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## **HIV MDR is still a relevant issue despite its dramatic drop over the years**

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1 **HIV multi-drug resistance is still a relevant issue despite its dramatic drop over the**  
2 **years**

3

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27 **Running title:** HIV multi-drug resistance over time

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30

31 **Abstract**

32 **Objectives:** To evaluate the prevalence and the therapeutic relevance of drug-resistance  
33 among isolates from ART-experienced HIV-1 infected patients over the past two decades in  
34 Italy.

35 **Methods:** Dynamics of resistance to 1, 2 and  $\geq 3$  antiretroviral-classes was evaluated over-  
36 time from 1999 to 2018. Virological success (VS) after the latest therapy switch was also  
37 evaluated according to cumulative class-resistance and cumulative genotypic-susceptibility-  
38 score (Stanford HIV\_DB algorithm).

39 **Results:** Among 13663 isolates (from 6739 patients) resistance to  $\geq 1$  drug-class decreased  
40 sharply from 1999 up to 2010 ( $\leq 2001$ :84.6%; 2010:43.6%;  $p < 0.001$ ), then remained  
41 relatively constant at around 40% over 2010-2018, with a proportion of isolates with  $\geq 3$   
42 class-resistance also stable (around 5%). After 2008, INI-resistance slightly increased from  
43 5.6% to 9.7% in 2018, and contributed to resistance particularly in isolates with  $\geq 3$  class-  
44 resistance (1 class:8.4%; 2 classes:15.3%;  $\geq 3$  classes:34.7%,  $p < 0.001$ ).

45 Among 1827 failing patients with an available follow-up, by one year after genotype-guided  
46 therapy start, the probability of VS was 87.6%. Patients with  $\geq 3$  cumulative class-resistance  
47 and receiving a poorly active regimen showed the lowest probability (62.6%) of VS  
48 ( $p < 0.001$ ) compared to all other patients ( $\geq 81.8\%$ ). By Cox regression analysis, cumulative  
49 multi-drug resistance and receiving poorly active antiretroviral regimens were associated with  
50 a lower hazard of VS compared to those without resistance.

51 **Conclusions:** A dramatic drop of HIV-1 drug-resistance at failure has been achieved over the  
52 last two decades in Italy; resistance to  $\geq 3$  classes is low but constantly present among  
53 currently failing patients. Its management still requires a rational and careful diagnostic and  
54 therapeutic approach.

55

## 56 **Introduction**

57 Drug-resistance is a major barrier to successful treatment and eradication of human  
58 immunodeficiency virus type 1 (HIV-1) infections <sup>1</sup>. Recent studies demonstrated that  
59 acquired drug-resistance markedly decreased over time in high income countries (HICs) <sup>2-4</sup>,  
60 thanks to several factors, such as: i) the use of a combination antiretroviral treatment (cART),  
61 which is highly effective at suppressing HIV replication in infected individuals; ii) the  
62 availability of treatment options with increasingly better tolerability/convenience and genetic  
63 barrier; iii) a proper management of HIV-1 infection <sup>5-7</sup>. Concerning this last point, it should  
64 be highlighted that, beyond the mere drug administration, virological failure and resistance  
65 selection can be avoided only with a strict viro-immunological monitoring (including viral-  
66 load, CD4 count and resistance), in conjunction with individualized treatment and prompt  
67 intervention in case of failure <sup>8</sup>.

68 Despite the current decrease of resistance prevalence in HICs in the large majority of  
69 patients, a minority of individuals still have difficulty in achieving and maintaining  
70 virological suppression. These patients, mainly with a long history of HIV infection and  
71 previously exposed to sub-optimal therapies, accumulated over time many resistance  
72 mutations to several drug-classes, that their treatment options are becoming exhausted <sup>9</sup>. Due  
73 to the current availability of new drug classes such as integrase inhibitors (INIs), it is  
74 important to verify if these drugs might be effective enough in patients harboring multi-  
75 resistant viruses. Beyond the fact that these patients are often immunologically compromised  
76 and at risk of death for the uncontrolled viral replication (and for this reason they can be  
77 considered a fragile population) <sup>10</sup>, they represent a potential source for spreading multi-  
78 resistant viral strains among newly infected individuals. In this context, an update of  
79 resistance prevalence focusing attention on patients with at least three class-resistance is of  
80 crucial clinical importance.

81 Based on all these considerations, the objective of the present manuscript was to evaluate the  
82 prevalence of resistance among isolates from HIV-1 infected ART-experienced patients over  
83 the past two decades in Italy. Moreover, we aimed to investigate the impact of cumulative  
84 class-resistance and cumulative genotypic-susceptibility score on the virological response  
85 after the last therapy switch recorded.

## 86 **Materials and methods**

### 87 *Study population*

88 This retrospective study included HIV-1 sequences of protease (PR), reverse transcriptase  
89 (RT) and integrase (IN, when available), from plasma genotypic resistance tests (GRTs) that  
90 were performed from January 1999 to July 2018. These tests were conducted in several  
91 clinical centres in Northern and Central Italy for routine clinical purposes on ART-  
92 experienced HIV-1 infected patients.

### 93 *Genotyping and evaluation of resistance over time*

94 GRT information was retrieved from an anonymous database in which all genotypic data  
95 from isolates of ART-experienced patients were stored. PR/RT and IN GRTs were previously  
96 performed using commercially available kits (ViroSeq HIV-1 Genotyping System, Abbott  
97 Molecular, Des Plaines, IL, USA; Trugene-HIV-1 Genotyping-Kit, Bayer HealthCare LLC,  
98 Tarrytown, NY, USA) and/or a homemade system, as previously described<sup>11,12</sup>.

99 Resistance to one, two and at least three drug-classes among NRTIs, NNRTIs, PIs, or INIs  
100 was evaluated per isolate over the years according to the presence of at least one major  
101 resistance mutation (MRM) paneled by Stanford HIV Drug-resistance database 2019 (HIVdb  
102 version 8.8). More than three class-resistance was defined as the occurrence of at least one  
103 MRM against three of the following four drug-classes: NRTIs, NNRTIs, PIs or INIs. INI-  
104 resistance was explored in isolates for whom IN GRTs were performed after 2007, period  
105 subsequent to the approval of the first INI raltegravir. Temporal trend of MRMs and  
106 PI/NRTI/NNRTI/INI resistance was also evaluated. Finally, resistance was evaluated  
107 according to HIV-1 subtype (B versus non-B).

### 108 *Evaluation of the impact of cumulative drug-resistance on virological outcome*

109 For patients with a complete therapeutic history and with an available viremia follow-up after  
110 the last recorded therapy switch due to virological failure (viremia >50 copies/mL), the  
111 impact of cumulative resistance on virological response was also evaluated.

112 For each patient, cumulative class-resistance and cumulative genotypic susceptibility score  
113 (cGSS) were calculated by cumulating all the mutations observed in all available GRTs  
114 before starting the last therapy recorded. More than three cumulative class-resistance was  
115 defined as above. cGSS was calculated using the HIV\_DB algorithm (version 8.8; Stanford  
116 University, Stanford, CA; <http://sierra2.stanford.edu/sierra/servlet/JSierra?action=hivalgs>)  
117 based on the sum of genotype susceptibilities to drugs prescribed at the last therapy recorded.  
118 The activity of enfuvirtide and maraviroc was assessed according to whether the drugs had  
119 been previously employed by the patients or not, assuming that maraviroc was newly

120 administered after confirming a pure CCR5 viral strain by the genotypic tropism prediction.  
121 A regimen was considered: i) fully active: when all the drugs were scored as fully active; ii)  
122 partially active: when at least one drug was scored as fully active; iii) poorly active: when  
123 none of the drugs were scored as fully active. Patients were stratified according to cumulative  
124 resistance and cGSS.  
125 Virological response after the last therapy switch recorded was evaluated through survival  
126 analysis. The risk of virological success (VS, defined as the achievement of plasma HIV-  
127 RNA <50 copies/mL after therapy switch) and virological rebound (VR, defined as the first  
128 of 2 consecutive plasma HIV-RNA >50 copies or a single HIV-RNA >1000 copies after VS)  
129 were evaluated according to the extent of resistance accumulated over time and the cGSS to  
130 the therapy received.

### 131 *Statistical analysis*

132 All the analyses were performed using R open source environment for statistical computing  
133 (version 3.4.3), and the software package SPSS (version 19.0) for Windows (SPSS Inc.,  
134 Chicago, Illinois). In all the analyses a p-value <0.05 was considered statistically significant.

#### 135 *i) Evaluation of the prevalence of resistance over time*

136 Potential differences over the years in the prevalence of resistance to one, two and at least  
137 three drug-classes, of each MRM and of each resistance class were evaluated by Chi-squared  
138 test for trend. The analyses were performed retaining all GRTs available. We performed  
139 several sensitivity analyses to confirm the robustness of the results obtained by this approach,  
140 by considering one GRT per patient per year in three different ways: i) retaining the first  
141 GRT; ii) retaining the last GRT; iii) retaining the GRT with the highest resistance (in case of  
142 more than one GRT having the same number of resistances, the last one was considered).

#### 143 *ii) Assessment of the parameters in relationship with cumulative resistance*

144 In the sub-group of patients selected for the evaluation of the impact of cumulative resistance  
145 on the virological outcome, differences in demographic, viro-immunological and treatment  
146 parameters according to level of resistance were evaluated. Chi-Squared test for trend (for  
147 categorical variables), and Jonckheere-Terpstra-Test (for quantitative variables) were used.

148

#### 149 *iii) Evaluation of virological outcome in relationship to resistance*

150 Survival analysis was used to estimate the cumulative probability and predictors of achieving  
151 VS and experiencing VR after VS after the last therapy switch subsequent to virological  
152 failure (see above). Analyses were performed by ignoring therapy changes, and patients'  
153 follow-up was censored at the date of their last available viremia measurement or at the time  
154 of eventual treatment interruption (ITT approach).  
155 Kaplan-Meier curves were performed to estimate the probability of VS and VR according to  
156 cumulative class-resistance and genotypic susceptibility. Cox regression analysis was  
157 performed to evaluate the association of cumulative resistance and genotypic susceptibility  
158 on the risk of achieving VS or experiencing VR after controlling for other potential  
159 demographic, viro-immunological and treatment confounding factors under the assumption  
160 of proportionality of the hazards.

## 161 **Results**

### 162 *Study population*

163 Analysis was performed on 13663 isolates from 6739 ART-experienced HIV-1 infected  
164 patients, for whom GRTs for PR/RT (N=13663) and IN (N=2257) were performed for  
165 routine clinical purposes. The median (IQR) number of GRTs analysed per patient was 1 (1-  
166 2) (Table 1). Patients were mainly male (4575, 67.9%), infected with HIV-1 B subtype (5210,  
167 77.3%) and Italian (3578, 53.1%). The median (IQR) year of starting first-line regimen was  
168 2000 (1997-2006). Concerning characteristics related to each isolate, median (IQR) year of  
169 genotyping was 2007 (2004-2011) (Supplementary table 1).

170 At the moment of genotyping, the median (IQR) plasma HIV-1 RNA was of 4.2 (3.2-5.0)  
171  $\log_{10}$  copies/mL, and the median (IQR) number of previous regimens before performing GRT  
172 was 3 (2-6).

### 173 *Evaluation of the prevalence of resistance over time*

174 Overall, 40.6% of GRTs showed no resistance, while the prevalence of resistance to one, two  
175 and at least three classes was 21.8%, 24.6% and 12.9%, respectively (Figure 1). The  
176 evaluation of the dynamic of resistance over the years showed that resistance to at least one  
177 drug-class dramatically decreased from 1999 up to 2010 ( $\leq 2001$ : 84.6%; 2010: 43.6%;  
178  $p < 0.001$ ), in conjunction with a remarkable increase of GRTs without resistance ( $\leq 2001$ :  
179 15.4%; 2010: 56.4%;  $p < 0.001$ ). In particular, from 1999 to 2010, the decrease of resistance  
180 was related to the drop of resistance to two classes ( $\leq 2001$ : 40.7%; 2010: 16.6%;  $p < 0.001$ )  
181 and at least three drug-classes ( $\leq 2001$ : 26.0%; 2010: 5.6%;  $p < 0.001$ ). By contrast, the

182 prevalence of at least one class-resistance remained constant settling at around 40% from  
183 2010 to 2018 (Figure 1). In particular, the proportion of isolates with at least three class-  
184 resistance remained constantly settled at around 5% ( $p=0.479$ ).

185 Concerning resistance according to HIV-1 subtype, overall the proportion of non-B isolates  
186 resistant to at least one class was lower (52.9%) compared to B isolates (60.9%,  $p<0.001$ ).  
187 Nevertheless, resistance dramatically decreased from 1999 to 2018 both in B (from 84.9% to  
188 36%) and in non-B isolates (from 81.3% to 35.5%,  $p<0.001$ ), with a similar rate (data not  
189 shown).

190 All the results of this analysis were confirmed by the sensitivity analyses described in the  
191 Materials and Methods section (data not shown).

192 Concerning the resistance to specific drug-classes, an overview of temporal trend of  
193 resistance per each class and of MRMs (with a prevalence  $>5\%$ ) is reported in Figure 2.

194 The proportion of isolates with at least one PI-MRM significantly decreased from 52.5% to  
195 3.9% from 1999 to 2018 (Figure 2, Panel A). Among PI-MRMs, the prevalence of L90M,  
196 M46I and I84V significantly decreased until 2018, while the prevalence of L33F, V82A and  
197 I54V, after a decrease until 2010, remained low ( $<3\%$ ) but stable in the last 8 years (2011-  
198 2018). All the other PI-MRMs detected with a prevalence  $<5\%$  in general, significantly  
199 decreased or completely disappeared from 1999 to 2018 (Supplementary Table 2).

200 Concerning NRTI-resistance (Figure 2, Panel B), the proportion of isolates with at least one  
201 NRTI-MRM dramatically decreased from 80.1% in 1999 to 15.2% in 2018. Among NRTI-  
202 MRMs, the prevalence of M41L, T215Y, K70R, L210W, T219Q and T215F significantly  
203 decreased from 1999 to 2018. The prevalence of M184V also significantly decreased from  
204 52.1% in 1999 to around 13% in 2014 ( $p<0.001$ ), then it subsequently settled at around 13-  
205 14% from 2014 to 2018 ( $p=0.692$ ). The temporal trend from 1999 to 2018 of prevalence of  
206 K65R (overall prevalence: 1.9%), T219E (overall prevalence: 3.3%) and Y115F (overall  
207 prevalence: 0.8%) was stable over time ( $p>0.05$ , Supplementary Table 3).

208 Concerning NNRTI-resistance (Figure 2, Panel C), the proportion of isolates with at least one  
209 NNRTI-MRM dramatically decreased from 50.7% to 28.7% from 1999 to 2010 ( $p<0.001$ ),  
210 but remained stable from 2011 to 2018 settling at around 27% ( $p=0.561$ ). Among NNRTI-  
211 MRMs, the prevalence of K103N and G190A dramatically decreased from 1999 to 2018. The  
212 prevalence of Y181C mutation also decreased from 15.5 to 4.5% from 1999 to 2011, but later  
213 its prevalence remained settled at around 4% after 2010 ( $p=0.315$ ). Noteworthy, mutations at  
214 the position E138 increased over time (Figure 2 and Supplementary Table 4); in particular,  
215 E138A mutation prevalence significantly increased from 3.5% in 1999 to 4.6% in 2010 and



216 continued to increase until 2018 ( $p<0.05$ ). Taken together, in the large majority of cases, the  
217 rate of resistance mutations in RT and protease sharply dropped in failing patients until 2010-  
218 2011, then it settled to level that remained pretty constant over the years.

219 Concerning INI-resistance, among isolates for whom IN genotyping was performed  
220 ( $N=2257$ ), the proportion of isolates with at least one INI-MRM slightly increased from 5.6%  
221 in 2008 to 9.7% in 2018 ( $p=0.208$ ) (Supplementary Table 5). Overall, N155H was the most  
222 prevalent INI-MRM (4.2%), followed by G140S (2.2%) and Q148H (2.1%). All the other  
223 INI-MRMs detected (T66I/A, E92Q, G140A/C, S147G, Q148R/K, R263K) showed a  
224 prevalence  $<1\%$ . Only E92Q and S147G MRMs, mainly associated to elvitegravir-resistance,  
225 increased their prevalence from 2008 to 2018 (E92Q: from 0% to 3.4%,  $p=0.003$ ; S147G:  
226 from 0% to 1%,  $p=0.019$ ).

227 Noteworthy, from 2008, INI-resistance contributed to resistance mostly in those isolates with  
228 at least three class-resistance (1 class: 8.4%; 2 classes: 15.3%;  $\geq 3$  classes: 34.7%,  $p<0.001$ ).

#### 229 *Evaluation of cumulative resistance among ART-experienced patients with an available* 230 *viremia follow-up after therapy switch*

231 Table 2 reports demographic, viro-immunological and therapeutic characteristics of 1827  
232 ART-experienced patients who switched treatment and for whom at least one viremia  
233 measurement was available after the switch, overall and according to cumulative resistance.  
234 Before the last therapy switch recorded, 360 (19.7%), 553 (30.3%) and 310 (17.0%) patients  
235 accumulated one, two and at least three class-resistance, respectively, while 604 (33.1%)  
236 harbored a virus without known resistance. Italian nationality and a perinatal infection were  
237 associated with increased cumulative resistance, while a higher proportion of homosexual  
238 route as risk factor was found in individuals harboring a virus without known resistance  
239 ( $p<0.05$ ).

240 Concerning viro-immunological parameters, the proportion of patients infected with B  
241 subtype and with a viremia zenith  $>500,000$  copies/mL increased by increasing cumulative  
242 resistance. Whereas, baseline viremia was higher in patients without resistance compared to  
243 those who had accumulated at least one class-resistance class ( $p<0.05$ ). Patients with at least  
244 three cumulative class-resistance had the highest proportion of CD4 cell count nadir  $<100$   
245 cells/ $\text{mm}^3$  and the lowest median (IQR) baseline CD4 count ( $p<0.05$ ).

246 Concerning treatment parameters, by increasing cumulative resistance, patients showed a  
247 longer treatment history and an increased number of previous regimens.

248 *Impact of cumulative drug-resistance on virological outcome*

249 Overall, among the 1827 patients analyzed, the probability of achieving virological success  
250 by one year after the last recorded therapy switch was 87.6%, reached in a median time (95%  
251 Confidence interval, C.I.) of 3.3 (3.1-3.5) months. By stratifying according to the number of  
252 cumulative class-resistance and the activity of the drugs administered at last switch recorded,  
253 patients were divided into seven groups: i) 604 (33.1%) without any cumulative resistance  
254 receiving a fully active regimen; ii) 271 (14.8%) with one or two cumulative class-resistance  
255 receiving a fully active regimen; iii) 27 (1.5%) with at least three cumulative class-resistance  
256 receiving a fully active regimen; iv) 630 (34.5%) with one-two cumulative class-resistance  
257 receiving a partially active regimen; v) 247 (13.5%) with at least three cumulative-class  
258 resistance receiving a partially active regimen; vi) 12 (0.7%) with one or two cumulative  
259 class-resistance receiving a poorly active regimen; vii) 36 (2.0%) with at least three  
260 cumulative-class resistance receiving a poorly active regimen.

261 By stratifying VS probability according to these susceptibility groups described above,  
262 patients with at least three class-resistance receiving a poorly active regimen showed the  
263 lowest probability (62.6%) and the longest median time (C.I.) of achieving VS (6.7 [4.0-9.3]  
264 months,  $p < 0.001$  Figure 3, Panel A). Patients with one or two class-resistance and receiving a  
265 poorly active regimen showed a long median time (C.I.) of achieving VS (6.1 [1.8-10.3]  
266 months), despite a high probability of VS (81.8%). All the other patients treated with fully or  
267 partially active regimens (including the multi-resistant patients), showed a good virological  
268 response with a probability of VS  $> 86\%$  and a shorter median time of achieving VS ( $\leq 3.5$   
269 months). By Cox regression analysis, only the patients who cumulated at least three class-  
270 resistance and received a poorly active regimen showed a lower adjusted hazard of achieving  
271 VS compared to those without cumulative resistance receiving fully active drugs with  
272 statistical significance in the univariable model and with a trend toward significance in the  
273 multivariable model (Table 3).

274 Concerning the maintenance of VS, overall, by four years after VS the probability of VR was  
275 29.7%. No significant differences in terms of probability of VR among the seven  
276 susceptibility groups examined were observed ( $p = 0.363$ , Figure 3, Panel B). Cox uni-  
277 multivariable regression models confirmed no association between resistance and regimen  
278 susceptibility with VR (Table 3). Thus, the presence of  $< 3$  class resistance does not preclude  
279 the achievement of controlled viral load, provided that the therapy is based upon active drugs.

280

## 281 Discussion

282 In the present manuscript we evaluated the trends of drug-resistance prevalence in the last  
283 two decades in a large cohort of cART-experienced patients in Italy. We observed a dramatic  
284 drop in drug-resistance (both in B and non-B isolates) from 80-85% from 1999 to around  
285 36% in 2018, as a result of a good management of HIV infection and the progressive  
286 improvement of antiretroviral drugs in terms of potency, efficacy, tolerability and genetic  
287 barrier, together with a rapid intervention in case of virological failures<sup>5-7</sup>. However, we  
288 should highlight that the dramatic drop in resistance was observed until 2010 (with a  
289 prevalence of 44%), while in the last 8 years (2011-2018), the proportion of isolates with at  
290 least one class-resistance has remained stable at around 40% (range: 36%-46%). In particular,  
291 the percentage of isolates with resistance to at least 3 classes has remained stably settled at  
292 around 5% (range 3%-6%). Several studies performed in different populations and in several  
293 geographic area in HICs, showed a similar decline of drug-resistance up to 2010-2014<sup>3,4,13-</sup>  
294<sup>15</sup>. A recent study including specimens processed for routine genotypic resistance testing  
295 between 2006 to 2017 in the USA, demonstrated a continuous general decrease of resistance  
296 with a prevalence settled at around 40% in 2017, similar to the one found in our study in the  
297 same year; this decrease was particularly associated to high-level dual- and triple-class  
298 resistance<sup>13</sup>. To our knowledge, there is no recent data about the clinical relevance of HIV-1  
299 drug-resistance in HIC. In the present manuscript we found that only patients who  
300 accumulated at least three class-resistance and received poorly active regimens showed a  
301 significant lower probability of achieving VS compared to those without resistance. Thus,  
302 despite the improvement in the management of HIV infection achieved in recent years, the  
303 phenomenon of multi-drug resistance, even though rare or almost ceased in some settings<sup>3</sup>,  
304 remains a clinical concern in HICs<sup>9,16,17</sup>.

305 Concerning specific drug-classes, we found that resistance to PIs, NRTIs and NNRTIs  
306 dramatically decreased over time. However, resistance to NNRTIs has been stable at around  
307 25% from 2011 to 2018 and NNRTI-MRMs such as Y181C, associated with wide cross-  
308 resistance to NNRTIs<sup>18,19</sup>, remained stable at around 4%. Moreover, we found that some  
309 rilpivirine and etravirine associated mutations at position 138 of RT increased their  
310 prevalence over time, as recently observed<sup>13</sup>. In light of forthcoming availability in clinic of  
311 the novel NNRTI inhibitor doravirine and of the current usage in clinical practice of dual  
312 regimen based on INIs plus rilpivirine<sup>5-7</sup>, these findings underline that genotypic resistance  
313 testing remains a crucial tool to guide the usage of these new treatment strategies.

314 In fact, even though *in vitro* doravirine susceptibility seems not affected by mutations at the  
315 RT position 138 <sup>20</sup>, few observational data about the impact of NNRTI resistance on  
316 doravirine response are so far available. Moreover, the impact of NNRTI mutations on the  
317 response to the dual therapy based on dolutegravir plus rilpivirine should be carefully  
318 investigated in real-life <sup>21</sup>.

319 Concerning NRTI-resistance, we found a dramatic and continuous drop of the prevalence of  
320 thymidine analogues MRMs from 1999 to 2018 and a generally stable prevalence of other  
321 NRTI-MRMs, such as K65R (1.9% overall). Concerning the emtricitabine/lamivudine-  
322 associated mutation M184V, its prevalence dramatically decreased from 52% in 1999 to 14%  
323 in 2014, but it was subsequently stable until 2018. In a recent study performed on  
324 virologically suppressed patients, the presence of previous M184V was associated with an  
325 increased risk to having viral blips under a dual therapy containing lamivudine <sup>22</sup>. Patients  
326 harbouring M184V with a previous time of virological suppression lower than three years  
327 had the highest probability to experience virological failure <sup>22</sup>. As far as NNRTI resistance,  
328 these findings suggest that the currently stable presence of M184V in isolates from failing  
329 patients still might be a concern in the future for patients for whom treatment optimization  
330 will be required.

331 If resistance to RT inhibitors still arouse suspicions, in the present study we found a dramatic  
332 decrease of PI-resistance with a complete disappearance of several mutations, as recently  
333 confirmed in other studies <sup>13</sup>.

334 Concerning INI-resistance, we found that the proportion of isolates with at least one INI-  
335 MRM increased over time from 5.6% in 2008 to 9.7% in 2018, and mostly contributed to  
336 resistance in those isolates with at least three class-resistance. The prevalence of elvitegravir-  
337 associated mutations such as E92Q and S147G slightly increased over time. This observation  
338 agrees with recent American data <sup>13</sup> and these signals of emerging INI-resistance, even  
339 though still marginal, should be considered with caution. Indeed, clinical trials demonstrated  
340 that a high percentage of patients harbouring resistance to elvitegravir, also showed NRTI-  
341 resistance, including M184V <sup>23</sup>.

342 Based on these considerations, currently genotypic resistance testing remains crucial to tailor  
343 a fully active therapy switch in failing patients, especially for those with a long history of  
344 treatment failures. Moreover, the fact that multi-drug resistance might be a concern on  
345 choosing an effective treatment, the need for developing new anti-HIV drugs remains crucial.

346 Our study might have some limitations. Firstly, our observations are based on routine clinical  
347 samples, thus such data as adherence or bias due to clinicians' decisions cannot be  
348 considered. We considered HIV subtype only comparing B vs non-B strains  
349 Moreover, due to the recent introduction of INIs in clinical practice, prevalence of integrase  
350 resistance was evaluated in a subset of isolates for whom integrase GRTs were requested.  
351 Concerning resistance outcome analyses, even though we considered cumulative resistance  
352 and around five years of follow-up, our evaluations might be under-estimated for patients  
353 who recently switched to treatment containing novel drugs such as dolutegravir.  
354 In conclusion, HIV-1 drug-resistance in failing patients has been stable since 2011, despite its  
355 dramatic decrease over time, from 1999 to 2010. Resistance to at least three classes is still  
356 present, even though at a lower rate. Its management requires an appropriate diagnostic and  
357 therapeutic approach.

358

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404

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469 **Legends to figure**

470 **Figure 1. Trend of resistance prevalence over the years 1999-2018.** Analysis performed  
471 on 13663 isolates from 6739 ART-experienced HIV-1 infected patients, for whom genotypic  
472 resistance tests for protease/reverse transcriptase (N=13663) and integrase (N=2257) were  
473 carried out for routine clinical purposes from January 1999 to July 2018. P-values were  
474 calculated by Chi-squared test for trend; statistically significant tests ( $p<0.05$ ) are indicated in  
475 boldface. Sequences performed from 1999 to 2001 were grouped.

476 **Figure 2. Trend of prevalence of major resistance mutations for protease inhibitors**  
477 **(PIs), nucleos(t)ide reverse transcriptase inhibitors (NRTIs), non-NRTI (NNRTIs) over**  
478 **the years 1999-2018.** Analysis performed on 13663 isolates from 6739 ART-experienced  
479 HIV-1 infected patients, for whom genotypic resistance tests for protease/reverse  
480 transcriptase (N=13663) were carried out for routine clinical purposes from January 1999 to  
481 July 2018. All the major resistance mutations paneled by Stanford HIV Drug resistance  
482 database 2019 (HIVdb version 8.8) and with an overall prevalence  $>5\%$  are reported in the  
483 figure. P-values were calculated by Chi-squared test for trend; statistically significant tests  
484 ( $p<0.05$ ) are indicated in boldface. Sequences performed from 1999 to 2001 were grouped.

485 **Figure 3. Kaplan Meyer estimates of virological success and virological rebound in**  
486 **cART failing patients switching treatment according to cumulative resistance and**  
487 **cumulative genotypic susceptibility score (cGSS).** Panel A: Kaplan Meier estimation of  
488 virological success (VS); table legend indicates the probability of VS by one year after switch  
489 and the median time (95% confidence interval, C.I.) of achieving VS, stratified according to  
490 cumulative resistance and cGSS. Panel B: Kaplan Meier estimation of virological rebound;  
491 table legend indicates the probability of virological rebound by 4 years after achieving VS,  
492 stratified according to cumulative resistance and cGSS. P values were calculated by using the  
493 Peto and Peto modification of the Gehan–Wilcoxon test.

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Characteristics	N=6739
<b>Gender, n (%)</b>	
<i>Male</i>	4575 (67.9)
<b>Subtype, n (%)</b>	
<i>B</i>	5210 (77.3)
<i>CRF02_AG</i>	480 (7.1)
<i>F</i>	205 (3.0)
<i>C</i>	261 (3.9)
<i>Other</i>	583 (8.7)
<b>Nationality, n (%)</b>	
<i>Italian</i>	3578 (53.1)
<i>Non-Italian</i>	1369 (20.3)
<i>Unknown</i>	1792 (26.6)
<b>Risk factor, n (%)</b>	
<i>Heterosexual</i>	1877 (27.9)
<i>Homosexual</i>	1066 (15.8)
<i>Drug abuser</i>	1780 (26.4)
<i>Sexual</i>	288 (4.3)
<i>Perinatal</i>	228 (3.4)
<i>Other/unknown</i>	1500 (22.3)
<b>Year of first-line regimen starting, median (IQR)<sup>a</sup></b>	2000 (1997-2006)
<b>Previous exposure to sub-optimal cART<sup>a</sup>, n (%)</b>	
<i>NRTI-based</i>	1746 (30.2)
<i>Unboosted PI-based</i>	2422 (41.8)
<b>No. of GRTs per patient, median (IQR)</b>	1 (1-2)
<b>Patients with 1 GRT, n (%)</b>	3931 (58.3)
<b>Patients with 2 GRTs, n (%)</b>	1288 (19.1)
<b>Patients with ≥3 GRTs, n (%)</b>	1520 (22.6)
<b>Time between first and last GRT, years, median (IQR)<sup>b</sup></b>	3.9 (1.6-7.2)

<sup>a</sup> Available information for 5880 patients with treatment history. <sup>b</sup> Values calculated on the total of patients with at least two GRTs available (N=2808). cART: combined antiretroviral therapy. GRTs: genotypic resistance tests. IQR: interquartile range. NRTI: nucleos(t)ide reverse transcriptase inhibitor. PI: protease inhibitor.

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504 **Table 2. Baseline characteristics of 1827 cART experienced patients switching to a new regimen**  
 505 **according to previous cumulative resistance**

Variables	Overall (N=1827)	Number of class resistance cumulated before therapy switch				P value
		0 (N=604)	1 (N=360)	2 (N=553)	≥3 (N=310)	
<b>Age, median (IQR)</b>	43 (38-49)	<b>43 (37-49)</b>	<b>43 (37-49)</b>	<b>43 (38-48)</b>	<b>44 (39-50)</b>	<b>&lt;0.001</b>
<b>Male, n (%)</b>	1255 (68.7)	402 (66.6)	252 (70.0)	391 (70.7)	210 (67.7)	0.406
<b>Nationality, n (%)<sup>a</sup></b>						
Italian	1411 (77.2)	<b>441 (73.0)</b>	<b>266 (73.9)</b>	<b>433 (78.3)</b>	<b>271 (87.4)</b>	<b>&lt;0.001</b>
Non-Italian	337 (18.4)	<b>128 (21.2)</b>	<b>69 (19.2)</b>	<b>103 (18.6)</b>	<b>37 (11.9)</b>	<b>0.002</b>
Unknown	79 (4.3)	<b>35 (5.8)</b>	<b>25 (6.9)</b>	<b>17 (3.1)</b>	<b>2 (0.6)</b>	<b>&lt;0.001</b>
<b>Risk factor, n (%)</b>						
Heterosexual	610 (33.4)	212 (35.1)	106 (29.4)	180 (32.5)	112 (36.1)	0.928
Homosexual	416 (22.8)	<b>162 (26.8)</b>	<b>82 (22.8)</b>	<b>107 (19.3)</b>	<b>65 (21.0)</b>	<b>0.006</b>
Drug abuser	538 (29.4)	155 (25.7)	116 (32.2)	176 (31.8)	91 (29.4)	0.099
Perinatal	17 (0.9)	<b>0 (0.0)</b>	<b>6 (1.7)</b>	<b>7 (1.3)</b>	<b>4 (1.3)</b>	<b>0.032</b>
Sexual	56 (3.1)	21 (3.5)	9 (2.5)	19 (3.4)	7 (2.3)	0.503
Other/unknown	190 (10.4)	54 (8.9)	41 (11.4)	64 (11.6)	31 (10.0)	0.373
<b>Subtype, n (%)</b>						
B	1548 (84.7)	<b>479 (79.3)</b>	<b>305 (84.7)</b>	<b>484 (87.5)</b>	<b>280 (90.3)</b>	<b>&lt;0.001</b>
CRF02_AG	68 (3.7)	<b>32 (5.3)</b>	<b>17 (4.7)</b>	<b>14 (2.5)</b>	<b>5 (1.6)</b>	<b>0.001</b>
F	38 (2.1)	17 (2.8)	7 (1.9)	9 (1.6)	5 (1.6)	0.144
C	42 (2.3)	16 (2.6)	7 (1.9)	13 (2.4)	6 (1.9)	0.563
Other	131 (7.2)	<b>60 (9.9)</b>	<b>24 (6.7)</b>	<b>33 (6.0)</b>	<b>14 (4.5)</b>	<b>0.001</b>
<b>Viremia zenith, copies/mL, n (%)</b>						
<100,000	697 (38.1)	<b>247 (40.9)</b>	<b>140 (38.9)</b>	<b>212 (38.3)</b>	<b>98 (31.6)</b>	<b>0.014</b>
100,001-500,000	688 (37.7)	231 (38.2)	127 (35.3)	217 (39.2)	113 (36.5)	0.921
>500,000	442 (24.2)	<b>126 (20.9)</b>	<b>93 (25.8)</b>	<b>124 (22.4)</b>	<b>99 (31.9)</b>	<b>0.004</b>
<b>Baseline viremia, median (IQR) copies/mL</b>	4.0 (2.9-4.9)	<b>4.3 (2.9-5.0)</b>	<b>4.0 (2.7-4.8)</b>	<b>3.7 (2.9-4.6)</b>	<b>4.1 (3.0-4.9)</b>	<b>0.011</b>
<b>Nadir CD4 count &lt;100 cells/mm<sup>3</sup>, n (%)</b>	680 (37.2)	<b>174 (28.8)</b>	<b>126 (35.0)</b>	<b>219 (39.6)</b>	<b>161 (51.9)</b>	<b>&lt;0.001</b>
<b>Baseline CD4 count, median (IQR) cells/mm<sup>3</sup></b>	322 (190-482)	322 (189-466)	334 (195-505)	336 (207-512)	279 (152-456)	0.209
<b>Year of treatment switch, n (%)</b>						
<2008	731 (40.0)	<b>161 (26.7)</b>	<b>118 (32.8)</b>	<b>286 (51.7)</b>	<b>166 (53.5)</b>	<b>&lt;0.001</b>
2008-2010	485 (26.5)	<b>179 (29.6)</b>	<b>94 (26.1)</b>	<b>135 (24.4)</b>	<b>77 (24.8)</b>	<b>0.049</b>
2011-2018	611 (33.4)	<b>264 (43.7)</b>	<b>148 (41.1)</b>	<b>132 (23.9)</b>	<b>67 (21.6)</b>	<b>&lt;0.001</b>
<b>Type of regimen administered, n (%)</b>						
Pib+ ≥2NRTIs	942 (51.6)	<b>283 (46.9)</b>	<b>176 (48.9)</b>	<b>329 (59.5)</b>	<b>154 (49.7)</b>	<b>0.010</b>
NNRTI+ ≥2NRTIs	379 (20.7)	<b>208 (34.4)</b>	<b>80 (22.2)</b>	<b>73 (13.2)</b>	<b>18 (5.8)</b>	<b>&lt;0.001</b>
INI containing (≥3 drugs)	260 (14.2)	86 (14.2)	53 (14.7)	61 (11.0)	60 (19.4)	0.394
Dual INI based	116 (6.3)	<b>14 (2.3)</b>	<b>22 (6.1)</b>	<b>46 (8.3)</b>	<b>34 (11.0)</b>	<b>&lt;0.001</b>
Dual PI based	76 (4.2)	<b>12 (2.0)</b>	<b>20 (5.6)</b>	<b>27 (4.9)</b>	<b>17 (5.5)</b>	<b>0.007</b>
Other without INIs (≥3 drugs)	54 (3.0)	<b>1 (0.2)</b>	<b>9 (2.5)</b>	<b>17 (3.1)</b>	<b>27 (8.7)</b>	<b>&lt;0.001</b>
<b>Activity of drugs at regimen switch, n (%)<sup>b</sup></b>						
Full	902 (49.4)	<b>604 (100)</b>	<b>204 (56.7)</b>	<b>67 (12.1)</b>	<b>27 (8.7)</b>	<b>&lt;0.001</b>
Partial	877 (48.0)	<b>0 (0.0)</b>	<b>156 (43.3)</b>	<b>474 (85.7)</b>	<b>247 (79.7)</b>	<b>&lt;0.001</b>
Poor	48 (2.6)	<b>0 (0.0)</b>	<b>0 (0.0)</b>	<b>12 (2.2)</b>	<b>36 (11.6)</b>	<b>&lt;0.001</b>
<b>Time from first-line regimen before switch, years, n (%)</b>						
≤1	282 (15.4)	<b>142 (23.5)</b>	<b>71 (19.7)</b>	<b>63 (11.4)</b>	<b>6 (1.9)</b>	<b>&lt;0.001</b>
1-5	372 (20.4)	<b>140 (23.2)</b>	<b>73 (20.3)</b>	<b>113 (20.4)</b>	<b>46 (14.8)</b>	<b>0.007</b>
5-10	471 (25.8)	<b>127 (21.0)</b>	<b>84 (23.3)</b>	<b>168 (30.4)</b>	<b>92 (29.7)</b>	<b>&lt;0.001</b>
10-15	357 (19.5)	<b>63 (10.4)</b>	<b>69 (19.2)</b>	<b>136 (24.6)</b>	<b>89 (28.7)</b>	<b>&lt;0.001</b>
>15	220 (12.0)	<b>50 (8.3)</b>	<b>43 (11.9)</b>	<b>57 (10.3)</b>	<b>70 (22.6)</b>	<b>&lt;0.001</b>
Unknown	125 (6.8)	<b>82 (13.6)</b>	<b>20 (5.6)</b>	<b>16 (2.9)</b>	<b>7 (2.3)</b>	<b>&lt;0.001</b>
<b>Number of regimens administered before switch, n (%)</b>						
1	335 (18.3)	<b>159 (26.3)</b>	<b>84 (23.3)</b>	<b>72 (13)</b>	<b>20 (6.5)</b>	<b>&lt;0.001</b>
2-4	615 (33.7)	<b>204 (33.8)</b>	<b>126 (35)</b>	<b>230 (41.6)</b>	<b>55 (17.7)</b>	<b>0.013</b>
≥5	659 (36.1)	<b>92 (15.2)</b>	<b>107 (29.7)</b>	<b>232 (42)</b>	<b>228 (73.5)</b>	<b>&lt;0.001</b>
Unknown	218 (11.9)	<b>149 (24.7)</b>	<b>43 (11.9)</b>	<b>19 (3.4)</b>	<b>7 (2.3)</b>	<b>&lt;0.001</b>
<b>Previous INI exposure</b>	173 (9.5)	46 (7.6)	43 (11.9)	44 (8.0)	40 (12.9)	0.080
<b>Previous T20/MVC exposure</b>	<b>94 (5.1)</b>	<b>4 (0.7)</b>	<b>7 (1.9)</b>	<b>19 (3.4)</b>	<b>64 (20.6)</b>	<b>&lt;0.001</b>

Analysis performed by cumulating mutations detected in 4028 GRTs from 1827 patients. INI: integrase inhibitor; IQR: interquartile range; MVC: maraviroc; NRTI: nucleos(t)ide reverse transcriptase inhibitors; NNRTI: non-NRTI; Pib: cobicistat/ritonavir boosted protease inhibitor; T20: enfuvirtide.

507 **Table 3. Hazard ratio of achieving VS and experiencing VR after therapy switch, according to**  
 508 **cumulative resistance and drug activity in cART-experienced patients**

Cumulative resistance & drug activity	Hazard ratio of achieving VS				Hazard ratio of experiencing VR			
	Crude		Adjusted <sup>a</sup>		Crude		Adjusted <sup>a</sup>	
	HR (95% C.I.)	P value	HR (95% C.I.)	P value	HR (95% C.I.)	P value	HR (95% C.I.)	P value
<i>No resistance &amp; fully active<sup>b</sup></i>	1		1		1		1	
<i>1-2 class &amp; fully active</i>	1.00 (0.86-1.16)	0.957	0.88 (0.72-1.08)	0.213	1.30 (0.99-1.72)	0.061	1.29 (0.96-1.74)	0.093
<i>≥3 classes &amp; fully active</i>	<b>2.26 (1.53-3.33)</b>	<b>&lt;0.001</b>	1.29 (0.84-2.00)	0.249	0.91 (0.40-2.05)	0.812	1.46 (0.60-3.52)	0.401
<i>1-2 class &amp; partially active</i>	0.97 (0.87-1.09)	0.656	1.10 (0.94-1.28)	0.230	1.14 (0.92-1.41)	0.228	0.95 (0.75-1.20)	0.669
<i>≥3 class &amp; partially active</i>	1.04 (0.89-1.21)	0.642	1.08 (0.86-1.35)	0.534	0.98 (0.73-1.30)	0.868	0.80 (0.57-1.12)	0.199
<i>1-2 class &amp; poor active</i>	0.58 (0.30-1.11)	0.101	0.73 (0.35-1.55)	0.416	1.84 (0.75-4.49)	0.180	1.48 (0.59-3.73)	0.402
<i>≥3 class &amp; poor active</i>	<b>0.53 (0.35-0.80)</b>	<b>0.002</b>	0.59 (0.35-1.00)	0.051	1.48 (0.82-2.67)	0.191	1.29 (0.70-2.39)	0.421

<sup>a</sup>Adjusted for: age, gender, risk factor, subtype, nationality, viremia zenith (as the highest plasma HIV-RNA value recorded in patients' history), CD4 cell count nadir (as the lowest CD4 count recorded in patient's history), baseline viremia and CD4 cell count (before last therapy switch recorded), type of regimen received at therapy switch, duration of previous cART exposure before therapy switch, previous exposure to integrase inhibitors, previous exposure to enfuvirtide or maraviroc, number of regimens experienced before switch, year of treatment switch. <sup>b</sup>Reference group (dummy). cART: combined antiretroviral therapy; CI: confidence interval; HR: hazard ratio; VS: virological success; VR: virological rebound. P values <0.05 were considered statistically significant and were highlighted in bold in the models.