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A Biological Perspective of CSF Lipids as Surrogate Markers for Cognitive Status in HIV

Norman J. Haughey^{1,2}, Zhu Xiaomao¹, and Veera Venkata Ratnam Bandaru¹

¹The Johns Hopkins University School of Medicine, Department of Neurology, Division of Neuroimmunology and Neurological Infections, Baltimore, MD

²The Johns Hopkins University School of Medicine, Department Psychiatry, Division of Neuroimmunology and Neurological Infections, Baltimore, MD

Abstract

The development and application of biomarkers to neurodegenerative diseases has become increasingly important in clinical practice and therapeutic trials. While substantial progress has been made at the basic science level in understanding the pathophysiology of HIV-Associated Neurocognitive Disorders (HAND), there are significant limitations in our current ability to predict the onset or trajectory of disease, and to accurately determine the effects of therapeutic interventions. Thus, the development of objective biomarkers is critical to further our understanding and treatment of HAND. In recent years, biomarker discovery efforts have largely been driven forward through the implementation of multiple “omics” approaches that include (but are not restricted to): Lipidomics, proteomics, metabolomics, genomics, transcriptomics, and advances in brain imaging approaches such as functional connectomics. In this paper we summarize our progress to date on lipidomic approaches to biomarker discovery, discuss how these data have influenced basic research on the neuropathology of HAND, and implications for the development of therapeutics that target metabolic pathways involved in lipid handling.

Overview

The introduction of combinational antiretroviral therapy (cART) in the early 1990s dramatically increased the expected lifespan of those infected the Human Immunodeficiency Virus (HIV) (Tozzi et al., 2007; Cardenas et al., 2009; Heaton et al., 2010). In addition to extending lifespan these therapeutics also decreased the incidence of cognitive impairment in HIV-infected patients. However, nearly two decades after the introduction of cART it is apparent that the prevalence of cognitive impairment is unchanged (and may be increasing), with approximately half of those infected with HIV likely to develop some form of cognitive impairment (Antinori et al., 2007; Chang et al., 2008; Valcour et al., 2008; Achim et al., 2009; Brew et al., 2009; Ances et al., 2010). Although cognitive impairments in cART treated patients tend to be less severe compared to untreated HIV-infected patients, they nonetheless can profoundly impact quality of life. Despite effective viral suppression with cART, brain volume loss, and evidence of age-influenced white matter damage are common in HIV-infected patients (Chang et al., 2008; McMurtray et al., 2008; Cardenas et al., 2009; Gongvatana et al., 2009). Examinations of brain tissues from cART treated patients shows evidence of metabolic disturbances, inflammation, synaptic and dendritic damage

Correspondence. Norman J. Haughey, Departments of Neurology and Psychiatry, Johns Hopkins University School of Medicine, Meyer 6-109, 600 North Wolfe Street, Baltimore MD, USA., 21287. nhaughe1@jhmi.edu. 443-287-3825 phone. 410-955-0672 fax. .

Conflict of interest

The authors have no conflicts of interest related to this work to report.

(McArthur et al.; Gelman, 2007; Pelle et al., 2008; Khanlou et al., 2009; Cohen et al., 2010; Nguyen et al., 2010; Kamat et al., 2012) These observations suggest that cART is not sufficient to prevent neural damage, and that an adjunctive neuroprotective therapy is required to protect the brain in HIV-infected patients. The development and validation of surrogate markers for brain damage are critical to facilitate therapeutic development, and could potentially identify HIV-infected patients at the earliest stages of neural damage, when a neuroprotective therapeutic would be most beneficial.

Cerebral Spinal Fluid Sphingolipid Content is a Surrogate Measure for Brain Sphingolipid Metabolism

In 2004 we first demonstrated that disruptions in brain sphingolipid metabolism are apparent in HIV infected patients. Accumulations of ceramide and sphingomyelin were notable in the frontal, parietal and temporal cortex of individuals infected with HIV, and there were anatomical differences in the particular species and sub-species of sphingolipid that accumulated. The degree of these metabolic disturbances in sphingolipid metabolism appeared to be related cognitive status (Haughey et al., 2004). In general, accumulations of ceramide and sphingomyelin were greater in patients with more severe forms of cognitive impairment. A significant discovery from this study was that the CSF sphingolipid content appeared to reflect a composite of the metabolic profile obtained from multiple brain regions. Thus, we reasoned that the sphingolipid composition of CSF could be a useful surrogate measure to estimate brain sphingolipid metabolism. As CSF can be collected from living patients, this approach allowed us to determine the cross sectional and temporal relationships of these lipid metabolites to changes in cognitive status. In a series of subsequent studies we measured CSF at one or more time points and quantified sphingolipid content. Data were then stratified according to longitudinal changes in cognitive functioning. The findings from these studies have begun to unravel complex temporal changes in sterol and sphingolipid metabolic profiles that are associated with HIV infection and temporal shifts in cognitive status. Recent evidence suggests that these metabolic disturbances in brains of HIV-infected patients may be a manifestation of underlying dysfunctions in endolysosomal systems (Bandaru et al., 2013).

Brain Sphingolipid Metabolism

Brain has one of the highest lipid contents of any organ in the human body. Our understanding of brain lipid metabolism, and in particular, roles that sphingolipids play in the regulation of neuronal and glial function is moving forward at a rapid pace. This current rate of discovery has been made possible due to a combination of technological advances in mass spectrometry, ongoing efforts to map pathways that regulate lipid metabolism (see lipidmaps.org), and an increased appreciation that dysregulations of sphingolipid metabolism play important roles in the pathogenesis of a number of different neurodegenerative conditions including AD, Parkinson's disease, Multiple Sclerosis, and HIV Associated Neurocognitive Disorders (HAND) (Cutler et al., 2004; Haughey et al., 2004; Bandaru et al., 2009; Mielke et al., 2010; Fabelo et al., 2011).

Sphingolipids are a class of lipid derived from the aliphatic amino alcohol sphingosine. The sphingosine backbone is O-linked to a charged head group such as ethanolamine, serine or choline, and amide-linked to an acyl group, such as a fatty acid. Ceramides are the simplest sphingolipid, consisting of a fatty acid chain attached by an amide linkage to sphingosine. Ceramide is a bioactive lipid that has been implicated in the regulation of numerous cellular processes including cell growth, survival, motility, protein traffic, neuronal plasticity, endolysosomal, autophagic and excitotoxic events, neurite outgrowth, synaptogenesis, neurotransmitter release, cytokine production, and the cellular response to inflammatory

stimuli ((Jana et al., 2009; Zhang et al., 2009)). Such a wide variety of cellular functions are regulated by this family of lipids due to a complex network of interconnected metabolic pathways that are tissue, cellular, sub-cellular, and organelle specific. These metabolic pathways regulate the spatial and temporal patterns of ceramide production that determine the effects on these diverse cellular processes (reviewed in (Bikman and Summers, 2011; Worgall, 2011; Mencarelli and Martinez-Martinez, 2013). Ceramides can be produced *de novo* by the condensation of palmitate and serine to form 3-keto-dihydrosphingosine (by serine palmitoyl transferase). 3-keto-dihydrosphingosine is then reduced to dihydrosphingosine, which is acylated to produce dihydroceramide and finally converted to ceramide (by dihydroceramide desaturase). Ceramide can also be rapidly produced by reacylation of sphingosine (the salvage pathway) and by the hydrolysis of sphingomyelin (via a family of sphingomyelinases). Sphingomyelinases are categorized based on optimal pH for activity into acidic- (aSMase), alkaline- (alkSMase) and neutral (nSMase). Neutral sphingomyelinase-2 (nSMase2) is highly expressed in the CNS (Fensome et al., 2000; Hofmann et al., 2000; Clarke and Hannun, 2006), and is primarily located to the Golgi apparatus (Tomiuk et al., 1998; Hofmann et al., 2000), but can translocate to perinuclear regions in response to the antioxidant glutathione and to the plasma membrane in response to oxidative stress (Andrieu-Abadie and Levade, 2002; Levy et al., 2006; Castillo et al., 2007). nSMase2 activity is regulated by TNF α , IL-1, FasL, the HIV coat protein gp120 and oxidative stress (Liu and Hannun, 1997; Visnjic et al., 1999; Clarke and Hannun, 2006; Jana and Pahan, 2007; Wheeler et al., 2009). nSMase2 has been implicated in the neuropathogenesis associated with HAND and other inflammatory-associated conditions (France-Lanord et al., 1997; Cutler et al., 2002; Cutler et al., 2004; Haughey et al., 2004). Sphingomyelin synthesis involves the transfer of choline from phosphatidyl choline to the head group of ceramide. This reaction is catalyzed by sphingomyelin synthases 1 and 2 that catalyze the conversion of ceramide and phosphatidylcholine to sphingomyelin and diacylglycerol. Human SMS1 is localized to the Golgi, while SMS2 resides primarily at the plasma membrane. Experimental evidence to date suggests that accumulations of ceramide and sphingomyelin in neurons may be related to the development and progression of HAND. The temporal order perturbations in sphingolipid metabolism provided clues that have fostered the development of therapeutics that target specific enzymes in these pathways.

Central Nervous System Inflammation is Associated with Ceramide Production

In multiple cohorts of HIV-infected patients we have consistently found that CSF accumulations of sphingomyelin and ceramide are associated with cognitive impairment (Haughey et al., 2004; Sacktor et al., 2004; Bandaru et al., 2007; Bandaru et al., 2013). However, deciphering the temporal relationship of these metabolites to cognitive status has proven to be more challenging.

Some of the earliest biochemical changes that may predict cognitive decline are consistent with the cellular response to an ongoing inflammatory process. Low-level neuroinflammation produced through the persistent activation of brain resident astrocytes and microglia is thought to be an important contributor to the neuropathogenesis of HAND. Brain and CSF levels of numerous inflammatory cytokines such as transforming growth factor- β (TGF- β), interleukins IL-1 α , IL-1 β , IL-6, tumor necrosis factor alpha (TNF α), and CD95 ligand have been repeatedly reported as elevated in HIV-infected patients in accordance with cognitive impairment (Grimaldi et al., 1991; Wahl et al., 1991; Tyor et al., 1992; Achim et al., 1993; Wesselingh et al., 1993; Saas et al., 1999; Avison et al., 2004). These effects were recapitulated by intact virions, or viral proteins in tissue culture, rodent,

and primate models of HAND (Burdo et al., 2010; Roberts et al., 2010; El-Hage et al., 2011; Weed et al., 2012; Marker et al., 2013). TNF α and IL-1 β are among the most frequently reported cytokines to be increased in brain and CSF of HIV-infected patients, and in pre-clinical models of HAND (Brabers and Nottet, 2006). These cytokines are known to be connected to ceramide associated stress signaling in neurons, and to cytokine/chemokine production in glia (Singh et al., 1998; Ayasolla et al., 2004; Davis et al., 2006; Wheeler et al., 2009; Martinez et al., 2012; Gu et al., 2013). TNF α and IL-1 β receptors are linked to the generation of ceramide through sphingomyelin hydrolases (aSMase and nSMase as discussed above in the section on sphingolipid metabolism) that rapidly convert membrane sphingomyelin into ceramide. These actions occur through adaptor proteins and signaling intermediates that directly link TNF and IL-1 receptors to these hydrolytic enzymes (Adam-Klages et al., 1996; Schwandner et al., 1998). In addition to cytokines the HIV-coat protein gp120 has been shown to increase neuronal ceramide through actions that involved binding to CXCR4, increases of ER calcium, and a redox-sensitive translocation of nSMase2 to the plasma membrane (Haughey et al., 2004; Jana and Pahan, 2004b; David Wheeler, 2009). When localized to the plasma membrane neutral sphingomyelinase catalyzes the conversion of sphingomyelin to ceramide. As ceramide is a highly saturated lipid that packs tightly together, increases of membrane ceramide stabilize the structure of membrane microdomains (Haughey et al., 2004; Jana and Pahan, 2004b; David Wheeler, 2009). These microdomains (also called lipid rafts) are regions of the plasma membrane that exhibit decreased lateral mobility due to a focal enrichment of saturated lipids including cholesterol, sphingomyelin and ceramide. Membrane microdomains play important roles in signal transduction as delivery docks for protein insertion, and through the creation of transient signaling platforms that regulate protein scaffolding and downstream signaling.

NMDA Receptors Localize to Ceramide Enriched Membrane Microdomains

The insertion and removal of *N*-methyl *D*-aspartate (NMDA) receptors from the neuronal synapse is critical for the modulation of synaptic plasticity (Roche et al., 2001; Barria and Malinow, 2002; Nong et al., 2003; Lavezzari et al., 2004; Scott et al., 2004; Washbourne et al., 2004). Transmembrane receptors traffic within cells encased into intracellular vesicles. The insertion of receptors into the plasma membrane requires the fusion of receptor-laden vesicles with the plasma membrane. A specific, transient and focal reorganization of the plasma membrane is necessary for the insertion and surface expression of NMDA receptors (Wheeler et al., 2009). For example, applications of TNF α to neuronal cultures rapidly increased plasma membrane ceramide by mechanisms that involved a translocation and activation of the sphingomyelin hydrolase nSMase2. These increases of ceramide temporarily stabilized membrane microdomains, and facilitated the fusion of NMDA receptor containing vesicles with the plasma membrane. These newly inserted NMDA receptors were located to synapses in small clusters, where NMDA-evoked calcium transients were enhanced. NMDA-evoked excitatory post-synaptic currents, and long-term potentiation recorded from CA1 pyramidal cells in brain slice preparations were also enhanced following activation of nSMase2. Pharmacological, molecular and transgenic approaches each confirmed that nSMase2 was critical for NMDA receptor surface expression and functional changes in synaptic activity. Consistent with a role in plasticity, these increases of plasma membrane ceramide and NMDA receptor clustering were rapid and transient. Activation of nSMase2 increased ceramide and clustering of NMDA receptors within 2 min, and these effects returned to baseline within 10 min. Similar effects were found *in vivo*, in which nSMase2 plays an important role in memory formation. Pharmacological blockade of nSMase2 decreased the ceramide content in hippocampus and cortex, altered the number and molecular composition of NMDA and AMPA receptors, and impaired spatial and episodic-like memory in mice (Tabatadze et al., 2010). Together these studies identified nSMase2-generated ceramide as an important regulator of membrane

microdomain stability, NMDA receptor trafficking, with associated effects that regulate cellular and behavioral manifestations of memory.

In the setting of HIV-infection, the virus in combination with inflammatory cytokines may over activate nSMase2 resulting in a chronic elevation of ceramide with prolonged stabilization of NMDA receptors to membrane microdomains. In gp120 treated neurons, clusters of NMDA receptors were trapped into stabilized membrane microdomains, where the receptors were unable to laterally disperse or internalize, even following strong agonist induction (Xu et al., 2011). Similar effects were observed in gp120 transgenic mice where accumulations of ceramide and over activation of nSMase2 were accompanied by modifications in the subunit composition of NMDA receptors, and hyper phosphorylation of NR1 subunits on serine 896 (indicative of increased surface expression). Isolation of lipid raft fractions from the cortex of these animals showed that NMDA receptors were preferentially located to flotilin-1 positive lipid rafts fractions. Daily administrations of a nSMase2 inhibitor to gp120 mice blunted nSMase2 activity, normalized brain ceramide content, and reduced NR1 serine 896 phosphorylation (Chen, 2012). Thus, prolonged increases of ceramide that are apparent in the brains and CSF of HIV-infected individuals may perturb cognitive function through a stabilization of NMDA receptors into membrane microdomains where excessive calcium signaling may evoke cellular stress pathways (Figure 2A).

Accumulations of Sphingomyelin may Provoke Endolysosomal Dysfunction

We have just recently completed a study that describes interactions between CSF lipidoses and temporal shifts in the cognitive status in a demographically diverse multicenter collection of HIV-infected subjects. These findings provided evidence that the onset and progression of HAND may involve a progressive disturbance in ceramide, sphingomyelin, and cholesterol metabolism that is reminiscent of the biochemical manifestations associated with a lysosomal storage disorder (Bandaru et al., 2013). Increases in a single ceramide species and reduced esterification of cholesterol were associated with prodromal stages of cognitive decline, while progressive cognitive impairments were characterized by accumulations in multiple sphingomyelin species. The implications of these findings are that perturbed endolysosomal function may contribute to cognitive decline in HIV-infected subjects, and is consistent with reports of enlarged endolysosomal systems and defects of autophagy in neuronal cultures, brain tissues of HIV-infected subjects, and in primates infected with SIV (Gelman et al., 2005; Alirezai et al., 2008b; Alirezai et al., 2008a; Chen et al., 2013). These pathological observations are consistent with our biochemical findings of perturbed sphingolipid balance, since the molecular response to an overabundance of sphingolipids is to sequester these products into lysosomes. These findings suggest that HIV-associated perturbations in sphingolipid metabolism may eventually lead to dysfunctions in endolysosomal systems (Figure 2A).

The Endolysosomal System

Organization of the endolysosomal pathway is complex and there are a various models explaining how organelles within the system relate to one another. One of the most accepted is the maturation model which suggests each organelle is a transient but distinct entity that matures along a defined pathway (Luzio et al., 2007). In this model, endocytosed material is first compartmentalized into early endosomes, which then mature into a transient late endosomes that continue to mature into lysosomes (a terminal organelle). Early endosomes are the major sorting station in the endolysosomal system that allows for the recycling of membrane receptors and other materials back to the membrane surface. From the early

endosomes internalized material designated for degradation is separated into clumps of vesicles called multivesicular bodies that mature into late endosomes, and eventually to lysosomes (Sachse et al., 2002). Substrates can enter the endolysosomal system through endocytosis, autophagy, and phagocytosis. Endocytosis allows the digestion of exogenous proteins through clathrin-mediated or clathrin independent actions. Clathrin-mediated endocytosis depends on surface membrane receptors that bind to substrates before invagination to form a vesicle. Although the majority of endocytosis occurs via the clathrin route, some substrates enter through clathrin-independent routes such as the CLIC/GEEC and flotillin-dependent pathways. Autophagy is responsible for the removal of endogenous proteins and is critical for metabolic and homeostatic function (Kroemer and Jaattela, 2005). Four autophagic mechanisms have been described that include macro-autophagy, micro-autophagy, crinophagy and chaperone-mediated autophagy. Macro-autophagy is the primary route of the autophagic pathway and occurs when large regions of the cytoplasm are sequestered into a double membrane autophagosome destined for degradation. The autophagosome travels through the cytoplasm and fuses with the lysosomal membrane where proteolysis of the cargo can occur. Proteolysis occurs primarily in the late endosomes and lysosomes as both contain lysosomal hydrolases (digestive enzymes). However there are distinct differences in morphology and proteolytic capacity of the two structures. Late endosomes have a complex morphology that is organized by microtubules while lysosomes are simpler, electron-dense organelles. Late endosomes contain only ~20% of the total hydrolase pool, yet are the primary site of proteolysis in the endolysosomal pathway. Lysosomes on the other hand contain the bulk of the total hydrolase pool but only contribute approximately 20% of total proteolytic capacity. Proper functioning of the endolysosomal system is critical for normal cellular metabolism, and perturbations that cause lipids to accumulate in these compartments disrupts endolysosomal function.

Typical and “Atypical” Disorders of Lysosomal Storage

Though rare, mutations in genes that code for lysosomal enzymes produce a series of diseases that are collectively known as lysosomal storage disorders. To date nearly 70 genetically distinct lysosomal storage disorders have been described, and many are associated with dysfunctions in macromolecular storage that result in physical disruption of the endolysosomal system. Although there is a great deal of variability in the severity of lysosomal storage disorders, those that involve sphingolipidoses are frequently associated with serious neurological and cognitive symptoms. Gaucher disease, Tay-Sachs disease, Sandhoff disease, and Niemann-Pick C disease are typical lysosomal storage disorders that result from genetic mutations in enzymes involved with lipid metabolism. In each of these disorders, sphingolipid metabolism is impaired and one or more lipid products accumulate in endolysosomal compartments. Neural degeneration is a universal feature of these disorders involving atrophy, dendritic, axonal, and white matter degeneration (Sun et al.; Nixon et al., 2008; Bellettato and Scarpa, 2010)

Neurodegenerative pathology caused by altered lipid metabolism and dysfunctions in the endolysosomal system are not restricted to these typical lysosomal storage disorders. A growing body of evidence supports the notion that accumulations in certain lipid metabolites play a role in the neuropathology of a number of neurodegenerative diseases in addition to HAND (as discussed above). For instance Alzheimer’s disease (AD) is a progressive and age dependent neurodegenerative disorder that is characterized by dementia and memory loss. The hallmarks of AD include widespread neuronal death associated with the accumulation of extracellular deposits of amyloid β -peptide ($A\beta$) into plaques and intraneuronal aggregates of the microtubule associated protein tau into neurofibrillary tangles. While the cause of sporadic AD remains elusive, there is evidence that altered sphingolipid and sterol metabolism may create conditions that are favorable for the

formation and aggregation of A β (van Echten-Deckert and Walter, 2012). One of the first studies to show that ceramides are elevated early in the pathogenesis of AD found nearly a three-fold elevation of ceramides in white matter of AD patients that peaked at the stage of very mild dementia (Han et al., 2002). A subsequent study showed that long-chain ceramides, especially ceramide C24, accumulates in brains of AD patients (Cutler et al., 2004). Ceramide has been shown to be important for A β formation by increasing the half-life of β -secretase. The addition of C6 ceramide to CHO cells stably transfected with APP (CHO-APP) increased A β by increasing β - but not γ -secretase cleavage of the amyloid precursor protein (Kalvodova et al., 2005). It has also been demonstrated that β -amyloid can increase ceramide levels by mechanisms that involve nSMase, lipid peroxidation and oxidative stress (Ayasolla et al., 2004; Jana and Pahan, 2004a; Ju et al., 2005; Sato et al., 2005; Malaplate-Armand et al., 2006). The addition of nSMase to CHO-APP cells increased ceramide and the formation of β -amyloid while the inhibition of ceramide synthesis with fumonisins B1 decreased ceramide and β -amyloid (Puglielli et al., 2003). Likewise, β -amyloid peptides induced the NADPH oxidase-mediated production of superoxide radicals in neurons that was involved in the activation of nSMase, but not aSMase, via hydrogen peroxide. (Jana and Pahan, 2004a). Thus, ceramide can promote β -amyloid formation through induction and stabilization of β -secretase, and β -amyloid can induce ceramide by redox-sensitive up regulation of nSMase. In addition to HIV and AD, disorders of lipid metabolism have also been linked to Parkinson's disease (Brugg et al., 1996; France-Lanord et al., 1997; Lwin et al., 2004; Cheng et al., 2011; Lee et al., 2011), and Multiple Sclerosis (Kim et al.; van Doorn et al.; Glabinski, 1993; Chen et al., 2005; Hait et al., 2006; Narayanan et al., 2006; Wheeler et al., 2008). If indeed sphingolipidoses are common to many neurodegenerative conditions (Haughey, 2010), this suggests that accumulations of sphingolipids in lysosomes with consequent disruption of endolysosomal systems may be a common neurodegenerative phenotype.

Surrogate Marker Guided Therapeutic Development

Reducing neuroinflammation has proven to be a difficult therapeutic target. Moreover, not all inflammation is detrimental or should be inhibited. Inflammation alerts the body to infection or damage. It is when the immunological response becomes non-specific or prolonged that inflammation can itself damage multiple organ systems including brain. However, blocking inflammatory pathways can have unanticipated consequences that damage neural, vascular and cardiac systems, depending on the drug, target, duration of dosing, and timing of treatment (Karplus and Saag, 1998; Sugaya et al., 2000; Hoozemans et al., 2008; Jaturapatporn et al., 2012). Alternate therapeutic approaches to blocking inflammation have been to target noxious downstream effectors of inflammatory signaling (Nixon, 2009; Marker et al., 2013). Lipidomic approaches discussed here have begun to identify several possible targets for therapeutic intervention that are downstream of inflammatory cytokine signaling. For example, nSMase2 is activated by inflammatory stimuli such as IL-1 β , TNF α , and the HIV-coat protein gp120. Inhibiting nSMase2 protects neurons and mitochondria by reducing increases of ceramide and normalizing NMDA receptor trafficking (Haughey et al., 2004; Tsakiri et al., 2008; Novgorodov and Guduz, 2011; Gu et al., 2013)(Figure 2B). Attempts to manufacture therapeutically useful inhibitors of nSMase2 have been difficult. As this enzyme regulates the hydrolysis of sphingomyelin to ceramide, small molecule inhibitors of the enzymes catalytic site are hydrophobic (ie they look like a lipid). Hence, solubility can be an issue with these compounds, and oral bioavailability is often poor. On the plus side, once in circulation, these compounds have good brain penetrance. Alternative methods of biodelivery such as intranasal and nanoformulations to increase absorption may be required to improve delivery of these more hydrophilic compounds. Thus, although nSMase2 is an attractive therapeutic target, its 'drugability' has yet to be determined.

A second potential therapeutic target that is downstream of inflammatory signaling is lysosomal located calcium channels. Inflammatory associated increases of ceramide can be converted to sphingomyelin. Accumulations of sphingomyelin in endolysosomal compartments results in intraluminal accumulations of calcium that are associated with reductions in cellular energetics. Overly abundant calcium in lysosomes increases positive charge in the lumen, and prevents acidification of the compartment by impairing the hydrogen pump (the increased ionic gradient prevents pumping of positive charge into the lumen). Agonists that induce calcium efflux from these stores through activation of TRPM1 may help to dissipate this transmembrane potential and restore the derivative capacity of lysosomes (Figure 2B). In pre-clinical models of lysosomal storage disease TRPML1 agonists have been shown to restore intraluminal pH, and clear sphingolipids from lysosomes (Shen et al., 2012). These results suggest that small molecule therapeutics designed to restore lysosomal function may have alternative uses as therapeutics for neurodegenerative disease.

Challenges for Biomarker Discovery in cART treated HIV-Infected Patients

Declines in cognitive status prior to the introduction of cART were frequent in patients with advanced HIV-infection, commonly involved an encephalitic process, were typically progressive, and a predicted death (Porwit et al., 1989; Wigdahl and Kunsch, 1989; Griffin et al., 1990). Biomarkers for cognitive impairment pre-cART were tied to infectious markers such as viral load, CD4 counts, immunological and inflammatory indicators (Brew et al., 1990; Griffin et al., 1990; McArthur et al., 1992; Tyor et al., 1992; Wiley et al., 1992; Portegies et al., 1993; Haughey et al., 2004). The introduction of cART shifted the neurological manifestations of HIV-infection so that the majority of cognitive impairments were mild (asymptomatic neurocognitive impairment), with bidirectional transitions in severity. The association of virological, immunological and inflammatory biomarkers to cognitive status in cART treated patients is not entirely clear in the era of cART therapy. For instance, although viral load seems to no longer be a useful surrogate for cognitive status (i.e. neurocognitive impairments can occur in patients with little or no detectable viral load), CSF inflammatory markers such as MCP-1, TNF α , ceramide, and immunological markers such as neopterin, may continue to be useful surrogate measures (Sevigny et al., 2004; Hagberg et al., 2010; Bandaru et al., 2013; Yilmaz et al., 2013). However, there is not currently a consensus on what markers (biological and/or imaging) are the most useful surrogate measures of cognitive status in HIV-infected patients. There are currently a number of complex technical, social and economic challenges that that will need to be resolved as the field refines its search for a consensus group of HAND biomarkers.

There has been a noticeable shift in biomarker discovery from cross sectional approaches to longitudinal designs. Early biomarker studies often grouped HIV-infected patients based on cognitive status at a single time point. A temporal course of events was sometimes inferred from differences in biomarkers obtained from a single time point that were based on statistical associations with cognitive status at that time point. Before cART it was a reasonable assumption that cognitive decline typically followed a forward progression. The temporal course of cognitive status is more complicated in cART treated patients. Hence, the findings from cross sectional studies are more limited, and should not be used to infer a temporal course of events. Longitudinal approaches are now more commonly used and can include samples collected at a single time point with longitudinal clinical and cognitive testing data, to multiple time points that include sample collections, clinical and cognitive testing data. While these longitudinal approaches are considerably more informative, they too have limitations. The vast majority of cognitive impairments in cART treated patients fall into the categories of asymptomatic neurocognitive impairments (abnormalities in two or more cognitive abilities), and minor cognitive disorder (cognitive impairment with mild functional

impairment). These mild to moderate forms of impairment in the spectrum of HAND can be influenced by psychiatric conditions, drug and alcohol use, nutrition, sleep disturbances, aging, alcohol drug use (including tobacco) and socioeconomic status. Similarly, some of the biomarkers isolated from CSF or blood can be influenced by these same risk factors. In addition, antiretroviral drugs can alter some biomarkers through effects on insulin sensitivity, lipid handling, and cellular stress responses. The effects of these variables on biomarker measures can be more or less prominent depending on the particular biomarker in question, characteristics of the cohort, population size, and the length of time that the study encompasses. Although many of these variables can be controlled for in statistical analyses, it is difficult to control for all possible variables in any given population.

While there is no easy answer to address these potential confounds in biomarker studies, examinations of larger populations with mixed demographic and risk factors may aid in the discovery of biomarkers that are less influenced by these variables. The future success of biomarkers in the HIV field will require a great deal of co-operation between centers that have banked and prospective sample collections (including brain imaging), laboratories who study biomarkers, federal and private support to harness these considerable resources into meaningful and directed approaches for the discovery, consolidation and validation of biomarkers specifically related to cognitive status.

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Figure 1. Metabolic pathways for sphingolipid metabolism

Sphingolipid products are shown in blue, and enzymes that catalyze these reactions are shown in red. SMase (Sphingomyelinase), SMS (Sphingomyelin synthase), DES (Dihydroceramide desaturase), CERK (Ceramide kinase), CerS (Ceramide synthase), KDS (reductase= 3-keto-dihydrosphingosine reductase), SPT (Serine palmitoyl transferase), S1P Pase (Sphingosine 1-phosphate phosphatase), S1P lyase (Sphingosine 1-phosphate lyase), SphK (Sphingosine kinase), Cer1P Pase (Ceramide 1-phosphate phosphatase), GluCerS (Glucosylceramide synthase), GluCeramidase (Glucosyl ceramidase), GalCerS (Galactosylceramide synthase), LacCerS (Lactosylceramide synthase), GlyCerS, (Glycosylceramide synthase), Sulfo-GalCerS (Sulfo galactosylceramide synthase), Palmitoyl CoA (Palmitoyl coenzyme A hydrolase).

Figure 2. Accumulation of Ceramide and Sphingomyelin Perturb Protein Trafficking and Endolysosomal Function

A) HIV, TNF α and IL-1 β increase the size and stabilize the structure of membrane microdomains through increased ceramide formation. NMDA receptor clusters are trapped into stabilized membrane microdomains. **B)** Ceramide is the direct precursor to sphingomyelin. When sphingomyelin is overproduced, it becomes sequestered into lysosomes. This sequestration of sphingomyelin in lysosomes impairs the degradative capacity of endolysosomes and undegraded proteins accumulate. **C)** Inhibition of nSMase2 can prevent IL1 β , TNF α and HIV from inducing ceramide formation. Reducing ceramide formation in this setting normalizes the lipid content of the plasma membrane and restores

normal receptor trafficking. **D)** Agonists of the lysosomal TRPML1 channel induce the release of calcium. The release of calcium from lysosomes reduces the ionic gradient and hydrogen to be pumped into the lumen. This lowers the luminal pH and helps to clear debris through restoration of the lysosomal derivative capacity (lysosomal hydrolases have a low pH optima).