Blood-Brain Barrier Disruption Is Initiated During Primary HIV Infection and Not Rapidly Altered by Antiretroviral Therapy

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Abstract

Background:
We explored the establishment of abnormal blood brain barrier (BBB) permeability and its relationship to neuropathogenesis in early HIV by evaluating CSF:serum albumin quotient (Q_{Alb}) prior to and after combination antiretroviral therapy (cART) in primary HIV infection (PHI).

Methods:
Q_{Alb} was measured in longitudinal observational studies of PHI and in HIV-negative controls. We analyzed trajectories of Q_{Alb} pre- and post-cART using mixed-effects models, and associations between Q_{Alb} and CSF neurofilament light chain (NFL), N-acetylaspartate:creatinine (NAA:Cr, a magnetic resonance spectroscopy neuronal integrity biomarker), and neuropsychological testing.

Results:
Age-adjusted Q_{Alb} was elevated in PHI vs. controls at baseline (n=106, median 91 days post infection, dpi; n=64; p=0.02). Longitudinally pre-cART, Q_{Alb} increased in 84 participants with normal baseline Q_{Alb} (p=0.006), and decreased in 22 with high baseline Q_{Alb} (p=0.011). Q_{Alb} did not change after a median 398 days of cART initiated at 225 dpi (p=0.174). Q_{Alb} correlated at baseline and longitudinally with NFL (r=0.497, p<0.001; r=0.555, p<0.001) and NAA:Cr in parietal grey matter (r=-0.352, p<0.001, r=-0.387, p=0.008), but not neuropsychological performance.

Conclusion:
Q_{Alb} rises during early HIV, associates with neuronal injury, and does not significantly improve over a year of follow-up on treatment, suggesting that BBB-associated neuropathogenesis is initiated in early infection.

Keywords:
HIV/AIDS; PHI; primary HIV infection; BBB; Blood brain barrier; neuropathogenesis
**Introduction**

Chronic exposure to HIV can lead to neurological complications, with one third of untreated individuals with advanced AIDS developing HIV-associated dementia (HAD) [1, 2], a syndrome of severe cognitive, motor, and behavioral disturbances associated with cerebral atrophy, particularly of subcortical areas [3]. Although the incidence of HAD has significantly decreased with the advent of combination antiretroviral therapy (cART), a milder spectrum of neurocognitive deficits persists despite treatment [1, 2, 4]. As this persisting impairment may at least in part represent irreversible alterations of central nervous system (CNS) integrity accrued prior to the initiation of cART, investigative efforts have been drawn towards elucidating neuropathogenesis during the early stages of HIV neuroinvasion, and examining the effects of early cART on these processes.

Primary HIV infection (PHI) refers to the first phase of infection, from time of transmission up to 12 months post-transmission [5]. HIV infiltrates the CNS during PHI[6-8], as indicated by the presence of HIV RNA in the cerebrospinal fluid (CSF) compartment, even in the absence of neurological symptoms [1, 8-11]. CNS immune activation accompanies this viral invasion as reflected by elevations of CSF white blood count, the soluble CSF biomarkers neopterin (reflecting macrophage activation) and CXCL-10/IP-10 (a lymphocyte chemokine), and T lymphocyte activation in CSF[1, 12-15]. Furthermore, markers of immune activation may reflect degree of viral load and neurocognitive impairment[16]. Magnetic resonance spectroscopy (MRS), a noninvasive quantitative MR technique that measures alterations in cerebral metabolism, demonstrates that inflammatory cerebral metabolites are elevated in acute HIV (prior to antibody seroconversion) and longitudinally increased over time in PHI prior to cART[1, 17, 18]. Thus, crucial processes during the primary phase of viral infection may underlie the initiation of HIV associated CNS injury.

It is speculated that increased blood brain barrier (BBB) permeability is a critical contributor to HIV neuropathogenesis as disruption of this regulatory interface facilitates CNS infiltration of potentially harmful substances from the periphery, resulting in compounding viral entry and susceptibility to the inflammatory assault of immune cells[19, 20]. In order to exert its neurological effects, HIV-1 and/or its viral products must first traverse the BBB. Although data suggest that at this initial stage, HIV is transported to the CNS via trafficking of infected immune cells across a largely intact BBB, increased permeability of the BBB has been implicated in the progression of HIV neurological dysfunction[19, 21-24]. The ratio of albumin in the CSF to serum albumin (CSF:serum albumin concentration quotient, or Q_{Alb}) is the best established fluid marker for BBB permeability[25]. Albumin is synthesized exclusively in the liver and is largely excluded from the CSF. Upon deregulation of the neurovascular unit and sequential loss of tight junctions, BBB permeability to albumin increases, resulting in an increased Q_{Alb}.

In this study we aimed to elucidate the natural history of BBB permeability during PHI, and to determine whether these changes, if any, were associated with biomarkers of neuropathogenesis. Additionally, we sought to determine whether BBB permeability was responsive to cART treatment initiated during early HIV infection. These studies provide novel understanding of the changes to the brain microenvironment that begin during initial HIV infection, and the persistence of these alterations in the setting of early, virologically suppressive cART.

**Materials and Methods**

**Experimental design**

Individuals with PHI were recruited into prospective longitudinal studies of CNS HIV in Gothenburg, Sweden, and San Francisco, USA, between 1986 and 2014, as previously described [9]. Participants
were within the first year of HIV transmission as confirmed by the standard serologic testing algorithm for recent HIV seroconversion (STAHRS) [26], and all but three were ART-naive. A subset began cART at variable times during follow up for reasons outside of the study. None of the participants had a prior neurological disease history. A history of substance abuse was not an exclusion criterion, but no participants reported same-day substance abuse, which would have led to censoring of data. Date of HIV transmission was approximated as 14 days prior to the onset of seroconversion symptoms, when present [27]; otherwise, it was approximated as midway between the dates of the last negative and first positive EIA test[28]. HIV-uninfected individuals from the community were enrolled at the San Francisco site for comparison of baseline parameters.

**Ethics**

The study protocol was approved by the institutional review board or an equivalent research ethics committee of each institution involved. All study participants gave written consent.

**Data collection and laboratory analysis**

CSF, blood, neuropsychological testing performance, and magnetic resonance spectroscopy (MRS) were obtained at each visit, and collection performed as previously described[23, 29]. Study intervals were scheduled at baseline (time 0), six weeks, and every six months thereafter, although there was participant variation in timing and duration of follow up.

Blood CD4+ and CD8+ T-lymphocytes, CSF white blood cells, and CSF total protein were measured by flow cytometry. CSF NFL was measured with the NF-light® ELISA kit (UmanDiagnostics AB, Umeå, Sweden), a sensitive immunoassay [21] with a lower limit of detection 50 ng/L, and reference values for upper limit of normal of 380 ng/L (18–30 years), 560 (30–39years), 890 (40–59 years), and 1850 (>59 years)[21]. CSF and plasma albumin were measured by nephelometry (Behring Nephelometer Analyzer, Behringwerke AG, Marburg, Germany). QAlb was calculated as the CSF/plasma albumin ratio: CSF albumin (mg/l) / plasma albumin (g/l) [22]. Reference values for upper limit of normal were based on previously established values of <6.8 for age <45 years, and <10.2 for age >45 years [30]. HIV RNA was measured with the Abbott RealTime HIV-1 PCR assay (Abbot Laboratories, Abbot Park, IL, USA). Viral loads below the lower limit of detection (<50 copies/mL) were assigned a value of 49 copies/mL (1.69 on log_{10} scale).

Neuropsychological performance was determined through the appraisal of gross and fine motor skills, processing speed, executive function, learning, and verbal memory through a battery of 11 tests. Performance was summarized as an aggregate total Z score and a brief NPZ-4 score (including grooved pegboard, digit symbol, finger tapping, and timed gait).

MRI/MRS was obtained at the San Francisco site only. A neuroradiologist reviewed MRI images to ensure that no significant non-HIV associated pathologies were present. MRS data was processed and analyzed with the spectral fitting software SITools, which uses a parametric model of known (metabolites) and modeled spectral components (macromolecules) to fit all resonances and nonparametric parameters to the baseline. Metabolite disturbances can indicate neuropathology, including inflammation and injury. The ratio of the cerebral metabolite N-acetylaspartate to creatinine (NAA:Cr) is a putative marker of neuronal viability and number. We focused on the parietal grey matter, as we have previously identified metabolite abnormalities in this region during primary infection in this cohort [17, 23].
Statistical analysis
Non-parametric and parametric (ANCOVA) statistics were used to summarize baseline characteristics and compare findings between PHI and HIV-uninfected participants (significance level was set at p < 0.05, two-sided). The mixed-effects model was used to analyze longitudinal change of Q\textsubscript{Alb} post transmission. This model permits both fixed and random effects in the same analysis, allowing for variation in the number and time interval of participant follow-up visits. Baseline age was included as a fixed-effect covariate in the model. To account for a possible non-linear trajectory of Q\textsubscript{Alb} over time, a quadratic term (t\textsuperscript{2}) was included as a fixed-effect covariate. The model included a personal intercept for each participant as a random effect, allowing baseline Q\textsubscript{Alb} to vary for each participant. An interaction term was initially added to assess whether the trajectory of Q\textsubscript{Alb} over time depended on the baseline Q\textsubscript{Alb}, but was found to be insignificant. As log-transformed results were comparable to non-log-transformed analysis, the latter results are reported for familiarity of Q\textsubscript{Alb} values. The graphed y-intercept was calculated as follows: [(parameter estimate of baseline age)*(median age of subgroup)]+parameter estimate of subgroup intercept. Statistical analyses employed SPSS 23.0 statistical package (IBM Corp., Armonk, NY).

Results

Study participant characteristics
106 PHI participants fulfilled the inclusion criteria and had available Q\textsubscript{Alb} values. Only 9 participants experienced neurological disorders during seroconversion; these included meningitis (n=2), headache with photophobia (n=2), brachial neuritis (n=2), Guillain-Barre syndrome, facial palsy, and encephalitis. Total visits ranged from 1 to 13 with a median of 2, and follow-up ranged up to 2940 days with a median of 49.5 days. The majority of participants were infected with subtype B virus [9].

The baseline characteristics of PHI and uninfected control participants are presented in Table 1. The median duration of HIV infection in PHI participants was 91 days; plasma viral load in PHI was 1.81 log\textsubscript{10} greater relative to that in the CSF compartment. As compared to the HIV-uninfected participants, the PHI cohort had a higher percentage of males, and was younger. As expected, PHI participants had a lower CD4 count, elevated CD8 count, and decreased CD4/CD8 ratio. As previously reported, CSF white blood cells were elevated in the PHI group, as well as CSF neopterin, a marker of macrophage activation. Despite the younger age, PHI participants had elevated NFL and equivalent CSF total protein to the uninfected group, two parameters that increase with normal aging [21, 31, 32].

Blood brain barrier permeability at baseline
At baseline, age adjusted Q\textsubscript{Alb} was elevated in the PHI cohort compared to controls (means 5.9, 95% CI 5.5 to 6.3 in PHI; and 5.0, 95% CI 4.4 to 5.6 in controls; p=0.02). Using previously published reference values [30], baseline Q\textsubscript{Alb} was above the age-specific upper limit of normal (ULN) in 22 PHI participants (20.8%), referred to as the "high baseline Q\textsubscript{Alb} subgroup." The remaining 84 PHI participants with baseline Q\textsubscript{Alb} values below the ULN are referred to as the "normal baseline Q\textsubscript{Alb} subgroup." The baseline clinical characteristics of these two subgroups are summarized in Table 2. 4/22, or 18%, in the high baseline Q\textsubscript{Alb} subgroup had neurosymptomatic seroconversion versus 5/84, or 6%, in the normal baseline Q\textsubscript{Alb} subgroup. Elevated NFL, CSF total protein, CSF neopterin (but not blood neopterin), CD8+ T cell count, and a decreased plasma:CSF HIV RNA ratio were found in the high baseline Q\textsubscript{Alb} as compared to normal baseline Q\textsubscript{Alb} group.
**Longitudinal blood brain barrier permeability in PHI prior to cART**

The individual trajectories of each PHI participant’s Q_{Alb} over the duration of the study prior to cART initiation are plotted in Figure 1. A mixed model analysis to evaluate the natural history of blood brain barrier integrity in the overall PHI group prior to cART did not reveal a significant change in Q_{Alb} over time (-0.000436/day, p=0.092). Figure 2 compares the trajectories of the high and normal baseline Q_{Alb} groups. The high baseline group showed a declining trend (-0.003/day, p=0.006) while the normal baseline group initially increased (0.00144/day, p= 0.004) and reached a plateau quickly (quadratic time effect p= 0.004).

**Correlation of blood brain barrier integrity with markers of neuropathogenesis**

To further evaluate the implications of an elevated Q_{Alb}, correlations between Q_{Alb} and markers of neuronal health were evaluated in pre-cART study intervals (Figure 3). Partial correlation coefficients were calculated to correct for the confounding effects of age, as Q_{Alb} and NFL both directly correlate with age. Q_{Alb} demonstrated a strong positive correlation with NFL, a marker of active neuronal injury, upon cross-sectional analysis at baseline (r=0.497, p<0.001), and longitudinally with both between-participant (r=0.555, p<0.001) and within-participant analysis (r=0.523, p=0.001). Q_{Alb} inversely correlated with NAA:Cr, a cerebral metabolite biomarker of neuronal health, upon cross-sectional analysis at baseline (r=0.352, p=0.015), and longitudinally with between-participant analysis (r=0.387, p=0.008) but not within-participant analysis (r=0.218, p=0.125). Q_{Alb} did not correlate with composite z-scores (total Z or NPZ4) of neuropsychological testing at baseline nor in longitudinal analysis.

**Characteristics of cART-treated study participants**

58 PHI participants initiated a cART regimen during study follow-up, although one participant was excluded for virologic failure (two consecutive plasma samples with HIV RNA >50 copies/mL after 6 months of ART). Treatment regimens were heterogeneous, consisting of a combination of ≥3 antiretroviral agents. cART was initiated at a median 225 days post infection, with 402 days median on-cART follow-up. Table 4 compares the cross-sectional laboratory parameters before (last visit before treatment) and after cART treatment (last visit of study) in those who initiated cART. There was improvement in most parameters after approximately a year of cART: suppression of plasma and CSF HIV RNA to the lower limit of PCR detection (p<0.001), increased CD4+ counts (p<0.001), decreased WBC count (p<0.001), and decreased blood and CSF neopterin (p<0.001). In this comparison, NFL and albumin ratio did not significantly change with cART treatment (640 vs 670, p=0.911; 5.18 vs 5.09, p=0.851).

**Longitudinal history of blood brain barrier integrity following cART initiation**

A mixed model analysis was performed to assess the longitudinal trajectory of Q_{Alb} over 13 months of cART (Figure 4). Three participants were recruited into the cohort already on cART (for 29, 27, and 19 days) and thus included in the linear mixed model (n=60) but excluded from Table 4. As cART was initiated at a median of 225 dpi (t=0 on Figure 4), this time-point corresponded with the linear portion of Figure 2, where the acute quadratic changes of the normal baseline subgroup are resolving and reaching a set-point. Thus, initial analysis was performed with the total cART-treated group rather than separating into subgroups of high and normal baseline Q_{Alb}. There was no significant change detected in Q_{Alb} over the median >1 year duration of cART treatment (slope=-0.00369/month, p=0.174). With group stratification, the high baseline subgroup (n=7) demonstrated no significant change in Q_{Alb} over time.
The low baseline subgroup (n=53) demonstrated a slope of effectively zero (slope=0.00008/month, p=0.004), similar to the pre-cART plateau.

Discussion
In this study, we aimed to analyze the natural history of BBB permeability during the course of primary HIV infection, and the influence of early cART on this trajectory. We initially show that albumin ratio is mildly elevated in PHI participants as compared to uninfected controls when correcting for age. This correction is relevant given that BBB permeability increases with normal aging[33], and may explain why previous studies have not reported abnormalities in BBB permeability during PHI when compared to controls, particularly given that most early HIV studies enroll young patients. That being said, we have previously identified moderate elevation of albumin ratio in PHI [9, 34], and in chronic HIV participants who are cART-naive and neuroasymptomatic[9]. Similarly, Li et al have reported a strong association between matrix metalloproteinases--enzymatic surrogate markers of BBB permeability--and neurocognitive status in early HIV[35].

The novelty of this study is our finding that BBB permeability is undergoing dynamic changes early in the course of HIV infection, even within days of transmission. Two distinct trajectories were noted for the PHI cohort when stratified by baseline albumin ratio. Those with a normal baseline albumin ratio (below the ULN) showed a mild initial increase that plateaued within the first 1000 days of infection. Despite the initial rise, the Q_{Alb} remains well below the ULN. As will be discussed below, it may be that there is an element of sub-clinical injury associated with this mild rise. The subgroup with high baseline albumin ratios demonstrated a marked decline in albumin ratio within the first 1000 days of infection. Presumably an early rise in albumin ratio occurred immediately following infection before participant recruitment, and is resolving during the follow up period. Notably, the subgroup with higher baseline albumin ratio was characterized by a higher percentage of neurosymptomatic seroconversion, elevations in CSF markers of axonal injury and immune activation, as well as a higher CSF-to-plasma HIV RNA ratio. These findings suggest that a subgroup of PHI participants is susceptible to marked BBB disruption, which persists even beyond 1000 days post infection, and is associated with signs of increased CNS involvement. Factors which predispose individuals to one trajectory versus the other warrant further investigation.

Upon revealing deregulation of BBB early in PHI, we were interested in determining the significance of these changes. Previous studies have expounded on the association of albumin ratio with biomarkers of CNS inflammation and injury[20]. We confirm that in PHI albumin ratio correlates strongly with the axonal injury marker, NFL[23], and newly demonstrate that it inversely correlates with the metabolic marker of neuronal health, NAA:Cr, which has not previously been shown. The utility of NFL is that it is a sensitive marker of active ongoing neuronal damage and its levels correlate with the severity of this damage [36-38]. We have previously shown NFL to be the most sensitive neuronal biomarker for assessing HIV neurodegeneration, as it can detect subclinical injury in neuroasymptomatic individuals, even in the early phase of infection [34, 39]. As disease progresses, it is also associated with overt clinical neurological disease, thus not only reflecting structural but functional changes[36]. However, it is not specific for HIV neurodegeneration, and thus must be interpreted with caution[34, 37]. We minimized this possibility by ensuring that none of the participants had comorbid neurological conditions at the study onset. Similar to Q_{Alb}, NFL was elevated in PHI but remained below the ULN (<560), possibly indicating subclinical damage, which may explain the lack of correlation with NPZ-4 testing. In line with this conclusion, we have previously shown a lack of correlation between NFL and NPZ-4 during PHI, despite showing moderate elevations when compared to uninfected controls [23, 34]. An additional disadvantage of NPZ-4 testing is that, unlike NFL, performance does
not distinguish active impairment from the residual defect of earlier pathologic neurological processes. Similar to the utility of NFL as a biomarker of early subclinical injury, MRS has been shown to detect early HIV neuropathogenesis prior to conventional MRI changes[40]. In a recent study, chronically infected HIV subjects with cognitive defects were shown to have reduced glutamate and NAA in several brain regions, but most pronounced in the parietal grey matter[41]. Here, we extend that finding to the earliest stage of infection.

Once we demonstrated that BBB permeability was altered in PHI, and associated with markers of neuronal pathology, we assessed whether early cART treatment could remediate these changes. Surprisingly, the effect of cART on BBB permeability has not been intensely evaluated. In an unpublished study, Crozier and colleagues observed the gradual diminishment of albumin ratio (median 6.48 to 6.09) in 16 neuroasymptomatic participants with chronic HIV infection after 200 days of cART therapy[42]; thus, although BBB integrity improved over time with cART therapy, a return to baseline or near baseline function may take years. In contrast, Abdulle and colleagues reported no significant reduction in BBB permeability after 2 years of cART treatment in 38 neuroasymptomatic participants[43]. Importantly, the median baseline albumin ratio of participants in the Crozier study was greater than that of participants in the Abdulle study (6.48 vs 4.45), potentially contributing to the discrepancy in cohort response to cART.

In our study, cART treatment, initiated at a median of 225 days post infection, was effective in suppressing CSF and plasma HIV RNA, suggesting medication compliance and effectiveness. Notably, the inflammatory marker neopterin improved to the upper level of normal limits both in the plasma and CSF. Despite this systemic (including CNS) suppression of viral replication and inflammation, NFL and albumin ratio were unchanged. The pre-cART measurement of albumin ratio is comparable to the age-matched uninfected controls, and thus may indicate that the acute changes of albumin ratio in the high baseline sub-group had largely resolved and reached near-baseline once cART was initiated at 225 days post infection. On the other hand, although NFL is below the age-specific ULN (<840), it is significantly elevated compared to uninfected controls and the baseline PHI cohort, given only a marginal age difference. Although the half-life of NFL is not established, there is a gradual normalization of NFL following axonal injury which is unlikely to persist for over a year[44]. Thus, this persistently elevated level of NFL may reflect continued subclinical injury despite cART treatment and what appears to be a largely normal albumin ratio.

We hypothesize that perhaps (1) the initially altered BBB permeability has initiated CNS injury which persists despite resolution of BBB integrity, (2) the mechanism of injury is independent of BBB integrity, or (3) BBB permeability is mildly elevated and has not fully returned to baseline resulting in persisting neuronal injury. Alternatively, it is possible that despite the large sample size, we still have insufficient power to detect a significant change in NFL and Q_{Alb} after cART. Further studies are necessary to elucidate the possible explanation. Notably, a previous study showed normalization of the CD4/CD8 ratio during PHI only when cART was initiated within 6 months of transmission[45]. Furthermore, in a cohort of individuals started on treatment during acute HIV, CSF NFL was not elevated at baseline nor after 6 and 24 months of cART[46]. The effects of earlier cART intervention on albumin ratio normalization should be investigated.

**Limitations**
The conclusions from our study depend on the reliability of Q_{Alb} as a marker of BBB permeability. As Q_{Alb} is a ratio, it is a relatively method-independent parameter, thus reference values continue to be reliable despite difference in laboratory equipment or technique[33]. Notably, total CSF protein is an absolute measurement and is influenced by intrathecal protein synthesis, unlike Q_{Alb}, thus accounting for
possible discrepancies between the two measurements in this study. $Q_{\text{AB}}$ is affected by many factors not accounted for in this study, including body weight and smoking[33]. Abuse of substances such as cocaine has been shown to at least transiently increase BBB permeability[47]; thus, misreporting of ongoing drug use or long-term effects of previous drug use cannot be discounted as confounding factors. Furthermore, given the observational nature of this study, cART regimens were heterogeneous which may result in distinct effects on the BBB. Some researchers caution against describing this measure as a blood-brain barrier test and state that it actually reflects the blood-CSF barrier at the choroid plexus (ref: Reiber, H. & Peter, J.B. Cerebrospinal fluid analysis: disease-related data patterns and evaluation programs. J Neurol Sci 184, 101-122, 2001). However, in for example stroke, leaving the choroid plexus intact but injuring cerebrovascular endothelial cells, the CSF/serum albumin ratio may be increased (ref: Brouns, R., Wauters, A., De Surgeloose, D., Marien, P. & De Deyn, P.P. Biochemical markers for blood-brain barrier dysfunction in acute ischemic stroke correlate with evolution and outcome. Eur Neurol 65, 23-31, 2011), suggesting that CSF/serum albumin ratio probably is a marker of both barriers.

**Conclusions**

Blood brain barrier permeability undergoes a dynamic process early in HIV infection, demonstrating acute changes within days. We identified two subgroups of PHI participants with different albumin ratio trajectories: one with a presumed acute increase and gradual improvement over the course of infection, and a second with a mild initial increase. BBB permeability correlated with markers of neuropathogenesis. Initiation of cART in the first year of infection did not significantly alter BBB permeability in our study. Further investigations should test the effects of earlier cART initiation, especially in individuals with signs of early BBB disruption.

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**Conflicts of interest**

All authors: No reported conflicts.

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