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Longitudinal Assessment Of Blood Brain Barrier Disruption In Primary Hiv Infection And Effect Of Cart Therapy

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LONGITUDINAL ASSESSMENT OF BLOOD BRAIN BARRIER DISRUPTION IN PRIMARY HIV INFECTION AND EFFECT OF CART THERAPY

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LONGITUDINAL ASSESSMENT OF BLOOD BRAIN BARRIER DISRUPTION IN PRIMARY HIV INFECTION. Elham Rahimy, Fang-Yong Li, Lars Hagberg, Dietmar Fuchs, Kevin Robertson, Dieter J. Meyerhoff, Henrik Zetterberg, Richard W. Price, Magnus Gisslén, and Serena Spudich. Department of Neurology, Yale University, New Haven, CT, USA.

Abnormal blood brain barrier (BBB) permeability has been implicated in the neuropathogenesis of chronic HIV infection. As neurocognitive impairment can persist despite effective combination antiretroviral therapy (cART), it is possible that irreversible central nervous system (CNS) processes are initiated in early infection, before cART is typically initiated. We analyzed the natural history of BBB permeability in primary HIV infection (PHI), and the effects of cART initiated during this period. CSF:Serum albumin quotient (Q_{Alb}), a marker of BBB permeability, was measured in longitudinal observational studies of PHI. 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 biomarker for neuronal integrity), and neuropsychological testing. 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). Before cART, Q_{Alb} increased over time 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} 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=0.015, r=-0.387, p=0.008), but not neuropsychological performance. Q_{Alb} did not change after a median 398 days of cART initiated at 225 dpi (p=0.174). Q_{Alb} rises during early HIV, associates with neuronal injury, and does not significantly improve over a year of treatment. HIV BBB-associated neuropathogenesis may be initiated in early infection.

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INTRODUCTION

As of 2016, the CDC reports more than 1.2 million individuals are living with human immunodeficiency virus (HIV) in the United States, with as many as 1 in 8 unaware of their diagnosis[1,2]. HIV has significant genetic diversity, exhibiting different strains, subtypes, and even sub-subtypes, internationally. HIV-1 is the most predominant form worldwide, apart from western Africa[3], and will be the focus of this thesis. Herein, 'HIV' refers to HIV-1.

Neurocognitive dysfunction in HIV

I. HIV-associated neurocognitive disorder (HAND)

HIV infection can have significant systemic ramifications, and the central nervous system (CNS) is no exception. Chronic exposure to HIV can frequently lead to devastating neurological complications, with approximately one third of untreated patients developing HIV associated dementia (HAD) [4]. HAD is characterized by severe cognitive, motor, and behavioral disturbances associated with global cerebral atrophy, with subcortical areas exhibiting particular susceptibility [5]. Given the morbidity of the illness in the absence of treatment, HAD is considered an AIDS-defining illness, with disease severity correlating with the degree of CD4+ suppression[6,7]. With the introduction of highly active antiretroviral therapy (HAART)/combination antiretroviral therapy (cART) in 1995, and thus restoration of CD4+ counts and effective viral load suppression, the incidence of HAD has significantly decreased to as low as 5%[8,9]. However, a milder spectrum of neurocognitive deficits persists despite successful cART treatment, effecting up to 50% of chronically infected patients[8-10]. HIV-associated neurocognitive disorders (HAND) is an umbrella term for this observed spectrum of neurocognitive complications,

comprised of three categories: asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HAD, in increasing severity[4,11].

As indicated above, HAND predominantly involves the subcortical region and frontostriatal circuits, often manifesting as cortical atrophy and white matter signal hyperintensities detectable on magnetic resonance imaging (MRI)[12], although these findings are neither specific nor sensitive[13,14]. Corresponding to the imaging findings, the cognitive domains most commonly affected include motor, psychological (agitation, apathy, depression), executive, speed of processing information, and attention, with very limited involvement of language, judgment, and reasoning[12,15]. Like any form of dementia, HAND is a clinical diagnosis, and the above domains may be assessed clinically with a battery of neuropsychological testing (ie, trail making, grooved pegboard), which allows for classification in one of the HAND categories. All categories of HAND require impairment in at least 2 tested domains, while the degree of impairment in functional performance determines the category: no impairment qualifies as ANI, mild impairment qualifies as MND, and moderate-to-severe impairment qualifies as HAD[16]. Undoubtedly, these neurocognitive deficits, even when mild, can have a severe impact on the patient's quality of life, and may even compromise cART adherence, resulting in viral resistance and disease progression[17]. Thus, the continued foothold of HAND in post-cART era is an issue necessitating better understanding and further investigation.

II. Neuropathogenesis in HAND

Since the mid-1980s, numerous autopsy studies have characterized the histological hallmarks of HAD with relative consensus: If HAD is the clinical manifestation of severe

neurocognitive deficits, HIV-encephalitis (HIVE) is the pathologic correlate. Apart from gross evidence of diffuse brain atrophy--indicative of neuronal loss--and correspondingly enlarged ventricles, tissue specimens in classic HAD demonstrate wide-spread inflammation. This characteristic inflammation, termed HIVE, is defined by the presence of activated resident macrophages (ie, microglia) and infiltrating peripheral macrophages, with specific findings as follows: multinucleated giant cells expressing viral antigens like p24 (likely representing viral fusion of macrophages), activated microglial nodules, perivascular 'cuffs' (leukocyte aggregation in the perivascular space), marked astrocytosis (astrocyte activation and dysfunction)[18], white matter gliosis and demyelination, synapto-dendritic injury, and the presence of detectable virus production[19-23].

Even in the post-cART era, post-mortem analysis of patients with mild forms of HAND surprisingly still shows marked neuroinflammation[24]. Although pathologically similar to HIVE, productive HIV infection is not detectable, and the structural sites of inflammation are different, now primarily involving the hippocampus and surrounding peri-entorhinal cortex with less involvement of the basal ganglia[6]. While some argue that the milder forms of HAND in the post-cART era may represent a different pathological process from pre-cART HAD, it is clear that neuroinflammation likely contributes to pathogenesis in both. In fact, the degree of histologic neuroinflammation correlates strongly with clinical progression of HAND[6]; in 1995, Glass et al showed a strong correlation between neurologic disease progression and macrophage staining, whereas HIV-1 staining (ie, gp41 expression) demonstrated a weak correlation at best[25]. These findings by Glass, and similar pathologic and experimental findings by other researchers

in years to come, implicated inflammation as a key player in HIV-mediated neurodegeneration, more so than viral load.

Neuronal toxicity is thought to be primarily due to indirect mechanisms, such as inflammation, as repeated histological and *in vitro* studies have shown HIV to have very limited, if any, infection of neurons. Few post-mortem PCR studies have shown that HIV has the capability to invade neurons[26,27], although the infected proportion is small (as neurons do not express required HIV receptors) and the infection is likely nonproductive (as neurons cannot regenerate)[28]. Although this putative neuronal infection is thought to have an inconsequential role in neuropathogenesis, replication-derived viral proteins can exert neurotoxic effects either directly via apoptotic activation or indirectly via induction of inflammatory cytokines: Tat (transcriptional transactivator), gp120 (envelope glycoprotein), and VPR (viral protein R) have been most strongly implicated in HIV-mediated neurodegeneration, stimulating apoptosis through activation of caspase-3 or -8, and up-regulating microglial synthesis of IL-1β and TNF-α[6,19,20,28,29]. Thus, HIV is thought to cause neuronal injury via induction of a pro-inflammatory state, but also via secretion of neurotoxic viral proteins, with a strong interplay and synergistic effect between these two mechanisms. Notably, excito-toxicity is thought to be the common endpoint of both pathways, as inflammatory cytokines and HIV-encoded proteins have been shown to increase extracellular glutamate concentration in the CNS, resulting in excess activation of the *N*-methyl-d-aspartate (NMDA) receptor, and subsequent apoptosis[20,30,31]. (*For a discussion on how HIV breaches the BBB and induces neuroinflammation, see section titled "Role of the blood brain barrier" below.*)

III. Theories underlying persistence of HAND despite successful cART

As indicated in the above sections, even in the context of successful cART therapy (ie, undetectable viral load on ultrasensitive tests), HAND persists in milder forms. Several explanations have been offered, although the answer is still unclear. A few theories will be discussed briefly below.

One possibility is the presence of continued low-grade neuroinflammation even with complete viral suppression, as suggested by the autopsy studies of Tavazzi et al demonstrating marked neuroinflammation without histologic signs of viral replication (referenced above in "*Neuropathogenesis of HAND*"). It is speculated that resolution of inflammation in the CNS is slower than the periphery[32], and may remain unchecked for years, with persistently abnormal inflammatory biomarkers[9].

A second possibility is continued low-grade viral replication in the CNS. As an immune privileged site, the brain may serve as a perfect viral sanctuary for HIV to escape exposure to cART circulating in the periphery[33]. cART penetration into the CNS is limited, even in regimens with a high 'CNS Penetration Effectiveness' (*see "cART therapy overview" below*), thus allowing for continued CNS replication even with viral eradication in the periphery[9]. Persistence of HIV in the CNS may also allow for evolution of drug-resistant strains, as the presence of genetically distinct populations in the CSF versus the plasma has been well documented in chronic infection[19,34].

A third possibility is the confounding effect of comorbidities prevalent in HIV+ patients[35]; although the resulting neurocognitive diseases are histologically distinct from HAND, they may be difficult to distinguish clinically. Such confounders include medical comorbidities (ie, cardiovascular disease, co-infection with hepatitis)[36-41], psychological disease (ie, depression)[42-44], drug abuse (ie, crack/cocaine)[41,45], medication-related side

effects, and increased susceptibility to age-related neurodegenerative processes (ie, acceleration of amyloid-driven diseases such as Alzheimer's and Parkinson's)[6,46-51]. In regards to medication-related side effects, ART therapy is known to cause neurotoxicity, the severity of which may correlate with CNS Penetration Effectiveness[6,44,52].

Perhaps most relevant to the purpose of this thesis, the fourth possibility is that this persisting neurocognitive impairment may represent irreversible neuronal damage accrued prior to the initiation of cART, even in early infection[53]. Thus, investigative efforts have been drawn towards elucidating and characterizing the earliest stages of HIV neuroinvasion and associated neuronal injury, as will be explored further below.

Initiation of neuropathogenesis in early HIV infection

Primary HIV infection (PHI) refers to the earliest stage of systemic infection, from time of transmission up to 12 months post-transmission, encompassing the time of seroconversion and establishment of virus load set point[54]. An acute retroviral syndrome (ARS) develops in over half of PHI patients, and is thought to represent either a direct cytotoxic effect or immunologic response to the high load viremia typical of the acute phase of infection. Clinical symptoms of ARS tend to be nonspecific, classically a mononucleosis- or influenza-like syndrome which can last several days to weeks; however, neurological symptoms, such as aseptic meningitis, encephalitis, or radiculopathy, develop in up to 17% during seroconversion, and may be associated with a more rapid progression of neurocognitive deficits[55,56]. Several studies have demonstrated HIV infiltration of the CNS during PHI[57-59], as indicated by the presence of HIV RNA in the CSF compartment, as early as eight days post-infection, even preceding the manifestation of neurological symptoms [4,59-62]. 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[4,63-66]. Furthermore, markers of immune activation may reflect degree of viral load and neurocognitive impairment[67].

Accumulating evidence suggests that this pro-inflammatory state coincides with neuronal damage during PHI. Neurofilament light chain (NFL) is one of three subunits which comprises the cytoskeletal protein neurofilament, an intermediate filament essential for axonal support of myelinated neurons. It is an established CSF biomarker of axonal damage in a variety of neurological processes, with growing prominence in HIV-related neurodegeneration. The reason for this trend is that, unlike other CSF biomarkers which are reflective of viral load or immune activation, NFL directly reflects the severity of active neuronal damage[32]. Elevations in NFL have also been demonstrated in PHI, even in neuroasymptomatic patients, indicative of subclinical injury[50,68].

Another useful method of detecting neuronal damage and CNS inflammation utilizes magnetic resonance spectroscopy (MRS), a noninvasive quantitative MR technique which measures alterations in cerebral metabolite levels. Previous MRS studies have shown elevation of inflammatory cerebral metabolites in acute HIV (prior to antibody seroconversion) which increases longitudinally over time in PHI prior to cART[4,69,70], as well as elevation in neuronal injury metabolites responsive to ART [69]. The cerebral metabolite N-acetylaspartate (NAA) is a marker of neuronal viability and number, and is often expressed as a value normalized to creatinine (NAA:Cr). We have previously shown a strong correlation between high NFL and low NAA:Cr in the parietal grey matter of neurosymptomatic PHI subjects [68]. Thus, crucial processes during the

primary phase of viral infection may underlie the initiation of HIV associated CNS injury.

Whether clinically overt signs of neurocognitive impairment begin in PHI is not clear, as studies have been limited. A meta-analysis has previously concluded that cognitive deficits in early HIV infection are rare and mild[15], although several primary studies have been published since then, either noting deficits in a large subgroup[71,72], or finding no deficits[73,74]. Notably, these studies varied in the timing of their analysis relative to date of transmission, a relevant point if the earlier phases of infection are dominated by sub-clinical neuronal injury.

Role of the blood brain barrier

I. Anatomy of the blood brain barrier

The highly restrictive blood brain barrier (BBB) defines almost the entirety of the brain's capillary endothelium, bestowing the CNS with a specialized microenvironment distinct from systemic circulation[75]. These endothelial cells are tightly opposed via junctional protein complexes, called zonulae occludentes, which prevents any paracellular passage[76]. Other components of the neurovascular unit (NVU) include peri-endothelial cells, specifically pericytes and astrocytes; the NVU also interacts with the resident CNS immune cells, called microglia[77,78](Figure 1). The ratio of albumin in the cerebrospinal fluid to albumin in the serum (CSF:serum albumin concentration quotient, or Q_{Alb}) is a specific marker for BBB permeability[79]. 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}. Some researchers caution against using Q_{Alb} as a blood-brain barrier marker and state that it actually reflects the permeability of the blood-CSF barrier at the choroid plexus[80], since the choroid vasculature is more prone to inflammation and 'leakiness' compared to the intraparenchymal vasculature. But in cases of stroke, which leaves the choroid plexus intact and injures the cerebrovascular endothelial cells, Q_{Alb} is increased[81], suggesting that Q_{Alb} is a marker of both barriers.

Figure 1: Anatomy of the blood brain barrier

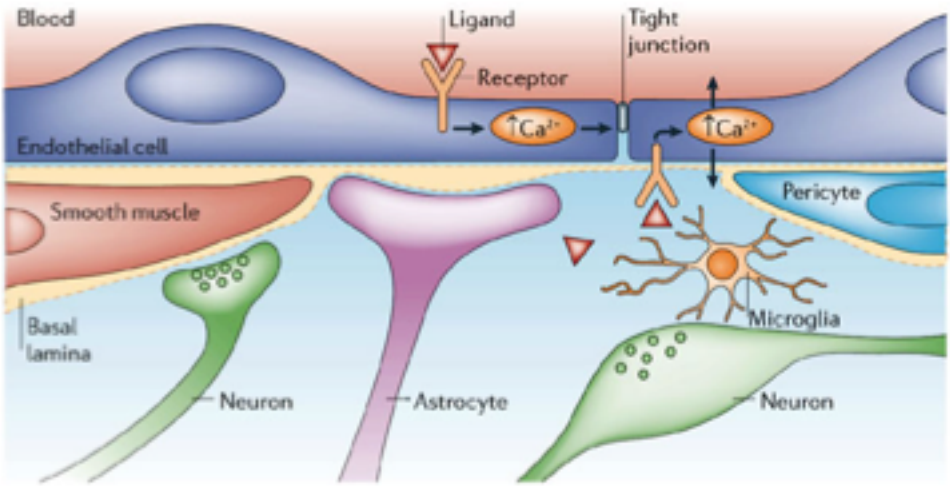

Figure 1. Key players of the blood brain barrier are shown. Endothelial cells are conjoined by tight junctional proteins, the expression of which are affected by ligand-receptor interactions. Pericytes closely encircle the endothelial cells within the basal lamina, while astrocyte endfeet provide support just outside of the basal lamina. The resident central nervous system macrophages, microglia, interact with the neurovascular unit within the perivascular space. Adapted with permission from Abbott NJ et al. (2006) Astrocyte–endothelial interactions at the blood–brain barrier, Nature Reviews Neuroscience. 7: 41–53.

Expression of the endothelial junctional proteins, such as occludin-1 and ZO-1, and the viability of endothelial cells are highly influenced by environmental factors, including expression of pro-inflammatory cytokines such as TNF-α and IL-1β [48,82,83], direct infection by various neurotropic viruses[39,84], or exposure to cytotoxic molecules in

the serum[85]. All three of these factors are thought to be involved in the pathogenesis of HAND *(See "Neuropathogenesis in HAND" above).* Is it possible that the BBB, the guardian of the CNS so susceptible to environmental factors, is implicated in the neuro-pathogenesis of HAND?

II. Dysregulation of the blood brain barrier in HIV

In order to exert its neurological effects, HIV and/or its viral products must first traverse the BBB. Although the mechanisms have not yet been fully clarified, several models have been proposed. The aptly named "Trojan horse theory" is perhaps the most widely accepted, suggesting the HIV virus crosses the BBB primarily via infection of peripheral monocytes destined to take up residence in the CNS as macrophages [20]. Although this model indicates that HIV is able to traverse the largely intact BBB, increased permeability of the BBB has been strongly implicated in the progression of HIV neurological dysfunction[68,86-89]. Since the early 1990s, studies have shown that Q_{Alb} is increased in infected individuals[90,91] and is strongly associated with the presence of neurological signs/symptoms[92]. Around this time, post-mortem studies of chronically infected AIDS patients provided tissue evidence of the correlation between BBB dysregulation and neurological impairment, as serum protein deposition in subcortical white matter was greater in those with neurological symptoms[89]. Peluso and colleagues reported a significant correlation between the axonal injury marker NFL and Q_{Alb}, further suggesting a possible relationship between a dysregulated BBB and neurological dysfunction[68]. In line with this observation, several studies have shown that the neuronal injury accrued upon CNS infiltration is not a direct result of cytolytic infection[61,88]. On the contrary, within the CNS, HIV infection is restricted to macrophages, microglia, and,

to a lesser extent, endothelial and peri-endothelial cells of the neurovascular unit (ie, astrocytes and pericytes) which compromise the BBB[85,93]. As will be discussed below, it is the downstream effects of HIV infection in these cells that will culminate in neuronal injury through a compromised BBB.

It is speculated that increased 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[88]. Gisslén and colleagues demonstrated a significant relationship between Q_{Alb}, CSF HIV-1 RNA, and the macrophage activation marker neopterin, thus suggesting a strong association between immune activation as an important factor in BBB permeability in HIV infection[87]. In line with this theory, monocyte infiltration has previously been found to correlate with loss of tight junction immunoreactivity in brain tissue of HAD patients[94]. Similar to the speculated mechanisms of neuropathogenesis outlined previously *(See "Neuropathogenesis in HAND" above)*, growing evidence suggests BBB permeability is the result of a multifactorial process involving immune-mediated mechanisms, as well as viral mechanisms. For example, the HIV-1 derived proteins Tat and gp120 exhibit direct neurotoxic effects, but also severely compromise the integrity of the BBB, permitting the entry of peripheral cytokines and additional infected monocytes and free virions [20]. Furthermore, infection of pericytes, which encircle and stabilize endothelial cells of the BBB, has been shown to diminish tight junction integrity and increase permeability *in vitro*[93]. Even in the presence of low HIV infection, astrocytes undergo altered end feet signaling, accelerating endothelial apoptosis[95]. The inflammatory cascade that results

from entry of peripheral cytokines and immune cells, further exacerbated by activation of residential CNS macrophages and microglial cells, results in a storm of reactive oxygen species, nitric oxide, glutamate, cytokines, and other neurotoxins that ultimately lead to neuronal damage and death[20]. Interestingly, it should be noted that albumin itself produces concentration-dependent neurotoxic effects in rat brain parenchyma *in vivo*[96], and induces expression of the pro-inflammmatory cytokines IL-1β and TNF-α possibly via MAP-K activation in astrocytes and microglial cells[97-99]. Thus, it may be these resident immune cells rather than infiltrating macrophages are the cause of neuro-inflammation and neuro-toxicity once the BBB is compromised.

Although BBB dysregulation is thought to be secondary to viral and immune-mediated processes directly related to HIV-infection, one must not discount the influence of HIV-related comorbidities. Concurrent infections may significantly alter BBB, either via direct NVU infection or induction of cytokines which indirectly cause BBB hyper-permeability. Most notably, up to one third of HIV-infected patients are concurrently infected with Hepatitis C, which directly infects NVU endothelial cells and may cause neurocognitive dysfunction[37]. Drug abuse is highly prevalent in HIV+ populations, with cocaine and methamphetamine implicated in BBB dysruption[38]. Cardiovascular disease is also commonly accelerated in HIV+ patients due to chronic systemic inflammation and metabolic side effects of cART therapy (ie, protease inhibitors)[35,100,101]. Given the presence of cardiovascular disease and its risk factors (hypertension, hypercholesterolemia) have been associated with neurocognitive decline in HIV[36], it likely contributes to BBB hyper-permeability.

III. Blood brain barrier status in early HIV infection

As indicated above, dysregulation of the BBB is a well-established event in chronic HIV infection and correlates with neurological injury and neuro-inflammation. However there is very limited data assessing blood brain barrier integrity during the primary phase of infection. Moderate elevations of albumin ratio in PHI have previously been shown in cross-sectional studies [50,60], and there exists a strong association between matrix metalloproteinases--enzymatic surrogate markers of BBB permeability--and neurocognitive status in early HIV[102]. Although studies assessing BBB status in PHI patients are limited, several studies have analyzed albumin ratio in neuro-asymptomatic patients (recruited regardless of chronicity/transmission date), often with mixed results, either demonstrating increased Q_{Alb} compared to uninfected controls[60,87] or no significant difference[32,103].

Role of cART therapy

I. cART therapy overview

Since clinical trials with azidothymidine (AZT) in 1987, it has been known that antiretroviral therapy can significantly reverse HAD, as assessed by clinical neuropsychological testing and positron emission tomography[104]. This improvement is clearly enhanced with highly antiretroviral therapy (HAART)/combination ART (cART)[10,105], the standard of treatment for HIV. There are 24 FDA-approved ARTs currently available with varying molecular actions: (1) nucleoside-analog reverse transcriptase inhibitors (NRTIs), (2) non-nucleoside reverse transcriptase inhibitors (NNRTIs), (3) protease inhibitors (PI), (4) integrase inhibitors, (5) fusion inhibitors, and (6) co-receptor antagonists. Drugs from at least two different molecular classes are

combined in a cART regimen (consisting of three or more drugs), with the underlying purpose of preventing drug resistance[106]. Most commonly, regimens consist of two NRTIs with one PI or one NNRTI. Nevertheless, cART regimens are highly heterogeneous and may require complex, specific regimens necessitating as many as 30 pills a day[17], though many patients are able to take single pill regimens in the modern era.

While certain regimens have a higher 'CNS Penetration Effectiveness' (CPE)[107], and thus better ability to cross the BBB and control CSF viral load, studies regarding the clinical impact of CPE have been mixed[52], primarily demonstrating unchanged or worsened neuropsychological performance associated with these regimens[108,109]. Apart from inherent bias involved in these study designs (ie, patients with more severe HAD are prescribed a higher CPE regimen)[34], there are other possible explanations. Not only are high CPE drugs intuitively more likely to be neurotoxic, but some researchers argue that the pathology of HAND is inflammation-mediated and not viral load-dependent (see above sections), as the viral load may simply reflect plasma spillover from a leaky BBB[110]. As cART medications directly target viral load and not the activated inflammatory cascade, neuropsychological damage may persist with the continued low-grade inflammation. Thus, as no specific cART regimen is considered superior for the treatment of HAND[16], individual regimens are based on the consideration of several factors (ie, regimen complexity and compliance, CPE, drug-resistance testing).

II. Effects of cART therapy on neuropathogenesis in early infection

Given the remarkable and indisputable effect of cART on HAD, more recent studies have aimed to assess cognitive benefits from earlier initiation of cART. In a longitudinal observational study of PHI patients, cART therapy was shown to attenuate, although not

fully reverse, abnormalities in MRS metabolite markers[69]. Although it was speculated that normalization may occur beyond the limited follow-up period (median of 6.0 months), persistent neuroinflammation is known to occur despite successful cART (*see "Theories underlying persistence of HAND despite successful cART" above*). Of note, MRS markers of excitotoxicity exhibited greater attenuation than markers of inflammation, perhaps suggesting persistent low-grade inflammation without significant neurotoxicity. A similarly designed study assessed MRS abnormalities, but this time in acute HIV infection (recruited within one month of transmission versus one year in PHI), and found normalization with cART therapy[70]; thus, it may be that reversibility is achieved with earlier cART initiation. Effects of cART on neuropsychological performance in early infection have also been examined, although limited to two studies[71,72]. The PHI study identified mild deficits with at least a partial response to cART therapy; the second study, this time in acute HIV infection, demonstrated no measurable neurocognitive deficits in the majority of patients, although a subgroup with severe deficits showed very limited response to cART. The above studies suggest that neuropathogenesis may have varied response to cART, as dictated by a combination of factors, including timing of cART initiation and the severity of neurologic disease.

III. Effect of cART therapy on the blood brain barrier

Surprisingly, the effect of cART therapy on BBB permeability has not been intensely evaluated. In an unpublished study, Crozier and colleagues reported the gradual diminishment of albumin ratio from a median baseline of 6.48 to a median endpoint value of 6.09 in 16 neuroasymptomatic patients with chronic HIV infection after 200 days of cART therapy[111]; thus, although BBB integrity improved over time with cART therapy, a

return to premorbid or near premorbid function may take years. In contrast, Abdulle and colleagues observed no significant reduction in BBB permeability after 2 years of cART treatment in 38 neuroasymptomatic patients[32]. Importantly, the median baseline albumin ratio of patients in the Crozier study was higher (6.48, range: 4.79-10.29,) than that of patients in the Abdulle study (4.45, range: 1.77-9.84), potentially contributing to the discrepancy in cohort response to cART. No studies have investigated the effect of cART therapy on BBB permeability in early stages of infection.

STATEMENT OF PURPOSE

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. Our specific study questions and accompanying aims are delineated below:

I. What is the natural history of blood brain barrier permeability during primary HIV infection?
    - Aim: To determine the longitudinal trajectory of blood brain barrier permeability, as measured by Q_{Alb}, in primary HIV infection, in the absence of cART treatment.

II. Is blood brain barrier status associated with neuropathogenesis during primary infection?

- Aim: To determine whether Q_{Alb} correlates with markers of neuronal injury (NFL), neuronal health (NAA:Cr), and neuropsychological testing (NPZ).

III. Does early combination antiretroviral treatment (cART) influence Q_{Alb}?

- Aim: To determine whether cART initiation effects the slope of the Q_{Alb} trajectory established in Aim I, and associated markers of neuropathogenesis in Aim II.

We hypothesized that a) Q_{Alb} will increase over time for cART-naive patients, and b) Q_{Alb} will correlate positively with markers for neuronal injury (NFL), and inversely with neuropsychological test performance (NPZ) and neuronal health (NAA:Cr). Furthermore, c) following initiation of effective cART regimen, as indicated by reduced CSF HIV-1 RNA, we expect Q_{Alb} to gradually diminish with improvement in aforementioned markers of early CNS injury/inflammation.

These results will 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.

METHODS

Study 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 [60] and outlined below. Participants were within the first year of HIV transmission as confirmed by the standard serologic testing algorithm for recent HIV seroconversion (STAHRS) [112], 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 [113]; otherwise, it was approximated as midway between the dates of the last negative and first positive EIA test[114]. HIV-uninfected volunteers were recruited from the San Francisco community, and had no history of neurological conditions nor active systemic diseases.

Ethics

The study protocol was approved by the institutional review board of each institution involved. All study participants gave written consent.

Data collection and laboratory analysis

Paired CSF and blood/plasma samples were obtained and neuropsychological testing and MRS were performed at each visit as described in detail below[42,68]. Study intervals were scheduled at baseline (t=0), six weeks, and every six months thereafter, although there was participant variation in timing and duration of follow up.

Following phlebotomy and lumbar puncture, CSF total WBC, lymphocyte counts, total protein, albumin, and blood/plasma albumin were measured from fresh samples. Frozen samples were prepared for assays of HIV RNA, neopterin, and NFL: Fresh samples were maintained on ice, and, after low-speed centrifugation, cell-free CSF and paired blood

plasma aliquots were stored within 2 hours of collection in –70°C to –80°C freezers. Previous studies have demonstrated neopterin and NFL to tolerate repeated freeze-thaw cycles and long-term storage with minimal compromise in integrity[115,116], although both conditions were minimized as much as possible throughout the course of this study.

CSF NFL was measured with the NF-light® ELISA kit (UmanDiagnostics AB, Umeå, Sweden), a sensitive immunoassay with a lower limit of detection of 50 ng/L [86], and reference values for upper limit of normal of 380 ng/L (18–29 years), 560 (30–39years), 890 (40–59 years), and 1850 (>59 years)[86]. CSF NFL assays were singularly performed in the Laboratory of Neurochemistry at the University of Gothenburg on previously frozen samples.

CSF and plasma albumin were measured by nephelometry (Behring Nephelometer Analyzer, Behringwerke AG, Marburg, Germany). Q_{Alb} was calculated as the CSF/plasma albumin ratio: CSF albumin (mg/l)/plasma albumin (g/l) [87]. Upper limits of normal were based on previously established values of <6.8 for age <45 years, and <10.2 for age >45 years [117]. CSF and plasma albumin were measured in local clinical laboratories. Given the inherent advantage of being a ratio, Q_{Alb} is laboratory- and method-independent.

CSF and plasma neopterin was measured in the laboratory of Dr. Fuchs by commercial immunoassays (BRAHMS, Berlin, Germany) on previously frozen samples. CSF white blood cells, lymphocytes, total protein, and HIV RNA were measured in local

laboratories as previously described[60] and outlined below. CD4+ and CD8+ lymphocytes and white blood cells were measured in fresh, paired CSF and blood/plasma samples using flow cytometry. HIV RNA (viral load) was quantified in previously frozen, paired cell-free CSF and plasma samples at local laboratories using either the ultrasensitive Amplicor HIV Monitor PCR (version 1.5; Roche Molecular Diagnostic Systems, Branchburg, NJ), Cobas TaqMan RealTime HIV-1 PCR (version 1 or 2; Hoffmann-La Roche, Basel, Switzerland), or the Abbott RealTime HIV-1 PCR assay (Abbot Laboratories, Abbot Park, IL, USA). Viral loads below 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).

A trained neuro-radiologist interpreted MRI data for exclusion of non-HIV associated pathologies and assignment of atrophy and white matter hyperintensity ratings. Brain MRI/MRS was obtained at the San Francisco site only. MRS data were 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 peak area

under the curve for the metabolite N-acetylaspartate to the peak area under the curve for creatine-containing metabolites (NAA:Cr) is a putative marker of neuronal viability and number. We focused spectral acquisition on the parietal grey matter, as we have previously identified metabolite abnormalities in this region during PHI [68,69].

Statistical analysis

Baseline characteristics were summarized as frequencies for categorical variables and median and IQR for continuous variables. Non-parametric, Chi-square, and Fisher's exact test were used for group comparisons. Specifically, comparison between independent groups was performed with the nonparametric method of Mann Whitney U-test for continuous variables, unpaired t-test for normal distribution, and the chi^2 test or Fisher's exact test for categorical variables; comparison between dependent samples (repeated measures of participants pre- and post-cART) was performed with Wilcoxon signed-rank test. Analysis of covariance (ANCOVA) was performed to compare Q_{Alb} between PHI and controls while adjusting for the potential confounders of age and sex.

The mixed-effects model was used to analyze longitudinal change of Q_{Alb} post-transmission, both pre- and post-cART. This model includes both fixed and random effects in the same analysis, allowing for variation in the number and time interval of participant follow-up visits. Because albumin ratio increases with normal aging, the equation was adjusted for baseline age by including it as a fixed-effect covariate in the model. To account for a possible non-linear trajectory of Q_{Alb} over time, a quadratic term (t^2) was also included as a fixed-effect covariate along with days post-transmission (t).

The model included a personal intercept for each subject as a random effect, allowing baseline Q_{Alb} to vary for each participant. An interaction term was initially added to assess whether the trajectory of Q_{Alb} over time depended on the baseline Q_{Alb}, but was found to be insignificant and thus excluded from the final model. Q_{Alb} values were log-transformed for normal distribution before longitudinal analysis. As transformed results were comparable to non-log-transformed analysis, the latter results are reported for familiarity of Q_{Alb} values. For the equations generated, the final y-intercept was calculated as follows: [(parameter estimate of baseline age)*(median age of subgroup)]+parameter estimate of subgroup intercept.

Partial correlation coefficients were calculated to determine potential relationships between Q_{Alb} and other measured parameters, as indicated, while adjusting for effects of age. Correlations were computed as a cross-sectional analysis using each participant's baseline values, as well as a longitudinal analysis using the intra- and inter-subject method of Bland and Altman[118,119]. Specifically, the intra-subject, or 'within subject', method determines the correlation of Q_{Alb} and a second variable within a subject over the course of the study, thus assessing the longitudinal relationship between the two variables while removing variation due to subjects. In other words, it assesses whether an increase in the Q_{Alb} of an individual subject is associated with a change in the second variable. For the inter-subject, or 'between subject', method, each subject's repeated measures over the course of the study are averaged for Q_{Alb} and the second variable, and a simple regression is performed with the weighted means. In other words, this approach assesses whether

individuals with elevated Q_{Alb} throughout the study also tended to have elevated/depressed values of the second variable.

Statistical analyses employed SPSS 23.0 statistical package (IBM Corp., Armonk, NY). Significance level was set as $p<0.05$, two-sided.

Author contributions

Patient recruitment, data collection, and laboratory analysis were performed previously by the research groups of Drs. Serena Spudich, Magnus Gisslén, and Richard W. Price in their cohort studies evaluating the CNS effects of HIV in PHI. This study of blood brain permeability in the context of the data available from these cohorts was designed by Elham Rahimy and Serena Spudich. Statistical analyses were performed by Elham Rahimy and confirmed by Fang-Yong Li. Additionally, Fang-Yong Li provided invaluable teaching on the more complex statistical analyses such as the mixed-effects model. Elham Rahimy also created figures, tables, and drafted the manuscript. All the authors assisted in revising the manuscript and approved the final version.

RESULTS

Study participant characteristics

106 PHI participants fulfilled the inclusion criteria and had available Q_{Alb} values. Nine participants experienced clinically overt neurological disorders during seroconversion: meningitis (n=2), headache with photophobia (n=5), 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 3572 days with a median of 50 days. The majority of participants were infected with subtype B virus [60].

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 1.8 $\log_{10}$ 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 compared to the uninfected group, two parameters that increase with normal aging [86,120,121].

Information regarding drug and alcohol use was available for participants from the San Francisco site (n=82) only: 33.0% reported recent alcohol abuse, and 49.1% reported recent drug use. The most frequently reported drug use were methamphetamine, marijuana, and cocaine, in descending order.

Table 1. Baseline demographic and clinical characteristics of study participants

	Primary HIV infection (n=106)	HIV uninfected (n=64)	p value
Sex (% male)	94	82	0.001
Age (y)	36 (29, 46)	43 (34, 50)	0.003
Site	SF (n=82) GOT (n=24)	SF	
Days post-HIV transmission	91 (53, 149)	---	---
CD4+ count (cells/μl)	567 (402, 709)	808 (678, 1009)	<0.001
CD8+ count (cells/μl)	954 (714, 1358)	487 (343, 733)	<0.001
CD4/CD8	0.528 (0.391, 0.791)	1.76 (1.32, 2.18)	<0.001
Plasma HIV RNA (log_{10}copies/ml)	4.69 (4.08, 5.34)	---	---
CSF HIV RNA (log_{10}copies/ml)	2.83 (2.14, 3.51)	---	---
Plasma:CSF HIV RNA ratio (log_{10}copies/ml)	1.81 (1.33, 2.28)	--	--
CSF WBC count (cells/mm^3)	6 (2, 11)	2 (0, 3)	<0.001
CSF total protein (mg/dl)	41 (31, 51)	41 (31, 54)	0.611
NFL (pg/ml)	518 (391, 819)	411 (320, 550)	<0.001
CSF neopterin (nmol/l)	9.6 (6.8, 20.4)	5.0 (4.1, 6.8)	<0.001
% neurosymptomatic ARS	8.5% (n=9)	--	--
Total number of visits	2 (1, 3)	---	---
Duration of follow up (days)	50 (0, 450)	---	---

Values are expressed as median and IQR (Q1, Q3).
ARS, acute retroviral syndrome; CSF, cerebrospinal fluid; GOT, Gothenburg, Sweden; NFL, neurofilament light chain; SF, San Francisco, USA; WBC, white blood cell count.

Blood brain barrier permeability at baseline

At baseline, age adjusted Q_{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[117], baseline Q_{Alb} was above the age-specific upper limit of normal (ULN) in 22 PHI participants (21%), referred to as the "high baseline Q_{Alb} subgroup." The remaining 84 PHI participants with baseline Q_{Alb} values

below the ULN are referred to as the "normal baseline Q_{Alb} subgroup." The baseline clinical characteristics of these two subgroups are summarized in **Table 2.** 4/17, or 24%, in the high baseline Q_{Alb} subgroup had neurosymptomatic seroconversion versus 8/64, or 13%, in the normal baseline Q_{Alb} subgroup, although statistically insignificant. 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_{Alb} as compared to normal baseline Q_{Alb} group.

Table 2: Baseline clinical characteristics of high and normal Q_{Alb} subgroups

	Subgroup with High Baseline Q_{Alb} (n=22)	Subgroup with Normal Baseline Q_{Alb} (n=84)	p value
Age (y)	36 (29, 45)	37 (28, 46)	0.797
Days post-HIV transmission	85 (60, 125)	92 (51, 150)	0.785
CD4+ count (cells/μl)	596 (484, 681)	550 (389, 730)	0.469
CD8+ count (cells/μl)	**1294 (792, 1620)**	**915 (706, 1200)**	**0.023**
CD4/CD8	0.463 (0.321, 0.791)	0.530 (0.391, 0.803)	0.376
Plasma HIV RNA (log_{10}copies/ml)	4.60 (3.91, 5.39)	4.69 (4.09, 5.32)	0.629
CSF HIV RNA (log_{10}copies/ml)	3.23 (1.77, 3.87)	2.73 (2.14, 3.43)	0.340
Plasma:CSF HIV RNA ratio (log_{10}copies/ml)	**1.27 (0.464, 2.16)**	**1.84 (1.50, 2.29)**	**0.020**
CSF WBC count (cells/mm^3)	7 (4, 13)	5 (2, 11)	0.087
CSF total protein (mg/dl)	**59 (52, 74)**	**37 (28, 42)**	**<0.001**
NFL (pg/ml)	**857 (468, 1474)**	**498 (360, 729)**	**0.008**
Blood neopterin (nmol/l)	18.0 (10.8, 28.9)	14 (9.4, 21.3)	0.183
CSF neopterin (nmol/l)	**14.3 (8.4, 32.0)**	**9.0 (6.5, 17.4)**	**0.035**
Baseline Q_{Alb}	**9.18 (7.5, 11.3)**	**4.66 (3.57, 5.75)**	**<0.001**
% neurosymptomatic ARS	**18% (n=4)**	**6.0% (n=5)**	**---**
Total number of visits	2 (1, 3)	2 (1,3)	0.617
Duration of follow up (days)	48 (0, 398)	51 (0, 455)	0.715

Values are expressed as median and IQR (Q1, Q3).
ARS, acute retroviral syndrome; CSF, cerebrospinal fluid; NFL, neurofilament light chain; WBC, white blood cell count.

Statistically significant parameters are bolded.

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 2**. 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 3** compares the trajectories of the high and normal baseline Q_{Alb} groups. The high baseline group showed a declining trend (-0.00305/day, p=0.011) while the normal baseline group initially increased (0.00144/day, p= 0.006) and reached a plateau quickly (quadratic time effect p= 0.004). These results indicated the heterogeneous time effect in two subgroups.

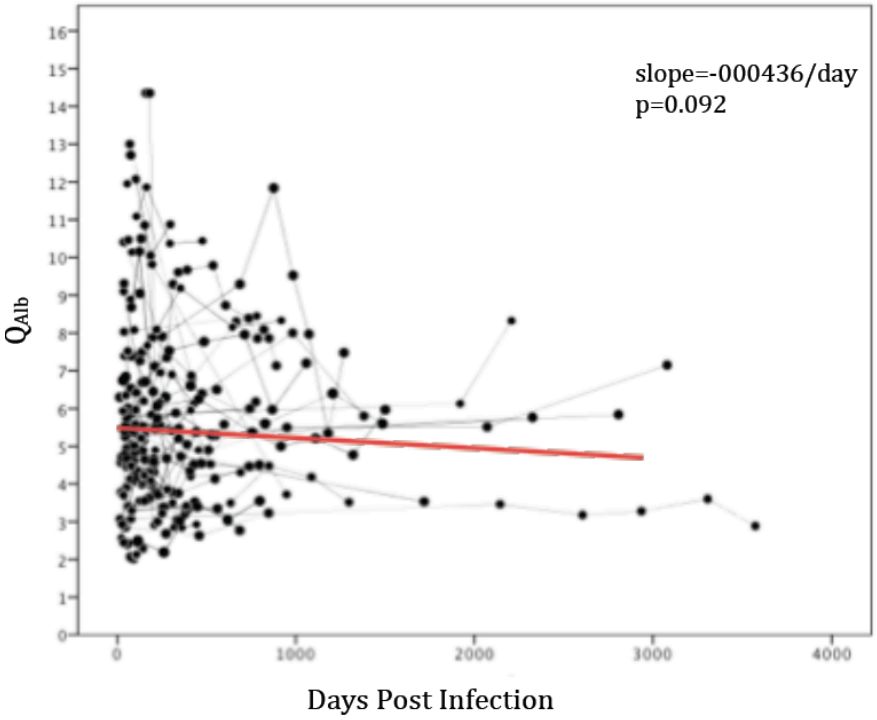

Figure 2*: Natural history of blood brain barrier integrity pre-cART in total cohort.*

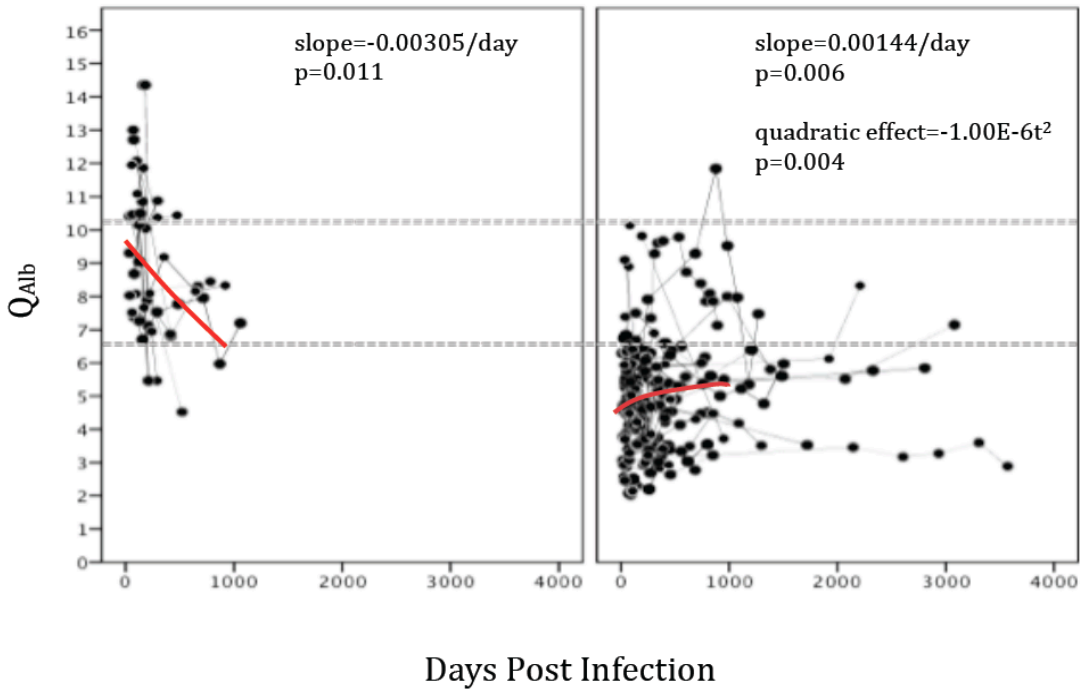

Figure 3*: Natural history of blood brain barrier integrity pre-cART upon cohort stratification.* Graphs demonstrate individual participant and overall trajectory of Q_{Alb} in cART-naive participants upon stratification into high and low baseline Q_{Alb} subgroups. Dashed gray lines simply indicate upper limit of normal for participants aged <45 years (at Q_{Alb}=6.5) and those aged >45 years (at Q_{Alb}=10.2).

Correlation of blood brain barrier integrity with markers of neuropathogenesis

To further evaluate the implications of elevated Q_{Alb}, correlations between Q_{Alb} and markers of neuronal health were evaluated in pre-cART study intervals (**Figure 4**). 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). MRS was performed at a median 114 days post infection (dpi). Q_{Alb} did not correlate with composite z-scores (total Z or NPZ4) of neuropsychological testing at baseline nor in longitudinal analysis (data not shown).

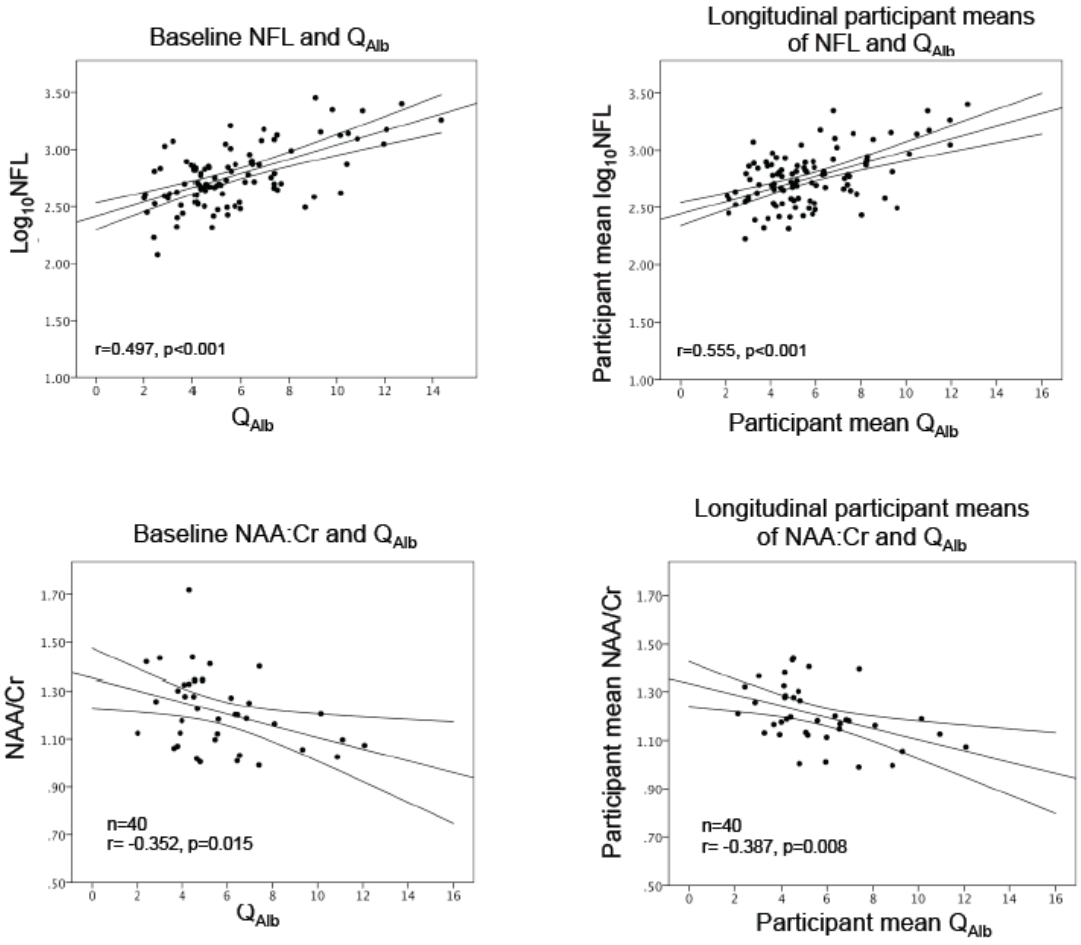

Figure 4*: Correlation of blood brain barrier permeability with clinical and laboratory indicators of neuropathogenesis.*

Characteristics of cART-treated study participants

Fifty-eight 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 10 integrase-based, 25 protease-based, and 22 NNRTI-based (15 of which were efavirenz-based), with 19 distinct combinations. cART was initiated at a median 225 dpi, with 402 days median on-cART follow-up. **Table 3** 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, $p=0.851$).

Table 3: Pre- and post-treatment characteristics of participants initiating cART

	Last pre-cART visit (n=57)	cART-treated endpoint	p value
Age (y)	41 (29, 46)	---	---
Days post-HIV transmission	225 (96, 760)	---	---
Days prior to ART initiation	19 (3, 85)	---	---
# follow-up visits	---	2 (1, 6)	---
Days on cART	---	402 (192, 1060)	---
CD4+ count (cells/μl)	**431 (282, 588)**	**643 (483, 787)**	**<0.001**
Plasma HIV RNA (log_{10}copies/ml)	**4.9 (4.4, 5.3)**	**1.69 (1.69, 1.69)**	**<0.001**
CSF HIV RNA (log_{10}copies/ml)	**3.4 (2.6, 4.0)**	**1.69 (1.69, 1.69)**	**<0.001**
Plasma:CSF HIV RNA ratio (log_{10}copies/ml)	**1.49 (0.71, 2.08)**	**0.00 (0.00, 0.20)**	**<0.001**
CSF WBC count (cells/mm^3)	**4 (6, 14)**	**2 (1, 3)**	**<0.001**
CSF total protein[A]	**40 (33, 50)**	**35 (29, 42)**	**0.001**
NFL (pg/ml)[B]	640 (515, 965)	670 (453, 1072)	0.911
QAlb	5.18 (3.92, 6.40)	5.09 (3.87, 6.21)	0.832
Blood neopterin (nmol/l)	**18.4 (8.4, 24.9)**	**7.6 (5.2, 12.9)**	**<0.001**
CSF neopterin (nmol/l)	**13.9 (7.8, 21.6)**	**5.2 (4.7, 7.7)**	**<0.001**

Values are expressed as median and IQR (Q1, Q3). Group comparisons were performed using non-parametric analysis for related samples.
CSF, cerebrospinal fluid; NFL, neurofilament light chain; WBC, white blood cell count.
[A]n=21 paired; [B]n=42 paired.
Statistically significant parameters are bolded.

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 5**). Three participants were recruited into the cohort already on cART (for 29, 27, and 19 days) and thus were included in the linear mixed model (n=60) but excluded from Table 3. As cART was initiated at a median of 225 dpi (t=0 on Figure 5), this time-point corresponded with the linear portion of **Figure 3**, where the 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 (p=0.783). 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.

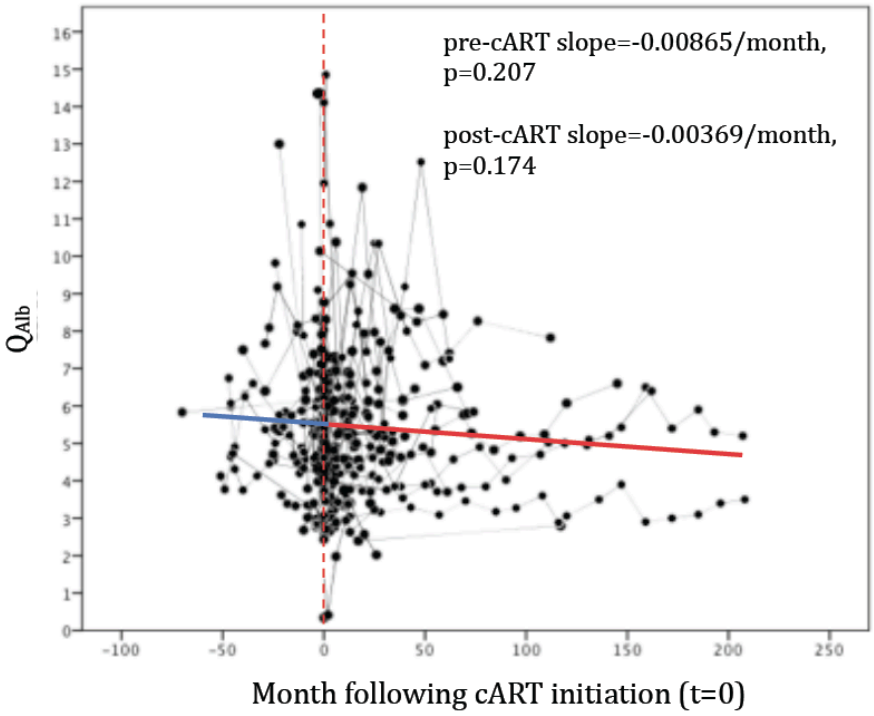

Figure 5*: Effects of cART on trajectory of blood brain barrier permeability.* Scatterplot shows individual participant and overall trajectory of Q_{Alb} pre- and post- cART initiation (indicated by dashed red line at t=0). Linear mixed model analysis generated the equations shown. Blue line shows trajectory of Q_{Alb} pre-cART initation and red line shows trajectory of Q_{Alb} post-cART initiation. Months pre-cART are negative values.

DISCUSSION

In this study, we analyzed the natural history of BBB permeability during primary HIV infection, and the influence of early cART. We showed that the albumin ratio is mildly elevated in PHI participants compared to uninfected controls when correcting for age. This correction is relevant given that BBB permeability increases with normal aging[122],

and may explain why previous studies have not reported abnormalities in BBB permeability during PHI when compared to controls, given that most early HIV studies enroll young patients. That being said, we have previously identified moderate elevation of albumin ratio in PHI [50,60], and in chronic HIV participants who are cART-naive and neuroasymptomatic[60]. Similarly, Li et al have reported a strong association between matrix metalloproteinases--enzymatic surrogate markers of BBB permeability--and neurocognitive status in early HIV[102].

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. 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, and 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.

Previous studies have expounded on the association of albumin ratio with biomarkers of CNS inflammation and injury[123]. We confirm that in PHI albumin ratio correlates strongly with the axonal injury marker NFL[68], and newly demonstrate that it inversely correlates with the metabolic marker of neuronal health, NAA:Cr. NFL is a sensitive marker of active neuronal damage, and its levels correlate with the severity of this damage [51,124,125]. 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 [50,126]. As disease progresses, it is also associated with overt clinical neurological disease, thus not only reflecting structural but functional changes[124]. Although NFL is not specific for HIV neurodegeneration[50,51], comorbid neurological conditions were excluded from the study onset. Similar to Q_{Alb}, NFL was elevated in PHI although 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 [50,68]. 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[127]. In a recent study, chronically infected HIV subjects with cognitive defects were shown to have reduced glutamate and NAA in several brain regions, most pronounced in the parietal grey matter[128]. Here, we extend that finding to the early 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[111]; 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[32]. 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 below 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 Q_{Alb} subgroup 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 elevated compared to uninfected controls and the baseline PHI cohort, given only a marginal age difference. There is a gradual

normalization of NFL following axonal injury which is unlikely to persist for over a year[129]. 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.

Notably, we have previously shown a reduction in NFL in response to cART[124]. However, there are important distinctions between the two studies. Although both cohorts demonstrate approximately equal proportion of patients with elevated age-specific NFL values (38% in the current study, 40% in the previous), the elevations are much less marked in the current study: the patients in the current study demonstrate a lower baseline NFL upon cART initiation (640 pg/mL [IQR 515, 965] vs 780 ng/L [IQR 480, 7300]) for a slightly older cohort (median 41 vs 38 years). Additionally, the previous study had a large proportion of neurologically symptomatic patients (ie, with ADC), whom were noted to have the more marked elevations in NFL. Furthermore, the current study uses a more sensitive NFL assay, possibly more accurately detecting subtle elevations in NFL that remain persistently elevated after cART. Our findings in the current study are consistent with a more recent study from the Gisslén/Zetterberg group using the same highly sensitive assay[86] that demonstrated that the subgroup of HIV-infected individuals with normal CSF NFL at baseline exhibited no significant reduction in CSF NFL after treatment initiation, and also that in the overall group studied, NFL levels did not completely normalize in the setting of long-term cART. Finally, the current study is assessing early stage primary HIV infection, while the previous study was assessing chronic HIV infection/AIDS, thus there may be inherent differences between the two disease stages (although beyond the scope of this study).

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[130]. 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[131]. The effects of earlier cART intervention on albumin ratio normalization should be investigated.

Limitations

In light of the genetic diversity of HIV, the findings of this study are most representative of infection with HIV-1 subtype B, the predominant form in Europe, Australia, and the Americas[132] and the subject of most *in vitro* experiments and antiretroviral drug experiments[133].

Q_{Alb} is affected by many factors not accounted for in this study, including body weight and smoking [122]. Comorbidities which are highly prevalent in HIV+ individuals, such as cardiovascular disease and diabetes mellitus, are known to influence the integrity of the BBB. In this study, cholesterol and other cardiovascular risk factors were not routinely screened for, although none of the participants had a known history of clinically apparent cardiovascular disease such as coronary artery disease, peripheral vascular

disease, or stroke. One participant, in the low baseline group, had a known diagnosis of diabetes mellitus type 2. Abuse of substances such as cocaine has been shown to at least transiently increase BBB permeability[45]. As indicated in the results, drug use was highly prevalent in this cohort, 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. The influence of distinct regimens is further complicated by the fact that several participants changed cART regimens throughout the course of the follow-up period for different reasons (ie, drug reactions). Therefore, the sample size we have is too small to support a meaningful comparison by therapy.

In light of the limitations delineated above, the ideal confirmatory study would recruit healthy subjects without confounding factors of BBB status--HBV/HCV negative, no previous history of drug or alcohol use, no history of cardiovascular disease or cardiovascular risk factors, including hypertension, diabetes, or even obesity. Participants would be recruited nationwide via different universities/institutions, and would include a greater female population (large enough for further analysis upon sex stratification) and larger age distribution (again, large enough for further analysis upon stratification). Follow-up would begin from day 1 of HIV transmission and follow-up time would be homogeneous for all participants (ie, at baseline, six weeks, and every six months thereafter until the last day without any loss to follow-up). Participants would have excellent access to health care throughout the observational study, so as to minimize interference of confounding health conditions on the different measured CSF and blood

parameters. A large portion of participants would then start an antiretroviral therapy regimen within the PHI phase and remain compliant with successful viral suppression. With an earlier cART initiation, we may thus be able to better assess the effect of cART on the BBB permeability trajectory compared to the cART-naive population. Additionally, if cART regimens were more homogeneous among participants, we may be able to stratify based on regimen characteristics (ie, CPE) to determine if unique regimens exhibit different effects on BBB permeability.

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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