

Paired HIV-1 RNA and Proviral DNA Next-Generation Genotyping in ART-Naïve People with HIV: A Pilot Study of Low-Frequency Drug Resistance Mutations

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Abstract

Background

Next-generation sequencing (NGS) and proviral DNA genotyping can detect low-frequency HIV-1 drug resistance mutations (DRMs) that may be missed by conventional Sanger sequencing, but their clinical interpretation remains uncertain. We characterized baseline paired plasma HIV-1 RNA and peripheral blood mononuclear cell (PBMC) DNA NGS genotypes in antiretroviral therapy (ART)-naïve people with HIV.

Methods

Thirty-five participants with no prior preexposure prophylaxis or ART exposure underwent paired baseline plasma RNA and PBMC DNA genotyping. Sequencing was performed using the Ion Torrent PGM platform, and sequences were analyzed with Geneious and the Stanford HIV Drug Resistance Database. DRMs were characterized by specimen type, antiretroviral class, variant frequency, and potential APOBEC association.

Results

A total of 264 DRMs were detected at a variant frequency of $\geq 1\%$; 82 (31%) were detected in RNA and 182 (69%) in DNA. Overall, 185 (70%) DRMs occurred at $< 5\%$ variant frequency, including 65/82 (79%) RNA and 120/182 (66%) DNA DRMs. NRTI and INSTI DRMs were most frequent. Multiclass DRM patterns decreased substantially when the detection threshold was increased from $\geq 1\%$ to $\geq 5\%$.

Conclusions

Paired NGS identified substantially more DRMs in proviral DNA than plasma RNA, with most variants occurring at low frequency. Interpretation of resistance profiles was highly dependent on specimen type and detection threshold, underscoring uncertainty regarding the clinical significance of low-frequency and potentially APOBEC-associated DRMs.

Background

Genotypic antiretroviral resistance testing has relied on Sanger sequencing of plasma HIV-1 RNA and has performed reliably for more than a quarter century.¹ Next generation sequencing (NGS) has gained more attention due to its greater sensitivity and potential cost savings.^{2,3} In November 2019, the US Food and Drug Administration approved the first NGS genotyping assay.⁴ While Sanger sequencing has served as the clinical standard, it typically fails to detect mutations in variants present in less than 20%

of the viral population, although the range of sensitivity varies;³ therefore, the assay may fail to detect important drug resistance mutations (DRMs) leading to virologic failure.^{3, 5-9} NGS can detect low-abundance DRMs present at less than 1%, but is confounded by amplification and sequencing error, increased detection of APOBEC-mediated hypermutation (APO), and unclear clinically relevant mutational frequency or burden cutoff thresholds, creating an interpretive dilemma.^{5, 10, 11}

This conundrum is made more complex with the introduction of peripheral blood mononuclear cells (PBMC)-derived HIV-1 DNA genotyping, which is typically performed when plasma HIV-1 RNA is too low to sequence or not detected.^{12, 13} HIV-1 DNA provides a glimpse into the evolutionary history of HIV-1 in a given individual and may persist for years as integrated provirus in latently infected cells.^{13, 14} Although there are clinical scenarios of benefit,¹⁵ the majority of PBMC virus is considered replication incompetent in part due to contribution of APOBEC-mediated DRMs.¹⁶ Additionally, sampling bias may limit the reproducibility and reliability of proviral mutation interpretation.¹⁷ Further, the clinical utility of proviral genotyping has not been established in a definitive clinical trial.^{13, 18} Thus, NGS genotyping, depending on the extent of filtering, can present clinicians with a myriad of DRMs of uncertain significance and confound the choice of a durable ART regimen.

We are conducting a longitudinal study, performed in the context of clinical care, examining serial paired plasma RNA and PBMC DNA NGS susceptibility testing specimens, among antiretroviral therapy (ART)-naive people with HIV (PWH), prior to the initiation of ART. The purpose is to determine the impact of DNA DRMs on NGS interpretation based on the Stanford University HIV Drug Resistance Database sequence analysis tool.¹⁹ In this initial analysis, we characterized baseline paired plasma HIV-1 RNA and PBMC DNA DRMs detected by NGS, compared the resulting resistance profiles across variant-frequency thresholds, and examined the potential clinical implications of these interpretive differences.

Methods

Design and Setting

This parent pilot study was designed to explore the role that proviral DNA genotyping might play in the evolution of drug resistance and prediction of treatment failure. Thirty-five preexposure prophylaxis/ART-naive PWH who presented to Henry Ford Health, a Detroit Ryan White funded inner-city HIV clinic, and who were ready to start ART agreed to participate in this study and signed informed consent. These individuals had baseline blood obtained for both RNA and DNA genotyping. Subsequently, additional samples were collected every 4 months for 48 weeks (and upon recurrent viremia, if observed) for repeat RNA and DNA genotyping. RNA samples were utilized for real-time clinical monitoring while paired additional RNA and DNA samples were reserved for batched sequencing assays for this study. Care providers-initiated ART based on standard-of-care considerations, including review of real-time genotypic resistance testing. Only those individuals with paired baseline RNA and DNA study genotypes were

included in these analyses. The laboratory has approximately two decades of experience with clinically targeted PBMC-extracted HIV-1 DNA genotyping.²⁰

Sample Collection and Processing

Peripheral blood specimens were collected in either plasma preparation EDTA blood tubes (BD Biosciences PPT, Franklin Lakes, NJ) for RNA or mononuclear cell preparation sodium citrate blood tubes (BD Biosciences CPT) for DNA. PPT tubes were centrifuged at 1500xg for 15 minutes within 2 to 6 hours of blood collection. CPT tubes were centrifuged at 1500xg for 15 minutes within 2 hours of blood collection. All blood tubes were frozen within 24 hours at -80°C until nucleic acid extraction. Prior to extraction, plasma was centrifuged at 23,000 x g for 60 minutes at 4°C. RNA (200 uL plasma) and DNA (200 uL buffy coat) were extracted from batched specimens using either the Qiagen Viral RNA kit or DNeasy Blood and Tissue kit (Qiagen, Germantown, MD). Nucleic acid samples were stored at -80°C until sequencing was performed.

Genotyping

Sequencing was performed using the Ion Torrent PGM System (Thermo Fisher Scientific, Detroit, MI). Geneious 2019.2.3 for Mac was used for bioinformatic sequence analysis. This system uses semiconductor sequencing to detect hydrogen ions released during nucleotide incorporation into growing DNA strands, enabling millions of sequences to be read in parallel.²¹ Generated sequences were aligned to the HIV-1 HXB2 reference genome (GenBank accession NC_001802.1).²² Automated mutation-analysis results, following manual review and verification of reads at Q20 and a depth of 500x, were entered into Stanford HIVdb version 9.4 to determine inferred levels of resistance to commonly used antiretroviral agents.¹⁹ DRMs were categorized by affected antiretroviral class: protease inhibitor (PI), nucleoside/nucleotide reverse transcriptase inhibitor (NRTI), non-nucleoside reverse transcriptase inhibitor (NNRTI), and integrase inhibitor (INSTI). DRMs were also grouped by variant frequency: 1% to <5%, 5% to <10%, and ≥10%. DRMs associated with APOBEC hypermutation were also noted.

APOBEC-associated DRMs defined by Stanford HIVdb version 9.4 are listed in Table 1.

Data Analysis

Analyses were descriptive. Continuous variables were summarized using means and standard deviations, and categorical variables using frequencies and percentages. DRMs were summarized by specimen type, antiretroviral drug class, variant-frequency category, and potential APOBEC association. Because this was an exploratory pilot study, no formal power calculation or inferential hypothesis testing was performed. Clinical expertise was used to examine the resulting categorizations and make inferences. Data were stored on a password-protected, HIPAA-compliant computer. Study procedures were conducted in accordance with applicable institutional and national ethical standards and the Declaration of Helsinki (protocol #14219). ChatGPT (OpenAI) was used to assist with manuscript

organization and language refinement. All scientific content, analyses, interpretations, and final wording were reviewed and approved by the authors.

Results

Demographics

From 6/1/2021-12/1/2024, 35 PWH who had never received ART were enrolled in this study. Their mean age was 37 years; 83% of participants were male and 80% were Black; mean baseline viral load was 553,000 copies/mL and mean initial CD4 count was 376 cells/mm³ (Table 2).

Description of HIV-1 RNA and DNA DRMs

Among 264 total DRMs detected at a variant frequency of $\geq 1\%$, 82 (31%) were detected in RNA and 182 (69%) in DNA. Of the RNA DRMs, 18/82 (22%) were potentially APOBEC-associated, compared with 64/182 (35%) of DNA DRMs.

Table 3 illustrates the mean number of mutations per patient by drug class and specimen type. A greater number of DRMs were detected in DNA than in RNA, and NRTI and INSTI DRMs were more common than NNRTI and PI DRMs in both specimen types. Only two participants had no DRMs in either RNA or DNA, five had DRMs but no APOBEC-associated DRMs, and three had APOBEC-associated DRMs only.

Overall, 185/264 (70%) DRMs occurred at a variant frequency of $< 5\%$, including 65/82 (79%) RNA DRMs and 120/182 (66%) DNA DRMs. NRTI DRMs were most frequent (31 in RNA and 55 in DNA), of which 26/31 (84%) and 40/55 (73%), respectively, occurred at $< 5\%$. This was followed by INSTI DRMs (26 in RNA and 51 in DNA), of which 20/26 (77%) and 32/51 (63%), respectively, occurred at $< 5\%$.

Absolute Frequency of Mutations by Drug Class

We explored hypothetical genotypic interpretive scenarios based on the criteria used to include or exclude mutations in susceptibility reports. Table 4 summarizes the distribution of DRMs (N=264) observed in RNA and DNA. The table indicates the absence or presence of at least one DRM (regardless of Stanford penalty score) affecting at least one antiretroviral agent in each class (PI, NRTI, NNRTI, and INSTI). DRMs were less prevalent in RNA than in DNA, and prevalence decreased as the detection threshold increased. At an RNA sequencing threshold of $\geq 1\%$, 20% of participants had DRMs affecting three or four drug classes, compared with none at a $\geq 5\%$ cutoff. At a $\geq 1\%$ variant-frequency threshold for DNA, 69% of participants had DRMs affecting three or four drug classes, compared with 17% at $\geq 5\%$. A four-class DRM pattern was the most common pattern detected by proviral DNA (n=13) and combined DNA/RNA analysis (n=16). Increasing the detection threshold to $\geq 5\%$ and eliminating DNA markedly reduced multiclass DRM patterns, demonstrating that these patterns were largely driven by low-frequency ($< 5\%$) DRMs, which were more commonly detected in DNA.

Discussion

While HIV Sanger genotyping has allowed clinicians to reliably select effective and suppressive ART, it lacks sensitivity, as revealed by the expanded detection of relevant DRMs from NGS and DNA sequencing assays.^{5, 6} Conversely, NGS assay results are accompanied by increased uncertainty in the interpretation of low-abundance DRMs and APO DRMs, which may lead to an over- or underestimation of ART resistance. This study highlights these interpretive dilemmas.

This pilot study found that in ART-naïve PWH, DRMs were commonly detected against NRTIs, INSTIs, NNRTIs, and PIs, in descending order, after NGS genotyping of plasma HIV-1 RNA and PBMC-extracted HIV-1 DNA. Interestingly, most DRMs were detected among NRTIs and INSTIs, the most used drug classes in our clinic for many years. Whether this pattern reflects transmitted resistance, biological differences between viral compartments, APOBEC-associated variation, or assay characteristics cannot be determined from this study. The greater detection of DRMs in DNA compared to RNA is consistent with previous comparative studies.²⁴

Given the prevalence of DRMs, approximately one per person per drug class, the selection of an appropriate initial ART regimen is less straightforward and depends on the criteria used to interpret the genotypes. The most straightforward interpretation uses RNA results present at $\geq 5\%$, excluding APOBEC-associated DRMs, which were concentrated at lower variant frequencies. While 94% of participants in this study were able to initiate INSTI+NRTI-based therapy, the question remains whether low-abundance DRMs found in RNA or DNA will affect the longevity of the ART regimen, predict treatment failure, or contribute to further transmitted drug resistance. During 48 weeks of clinical follow-up, no participant had HIV-1 RNA >50 copies/mL or discontinued or switched ART because of adverse effects.

Low-abundance resistant HIV-1 RNA variants have been shown to be clinically important, particularly with NNRTIs. In a pooled analysis of 985 participants from 10 studies, low-frequency DRMs were associated with a two- to threefold increased risk of virologic failure.^{25, 26} The highest risk was associated with NNRTIs and increased with variant copy number. The risk associated with low-frequency DRMs was observed among participants with both $\geq 95\%$ and $<95\%$ medication adherence, compared with those without low-frequency drug-resistant variants and $\geq 95\%$ adherence. In a case-control study of efavirenz-based ART, low-frequency DRMs were found in 7/95 (7%) treatment failures compared with 2/221 (0.9%) treatment successes.²⁷

Milne and colleagues also found that low-frequency (1%–9%) DRMs were associated with treatment failure of an efavirenz-based regimen.²⁸ In another study, 13/22 participants starting efavirenz/tenofovir disoproxil fumarate/lamivudine with DRMs detected at $\leq 1\%$ experienced treatment failure. However, the study design, small sample size, and lack of viral samples at treatment failure limit attribution of these failures to the detected DRMs.²⁹ In these studies, first-generation NNRTIs, or regimens with lower barriers to resistance, were most strongly implicated in treatment failure associated with plasma RNA drug-resistant variants. The role of DRMs in other ART classes is less clear.

The clinical significance of circulating PBMC DNA genotyping remains incompletely defined. DNA genotyping has demonstrated utility in specific clinical scenarios, such as evaluating archived resistance before switching from an oral regimen to long-acting injectable cabotegravir/rilpivirine.^{15,30} Interpretation of PBMC DNA results is challenging because more than 97% of intracellular HIV proviruses have been shown to have fatal defects.³¹ Therefore, DRMs may originate from non-replicative or less efficient replicative virus.^{30,31} As a result, DNA genotyping is believed to overestimate resistance.

Emerging evidence suggests that at least a subset of proviral DRMs detected in PBMC DNA are clinically relevant.¹⁸ In one study of 12 PBMC DNA samples from 11 virologically suppressed individuals, 70 DRMs spanning PI, NRTI, NNRTI, and INSTI resistance were identified; 47 (67%) were subsequently recovered in replication-competent virus using quantitative viral outgrowth assays.³⁶ These findings suggest that at least some DRMs identified by proviral DNA genotyping may be present in replication-competent virus and therefore should not be dismissed solely because they are detected in proviral DNA.

While antiretroviral resistance surveillance studies in the United States have not shown the level of DRMs observed in this study,^{32,33} many comparative studies of Sanger sequencing and NGS have reported similar findings,^{6,12,13,20,34} although the distribution of DNA and RNA DRMs by drug class in Sanger-based studies differed from our findings.¹³ The extent to which low-abundance mutations in DNA and RNA, as well as APOBEC-mediated hypermutation, may foreshadow HIV evolution and treatment longevity remains unresolved. Inzaule et al. evaluated diagnostic accuracy at different detection thresholds in a case-control study to predict virologic failure. They found that ultrasensitive resistance testing increased identification of individuals at risk for virologic failure, but at thresholds <1% there was potential for misclassification and no optimal detection threshold could be identified.³⁵ Therefore, the clinical relevance of these low-abundance DRMs would best be established in a formal clinical trial. DRMs were common in NRTIs and INSTIs, which are widely used to treat PWH. Although most DRMs occurred at <5% variant frequency and a substantial minority were potentially APOBEC-associated, the long-term clinical implications of multidrug low-abundance DRMs remain unresolved. At a frequency of approximately one DRM per patient per drug class, these findings may make drug selection less straightforward.

There are several limitations to this study. Residual DNA contamination in RNA specimens is possible, although this risk was minimized through the use of plasma preparation tubes, timely centrifugation, storage at -80°C, and additional high-speed centrifugation for plasma clarification before viral RNA extraction. The sample size was small and the analysis included only baseline DRMs. Because the quantity of HIV-1 DNA extracted from PBMCs in a typical blood draw is limited, sampling may contribute to poor reproducibility or inconsistent results.³⁰ NGS can increase DRM detection by identifying low-frequency variants, but this advantage is counterbalanced by amplification and sequencing error and the uncertain significance of some low-frequency DRMs.³⁰ Very low-frequency variant calls may therefore be vulnerable to sequencing and amplification error and should be interpreted cautiously, particularly near the analytical limits of the platform.²⁰ Given the limited number of intracellular HIV-1 copies, DNA

genotyping is also subject to selection bias.¹⁷ The mean viral load of participants exceeded 550,000 copies/mL, and participants generally had high levels of RNA mutations; however, we did not calculate RNA mutational load for this report. This decision was partly due to the challenge of aligning RNA mutational load with an accurately computed DNA mutational load that reflects active resistance or transmission potential because defective hypermutated sequences were not bioinformatically removed. Finally, we did not analyze specific DRMs in this pilot study; however, we plan to report their clinical impact in a follow-up analysis.

Clinical experience, notably with codon 184V/I mutations, shows that some DRMs in appropriately constructed ART regimens have not led to treatment failure after several years of observation.³⁷ Yet there are examples in which even a low-abundance resistance mutation has been associated with recurrent viremia during therapy.^{9, 16} Large population-based and rigorously developed interpretive databases provide useful guidance for clinicians confronted with the wealth of information generated by NGS-based genotypic resistance testing.^{19, 23} For an individual patient, however, all DRMs should be considered, even if they do not necessarily prompt a change in therapy, because pathways may exist for these mutations to become clinically relevant. Given the success of conventional Sanger RNA genotyping in selecting initial ART regimens, the incremental value of identifying low-abundance DRMs and using DNA sequencing requires further study.

Conclusions

Paired NGS of plasma HIV-1 RNA and PBMC proviral DNA in ART-naïve people with HIV identified numerous DRMs, most of which occurred at low variant frequencies and were more commonly detected in DNA. Apparent multiclass DRM patterns varied substantially according to specimen type and detection threshold, highlighting the interpretive challenges associated with low-frequency and potentially APOBEC-associated variants. The clinical significance of these variants, particularly those detected in proviral DNA, requires further study before they are routinely used to guide initial ART selection.

Abbreviations

APO/APOBEC: apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like

ART: antiretroviral therapy

DRM: drug resistance mutation

INSTI: integrase strand transfer inhibitor

NGS: next-generation sequencing

NNRTI: non-nucleoside reverse transcriptase inhibitor

NRTI: nucleoside/nucleotide reverse transcriptase inhibitor

PBMC: peripheral blood mononuclear cell

PI: protease inhibitor

PWH: people with HIV

Declarations

Ethics approval and consent to participate

This study was approved by the Henry Ford Health Institutional Review Board (protocol #14219). All participants provided written informed consent prior to enrollment. All study procedures were conducted in accordance with applicable institutional and national ethical standards and the Declaration of Helsinki.

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to participant privacy and confidentiality considerations but may be available from the corresponding author upon reasonable request and subject to applicable institutional requirements.

Competing interests

JCL is an employee of Gilead Sciences and owns stock in Gilead Sciences. SG, DB, JJ, and NM declare that they have no competing interests.

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Authors' contributions

SG conducted the study and drafted the manuscript. DB contributed to data analysis and was a major contributor to manuscript preparation and revision. JJ contributed to the statistical analysis. JCL contributed to manuscript preparation and revision and served as the liaison with Gilead Sciences. NM conducted the study and contributed to manuscript preparation and revision. All authors reviewed and approved the final manuscript.

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Tables

Table 1. APOBEC-associated drug resistance mutations defined by Stanford HIVdb version 9.4

Protease APOBEC mutations	30N, 46I, 48S, 73S
Reverse transcriptase APOBEC mutations	67N, 138K, 184I, 190E, 190S, 230I,
Integrase APOBEC mutations	118R, 138K, 140R, 140S, 163K, 163R, 232N, 263K

Table 2. Baseline demographics

Demographics	N=35
Age, mean $\pm$ SD (years)	37.3 $\pm$ 13.9
Sex, n (%)	
Female	6 (17.1)
Male	29 (82.9)
Race/Ethnicity, n (%)	
Arab	1 (2.9)
Black	28 (80)
Hispanic	4 (11.4)
White	2 (5.7)
Treatment, n (%)	
INSTI + NRTI	33 (94.3)
PI + NRTI	2 (5.7)
Baseline viral load, mean $\pm$ SD (copies/mL)	553,051 $\pm$ 1,843,541
Initial CD4 cell count, mean $\pm$ SD (cells/mm³)	376 $\pm$ 310

INSTI, integrase inhibitor; NRTI, nucleoside/nucleotide reverse transcriptase inhibitor; PI, protease inhibitor; SD, standard deviation.

Table 3. Number of DRMs per patient by drug class and specimen type

Sample Type	PI	NRTI	NNRTI	INSTI
RNA, mean $\pm$ SD	0.2 $\pm$ 0.53	0.89 $\pm$ 1.16	0.51 $\pm$ 0.74	0.74 $\pm$ 0.82
DNA, mean $\pm$ SD	1.09 $\pm$ 1.5	1.57 $\pm$ 1.88	1.09 $\pm$ 1.15	1.46 $\pm$ 1.24

INSTI, integrase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside/nucleotide reverse transcriptase inhibitor; PI, protease inhibitor; SD, standard deviation.

Table 4. DRM patterns by specimen type and variant-frequency threshold

Drug Class	RNA ≥1%	RNA≥5%	DNA≥1%	DNA≥5%	DNA+RNA ≥1%	DNA+RNA ≥5%
No DRM detected	7	22	4	12	2	9
PI	0	1	0	2	0	2
NRTI	2	2	1	1	1	3
NNRTI	1	4	1	4	0	4
INSTI	7	3	2	3	2	4
PI+NRTI	0	1	0	1	0	1
PI+NNRTI	0	0	0	1	0	1
PI+INSTI	1	0	0	1	1	1
NRTI+NNRTI	6	0	1	1	2	0
NRTI+INSTI	4	2	2	3	3	3
NNRTI+INSTI	0	0	0	0	0	0
PI+NRTI+NNRTI	0	0	1	2	1	3
PI+NRTI+INSTI	0	0	5	1	3	1
PI+NNRTI+INSTI	1	0	1	0	1	0
NRTI+NNRTI+INSTI	3	0	4	3	3	3
PI+NRTI+NNRTI+INSTI	3	0	13	0	16	0

INSTI, integrase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside/nucleotide reverse transcriptase inhibitor; PI, protease inhibitor.

Figures

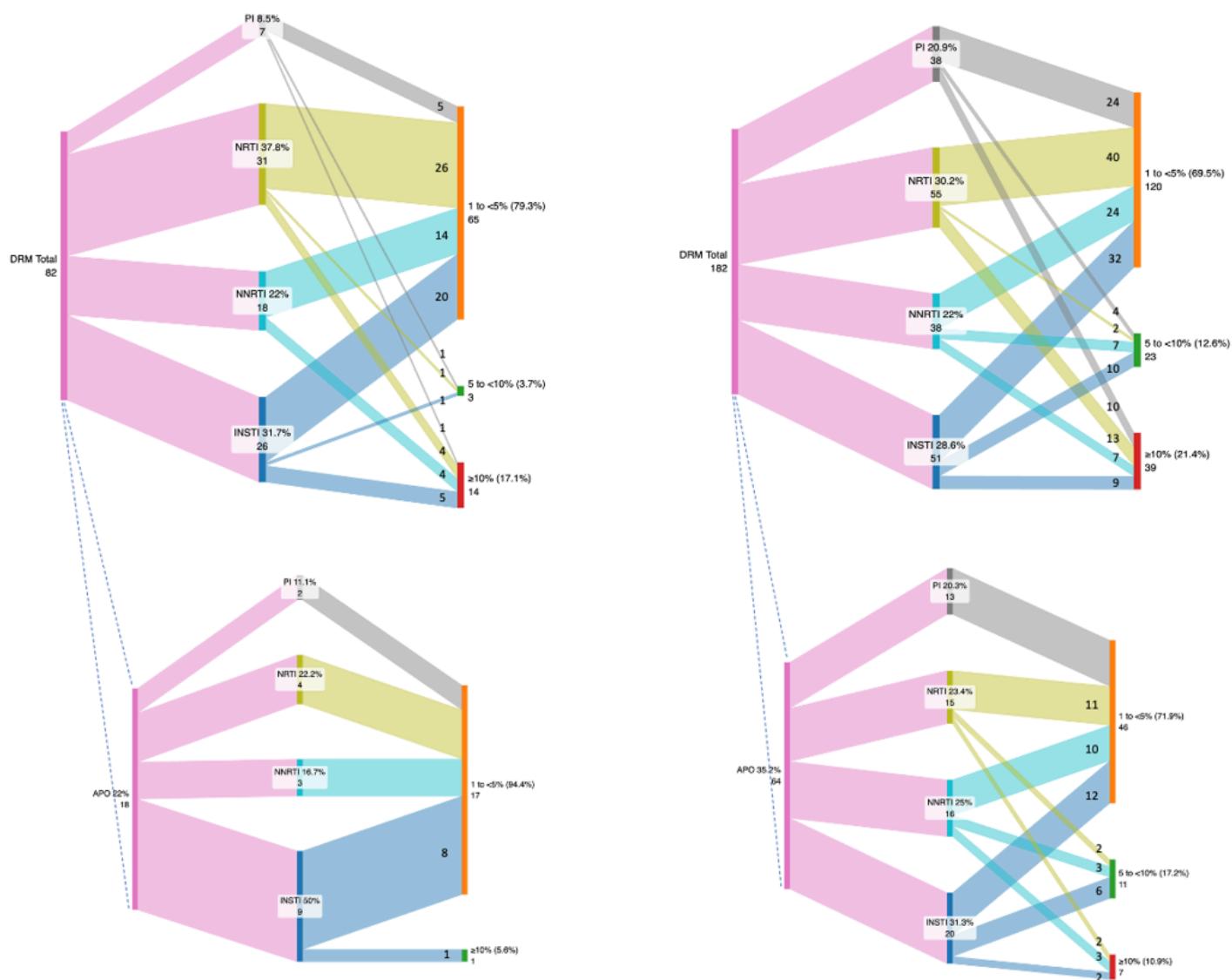


Figure 1

Sankey diagrams of HIV-1 RNA (A) and DNA (B) drug resistance mutations (DRMs, top) and APOBEC-related mutations (APO, bottom), demonstrating the number and percentage of mutations in each drug class: protease inhibitor (PI, grey), nucleoside/nucleotide reverse transcriptase inhibitor (NRTI, olive), non-nucleoside reverse transcriptase inhibitor (NNRTI, aqua), and integrase inhibitor (INSTI, blue). The diagrams also show the absolute number of mutations stratified by variant frequency: 1% to <5% (orange), 5% to <10% (green), and ≥10% (red).