EFFECT OF ANTIRETROVIRAL THERAPY ON BLOOD-BRAIN BARRIER INTEGRITY AND CENTRAL NERVOUS SYSTEM INFLAMMATION

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BY
JOANNA MARIE KETTLEWELL

DISSERTATION COMMITTEE:
BRUCE SHIRAMIZU, Chairperson
F. DEWOLFE MILLER, IV
RICHARD YANAGIHARA
SAGUNA VERMA
RANDAL K. WADA, External Member

DEDICATION

This work is dedicated to my parents,

Mr. JOHN HENRY KETTLEWELL

and

Mrs. BARBARA SUE KETTLEWELL

You always placed an emphasis on education and made abundant sacrifices to invest in my academic journey. Thank you for impressing upon me the importance of resilience in achieving my goals and making sure I never forget that home is only a phone call away.

"Now, I know we have still not shattered that highest and hardest glass ceiling, but someday someone will — and hopefully sooner than we might think right now. And to all of the little girls who are watching this, never doubt that you are valuable and powerful and deserving of every chance and opportunity in the world to pursue and achieve your own dreams. Finally, finally, I am so grateful for our country and for all it has given to me. I count my blessings every single day that I am an American, and I still believe, as deeply as I ever have, that if we stand together and work together with respect for our differences, strength in our convictions, and love for this nation, our best days are still ahead of us."

--- Hillary Rodham Clinton

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ABSTRACT

Human immunodeficiency virus type 1 (HIV) enters the central nervous system (CNS) as early as eight days after infection and crosses the blood-brain barrier (BBB) primarily via infected monocytes. Even with suppressive antiretroviral therapy (ART), HIV infection of the CNS causes persistent inflammation, neuronal injury, and BBB breakdown leading to neurocognitive impairment, categorized as HIV-associated neurocognitive disorders (HAND). HAND is a common, debilitating complication of HIV infection. Diagnosing and treating HAND remains challenging. Multiple biomarkers have been proposed to aid in the management of patients with HAND, however, their clinical relevance is undetermined. To address this gap, this study analyzed changes in neuroinflammatory mediators in serum and cerebrospinal fluid (CSF) of HIV-infected study participants with HAND on suppressive ART receiving CCR5-inhibitor maraviroc for 48 weeks. Due to inhibition of the CCR5 receptor, which is important for HIV entry into monocytes, the central hypothesis was maraviroc would reduce neuroinflammation and improve BBB integrity *in vivo* and *in vitro*, corresponding to improved neuropsychological performance. The effect of maraviroc on BBB integrity was assessed using both *in vivo* and *in vitro* functional assays. The study demonstrated a reduction in some neuroinflammatory mediators, but none that corresponded to improved neuropsychological performance. Decreased *in vivo* BBB integrity corresponded to increased CSF tumor necrosis factor α and serum calcium-binding protein B of the S-100 protein family. This study also assessed maraviroc as an addition to current pre-exposure prophylaxis (PrEP) therapy [tenofovir disoproxil fumarate, emtricitabine] due to concerns of drug resistance. PrEP with and without *in vitro* addition of maraviroc showed a reduction in monocyte trafficking across the BBB in two out of three study participants. *In vitro* PrEP exposure of BBB endothelial cells with and without maraviroc showed an increased

presence of tight junction protein occludin. These findings indicate CCR5 inhibition with maraviroc may reduce some neuroinflammation and current PrEP drugs tenofovir and emtricitabine, with and without maraviroc, may be neuroprotective. This study contributes to the field on potential treatment and prevention strategies for HIV infection and HAND. Future research will increase the clinical and translational impact of these findings.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

CHAPTER 2

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

LIST OF ABBREVIATIONS AND SYMBOLS

AAN	American Association of Neurology
AIDS	Acquired immunodeficiency syndrome
ANI	Asymptomatic neurocognitive impairment
ART	Antiretroviral therapy
BBB	Blood-brain barrier
BMVEC	Brain microvascular endothelial cells
CCL2	C-C motif chemokine ligand 2
CCL3	C-C motif chemokine ligand 3
CCR1	Chemokine receptor type 1
CCR3	Chemokine receptor type 3
CCR4	Chemokine receptor type 4
CCR5	Chemokine receptor type 5
CSF	Cerebrospinal fluid
CXCL10	C-X-C motif chemokine ligand 10
CX_2CL1	C-X2-C motif chemokine ligand 1
CXCR4	C-X-C chemokine receptor type 4
DNA	Deoxyribonucleic acid
EBA	0.45% Evans Blue dye-conjugated bovine serum albumin
ELISA	Enzyme-linked immunosorbent assay
EVOM	Epithelial voltohmmeter
FTC	Emtricitabine

FITC	Fluorescein isothiocyanate
Global NPZ	Aggregation of neuropsychological performance test domain scores
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorders
hCMEC/D3	Human brain microvascular endothelial cell immortalized cell line
HICFA	Hawaii Center for AIDS
HIV	Human immunodeficiency virus type 1
ICAM-1	Intercellular adhesion molecule 1
INSTI	Integrase strand transfer inhibitor
IL-1α	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IRB	Institutional review board
JABSOM	John A. Burns School of Medicine
LPS	Lipopolysaccharide
LRA	Latency reversal agent
MCP-1	Monocyte chemoattractant protein 1
MIP-1α	Macrophage inflammatory protein 1 α
MMP-2	Matrix metalloproteinase 2
MMP-9	Matrix metalloproteinase 9
MND	Mild neurocognitive disorder

NIH	National Institutes of Health
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
P	p-value
PBMC	Peripheral blood mononuclear cells
PECAM-1	Platelet endothelial cell adhesion molecule 1
PET	Polyethylene terephthalate
PI	Protease inhibitor
PLWH	Persons living with HIV
PrEP	Pre-exposure prophylaxis
RNA	Ribonucleic acid
S100B	Calcium-binding protein B of the S-100 protein family
sCD14	Soluble cluster of differentiation 14
sCD163	Soluble cluster of differentiation 163
TDF	Tenofovir
TEER	Trans-endothelial electrical resistance
TNFα	Tumor necrosis factor α
UH	University of Hawai‘i
Qalb	CSF-to-serum albumin ratio
ZO-1	Zonula occludens 1
ZO-2	Zonula occludens 2
ZO-3	Zonula occludens 3

CHAPTER 1

INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Human immunodeficiency virus type 1 (HIV) is an enveloped virus which contains two strands of positive-sense ribonucleic acid (RNA) for its genome and is a member of the *Lentivirus* genus within the *Retroviridae* family [1, 2]. Viruses within the *Lentivirus* genus, including HIV, are known to be associated with prolonged, chronic illness [2].

The cell types that HIV infects and replicates in include cells of the T-helper lymphocyte linage and cells of the myeloid lineage due to HIV binding to the cluster of differentiation (CD4) receptor and the co-receptor C-C chemokine type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) [3]. Binding to both the CD4 and co-receptor CCR5 or CXCR4 leads to viral fusion with the host cell membrane and release of the RNA genome into the cytoplasm where reverse transcription occurs [1]. Once transcribed to double-stranded deoxyribonucleic acid (DNA), HIV integrase cleaves the 3' end and integrates the viral genome into the DNA of the host cell. HIV genes are then transcribed, translated, and HIV protease cleaves the resulting polypeptides. Viral proteins assemble at the host cell membrane and progeny virus buds from the host cell [4]. At the tissue site of initial exposure and infection, innate immune responses result in the release of chemokines and cytokines that attract susceptible immune cells, such as macrophages and dendritic cells [5, 6]. Systemic dissemination occurs rapidly within days of initial infection, including to the CNS [7, 8].

Heterosexual intercourse remains the predominant mode of transmission of HIV worldwide, but in the United States, HIV is primarily transmitted by anal intercourse among men who have sex with men [9, 10]. Acquired immunodeficiency syndrome (AIDS), the disease

caused by HIV infection, was first described by Gottlieb and colleagues, who reported treating four previously healthy homosexual men for Pneumocystis pneumonia (caused by the yeast-like fungus, *Pneumocystis jiroveci*), which is now recognized as one of several AIDS-defining illnesses [11, 12]. Since the discovery of the disease, recent data from 2018 indicates that 74.9 million people worldwide have been infected by the HIV virus, 32 million have died, and 37.9 million people are currently living with HIV [13]. Despite only accounting for approximately 2% of the world's population, 2018 data showed eastern and southern Africa had the highest prevalence of HIV with 20.6 million cases [10, 13]. In the United States, most recent estimates from 2016 of HIV prevalence were at 1.1 million people [14]. According to the 2019 HIV/AIDS Surveillance Annual Report released by the Hawaii State Department of Health, the state of Hawaii had a total of 2,393 individuals living with HIV/AIDS at the end of 2016 [15]. The rate of new HIV/AIDS diagnoses in Hawaii in 2018 was 5.5 per 100,000 individuals, which is relatively low in comparison to states within the southern region of the United States (examples: Georgia 29.9/100,000; Louisiana 25.5/100,000) but not the lowest (examples: Maine 2.4/100,000; Wyoming 2.5/100,000) [16].

The Hawaii Center for AIDS (HICFA) includes the Hawaii AIDS Clinical Trial Unit, several laboratories, and the Clint Spencer Clinic, which serves to treat persons living with HIV (PLWH) and prevent new infections [9]. Due to the low number of cases and the quality of scientific, clinical, and community infrastructure in Hawaii, HICFA believes it can transform Hawaii into the first HIV-free state. The initiative has been named the "Hawaii to Zero Cure Initiative" [9]. This study supports the goal and mission of the initiative by researching

strategies to prevent new HIV infections and utilizing clinical trials research (NCT 02159027) to assess potential treatments for HIV-associated morbidity and mortality [17, 18].

HIV INFECTION OF THE CENTRAL NERVOUS SYSTEM

There are three major separations and interfaces of the vascular system and the CNS that serve as potential entry sites for HIV. The arachnoid epithelium is the middle layer of three protective membranes that surround the brain, collectively known as the meninges. The arachnoid epithelium separates cerebrospinal fluid (CSF) in the subarachnoid space from blood, but is not considered a significant site for potential HIV infection [19]. A second interface between the CSF and blood is known as the blood-CSF barrier. The blood-CSF barrier is formed by the choroid plexus epithelium that produces CSF in the cerebral ventricles and separates it from peripheral blood. Although the blood-CSF barrier is a site of cerebrovascular exchange, the most significant site for cerebrovascular exchange, and gateway for HIV infection of the CNS, is the blood-brain barrier (BBB) [19, 20].

At the site of the BBB, astrocytic perivascular end feet are in contact with epithelium of cerebral capillaries [21-24]. Astrocytes in sync with microglia and neurons form a dynamic system known as the neurovascular unit that helps maintain a tightly regulated and intact BBB [21]. Since no brain cell is further than 25 μm from a brain capillary, maintenance of the BBB is vital. Dysregulation, such as the kind that occurs in HIV infection, can contribute to sustained neuroinflammation and damage to cells comprising the neurovascular unit [19]. During both acute and chronic HIV infection, HIV-infected CD4+ T cells and monocytes traverse the BBB. Once inside the brain, monocytes become activated perivascular macrophages. These activated

perivascular macrophages produce HIV virions, infecting other cells in the brain such as astrocytes and microglia (Figure 1). This results in the production of neurotoxic molecules and inflammatory cytokines and chemokines, which damage neurons and oligodendrocytes [21, 24-28]. This cascade of events is thought to begin early after infection due to data showing HIV RNA in the CNS as early as eight days post-infection, corresponding to CNS inflammation by analysis of CSF and magnetic resonance spectroscopy [7]. Early HIV seeding in the CNS and resultant neuroinflammation is thought to contribute to the development of HIV-associated neurocognitive disorders (HAND) [24, 25, 29-33].

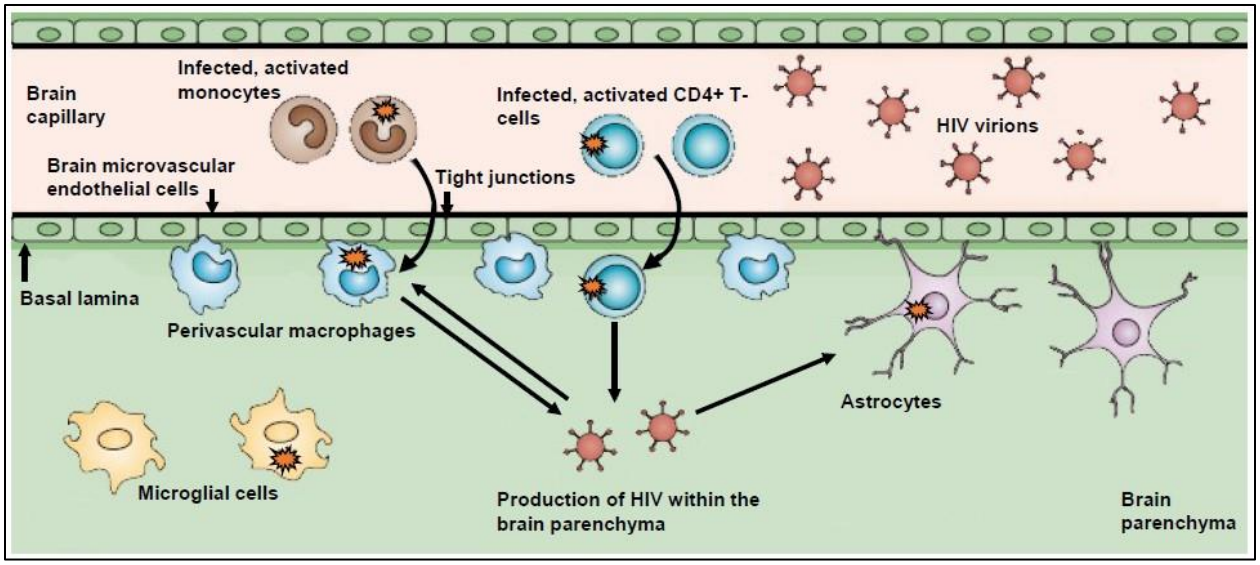

Figure 1. HIV infection of the CNS. Infected monocytes and CD4+ T cells in brain capillaries traverse the BBB. Once inside the brain parenchyma, progeny virions are produced that infect microglial cells and astrocytes [25].

BLOOD-BRAIN BARRIER (BBB)

The BBB is a selective barrier formed by the endothelial cells that line cerebral microvessels and restricts the movement of potentially toxic or harmful substances from the

blood to the brain while maintaining the transport of nutrients and the removal of metabolites [21, 34]. The brain microvascular endothelial cells (BMVEC) form a physical barrier due to the presence of tight junction proteins that occlude the intercellular space by forming a multi-protein complex composed of transmembrane proteins (claudins, occludin, and junctional adhesion molecules) and cytoplasmic proteins (zonula occludens (ZO)-1, ZO-2, ZO-3, and cingulin) which are linked to the actin cytoskeleton (Figure 2) [19, 21, 26, 35]. Tight junctions are responsible for the restriction of the paracellular diffusional pathway to ions (such as Na^+ and Cl^-) and other polar ions, and effectively block penetration of macromolecules [19, 21]. The presence of these tight junctions results in a high trans-endothelial electrical resistance (TEER) which is >1000 Ω/cm^2 in comparison to the TEER of peripheral capillaries which is approximately 2–20 Ω/cm^2 [21, 35]. BMVEC also have a lower pinocytic activity and a greater number of mitochondria compared to peripheral endothelial cells, which is thought to be required for active transport of nutrients into the brain [26].

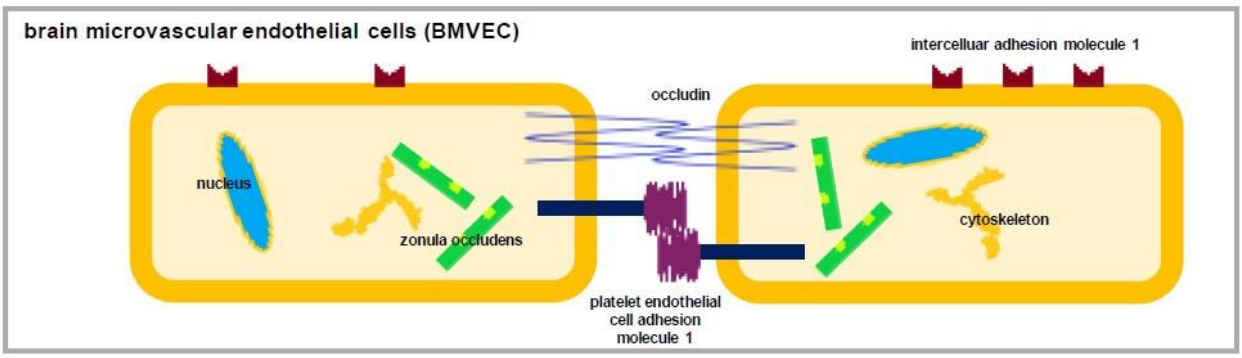

Figure 2. Brain microvascular endothelial cells (BMVEC). Tight junction proteins such as occludin are anchored to the cytoskeleton via the cytoplasmic scaffolding proteins, such as zonula occludens 1 (ZO-1). Intercellular adhesion molecule 1 (ICAM-1) and platelet endothelial cell adhesion molecule 1 (PECAM-1) are expressed on BMVEC and are crucial to the movement of leukocytes across the BBB [19, 26].

Intercellular adhesion molecule 1 (ICAM-1) (Figure 2), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin are expressed on the surface of BMVEC and are crucial for the transmigration of lymphocytes and monocytes [36]. In the instance of HIV infection, disruption of tight junction proteins and increased presence of adhesion molecules, including pPECAM-1, on inflamed BMVEC facilitates diapedesis of leukocytes via a paracellular route (as opposed to transcellular route directly through the cytoplasm under homeostatic conditions) [19, 26, 36, 37].

Other cell types help maintain BBB characteristics. Pericytes are engulfed in the basal lamina of the BBB and cover approximately 22–32% of the BBB circumference [26, 34]. Pericytes contribute to vascular stability and induce the polarization of astrocytic perivascular end feet, helping to coordinate signaling between cells of the neurovascular unit [35, 38, 39]. However, additional functional roles of pericytes remain largely unknown [35]. Astrocytic perivascular end feet envelop approximately 99% of the total BBB endothelium and interact with BMVEC to increase expression of tight junctions, reduce the gap junctional area between BMVEC, and regulate expression of transporters and enzyme systems [21, 26, 35].

BBB impairment is a common finding in PLWH and has been shown to occur even in PLWH with undetectable viral loads at a proportion as high as 22% [28, 40]. In autopsy samples of patients with AIDS, BBB breakdown is present in 50% of samples regardless of pathology and in 100% of samples from those diagnosed with HIV-associated dementia [40-42]. BBB breakdown in HIV infection is considered to be chronic and slowly progressive as a result of ongoing HIV-infected monocyte trafficking into the CNS and entry of serum-derived factors,

such as tumor necrosis factor α (TNFα) and viral proteins that damage neurons and activate perivascular macrophages and microglia within the CNS causing secretion of neurotoxic substances [40, 43]. However, recent data suggest that BBB impairment occurring early in infection is significant as it may take years to return to baseline or near baseline with suppressive antiretroviral therapy (ART) [43]. Even though astrocytes are latently infected at proportions as low as 1–3%, HIV infection of astrocytes may play a prominent role in BBB breakdown as infection has been shown to interfere with astrocyte-BMVEC regulation of BBB integrity resulting in increased BBB permeability [25, 44]. Additionally, infected astrocytes have been shown to secrete chemokines including C-C motif chemokine ligand 2 (CCL2), also referred to as monocyte chemoattractant protein 1 (MCP-1), C-C motif chemokine ligand 3 (CCL3), also referred to as macrophage inflammatory protein 1 α (MIP-1α), C-X-C motif chemokine ligand 10 (CXCL10), and C-X2-C motif chemokine ligand 1 (CX_2CL1) that upregulate monocyte recruitment into the CNS, exacerbating CNS inflammation [44]. Since data suggest PLWH with impaired BBB integrity have a higher prevalence of neurocognitive deficits, the BBB is a crucial component of HIV neuropathogenesis research [42].

MEAURES OF BBB INTEGRITY

The current gold standard method for evaluating BBB integrity *in vivo* is to determine CSF-to-serum albumin ratios (Qalb) [43, 45-48]. Albumin is produced exclusively in the liver and is largely excluded from the CSF, but when the BBB becomes dysregulated and the presence of tight junctions is reduced, permeability increases and the albumin levels in the CSF increase [43]. Although Qalb is known to increase slightly with age, drastic increases in Qalb are seen in

neurological diseases for which BBB breakdown is a component of pathogenesis, such HAND [43, 46].

Utilizing an *in vitro* model of the BBB provides an additional tool for researchers to analyze molecular and physiological effects to the BBB. Ideally, any *in vitro* BBB model is reproducible and comes as close to an *in vivo* system as possible with functional characteristics of specific BBB properties [49]. There are a significant number of publications utilizing different cell types from both primary and immortalized cell cultures of human, primate, bovine, porcine, rodent and murine species utilizing a single-layer endothelial model or a co-culture model with astrocytes and/or pericytes [34, 49]. The diversity of systems leads to differential expression of BBB characteristics and may make comparison of results difficult [34, 49]. However, HIV studies using a co-cultured model of human BMVEC and human astrocytes on 3.0-μm pore size tissue culture inserts have been shown to allow astrocytic end feet to remain in direct contact with BMVEC (Figure 3) [27, 50]. BMVEC in this co-culture *in vitro* model has also been shown to retain BBB properties and be suitable for use in assessing leukocyte transmigration [27, 50-52].

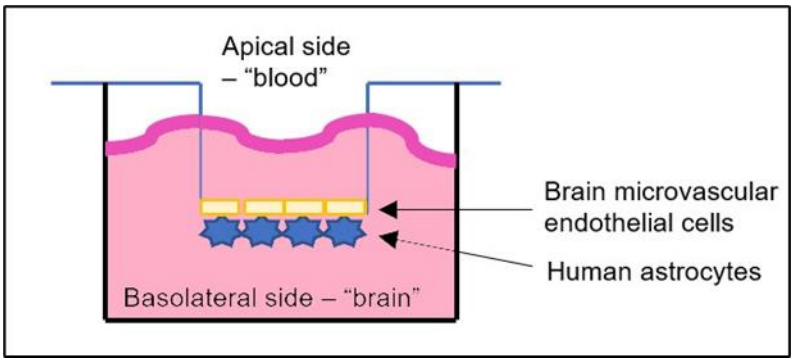

Figure 3. Schematic of an *in vitro* bilayer BBB co-culture system. The model used in this study was constructed with $10x10^5$ primary adult human astrocytes and $2x10^5$ primary

BMVEC co-cultured to confluence over six days on opposite sides of polyethylene terephthalate inserts containing 3-μm pores and coated with rat-tail collagen type I [35].

There are several methods to determine the integrity of *in vitro* BBB models. TEER is measured by having one electrode on the apical side of the *in vitro* BBB and one on the basolateral side utilizing either chopstick electrodes via an epithelial volt ohmmeter (EVOM) (world Precision Instruments, Sarasota, FL, USA) or the cellZscope system (NanoAnalytics, Munster, Germany) [34, 51]. The cellZscope system allows for continuous monitoring of TEER for up to 24 wells whereas the EVOM only allows for manual measurements during which the cultures must be removed from incubation [34, 53]. *In vitro* bilayer BBB models over 100–120 Ω/cm^2 have been shown to be suitable for experiments assessing leukocyte transmigration [34]. BBB integrity can also be measured by Evans blue-labeled fetal bovine serum albumin (EBA, molecular weight = 67 kDa) as a large molecule tracer read spectrophotometrically at 620 nm [34, 50, 51]. Varying protocols exist using small molecule tracers sodium fluorescein (molecular weight = 376 Da) and fluorescein-isothiocyanate (FITC)-dextran (molecular weight = 4 kDa) are also used to measure BBB permeability and are assessed spectrophotometrically at 520 nm [34, 54]. These methods to assess BBB integrity are commonly used in *in vitro* studies of HIV neuropathogenesis and have been utilized for *in vitro* bilayer BBB portions of this study [27, 51, 55].

HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS (HAND)

HAND are a spectrum of neurocognitive impairment affecting up to 69% of PLWH [56, 57]. The original defining criteria for HAND was created in 1991 by the American Academy of

Neurology (AAN), but new framework, referred to as the Frascati criteria for HAND (Table 1), was published in 2007 by a working group at the HIV Neurobehavioral Research Center at the University of California San Diego under the direction of the National Institute of Mental Health and the National Institute of Neurological Disorders and Stroke. To critically review and update the framework for HAND diagnosis, the group took the 1991 AAN criteria and introduced changes based on research, observations, and published data [58].

Diagnosis of HAND with the 2007 Frascati criteria requires a clinical determination of neurocognitive impairment as a result of HIV CNS infection, rather than comorbid conditions, including substance abuse, psychiatric disorders, epilepsy, brain trauma, cardiovascular disease, coinfections, such as hepatitis C, ageing, or low educational achievement [24, 58]. Evaluation of neuropsychological performance and diagnosis of HAND is made through a battery of tests and functional status assessments administered by neuropsychologists [24, 28]. Five to seven neurocognitive subdomains are analyzed to determine overall neuropsychological performance (global NPZ), including: verbal/language fluency, attention/working memory, abstraction/executive functioning, learning/recall, speed of information processing, and motor function (perceptual-motor speed) [28, 59].

The mildest form of HAND is referred to as asymptomatic neurocognitive impairment (ANI). Reports of prevalence vary with ANI accounting for 20-70% of HAND diagnoses [28, 56]. Individuals diagnosed with ANI show impairment on neuropsychological performance testing with impairment in at least two neurocognitive subdomains (>1 standard deviation below the mean for age-education-appropriate norms) but are asymptomatic in the sense that specified

criteria for establishing negative effects on daily life have not been met [58, 60]. Criticism of the clinical applicability of the ANI diagnosis has been made due to data indicating up to 16-19% of HIV-negative individuals would be categorized with HAND upon neuropsychological performance testing [60, 61]. However, some evidence suggests individuals with ANI have a two-fold greater risk of poor medication adherence and may progress to more severe forms of HAND, such as mild neurocognitive disorder (MND) or HIV-associated dementia (HAD) [59, 62, 63]. This information further suggests the need for additional research into mechanisms involved in the progression of HAND.

MND must also be attributable to HIV rather than a comorbid condition, with impairment in at least two neurocognitive subdomains (>1 standard deviation below the mean for age-education-appropriate norms) [59, 64]. Unlike ANI, MND diagnosis is accompanied by evidence of mild interference in >1 activities of daily living such as general mental acuity, inefficiency at work, homemaking or social functioning by either self-report or observation by others familiar with the individual [28, 58, 59]. It is notable that for diagnosis with MND that interference with daily living must be mild and impairment does not meet criteria for dementia or delirium [58]. Prevalence of MND varies by study cohort but has been noted to be between 12-52% [28, 63].

The most severe form of HAND, HAD, is synonymous with the terms HIV encephalopathy and AIDS dementia complex [65]. Factors that increase risk of the development of HAD include experiencing systemic symptoms, a high viral load early in infection, low CD4 counts (<200 cells/mm^3), low body mass index, anemia, injection drug use, increased age, and

female sex [24, 64, 65]. HAD is defined by impairment in at least two neurocognitive subdomains with two of the domains containing deficits to be >2 standard deviations below the mean for age-education-appropriate norms [58]. For HAD, neurocognitive deficits are frequently seen in learning of new information, information processing, and attention and concentration [28, 58]. Individuals diagnosed with HAD have marked interference with activities of daily living and diagnosis is associated with rapid progression to death [28, 58, 64, 65]. HAD diagnosis must rule out another cause for the dementia [58]. Due to the advent of combined ART in 1996, defined as at least three antiretroviral drugs targeting multiple steps of the HIV life cycle used to maximally suppress replication of virus and disease progression, the prevalence of HAD has dropped from approximately 20% to 5% or less, with some studies identifying zero people with HAD [28, 59, 63, 66-68].

TABLE 1 Frascati criteria for the classification of HIV-associated neurocognitive disorders (HAND)

HAND designation	Estimated Prevalence	Diagnostic Criteria		
		Neuropsychological testing	**Interference in daily life**	**Additional criteria**
Asymptomatic neurocognitive impairment (ANI)	20-70%	Involvement in at least two neurocognitive subdomains with performance at least 1.0 standard deviation below the mean for age-education-appropriate norms.	No interference.	Does not meet criteria for delirium or dementia. There is no evidence of another pre-existing cause.
Mild Neurocognitive Disorder (MND)	12-52%	Involvement in at least two neurocognitive subdomains with performance at least 1.0 standard deviation below the mean for age-education-appropriate norms	Mild interference defined by reduced mental acuity, inefficiency in work, homemaking, or social functioning documented by at least one of the following: a) Self-report b) Observation by knowledgeable others	Does not meet criteria for delirium or dementia. There is no evidence of another pre-existing cause.
HIV-associated dementia (HAD)	0-5%	Impairment is typically in multiple neurocognitive subdomains, especially learning of new information, slowed information processing, and defective attention/concentration. Impairment must be in at least two neurocognitive subdomains with at least 2.0 standard deviation or greater than age-education-appropriate norms.	Marked interference.	Does not meet criteria for delirium. There is no other pre-existing cause for dementia.
If the individual also satisfies criteria for a severe episode of major depression, substance dependence, or MND/HAD- with significant functional limitations or psychotic features, the diagnosis should be deferred to a subsequent examination conducted at a time when the major depression has remitted or at least one month has elapsed following cessation of substance use.				
Neuropsychological testing must include the components of verbal/language, attention/working memory, abstraction/executive function, memory (learning, recall), speed of information processing, sensory-perceptual, motor skills				
Table adapted from Antorini et al, 2007; Brew et al, 2009; Carroll and Brew, 2017; Heaton et al, 2010; Sanmarti et al 2014				

Although neuropsychological performance testing is a critical component in the diagnosis of HAND, it cannot predict improvement or decline [69]. Testing is time consuming and language and educational achievement may impact testing outcomes [70]. Furthermore, while HAND diagnosis is confirmed to be a result of HIV rather than comorbidities, comorbidities have a compounding detrimental impact on cognitive function, making it difficult to decipher HIV-specific effects [24, 68]. Research seeking to identify specific biological markers (biomarkers) related to HAND pathogenesis would help to decipher HIV-specific effects, determine prognosis, evaluate an individual's response to therapy, and inform potential interventions to treat and prevent HAND.

BIOMARKERS IN HAND

Neuropsychological performance testing is a critical component in the diagnosis of HAND, but cannot efficiently and singularly predict improvement or decline [61, 69]. Since impairment in neuropsychological performance testing has been correlated with persistent immune activation in the CSF and periphery, attempts have been made to identify a biomarker or biomarker panel that would be prognostic for HAND [24, 61, 69]. Potential biomarkers have been identified, but there is currently no standard biomarker or biomarker panel in use for prognostic purposes [24, 69, 71]. The search for novel biomarkers is ongoing due to the current lack of biomarker specificity to HAND and the potential overlap with HIV comorbid conditions [71, 72]. To inform the development of therapeutic interventions, this study analyzed changes in inflammatory biomarkers previously linked to critical components of HAND pathogenesis (monocyte infection and seeding in the CNS, microglial and astrocyte activation, neuronal

damage, and BBB impairment) as they related to changes in neuropsychological performance testing and BBB integrity in the setting of drug intervention [24, 73, 74]. Thus, the scope of biomarkers discussed and analyzed in this study (Table 2) is specific in nature, derived from a body of literature in which associations have been made between the presence of inflammatory biomarkers and HAND [21, 24, 32, 40, 68, 69, 75-86].

TABLE 2 Biomarker Panel

Biomarker	**Source**	**Association**
TNFα (Brew *et al*, 2009)	serum, CSF	systemic inflammation, microglial activation, astrocytosis, neuronal death
IL-6 (Brew *et al*, 2009; Kamat *et al*, 2012)	serum, CSF	systemic inflammation, microglial activation, astrocytosis
sCD14 (Kamat *et al*, 2012; Ndhlovu *et al*, 2014)	serum, CSF	systemic inflammation, monocyte activation
sCD163 (Nhdlovu *et al*, 2014)	serum, CSF	systemic inflammation, monocyte activation
Neopterin (Barber *et al*, 2018)	serum, CSF	linked to HAD severity and BBB dysfunction indicated by CSF to serum albumin ratios (Qalb)
S100B (Barber *et al*, 2018; Brew *et al*, 2009)	serum, CSF	astrocytosis, neuronal death, BBB dysfunction, decreased executive functioning
MMP-9 (de Almeida *et al*, 2017; Xing *et al*, 2017)	serum, CSF	BBB dysfunction
MMP-2 (de Almeida *et al*, 2017; Xing *et al*, 2017)	serum, CSF	BBB dysfunction
Abbreviations: Tumor necrosis factor α (TNFα), Interleukin 6 (IL-6), Soluble cluster of differentiation 14 (sCD14), Soluble cluster of differentiation 163 (sCD163), Calcium-binding protein B of the S-100 protein family (S100B), Matrix metalloproteinase 9 (MMP-9), Matrix metalloproteinase 2 (MMP-2)		

TNFα is a systemically-acting cytokine involved in immune regulation and inflammation and is produced by monocytes, activated macrophages and microglia [87]. Certain monocyte

subsets, such as "nonclassical" $CD14_{lo}CD16_{hi}$, are major producers of TNFα and are shown to expand to greater than 20-40% of the circulating monocyte population in response to inflammatory states such as HIV infection (5-10% non-HIV) and to preferentially migrate into tissues [32, 80, 88]. Increased levels of systemically acting cytokines such as TNFα and interleukin 6 (IL-6) exacerbate a cycle of sustained neuroinflammation that persists even with suppressive ART and is linked to increased morbidity and mortality [80, 89]. Both TNFα and IL-6 are consistently reported in the CSF and serum of study participants with HAND and linked to brain injury, although some reports indicate elevated IL-6 regardless of neurocognitive status [68, 77, 82, 87].

Soluble monocyte markers of activation have been consistently linked to HAND [32, 40, 77, 78, 82-84]. Soluble cluster of differentiation 163 (sCD163) is a scavenger receptor found on monocytes and is shed by proteolytic cleavage after pro-inflammatory stimulation with toll-like receptors and lipopolysaccharide (LPS) in inflammatory states such as HIV infection [32]. sCD163 has been directly correlated with monocyte expansion in HIV infection and is associated with neurocognitive impairment [32, 78]. Neopterin is a soluble aromatic chemical compound composed of fused pyrimidine and pyrazine rings, also known as a pteridine, that is produced by myeloid-derived cells such as monocytes, macrophages, and microglia [32, 87]. High levels of CSF neopterin are found in individuals with HAD and are shown to markedly reduce with ART, but even with viral suppression, only 55% of PLWH exhibit a reduction in neopterin levels comparable to non-HIV infected individuals [32, 77, 90]. It is estimated that 97.5% of neopterin originates within the CNS [91]. Furthermore, due to the pteridine structure, neopterin can more easily penetrate membranes and tissues and could explain why the inflammatory biomarker

remains elevated in CSF despite ART [90]. Soluble cluster of differentiation 14 (sCD14) is released primarily by activated monocytes and is an LPS-binding protein thought to mediate LPS-induced activation of non-CD14-expressing cells such as epithelial and endothelial cells and to play a role in immune modulation through direct interaction with T-cells and B-cells [87, 92]. Increased LPS as a result of microbial translocation from the gut of PLWH contributes to the activation of monocytes [93]. Studies have correlated elevated levels of plasma sCD14 to HAND, specifically to deficits in attention and learning evaluated by neuropsychological testing [83, 89, 93]. CSF levels of sCD14 have been associated with persistent CNS immune activation despite suppressive ART and are considered to be a result of trafficking monocytes into the CNS and perivascular macrophages, rather than native microglia in the brain [87, 89].

Calcium-binding protein B of the S-100 protein family (S100B), produced primarily by astrocytes, is a marker for astrocyte activation [87]. Astrocyte activation, also known as astrocytosis, can lead to BBB breakdown. Increased CSF S100B is linked specifically to deficits of executive function and language evaluated by neuropsychological performance testing. Furthermore, elevated CSF S100B is associated with neuronal death [77, 87]. Increased presence of CSF S100B is associated with decreased verb word generation counts and executive functioning evaluated by neuropsychological performance testing [77]. Elevated levels of CSF S100B have been shown to be present in study participants with HAD and be indicative of rapid progression to death [77, 94]. Inconsistent data exists, however, showing decreased risk of ANI and MND with increased S100B in CSF ($p= 0.02$) in PLWH in Uganda [75]. This inverse association was confirmed with S100B in serum of PLWH exhibiting neurocognitive impairment in the Hawaii Aging with HV-Cardiovascular study [95].

Matrix metalloproteinases (MMPs) are a family of neutral proteases involved in tissue remodeling but are implicated in processes of neuroinflammation [86, 87]. MMP-2 and MMP-9 specifically are referred to as gelatinases [96]. Endothelial cells and vascular smooth muscle cells can release MMP-9 when injured or exposed to certain stimuli, such as the systemically acting pro-inflammatory cytokine interleukin-1α (IL-1α), which can be produced by activated monocytes and macrophages [96, 97]. Exposure to the HIV envelope protein gp120 has also been shown to increase production of MMP-2 and MMP-9 [96]. In the CNS, MMP-2 and MMP-9 can degrade components of the basal lamina such as Type IV collagen, fibronectin, laminin, and digest tight junction proteins, leading to BBB breakdown [86, 87, 96]. This has been shown with elevated MMP-9 corresponding to higher Qalb, an indicator of reduced *in vivo* BBB integrity [87]. Both MMP-2 and MMP-9 have been shown to be elevated in the plasma and CSF of individuals diagnosed with HAND, particularly individuals with HAD [87]. CSF MMP-9 has been measured as high as 1050 times in HAND study participants compared to controls. Studies have shown MMP-2 to have a positive correlation with HIV viral load and a negative correlation with CD4-counts, although no such correlations exist for MMP-9 [86].

There is currently no specific treatment for HAND [73, 74]. By correlating changes in inflammatory biomarkers previously linked to HAND with changes in neuropsychological performance testing and BBB integrity in the setting of drug intervention, the results of this study give further insight into the underlying molecular mechanisms of HAND pathogenesis and inform the development of targeted prevention and treatment interventions for HAND.

ANTIRETROVIRAL THERAPY AND HAND

Dramatic strides in HIV prevention and treatment have occurred since the beginning of the HIV epidemic in the early 1980's, including a 50% decline in deaths from AIDS upon the introduction of ART in 1996 and a 40-50% decline in the incidence of HAD [24, 98]. The estimated life expectancy of a 20-year old PLWH in the United States or Canada on adherent, life-long ART is now in the early seventies, approximately five years less than the estimated life expectancy of an uninfected individual in the general population [99, 100]. Furthermore, the rare event of CNS escape in PLWH on adherent, life-long ART provides evidence that current ART regimens control HIV in the CNS, despite low-level viremia that may drive the development of HAND [101].

According to the National Institutes of Health (NIH), standard ART regimens (Figure 4) generally include two nucleoside reverse transcriptase inhibitors (NRTIs) plus an integrase strand transfer inhibitor (INSTI), a non-nucleoside reverse transcriptase inhibitor (NNRTI), or a protease inhibitor (PI) [102]. However, the World Health Organization defines standard ART as at least three drugs that maximally suppress HIV replication [67]. ART regimens vary based upon side effects, comorbidities, drug resistance, drug-drug interactions or even cost [102]. Including drugs from classes other than NRTIs, NNRTIs, INSTIs, and PIs may help reduce inflammation that persists even with viral suppression: fusion inhibitors, post-attachment inhibitors, and a relatively new class of drugs referred to as CCR5-antagonists or CCR5-inhibitors [102, 103].

ART regimens are rated on their CNS penetration-effectiveness, with "neurologically active" regimens considered to be the most efficient in blocking ongoing replication in the CNS [101, 104, 105]. However, some studies suggest ART results in neurotoxic effects, with one study assessing 15 ART of different classes showing evidence of neuronal toxicity *in vivo* (highest neurotoxicity: abacavir, efavirenz, etravirine, nevirapine, and atazanavir; lowest neurotoxicity: darunavir, emtricitabine, tenofovir, and maraviroc) and another finding that the commonly used NNRTI efavirenz causes neuronal injury *in vitro* [105, 106]. However, the clinical implications of ART neurotoxicity are not known and no cessation of ART is recommended based upon studies of neurotoxicity [24, 104].

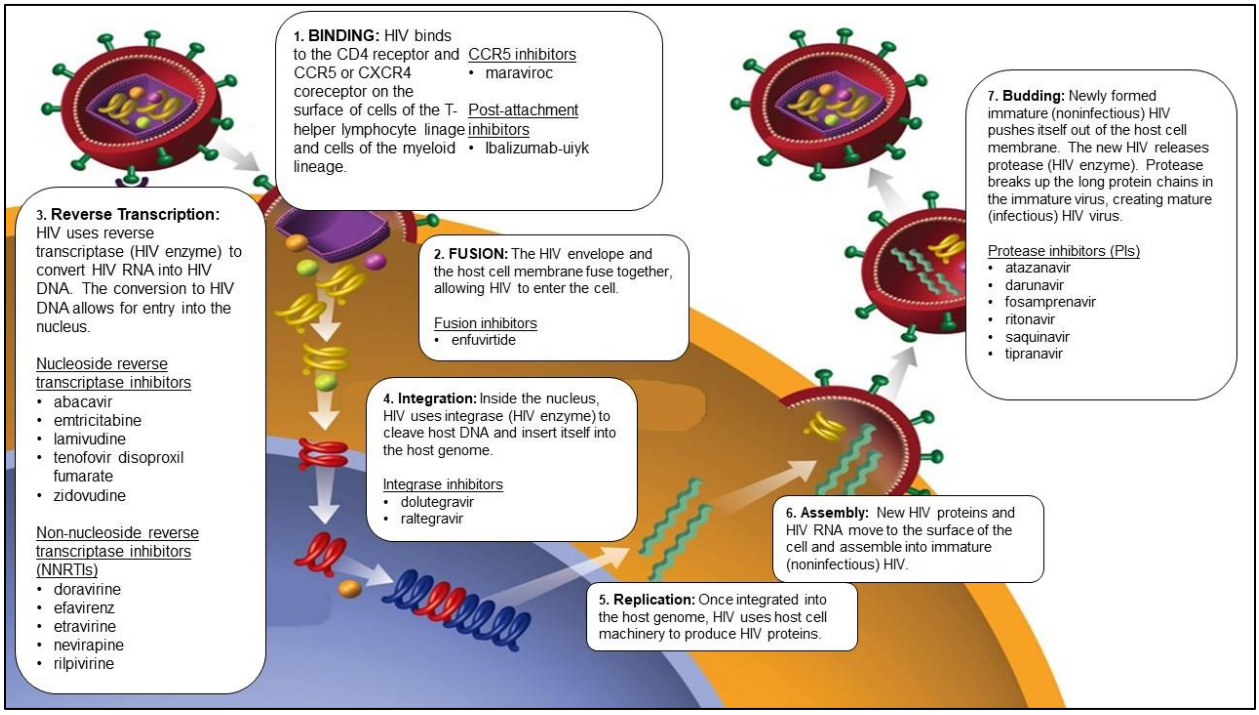

Figure 4. HIV replication cycle and corresponding ART. CCR5-inhibitors and post-attachment inhibitors stop HIV virus from binding to the host cell. Fusion of the HIV envelope with the host cell membrane is halted by fusion inhibitors. Conversion of HIV

RNA into HIV DNA, also known as reverse transcription, is obstructed by NRTIs and NNRTIs. Integration of HIV DNA into the host genome is impeded by integrase inhibitors. Protease inhibitors stop the HIV enzyme protease from cleaving long protein chains in immature viral particles [73, 107].

A strategy that has been recently explored to reduce the HIV CNS reservoir has been the use of latency reversal agents (LRAs). However, LRAs are not efficient at penetrating the CNS and are found to be less effective on viral strains present in the CNS [104]. A promising alternative strategy to LRAs to reduce the HIV CNS reservoir is to target monocytes to prevent CNS viral seeding, ongoing viral replication, and inflammation as a result of HIV-infected monocyte trafficking across the blood-brain barrier. Studies have shown that ART with high effectiveness in inhibiting monocyte infection, such as CCR5-inhibitor maraviroc, has correlated with positive neuropsychological performance outcomes [32, 33, 101, 108-111].

CCR5-INHIBITOR MARAVIROC

CCR5 is a receptor that is expressed on immune cells including monocytes, macrophages, dendritic cells, T-helper cells, astrocytes, microglia, neurons, and is also expressed on the vascular endothelium [29]. CCR5 plays a role in immune signaling and is an important co-receptor for HIV viral entry [29, 107]. HIV isolates from brain tissue show a greater tropism towards monocytes and an ability to infect cells expressing low levels of CD4 and CCR5 [25, 112-114]. Furthermore, it has been shown that individuals who are homozygous for the CCR5Δ32 mutation abolishing CCR5 aren't susceptible to HIV viral strains that utilize the CCR5-receptor (M-tropic, R5 strains). Thus, PLWH who are heterozygous for CCR5Δ32

progress more slowly to AIDS [107, 110]. This information suggests that blocking CCR5 could benefit PLWH.

To mimic the reduction of CCR5 expression, oral-dose, non-competitive CCR5-inhibitor maraviroc could potentially be used (Figure 5). Biochemical studies suggest that maraviroc is small-molecule allosteric modulator that stabilizes CCR5 in an inactive confirmation [115]. Maraviroc is known to efficiently penetrate the CNS (CSF concentration: 1.83–12.2 ng/mL) and reduce viral load [25, 29, 32, 109, 110]. Furthermore, maraviroc has been shown in preliminary studies to show improvements in neuropsychological performance in PLWH who are on suppressive ART [32, 111]. However, similar improvement has not been shown in ART-naïve PLWH [116, 117].

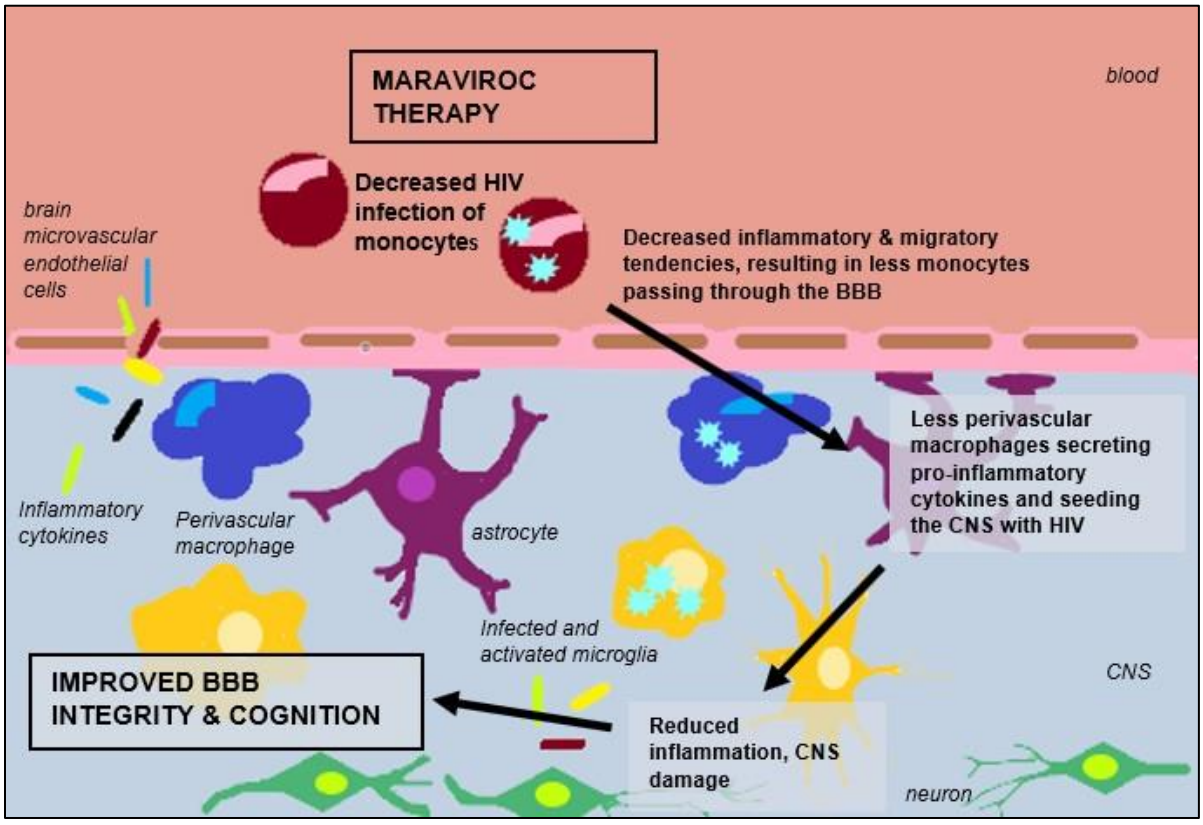

Figure 5. Proposed maraviroc effect on HIV-infected monocyte trafficking across the BBB in CNS infection [25, 29-32].

Of the two studies assessing impact of maraviroc on neuropsychological performance in virally suppressed PLWH (HIV RNA <50 copies/ml), Ndhlovu *et al* assessed outcomes at 24 weeks and Gates *et al* assessed outcomes at six months and twelve months [32, 111]. At 24 weeks, Ndhlovu *et al* observed no significant changes in overall neuropsychological performance (global NPZ) but improvement in executive functioning ($p= 0.08$) in twelve study participants [32]. However, in analyses of the six study participants who entered the study with neuropsychological impairment (global NPZ $\leq$ -0.5), maraviroc showed significant improvement on global NPZ scores ($p=0.03$), neurocognitive subdomains of learning and memory ($p=0.03$), and executive function ($p=0.046$) [32]. Analysis of the inflammatory biomarker sCD163 was

observed to decline in the plasma in all individuals over the study (p=0.0039) [32]. Gates *et al* showed improved global NPZ scores in nine study participants with HAND at six months ($p<0.05$) and at twelve months ($p <0.77$) of maraviroc therapy, but no changes in CSF biomarkers, including neopterin [111].

Maraviroc blockage of CCR5 is theorized to inhibit an inflammatory cascade leading to positive clinical outcomes in the form of improved neuropsychological performance (Figure 5). It is hypothesized that through inhibition of CCR5, maraviroc will decrease HIV infection of monocytes and inflammatory and migratory tendencies of monocytes crossing BBB [29, 30]. As a result of less infected monocyte trafficking across the BBB, less HIV viral seeding in the brain will occur, along with a reduction in the secretion of pro-inflammatory cytokines and chemokines [25, 32]. Due to less viral seeding and secretion of pro-inflammatory cytokines and chemokines, overall brain inflammation and CNS damage will be reduced, resulting in improved BBB integrity and neuropsychological performance in PLWH on a maraviroc regimen [31, 32].

MARAVIROC AS PRE-EXPOSURE PROPHYLAXIS (PrEP)

In lieu of an effective HIV vaccine, preventative measures using NRTIs have been adopted for clinical use. Combination once-daily PrEP drugs, consisting of emtrictabine (FTC, 200 mg) and tenofovir disoproxil fumarate (TDF, 300 mg), have been shown to be 99% effective in HIV prevention [118]. However, efficacy drops significantly with non-adherence (44–75%) [119, 120]. Furthermore, although the majority of drug resistance noted in PrEP trials has been a result of previously unrecognized acute HIV infection, rare instances of resistance to PrEP have occurred in those infected after study enrollment [60, 121, 122]. Since PrEP drugs (TDF and

FTC) are recommended among initial ART regimens, increased drug resistance could compromise treatment for PLWH and utilization as PrEP [66, 123, 124]. Therefore, additional strategies for PrEP such as replacement with or inclusion of a CCR5-inhibitor, such as maraviroc, have been proposed [6, 85, 122, 123, 125].

Studies assessing maraviroc alone as PrEP have appeared to be ineffective in preventing HIV infection, both in an *ex vivo* rectal biopsy model and in a macaque simian HIV model in which five of six animals became infected after rectal challenge despite measured peak maraviroc concentrations in rectal sections [126, 127]. However, a recent phase 2 clinical trial assessing maraviroc in combination with TDF or FTC showed promising results by preventing infection in individuals with adequate drug plasma concentrations [123]. While many future HIV treatment and PrEP strategies focus on long-acting, slow-release nanoparticle therapy due to an ability to maintain higher plasma concentrations for a sustained period, these therapies are still in development [128, 129]. TDF, FTC, and maraviroc are approved therapies routinely prescribed in HIV prevention and infection and have proven to be safe in clinical trials, which may allow for quicker approval of clinical use as alternative PrEP strategies [128].

SCOPE OF DISSERTATION RESEARCH

HIV entry into the CNS is considered to occur early in infection with the primary route of entry being HIV-infected monocytes that traverse the BBB. Neuroinflammation persists even with suppressive ART, which contributes to the development of HAND affecting more than half of PLWH. While biomarkers for HAND could be clinically valuable, gaps in knowledge remain regarding the clinical relevance of certain biomarkers of HAND and how they might be useful in

strategies to prevent HAND. Since the CCR5-receptor is a co-receptor for HIV entry into monocytes, inhibition of the receptor by the non-competitive, receptor antagonist maraviroc might reduce neuroinflammation and improve BBB integrity and neuropsychological performance in PLWH.

Therefore, the objective of the proposed research was to identify biological markers (biomarkers) of neuroinflammation impacted by CCR5 inhibition, which could identify changes in neuropsychological performance and BBB integrity. The central hypothesis was that 48 weeks of maraviroc intensification would reduce neuroinflammatory biomarkers and improve BBB integrity and thus correlate with improved neuropsychological performance. In parallel, *in vitro* BBB exposure to PrEP drugs with maraviroc would reduce transmigration of uninfected monocytes.

SPECIFIC AIMS

The following specific aims will test the central hypothesis:

Specific Aim 1: Measure neuroinflammatory biomarkers which correspond to disruption of BBB integrity and neurocognitive impairment in HIV-positive study participants pre- and post-maraviroc intensification.

Hypothesis: Maraviroc intensification in HIV-infected study participants with mild to moderate HAND defined as global neuropsychological performance test (global NPZ) scores of < -0.5 or an abnormality (< -0.5) in at least one neurocognitive domain typically affected by HIV

(executive function, psychomotor speed and attention, learning and memory) will result in a reduction of neuroinflammatory biomarkers and improvements in neuropsychological performance after 48 weeks.

Experimental plan: TNFα, IL-6, MMP-2, and MMP-9, and S100β were analyzed in serum (diluted 1:2) and CSF (undiluted) utilizing an antibody-based, magnetic bead multiplex assay (R&D Systems, Minneapolis, MN, USA). MMP-2 and MMP-9 were analyzed in serum (diluted 1:50) and CSF (undiluted) utilizing an antibody-based, magnetic bead multiplex assay (R&D Systems, Minneapolis, MN, USA). sCD14 was analyzed in serum (diluted 1:200) and CSF (diluted 1:50) via sandwich ELISA (R&D Systems, Minneapolis, MN, USA). sCD163 was analyzed in serum (diluted 1:500) and in CSF (diluted 1:50) via sandwich ELISA (IQ Products, The Netherlands). Neopterin was analyzed (1:2) in serum and undiluted in CSF via competitive ELISA (Biomatik Corporation, ON, Canada). Neuropsychological performance testing was completed by neuropsychologists and evaluated the neurocognitive subdomains of executive function, learning and memory, working memory, and psychomotor speed. Based on performance in each neurocognitive subdomain along with assessment of gross motor skills, a global NPZ score was calculated to reflect overall neuropsychological performance. Study participants were grouped into two groups reflecting overall performance outcome after 48 weeks of maraviroc treatment, improved global NPZ score or declined global NPZ score. Spearman rank correlation coefficients and corresponding p-values were calculated to determine relationships between changes in neuroinflammatory biomarkers and changes in neuropsychological performance to determine which changes in biomarkers, if any, correlated to improved neuropsychological performance.

***Specific Aim 2*: Determine if maraviroc intensification impacts BBB permeability in vivo and in vitro.**

Hypothesis: Reduction of neuroinflammation as a result of maraviroc treatment for 48 weeks in HIV-infected individuals will result in improved BBB integrity.

Experimental plan: Albumin was measured in CSF (diluted 1:5,000) and serum (diluted 1:5,000,000) using a sandwich ELISA (Bethyl Laboratories Inc., Montgomery, TX). A ratio of CSF albumin to serum albumin was calculated to determine Qalb. *In vitro* BBB integrity was assessed by TEER and by FITC-dextran permeability. Neuropsychological performance testing was completed and neuroinflammatory biomarkers were analyzed in serum and CSF at entry and week 48 as described. Spearman rank correlation coefficients and corresponding p-values were calculated to examine relationships between changes in neuroinflammatory biomarkers and changes in *in vitro* and *in vivo* BBB integrity as well as the relationship between *in vivo* and *in vitro* BBB integrity. The relationships between Specific Aims 1 and 2 are described in Figure 6.

***Specific Aim 3*: Assess molecular and physiological impact of PrEP drugs with and without maraviroc on an in vitro BBB model.**

Hypothesis: Presence of BBB structural and functional proteins in BMVEC of the BBB will not be affected by either PrEP or maraviroc. We hypothesize no reduction in HIV-negative monocyte transmigration with PrEP alone, but reduced transmigration with maraviroc treatment.

Experimental plan: BMVEC grown to confluence either without the presence of drug or in the presence of 0.1 μM PrEP drugs with and without 0.2 μM maraviroc and assessed for expression of PECAM-1, ZO-1, ICAM-1, and occludin by ELISA and immunofluorescence. Mann-Whitney tests were completed to assess differences between conditions. To assess whether PrEP

with and without an *in vitro* addition of maraviroc impacted transmigration of monocytes across an *in vitro* BBB model, peripheral blood mononuclear cells (PBMC) from healthy volunteers before and after 12 weeks of oral PrEP were transmigrated for 24 hours at 37°C, 5% CO_2, across an *in vitro* BBB model with MCP-1 used as a chemoattractant for monocytes. After 24 hours, transmigrated cells were collected and analyzed via flow cytometry for the number of CD3-CD14+ cells (monocytes). The percentage of monocytes transmigrated was calculated based on the total number transmigrated by the total number of monocytes in an aliquot of PBMC in a pre-transmigration sample. Mann-Whitney tests were completed to assess differences between conditions.

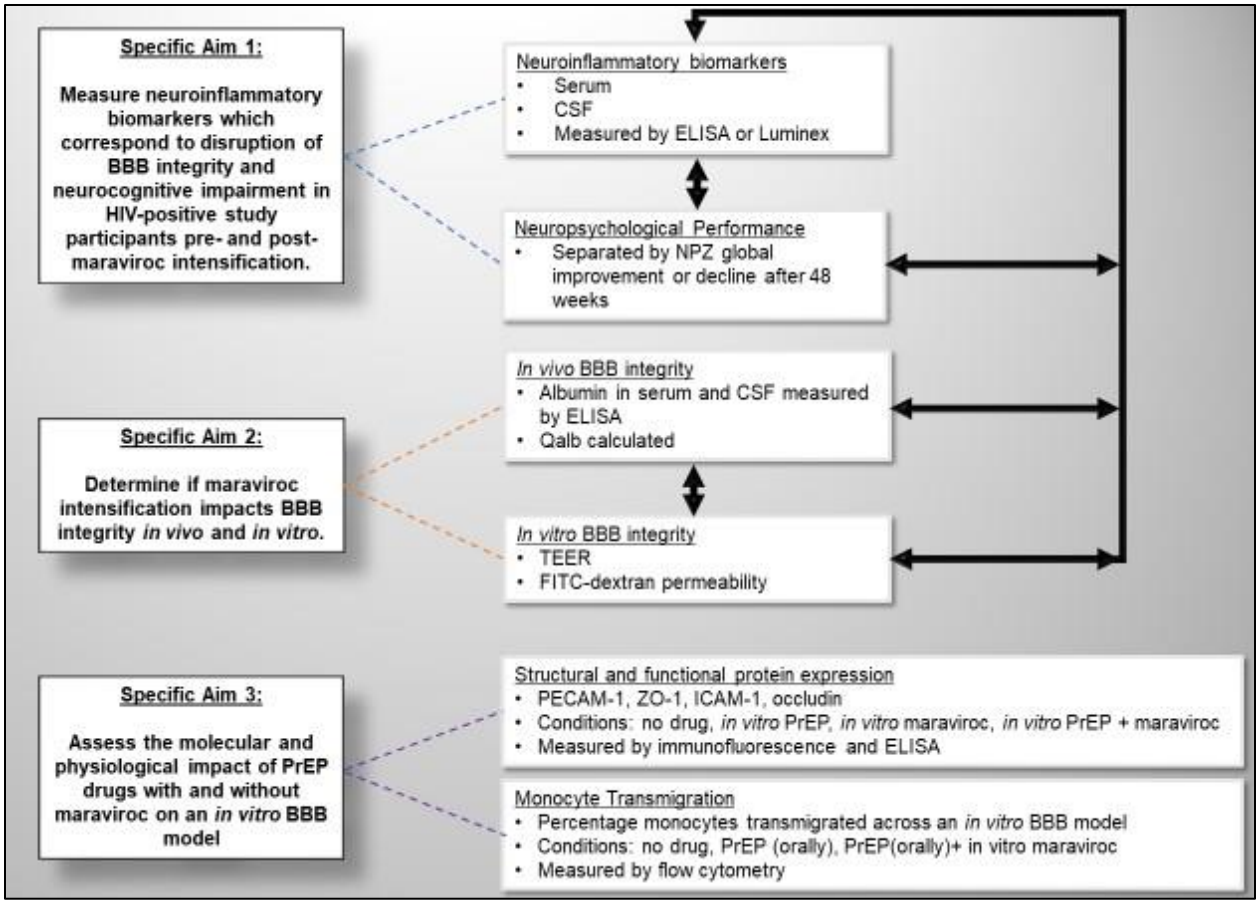

Figure 6. Specific Aims Summary. Neuroinflammatory biomarkers in serum and CSF, neuropsychological performance, and measures of *in vivo* and *in vitro* BBB integrity were analyzed before and after maraviroc intensification. Changes in neuroinflammatory biomarkers in serum and CSF were correlated with changes in neuropsychological performance and changes in *in vivo* and *in vitro* measures of BBB integrity. Changes in i*n vivo* and *in vitro* measures of BBB integrity were also assessed for strength of correlation.

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CHAPTER 2

IN VIVO AND *IN VITRO* IMPACT OF CCR5-INHIBITOR MARAVIROC

ABSTRACT

There is no treatment for HAND and the clinical relevance of biomarkers related to HAND is unclear. This study determined the impacts of the C-C chemokine receptor type 5-inhibitor maraviroc on biomarkers of inflammation and blood-brain barrier (BBB) integrity to potentially inform new HAND interventions. Improved global neuropsychological performance (NPZ) scores did not correlate to reduced inflammation and decreased *in vivo* BBB integrity was observed, correlating to increased cerebrospinal fluid tumor necrosis factor α and serum calcium-binding protein B of the S-100 protein family. Reduced inflammation (periphery vs. central nervous system) with increased inflammation in the alternate compartment indicates the challenge of identifying treatments to reduce inflammation, which may impact HAND progression.

INTRODUCTION

More than half of the 37.9 million PLWH worldwide experience neurocognitive impairment, defined as HAND [1, 2]. Since the introduction of ART, PLWH are less likely to have HAD which severely impacts activities of daily living [3]. However, there is increased prevalence of individuals with less severe forms of HAND who exhibit mild to moderate deficits defined as MND and those with ANI [4, 5].

HAND likely results from processes of inflammation, neuronal injury, and BBB dysfunction in part due to HIV trafficking to the CNS even in the face of undetectable viral loads [6, 7]. HAND is diagnosed by performance on neuropsychological tests, which may also be accompanied by evidence of interference in activities of daily living such as general mental acuity, inefficiency at work, homemaking or social functioning by either self-report or observation by others familiar with the individual [8, 9]. Multiple studies have attempted to identify biomarkers with prognostic significance or to utilize for HAND diagnosis [8]. However, the clinical relevance of many potential HAND biomarkers remains unclear and none have been confirmed to be predictive or diagnostic [5, 10]. With no specific treatments for HAND, clarifying the clinical relevance of HAND biomarkers could inform new treatment targets or interventions [5, 9]. Furthermore, discovering preventative strategies for HAND could reduce morbidity for PLWH.

One proposed approach is to block CCR5, an HIV co-receptor for virus entry into monocytes, which traverse the BBB causing CNS infection [10, 11]. Pharmaceutical agents that block the CCR5 co-receptor have the potential to interrupt these events which occur early in infection [10].

Maraviroc is a non-competitive CCR5 antagonist [11]. In addition to the ability to block the CCR5 co-receptor that HIV uses for viral entry into cells such as monocytes, maraviroc penetrates the CNS and preliminary studies suggest improvements in neuropsychological performance in patients treated with maraviroc [7, 12]. Through either direct blockage of monocyte infection or BBB penetration, maraviroc could reduce neuroinflammation and improve neurocognition in PLWH [10].

MATERIALS AND METHODS

Cohort and Clinical Specimens

Research samples were collected from participants enrolled in a randomized, double-blind clinical trial, called "Maraviroc and NeuroAIDS Pathogenesis" (NCT 02159027), which was conducted at the Hawaii Center for AIDS (HICFA), John A. Burns School of Medicine (JABSOM), University of Hawaii (UH) at Manoa, Honolulu, HI. Entry criteria were: HIV-positive patients on uninterrupted ART regimens defined by Department of Health and Human Services guidelines for greater than or equal to one year with plasma HIV <50 copies/mL and mild to moderate neurocognitive impairment defined as global neuropsychological performance test (global NPZ) scores of <-0.5 or an abnormality (< -0.5) in at least one neurocognitive domain typically affected by HIV (executive function, psychomotor speed and attention, learning and memory) [13]. Following informed consent per guidelines approved by the UH Institutional Review Board, eligible study participants were randomized to either placebo or treatment (maraviroc). Maraviroc dose was based on the participant's current ART regimen (Table 1).

Table 1 Study participant dosage and neuropsychological performance

Participant	ART regimen	Maraviroc dosage	Entry Global NPZ	Week 48 Global NPZ	Change Global NPZ
1	cobicistat, elvitegravir, emtricitabine, tenofovir disoproxil fumarate	150 mg/twice daily	-0.57	-1.43	-0.86
2	emtricitabine, rilpivirine, tenofovir alafenamide	300 mg/twice daily	-1.23	-0.70	+0.53
3	abacavir, efavirenz, lamivudine	600 mg/twice daily	-1.84	-0.97	+0.87
4	emtricitabine, etravirine, raltegravir, tenofovir alafenamide	600 mg/twice daily	-0.95	-0.37	+0.58
5	cobicistat, darunavir, emtricitabine, tenofovir alafenamide	150 mg/twice daily	-1.32	-0.96	+0.36

Neuropsychological performance testing was completed at entry and week 48 testing was completed within two weeks of the final study visit, while study participants were still taking maraviroc. For the current report, eligible participants were those with paired serum and CSF at entry and week 48 in the treatment arm only. Serum was collected, processed, and stored at -80°C until thawed for assays. CSF was collected from lumbar punctures, processed, and stored at -80°C until thawed for assays.

Biomarker Assays

The biomarkers assayed are summarized in Table 2. TNFα, IL-6, MMP-2, MMP-9, and S100B were measured in serum and CSF in duplicate, using an antibody-based, magnetic bead multiplex Luminex assay (R&D Systems, Minneapolis, MN, USA).

Table 2 Biomarker Panel

Biomarker	Source	Association
TNFα (Brew *et al*, 2009)	serum, CSF	systemic inflammation, microglial activation, astrocytosis, neuronal death
IL-6 (Brew *et al*, 2009; Kamat *et al*, 2012)	serum, CSF	systemic inflammation, microglial activation, astrocytosis
sCD14 (Kamat *et al*, 2012; Ndhlovu *et al*, 2014)	serum, CSF	systemic inflammation, monocyte activation
Neopterin (Barber *et al*, 2018)	serum, CSF	linked to HAD severity, indicates BBB dysfunction with increased CSF to serum albumin ratios (Qalb)
S100B (Barber *et al*, 2018; Brew *et al*, 2009)	serum, CSF	astrocytosis, neuronal death, BBB dysfunction, decreased executive functioning
MMP-9 (de Almeida *et al*, 2017; Xing *et al*, 2017)	serum, CSF	BBB dysfunction
MMP-2 (de Almeida *et al*, 2017; Xing *et al*, 2017)	serum, CSF	BBB dysfunction

sCD14, sCD163, neopterin, and albumin were measured by ELISA. sCD14 was quantified in serum (diluted 1:200) and CSF (undiluted) via sandwich ELISA (R&D Systems, Minneapolis, MN, USA). Neopterin was measured in serum (diluted 1:2) and CSF (undiluted) via competitive ELISA (Biomatik Corporation, ON, Canada). sCD163 was analyzed in serum (diluted 1:500) and CSF (diluted 1:50) via sandwich ELISA (IQ Products, The Netherlands). Albumin was measured in serum (diluted 1:5,000,000) and CSF (diluted 1:5,000) to calculate Qalb. A sandwich ELISA (Bethyl Laboratories, Inc., Montgomery, TX, USA) was used. All samples were assayed in duplicate. Reactions were quantified spectrophotometrically at 450 nm.

Blood-brain Barrier (BBB)

BBB bilayers were constructed using human adult primary brain microvascular endothelial cells ($2x10^4$ cells/well) (Angio-Proteomie, Boston, MA) and astrocytes ($10x10^4$ cells/well) (Angio-Proteomie, Boston, MA) cultured on opposite sides of 24-well polyethylene terephthalate (PET) inserts containing 3-μm pores (Corning, Corning, NY) coated with rat-tail collagen type I (Sigma-Aldrich, St. Louis, MO) at 50 μg/mL and grown to confluence over six days. Approximately 12–16 hours prior to experiments, bilayers were switched to basal medium without growth factors.

In vitro BBB Serum Incubation Assay

To determine the impact of participant serum on *in vitro* BBB integrity, 20% sera from entry and week 48 was incubated on the apical side of bilayers for 24 hours at 37°, 5% CO_2. BBB TEER, measured in duplicate, was determined using an epithelial voltohmmeter (World Precision Instruments, Sarasota, FL). TEER was adjusted to PET inserts in growth medium without cells. BBB permeability, measured in duplicate, was determined using 4 kDa FITC–dextran. After 24 hours of serum incubation at 37°C, 5% CO_2, 150 μL of 100 μg/mL FITC-dextran was added to the apical side of bilayers and incubated for 2 hours at 37°C, 5% CO_2. After incubation, 150 μL media was removed from the basolateral side of the well and analyzed on Victor 1420 fluorescence microplate reader (PerkinElmer, USA) [14].

Statistical Methods

Spearman rank correlation coefficients and corresponding p-values (p) were calculated to examine correlations between biomarkers, measures of BBB integrity *in vitro* and *in vivo*, and outcomes of neuropsychological performance testing at baseline and week 48. Due to the small sample size, a p-value of 0.05 was unattainable and thus, $p < 0.1$ was used as the criteria for statistical significance. R 3.5.1 was used for all analyses. GraphPad Prism 8 (GraphPad Software, San Diego, CA) was used to create all figures.

RESULTS

This study assessed five male participants who were between 44 and 66 years of age and received maraviroc for 48 weeks. Participants were assessed for differences in inflammatory biomarkers and BBB integrity based on global NPZ improvement or decline.

Four participants who had improved global NPZ scores after starting maraviroc demonstrated slight decreases in *in vivo* BBB integrity (increased Qalb, mean change: +0.18) (Figure 1). These same study participants had slight increased CSF TNFα (mean change: +0.03 pg/mL; p=0.08), but decreased CSF IL-6 (mean change: -0.74 pg/mL; p=0.08) (Figure 1 and Figure 2b). However, the associations disappeared when the participant who demonstrated a decreased global NPZ score was included in analyses with the other four participants (Figure 1 and Figure 2b).

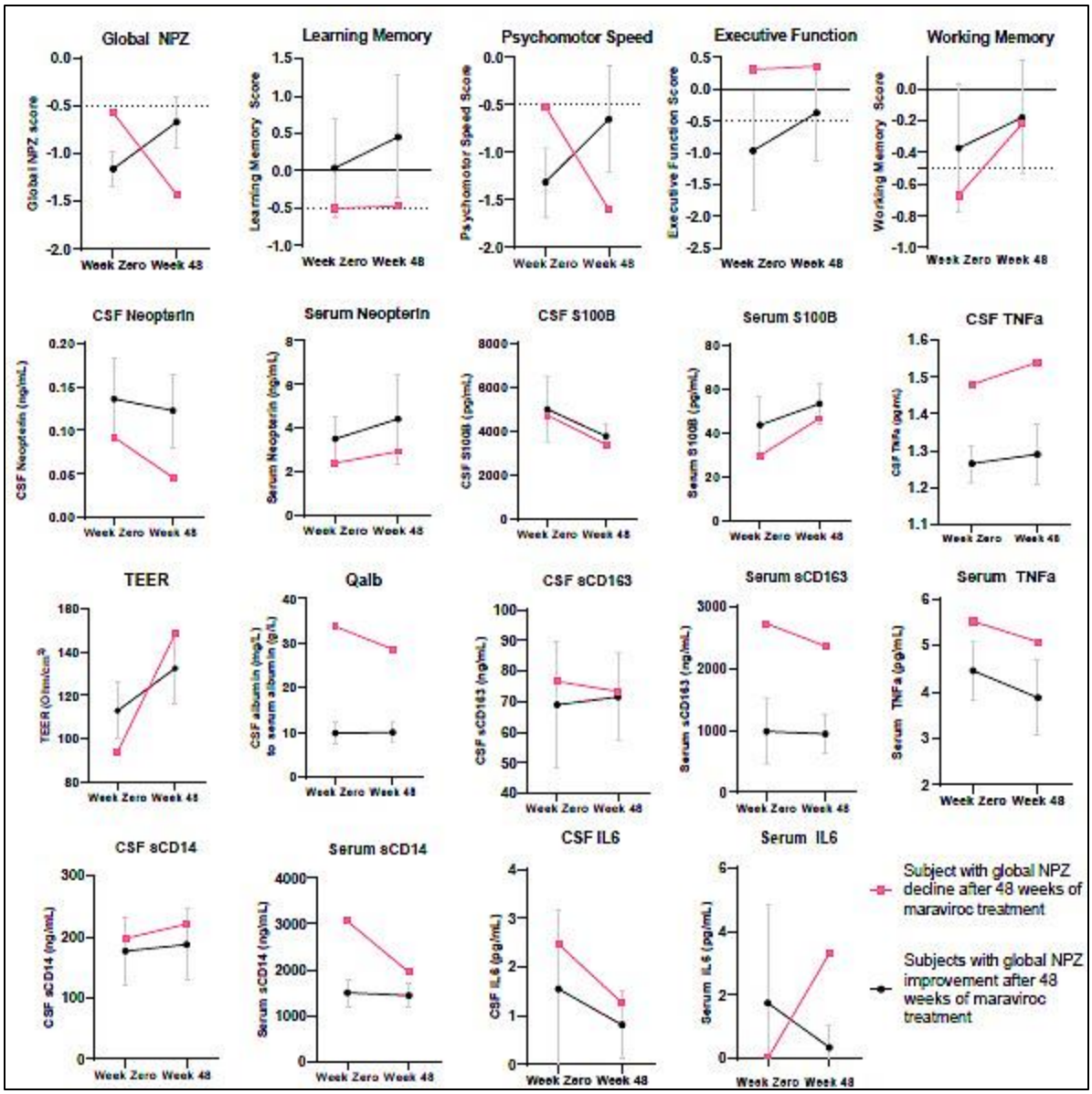

Figure 1. Graphical depiction of change in neuropsychological performance, biomarkers of inflammation, and *in vivo* BBB integrity 48 weeks after maraviroc treatment. Four participants with improved global NPZ scores (black) versus single participant with declined global NPZ score (red).

These four participants had a stronger correlation with increased serum S100B (mean change: +9.78 pg/mL) to improved working memory (mean change: +0.19; p=0.08), but also to

slightly increased Qalb (mean change +0.18; p=0.08) (Figure 1 and Figures 2a, 2b). An increase in CSF sCD163 (mean change: +2.53 ng/mL) correlated with improved psychomotor speed (mean change: +0.66; p=0.08) and global NPZ (mean change: +0.49; p=0.08) (Figure 1 and Figure 2a). Furthermore, an increase in *in vitro* BBB integrity as measured by TEER (mean change: 19.70 Ω/cm^2) correlated with increased CSF sCD14 (mean change: +10.75 ng/mL; p=0.08) and improved learning memory (mean change: +0.42, p=0.08) (Figure 1 and Figures 2b, 2c). These associations disappeared when considering all five participants (Figure 1 and Figures 2b, 2c).

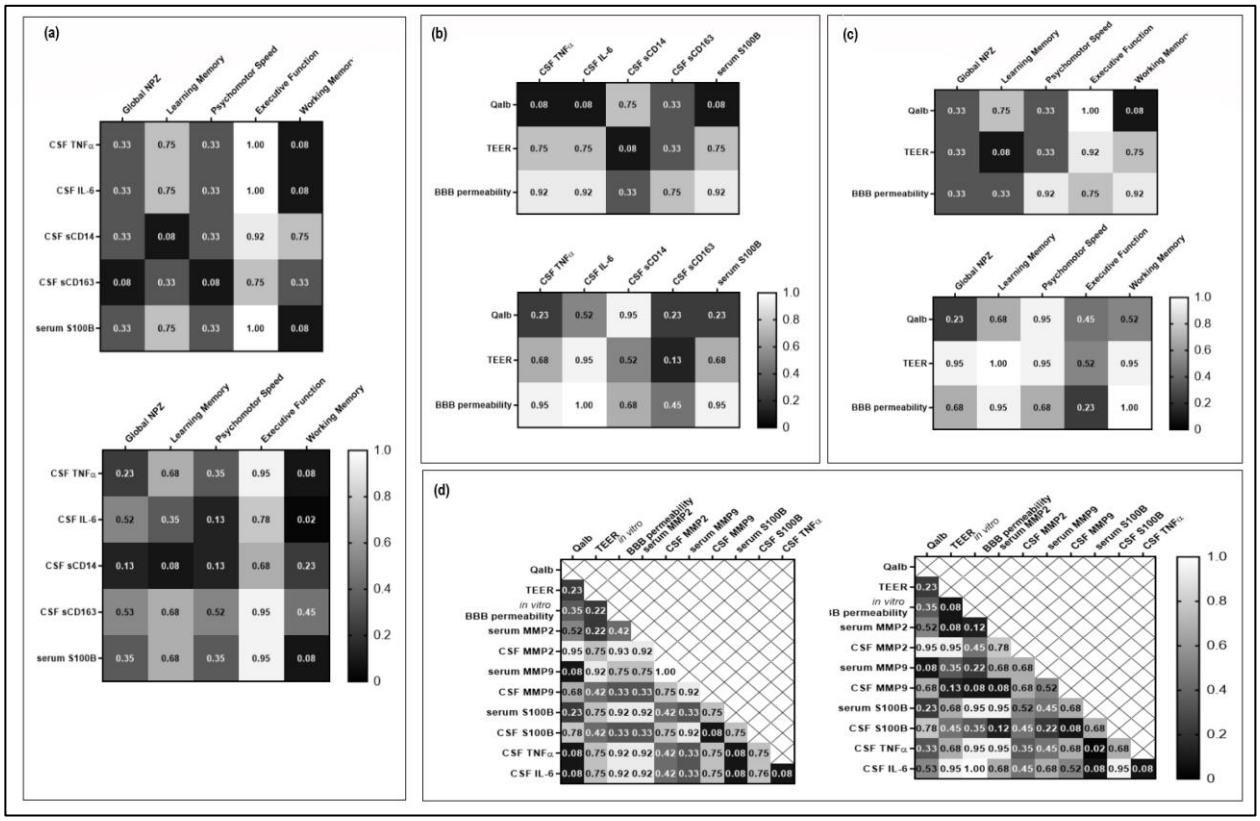

Figure 2. Spearman correlation p-values between changes in neuropsychological performance, biomarkers of inflammation, and BBB integrity. **a)** Changes in neurocognitive performance versus changes in biomarkers of inflammation CSF TNFα, CSF IL-6, CSF sCD14, CSF sCD163, and serum S100B in four individuals with improved

global NPZ scores (top) versus all five individuals (bottom). **b)** Changes in biomarkers of inflammation CSF TNFα, CSF IL-6, CSF sCD14, CSF sCD163, and serum S100B versus changes in BBB integrity *in vivo* and *in vitro* in four individuals with improved global NPZ scores (top) versus all five individuals (bottom). **c)** Changes in neuropsychological performance versus changes in BBB integrity *in vivo* and *in vitro* in four individuals with improved global NPZ scores (top) versus all five individuals (bottom). **d)** Relationship between changes in biomarkers of inflammation and changes in BBB integrity *in vivo* and *in vitro* in four individuals with improved global NPZ scores (left) versus all five individuals (right).

This study showed no correlations between changes in biomarkers of monocyte activation (neopterin, sCD14) and neuropsychological performance after 48 weeks of maraviroc except for increased CSF sCD163 corresponding to improved (rather than declined) psychomotor speed and global NPZ scores (Figure 2a).

While TEER for all study participants correlated with 4-kDa FITC-dextran BBB permeability, neither measure of *in vitro* BBB integrity correlated with *in vivo* BBB integrity as measured by Qalb (Figure 2d).

Biomarkers MMP-2 and MMP-9 in serum and CSF showed no correlation with neurocognitive changes (data not shown). However, changes in serum MMP-9 correlated to changes in Qalb ($p= 0.08$) for all participants regardless of neuropsychological performance outcomes (Figure 2d). Increased TEER, which was noted for all participants, was correlated to changes in serum MMP-2 ($p= 0.08$) (Figure 2d). Changes in *in vitro* BBB permeability were correlated to changes in CSF MMP-9 ($p= 0.08$) (Figure 2d).

The largest quantitative differences in biomarkers assessed at week 48 between the participant with declined global NPZ (change: -0.8) and the four participants with improved global NPZ scores (mean change: +0.49) were seen in CSF TNFα (1.59 pg/mL v. mean 1.29 pg/mL), serum TNFα (5.08 pg/mL v. mean 3.88 pg/mL), serum IL-6 (3.32 pg/mL v. mean 0.35 pg/mL), serum sCD163 (2360 ng/mL v. mean 944 ng/mL), serum sCD14 (1953 ng/mL v. mean 1445 ng/mL), CSF neopterin (0.05 ng/mL v. mean 0.12 ng/mL), and Qalb (28.66 v. mean 10.02) (Figure 1).

A trend was observed for all five participants in whom there was a reduction of inflammation in one compartment (CNS, CSF; periphery, sera) and an increase of the same biomarker in the alternate compartment. This was noted with reduced CSF neopterin (mean change: -0.02 ng/mL), but increased serum neopterin (mean change: +0.83 ng/mL), as well as reduced CSF S100B (mean change: -1245 pg/mL) but increased serum S100B (mean change: +11.20 pg/mL). Maraviroc treatment resulted in reduced serum sCD14 (mean change: -270 ng/mL) and increased CSF sCD14 (mean change: +13.34 ng/mL) as well as a reduced serum TNFα (mean change: -0.55 pg/mL) but increased CSF TNFα (mean change: +0.03 pg/mL) (Figure 1).

DISCUSSION

Although four of five participants were observed to have improved global NPZ scores over the course of the study, no reductions in inflammatory biomarker(s) correlated with improved global NPZ scores. For these four participants, reduced *in vivo* BBB integrity correlated with increases in inflammatory markers CSF TNFα and serum S100B. However, for

the participant whose global NPZ score declined, Qalb and other biomarkers of inflammation (serum and CSF TNFα, serum IL-6, serum sCD14, serum sCD163) were observed to be higher throughout the course of the study than the four individuals whose global NPZ scores improved.

As a potential biomarker, TNFα, a systemically-acting inflammatory cytokine, is often increased in PLWH [15]. Increased TNFα expression results in dysregulated cytokine secretion, exacerbating a cycle of sustained inflammation. While both TNFα and IL-6 levels are increased in CSF and serum of HAND patients, some reports have indicated increased CSF IL-6 regardless of neurocognitive status [16, 17]. For the four participants whose global NPZ scores improved after 48 weeks of maraviroc treatment, an increase in CSF TNFα, but a decrease in CSF IL-6, correlated to decreased *in vivo* BBB integrity (increased Qalb), indicating the sensitivity of the BBB to CSF TNFα. This was further supported by higher Qalb and higher CSF TNFα exhibited at week 48 by the individual whose global NPZ score declined. When considering all study participants regardless of global NPZ change, there was an exhibited decrease in CSF IL-6, which is consistent with previous reports indicating a lack of correlation between neurocognitive status and IL-6 [3, 17].

S100B, produced primarily by astrocytes, is a biomarker of astrocytosis [18]. Increased CSF S100B has been previously correlated with deficits of executive function and language [19]. Although not significant through statistical analyses, maraviroc treatment for 48 weeks resulted in reduced CSF S100B, as well as improved executive function in all study participants, indicating a consistent trend with results reported by Woods and colleagues [19]. In contrast, a study in PLWH in Uganda found decreased risks of ANI and MND with increased CSF S100B [20]. While the results of this study showed a decrease in CSF S100B, an increase in serum S100B was correlated with improved working memory. Previously reported data from the

Hawaii Aging with HIV-Cardiovascular study found lower serum S100B levels corresponded to neurocognitive impairment [21]. Our study results were consistent with this inverse trend of serum S100B and neurocognition. Disparate links between S100B and neuropsychological performance outcomes support the need for future research regarding the relevance of S100B to HAND.

Biomarkers of monocyte activation consistently linked to HAND include sCD14, sCD163, and neopterin [7, 17, 18]. While it may seem counterintuitive to observe an increase in monocyte markers of activation, such as CSF sCD14 or serum neopterin, occurring in individuals receiving CCR5-inhibitor maraviroc, increased expression of monocyte markers of activation has been noted in the instance of CCR5 blockage, hypothesized to occur due to CCR5-ligands interacting with immune receptors chemokine receptor type 1 (CCR1), CCR3, and CCR4 [22, 23].

The correlation between TEER and 4-kDa FITC-dextran BBB permeability and the lack of correlation between either measurement of *in vitro* BBB integrity with *in vivo* BBB integrity as measured by Qalb indicates the need for improvement of *in vitro* BBB models, so that models may more closely mimic the BBB.

Although there were no correlations between MMP-2 and MMP-9 and neuropsychological performance outcome, changes in serum MMP-2, serum MMP-9, and CSF MMP-9 all correlated to changes in measures of BBB integrity. These results are consistent with literature indicating that increased presence of these inflammatory mediators is closely linked to breakdown of the BBB and thus MMPs may play an important role in compromise of the BBB that contributes to HAND progression [24, 25].

Although descriptive, the observation that the individual with worsening global NPZ score was observed to have higher levels of certain biomarkers of inflammation throughout the study indicates that neuropsychological outcomes are multifactorial and not dependent upon the reduction of one specific biomarker of inflammation. Furthermore, the observations that maraviroc treatment resulted in reductions of inflammation in one compartment (CNS or peripheral) and an increase of the same biomarker in the other compartment speak to the challenges regarding the pharmaceutical strategies for HAND to reduce inflammation in both the CNS and periphery which may impact neurocognition.

The very low number of study participants and the lack of a control group in the current study severely limited the ability to draw clinically relevant conclusions. However, the suggestion that neuropsychological performance outcomes with maraviroc might correspond to reductions in some biomarkers of inflammation warrants further investigation.

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CHAPTER 3

PRE-EXPOSURE PROPHYLAXIS THERAPY AND THE BLOOD-BRAIN BARRIER: IS PREP NEUROPROTECTIVE?

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ABSTRACT

Background: HAND cause morbidity for PLWH. *In vitro* BBB models allow assessment of therapeutics on HIV neuroinvasion. Due to concerns of drug resistance, addition of a CCR5-inhibitor such as maraviroc to current PrEP therapy, TDF and FTC, has been proposed. The effects of PrEP, with and without maraviroc, on the BBB are unknown.

Objective: Compare functional protein expression and TEER in primary human BMVEC to the immortalized cell line hcMEC/D3 for utilization in a bilayer BBB model with primary human astrocytes. Determine the effects, if any, of PrEP, with and without maraviroc, on functional protein expression in the BMVEC monolayer of the BBB and the impact on monocyte transmigration across an *in vitro* bilayer BBB model.

Methods: Immunofluorescence and ELISA were completed to compare expression of PECAM-1, ICAM-1, occludin, and ZO-1 in primary BMVEC compared to hCMEC/D3. TEER was also compared. Immunofluorescence and ELISA assays were completed to assess the impact of PrEP, maraviroc, and maraviroc in combination with PrEP on expression of PECAM-1, ICAM-1, occludin, and ZO-1 in primary BMVEC. Transmigration assays across an *in vitro* bilayer BBB model were completed using peripheral blood mononuclear cells from healthy volunteers before and after beginning a PrEP regimen, with and without the *in vitro* addition of maraviroc, to determine effects on transmigration of monocytes.

Results: Primary BMVEC showed equivalent protein expression and increased TEER in comparison to hCMEC/D3. Exposure of PrEP, maraviroc, and PrEP with maraviroc on primary BMVEC exhibited increased expression of the tight junction protein occludin. In two of three participants instituting a PrEP regimen, PrEP, both with and without *in vitro* addition of maraviroc, resulted in reduction of transmigration of monocytes.

Conclusions: Results indicate PrEP, both with and without maraviroc, may be neuroprotective through the upregulation of the tight junction protein occludin and reduction in transmigration of monocytes. Further research is needed to verify results.

INTRODUCTION

HIV enters the CNS as early as eight days post-infection resulting in viral replication, CNS immune activation, and BBB compromise contributing to HAND [1, 2]. HAND develops in more than 50% of PLWH even with suppressive ART [3]. Therefore, identifying strategies to decrease neuroinvasion could profoundly improve HIV-associated complications.

Brain capillaries, a major component of the BBB, provide a vast area for cerebrovascular exchange, ~12 m^2, which is tightly under homeostatic conditions [4]. BMVEC cover cerebral vessels in a continuous layer on the surface of the basal lamina and exhibit a low pinocytic activity and an increased metabolic rate compared to peripheral endothelial cells [5]. BMVEC are also notable in the ability to form connections between adjacent BMVEC known as adherens and tight junctions. Tight junction proteins (occludin, claudins, junctional adhesion molecules) barricade intercellular space by forming a multi-protein complex with cytoplasmic proteins such as ZO-1, ZO-2, ZO-3 and cingulin, which are anchored to the cytoskeleton. Tight junctions of the BBB cause a high TEER (>1000 Ω/cm^2) compared to peripheral capillaries (2-20 Ω/cm^2) and reduce paracellular and transcellular permeability [4, 5].

Pericytes and astrocytes are important supportive components of the BBB by modulating BMVEC maturation and protein expression, such as tight junctions. During acute and chronic HIV neuroinvasion, infected blood monocytes traverse the BBB and become activated perivascular macrophages, which can produce HIV virions, infecting astrocytes and microglia [6]. Production of neuroinflammatory mediators by perivascular macrophages, microglia, and astrocytes damages neurons and oligodendrocytes, dysregulates cytokine secretion, and causes sustained neuroinflammation. Resultant disruption of BBB regulation allows for inflammatory

cytokines to enter from the periphery and results in an increase in transmigration of infected monocytes, contributing to HAND progression [1, 6, 7]. Data has shown BBB compromise occurs in up to 22% of PLWH with undetectable viral loads, which shows CNS damage and HAND progression occurs even with suppressive ART [8].

In vitro BBB models provide a tool to study HIV neuroinvasion and analyze molecular and physiological effects of pharmacological interventions. However, there is no "gold standard" *in vitro* BBB model designed to study HIV infection of the CNS and pharmaceutical interventions. Existing models incorporate single and multi-layer designs with varying cellular matrices and culture conditions. Cell sources vary and may include primary human cells, rat, bovine, porcine, and the human BMVEC immortalized cell line, hCMEC/D3 [5, 9]. hCMEC/D3 cells originated from vessels of the temporal lobe from a patient with epilepsy, transduced by lentiviral vectors incorporating human telomerase or SV40 T antigen. Due to the difficulty of isolating primary BMVEC, hCMEC/D3 cells have provided a reliable source for *in vitro* BBB models due to stable growth and BMVEC characteristics at least through the 35^{th} passage with comparable leukocyte transmigration dynamics [9]. However, hCMEC/D3 cells have low and varied TEER (from 30 Ω/cm2 to >100Ω/cm2), suggesting that primary BMVEC provide a more biologically relevant cell type for use in *in vitro* BBB models. The commercial availability of human primary adult BMVEC removes technical barriers to using primary BMVEC in *in vitro* BBB models. This study is the first to compare commercial human adult primary BMVEC to hCMEC/D3 cells for use in an *in vitro* bilayer BBB model for studies examining HIV infection of the CNS. These comparative analyses validate commercial human adult primary BMVEC to show similar or increased BMVEC protein expression to hCMEC/D3 cells and increased TEER,

providing an accessible human primary BMVEC source for use in *in vitro* BBB models including the bilayer model utilized in this study for transmigration experiments.

PrEP, consisting of FTC (200 mg) and TDF (300 mg), prevents HIV infection with 99% effectiveness when taken daily [10]. However, there are concerns about drug resistance, and little is known about the effects of TDF and FTC on the blood-brain barrier (BBB) [12]. The vast area of the BBB (~12m^2) is thought to be the main route of HIV seeding in the brain through infected blood monocytes, contributing to the development of HAND [4, 6]. Since HAND causes morbidity for more than half of PLWH and PrEP drugs TDF and FTC are also two drugs commonly used in regimens for PLWH, it is important to determine the impact of PrEP drugs on the BBB [2].

Efficacy of PrEP drops significantly with non-adherence (44-75%) and due to concerns of drug resistance, additional strategies have been proposed, such as replacement with or inclusion of a CCR5-inhibitor, such as maraviroc [10-13]. Data suggests maraviroc reduces HIV-infected monocyte transmigration across the BBB and improves cognition in PLWH [3, 7]. Although maraviroc alone is insufficient as PrEP, maraviroc in combination with TDF or FTC appears to be safe and potentially effective in a recent phase 2 clinical trial, meriting further exploration as a potential addition to current PrEP to guard against potential drug resistance [11, 12, 14].

This study is the first to compare the effects of PrEP drugs TDF and FTC, maraviroc, and PrEP drugs with maraviroc, on primary BMVEC as well as the effects of PrEP therapy with and without an *in vitro* addition of maraviroc on HIV-negative monocyte transmigration across an *in vitro* bilayer BBB model [15].

MATERIALS AND METHODS

Blood-brain Barrier (BBB)

Monolayers were constructed using commercially acquired human adult primary BMVEC (Angio-Proteomie, Boston, MA) and hCMEC/D3 cells (courtesy of Dr. Monique Stins, Johns Hopkins University, Baltimore, MD). BMVEC and hCMEC/D3 ($2x10^4$ cells/well) were cultured on 24-well PET inserts containing 3-μm pores (Corning, Corning, NY) coated with rat-tail collagen type I (Sigma-Aldrich, St. Louis, MO) at 50 μg/mL and grown to confluence over 5.5 days. Bilayers were constructed using commercial human adult primary BMVEC ($2x10^4$ cells/well) and commercial astrocytes ($10x10^4$ cells/well) (Angio-Proteomie, Boston, MA) cultured on opposite sides of 24-well PET inserts containing 3-μm pores coated with rat-tail collagen type I at 50 μg/mL and grown to confluence over six days.

Trans-endothelial Electrical Resistance (TEER)

TEER of primary BMVEC and hcMEC/D3 monolayers were assessed every 5 minutes over 5.5 days using CellZscope technology (NanoAnalytics, Munster, Germany) for a total of 1,584 readings. Controls included PET inserts and growth medium. TEER was normalized to baseline resistance values [16]. TEER of bilayers (BMVEC passage 10, astrocytes passage 10) was assessed over 6 days using EVOM (World Precision Instruments, Sarasota, FL) every 24 hours. TEER was adjusted to control PET inserts.

Immunofluorescence

Primary BMVEC (passage 10) and hCMEC/D3 (passage 23) were grown to confluence on a rat-tail collagen type I-coated (50 μg/mL) (Sigma-Aldrich, St. Louis, MO) glass coverslips with or without PrEP (0.1 μM TDF, 0.1 μM FTC) and/or maraviroc (0.2 μM). Cells were fixed with 4% paraformaldehyde or mixture of equal parts methanol and acetone. Blocking was completed using 5% goat or horse serum. Primary antibodies were used at the following dilutions according to manufacturer instructions: occludin (diluted 1:166) (ThermoFisher, USA), ZO-1 (diluted 1:100) (Abcam, Cambridge, UK), PECAM-1 (diluted 1:250) (ThermoFisher, USA), and ICAM-1 (diluted 1:1,000) (Santa Cruz Biotechnology, Inc., Dallas, TX) followed by incubation with secondary anti-mouse (ThermoFisher, USA) or anti-rabbit (ThermoFisher, USA) IgG antibody conjugated to a fluorophore. Coverslips were mounted and imaged using a fluorescent microscope at 20X (Nikon, Melville, NY) (Leica, Buffalo Grove, IL) and analyzed using ImageJ Software (National Institutes of Health, USA).

Enzyme-linked Immunosorbent Assay (ELISA)

Occludin (ThermoFisher, USA), ZO-1 (Abcam, Cambridge, UK), PECAM-1 (ThermoFisher, USA), and ICAM-1 (Santa Cruz Biotechnology, Inc., Dallas, TX) were analyzed via indirect ELISA. Primary BMVEC (passages 9 and 10) and hCMEC/D3 (passage 23) were grown to confluence on a rat-tail collagen type I-coated (50 μg/mL) (Sigma-Aldrich, St. Louis, MO) flat-bottomed 96-well plate with or without PrEP (0.1 μM TDF, 0,1 μM FTC) and/or maraviroc (0.2 μM). Cells were fixed with 4% paraformaldehyde or mixture of equal parts methanol and acetone and blocked using 5% goat or horse serum. Primary antibodies were used at dilutions: occludin (diluted 1:500), ZO-1 (diluted 1:50), PECAM-1 (diluted 1:1,000), and ICAM-1 (diluted 1:200) followed by incubation with biotinylated secondary anti-mouse

(ThermoFisher, USA) or anti-rabbit (R&D Systems, Minneapolis, MN) IgG antibody. Solution containing Avidin DH and Biotinylated Alkaline Phosphatase H of the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA) was used and the reaction was developed with p-nitrophenyl-phosphate as substrate. Nitrophenol was quantified spectrophotometrically at wavelength 405 nm. Assays were performed in triplicate. Results were adjusted to control wells containing secondary antibody only. Bradford assays were used to normalize to total protein concentration in each well and analyzed using a 10-parameter logistical standard curve with GraphPad Prism 8 (GraphPad Software, San Diego, CA).

Monocyte Transmigrations

All study participants were HIV-negative male patients of the Clint Spencer Clinic of the Hawaii Center for AIDS (HICFA) at the University of Hawaii (UH) at Manoa, identified and selected because of the intention to begin a PrEP regimen. Per guidelines approved by the UH Institutional Review Board, informed consent was received prior to blood draw. PBMC were isolated from study volunteers prior to initiating PrEP and at 12 weeks post-PrEP initiation while still taking PrEP. PBMC were resuspended at a concentration of $1x10^6$ cells/mL in BMVEC medium without added growth factors (Angio-Proteomie, Boston, MA). Attune NxT Flow Cytometry Software (ThermoFisher, USA) was used to analyze CD3-CD14+ cells (monocytes) in PBMC samples at entry and 12 weeks post-PrEP initiation. *In vitro* bilayer BBB models were grown for 6 days and switched to medium without growth factors 12-16 hours prior to experimental manipulation. $0.5x10^6$ PBMC were added to the apical side of each BBB. Transmigrations were 24 hours at 37°C, 5% CO_2 with 100 ng/mL MCP-1 (R&D Systems, Minneapolis, MN) as a chemoattractant for monocytes. To assess the effect of maraviroc on monocyte transmigration, 0.2 μM maraviroc was added *in vitro* to a subset of BBB wells for the

12 weeks post-PrEP transmigrations. Each condition was assayed in at least triplicate: Entry (no drug) and Post- (PrEP, PrEP with the *in vitro* addition of 0.2 μM maraviroc). Transmigrated cells were harvested and stained with CD3 (Biolegend, San Diego, CA) and CD14 antibodies (ThermoFisher, USA) and analyzed using Attune NxT Flow Cytometry Software and FlowJo Software (FlowJo, USA) (Figure 1). Post-transmigration integrity assessments of BBB wells were completed using 0.45% EBA.

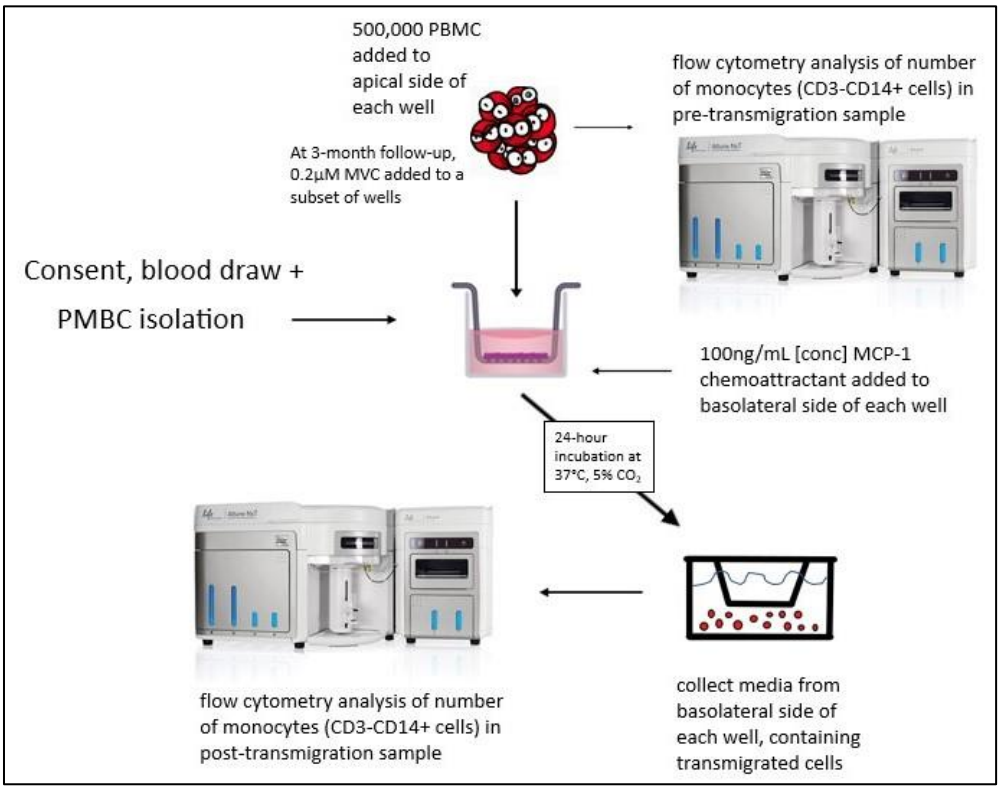

Figure 1. Evaluation of PrEP treatment with and without maraviroc on monocyte transmigration across an *in vitro* BBB model. Experimental workflow of transmigration assays for HIV-negative blood donors beginning a PrEP regimen. Consent and blood draw were completed prior to beginning PrEP and after 12 weeks of PrEP. Maraviroc (0.2 μM) was added in *vitro* to a subset of BBB wells at 12-weeks to assess differences in transmigration of uninfected monocytes with the addition of maraviroc.

Statistical Methods

To assess differences in TEER of BMVEC and hcMEC/D3 cells at 5.5 days of growth determined by CellZscope and differences in protein expression of PECAM-1, ZO-1, occludin, and ICAM-1 determined by ELISA, Mann-Whitney tests were completed using GraphPad Prism Software. Statistical significance was determined at $p \leq 0.05$.

To assess differences in monocyte transmigration under the conditions of no drug, PrEP, and PrEP with the *in vitro* addition of maraviroc, determined by Attune NxT Flow Cytometry Software and FlowJo Software, Mann-Whitney tests were completed. Statistical significance was determined at $p \leq 0.05$.

RESULTS

Primary BMVEC (passage 10) showed increased expression of PECAM-1 ($p<0.01$) compared to hcMEC/D3 (passage 23), decreased expression of ICAM-1 ($p<0.01$) even when hcMEC/D3 were treated with hydrocortisone, and similar expression of occludin and ZO-1 as shown by immunofluorescence (Figure 2a) and verified by ELISA (Figure 2b). Normalized TEER of primary BMVEC ($2.0x10^4$cells/well, passage 10) was increased with a trend towards significance ($p=0.1$) over hcMEC/D3 ($2x10^4$ cells/well, passage 23) over 133.8 hours or 5.5 days for a total of 1.584 readings (n=3). TEER of bilayer BBB using primary BMVEC ($2x10^4$ cells/well, passage 10) and primary astrocytes ($10x10^4$ cells/well, passage 10) co-cultured for 144 hours or six days (n=12) was 165.54 Ω/cm^2.

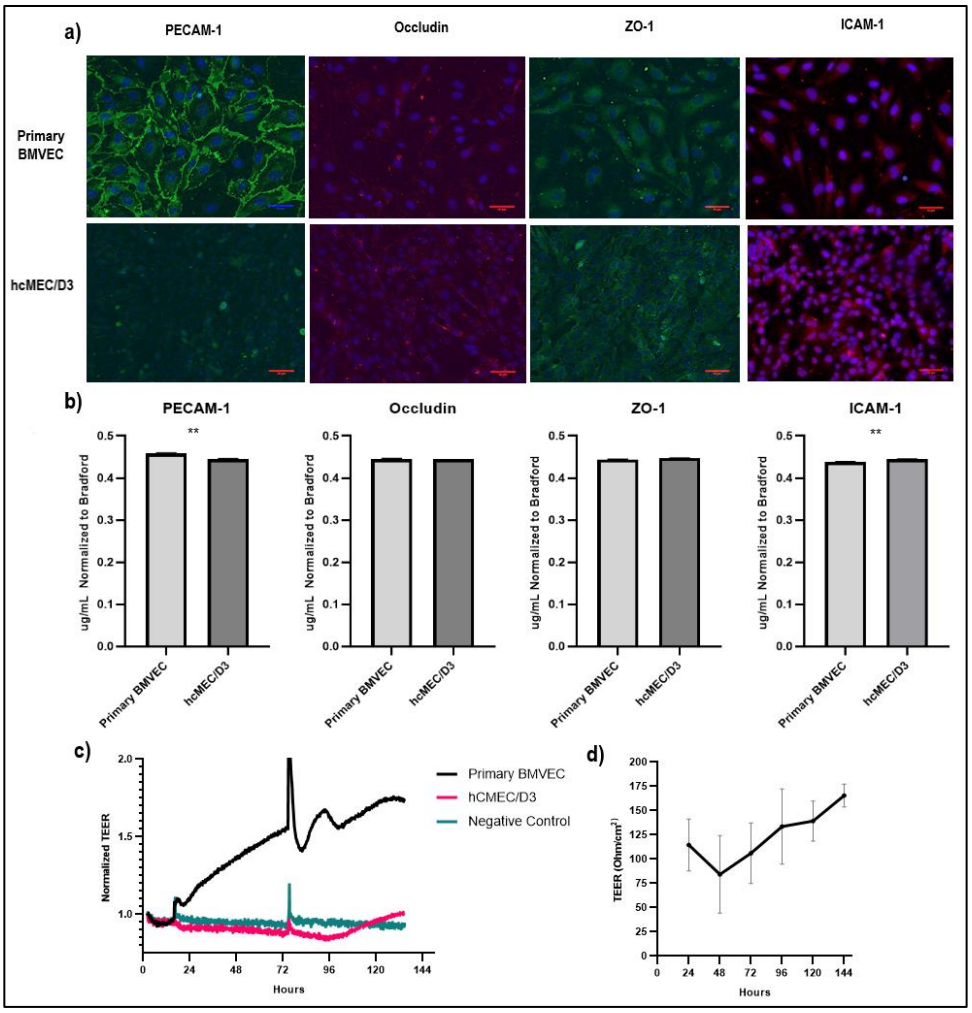

Figure 2. Comparison between primary BMVEC and human BMVEC immortalized cell line hcMEC/D3 **a)** Indirect ELISA of protein expression in primary BMVEC compared to hcMEC/D3 cells normalized to total concentration per well (µg/mL) via Bradford assay. **b)** Immunofluorescence of protein expression in primary BMVEC compared to hcMEC/D3 cells. **c)** Growth curve comparison of primary BMVEC ($2x10^4$ cells) to hcMVEC/D3 cells ($2x10^4$ cells) over the span of 133 hours (n=3). TEER was taken over 5.5 days for a total of 1,584 readings and normalized to the first five readings at baseline. **d)** Growth curve of bilayer BBB *in vitro* model over the span of six days. Barriers (n=12) were cultured on opposite sides of PET inserts with 3µm pores using primary BMVEC

($2x10^4$ cells) and primary astrocytes ($10x10^4$ cells) over the span of 6 days or 144 hours with TEER taken every 24 hours. TEER was adjusted to controls. *p<0.05, **p<0.01

Immunofluorescence (Figure 2b) and ELISA (Figure 2a) analysis of the effects of PrEP, maraviroc, and PrEP with maraviroc showed no changes in BBB protein expression in commercial human adult primary BMVEC (passage 9) except for increased expression of the tight junction protein occludin (p<0.01) with PrEP, maraviroc, and PrEP with maraviroc treatment.

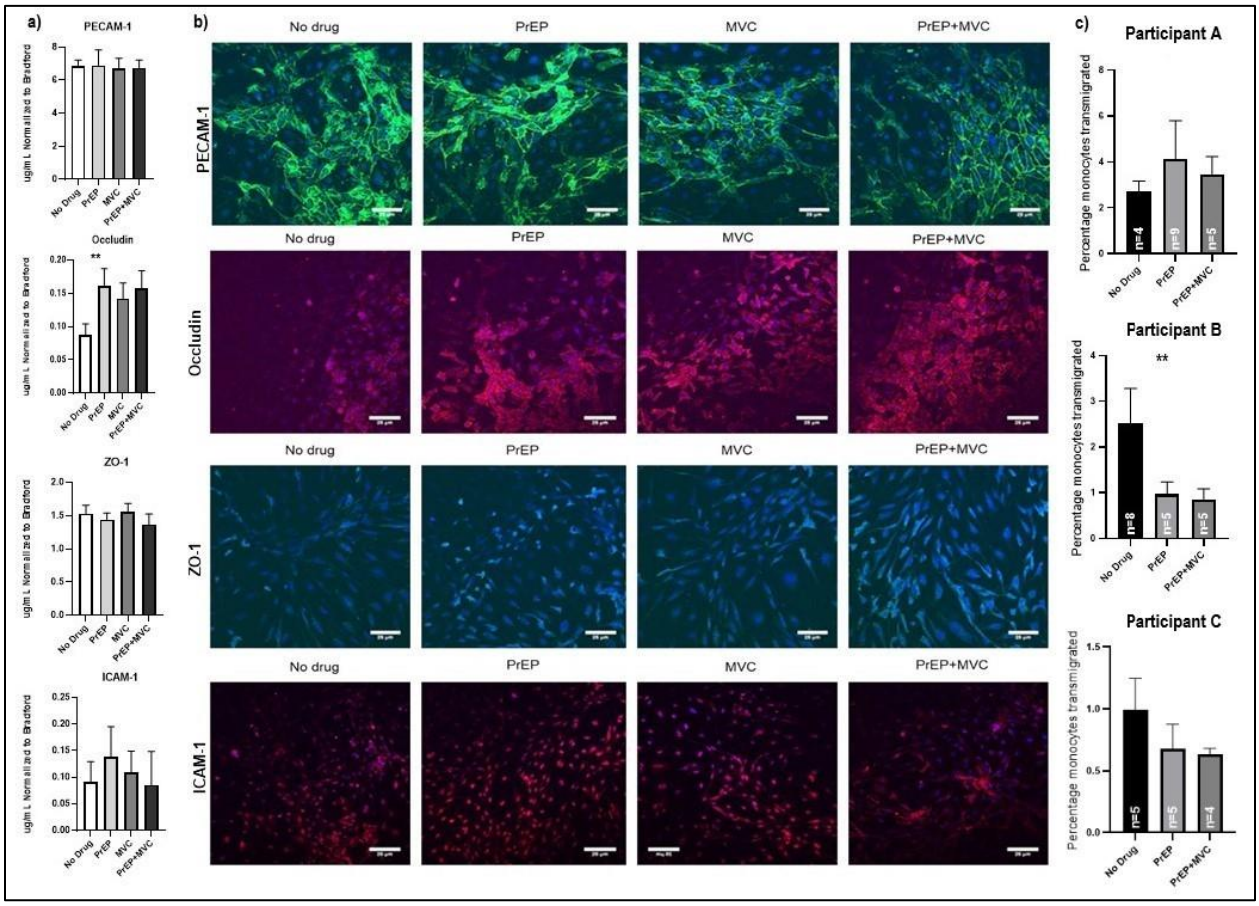

Figure 3. Impact of PrEP, maraviroc, and PrEP with maraviroc on BMVEC and uninfected monocyte transmigration **a)** ELISA analysis of PrEP, maraviroc, and PrEP with maraviroc on primary BMVEC characteristics (passage 9). **b)** Immunofluorescence

analysis of PrEP, maraviroc, and PrEP with maraviroc on primary BMVEC characteristics (passage 9). **c)** Percentage monocytes transmigrated in a subset of three study participants pre- and post- 12-weeks of a PrEP regimen, with an *in vitro* addition of maraviroc.

In a subset of three participants instituting a PrEP regimen, Participant B ($p= 0.016$) and C ($p= 0.0952$) showed reductions in the percentage of monocytes transmigrated across an *in vitro* bilayer BBB model with PrEP and PrEP with the *in vitro* addition of maraviroc while participant A showed no significant changes (Figure 3).

DISCUSSION

The results of this study determine commercial human adult primary BMVEC as an acceptable option for use in *in vitro* BBB models, comparable to or exceeding hcMEC/D3 cells in measured BMVEC characteristics and further supports their use as noted in other studies [15].

Although the study involving individuals instituting a PrEP regimen is limited by the small number of participants and the inability to determine adherence to PrEP via analysis of plasma concentration, two of three participants showed a reduction in monocyte transmigration across an *in vitro* bilayer BBB model with and without *in vitro* addition of maraviroc after 12 weeks. These results in combination with ELISA analysis and corresponding immunofluorescence showing an increase in expression of tight junction protein occludin with PrEP and PrEP with the addition of maraviroc suggests PrEP drugs TDF and/or FTC with and without maraviroc may be neuroprotective. To our knowledge, this has not been previously reported.

PrEP drugs TDF and FTC are both nucleoside reverse transcriptase inhibitors (NRTIs) which function to block HIV reverse transcriptase from transcribing HIV RNA into DNA [17]. While this function should have no direct impact on BMVEC, astrocytes, or uninfected monocytes, an impact on inflammatory pathways may explain the upregulation of occludin and decrease in monocyte transmigration observed in this study. Although some studies have indicated that TDF increases inflammatory cytokine expression such as interleukin 8 (IL-8), CCL3, interleukin 10 (IL-10), or IL-6, Melchjorsen *et al.* found that that TDF reduced IL-10 expression coinciding with increased interleukin 12 (IL-12) expression in human PBMC, an anti-inflammatory shift [18]. This suggests that TDF may play a role in limiting inflammatory responses. While a potential anti-inflammatory role of TDF on endothelium and epithelium has been unexplored in detail, a potential anti-inflammatory explanation may additionally be supported by data showing that TDF microbicide gel does not contribute to mucosal inflammation and shows efficacy in preventing HIV infection [19]. Furthermore, Melchjorsen *et al.* also showed TDF decreased levels of IL-8 and CCL3 expressed by human PBMC, which potentially explains the reduced transmigration of monocytes across an *in vitro* BBB in the presence of PrEP without the addition of maraviroc seen in this study [18].

Due to preliminary nature and limited scope of this study, additional research is needed to verify results and explore the potential mechanisms of PrEP drugs, both with and without maraviroc, impacting expression of occludin in BMVEC and transmigration of monocytes across the BBB.

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CHAPTER 4

STUDY SUMMARY AND FUTURE DIRECTIONS

STUDY SUMMARY

HAND impact more than 50% of PLWH. The mechanisms leading to the development of HAND are thought to be a result of HIV entering the CNS primarily through trafficking of HIV-infected monocytes across the BBB. Once inside the brain parenchyma, monocytes become activated perivascular macrophages that produce HIV, which infects astrocytes and microglia. Dissemination of infection within the brain results in production of neurotoxic molecules and inflammatory cytokines and chemokines which results in dendritic beading and the retraction of dendritic spines of neurons as well as neuronal apoptosis. Breakdown of the BBB as a result of neuroinflammation and production of HIV viral particles allows for increased entry of inflammatory cytokines and chemokines as well as infected monocytes, exacerbating inflammatory processes and resulting in neurocognitive decline for PLWH. Data has shown HIV RNA in CSF as early as eight days post-infection. This suggests that CNS viral seeding which precedes inflammatory events contributing to HAND occurs early after initial infection.

The development and broad implementation of suppressive ART has resulted in a reduction in the prevalence of the most severe form of HAND, referred to as HAD. However, the prevalence of milder forms of HAND, mild neurocognitive disorder, and asymptomatic neurocognitive impairment, have increased. There is currently no ART available specifically for HAND and no biomarker or set of biomarkers have been discovered that indicate HAND progression or improvement. Identifying biomarker(s) which do so would aid in the development of preventative or therapeutic interventions.

CCR5 is a co-receptor that HIV uses for viral entry into cells including monocytes that traverse the BBB resulting in HIV infection of the CNS and CCR5 is also utilized for immune signaling. Preliminary data has shown that an addition of the CCR5-inhibitor maraviroc to ART regimens in virally suppressed (HIV viral RNA <50 copies/mL) improves neurocognition. Thus, assessing the impacts of blocking CCR5 with a CCR5-inhibitor such as maraviroc, which may reduce or prevent HIV CNS seeding and inflammation that contributes to the development of HAND, warrants further study. Furthermore, maraviroc has been shown to be safe and potentially effective at preventing HIV infection in a phase II clinical trial with PrEP drugs TDF and FTC, suggesting maraviroc may be utilized as an addition to current PrEP to alleviate concerns of drug resistance.

Due to the lack of clinically relevant biomarkers of HAND, the promising preliminary data that blocking the CCR5 receptor with CCR5-inhibitor maraviroc may prevent and treat HAND, and the current lack of approved therapies specifically for HAND, the objective of the this study was to identify biomarkers of inflammation impacted by CCR5-inhibition related to changes in neuropsychological performance and BBB integrity in order to inform potential prevention and treatment modalities for HAND. Furthermore, while current PrEP drugs TDF in combination with FTC have been in use since 2012, no studies have evaluated their effects on the BBB and transmigration of monocytes.

This study did not demonstrate a reduction in an inflammatory biomarker or set of biomarkers correlated to improvements in global NPZ scores after 48 weeks of maraviroc therapy. However, differences in biomarkers of inflammation observed between the participants whose neuropsychological performance improved over the course of the study and the single participant whose neuropsychological performance declined may give insight into how

biomarkers of neuroinflammation may impact neuropsychological outcomes. The individual with declined global NPZ score was noted to have higher levels of CSF TNFα, serum TNFα, serum IL-6, serum sCD163, and serum sCD14. While these observations are descriptive due to the number of study participants, these results suggest that overall levels of neuroinflammation play a more significant role in neuropsychological outcomes than any single biomarker.

An additional observation was noted in this study in which there was a reduction in inflammation in one compartment (CNS, CSF; periphery, sera) and an increase of the same biomarker in the alternate compartment. While this data is also descriptive due to the limited number of study participants, the presence of this trend speaks to the challenges regarding pharmaceutical strategies for HAND to reduce inflammation in both the CNS and periphery which may impact neurocognition. Due to the complexity and interconnectedness of the human immune system, inhibiting one receptor, such as CCR5 with maraviroc, may result in the activation of alternate inflammatory pathways. This has been noted in previous studies in which CCR5 inhibition with maraviroc resulted in increased measures of sCD14 and sCD163, which is consistent with the results of this study. It has been hypothesized that these results occur due to CCR5-ligands interacting with immune receptors CCR1, CCR3, and CCR4 rather than CCR5. Therefore, it might be beneficial for future studies of potential HAND interventions to pair clinical outcomes with basic science research examining the effects on inflammatory pathways. Future HAND treatment strategies may need to target more than one potential inflammatory pathway.

The results of this study indicate a lack of significant relationship between *in vivo* BBB integrity as measured by Qalb and *in vitro* BBB as measured by TEER or 4-kDa FITC-dextran permeability. However, the *in vitro* BBB model used in this study is a static model in which

TEER at peak growth and confluence of 165 Ω/cm^2 falls below that of TEER in *in vivo* brain capillaries at >1000 Ω/cm2. Microfluidic *in vitro* BBB systems mimicking shear stress that results from blood flow have been shown to have a much higher TEER than static model systems, suggesting they may provide a better comparison to Qalb. However, microfluidic systems are inadequate for transmigration experiments such as those completed in this study. The development of more versatile *in vitro* BBB systems, which closely mimic *in vivo* characteristics, is necessary to improve the translational capacity of research utilizing *in vitro* BBB models.

In vitro BBB models do have translational limits, yet they provide a valuable tool to explore research questions that cannot currently be examined in living people, such as the impact of pharmaceutical agents on cells of the BBB or transmigration of cells across the BBB. To our knowledge, the impact of PrEP drugs, with and without maraviroc, on the BBB had not been previously assessed. Results are preliminary and warrant further research, however, the observed reduction in transmigration of uninfected monocytes with PrEP alone indicates that PrEP drugs TDF and/or FTC may act to reduce inflammation through a currently unknown mechanism. While it was hypothesized that maraviroc may reduce transmigration of monocytes across an *in vitro* BBB model due to the mechanism of action in blocking CCR5, similar results were unexpected with PrEP alone. Previous studies examining the effects of TDF, a component of PrEP, indicate TDF may reduce inflammation in human peripheral blood mononuclear cells, which may account for the observed reduction in transmigration of monocytes across an *in vitro* BBB model with PrEP alone. These findings are important and warrant further research because PrEP drugs TDF and FTC are utilized in in ART regimens for people living with HIV and not just for HIV-prevention.

In summary, this study concluded that no biomarker(s) of inflammation were specifically linked to improved neuropsychological performance in the setting of CCR5 inhibition and there was no correlation between *in vitro* and *in vivo* BBB integrity. When evaluating the impact of drug treatment on HAND and biomarkers of inflammation in a clinical setting, it would be beneficial to pair studies with a basic science component to examine effects of blocking specific immune pathways in a controlled setting. Furthermore, a combined approach to HAND treatment is needed: therapies to prevent and reduce HIV viral seeding in the CNS and treatment modalities to inhibit HAND progression and improve neuropsychological performance outcomes. The findings of this study have the potential to inform future research for both HAND prevention and treatment interventions.

FUTURE DIRECTIONS

The following future experiments would build upon the framework and outcomes of this study:

To determine the extent of neuronal damage rather than just assess biomarkers related to systemic inflammation, BBB impairment, and monocyte activation, it would be beneficial to include biomarkers related to neuronal damage, such as neurofilament light chain. Likewise, to assess the impact of participant serum on not only the BBB, but subsequent downstream impact to neurons, a tri-culture system could be utilized with an *in vitro* bilayer BBB with the addition of neurons cultured on the basolateral side of the BBB on the bottom surface of the 24-well plate. Furthermore, the medium located on the basolateral side of the BBB could be analyzed for the impact of an individual's serum on astrocytic signaling processes.

This study indicated a correlation between matrix metalloproteinases, S100B, and BBB impairment, so future experiments could verify this role through serum incubation on an *in vitro* BBB model both with and without the presence of specific inhibitors for these mediators. Since CD14+CD16+ monocytes preferentially migrate into tissues, assessing the impact of PrEP drugs, with and without maraviroc, on the presence and transmigration of monocyte subsets across an *in vitro* BBB model may give more of an insight into the outcomes seen in this study. Finally, because this study noted increased expression of the tight junction protein occludin in primary human BMVEC with PrEP drugs, maraviroc, and PrEP with maraviroc via ELISA and immunofluorescence, it would be beneficial to also assess if these drugs impact occludin transcription within BMVEC.

While this study compared changes in neuroinflammatory biomarkers to changes in neuropsychological performance and BBB integrity, assessing these outcomes alongside results of neuroimaging both before and after drug-intervention may provide more insight into structural and metabolic brain changes that occur as a result of drug intervention. Due to the small study population, it would be valuable to assess outcomes of neuropsychological performance, BBB integrity, and changes in neuroinflammatory biomarkers in larger and diverse cohorts. Additional statistical analyses of the outcomes of this research, such as reviewing these results as changes in biomarker ratios (CSF/serum) over the course of the study may be a more sensitive measure to determine the impact of treatment outcomes on biomarkers of neuroinflammation as they relate to neuropsychological performance outcomes and BBB integrity.