Cerebrospinal fluid biomarkers of neurovascular dysfunction in mild dementia and Alzheimer’s disease

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INTRODUCTION
Alzheimer’s disease (AD) is the most common form of age-related dementias. In addition to genetics, environment, and lifestyle, growing evidence supports vascular contributions to dementias including dementia because of AD. Alzheimer’s disease affects multiple cell types within the neurovascular unit (NVU), including brain vascular cells (endothelial cells, pericytes, and vascular smooth muscle cells), glial cells (astrocytes and microglia), and neurons. Thus, identifying and integrating biomarkers of the NVU cell-specific responses and injury with established AD biomarkers, amyloid-β (Aβ) and tau, has a potential to contribute to better understanding of the disease process in dementias including AD. Here, we discuss the existing literature on cerebrospinal fluid biomarkers of the NVU cell-specific responses during early stages of dementia and AD. We suggest that the clinical usefulness of established AD biomarkers, Aβ and tau, could be further improved by developing an algorithm that will incorporate biomarkers of the NVU cell-specific responses and injury. Such biomarker algorithm could aid in early detection and intervention as well as identify novel treatment targets to delay disease onset, slow progression, and/or prevent AD.

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NEUROVASCULAR DYSFUNCTION
The influence of vascular dysfunction has been an increasing focus in the AD field with notable support in the last decade.4,7-13,15-21 According to pathologic studies, ~50% of patients diagnosed with AD and mild cognitive impairment (MCI) have mixed AD/vascular pathologies, which increases with age,22 including microinfarcts, arteriosclerosis, or atherosclerosis.23,24 Autopsies from AD patients further revealed the presence of cerebrovascular disease suggesting that vasculature plays a notable role in neurodegeneration.25

In this section, we review studies describing neurovascular dysfunction in early dementia and AD, particularly in regard to the blood–brain barrier (BBB) integrity, cerebral blood flow (CBF), and glucose transport into the brain. We also briefly discuss findings in the corresponding transgenic animal models. Finally, we discuss the two-hit vascular hypothesis of AD.

Blood–Brain Barrier Integrity
Endothelial cells and mural perivascular pericytes form the BBB.5,12,21 The BBB is a highly selective barrier between blood and the central nervous system that limits entry into the brain of potentially toxic plasma-derived proteins, circulating metals, cells, and pathogens. In contrast to nutrients (e.g., glucose and amino acids) that are transported across the BBB via specialized transport systems,5 peptides and other larger molecules do not cross the BBB unless they have specific carriers and/or receptors expressed in the brain endothelium.28,29 Quite the reverse of brain capillaries,
Figure 1. Diagram of the neurovascular unit. The neurovascular unit represents an interactive network of vascular cells (pericytes and endothelial cells), glia (astrocytes and microglia), and neurons.

systemic capillaries have a permeable vascular barrier allowing transport of solutes and larger molecules to parenchymal tissue.20

Maintaining the BBB integrity is vital for proper physiologic functioning of neurons and brain circuitries. Studies in transgenic mice have shown that pericytes maintain the BBB integrity and that loss of pericytes leads to a chronic BBB breakdown associated with accumulation of blood-derived neurotoxic proteins in the CNS causing neuronal dysfunction, injury, and loss.21,31–35 Multiple postmortem studies in AD brains have shown BBB breakdown including accumulation in the hippocampus and cortex of blood-derived proteins (e.g., immunoglobulins, albumin, fibrinogen, and thrombin)36–41 and degeneration of BBB-associated pericytes.41–43 Neuroimaging studies have shown microbleeds and accumulation of blood-derived iron in AD,44–46 particularly in the hippocampus.47

Early neuroimaging attempts to measure the BBB permeability Ktrans constant in the brain in individuals with MCI and AD failed to detect significant changes in BBB integrity.48,49 These studies might have been hampered by their relatively long time resolutions and the semi-quantitative nature of their analyses. More recent studies acquired at higher field strengths and using more sophisticated kinetic modeling have determined that the Ktrans of healthy BBB is low but not zero and detected differences in the white matter (WM) in patients with multiple sclerosis.50–52

A recent study using an advanced dynamic contrast-enhanced magnetic resonance imaging (MRI) protocol has determined for the first time low levels of regional BBB permeability (i.e., Ktrans constant) in the living human brain simultaneously in multiple gray and WM regions.53 This study found an age-dependent BBB breakdown in the hippocampus and its CA1 and dentate gyrus subdivisions, but not other brain regions, during normal aging, and worsening in individuals with mild dementia (i.e., MCI) compared with age-matched controls with no cognitive impairment (NCI).53 Interestingly, the CSF biomarkers of BBB breakdown (e.g., albumin quotient (Qalbum)) and pericyte-specific injury (e.g., soluble platelet-derived growth factor receptor-β, sPDGFβR) were both increased in MCI compared with NCI individuals and correlated well with the Ktrans measure of BBB breakdown in the hippocampus and its CA1 and dentate gyrus regions.53 This study also found that MCI individuals with increased Ktrans BBB permeability had no significant alterations in inflammatory, neuronal (total and phosphorylated tau, pTau), or Aβ biomarkers in the CSF compared with age-matched NCI controls53 suggesting that excessive vascular leakage between the brain and the blood may be an early critical step toward age-related dementias that starts in the hippocampus, a learning and memory center of the brain.

Notably, the Ktrans values from recent studies in the normal living human brain53 were within a range of previously reported BBB Ktrans values determined in mammals.28,33,54 Owing to the improved technology, future in vivo human studies are likely to emerge in the coming years to further assess BBB integrity in the context of AD and other neurologic conditions. In addition to pericyte-deficient mice,31–33 the BBB breakdown has been shown in three different lines of AD transgenic mice overexpressing Aβ-precursor protein (APP)55 and in mice overexpressing Slit-2, a gene involved in cell migration,56 that develop behavioral deficits associated with increased BBB permeability and Aβ accumulation.

Cerebral Blood Flow

Resting CBF is diminished in older cognitively normal individuals at risk for AD,57,58 in AD patients,59 and during normal aging in apolipoprotein E e4 (APOE e4) carriers, that is a major genetic risk factor for late onset AD.60 Cerebral blood flow dysfunction can occur prior to cognitive impairment.61 Cerebral hypoperfusion has been confirmed in AD patients by the arterial spin labeling MRI studies.62 Dysregulated CBF responses were found in AD as reviewed elsewhere.13,15 Microvascular and CBF reductions were also found in early disease stage in different APP transgenic models,14,64,66 senescence-accelerated prone mouse strain 8 mice,65 and pericyte-deficient mice.33,66 Cerebral microvascular Aβ deposits have been shown in different APP transgenic models.67

Glucose Transport

The glucose transporter GLUT1 is exclusively expressed at the BBB.5,52 GLUT1 mediates delivery of glucose, a key energy metabolite, to the brain. The crystal structure of human GLUT1 has been recently reported.56 Local glucose uptake by the brain closely correlates with GLUT1 levels in cerebral microvessels that are increased in response to increased neuronal activity and metabolic demand.69–71 Reduced glucose uptake in the hippocampus, parietotemporal cortex, and/or posterior cingulate cortex was found by 2-[18F] fluoro-2-deoxy-D-glucose positron emission tomography (PET) in AD APOE e4 carriers,72,73 individuals with positive family history,74 and/or MCI or NCI who develop AD later in life. 2-[18F] Fluoro-2-deoxy-D-glucose PET changes can precede brain atrophy and neuronal dysfunction in humans75,76 and transgenic APP models.76 Moreover, reductions in BBB glucose transport have been suggested to lead to 2-[18F] fluoro-2-deoxy-D-glucose PET changes.77,78 Consistent with these findings, diminished GLUT1 levels in brain microvessels have been reported in AD patients.79–82 Whether reduced BBB transport of glucose contributes to brain hypometabolic state and neurodegeneration or is the by-product of neurodegeneration is unclear at present. It is also elusive whether loss of GLUT1 can lead to BBB breakdown in AD as it does in the lower vertebrates.83

Two-Hit Vascular Hypothesis of Alzheimer’s Disease

The two-hit vascular hypothesis of AD proposes that microvascular damage is an initial insult through which BBB dysfunction and/or diminished brain perfusion lead to secondary neuronal injury and prime the brain for Aβ accumulation.1,12 The BBB breakdown results in leakage of neurotoxic proteins into the brain that is followed by astrocyte and microglia response, aberrant vascular remodeling (i.e., angiogenesis), and inflammatory response12 that all can promote neuronal injury (e.g., formation of toxic tau neurofibrillary tangles, WM damage, and decreased dendritic spine density) and Aβ accumulation. There is notable interplay between Aβ-independent and Aβ-dependent pathways in AD development,
Reliable biomarkers that define the clinical severity of CAA, which could be similarly informative in AD development.

Early Onset Familial Alzheimer’s Disease

Genetic mutations within APP, presenilin-1 (PS1), and PS2 result in early onset FAD.93,94 To date, only a few studies investigate injury to the NVU cell types in FAD. For instance, postmortem studies have shown degeneration of pericytes and vascular smooth muscle cells and perivascular amyloid deposits in the brains of PS1 mutation carriers.95 Multiple types of Aβ deposits (i.e., preamyloid, neuritic, and dense cored) were found associated with arteries and capillaries in FAD.96 Cerebral amyloid angiopathy is frequently present in individuals with FAD including Arctic APP and PS1 mutations.97,98 One case study reports disruption in meningeal, subpial, and cortical arterioles in a patient with a PS1 mutation.99

Studies in transgenic mice have informed FAD pathophysiology. For example, APPswswe PS1M146V mice (i.e., APPswswe mice crossed with PS1M146V mutant line) have BBB and microvascular dysfunction causing leakage of blood-derived proteins and the dense-core Aβ deposited in perivascular areas and/or directly attached to vessel walls.67 Recently, disrupted cerebral microvasculature, BBB breakdown, and microaneuysms have been described in APPswswe PS1E9 mice.99 In addition to amyloid deposits and tau accumulation, mice expressing human PS1117 and PS1M146V exhibited age-related vascular changes such as thinning capillaries with abnormal loops, string vessels, cortical surface microhemorrhages, and endothelial cell injury.100 The reduced microvasculature in the hippocampus was associated with higocampal atrophy.101,102 Interestingly, expression of PS1117 and PS1M146V in these mice was driven by a neuronal promoter103 suggesting that aberrant signaling between neurons and vascular cells may induce vascular pathophysiology in a mouse model of FAD.

Given the presence of vascular disruption in combination with Aβ and tau, future longitudinal studies in FAD patients investigating additional NVU biomarkers would help define FAD pathologic progression and could ultimately aid in research efforts to better understand pathogenesis and therapeutic targets in FAD, and by extension in AD.

Late Onset Alzheimer’s Disease

The large majority of AD cases are sporadic, late onset. The strongest genetic risk factor identified for late onset AD is APOE ε4.104 Apolipoprotein E ε4 increases risk for AD by 7% and 28% and 30% and 60% in carriers with one and two APOE ε4 alleles, respectively, at ages 75 and 85.105 Studies in transgenic mice have shown that APOE ε4 regulates cerebrovascular integrity4 and Aβ clearance from the brain.102 Postmortem human studies have shown that APOE ε4 genotype accelerates the BBB damage in AD patients37,38,40,46,103,104 and increases CAA severity43 and fibrinogen deposition associated with microvascular Aβ deposits.106 In addition, young cognitively normal APOE ε4 carriers have impaired cerebrovascular reactivity in response to memory task and CO2 inhalation107.

Studies in mice with targeted replacement of murine APOE with each human APOE isoform and in mice expressing each human APOE isoform under control of the astrocyte-specific glial fibrillary acidic protein promoter on an APOE null background suggest that human ApoE impacts cerebrovascular and BBB integrity via brain pericytes in an isoform-dependent manner.34 ApoE4, but not ApoE3 and ApoE2, increases the levels of proinflammatory cyto- kine cyclophilin A (CypA) in pericytes, which leads to increased levels of the matrix metalloproteinase-9 (MMP-9) resulting in degradation of BBB tight junction and basement membrane proteins and BBB breakdown causing secondary neuronal dysfunction and degenerative changes.35 Reduced cerebral vascularization and BBB breakdown in human APOE ε4 targeted
replacement mice compared with mice expressing APOE ε3 and APOE ε2 have been recently confirmed by an independent study. 107 Interestingly, a recent study in cognitively normal human APOE ε4 carriers compared with APOE ε3 carriers has shown an age-dependent increase in CypA and active MMP-9 levels in the CSF suggestive of activation of the CypA-MMP-9 pathway that correlated with increased CSF/plasma albumin ratio indicating BBB breakdown. 103

In addition to confirming APOE ε4 as a major genetic risk factor for AD, genome-wide association studies have identified multiple single-nucleotide polymorphisms in different genes associated with AD. 108–110 The biology of these AD-associated genes and allelic variants remains, however, elusive for the majority of genes and single-nucleotide polymorphisms. Some AD genes can potentially directly affect the cerebrovascular system as, for example, the transvascular Aβ clearance across the BBB (e.g., APOE 102 and CLU 111), and/or Aβ production (PS1, PS2, PICALM, BIN1, ATXN1, and ADAM10 112), Aβ aggregation (APOE), and Aβ degradation (CD33, CR1, and EPHA1). 113 The relationship between neurovascular dysfunction and genes and single-nucleotide polymorphisms associated with late onset AD remains to be investigated by future studies.

LIFESTYLE AND VASCULAR RISK

Lifestyle (e.g., diet and exercise) and vascular-related risk factors (e.g., hypertension, atherosclerosis, type 2 diabetes mellitus, and obesity) influence cognitive impairment in age-related dementias and AD. 10,14,114 Arterial stiffening is associated with reduced vascular clearance of Aβ. 12,91 Hypertension and an altered CSF AD biomarker profile (low Aβ42, high total tau, and high pTau) leads to increased gray matter degeneration in a presymptomatic period. 115

Diet

The major component of essential omega-3 fatty acids, docosahexaenoic acid (DHA), has long been known to be beneficial to cognition and overall brain health. 116 Alzheimer’s disease patients have lower DHA lipid levels in the CSF. 117 The primary DHA transporter at the BBB is the major facilitator superfamily domain containing 2A transporter, recently shown to have a dual function at the BBB mediating transport of DHA into the brain and also regulating the BBB integrity. 118–120 Its role in AD and dementia remains, however, unknown. Interestingly, transgenic APOE ε4 mice show reduced brain uptake of DHA compared with transgenic APOE ε2 mice. 121

High dietary consumption of cocoa flavonoids has been recently shown to enhance dentate gyrus–associated cognitive function in the hippocampus of healthy cognitively normal individuals by increasing capillary density, as shown by cerebral blood volume functional MRI measurements, suggesting the hippocampus likely has a significant vasculoplastic reserve. 122 The relationship between vasculoplasticity and neuronal plasticity during normal aging and dementia, and how it is impacted by diet and risk factors (i.e., genetic, vascular, environment, and lifestyle), remains largely unknown and should be addressed by future studies. 123

Exercise

Exercise and increased physical activity and higher midlife fitness levels are associated with reduced risk of all-cause dementias including AD. 124 Previous studies in laboratory animals have shown that environmental enrichment or functional enriched high activity promote Aβ clearance from the brain by accelerating Aβ enzymatic degradation and enhancing Aβ transvascular transport, which reduces Aβ levels and amyloid deposition in transgenic mouse models of AD. 125,126 It has been demonstrated that enriched activity upregulates expression of the low density lipoprotein receptor-related protein 1, 127 a major Aβ clearance receptor at the BBB 125,126,128 and in smooth muscle cells of small penetrating brain arteries, 128 and downregulates expression of the receptor for advanced glycation endproducts (RAGEs) at the BBB, which mediates reentry and influx of Aβ from circulation into the brain. 129 These molecular changes in brain vascular system create favorable conditions for Aβ clearance counteracting Alzheimer’s vascular dysfunction.

Though the precise mechanisms remain still largely elusive, lifestyle and vascular health have been increasingly recognized as key modulators of one’s risk for developing AD.

CEREBROSPINAL FLUID BIOMARKERS OF THE NEUROVASCULAR UNIT

The lack of presymptomatic detection with reliable biomarkers is a major limitation for developing and implementing successful treatments for AD. Established AD biomarkers include tau (total and phosphorylated at threonine 181 and 213) and 42-amino acid Aβ. Decreased Aβ42 levels and increased tau and pTau levels in the CSF are reproducibly shown at different stages during AD. 130–132 As discussed below, Aβ42 is also shown to be decreased in the CSF during preclinical stages of AD, particularly in APOE ε4 carriers. 133 It has been suggested that individuals with abnormal CSF Aβ42 and pTau 11P levels likely to have an asymptomatic period of 7 years before the onset of cognitive impairment and AD clinical diagnosis. 134 Whether CSF biomarkers reflecting NVU cell-specific injury are altered before or after Aβ and tau remains elusive, at present. 135 Identifying novel NVU biofluid-based biomarkers associated with early cognitive impairment in individuals at risk for AD has the potential to provide a molecular phenotype associated with early stages of AD development.

Cerebrospinal Fluid as a Source of the Neurovascular Unit Biomarkers

Cerebrospinal fluid is ideal for molecular biomarker studies because its juxtaposition with brain interstitial space may reflect a measure of brain biochemical changes. In addition, the CSF compartment is isolated from systemic influences by the BBB and the blood–CSF barrier. 12 There is, however, a large degree of inconsistency in AD biomarker studies (with the exception of the established biomarkers, Aβ and tau). This could be attributed to the (1) lack of sample/procedural standardization (discussed in Standardization and Validity section), (2) failure to adequately account for risk factors, and (3) cross-sectional study design. The heterogeneity in AD patients is becoming increasingly apparent because of differential contributions of lifestyle and genetic, vascular, and environmental risk factors. In addition, in the prodromal stage of AD, namely MCI, some individuals remain arrested at this stage while others convert to AD. Given this prodromal phase, dichotomizing subjects into only two categories, cognitively normal and cognitively impaired, does not completely represent the molecular and phenotypic stages of cognitive impairment in AD. Cross-sectional studies should carefully distinguish cognitive status; however, longitudinal studies are ideal to account for individual variations as related to the progression of cognitive decline and AD pathophysiologic changes. Although established biomarkers Aβ42, pTau, and total tau are altered in preclinical stages and throughout AD, the clinical use of these markers is still relatively limited. Importantly, AD progresses through overlapping Aβ-independent and Aβ-dependent pathways (Figure 2), and numerous cell types are affected within the NVU (Figure 1). There are few existing studies that have been conducted to characterize markers of nonneuronal cell types of the NVU. 20,135 Moreover, majority of biomarker studies are narrow in scope and investigate a single category of injury in AD. Conducting simultaneous biomarker measurements would importantly allow
for direct comparison across multiple cell types within the NVU as related to cognitive impairment and decline. Below, we discuss a breakdown of CSF biomarkers into categories of NVU cell- and system-specific injury. Evidence is provided to suggest there is differential regulation of these markers in early cognitive impairment and AD.

**Biomarkers of Blood–Brain Barrier Breakdown and Vascular Injury**

Given the early occurrence of vascular dysfunction in AD, investigating biomarkers of BBB breakdown and vascular injury has the potential to importantly aid in early diagnosis of vascular dysfunction in AD.

**Albumin cerebrospinal fluid/plasma ratio.** Earlier studies using $Q_{ab}$ or the CSF to plasma ratio of blood-derived albumin have shown BBB breakdown in AD, particularly associated with vascular risk factors, WM lesions, subcortical vascular dementia, or accompanying vascular disorders (e.g., arterial hypertension, diabetes mellitus, and ischemic heart disease), but not in AD cases without vascular factors.$^{137-139}$ Others found that $Q_{ab}$ is higher in all dementias including AD$^{140}$ suggesting that BBB dysfunction is an early event in the disease process regardless of the type of dementia, i.e., AD or vascular dementia. More recent studies have confirmed these findings by showing elevated $Q_{ab}$ with age in individuals with NCI carrying an APOE $\varepsilon 4$ allele, but not an APOE $\varepsilon 3$ allele$^{103}$ and in MCI patients, which correlated with elevated $K_{trans}$ BBB permeability constant in the hippocampus as determined by dynamic contrast-enhanced MRI.$^{59}$

Increasing evidence suggests clinical and pathologic overlap between AD and other AD-related dementias including vascular dementia. Thus, though $Q_{ab}$ as a marker of BBB breakdown is elevated in dementias including AD, it does not appear to be specific to AD. Overall, these data highlight the heterogeneity observed in AD patients, and the importance of accounting for the presence and impact of vascular risk factors, APOE genotype, and other potential risk factors in analyses.

**Other biomarkers of blood–brain barrier breakdown.** Based on experimental and postmortem human studies, additional proposed molecular markers of BBB breakdown include increased levels of the proinflammatory cytokine CypA, active MMP-9, and blood-derived fibrinogen and plasminogen.$^{34,39,104}$ Albumin quotient, CypA, and active MMP-9 in CSF were all shown to be increased in older cognitively normal APOE $\varepsilon 4$ carriers or APOE $\varepsilon 4$ noncarriers.$^{103}$ In addition, CSF levels of fibrinogen are increased in mild dementia.$^{143}$ Postmortem analysis of AD brain tissue compared with control brains showed increased extravascular fibrinogen, IgG, and Aβ deposits located close to blood vessels.$^{39}$ Interestingly, MMP-9 is activated in fibrinogen binding to vascular endothelium, which may result in BBB breakdown.$^{142}$ Plasminogen CSF levels were not altered in AD$^{143,144}$ whereas MCI individuals had elevated$^{144}$ CSF plasminogen (Table 1). The BBB breakdown and the resulting infiltration of blood-derived neurotoxic proteins can subsequently lead to neuronal injury as shown in experimental models.$^{21,31-35}$

**Pericyte markers.** Experimental studies have demonstrated that brain pericytes are key to maintaining BBB integrity,$^{21,31-35}$ and that loss of pericytes leads to a chronic BBB breakdown followed by secondary neurodegenerative changes.$^{12,32,33,66,145}$ Pericytes degenerate in AD$^{41-43}$ and AD models.$^{66,146}$ Postmortem studies have shown that BBB breakdown in AD patients closely correlates with loss of pericytes$^{51}$ and is accelerated by APOE $\varepsilon 4$ genotype.$^{104}$ Injury to cultured human pericytes results in shedding of soluble form of the pericyte marker PDGFRβ (sPDGFRβ), and CSF sPDGFRβ levels are increased in experimental models with

| Table 1. Cerebrospinal fluid biomarkers of BBB breakdown, vascular cells and astrocytes in mild dementia (i.e., MCI) and AD compared with cognitively normal individuals |
|-----------------|-----------------|-----------------|
| **BBB breakdown** | **Mild dementia** | **AD** |
| Albumin quotient$^{a}$ | ↑Upregulation$^{53}$ | ↑Upregulation$^{137,140}$ and in AD with vascular risk factors$^{137,138}$ |
| CypA$^{a}$ | No existing literature | No existing literature |
| Active MMP-9$^{a}$ | No existing literature | No existing literature |
| Fibrinogen | ↑Upregulation$^{144}$ | No existing literature |
| Pericyte markers | | |
| sPDGFRβ | ↑Upregulation$^{53}$ | No existing literature |
| Endothelial markers | | |
| PDGF-BB | No existing literature | ↑Upregulation$^{146}$ |
| sVCAM-1 | No change$^{141}$ | ↓Downregulation$^{151}$ |
| sICAM-1 | No change$^{141}$ | No change$^{151}$ |
| Vascular growth factors | | |
| VEGF-A | ↓Downregulation$^{141}$ | ↑Upregulation$^{155}$ |
| VEGF-C, VEGF-D, and VEGFR1 | No existing literature | ↑Upregulation$^{148}$ |
| Plasminogen | No change$^{141}$ | No existing literature |
| Tie-2 | No existing literature | No existing literature |
| Astrocyte markers | | |
| S100B | No existing literature | No change$^{162}$ |
| | | ↑Upregulation$^{161}$ |

Abbreviations: AD, Alzheimer’s disease; BBB, blood–brain barrier; CypA, cyclophilin A; MCI, mild cognitive impairment; MMP-9, matrix metalloproteinase-9; PlGF, placental growth factor; sICAM-1, soluble intercellular adhesion molecule 1; sPDGFRβ, soluble platelet-derived growth factor receptor-β; sVCAM-1, soluble vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor. *Upregulation in cerebrospinal fluid of cognitively normal subjects with genetic risk for AD.$^{103}$
pericyte degeneration and chronic BBB breakdown including pericyte-deficient and APP<sub>Swe</sub> mice. Pericytes are extremely susceptible to changes in the CBF and die rapidly under hypoxic conditions associated with diminished CBF. 147 Interestingly, a recent study found increased CSF sPDGFRβ levels in individuals with mild dementia compared with controls, which correlated with increased BBB breakdown in the hippocampus. Cerebrospinal fluid levels of endothelial-derived growth factor PDGF-BB are also increased in AD, 148 but whether this reflects a compensatory response to alleviate the loss of PDGF-B signaling in pericytes remains unknown (Table 1). Although relatively new and not yet fully validated, the CSF markers of pericyte injury hold promise for detecting early vascular changes associated with early cognitive impairment or impairment in individuals at increased genetic risk for AD, such as APOE ε4 carriers.

**Endothelial markers.** Expression of endothelial adhesion molecules is suggested to reflect BBB dysfunction. 149 For example, in a transgenic mouse model with BBB dysfunction, both endothelial intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 are strongly associated with vessel albumin extravasation, 150 supporting that increased expression of endothelial adhesion molecules is related to vascular injury. In individuals with neuroinflamatory conditions, an increase in CSF soluble ICAM-1 (sICAM-1) levels correlated with Q<sub>Alb</sub>. Similarly in AD subjects, CSF levels of sICAM-1 and soluble vascular cell adhesion molecule 1 correlate highly with each other and with elevated BBB permeability measured by Q<sub>Alb</sub>. Soluble vascular cell adhesion molecule 1 is also reported to be decreased in AD patients 151 (Table 1). No change in sICAM-1 is reported in MCI 141 or AD patients 151 (Table 1). Given the evidence of vascular dysfunction in AD, studies on vascular injury markers could aid in better understanding whether brain endothelial responses are involved in early cognitive impairment and/or in a subset of AD patients with vascular risk factors or at genetic risk for AD.

**Vascular Growth Factors**

Vascular dysfunction and angiogenesis may collectively contribute to neurodegeneration. 152 Growing research in AD suggests that angiogenesis driven by pathologic events provides an additional avenue in promoting Aβ accumulation. 153 Markers known to be involved in both vasculogenesis and angiogenesis include the vascular endothelial growth factor family (VEGF): VEGF-A, VEGF-C, VEGF-D, VEGFR1, and placental growth factor. 154 Cerebrospinal fluid levels of VEGF-A are decreased in MCI 141 and either increased 155 or decreased 148 in AD (Table 1). Surprisingly, CSF levels of VEGF-C, VEGF-D, and VEGFR1 have not been reported in cognitively impaired individuals. Similar to VEGF family members, both basic fibroblast growth factor and tyrosine-kinase signaling through the Tie-2 receptor are involved in vessel growth, maintenance, and repair, 152 and could be potential biomarkers. Brain endothelium from AD patients has been shown to have a diminished ability to respond to angiogenic factors VEGF and basic fibroblast growth factor because of extremely low levels of expression of homeodomain transcription factor mesenchyme homeobox gene 2, which leads to aberrant angiogenesis and death of newly formed capillary tubes in response to angiogenic stimulation. 156

In neurologic conditions such as AD there is mechanistic overlap in cell signaling within the NVU that can signify both (1) an acute injury response and (2) an endogenous response to initiate NVU repair and reorganization. 157 Thus, during the development of AD, it would be informative to clarify when gradients of angiogenic markers alternatively signal an injury versus repair phenomenon. This would help elucidate the molecular phenotype associated with AD progression, and could potentially inform the transition from prodromal stages to AD.

**Astrocyte Markers**

Astrocytes can regulate vasodilation and vasoconstriction through VEGF and basic fibroblast growth factor signal transduction to endothelial cells. 158 In addition, astrocyte-secreted factors transmit signal transduction to pericytes that critically maintains the BBB integrity. 34 Prolonged astrocyte activation in AD brains disrupts neuronal survival 159 suggesting that astrocytes are an intermediate player in AD pathogenesis. Furthermore, astrocyte-secreted cytokine beta-calganulin (S100B) is involved in the innate immune response to AD. 160 S100B CSF levels are reported to be increased 161 or not altered 162 in AD compared with age-matched NCI individuals, whereas CSF S100B has not been reported in MCI (Table 1). Although the effect of elevated CSF S100B remains unknown, S100B can promote overexpression of neuronal APP and expression of interleukin-6 (IL-6) and IL-1β. 163 In addition, overexpressing human S100B in APP<sub>Swe</sub> mice resulted in both parenchymal and cerebrovascular Aβ deposits. 160 Incorporating astrocyte markers in an algorithm with other biomarkers of the NVU would aid in clarifying molecular phenotypes of AD progression.

**Inflammatory Response**

Glial inflammation is a hallmark feature of AD and refers to inflammatory response mounted by the brain’s glial cells. Once thought to be a by-product of AD disease process, increasing research suggests the role of reactive astrocytes and microglia 164 and elevated levels of some cytokines in the plasma, serum, and/or CSF 165 in AD progression. However, several cytokines have not been analyzed in the CSF from MCI cases including IL-2, IL-6, IL-12, and interferon-γ. Furthermore, CSF cytokine levels of tumor necrosis factor-α, IL-10, and IL-1β have only been conducted in few studies in MCI cases 141, 166 Table 2 describes changes in protein levels reported in CSF for inflammatory markers in both MCI and AD, relative to NCI.

**Table 2.** Cerebrospinal fluid biomarkers of inflammatory response in mild dementia (i.e., MCI) and AD compared with cognitively normal individuals

| Inflammatory response | Mild dementia | AD |
|-----------------------|--------------|----|
| TNF-α                 | ↑Downregulation<sup>166</sup> | No change<sup>169</sup> |
| IL-1β                 | No change<sup>166</sup> | ↑Downregulation<sup>167</sup> |
| IL-2                  | No existing literature | No change<sup>168,171</sup> |
| IL-8                  | ↑Upregulation<sup>172</sup> | No change<sup>169</sup> |
| IL-10                 | ↑Upregulation<sup>141</sup> | No change<sup>169</sup> |
| IL-6                  | No existing literature | No change<sup>167,169,173</sup> |
| IFN-γ                 | No existing literature | No change<sup>169</sup> |
| S100B                 | ↑Upregulation<sup>170</sup> | No change<sup>169</sup> |

Abbreviations: AD, Alzheimer’s disease; IFN-γ, interferon-γ; IL, interleukin; MCI, mild cognitive impairment; TNF-α, tumor necrosis factor α.
an increase\textsuperscript{171} or no change\textsuperscript{166,167,169} is reported for IL-1\textbeta (Table 2). Although these studies found interesting changes, the findings are not always consistent. Thus, there is a need for standardization of sample collection, processing, and analyses as well as simultaneous biomarker measurements to help remedy these inconsistencies.

Biomarkers of White Matter Injury

Myelin integrity is damaged in AD as shown through diffusion-tensor imaging and is thought to contribute to impaired cognition.\textsuperscript{174} A recent study showed that diffusivity measures could detect more subtle differences in MCI and AD brains compared with traditional fractional anisotropy measurements, which is a ‘gold standard’ postprocessing paradigm used to assess WM integrity.\textsuperscript{175} In addition, MCI individuals with both increased WM hyperintensities (WMHs) and increased temporoparietal glucose metabolism converted to AD, whereas MCI individuals without WMHs and metabolic disruption remained in prodromal stages.\textsuperscript{176} Similarly, AD patients have increased WMHs compared with normal aging.\textsuperscript{177} Interestingly, hypertensive individuals have heightened WMHs restricted to the periventricular region.\textsuperscript{178} These data support the role of vascular dysfunction in AD etiology.

Myelin basic protein is degraded in periventricular WM and was identified in vessels surrounding these regions in AD brains.\textsuperscript{179} However, no change in myelin basic protein expression was detected in AD compared with NCI in past CSF biomarker studies\textsuperscript{162,179} (Table 3). In addition, no studies have determined CSF levels of myelin basic protein in MCI, or myelin-associated glycoprotein in either MCI or AD (Table 3). The WMHs found in AD could be either a by-product of normal aging or the concurrent presence of vascular conditions such as cerebrovascular disease or ischemic injury. Given the association of hypertension and vascular changes with WMHs,\textsuperscript{177} it would be interesting to see whether biomarkers of WM injury correlate with markers of vascular injury. Nevertheless, incorporating WM markers in an algorithm of other NVU biomarkers could help define the heterogeneity of AD patients and stages of disease progression.

Amyloid-β Peptide

Amyloid deposits are visualized with a PET tracer Pittsburgh compound B, which detects both microvascular and parenchymal Aβ.\textsuperscript{180} Amyloid can deposit in the brain years prior to AD clinical onset.\textsuperscript{181} In NCI individuals, CSF Aβ42 levels decrease prior to amyloid deposition in the brain, particularly in APOE ε4 carriers.\textsuperscript{133} Literature consistently reports lower CSF levels of Aβ42 in MCI,\textsuperscript{141,182,183} which is further decreased in AD\textsuperscript{148,184} (Table 3). NCI individuals with abnormal CSF Aβ42 and pTau\textsubscript{181P} levels develop cognitive impairment faster than those with normal CSF Aβ42 and pTau\textsubscript{181P} levels; however, some individuals with abnormal AD-injury biomarkers remain cognitively normal for up to 7 years.\textsuperscript{134} Thus, identifying a cutoff level of CSF Aβ42 and confirming this marker’s ability to predict cortical Aβ deposition is important for establishing the clinical usefulness of CSF Aβ42 for routine clinical practice.\textsuperscript{185} How Aβ CSF levels relate to BBB breakdown and vascular and inflammatory biomarkers in the CSF remains elusive at present. A recent study found that CSF sPDGFβR, a marker of pericyte injury, is elevated in mild dementia prior to changes in CSF Aβ42 levels and inflammatory changes.\textsuperscript{53} Incorporating biomarkers of the NVU in prodromal stage along with established AD biomarkers could greatly enhance the ability to predict cell-specific involvement in the development of dementia, which may point to novel therapeutic targets.

**Table 3.** Cerebrospinal fluid biomarkers of white matter damage, amyloid-β (Aβ), and neuronal injury in mild dementia (i.e., MCI) and AD compared with cognitively normal individuals

| Wild matter damage | AD |
|--------------------|----|
| MBI | No existing literature | No change\textsuperscript{162,179} |
| MAG | No existing literature | No existing literature |
| | | |
| Aβ\textsubscript{4-2 peptide} | Downregulation\textsuperscript{141,183} | Downregulation\textsuperscript{130,148,184} |
| | | |
| Aβ\textsubscript{42} | Upregulation\textsuperscript{141,183} | Upregulation\textsuperscript{130,148,184} |
| pTau | No existing literature | No existing literature |
| | | |
| Total tau | Upregulation\textsuperscript{130,184} | Upregulation\textsuperscript{130,148,184} |
| NSE | No existing literature | No existing literature |
| | | |
| Neurofilament-L | Upregulation\textsuperscript{192,193} | Upregulation\textsuperscript{190,191} |

Abbreviations: AD, Alzheimer’s disease; Aβ, amyloid-β; MAG, myelin-associated glycoprotein; MBI, myelin basic protein; MCI, mild cognitive impairment; NSE, neuron-specific enolase; pTau, phosphorylated tau.

**Neuronal Injury**

Literature reports a consistent upregulation of both total and pTau\textsubscript{231P} in MCI,\textsuperscript{141,182,183} which is further increased in AD\textsuperscript{148,184} (Table 3) and correlates with hippocampal atrophy.\textsuperscript{189} Also, the CSF levels of neuronal markers neurofilament-L\textsuperscript{190,191} and neuron-specific enolase\textsuperscript{192,193} are elevated in AD (Table 3). In MCI, CSF levels have not been reported for either neuron-specific enolase or neurofilament-L (Table 3). No study, however, describes how markers of neuronal injury relate to markers of vascular injury during prodromal stage of dementia and AD, and whether alterations in the CSF levels occur simultaneously or have a differential time course for different cell-specific biomarkers.

**Oxidative and Metabolic Stress**

Oxidative stress likely contributes to different stages of AD pathogenesis by damaging cell proteins and cell membrane lipids.\textsuperscript{194} For example, oxidative damage to low density lipoprotein receptor-related protein 1, a key clearance receptor for Aβ,\textsuperscript{12} leads to formation of oxidized low density lipoprotein receptor-related protein 1 that cannot bind Aβ and mediate its efflux at the BBB.\textsuperscript{195} Cerebrospinal fluid lactate levels are higher in mild AD compared with moderate/severe AD\textsuperscript{196} suggesting either metabolic stress or compensatory changes in brain metabolism. Cholesterol metabolism depends on intact BBB.\textsuperscript{197} Decreased CSF cholesterol levels correlate with increased CSF APP\textalpha and APP\beta (products of APP processing) levels and a robust decrease in CSF Aβ42 suggesting a possible relationship between cholesterol metabolism and increased amyloidogenesis.\textsuperscript{198} Cerebrospinal fluid levels of ApoA-I, the major component of high-density lipoproteins involved in cholesterol transport and lipid metabolism, are also decreased in AD patients.\textsuperscript{199} Moreover, AD patients have lower CSF levels of DHA, whereas MCI subjects have lower levels of α-linolenic acid.\textsuperscript{177} Both, DHA and α-linolenic acid are components of omega-3 fatty acids, suggesting that disrupted polyunsaturated fatty acid metabolism may contribute to AD and could be a result of reduced neurovascular integrity through the dual role of the major facilitator superfamily domain containing 2A\textsuperscript{118–120} (discussed in Lifestyle and Vascular Risk section). Given the possible impact of oxidative and metabolic stress on the NVU and brain functions in AD, markers of oxidative stress, mitochondrial, and metabolic changes could be useful for early detection of vascular-mediated injury in AD.\textsuperscript{200}
Current Status of Cerebrospinal Fluid Neurovascular Unit Biomarker Studies Collectively, the reviewed CSF biomarker studies raise a possibility that differential expression of biomarkers in multiple cell types within the NVU may relate to the disease process and cognitive decline. For example, biomarkers of pericyte-specific vascular injury (elevated sPDGFβ) and loss of cerebrovascular integrity (increased plasminogen and fibrinogen) and early growth factors mediating angiogenic response (VEGF-A and IL-8) show large changes associated with mild dementia (i.e., MCI) but have less or no regulation in advanced disease stages (Tables 1 and 2), suggesting possibly early involvement and responses of the vascular system. Similarly, BBB damage is seen early in older individuals with genetic risk for AD before cognitive impairment. However, neuronal injury markers (total tau and pTau) and Aβ42 reveal relatively moderate changes in early cognitive impairment stage, but are greatly enhanced during AD disease progression (Table 3). Surprisingly, Aβ42 and tau are infrequently studied in relation to biomarkers of the NVU responses and/or injury. Nor has the relationship between the NVU biomarkers and imaging biomarkers of neurovascular function and brain function, and/or risk factors been thoroughly investigated. All these factors may influence interpretation of the results even for established AD biomarkers. For example, elevated arterial pulse pressure in NCI individuals correlates with decreased Aβ42 and increased pTau levels, suggesting an AD CSF profile. Moreover, CSF pTau1231p levels are increased in hypertensive elderly NCI individuals suggesting AD-like CSF changes.

Current limitations. Several factors limit comparison and meta-analysis of the current biomarker studies. This includes, but is not limited to, differences in cross-sectional design, failure to account for known risk factors, unstandardized sample collection and processing, and inconsistent protein detection methods across laboratories. Majority of existing CSF biomarker studies used traditional enzyme-linked immunosorbent assays, which in some cases might have a limited range of detection and may lack the needed sensitivity to detect changes in CSF protein levels during disease states. The mini-mental state examination (MMSE) was largely used as the clinical criteria for categorizing cognitive status of NCI, MCI, and AD using a cross-sectional design, with scores ranging from 27 to 30, 22 to 27, and <22, respectively, but often overlooking potential lifestyle, vascular, and genetic risk factors, and/or environmental influences, which may alter the molecular profile of dementia and AD progression. Overcoming these existing limitations and performing longitudinal studies would aid in the ability to relate risk factors to disease development and ideally allow for preclinical detection of AD.

BLOOD-BASED BIOMARKERS

Multiple blood-based (i.e., serum and plasma) biomarker studies have been conducted to detect cognitive impairment because of AD. Many of the markers studied in blood overlap with those studied in CSF. Blood-based biomarkers are advantageous owing to ease of collection, large obtainable volume, and their ability to be easily implemented into clinical practice. The Blood-Based Biomarker Interest Group was established with the intent to identify novel, reliable biomarkers specific for AD and to overcome existing challenges in biomarker studies. The topic of blood-based biomarkers has been reviewed by several recent excellent reviews.

The limitations of the length and focus of this review on CSF biomarkers, however, does not allow us to discuss in greater detail how blood-based biomarkers relate to NVU injury.

STANDARDIZATION AND VALIDITY

One major factor affecting the current inability to identify novel, reliable biomarkers in AD involves the lack of standardization of biofluid collection, processing, and analyses across hospitals and research centers. Several programs were initiated to investigate multicenter biomarker comparison including monitoring commercial assay variation between lots, the variability of biomarker measurements across cohorts, the effects of fasting, material of sample collection tubes, centrifugation conditions, time before storage, storage temperature, and repeated freeze/thaw cycles for CSF. Recent efforts are aimed at establishing guidelines for reporting biomarker results to enhance comparability between studies. Until these standardization procedures are implemented into clinical and experimental practice, the field will lack valid comparability across research institutes and patient cohorts.

TARGETING VASCULATURE FOR ALZHEIMER’S DISEASE TREATMENT

There is overwhelming evidence supporting the role of vascular dysfunction in the etiology of AD and the influence of vascular risk factors in the onset and progression of AD. Targeting the vasculature for potential AD treatment has been, however, largely underresearched. Blood–brain barrier breakdown is thought to impact AD development through leakage of toxic blood-derived proteins (i.e., albumin, plasmin, thrombin, and fibrin) into the brain and disruption of CBF. Whether repairing BBB integrity could successfully arrest and/or reverse disease progression in a heterogeneous population of AD patients, as shown for example in animal models of neurodegeneration, remains elusive at present. Here, we discuss briefly some examples of vascular-directed strategies.

Activated Protein C

One potential vasculoprotective compound is activated protein C, which acts through brain endothelium via endothelial protein C receptor and protease-activated receptor-1. Activated protein C stabilizes the BBB through Rac1-dependent stabilization of endothelial cytoskeleton, suppression of proinflammatory cytokines, inhibition of cerebrovascular MMP-9 activity, and inhibition of apoptosis in injured vascular cells. Sealing endothelial barriers, including a leaky BBB, with activated protein C might have beneficial implications for multiple systemic and neurodegenerative conditions involving vascular dysfunction.

Cyclophilin A Inhibition

Activation of the proinflammatory signaling cascade by CypA leads to increased MMP-9 activity resulting in tight junction and basement membrane protein degradation. Cyclophilin A is also known to promote vascular oxidative stress. Thus, CypA inhibitors could be used to prevent oxidative damage and downregulate MMP-9, which could potentially alleviate BBB breakdown in dementias including AD, particularly in APOE ε4 carriers.

Fibrinogen/Aβ Inhibition

Fibrinogen binding to Aβ can structurally alter fibrin clots and reduce clot degradation. An inhibitor of this interaction RU-505 was able to restore the fibrin structure and reduce the time for clot degradation in vitro. Further, administration of RU-505 to AD transgenic mice resulted in reduced Aβ plaque burden and associated toxicity as well as improvement in cognitive function.
Autoantibodies

Recently, it was reported that N-methyl-D-aspartate receptor autoantibodies have increased seroprevalence in neuropsychiatric diseases.216 When APOE ε4 homozygous mice were injected with N-methyl-D-aspartate receptor autoantibody-positive serum, they developed animal models of sporadic AD.129 Similarly, the drug pinocembrin, which inhibits RAGE signaling to protect against Aβ toxicity,218 particularly Aβ40 and Aβ42 in APPswe mice.129

Receptor for Advanced Glycation Endproduct Inhibitors

Receptor for advanced glycation endproduct is involved in mediating reentry of circulating Aβ across the BBB.217 Animal studies suggest a multimodal action of Aβ/RAGE-specific inhibitors. Treatment with FPS-ZM1, a RAGE inhibitor, resulted in both reduced inflammatory cytokines and blocked Aβ binding to RAGEs at the BBB, which reduced levels of Aβ40 and Aβ42 in APPswe mice.129 Similarly, the drug pinocembrin, which inhibits RAGE signaling was shown to protect against Aβ toxicity,218 particularly in relation to reducing BBB injury and improving CBF.219 A recent clinical trial with the low-dose RAGE inhibitor PF-04494700 slowed cognitive decline after 18 months of treatment.220

Given the early occurrence of vascular-related events in AD, targets of BBB breakdown and vascular damage have the potential to be considered as alternative treatment options for AD patients or subjects at risk for AD exhibiting vascular dysfunction.

CONCLUSIONS AND FUTURE DIRECTIONS

The lack of preclinical detection is a major limitation for AD treatment efforts. There is a pronounced need in the field to identify molecular, structural, and functional phenotypes associated with defined stages of disease progression that are specific to AD. Clinically, many types of AD-related dementias (i.e., frontotemporal, vascular, mixed, and Lewy-body dementias) in addition to normal aging exhibit both vascular dysfunction and amyloid accumulation. Though amyloid changes occur during preclinical stages of AD, measures of amyloid (CSF levels of Aβ42 and Pittsburgh compound B PET) and tau do not appear to be sensitive enough to predict the onset of cognitive impairment and AD clinical diagnosis. Recent guidelines have been established for the clinical use of CSF markers Aβ42, pTau181, and total tau209 and the identification of early, reliable, and validated AD biomarkers.221 The Alzheimer’s Biomarkers Standardization Initiative is overseeing these efforts with the goal to identify and standardize biomarkers for the diagnostic accuracy of MCI and AD.209 Reliable biomarkers are essential for (1) early disease detection and intervention and (2) evaluating the effectiveness of clinical trials. It is apparent from existing Aβ treatment efforts that intervening during moderate to advanced AD may be too late in the disease process to effectively reduce and/or prevent pathologic progression and cognitive decline.222 Recent AD prevention efforts have emphasized the importance for targeting not only neurons but also nonneuronal cell types.135 Detecting AD during preclinical stages as well as predicting prodromal conversion to AD is essential for successful intervention. Incorporating multiple biomarkers of the NVU with the currently established AD biomarkers, Aβ and tau, could potentially increase the clinical usefulness of CSF biomarkers for early AD-specific diagnosis.

The impact of vascular dysfunction in influencing the etiology of sporadic AD is apparent from clinical studies, human AD autopsy reports, neurovascular MRI studies, and transgenic animal model studies. Altogether, research supports a temporal alteration of injury to cells of the NVU in AD. Thus, in light of observed detectable changes, we propose a hypothetical model suggesting differential temporal involvement of cell-specific biomarkers of vascular injury, inflammatory response, and neuronal injury throughout cognitive decline (Figure 3). Additional research is necessary to confirm or amend this general model.

In summary, simultaneous detection of molecular biomarkers in combination with structural and functional imaging biomarkers is necessary to identify a biomarker algorithm associated with defined stages of AD development. Future longitudinal CSF and imaging (i.e., BBB and CBF) biomarker studies in human subjects with NCI and/or MCI that incorporate risk factors for AD (i.e., genetic, vascular, environmental, and lifestyle) should continue to interrogate the role of neurovascular mechanisms in the pathogenesis of dementia due to AD and other causes. Elucidating the precise mechanism through which vascular insults influence AD development would be beneficial and might help identify novel biologic targets for drug development and aid in patient-directed treatment efforts.

DISCLOSURE/CONFlict OF INTEREST

The authors declare no conflict of interest.

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