CHARACTERIZATION OF THE HIV-1 PROVIRAL LANDSCAPE IN PERINATALLY INFECTED EARLY TREATED CHILDREN ON LONG-TERM ANTIRETROVIRAL THERAPY

by

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DEDICATION

I dedicate this work to parents, MH and HA, thank you for always supporting me in my education, career, and life. Your love and encouragement have made me the person I am today. To my siblings, YH, AH, and AH, thank you for always sticking by my side, I know that I can always rely on you. To my closest friends, EM, CS, LP, MC, and SP, thank you for reassuring me through all my worries and reminding me that I am a capable young scientist.

Thank you all for being my support system and never failing to remind me that I am not alone.

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I would like to thank my committee members, Dr. Mary Kearney, Dr. Sean Patro, and Dr. Ann Boyd. Thank you for all your support, you have molded me into the scientist I am today. Without your support, my accomplishments in this program and with this project would not have been possible. I would also like to thank the entirety of the Translation Research Section for their feedback and support on this project.

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ABSTRACT

Antiretroviral therapy (ART) prevents disease progression in people living with Human Immunodeficiency Virus (HIV) (PLWH). However, off-target effects can result in liver disease, heart disease, diabetes, and more, and there is no accessible cure for the virus. Therefore, understanding the mechanisms for HIV persistence on ART is important to inform strategies for the development of future potential curative inventions. Studies investigating the genetics of integrated HIV genomes (proviruses) in adults revealed the sources of low-level viremia and informed new targets for possible curative strategies. However, little is known about the effect of HIV and long-term ART on the developing immune systems of infants and children. Differences between the immune systems of adults and children may influence mechanisms of viral persistence and immune control, which may be reflected by differences in the HIV proviral genetics. The aim of this thesis research was to characterize the HIV proviral genetic landscape in children on long-term ART and compare it to infants on short-term ART, children on short-term ART, and adults on long-term ART to elucidate any potential differences in the proviral populations that may inform new targets for future cure interventions.

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LIST OF ABBREVIATIONS

3TC	Lamivudine (nucleoside reverse transcriptase inhibitor)
AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral Therapy
AZR	Zidovudine (nucleoside reverse transcriptase inhibitor)
CHER	Children with HIV Early Antiretroviral Therapy
dsDNA	Double stranded DNA
EAST	Early ART Short Term
EIT	Early Infant Treatment
env	Envelope encoding gene
gag	Matrix, capsid, and nucleocapsid encoding genes
gDNA	Genomic DNA
HIV	Human Immunodeficiency Virus
LPV/r	Lopinavir/ritonavir (protease inhibitors)
LTR	Long terminal repeats
MSD	Major splice donor site
nef	HIV accessory protein
NFL	Near full-length
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
PCR	Polymerase chain reaction
PI	Protease inhibitor
PLWH	People living with HIV

pol	Protease, reverse transcriptase, and integrase encoding genes
PSD	Proviral Sequence Database
RRE	Rev Response Element
SGS	Single genome sequencing
tat	HIV regulatory protein

INTRODUCTION

Human Immunodeficiency Virus Type 1 (HIV-1) is a retrovirus that infects CD4+ T cells and macrophages. HIV-1 infection is initiated when the viral glycoprotein, Env, of a mature virion binds to the CD4 receptor and CCR5 or CXCR4 coreceptors on the surface of target cells. Once the virion and immune cell have fused, the viral capsid enters the cytoplasm and is transported to the nucleus (Burdick et al. 2020; Francis et al. 2020; Li et al. 2021). Reverse transcription of the HIV-1 RNA genome into double stranded (ds)DNA occurs during this process. After the capsid enters the nucleus, it is uncoated, and the viral integrase inserts the double-stranded HIV-1 DNA genome into the human genome. An integrated dsDNA viral genome is referred to as a "provirus." The provirus can be transcribed and translated by host cellular machinery. Viral gene expression allows for the ongoing infection and viral replication as new virus particles are generated and released (Kleinpeter and Freed 2020).

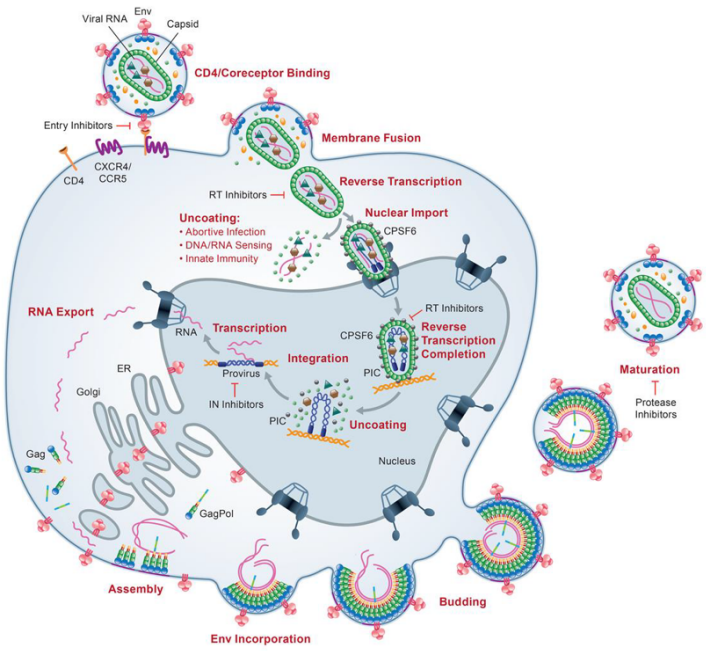


Figure 1: HIV-1 Replication Cycle (Klienpeter and Freed 2020)

After the initial transmission event, the virus replicates exponentially, and mutations that accumulate during replication result in rapid genetic evolution (Coffin 1995). Errors during the replication cycle result in mutations that can benefit the virus in terms of immune evasion and development of drug resistance or that can potentially preclude replication competency. Most of the mutations that arise are due to errors of HIV-1 reverse transcriptase enzyme. Reverse transcriptase is highly error prone ($\sim 1.4 \times 10^{-5}$ mutations/base pair/cycle) and lacks proofreading ability (Abrams 2010; Preston et al. 1988). Additionally, reverse transcription requires a strand transfer, in which the enzyme dissociates from one RNA template and re-initiates on a second template, resulting in truncated DNA genomes containing large internal deletions (Abrams 2010; Hu and Hughes 2012). Several host factors also contribute to the accumulation of defective HIV genomes. APOBEC3 proteins play a role in intracellular anti-viral immunity by editing the reverse transcribed viral genome (Chiu et al. 2005; Harris et al. 2003; Mangeat et al. 2003). APOBEC3 proteins are cytosine deaminases, resulting in a C→U nucleoside conversion in single stranded DNA (Bishop et al. 2004; Henderson and Fenton 2015). These conversions result in subsequent G→A in the coding strand, often resulting in premature stop codons that prevent transcription of the hypermutant viral genomes (Bourara et al. 2007; Sadler et al. 2010; Smyth et al. 2012).

Currently, there is no scalable cure for HIV-1 due to the irreversible integration of the provirus into the host genome. The current standard care is lifelong antiretroviral therapy (ART) (Coffin 1995; Finzi et al. 1999). ART is a combination of compounds that target multiple steps of the viral replication cycle such as entry, reverse transcription, integration, assembly, and maturation, effectively preventing the infection of new cells and

allowing for recovery of CD4+ T cell count and immune function (Arts and Hazuda 2012; Kearney et al. 2014; Lee et al. 2019; McManus et al. 2019; Pau and George 2014; van Zyl et al. 2017). However, if ART is interrupted, viral replication will start again, resulting in rebound of viremia to pre-therapy levels, loss of CD4 count, and decline of immune function (Chun et al. 1999; Kearney et al. 2015; Skiest et al. 2007). ART allows people living with HIV (PLWH) to regain near normal lifespan, but many toxicities and comorbidities have been observed as effects of long-term infection and antiretroviral drugs (Chawla et al. 2018; Guaraldi et al. 2019; Webel et al. 2021).

Currently, there are approximately 37.7 million PLWH and about 1.5 million new infections every year. Only 28.2 million PLWH have access to ART (UNAIDS, https://www.unaids.org/en/resources/fact-sheet). Untreated HIV-1 infection results in depletion of CD4+ T cells leading to the development of Acquired Immunodeficiency Syndrome (AIDS), a state of severe immune impairment that leaves the sufferer at extreme risk of opportunistic diseases. In 2020, 680,000 infected individuals died of AIDS-related illnesses (UNAIDS, https://www.unaids.org/en/resources/fact-sheet).

Of the millions of PLWH, 1.7 million are children between the ages of 0 and 14, with 150,000 new children born with HIV-1 every year. Of these children, only half have access to ART. Most infections in children occur through vertical transmission, that is in utero, intrapartum, or during breastfeeding. The treatment of mothers with HIV-1 with antivirals has drastically decreased the incidence of vertical transmissions (Hurst et al. 2015; UNAIDS, https://www.unaids.org/en/resources/fact-sheet). However, due to the lack of a cure, children with HIV-1 must remain on ART for their entire lives. Life-long adherence to ART is challenging due to economic and social factors. In addition, the effects

of HIV infection and long-term ART on the developing immune system are largely unknown.

Due to differences in the immune dynamics of children and adults (Katusiime et al. 2021), different strategies may be required for curative interventions. The pediatric immune system is predominantly antigenically inexperienced, relying heavily on innate immunity and influenced by the maternal immunological experience (Cinicola et al. 2021; Shearer et al. 1997). The pediatric immune response favors an anti-inflammatory response and antibody responses are limited as compared to adults (Lilic et al. 1997; Payne et al. 2015; Siegrist 2009; Tobin and Aldrovandi 2013). Despite existing at greater total levels than observed in adults, CD4+ T-cells in infants are mostly naïve and lack coreceptor expression (Shalekoff et al. 2004; Tuttle et al. 2004). This results in a lack of effective CD8+ T-cell response and stimulation of B-cells (Tobin and Aldrovandi 2013). Studies investigating the effects of long-term ART and life-long HIV-1 persistence in adults suggest that PLWH are more at risk for non-AIDS related co-morbidities (Horvat et al. 2015), but little work has been done to understand the effects on developing immune systems and on the overall health of children born with HIV-1.

As in adults, HIV-1 cell-associated DNA persists in children despite ART. In children with viremia fully suppressed on ART, there is no evidence of viral replication (van Zyl et al. 2017). Also as in adults, during long-term viral suppression, only 0.1-2% of proviruses are genetically intact and capable of fueling recrudescence of viremia upon therapy cessation (Bruner et al. 2016; Heiner et al. 2017; Ho et al. 2013, Katusiime et al. 2020). In depth characterization of intact HIV-1 proviruses, referred to as the HIV-1 reservoir, and the defective proviral population that persists on ART may reveal potential

sources of persistent low-level viremia and potential targets for curative interventions. As there is currently no accessible cure for HIV-1, understanding the genetics of proviruses that persist despite ART is important for informing studies toward the design of curative strategies. Therefore, for my thesis research, I characterized the genetic structures of HIV-1 proviruses that persist in children on long-term ART and compared them to proviral structures that were present in children before ART and on short-term ART. I also compared the proviral structures in children on long-term ART to those that persist in adults on long-term ART. I hypothesized that the proviral structures that persist on long-term ART in children will be different than those in pre-ART and on short-term ART, revealing a continual evolution of HIV-1 on ART that results, not from viral replication, but from the elimination of proviruses that express viral proteins. To test this hypothesis, I isolated and PCR amplified hundreds of single proviruses from children on long-term ART in separate wells of microtiter plates (to ensure that there would be no artifactual recombination between HIV-1 genomes), sequenced the products of each amplicon using Illumina Miseq technology, characterized the structure of each, and compared the structures to those in children not on ART or on short-term ART.

MATERIALS AND METHODS

Donor Samples

Genomic DNA extracted from 1.25×10^6 peripheral blood mononuclear cells (PBMCs) of 4 perinatally infected children living with HIV-1 from the Children with HIV and Early Retroviral therapy (CHER) cohort was obtained (NCT00102960) (Violari 2008) (**Table 1**).

Table 1: Donor Characteristics

Participant ID	Sex	Age at ART initiation (months)	Years on suppressive ART	HIV DNA copies/PBMC at sample collection	ART regimen
ZA-004	Male	2.7	7.92	23.6	AZR/3TC/LPV/r
ZA-006	Female	9.0	7.45	46.7	AZR/3TC/LPV/r
ZA-011	Female	9.3	7.35	181.5	AZR/3TC/LPV/r
ZA-012	Male	5.1	8.6	11.9	AZR/3TC/LPV/r

Genomic DNA Extraction

Genomic DNA extraction was performed as in Katusiime et al., (Katusiime et al. 2020). In brief, 100µL of 3M guanidine HCl (Sigma-Aldrich: Cat#G9284) and 5µL Proteinase K (Applied Biosystems: Cat#AM2548) were added to each cell pellet. To ensure resuspension, the pellets were vortexed for 10 seconds and incubated for 1 hour at 42°C. Next, 400µL of 6M guanidine isothiocyanate (GuSCN) (Sigma-Aldrich: Cat#50983) and 8µL of 20mg/mL glycogen (Roche: Cat#10901393001) were added to each sample, vortexed, and incubated at 42°C for 10 minutes. Next, 500µL of 100% isopropanol was added to each sample, then vortexed and centrifuged at 21,000×g for 10 minutes in order to precipitate nucleic acids. The supernatant was removed from each sample and pellets

were washed with 750µL of 70% ethanol. Following an additional 10-minute centrifugation at 21,000×g, residual ethanol was removed and the pellet was air dried for 5 minutes. Finally, the pellet was resuspended in 100µL of 5mM Tris-HCl (pH 8.0). The resuspended DNA was incubated at 42°C for 2 hours then stored at -80°C.

Near Full-Length Single Genome Sequencing (NFL-SGS) Workflow

Extracted gDNA was diluted to an endpoint (<0.3 proviral genomes per well in a 96-well plate) with 5 mM Tris-HCl (pH 8.0). Near full-length (NFL) proviruses were amplified with polymerase chain reaction (PCR) using a nested PCR approach (**Figure 2**). The PCR primers anneal to the *gag* leader downstream from the 5'LTR as specified in **Table 2**. NFL amplification of proviruses in samples from donors ZA-004, ZA-006, and ZA-011 was performed using Ranger mix (Bioline: Cat#BIO-25052). A 200µL volume of diluted gDNA was added to a PCR master mix comprised of 500µL 2x Ranger Mix, 284µL nuclease free water, 8µL each of 50µM forward and reverse primer (**Table 2**). The total volume was spread across a 96-well plate (10µL per well). PCR cycling was performed as described in **Table 3**. Due to discontinuation of Ranger polymerase from Bioline, NFL amplification from donor ZA-012 was performed using Platinum SuperFi II DNA polymerase (Thermofisher Scientific: Cat#12361010). A master mix containing 210µL 5x SuperFi buffer, 20µL of 10µM dNTP mix, 20µL each of 10µM forward and reverse primer (**Table 2**), 559.5µL nuclease free water, and 10.5µL SuperFi II DNA Polymerase was prepared, combined with 220µL of diluted gDNA, spread across a 96-well plate (10µL per well), and exposed to PCR cycling described in **Table 3**. Nested PCR was performed under the same conditions but with primers shown in **Table 2**.

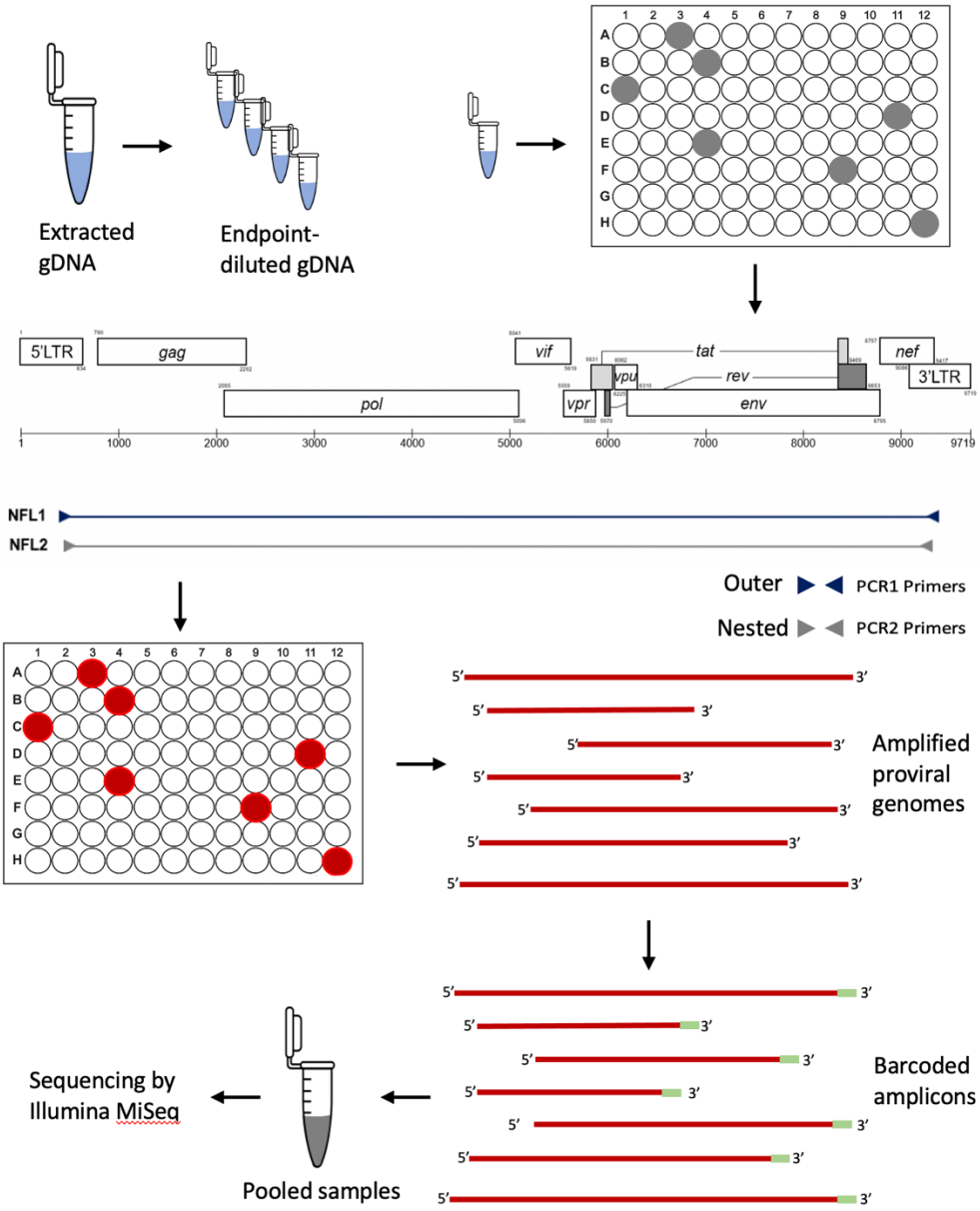


Figure 2: NFL-SGS Workflow. Endpoint-diluted gDNA was spread across a 96-well plate. Near full-length HIV-1 proviral genomes were amplified via sequential nested PCR. Amplicons were then barcoded, pooled, and sequenced by Illumina MiSeq.

Table 2: List of Primers Used for NFL PCR Amplification

Gene	**Primer Location (HXB2 Reference)**	**Sequence**
5' LTR	623+	AAATCTCTAGCAGTGGCGCCCGAACAG
gag	642+	CCGAACAGGGACBHGAAAGCGAA
3' LTR	9604-	GACTCTGGTAACTAGAGATCCCTCA
3' LTR	9662-	TAAGCCTCAATAAAGCTTGCCTTGAGTGC

Table 3: NFL-SGS PCR Cycling Conditions

NFL Enzyme	PCR1	PCR2
Ranger	1. 95ºC for 2 min 2. 98ºC for 10 sec 3. 61.5ºC for 10 min 4. 72ºC for 10 min 5. Go to #2 30x cycles 6. 4ºC hold	1. 95ºC for 2 min 2. 98ºC for 10 sec 3. 65.5ºC for 10 min 4. 72ºC for 10 min 5. Go to #2 30x cycles 6. 4ºC hold
Superfi II	1. 98ºC for 3 min 2. 98ºC for 10 sec 3. 61.5ºC for 30 sec 4. 72ºC for 5 min 5. Go to #2 30x cycles 6. 72ºC for 5 minutes 7. 4ºC hold	1. 98ºC for 3 min 2. 98ºC for 10 seconds 3. 65.5ºC for 30 seconds 4. 72ºC for 5 minutes 5. Go to #2 30x cycles 6. 72ºC for 5 minutes 7. 4ºC hold

Invitrogen 96 well 1% agarose E-Gels (Thermofisher Scientific: Cat#G700801) were used to identify positive wells. Amplicons were then run on 12 well 0.8% agarose E-Gels along with a 1 Kb plus DNA ladder to determine amplicon size (Thermofisher Scientific: Cat#G501808, Cat#10488090).

Illumina Sequencing

The resulting NFL amplicons were sequenced by next generation sequencing (NGS) using the Illumina MiSeq platform. Due to low fidelity 5' phosphorylation by the prep kit, a single round of PCR was performed using pre-phosphorylated primers (FWD: CCGAACAGGGACTTGAAAGCGAA, REV: TAAGCCTCAATAAAGCTTGCCTTGAGTGC) using the Ranger Mix PCR2 cycling conditions, with two changes; step 3: 55ºC for 30 sec, step 5: go to #2 20x cycles. The amplicons were then purified using AMPure XP beads (Beckman Coulter: Cat#A63881)

according to the manufacturer protocol and quantified via FluoroSkan (ThermoFisher: Cat#5200110). Sequencing libraries of individual amplicons were prepared according to the manufacturer protocol using the NEBNext Ultra II FS DNA Library Preparation Kit (New England Bio Labs: Cat#E7805L). In brief, the amplicon DNA was fragmented, blunted, and underwent end repair, 5' phosphorylation, and 3' dA-tailing. Hairpin loop adapters were then ligated to the fragments and USER enzyme was then used for U excision to free the adapter ends (**Figure 3**). The sample was then enriched for adaptor ligated fragments of 150-250 base pairs via size exclusion AMPure XP bead purification according to the manufacturer protocol. The libraries were then enriched by PCR and barcoded. The libraries undergo a final PCR cleanup AMPure XP bead purification and were assessed for quality control and concentration via TapeStation (Agilent: Cat#G2992AA). Equal molar concentrations of each library were then pooled for NGS. The pooled libraries were run on the Illumina MiSeq using 300 cycle MiSeq Reagent Nano Kit v2 (Illumina: Cat#MS-103-1001).

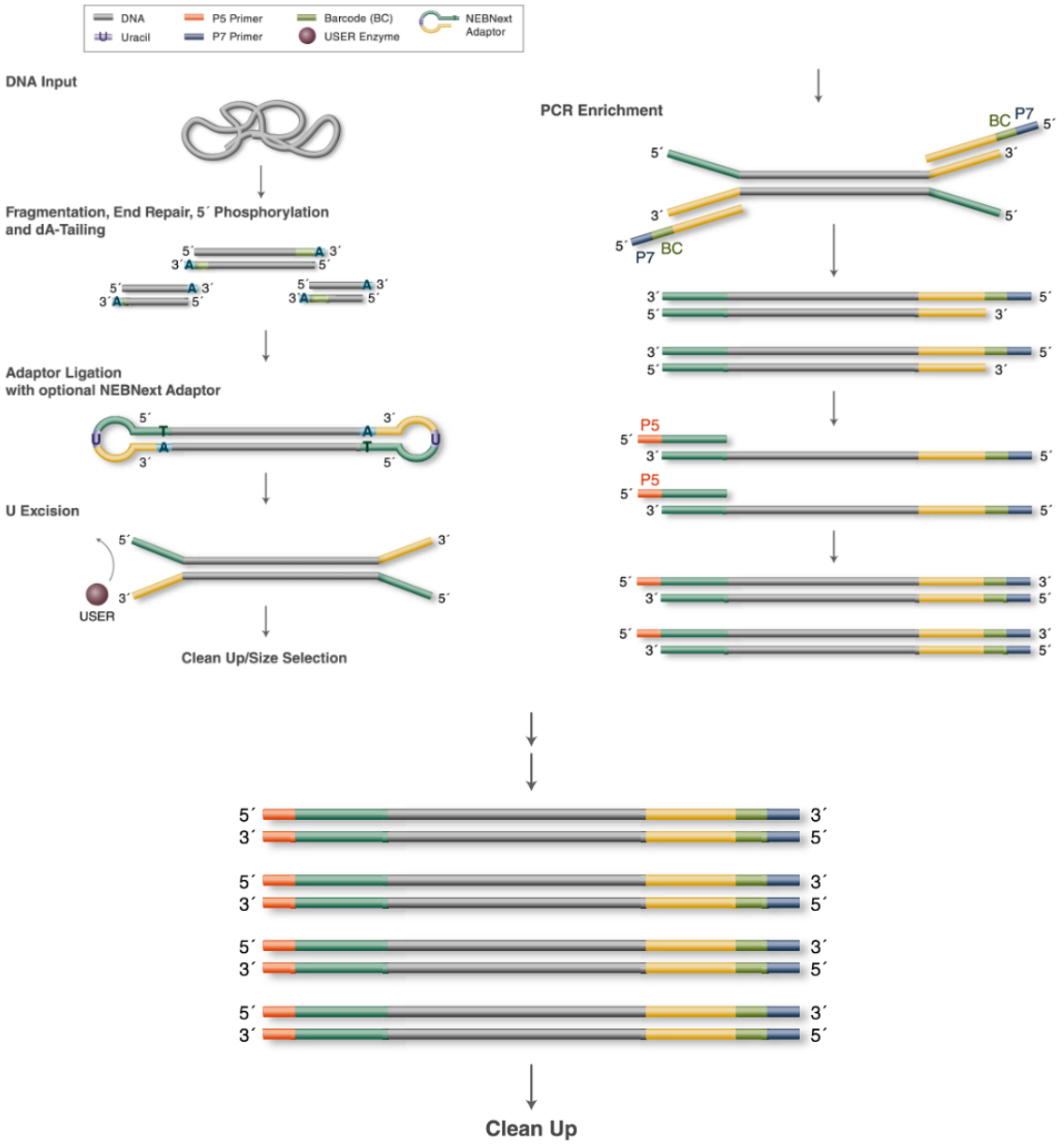


Figure 3: NEBNext Ultra II FS DNA Library Preparation Kit Workflow (Figure from New England Biolabs NEBNext Ultra II FS DNA Library Prep Kit).

Sequence Analysis

Resulting NFL sequences were assembled and aligned using the MAFFT alignment program (Katoh and Standley 2013). The Proviral Sequence Annotation & Intactness Test (ProSeq-IT) tool was used to annotate defects throughout the provirus and to infer genetic

intactness (Shao et al. 2020). The tool infers intactness based on sequence length, major splice donor (MSD) site, packaging signal, rev response element (RRE) region status, insertions, deletions and premature stop codons in *gag*, *pol*, and *env*. The fraction of intact proviruses and those with major internal deletions or mutations in each donor was determined. Proviral populations were also analyzed based on the types and abundance of defects (**Figure 4**).

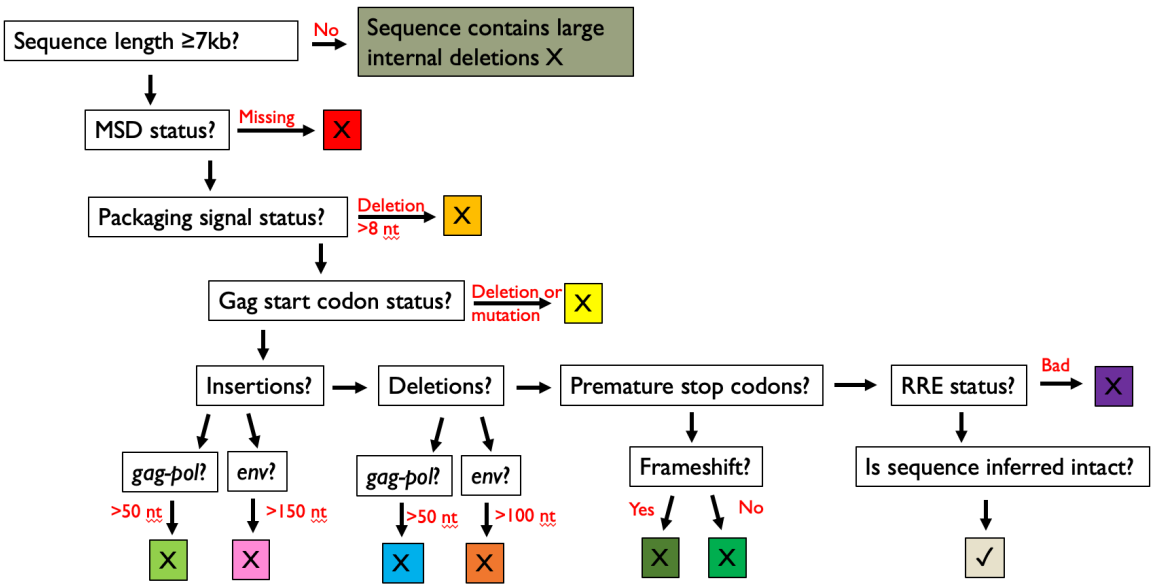


Figure 4: ProSeq-IT defined defects.

The fraction of proviruses with defects or deletions in *tat* and *nef* were also determined and compared. Intact proviruses were analyzed for drug resistance using the Stanford University HIV Drug Resistance database (https://hivdb.stanford.edu), receptor tropism using Geno2Pheno (Lengauer et al. 2007), and potential clonality by phylogenetic analysis. These analyses were also performed on previously published data from infants treated shortly after birth (Early Infant Treatment Study (EIT) (Garcia-Broncano et al. 2019)), children on short-term ART (Early ART Short Term (EAST) (Garcia-Broncano et. al 2019)), and adults on long-term ART (Ho et al. 2013, Bruner et al. 2016, Heiner et al.

2017) (**Table 4**) and compared to the sequences characterized from the children in this study.

Table 4: Donor characteristics of previously published NFL sequences

Publication	Age group	Subtype	Donors (n)	Time until ART initiation (months)	Time on suppressive ART (months)
Ho YC et al. *Cell*. 2013	Adult	B	8	6-141	28-108
Bruner KM et al. *Nat Med*. 2016	Adult	B	19	17 days-6 months	10-203
Hiener B et al. *Cell Rep*. 2017	Adult	C	6	6-12	38.4-212.4
Garcia-Broncano P et al. *Sci Transl Med*. 2019	EIT	C	10	-	Pre-ART
				0.2 hrs-4.77 days	1-24
Garcia-Broncano P et al. *Sci Transl Med*. 2019	EAST	C	10	2.6-11.7	16-31

RESULTS

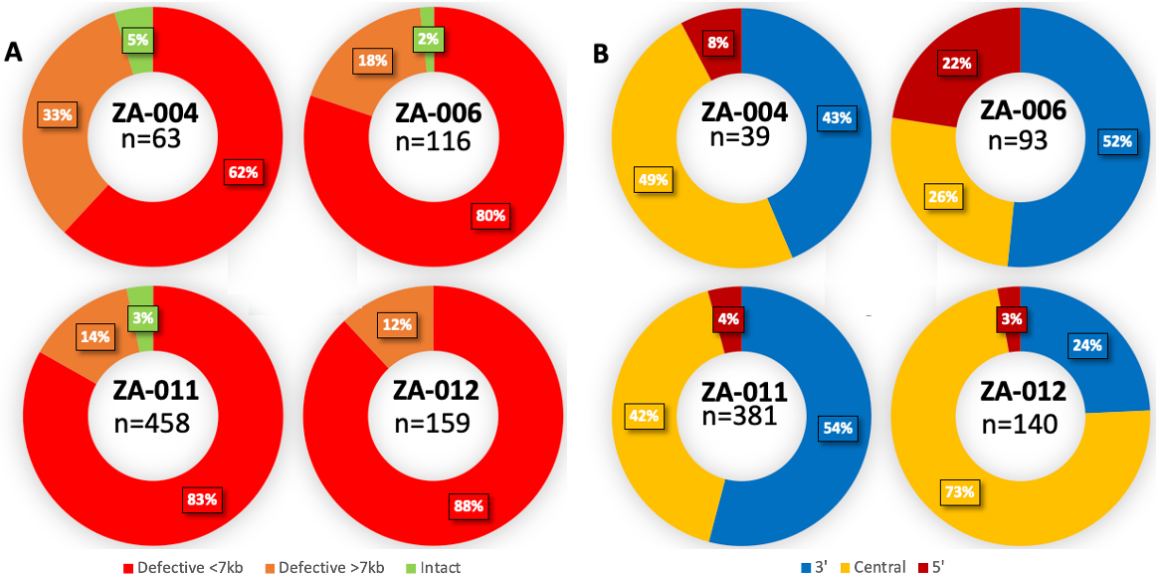
CHER Cohort HIV Proviral Genetic Characterization

Across the 4 donors, a total of 796 single proviral genome amplicons were obtained by near full-length single-genome sequencing (NFL-SGS) (**Table 5**). Twenty were inferred intact (2.6%) and 776 were found to be defective using the ProSeq-IT tool (**Table 5** and **Figure 5A**).

Table 5: NFL sequences per CHER donor

Participant ID	Number of sequences obtained	Number of sequences		
		Intact	>7kb defective	<7kb defective
ZA-004	63	3	21	39
ZA-006	116	2	21	93
ZA-011	458	15	62	381
ZA-012	159	0	19	5
Total	796	**20**	123	653

Of the defective population, 653 had large internal deletions resulting in sequences smaller than 7kb in size. The majority of large internal deletions were found to span the 3' and/or central regions of the genome (47% and 47%) (**Figure 5B**). The remaining 123 were deemed defective due to defects or deletions in their major splice donor site (MSD), packaging signal, *gag* start codon, RRE, or in one or multiple of the coding genes- *gag*, *pol*, and/or *env*. Proviruses were found to have up to 6 different defects, but 2 defects were seen most commonly (37%) (**Figure 5C**). Of the observed defects, frameshift induced stop codons in *gag*, *pol*, and *env* (59%) and defects in the *gag* start codon (46%) were observed most often (**Figure 5D**). Deletions in *tat* and *nef* were uncommon in proviruses that were greater than 7kb in size, however, were commonly observed in those smaller than 7kb (**Figure 5E**).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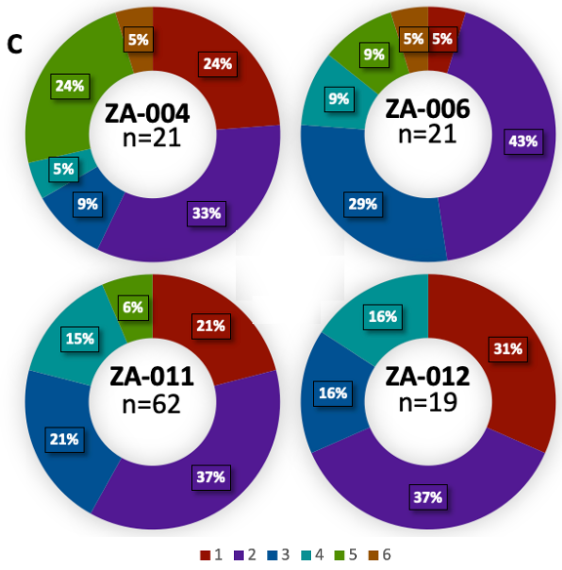
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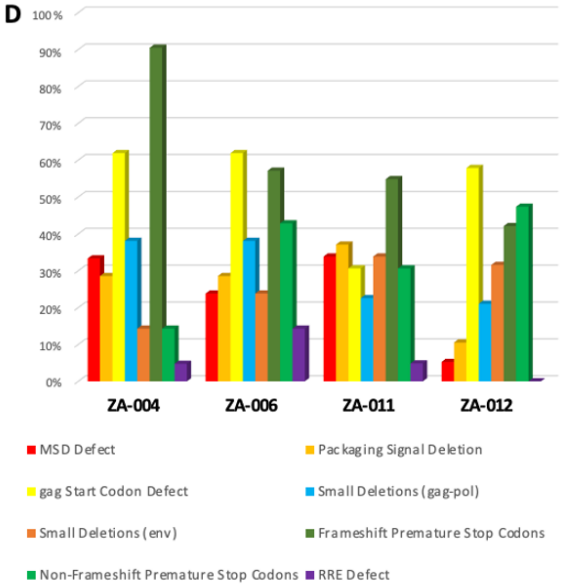
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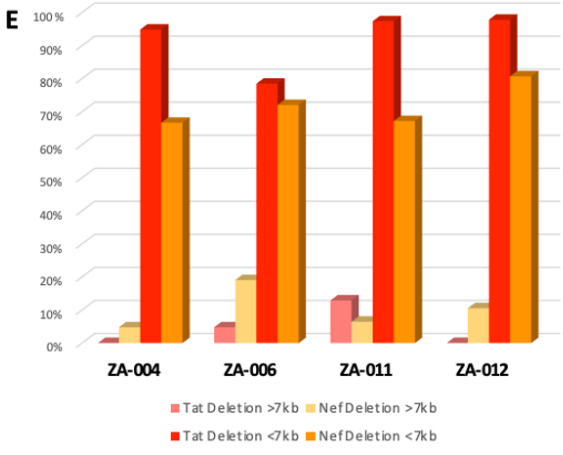
ZA-011
ZA-012
MSD Defect
Packaging Signal Deletion
gag Start Codon Defect
Small Deletions (gag-pol)
Small Deletions (env)
Frameshift Premature Stop Codons
Non-Frameshift Premature Stop Codons
RRE Defect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ZA-004
ZA-006
ZA-011
ZA-012
Tat Deletion >7kb
Nef Deletion >7kb
Tat Deletion <7kb
Nef Deletion <7kb

Figure 5: Characterization of proviral genetics in the CHER cohort. A. The fraction of total intact and defective sequences. Red: Defective <7kb. Orange: Defective >7kb. Green: Intact. B. The distribution proviruses with large internal deletions. Blue: 3' deletions. Yellow: Central deletions. Red: 5' deletions. C. The number of defects per sequence. Maroon: 1 defect. Purple: 2 defects. Blue: 3 defects. Teal: 4 defects. Green: 5 defects. Brown: 6 defects. D. The frequency of the observed defects. Red: MSD defect. Orange: Packaging signal defects. Yellow: gag start codon defect. Blue: Small deletions (gag-pol). Orange: Small deletion (env). Dark green: Frameshift premature stop codons. Light green: Non-frameshift premature stop codons. Purple: RRE defect E. The fraction of observed tat/nef deletions. Pink: tat deletion >7kb. Yellow: nef deletion >7kb. Red: tat deletion <7kb. Orange: nef deletion <7kb.

Defects in HIV-1 proviruses persisting in children and adults before and during ART

To compare proviral sequences among donors with different times of ART initiation and different ART durations, published NFL sequences were obtained from the HIV Proviral Sequence Database (PSD) and from GenBank (summarized in **Table 6**) and were compared to our dataset obtained from children in the CHER cohort. These sequences originate from previously published data from infants treated shortly after birth (Early Infant Treatment Study (EIT) (Garcia-Broncano et al. 2019)), children on short-term ART (Early ART Short Term (EAST) (Garcia-Broncano et. al 2019)), and adults on long-term ART (Ho et al. 2013, Bruner et al. 2016, Heiner et al. 2017) (**Table 4**).

Table 6: Number of NFL sequences per dataset

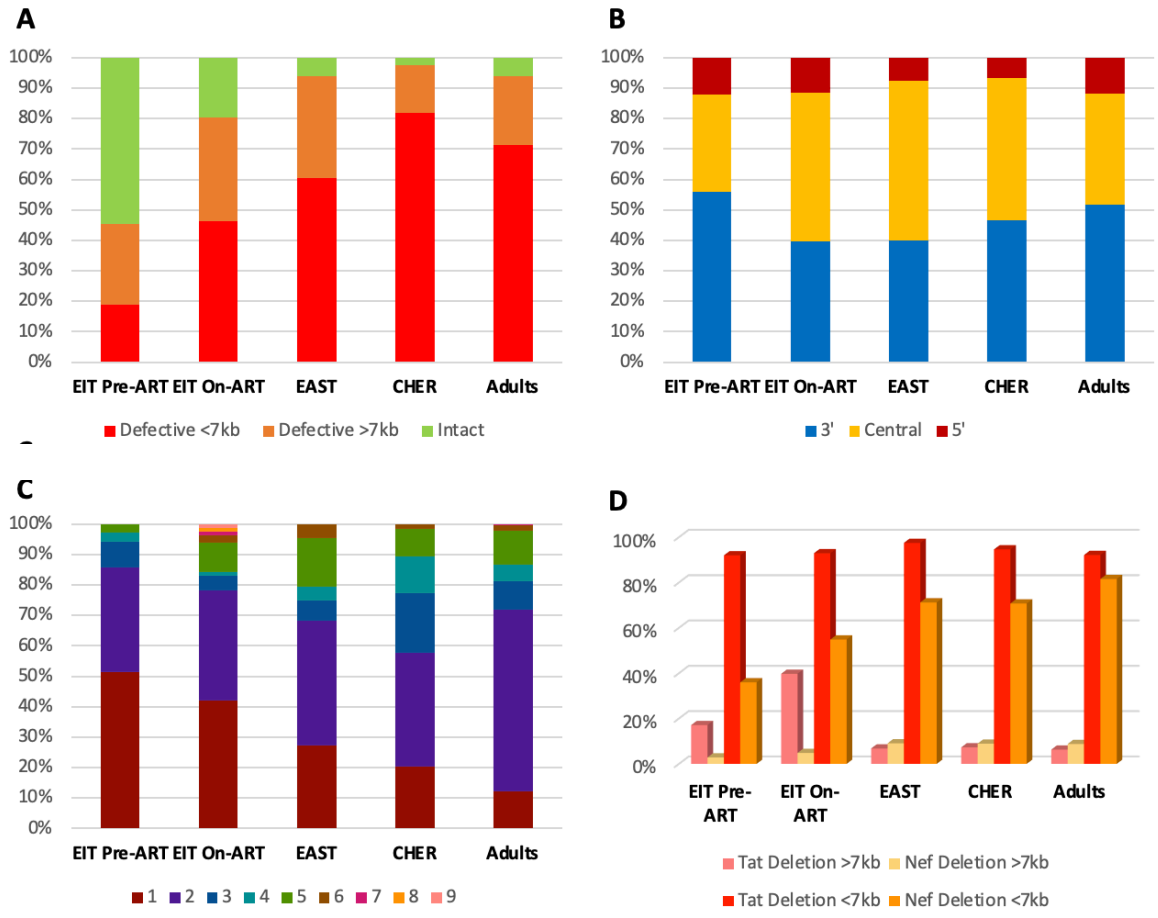
Group	Total # of sequences	# of sequences		
		Intact	>7kb defective	<7kb defective
EIT Pre-ART (Garcia-Broncano P et al. 2019)	132	72	35	25
EIT On-ART (Garcia-Broncano P et al. 2019)	244	48	83	113
EAST (Garcia-Broncano P et al. 2019)	132	8	44	80
Adults (Ho YC et al. 2013, Bruner	1056	63	239	754

KM et al. 2016, Hiener B et al. 2017)				
Total	1564	191	401	972

The majority (55%) of sequences obtained from the EIT Pre-ART group were found to be intact (**Figure 6A**). However, the majority of sequences obtained from the EIT On-ART group, the EAST group, and the Adults group were defective, with only 2-19% being intact (**Figure 6A**), similar to what was found in the CHER cohort. Of all the on-ART sequences in all groups, most contained large internal deletions. However, pre-ART sequences were observed with more defects resulting from small deletions or mutations (especially frameshifts) in MSD, packaging signal, *gag* start codon, RRE, and/or *gag*, *pol*, or *env* coding regions (**Figure 6B**). Of the sequences with large internal deletions, most were in the 3' and central regions of the genome regardless of ART status, ART duration, or age (**Figure 6B**). Like the proviruses in the CHER group, sequences that were greater than 7kb were often found to contain more than one defect; some had up to 9 different defects. However, the pre-ART and on-ART proviruses in the EIT group were observed most often with only 1-2 different defects whereas most sequences in the CHER, EAST, and Adult groups were observed with 2 or more defects (**Figure 6C**). **Figure 6E** summarizes the frequency of each defect in the defective proviruses that were greater than 7kb.

G>A hypermutation of HIV-1 proviruses, caused primarily by the host enzyme APOBEC3G, can result in premature stop codons and were the most commonly seen defects in the EIT Pre-ART group. The EIT On-ART sequences exhibited more variance in the types of defects observed, including *gag* start codon defects, small deletions in *gag*,

pol, and *env*, and stop codons resulting from frameshifts and hypermutation, all of which were detected in similar frequencies. Proviruses in the EAST group most commonly contained *gag* start codon defects and hypermutations while proviruses in the Adult group were dominated by MSD defects and packaging signal deletions. Deletions of *tat* were typical in proviruses with large deletions in all groups. In the EAST and Adults groups, deletions of *nef* were observed in the majority of the proviruses with large internal deletions (71-81%) but, in the EIT pre-ART and on-ART groups, a smaller fraction had deletions of *nef*, 36% and 55% respectively (**Figure 6D**). In the >7kb sequences, *tat* deletions were observed in 17-39% of the proviruses and *nef* deletions were uncommon.



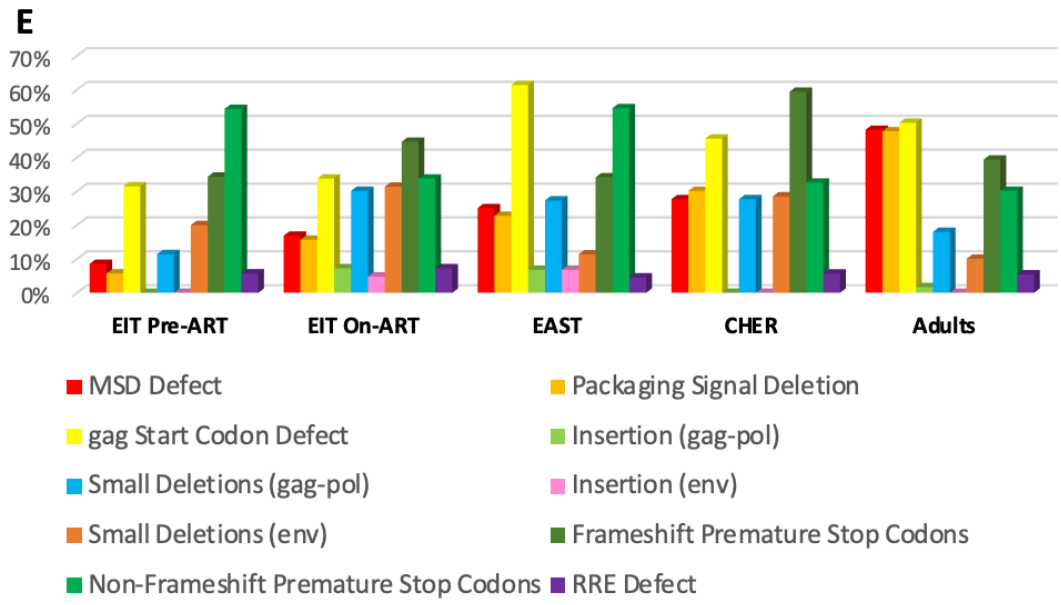


Figure 6: Proviral landscape comparison between EIT Pre-ART, EIT On-ART, EAST, CHER, and Adult proviruses. A. The fraction of intact and defective proviruses in the samples. Red: Defective <7kb. Orange: Defective >7kb. Green: Intact. B. The distribution of proviruses with large internal deletions. Blue: 3' deletions. Yellow: Central deletions. Red: 5' deletions. C. The number of defects per sequence. Maroon: 1 defect. Purple: 2 defects. Blue: 3 defects. Teal: 4 defects. Green: 5 defects. Brown: 6 defects. Dark pink: 7 defects. Orange: 8 defects. Light pink: 9 defects. D. The fraction of observed tat and nef deletions. Pink: tat deletion >7kb. Yellow: nef deletion >7kb. Red: tat deletion <7kb. Orange: nef deletion <7kb. E. The frequency of different types of defects. Red: MSD defect. Orange: Packaging signal defects. Yellow: gag start codon defect. Blue: Small deletions (gag-pol). Orange: Small deletion (env). Dark green: Frameshift premature stop codons. Light green: Non-frameshift premature stop codons. Purple: RRE defect.

Characterization of Intact Proviruses

Intact proviruses were only observed in 3 of the 4 donors from the CHER cohort (**Table 5**). None were found to carry drug resistance mutations. Geno2Pheno coreceptor tropism analysis revealed that all intact sequences likely utilize the CCR5 coreceptor. Similarly, no drug resistance mutations were found in the intact sequences from the EIT and EAST groups. However, several drug resistance mutations were observed in intact sequences from the Adults group including protease inhibitor (PI), nucleoside reverse transcriptase inhibitor (NRTI), and non-nucleoside reverse transcriptase inhibitor (NNRTI)

resistance-engendering mutations. All intact proviruses in all groups were predicted to utilize the CCR5 coreceptor, as was observed in the CHER cohort.

A phylogenetic tree was constructed from the CHER donor with the most intact sequences, ZA-011 (**Figure 7**). Of the 13, 4 identical sequences were observed. In the same rake, 3 sequences were found to be closely related with 3-4 nucleotide differences. In the other two donors no clones of intact sequences were found.

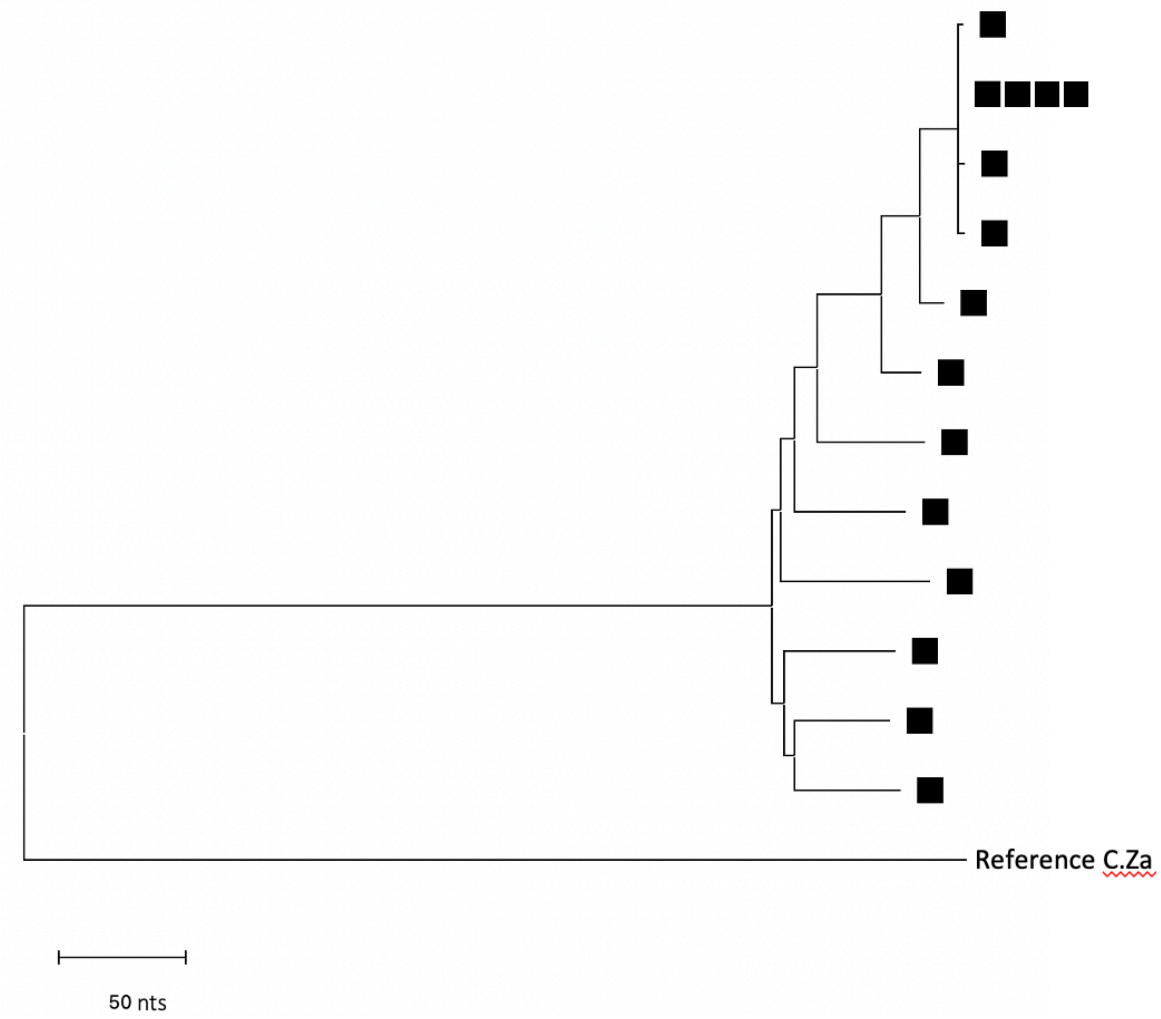


Figure 7: Neighbor-joining phylogenetic tree of intact proviruses detected in PBMCs from donor ZA-011. Each square represents a single provirus. The tree is rooted on a reference HIV-1 subtype C sequence from South Africa.

Reconstructing Inferred Intact Proviruses

Utilizing defective sequences from the CHER cohort, the predicted intact sequences of their ancestors could be reconstructed. Candidates for reconstruction were chosen based on their sequence content- sequences that were identical but contained non-overlapping deletions were aligned to reconstruct potential ancestors. Using this approach, a total of 3 potential ancestral HIV-1 variants from donor ZA-004 were reconstructed and all 3 sequences were inferred intact using the ProSeq-IT tool (**Figure 8A**). In donor ZA-012, 1 intact sequence was reconstructed from a pair of defective proviruses (**Figure 8B**). This approach is a unique method to characterize the genetics of the HIV-1 reservoir in donors on ART that may eliminate the need for deeper sequencing.

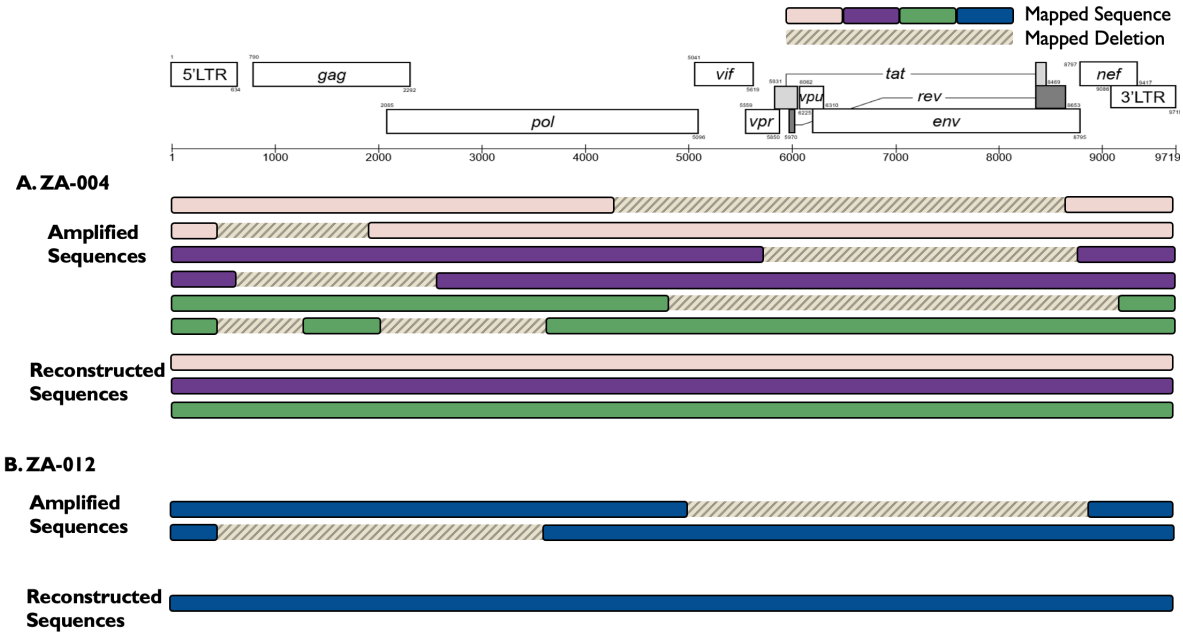


Figure 8: Reconstruction of intact ancestral viral genomes. A. 3 intact sequences reconstructed in donor ZA-004. B. One intact sequences reconstructed in donor ZA-012.

DISCUSSION

This study aimed to characterize the proviral landscape of children treated within 9 months of birth and on suppressive ART for up to 9 years and compare it to previously published data from children on shorter-term ART and from adults on long-term ART. The proviral population in these children was found to contain mostly defective proviruses, with only 3% of the viral genomes being intact. This finding is consistent with previously published data showing that only about 1% of proviruses in children and 0.2-2% in adults are sequence intact (Bruner et al. 2016; Heiner et al. 2017; Ho et al. 2013, Katusiime et al. 2020). However, in pre-ART samples from early treated infants on short-term ART (EIT), this fraction was found to be significantly larger at 54%. In EIT on-ART, 20% of HIV proviral sequences were intact. This difference in the fraction of intact proviruses correlates with time on ART, suggesting that the fraction of intact proviruses in children decreases over time on treatment as has been shown to occur in adults (Ganghi 2021; Peluso 2020).

Of the defective proviruses in the CHER cohort, the majority (82%) contained large internal deletions. Our analysis of previously published datasets found 46-71% to contain large internal deletions. Large internal deletions are thought to result from errors in reverse transcription (Abrams 2012). Interestingly, the majority of sequences in all datasets regardless of ART status, time of ART initiation, or duration of ART were found to have deletions in the 3' end of the genome encompassing part or all of *env* (88-93%). Furthermore, of the sequences with major internal deletions, in all four datasets, the majority contained a partial or whole deletion of *tat* (92-97%), the transcription factor used for viral expression. This finding suggests that there may be a selection pressure against proviruses with the ability to express genes in the 3' region.

Deletions in *nef* were also investigated as *nef* has been found to play a role in immune evasion, potentially allowing proviruses to persist on ART (brumme ref). In EIT pre-ART and on-ART sequences, deletions in *nef* were less frequent than in proviruses persisting from a longer duration of ART. This may be because while *nef* may aid proviral persistence, deletions of the 3' region of the genome including *tat* and *nef* are selected for because they also limit/prevent the expression of upstream genes.

The majority of defective sequences in pre-ART samples (27%) did not contain large internal deletions. Hypermutation by host APOBEC enzymes resulting in premature stop codons were the most common defects observed in these samples. The stop codons are the result of G-to-A mutations in the HIV DNA from the deamination of cytosines in the viral RNA induced primarily by APOBEC-3G (Keiffer et al. 2005 and Bruner et al. 2016). This observation suggests that APOBEC-3G may contribute to pre-ART control of HIV replication. Most sequences with multi-defects and high variance of defect types were found in the EIT on-ART proviruses, suggesting that the greatest change in the proviral landscape occurs after ART initiation. This may be because upon ART initiation, infected cells expressing viral proteins are rapidly culled from the population, shifting the population majority to those with defects.

In adults, mutations or deletions in the MSD, the packaging signal, and the *gag* start codon are common. The results of this thesis project demonstrates that defects in the *gag* start codon were also common in the EAST and CHER cohorts, suggesting that there is selection for proviruses over time on ART that are not able to express *gag*, potentially making a sub-genomic target of interest. This may be because early cellular detection of

the transcripts or protein leads to cell death, resulting in selection for viral genomes with small deletions spanning these regions.

The low viral genetic diversity in children who are born with HIV and treated with ART soon after birth may make them more susceptible to future curative interventions. Phylogenetic analyses indicate that there is very low viral genetic variance in some of these children, including a set of clonal sequences in donor ZA-011. These proviruses may be candidates for further study, such as testing of bNAB susceptibility and potential therapeutic vaccine targets. The detection and sequencing of defective proviruses in the children allowed for the reconstruction of inferred intact proviruses that may persist at levels or in tissues not sampled in this study. Initial analyses found an identical match between a reconstructed genome and its ancestor, supporting this idea. Reconstructed proviruses may provide targets for curative strategies in cases where replication competent proviruses are not found but may still be present at low levels or may emerge through recombination if ART is interrupted.

While this study provides further data and insight into the proviral landscape of early treated children, larger studies are needed to determine statistical differences across populations of donors. A limitation of this thesis research is the method used for NFL PCR amplification. A recent study comparing methodologies for HIV-1 reservoir characterization demonstrated amplification bias for differently sized amplicons (White 2022) with higher efficiency for amplifying proviruses with large internal deletions and a lower efficiency for amplifying intact proviruses. Future studies should control for this potential bias.

My findings provide valuable information for the design of new curative strategies for children with HIV-1 and on long-term ART, such as inferring the sequences of potential intact sequences that may persist below levels of detection and may be susceptible to broadly neutralizing antibodies or therapeutic vaccination. Future studies will genotype and phenotype such variants for their response to these interventions. Because deletions in the 3' half of the HIV-1 proviruses that persist on ART were most common, futures investigations into the diversity of *env* could shed light on other potential targets for future curative interventions.

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