Mechanisms for HIV persistence during effective treatment

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Abstract

Mechanisms for HIV persistence during effective treatment

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Despite antiretroviral therapy (ART) that suppresses HIV replication to below the limit-of-quantification of commercial assays, small numbers of virions can often still be detected in the plasma, termed residual viremia (RV). The cells producing infectious RV must be eliminated to cure the infection. The dynamics of RV production was characterized by determining viral C2V5*env* sequences from longitudinally-derived plasma specimens, comparing RV sequences to those from replicating viruses in pre-ART plasma, in quantitative viral outgrowth assays (QVOA) of virus present in $CD4^+$ T cells collected during ART, and in plasma from post-ART-interruption. Specimens from the time of acute infection and spanning more than 13 years of infection were examined from 3 participants in which ART was initiated after ~3 or more years of infection. Following ART-suppression for ~7 years, participants self-initiated ART-interruptions. RV was found to be composed of varying fractions (8-84%) of monotypic (i.e.,

clonally-derived) versus unique HIV genotypes that were most closely related to viruses produced throughout the pre-ART period, and these monotypic RV variants persisted for a median of 3 years (range:0.3-8.2) of ART-suppression. RV sequences were found to be identical to replicating viruses found during pre-ART and infectious variants induced in QVOA and rebound viremia (n=21/154 (14%)) across the 3 participants. The frequency of RV variants during ART was not always predictive of the composition to rebound viremia after ART-interruption. These findings are consistent with a dynamic “active” HIV reservoir during ART that can contribute to the rekindling of infection upon ART interruption. The persistence of monotypic RV variants over time suggests that the cells producing these virions either resist immune clearance or represent the occasional emergence from latency of only subpopulations of clonally proliferating, infected cell populations, which are only slowly cleared over time.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Chapter 1. INTRODUCTION

1.1 VIROLOGY AND LIFE CYCLE OF HIV

HIV-1 (HIV) is a retrovirus with two (+) single-stranded RNA genomes that encode 9 genes including 3 major genes: glycoproteins (***gag***), polymerase (***pol***) and envelope (***env***). Retroviruses are capable of reverse transcribing their RNA genome into complementary DNA (**cDNA**) using their virally-encoded reverse transcriptase (**RT**) enzyme encoded by the *pol* locus. The HIV RT is a highly error-prone polymerase with low fidelity [1], resulting in multiple mutations in the cDNA during the reverse transcription step [2, 3]. As a result of the errors introduced by the RT, every cycle of HIV replication may result in an average of 1-10 base pair (**bp**) mutations in the viral genome [4].

HIV virions primarily infect CD4+ T cells by attaching to the CD4 receptor and either the CCR5 and/or CXCR4 co-receptor [5]. Upon binding, the viral envelope fuses with the cell membrane and the HIV capsid carrying the RNA genome is delivered within the cell. During this process, reverse transcription of the HIV RNA begins and upon completion of the double stranded cDNA, the cDNA may be imported into the nucleus for further integration of the HIV genome within the human genome, persisting as a "provirus," and becoming a permanent component of the host genome [6]. Furthermore, host cell DNA replication and cell division allow the HIV genome to be carried into future daughter cells. HIV transcription from the provirus may occur, which results in viral protein production [7] in the cytoplasm and assembly of the HIV virion at the cell membrane. Upon assembly, the virion buds from the membrane and undergoes maturation, completing the infectious cycle.

1.2 ANTIRETROVIRAL THERAPY (ART)

Multiple drugs were developed to target different stages in the HIV life cycle to further block viral replication and release. These drugs are collectively referred to as antiretroviral therapy (**ART**) and can target viral fusion, reverse transcription, integration, viral transcription, and virion maturation (**Figure 1-1**). Individuals taking effective ART generally have suppressed levels of HIV RNA below the limit of detection of commercial assays (<50c/mL). ART is a life-saving intervention for millions of people worldwide and has extended the lifespan of many infected individuals, giving them the opportunity to live a normal life [8, 9]. However, despite the efficacy of these drugs, HIV is capable of developing mutations in the genome during reverse transcription that allow it to become resistant to one or all drugs. Furthermore, with multiple virions infecting cells, recombination of the independent viral genomes can result in even more genetic variation that could lead to drug resistance [4].

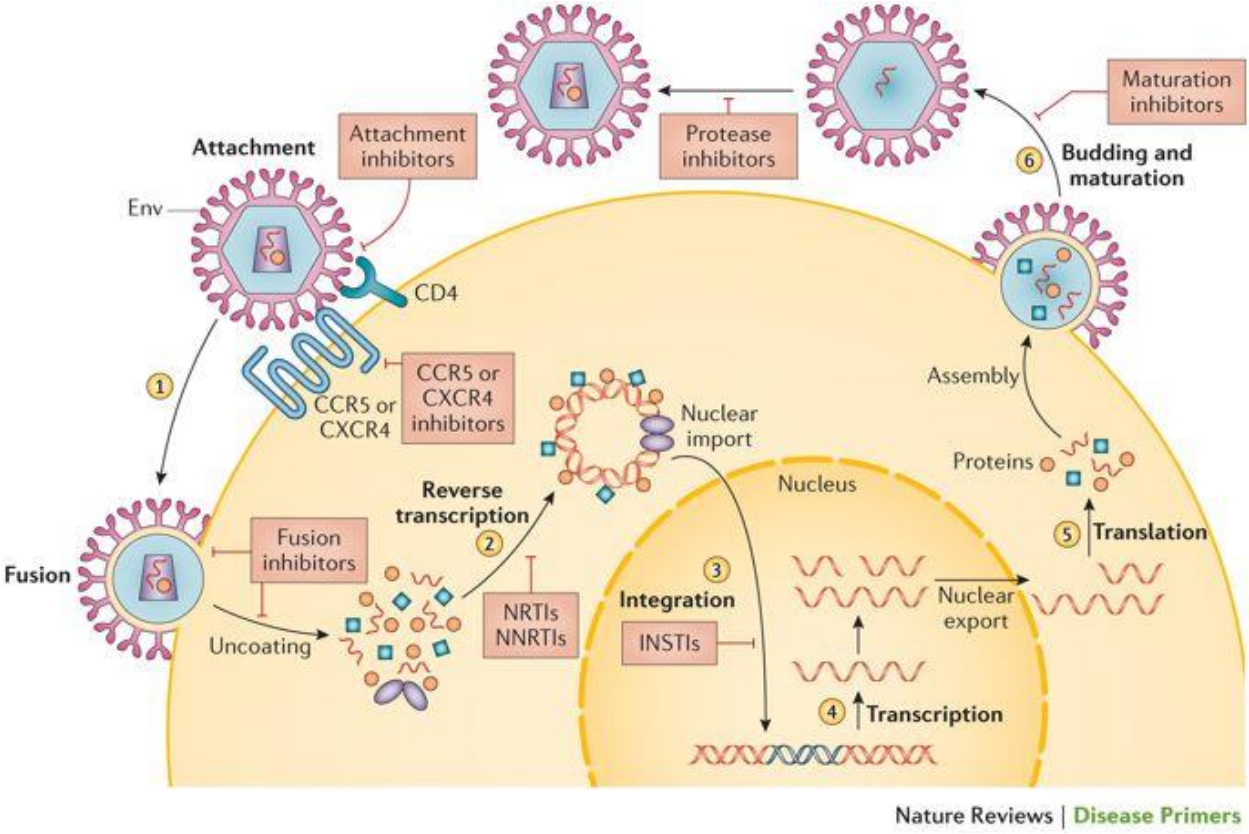


Figure 1-1: HIV life cycle and viral events inhibited by antiretroviral drugs

Retroviruses including HIV require attachment and binding to a CD4 receptor and a coreceptor such as CCR5 and/or CXCR4 as the first step of infection. Antiretroviral drugs such as CCR5 or CXCR4 inhibitors may interfere with this step and inhibit HIV attachment by binding to these receptors. Attachment is followed by fusion of the viral and host cell membranes resulting in delivery and uncoating of the viral capsid. Antivirals termed fusion inhibitors can interfere with this step. Subsequently, the viral RNA is reverse transcribed into cDNA, which can be inhibited by two classes of drugs with separate actions, the nucleoside and non-nucleoside reverse transcriptase inhibitors (**NRTI/NNRTI**). The double stranded viral cDNA can then enter the nucleus for integration into the host genome. Antivirals termed integrase strand transferase inhibitors (**INSTIs**), however, can interfere with the integration process. Upon integration, the viral genome can be transcribed and exported into the cytoplasm where it can be translated into viral proteins. Assembly of the viral proteins occurs in the cell membrane which can form budding virions that are released from the cell in the final step of the viral life cycle. During the release, the capsid undergoes maturation using the virally encoded protease enzyme which renders the virion capable of starting new rounds of infection. Drugs called protease or maturation inhibitors can interfere with this step of the viral life cycle. In general, current drugs block new infections but do not excise the provirus or block virus transcription and production from previously infected cells. Figure source [10].

1.3 HIV LATENCY

HIV that is integrated within the host genome and which can undergo viral transcription upon cell activation is referred to as a latent provirus [11]. Latency allows HIV to persist within the host, and reflects infected, activated cells that may have avoided host cytopathic effects and reverted to a resting state [12]. HIV latency represents a major block to an HIV cure, allowing the virus to survive for many years on effective therapy and remain hidden from immune detection by the lack of HIV transcriptional activity and protein expression. One of the current HIV cure strategies operates on the premise of latency reversal to enable infected cell death via a method called "kick and kill," whereby latently infected cells are activated to promote HIV transcription and further enable host immune detection and cytotoxic T lymphocyte (**CTL**)-mediated elimination [13]. This strategy, however, has had mixed success and was observed to be ineffective at reducing the number of infected cells *in vivo* [14, 15].

1.4 HIV RESERVOIR

One of the major obstacles to an HIV cure is persistence of intact proviruses as the latent HIV reservoir. The HIV reservoir is defined as immune cells harboring stably integrated, replication-competent HIV which may contribute to infectious virion production [16]. The HIV reservoir is in large part established during acute infection, as early as day 3 post-infection in macaque studies, and may be reversibly latent [16, 17]. Although resting memory CD4+ T cells contain the majority of the HIV reservoir in the blood [18, 19], other cell types can serve as a reservoir for HIV including naïve T cells (**Tn**) [18, 20], stem cell memory (**Tscm**) [21], central/transitional/effector memory T cells (**Tcm/Ttm/Tem**) [18, 19, 21], terminally differentiated (**Ttd**) [22], regulatory T (**Treg**) [23, 24], follicular helper T (**Tfh**) [25-27], monocytes/macrophages [28-30], and

CD3+CD4- T cells [31-33]. Infected CD4-expressiong cells have shown differential susceptibility to HIV infection and can differentially contribute to the infectious reservoir (**Figure 1-2**). Many of the cells making up the HIV reservoir are categorized as reversibly latent cells where upon cell stimulation, a latent provirus can be activated to transcribe and produce virions. During this stage of active virus production, the reservoir is referred to as the "active" reservoir which is a major driver of low-level virus production during effective treatment. A major block in HIV cure studies is the inability to reduce or eliminate the infectious HIV reservoir. Even long-term effective therapy is deemed highly unlikely to eradicate HIV due to the observation that the infectious reservoir appears relatively stable with decay of HIV estimated to be $t_{1/2}$ of 44 months [34], suggesting that the virus may be potentially eliminated after at least 70 years, or the average lifespan of many individuals. Given that it is not feasible to wait for at least 70 years, treatment options that eliminate the infectious reservoir are urgently needed.

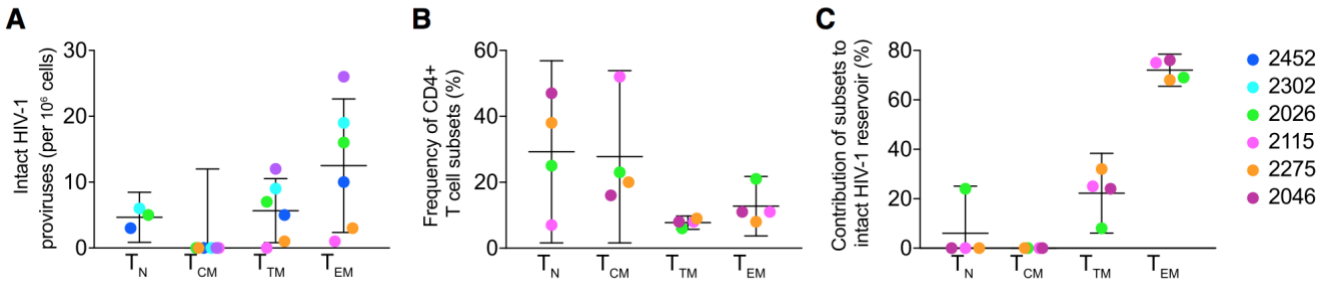


Figure 1-2: Differential contribution of peripheral immune cell subsets to the intact, infectious HIV reservoir in individuals undergoing long-term antiretroviral therapy

Examination of the total number of intact HIV proviruses per million cells among Tn, Tcm, Ttm, and Tem cells in 6 ART-suppressed individuals using a full-length proviral genome sequencing assay shows a higher frequency of intact viruses among Tem cells (**A**). Evaluation of the frequency of each T cell subset among total CD4+ T cells in the 6 individuals reveals Tn and Tcm subsets make up the majority of the total CD4+ T cell population in the blood (**B**). The contribution of each T cell subset to the infectious HIV reservoir reveals Tem contribute at least 70% of the infectious HIV reservoir relative to the other subsets (**C**). Figure source [35].

1.5 INFECTIOUS HIV RESERVOIR

During ART that suppresses HIV replication, it is estimated that 1 per 100-1,000 peripheral blood lymphocytes have an integrated provirus [36]. However, not all HIV cDNA result in proviruses, as some cDNA can form 1 or 2-LTR circles [37, 38], and not all proviruses are infectious [39]. This is primarily due to the process of reverse transcription and integration, which can lead to insertions, deletions, or recombination, resulting in defective proviruses [40]. Furthermore, host cellular proteins like apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (**APOBEC3G**) can create G-A hypermutations in the viral cDNA, which can lead to stop codons and a defective proviral genome [41].

One method used to measure the infectious reservoir is the quantitative viral outgrowth assay (**QVOA**) assay [42]. QVOA is an *in vitro* culture system involving serial dilutions of autologous infected total or resting CD4+ T cells co-cultured with irradiated feeder cells (**Figure 1-3**). Latent, infected cells can be activated to produce virions using anti-CD3/CD28 antibodies and cytokines such as IL-2. Virion production can then be measured longitudinally over a period of 28 days by sampling the supernatant for the HIV p24 antigen using an enzyme-linked immunosorbent assay (**ELISA**). The number of infectious units per million (**IUPM**) cells can then be calculated using statistical methods providing an estimated measurement of the infectious reservoir in cells.

The majority of proviruses in the HIV reservoir are defective. In fact, one study suggests that intact, infectious proviruses are limited to 1 per million peripheral CD4+ T cells [43]. Comparisons of various methods to measure the infectious reservoir revealed a stark difference between typical PCR methods and QVOA in accurately estimating the intact, infectious reservoir (**Figure 1-4**) [44, 45]. PCR methods tend to overestimate the true HIV DNA load by measuring the copy number of

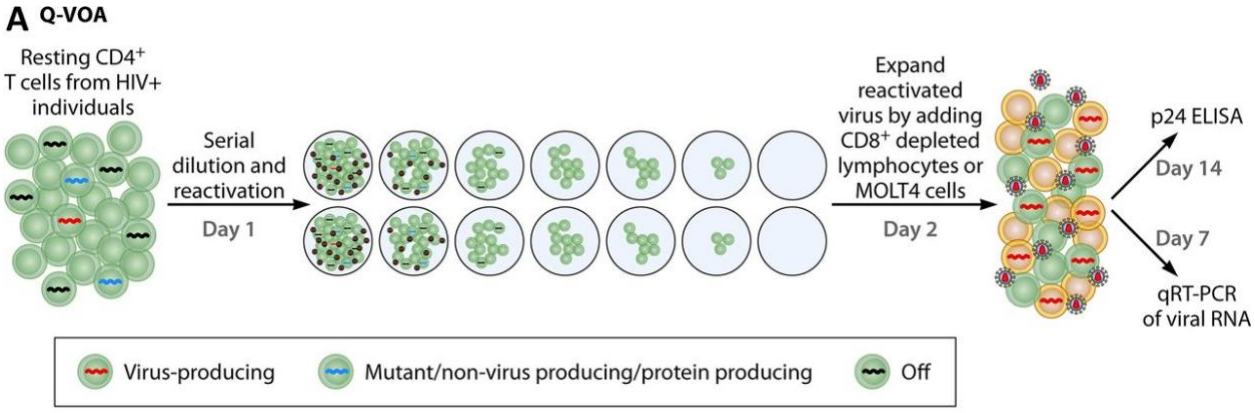


Figure 1-3: Overview of the QVOA protocol

Resting CD4+ T cells from HIV+ individuals are cultured *in vitro* after serial dilution and activation with added cytokines or co-stimulatory molecules. On day 2, the reactivated cells are expanded with autologous CD8+ depleted lymphocytes or feeder cells such as MOLT4. On days 7, 14, 21, or 28, the supernatant is sampled for viral RNA production using quantitative reverse-transcriptase-PCR (**qRT-PCR**) and for virion release by measuring the p24 antigen in an ELISA, respectively. Figure source [46].

1 gene, while deletions may exist beyond that gene that would not be detected [47, 48]. Conversely, QVOA underestimates the true intact infectious reservoir because most QVOAs are performed with one round of stimulation, while it was observed that multiple rounds of latent cell stimulation could enhance cell activation and allow for more virus production [49].

While defective proviruses may seem irrelevant for HIV cure studies, defective proviruses have been observed to transcribe proviral RNA at levels similar to intact proviruses [50] and may contribute to HIV-associated disease through production of novel proteins [7] proposed to contribute to immune activation/inflammation and other comorbidities [51]. Furthermore, defective proviruses can be recognized by CTLs and can shape the proviral landscape [52]. In fact, it was observed that defective proviruses can recombine to produce infectious virus [53, 54]. These findings suggest that defective proviruses, in addition to intact proviruses, can play a role in the pathogenesis of HIV despite deletions/mutations in the viral genome.

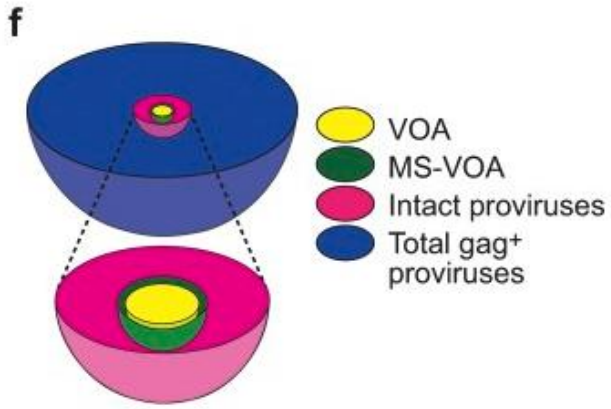


Figure 1-4: Multiple assays used to measure the HIV infectious reservoir do not reflect the true level of intact, infectious genomes

Current methods to measure the HIV infectious reservoir include viral outgrowth assays (VOA) and traditional PCR measuring HIV genes such as *gag*. Traditional PCR methods overestimate the true level of intact, infectious proviruses due to the presence of deletions outside of the PCR-amplified gag region, while VOA methods underestimate the infectious reservoir due to the inability of all latent, infectious proviruses to be stimulated after 1 round of stimulation. Even multiple stimulation-VOA (MS-VOA), which allows for a better estimate of the infectious reservoir than VOA due to multiple stimulation rounds activating more latently infected cells, underestimates the true level of infectious reservoir because some cells may harbor infectious provirus but still do not respond to stimulation. Figure source [44].

1.6 RESIDUAL VIREMIA DURING ART

While ART may limit viremia to below the limit of detection, it cannot block transcription from proviral DNA that can result in low-level production of virions [55, 56] (**Figure 1-5).** HIV-infected individuals on suppressive ART may still have "residual viremia (**RV**)" or low-levels of virus production, below the limit of detection of clinical assays, regardless of their ART regimen and level of adherence [57-61]. Furthermore, upon ART interruption, infected individuals usually have a rebound in viral replication, suggesting the persistence of an infectious HIV reservoir. Although individuals on ART may have improved life quality and longer life spans, residual viremias may be associated with adverse health outcomes including low CD4 counts, persistent immune activation and microbial translocation [62].

RV represents virus production from "active" reservoirs. The cells or cell clones producing the RV can remain stable for at least 7 years on ART [63, 64] and are unaffected by intensified treatment [57], suggesting that the persistent RV variants may be capable of evading immune detection/elimination [58, 65]. A median of 1.5 copies of residual virus can be found per milliliter of plasma during suppressive ART [57, 63], with residual viremia levels found to correlate with the size of the reservoir established before ART [57, 66]. Studies of RV sequences have generally not detected genetic evidence of accumulating mutations that would support full cycles of viral replication [57, 67]. However, monotypic RV sequences have been observed suggesting release from infected cells that are clonally expanding or the release of identical virions from the clonal cells [68]. The level of clonally expanding cells appears to increase over time of treatment [69, 70], suggesting these infected cells and the resulting RV released from them may be capable of resisting antibodies and/or CTLs. Many of these proliferating infected cells have been observed to

harbor potentially infectious viruses [44, 64, 68, 71-75], which suggests RV may be infectious and may contribute to rebound viremia when ART is suspended [50, 76].

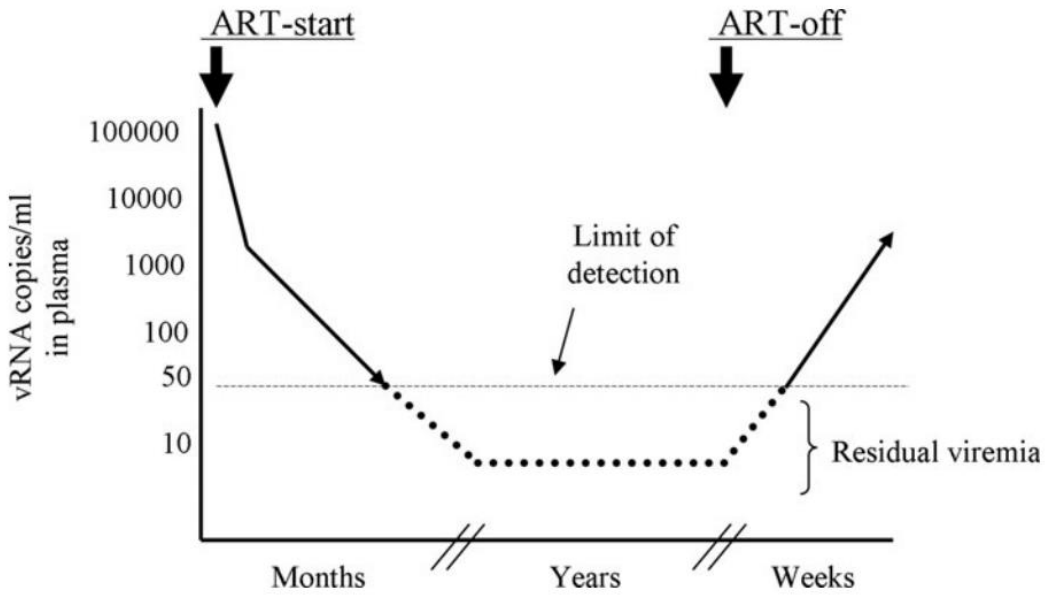


Figure 1-5: Residual viremia is commonly observed during suppressive ART

Residual viremia, or levels of HIV RNA below the limit of detection of clinical assays, is commonly observed in ART-treated individuals. With effective ART, HIV RNA copy number decreases below the limit of detection, but upon ART cessation, virus generally rebounds within a few weeks. Figure source [65].

1.7 PREVALENCE OF MONOTYPIC HIV RNA FROM VIRIONS COMPRISING RESIDUAL VIREMIA

Amplifying long fragments of HIV RNA present in the plasma at low concentrations is challenging. As a surrogate to sequencing the full HIV genome, which is ~10kb, most studies of RV have compared viral sequences of the hypervariable C2V5 region of *envelope* (***env***) or, for convenience, the region of polymerase (***pol)*** used to evaluate drug-resistance [44, 49, 50, 55, 64, 68, 73, 77-85]. Longitudinal studies of RV *pol* (546 bp) sequences over a period of 2.5 years of effective antiretroviral therapy found subpopulations of identical (i.e., monotypic) 546bp *pol* HIV sequences within 9 individuals [64] that comprised >50% of the total RV sequences [55, 64, 80, 81]; which was termed a predominant plasma clone (**Figure 1-6)**. More recent studies spanning 4 years of ART observed waxing and waning of genotypic variants found in the RV over time [73]. Residual viremia variants, including predominant plasma clones, have been genetically linked to replication-competent variants from QVOA, suggesting that monotypic variants may represent release from infectious proviruses that persist during ART [64, 75]. Longitudinal comparisons of RV sequences generally do not show genetic evidence of viral replication by divergence analysis from the founder virus [57, 67], and thus a current hypothesis is that RV represent active production and release of virions from infected cells, a process that is not impeded by current ART regimens [60, 86]. If this hypothesis is true, then the finding of monotypic RV sequences within individuals suggests that infected cells that persist and clonally expand since early infection [87] can produce RV [69], including potentially infectious viruses that may contribute to rebound viremia when ART is suspended [44, 50, 57, 64, 68, 69, 71-76]. The persistence of an active, proliferating reservoir that releases monotypic RV variants over years of effective treatment [81] suggests that cells producing these RV resist immune clearance, implying HIV cure strategies such

as “kick-and-kill” may be ineffective against the active reservoir, and underscores the need for newer strategies to target the elimination of the proliferating, persistent cells that give rise to the RV [88].

1.8 HIV PERSISTENCE

Multiple mechanisms may contribute to cells with infectious proviruses persistence including: 1) long-lived memory T cells, 2) anatomical ART sanctuaries, 3) immune escape and exhaustion [89], 4) homeostatic T cell proliferation, 5) antigen-induced T cell proliferation, and/or 6) integration site-induced T cell proliferation [19, 69, 90]. While some of these mechanisms may work synergistically to promote HIV persistence, recent findings from our lab and others suggest the integration site may drive cellular proliferation and persistence [69, 91-93].

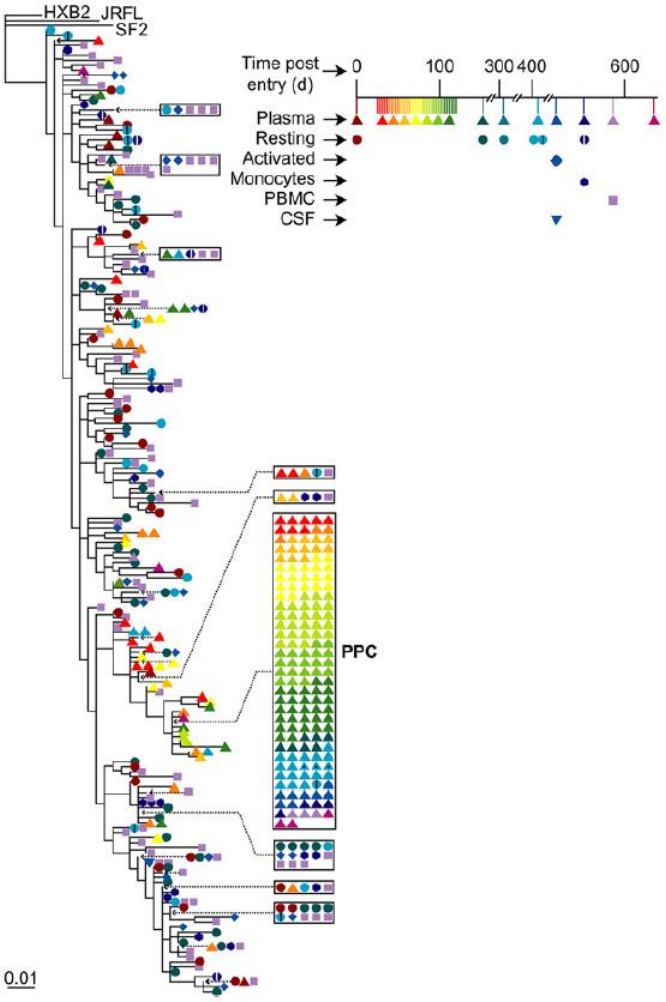


Figure 1-6: Residual viremia includes monotypic virions that persist over time in an individual initiating ART during chronic infection

Representative maximum likelihood phylogenetic tree showing the diversity among HIV *RT* (546bp) sequences from plasma viruses and proviruses sampled longitudinally over 600 days of ART suppression in 1 individual. Residual plasma virus was compared to resting CD4+ T cells, activated CD4+ T cells, monocytes, bulk PBMC, and cerebrospinal fluid (**CSF**). The presence of a predominant plasma clone is evident in this individual and this variant persisted for over 600 days and the *RT* sequence matched the proviral *RT* identified in resting CD4+ T cells at one timepoint. Figure source [64].

In ART-treated individuals, it was observed that HIV-infected cells can undergo cell proliferation, and that over time on ART, proliferating cells comprise an increasing proportion of the HIV reservoir [44, 69, 70, 72, 87]. Proliferation could also be driven by dysregulation of gene transcription or activation of proto-oncogenes as has been seen with other retroviruses like murine leukemia virus (**MLV**) [94, 95]. Indeed, it was shown that the site of HIV integration influences basal HIV transcription and inducibility by Tat [96]. In one HIV-infected individual with cancer, a single, infected cell clone was shown to make up >50% of the infected cell population and this clone was shown to be replication-competent [71], further supporting the role of clonal expansion in contributing to infectious virus production [71, 73, 74]. Since cancer is one of the leading causes of death for HIV-infected, ART-treated individuals [97], analyzing the impact of HIV integration into the human genome can aid efforts to understanding not only HIV persistence but HIV-associated morbidities as well.

1.9 HIV INTEGRATION SITES

After integration of the HIV genome within the host genome, the integration site (**IS**) becomes a unique identifier for infected cells due to the random nature for HIV integration into the human genome where it is highly unlikely that HIV integrates in the same spot in two different cells. Every new infection cycle and provirus formation generally results in a new and unique IS. HIV is predominately (80%) integrated in genic regions, specifically in introns [98-101]. Gene-dense regions and highly expressed genes are common sites for HIV integration [102]. There are tissue-specific differences in HIV integration where genes more highly expressed in one tissue had more frequent HIV IS [102]. Infected cells found to harbor the same IS and HIV sequence are referred to as clonally expanded cells due to cell proliferation. Biological pathway analyses of HIV IS in infected individuals revealed an enrichment for HIV integrations in genes involved with cancer

and cell cycle regulation [69, 91]. These findings led to the hypothesis that the site of HIV integration may impact cell functions and promote cell proliferation and persistence during ART by dysregulating host gene expression [69, 91, 103, 104]. Similarly, HIV integration into genes promoting immune evasion/dysregulation (i.e., genes involved in MHC I antigen processing, Treg function) may extend the longevity of the infected cell [104].

HIV IS location and orientation relative to a nearby gene can affect proviral expression and HIV latency during ART [105-107]. Indeed, it was observed that major changes in HIV expression and HIV *tat* inducibility can occur due to the IS and not the chromatin environment such as DNA methylation or histone acetylation [96]. Studies show the integration site can affect both proviral and host gene expression due to cis-regulation by the HIV *LTR* promoter [104, 108]. With respect to proviral expression, the site of HIV insertion can impact proviral transcriptional activity and cause up to a ~1000-fold difference in expression levels; underscoring the relevance of the IS in affecting proviral expression and latency [109, 110]. Integration in genes can promote transcriptional interference, which can result from convergent, tandem, or divergent promoters [111]. HIV LTR can act as a promoter and depending on the site and proviral orientation, the target gene expression could be up or downregulated [112-114]. In fact, retroviral integration can affect expression of genes even if the gene is several hundred kilo-bases away from the provirus [112]. One study demonstrated that expression of genes upstream of a provirus can interfere with HIV transcription from the 5' LTR [115], which explains why proviruses integrated into highly expressed genes are associated with lower HIV expression [105]. However, removing the interference caused by the upstream promoter by inhibiting upstream transcription or by activating viral transcription initiation and elongation, enabled HIV transcription and reversion of latency [115]. Convergent transcription, whereby HIV provirus and host gene transcription machinery

collide, can lead to the formation of dsRNA and RNA interference, further promoting latency [116]. Integration can have the effect of activating alternative promoters to upregulate expression of splice variants as shown for MLV [113] and HIV [104] integration into BACH2. This former study found that MLV integration into BACH2, a transcription factor, caused downregulation of BACH2 expression and further contributed to B-cell lymphomagenesis [113]. The latter study found HIV integration into BACH2 and STAT5b in Treg cells, which are associated with cell proliferation, caused insertional activation of chimeric mRNAs that promoted infected cell proliferation [104]. Similar findings were reported by others using cell lines where Epstein Barr virus (**EBV**) integration in intron 1 of BACH2 led to a loss of BACH2 expression and promotion of lymphomagenesis [117]. Collectively, these studies show that integration of HIV can activate proto-oncogenes or repress tumor suppressor genes.

Not only does the site of HIV integration affect proviral expression and latency, but the proviral orientation also appears to affect gene expression. Multiple studies have reported an HIV integration orientation bias relative to the gene or chromosome [98, 105], with the majority of HIV integrations found in the reverse orientation relative to the gene while others found no orientation bias [118]. One study demonstrated that a latently infected primary CD4+ T cell line had significantly more proviruses in the forward orientation than acutely, persistently infected cells which had a bias for the reverse orientation [105]. Forward integration relative to the gene can boost proviral transcription >10 fold, while reverse integration could decrease expression by 4-fold [106]. These findings have implications for infected cell persistence and potential immune detection and elimination. The proviral orientation may promote or inhibit host and HIV expression causing potential recognition by CTLs or promoting latency [104]. In summary, by changing the transcriptional landscape of an infected host cell, the site and orientation of HIV

integration may promote persistence of the infected cell further affecting cell cycle regulation, apoptosis, immune detection, and ultimately proviral latency [96, 119, 120].

1.10 UNANSWERED QUESTIONS

Due to the persistence of HIV reservoirs that include infectious variants, HIV-infected individuals must remain on ART for their lifetime. ART is not only costly, but it can cause acute and chronic adverse reactions. Novel treatment strategies are urgently needed to target the persistent reservoir. Residual viremia during ART appears to provide a "window" into the active HIV reservoir allowing for the identification of viral variants that are being actively expressed from the HIV reservoir, and a subset of these appear infectious. It is unclear if the expression of viral variants detected as RV is impacted by treatment interruption and whether the RV are infectious viruses that contribute to rebound viremia upon ART interruption.

1.11 AIM AND HYPOTHESIS

Since proliferating cells have been shown to increase over time on ART and can harbor infectious virus [44, 50, 68, 69, 71-74], I aim to examine if RV originate from these proliferating cells. Given that infected cell clones that appear to defy immune clearance can produce RV [121], I aim to examine whether how long RV variants can persist over time and whether the frequency of specific viral variants in the plasma is associated with persistence. I am also interested in determining when the cells carrying the persistent proviruses contributing to the RV were infected "seeded" with HIV. Furthermore, I aim to determine whether the RV produced over time represent potentially infectious viral variants that can rekindle infection upon ART interruption. Examining these questions may provide evidence for useful targets and strategies for HIV cure studies by allowing

for targeted interference of the proviruses associated with persistent RV production. Therefore, my study aim is to:

Aim: Evaluate the longitudinal stability and infectivity of residual virus during long-term ART and after ART interruption

Aim hypothesis: RV will include multiple HIV variants that fluctuate over time with some monotypic variants persisting over time and these variants will be linked by identical C2V5 env genotypes to infectious viruses in plasma pre-ART, in QVOA and contribute to virus rebound upon ART interruption.

Chapter 2. HIV RESIDUAL VIREMIA DYNAMICS DURING LONG-TERM ART

2.1 INTRODUCTION

Antiretroviral therapy (ART) has modified the course of human immunodeficiency virus type-1 (HIV) infection from a largely fatal disease to a chronic, treatable infection with a near-normal lifespan [8, 9]. Treatments for HIV infection continue to improve, allowing for less frequent dosing of medicines and fewer adverse reactions. However, despite ART-suppression of HIV replication for decades, ART does not cure the infection [122, 123]. HIV persists and if ART is interrupted, the infectious variants resume replication. Given that these infectious variants must be eliminated to cure the infection, many studies have focused on understanding the establishment of this reservoir [17, 34, 43, 124] and the mechanisms that allow it to persist [19, 51, 69, 91]. Studies have revealed essentially stable levels of HIV DNA in the blood [44], although viral variants within the HIV reservoirs are dynamic [73], with the complexity of the reservoir decreasing over time, consistent with replacement with clonally proliferating cells [68, 69]. These cells harbor mostly defective viruses [35, 39, 45, 52, 71, 72, 74, 87, 125], and those with infectious proviruses progressively diminish in frequency during effective ART [70, 126], presumably due to lytic infection or elimination by cytotoxic-T-lymphocytes (CTL). Exceptions include intact proviruses integrated in regions of the genome that are rarely transcribed [126, 127], which are hypothesized to be “deeply” latent.

During ART-suppression, not all proviruses persist in a latent state. Among infected individuals with plasma HIV RNA levels below the limit-of-quantification of clinical assays (<20-50 copies per milliliter), virions are produced (mean 1.5 copies per milliliter), termed residual viremia (RV) [57, 77]. RV is of interest as it can arise from proviruses capable of

making virions that have been genetically-linked to replication-competent variants from quantitative viral outgrowth assays (QVOA) [64, 75]. Analyses of RV, therefore, can provide insights to the persisting reservoir that escape clearance by lytic infection or immune elimination.

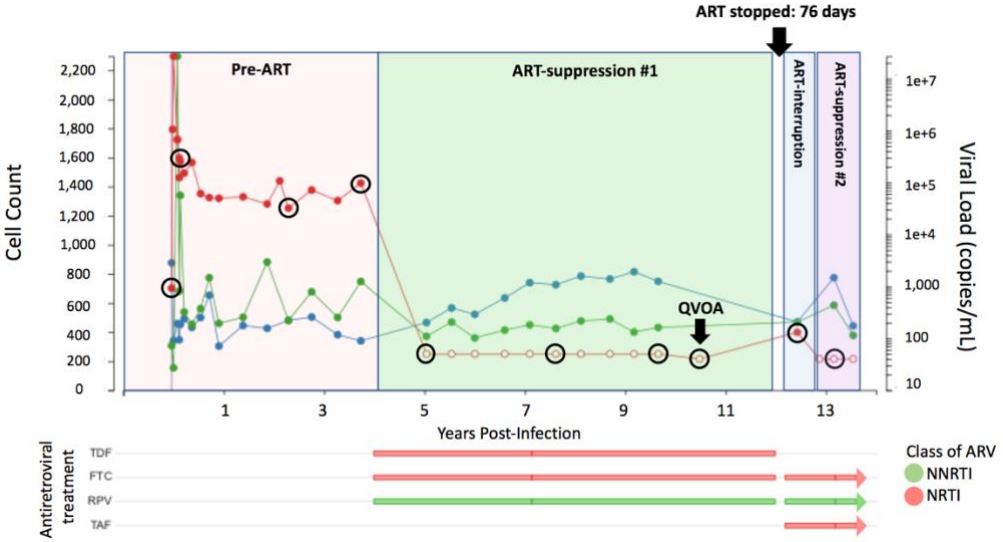
RV has been found to include identical (i.e., monotypic) HIV *env* and *pol* sequences [64, 75, 78] that generally do not diverge from those found immediately pre-ART [57, 67]. Thus, RV is thought to arise from a subset of cells infected before the time of ART-initiation, that persist, proliferate, and produce virions during ART [60, 86]. While current treatment regimens do not prevent the production of virions [57], they do block these virions from productively infecting additional cells [60, 68, 77, 86]. While RV variants can vary over time [64, 73], monotypic RV *pol* variants can comprise >50% of total viral sequences at multiple timepoints and have been reported to persist for up to 2.5 years of effective ART [64, 73]; suggesting that not all RV are effectively killed by cytopathic effects or targeted for immune elimination.

The recognition that HIV-infected cells that proliferate during ART [68, 69, 77, 87, 91] produce infectious RV [64, 75] emphasizes their importance as a barrier to curing HIV infection. To better understand this "active" reservoir, we studied RV in individuals followed since the time of acute infection, evaluating longitudinal specimens from prior to and during a prolonged period of ART, and following ART-interruptions. To inform the design of effective cure strategies, our study focused on the duration that specific monotypic RV were produced and the genetic linkage of RV to potentially infectious HIV variants replicating prior to ART, found in QVOA of total $CD4^+$ T cells after prolonged ART-suppression, and to plasma variants detected following ART-interruption.

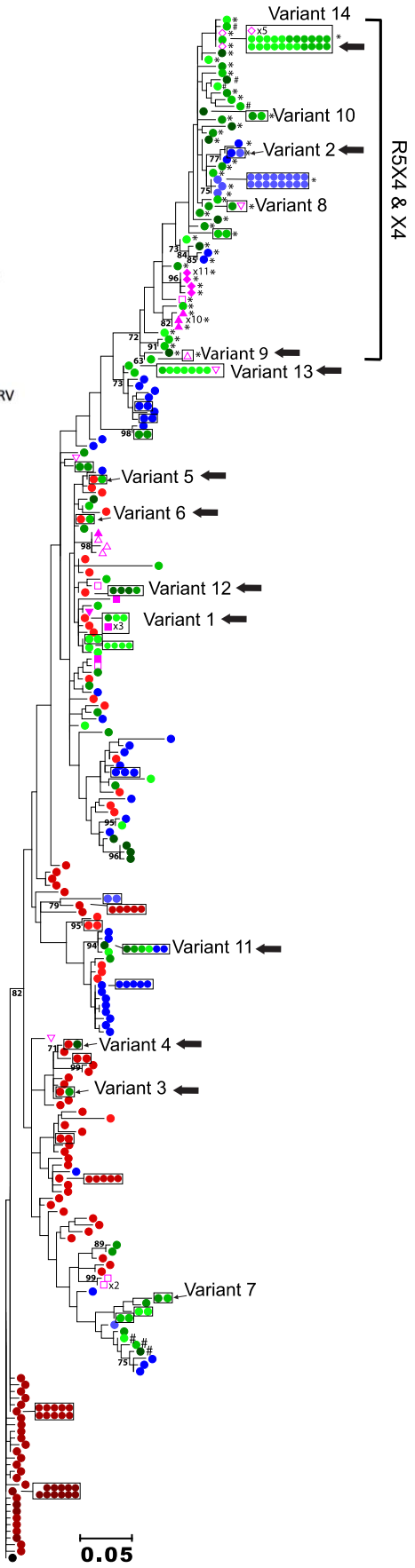
2.2 STUDY DESIGN AND PARTICIPANT CLINICAL HISTORY

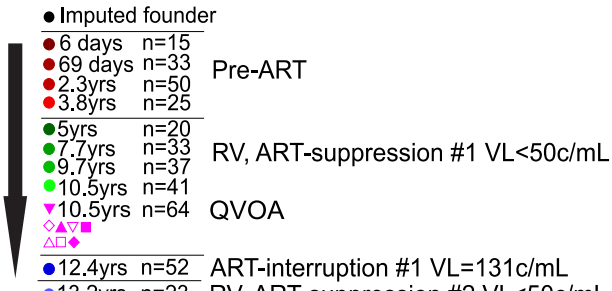
Virions present in RV were genetically characterized by sequencing of single viral amplicons (or single genome sequencing, SGS) derived from the C2V5 region of the HIV *env* gene (C2V5*env*) from three participants followed since the time of acute HIV infection for 13.2, 13, and 13.7 years. Three men, Participants 83747 (hereafter referred to as "1"), 59530 ("2"), and 50877 ("3"), respectively), from the Seattle Primary Infection Cohort met inclusion criteria for this study: 1) initiating ART during chronic infection, 2) maintained suppression of viremia at <50 copies/mL of viral RNA for ≥5 years, and 3) ART-interruption followed by ART-re-initiation (**Figure 2-1A, C, E**). Participants 1-3 were enrolled in the study during Fiebig stages I, I/II, IV and were diagnosed at the ages of 31, 46, and 33, respectively. ART was initiated during chronic infection after 4, 2.9, and 3.6 years of infection when they had $CD4_+$ T-cell counts of 340, 287, and 643 cells/uL, respectively. They were ART-suppressed (VL<50c/mL) for 8, 7.6, and 6.8 years prior to self-initiated ART interruptions spanning 76 days, 20-31 days, and 3 years, respectively, and subsequent re-initiation of ART.

A

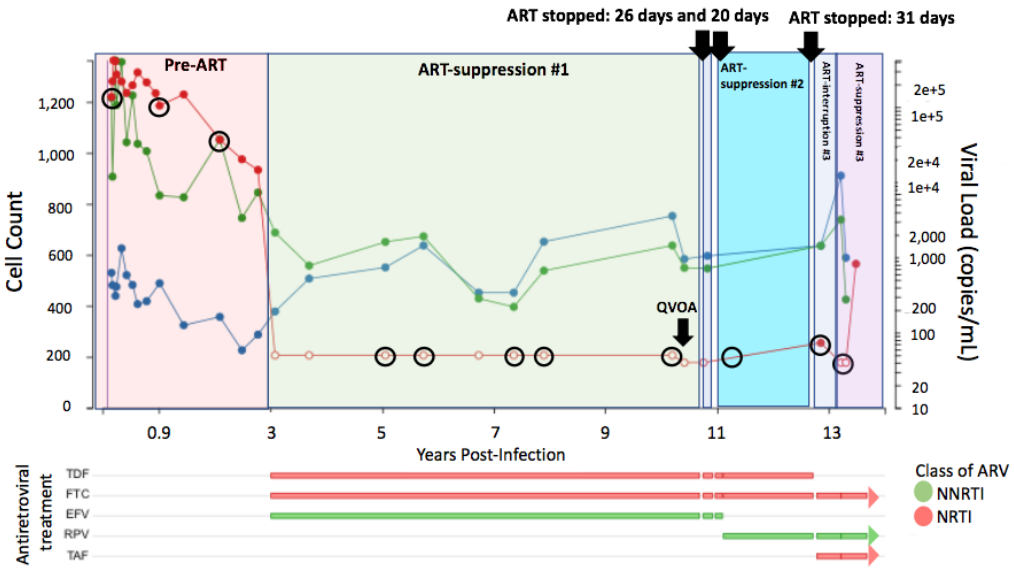

ART stopped: 76 days
Pre-ART
ART-suppression #1
ART-interruption
ART-suppression #2
QVOA
Cell Count
Viral Load (copies/mL)
Years Post-Infection
Antiretroviral treatment
TDF
FTC
RPV
TAF
Class of ARV
NNRTI
NRTI

B

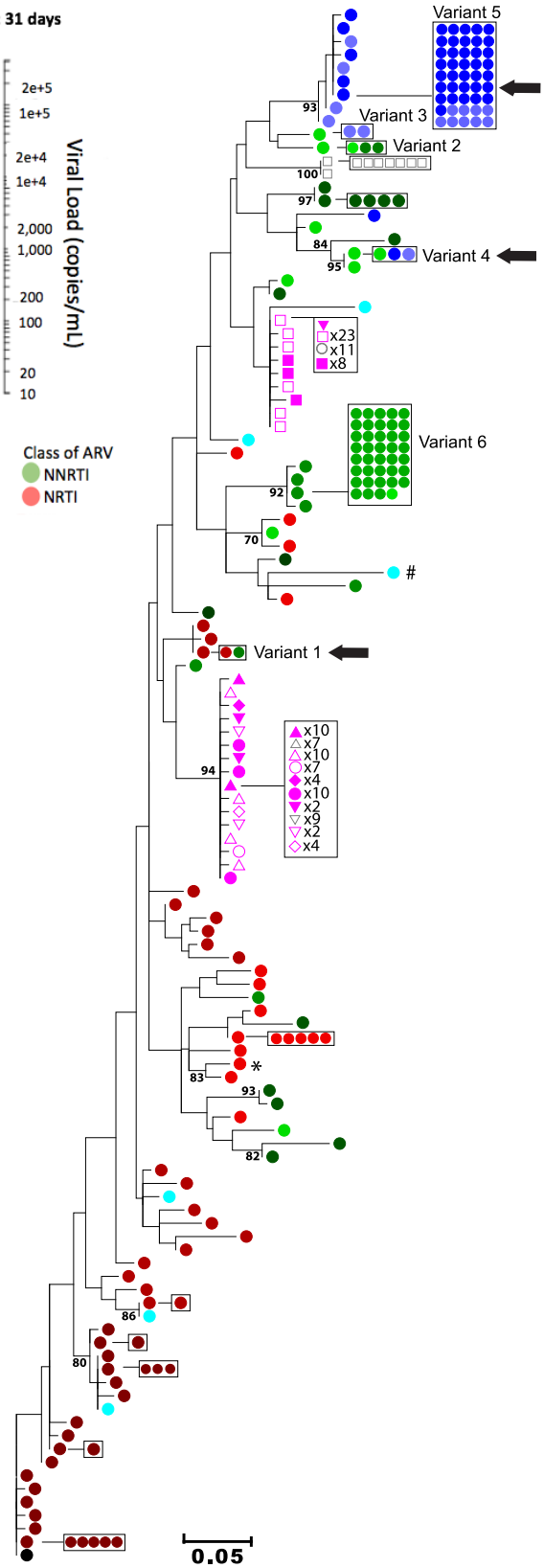

Variant 14
Variant 10
Variant 2
Variant 8
R5X4 & X4
Variant 9
Variant 13
Variant 5
Variant 6
Variant 12
Variant 1
Variant 11
Variant 4
Variant 3
Variant 7
0.05


Imputed founder
6 days n=15
69 days n=33
2.3yrs n=50
3.8yrs n=25
Pre-ART
5yrs n=20
7.7yrs n=33
9.7yrs n=37
10.5yrs n=41
RV, ART-suppression #1 VL<50c/mL
10.5yrs n=64
QVOA
12.4yrs n=52
ART-interruption #1 VL=131c/mL
13.2yrs n=23
RV, ART-suppression #2 VL<50c/mL

C



D



- Imputed founder
- 28 days n=26
- 0.9yr n=21
- 2yrs n=17

Pre-ART

- 4.9-5.6yrs n=19
- 7.3-7.8yrs n=45
- 10.1yrs n=10
- 10.2yrs n=158

RV, ART-suppression #1 VL<50c/mL

QVOA

- 11.1yrs n=6

RV, ART-suppression #2 VL<40c/mL

- 12.8yrs n=43

ART-interruption #3 VL=73c/mL

- 13.1yrs n=16

RV, ART-suppression #3 VL<40c/mL

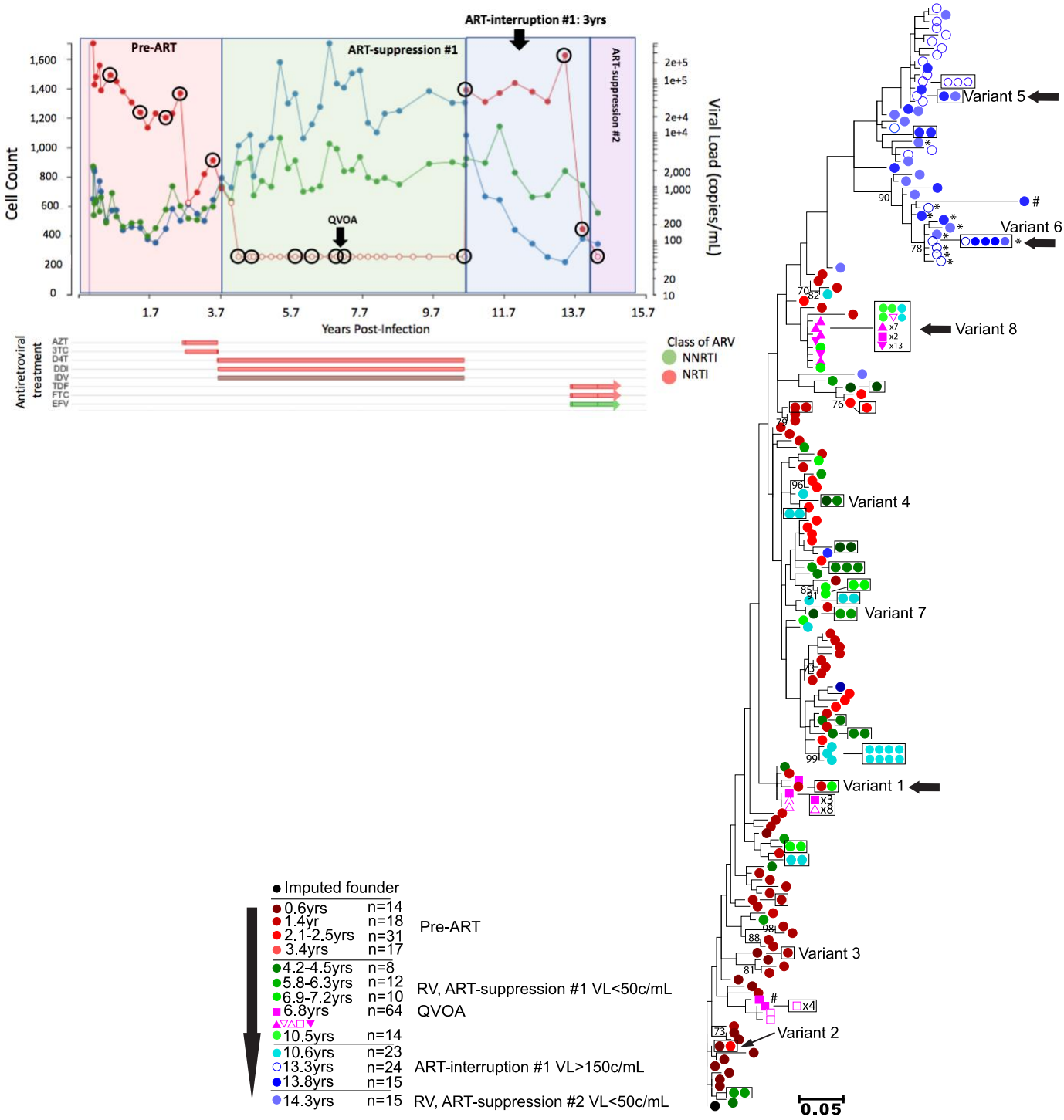


Figure 2-1: HIV loads, CD4/8 T-cell counts, antiretroviral treatments of 3 study participants and phylogenetic analyses of HIV C2V5*env* gene sequences from viruses in their plasmas and induced in QVOA.

Each participant's plasma HIV RNA values (red), $CD4_+$ (blue), and $CD8_+$ (green) T-cell counts, antiretroviral treatment across time (x-axis) and phylogenetic analyses are shown in separate

panels (Participant 1 (**A,B**), Participant 2 (**C,D**), and Participant 3 (**E,F**)). Plasma HIV RNA loads (**VL**) are filled with red when quantifiable and open circles when below the lower-limit-of-quantification (**LLOQ**); 40-50c/mL, depending on clinical assay employed. Timepoints selected for single genome amplification (**SGA**) of HIV C2V5*env* from plasma have red circle of VL encircled in black and QVOA indicated by a black arrow. Antiretrovirals (**ARV**) used for treatment and time intervals prescribed are shown by horizontal bars at the bottom. The durations of self-initiated ART-interruptions are indicated by black arrows at top of each panel. HIV C2V5*env* SGA sequences from the longitudinal plasma viruses and viruses induced from QVOA from the 3 participants were assembled into a maximum likelihood phylogenetic tree rooted with the consensus sequence of each participant's founder virus (consensus sequence of 1st pre-ART timepoint). The key indicates the color code for the time that specimens were collected from the estimated date of HIV infection, the antiretroviral treatment status, and the number of sequences derived from plasma or QVOA. Viral sequences from each unique QVOA well are represented by different symbols. Identical (monotypic) sequences are shown laterally and boxed for clarity. Significantly G-A hypermutated sequences are noted by #. CXCR4 and dual-tropic (X4) sequences are noted by *. Remaining sequences are predicted to be CCR5 (R5)-tropic, except for all sequences in the uppermost marked clade in Participant 1 which are X4/dual-tropic. Residual viremia (**RV**) sequences identical to sequences from replicating, potentially infectious, viruses are shown with a horizontal black arrow, including those from pre-ART plasma, viruses induced from QVOA cultures, or ART-interruption. Clades with bootstraps >70 are shown.
Abbreviations: ARV: antiretroviral; AZT: zidovudine; DDI: didanosine; EFV: efavirenz; FTC: emtricitabine; 3TC: IDV: indinavir**;** lamivudine; NNRTI: non-nucleoside reverse transcriptase inhibitor; NRTI: nucleoside reverse transcriptase inhibitor; RLP: rilpivirine; D4T: stavudine; TAF: tenofovir alafenamide; TDF: tenofovir.

2.3 Quantification of residual viremias and infectious viruses during ART

Plasma specimens, each totaling 1-21 mL, were used for analysis. These included from "pre-ART" from 3-5 timepoints, during "ART-suppression" from 4-7 timepoints when plasma HIV RNA was <50c/mL, from "ART-interruption" when the participant was viremic at >50c/mL at 1-3 timepoints following suspension and shortly after re-initiation of ART, and a second period of "ART-suppression" from 1-2 timepoints after plasma HIV RNA was again <50c/mL (**Figure 2-1A, C, E**). Negatively selected $CD4_+$ T cells collected 6.6, 7.3, and 3.1 years following ART-initiation were used in a QVOA for 28 days (**Figure 2-2**), with C2V5*env* SGS derived from p24-antigen (+) wells.

The total number of SGS derived from these specimens are shown in **Table 2-1**. Plasmas collected during ART-suppression yielded a mean of 3.3, 1.8, 1.5 SGS/mL from Participants 1-3, respectively (**Figure 2-3**). For quality control, all sequences were included in a maximum likelihood phylogenetic tree that showed a lack of cross-contamination between the 3 participants' specimens or from extraneous sources of viruses in our laboratories (**Figure 2-4**).

Table 2-1: Number (%) of plasma- and QVOA-derived HIV C2V5*env* SGA sequences derived from participants by antiretroviral status

	Pre-ART (VL: 923-1.4 million c/mL)				RV, ART-suppression #1 (VL<50c/mL)									ART-interruption (VL: 73-268,940c/mL)				RV, ART-suppression #2/3 (VL<50c/mL)			
					Plasma				QVOA			Linked infectious viruses									
Participant	Total plasma	Unique plasma	# (%) monotypic	# (%) unique X4-tropic	Total plasma	Unique plasma	# (%) monotypic	# (%) unique X4-tropic	Total QVOA	Unique QVOA	# (%) unique QVOA X4 tropic	# (%) unique RV linked to infectious pre-ART	# (%) unique RV linked to infectious QVOA	# (%) unique RV linked to infectious rebound	Total plasma	Unique plasma	# (%) monotypic	# (%) unique X4-tropic	Total plasma	Unique plasma	# (%) monotypic	% unique X4-tropic
1	123	87	36 (29%)	0	131	75	56 (43%)	41 (55%)	64	28	10 (36%)	6 (8%)	5 (6%)	2 (3%)	52	41	11 (21%)	7 (17%)	23	5	18 (78%)	4 (80%)
2	64	47	17 (27%)	1 (2%)	74	25	49 (66%)	0	158	27	0	1 (3%)	0	2 (6%)	43	7	36 (84%)	0	22	10	12 (55%)	0
3	80	74	6 (8%)	0	44	26	18 (41%)	0	64	15	0	1 (3%)	1 (3%)	3 (8%)	62	38	24 (38%)	7 (18%)	15	13	2 (13%)	3 (23%)

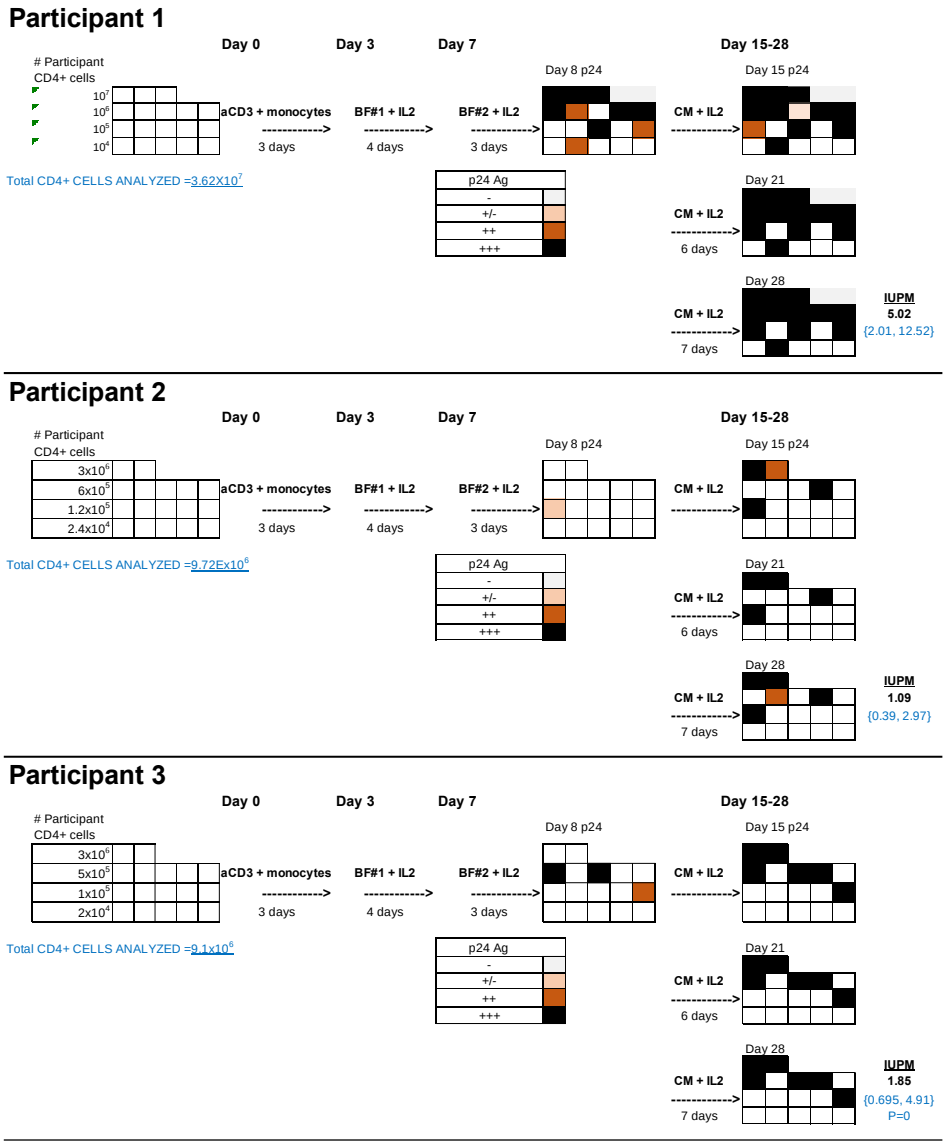


Figure 2-2: Schema of quantitative viral outgrowth assay (QVOA) workflow and results for each participant.

Panels show QVOA results for each Participants 1 (Panel **A**), 2 (**B**), and 3 (**C**). Co-cultures utilized total negatively-selected CD4+ T-cells that, following serial dilution, were co-cultured with monocytes from a healthy, HIV-seronegative donor, activated with anti-CD3 and maintained with IL-2 in culture for up to 28 days. HIV wells yielding virus were detected by testing supernatants for p24 antigen (Ag) by ELISA on days 8, 15, 21, and 28. The infectious units per million (IUPM) cells and 95% confidence intervals were calculated at day 28 of culture. Colors indicate semi-quantitative amount of p24Ag detected and show that in Participant 2, extension of the culture for 28 days increased number of wells yielding viruses. Abbreviations: CM: culture media. BF: PHA stimulated CD8-depleted PBMC 72hour Blast Feed.

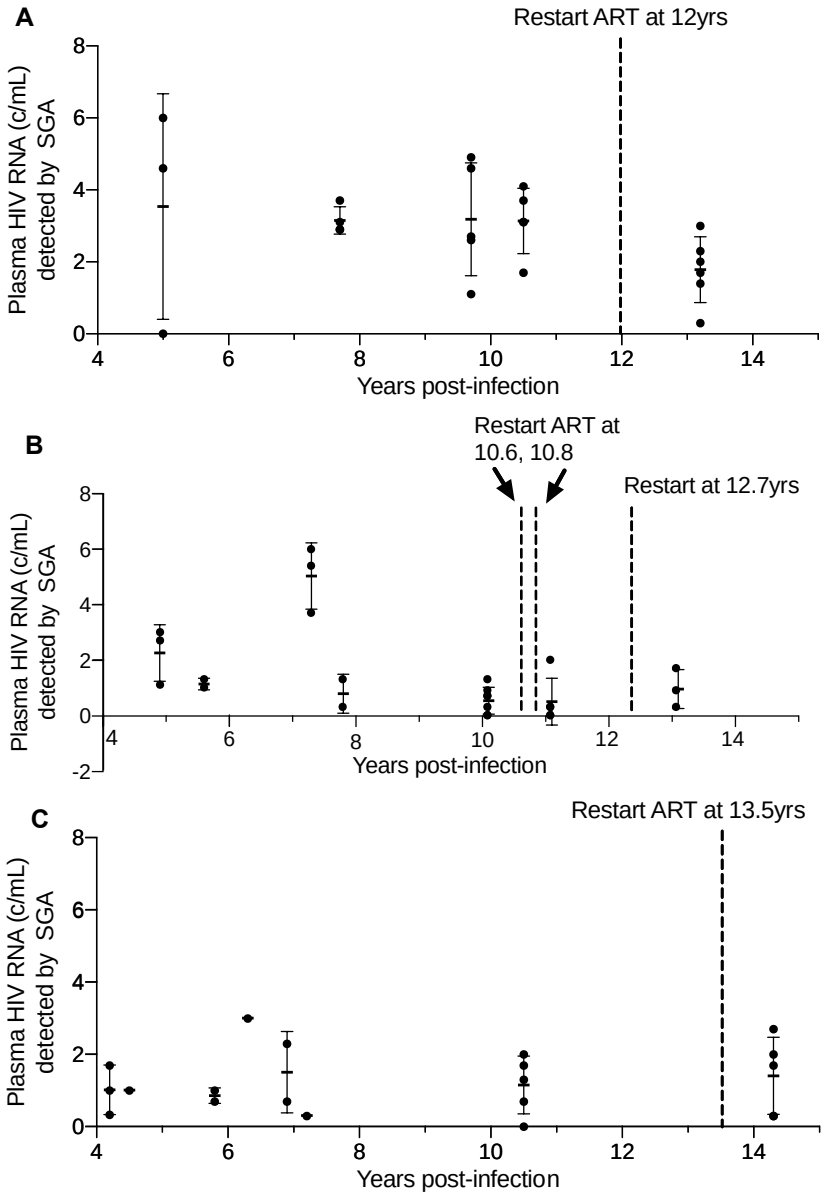


Figure 2-3: Mean HIV virion concentrations in plasma specimen analyzed at each timepoint

Each panel shows mean concentration of virions (y-axis) estimated in plasma specimens during ART-suppression (VL<50c/mL) analyzed from Participants 1 (**A**), 2 (**B**), and 3 (**C**) at indicated timepoints (x-axis) following the estimated date of HIV infection. Data points within a timepoint represents a different aliquot. Each data point represents the total number of C2V5*env* sequences derived by single genome amplification divided by the volume of each plasma aliquot, with means and standard deviations across aliquots from each timepoint shown. Levels of residual viremia were relatively consistent within each participant over time, except for one outlier timepoint in Participant 2 at 7.3 years of infection that yielded a disproportionately large monotypic population and an outlier timepoint in Participant 3 at 6.3 years of infection.

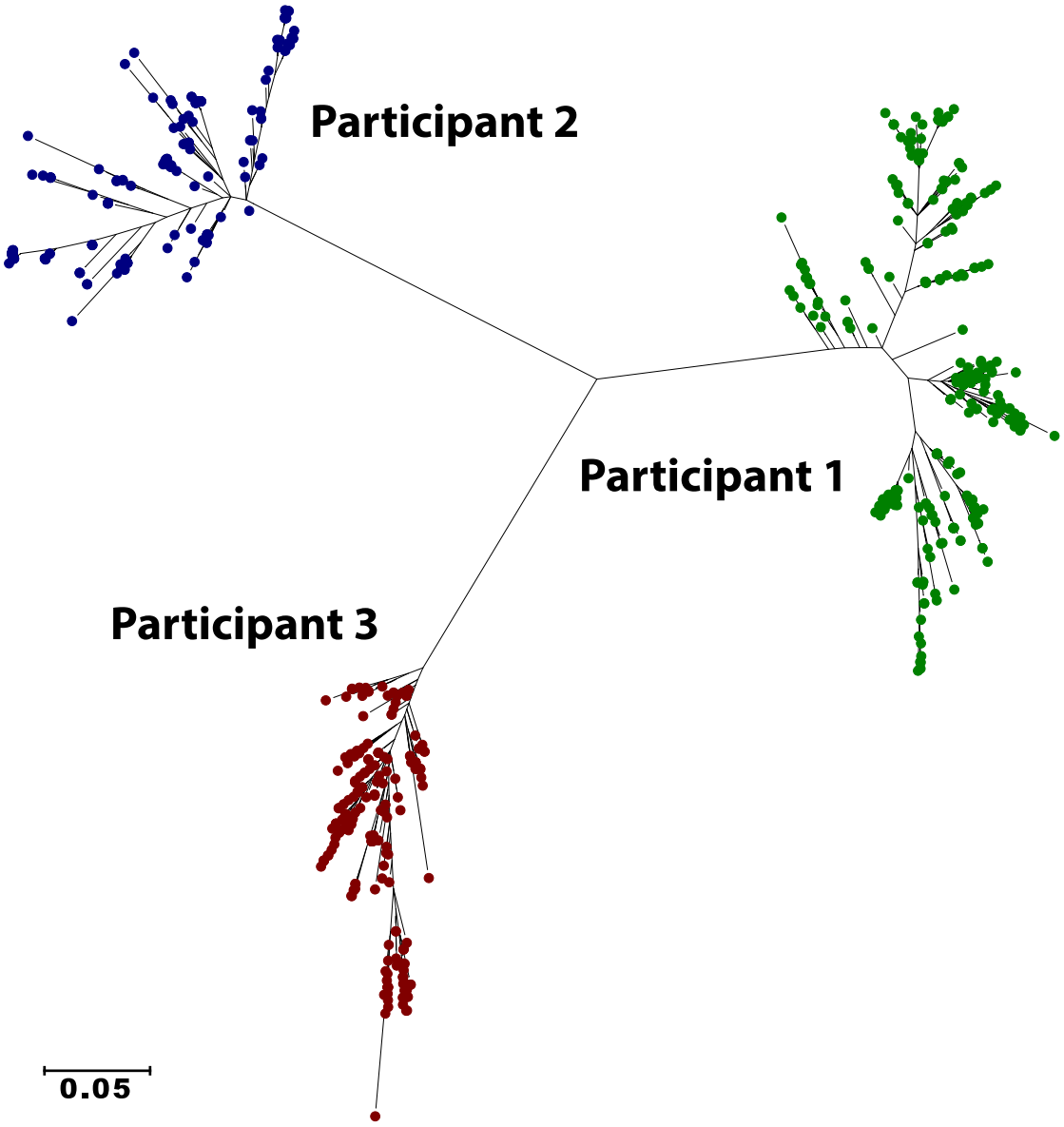


Figure 2-4: Phylogenetic analysis of HIV C2V5*env* gene single genome sequences across 3 participants reveals no cross-contamination or extraneous sequences.

Participant's 1-3 plasma and QVOA-derived single genome sequences (590bp) are represented by green, blue and red, respectively, with each dot representing one sequence. A maximum likelihood phylogenetic tree of all 3 participants' sequences shows segregation and clustering of sequences by participant suggesting a lack of cross-contamination among participants.

2.4 PHYLOGENETIC ANALYSIS AND SEEDING OF THE HIV RESERVOIR

Phylogenetic analysis revealed that RV sequences clustered across pre-ART clades within each participant (**Figure 2-1**). The distribution of RV across clades varied, but only included founder sequences in Participant 3 (**Figure 2-1B, D, F**). Pairwise distances between RV/QVOA-derived viruses and pre-ART or ART-interruption variants were also compared (**Figure 2-5**). Proviruses contributing to the RV throughout the first period of ART suppression were estimated to have seeded the reservoir during pre-ART, especially before ART initiation for all 3 participants (**Figure 2-5**) and as early as 28 days post-infection for Participant 2 (**Figure 2-5C**). RV detected after ART was interrupted and re-initiated were estimated to also have been derived from proviruses that seeded the reservoir during the pre-ART period in Participants 1 and 3. This included viruses of both predicted CCR5 and CXCR4 cell tropisms (**Figure 2-5A,B, D**). However, all of the RV sequences from ART-suppression #3 in Participant 2 and 80% of the RV from ART-suppression #2 in Participant 3 appeared to be derived from proviruses seeded during the ART-interruptions (**Figure 2-5C, D**). Following the ART-interruptions, RV was composed primarily of variants, often monotypic, genetically similar to the clades that evolved during ART-interruption (**Figure 2-1B, D, F**).

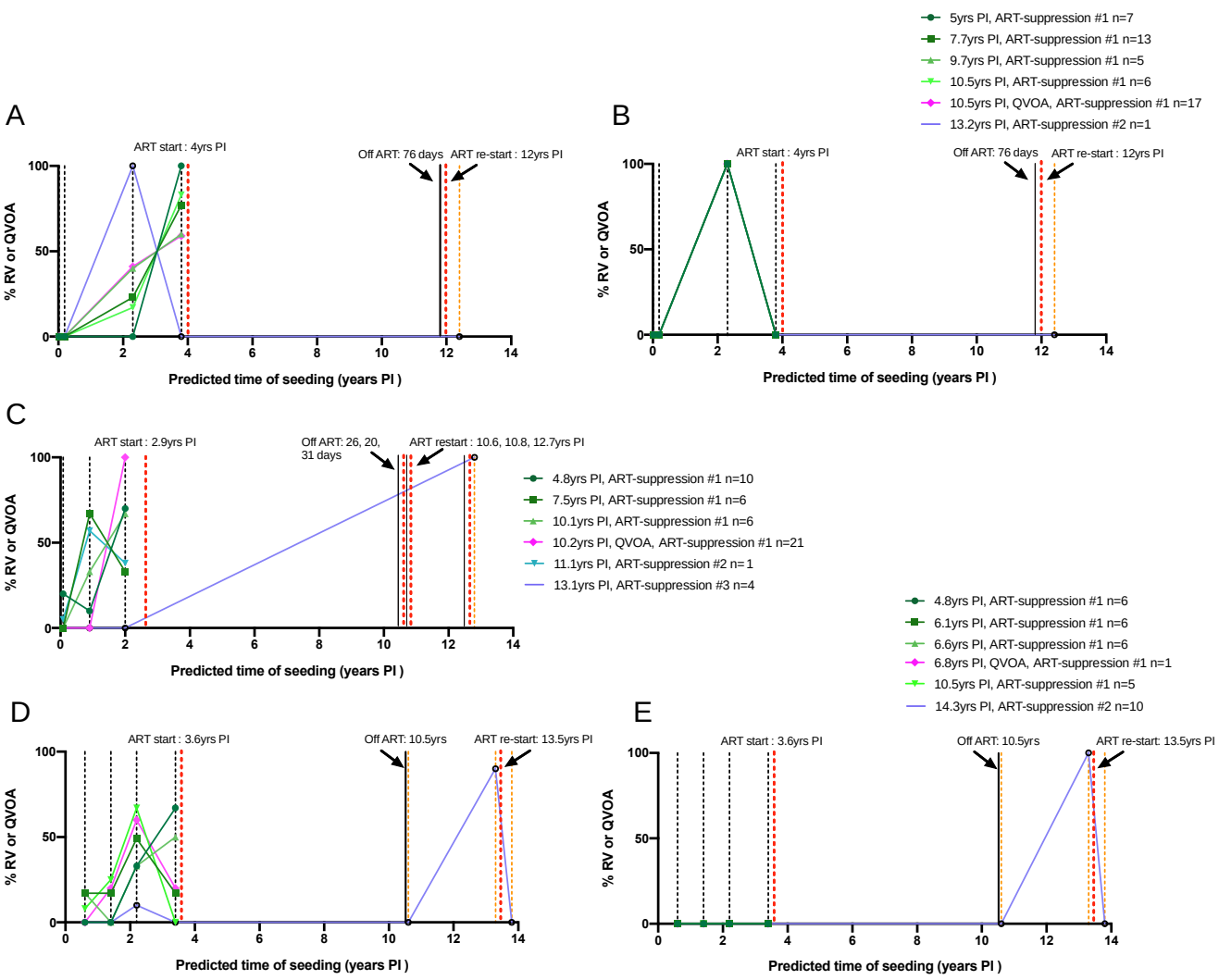


Figure 2-5: Pairwise distances reveal that RV and QVOA-producing proviruses during ART-suppression were seeded throughout pre-ART

The percentage of sequences from each RV/QVOA timepoint shown in the key predicted to be seeded at a specific pre-ART or ART-interruption timeframe is shown on the Y-axis **for** Participant 1 (**A,B**), Participant 2 (**C**), and Participant 3 (**D,E**). Estimated seeding of imputed unique R5- (**A, C, D**) and X4-tropic RV (**B** and **E**) are shown separately. Pairwise distances between each unique RV during ART-suppression #1 (all 3 Participants) and ART-suppression #2 (Participant 2) from the timepoints shown in the key and each of the examined pre-ART timepoints for each participant (dotted black line) were compared. Pairwise distances between RV during ART-suppression #2 (Participants 1, 3) and ART-suppression #3 (Participant 2) shown in the key and each of the examined pre-ART timepoints and examined ART-interruption timepoints (dotted orange line) were compared. The pre-ART or ART-interruption timepoint with the shortest pairwise distance with each unique RV/QVOA-derived virus was predicted to be the time for reservoir seeding of the proviruses contributing to these RV/QVOA-derived viruses. A total 4, 3, 4 pre-ART and 1, 1, 3 ART-interruption timepoints were used to infer

reservoir seeding time for Participants 1-3, respectively. ART-interruption timepoints for Participants 1 and 2 included 1 timepoint after ART re-initiation when viral levels were approaching the limit of detection of our assay (50c/mL). Red dotted line indicates the timepoint ART was initiated. Black solid line indicates the timepoint ART was interrupted. Black dotted line indicates the pre-ART plasma timepoints examined. Orange dotted line indicates the ART-interruption plasma timepoints examined. No X4-tropic viruses were detected among the RV or QVOA-derived sequences for Participant 2. Seeding of the reservoirs contributing to the RV and QVOA-derived viruses are plotted using colors corresponding to time detected post-infection (same color code as in phylogenetic trees). Total (n) numbers of unique RV and QVOA-derived sequences plotted are shown in the key.

2.5 LACK OF ONGOING EVOLUTION AMONG RV DURING SUPPRESSIVE ART

Genetic pairwise diversity and divergence of sequences from the imputed founder of infection found the expected pattern of progressive genetic diversification and divergence prior to ART-initiation (**Figures 2-6 and 2-7**). Notable nuances in the observed patterns included an increase in RV divergence and diversity compared to pre-ART timepoints potentially due to the first-time detection of a population of X4-tropic variants in Participant 1, who had no X4-tropic variants detected among pre-ART sequences (**Figures 2-1B and 2-6**). When these X4-tropic variants were excluded in Participant 1, the mean divergence distance of the latest pre-ART and RV sequences from the founder were similar (**Figure 2-6A**). Divergence among ART-interruption sequences appeared to depend on the frequency and duration that ART was suspended. The ART-interruption sequences from Participant 1, who had one brief (76-day) interruption, had no significant increase in divergence compared to RV sequences (**Figure 2-6A**). Whereas Participant 2, who had three brief ART-interruptions (26, 20, and 31 days), his RV sequences during ART-suppression #2 and during a viremic timepoint following ART-interruption #3, included plasma sequences identical to RV from ART-suppression #1, and the latter viremic timepoint included a new clade (**Figure 2-1D**). Variants detected during ART-suppression #2 in Participant 2 did not significantly diverge from the latest RV timepoint while variants detected during ART-interruption #2 and ART-suppression #3 were significantly diverged (**Figure 2-6B**). Participant 3, who suspended ART for 3 years, had ART-interruption sequences very early in the interruption (28 days post-interruption) with divergence similar to RV during the first ART-suppression, despite a high plasma HIV RNA (61,308 copies/mL), and sequences from later in the interruption (2.8 years) showing a significant increase in divergence relative to the latest RV timepoint (**Figure 2-6C**). Of note, despite a lack of increased distances in the early ART-

interruption sequences and the clustering of some of these sequences with pre-ART and RV sequences in Participants 1 and 3, a subset of these sequences appeared to be located on the most evolved branch tips (**Figure 2-1B, F**). These observations indicate that divergence analyses of sequences following brief ART-interruptions in these two participants were not sufficiently sensitive to detect HIV evolution. Whereas, the new clades detected from the later ART-interruption sequences of Participant 3, included first-time detection of X4-tropic variants, at which time his CD4+T-cell counts had dropped to a nadir of 221 cells/uL. Also notable, was that following the ART-interruptions, subsequent RV included primarily monotypic variants or variants genetically similar to the new clades that evolved during the ART-interruption in all 3 participants (**Figure 2-1B, D, F**).

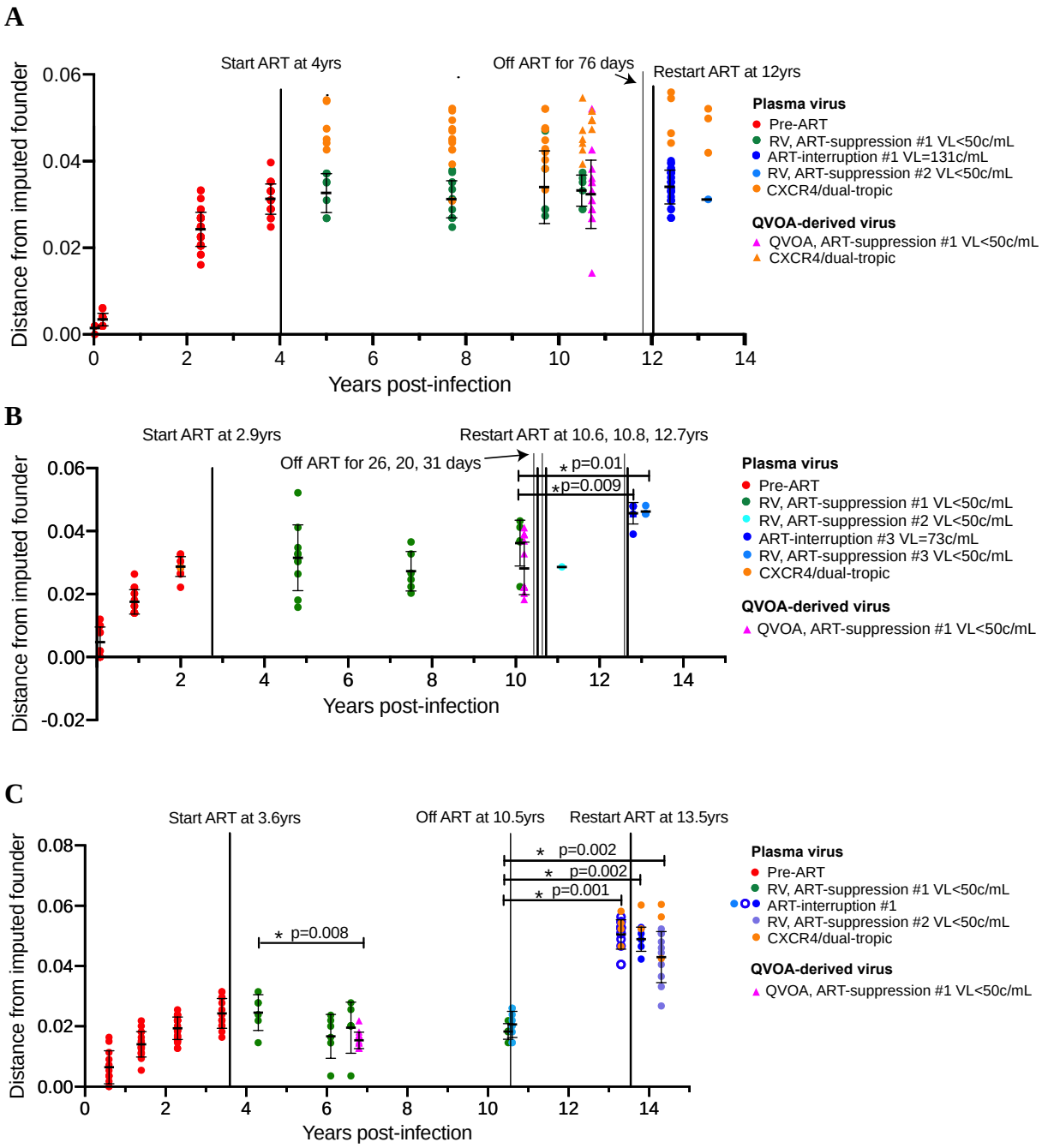


Figure 2-6: Divergence of HIV C2V5env unique and monotypic sequences from participants' founder virus

Time-ordered divergence from founder detected among unique C2V5*env* gene sequences pre-ART was not detected in RV or QVOA-derived sequences but resumes with ART interruptions. Each panel shows pairwise distances (y-axis) of unique plasma and QVOA-derived sequences from the imputed founder (means ±SD indicated for each timepoint in black) for Participant 1

(**A**), 2 (**B**), and 3 (**C**) over time (x-axis). Treatment status or imputed sequence tropism is indicated by color, shown in key. The time when ART was initiated or suspended are shown with thick and thin black vertical lines, respectively. ART was suspended for 76 days, 26-31 days, and 3 years in Participants 1-3, respectively. Statistically significant differences (*$p<0.05$) in divergence of the RV/QVOA-derived viruses relative to the first RV timepoint as determined by 2-sample Wilcoxon rank-sum test are indicated. Also indicated are significant differences between the ART-interruption and RV after re-suppression relative to the latest RV timepoint during ART-suppression #1.

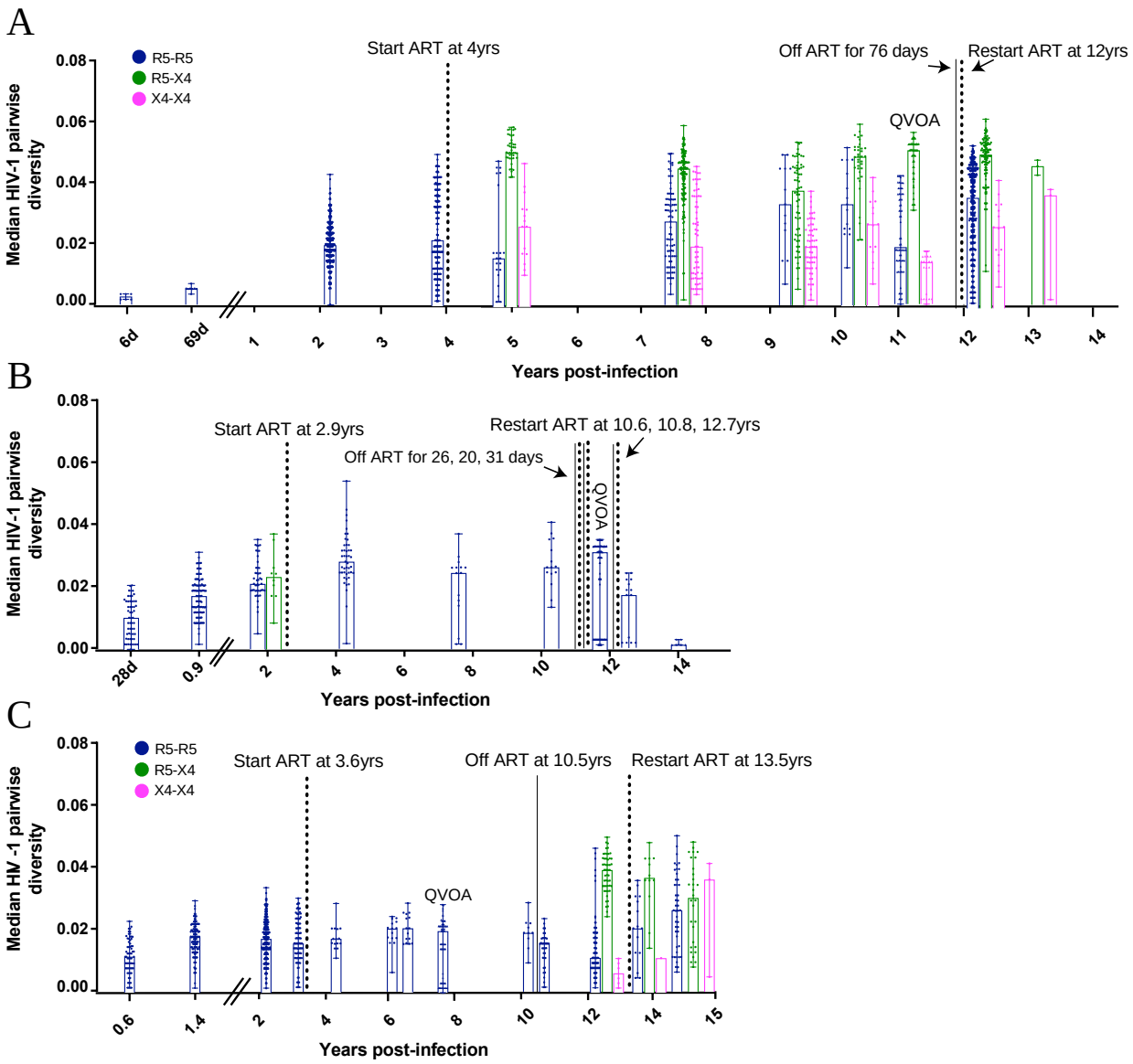


Figure 2-7: Diversity of unique C2V5*env* genotypes from plasma and QVOA-derived viruses over time of infection reveals general greater diversity in RV relative to pre-ART

An increase in diversity was more evident when comparing R5 and X4/dual-tropic sequences. HIV-1 pairwise diversity within each specimen is shown with median and range of longitudinal unique plasma and QVOA-derived sequences for Participant 1 (A), Participant 2 (B), and Participant 3 (C). Colors represent comparisons among sequences from CCR5-CCR5 (blue), CCR5-CXCR4/dual (green), and CXCR4/dual-CXCR4/dual (magenta) tropisms. QVOA sequences are shown as a triangle and are indicated in the figure. The time when ART was initiated or interrupted are shown with dotted and black vertical lines, respectively.

2.6 RV INCLUDE POTENTIALLY INFECTIOUS VARIANTS

RV C2V5*env* SGA sequences were identical to sequences derived from replicating, potentially infectious, viruses from pre-ART plasma, QVOA, and rebound viruses in plasma (ART-interruption), including 16%, 9%, 13% of the unique RV variants amplified from Participants 1-3 (**Table 2-1**), respectively. This included 14 of 126 (11%) unique RV genotypes from ART-suppression-1, but with RV from subsequent ART-suppressions the total is 7/28 (25%) across the three participants. The majority of these potentially infectious RV genotypes (17/19; 89%) had monotypic sequences detected in specimens from multiple timepoints indicating that likely they came from proliferating cells.

2.7 DYNAMICS OF RESIDUAL VIREMIA VARIANTS OVER TIME

Cell clones imputed to harbor infectious viruses have been reported to persist over years [44, 71, 73, 74]. RV during the first ART-suppression appeared to be produced in large part by cell clones as monotypic variants comprised 41%, 43% and 66% of the total sequences observed in Participants 1-3, respectively. The detection and persistence of specific monotypic RV genotypes over time fluctuated significantly in Participants 1 and 3 ($p<0.0001$, 2xC Fisher's exact test), revealing the dynamic nature of RV during ART-suppression. The persistence of RV variants was not associated with imputed cell tropism. The within timepoint frequency of monotypic RV genotypes occasionally comprised the majority (>50%) of RV sequences (Participant 2's Variants 5 and 6; **Figure 14B**). However, most monotypic RV genotypes were minority variants. The number of unique monotypic RV genotypes that persisted (detected at two or more timepoints) totaled of 14, 6, and 8 genotypes in Participants 1-3, respectively (**Figure 14**). A subset of these unique RV genotypes (total 13, 3, and 5, respectively) were among those linked

by identical C2V5*env* sequences to potentially infectious plasma viruses from pre-ART, QVOA, or ART-interruption, and these were detected over a median timespan of 3.9 years (range: 0.8-7.4), 3 years (range: 3-3.9), and 1 year (range: 0.1-8.2) in Participants 1-3, respectively (**Figure 2-8, Table 2-1**). RV genotypes linked to unique QVOA genotypes included 6/154 (4%) across the three participants (Participant 1, Variants 1, 8, 9, 13, 14 and Participant 3, Variant 8) (**Figure 2-1B, F and Figure 2-8A, C)**; (note Participant 2's QVOA (1.09) had the lowest infectious units per million (IUPM) cells, which likely affected our ability to link his sequences (**Figure 2-2**)). Several variants detected as RV or from QVOA were notable for identity with viral sequences detected during ART-interruption (Participant 1, Variant 2 and 11; Participant 2, Variants 4 and 5; and Participant 3, Variants 5, 6, and 8) (**Figure 2-8**). Notably, these included genotypes detected across long timespans; 7.4 years for Participant 1's Variant 11) and ~ 3 years for Participant 2's Variants 4 and 5) (**Figure 2-8A, B**). This suggests that these RV and rebound viruses were produced by long-lived clonal cell populations. The RV following ART-interruption and re-suppression in all 3 participants varied from those found during the first ART-suppression, suggesting that HIV replication had reset and/or obscured the long-lived reservoir (**Figure 2-9**), except for the variants detected during ART interruption as mentioned above that were detected after ART re-suppression.

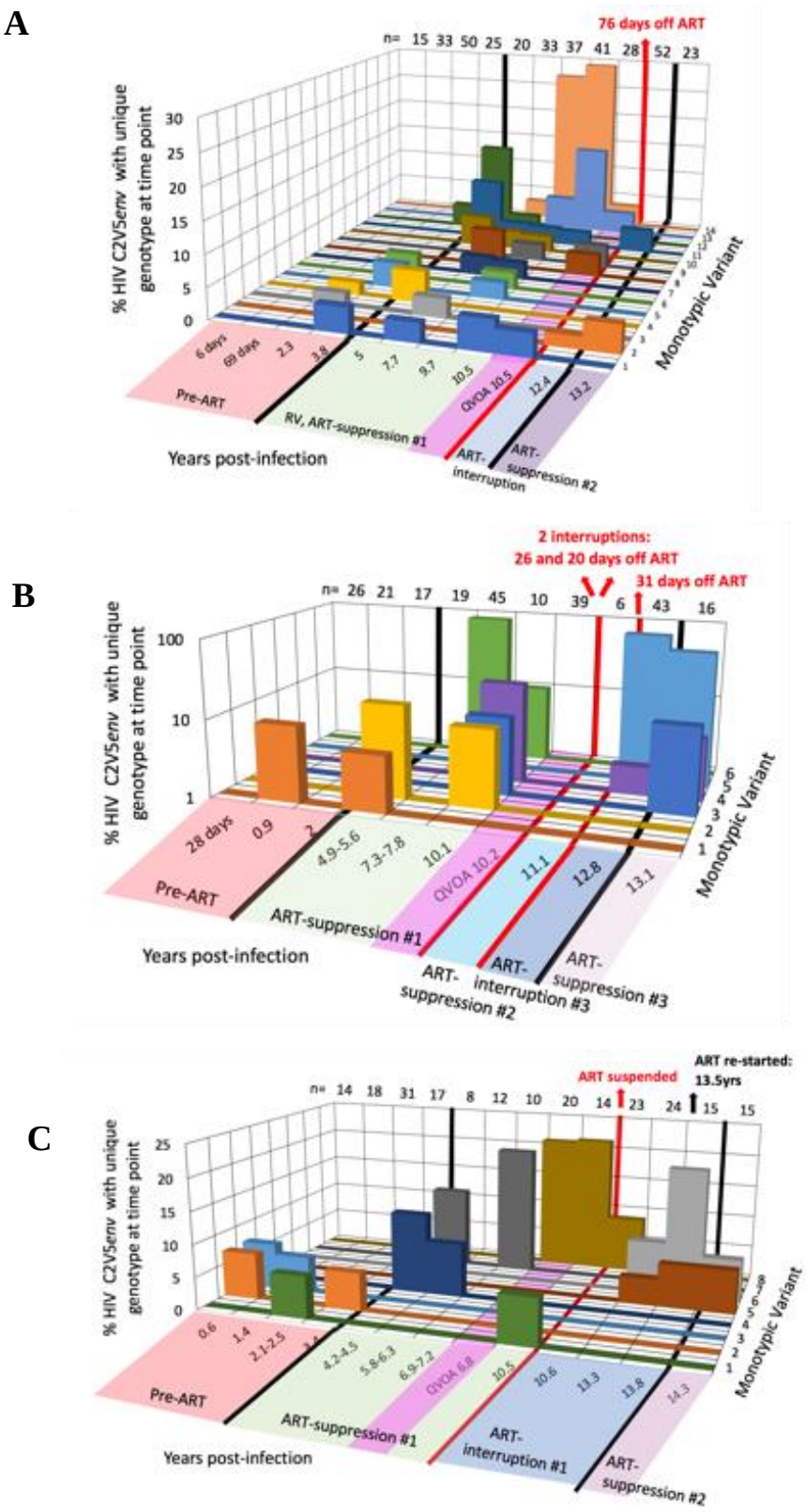
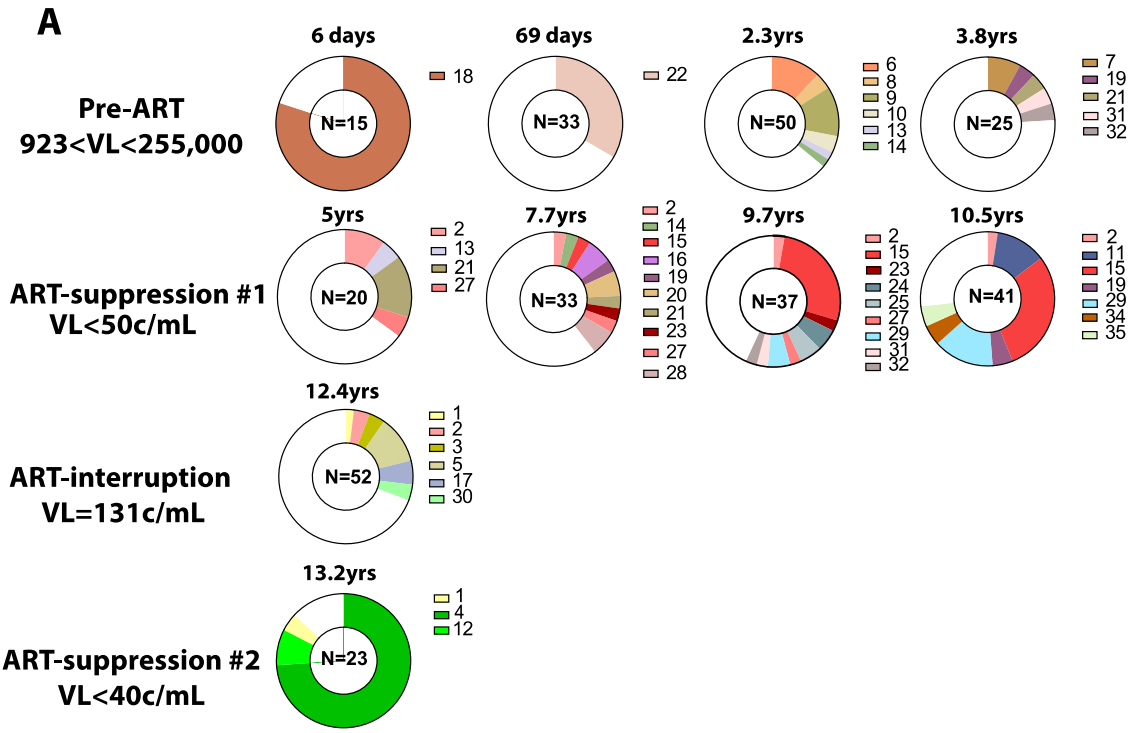
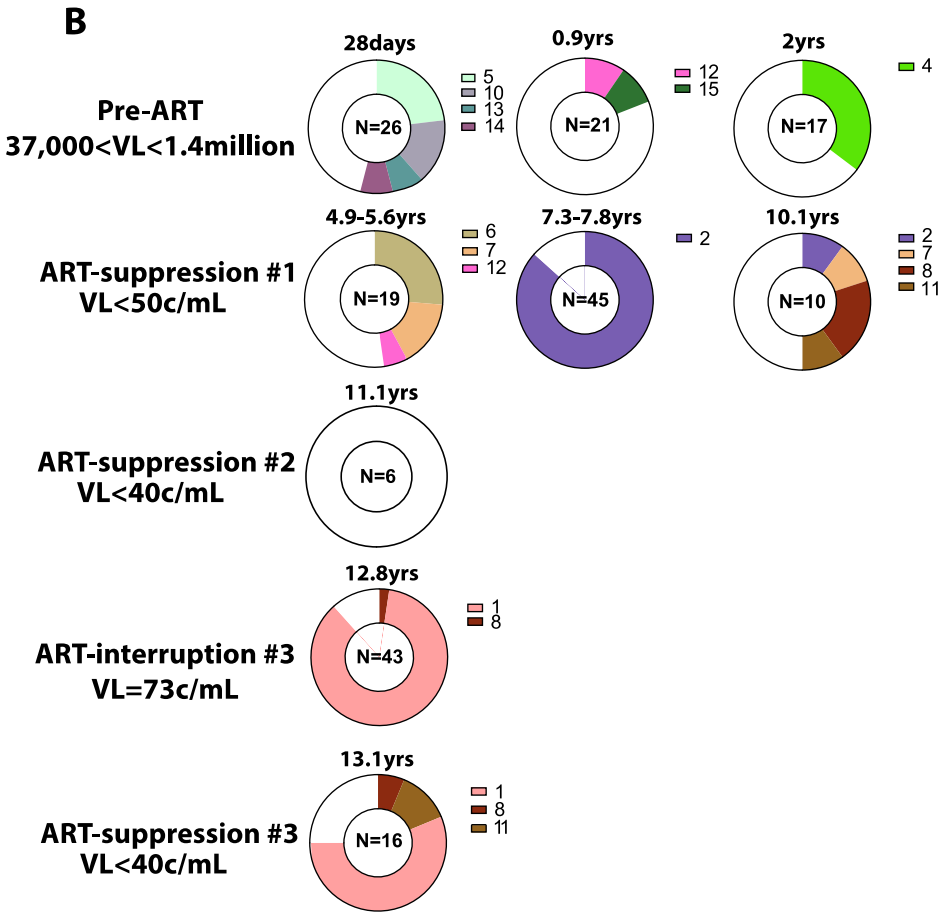


Figure 2-8: 3-dimensional depiction of monotypic HIV C2V5*env* clonal variants that persist over a median of 3 years (ranges: 0.1-8.2 years) for all 3 participants.

Each panel shows data for one study participant (Participant 1 (**A**), 2 (**B**), and 3 (**C**)). Persisting unique plasma and QVOA-derived variants detected at ≥2 plasma timepoints, and thus imputed to be from a clonal cell population, are shown by a different colors (z-axis) with proportion (y-axis) of the variant among all sequences obtained at that timepoint shown. Multiple monotypic plasma variants detected at only one timepoint are not graphed. Time since the estimated date of HIV infection and ART status is indicated along bottom of x-axis. ART-interruptions are noted by red lines transecting z- and y-axes with durations noted above red arrows. Numbers along the top of each panel represent the total number of sequences derived during each timepoint.


A
Pre-ART
923<VL<255,000
6 days
N=15
18
69 days
N=33
22
2.3yrs
N=50
6
8
9
10
13
14
3.8yrs
N=25
7
19
21
31
32
ART-suppression #1
VL<50c/mL
5yrs
N=20
2
13
21
27
7.7yrs
N=33
2
14
15
16
19
20
21
23
27
28
9.7yrs
N=37
10.5yrs
N=41
2
11
15
19
29
34
35
ART-interruption
VL=131c/mL
12.4yrs
N=52
1
2
3
5
17
30
ART-suppression #2
VL<40c/mL
13.2yrs
N=23
1
4
12


B
Pre-ART
37,000<VL<1.4million
28days
N=26
5
10
13
14
0.9yrs
N=21
12
15
2yrs
N=17
4
ART-suppression #1
VL<50c/mL
4.9-5.6yrs
N=19
6
7
12
7.3-7.8yrs
N=45
2
10.1yrs
N=10
2
7
8
11
ART-suppression #2
VL<40c/mL
11.1yrs
N=6
ART-interruption #3
VL=73c/mL
12.8yrs
N=43
1
8
ART-suppression #3
VL<40c/mL
13.1yrs
N=16
1
8
11

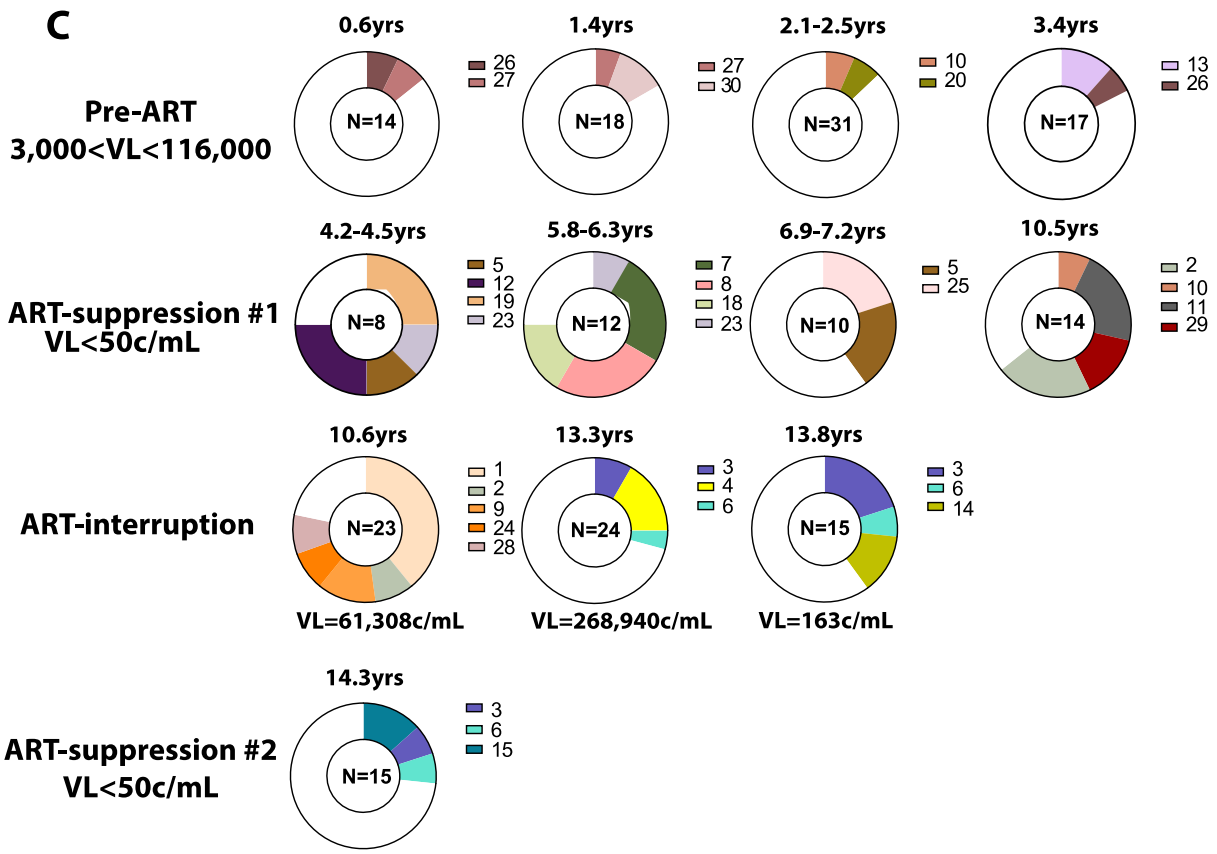


Figure 2-9: Proportions of monotypic and unique HIV C2V5*env* single genome sequences amplified from longitudinal plasma.

Each panel shows data for one participant: Participant 1 (**A**); Participant 2 (**B**); and Participant 3 (**C**). Each "donut" shown horizontally within the panels represents a timepoint with the segmented colors within the donut representing monotypic variants at 1 or >1 timepoint. The donuts are organized horizontally within each panel by antiretroviral status, as described to the left of each panel. Numbers corresponding to each color in the key represent the specific variant with the same number and color maintained for persistent variants detected over time. Shown is the proportion of HIV C2V5*env* single genome sequences with each unique genotype (i.e., variant) among all sequences amplified from that timepoint, with the total number of single genome templates sequenced noted in the center of each donut. All unique variants detected only once are grouped in the white section of the donut. The time since the estimated date of HIV infection is indicated above the donut, and plasma HIV RNA load, if detectable, is noted either above the donut or to the left side of each panel. Variants detected at ≥2 specimens are shown by the same color across longitudinal specimens.

2.8 LACK OF AN EFFECT OF N-LINKED GLYCOSYLATIONS ON RV PERSISTENCE

To assess whether RV or rebound sequences during ART-interruption were heavily glycosylated promoting these variants to escape antibody targeting and allow for persistence [128, 129], the number of potential N-linked glycosylation sites (PNLGS) were tallied and compared over time for each participant. No patterns in PNLGS distributions were detected across the three participants over time and PNLGS distributions were relatively stable over time (**Figure 2-10**).

Overall, these observations suggest that some clones have features favoring persistence and production of virions. These findings further suggest RV include multiple unique and monotypic genotypes, some of which appear potentially infectious based on sequence identity with plasma viruses during periods of viral replication or from QVOA.

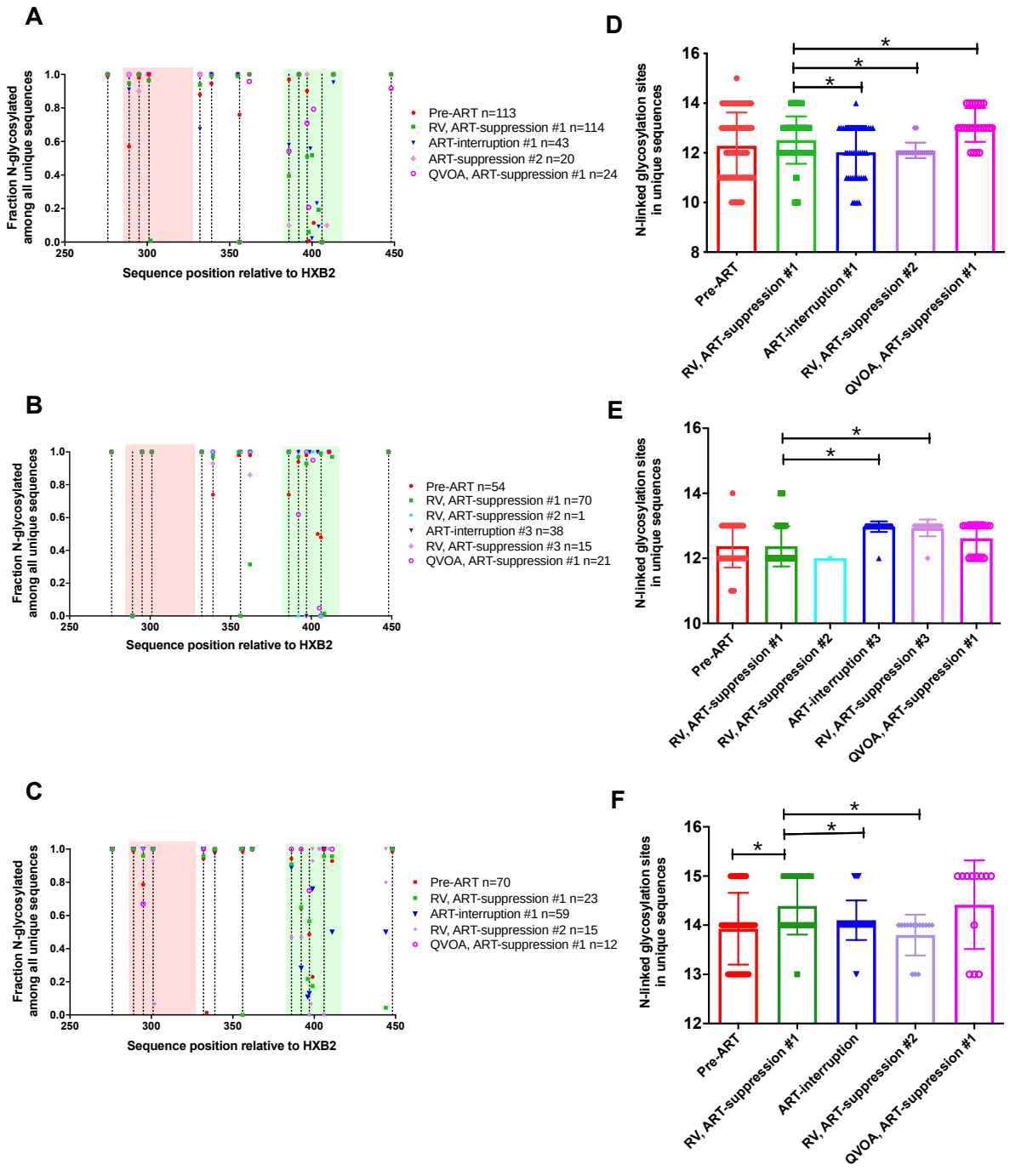


Figure 2-10: Predicted N-linked glycosylation sites (PNLGS) in unique plasma and QVOA C2V5*env* variants reveals a lack of a consistent pattern among the RV PNLGS level in all 3 participants.

"N-glycosite" at Los Alamos National Laboratory (LANL) website (URL: https://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html; accessed on 9-12-19)

was used to determine the fraction of N-linked glycosylation sites among all unique plasma and QVOA-derived C2V5*env* single genome sequences from Participants 1 (**A, D**), 2 (**B, E**), and 3 (**C, F**). Panels A-C show the fraction of N-linked glycosylation sites (Y-axis) among unique sequences relative to HXB2, the reference, full-length HIV genome. Colors and symbols represent different plasma and QVOA timepoints. Dotted lines represent known HXB2 N-linked glycosylation sites (n=12 sites) [130]. Red and green boxes represent the V3 and V4 regions in the HXB2 C2V5env amino acid sequence, respectively. Only unique C2V5*env* gene sequences that did not contain stop codons and that were not significantly G-A hypermutated as determined by LANL Hypermut 2.0 are shown. Panels D-F show the mean +/- standard deviation of N-linked glycosylation sites among all unique plasma virus C2V5*env* amino acid sequences and sequences of viruses induced from QVOA. Number of unique sequences included in the analysis is shown in the legend. *$p<0.05$, 2-sample Wilcoxon rank sum test, adjusted for multiple comparisons using the Holm method, was performed comparing levels of glycosylation among RV during ART-suppression #1 relative to sequences from all other timeframes.

2.9 DISCUSSION

Residual viremias provide a window into the segment of the HIV reservoir that actively produces virions during ART-suppression. Our study examined the longitudinal dynamics of RV released from this "active" HIV reservoir over a prolonged period of ART-suppression and ART-interruption, and to our knowledge is the first to report multiple genetic linkages between RV genotypic variants that persist over many years of ART-suppression and to rebound viruses from ART-interruption. These observations indicate that RV likely reveal a HIV reservoir that is critical for cure strategies to target.

Our examination of RV dynamics and infectivity inferred by C2V5*env* genetic linkages revealed the following findings: (1) RV genotypes were identical (i.e., monotypic) to replicating plasma variants from pre-ART, to infectious variants induced in QVOA from $CD4_+$ T-cells collected during ART-suppression, and to genotypes detected following ART-interruption, which suggests that RV reveal proviruses likely to constitute a segment of the infectious HIV reservoir; (2) RV and infectious viruses released from QVOA were genetically-linked via short pairwise distances to pre-ART plasma variants that spanned from near the time of acute infection to near the time of ART-initiation; which suggests seeding of the persistent HIV reservoir begins in early infection and continues throughout untreated infection; (3) ART-interruptions selected new variants that were detected along with past variants in RV following ART re-suppression, suggesting that viruses produced by recently infected cells that were decaying can be detected as RV, but it was unclear whether these variants markedly reshaped the reservoir; (4) Monotypic RV variants predominated as majority variants at single but not across multiple timepoints, confirming that the HIV reservoir producing RV includes a dynamic component; (5) Potentially infectious, monotypic RV variants were documented to persist for a median of 3.9 years across

participants, including after ART-interruption and during ART-re-suppression, suggesting selective survival of certain clones; (6) Prevalent and rare monotypic RV variants did not differ in the duration of persistence or in the contribution to rebound viremia; suggesting that clone size may not be a determinate of infectious fitness or persistence; 7) X4-tropic variants were prevalent in RV and QVOA of one participant who had relatively high $CD4_+$ T-cells and no X4-tropic variants detected prior to ART, suggesting a survival advantage for X4-tropic variants within the HIV infectious reservoir; and (8) PNLGS did not appear increased among RV or ART-interruption variants nor associated with persistence of monotypic variants, indicating that escape from neutralizing antibodies are unlikely to shape the infectious and persistent reservoirs. While our study participants provided a uniquely vast specimen bank compared to previous studies of RV [64, 73] that allowed examination of a relatively long timespan, our studies of ART-interruptions and ART-re-suppression, while unique, did not determine the extent to which interruptions reshape the persistent HIV reservoir, which will require even longer studies.

Genotypic identity between RV C2V5*env* sequences and replicating viral variants in pre-ART plasma, viruses induced to replicate in QVOA and rebound plasma viruses following ART-interruption suggest that RV were produced by clonal cell populations that include infectious HIV. Our finding that RV variants may be produced by clones that include infectious proviruses is supported by others' findings of RV sequences genetically-linked to infectious proviruses in resting $CD4_+$ T-cells by QVOA [55, 64, 78], genetically-intact proviruses [35, 44, 71-74], and linkage of on-ART proviruses to viral rebound after ART-interruption [85]. While studies have compared rebound viruses to pre-ART plasma viruses [85, 131, 132], to proviral DNA [85], or viruses induced from QVOAs [133], to our knowledge, studies have not previously compared

RV to rebound viruses, which revealed that RV may contribute to rebound viremia and persist after ART re-suppression.

Despite differences in the frequency and duration of ART-interruptions across the participants in our study, with rebound sequences from varied times following ART suspension, the ART-interruption C2V5*env* sequences identical to RV during the first period of ART-suppression were detected in all three participants. However, only 2 of 3 had identical C2V5*env* sequences detected between RV and QVOA and only 1 of 3 between QVOA and ART-interruption/rebound. The paucity of C2V5*env* genotypic linkages detected between RV and QVOA-derived viruses is similar to others' reports of little to no overlap between sequences from these specimens [55, 64, 78], and similar to others' reports comparing QVOA-derived viruses and rebound viruses [133, 134]. The lack of an overlap in C2V5*env* sequence identity of RV with QVOA-derived viruses may reflect RV production by tissues other than the peripheral blood, such as the lymph nodes as others' have postulated [135], or simply our sparse sampling in QVOA. Only 14% of the unique RV C2V5*env* sequences among all 3 participants were identical to potentially infectious viruses sequenced from pre-ART plasma, QVOA supernatants, and/or from plasma during ART-interruption. This low rate of sequence identity may reflect sparse sampling of viruses, however, if these linkages are representative of infectious, archived variants as we hypothesize, then the detection of 14% would represent a significantly greater yield compared to sequencing of proviruses among $CD4_+$ T-cells [43].

While previous studies of low-level intermittent viremias (50<VL<400 c/mL) [77, 136] occasionally detected evidence of HIV replication, the genetic divergence analyses of our participants' sequences indicated HIV replication only after frequent or prolonged ART-interruptions. Following short durations of ART-interruption, the sequences we generated from

Participants 1 and 3 did not demonstrate a significant increase in divergence despite phylogenetic analyses showing these ART-interruption sequences on the branch tips and a high plasma HIV RNA in Participant 3. These observations emphasize that comparison of genetic distances between timepoints in individuals with genetically diverse virus populations may not be sufficiently sensitive to detect short periods of HIV replication that were documented in these participants by virtue of plasma HIV RNA levels. Furthermore, it underscores that that a lack of a significant increase in divergence should not be used to exclude virus replication.

Divergence analyses across our participants suggest that the HIV-infected cells producing RV and those reactivated in QVOA seeded the persistent HIV reservoir early in infection and across the timespan that virus was replicating prior to ART initiation. The longer the duration of ART-suppression, the more RV tended to cluster in phylogenetic analyses with plasma variants that evolved closer to the time of acute infection as was observed in Participants 2 and 3, where the distribution of pairwise distances were smallest between their RV and pre-ART sequences. This contrasts with previous observations [60, 137] of genetic linkages of the infectious reservoir in the blood to viruses that primarily evolved within the year prior to ART initiation [137]. We hypothesize that the observed genetic linkage to cells infected just prior to ART-initiation may indicate detection of the decaying populations of recently replicating viruses [63] and/or may be due to low-levels of ongoing replication. Decay of recently replicating viruses or HIV-replication is suspected due to the expected representation of virions from newly infected cells based on the decay half-lives of HIV-infected cells predicted by studies following ART-initiation [43, 138], data from our previous studies [123], and our observation in this study that genotypes prevalent during ART-interruption were also prevalent in RV shortly after ART-re-suppression.

The variations in RV genotypes detected over time in each of our participants confirms a pattern of genotypes waxing and waning in prevalence as observed by others [19, 55, 64, 73], which could reflect variations in the activation and proliferation of cell clones to produce virions due to immune elimination of specific infected clones, homeostatic proliferation and/or a gain or loss of gene function due to the location of the HIV integration site [104]. Additionally, shortly after ART initiation, one would expect rapid decay of actively replicating cells [138], which would change the frequency of RV, as suggested above.

Monotypic C2V5*env* sequences were observed to persist across long spans of time, and across specimen types including prior to and during ART-suppression and following ART-interruptions. Our observation that some variants persisted for nearly a decade suggests not only a relatively high capacity for certain infected clones to produce virions, but the longevity of these virion-producing clones suggests a resistance to either viral cytopathic lysis or to clearance by CTLs as previously proposed [87, 121, 139]. Interestingly, the duration of persistence or contribution to rebound viremia did not differ between the genotypic variants we found to be prevalent or rare, suggesting that multiple factors likely contribute to virion production by HIV-infected clones.

Prevalent X4-tropic variants were detected in 2 of the 3 participants but at times that their immunologic statuses differed. X4-tropic RV and QVOA variants were detected in Participant 1, who had relatively high $CD4_+$ T-cells and no X4-tropic variants detected prior to ART; these observations suggest that X4-tropix variants may preferentially populate the reservoir that persists during ART. Whereas, X4-tropic variants were detected in Participant 3 near the end of his 3-year ART-interruption, when his $CD4_+$ T-cells had dropped to 221cells/uL, suggesting that these variants evolved during the ART-interruption when an immunologic niche supported their

replication [140]. We expected to detect R5-tropic viruses nearly exclusively in the RV given that founder strains are nearly universally R5-tropic [141], and these participants' $CD4_+$ T-cell nadirs prior to ART-initiation exceeded 200 cells/uL, the value below which X4-tropic viruses generally become detectable [140, 142]. The detection of X4-tropic viruses among the RV [136] has been previously observed in individuals with low-level viremias, which suggests the reservoirs producing these X4-tropic RV and QVOA-derived variants may include X4-expressing naïve T cells [136, 143] or X4-expressing hematopoietic stem and progenitor cells [80]. The survival advantage of HIV-infected cells with X4-tropic proviruses in Participant 1 suggests that cell-specific eradication approaches should target diverse cell types to eliminate the active HIV reservoirs.

Our comparisons of PNLGS between individual's sequence sets revealed significant differences between timepoints, but no patterns were observed across the participants. In 2 participants, PNLGS were increased in RV sequences relative to ART-interruption sequences, providing a weak suggestion that antibody escape may contribute to the selection of plasma variants that persisted during ART. The lack of an increase in PNLGS among the ART-interruption sequences was not surprising given that purifying selection by neutralizing antibodies [144] likely occurred across the timeframes that these individuals were studied.

Our study has some limitations. First, given that we only examined a 590bp region of the HIV genome, the genetic-linkage between two or more identical sequences may have overestimated "monotypic" populations due to sequence differences outside of this region. However, given the hypervariability of the C2V5*env* region, the Clonal Prediction Score Calculator [145] (https://silicianolab.johnshopkins.edu/cps/#/) estimates that identical sequences amplified by our primers provide an 83% certainty that the sequences are identical throughout

the genome of persons, such as our participants, who initiated ART during chronic infection. Furthermore, an alignment of all 1,071 subtype B HIV-1 sequences from the Los Alamos National Laboratory database revealed that our first round forward and reverse C2V5*env* primers were conserved in 91% and 92% of sequences in the database, respectively; and conserved in 99% and 91%, respectively, of the critical 5-basepairs where the 3' ends of these primers anneal. Thus, this region has been used across phylogenetic studies of HIV [29, 55, 64, 78, 80, 146, 147], and suggests that minimal bias was introduced by our primers. Second, our sampling of unique and monotypic viruses was limited by the number and volume of banked plasma specimens, which suggests that we amplified majority variants and that our detection of less prevalent viruses was stochastic, which may have exaggerated the variability in RV variants across time. Third, specimens were not collected at ideal times during the brief ART-interruptions in 2 of the 3 participants (i.e., at the beginning and end of the time that ART as suspended), thus sequences from viremias shortly after ART was re-initiated were used to capture the HIV variants replicating during ART-interruption. Fourth, we did not examine CTL activity or assess RV for escape variants.

Our findings suggest that despite years of effective ART, multiple RV variants selectively persist over time, even after ART-interruption. These variants appears to evade immune targeting and may rekindle infection upon ART interruption. The persistence of RV variants for at least 8.2 years during effective ART suggests that cure strategies such as "kick and kill" may be ineffective in eliminating the active reservoirs. Instead, multiple eradication strategies targeting different persisting populations (e.g., latent viruses integrated in "silenced" regions of the human genome, cells actively producing virions not effectively targeted by the

host, various host cell subsets and immune micro-environments, etc.) or immunotherapies that persistently and effectively target expressed virions may be necessary to cure HIV infection.

2.10 MATERIALS AND METHODS

2.10.1 *Study participants*

Specimens from the Seattle Primary Infection Program (SeaPIP) were selected based on the following inclusion criteria for this study: 1) initiated ART during chronic infection; 2) ART-suppressed for ≥5 years with all plasma HIV RNA loads <50c/mL; 3) cryopreserved leukapheresis and multiple plasma specimens available each year prior to and during ART-suppression; and 4) a treatment interruption with plasma specimens available following re-initiation of treatment. SeaPIP enrolled individuals with primary HIV into an observational cohort from 1992-2015 [148-151]. The University of Washington Institutional Review Board approved this study, and all participants provided written consent. Participants initiated treatment under the care of their clinicians as recommended by guidelines and personal preferences. At the time of cohort entry, all participants had either acute or early HIV infection, with days post-infection representing 11-13 days post-exposure [151]. ART was initiated based on the availability of potent antiretroviral therapy, the strength of consensus treatment recommendations, and personal preferences. Participants' study visits and specimen collection were as frequently as weekly for the month following enrollment, monthly for three months, at eight-week intervals through the first year of observation, and then at a minimum of six-month intervals, although the frequency varied somewhat within the cohort. Self-initiated treatment interruptions for the 3 participants including in our study for reasons including insurance lapse (Participants 1, 2) and a decision made in conjunction with a clinical care provider (Participant 3).

2.10.2 *QVOA*

Total $CD4^+$ T cells (~10^7) were purified by negative selection (Miltenyi, Catalog # 130-096-533) from participant PBMC, serially diluted (3-5-fold) into 48-well plates (2-8 replicates/$CD4^+$ input) containing monocytes (5^5/well) purified from a healthy HIV-seronegative donor. Cocultures were maintained for 3 days in 1mL culture media (Iscoves with 10% FCS) containing 1ug/mL anti-CD3 OKT3 (Miltenyi, Catalog # 130-093-387). At days 3 and 10 of coculture, the supernatant was removed, and CD8-depleted allogeneic PHA-blasts from a healthy HIV-seronegative donor (5^5/well) were added to the cocultures in media containing IL-2 (PeproTek, Catalog # 200-02). Cocultures were maintained for 28 days and supernatants harvested weekly for virus outgrowth by HIV p24 antigen. The infectious units per million $CD4^+$ T cells (IUPM) was calculated using the maximum likelihood estimate on days 15, 21, and 28 of culture [152].

2.10.3 *Plasma viral RNA extraction*

RNA was extracted from 1-21mL aliquots of participants' plasma or 50-140uL of QVOA supernatants. When plasma contained HIV RNA at <50c/mL, the virus particles were pelleted by centrifugation at 14,000rpm for 90min at 4°C, with removal of all but ~140uL of plasma over the pellet. The Qiagen QIAmp Viral RNA Mini kit (Qiagen, Catalog # 52906) was used as directed by the manufacturer to extract the RNA from 140uL of plasma. When viruses were pelleted, lysis buffer was added directly to the pellet and remaining plasma and once resuspended, was transferred to a new 1.5mL Eppendorf tube for the extraction, along with ~500uL of PBS used to wash any remaining virions from the tube. A positive control plasma sample containing >100,000 copies/mL of HIV RNA was similarly processed with each extraction. RNA was eluted into 60uL of AVE buffer (Qiagen) and was immediately DNase-treated to degrade contaminating HIV proviral DNA using Invitrogen DNase I, Amplification Grade (Catalog #

18068-015) following the manufacturer's instructions (1uL of 10X buffer was added to the eluted RNA followed by 1uL DNase I). The mixture was incubated at room temperature for 15 minutes (min), then the enzyme was deactivated with the addition of 1uL 25mM EDTA, incubated for 10 min at 65° and then put on ice. RNA from the QVOA supernatants were similarly DNase I-treated with the exception of 6/28 (21.4%) QVOA p24Ag(+) wells from all 3 participants when DNase I was not available. RNA was immediately converted to cDNA (see below).

2.10.4 *cDNA synthesis*

All 60uL of DNase-treated RNA was converted into cDNA using the Invitrogen Superscript IV (SSIV) enzyme kit (Catalog # 18090200). Briefly, to the 60uL RNA, 5.5uL of 10mM dNTP, 5.5ul of each of the 20uM of HIV *pol*-specific forward (HXB2 positions 3328-3303);RTA: 5'-AACTTCTGTATGTCATTGACAGTCCA-3') and *env*-specific reverse (HXB2 positions 7697-7725); BH2: 5'-CCTTGGTGGGTGCTACTCCTAATGGTTCA-3') primers were added and the mixture was incubated in a thermocycler at 65° for 5 min and then immediately put on ice for at least 1min. After ≥1 minute on ice, 2.75uL of molecular grade water, 22uL 5X RT buffer (Invitrogen, catalog # 18090200, 5.5uL 0.1M DTT, 2.75uL Promega RNasin Plus RNAse inhibitor, 10,000U (Catalog # N2615), and 1uL of the SSIV enzyme was added to the tube for one cycle at: 55° for 90min, 80° for 10min, and held at 12° prior to storing at -20°.

2.10.5 *Endpoint nested PCR of C2V5env*

Sequences of the C2V5 region of *env* were derived from specimens diluted to single HIV templates [153, 154]. These C2V5*env* gene sequences were derived following limiting dilution (i.e., by single genome amplification, SGA) to minimize recombination, resampling and

sequencing errors [155, 156]. The first-round 50uL PCR included: 2-3uL diluted cDNA, molecular grade water,10uL Bioline 5x MyTaq Reaction Buffer (Catalog # BIO-21107), 1ul each of the 20uM forward (HXB2 positions 6865-6831; ED31X 5'-CAGGCCTGTCCAAAGGTATCCTTTGAGCCAATTCC-3') and reverse *env*-specific primer (BH2, see above for sequence) and 0.5ul Bioline MyTaq DNA polymerase (5U/ul). The nested second-round 25uL PCR reaction included: 2uL of first-round PCR product, molecular grade water, 5uL 5x MyTaq Reaction Buffer, 0.5uL each of the 20uM forward (HXB2 positions 6990-7021); DR7; 5' TCAACTCAACTGCTGTTAAATGGCAGTCTAGC-3') and reverse (HXB2 positions 7631-7653;DR8new; 5'- CCCTCATATCTCCTCCTCCAGGTCTGAA-3') primers, and 0.25uL MyTaq DNA polymerase (5U/uL). A PCR positive control (10 copies of 8E5 cellular DNA) provided by NIH AIDS Program and a negative control (water) were included with each run. The cycling conditions for both rounds 1 and 2 were: 94° for 5min, 34 cycles of (94° for 20 sec, 55° for 20sec, 72° for 1min), 1 cycle at 72° for 7 min and a hold at 8°C. In some cases, an alternate second-round PCR reverse primer was used (HXB2 positions 7656-7690; env7656; 5'-ACTACTTTATATTTATATAATTCACTTCTCCAATT-3'), in which case the annealing temperature was 52° instead of 55°.

2.10.6 *DNA sequencing and phylogenetic tree analyses*

The 609bp C2V5*env* gene amplicons from the 2nd round nested PCR were directly sequenced by Sanger using DR7 and DR8new or env7656 primers, above. All chromatograms were evaluated to ensure the lack of any double peaks to confirm single genome sequences, with sequences with >1 peak eliminated from analyses. The forward and reverse sequences were aligned to a reference HXB2 sequence (Genbank, K03455.1) using Geneious R11.1.5 (https://www.geneious.com) and base calls reviewed for bi-directional consistency. Sequences

with significant levels of APOBEC-induced G-A hypermutations were determined by Hypermut, LANL [157]. Briefly, all plasma and QVOA-derived *C2V5env* sequences were aligned in each Participant using the most common plasma variant found at the first pre-ART plasma timepoint. Geneious R11.1.5 was used to create the Muscle alignment which was then imported into Hypermut, LANL using the default settings. Hypermut 2.0 was selected as the output to specifically examine APOBEC3G and APOBEC3F-induced hypermutations. Sequences with a Fisher's exact P value <0.05 were considered significantly hypermutated and were noted on the tree as such.

All plasma and QVOA-derived virus C2V5*env* nucleotide sequences for each participant were aligned relative to the founder sequence from each participant (consensus sequence of the first pre-ART timepoint (Participant 1, 6 days post-infection (PI); 2, 28 days PI; 3, 0.6 years PI). Nucleotide sequences were codon-aligned using Muscle 3.8.425 in Geneious R11.1.5 using the default settings. Alignments were manually edited and evaluated to ensure minimal gaps and identical sequence lengths. To ensure all sequences in the alignment were of identical length, sequences were trimmed to ~590bp. Sequences with stop codons or significant levels of APOBEC-induced G-A hypermutations were not removed from the alignment. UniSeq (https://indra.mullins.microbiol.washington.edu/cgi-bin/UniSeq/uniseq.cgi) was used to remove all identical sequences from the alignment prior to creating the phylogenetic trees. The nucleotide alignment containing all unique sequences was imported into DIVEIN [158] (https://indra.mullins.microbiol.washington.edu/DIVEIN/diver.html) to create a maximum likelihood phylogenetic tree using the GTR substitution model. The following settings were changed from the default: type of tree improvement→Best of NNI and SPR, perform bootstrap→Yes. MEGA version 7 [159] was used to format the output phylogenetic tree from

DIVEIN. Taxa was rearranged for a balanced shape when formatting the phylogenetic tree in MEGA7.

2.10.7 *Divergence from the founder virus*

To evaluate divergence of plasma and QVOA sequences from imputed founder virus of each participant, either total or unique C2V5*env* nucleotide sequences were codon-aligned relative to the consensus sequence of the first pre-ART timepoint. Alignments were created using Geneious R11.1.5. C2V5*env* sequences that were significantly hypermutated by LANL Hypermut 2.0 [157] or contained stop codons were removed from alignments. DIVEIN [158] was used to estimate pairwise distance from the imputed founder. Mean and standard deviations were determined using GraphPad Prism v6.07. Two-sample Wilcoxon rank sum test was performed to evaluate significant changes in RV divergence comparing the first ART-suppression timepoint to each subsequent RV timepoint. Two-sample Wilcoxon rank sum test was also performed comparing sequences at the latest RV timepoint during ART-suppression #1 relative to all subsequent timepoints.

2.10.8 *Reservoir seeding*

For each ART-suppressed sequence, the distance between that sequence and each pre-ART sequence from the same participant was examined, using the phylogenetic trees constructed and pairwise distances obtained from the divergence analysis above. The estimated time of seeding of the provirus contributing to the RV was estimated as the pre-ART or ART-interruption (Participant 3) timepoint with the smallest pairwise distance relative to the RV. In case of a tie, the seeding time was estimated to be the earliest time at which we observed pre-ART sequences tied for the smallest distance.

2.10.9 *Diversity of plasma and QVOA virus*

To evaluate diversity among viruses collected from the QVOA supernatants and at each plasma timepoint, each participant's unique and total plasma and QVOA C2V5*env* nucleotide sequences were codon-aligned relative to their imputed founder sequence. Alignments were created using Geneious R11.1.5. C2V5*env* gene sequences that were significantly hypermutated by LANL Hypermut 2.0 [157] (5 sequences from all 3 Participants) or contained stop codons (Participant 1, 18 sequences; 2, 25; 3, 15) were removed from alignments. DIVEIN [158] was used to estimate pairwise distance between sequences from each timepoint. The median pairwise distance and ranges were determined using GraphPad Prism v6.07 for each timepoint.

2.10.10 *Cellular tropism*

To predict viral co-receptor use, or "tropism", the plasma and QVOA viral nucleotide sequences were codon-aligned relative to the V1-V3 region (7,110-7,218bp) of HXB2 (Genbank, K03455.1). Any insertions found in the alignment of plasma virus sequences and sequences of viruses induced from QVOA that were not found in HXB2, were removed from tropism analysis performed using PSSM [140]. Sequences with a percentile >95 or that contained stop codons were excluded from tropism analysis. Tropism predictions are reported as previously described [140]; scores $\leq$ -7 as CCR5-tropic, -7 to -3 as dual-tropic, and $\geq$ -3 as CXCR4-tropic.

2.10.11 *N-linked glycosylation*

To evaluate the number of predicted N-linked glycosylation sites for each RV variant, unique C2V5*env* nucleotide sequences were codon-aligned relative to the founder reference sequence. Separate alignments were created of sequences from each timeframe (pre-ART, ART-suppression, ART-interruption, QVOA). Alignments were created using Muscle in Geneious

R11.1.5 and input into the N-glycosite program [160] in LANL using the default settings. The output file “A summary of a tally of N-linked glycosylation sites in each sequence” was used to create the bar graphs in Figure 2-10. The output file “Number of N-linked glycosylation sites by position” was used to determine the fraction of glycosylation sites among sequences at each amino acid position in Figure 2-10. C2V5*env* gene sequences that were significantly hypermutated by LANL Hypermut 2.0 [157] (5 sequences from all 3 Participants) or contained stop codons (Participant 1, 18 sequences; 2, 25; 3, 15) were removed from alignments

2.10.12 *Statistical analyses*

Fisher's exact test was used to determine whether the distribution of observed variants during ART suppression was homogeneous over time. For each participant, we determined the most common variant (MCV) by pooling variant counts during the ART suppression period. In the case of a tie, the MCV was the variant with the greatest number of counts during the entire study period. Each participant thus contributed a 2xC contingency table, where C is the number of timepoints (non-ordered) during ART suppression for that participant, and observations at each timepoint were dichotomized as being MCV or non-MCV. In Participants 1 and 2, we found that the odds of observing the MCV was not homogeneous across time during the ART suppression period ($p = 0.0006$ and <0.0001, respectively). For Participant 3, we did not find statistically significant evidence of heterogeneity in the odds of observing the MCV across time during the same period ($p=0.131$).

In order to determine the degree to which viruses seen during pre-ART contribute to residual viremia during ART suppression and re-suppression, we examined the proportion of sequences seen during ART suppression or re-suppression that were also seen pre-ART for each participant. We calculated 95% exact binomial confidence intervals for each proportion reported.

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Chapter 3. DISSERTATION CONCLUSIONS

3.1 SUMMARY

HIV-infected individuals undergoing suppressive ART must remain on life-long treatment due to the possibility of rebound viremia. While ART is generally effective at limiting viral replication, it can be toxic and still result in persistent immune activation due to ongoing low-level RV production [67, 161]. Newer strategies are urgently needed to target the HIV reservoir that is promoting this low-level persistent viremia.

My dissertation was able to show the dynamic state of residual viremia during long-term suppressive ART and I observed a prevalence of monotypic virions that can persist for at least 8 years regardless of predicted cell tropism. I observed that the persistent proviruses contributing to the RV may be seeded throughout pre-ART and as early as 28 days post-infection for Participant 2. Furthermore, I observed residual viremia can represent infectious monotypic viruses that may potentially contribute to rebound virus upon treatment cessation. These observations suggest that even with effective ART, RV represent a disproportionate number of replicating, potentially infectious, variants that can propagate infection in the instance individuals are not adherent to ART.

3.2 SIGNIFICANCE AND FUTURE DIRECTIONS

To our knowledge, my study is the first to report multiple genetic linkages between RV variants that persist over ~8 years of ART-suppression and infectious variants from ART-interruption. The fluctuation and persistence of RV suggests multiple episodes of antigen-mediated stimulation may promote residual viremia expression over time. Immune evasion and longevity

of infected cells may contribute to long-term persistence of residual viruses since early infection and after ART-interruption.

The work presented in this dissertation contributes to the field in multiple ways. Our ability to obtain >300 single genome plasma virion sequences from timepoints with undetectable levels of virus in all 3 participants is not an ordinary feat and will provide a better understanding of the diversity and persistence of the active reservoir contributing to RV production even in the face of effective ART. Our observation that at least 14% of all unique RV in all 3 participants may be potentially infectious and contribute to rebound viremia suggests the active reservoir in the peripheral blood appears to evade immune targeting and may rekindle infection upon ART interruption or non-adherence. Since the infected cells contributing to the RV were seeded throughout pre-ART, HIV cure studies should limit the infectious reservoir established early post-infection.

Further studies are needed to define mechanisms for the maintenance of the active reservoir contributing to the RV and whether immune escape or resistance to CTLs promotes persistence of the RV. Multiple and different eradication strategies may be necessary to target the various sources of the R5 and X4/dual-tropic RV observed in our study. The persistence of RV variants for at least 8.2 years during effective ART suggests cure strategies such as “kick and kill” may be ineffective at eliminating the active reservoirs [49]. Further understanding the dynamics of residual viremia may allow for targeted cure strategies against the persistent, proliferating infected cells contributing to infectious residual viremia despite successful ART.

Since a large proportion of the RV were monotypic, suggesting release from proliferating cells, HIV cure studies can benefit from the design of pharmacological inhibitors that could block

infected cell proliferation. Current studies in other labs are examining methods to block clonal proliferation by using JAK/STAT inhibitors in a clinical trial (NCT02475655) [74].

Our future work is focused on examining the HIV integration sites associated with the proviruses contributing to the RV. Examination of HIV integration sites is especially informative for HIV therapeutic studies using gene therapy. Retrovirus integration is non-specific at the gene level but can impact cell function and survival by insertional mutagenesis and gene activation [95]. Furthermore, deleterious problems to the cell and host can occur if HIV integration is found in oncogenes, for example. A previous study in a human gene therapy trial observed insertional activation of oncogenes as a result of the therapeutic retroviral vector integration near a proto-oncogene, suggesting integration can have significant effects on the fate of the cell and potential cancer development/proliferation [162].

By examining HIV integration sites associated with the proviruses contributing the RV, we can identify patterns for enrichment in genes associated with specific cellular pathways. For example, if there is an enrichment for HIV integration in genes associated with transcription regulation, drugs can be designed to either promote or inhibit HIV expression through “block and lock” by targeting the drugs to bind to certain IS genes and turn off viral expression. Alternatively, pharmacological inhibitors can be designed to alter or activate cell death pathways to promote infected cell death [163, 164]. One study observed that HIV infection upregulated BIRC5, an apoptosis inhibitor, and enabled survival of the infected cell and by pharmacologically inhibiting BIRC5, they selectively decreased the number of infected cells *in vitro* [165]. Others found inhibiting metabolic activities can block HIV replication whereby suboptimal inhibition of metabolic activities such as glycolysis resulted in an early block in HIV replication and induced cell death [166]. In short, future studies may choose to determine the IS landscape among infected

individuals to further identify shared enriched biological processes or regions associated with HIV integration to make a more informed decision about potential drug targets.

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APPENDIX A

Participant 1	ART-suppressed				Post-ART interruption and restart
Years post-infection	5	7.8	9.8	10.6	13.2
Date	1-13-10	8-15-12	8-27-14	6-26-15	2-28-18
Years post-ART start	1	3.7	5.7	6.5	9.2
Viral load	<50	<50	<50	<40	<40
Mean # virions/mL plasma	3.52	3.13	3.16	3.17	1.79
extraction 1 (positive gel bands/mL extracted)	18/3ml	11/3ml	8/3ml	6/3.5ml	8/3.5ml
extraction 2	0/3.5ml	11/3.5ml	16/3.5ml	11/3.5ml	5/3.5ml
extraction 3	16/3.5ml	10/3.5ml	4/3.5ml	29/7ml	5/3ml
extraction 4		10/3.5ml	9/3.5ml	11/3.5ml	9/3ml
extraction 5			17/3.5ml	13/3.5ml	6/3ml
extraction 6					1/3ml
Total plasma mL used for RNA extraction	10	13.5	17	21	19
Total C2V5*env* sequences obtained	34	42	54	70	34

Participant 2	ART-suppressed						Post-ART interruption and restart
Years post-infection	4.11	5.7	7.3	7.9	10.1	11.1	13.10
Years post-ART start	2	2.7	4.3	4.8	7.2	8.2	10.2
Date	1-25-10	9-27-10	5-14-12	11-26-12	3-16-15	3-24-16	3-21-18
Viral load	<50	<50	<50	<50	<50	<40	<40
Mean # virions/mL plasma	2.27	1.17	5.03	0.83	0.55	0.52	1.81
extraction 1 (positive gel bands/mL extracted)	9/3ml	4/3ml	19/3.5ml	4/3ml	0/3.5ml	7/3.5ml	3/3.5
extraction 2	4/3.5ml	3/3ml	21/3.5ml	1/3ml	3/3.5ml	1/3.5ml	6/3.5
extraction 3	8/3ml		11/3ml		4/3ml	1/3ml	1/3ml
extraction 4					2/3ml	0/1.5ml	
extraction 5					2/3ml	0/1.5ml	
extraction 6					0/1.5ml		
extraction 7					1/3ml		
Total plasma mL used for RNA extraction	9.5	6	10	6	20.5	13	13
Total C2V5*env* sequences obtained	21	7	51	5	12	9	10

Participant 3	ART-suppressed							Post-ART interruption and restart
Years post-infection	4.2	4.6	5.9	6.3	6.11	7.2	10.6	14.4
Years post-ART start	6 months	10 months	2.2	2.6	3.3	3.5	6.9	10.7
Date	7-8-98	11-2-98	2-14-00	7-31-00	4-12-01	6-25-01	11-22-04	8-27-08
Viral load	<50	<50	<50	<50	<50	<50	<50	<50
Mean # virions/mL plasma	1.00	1.00	0.83	3.00	1.50	0.33	1.13	1.40
extraction 1 (positive gel bands/mL extracted)	5/3ml	3/3ml	3/3ml	9/3ml	7/3ml	1/3ml	4/3ml	6/3ml
extraction 2	1/3ml		2/3ml		2/3ml		0/1.5ml	1/3ml
extraction 3							6/3ml	8/3ml
extraction 4							5/3ml	5/3ml
extraction 5							2/3ml	1/3ml
Total plasma mL used for RNA extraction	6	3	6	3	6	3	13.5	15
Total C2V5*env* sequences obtained	6	3	5	9	9	1	17	21

VITA

Hadega Aamer is an American-Egyptian who was born and raised in Spokane, WA. She completed her Bachelor's of Science at Eastern Washington University in Cheney, WA and then pursued a Master's of Science at the University of Pittsburgh in Pittsburgh, PA. After living on the East coast for several years, she missed the beautiful Northwest and came to the University of Washington to start her PhD in the Pathobiology program. During her time away from the lab, she loves to read fiction novels and lifestyle magazines. She also enjoys spending time in nature, camping and kayaking. She is obsessed with trying new food and exploring cafes and restaurants and plans to start a "Foodie" podcast with her foodie buddy, reviewing local eateries in Seattle. Hadega loves to work in the lab and hopes to pursue a career in industry, researching therapeutics for infectious diseases.