Cerebrospinal Fluid Markers of Synaptic Injury and Functional Connectivity in Alzheimer’s Disease

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A. Background and Scientific Rationale

Amyloid plaques (AP) and neurofibrillary tangles (NFT) are early pathological substrates of Alzheimer’s disease (AD) that precede symptom onset by 10-15 years [1, 2]. However, it is only after significant synaptic loss has occurred in vulnerable brain regions that the first signs of cognitive impairment appear [3]. Synaptic dysfunction is an early and prominent pathological feature of AD that precedes frank neuronal loss in several brain regions [4-8]. Cortical synaptic density is reduced by as much as 35%, and synaptic density per neuron by 15-35%, in even the earliest symptomatic stages of AD [9, 10]. Clinico-pathological studies suggest that synaptic loss is the best surrogate for cognitive decline and disease progression in AD [6, 9-12], and correlates more closely with cognitive deficits than the number of plaques or tangles, or the extent of cortical gliosis in postmortem AD brains [9]. In addition to AP and NFT pathology, soluble Aβ oligomers are thought to contribute to synaptic dysfunction in AD [13, 14]. Significant synaptic pathology is observed in areas devoid of, or distant from, insoluble amyloid deposits [8, 14-16]. Together, these findings support the notion that synaptic loss reflects the cumulative outcome of different pathological substrates in AD (soluble and insoluble Aβ, tau, and inflammation), and mediates effects of these pathologies on memory and cognitive functions [12, 17].

We have recently identified neurogranin (Ng), synaptosomal-associated protein-25 kD (SNAP-25), and synaptotagmin as potential biomarkers of synaptic injury in AD [17, 18]. Ng, SNAP-25, and synaptotagmin are synaptic proteins which are abundantly and preferentially expressed in the pre-synaptic (SNAP-25 and synaptotagmin) or post-synaptic (Ng) membranes, and are widely distributed in the brain [19-21]. Ng is a neuron-specific [19] calmodulin-binding post-synaptic protein [22] which is abundantly expressed in neuronal dendritic spines. Several studies have implicated Ng in activity-dependent synaptic plasticity, memory, and learning [22-26]. Ng enhances synaptic function [19] and facilitates long-term potentiation through its ability to regulate the availability of calmodulin at synaptic sites [27-29]. SNAP-25 is a widely distributed pre-synaptic protein which is involved in docking and fusion of vesicles, a process essential for exocytosis [30]. SNAP-25 has been implicated in axonal outgrowth and nerve growth-induced neurite elongation [31]. Synaptotagmin is a pre-synaptic calcium-sensor protein which is involved in exocytosis and regulates neurotransmitter release in hippocampal neurons through its interaction with SNARES (soluble NSF attachment protein receptors) [32].
Studies by our group, and others, have shown that cerebrospinal fluid (CSF) levels of Ng [17, 33], SNAP-25 [20], and synaptotagmin [21] are elevated in AD compared to controls. Elevated CSF levels of these synaptic proteins in AD likely reflect the release of abundant synaptic constituents into the extracellular space in the setting of neurodegeneration [17]. Our findings that CSF Ng levels are not elevated in a small cohort of individuals with non-AD dementias suggest that CSF Ng levels may offer diagnostic specificity for AD, and may be influenced by relatively disease-specific mechanisms of synaptic injury or alterations in signaling pathways [17, 34].

We have previously shown that CSF Ng levels strongly correlate with CSF tau and p-tau181 levels, whole-brain and regional atrophy, cortical amyloid deposition, and rates of cognitive decline in a large well-characterized cohort of individuals with pre-symptomatic and early symptomatic AD, and healthy controls who were followed longitudinally over 3 years [17]. In this cohort, CSF Ng levels offered predictive value for future cognitive impairment in cognitively normal individuals over a 2-3 year follow-up period that was comparable to other biomarkers of AD pathology (tau, p-tau181, and Aβ42), and complemented the collective ability of these markers to predict AD pathology in cognitively normal elderly (i.e. pre-symptomatic AD). Furthermore, data from our group, and others [20, 21], suggest that CSF levels of the synaptic proteins, SNAP-25 and synaptotagmin, offer value as diagnostic and predictive markers in early AD, and correlate with other CSF biomarkers of AD and brain atrophy. Together, these findings support the value of CSF Ng, SNAP-25, and synaptotagmin as CSF surrogates of synaptic injury in AD.

Functional magnetic resonance imaging (fMRI) studies have been instrumental in the identification of networks of cortical regions which demonstrate highly synchronized activity during the resting state (i.e. the absence of any specific cognitive activity) or during the performance of specific cognitive tasks (e.g. sensory, motor, memory, attention, or language-related tasks) [35, 36]. Importantly, fMRI studies have allowed the detection of specific patterns of altered functional connectivity (FC) in these networks which may occur as a result of healthy aging or disease processes such as AD [36]. The importance of fMRI as an imaging biomarker in AD is highlighted by its ability to detect functional alterations in neural networks that are vulnerable to early stages of AD pathology (e.g. resting state, episodic memory, and semantic memory networks) [37, 38], which often precede the onset of clinically-detectable cognitive impairment or structural changes on MRI. Several lines of evidence suggest that FC alterations mirror to a large extent the structural, molecular, and metabolic changes in vulnerable networks in disease-specific patterns [39].

Importantly, fMRI studies have highlighted an intrinsic network of brain regions (posterior cingulate, precuneus, medial temporal, medial pre-frontal, and inferior parietal regions) which shows highly synchronized activity at rest, and increased activity in a task-free state compared to cognitively demanding tasks, referred to as the default-mode network (DMN) [40, 41]. Perturbations in the DMN have been well described in mild cognitive impairment (MCI) due to AD and AD dementia on resting-state fMRI [36, 42, 43]. Several studies have demonstrated decreased FC between the posterior cingulate/precuneus and hippocampus/medial temporal lobe in individuals with MCI due to AD and AD dementia [19, 44, 45]. Such changes are thought to be the result of structural alterations in the hippocampus and disruption of the cingulum bundle leading to a “disconnection” between these regions, which precedes radiologic evidence of hippocampal atrophy [43]. The magnitude of such asynchronies correlates well with the degree of pathology [46, 47]; individuals with AD dementia have more severe alterations in FC among these regions than individuals with MCI due to AD, and more severe alterations are noted in MCI than in healthy elderly.
Semantic memory refers to the recall of general facts and knowledge that are not contextually-specific (e.g. making a categorical or attributional judgment to a presented item) [48]. We [49], and other groups [48, 50], have shown that significant impairment in semantic memory functions can be seen in even the earliest clinical stages of AD, including MCI. Functional MRI studies have identified a left lateralized network of cortical regions involved in semantic memory processing, including the posterior cingulate, posterior inferior parietal, middle temporal, fusiform, parahippocampal gyrus, dorsomedial prefrontal, ventromedial prefrontal, and inferior frontal cortices [51, 52]. Reduced FC among these regions has been reported in MCI due to AD during the performance of a semantic memory task which consists of the identification of famous names (Famous Name Discrimination Task [FNDT]) [38]. This semantic memory task has been shown to activate several brain regions which overlap with the episodic memory network [37, 51], and can be reliably performed by individuals with MCI or mild AD dementia [38].

The main purpose of this study is to examine cross-sectional associations between CSF markers of synaptic injury (Ng, SNAP-25, and synaptotagmin) and functional connectivity (FC) in the default-mode and semantic memory networks using 3T-functional MRI in early symptomatic AD (MCI due to AD and mild AD dementia; Clinical Dementia Rating [CDR] 0.5 and 1, respectively) (n=15) and cognitively normal controls (CDR 0; n=15).

The identification of CSF biomarkers that reflect functional alterations in neural networks targeted by early AD pathology (i.e. default-mode and semantic memory networks) will provide a valuable tool to monitor disease progression and response to disease-modifying therapies directed against different pathological substrates of AD, independently of changes to amyloid or tau pathology. Results from this study will shed light on the potential utility of synaptic proteins as CSF surrogates of functional connectivity within neural networks, and provide useful information regarding their value as potential outcome measures or stratification tools in clinical trials of AD therapeutics. CSF biomarkers of amyloid and tau have limited value as markers of disease progression and do not change considerably over time in symptomatic and asymptomatic individuals [53, 54]. Furthermore, imaging methods that utilize amyloid-binding ligands do not reliably reflect soluble Aβ species which contribute significantly to synaptic damage and cognitive impairment in AD. Therefore, synaptic markers may offer useful measures for disease outcomes and therapeutic response at an earlier stage, and to a better degree, than CSF or imaging markers of amyloid or tau pathology. Importantly, this study will provide insight into the molecular mechanisms that underlie the radiologic correlates of neural activity in different stages of disease, and will improve our understanding of the dynamic interface between CSF and imaging surrogates of synaptic activity in the presence and absence of AD pathology.

To our knowledge, this will be the first study to investigate associations between CSF markers of synaptic injury and FC in individuals with AD or controls. We have previously demonstrated correlations of CSF Ng levels with whole-brain and regional atrophy in AD [17]. However, we are not aware of any studies which have investigated correlations between CSF Ng, SNAP-25, or synaptotagmin levels and fMRI measures of functional connectivity in MCI or AD. While associations of CSF tau, p-tau181, or Aβ42 with FC on resting-state fMRI in AD [55], and with task-activated fMRI in cognitively normal elderly [56] have been reported, we have not been able to identify any published studies which have investigated correlations between any CSF biomarkers of AD pathology and FC on task-activated fMRI in individuals with MCI or AD. Therefore, this will also be the first study to investigate associations between CSF biomarkers of AD pathology (including CSF markers of synaptic injury) and FC during the performance of a cognitive task (i.e. the semantic memory task, Famous Name Recognition Test) which can be reliably performed by individuals with MCI and mild AD dementia. Our study will provide novel
CSF biomarker and fMRI data which is not available in databases such as ADNI and the Dominantly Inherited Alzheimer’s Network (DIAN).

B. Study Objectives:

Primary Objectives:

Primary Objective 1. Investigate correlations between CSF biomarkers of synaptic injury (Ng, SNAP-25, and synaptotagmin) and functional connectivity in the default-mode network using resting-state fMRI (adjusting for age, gender, apolipoproteinE4 [APOE ε4] genotype, task performance, and regional brain atrophy) in AD (MCI due to AD and mild AD dementia) and controls.

Primary Objective 2. Examine correlations between CSF biomarkers of synaptic injury and functional connectivity in the semantic memory network on task-activated fMRI during the performance of the Famous Name Discrimination Task (adjusting for age, gender, APOE ε4 genotype, task performance, and regional brain atrophy) in AD (MCI due to AD and mild AD dementia) and controls.

We hypothesize that higher CSF Ng, SNAP-25, and synaptotagmin levels (i.e. reflective of more severe synaptic injury) will be associated with lower FC between the posterior cingulate/precuneus seed and other regions in the DMN, including the hippocampus/medial temporal, medial prefrontal, and inferior parietal regions, on resting-state fMRI in individuals with MCI and mild AD dementia. Similarly, we hypothesize that CSF Ng, SNAP-25, and synaptotagmin levels will negatively correlate with FC between the posterior cingulate seed and other regions in the semantic memory network (posterior inferior parietal, middle temporal, fusiform, dorsomedial prefrontal, and inferior frontal regions) during the performance of a semantic memory task in MCI due to AD and mild AD dementia. Conversely, we hypothesize that no significant correlations between CSF Ng, SNAP-25, and synaptotagmin levels and FC will be observed in the default mode or semantic memory networks during resting-state or task-activation fMRI, respectively, in cognitively normal controls.

Secondary Objectives:

Secondary Objective 1. Investigate correlations between established AD biomarkers (CSF tau, p-tau181, and Aβ42) and functional connectivity in the default-mode network using resting-state fMRI (adjusting for age, gender, APOE ε4 genotype, task performance, and regional brain atrophy) in AD (MCI due to AD and mild AD dementia) and controls.

Secondary Objective 2. Examine correlations between established AD biomarkers (CSF tau, p-tau181, and Aβ42) and functional connectivity in the semantic memory network on task-activated fMRI during the performance of the Famous Name Discrimination Task (adjusting for age, gender, APOE4 genotype, task performance, and regional brain atrophy) in AD (MCI and mild AD dementia) and controls.

Secondary Objective 3: Compare correlations of novel and established AD biomarkers (CSF Ng, SNAP-25, synaptotagmin, tau, p-tau181, and Aβ42), individually or as combinations of biomarkers, with FC in the default-mode and semantic memory networks in AD (MCI due to AD and mild AD dementia) and controls.
C. Methods

This will be a cross-sectional study of individuals with amnestic MCI due to AD or mild AD dementia (n=15), and cognitively normal controls (n=15). All participants will undergo clinical and detailed cognitive assessments, lumbar punctures (LPs), structural MRI, and fMRI. Functional MRI studies will be obtained during rest and the performance of a semantic memory task (i.e. FNDT). Cognitive, CSF, and MRI evaluations will be completed within 4 months of enrollment in all participants. Resting-state and task-activated fMRI analyses will be conducted in the same setting to minimize effects of environmental factors on fMRI parameters.

Participants: Participants will be recruited from the community and the Memory Disorders Clinic of The OSU Wexner Medical Center Department of Neurology. This study will include cognitively normal individuals (CDR 0; n=15), individuals with a clinical diagnosis of single-domain or multi-domain amnestic MCI due to AD or mild AD dementia (CDR 0.5 or 1; n=15). Written informed consent will be obtained from all participants or their legally authorized representatives when appropriate. Additionally, written informed assent will be obtained from all participants with mild AD dementia (Consent Forms are enclosed).

Inclusion criteria: Participants included in the study will be i) 60 years of age or older, with a clinical diagnosis of amnestic MCI due to AD, mild AD dementia, or normal cognition (See Criteria for Diagnostic Classification), who have ii) no significant medical or surgical co-morbidities, iii) no contraindications to LP or MRI (see Appendix), and iv) adequate visual and auditory acuity for testing. Participants must have a responsible study partner who either lives with them or is in regular contact with them for at least 10 hours per week.

Exclusion criteria: i) participants with MCI due to AD or mild AD dementia who have been started on cholinesterase-inhibitors (CHEI) and/or memantine within 3 months of study enrollment, or whose dosage of these medications had been adjusted in the 3 months prior to enrollment, ii) individuals with any past history of ischemic or traumatic brain injury (including concussion), iii) individuals with imaging evidence of significant cerebrovascular disease or structural brain lesions (e.g. tumor, demyelinating disorders, infection, or congenital anomalies), iv) active mood or psychiatric disorder, v) active daily alcohol use, past or current history of alcohol abuse/dependence, or vi) active daily or frequent (≥ 2 times/week) use of benzodiazepines, barbiturates, anticholinergics, anti-histamines, sedatives, sleep aids, or anti-epileptic medications in the 3 months prior to the study will be excluded from participation.

Eligible participants who have been on stable doses of CHEI and/or memantine for ≥ 3 months at the time of enrollment, and who meet the other eligibility criteria for the study, will be included. Eligible study participants will be instructed to avoid use of alcohol, benzodiazepines, over-the-counter sleep aids, anti-histamines, and anticholinergic medications for at least 2 weeks prior to the time of their enrollment and for the whole duration of the study.

Participant Evaluation:

Clinical Diagnosis:

Criteria for Diagnostic Classification:

- The clinical diagnoses of amnestic MCI due to AD will be made according to standard clinical criteria as described by the National Institute of Aging –Alzheimer’s Association Working Group [57, 58] and supported by CSF biomarker data for tau, p-tau181, and Aβ42 (i.e. elevated CSF tau, elevated p-tau181, and low CSF Aβ42 levels). This
includes evaluation for other systemic or neurological disorders which could account for the cognitive impairment, and inclusion of results from ancillary structural imaging (CT or structural MRI), neuro-psychometric testing, and FDG-PET imaging (when available) into the diagnostic scheme [57].

- The diagnosis of amnestic MCI will be based on impairment in episodic memory with or without impairment in other cognitive domains (i.e. multi-domain and single-domain amnestic MCI, respectively), which exceeds 1.5 SD of age-, gender-, and education-matched norms, and is not associated with significant functional decline [57].

- The clinical diagnosis of mild AD dementia will be made according to standard clinical criteria as described by the National Institute of Aging –Alzheimer's Association Working Group [57, 58] and supported by CSF biomarker data for tau, p-tau181, and Aβ42. This includes evaluation for other systemic or neurological disorders which could account for the cognitive impairment, and inclusion of results from ancillary structural imaging (CT or structural MRI), neuro-psychometric testing, and FDG-PET imaging (when available) into the diagnostic scheme [57].

- In individuals who meet standard criteria for AD dementia, the CDR will be used to determine the severity of dementia; a CDR designation of 1, 2, and 3 denotes mild, moderate, and severe AD dementia, respectively [59].

- Normal cognition will be defined as cognitive performance on detailed neuropsychometric testing that falls within 1 SD of age-, gender-, and education-matched norms in all cognitive domains, and no subjective report of cognitive decline from an individual's baseline (i.e. CDR 0). The clinical diagnosis of controls will be confirmed by normal CSF tau, p-tau181, and Aβ42 levels (CSF tau<93 pg/ml, p-tau181<23 pg/ml, and Aβ42>192 pg/ml) (See CSF Methods).

- All clinical diagnoses will be reached by consensus of the cognitive neurologists involved in the study.

Clinical and Cognitive Assessments:

Clinical evaluations will be performed by cognitive neurologists in the Memory Disorders Clinic of The Ohio State University (Tarawneh and Scharre).

- Clinical assessments will include a detailed review of history of present illness, past medical, past surgical, social, and family history, medications, and allergies, and a detailed physical and neurological exam

Cognitive assessments will include:

- The Clinical Dementia Rating
- The Mini-Mental Status Examination or equivalent
- The Self-Administered Gerocognitive Examination (SAGE)
- The Geriatric Depression Scale (GDS)

Neuropsychometric Assessments:

Neuropsychometric assessments will be performed by experienced OSU neuropsychometricians. These evaluation will include the following tests:

- Associate Learning subtest of the Wechsler Memory Scale [WMS]
- Immediate recall of the WMS Logical Memory (I and II)
- Hopkins Verbal Learning test
- Information subtest from the Wechsler Adult Intelligence Scale [WAIS]
- Boston Naming Test
- Animal Naming
- WMS Mental Control
- Digit Span Forward and Digit Span Backward
- Letter Fluency for F and S
- WAIS Block Design
- Digit Symbol subtests
- Trail-making Tests A and B
- WAIS and Boston Naming Test
- The behavioral component of the Neuropsychiatric Inventory
- The Functional Activity Questionnaire

Outcome Measures:

Blood Sample Analyses

- APOE4 genotype
- PT, PTT, INR (as screening for coagulopathy prior to LP)
- CBC and platelets (as screening for coagulopathy prior to LP)
- (optional) platelet functional assays when indicated

Lumbar Puncture:

All participants will undergo LPs within 0-4 months of the clinical and cognitive assessments. A total of 20-25 ml will be obtained from each participant in the lateral decubitus position under sterile conditions, collected in a sterile polypropylene tube, placed on dry ice, centrifuged, and stored at -80°C prior to analysis. CSF aliquots will be thawed and analyzed for Ng, SNAP-25, synaptotagmin, tau, p-tau_181, and Aβ42 using multiplex enzyme-linked immunosorbent assays (ELISAs) on a Luminex xMAP platform as described in the manufacturer’s protocol (Luminex Corp, Austin, TX)[60].

Cerebrospinal Fluid (CSF) Biomarker Assessments:

- CSF neurogranin levels (ng/ml)
- CSF SNAP-25 levels (ng/ml)
- CSF synaptotagmin levels (ng/ml)
- CSF tau levels (pg/ml)
- CSF p-tau_181 levels (pg/ml)
- CSF Aβ42 levels (pg/ml)

Structural MRI measures

- Normalized whole-brain volume
- Regional volumes of the hippocampus, entorhinal cortex, parahippocampal gyrus, precuneus, posterior cingulate, fusiform, and occipital regions.

Functional MRI measures:

- Functional connectivity between the posterior cingulate seed and other regions in the default mode network on resting-state fMRI.
**Functional connectivity** Functional connectivity between the posterior cingulate seed and other regions in the semantic memory network on task-activated fMRI during the performance of the Famous Name Discrimination Task.

**Case Report Form:**

1. Digital data collection will be collected on each subject.
2. The subject identification number must be noted on the digital data collection form as well as on the blood tubes.
3. The digital data collection includes the following for each subject: name, identification number, date blood collected, diagnosis, age, gender, race, education level, family history of neurologic disorder, list of active medications, behavioral, sleep, and functional questionnaire results, vital signs, physical examination and neurologic examinations, APOE status, neuroimaging results, neuropsychological test results, CSF results, miscellaneous comments section, and medical record number if available.

**D. Study Design:**

This will be a cross-sectional study of individuals with amnestic MCI due to AD or mild AD dementia (n=15) and cognitively normal controls (n=15). All participants will undergo one clinical and detailed cognitive assessment during the first visit, one lumbar puncture (LP) during the second visit, and one structural and functional MRI evaluation during the third visit. Functional MRI studies will be obtained during rest and the performance of a semantic memory task (i.e. FNDT). Cognitive, CSF, and MRI evaluations will be completed within 4 months of enrollment. Resting-state and task-activated fMRI analyses will be conducted in the same setting to minimize effects of environmental factors on fMRI parameters.

<table>
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<tr>
<th>Study Visit</th>
<th>Evaluations/procedures to be completed</th>
<th>Estimated Visit Duration</th>
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| 1           | - Detailed history and review of past medical history from study participant and study partner  
- Detailed physical and neurological exam  
- Detailed assessment of cognitive functions including memory, language, attention, processing speed, judgment and problem-solving, and visuospatial functions  
- Blood sample for genetic testing and to rule out bleeding disorders prior to the lumbar puncture (i.e. spinal tap)  
- Detailed review of checklist for safety/eligibility to perform the magnetic resonance imaging (MRI) and the lumbar puncture (LP) | 3-4 hours |
| 2           | Lumbar puncture (i.e. spinal tap) | 60-90 minutes |
Magnetic Resonance Imaging (MRI) while resting and during the performance of a cognitive task that involves identifying names of famous people (i.e. semantic memory task) 90 minutes

E. Participants:

Participants will be recruited from the community and the Memory Disorders Clinic of The OSU Wexner Medical Center Department of Neurology. This study will include cognitively normal individuals (CDR 0; n=15), and individuals with a clinical diagnosis of single-domain or multi-domain amnestic MCI due to AD, or mild AD dementia (CDR 0.5 or 1; n=15). Clinical diagnoses will be made as described in “Criteria for Diagnostic Classification”.

F. Informed Consent

For those individuals who meet inclusion and exclusion criteria and who agree to participate in this study, informed consent will be obtained from all participants or legally authorized representatives. As this proposed study includes cognitively normal individuals and individuals with only mild cognitive impairment or mild AD dementia, we anticipate that all study participants will be able to fully participate in the consent or assent process.

Permission from the participant will be obtained to interview a study partner who knows the subject well and can provide collateral information. The study partner must be willing to come to the study visit and answer questions about the participant. Study partners can be friends or family members who either live with the participant or are in regular contact with him/her (i.e. at least 10 hours per week). The study partner will be asked questions about the participant’s cognitive, behavioral, and functional abilities and medical history.

Written informed consent will be obtained from all study participants (or when appropriate, their legally authorized representatives [LARs]), and all study partners. For participants with mild AD dementia, a written assent will also be obtained from the study participant. For the purpose of this study, a written assent form has been created, which has been adapted from assent forms previously approved by the IRB for other studies involving lumbar punctures, such as the ADNI-2, ADNI-GO, ADNI-3, and the Buck-Eye Biospecimen Repository (protocol number 94H0440).

G. Procedures

All subjects will have a clinical evaluation (including a detailed history and review of medical records), physical examination including vital signs and orthostatic blood pressure (i.e. blood pressure during the lying, sitting, and standing positions) and pulse, neurological examination, clinical and cognitive assessments (as detailed above), detailed neuropsychometric testing 9as detailed above), blood sample collection, structural MRI, functional MRI during rest and task-activation, and lumbar puncture for CSF biomarker assessment. Clinical, cognitive, and neuropsychometric assessments will be performed by study personnel who are trained and experienced in their administration. The study partner will be interviewed for completion of all of the behavioral and functional measures listed above by trained study personnel trained. Participants will also have the option of participating in other IRB approved research studies.
including the Buckeye Biospecimen repository and the brain donation program. Study personnel will access IHIS (OSU Electronic Medical Record) only for the purpose of supplementing the consented subject’s medical history.

H. Outcomes, Results, and Analysis

Statistical Analysis:

Student’s t-tests, chi-square ($\chi^2$) analyses, and Analysis of Co-variance (ANCOVA) will examine differences in demographic, clinical, cognitive, CSF biomarker, and fMRI parameters between the study groups (SPSSv15, SPSS, IL). Partial correlation analyses and linear regression models will examine associations between CSF biomarker levels and FC measures on fMRI, adjusting for age, gender, APOEɛ4 genotype, task performance, regional brain atrophy, and treatment with CHEI or memantine (SPSSv15, SPSS, IL). Bootstrap analyses will compare correlations between CSF biomarker measures (individually or as combinations of markers [using Principal Components Analysis]) and FC in the DMN and semantic memory networks in AD and controls (R Statistical Software, www.r-project.org). Statistical analyses will be performed by Juan Peng, MS in the OSU Center for Biostatistics.

CSF Collection, Processing, and Analysis:

All participants will undergo LPs within 0-4 months of the clinical and cognitive assessments. A total of 20-25 ml will be obtained from each participant in the lateral decubitus position under sterile conditions, collected in a sterile polypropylene tube, placed on dry ice, centrifuged, and stored at -80 C prior to analysis.

Lumbar punctures will be performed in the outpatient Memory Disorders Clinic of the Ohio State University by study investigators (Tarawneh and Scharre) per standard procedures. CSF sample processing and analyses will be performed in the laboratory of study investigator Dr Stephen Kolb at Ohio State University by personnel with experience in immunoassays. Study investigators, Kolb and Tarawneh, have extensive experience in the development and optimization of immunoassays for biomarker development and validation [17, 49, 61, 62].

CSF aliquots will be thawed and analyzed for Ng, SNAP-25, synaptotagmin, tau, p-tau181, and Aβ42 using multiplex enzyme-linked immunosorbent assays (ELISAs) on a Luminex xMAP platform as described in the manufacturer’s protocol (Luminex Corp, Austin, TX)[60]. All samples will be centrifuged (11,000 g x 3 minutes) to remove particulates prior to analysis. Assay steps will be performed at room temperature unless otherwise indicated.

CSF tau, p-tau181, and Aβ42

CSF tau, p-tau181, and Aβ42 levels will be measured on a multiplex xMAP Luminex platform (Luminex Corp, Austin, TX) using the Fujirebio (formerly “Innogenetics”, Ghent, Belgium) AlzBio3 immunoassay kit–based reagents per the manufacturer’s protocol as described [63-65]. The Fujirebio AlzBio3 kit reagents include well-characterized capture monoclonal antibodies specific for Aβ42 (4D7A3), t-tau (AT120), and p-tau181 (AT270), which are each chemically bonded to unique sets of color-coded beads, and analyte-specific detector antibodies (HT7, 3D6).

For CSF biomarker assessments for tau, p-tau181, and Aβ42 in this study, we will use the cut-off values (93 pg/ml, 23 pg/ml, and 192 pg/ml, respectively) proposed by ADNI studies [64].
which used similar methods and platforms for CSF analyses in autopsy-confirmed cases of AD and controls (https://ida.loni.usc.edu for ADNI_Methods_UPENN_Biomarker_20120710.pdf). These proposed cut-off values offer a diagnostic sensitivity of 70%, 68%, and 96%, and a specificity of 92%, 73%, and 77% (for tau, p-tau181, and Aβ42, respectively) in differentiating AD from controls.

**CSF Ng**

CSF samples will be analyzed for Ng levels using an affinity-purified polyclonal rabbit anti-human Ng antibody directed against a C-terminal epitope (P-4793) as the capture antibody, and a rabbit polyclonal anti-human Ng antibody directed against an N-terminal epitope (P-4794) as a detection antibody (provided by the Laboratory of Dr Jack Ladenson in Washington University)[17]. The xMAP Antibody Coupling kit will be used to couple the capture antibodies to MagPlex low concentration microspheres to allow analysis on the Luminex xMAP platform per manufacturer's protocol [60]. We have previously demonstrated the efficacy of these antibodies for CSF Ng measurements in a large longitudinal study of AD and controls enrolled in the Washington University ADRC [17], and 152 CSF samples obtained from ADNI [66] using the Erenna immunoassay system (Singulex).

**CSF SNAP-25**

CSF SNAP-25 levels will be measured using a mouse monoclonal anti-human SNAP-25 antibody (6H07-2C12) as a capture antibody, and a mouse monoclonal anti-human SNAP-25 9E11 as a detection antibody (provided by the Laboratory of Dr Jack Ladenson in Washington University). The capture antibodies will be coupled to MagPlex microspheres to allow analysis on the Luminex xMAP platform using the xMAP Antibody Coupling Kit as described [60]. Both of these antibodies have been used for CSF biomarker assessments of 152 samples from the ADNI cohort using the Erenna immunoassay system (Singulex)[66].

**CSF synaptotagmin**

CSF synaptotagmin levels will be measured using a mouse monoclonal anti-human synaptotagmin antibody (ab77314, Abcam) as a capture antibody, and a rabbit polyclonal anti-human synaptotagmin antibody (ab51164, Abcam) as a detection antibody. The capture antibodies will be coupled to MagPlex microspheres to allow analysis on the Luminex xMAP platform using the xMAP Antibody Coupling Kit as described [60].

The capture antibodies for the CSF Ng, SNAP-25, and synaptotagmin analyses will be coupled to MagPlex microspheres to allow analysis on the Luminex xMAP platform per manufacturer’s instructions as described [60]. The antibodies for Ng and SNAP-25 have been evaluated on the Luminex xMAP platform in previous pilot analyses with comparable results to the Erenna/Singulex system. The Luminex xMAP platform is available at OSU, and offers several technical advantages to the Erenna system. The Luminex xMAP technology allows multiplex immunoassays on single samples which reduces non-specific binding and allows use of smaller sample volumes, resulting in increased throughput and efficiency.

The CSF samples will be stored and analyzed in the Laboratory of Dr Stephen Kolb at OSU. The Kolb Laboratory has the equipment, personnel, and expertise in CSF analysis using the Luminex xMAP platform, and has been successful in applying this immunoassay system for the identification of novel CSF biomarkers for neuromuscular disorders. Additionally, the study PI (Tarawneh) has multiple collaborations with the Washington University Alzheimer’s Disease Research Center and the Department of Pathology (previous mentors: Dr David Holtzman, Dr John Morris, and Dr Jack Ladenson) who are available for consultation and assistance with CSF biomarker analyses.
Plasma collection and APOE genotyping:
A total of 10 ml of blood will be obtained from each participant, collected in EDTA tubes, stored at room temperature, and shipped within 24 hours of collection to Athena Diagnostics for APOE genotyping using an RFLP (restriction fragment length polymorphism method) (Athena Diagnostics, ADmark™ Assays, USA).

Structural Magnetic Resonance Imaging:
Structural MRI will be performed using a Siemens 3.0 Tesla Prisma scanner (Siemens, Erlangen, Germany). Structural MRIs done for the purpose of this study will not include the use of gadolinium contrast. One to four T1-weighted sagittal magnetization prepared rapid gradient-echo (MPRAGE) scans will be acquired for each participant, and one MPRAGE will be needed for each scanning session. Image processing will be performed as previously described [67, 68]. Whole-brain volume will be obtained using freely available Freesurfer 5.0 software [69, 70], with segmentation classifying each voxel of the MR image as CSF, gray matter, or white matter. Normalized whole-brain volumes (nWBVs) will be computed as the proportion of all voxels occupied by gray and white matter (equivalent to 100% minus the percentage of CSF) voxels, yielding a unit that represents the proportion of estimated total intracranial volume (ICV). Regional volume estimates will be obtained via the Freesurfer 5.0 image analysis suite, which implements an automated probabilistic labeling procedure [71, 72]. Regions of interest (ROIs) will include the hippocampus, entorhinal cortex, and parahippocampal gyrus, precuneus, fusiform gyrus, and posterior cingulate cortex. The pericalcarine cortex will be included as a control region. Estimated ICV will be used to adjust ROIs for head size variation based on a covariance approach as described. To reduce the number of comparisons, the ROI volumes will be averaged across hemispheres.

Functional Magnetic Resonance Imaging: Whole-brain resting-state and task-activated fMRI will be conducted on a Siemens 3.0 Tesla Prisma scanner (Siemens, Erlangen, Germany) equipped with a 32-channel head array coil. Echo planar images will be collected using a pulse sequence (TE = 28 ms; flip angle = 72 degrees; field of view (FOV) = 240 mm; matrix size = 64 × 64). Thirty-six contiguous axial 4-mm-thick slices will be selected to provide coverage of the entire brain (voxel size = 3.75 × 3.75 × 4 mm). The inter-scan interval (TR) will be 2 seconds. High-resolution, three-dimensional anatomic images will be acquired using the MPRAGE sequence (TE = 4.44 ms; TR = 1900 ms; inversion time = 950 ms; flip angle = 12 degrees; slice thickness = 1.0 mm; FOV = 256 mm; matrix size = 256 × 224; resolution 1 × 1 × 1 mm). Foam padding will be used to reduce head movement within the coil.

Functional images will be analyzed using FSL software package (FMRIB software library, version 5.0.8, www.fmrib.ox.ac.uk/fsl). A general linear model (GLM) analysis will be used to extract brain activation to famous and unfamiliar names from the time-series. Motion parameters will be incorporated into the model as nuisance regressors. Individual anatomical and functional scans will be transformed into standard stereotaxic Talairach space. To compensate for normal variation in anatomy across participants, functional images will be blurred using a 6-mm Gaussian full-width half-maximum filter. Functional connectivity within the default and semantic memory networks will be examined using a region-of-interest (ROI, i.e. seed) model. Using voxel-wise t-tests, resting-state and task activations will be calculated separately. Significant cluster volumes will be used to create functional regions of interest (fROI) for each state. The
average AUC of all voxels within each fROI will be calculated for each participant. Structural MRI and fMRI evaluations will be performed in the Center for Cognitive and Behavioral Brain Imaging (CCBBI) of the Ohio State University under the supervision of the center director and study investigator, Dr Zhong-Lin Lu. Image acquisition and analysis will be performed by CCBBI imaging personnel who are trained and experienced in these procedures.

Semantic Memory Task:

The task-activated fMRI will be obtained during performance of a semantic memory task that consists of the presentation of 30 highly recognizable famous names and 30 unfamiliar names (referred to as the Famous Name Discrimination Task [FNDT]). Accuracy and reaction time will be recorded. The use of a semantic memory task offers several advantages over episodic memory tasks in MCI and mild AD. In contrast to episodic memory tasks which may be impaired with healthy aging, semantic memory tasks remain relatively intact in healthy elderly but are impaired in the presence of AD pathology. Furthermore, semantic tasks are easier and less frustrating for elderly to perform, therefore, allowing for more accuracy in interpreting test results by eliminating confounding effects of increased mental effort on fMRI signal. This semantic task has been successfully applied in multiple fMRI studies of MCI and AD [38]. The semantic memory task will be performed by clinical research personnel of the Memory Disorders Clinic at the Ohio State University, who are trained and experienced in the performance of this test [38].

I. Risk-Benefit Analysis

The risks of this study, involving lumbar punctures and MRI, represent no more than a minor increase over minimal risk.

For the purpose of this study, one brain MRI session will be performed for each study participant (total duration 60-90 minutes). The performance of MRI does not include any risk of radiation exposure. The MRIs performed in this study will be performed without contrast, and therefore, there will be no intravenous injection or exposure to contrast agent (i.e. gadolinium). All participants will be screened by the study PI as well as the radiology staff performing the MRI to determine the safety of MRI, and the absence of any absolute or relative contra-indications for MRI through a detailed checklist that is provided with this application (Study Protocol Appendix and separate document uploaded online under “Other Documents”). Participants will be given head-phones to wear to minimize discomfort due to loud noise of the MRI machine during the performance of the resting-state MRI. MRIs are routinely performed as part of several research-based efforts in dementia and cognitive disorders across different centers including the ADNI studies, which include over 20 research centers across the US. Therefore, based on these screening criteria to ensure safety of MRI and the use of non-contrast MRI, we consider the use of MRI in this study as minimal risk.

For the purpose of this study, one lumbar puncture will be performed for each participant in the lateral decubitus position. Participants will be screened by the PI to determine the safety of the LP prior to performance of the procedure; participants with any of the listed absolute or relative contraindications for LPs will be excluded from participation. Screening will also include a review of current medications and a blood test to exclude a coagulopathy (i.e. coagulation profile: platelets, PT/INR, and PTT). Furthermore, aspirin will be held for an appropriate duration of time prior to the LP, if deemed safe by treating physicians, to further minimize the risk of bleeding complications (<1%) that may be associated with the LP.
There is a 5-15% risk of a post-LP headache, which resolves spontaneously within a few hours in 90% of cases, or within a few days, in 95% of the cases. Several precautions will be undertaken to further minimize the risk of post-LP headache; including the use of Sprotte needles, performance of the LP in the latera decubitus (rather than upright) position, removal of a small amount of fluid through a drip method for diagnostic purposes, and having the patients remain in the supine position for about 30 minutes following the procedure. Additionally, post-LP headaches are less common in elderly (above the age of 65 years) than in younger populations. Furthermore, post-LP headaches are much less common in diagnostic LPs which involve removal of a small amount of CSF through the drip method (as proposed in this study), than they are with therapeutic LPs which involve the removal of more than 50ml. The small amount of CSF is usually replaced by the body within 2-3 hours. There will be no injection of material or medications into the spinal canal. As the procedure is done under sterile conditions, the risk of introducing an infection is very rare (<1%). In rare occasions (<1%), an LP performed in an incorrect locations may be associated with nerve damage. To minimize these risks, the lumbar puncture will be performed by Dr. Tarawneh or by Dr Scharre who have extensive experience in performing LPs.

Diagnostic LPs are routinely performed in the clinic as part of the dementia evaluation (to rule out reversible causes of cognitive decline and assess for Alzheimer’s disease biomarkers). Furthermore, there is a long tradition of the OSU-IRB acceptance of lumbar punctures as a procedure with a risk that is no more than a minor increase over minimal risk in cognitively impaired elderly, as there are currently several ongoing research studies at OSU, which involve the performance of LPs in similarly aged populations with cognitive impairment, including ADNI I, ADNI II, ADNI-GO, ADNI-III, and the Buckeye CSF and Plasma Biorepository (protocol number 94H0440). Multiple Alzheimer Disease Research Centers across the country include LPs as part of their standard research evaluation of participants with cognitive impairment, in order to identify markers of disease progression and/or identify markers associated with specific pathologies.

Although very rare, it is possible that participants may have an allergic reaction to the local anesthetic, like lidocaine, used for the lumbar puncture. An allergic reaction would cause swelling and a rash on the skin where the anesthetic was injected. Participants will be instructed to alert the study staff of any adverse reactions to local anesthetics in the past (especially if this occurred with a dental procedure). Any participants with an allergic reaction to lidocaine or other local anesthetics in the past will be excluded from the study (See Appendix for LP contraindications).

Potential psychological risks would include any stress a subject may normally have in having a history, examination, cognitive testing, blood tests, and neuroimaging performed. The seriousness of these risks is very low. The risks of any breach of confidentiality are also small as the subject’s diagnoses are already established, and all efforts will be made to maintain strict confidentiality of participant data in accordance with HIPAA and research conduct guidelines.


Appendix 1

Contraindications to Magnetic Resonance Imaging:

**Absolute contraindications:**

- Permanent pacemakers and Implantable Cardiac Defibrillators (ICD)
- Pulmonary artery monitoring and thermodilution devices (i.e. Swan-Ganz catheters)
- Temporary cardiac pacemaker (i.e. external pulse generator or temporary pacing leads)
- Hemodynamic monitoring and support devices (i.e. intra-aortic balloon pump and ventricular assist devices)
- Retained transvenous pacemaker and defibrillator leads
- Nerve stimulators
- Cochlear implants (implanted hearing aids)
- Ferromagnetic aneurysm clips
- Transdermal patches that contain aluminum or other metals (i.e. scopolamine, clonidine, nicotine, and fentanyl patches)
- Insulin pump
- Metallic body in the eye

**Relative contraindications and precautions:**

Safety of MRI in these cases will be determined by the supervising radiologist according to the type of device and individual participant factors (in accordance with clinical practice guidelines followed by Radiology in these situations).

In these cases, structural and functional MRIs will only be performed if the radiologist and study investigators determine that these devices are MRI-compatible, and that performance of the MRI poses *no* risk to the participant.

- Metal hip replacements, sutures or foreign bodies in other sites
- Aortic stent grafts
- Cardiac closure and occluder devices
- Coronary artery stents
- Loop recorders (event monitor)
- Cardiac embolization coils
- IVC filters
- Peripheral vascular stents

Contraindications to Lumbar puncture:

**Absolute contraindications to lumbar puncture:**

- Allergy to lidocaine or other local anesthetics
- Possible raised intracranial pressure
- Thrombocytopenia (< 100,000/µL) or other bleeding diathesis (including ongoing anticoagulant therapy) with INR >1.4, PTT >40
- Treatment with oral or intravenous anticoagulants (heparin, LMWH, warfarin-Coumadin, Dabigatran-Pradaxa®, Rivaroxaban-Xarelto®, Apixaban-Eliquis)
- Suspected spinal epidural abscess
- Active skin or subcutaneous infection at the site of the procedure

*Relative contraindications to lumbar puncture:*

For the purposes of this study, eligible participants who are on treatment with oral antiplatelet agents other than aspirin (e.g. clopidogrel (Plavix), ticlopidine, plasugrel) will be excluded from the study.

There is no evidence that treatment with aspirin (i.e. ASA, acetylsalicylic acid) increases the risk of bleeding or other side effects of LPs. Participants who are being treated with aspirin (160-325 mg/day), and who are otherwise eligible for the study, will be included. In these participants, efforts will be made to discontinue the aspirin for 5 days prior to the LP if deemed safe by the prescribing physician and when risk of discontinuation is considered minimal (e.g. taking aspirin for primary prophylaxis). For participants who take aspirin for secondary prophylaxis or other clinical indications, the decision to continue or discontinue the aspirin at the time of the LP will be discussed with the participant’s physicians to determine the course of action with the most favorable safety profile.