25141905	15	Philippines, Thailand

Riddler, SA	18480202	12	U.S.
Abdissa, A	24708645	12	Ethiopia
Rey, D	19036752	8	France
Avidor, B	23469241	8	Israel
Non-B Workgroup	15839752	7	Portugal, U.K.
Khairunisa, SQ	25348045	5	Indonesia

TDF - tenofovir disoproxil fumarate; TenoRes Studies – studies included in the TenoRes analysis (TenoRes Study Group, Lancet Infect Dis. 2016).

SUBTYPE	L74L	L74I	L74V
A	42 (95.5%)	1 (2.3%)	1 (2.3%)
B	107 (89.2%)	9 (7.5%)	4 (3.3%)
C	349 (83.3%)	49 (11.7%)	21 (5.0%)
CRF01_AE	115 (82.7%)	20 (14.4%)	4 (2.9%)
CRF02_AG	18 (100.0%)	0 (0%)	0 (0%)
D	36 (94.7%)	1 (2.6%)	1 (2.6%)
F	3 (100.0%)	0 (0%)	0 (0%)
G	31 (93.9%)	1 (3.0%)	1 (3.0%)
K	3 (100.0%)	0 (0%)	0 (0%)

Supplementary table 1: Subtype distribution of mutations at RT position 74 in participants with RT M184V/I detected by Sanger sequencing at virological failure.

Supplementary Figure 1: Difference in viral load at virological failure in the presence of M184I (left panel) or M184V (right panel) versus M184M within study groups with 95% confidence interval using random effects meta-analysis. Boxes represent mean with 95% CI.