Association of antiretroviral therapy with brain aging changes among HIV-infected adults

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Objective: Antiretroviral therapy (ART) is currently recommended for all persons living with HIV (PLWH), regardless of their $CD4^+$ T-cell count, and should be continued throughout life. Nonetheless, vigilance of the safety of ART, including its neurotoxicity, must continue. We hypothesized that use of certain ART drugs might be associated with aging-related cerebral degenerative changes among PLWH.

Design: Clinicopathological study of PLWH who were using ART drugs at the last clinical assessment.

Methods: Using multivariable logistic regression, we tested associations between use of each specific ART drug (with reference to use of other ART drugs) and cerebral degenerative changes including neuronal phospho-tau lesions, β-amyloid plaque deposition, microgliosis, and astrogliosis in the frontal cortex and putamen (immunohistochemistry), as well as cerebral small vessel disease (CSVD) in the forebrain white matter (standard histopathology), with relevant covariates being taken into account. The Bonferroni adjustment was applied.

Results: Darunavir use was associated with higher likelihood of neuronal phospho-tau lesions in the putamen [odds ratio (OR) 15.33, $n = 93$, $P = 0.005$]. Ritonavir use was associated with marked microgliosis in the putamen (OR 4.96, $n = 101$, $P = 0.023$). On the other hand, use of tenofovir disoproxil fumarate was associated with lower likelihood of β-amyloid plaque deposition in the frontal cortex (OR 0.13, $n = 102$, $P = 0.012$). There was a trend toward an association between emtricitabine use and CSVD (OR 13.64, $n = 75$, $P = 0.099$).

Conclusion: Our findings suggest that PLWH treated with darunavir and ritonavir may be at increased risk of aging-related cerebral degenerative changes.

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Introduction

Early diagnosis of HIV infection followed by initiation of combination antiretroviral therapy (ART) has a clear clinical benefit in preventing the complications of HIV disease [1,2]. According to the United States Department of Health and Human Services (DHHS), guidelines for the use of ART drugs in adults and adolescents [3], ART

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is recommended for all persons living with HIV (PLWH), regardless of their $CD4^+$ T-cell count. As currently available ART regimens do not cure HIV infection [4], ART should be continued throughout life [3], and therefore, understanding the long-term consequences of ART is critically important. Certain ART drugs may cause chronic toxicity to parenchymal and vascular components of the central nervous system (CNS), contributing to neurobehavioral symptoms [5–16]. Although ART drugs are used in combination, individual ART drugs are associated with distinct adverse events. For instance, efavirenz use can cause acute and chronic CNS adverse effects [12,13,17] even though it is commonly used in combination with tenofovir and emtricitabine. Many ART drugs, even within the same class, appear to differ in their toxicity profile [16].

Experimental studies on ART drug toxicity have been conducted in cell cultures and animal models. Several cell types were used in cell-culture experiments [15], such as neuronal cells [7,18–21], astroglia [22], oligodendroglia [23], endothelial cells [24–37], and vascular smooth muscle cells [35,38]. Brown *et al.* [19] reported that efavirenz induced β-secretase-1 protein expression and promoted soluble β-amyloid production in murine N2a neuronal cells [transfected with the Swedish mutant form of human β-amyloid precursor protein (*APP*) gene] and in Tg2576 mice expressing Swedish mutated APP. Efavirenz exposure also reduced primary mouse microglial phagocytosis of β-amyloid peptide [19].

These experimental studies provide important insights into potential ART neurotoxicity. However, there have been limited clinicopathological studies using postmortem brain samples to explore associations between ART and neuropathologic changes [23,39–41]. Regarding aging-related cerebral degenerative changes [42], Anthony *et al.* [41] reported an increase in hippocampal phospho-tau lesions (using AT8 antibody) in ART-treated PLWH compared with age-matched non-HIV participants. The same group [39] also observed markedly increased CD68-immunoreactive microglial activation in the hippocampus and basal ganglia in the absence of other significant neuropathologic changes in ART-treated PLWH compared with non-HIV participants. β-Amyloid plaque deposition, another aging-related cerebral degenerative change [42], was described in the cerebral neocortex [43–50] and hippocampal formation [41,51] of PLWH in autopsy studies. However, the association between ART and β-amyloid plaque deposition has not been studied systematically.

The potential effects of ART on cerebral small vessel disease (CSVD), commonly associated with aging, hypertension, and diabetes mellitus [52], have been explored in few autopsy studies [14,53]. A clinicopathological study of PLWH by our group [14] revealed an association between protease inhibitor-based ART and a higher risk of CSVD after statistically adjusting for diabetes mellitus. Similarly, an autopsy study by Morgello *et al.* [53] showed that protease inhibitor-based ART was associated with more severe CSVD compared with nonnucleoside reverse transcriptase inhibitor (NNRTI)-based ART.

Taken together, aging-related cerebral degenerative changes have been observed in the postmortem brains of PLWH. Nonetheless, the occurrence of these changes in relation to specific ART drugs has not been strongly examined. In the present clinicopathological study, we aimed to analyze associations between specific ART drug use and aging-related cerebral parenchymal and vascular degenerative changes, including neuronal phospho-tau lesions, β-amyloid plaque deposition, microgliosis, astrogliosis, and CSVD [42,52]. Our study focused on ART drugs included in recommended initial regimens in the DHHS guidelines [3]. We chose to examine parenchymal degenerative changes in the middle frontal gyrus and putamen because changes in brain metabolite levels in these brain regions on magnetic resonance spectroscopy were shown to correlate with cognitive impairment in PLWH [54,55]. We hypothesized that use of certain ART drugs might be associated with aging-related cerebral degenerative changes among PLWH.

Methods

Study cohort

We assembled 187 PLWH autopsy cases that were using ART drugs at the last clinical assessment in the National NeuroAIDS Tissue Consortium (NNTC, R432 and R458). The data on ART drug use were recorded within a median of 16.36 weeks (interquartile range 6.87–29.48 weeks, $n = 187$) before death. The University of California San Diego Human Research Protections Program approved the project and all study participants provided written informed consent to participate. A written consent to autopsy was also obtained. The patients died between 1999 and 2014. The demographic data, potential covariates, and ART drug use are summarized in Table 1. Only ART drugs that were currently used at the last clinical assessment and included in recommended initial regimens in the current DHHS guidelines [3] were analyzed as predictors, including four nucleoside reverse transcriptase inhibitor (NRTI: abacavir, emtricitabine, lamivudine, tenofovir disoproxil fumarate), one NNRTI (efavirenz), four protease inhibitor (atazanavir, darunavir, lopinavir, ritonavir), and one integrase strand transfer inhibitor (INSTI: raltegravir) drugs. We tested associations between use of each specific ART drug and aging-related cerebral degenerative changes with reference to use of other

Table 1. Summary of demographic data, potential covariates, and antiretroviral drug use.

Age at death (years): range, median [IQR], *n*	27–68, 47 [40–55], 187
Female: *n* (%)	40 (21.4)
Race/ethnicity: *n* (%)	
White	90 (48.1)
Black	40 (21.4)
Hispanic	48 (25.7)
Others	9 (4.8)
APOE ε4 carrier status: *n* (%)[a]	37 (23.1)
APOE ε2 carrier status: *n* (%)[a]	14 (8.8)
Hepatitis C virus serostatus: *n* (%)	55 (43)
Diabetes mellitus: *n* (%)	19 (14.3)
Hypertension: *n* (%)	41 (30.4)
Hyperlipidemia: *n* (%)	24 (18.9)
Lifetime alcohol dependence: *n* (%)	55 (37.7)
Lifetime cannabis dependence: *n* (%)	16 (11)
Lifetime psychostimulant dependence: *n* (%)	24 (16.4)
Lifetime cigarette smoking: *n* (%)	45 (39.5)
Lower last-visit $CD4^+$ T-cell count (<200 cells/μl): *n* (%)	137 (74.9)
Last-visit plasma HIV-1 RNA (log_{10} copies/ml): range, median [IQR], *n*	1.602–6.295, 3.624 [2.341–4.875], 180
Longer postmortem interval (>12 h): *n* (%)	75 (40.1)
HIV encephalitis or white matter lesions: *n* (%)	35 (18.7)
Opportunistic brain diseases: *n* (%)	16 (8.6)
National NeuroAIDS Tissue Consortium (NNTC) site: *n* (%)	
California NeuroAIDS Tissue Network (CNTN)	80 (42.8)
National Neurological AIDS Bank (NNAB)	58 (31.0)
Texas NeuroAIDS Research Center (TNRC)	25 (13.4)
Manhattan HIV Brain Bank (MHBB)	24 (12.8)
NRTI drug use: *n* (%)	
Abacavir (ABC)	50 (26.7)
Emtricitabine (FTC)	55 (29.4)
Lamivudine (3TC)	101 (54)
Tenofovir disoproxil fumarate (TDF)	88 (47.1)
NNRTI drug use: *n* (%)	
Efavirenz (EFV)	42 (22.5)
PI drug use: *n* (%)	
Atazanavir (ATV)	26 (13.9)
Darunavir (DRV)	13 (7)
Lopinavir (LPV)	46 (24.6)
Ritonavir (RTV)	94 (50.3)
INSTI drug use: *n* (%)	
Raltegravir (RAL)	8 (4.3)
CPE score: range, median [IQR], *n*	2–27, 8 [6–9], 187

APOE, apolipoprotein-E gene; IQR, interquartile range; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor; INSTI, integrase strand transfer inhibitor; CPE, central nervous system penetration-effectiveness.
[a]With one or two *APOE* ε2 or ε4 alleles, excluding *APOE* ε2/ε4 carrying cases.

ART drugs. Included as a separate predictor was ART penetration or effectiveness in the CNS estimated by using the CNS penetration-effectiveness (CPE) method [56].

We evaluated 17 biological covariates: age at death, sex, race/ethnicity, apolipoprotein-E (*APOE*) ε4 carrier, *APOE* ε2 carrier, hepatitis C virus (HCV) serostatus, diabetes mellitus, hypertension, hyperlipidemia, lifetime alcohol dependence, lifetime cannabis dependence, lifetime psychostimulant dependence, lifetime cigarette smoking, lower last-visit $CD4^+$ T-cell count (<200 cells/μl), last-visit plasma HIV-1 RNA (log_{10} copies/ml), presence of HIV encephalitis (HIVE) or forebrain white matter lesions (WML), and evidence of opportunistic brain diseases (e.g. progressive multifocal leukoencephalopathy (PML) and primary CNS lymphoma). The longer postmortem interval (>12 h) and operating sites in the NNTC (Table 1) were included as technical covariates because of possible site differences.

Histopathological examination and apolipoprotein-E genotyping

Histopathologic diagnoses made by site neuropathologists were available from the NNTC. CSVD (also referred to as arteriolosclerosis) was defined as concentric intramural hyalinization of small arteries or arterioles in the forebrain white matter [14,52]. HIVE was defined as multiple foci of microgliosis, multinucleated giant cells, and astrogliosis [57]. WML was defined as foci of white matter rarefaction with macrophage infiltration or astrogliosis in the forebrain in the absence of HIVE and PML. The *APOE* genotypes (ε2, ε3, and ε4 alleles) were determined by using the allelic discrimination assay (*rs*429358 and *rs*7412 single nucleotide polymorphisms) of DNA extracted from frozen brain samples (Taqman SNP

Genotyping Assays; Applied Biosystems, Carlsbad, California, USA) [50].

Immunohistochemistry

We performed immunohistochemical staining on 5-μm thick tissue sections prepared from two formalin-fixed paraffin-embedded brain blocks: middle frontal gyrus and putamen. We used primary antibodies to phospho-tau (clone AT8; MN1020; Pierce Biotechnology, Rockford, Illinois, USA; 1 : 1000 dilution), β-amyloid (clone 4G8; SIG-39220; Covance, Princeton, New Jersey, USA; 1 : 20000), ionized calcium-binding adapter molecule-1 (Iba1, microglia/macrophage marker; 019–19741; Wako, Richmond, Virginia, USA; 1 : 1000), and glial fibrillary acidic protein (GFAP, astroglia marker; Z0334; Dako, Carpinteria, California, USA; 1 : 1000). The immunohistochemical stainings with diaminobenzidine (DAB) were conducted as previously described [50,58]. For phospho-tau and β-amyloid immunohistochemistry, the tissue sections were counterstained with Mayer hematoxylin, and cerebral cortex tissue sections from an Alzheimer's disease brain were used as positive tissue controls in all staining batches.

In addition, we performed sequential double-label immunofluorescence staining on 5-μm-thick formalin-fixed paraffin-embedded brain tissue sections with primary antibodies to phospho-tau (clone AT8; MN1020; Pierce Biotechnology; 1 : 500) and microtubule-associated protein-2 (MAP2, somatodendritic neuronal marker; PRB-547C; Covance; 1 : 250), and Alexa Fluor 488 (A-11017) and 568 (A-11036) conjugated secondary antibodies (Molecular Probes, Eugene, Oregon, USA; 1 : 200), respectively. Cellular nuclei were stained with Hoechst 33342 (H3570; Molecular Probes; 1 : 2000). To quench autofluorescence, Vector TrueVIEW (SP-8400, Vector Laboratories, Burlingame, California, USA) and 0.3% Sudan Black B/70% ethanol were used, as previously described [59].

Assessments of neuronal phospho-tau lesions and β-amyloid plaque deposition

Neuronal phospho-tau lesions were designated as present when there were phospho-tau-immunoreactive neuropil threads, pretangle neuronal soma, neurofibrillary tangles, or their combinations [50,60]. The neuronal nature of cells involved by phospho-tau lesions was confirmed on double-label immunofluorescence staining for phospho-tau and MAP2 (Fig. 1a–a"). No phospho-tau lesions were observed in other CNS cell types in either the frontal cortex or putamen. The density of neuropil threads was graded as 1 (barely present at ×100 magnification on light microscopy, Fig. 1b), 2 (easily noted at ×100 magnification on light microscopy, Fig. 1c), or 3 (notable with naked eye inspection, Fig. 1d), a grading system adapted from a BrainNet Europe Consortium study [61].

β-Amyloid plaque deposition was designated as present when extracellular β-amyloid-immunoreactive plaques were observed (Fig. 1e), regardless of their type [60] or density. The density of β-amyloid plaques was graded as focal or widespread [50]. We did not take into account intracellular β-amyloid immunoreactivity because the immunohistochemical visualization of intracellular β-amyloid appears to depend on antigen retrieval methods used [60].

Assessments of ionized calcium-binding adapter molecule-1 microgliosis and glial fibrillary acidic protein astrogliosis

Immunohistochemistry for Iba1 in the frontal cortex and putamen showed resident microglia and perivascular macrophages (Fig. 1f), and that for GFAP showed diffusely distributed astroglia of variable density (Fig. 1g), as previously described [58]. For quantification of Iba1 and GFAP immunoreactivity density, the DAB tissue slides were scanned using a slide scanner (20× objective, Aperio ScanScope GL; Leica Biosystems, Buffalo Grove, Illinois, USA), and a square of $3000 \times 3000\ \mu m^2$ was extracted from the frontal cortex and putamen. Using the Image-Pro Analyzer software (Version 6.3; Media Cybernetics, Bethesda, Maryland, USA), the DAB intensity was quantified within each area of interest, that is, the frontal cortical layers II–VI and putamen [59]. The DAB intensity per unit area (i.e. DAB density) was calculated [62]. To adjust for between-batch variation, one brain section from the same positive tissue control block was included in each immunostaining batch. The control DAB density value (in a specified area) was used to normalize all the DAB density values of studied cases in the same batch, giving rise to the immunoreactivity density values (continuous). Finally, each of the Iba1 and GFAP immunoreactivity density values was graded as mild (the median or less) or marked (greater than the median) gliosis (categorical).

Statistical analysis

We used binary logistic regression to test associations between 11 ART predictors and nine neuropathologic outcomes. To reduce the small-sample bias in (conventional) maximum likelihood estimation, we employed the Firth penalized maximum likelihood method. To select covariates for the inclusion in the multivariable regression model for each pair of the ART predictor and neuropathologic outcome, we first ran a regression analysis [Model (Firth method): outcome = biological covariate + two technical covariates]. The biological covariates having P less than 0.20 were subsequently included in the initial multivariable analysis of each ART predictor and that outcome [Model (backward stepwise, entry probability 0.05, removal probability 0.10, likelihood ratio test): outcome = ART predictor (forced entry) + biological covariates + two technical covariates]. The main effects were analyzed. Then, the biological or technical covariates that remained in the initial multivariable model were included in the final multivariable analysis [Model (Firth method): outcome =

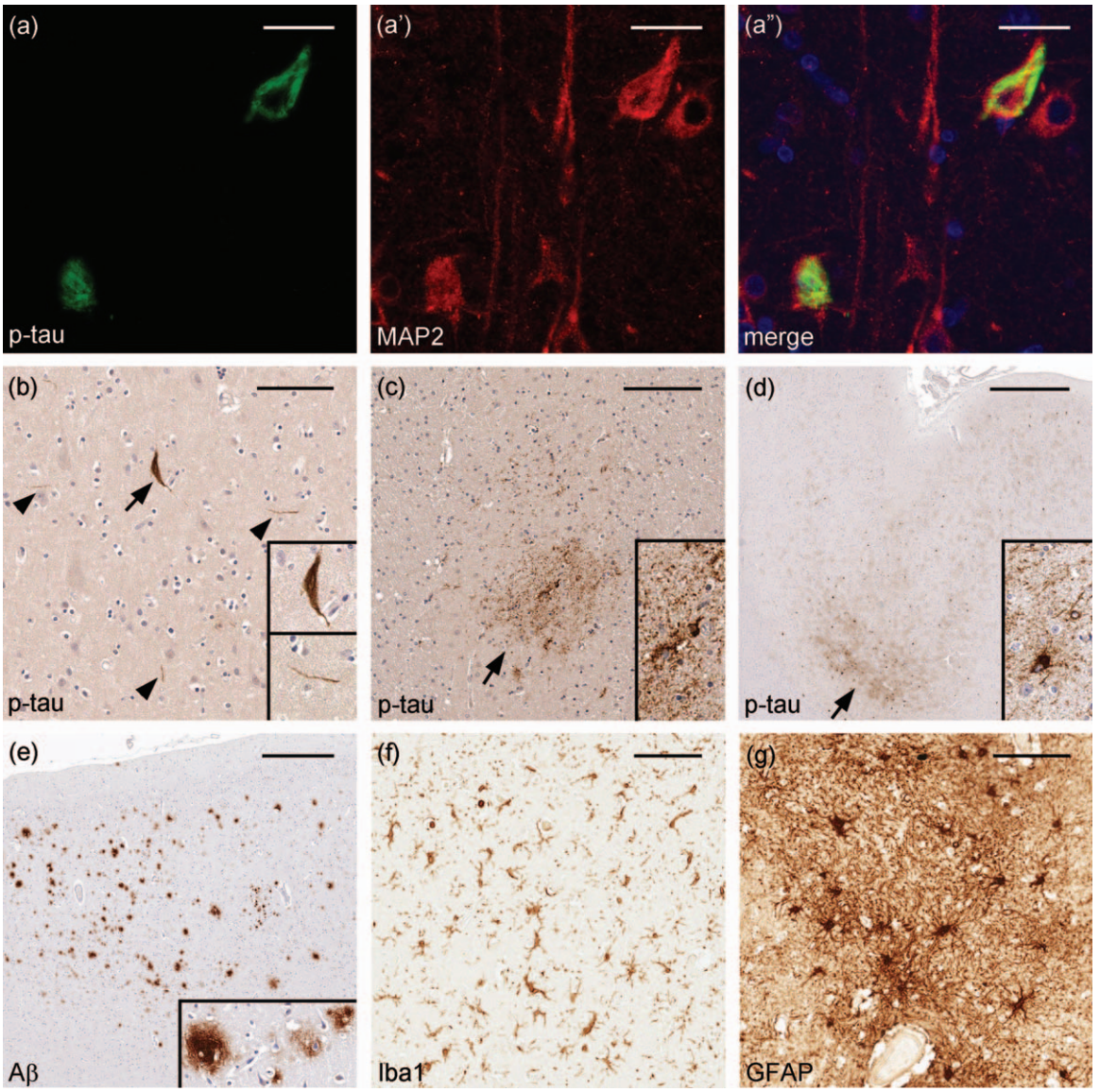

Fig. 1. Immunohistopathologic changes in the frontal cortex and putamen of HIV-infected adults. Double-label immunofluorescence staining for phospho-tau (p-tau, a) and microtubule-associated protein-2 (MAP2, a′) shows p-tau-immunoreactive neurofibrillary tangles within MAP2-immunoreactive neurons (merge, a″) in the frontal cortex of a 44-year-old black man; scale bars 40 μm. Immunohistochemical staining for p-tau (b–d) shows neuropil threads of grade-1 density in the frontal cortex of 43-year-old white man (b, scale bar 100 μm; arrow and upper inset showing a neuron, arrowheads and lower inset showing neuropil threads), grade-2 density in the putamen of 44-year-old white man (c, scale bar 150 μm; arrow and inset showing an area involved by neuronal p-tau lesions), and grade-3 density in the frontal cortex of a 44-year-old black man (d, scale bar 1000 μm; arrow and inset showing an area involved by neuronal p-tau lesions; the same case as in a–a″). Immunohistochemical staining for β-amyloid (Aβ, e) shows diffuse plaques of widespread density in the frontal cortex of 57-year-old Hispanic woman; scale bar 600 μm. Immunohistochemical staining for ionized calcium-binding adapter molecule-1 (Iba1, f) shows ramified microglia scattered in the frontal cortex of 44-year-old black man (the same case as in a–a″); scale bar 100 μm. Immunohistochemical staining for glial fibrillary acidic protein (GFAP, g) shows astroglia scattered in the putamen of 35-year-old white man; scale bar 100 μm.

ART predictor + covariates]. The odds ratio (OR) [95% confidence interval (CI)] measured the effect size.

Of 187 cases in the present study, not every case had all tissue samples and biological data (Table 1). The statistical models were analyzed on available cases, with pairwise exclusion. For each ART predictor, the Bonferroni method was used to calculate adjusted *P* values to account for the type-I error in multiple comparisons in the family of nine neuropathologic outcomes. Two-tailed *P* values were considered significant at *P* less than 0.05. The 95% CI of OR values were not adjusted for multiple comparisons. The statistical analyses were performed using IBM SPSS Version 24 (IBM Corp., Armonk, New

York, USA) with Essential for R version 3.2 (R Foundation, Vienna, Austria).

Results

The frequency distribution of neuropathologic outcomes within ART predictors and covariates is shown in Table, Supplemental Digital Content 1, http://links.lww.com/QAD/B309. The results of logistic regression models testing associations between biological covariates and neuropathologic outcomes, after adjusting for the two technical covariates, are shown in Table, Supplemental Digital Content 2, http://links.lww.com/QAD/B309. The results of final multivariable models testing associations between ART predictors and neuropathologic outcomes, after adjusting for applicable covariates, are shown in Table 2 and summarized in the following subsections.

Table 2. Final multivariable logistic regression models testing associations between antiretroviral therapy predictors and neuropathologic outcomes.

OR, *P* value, *n*	Neuronal phospho-tau lesions[a]		β-Amyloid plaque deposition[a]	
Covariates	Frontal cortex	Putamen	Frontal cortex	Putamen
NRTI drug use				
Abacavir (ABC)	2.16, 0.216, 96 *Site, PVL, Stim, HT*	1.05, 0.928, 80 *Stim*	0.69, 0.540, 102 *Site, APOE4, Age, DM*	0.54, 0.362, 119 *Site, Age*
Emtricitabine (FTC)	0.29, 0.058, 96 *Site, PVL, Stim, HT*	2.39, 0.124, 80 *HIVE, Stim*	0.98, 0.966, 102 *Site, APOE4, Age, DM*	2.34, 0.139, 119 *Site, Age*
Lamivudine (3TC)	1.89, 0.228, 96 *Site, PVL, Stim, HT*	0.50, 0.164, 80 *HIVE, Stim*	1.14, 0.783, 102 *Site, APOE4, Age, DM*	0.94, 0.906, 119 *Site, Age*
Tenofovir DF (TDF)	0.42, 0.130, 96 *Site, PVL, Stim, HT*	1.40, 0.497, 80 *HIVE, Stim*	**0.13, 0.001**, 102** *Site, APOE4, Age, DM*	2.17, 0.180, 119 *Site, Age*
NNRTI drug use				
Efavirenz (EFV)	1.44, 0.560, 96 *Site, PVL, Stim, HT*	1.13, 0.835, 80 *Stim*	0.94, 0.918, 102 *Site, APOE4, Age, DM*	2.54, 0.166, 119 *Site, Age*
PI drug use				
Atazanavir (ATV)	0.48, 0.312, 96 *Site, PVL, Stim, HT*	2.72, 0.120, 80 *HIVE, Stim*	0.27, 0.087, 101 *Site, APOE4, Age, DM, HT*	1.46, 0.576, 119 *Site, Age*
Darunavir (DRV)	0.54, 0.558, 96 *Site, PVL, Stim, HT*	**15.33, 0.0005***, 93** *APOE4*	0.37, 0.261, 102 *Site, APOE4, Age, DM*	0.88, 0.882, 119 *Site, Age*
Lopinavir (LPV)	0.50, 0.319, 96 *Site, PVL, Stim, HT*	1.34, 0.634, 72 *APOE4, Stim*	0.56, 0.331, 102 *Site, APOE4, Age, DM*	2.78, 0.083, 119 *Site, Age*
Ritonavir (RTV)	0.53, 0.230, 96 *Site, PVL, Stim, HT*	2.15, 0.125, 80 *HIVE, Stim*	**0.30, 0.023, 102** *Site, APOE4, Age, DM*	1.49, 0.483, 119 *Site, Age*
INSTI drug use				
Raltegravir (RAL)	8.56, 0.108, 96 *Site, PVL, Stim, HT*	5.73, 0.083, 80 *HIVE, Stim*	1.48, 0.687, 102 *Site, APOE4, Age, DM*	3.84, 0.193, 119 *Site*
CPE score[c]	1.07, 0.414, 96 *Site, PVL, Stim, HT*	1.04, 0.520, 80 *HIVE, Stim*	0.89, 0.102, 101 *Site, APOE4, Age, DM, HT*	1.05, 0.397, 119 *Site, Age*

OR, *P* value, *n*	Iba1 microgliosis[b]	
Covariates	Frontal cortex	Putamen
NRTI drug use		
Abacavir (ABC)	**3.09, 0.039, 99** *Site, Ciga*	1.34, 0.592, 101 *Site, APOE4*
Emtricitabine (FTC)	2.28, 0.086, 124 *Site, APOE4, Stim, PVL*	2.11, 0.170, 101 *Site, APOE4, Sex*
Lamivudine (3TC)	0.55, 0.162, 124 *Site, APOE4, Stim, PVL*	0.58, 0.274, 101 *Site, APOE4*
Tenofovir DF (TDF)	1.24, 0.558, 148 *Site, APOE4*	1.68, 0.297, 101 *Site, APOE4, Sex*
NNRTI drug use		
Efavirenz (EFV)	0.75, 0.480, 148 *Site, APOE4*	0.38, 0.118, 101 *Site, APOE4, Sex*
PI drug use		
Atazanavir (ATV)	2.32, 0.175, 124 *Site, APOE4, Stim, PVL*	1.07, 0.913, 101 *Site, APOE4*
Darunavir (DRV)	1.64, 0.528, 124 *Site, APOE4, Stim, PVL*	1.39, 0.772, 101 *Site, APOE4*
Lopinavir (LPV)	1.28, 0.550, 148 *Site, APOE4*	**4.44, 0.013, 101** *Site, APOE4, Sex*
Ritonavir (RTV)	1.74, 0.128, 148 *Site, APOE4*	**4.96, 0.003*, 101** *Site, APOE4, Sex*

Table 2 (*continued*)

OR, *P* value, *n*	Iba1 microgliosis[b]	
Covariates	Frontal cortex	Putamen
INSTI drug use		
Raltegravir (RAL)	1.42, 0.647, 148 *Site, APOE4*	1.85, 0.649, 101 *Site, APOE4*
CPE score[c]	1.01, 0.852, 148 *Site, APOE4*	1.06, 0.297, 101 *Site, APOE4*

OR, *P* value, *n*	GFAP astrogliosis[b]		CSVD[a]
Covariates	Frontal cortex	Putamen	Forebrain white matter
NRTI drug use			
Abacavir (ABC)	0.65, 0.296, 138 *Site, Stim*	1.34, 0.546, 99 *Site, APOE4, CD4*	0.75, 0.718, 55 *HIVE/WML, Alco*
Emtricitabine (FTC)	1.58, 0.241, 138 *Site, Stim*	1.08, 0.866, 99 *Site, APOE4, CD4*	**13.64, 0.011, 75** *HIVE/WML, DM*
Lamivudine (3TC)	0.91, 0.802, 138 *Site, Stim*	1.06, 0.898, 99 *Site, APOE4, CD4*	0.75, 0.694, 55 *HIVE/WML, Alco*
Tenofovir DF (TDF)	0.85, 0.662, 138 *Site, Stim*	1.14, 0.767, 99 *Site, APOE4, CD4*	1.42, 0.623, 55 *HIVE/WML, Alco*
NNRTI drug use			
Efavirenz (EFV)	1.29, 0.539, 138 *Site, Stim*	0.49, 0.217, 99 *Site, APOE4, CD4, Age*	1.04, 0.956, 55 *HIVE/WML, Alco*
PI drug use			
Atazanavir (ATV)	0.74, 0.552, 138 *Site, Stim*	1.37, 0.576, 99 *Site, APOE4, CD4*	0.23, 0.195, 55 *HIVE/WML, Alco, DM*
Darunavir (DRV)	1.26, 0.709, 138 *Site, Stim*	2.10, 0.392, 99 *Site, APOE4, CD4*	0.47, 0.675, 55 *HIVE/WML, Alco*
Lopinavir (LPV)	1.12, 0.792, 138 *Site, Stim*	0.97, 0.949, 99 *Site, APOE4, CD4*	0.90, 0.909, 55 *HIVE/WML, Alco*
Ritonavir (RTV)	0.81, 0.552, 138 *Site, Stim*	1.85, 0.174, 99 *Site, APOE4, CD4*	0.92, 0.911, 55 *HIVE/WML, Alco*
INSTI drug use			
Raltegravir (RAL)	1.53, 0.556, 138 *Site, Stim*	1.73, 0.648, 99 *Site, APOE4, CD4*	Inadequate samples
CPE score[c]	1.04, 0.410, 138 *Site, Stim*	0.99, 0.863, 99 *Site, APOE4, CD4*	0.98, 0.859, 55 *HIVE/WML, Alco*

[a]Presence (with reference to absence).
[b]Marked gliosis (with reference to mild gliosis).
[c]Each unit increase.
In boldface: *P* less than 0.05 (Firth penalized maximum likelihood). Bonferroni family-wise adjusted $^{*}P = 0.023$, $^{**}P = 0.012$, $^{***}P = 0.005$ (in the family of nine neuropathologic outcomes). CPE, central nervous system penetration-effectiveness; CSVD, cerebral small vessel disease; Iba1, ionized calcium-binding adapter molecule-1; INSTI, integrase strand transfer inhibitor; GFAP, glial fibrillary acidic protein; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; OR, odds ratio; PI, protease inhibitor. *Covariates: Age*, age at death (each year increase); *Alco*, lifetime alcohol dependence; *APOE4*, apolipoprotein-E ε4 carrier status (excluding ε2/ε4 carrying cases); *CD4*, lower last-visit $CD4^+$ T-cell count (<200 cells/μl); *Ciga*, lifetime cigarette smoking; *DM*, diabetes mellitus; *HIVE/WML*, HIV encephalitis or white matter lesions; *HT*, hypertension; *PVL*, last-visit plasma HIV-1 RNA ($\log_{10}$ copies/ml); *Site*, National NeuroAIDS Tissue Consortium (NNTC) site; *Stim*, lifetime psychostimulant dependence.

Neuronal phospho-tau lesions

In the frontal cortex, neuronal phospho-tau lesions were present in 74 of 171 cases (43.3%). Of these 74 cases, 72 cases (97.3%) had neuropil threads of grade-1 density, another case with grade-2 density, and the remaining case with grade-3 density. In the putamen, neuronal phospho-tau lesions were present in 33 of 107 cases (30.8%). Of these 33 cases, 32 cases (97%) had neuropil threads of grade-1 density, and the remaining case had grade-2 density.

Darunavir use was associated with a higher likelihood of neuronal phospho-tau lesions in the putamen (OR 15.33; 95% CI 3.03–153.85; $n = 93$, adjusted $P = 0.005$; Table 2). In this analysis model, there were eight darunavir users (8.6%). Darunavir was used in combination with other ART drugs, such as ritonavir (eight cases, 100%), tenofovir DF (seven cases, 87.5%), and emtricitabine (six cases, 75%). Of 85 nondarunavir users, 40 (47.1%) used ritonavir, 45 (52.9%) used tenofovir DF, and 24 (28.2%) used emtricitabine. In the frontal cortex, none of the ART predictors was significantly associated with neuronal phospho-tau lesions (Table 2).

β-Amyloid plaque deposition

Almost all β-amyloid plaques found in the present study were of diffuse type [60]. In the frontal cortex, β-amyloid plaque deposition was present in 48 (focal in 33 and

widespread in 15) of 171 cases (28.1%). In the putamen, β-amyloid plaque deposition was present in 17 (focal in 16 and widespread in 1) of 119 cases (14.3%).

Tenofovir DF use was associated with a lower likelihood of frontal β-amyloid plaque deposition (OR 0.13; 95% CI 0.03–0.48; $n = 102$, adjusted $P = 0.012$; Table 2). In this analysis model, there were 54 tenofovir DF users (52.9%), 34 of whom (63%) also used emtricitabine. Of 48 nontenofovir DF users, 3 (6.3%) used emtricitabine. Ritonavir use appeared to show a similar association (OR 0.30; 95% CI 0.10–0.85; $n = 102$), but this association did not survive the Bonferroni correction (adjusted $P = 0.207$). Note that *APOE* ε4 carrier status appeared to be associated with frontal β-amyloid plaque deposition in the final multivariable models ($P < 0.020$, data not shown), as did older age ($P < 0.048$, data not shown). In the putamen, none of the ART predictors was significantly associated with β-amyloid plaque deposition (Table 2).

Ionized calcium-binding adapter molecule-1 microgliosis

Ritonavir use was associated with marked Iba1 microgliosis in the putamen (OR 4.96; 95% CI 1.71–16.31; $n = 101$, adjusted $P = 0.023$; Table 2). In this analysis model, there were 51 ritonavir users (50.5%), 27 of whom (52.9%) also used lopinavir, 13 (25.5%) also used atazanavir, and 8 (15.7%) also used darunavir. Of 50 nonritonavir users, 5 (10%) used atazanavir and none used lopinavir or darunavir. Lopinavir use appeared to show a similar association (OR 4.44; 95% CI 1.36–17.39; $n = 101$), but this association did not survive the Bonferroni correction (adjusted $P = 0.113$). The *APOE* ε4 carrier status was associated with marked Iba1 microgliosis in the putamen in the final multivariable models ($P < 0.0005$, all models, data not shown). In the frontal cortex, the apparent association between abacavir use and marked Iba1 microgliosis (OR 3.09; 95% CI 1.06–10.03, $n = 99$; Table 2) did not remain significant after the Bonferroni correction (adjusted $P = 0.353$).

Glial fibrillary acidic protein astrogliosis

In either the frontal cortex or putamen, none of the ART predictors was significantly associated with marked GFAP astrogliosis (Table 2). The *APOE* ε4 carrier status appeared to be associated with marked GFAP astrogliosis in the putamen in the final multivariable models ($0.021 < P < 0.029$, data not shown).

Cerebral small vessel disease

In a subset of 77 cases from California NeuroAIDS Tissue Network where CSVD was evaluated, CSVD (of any degrees of severity) was diagnosed in 62 cases (80.5%). The two technical covariates were not included in analyses. There was a trend toward an association between emtricitabine use and CSVD (OR 13.64; 95% CI 1.61–1782.91; $n = 75$; Table 2; adjusted $P = 0.099$). In this analysis model, there were 19 emtricitabine users (25.3%), 17 of whom (89.5%) also used tenofovir DF. Of 56 nonemtricitabine users, 21 (37.5%) used tenofovir DF.

Discussion

We tested associations between specific ART drug use and nine neuropathologic changes denoting aging-related cerebral parenchymal and vascular degeneration in the brains of PLWH. We found that darunavir use was associated with neuronal phospho-tau lesions in the putamen. Ritonavir use was associated with marked Iba1 microgliosis in the putamen. On the other hand, tenofovir DF use was associated with a lower likelihood of β-amyloid plaque deposition in the frontal cortex. A trend toward an association between emtricitabine use and CSVD in the forebrain white matter was also observed.

The finding of an association between darunavir use and neuronal phospho-tau lesions in the putamen was based on the multivariable regression analysis of the relatively small number of darunavir users (eight cases, 8.6% of 93 cases). Hence, larger studies are warranted to confirm our finding. Also, there might be a sampling bias related to the possibility that PLWH received a darunavir/ritonavir (with the CPE rank of 3) [56] prescription because they had increased neurobehavioral symptoms.

Previous reports by Anthony *et al.* [39,41] showed that ART history was associated with neuronal phospho-tau lesions and microglial activation in postmortem brain samples. However, data on the use of individual ART drugs were not available in these two reports. CNS adverse events associated with ritonavir-boosted darunavir use were not common in clinical trials and postmarketing experience [63]. In cell-culture experiments using primary rat fetal brain neuronal cells, Robertson *et al.* [7] reported that 1-week exposure to darunavir or ritonavir led to a relatively lower risk of neuronal toxicity assessed by MAP2 immunoreactivity. Nonetheless, comparing the findings from various studies using different end points is not straightforward. Neuronal phospho-tau lesions, which progress from neuropil threads and pretangle neuronal soma to intracellular neurofibrillary tangles and extracellular (ghost) neurofibrillary tangles (indicative of neuronal loss), follow a chronic course that may be irreversible especially in later stages [60]. In the present study, 97% of 33 cases that showed neuronal phospho-tau lesions in the putamen had neuropil threads of grade-1 density (i.e. in the earliest stage). Neuronal phospho-tau lesions likely do not clinically manifest until they reach a critical threshold.

Among 77 cases with CSVD data, all 20 emtricitabine users had CSVD (see Table, Supplemental Digital

Content 1, http://links.lww.com/QAD/B309). However, in the final multivariable analysis (19 emtricitabine users vs. 56 nonemtricitabine users), there was only a trend toward an association between emtricitabine use and CSVD after the Bonferroni correction (adjusted $P = 0.099$). The lack of non-CSVD cases among emtricitabine users is potentially an important finding, but it also makes the accurate estimation of OR computationally more challenging. Future studies with larger sample sizes are warranted to verify this finding. In our previous study [14], we focused on the protease inhibitor class of ART drugs and categorized ART regimens used (at the last clinical assessment) into protease inhibitor-based, nonprotease inhibitor-based, and no ART [i.e. use of (single-drug or dual-drug) non-ART regimens, discontinuation of ART, or being ART-naive). We found an association between protease inhibitor-based ART (with reference to no ART) and a higher risk of CSVD [14]. In the present study, however, we did not find any significant association between the use of any protease inhibitor drugs analyzed (i.e. atazanavir, darunavir, lopinavir, and ritonavir) and CSVD (with reference to the use of other ART drugs). The apparent discrepancy may be explained by a difference in the reference groups, in which ART-naive or ART-discontinuing cases were not included in the present study.

We did not find any significant association between the CPE score and neuropathologic changes examined, which is not surprising. The CPE system has been designed to predict antiviral efficacy, not toxicity [9]. The CPE ranking does not take into account either CNS tissue drug concentrations or toxicity. In addition, the relationship between drug concentrations and toxicity can be completely different from that between drug concentrations and antiviral efficacy [11].

In the present study, associations between specific ART drug use and neuropathologic changes were observed in either the frontal cortex or putamen but not simultaneously. Variation in susceptibility to chronic ART toxicity may exist between different brain regions. During the human lifespan in general, the brain regional expansion of neuronal phospho-tau lesions and β-amyloid plaque deposition progresses with advancing age in characteristic sequences [42,64]. In addition, these neuropathologic changes can develop in association with the altered neural microenvironment in particular brain regions, for example, neuronal phospho-tau lesions in chronic traumatic encephalopathy [65].

The present study had limitations inherent to research using postmortem human samples. According to the current DHHS guidelines [3], ART drugs in the INSTI class (i.e. dolutegravir, elvitegravir, and raltegravir) are principal components of the recommended initial regimens. Nonetheless, INSTI-using cases have been relatively underrepresented in the NNTC cohort (personal communication with the NNTC Data Coordinating Center). Fewer PLWH are dying with INSTI-based ART, and therefore, fewer brains are available to research. This led to limited statistical power for testing associations between raltegravir (the only INSTI drug used in the present study) use and neuropathologic changes. As some ART drugs were always (e.g. darunavir and ritonavir) or often (e.g. emtricitabine and tenofovir DF) prescribed together, we cannot exclude the possibility that the effects of one ART drug resulted from the interaction effects of multiple ART drugs used simultaneously.

The duration of specific ART drug use might affect chronic neurotoxicity. Many PLWH change their ART regimen during their clinical course because of virologic failure or adverse events. In a given PLWH, the duration of specific ART drug use varied from one drug to another (data not shown). We selected cases based on the availability of complete data on the list of ART drugs currently used at the last clinical assessment. However, data on the duration of specific ART drug use were available in only a subset of these cases, and therefore could not be readily analyzed. We did not include brain HIV-1 RNA levels in the two brain regions examined as covariates because these data were available only in a small subset of cases. To compensate for this limitation, we included a histopathologic diagnosis of HIVE, which indicated productive HIV infection in the brain [57], and last-visit plasma HIV-1 RNA levels, as covariates.

In conclusion, our findings suggest that PLWH treated with darunavir and ritonavir may be at increased risk of particular aging-related cerebral degenerative changes including neuronal phospho-tau lesions and microgliosis. Larger autopsy cohorts of PLWH are needed to confirm an association between emtricitabine use and CSVD. Also justified are ART drug toxicity studies using multilayer cultures of vascular endothelial cells, smooth muscle cells, and pericytes, which mimic the in-vivo small vessel wall. The results of clinicopathological analyses can be used to guide experimental studies using animal models and cell systems to explore cause–effect relationships.

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Conflicts of interest

There are no conflicts of interest.

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