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Journal

Journal of NeuroVirology, 29(2)

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Publication Date

2023-04-01

DOI

10.1007/s13365-023-01122-6

Peer reviewed

HHS Public Access

Author manuscript

J Neurovirol. Author manuscript; available in PMC 2024 February 24.

Published in final edited form as:

J Neurovirol. 2023 April ; 29(2): 218–224. doi:10.1007/s13365-023-01122-6.

Plasma extracellular vesicles and cell-free mitochondrial DNA are associated with cognitive dysfunction in treated older adults with HIV

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Abstract

Extracellular vesicles (EVs) are nanoparticles with a role in intercellular communication. Cell-free mitochondrial DNA (cf-mtDNA) has been associated with cognitive dysfunction in people with HIV (PWH). We conducted a nested case–control study to test the hypothesis that plasma EVs are associated with cf-mtDNA and cognitive dysfunction in older PWH. A machine learning-based model identified total EVs, including select EV subpopulations, as well as urine cf-mtDNA and 4-meter walk time carry power to predict the neurocognitive impairment. These features resulted in an AUC-ROC of 0.845 + / − 0.109 (0.615, 1.00).

Keywords

HIV; Aging; Cognition; Inflammation; Frailty; Mitochondria; Extracellular vesicles

Background

As people with HIV (PWH) age, they often experience a greater degree of multimorbidity and geriatric syndromes than their HIV-uninfected peers (Guaraldi et al. 2011; Pelchen-Matthews et al. 2018), including neurocognitive impairment and frailty (Guaraldi et al. 2011; Brothers et al. 2014; Clifford 2017). HIV-associated neurocognitive disorders

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Declarations

Conflict of Interest E.G.M., S.B., and P.J.N. have no conflicts to report.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13365-023-01122-6.

(HAND) represent a spectrum of pathology ranging from asymptomatic neurocognitive impairment to HIV-associated dementia, with a prevalence of up to 50% of PWH, despite suppression of HIV viremia (Heaton et al. 2010). Specific cell types within the brain, including astrocytes and microglia, have been identified to harbor HIV (Ash et al. 2021), and subsequently, HIV persists within the central nervous system (CNS) and can be detected in cerebrospinal fluid (CSF) (Anderson et al. 2016). In addition, an expanded HIV reservoir may exist within the CNS despite antiretroviral therapy (ART) (Ash et al. 2021; Ho et al. 2013). Despite the persistence of HIV within such privileged sites, biomarkers of ongoing pathology that underlie HAND remain limited, and frailty and cognitive impairment contribute to significant morbidity and mortality in PWH (Piggott et al. 2020; Hosaka et al. 2019).

Extracellular vesicles (EVs)—small structures with a range of functions including cell-to-cell communication and inflammation—have been associated with inflammation in the setting of HIV and can provide diagnostic and predictive value for CNS injury in HIV (Guha et al. 2019). Neurons and microglia can secrete EVs that cross the blood–brain barrier, suggesting plasma EVs may provide a window into the CNS environment.

Recent advances in understanding of cellular inflammation and death processes, including necroptosis (necrosis-mediated cell death), have allowed for measurement of novel biomarkers of cellular death and inflammation, including cell-free mitochondrial DNA (cf-mtDNA) (Cossarizza et al. 2011; Picca et al. 2019). Intrinsic cell death pathways, specifically necrosis-mediated cell death due to RIPK3 phosphorylation of MLKL, result in programmed cell death in which rupture of cellular membranes releases intracellular substances, including cfmtDNA, and MLKL phosphorylation increases generation of EVs (Yoon et al. 2017). Additionally, PWH have been demonstrated to have greater quantities of pro-inflammatory EVs (Poveda et al. 2022), and in an in vitro model, EVs derived from PWH induced significantly more endothelial cell necrosis and death than EVs from HIV-uninfected individuals (de Menezes et al. 2022a).

We have previously demonstrated that higher levels of plasma cf-mtDNA were associated with cognitive dysfunction and key components of frailty including slow walk and exhaustion (Johnston et al. 2022), and urine cf-mtDNA was associated with the frailty component of unintentional weight loss and lower indices of skeletal muscle (Johnston et al. 2021) in older PWH. As the overlap of cognitive impairment and physical frailty, termed “cognitive frailty,” is associated with significant morbidity and mortality (Erlandson et al. 2019), we included plasma and urine cf-mtDNA as candidate biomarkers in this study. Building upon these findings, we hypothesized that plasma EV concentrations would be associated with cognitive dysfunction in older PWH and sought to correlate EVs with cf-mtDNA.

Methods

Study design

PWH age 50 and older were randomly selected (with oversampling of those age 60 and older) from a large urban academic medical center outpatient HIV clinical practice and

invited to complete a detailed questionnaire focusing on health status, quality of life, psychosocial factors, and current substance use (Erenrich et al. 2018). Participants age 55 and older who completed the questionnaire were then invited to participate in a substudy consisting of a cognitive evaluation using the Montreal Cognitive Assessment (MoCA) (Nasreddine et al. 2005) and frailty phenotype testing (Fried et al. 2001). We conducted a nested case–control study within this substudy that compared cases with cognitive dysfunction (Montreal Cognitive Assessment [MoCA] score < 23) to demographically similar controls (MoCA > 26) [Supplemental Fig. S1]. Participants with HIV-1 viral load > 40 copies/ml were excluded from the nested case–control study. Written informed consent was obtained, and this study was approved by the Weill Cornell Medicine IRB.

Montreal cognitive assessment (MoCA) testing

The MoCA was administered by trained staff, and scores on individual domains of the MoCA were tabulated to compute the composite MoCA score, with one point added if the participant reported an education level of high school or less (Nasreddine et al. 2005). The MoCA was scored out of a total of 30 points, and scores < 23 were categorized as cognitively impaired (Nasreddine et al. 2005; Carson et al. 2018).

Frailty testing

The frailty phenotype was assessed as previously described (Fried et al. 2001). Participants completed a timed 4-m walk, and slowness was determined by the average of two readings of the 4-m walk: men ≤ 173 cm and women ≤ 159 cm in height who required > 6.22 s, or men > 173 cm and women > 159 cm who required ≥ 5.33 s to complete the walk met the criterion for slowness (Erlandson et al. 2017). Measurement of dominant-hand grip strength was assessed by dynamometry; participants completed three trials, and the average was computed. Questions about exhaustion, level of physical activity, and unintentional weight loss were completed in the questionnaire. Missing weight data were obtained from chart review. If 0 criteria were met, the participant was classified as nonfrail; if 1–2 criteria were met, they were classified as pre-frail, and if 3–5 criteria were met, they were classified as frail (Fried et al. 2001).

Cell-free mitochondrial DNA measurement

Fasting blood samples were drawn into chilled tubes, and spot urine samples were collected; all samples were immediately placed on ice. Plasma and urine samples were processed within four hours of collection, and cf-mtDNA levels were measured by SYBR green dye-based qPCR assay using a PRISM 7500 sequence detection system (Applied Biosystems) as previously described (Nakahira et al. 2013; Johnston et al. 2022, 2021). The concentration of cf-mtDNA in plasma was calculated in copies per μL, and in urine, cf-mtDNA is reported as copies per gram of urine creatinine.

EV measurement

Blood samples were collected into EDTA tubes, and separated plasma was stored at −80 °C. After thawing, blinded plasma samples were centrifuged at 2000 g for 10 min at 4 °C and stained using pre-titrated volumes of fluorochrome-conjugated monoclonal

antibodies in five separate panels, purchased from BioLegend except as noted and listed here according to either their cell of origin or functional activity: 1/lymphocytes: CD4-PE (SK3), CD19-PECy7 (SJ25CI, BD Biosciences), and CD73-BV41 (AD2), 2/monocytes: CD14-APC (63D3), CD16-PerCP-Cy5.5 (3G8), CD68-FITC (Y1/82A), CD163-PECy7 (GHI6I), 3/chemokine receptors: CD192-V421 (K036C2) CCR2, CD195-PE (2D7) CCR5, BD Biosciences) and CX3CR1-BV421 (2A91), 4/metabolism: GLUT1-PE (glucose transport 1, 202,915, R&D Systems), 5/granulocytes: CD66b-PerCP/Cy5.5 (G10FS), 6/platelets: CD41a-PerCP/Cy5.5 (HIP8), CD36-PE (5–271), and CD62P-APC (AK-4, BD Biosciences), 7/EVs: tetraspanin protein CD63-PerCP/Cy5.5 (H5C6), and CD9-FITC (H19a), 7/lectins: WGA-FITC (wheat germ agglutinin, FL-1021, Vector Laboratories), and MAL I-FITC (maackia amurensis lectin I, FL-1311, Vector Laboratories), 8/antigen-presenting cells: MHC Class II-BV510 (major histocompatibility complex class II, G10F5), 9/astrocytes GFAP-V421 (glial fibrillary acidic protein, 2E1E9), 10/macrophage/microglia: CD11b-PECy7 (ICRF44, BioLegend), and 11/neuronal: S100B-PE (4C4.9 + S100B/102, Novus Biologicals), neurons CD200-PerCP/Cy5.5 (OX104), NFL-Alexa 647 (neurofilament light chain, 8A1, Santa Cruz Biotechnology), and MAP2-Alexa 488 (microtubule-associated proteins, 18MAP2B, BD Biosciences). Prior to testing EV samples, each antibody was filtered using a 0.22 μm centrifugal filter (Millipore) to remove aggregates, and the filtrate was used for staining. One to 3 μL were added to 10 μL of EVs and incubated at 4 °C for 30 min. EVs were diluted in 0.22 μm-filtered PBS to appropriate dilutions to avoid swarm detection, as previously described (de Menezes et al. 2022b).

Acquisition was performed using a five-laser Aurora spectral flow cytometer (Cytek Biosciences). Quality control was performed using SpectroFlo QC Beads to normalize sensor gain as recommended by the manufacturer (Cytek Biosciences). A clean flow cell procedure was conducted prior to sample analysis to minimize EV carryover. Side scatter was measured using the 405 nm violet laser at a threshold of 1000 arbitrary units. A 0.22 μm-filtered PBS control was recorded to estimate the background signal. Samples were acquired for 1 min at a low flow rate (~ 15 μL/min). The reference bead mix (Apogee Flow Systems) composed of a mixture of 110 nm and 500 nm polystyrene (PS) beads and 180, 240, 300, 590, 880, and 1300 silica beads was used to evaluate the fluorescence performance (both sensitivity and resolution). EV gates were established using PS beads ranging from 80 to 1300 nm (Apogee and NIST-traceable PS beads). EV counts/μL were calculated using the flow rate of the cytometer. Analysis was performed using SpectroFlo software (version 2.2.0.4; Cytek Biosciences).

Machine learning-based modeling

Removing correlated predictor variables is essential for accurate interpretation of machine learning models. Spearman's correlation coefficient was used to eliminate redundant features (rho ≥ 0.93). We utilized recursive feature elimination (RFE), which fits a model and removes the features which carry the weakest predictive power until a specified number of features remain. 3-repeated, fivefold cross-validation (CV) was used to optimize the number of predictors from our dataset.

In this study, we implemented a Python-based Support Vector Machine (SVM) supervised learning model to discriminate the cognitive status of PWH utilizing the RFE-selected features. SVM is an effective classification method based on structural risk minimization (Yoon and Kim 2008). Briefly, SVM maps input vectors nonlinearly to high-dimension feature space, then searches for an optimal hyperplane which maximizes the distance between the hyperplane and the closest samples of each class. Finally, we plotted the receiver operating characteristic (ROC), then calculated the area under the curve (AUC) to examine the probability of discriminating cognitive function in PWH correctly.

Statistical methods

Variables were summarized using descriptive statistics. The median and interquartile range were used for continuous variables, and frequencies and proportions for categorical measures. The Mann–Whitney U test analysis evaluated differences between groups. Statistical analyses were performed using SPSS software version 27 (IBM, Armonk, NY). A two-sided $p \leq 0.05$ was regarded as statistically significant for all tests.

Results

The study consisted of 50 participants (25 cases) whose characteristics are summarized in Table 1. Briefly, participants had a median age of 60 (Q1, Q3: 57, 65) years, were 38% female, 53% black, and 25% Hispanic, with a median history of living with HIV for 24 (21, 28) years, and a median CD4 T-cell count of 596 (479, 859) cells/μL. Plasma cf-mtDNA levels were higher in participants with cognitive impairment compared to without (geometric mean 5.72 [5.32, 63.0] vs 5.28 [4.92, 5.75], respectively, $p = 0.05$). Urine cf-mtDNA levels did not statistically differ between the two groups ($p = 0.85$). Frailty scores were calculated for 48 of the 50 participants; two participants without cognitive impairment had missing grip strength data. Overall, 65% met the criteria for pre-frail/frail state, without significant differences between the two groups by Mann–Whitney U test ($p = 0.13$). Gait speed was slower in participants with cognitive impairment compared to those without (5.21 s [4.44, 5.87] vs 4.21 [3.58, 5.13], respectively, $p = 0.01$).

In order to investigate plasma EVs as potential biomarkers of cognitive dysfunction in older PWH, two models were created that consisted of just EV measures or included physical function measures from frailty testing and plasma and urine cfmtDNA levels in addition to EVs. Spearman correlation analysis identified CD4-, MAP2-, CD73-, and CD68-expression on EVs being highly correlated (Spearman's rho ≥ 0.93) with expression of other EV surface markers (Supplemental Fig. S1).

These redundant features were then eliminated from analysis but may serve as surrogate markers and be substituted into the model with correlated counterparts. Substitutions should have a minimal impact on model accuracy.

Utilizing only EV measurements, RFE identified total EVs, CD19 +, WGA +, CD41a +, CD62P +, CD163 +, CCR2 +, CCR5 +, MAL-1 +, CD11b +, CD200 +, S100B +, GFAP +, CD9 +, CD63 +, MHC-II +, GLUT-1 +, and CD36 + EV subsets as predictive of cognitive impairment in PWH on ART aged ≥ 55 years. This EV-only model resulted in an AUC-ROC

of 0.632 + / − 0.136 [95% CI: 0.308, 0.846] (Fig. 1). When the additional clinical measures including age, sex, race, physical function and plasma and urine cf-mtDNA measures were included, total EVs, CD16 +, WGA +, CD41a +, CD163 +, CCR5 +, MAL-1 +, CD200 +, NFL +, S100B +, CD9 +, CD63 +, MHC-II +, and CD36 + EV subsets, as well as, urine cf-mtDNA and mean 4 m walk time were identified by RFE to carry predictive power to classify PWH by cognitive status. The 16-feature model trained using these predictors resulted in an AUC-ROC of 0.845 +/− 0.109 [0.615, 1.00] (Fig. 1), an improvement over the EV-only model.

We observed greater numbers of CCR5 + EVs and longer 4 m walk time durations and lower numbers of CD16 +, CD41a +, and WGA + EVs amongst PWH with cognitive impairment compared to PWH with normal cognition (Supplemental Fig. S3). When the model was retrained with only these significantly different features, the resulting AUC-ROC was 0.696 ± 0.115 [0.462, 0.923], a reduced performance compared to the 16-feature model. These results suggest that inclusion of the non-significantly different features may insulate the model from outliers and noise within the data.

Conclusions

A support vector machine model, comprised of total EVs, CD16 +, WGA +, CD41a +, CD163 +, CCR5 +, MAL-1 +, CD200 +, NFL +, S100B +, CD9 +, CD63 +, MHC-II +, and CD36 + EV subsets, as well as urine cf-mtDNA and mean 4 m walk time, accurately predicted cognitive dysfunction with 85% certainty within an older population of PWH. This was an improvement over EV measures alone (63% certainty). Slow gait has previously been associated with cognitive dysfunction in older PWH, and our results support these prior findings in our larger study of older PWH (Derry et al. 2020) and by others (Masters et al. 2019).

Considering the specific EV components common between the EV-only and 16-feature models, CCR5 plays a critical role in immune cell trafficking and is expressed on activated T cells, as well as serving as an HIV co-receptor and mediator of inflammatory activity in the CNS. Our group previously observed EVs expressing the monocyte-derived markers CCR5 and CD163 to be significantly more abundant in PWH with cognitive impairment compared to PWH with normal cognition (Marques de Menezes et al. 2018). The platelet biomarker CD41a has been suggested as an inflammatory marker in HIV (Falasca et al. 2021), and our group reported that CD41a + EVs were correlated with CCR5 expression on total monocytes (de Menezes et al. 2022a). The same study found that CD16 + EVs were associated with cognitive impairment in PWH. Regarding other notable EVs in our model, CD36 is a scavenger receptor involved in metabolism, atherosclerosis, and inflammation and has been demonstrated to be vital to HIV transmission to T-cells by infected macrophages (Berre et al. 2013). WGA and MAL-1 have been linked to blood–brain barrier (BBB) transcytosis (Abulrob et al. 2005). S100B is considered a peripheral marker of CNS injury and BBB permeability (Bandera et al. 2019).

Plasma cf-mtDNA was higher in older PWH with cognitive dysfunction in this subset of our larger study, as previously reported (Johnston et al. 2022). Interestingly, plasma

cf-mtDNA was not included in the best model; however, urine cf-mtDNA, which was not different between cognitive groups ($p = 0.846$), was included. We believe feature selection identified plasma cf-mtDNA as a redundant variable which did not provide additional unique information to further power the classification model, while substituting urine cf-mtDNA, a non-significant variable, likely insulated the model from noise. Furthermore, the inclusion of stabilizing non-significant variables has been shown to reduce the risk of over-fitting, suggesting that our model, while comparable to those that included plasma cf-mtDNA, is also more likely to be applicable to other cohorts. We have previously reported on the linkage between higher plasma cf-mtDNA and cognitive dysfunction, including measures of frailty (Johnston et al. 2022). WGA and CCR5 both serve as key receptors in cellular migration which may permit EVs to transmigrate from the periphery to the CNS compartment (Kuo et al. 2017). From our data in Supplementary Fig. S2, quantification of potentially unique combinations of EVs and measures of frailty and cell death may be indicative of a cascade leading to neuroinflammation, neuronal cell death increasing cf-mtDNA and worsening cognition.

This study was limited by the lack of an external dataset to validate model quality, despite steps taken to reduce overfitting. These results would benefit from a larger study to validate our findings and the inclusion of a battery of neuropsychological assessments which may identify specific neurological domains associated with EVs, as well as measurement of plasma EVs in a HIV negative and demographically similar cohort.

Our findings suggest a role of EVs and cf-mtDNA as potential biomarkers of cognitive dysfunction, and the strongest predictive model included monocyte, platelet, microglia, and neuronal-derived EVs specific to cellular inflammation and metabolism, as well as the physical function parameter of slow gait, and urine cf-mtDNA. These findings warrant further study, including longitudinal analysis and deeper investigation of EV markers of cellular origin (Li et al. 2020), which could provide enhanced insight into tissue-level intercellular communication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding

This work was supported by the NIH (National Institute of Aging (K23 AG 072960), National Center for Advancing Translational Sciences (UL1TR000457), National Institute of Neurological Disorders and Stroke (R21NS106970), National Institute of Neurological Disorders and Stroke (R01 NS117458), Gilead Sciences, and Weill Cornell Fund for the Future Award.

C.D.J served as a consultant for TheraTechnologies. M.J.G. reports research support to his institution (Weill Cornell Medicine) from Gilead Sciences and Regeneron, has served as a consultant to Enzychem, Regeneron, ReAlta Life Sciences, and Sobi, and receives royalties from Springer and UpToDate. L.C.N. reports grants from the NIH and has received consulting fees from work as a scientific advisor for AbbVie, ViiV Healthcare, and Cytodyn and for service on the Board of Directors of CytoDyn, all for work outside of the submitted work.

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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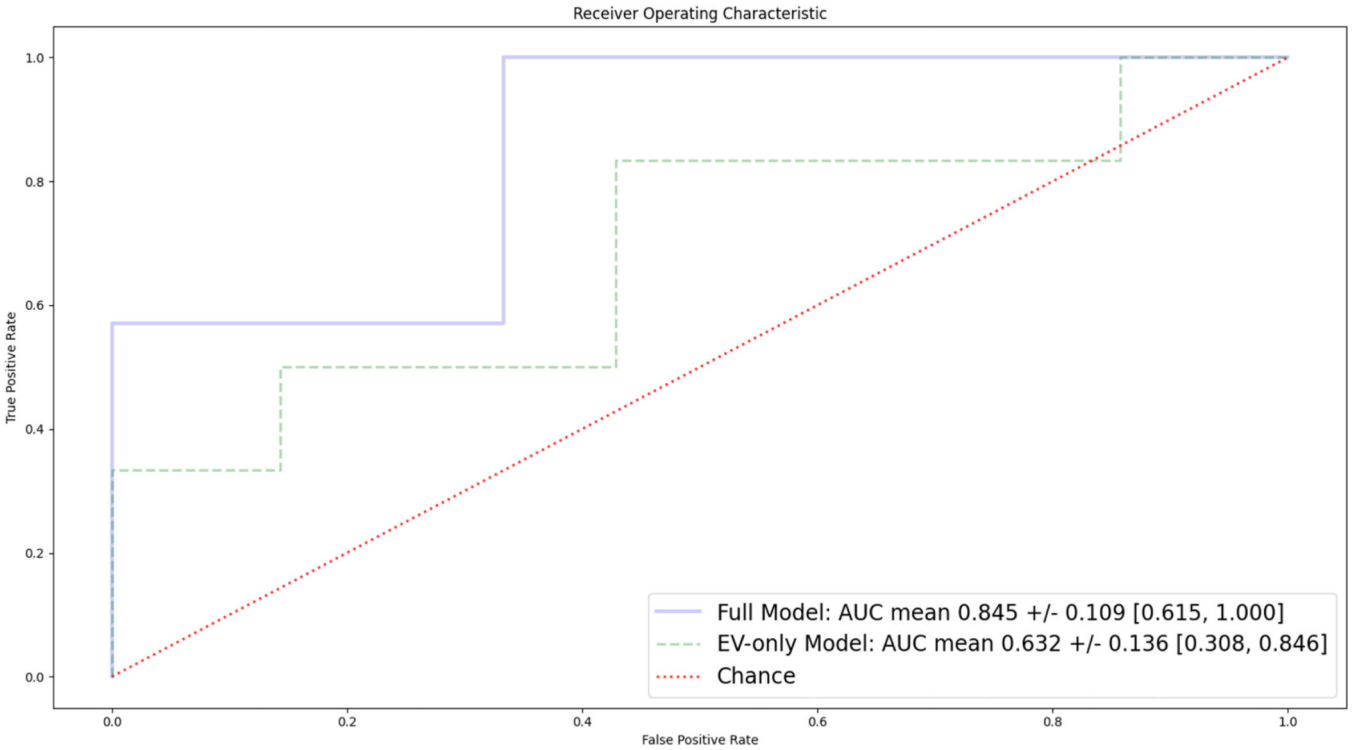

Fig. 1.

Model performance. Fig. 1 support vector machine (SVM)-derived model comprised of total EVs and CD19, WGA, CD41a, CD62p, CD163, CCR2, CCR5, MAL-1, CD11b, CD200, S100B, GFAP, CD9, CD63, MHC-II, GLUT-1, and CD36 + EV subsets (EVonly, dashed green line) resulted in an area under the curve (AUC) of the receiver operating characteristic (ROC) of 0.632 ± 0.136 [0.308, 0.846]. When clinical, frailty, and cell-free mitochondrial DNA measures were included in feature selection, the resulting SVM model comprised of total EVs, CD16, WGA, CD41a, CD163, CCR5, MAL-1, CD200, NFL, S100B, CD9, CD63, MHC-II, and CD36 EVs, as well as urine cfmtDNA and mean 4 m walk time (full model, solid blue line) resulted in an AUC-ROC of 0.845 ± 0.109 (0.615, 1.000)

Table 1

Participant characteristics

	Total (*n* = 50)	**Cognitively impaired (*n* = 25)**	**Normal cognition (*n* = 25)**	***p*-value**
Age, years, median [IQR]	60.0 (57.0, 64.8)	61.0 (57.0, 66.0)	58.0 (57.0, 63.0)	0.263
Sex, Male, n (%)	31 (62.0%)	15 (60.0%)	16 (64.0%)	1
HIV Duration, years, median [IQR]	24.0 (21.3, 27.8)	23.0 (22.0, 27.0)	24.0 (20.0, 29.0)	0.861
CD4 T cell count, median [IQR]	596 (479, 859)	593 (498, 750)	703 (476, 962)	0.541
Plasma cf-mtDNA[a] copies/mL (Ln transformed), geometric mean [IQR]	5.51 (5.08, 5.89)	5.75 (5.32, 6.30)	5.28 (4.92, 5.77)	0.051
Urine cf-mtDNA[a] copies/g of urine creatinine (Ln transformed), geometric mean [IQR]	19.34 (18.34, 20.25)	19.29 (18.59, 20.16)	19.40 (18.11, 20.27)	0.846
Frailty, n (%)				0.132
Not Frail	17 (35.1%)	6 (24.0%)	11 (47.8%)*	
Pre-frail/frail	31 (64.6%)	19 (76.0%)	12 (52.2%)*	
4 m walk time, seconds, median [IQR]	4.84 (3.90, 5.63)	5.21 (4.44, 5.87)	4.21 (3.58, 5.13)	0.013

cognitive impairment was defined as montreal cognitive assessment < 23 (of maximum 30 points)

[a]Cell-free mitochondrial DNA (cf-mtDNA) is reported as Ln-transformed copies/ml of plasma and copies/gram of urine creatinine

*23/25 non-cognitively impaired individuals had available frailty classification