

**Performance of HIV-1 Drug Resistance Testing at Low Level Viraemia and Its Ability to Predict Future Virologic Outcomes and Viral Evolution in Treatment-Naïve Individuals**

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Summary: This study shows that routine HIV genotyping of low-level viraemia samples can be performed with a reasonably high success rate and the results are predictive of future virologic outcomes and viral evolution in treatment-naïve individuals.

## Abstract

**Background:** Low-level HIV viraemia (LLV; 50-999 copies/mL) occurs frequently in patients receiving antiretroviral therapy (ART), but there are little or no data available demonstrating that HIV drug resistance testing at viral loads (pVL) <1000 provides potentially clinically useful information. Here, we assess the ability to perform resistance testing by genotyping at LLV and whether it is predictive of future virologic outcomes in patients beginning ART.

**Methods:** Resistance testing by genotyping at LLV was attempted on 4915 plasma samples from 2492 patients. A subset of previously ART-naïve patients was analyzed who achieved undetectable pVL and subsequently rebounded with LLV (N=212). A genotypic sensitivity score (GSS) was calculated based on therapy and resistance testing results by genotyping, and stratified according to number of active drugs.

**Results:** 88% of LLV resistance assays produced useable sequences, with higher success at higher pVL. Overall, 16/212 (8%) patients had pre-therapy resistance. 38/196 (19%) patients without pre-therapy resistance evolved resistance to 1 or more drug classes – primarily the nRTIs (14%) and/or NNRTIs (9%). Patients with resistance at LLV (GSS<3) had a 2.1-fold higher risk of virologic failure (95%CI 1.2-3.7) than those without resistance (p=0.007). Progressively lower GSS scores at LLV were associated with a higher increase in pVL over time (p<0.001). Acquisition of additional resistance mutations to a new class of antiretrovirals during LLV was not found in a subset of patients.

**Conclusions:** Routine HIV genotyping of low-level viraemia samples can be performed with a reasonably high success rate and the results appear predictive of future virologic outcomes.

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## Introduction

An increasing proportion of persons living with HIV are receiving suppressive antiretroviral therapy (ART) [1, 2]. However, despite stable ART, many patients experience episodes of low-level viraemia (LLV), defined as plasma viral load (pVL) measurements between 50 and 1000 HIV-RNA copies/mL which may include repeated pVL episodes, intermittent pVL and blips. An increased risk of virologic failure has been associated with episodes of LLV in several studies [3-6], but not in others [7, 8]. In addition, LLV episodes have been associated with higher immune activation [9, 10] and even possible increased mortality [11]. A main factor in increased risk of virologic failure appears to be the accumulation of drug resistance mutations, either released from stable HIV reservoirs [12] and/or from ongoing cycles of replication [7].

However, data on resistance during low-level viraemia is limited, in part because FDA-approved genotypic HIV resistance assays require at least 1,000 copies/ml (TRUGENE HIV-1 Genotyping Assay) or 2000 copies/mL (ViroSeq® HIV-1 Genotyping System) [13]. Another concern is the possibility that amplification of a very small number of HIV copies may render inaccurate genotypic results. Thus, studies of antiretroviral resistance during low-level viraemia on patients receiving first-line ART are scarce [14]. The aims of our study were to evaluate emergence of HIV drug resistance mutations during periods of low-level viraemia, assess the ability to successfully sequence them using an in-house assay and evaluate the association of LLV resistance with subsequent virologic outcomes in a cohort of patients beginning their first antiretroviral therapy regimen.

## Materials and Methods

### Study population

We evaluated genotype success rates of all HIV-infected adults who enrolled in the British Columbia (BC) Drug Treatment Program between 1996 and 2012 with any detectable plasma viral load (pVL) <1000 copies/mL by Roche COBAS® Amplicor™ HIV-1 Monitor® Test v1.5 (detection limit of 400 copies/mL from 1996 to 1999, and 50 copies/mL from 1999-2009) or Roche COBAS TaqMan HIV-1 v1.0 or v2.0 (detection limit set to 50 copies/mL, and in use from 2009-2012). A total of 4915 results were obtained from samples with detectable pVL <1000 copies/mL. These samples came from a total of 2492 patients. Many of these results (27%) were obtained retrospectively for research purposes on stored specimens, since the cutoff for ordering a low-level viraemia resistance genotype was initially 500 copies/mL, lowered subsequently to 250 copies/mL, though samples with pVL <250 could be tested by physician request.

### HIV RNA extraction and drug resistance analysis

Drug resistance testing was performed on physician-requested samples with pVL as defined above. In addition to changes in the viral load assay over the years, various methods and equipment have also been used for resistance genotyping in BC. From 1998 to 2006, HIV RNA was manually extracted from frozen plasma samples using guanidinium based lysis buffer followed by isopropanol/ethanol washes (Qiagen), or automatically using a BioRobot (Qiagen), and from 2006 to 2012 by automated

extraction using a NucliSENS easyMAG (bioMerieux). Amplification of the protease (PR) and reverse transcriptase (RT) regions was performed using nested RT-PCR followed by sequencing in both the 5' and 3' directions on an ABI 3100, 3130, or 3700 sequencer from 1996 to 2006, and an ABI 3730 sequencer from 2006 to 2012. Primers used span all of protease, and to codon 400 of RT (Primary PCR product). Amplification was repeated with different primers (spanning to codon 250 of RT; "Backup PCR product") when the first attempt was unsuccessful. Although a second PCR attempt must be made, this "backup" method simply uses a different primer set which spans a smaller region, so is no more resource intensive than the primary method. A test was reported as failed when a second attempt with re-extraction and back-up primers was unsuccessful. Sequence data were analyzed using Sequencher (Genecodes) from 1996 to 2007 and RECall (BC Centre for Excellence in HIV/AIDS) automated sequencing software from 2007 to 2012 [15-17]. Nucleotide mixtures were identified if the secondary peak height exceeded approximately 17.5% of the dominant peak height.

### **Genotypic Sensitivity Scores**

The genotypic sensitivity score (GSS) was obtained using the Stanford HIVdb genotypic resistance interpretation system [18]. The Stanford algorithm generates five levels of resistance to a drug, ranging from fully susceptible (i.e., wild-type), low to intermediate resistance, and high level resistance. Here, we assigned a GSS value of 1 to each drug categorized as susceptible, potential low-level resistance and low-level resistance; a GSS value of 0.5 to the intermediate resistance category; and a GSS

value of 0 to the high-level resistance category. The GSS values for all drugs in a regimen were added together to give a final total GSS. Patients were grouped into 4 categories depending on their GSS scores at LLV, corresponding to the number of active drugs prescribed: 0-0.5; 1-1.5; 2-2.5; and  $\geq 3$ . In a subsequent analysis, patients were grouped into resistant (GSS<3) and not resistant (GSS $\geq 3$ ) categories. The virtual phenotypic sensitivity score Virco@TYPE HIV-1 (vPSS) was also used to re-evaluate the results [19].

### **Patient outcome analysis**

To evaluate the effect of LLV resistance on subsequent virologic outcome, further analyses were restricted to previously ART-naïve patients who achieved undetectable viral loads but whose virus rebounded with repeated pVL between 50-999 copies/mL. Patients were included only if they had not had a previous blip ( $\geq 1000$  copies/mL), and they were followed as long as they were receiving constant therapy without any changes or interruptions. Many of these results (24%) were obtained retrospectively.

### **Statistical analysis**

Kaplan-Meier methods were used to monitor time from LLV to virologic failure  $\geq 1000$  copies/mL. Subjects without virologic failure were right censored at the date of last observation while on the same therapy. Linear mixed-effect models were used to compare the change in pVL over time between GSS categories. A Cox proportional

hazards model was used to estimate the hazard ratios of virologic failure, adjusting for other explanatory variables, such as gender, age, Hepatitis C status, and pVL at LLV. A backward stepwise technique was used in the selection of covariates for an explanatory model. The selection of variables was based on two criteria: Akaike Information Criterion (AIC) and Type III p-values. Tests for statistical significance were performed using Fisher's exact test or Pearson's chi-square test for categorical variables and the Wilcoxon rank-sum test for continuous variables. Analyses were performed using SAS software version 9.3 (SAS, Cary, NC).

## **Ethics Statement**

This study was approved by the Committee on Human Research and the University of British Columbia/ Providence Health Care Research Ethics Board.

## **Results**

### **Genotyping success**

Overall, 4312 of 4915 (88%) low-level viraemia assays attempted produced usable sequences (Table 1). When  $\geq 2$  different viral strains are amplified within the same sample  $>1$  mixtures will be found in the final sequence. Successful genotypes were obtained more frequently at higher pVL strata approaching 1000 copies/mL. These higher viral load samples tended to have more observed sequence mixtures, suggesting

>1 viral input copies amplified from the samples. Successful results were obtained from 74% of samples with viral loads <250 c/mL and from approximately 90% of samples with viral loads above 250 c/mL. Results were similar regardless HIV subtype (data not shown). Unsuccessful genotypes and the use of Backup PCR product progressively increased with decreasing viral load (Figure 1a). In addition, we analyzed whether the age of the sample (time from collection to testing) affected the success in testing finding that the longer age of the samples the success in testing was slightly lower (Figure 1b). However, even in samples stored at -20°C for more than four years, success in testing remained around 70-80%.

### **Patient characteristics and resistance testing by genotyping**

Patient characteristics are shown in Table 2 for 212 subjects previously drug naïve who rebounded with LLV during their first ART regimen. At time of resistance testing, patients had moderately high CD4 counts (median 415 cells/mm<sup>3</sup>; 25<sup>th</sup>-75<sup>th</sup> percentile: 260-580 cells/mm<sup>3</sup>) and low pVL (median 374 copies/mL; 25<sup>th</sup>-75<sup>th</sup> percentile: 267-559 copies/mL). Resistance testing by genotyping before treatment (baseline) and at time of genotype testing are shown in Table 3. Overall, 16 of the 212 patients (8%) had baseline resistance prior to therapy. Of those without baseline resistance (N=196), 38 patients (19%) evolved resistance to any class of medication at follow up with LLV, a median of 6.9 months (25<sup>th</sup>-75<sup>th</sup> percentile: 3.3–18) after their pVL became undetectable. In these patients, resistance was most common to the NRTI

(N=28; 14%) and/or NNRTI (N=18; 9%) drug classes. Of note, only 2 cases (1%) of emerging PI mutations arose (D30N), both in patients taking nelfinavir, despite 67% of patients receiving a PI. No patients evolved triple-class drug resistance during the study period. The most common mutations found at LLV were M184V/I (10%V, 4%I), K103N (6%), T215Y/F/C/D/E/S/I (4%), M41L (4%), Y181C (3%), K70R/E (3%) and T69D/N/S (3%).

Before treatment, baseline (i.e., transmitted) resistance was marginally more common in patients who were male ( $p=0.02$ ) and slightly older (median 46 vs 43 years;  $p=0.04$ ). Analyses were then further restricted to the 196 patients without baseline resistance. Those patients who evolved resistance at LLV had a non-statistically significant trend towards higher viral load levels at LLV versus those who did not evolve resistance (median 472 vs 369 copies/mL,  $p$ -value=0.067). Moreover, we observed that the prevalence of resistance increased at higher viral load strata at LLV. Only 5% of patients (N=2) with 50-249 c/mL at LLV had resistance whereas 24% (N=22), 17% (N=7) and 30% (N=7) had LLV resistance at 250-499, 500-749 and 750-999 c/mL respectively ( $p$ -value=0.041). Other patient characteristics (risk group, HCV coinfection, CD4, ethnicity, time from undetectable viraemia to LLV) were not associated with resistance at LLV ( $p$ -value  $\geq 0.1$ ).

## Virologic outcomes

Kaplan-Meier curves were used to evaluate time to virologic failure ( $\geq 1000$  copies/mL) while remaining on the same therapy. Figure 2A indicates that patients with resistance ( $GSS < 3$ ) at LLV had significantly increased risk of subsequent virologic failure compared to those without resistance ( $GSS \geq 3$ ) ( $p = 0.007$ ). Furthermore, linear mixed-effect models showed that progressively lower GSS scores at LLV were significantly associated with an increased change in median pVL over time (overall  $p$ -value  $< 0.001$ ) (Figure 2B).

Bivariate analysis of patient characteristics associated with virologic failure after LLV indicated that higher pVL at LLV ( $p = 0.02$ ), history of injection drug use ( $p = 0.03$ ), HCV coinfection ( $p = 0.04$ ) and female gender ( $p = 0.05$ ) were associated with an increased likelihood of a subsequent pVL  $\geq 1000$  copies/mL. To determine predictors associated with the hazard of virologic failure, unadjusted and adjusted Cox proportional hazards models were applied to all the variables related to virologic failure. Among covariates considered, only gender and pVL at LLV were included in the final model. Adjusted hazard ratios were 2.12 (95% CI 1.23-3.66) for patients with resistance at LLV ( $GSS < 3$ ), 2.34 (95% CI 1.11-4.93) for patients with pVLs 500-749 copies/mL vs. 50-249 copies/mL and 1.64 (95% CI 0.97-2.78) for patients reporting female gender. The other pVL strata had  $p$ -values  $> 0.1$  compared with pVLs 50-249 copies/mL.

Additionally, in a subset of 29 patients maintaining LLV with follow-up resistance test results, there was no evidence of acquisition of additional resistance mutations to a new class of antiretrovirals (data not shown) suggesting that the selection of resistance

to an additional family drug during a LLV episode on the same regimen may not be a common event, though the follow-up times were relatively short (8 months). Moreover, when the evolutionary distance between the first and last genotype was analyzed by the TN93 model [20] a slightly trend towards increased genetic diversity over time was observed. However, this did not reach statistical difference, probably because of the low number of patients (data not shown). This trend may suggest that even with no apparent resistance evolution, HIV-1 may be able to evolve at LLV.

Data were also stratified by whether or not any nucleotide mixtures were observed in the sequence chromatograms. An absence of mixtures suggested that only a single molecule may have been successfully reverse transcribed and amplified during sample processing. As is shown in Figure 3, the time to virologic failure curves are different between patients with resistance and no resistance, whether or not mixtures were observed ( $p=0.007$ ). Cox proportional hazards model adjusting for an indicator for mixture still showed a significant  $p$ -value for resistance ( $p$ -value= $0.007$ ), implying that even clonal products with only one sequence (no mixtures) gave useful results for predicting virologic failure. Furthermore, all the above analyses were performed using Virco@TYPE virtual phenotype interpretation instead of the Stanford algorithm, and very similar results were obtained (Supplementary data).

## Discussion

We have shown that routine HIV genotyping of low-level viraemia samples can be performed with a reasonably high success rate (74% in samples <250 copies/mL and 90% >250 copies/mL). In addition, we have shown that genotyping of low-level viraemia samples is predictive of future virologic outcomes in treatment-naïve patients on their first antiretroviral therapy regimen.

Diagnosis and management of emerging drug resistance during LLV is a clinical challenge, as FDA-approved genotyping assays require at least 1000 copies/ml [13] and some standard genotypic tests (including ours) have higher failure rates at amplifying HIV-1 RNA at low-level viraemia [21]. However, the in-house PCR method used in this study shows a high success rate for genotyping LLV samples (about 74% at 50-249 copies/mL and 90% at 250-999 copies/mL) suggesting that genotyping can be performed routinely on clinical low-level viraemia samples. Actually, based on the pVL and the volume used for testing, genotypes sequences were obtained ranging from 2 to 33 actual input copies. In addition to lower assay success in samples with especially low pVL levels, we found that the longer time elapsed between sample collection and testing also negatively affected success of genotyping. However, success was still relatively high, at 70-80%, even for samples older than 4 years old. The relatively high success rate of resistance genotyping at low-level viraemia has also been demonstrated by an independent laboratory, who report strikingly similar success rates to ours [22].

The results obtained in this study suggest that resistance detected during LLV may be clinically relevant to future outcomes and this information should be provided to healthcare practitioners to better monitor their patients. In fact, use of resistance testing by genotyping testing has been shown to improve virologic outcomes in several prospective studies of patients failing ART [23-25]. To infer resistance in this study, we used the Stanford HIVdb genotypic resistance interpretation system [18]. Other rules-based systems as ANRS, Rega and AntiRetroScan are available, and similar results are often obtained regardless of the system used [26, 27]. In addition, all our results were confirmed by using the Virco@TYPE HIV-1 as an alternative classification algorithm. Although some studies have reported that GSS had no impact on viral responses [28-30], several other investigations support genotypic or phenotypic sensitivity scores as predictors of viral response [4, 26, 27, 31, 32].

Here we show a strong association between risk of virologic failure and GSS scores (p-value <0.007). This is in agreement with a previous study analyzing LLV of 50-500 copies/mL [33]. We also found a weak association between virologic failure and injection drug use history, HCV co-infection, and female gender. This may be explained because all three of these patient characteristics co-associate in British Columbia and have been previously linked to sub-optimal adherence to ART [34]. However, after adjusting the model for these variables, the only variables independently associated with virologic failure were GSS<3 (p=0.007) and pVL 500-749 copies/mL vs. 50-249 (p=0.026), while female gender was no longer significant (p=0.069).

Only 1% of patients evolved resistance to protease inhibitors (specifically D30N mutations, while taking nelfinavir), despite 67% of patients receiving a PI. This result

confirms that emerging PI resistance rarely occurs during LLV, probably due to their high genetic barrier to resistance [35]. Also, our results showed that patients with higher pVL at LLV were more likely to have resistance mutations (mainly M184V/I and K103N) which is in agreement with a previous study analyzing 59 patients with LLV on first-line therapy [12].

A previously unaddressed concern of genotyping LLV samples has been whether the higher likelihood of amplifying only one viral input copy would still be informative. To clarify that concern, we analyzed our results by whether or not mixtures were found in the sequence chromatograms, and found no difference in the outcome between these two groups. This indicates that even clonal products, where only one viral input copy appeared to have been amplified, are informative of the future outcome of patients.

It has been recently reported that carrying a non-B HIV subtype was a predictor of very low-level viremia (<50 copies/mL) following initiation of HAART in 57 patients [36]. However, in our study with samples between 50 and 999 copies/mL, we found no difference either in genotypic testing success or patient outcomes for patients with B or non-B HIV subtypes (data not shown). It is possible that the low viral loads (<50), low number of patients and differences in patient characteristics between the B and non-B groups in that study are impacting its results.

Strengths of our study include its large sample size (about 5000), the duration of the study (from 1996 to 2012) and the duration of clinical follow-up (up to 8 years). The analysis of only previously ART-naïve patients achieving undetectable viral load before LLV and remaining on the same regimen during the entire follow-up were also major

strengths of the study. Moreover, when calculating the GSS we considered only intermediate and high-level resistance mutations, leaving aside low-level and potential low-level resistance mutations, which have a weaker influence on regimen efficacy. Furthermore, different from other groups [4], we considered only the resistant mutations found at sampling and did not assume that mutations detected in any previous analysis (e.g., before therapy) were still present even if not detected [37]. All these factors provide a more consistent and cleaner picture of LLV resistance.

Nevertheless, our study has several limitations. It was an observational study, and some our results were obtained retrospectively. A randomized controlled trial would be more definitive in proving that resistance testing at low-level viraemia is a useful strategy. We did not exclude patients based on the therapies they were receiving, so two patients with suboptimal regimens (<3 full-dose drugs) were included in our study. Resistance was assessed only through sequencing of the protease and RT regions of HIV, though other regions may also play a role in drug susceptibility [38]. We also did not address adherence in this study. Nevertheless, although adherence levels may play a hypothetical role in LLV, a previous study showed that adherence levels did not modify the associations between LLV resistance and virologic outcome [31]. Furthermore, the low viral loads of these patients likely indicate some level of adherence.

In conclusion, we show that routine HIV genotyping of low-level viraemia samples can be performed with a reasonably high success rate and the results obtained appear predictive of future virologic outcomes.

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**Potential conflicts of interest.**

All authors: No reported conflicts.

## References

1. Gill VS, Lima VD, Zhang W, et al. Improved virological outcomes in British Columbia concomitant with decreasing incidence of HIV type 1 drug resistance detection. *Clin Infect Dis*, **2010**; 50: 98-105.
2. Bansi L, Sabin C, Delpech V, et al. Trends over calendar time in antiretroviral treatment success and failure in HIV clinic populations. *HIV Med*, **2010**; 11: 432-8.
3. Cohen C. Low-level viremia in HIV-1 infection: consequences and implications for switching to a new regimen. *HIV Clin Trials*, **2009**; 10: 116-24.
4. Delaugerre C, Gallien S, Flandre P, et al. Impact of low-level-viremia on HIV-1 drug-resistance evolution among antiretroviral treated-patients. *PLoS One*, **2012**; 7: e36673
5. Karlsson AC, Younger SR, Martin JN, et al. Immunologic and virologic evolution during periods of intermittent and persistent low-level viremia. *AIDS*, **2004**; 18: 981-9.
6. Pilcher CD, Miller WC, Beatty ZA, Eron JJ. Detectable HIV-1 RNA at levels below quantifiable limits by amplicor HIV-1 monitor is associated with virologic relapse on antiretroviral therapy. *AIDS*, **1999**; 13: 1337-42.
7. Nettles RE, Kieffer TL, Kwon P, et al. Intermittent HIV-1 viremia (Blips) and drug resistance in patients receiving HAART. *JAMA*, **2005**; 293: 817-29.
8. Mira JA, Macías J, Nogales C, et al. Transient rebounds of low-level viraemia among HIV-infected patients under HAART are not associated with virological or immunological failure. *Antivir Ther*, **2002**; 7: 251-6.

9. Karlsson AC, Younger SR, Martin JN, et al. Immunologic and virologic evolution during periods of intermittent and persistent low-level viremia. *AIDS*, **2004**; 18: 981-9.
10. Taiwo B, Hunt PW, Gandhi RT, et al. CD8+ T-cell Activation in HIV-1-Infected Patients Experiencing Transient Low-level Viremia During Antiretroviral Therapy. *J Acquir Immune Defic Syndr*, **2013**; 63: 101-104.
11. Mark Hull, M Loutfy, W Zhang, et al. Persistent Low-level Viremia Is Associated with Increased Risk of Virologic Failure and Mortality, [Abstract 504] 17th Conference on Retroviruses and Opportunistic Infections, San Francisco 2010.
12. Ho DD. Toward HIV eradication or remission: the tasks ahead. *Science*, **1998**; 280: 1866-7.
13. Department of Health and Human Services (2011) Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. January 10, 2011; 1–166. Available at: <http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf>
14. Taiwo B, Gallien S, Aga E, et al. Antiretroviral drug resistance in HIV-1-infected patients experiencing persistent low-level viremia during first-line therapy. *J Infect Dis*, **2011**; 204: 515-20.
15. Dong W, Wynhoven B, Mo T, et al. Performance of RE\_Call basecalling software for high-throughput HIV Drug resistance basecalling using in-house methods. [Abstract TuPeB4598]. XIV International AIDS Conference; 2002; Barcelona, Spain.

16. Brooks JI, Woods CK, Merks H, et al. Evaluation of an automated sequence analysis tool to standardize HIV genotyping results. [Abstract O061]. Canadian Conference on HIV/AIDS Research; 2009; Vancouver, BC, Canada.
17. Brooks JI, Woods C, Merks H, et al. Evaluation of a Web-Based Automated Sequence Analysis Tool to Standardize HIV Genotyping Results. [Abstract]. 49th International Conference on Antimicrobial Agents and Chemotherapy, San Francisco, September 12-15, 2009.
18. Tang MW, Liu TF, Shafer RW. The HIVdb system for HIV-1 genotypic resistance interpretation. *Intervirology*, **2012**; 55: 98-101.
19. Van Houtte M, Picchio G, Van Der Borght K, Pattery T, Lecocq P, Bachelier LT. A comparison of HIV-1 drug susceptibility as provided by conventional phenotyping and by a phenotype prediction tool based on viral genotype. *J Med Virol*, **2009**; 81: 1702-9.
20. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol*, **1993**; 10: 512-26.
21. Assoumou L, Descamps D, Yerly S, et al. Prevalence of HIV-1 drug resistance in treated patients with viral load >50 copies/mL in 2009: a French nationwide study. *J Antimicrob Chemother*, **2013**; 68: 1400-1405.
22. Santoro MM, Alteria C, Armenia D et al. Reliability of HIV-1 drug-resistance test in patients with low viraemia levels. International Workshop on HIV and Hepatitis Virus Drug Resistance and Curative Strategies, Toronto, Canada, June 4-8, 2013.

- 23.** Durant J, Clevenbergh P, Halfon P, et al. Drug-resistance genotyping in HIV-1 therapy: the VIRADAPT randomised controlled trial. *Lancet*, **1999**; 353: 2195-9.
- 24.** Tural C, Ruiz L, Holtzer C, et al. Clinical utility of HIV-1 genotyping and expert advice: the Havana trial. *AIDS*, **2002**; 16: 209-18.
- 25.** McConnell MJ, Mier-Mota J, Flor-Parra F, et al. Improved viral suppression after treatment optimization in HIV-infected patients with persistent low-level viremia. *J Acquir Immune Defic Syndr*, **2011**; 58: 446-9.
- 26.** Frentz D, Boucher CA, Assel M, et al. Comparison of HIV-1 genotypic resistance test interpretation systems in predicting virological outcomes over time. *PLoS One*, **2010**; 5: e11505
- 27.** Zazzi M, Prosperi M, Vicenti I, et al. Rules-based HIV-1 genotypic resistance interpretation systems predict 8 week and 24 week virological antiretroviral treatment outcome and benefit from drug potency weighting. *J Antimicrob Chemother*, **2009**; 64: 616-24
- 28.** Cingolani A, Antinori A, Rizzo MG, et al. Usefulness of monitoring HIV drug resistance and adherence in individuals failing highly active antiretroviral therapy: a randomized study (ARGENTA). *AIDS*, **2002**; 16: 369-79.
- 29.** De Luca A, Cingolani A, Di Giambenedetto S, et al. Variable prediction of antiretroviral treatment outcome by different systems for interpreting genotypic human immunodeficiency virus type 1 drug resistance. *J Infect Dis*, **2003**; 187: 1934-43.

- 30.** Mazzotta F, Lo Caputo S, Torti C, et al. Real versus virtual phenotype to guide treatment in heavily pretreated patients: 48-week follow-up of the Genotipo-Fenotipo di Resistenza (GenPheRex) trial. *J Acquir Immune Defic Syndr*, **2003**; 32: 268-80.
- 31.** Castor D, Vlahov D, Hoover DR, et al. The relationship between genotypic sensitivity score and treatment outcomes in late stage HIV disease after supervised HAART. *J Med Virol*, **2009**; 81: 1323-35.
- 32.** Li JZ, Gallien S, Do TD, et al. Prevalence and significance of HIV-1 drug resistance mutations among patients on antiretroviral therapy with detectable low-level viremia. *Antimicrob Agents Chemother*, **2012**; 56: 5998-6000.
- 33.** García-Gascó P, Maida I, Blanco F, et al. Episodes of low-level viral rebound in HIV-infected patients on antiretroviral therapy: frequency, predictors and outcome. *J Antimicrob Chemother*, **2008**; 61: 699-704.
- 34.** O'Neil CR, Palmer AK, Coulter S, et al. Factors Associated with Antiretroviral Medication Adherence among HIV-Positive Adults Accessing Highly Active Antiretroviral Therapy (HAART) in British Columbia, Canada. *J Int Assoc Physicians AIDS Care (Chic)*, **2012**; 11: 134-41.
- 35.** Sax PE, Tierney C, Collier AC, et al. Abacavir/lamivudine versus tenofovir DF/emtricitabine as part of combination regimens for initial treatment of HIV: final results. *J Infect Dis*, **2011**; 204: 1191-201.

**36.** Saison J, Tardy JC, Scholtes C et al. Low-level viremia is associated with non-B subtypes in patients infected with HIV with virological success following HAART introduction. *J Med Virol*, **2013**; 85: 953-8.

**37.** Lambotte O, Chaix ML, Gubler B, et al. The lymphocyte HIV reservoir in patients on long-term HAART is a memory of virus evolution. *AIDS*, **2004**; 18: 1147-58.

**38.** Rabi SA, Laird GM, Durand CM, et al. Multi-step inhibition explains HIV-1 protease inhibitor pharmacodynamics and resistance. *J Clin Invest*. **2013**; 123:3848-60

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**Figure Legends:****Figure 1: a) Success at testing depending on the viral load of the sample and PCR attempted.**

Successful results were obtained in approximately 90% in samples with viral loads above 250 c/mL and decreased to 79% and 62% in samples with viral loads of 200-249 c/mL or 50-99 c/mL. Backup PCR (repetition of amplification with different primers when first attempt was unsuccessful) products were more commonly used at lower viral loads.

**b) Effect of duration of storage on successful PCR and sequencing.**

The samples with a longer age (time from collection to testing) had marginally lower success at testing. Samples less than 12 months old stored at -20°C had slightly greater testing success compared to those older than, however success in testing remained around 70-80% in all of them.

**Figure 2: a) Time to virologic failure more rapid in patients with resistance during low level viraemia.**

Kaplan-Meier curve of time to virologic failure >1000 copies/mL while remaining on constant therapy, as a function of whether patients had resistance at LLV (GSS<3) or not (GSS ≥3). Patients with resistance at LLV had a more rapid rate of virologic failure (p=0.007).

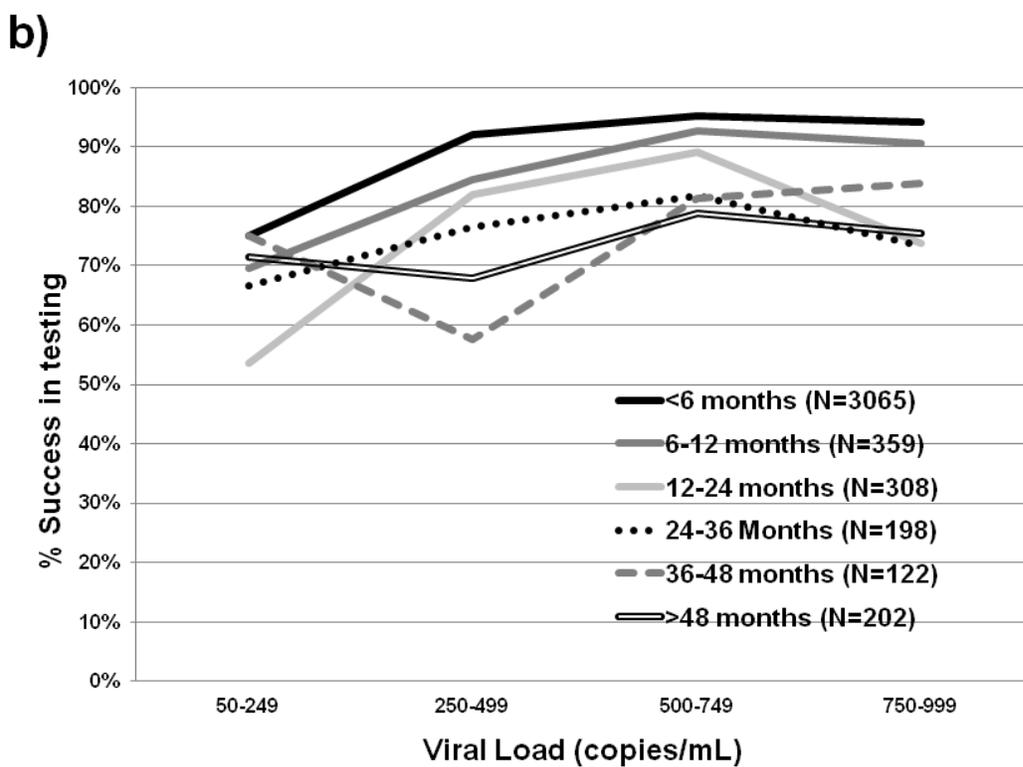
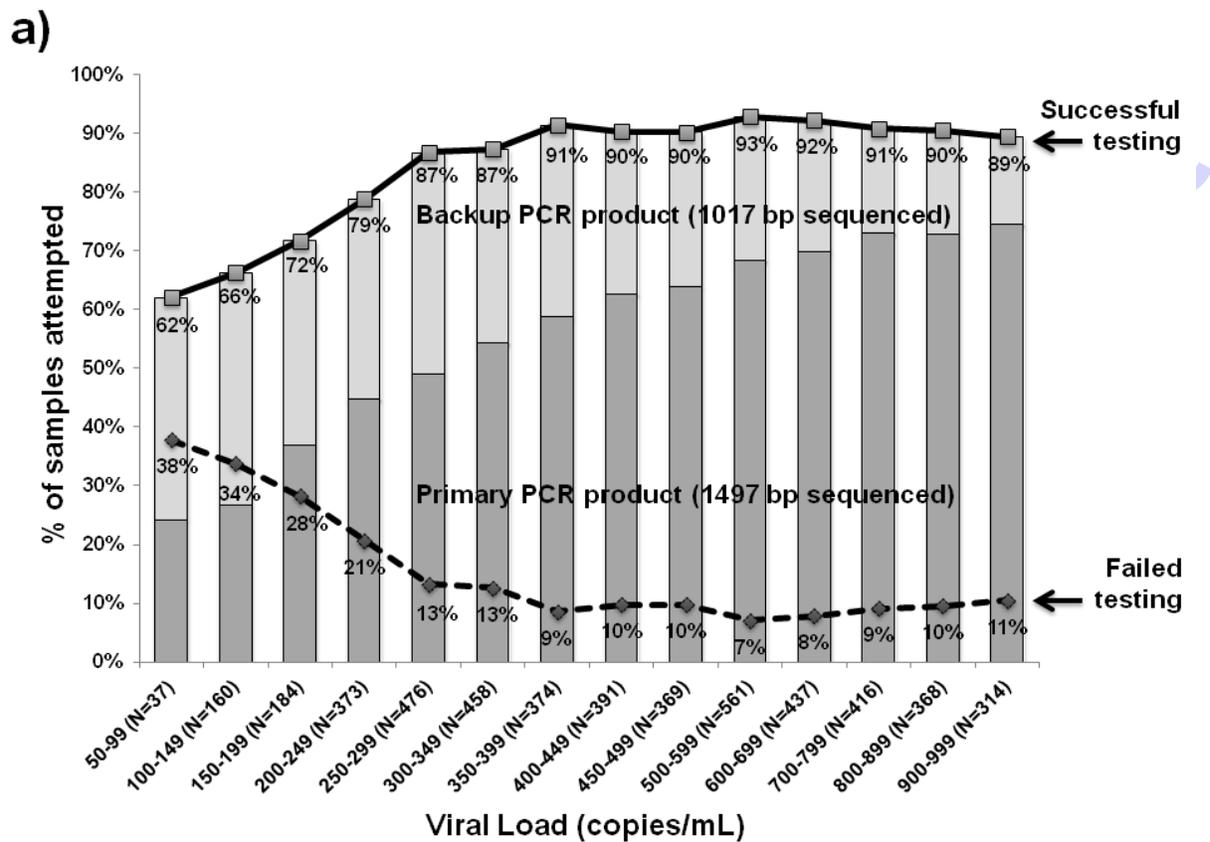
**b) More extensive resistance at LLV leads to higher viral load rebounds**

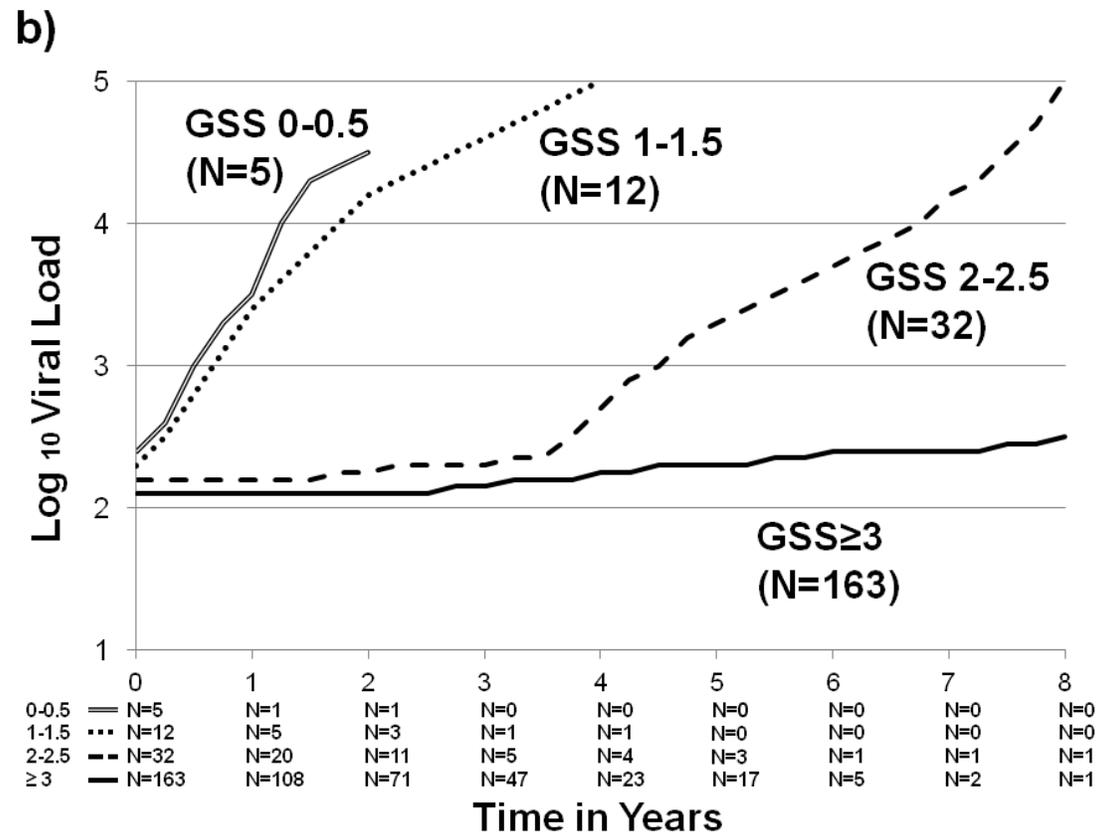
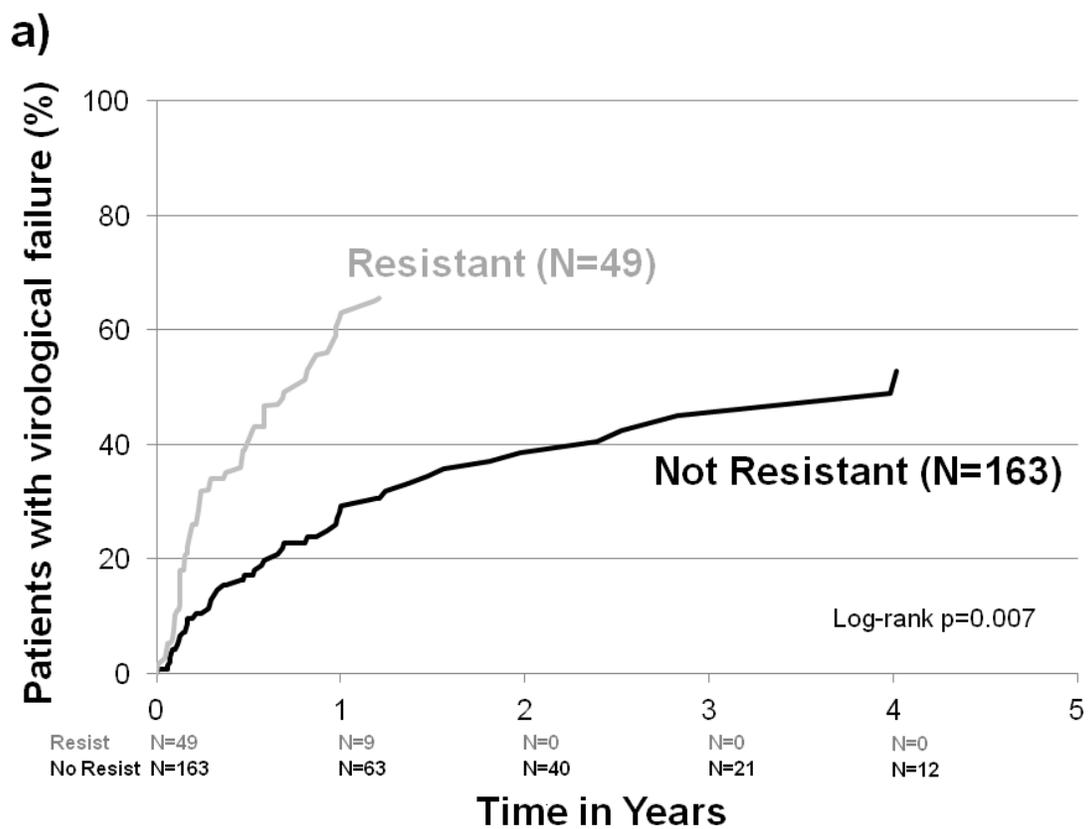
Progressively lower GSS scores at LLV were significantly associated with a higher increase in median pVL over time in patients while remaining on constant therapy (overall p-value <0.001).

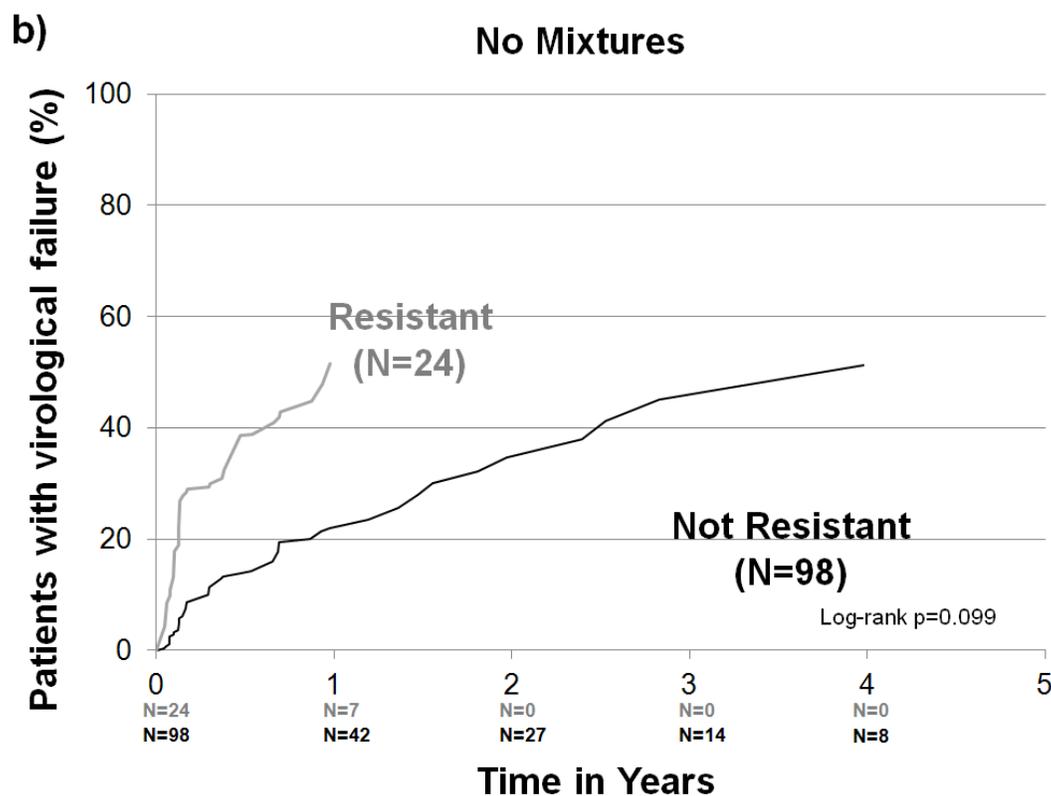
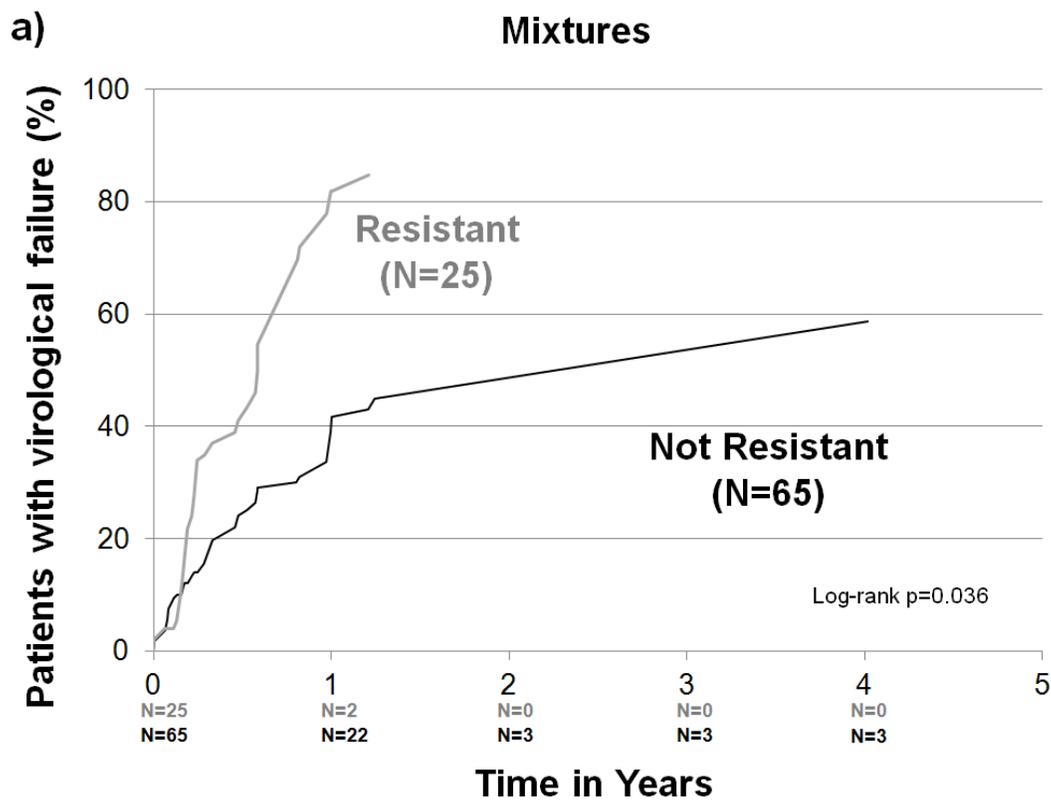
**Figure 3: a) Time to virologic failure (Patients with >1 viral input copies amplified, and sequence mixtures).**

**b) Time to virologic failure (Patients with 1 viral species amplified, and no sequence mixtures).**

Time to virologic failure curves are different between patients with resistance and no resistance whether virologic mixtures were observed in the sequence chromatograms (a) or not (b) (p-value=0.007).







**Table 1.** Success at low-level viraemia genotyping.

<b>Input Viral Load (copies/mL)</b>	<b>50 - 249</b>	<b>250 - 499</b>	<b>500 - 749</b>	<b>750 - 999</b>
<b>Actual input copies<sup>1</sup></b>	<b>1.7 - 8.3</b>	<b>8.3 - 16.6</b>	<b>16.7 - 24.9</b>	<b>25 - 33.3</b>
<b>Number attempted</b>	751	2068	1212	884
<b>Number successful</b>	555	1841	1118	798
<b>% Successful</b>	74%	89%	92%	90%
<b>% Sequences with any mixtures</b>	36%	51%	64%	71%
<b>Median number of mixtures per sequence</b>	0	1	5	9

<sup>1</sup>Actual input copies is a 30-fold dilution from the pVL based on the use of 500 µl from 1000 µl of plasma, utilized to obtain 60 µl of RNA extract from which only 4 ul were finally used for PCR.

**Table 2.** Patient characteristics at time of LLV genotype

Characteristics at LLV	Category	n (%)
Gender (male)		162 (76)
Age (Median years, 25 <sup>th</sup> -75 <sup>th</sup> )		44 (37-49)
CD4 cells/mm <sup>3</sup> at LLV (Median, 25 <sup>th</sup> -75 <sup>th</sup> ) (N=202)		415 (260-580)
HIV-RNA copies/ml at LLV (Median, 25 <sup>th</sup> -75 <sup>th</sup> )		374 (267-559)
HIV B subtype		200 (94)
Hepatitis C antibody positive (n=206)		90 (44)
Risk group (N=162)	IDU	95 (59)
	MSM	62 (38)
	Heterosexual sex	83 (51)
	Blood	2 (1)
	Other	5 (3)
	Ethnicity (N=139)	White
	Aboriginal	34 (24)
	Other	22 (16)
Regimen at LLV (N=212)	2 nRTI + boosted PI	81 (38)
	2 nRTI + unboosted PI	50 (24)
	2 nRTI + NNRTI	50 (24)
	other ≥4 ARVs	17 (8)
	other ≤3 ARVs	14 (7)
pVL at LLV (N=212)	50-249	44 (21)
	250-499	99 (47)
	500-749	45 (21)
	750-999	24 (11)

IDU, intravenous drug use; MSM, Men who have sex with men

**Table 3.** Resistance testing by genotyping at baseline and LLV

<b>Resistance mutations (N=212) N (%)</b>	<b>At baseline (before therapy) N (%)</b>	<b>At LLV N (%)</b>	<b>At LLV for subset with no pretreatment resistance (N=196) N (%)</b>
PI	5 (2.4)	8 (3.8)	2 (1)
NRTI	5 (2.4)	33 (15.6)	28 (14.3)
NNRTI	12 (5.7)	30 (14.2)	18 (9.2)
PI or NRTI or NNRTI	16 (7.5)	52 (24.5)	38 (19.4)

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