UC San Diego

UC San Diego Previously Published Works

Authors

Oliveira, Michelli F
Pankow, Alec
Vollbrecht, Thomas
et al.

Check for updates

Evaluation of Archival HIV DNA in Brain and Lymphoid Tissues

Michelli F. Oliveira,[a] Alec Pankow,[b*] Thomas Vollbrecht,[a,c] Nikesh M. Kumar,[a] Gemma Cabalero,[a] Caroline Ignacio,[a] Mitchell Zhao,[a] Andrej Vitomirov,[a] Ben Gouaux,[a] Masato Nakawawa,[a] Ben Murrell,[a] Ronald J. Ellis,[d] Sara Gianella[a]

aDepartment of Medicine, University of California San Diego, La Jolla, California, USA

bDepartment of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden

cVeterans Affairs San Diego Healthcare System, San Diego, California, USA

dDepartment of Neurosciences and Psychiatry, University of California San Diego, La Jolla, California, USA

Ronald J. Ellis and Sara Gianella are co-senior authors and contributed equally.

ABSTRACT HIV reservoirs persist in anatomic compartments despite antiretroviral therapy (ART). Characterizing archival HIV DNA in the central nervous system (CNS) and other tissues is crucial to inform cure strategies. We evaluated paired autopsy brain—frontal cortex (FC), occipital cortex (OCC), and basal ganglia (BG)—and peripheral lymphoid tissues from 63 people with HIV. Participants passed away while virally suppressed on ART at the last visit and without evidence of CNS opportunistic disease. We quantified total HIV DNA in all participants and obtained full-length HIV-envelope (FL HIV-*env*) sequences from a subset of 14 participants. We detected HIV DNA (*gag*) in most brain (65.1%) and all lymphoid tissues. Lymphoid tissues had higher HIV DNA levels than the brain ($P < 0.01$). Levels of HIV *gag* between BG and FC were similar ($P > 0.2$), while OCC had the lowest levels ($P = 0.01$). Females had higher HIV DNA levels in tissues than males (*gag*, $P = 0.03$; 2-LTR, $P = 0.05$), suggesting possible sex-associated mechanisms for HIV reservoir persistence. Most FL HIV-*env* sequences ($n = 143$) were intact, while 42 were defective. Clonal sequences were found in 8 out of 14 participants, and 1 participant had clonal defective sequences in the brain and spleen, suggestive of cell migration. From 10 donors with paired brain and lymphoid sequences, we observed evidence of compartmentalized sequences in 2 donors. Our data further the idea that the brain is a site for archival HIV DNA during ART where compartmentalized provirus may occur in a subset of people. Future studies assessing FL HIV-provirus and replication competence are needed to further evaluate the HIV reservoirs in tissues.

IMPORTANCE HIV infection of the brain is associated with adverse neuropsychiatric outcomes, despite efficient antiretroviral treatment. HIV may persist in reservoirs in the brain and other tissues, which can seed virus replication if treatment is interrupted, representing a major challenge to cure HIV. We evaluated reservoirs and genetic features in postmortem brain and lymphoid tissues from people with HIV who passed away during suppressed HIV replication. We found a differential distribution of HIV reservoirs across brain regions which was lower than that in lymphoid tissues. We observed that most HIV reservoirs in tissues had intact envelope sequences, suggesting they could potentially generate replicative viruses. We found that women had higher HIV reservoir levels in brain and lymphoid tissues than men, suggesting possible sex-based mechanisms of maintenance of HIV reservoirs in tissues, warranting further investigation. Characterizing the archival HIV DNA in tissues is important to inform future HIV cure strategies.

KEYWORDS brain, CNS, lymphoid tissue, archival HIV, sex differences, sequencing

Antiretroviral therapy (ART) suppresses viral replication in most people with HIV (PWH) and drastically reduces morbidity and mortality. However, since ART does not eradicate cells harboring HIV DNA (1, 2), plasma viremia generally rebounds quickly after treatment

TABLE 1 Demographics and clinical characteristics of the study population

Parameter	Values by category			
	All ($n = 63$)	Female ($n = 12$, 19%)	Male ($n = 51$, 81%)	P value[a]
Age[b]	55.0 (48.5, 61.0)	56.0 (49.5, 62.3)	54.0 (48.0, 61.0)	0.563
White (%)	60.3	25	68.6	0.005
Current CD4 T cell counts[b]	164 (80, 390)	330 (126, 453)	155 (77, 285)	0.134
EDI (yrs)[b]	14.0 (9.75, 19.0)	17.0 (15.5, 19.0)	13.0 (8.75, 18.3)	0.085
Estimated LT ART (yrs)[b]	6.07 (2.74, 9.43)	9.09 (6.15, 13.45)	5.04 (2.32, 8.50)	0.076

[a]P values were calculated for continuous variables using Mann-Whitney U test and for categorical variables using the χ^2 test between female and male sex.
[b]Data are expressed as median (interquartile range). ART, antiretroviral therapy; LT, lifetime; EDI, estimated duration of infection.

interruption (3–6). Replication-competent HIV DNA that remains in cellular reservoirs despite effective ART represents a major challenge for an HIV cure (7, 8). Most research trying to understand the characteristics and dynamics of these reservoirs focuses on blood CD4$^+$ T cells (9–11). However, characterizing residual sources of virus in other cell types and anatomic compartments, including the central nervous system (CNS) (12), is a crucially relevant area of investigation. Several considerations motivate a better understanding of the HIV reservoir in the CNS, as follows: (i) HIV enters the CNS immediately after systemic infection and creates a site of persistence that harbors replication-competent HIV that can rebound upon ART interruption (4, 13, 14); (ii) HIV infection of the CNS can lead to neurocognitive (NC) impairment, which still has a prevalence up to 50% among PWH, despite sustained viral suppression (15); (iii) any pharmacological interventions must overcome the blood-brain barrier, which may limit the ability of a drug to achieve effective concentrations in the CNS; (4) the CNS hosts unique cellular targets for HIV, including microglia, perivascular macrophages, and possibly astrocytes, that may enable the persistence of a distinctive HIV reservoir and may respond differently to interventions compared with cellular targets in other tissues (16–18); and (v) the CNS contains viral variants, including some resistant to antiretroviral drugs, that may influence the effectiveness of cure interventions (19–24). The factors associated with the maintenance of the HIV reservoirs and its effects in the CNS during ART remain to be fully understood. In this study, we sought to characterize the archival HIV DNA in the CNS by studying a unique set of postmortem samples collected in the National NeuroAIDS Tissue Consortium (NNTC) from PWH with viral suppression. Notably, viral suppression in PWH is a characteristic inadequately explored in prior publications. We quantified the levels of HIV DNA in three different brain regions and lymphoid tissues, and for a subset of these participants, we evaluated full-length HIV envelope (FL HIV-*env*) sequences.

RESULTS

Study population. Table 1 summarizes the clinical and demographic characteristics of the entire study sample, which was divided by sex at birth. The sample included 63 cases, who were enrolled into the NNTC between 1999 and 2014, with a median age of 55 years, with an estimated duration of infection of 14 years and 5.6 years of lifetime exposure to ART by the time of death, and without evidence of CNS opportunistic disease. In this study, we had 12 (19%) women and 51 (81%) men, and the two groups did not differ significantly for most characteristics (Table 1). Participants were considered virally suppressed at last visit prior to death (median 3 months prior to death) with viral loads of <50 cps/mL of plasma ($n = 37$ participants, $n = 9$ female) or <400 cps/mL ($n = 26$ participants, $n = 4$ female), which were assay dependent, according to the NNTC guidelines.

HIV DNA in brain and lymphoid tissues. We measured HIV DNA levels in a total of 237 autopsy tissue samples, from paired postmortem frontal cortex (FC; $n = 63$), occipital cortex (OCC; $n = 56$), basal ganglia (BG; $n = 59$), and peripheral lymphoid tissue ($n = 59$) across 63 participants (Table 2). We detected HIV DNA *gag* in a majority (65.1%) of brain tissues, ranging from 1.5 to 2,968 copies per 10^6 cells, and in all lymphoid tissues evaluated, ranging from 7.3 to 3,741 copies per 10^6 cells. We further detected 2-LTR circles in 23.6% of brains and 57.6% of lymph tissues. To investigate differences in levels among brain and

TABLE 2 Levels of HIV DNA in brain and lymphoid tissues

| | Data by category | | | | | |
| | All (n = 63) | | Female (n = 12, 19%) | | Male (n = 51, 81%) | |
Parameter	gag	2-LTR	gag	2-LTR	gag	2-LTR
HIV DNA gag and 2-LTR[a]						
Frontal cortex	69.8 (44/63)	22.2 (14/63)	75 (9/12)	41.6 (5/12)	68.6 (35/51)	17.6 (9/51)
Occipital cortex	55.4 (31/56)	21.4 (12/56)	75 (9/12)	33.3 (4/12)	50 (22/44)	11.4 (5/44)
Basal ganglia	69.5 (41/59)	27.1 (16/59)	81.8 (9/11)	54.5 (6/11)	66.7 (32/48)	20.8 (10/48)
Lymphoid tissue	100 (59/59)	57.6 (34/59)	100 (9/9)	55.5 (5/9)	100 (50/50)	58 (29/50)
HIV DNA gag and 2-LTR (cps/million cells)[b]						
Frontal cortex	186.3 (1.5–2,968.1)	44.4 (1.4–284.7)	187.8 (4.2–1,270.7)	22.4 (5.2–78.3)	163.7 (1.5–2,968.1)	56.6 (1.4–284.7)
Occipital cortex	78.5 (2.5–1,401.6)	15.9 (2.7–50.9)	177.6 (2.8–1,401.6)	14.9 (2.7–40.3)	37.9 (2.5–283.9)	16.6 (3.3–50.9)
Basal ganglia	230.5 (1.6–2,120.2)	32.4 (2.1–172.8)	423.4 (2.5–2,120.2)	39 (2.1–172.8)	183.5 (1.6–2,096.4)	28.4 (3.2–107.6)
Lymphoid tissue	364.7 (7.3–3,741.9)	29.9 (1–171)	501.1 (12.2–2,801.6)	53.5 (2–171)	313.7 (7.3–3,741.9)	26.5 (2.6–149.1)

[a]Data are shown as % (n of positive/available processed samples).
[b]Data are shown as mean (range) of detectable samples.

lymphoid tissues, we performed a contrast analysis, as described in the Materials and Methods. Lymphoid tissue had significantly higher levels of HIV DNA than brain tissues in both HIV gag and 2-LTR ($P < 0.01$), regardless of sex. HIV DNA levels in brain and lymphoid tissues were not correlated ($P > 0.2$). Next, we evaluated for the differential distribution of HIV DNA in brain tissues. We found that levels of HIV gag in BG and FC were similar ($P > 0.2$), while OCC had lower levels than the other two brain regions ($P = 0.01$). The 2-LTR levels were similar between the 3 brain regions ($P > 0.2$). We tested the potential confounding effect of relevant covariates, such as age, CD4 T cells, time on ART, race (white, black, and other), ethnicity (Hispanic and non-Hispanic), and EDI that were included in the model one at a time. None of these covariates was associated with HIV gag levels ($P > 0.14$), while all P values for the above contrast analysis remained significant, regardless of which covariate was included in the model.

HIV DNA in tissues between sexes. Since previous studies support sex differences in viral loads and HIV persistence, we included sex as a predictor in our statistical models to evaluate if levels of HIV DNA in tissues were associated with sex. First, we observed that among women's brain tissues, HIV DNA gag was detected in 75% of frontal and occipital cortexes and 81.8% of basal ganglia. Levels of total HIV DNA (gag) in women's brains ranged from 2.5 to 2,802 copies per 10^6 cells. For men's brain tissues, we detected HIV DNA (gag) in 68.6% of frontal cortex and 50% of occipital and 66.7% of basal ganglia (Table 2). In men's brains, levels of HIV DNA ranged from 1.5 to 3,742 copies per 10^6 cells. Overall, we found that female sex was associated with higher levels of HIV gag ($P = 0.03$) and a trend in higher 2-LTR levels ($P = 0.05$) in brain and lymphoid tissues, regardless of brain region (Fig. 1). The female and male patients did not significantly differ in the distributions of HIV gag and 2-LTR among the three brain regions and the lymphoid tissues (all $P > 0.27$ and $P > 0.52$, respectively).

Full-length HIV envelope (FL HIV-env) sequences. To evaluate genetic composition and relationships of HIV provirus, we performed single-genome amplification (SGA) coupled with PacBio sequencing to obtain FL HIV-env sequences. We obtained a total of 185 individual FL HIV-env sequences across 14 participants (FC, $n = 9$; OCC, $n = 5$; lymph node (LN), $n = 3$; spleen (SP), $n = 8$) (Fig. 2 and Table 3). Overall, 143 FL HIV-env sequences were genetically intact, while 42 sequences were nonfunctional, with major deletions and frameshift and stop codon mutations (Fig. 2 and Table 3). Table 3 summarizes the demographics and HIV DNA features of participants with available FL HIV-env sequences. Clonal sequences were found in brain or lymphoid tissues from 8 participants. Interestingly, for donor 54, we found the same clonal sequence with a frameshift mutation in both the brain (15 sequences) and spleen (7 sequences), suggesting a migration of cells with a defective clonal provirus between tissue compartments. For 10 donors, FL HIV-env sequences were
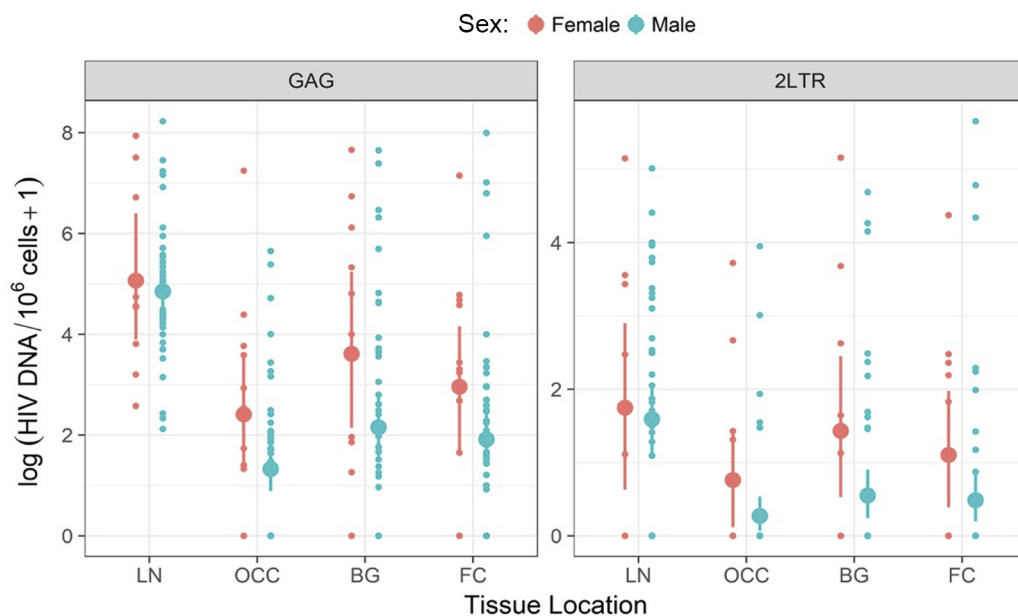
FIG 1 HIV DNA levels by tissue location and sex. Small dots represent individual observations. Large dots and vertical bars represent means and their 95% confidence intervals.

obtained from paired brain and lymphoid tissues. Sequence diversity within each tissue was assessed both including and excluding sequences with identified apolipoprotein B mRNA-editing enzyme, catalytic polypeptide (APOBEC) hypermutation. HIV compartmentalization patterns differed between some donors, with two (cases 2, viral load [VL] of <50 cp/mL; case 49, VL of <400 cp/mL) showing evidence of HIV DNA compartmentalization between brain tissues and lymph node (Fst test, $P < 0.05$). *In silico* tropism prediction identified likely R5-tropic sequences in both brain and lymphoid tissue in all but one participant with sequences from both tissue types (case 60). Four participants yielded sequences which were either likely X4-tropic or with a tropism prediction in an indeterminate range (cases 21, 54, 60, and 690) (Fig. 2; see Fig. S1 in the supplemental material; Table 3).

DISCUSSION

In this study, we sought to characterize the archival HIV DNA by studying a unique set of postmortem brain samples. Assessing tissue samples from different brain regions and lymphoid tissues across a total of 63 PWH and using the sensitive droplet digital PCR (ddPCR) method, we detected HIV DNA in a majority of these tissues (65% of brain and 100% of lymphoid samples). Few previous studies have evaluated brain tissues using quantitative PCR methods or tissue hybridization techniques, and the number of cases was comparatively small. In a study from Lamers et al. (25), HIV DNA was quantified in different autopsy tissues, including brain and lymphoid tissues, from 20 virally suppressed participants (plasma VL prior death of <40 or <400 copies/mL, assay dependent). In that study, HIV DNA was detected in 55% of brain tissues (48/87), with levels ranging from 266 to 49,401 copies per 10^6 cells using ddPCR for HIV *gag* (detection limit of 200 copies per 10^6 cells), which are levels similar to those seen in the current study. A second study from Ko et al. (12) investigated the presence of cells harboring HIV RNA and DNA in brains from 16 virally suppressed cases using RNAscope and DNAscope ISH technologies, respectively. In that study, cells harboring HIV DNA were detected in brains tissues from all virally suppressed individuals evaluated. Recently, our group evaluated autopsy tissues, including brain and lymphoid tissues, from six participants from the Last Gift (LG) cohort, of which four had confirmed viral suppression (<20 copies/mL) until the time of death (26). HIV DNA was detected in brain tissues from three out of the four LG participants with an undetectable plasma viral load,
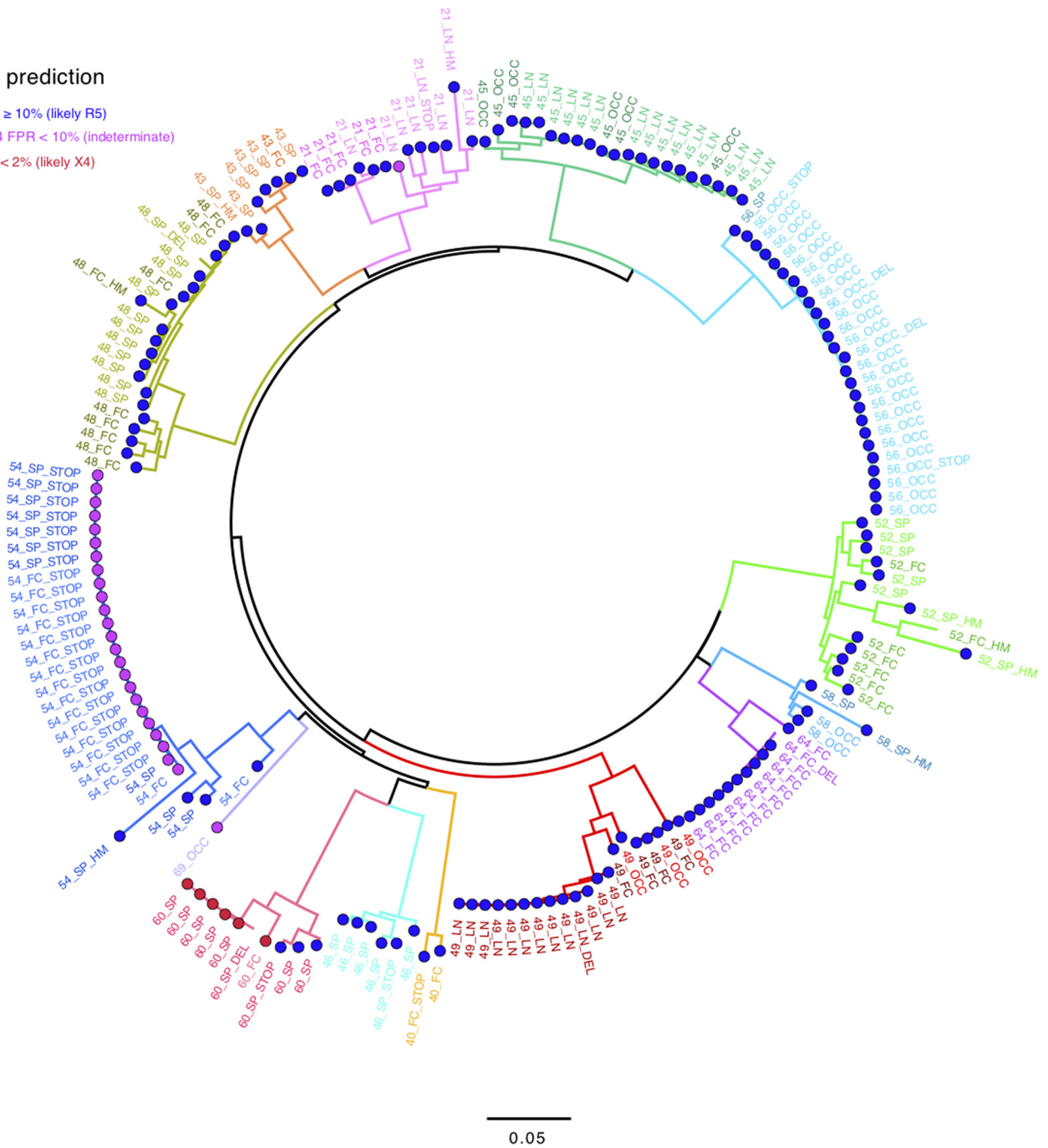
FIG 2 Maximum likelihood phylogeny of 185 FL HIV-*env* sequences obtained from postmortem brain and lymphoid tissues from 14 PWH donors enrolled as part of the NNTC cohort. Each taxon is an individual *env* sequence. Each sequence is labeled with the participant's number, followed by the tissue type (FC, frontal cortex; OCC, occipital cortex; LN, lymph node; SP, spleen). Individual participants are also represented by colors, and different tissues within a participant are represented by a shade of that color. Nonintact *env* sequences are indicated for APOBEC hypermutations (HM), deletions (DEL), and frameshifts due to stop codon (STOP), following the sequence name. Sequence nodes are colored based on *in silico* Geno2Pheno predictions of coreceptor tropism (see Materials and Methods). Cutoffs for classifying sequences based on the X4 FPR were defined as follows: less than 2% for likely CXCR4-tropic, 2% to 10% for an "indeterminate" range, and greater than or equal to 10% as likely CCR5-tropic. A missing node indicates the V3 loop was not identifiable by the Geno2Pheno tool (FPR, false-positive rate).

with HIV DNA levels in brain tissues up to 9.1 copies per 10^6 cells in the occipital cortex and up to 33.6 copies/10^6 cells in the frontal cortex. In the present study, we found a higher range of HIV DNA levels in brain tissues (Table 2) than that in our LG study. While participants from the Last Gift cohort had confirmed viral suppression at the time of or within days close to death, the NNTC cohort participants included in this

TABLE 3 Demographics and HIV DNA features for the subset of participants with available FL HIV-*env* sequences

Case no.	Sex	Age at death	EDI (yrs)	VL (mo prior death)	Last known ART regimen	Tissue type[a]	HIV DNA *gag* (cps/10⁶ cells)	HIV DNA *2-LTR* (cps/10⁶ cells)	No. of intact sequences/total	No. of clonal sequences/total	Diversity[b]	Fst P value
21	M	54	10	<400 (4)	3TC/D4T/FTV/NVP	FC	24.2	0	5/5	0/5	0.003	0.002
						LN	83.3	2.0	6/8	0/8	0.032	
40	M	41	14	<50 (3)	EFV/FTC/TDF	FC	18.2	0	1/2	0/2	0.011	NA
43	M	51	13	<400 (1)	3TC/AZT/NFV	FC	3.3	0	1/1	NA	NA	NA
						SP	13.1	0	5/6	0/6	0.016	
45	F	64	14	<50 (5)	3TC/ZDV/EFV	OCC	3.1	0	6/6	2/6	0.017	0.332
						LN	2,801.6	2.0	15/15	0/15	0.017	
46	M	59	19	<50 (6)	3TC/ABC/EFV	SP	1,338.1	26.4	5/6	0/6	0.013	NA
48	F	64	6	<50 (4)	3TC/D4T	FC	118.2	7.9	8/9	2/9	0.025	0.424
						SP	826.4	29.9	11/12	2/12	0.009	
49	M	50	10	<50 (3)	ABC/NVP/ZDV	FC	27.5	0	4/4	0/4	0.024	0.001
						OCC	25.2	0	3/3	0/3	0.028	0.007
						LN	1,726.9	149.2	12/13	9/13	0.002	
52	M	66	7	<50 (4)	3TC/ABC/EFV	FC	5.5	0	6/7	0/7	0.021	0.215
						SP	75.2	0	5/7	2/7	0.018	
54	M	52	15	<50 (3)	EFV/FTC/TDF	FC	12.4	0	2/17	15/17	0.006	0.133
						SP	39.4	0	3/11	7/11	0.011	
56	M	52	13	<50 (3)	3TC/D4T/EFV	OCC	283.9	50.9	21/25	17/25	0.003	NA
						SP	9.3	0	1/1	NA	NA	
58	F	40	16	<400 (2)	TDF/FTC/RGV	OCC	11.6	3.2	2/2	0/2	0.007	0.333
						SP	113.3	0	1/2	0/2	NA	
60	M	50	19	<50 (1)	TDF/FTC/ATV/RTV	FC	10.8	0	1/1	NA	NA	NA
						SP	185.9	53.6	7/9	3/9	0.020	
64	M	66	24	<50 (unknown)	ABC/EFV/LPV/RTV	FC	24.4	9.5	11/12	7/12	0.008	NA
69	M	69	26	<50 (unknown)	3TC/ABC/ATV	OCC	3.4	0	1/1	NA	NA	NA

[a]FC, frontal cortex; OCC, occipital cortex; LN, lymph node; SP, spleen; NA, not applicable.
[b]Mean normalized hamming distance after removing hypermutated sequences.

study had reported viral loads at a median 3 months prior to death. It is possible that some NNTC participants had detectable viral loads between their last visit and time of death, which could have resulted in a replenishment of HIV reservoirs in their tissues. In our study, we also found that total HIV DNA levels, normalized to estimated numbers of host cellular input (RPP30 gene), were typically 1 to 2 log lower in the brain than in lymphoid tissue, which is consistent with previous reports (25, 26). This is not unexpected since the density of target cells is much lower in the CNS than that in lymphoid tissues, which are considered the major site for HIV persistence (26–28). The lack of correlation between brain and lymphoid tissues suggests that brain HIV DNA levels contain information that is not redundant with lymphoid tissue and therefore might reflect biological differences in target cells and viral variants. The brain reservoir has some other unique characteristics relative to lymphoid tissue. Target cells for HIV in brain tissue are mostly long-lived cells such as macrophages, microglia, and possibly astrocytes (29–32), which might influence the timing (later) and extent of rebound after antiretroviral withdrawal. Also, brain HIV DNA can affect the expression of host genes and epigenetics depending on the integration site, even in those with no viral RNA (29, 30). The relatively lower HIV DNA levels in the occipital cortex than those in the frontal lobe and basal ganglia may reflect biological differences, such as the density or activation states of target cells (microglia and macrophages) in the different tissues.

To the best of our knowledge, this is the largest study of HIV DNA quantification in brain tissues from virally suppressed PWH. Our current findings are consistent with the previously published reports with respect to brain HIV DNA detection and quantitation in virally suppressed PWH. These data support the idea that the brain may serve as a reservoir, albeit significantly smaller than lymphoid tissues, from which HIV might possibly re-emerge after discontinuation of ART (4). The majority of HIV proviruses in the periphery have been shown to be defective (33), making the characterization of replication competence key to understanding the potential impact of viral reservoirs in tissues, including the CNS. To partially address this question, recognizing the inability to perform quantitative viral outgrowth assays on archived snap-frozen brain tissues, we obtained FL HIV-*env* sequences to estimate the proportion of intact proviruses in brain and lymphoid tissues. We found that most FL HIV-*env* sequences obtained from brain and lymphoid tissues in this study were intact. Overall, this result is similar to what we found across LG tissues (including brain and lymphoid tissues) and peripheral blood mononuclear cell (PBMC), using FL HIV-*env* SGA coupled with Illumina sequencing, where 89.5% of all sequences were intact (26). Particularly, in that study, a total of 10 FL HIV-*env* sequences from brain tissues were obtained across three out of the four LG participants who were consistently HIV suppressed until the time of death, and all sequences were intact. For lymphoid tissues (lymph node and spleen), a total of 107 out of 115 sequences obtained across the 4 participants were intact (26). In contrast, previous studies in blood (33) reported that near FL-HIV provirus sequences have a considerably larger proportion of defective HIV genomes containing large deletions, point mutations, and APOBEC hypermutations, likely upward of 90% (33–36). This discrepancy could be explained by a limitation of this and previous studies (26), where sequencing analysis was focused on the FL HIV-*env* gene only, which has been shown previously to be frequently deleted in HIV proviruses in blood (33). By targeting the HIV FL-*env* gene for sequencing, we likely enriched our analysis for proviruses containing intact *env* genes, while we might have missed proviruses with a deleted, nonintact envelope gene. We cannot affirm that other regions of the HIV genomes we detected were intact. We have attempted to obtain near FL HIV genome sequences from these samples, however unsuccessfully, likely due to long-term storage which can lead to DNA shearing over time, posing a challenge to obtaining longer DNA amplicons.

We also found clonal sequences across both tissues, which varied in proportion between participants and tissues, consistent with the known ability of HIV-infected cells to undergo clonal expansion as an important mechanism of HIV persistence (37). Although this idea could be a possible explanation for our findings, our conclusions

are limited by only evaluating HIV FL-*env* gene sequences. While we did not evaluate integrations sites or FL HIV genome sequences to directly assess clonal expansion in this study, intriguingly, one participant had 22 clonal sequences containing the same frameshift mutation in both spleen and frontal cortex tissues, which suggests a clonal expansion of cells harboring the HIV provirus that migrated to different body tissues in this participant. An additional technical limitation is the fact that at the time of collection, tissues has not been perfused which could result in a potential contamination of tissues with blood. This possible contamination is likely to have a small impact on our analysis, given the small size of capillaries compared with that of the overall tissue mass and the settling of blood in tissues, i.e., livor mortis. We also found that a subset of two out of seven cases showed evidence of virus compartmentalization between brain and lymphoid tissues and *in silico* tropism predictions of R5-tropic viruses in most tissues. As the power to detect compartmentalization increases with the number of sequences, the limited number of sequences obtained for some participants is a limitation of the study and likely represents an underestimated observation. Our results further suggest that HIV can independently evolve under the unique selective pressures of those tissue environments and is stored in tissues, at least in a subset of people (4, 38, 39).

In this study, we also found that women had higher levels of total HIV DNA in tissues than men. The consistency of these differences across brain regions and lymphoid tissues warrants further investigation. Sex differences in HIV-1 disease phenotypes and the risk of acquisition have long been noted by epidemiologic studies (40) and include lower viral loads during acute infection and stronger antiviral responses in women than those in men. Sex-based differences in HIV-1 infection are partially due to differences in socioeconomic factors and in comorbid risks. More recently, there has been increasing evidence that biological differences between the sexes, such as immune cell phenotypes, drug penetration to mucosal sites, and microbiome features, may also play an important role during HIV infection (41). One recent study has shown that women have a lower inducible HIV reservoir in blood (as measured by inducible cell-associated HIV RNA) than men and suggested significant sex differences in HIV reactivation response to *ex vivo* exposure to hormones (42–45). While the factors that underlie the establishment and maintenance of the HIV reservoirs in tissues are largely unknown and require investigation, it is possible that they could be impacted by sex-based social and biological variables, supporting the idea that HIV cure approaches may be impacted by factors associated with sex differences. Future studies should evaluate the mechanisms by which sex could affect the establishment of HIV reservoirs both in the circulation and in tissues, including the brain.

Additional limitations of our study must further be considered, including the small sample size, particularly the limited number of available female participants. The limited number of sequences obtained from a subset of tissues/participants could also have been a consequence of DNA shearing from long-term stored tissues, impairing our ability to obtain long amplicons. Moreover, the use of a 400-copy HIV RNA detection limit assay leaves the possibility of residual low-level replication in some cases, which could have confounded our viral compartmentalization analysis. Although most participants with available sequences had last antemortem viral loads below 50 cps/mL of plasma, single-copy assays have shown evidence of low-level replication in a substantial fraction of PWH with virologic suppression by the 50-copy assay (46–48). It is also possible that some individuals could have discontinued antiretroviral treatment between the last clinical assessment and death with potential antemortem virus rebound, and if it has happened, it could have reseeded brain and lymphoid tissue HIV DNA in these cases. The duration of viral suppression prior to death was not available for these cases. It has not yet been established whether HIV replication can rebound from brain tissue. Indeed, it is unclear how one could prove unique rebound from the CNS reservoir. We have shown previously using phylogenetics that viral variants rebounding in the CSF following ART interruption are often distinguishable from blood, suggesting a separate origin (4). An important issue is whether the numbers of

replication-competent viruses in the brain are sufficient to support systemic rebound. Here, we show using FL HIV-*env* that at least some CNS-derived variants may be nondefective, suggesting that they are indeed capable of supporting replication and could be a source for systemic viral rebound. We did not evaluate the relationship between neurocognition and brain HIV DNA levels here due to its complexity; the high prevalence of confounding neurocognitive conditions in these participants with a history of advanced HIV disease (49) makes interpretation difficult. The mechanisms and factors involved in the establishment and maintenance of the HIV reservoirs in the brain and other body tissues require future systematic investigation, as the presence of complete and replication competent provirus in the CNS need to be confirmed, which is critical in the context of HIV cure strategies.

MATERIALS AND METHODS

Ethics statement. The study was approved by the Institutional Review Board at the University of California and at each of the other respective NNTC sites at the University of California, Los Angeles, Icahn School of Medicine at Mount Sinai—New York City and The University of Texas Medical Branch at Galveston. All adult participants (age, ≥18 years) provided written informed consent. No children were included in this study.

Study design. (i) Study population and samples. We retrospectively selected participants and included postmortem paired brain and lymphoid tissues that were collected and stored at −80°C as part of the NNTC. The selection criteria of cases were as follows: participants had undetectable levels of HIV RNA (<50 or <400 copies/mL, assay dependent) in blood plasma and were on ART at the last visit, at least within 6 months prior to death, and had no evidence of opportunistic CNS disease at the time of autopsy. For all participants, information on demographics and clinical data, such as sex at birth, age, ethnicity, years of education, estimated duration of HIV infection (EDI), blood T lymphocyte counts, HIV RNA viral load, time on ART, and ART regimen, were available as part of the NNTC cohort. From each participant, we requested brain tissues, including frontal cortex (FC), occipital cortex (OCC), and basal ganglia and paired peripheral lymphoid tissues, including lymph nodes (LN) or spleen (SP), as available.

(ii) Quantification of HIV DNA in tissues. Total genomic DNA was extracted from 60 to 90 mg of tissues for all participants using magnetic beads per the manufacturer's protocol (MagMax DNA multisample; Ambion, Austin, TX). Levels of HIV *gag* DNA (HXB2 coordinates 1366 to 1619) and 2-LTR circles (HXB2 coordinates 9585 to 51) were measured by droplet digital PCR (ddPCR) (4, 50). Briefly, 1 μg of total genomic DNA per replicate was digested with the BanII enzyme (New England BioLabs, Ipswich, MA). ddPCR was performed with the following cycling conditions: 10 min at 95°C, 40 cycles of a 30 s denaturation at 94°C followed by a 60°C extension for 60 s, and a final extension at 98°C for 10 min. HIV copy numbers were calculated as the mean of triplicate measurements and were normalized to cellular input, estimated by measuring the human gene RPP30 levels, as described previously (50). HIV *gag* levels were used as an estimate of total HIV DNA, and 2-LTR circles were used as an estimate of unintegrated HIV DNA and a proxy of active HIV replication.

(iii) Statistical analysis. The Bayesian hierarchical regression model was used to evaluate the differences between HIV DNA levels (the outcome) in brain regions, lymphoid tissues, and sex (predictors). The model used a zero-inflated negative-binomial family with a logit and log link functions. Levels of HIV DNA were square-root-transformed to approach normality. Significance was assessed with the Bayesian P value of $2*p(\theta > 0|X)$, indicating a portion of 2-tailed posterior predictive distribution that is greater than 0, conditioned on X (a vector of other predictors and covariates). To investigate differences in HIV *gag* DNA levels among brain and peripheral tissues, we performed contrast analysis. For that analysis, we computed 3 orthogonal contrast coefficients and included in the same model (i) the average DNA levels in the 3 brain regions versus the peripheral DNA level, (ii) the average in the BG and FC versus OCC, and (iii) BG versus FC. Based on the notion that potential biological sex differences could be associated with HIV measures, we included sex as a predictor in our statistical models to evaluate if levels of HIV DNA in tissues were associated with sex.

(iv) FL HIV-*env* sequences and sequence analysis. The genotypic composition of FL HIV-*env* populations was evaluated via high-throughput near-single genome amplification (near-SGA) using the PacBio platform. Briefly, up to 10 μg of genomic DNA per sample was diluted to reach up to 80% positive wells and used in a nested PCR with the outer primer pair 5′FENV_MFO (TTAGGCATCTCCTATGGC AGGAA) and 3′RENV_MFO (TCTTAAAGGTACCTGAGGTCTGACTGG) and an inner primer pair described previously (51). After positive PCR wells were identifier, the corresponding 1st round PCRs were diluted 1:100 to eliminate both excess 1st round primers and nonspecific amplification products. Diluted 1st round products were used for the 2nd round PCR using inner primers with custom-made barcodes to generate uniquely barcoded FL HIV-*env* amplicons. All barcoded amplicons obtained in this study were quantified and multiplexed in equimolar amounts to generate PacBio library preparations for sequencing. Single FL HIV-*env* sequences were reconstructed using our previously described amplicon denoising approach with adaptations for near-SGA amplicon sequencing (52). All computational code for sequence postprocessing, including intact/defective determination, alignment, and phylogenetic tree construction, and Fst permutation testing is available online at https://github.com/alecpnkw/nntc_hiv_brain_lymphoid_reservoirs (53). Briefly, sequences were aligned using MAFFT (54), and the maximum likelihood phylogeny was constructed using FastTree (55). Sequences with a stop codon before residue 750 (premature stop) or a total translated length of less than 800 residues

(deletion) were interpreted as likely defective. G-to-A hypermutations were detected using Hypermut2 (Los Alamos National Laboratory) (56). Compartmentalization and diversity analyses were conducted via a nonparametric permutation test reporting a one and two-sided *P* value, respectively, in participants from which we obtained at least two sequences in brain and lymphoid tissues. Mean pairwise hamming distance was used as the measure of nucleotide diversity. The phylogenetic tree was annotated in FigTree (http://tree.bio.ed.ac.uk/software/figtree), and the viral tropism of the FL HIV-*env* sequences was predicted *in silico* using the Geno2Pheno online tool (https://coreceptor.geno2pheno.org/).

Data availability. All sequences are accessible though the link https://datadryad.org/stash/dataset/doi:10.5061/dryad.931zcrjqb.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
SUPPLEMENTAL FILE 1, TIF file, 7.1 MB.

REFERENCES

1. Chun T-W, Stuyver L, Mizell SB, Ehler LA, Mican JAM, Baseler M, Lloyd AL, Nowak MA, Fauci AS. 1997. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc Natl Acad Sci U S A 94:13193–13197. https://doi.org/10.1073/pnas.94.24.13193.
2. Wong JK, Hezareh M, Günthard HF, Havlir DV, Ignacio CC, Spina CA, Richman DD. 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science 278:1291–1295. https://doi.org/10.1126/science.278.5341.1291.
3. Calin R, Hamimi C, Lambert-Niclot S, Carcelain G, Bellet J, Assoumou L, Tubiana R, Calvez V, Dudoit Y, Costagliola D, Autran B, Katlama C, the ULTRASTOP study group. 2016. Treatment interruption in chronically HIV-infected patients with an ultralow HIV reservoir. AIDS 30:761–769. https://doi.org/10.1097/QAD.0000000000000987.
4. Gianella S, Kosakovsky Pond SL, Oliveira MF, Scheffler K, Strain MC, De la Torre A, Letendre S, Smith DM, Ellis RJ. 2016. Compartmentalized HIV rebound in the central nervous system after interruption of antiretroviral therapy. Virus Evol 2:vew020. https://doi.org/10.1093/ve/vew020.
5. Chun T-W, Justement JS, Murray D, Hallahan CW, Maenza J, Collier AC, Sheth PM, Kaul R, Ostrowski M, Moir S, Kovacs C, Fauci AS. 2010. Rebound of plasma viremia following cessation of antiretroviral therapy despite profoundly low levels of HIV reservoir: implications for eradication. AIDS 24:2803–2808. https://doi.org/10.1097/QAD.0b013e328340a239.
6. Li JZ, Etemad B, Ahmed H, Aga E, Bosch RJ, Mellors JW, Kuritzkes DR, Lederman MM, Para M, Gandhi RT. 2016. The size of the expressed HIV reservoir predicts timing of viral rebound after treatment interruption. AIDS Lond Engl 30:343–353. https://doi.org/10.1097/QAD.0000000000000953.
7. Ho Y, Shan L, Hosmane NN, Wang J, Laskey SB, Rosenbloom DIS, Lai J, Blankson JN, Siliciano JD, Siliciano RF. 2013. Replication-competent non-induced proviruses in the latent reservoir are barrier to HIV-1 cure. Cell 155:540–551. https://doi.org/10.1016/j.cell.2013.09.020.
8. Richman DD, Margolis DM, Delaney M, Greene WC, Hazuda D, Pomerantz RJ. 2009. The challenge of finding a cure for HIV infection. Science 323:1304–1307. https://doi.org/10.1126/science.1165706.
9. Pallikkuth S, Sharkey M, Babic DZ, Gupta S, Stone GW, Fischl MA, Stevenson M, Pahwa S. 2015. Peripheral T follicular helper cells are the major HIV reservoir within central memory CD4 T cells in peripheral blood from chronically HIV-infected individuals on combination antiretroviral therapy. J Virol 90:2718–2728. https://doi.org/10.1128/JVI.02883-15.
10. Bruner KM, Cohn LB. 2019. HIV-1 reservoir dynamics in CD4+ T cells. Curr Opin HIV AIDS 14:108–114. https://doi.org/10.1097/COH.0000000000000521.
11. Lee GQ, Lichterfeld M. 2016. Diversity of HIV-1 reservoirs in CD4+ T-cell subpopulations. Curr Opin HIV AIDS 11:383–387. https://doi.org/10.1097/COH.0000000000000281.
12. Ko A, Kang G, Hattler JB, Galadima HI, Zhang J, Li Q, Kim W-K. 2019. Macrophages but not astrocytes harbor HIV DNA in the brains of HIV-1-infected aviremic individuals on suppressive antiretroviral therapy. J Neuroimmune Pharmacol 14:110–119. https://doi.org/10.1007/s11481-018-9809-2.
13. Joseph SB, Trunfio M, Kincer LP, Calcagno A, Price RW. 2019. What can characterization of cerebrospinal fluid escape populations teach us about viral reservoirs in the central nervous system? AIDS 33:S171–S179. https://doi.org/10.1097/QAD.0000000000002253.

14. Lutgen V, Narasipura SD, Barbian HJ, Richards M, Wallace J, Razmpour R, Buzhdygan T, Ramirez SH, Prevedel L, Eugenin EA, Al-Harthi L. 2020. HIV infects astrocytes in vivo and egresses from the brain to the periphery. PLoS Pathog 16:e1008381. https://doi.org/10.1371/journal.ppat.1008381.

15. Heaton RK, Clifford DB, Franklin DR, Woods SP, Ake C, Vaida F, Ellis RJ, Letendre SL, Marcotte TD, Atkinson JH, Rivera-Mindt M, Vigil OR, Taylor MJ, Collier AC, Marra CM, Gelman BB, McArthur JC, Morgello S, Simpson DM, McCutchan JA, Abramson I, Gamst A, Fennema-Notestine C, Jernigan TL, Wong J, Grant I, CHARTER Group. 2010. HIV-associated neurocognitive disorders persist in the era of potent antiretroviral therapy: CHARTER Study. Neurology 75:2087–2096. https://doi.org/10.1212/WNL.0b013e318200d727.

16. Schnell G, Joseph S, Spudich S, Price RW, Swanstrom R. 2011. HIV-1 replication in the central nervous system occurs in two distinct cell types. PLoS Pathog 7:e1002286. https://doi.org/10.1371/journal.ppat.1002286.

17. Churchill MJ, Gorry PR, Cowley D, Lal L, Sonza S, Purcell DFJ, Thompson KA, Gabuzda D, McArthur JC, Pardo CA, Wesselingh SL. 2006. Use of laser capture microdissection to detect integrated HIV-1 DNA in macrophages and astrocytes from autopsy brain tissues. J Neurovirol 12:146–152. https://doi.org/10.1080/13550280600748946.

18. Churchill MJ, Wesselingh SL, Cowley D, Pardo CA, McArthur JC, Brew BJ, Gorry PR. 2009. Extensive astrocyte infection is prominent in human immunodeficiency virus-associated dementia. Ann Neurol 66:253–258. https://doi.org/10.1002/ana.21697.

19. Chen H, Wood C, Petito CK. 2000. Comparisons of HIV-1 viral sequences in brain, choroid plexus and spleen: potential role of choroid plexus in the pathogenesis of HIV encephalitis. J Neurovirol 6:498–506. https://doi.org/10.3109/13550280009091950.

20. Holman AG, Mefford ME, O'Connor N, Gabuzda D. 2010. HIVBrainSeqDB: a database of annotated HIV envelope sequences from brain and other anatomical sites. AIDS Res Ther 7:43. https://doi.org/10.1186/1742-6405-7-43.

21. Gray LR, Cowley D, Welsh C, Lu HK, Brew BJ, Lewin SR, Wesselingh SL, Gorry PR, Churchill MJ. 2016. CNS-specific regulatory elements in brain-derived HIV-1 strains affect responses to latency-reversing agents with implications for cure strategies. Mol Psychiatry 21:574–584. https://doi.org/10.1038/mp.2015.111.

22. Gama L, Abreu CM, Shirk EN, Price SL, Li M, Laird GM, Pate KAM, Wietgrefe SW, O'Connor SL, Pianowski L, Haase AT, Van Lint C, Siliciano RF, Clements JE, LRA-SIV Study Group. 2017. Reactivation of simian immunodeficiency virus reservoirs in the brain of virally suppressed macaques. AIDS 31:5–14. https://doi.org/10.1097/QAD.0000000000001267.

23. Beck SE, Queen SE, Viscidi R, Johnson D, Kent SJ, Adams RJ, Tarwater PM, Mankowski JL. 2016. Central nervous system-specific consequences of simian immunodeficiency virus Gag escape from major histocompatibility complex class I-mediated control. J Neurovirol 22:498–507. https://doi.org/10.1007/s13365-015-0420-5.

24. Power C, McArthur JC, Johnson RT, Griffin DE, Glass JD, Perryman S, Chesebro B. 1994. Demented and nondemented patients with AIDS differ in brain-derived human immunodeficiency virus type 1 envelope sequences. J Virol 68:4643–4649. https://doi.org/10.1128/JVI.68.7.4643-4649.1994.

25. Lamers SL, Rose R, Maidji E, Agsalda-Garcia M, Nolan DJ, Fogel GB, Salemi M, Garcia DL, Bracci P, Yong W, Commins D, Said J, Khanlou N, Hinkin CH, Sueiras MV, Mathisen G, Donovan S, Shiramizu B, Stoddart CA, McGrath MS, Singer EJ. 2016. HIV DNA is frequently present within pathologic tissues evaluated at autopsy from combined antiretroviral therapy-treated patients with undetectable viral loads. J Virol 90:8968–8983. https://doi.org/10.1128/JVI.00674-16.

26. Chaillon A, Gianella S, Dellicour S, Rawlings SA, Schlub TE, De Oliveira MF, Ignacio C, Porrachia M, Vrancken B, Smith DM. 2020. HIV persists throughout deep tissues with repopulation from multiple anatomical sources. J Clin Invest 130:1699–1712. https://doi.org/10.1172/JCI134815.

27. Thompson CG, Gay CL, Kashuba ADM. 2017. HIV persistence in gut-associated lymphoid tissues: pharmacological challenges and opportunities. AIDS Res Hum Retroviruses 33:513–523. https://doi.org/10.1089/aid.2016.0253.

28. Wong JK, Yukl SA. 2016. Tissue reservoirs of HIV. Curr Opin HIV AIDS 11:362–370. https://doi.org/10.1097/COH.0000000000000293.

29. Desplats P, Dumaop W, Cronin P, Gianella S, Woods S, Letendre S, Smith D, Masliah E, Grant I. 2014. Epigenetic alterations in the brain associated with HIV-1 infection and methamphetamine dependence. PLoS One 9:e102555. https://doi.org/10.1371/journal.pone.0102555.

30. Desplats P, Dumaop W, Smith D, Adame A, Everall I, Letendre S, Ellis R, Cherner M, Grant I, Masliah E. 2013. Molecular and pathologic insights from latent HIV-1 infection in the human brain. Neurology 80:1415–1423. https://doi.org/10.1212/WNL.0b013e31828c2e9e.

31. Gray LR, Roche M, Flynn JK, Wesselingh SL, Gorry PR, Churchill MJ. 2014. Is the central nervous system a reservoir of HIV-1? Curr Opin HIV AIDS 9:552–558. https://doi.org/10.1097/COH.0000000000000108.

32. Clements JE, Li M, Gama L, Bullock B, Carruth LM, Mankowski JL, Zink MC. 2005. The central nervous system is a viral reservoir in simian immunodeficiency virus–infected macaques on combined antiretroviral therapy: a model for human immunodeficiency virus patients on highly active antiretroviral therapy. J Neurovirol 11:180–189. https://doi.org/10.1080/13550280590922748-1.

33. Bruner KM, Murray AJ, Pollack RA, Soliman MG, Laskey SB, Capoferri AA, Lai J, Strain MC, Lada SM, Hoh R, Ho Y-C, Richman DD, Deeks SG, Siliciano JD, Siliciano RF. 2016. Defective proviruses rapidly accumulate during acute HIV-1 infection. Nat Med 22:1043–1049. https://doi.org/10.1038/nm.4156.

34. Lee GQ, Reddy K, Einkauf KB, Gounder K, Chevalier JM, Dong KL, Walker BD, Yu XG, Ndung'u T, Lichterfeld M. 2019. HIV-1 DNA sequence diversity and evolution during acute subtype C infection. Nat Commun 10:2737. https://doi.org/10.1038/s41467-019-10659-2.

35. Addo MM, Altfeld M. 2014. Sex-based differences in HIV type 1 pathogenesis. J Infect Dis 209:S86–S92. https://doi.org/10.1093/infdis/jiu175.

36. Harris RS, Liddament MT. 2004. Retroviral restriction by APOBEC proteins. Nat Rev Immunol 4:868–877. https://doi.org/10.1038/nri1489.

37. Kwon KJ, Siliciano RF. 2017. HIV persistence: clonal expansion of cells in the latent reservoir. J Clin Invest 127:2536–2538. https://doi.org/10.1172/JCI95329.

38. Bourgeois C, Gorwood J, Barrail-Tran A, Lagathu C, Capeau J, Desjardins D, Le Grand R, Damouche A, Béréziat V, Lambotte O. 2019. Specific biological features of adipose tissue, and their impact on HIV persistence. Front Microbiol 10:2837. https://doi.org/10.3389/fmicb.2019.02837.

39. Gianella S, Chaillon A, Chun T-W, Sneller MC, Ignacio C, Vargas-Meneses MV, Caballero G, Ellis RJ, Kovacs C, Benko E, Huibner S, Kaul R. 2020. HIV RNA rebound in seminal plasma after antiretroviral treatment interruption. J Virol 94:e00415-20. https://doi.org/10.1128/JVI.00415-20.

40. Griesbeck M, Scully E, Altfeld M. 2016. Sex and gender differences in HIV-1 infection. Clin Sci (Lond) 130:1435–1451. https://doi.org/10.1042/CS20160112.

41. Scully EP. 2018. Sex differences in HIV infection. Curr HIV/AIDS Rep 15:136–146. https://doi.org/10.1007/s11904-018-0383-2.

42. Das B, Dobrowolski C, Luttge B, Valadkhan S, Chomont N, Johnston R, Bacchetti P, Hoh R, Gandhi M, Deeks SG, Scully E, Karn J. 2018. Estrogen receptor-1 is a key regulator of HIV-1 latency that imparts gender-specific restrictions on the latent reservoir. Proc Natl Acad Sci U S A 115:E7795–E7804. https://doi.org/10.1073/pnas.1803468115.

43. Fourati S, Flandre P, Calin R, Carcelain G, Soulie C, Lambert-Niclot S, Maiga A, Ait-Arkoub Z, Tubiana R, Valantin M-A, Autran B, Katlama C, Calvez V, Marcelin A-G. 2014. Factors associated with a low HIV reservoir in patients with prolonged suppressive antiretroviral therapy. J Antimicrob Chemother 69:753–756. https://doi.org/10.1093/jac/dkt428.

44. Scully EP, Gandhi M, Johnston R, Hoh R, Lockhart A, Dobrowolski C, Pagliuzza A, Milush JM, Baker CA, Girling V, Ellefson A, Gorelick R, Lifson J, Altfeld M, Alter G, Cedars M, Solomon A, Lewin SR, Karn J, Chomont N, Bacchetti P, Deeks SG. 2019. Sex-based differences in human immunodeficiency virus type 1 reservoir activity and residual immune activation. J Infect Dis 219:1084–1094. https://doi.org/10.1093/infdis/jiy617.

45. Prodger JL, Capoferri AA, Yu K, Lai J, Reynolds SJ, Kasule J, Kityamuweesi T, Buule P, Serwadda D, Kwon KJ, Schlusser K, Martens C, Scully E, Choi Y-H, Redd AD, Quinn TC. 2020. Reduced HIV-1 latent reservoir outgrowth and distinct immune correlates among women in Rakai, Uganda. JCI Insight 5:e139287. https://doi.org/10.1172/jci.insight.139287.

46. Sarmati L, D'Ettorre G, Parisi SG, Andreoni M. 2015. HIV replication at low copy number and its correlation with the HIV reservoir: a clinical perspective. Curr HIV Res 13:250–257. https://doi.org/10.2174/1570162x13666150407142539.

47. Tosiano MA, Jacobs JL, Shutt KA, Cyktor JC, Mellors JW. 2019. A simpler and more sensitive single-copy HIV-1 RNA assay for quantification of persistent viremia in individuals on suppressive antiretroviral therapy. J Clin Microbiol 57:e01714-18. https://doi.org/10.1128/JCM.01714-18.

48. Pascual-Pareja JF, Martínez-Prats L, Luczkowiak J, Fiorante S, Rubio R, Pulido F, Otero JR, Delgado R. 2010. Detection of HIV-1 at between 20 and 49 copies per milliliter by the Cobas TaqMan HIV-1 v2.0 assay is associated with higher pretherapy viral load and less time on antiretroviral therapy. J Clin Microbiol 48:1911–1912. https://doi.org/10.1128/JCM.02388-09.

49. Soontornniyomkij V, Umlauf A, Chung SA, Cochran ML, Soontornniyomkij B, Gouaux B, Toperoff W, Moore DJ, Masliah E, Ellis RJ, Grant I, Achim CL. 2014. HIV protease inhibitor exposure predicts cerebral small vessel disease. AIDS 28:1297–1306. https://doi.org/10.1097/QAD.0000000000000262.

50. de Oliveira MF, Gianella S, Letendre S, Scheffler K, Kosakovsky Pond SL, Smith DM, Strain M, Ellis RJ. 2015. Comparative analysis of cell-associated HIV DNA levels in cerebrospinal fluid and peripheral blood by droplet digital PCR. PLoS One 10:e0139510. https://doi.org/10.1371/journal.pone.0139510.

51. Laird Smith M, Murrell B, Eren K, Ignacio C, Landais E, Weaver S, Phung P, Ludka C, Hepler L, Caballero G, Pollner T, Guo Y, Richman D, Protocol TI, Network I, Poignard P, Paxinos EE, Pond SLK, Smith DM, The IAVI Protocol C Investigators, The IAVI African HIV Research Network. 2016. Rapid sequencing of complete env genes from primary. Virus Evol 2:vew018. https://doi.org/10.1093/ve/vew018.

52. Kumar V, Vollbrecht T, Chernyshev M, Mohan S, Hanst B, Bavafa N, Lorenzo A, Kumar N, Ketteringham R, Eren K, Golden M, Oliveira MF, Murrell B. 2019. Long-read amplicon denoising. Nucleic Acids Res 47:e104. https://doi.org/10.1093/nar/gkz657.

53. Pankow A, Murrell B. 2023. BarcodedAmpliconDenoising.jl. GitHub repository. https://github.com/alecpnkw/nntc_hiv_brain_lymphoid_reservoirs.

54. Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30:3059–3066. https://doi.org/10.1093/nar/gkf436.

55. Price MN, Dehal PS, Arkin AP. 2010. FastTree 2—approximately maximum-likelihood trees for large alignments. PLoS One 5:e9490. https://doi.org/10.1371/journal.pone.0009490.

56. Rose PP, Korber BT. 2000. Detecting hypermutations in viral sequences with an emphasis on G –> A hypermutation. Bioinformatics 16:400–401. https://doi.org/10.1093/bioinformatics/16.4.400.