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We hypothesized that HIV-1 with dual-class but not single-class drug resistance mutations linked on the same viral genome, present in the virus population prior to initiation of antiretroviral therapy (ART), would be associated with failure of ART to suppress viremia. To test this hypothesis, we utilized an ultrasensitive single genome sequencing assay that detects rare HIV-1 variants with linked drug resistance mutations (DRMs). A case (ART failure) – control (non-failure) study was designed to assess whether linkage of DRMs in pre-ART plasma samples was associated with treatment outcome in the nevirapine /tenofovir/emtricitabine arm of the AIDS Clinical Trials Group A5208/OCTANE Trial 1 among women who had received prior single dose nevirapine. Ultrasensitive single genome sequencing revealed a significant association between pre-ART HIV variants with DRMs to 2 drug classes linked on the same genome (dual-class) and failure of three drug ART to suppress viremia. By contrast, linked, single-class DRMs were not associated with ART failure. We conclude that linked dual-class DRMs present before the initiation of ART are associated with ART failure, whereas linked single-class DRMs are not.

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## Linked Dual-Class HIV Resistance Mutations Are Associated with Treatment Failure

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## Abstract:

We hypothesized that HIV-1 with dual-class but not single-class drug resistance mutations linked on the same viral genome, present in the virus population prior to initiation of antiretroviral therapy (ART), would be associated with failure of ART to suppress viremia. To test this hypothesis, we utilized an ultrasensitive single genome sequencing assay that detects rare HIV-1 variants with linked drug resistance mutations (DRMs). A case (ART failure) – control (non-failure) study was designed to assess whether linkage of DRMs in pre-ART plasma samples was associated with treatment outcome in the nevirapine /tenofovir/emtricitabine arm of the AIDS Clinical Trials Group A5208/OCTANE Trial 1 among women who had received prior single dose nevirapine. Ultrasensitive single genome sequencing revealed a significant association between pre-ART HIV variants with DRMs to 2 drug classes linked on the same genome (dual-class) and failure of three drug ART to suppress viremia. By contrast, linked, single-class DRMs were not associated with ART failure. We conclude that linked dual-class DRMs present before the initiation of ART are associated with ART failure, whereas linked single-class DRMs are not.

## Introduction

Despite the remarkable success of combination antiretroviral therapy (ART) in preventing morbidity and mortality from HIV-1 infection, the development of HIV-1 resistance to antiretroviral (ARV) drugs remains a threat to its continued effectiveness. Consequently, drug resistance testing is recommended as part of the standard of care in settings where resources are sufficient (1, 2). Recently, more sensitive technologies have been developed that can detect subpopulations of drug-resistant variants at frequencies of 1% or less in clinical samples, but the clinical significance of such low frequency variants is controversial. Some analyses have shown that low frequency, drug-resistance mutations (DRMs), especially those conferring resistance to non-nucleoside reverse transcriptase inhibitors (NNRTI), are associated with virologic failure after initiating combination ART containing NNRTIs (3-11). By contrast, other studies have found no association with treatment outcomes when low frequency resistance mutations are detected that confer resistance to an antiretroviral drug used in the regimen (12-29). These conflicting results make interpretation of resistance results from newer, more sensitive resistance assays difficult (30).

We hypothesized that the context in which low frequency drug-resistant variants exist can help explain their variable impact on treatment outcome. Specifically, prior exposure to a single, partially suppressive antiretroviral, such as single dose nevirapine (sdNVP), is known to select for outgrowth of NVP-resistant variants within the virus population (31, 32). We and others have shown an association between NVP-resistant variants arising from prior sdNVP and subsequent virological failure of initial NVP-based ART (16, 33, 34). In such a scenario, the expanded population of resistant variants would provide a genetic background on which

additional mutations could occur stochastically leading to dual-class resistance at the time of ART initiation. If such combinations of DRMs on the same viral genomes confer resistance to more than one drug in an ART regimen, the risk of ART failure would be increased (illustrated in Figure 1). By contrast, if low-frequency, resistant variants arise stochastically (under no selection pressure), they would pose little to no risk of ART failure because the chance of DRMs to two different drug classes becoming linked on the same genome would be much lower. An appropriate context to test our hypothesis about the association between the type of low frequency drug-resistant variants and treatment outcome is the AIDS Clinical Trials Group (ACTG) 5208/OCTANE Trial 1 (35, 36), which assessed the response to nevirapine-containing ART [nevirapine/tenofovir/emtricitabine (NVP/TFV/FTC)] among women who had prior exposure to single dose nevirapine (sdNVP) 6-24 months before study entry and initiation of ART (ClinicalTrials.gov NCT00089505) (13, 33).

To test our hypotheses, we used a method called ultrasensitive single-genome sequencing (uSGS) that detects not only rare drug resistance mutations but also linkage of mutations on the same viral genome (37). The uSGS method uniquely tags each cDNA molecule and subsequently removes mutation and recombination artifacts arising from PCR and sequencing [33]. Using this approach, the frequencies of linked and unlinked HIV-1 drug resistance mutations conferring resistance to the NVP/TFV/FTC regimen were determined in plasma samples obtained at study entry (following previous sdNVP exposure but prior to ART initiation) from women enrolled in the AIDS Clinical Trials Group 5208/OCTANE Trial 1 to assess their association with treatment outcome.

## Results

### *Participants Studied*

Our study encompassed stored, pre-ART plasma samples obtained from AIDS Clinical Trials Group 5208 Trial 1, which included only women with prior exposure to sdNVP for prevention of mother to child transmission of HIV-1. Thirty-two women experienced ART failure in the NVP/TFV/FTC treatment arm (36) with failure defined as less than 1.0 log<sub>10</sub> drop in plasma HIV-1 RNA copies/ml from baseline by week 12 or confirmed plasma HIV-1 RNA >400 copies/ml at or after week 24. Pre-ART plasma samples from study entry were available from 27 of the 32 ART failure cases for uSGS testing (Figure 2). Twenty-one (88%) of these samples had plasma HIV-1 RNA >100,000 copies/ml. For comparison, pre-ART plasma samples from 34 women who did not experience ART failure (ART non-failure controls), 24 (71%) of whom had plasma HIV-1 RNA >100,000 copies/ml, were analyzed by uSGS (Figure 2). In total, we analyzed pre-ART plasma samples from 61 women from Trial 1.

### *Tabulation of uSGS Results*

A total of 755,155 ultrasensitive single-genome sequences (uSGS) was obtained from the 61 pre-ART plasma samples. Overall, a median of 4531 uSGS (range 46 to 57278) were obtained from each sample. The median number of uSGS obtained from participants who experienced ART failure was 6165 (46 to 57278; Q1, Q3: 3014, 18350), not significantly different (Mann-Whitney  $p = 0.52$ ) from the median number of uSGS obtained from non-failure controls (4071; range 157-46163; Q1, Q3: 2093, 13977), indicating no significant sampling bias (Table 1).

### *Frequency of DRMs*

All major NVP resistance mutations (100I, 101E, 103NS, 106AM, 181CIV, 184IV, 188LCH and 190AE) and major TFV and FTC resistance mutations (65R, 184IV) were assessed. Fifty-eight of the sixty-one pre-ART samples tested (95%) had one or more mutations conferring resistance to at least one of the drugs in the ART regimen (NVP/FTC/TFV) (Table 2). Three samples had no DRMs detected, one in the ART failure group and two in the non-failure group, most likely because of the low number of single genome sequences obtained (262, 157 and 358 single genome sequences, respectively). We compared the cumulative frequencies of resistance mutations to NVP, FTC and TFV detected at study entry by treatment outcome (Table 2). NVP resistance mutations were detected pre-treatment in 93% of women with treatment failure and 94% of non-failures. Pre-treatment FTC resistance mutations (largely M184I) were also evenly distributed between the two outcome groups: 21 of 27 (78%) in the treatment failure group and 25 of 34 (74%) in the non-failure group. TFV resistance mutations were detected somewhat more frequently in the treatment failure group (14 of 27 [52%]) than in the non-failure group (13 of 34 [38%]) but not significantly so ( $p = 0.38$  Table 2). Overall, there were no significant differences in the proportions of women with NVP, FTC and TFV resistance mutations or the frequencies of the resistant mutants between the two groups ( $p=0.41$ ) (Table 2). In addition, the frequency of NVP resistance was not associated with failure in either group by logistic regression;  $p=0.56$ , OR=2.1 [0.16,27.82]. The drug-resistant mutant “viral load” (mutant plasma HIV-1 RNA copies/ml) between those participants who experienced ART failure (median 1275 copies/ml) and those who did not (median 969 copies/ml), were similar suggesting that neither the raw frequencies of

drug resistant mutants nor the mutant “viral load” explained the difference in treatment outcome (ART failure vs. non-failure).

#### *Linkage of DRMs on the same viral genome*

Next, we analyzed the uSGS data from the pre-ART samples for the presence of linked DRMs (Figure 3; Table 3). We identified linked DRMs in samples from 15 (26%) of the 58 women with mutations identified. Ten of the 15 samples had linkage of dual-class DRMs (linkage of an NVP resistance mutation with an FTC or TFV resistance mutation on the same genome), and 5 other samples had linkage of single-class DRMs, i.e. 2 or more NVP resistance mutations on the same genome. None of the 43 remaining samples had linked mutations.

The presence pre-ART of linked dual-class DRMs was significantly associated with ART failure: 8 of 27 (30%) ART failures had pre-ART linked dual-class DRMs vs. 2 of 34 (6%) non-failures (OR 6.7 [95% CI:1.3,35.1],  $p=0.013$  (Figure 3A). The association of dual-class linked mutations with failure remained significant after adjustment for the number of single genomes obtained per sample  $p=0.03$  (Supplemental Table 1), arguing against an influence of detection bias in the association. In addition, analyses of the ART failure and non-failure groups by stratifying NVP DRM frequency into quartiles revealed that dual-class linked mutations were not associated with NVP DRM frequency (Supplemental Figure 1A), confirming the data in Table 2. Furthermore, the proportion failing treatment stratified by quartile of NVP DRM frequency was higher with than without dual-class linked mutations, indicating that dual-class resistance was associated with a higher risk of treatment failure independent of NVP DRM frequency (Supplemental Figure 1B).

Linked single-class DRMs were not significantly associated with treatment outcome: 2 of 27 (7%) ART failures had linked single-class DRMs vs 3 of 34 (9%) non-failures (OR=0.83 [95%

CI:0.13,5.3]  $p=0.84$ ) (Figure 3B). Linked, single class DRMs always consisted of 2 NVP resistance mutations, most frequently the 190A or E mutation linked to 103N or 101E. Less frequently, mutations associated with codon 103 were linked to mutants associated with codons 101 and 181 (Table 3). The K65R tenofovir resistance mutation was never found linked to 184I/V FTC resistance mutations (Table 3). In addition, 3 DRMs from one or two drug classes were never found on the same viral genome (Table 3).

Of the 43 pre-treatment samples in which no linkage of DRMS was observed, 31 (72%) had DRMs to both NVP and FTC or NVP and TFV in the population; 10 in the ART failure group and 21 in the non-failure outcome group. There was no association of unlinked mutations with ART failure ( $p=0.649$ , calculated by Chi-square test with Yates correction). To further investigate if ART failure was associated with the pre-ART presence of unlinked dual class DRMs, we analyzed all pre-ART samples for the presence of mutations to 2 drug classes in different sequences (i.e., unlinked mutations). In the ART failure group, there were 20/27 (74%) samples with unlinked mutations to 2 drug classes, and 25/34 (74%) in the non-failure group ( $p=1.0$ ), indicating no association of unlinked dual-class resistance with treatment failure.

#### *Validation of rare frequency of DRMs linked on the same viral genome*

The frequencies within the virus populations in pre-ART plasma samples of both single- and dual-class linked DRMs were extremely low, averaging just 0.01% with a median of 0.007% and ranging from 0.002% to 0.04% (Table 3). Although our bioinformatics pipeline was designed to ensure that technical artifacts were eliminated from the dataset as reported previously (37), there remains a measurable assay error rate, attributable to the RT step. Because participants

had prior exposure to sdNVP resulting in high frequencies of resistant variants to this drug, we investigated if background errors in the uSGS technique could have resulted in spurious detection of dual-class resistant sequences. We addressed this possibility by comparing the number of linked resistance mutations expected as the result of assay error on a template already containing a NVP resistance mutation, to the observed number of linked mutations. In addition, because 184I was the most common mutation found in genomes with linked dual-class DRMs genomes and is a very common RT error in vitro and in vivo, we determined the assay error rate to be  $1.33 \times 10^{-4}$  at this position (ATG to ATA) from the experiment shown in Table 3 of our previous study (37). This error rate was used to estimate the number of linked resistance mutations expected as the result of an assay error on a template already containing an NNRTI resistance mutation (Supplemental Table 2). The number of linked dual-class DRMs observed in the ART failure group was significantly different than expected ( $p < 0.000001$ ; Binomial test) indicating that the linked dual-class mutants detected were highly unlikely to have arisen from assay error (Supplemental Table 2).

#### *Association between DRMS in pre-ART and DRMs at treatment failure*

Of the 8 participants experiencing treatment failure who had linked DRMs, 7 also had plasma population genotypes available at the time of ART failure (Table 3). Five of 7 (71%) (Table 3, boldface type) had virus at treatment failure with one or two of the DRMs that were found to be linked in the pre-ART plasma. In the other two donors, the linked, dual-class DRMs detected pretherapy were the dominant virus detected by population sequencing at treatment failure.

Donor T1F03 (Table 3) was one of a few participants that had plasma samples both from pre-ART and treatment failure available for uSGS. Plasma from this donor sampled at the time of

failure was used to validate the bioinformatics linkage pipeline in an ART failure clinical sample prior to the beginning of the present study. uSGS revealed many different genomes containing linked dual-class DRMs in a total of 2801 single genome sequences, 130 of which were 181C/184I at the time of failure (Supplemental Table 3). Of note, pre-ART plasma uSGS detected 2 genomes with linked dual-class 65R/181C DRMs and 1 genome with 184I/103N (Table 3). These findings make a strong case for the occurrence of in vivo recombination between genomes found pre-ART (65R/181C and 184I/103N) resulting in 4.6% of the genomes at ART failure having 181C/184I mutations. However, the majority of the sequences at failure (2599 genomes) contained 181C/184V indicating that once the M184V mutant appears, whether by mutation or recombination, it has a strong selective advantage over variants with M184I (38) . This example illustrates continued evolution and recombination of virus populations with ART-imposed selection. This finding may help to explain why there were inconsistent matches (2 of 8) between the linked dual-class resistant variants at pre-therapy and the dominant virus population at treatment failure.

## **Discussion**

Using powerful new uSGS technology, we have shown that, among women with prior exposure to a single partially suppressive antiretroviral (single dose nevirapine) to prevent mother-to-child HIV transmission, pre-treatment linked dual-class resistant variants can be detected at frequencies as low as 0.002% of viral genomes. Detection of such rare linked mutations has not been reported previously. Indeed, such rare linked mutations could not have been detected using previously available NGS technology, due to technological limitations,

particularly high background error and PCR recombination rates. Importantly, we found that dual drug class HIV-1 resistance mutations linked on the same viral genome are significantly associated with higher risk of failure of combination ART containing nevirapine. By contrast, we found that two single-class NNTRI resistance mutations linked on the same genome or unlinked mutations were not associated with ART failure.

These findings provide proof of concept that the risk of ART failure is higher in individuals with pre-treatment dual class resistance mutations linked on the same viral genome, even if the dual mutants comprise only a very small fraction of the virus population. It is universally accepted that therapy with a single antiretroviral drug can rapidly fail because of the outgrowth of drug resistant HIV-1 variants that exist in the virus population before therapy is started (39-41). Consequently, combination ART with two or three ARVs is needed to prevent the breakthrough of pre-existing resistant variants (40). In the context of combination ART, variants resistant to a single drug are unlikely to result in treatment failure. However, a single variant that is resistant to two drugs in the three-drug regimen is more likely to result in treatment failure, as was shown in the current study. These observations support the argument that these dual-class drug resistant variants continue to replicate after ART initiation and evolve through many cycles of mutation and recombination resulting in selection of the most fit variants for the conditions present at treatment failure.

We previously hypothesized that it is the context or clinical history in which these low-frequency DRMs are present that influences their effect on treatment outcomes (13). For example, as illustrated in figure 1, when the replicating population size of a drug resistant variant is large due to selection by prior drug treatment or by transmission of a resistant variant, there

is a greater chance of additional mutations to occur stochastically, becoming linked on the same viral genome. We validated this concept here by finding linked DRMs conferring resistance to either single or dual class inhibitors in women in trial 1 who had previously been exposed to sdNVP (i.e., positive selection for resistance); however, a limitation of this work is that sufficient numbers of comparable samples from women who had not received sdNVP were not available for testing. Validation of our hypothesis is also needed in an antiretroviral drug naïve population in which low frequency drug-resistant mutants likely arise but are also lost over time to stochastic processes. Results from the present study, however, imply that analysis of extremely large number of viral sequences, probably requiring impractically large blood volumes, would be required for such a study. Another limitation is that the study population is relatively small thus the confidence interval around the odds ratio for risk of treatment failure is broad. Finally, our results explain only a subset (30%) of virological failures. This result was not unexpected because it is likely that some participants had levels of linked DRMs below what we could detect, which nonetheless led to failure as well. In addition, other common causes of treatment failure, including medication non-adherence or suboptimal drug pharmacokinetics, could have contributed to an unfavorable outcome. Nevertheless, a significant association of treatment failure with linked, dual-class mutations was revealed. By contrast, variants with linked single-class resistance to NVP were not associated with failure of a NVP containing regimen likely because these variants would remain susceptible to both tenofovir and emtricitabine, the two other components of the regimen. It is not clear from our study why some women had detectable dual-class linked mutations, while others exhibited only single class linked mutations or unlinked mutations. Possibilities include variation in the size or persistence of the replicating nevirapine-

resistant virus population as a consequence of differences in nevirapine pharmacokinetics across women (31, 32, 42), or stochastic variation related to the occurrence of point mutations conferring resistance to tenofovir or emtricitabine on the same genome that already encodes a nevirapine resistance mutation.

One or more of all the major NNRTI resistance mutations (100I, 101E, 103NS, 106AM, 181CIV, 184IV, 188LCH and 190AE) were identified at low frequency in pre-ART samples by uSGS in more than 93% of the women tested (all of whom previously took sdNVP). Similarly, and consistent with other studies (15), in about 70% of participants, uSGS revealed low frequency variants having a single, major nucleoside/tide analog DRM (65R, 184IV) in pre-ART plasma samples. These findings are consistent with evolutionary theory that drug resistance mutations accumulate to a low level in replicating virus populations before the initiation of ART (39), thus making resistance ever-present despite counter-selective pressure. The current study confirms that if many single genomes are analyzed, low frequency unlinked DRMs can be found in most antiretroviral naive persons, which are most often not associated with virologic failure.

These new insights into the existence of rare linked and unlinked drug resistance mutations in ART-experienced individuals and their impact on treatment outcome have been enabled by uSGS technology that uses primer IDs to tag individual viral templates and a stringent bioinformatics pipeline that removes sequencing errors and PCR-related artifacts, including contaminants, recombinants, and template resampling (37). However, our study was limited to resistance to nevirapine containing ART, which has since been replaced by more convenient, safe and effective ART regimens; the specific associations observed in the current study may not apply to newer combinations of antiretrovirals which may require more than one mutation to confer

resistance to each drug. Nevertheless, the principles derived from the current study are likely to apply to all regimens, including the presence of rare drug-resistant variants to most antiretrovirals before therapy is started and the importance of such preexisting linked dual- or multi-class drug resistance to failure of combination ART. They also reinforce the principle that treatment history, along with resistance testing, is essential to guide the choice of ARV regimen. Of the 27 participants who failed ART in this study, only 5 had preexisting NVP resistance at levels (> ca 10% of genomes) that would have been detectable by standard clinical resistance assays.

Overall, our study shows that linked, dual class DRMs are significantly associated with ART failure, whereas low frequency unlinked resistance to NVP or a single NRTI alone is not, helping to explain why studies that detect resistance to a single ARV can draw different conclusions about the association between low frequency drug-resistant variants and ART failure. We propose that it is not single, low-frequency drug-resistant variants that are clinically relevant, but rather, rare dual-class resistant variants that predispose to ART failure.

## Methods

### *Study Design and Sample Testing:*

The AIDS Clinical Trials Group A5208/OCTANE trials have been reported in detail by Lockman et al. (35, 36). In brief, A5208 enrolled African women who self-reported having received sdNVP during labor for prevention of mother to child transmission, 73% of whom had written documentation of sdNVP receipt (Figure 2) (33). Baseline plasma samples were collected, and the women were randomized to receive cART of either NVP/TFV/FTC or LPV/r/TFV/FTC.

The current study aimed to compare the number of single-genome sequences with linked single and dual class DRMs in entry plasma samples from the NVP/TFV/FTC treatment arm, including samples from women who did (cases) and did not meet protocol definitions of ART failure (controls). All the available pre-ART samples were tested from participants with ART failure. In addition, a similar number of pre-ART samples were tested from participants who did not experience ART failure. All samples were selected based on availability and plasma HIV-1 RNA  $\leq$  or  $>100,000$  copies/ml, the latter to ensure deep representation of variants.

### *uSGS Testing:*

Viral RNA was extracted from baseline plasma samples and cDNA libraries were generated with primer IDs for next-generation uSGS as previously described (37). Overall, the efficiency of cDNA synthesis ranges between 20% and 30% of the viral population. The proportions of samples with plasma HIV RNA  $>100,000$  copies/ml were balanced between the failure on non-failure groups for this study.

In brief, the primer IDs uniquely tag each cDNA molecule which are then amplified with primers containing deoxyuridine (dU) residues. These residues are subsequently removed leaving long, single-stranded overhangs available for efficient directional ligation of Illumina adaptor sequences and library construction. Libraries were sequenced using paired-end MiSeq next generation sequencing (NGS) technology, and raw sequencing reads were processed through an analytical pipeline where sequence reads from samples were separated according to their indexes and binned by common primer IDs (37). A bioinformatics pipeline was designed to ensure that technical artifacts were eliminated from the dataset. A single genome sequence had to be derived from a set of common primer IDs that satisfied the “supermajorities rule” of >80% consensus at each nucleotide position to be included in the dataset. After generating the supermajority sequences from the alignments of reads sharing common primer IDs and applying the consensus cutoff model, the PCR/sequencing error rates were reduced to ca  $10^{-4}$ , an error rate comparable to that of the RT step (37). This stringent bioinformatics program eliminated all sequences that had undergone PCR recombination, sequence or PCR error and where the primer IDs had been mutated (43). Hundreds to thousands of unique single-genome sequences covering amino acids 60 thru 200 in the reverse transcriptase (RT) gene were generated from each sample allowing for the detection of rare variants and characterization of the population genetics of the parent HIV-1 RNA in plasma.

#### *Data Analysis:*

Fastq files containing raw reads were exported for bioinformatics analyses. Paired-end reads were concatenated, and low-quality reads were removed using a program available at

[http://hannonlab.cshl.edu/fastx\\_toolkit](http://hannonlab.cshl.edu/fastx_toolkit) with parameters set to base call qualities of  $\geq 90\%$  at Q20 or above (Q20–P90), and default for all other settings. The filtered fastq sequences were then converted to fasta format and the fasta reads were sorted by indexes using programs available on the same website. Qualified Primer IDs were identified and sorted as described (43). Reads with identical primer IDs were aligned and the percent consensus determined at each nucleotide. Individual Primer ID sequence alignments with  $\geq 80\%$  identity at each nucleotide were included for subsequent analyses. The individual genomes were then analyzed for frequencies of the most common clinically significant NNRTI and NRTI resistant mutations to 1 or more of the inhibitors in the treatment arm of NVP/TFV/FTC, as determined by the Stanford HIV Drug Resistance Database, (<https://hivdb.stanford.edu/>). The mutations include: 65R, 100I, 101PE, 103NS, 106AM, 181CIV, 184IV, 188LCH and 190ASE. Genomes containing more than one of these mutations were categorized as linked. Allele frequencies of linked and unlinked mutations were calculated across all consensus sequences generated from each plasma sample. Perl scripts used in the above analyses are available at the GitHub code repository at [https://github.com/ShaoFred/MiSeq\\_consensus\\_builder.git](https://github.com/ShaoFred/MiSeq_consensus_builder.git). The sequence dataset (approximately 1.5 million consensus sequences) was subjected to a bioinformatics test to determine cross-contamination between patients where each sequence from a sample was compared to sequences from all other samples. All sequences are available on the National Cancer Institute HIV Dynamics and Replication Program's website found at <https://home.ncifcrf.gov/hivdrp/resources.html>.

### *Statistical Analyses:*

To check for bias in the sampling, the Mann-Whitney test was used to determine if there were significant differences among median and interquartile numbers of uSGS obtained for each sample when comparing outcome groups. This test generated the p-values for differences in median frequencies in each resistance category. In addition, logistic regression was used to investigate if the frequency of NVP resistance was associated with failure in either group. Logistic regression tests were also used to compute p values when comparing associations between linkage of DRMs and failure as well as when adjusting for the number of genomes per sample in each outcome group. All summary statistics were performed using R statistical language and p values of less than 0.05 were considered significant. When computing associations of unlinked mutations with ART failure Chi-squared test was used. A Binomial test was used to compare the observed frequency of linked, dual-class DRMs to that expected from the measured assay error (ATG to ATA at RT codon 184).

### *Study Approval*

All participants provided written informed consent to participate in the study. Testing of samples by uSGS was approved by the National Institutes of Health Office of Human Subjects Protection. Clinical Trials Registration. NCT00089505.

### *Author contributions*

VFB conducted the experiments, acquired and analyzed the data and wrote the manuscript. MJB, BL and MDH calculated the statistics. WS and MJB analyzed the data and EH provided the reagents and conducted the population genotyping. JAM, RTS, SL, JSC and FS designed the A5208 OCTANE Trial research studies, MDH analyzed the data and wrote the manuscript. MFK and JMC contributed to the study design and wrote the manuscript. JWM designed the research studies and wrote the manuscript.

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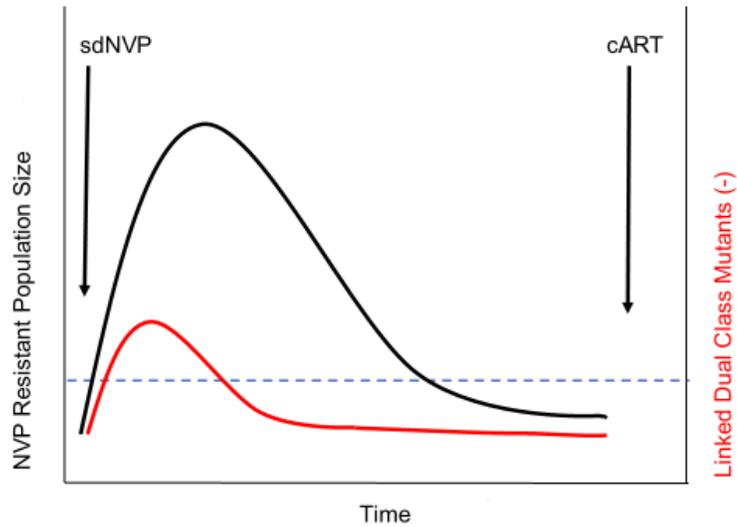
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Figure 1.



**Figure 1. Hypothetical Model Explaining Selection of Linked Mutations Among Women**

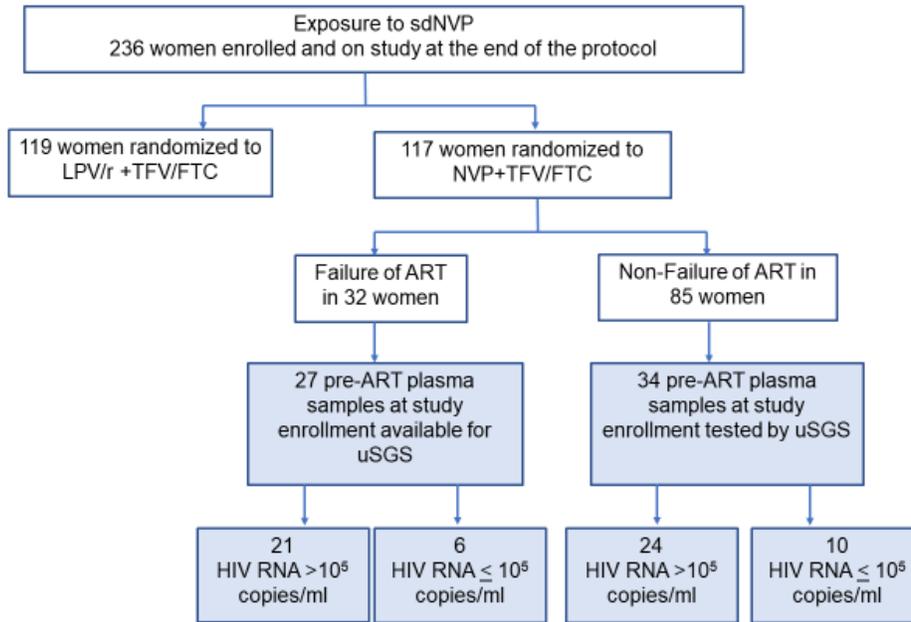
**Exposed to Single Dose Nevirapine.** The large replicating population of resistant variants

selected by single dose NVP is depicted in black. Linkage of dual-class resistance mutations,

occurring at that time either stochastically or through recombination, is shown in red. The blue

dashed line is the limit of detection of standard single genome sequencing.

Figure 2.



**Figure 2. Consort Diagram and sample selection from the NVP arm of A5208/OCTANE Trial 1, in women with prior sdNVP exposure.** The blue shaded boxes show plasma samples obtained from women at study entry randomized to the NVP/TFV/FTC arm and who were selected for analysis by ultra-sensitive single genome sequencing (uSGS) n=61, 27 ART failures and 34 non-ART failures. Samples were selected based on availability and plasma HIV RNA above or below 100,000 copies/ml. Samples from a given participant with plasma HIV RNA > 100,000 copies/ml were selected when available to increase the depth of representation of the viral population.

Figure 3A.

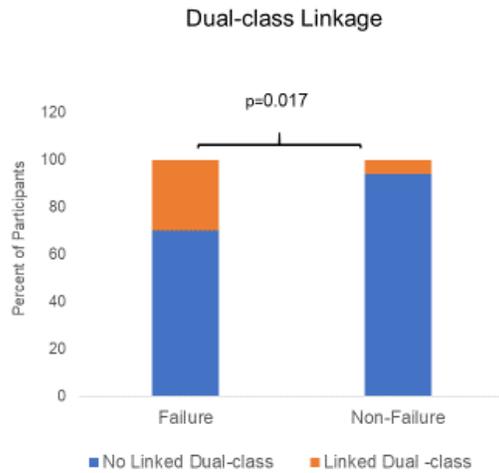
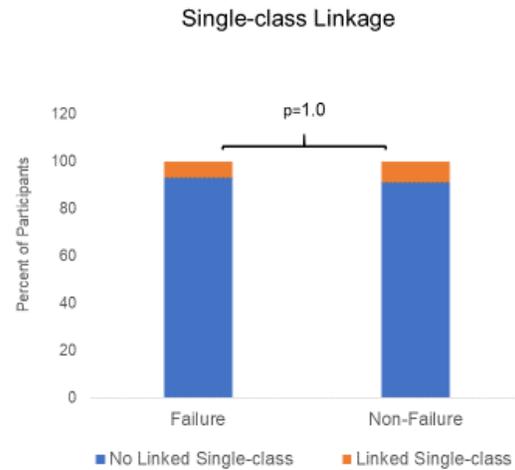


Figure 3B.



**Figure 3. Association of dual-class but not single-class linked drug resistance mutations with treatment failure.** (A) Shown are the proportions of participants with linked dual-class resistant mutants in pre-therapy plasma samples according to treatment outcome: 8/27 failure, 2/34 nonfailure. Linked dual-class resistance mutations were significantly associated with treatment failure: OR 6.7 [95% CI: 1.3-35.1];  $p = 0.013$  (logistic regression). (B) The proportions of participants with linked single-class resistant mutants in pre-therapy plasma samples according to treatment outcome were 2/27 failure and 3/34 non-failure OR=0.83, [95% CI: 0.13 to 5.3]  $p=0.841$  (logistic regression).

Table 1. Tabulation of ultrasensitive single-genome sequences from pre-ART plasma samples from 61 participants of AIDS Clinical Trials Group A5208/OCTANE receiving single-dose nevirapine.

| Number participants studied | Prior sdNVP | cART Outcome (N)  | Median # of single genomes obtained per sample (IQR) |
|-----------------------------|-------------|-------------------|------------------------------------------------------|
| 61                          | Yes         | Failures (27)     | 6,165 (3014, 18350)                                  |
|                             |             | Non-failures (34) | 4,071 (2093, 13977)                                  |

Sampling between groups was not significantly different by the Mann-Whitney test ( $p=0.52$ )

Table 2. Frequency of pre-treatment resistance mutations detected categorized by treatment outcome

| Resistance To:    | Outcome (N)      | Participants with pre-ART Mutations (%) | Median Mutant Frequencies | p value <sup>A</sup> | Range        | Q1, Q3         |
|-------------------|------------------|-----------------------------------------|---------------------------|----------------------|--------------|----------------|
| NVP or FTC or TFV | Failure (27)     | 26/27 (96%)                             | 0.73%                     | 0.41                 | 0.008%-71%   | 0.19%, 6.91%   |
|                   | Non-Failure (34) | 32/34 (94%)                             | 0.44%                     |                      | 0.008%-100%  | 0.18%, 3.42%   |
| NVP               | Failure          | 25/27 (93%)                             | 0.73%                     | 0.62                 | 0.008%-71%   | 0.12%, 6.83%   |
|                   | Non-Failure      | 32/34 (94%)                             | 0.41%                     |                      | 0.008%-100%  | 0.12%, 3.40%   |
| FTC               | Failure          | 21/27 (78%)                             | 0.05%                     | 0.34                 | 0.008%-0.73% | 0.02%, 0.07%   |
|                   | Non-Failure      | 25/34 (74%)                             | 0.04%                     |                      | 0.008%-0.27% | 0.008%, 0.06%  |
| TFV               | Failure          | 14/27 (52%)                             | 0.008%                    | 0.38                 | 0.002%-0.04% | 0.008%, 0.009% |
|                   | Non-Failure      | 13/34 (38%)                             | 0.008%                    |                      | 0.002%-0.05% | 0.008%, 0.009% |

<sup>A</sup>Mann-Whitney test

Table 3. Linked dual-and single-class resistance mutations detected in pre-ART samples from 8 donors with ART failure

| PID   | Total # genomes obtained | Pre-ART linked mutations conferring dual class resistance (% frequency) | Pre-ART linked mutations conferring single class resistance (% frequency) | Population genotype at failure |
|-------|--------------------------|-------------------------------------------------------------------------|---------------------------------------------------------------------------|--------------------------------|
| T1F03 | 4687                     | 65R/ <b>181C</b> <sup>A</sup> (0.04),<br>184I/103N (0.02)               | 101E/103N(0.02),<br>103N/190E (0.02)                                      | <b>181C,184V</b>               |
| T1F04 | 13747                    | 184I/190E (0.007)                                                       | none                                                                      | 65R,103N,181C,184V             |
| T1F07 | 31531                    | 184I/ <b>103N</b> (0.003),<br><b>184V/103N</b> (0.003)                  | <b>103N/181C</b> (0.003),<br>103N/190A (0.006)                            | <b>103N,181C,184V</b>          |
| T1F08 | 27132                    | <b>184I/103N</b> (0.004)                                                | none                                                                      | <b>184I, 103N</b> ,106M        |
| T1F18 | 12692                    | 65R/103N (0.008)                                                        | none                                                                      | Not available                  |
| T1F23 | 12155                    | 184I/ <b>103N</b> (0.008),<br>184I/190E (0.008)                         | none                                                                      | <b>103N</b>                    |
| T1F44 | 44124                    | 184I/ <b>103N</b> / (0.002),<br>184I/190E (0.005)                       | none                                                                      | 65R, <b>103N</b> ,181C         |
| T1F47 | 57278                    | 184I/190E (0.002)                                                       | none                                                                      | 181C,184V                      |

<sup>A</sup>**Bold face** indicates the same mutations detected pre-ART and at treatment failure. ***Italicized bold face*** indicates matches between dual class mutations in samples from pre-ART and at treatment failure.