Patterns of pretreatment drug resistance mutations of very early diagnosed and treated infants in Botswana

Natasha Onalenna Moraka[a,b,*], Pilar Garcia-Broncano[c,*], Zixin Hu[d,*],
Gbolahan Ajibola[a], Ontlametse T. Bareng[a], Molly Pretorius-Holme[e],
Kenneth Maswabi[a], Comfort Maphorisa[a], Terence Mohammed[a],
Simani Gaseitsiwe[a,e], Gert U. VanZyl[b], Daniel R. Kuritzkes[d],
Mathias Lichterfeld[c,d,f], Sikhulile Moyo[a,e,†] and Roger L. Shapiro[a,e,†]

Objective: To describe the occurrence of HIV drug resistance mutations (DRMs) in both intact and defective HIV-1 cell-associated DNA (HIV-1 CAD) among early-treated infants.

Design: The Botswana EIT Study (ClinicalTrials.gov NCT02369406) initiated antiretroviral therapy (ART) in the first week of life and evaluated HIV-1 in plasma and peripheral blood mononuclear cells (PBMCs).

Methodology: We analyzed 257 near-HIV-1 full-length sequences (nFLS) obtained by Illumina next-generation sequencing from infants near birth. Sanger sequencing of *pol* was performed for mothers at delivery and children with clinical failure through 96 weeks. DRMs were identified using the Stanford HIV Drug Resistance Database.

Results: In 27 infants, median PBMC HIV-1 proviral load was 492 copies/ml [IQR: 78–1246 copies/ml] at a median of 2 days (range 1–32); 18 (66.7%) had no DRMs detected; six (22.2%) had DRMs detected in defective DNA only, and three (11.1%) had DRMs in both defective and intact DNA ($P = 0.09$). A total of 60 of 151 (37.7%) defective sequences had at least one DRM: 31.8% NNRTI, 15.2% NRTI, 5.3% protease inhibitor, and 15.5% INSTI-associated mutations. In intact sequences, 33 of 106 (31.1%) had at least 1 DRM: 29.2% NNRTI, 7.5% NRTI, 0.9% protease inhibitor, and no INSTI-associated mutations. For all three infants with intact sequence DRMs, corresponding DRMs occurred in maternal plasma at delivery. Archived DRMs were detectable at a later clinical rebound on only one occasion.

Conclusion: Defective HIV-1 cell-associated DNA sequences may overestimate the prevalence of drug resistance among early-treated children. The impact of DRMs from intact proviruses on long-term treatment outcomes warrants further investigation.

[a]Botswana Harvard AIDS Institute Partnership, Gaborone, Botswana, [b]Division of Medical Virology, Stellenbosch University Tygerberg, Cape Town, South Africa, [c]Ragon Institute of MGH, MIT and Harvard, Cambridge, Massachusetts, [d]Division of Infectious Diseases, Brigham and Women's Hospital, Harvard Medical School, [e]Department of Immunology and Infectious Diseases, Harvard TH Chan School of Public Health, and [f]Harvard Medical School, Boston, Massachusetts, USA.

Correspondence to Sikhulile Moyo, PhD, Plot 1836 North Ring Road, Gaborone Botswana.

Tel: +267 3902671; fax: +267 3901284; e-mail: smoyo@bhp.org.bw; sikhulilemoyo@gmail.com

[*]N.O.M., P.G.-B., and Z.H. contributed equally.

[†]S.M. and R.L. contributed equally.

Received: 19 March 2021; revised: 9 July 2021; accepted: 26 July 2021.

Introduction

The use of antiretroviral therapy (ART) to prevent mother-to-child HIV transmission is one of the most successful public health interventions of the HIV pandemic. However, among the small number of infants who become infected, HIV drug resistance mutations (DRMs) are an important concern and may occur either through transmitted drug resistance (TDR) from the founder virus or through the development of de novo resistance in the setting of infant prophylactic agents [1] or ART in the setting of low genetic barrier regimens and inadequate adherence. DRMs threaten the effectiveness of HIV treatment during the critical period of early infancy when HIV viral reservoir is being established [2–4] and when HIV progression may be rapid.

In sub-Saharan Africa, a meta-analysis including data from 13 different countries reported a pooled pretreatment drug resistance (PDR) prevalence of 42.7% among prevention of mother to child transmission (PMTCT)-exposed children and 12.7% among PMTCT-unexposed children in 2016 [5]. Although all data for TDR to date have come from the evaluation of plasma, there is vast evidence of persistence of replication-competent proviruses in different anatomical sites (reservoirs) during prolonged ART [6–8]. Recently, the use of single-genome near full-length proviral sequencing has allowed the characterization of the evolution of proviral reservoir cells in great detail. This technique can distinguish the small proportion of genome-intact proviruses from the much larger number of defective proviruses, which are typically regarded as fossils of the replicative history of HIV-1 in a given patient that do not represent the active replication-competent viral reservoir. Single-genome near full-length proviral sequencing, therefore, provides an opportunity to evaluate antiretroviral drug resistance mutations that selectively occur in genome-intact proviruses.

In the present study, we conducted a detailed investigation of antiretroviral drug resistance mutations in the Early Infant Treatment (EIT) cohort, a unique group of children living with HIV in Botswana who started ART in the first week of life. We evaluated plasma mutations in transmitting mothers, HIV-1 cell-associated DNA (HIV-1 CAD) sequences in peripheral blood mononuclear cells (PBMCs) from neonates near the time of birth, and subsequent plasma mutations at the time of viral failure during infancy.

Methodology

Study design and study population

HIV-exposed infants with gestational age at least 35 weeks and birth weight at least 2000 g were screened for HIV at less than 96 h of age in the Gaborone and Francistown regions of Botswana as part of the Botswana–Harvard Partnership EIT Study (ClinicalTrials.gov NCT02369406). The EIT study has been described elsewhere [9]. In brief, participants were recruited from five hospital maternity wards and surrounding maternity clinics and initiated ART within 7 days after birth. In this study, virologic failure was defined as HIV-1 RNA more than 40 copies/ml (Coulter Abbott m2000sp/m2000rt; Abbott Molecular, Des Plaines, Illinois, USA; limit of quantitation 40 copies/ml). The study was approved by the ethical review boards in Botswana (Health Research Development Committee) and Boston (Harvard T.H. Chan School of Public Health Office of Human Research Administration).

Infants sample processing

Viable infant PBMCs were collected within 2 days of life. PBMCs were isolated and cryopreserved using standard Ficoll-Paque density gradient centrifugation following the ACTG/IMPAACT Cross-network PBMC procedures [10]. Samples were cryopreserved in liquid nitrogen.

Infant droplet digital PCR and next-generation sequencing from peripheral blood mononuclear cells

PBMCs were subjected to DNA extraction using commercial kits (QIAGEN AllPrep DNA/RNA Mini Kit or QIAGEN DNeasy kit; Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Total HIV-1 DNA was amplified using the QX100 Droplet Digital PCR System (ddPCR; Bio-Rad, Hercules, California, USA) using primers and probes described previously [11].

Near full-length single-genome HIV-1 sequencing

Genomic DNA was extracted from PBMC and diluted to single HIV-1 genome levels [12], followed by near full-length proviral amplification using primer sets adjusted to clade C sequences by Illumina MiSeq sequencing at the Massachusetts General Hospital (MGH) DNA Core facility, using an in-house standard protocol for library construction [11,13]. Resulting short reads were de novo-assembled using Ultracycler v1.0 and aligned to HXB2 to identify large deleterious deletions (<8000 bp of the amplicon aligned to HXB2), out of-frame indels, premature/lethal stop codons, internal inversions, or 5′-LTR defects (≥15 bp insertions and/or deletions relative to HXB2), using an automated in-house pipeline written in R scripting language. The presence/absence of APOBEC3G/3F-associated hypermutations were determined using the Los Alamos HIV Sequence Database Hypermut 2.0 program. Viral sequences that lacked all mutations listed above were classified as 'genome-intact'. Viral sequences were deposited in GenBank (accession numbers MK457765–MK458272).

Maternal HIV genotyping from plasma

Viral RNA was extracted from patient plasma using viral RNA Mini kit protocol (Qiagen, Venlo, The Netherlands). The reverse transcription and first-round PCR were performed using the Superscript IV One-Step RT-PCR System with Platinum High Fidelity Taq (Invitrogen Corporation, Carlsbad, California, USA). The RT-PCR reactions were performed in triplicate for each sample in 25 μl reactions. Three microliters of the extracted viral RNA as template was added into 12.5 μl of 2× buffer, 1.0 μl of SuperScript IV RT/Platinum Taq mix, 0.5 μl of 10 μmol/l of forward and reverse primers: PF1, 5′-AAAGGGCTGTTGGAAATGTGG-3′ and PR1, 5′-CCCCAATATGTTGTTATTAC-3′ and 7.5 μl of distilled water. The RT-PCR conditions were as follows: 55 °C for 30 min; 94 °C for 2 min; 25 cycles of 94 °C for 30 s, 56 °C for 30 s and 68 °C for 3.5 min; 68 °C for 5 min. Triplicate reactions for each sample were combined and mixed well to use as a template for the second-round PCR reaction, which was carried out in three separate 25 μl reactions using High Fidelity DNA Polymerase kit with forward and reverse primers: PF2, 5′-AAGGAAGGACACCAAATGAAAGA-3′ and PR2, 5′-TGGGATGTGTACTTCTGAACTTA-3′. The 25 μl PCR reaction contained 2.5 μl of 10× HF buffer, 0.5 μl of 10 mmol/l dNTP, 1.0 μl of 50 mmol/l Mg$_2$SO$_4$, 0.2 μl of Platinum Taq High Fidelity, 0.5 μl of 10 μmol/l of forward and reverse primers, 2 μl of the RT-PCR amplicon, and 17.8 μl of distilled water. Amplification was carried out with the following conditions: at 94 °C for 2 min, then 32 cycles of at 94 °C for 30 s, at 58 °C for 30 s and 68 °C for 3 min followed by a final extension at 68 °C for 5 min. The triplicate PCR reactions were combined and the presence of an approximately 3.1 kb amplicon was verified by agarose gel electrophoresis. Amplification products were subjected to Sanger sequencing at the Massachusetts General Hospital (MGH) DNA Core facility. Sequence alignments were performed using Geneious, presence/absence of drug resistance mutations was determined using Stanford University-HIV Drug Resistance Database (https://hivdb.stanford.edu/).

HIV drug resistance analysis and genotyping

All full-length proviral (from infant PBMCs) and HIV pol (from maternal plasma) sequences were analyzed for the presence of drug resistance mutations using the Stanford HIV Drug Resistance database for analysis (hivdb.stanford.edu). Sequences were subtyped by on-line tools REGA HIV-1 subtyping tool ver. 3 and Context-based Modeling for Expeditious Typing (COMET HIV-1) [14,15].

Statistical considerations

Microsoft Excel and SAS software (SAS Institute, Cary, North Carolina, USA) were used to sort, describe patient characteristics, and determine frequencies of DRMs present. We determined the difference in the proportion of HIV DRMs present in intact vs. defective HIV-1 CAD sequences using a two-sample z test. Because of the small sample size, comparisons were descriptive in nature. Proportion comparison of HIV DRMs detected in intact vs. defective proviral sequences was determined using two proportion z test. For purposes of this analysis, we assumed that all identified resistance mutations in the first infant proviral sample available within 1 month of life were TDR rather than de novo mutations. Statistical analysis was conducted using GraphPad Prism version 9 for Mac (GraphPad Software, San Diego, California, USA; www.graphpad.com) and Stata version 14 (StataCorp LP, College Station, Texas, USA).

Results

Twenty-seven infants from the EIT study with available proviral sequences were included in this analysis, and maternal plasma genotyping was available for a total of 22 mothers. The median age at enrollment for infants was 2 days, and the first available HIV-1 DNA was at a median of 2 days (range 1−32) with a median digital droplet PCR proviral load value of 492 (IQR: 78−1246) copies/ml. Maternal and infant HIV regimen was available for all infants. All 27 infants were started on zidovudine (ZDV)/lamivudine (3TC)/nevirapine (NVP), with a transition to ZDV/3TC/lopinavir-ritonavir (LPV-r) at 2−5 weeks. At delivery, mothers were receiving either efavirenz (EFV)/emtricitabine (FTC)/tenofovir (TDF) (36.4%), dolutegravir (DTG)/FTC/TDF (13.6%), lopinavir/ritonavir/FTC/TDF (4.5%), or no ART (40.9%). (Table 1).

HIV-1 cell-associated DNA drug resistance mutations in infants by 1 month of life

We analyzed a total of 257 sequences from 27 infants with successful NGS HIV-1 CAD genotyping in the first month, with a median of 24 (Q1, Q3: 11, 47) sequences per infant. All sequences were HIV-1 subtype C (100%). Cell-associated HIV DRM were detected in nine (33.3%) of 27 infants. When comparing intact and defective sequences, a total of three (11%) infants had DRMs detected in both intact and defective HIV-1-cell associated DNA sequences, whereas six (22.2%) infants had mutations detected only in their defective sequences. Table 2 shows the specific overall DRMs detected in these nine infants, as well as the mutations identified in the plasma of 22 mothers by population-based Sanger sequencing.

Among all infants with any DRMs detected in overall sequences, the most common mutations detected were NNRTIs and protease inhibitors (Table 2). In infants who had DRMs in their defective genomes only, the most common mutations conferred resistance to NNRTIs, protease inhibitors, and INSTIs. The most common protease inhibitor-associated DRMs observed in defective HIV-1 CAD sequences were D30N and M46I

Table 1. Baseline demographics of Early Infant Treatment infants and mothers at enrolment.

Maternal characteristics ($N = 22$)	
Maternal age (years), median (IQR)	28 (23–30)
Maternal absolute $CD4^+$ cell count (cell/µl), Median (IQR)	377 (220–634)
Maternal viral load (log_{10} copies/ml), median (IQR)	4.4, (3.3–4.9)
Maternal marital status [n (%)]	
Cohabiting	1 (4.5)
Single	21 (95.5)
Maternal highest education level [n (%)]	
Primary	1 (4.5)
Junior secondary	12 (54.5)
Senior secondary	7 (31.8)
Tertiary	2 (9.1)
Household electricity [n (%)]	
No	7 (31.8)
Yes	15 (68.2)
Household car [n (%)]	
No	18 (81.8)
Yes	4 (18.2)
Maternal ARV regimen at delivery [n (%)]	
None	9 (40.9)
EFV+FTC+TDF	8 (36.4)
TRU+DTG	3 (13.6)
TRU+ LPV/r	1 (4.5)
Maternal mode of delivery [n (%)]	
SVD	16 (72.7)
C-section	6 (27.3)
Enrolment site [n (%)]	
Francistown	10 (45.5)
Gaborone	12 (54.5)

Infant characteristics ($N = 27$)	
Sex [n (%)]	
Female	19 (70.4)
Male	8 (29.6)
Age at enrollment and ART initiation (days), median (IQR)	2.0 (2.0–3.0)
ZDV after birth [n (%)]	
No	1 (3.7)
Yes	26 (96.3)
sdNVP after birth [n (%)]	
No	4 (14.8)
Yes	23 (85.2)
Feeding method immediately after birth [n (%)]	
Breastfeeding only	7 (25.9)
Formula feeding only	20 (74.1)
ART regimen initiated [n (%)]	
ZDV-3TC-NVP	27 (100.0)
Absolute $CD4^+$ cell count (cells/µl), median (IQR)	1727 (1538–2177)
Infant viral load (log_{10} copies/ml), median (IQR)	3.5 (3.0–4.5)
Cell associated HIV DNA_ ddPCR (copies/ml), median (IQR)	492 (78–1246)

ARV, antiretroviral drugs; ART, antiretroviral treatment; ddPCR, digital droplet PCR; DTG, dolutegravir; EFV, efavirenz; FTC, emtricitabine; IQR, interquartile range; NVP, nevirapine; LPV/r, Lopinavir/Ritonavir; sdNVP, single-dose nevirapine; SVD, spontaneous vaginal delivery; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine; TRU, Truvada (FTC/TDF), ZDV, zidovudine.

occurring in five of six infants; the most common NNRTI–associated DRM was M230I, observed in four of six infants; and the most common major INSTI mutation was G140R, which is a Cabotegravir resistance mutation, observed in four of six infants. When considering only DRMs in infants with intact HIV-1 CAD sequences, the most common mutations were to NNRTIs, with K103N being the most commonly observed, in two of three infants (Table 3).

Among infants exposed to NNRTI regimens *in utero* (EFV/3TC/TDF), five of six had NNRTI mutations detected in their overall HIV-1 CAD within 1 month of life. When looking at only intact HIV-1 CAD sequences, two of three infants with NRTI and NNRTI (EFV/FTC/TDF) exposure *in utero* had DRMs associated with these regimens. One of these infants had K103N mutation without prior exposure to ART. The single infant whose mother received a protease inhibitor-containing regimen did not have any protease inhibitor mutations detected. All protease inhibitors and INSTI mutations were detected without prior ART exposure and occurred in defective HIV-1 CAD only.

Table 2. Drug resistance mutations detected in infant PBMCs within 1 month of life and maternal plasma at enrollment.

HIV DRMs	Genotyped infants ($N = 27$) Frequency (%)	Genotyped mothers ($N = 22$) Frequency (%)
Any DRM	9/27 (33.3)	5/22 (22.7)
PI mutations		
Any PI mutation	8/9 (88.9)	1/5 (20.0)
D30N	5/8	0
M46I	3/8	0
G48R[a]	5/8	0
G73S[a]	7/8	0
G48E[a]	1/8	0
L23LFIV[a]	0	1/5 (20.0)
NRTI mutations		
Any NRTI mutations	6/9 (66.7)	2/5 (40.0)
M184I	5/6	0
M184V	1/6	1/5
K65R	1/6	1/5
Y115F	1/6	1/5
NNRTI mutations		
Any NNRTI mutations	8/9 (88.9)	4/5 (80.0)
E138K	4/8	0
E138A	0	1/5
M230I	6/8	0
A98G	1/8	1/5
K103N	2/8	2/5
F227L	1/8	0
V106I/M	2/8	0
INSTI mutations		
Any INSTI mutations	7/9 (77.8)	
G140R	6/7	NA
E138K	1/7	NA
R263K	1/7	NA
G163R[a]	5/7	NA
G140E[a]	1/7	NA
G140K[a]	2/7	NA

ARV, antiretroviral drugs; HIV DRMs, HIV-associated drug resistance mutations; INSTI, integrase strand transfer inhibitors; NA, data not available; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, nonnucleoside reverse transcriptase inhibitors; PI, protease inhibitors'.
[a]Accessory mutations.

Of the 22 mothers with successful plasma genotypes, five (22.7%) had detectable DRMs (Table 2). None of the mothers were genotyped for INSTI DRMs. The most commonly occurring mutations were to NNRTIs (K103N, E138A, and A98G) detected in four (18.1%) of the mothers. Only one mother had a detectable protease inhibitor accessory mutation in plasma. When the genotyped mothers were compared with their infants, three of the five mothers with detectable plasma mutations may have transmitted these to their infants, based on detectability in intact HIV-1 CAD within 1 month. Of these, three pairs had at least one matched NNRTI mutation detected (including K103N, V106I/M, A98G, and E138K), and one pair had the same NRTI mutations detected (K65R, Y115F, and M184V). INSTI mutations in children could not be evaluated for TDR because of limited maternal sampling. We observe an M184I mutation in one defective infant sequence, and the matching maternal *pol* sequence at delivery had mutation M184MR (see Supplemental Digital Content 1, http://links.lww.com/QAD/C254).

The results in Table 3 show DRMs detected only in intact HIV-1 CAD sequences of infants within 1 month of life. We show that all intact infant sequences harboured the same mutations, the majority of which were DRMs associated with NNRTIs. Table 3 also compares these mutations with those found in maternal plasma at baseline using population-based Sanger sequencing; indicating a high concordance to what is found in infant HIV-1 CAD sequences within 1 month of life. Of these matched pairs, NNRTI mutations detected among both infants and mothers included K103N, V106M, and A98G and one pair had the same NRTI mutations detected (K65R, Y115F, and M184V). The mutation F227L was detected in an infant but not observed in corresponding maternal plasma.

Among 257 sequences analyzed from nine infants (Fig. 1), we observed overall lower frequencies of mutations within intact infant HIV-1 CAD sequences [33 (31.1%) of 106 sequences] as compared with defective HIV-1 CAD sequences [(39.7%) of 151 sequences] (Fig. 1a), $P = 0.14$. Among the intact sequences with DRMs detected, 29.2% had NNRTI, 7.5% NRTI, 0.9% PI, and none had INSTI-associated mutations.

Table 3. HIV-associated drug resistance mutations detected among intact HIV-1 cell-associated DNA sequences of infants and maternal HIV RNA in plasma.

Mothers[a]					Infants[b]					
Mother ID	PI	NRTI	NNRTI	INSTI	Infant ID	No. of intact sequences	PI	NRTI	NNRTI	INSTI
M-104	None	None	A98G	NA	AP-104	4	None	None	A98G	None
M-109	None	K65R, Y115F, M184V	K103N, V106M	NA	AP-109	10	None	K65R, Y115F, M184V	K103N, V106M, F227L	None
M-119	None	None	K103N	NA	AP-119	22	None	None	K103N	None

All mutations were found across all 36 infant sequences. INSTI, integrase strand transfer inhibitors; NA, not applicable (not tested); NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, nonnucleoside reverse transcriptase inhibitors; PI, protease inhibitors.
[a]Generated using population-based sanger sequencing of HIV pol from plasma.
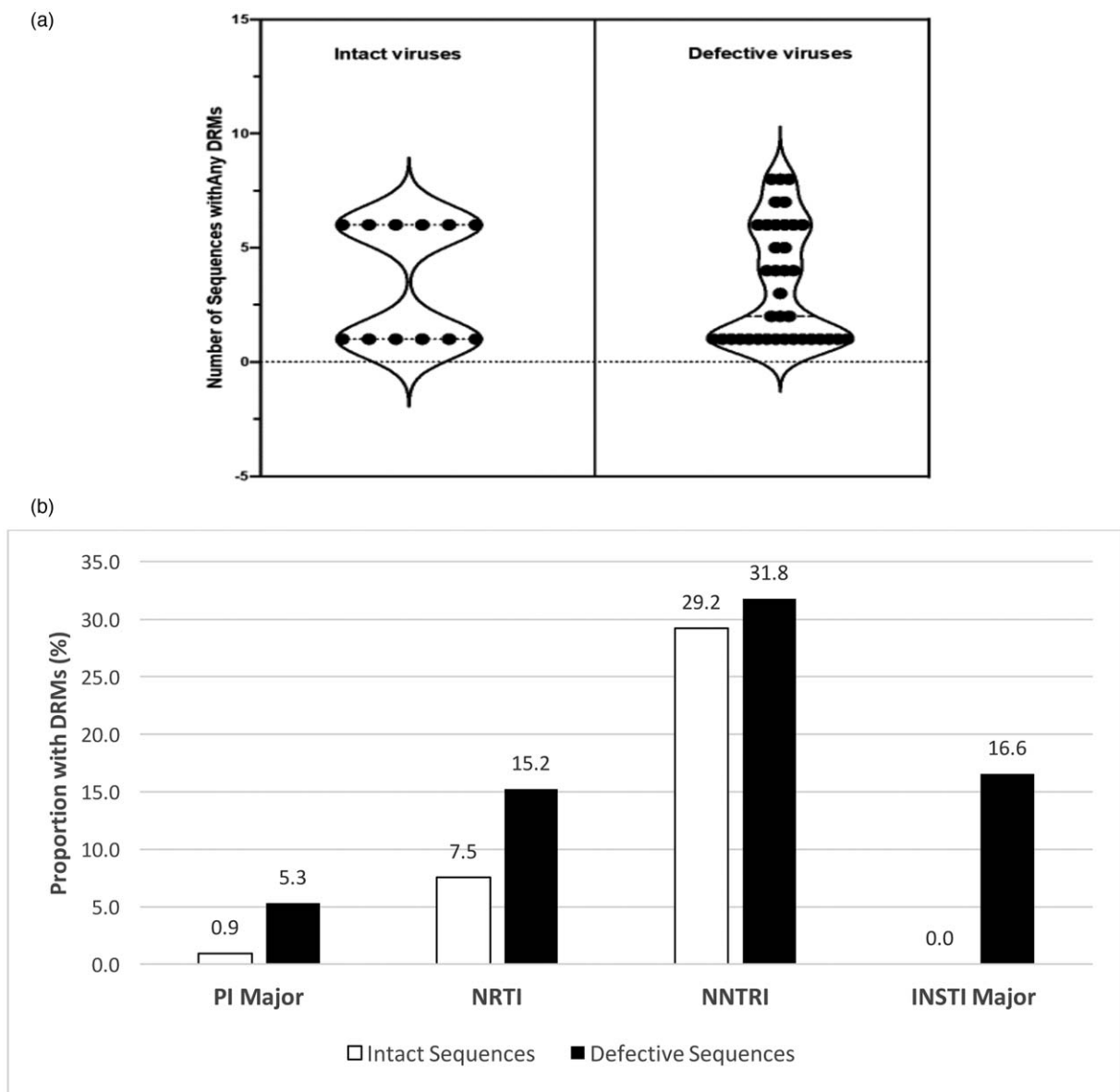[b]Generated using NGS FLIP sequencing from PBMCs.

(a)

(b)

Fig. 1. Frequency of any drug resistance mutations between intact and defective proviruses of infants within 1 month of life. (a) A violin plot that summarizes the differences in distribution of total HIV DRMs detected in intact and defective proviruses of nine EIT neonates (determined by single-genome, near-full- length next-generation sequencing from PBMCs) within 1 month of life; where each dot represents a sequence. Defective sequences were either: hypermutated, had a premature stop codon, internally inverted with large deletions or harbored large deletions. (b) Further represents total number of DRMs detected in intact vs. defective proviruses by drug regimen. DRMs, drug resistance mutations; EIT, Early Infant Treatment; PBMCs, peripheral blood mononuclear cells.

HIV-1 drug resistance mutations and clinical virologic failure within 96 weeks

Among all 27 children in this analysis, 16 (59.3%) had at least one visit with detectable HIV RNA from 24 to 96 weeks, including eight (88.7%) of the nine children with a transmitted mutation identified in PBMCs within the first month of life (see Supplemental Digital Content 1, http://links.lww.com/QAD/C254). Clinical genotyping of the virus from plasma using Sanger sequencing was performed and successful in eight (50%) of the 16 children at the time of viral rebound; seven (87.5%) had one or more identified mutation. Of the eight successful clinical genotypes performed, three (37.5%) were from children with a history of possibly archived resistance mutations in PBMCs and per records did not miss any medication during follow-up (see Supplemental Digital Content 1, http://links.lww.com/QAD/C254). Among these, there was one archived mutation in one (33.3%) child (A98G), and only novel mutations in two (66.7%). Thus, archived proviral TDRMs were detectable at a later clinical rebound on only one occasion.

Discussion

Although HIV TDR prevalence has been well described in adult populations worldwide [21], studies of HIV TDR mutations in infants with perinatally acquired HIV infection remain limited [1,16,17]. This is the first study that has investigated HIV drug resistance mutations in HIV-1 CAD sequences of very early diagnosed and treated infants. DRMs were detected in 11.1% of the participants in intact viruses but in 33.3% when defective viruses were included. The advantage of the whole-genome sequencing approach allowed us to detect defective viruses using all genomic regions of the virus rather than just the HIV *pol* gene commonly used in population-based Sanger sequencing. HIV DRMs found in defective proviral sequences may have little to no bearing on future clinical outcomes as these are most likely to be observed in genomes that do not contribute to the pool of replication-competent viruses. This finding emphasizes the importance of taking into account defective viruses when using proviral sequences or sample types, such as dried blood spots and PBMCs.

Mismatched drug resistance mutations between maternal−infant pairs were not common when assessing only DRMs in intact HIV-1 CAD. We observed only one mismatch between mother and child DRMs, and believe this is likely to represent TDR of minor variant F227L (rather than an early infant de novo mutation) as the infant PBMCs were drawn within 2 days of life. There were no other NNRTI mutation mismatches when considering only intact HIV-1 CAD sequences, supporting our assumption that the DRMs identified in PBMCs with intact HIV-1 CAD sequences represented TDR. It is good to note that NNRTI-associated mutations persist as a result of their viral fitness as compared with other HIV DRMs. A large number of studies have revealed TDR of NNRTIs to be one of the predictors of virologic failure in infants on ART, and resistance to NVP and EFV has been reported in treatment-experienced children [18,19]. There were no protease inhibitor or INSTI-associated resistance mutations in the infants with intact HIV-1 CAD.

We observed higher frequencies of HIV DRMs within HIV-1 CAD sequences with defects. The mutation D30N, which is a well known APOBEC-induced mutation [20], was the most common protease inhibitor major mutation and was not observed in any of the intact HIV-1 CAD sequences. This supports the hypothesis that HIV DRMs may occur more frequently in defective viruses if there are altered phenotypic characteristics that impact functionality. Similarly, the NNRTI and INSTI mutations E138K are known APOBEC-induced mutations and commonly occur in high frequencies when assessing mutations from HIV-1 CAD [20]. Furthermore, we observed INSTI mutations only in HIV-1 CAD sequences with defects, and none of the infants with these

mutations were exposed to INSTI regimens in utero or postpartum. However, we did observe some DRMs in defective HIV-1 CAD that were also observed in the plasma of their mothers, a finding that warrants further investigation into the mechanisms that drive defective HIV-1 CAD accumulation in early infection.

There is a possibility that the HIV DRMs detected in intact HIV-1 CAD sequences before 1 month may have had an impact on future clinical failure in this cohort. The mutation A98G was observed across all intact HIV-1 CAD sequences of the infant who had a viral rebound after 84 weeks, with the same clinical genotype detected by Sanger sequencing at failure (indicating that it was likely to be clinically relevant). Although this observation supports the possibility that early archived mutations in children may be implicated in future clinical failure [21], there were also two children with only novel mutations at the time of clinical failure. The majority of children with archived mutations did not provide evidence in either direction for the re-emergence of TDR because no genotype was obtained at failure (in most cases, as the clinical failure resolved with improved adherence to ART) or because failure did not occur.

The strengths of our study included the use of a carefully followed cohort and the ability to evaluate proviral sequences in early PBMC samples. Most prior studies have traditionally used either plasma or dried blood spots to describe TDR among infants. However, with very early treatment, it is increasingly difficult to sequence and genotype in HIV from plasma using current methods, and the availability of early PBMCs was an advantage. These archived sequences are very likely to represent TDR, given current knowledge of the early establishment of the latent proviral reservoir following infection − including in-utero infection [4,22,23]. Limitations of the study are that none of the mothers were genotyped for INSTI DRMs, and thus comparisons between maternal and infant INSTI DRMs could not be performed in the EIT cohort. We were also not able to show that sequence-intact HIV-1 CAD genomes were actually representative of replication-competent, and thus definitive proviral reservoirs. Lastly, not all mothers were genotyped for HIV DRMs, and therefore, we did not have a complete match between maternal and infant specimens. Our sample size was very small within both cohorts, precluding statistical comparisons.

In conclusion, we investigated the frequency of HIV DRMs among intact and defective HIV-1 CAD sequences from very early treated infants and observed higher rates of mutations among defective HIV-1 CAD sequences than intact sequences. This finding may indicate that use of whole blood or DBS for genotyping in infants overestimates the breadth of mutations, which are capable of future viral rebound. Therefore, when performing drug resistance testing, we believe

bioinformatic pipelines that exclude defective HIV-1 CAD sequences should precede any interpretation of drug resistance. It is not known whether the presence of DRMs in these intact sequences drive the emergence of DRMs in plasma at the time of clinical failure but our initial data indicate that this may not be common. The diversity of TDR patterns identified in infants, and the high frequency of multiple mutations within defective infant HIV-1 CAD sequences, warrant further study as this may overestimate the presence of clinically meaningful DRMs when defective and intact proviruses are not differentiated.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

References

1. Rojas Sánchez P, Holguín A. **Drug resistance in the HIV-1-infected paediatric population worldwide: a systematic review.** *J Antimicrob Chemother* 2014; **69**:2032–2042.
2. van Zyl GU, Bedison MA, van Rensburg AJ, Laughton B, Cotton MF, Mellors JW. **Early antiretroviral therapy in South African children reduces HIV-1-infected cells and cell-associated HIV-1 RNA in blood mononuclear cells.** *J Infect Dis* 2015; **212**:39–43.
3. Van Zyl GU, Katusiime MG, Wiegand A, McManus WR, Bale MJ, Halvas EK, et al. **No evidence of HIV replication in children on antiretroviral therapy.** *J Clin Invest* 2017; **127**:3827–3834.
4. Rainwater-Lovett K, Luzuriaga K, Persaud D. **Very early combination antiretroviral therapy in infants: prospects for cure.** *Current opinion in HIV and AIDS* 2015; **10**:4–11.
5. Boerma RS, Sigaloff KCE, Akanmu AS, Inzaule S, Boele van Hensbroek M, Rinke de Wit TF, Calis JC. **Alarming increase in pretreatment HIV drug resistance in children living in sub-Saharan Africa: a systematic review and meta-analysis.** *J Antimicrob Chemother* 2016; **72**:365–371.
6. Takata H, Buranapraditkun S, Kessing C, Fletcher JL, Muir R, Tardif V, et al., RV254/SEARCH010 and the RV304/SEARCH013 Study Groups. **Delayed differentiation of potent effector CD8(+) T cells reducing viremia and reservoir seeding in acute HIV infection.** *Sci Translat Med* 2017; **9**:eaag1809.
7. Kalams SA, Goulder PJ, Shea AK, Jones NG, Trocha AK, Ogg GS, Walker BD. **Levels of human immunodeficiency virus type 1-specific cytotoxic T-lymphocyte effector and memory responses decline after suppression of viremia with highly active antiretroviral therapy.** *J Virol* 1999; **73**:6721–6728.
8. Sadowski I, Hashemi FB. **Strategies to eradicate HIV from infected patients: elimination of latent provirus reservoirs.** *Cell Mol Life Sci* 2019; **76**:3583–3600.
9. Maswabi K, Ajibola G, Bennett K, Capparelli EV, Jean-Philippe P, Moyo S, et al. **Safety and Efficacy of Starting Antiretroviral Therapy in the First Week of Life.** *Clin Infect Dis* 2020; **72**:388–393.
10. Group C-NPSW. Cross-Network PBMC processing standard operating procedure. HIV/AIDS Network Coordination. 2018.
11. Garcia-Broncano P, Maddali S, Einkauf KB, Jiang C, Gao C, Chevalier J, et al. **Early antiretroviral therapy in neonates with HIV-1 infection restricts viral reservoir size and induces a distinct innate immune profile.** *Sci Transl Med* 2019; **11**:eaax7350.
12. Bui JK, Mellors JW, Cillo AR. **HIV-1 virion production from single inducible proviruses following T-cell activation ex vivo.** *J Virol* 2015; **90**:1673–1676.
13. Lee GQ, Orlova-Fink N, Einkauf K, Chowdhury FZ, Sun X, Harrington S, et al. **Clonal expansion of genome-intact HIV-1 in functionally polarized Th1 CD4+ T cells.** *J Clin Invest* 2017; **127**:2689–2696.
14. Pineda-Peña A-C, Faria NR, Imbrechts S, Libin P, Abecasis AB, Deforche K, et al. **Automated subtyping of HIV-1 genetic sequences for clinical and surveillance purposes: Performance evaluation of the new REGA version 3 and seven other tools.** *Infect Genet Evol* 2013; **19**:337–348.
15. Struck D, Lawyer G, Ternes A-M, Schmit J-C, Bercoff DP. **COMET: adaptive context-based modeling for ultrafast HIV-1 subtype identification.** *Nucleic Acids Res* 2014; **42**:e144–e1144.
16. Poppe LK, Chunda-Liyoka C, Kwon EH, Gondwe C, West JT, Kankasa C, et al. **HIV drug resistance in infants increases with changing prevention of mother-to-child transmission regimens.** *AIDS (London, England)* 2017; **31**:1885–1889.
17. Inzaule SC, Jordan MR, Bello G, Wadonda-Kabondo N, Mounerou S, Mbulli IA, et al., Infant HIV Drug Resistance Survey Team. **High levels of resistance to nucleoside/nucleotide reverse transcriptase inhibitors in newly diagnosed antiretroviral treatment-naive children in sub-Saharan Africa.** *AIDS* 2020; **34**:1567–1570.

18. Kuhn L, Hunt G, Technau K-G, Coovadia A, Ledwaba J, Pickerill S, *et al.* **Drug resistance among newly diagnosed HIV-infected children in the era of more efficacious antiretroviral prophylaxis.** *AIDS* 2014; **28**:1673–1678.
19. WHO. Surveillance of HIV drug resistance in children newly diagnosed with HIV by early infant diagnosis. 2017. Available at: https://apps.who.int/iris/bitstream/handle/10665/259732/9789241512541-eng.pdf [Accessed 4 February 2021]
20. Tzou PL, Kosakovsky Pond SL, Avila-Rios S, Holmes SP, Kantor R, Shafer RW. **Analysis of unusual and signature APOBEC-mutations in HIV-1 pol next-generation sequences.** *PloS One* 2020; **15**:e0225352.

21. Derache A, Shin H-S, Balamane M, White E, Israelski D, Klausner JD, *et al.* **HIV drug resistance mutations in proviral DNA from a community treatment program.** *PloS One* 2015; **10**:e0117430–e1117430.
22. Dickover RE, Garratty EM, Plaeger S, Bryson YJ. **Perinatal transmission of major, minor, and multiple maternal human immunodeficiency virus type 1 variants in utero and intrapartum.** *J Virol* 2001; **75**:2194–2203.
23. Ananworanich J, Robb M. **The transient HIV remission in the Mississippi baby: why is this good news?** *J Int AIDS Soc* 2014; **17**:19859.