

Title (90, max 100 char): Biomarker clusters are differentially associated with longitudinal cognitive decline in late mid-life

Running title (40, max 40 char) Biomarker clusters predict cognitive decline

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ABSTRACT (272 words, max 400 words)

The ability to detect preclinical Alzheimer's disease has incredible import, as this stage of the Alzheimer's continuum is believed to provide a key window for intervention and prevention. As Alzheimer's disease is characterized by multiple pathological changes, biomarkers for simultaneous pathology will likely be most useful for early detection. Towards this end, 175 late middle-aged participants (mean age 58.97, SD 5.8; 70% female) were recruited from two longitudinally followed cohorts to undergo magnetic resonance imaging and lumbar puncture. Cluster analysis then grouped individuals based on biomarkers of amyloid pathology (cerebrospinal fluid A β 42/A β 40 levels), neurodegeneration (cerebrospinal fluid-to-brain volume ratio and hippocampal volume), neurofibrillary tangles (cerebrospinal fluid phosphorylated tau 181 levels), and a brain-based marker of vascular risk (total white matter hyperintensity lesion volume). Clusters were then compared on their cognitive profile using longitudinally measured tests of episodic memory, semantic memory, executive function, and global function with linear mixed effects modeling. Four clusters emerged consistent with pre-clinical features of Alzheimer's disease, mixed dementia, atrophy, and healthy aging. Clusters demonstrated important differences in cognitive decline. Compared to the healthy cluster, all other clusters showed increased decline on a test of global functioning. Additionally, the preclinical Alzheimer's dementia-like cluster showed steeper worsening performance on tests of semantic memory and executive function; the mixed dementia-like cluster showed steeper worsening performance on an executive functioning test; and the atrophy cluster showed steeper worsening performance on tests of episodic and semantic memory. Our results demonstrate that pathology, as indicated by biomarkers, in a preclinical timeframe is related to patterns of cognitive decline

over time. Such biomarker patterns may be could be useful for identifying at-risk populations to recruit for clinical trials.

Key words (max 5): preclinical, Alzheimer's disease, cluster analysis, neuroimaging, cerebrospinal fluid

Abbreviations:

A β = amyloid-beta

APOE4 = apolipoprotein E4 allele

CSF = cerebrospinal fluid

FH = family history

GM = gray matter

ICV = intracranial volume

LM = Logical Memory

LME = linear mixed effects

LP = lumbar puncture

MCI = mild cognitive impairment

MMSE = Mini Mental State Exam

MRI = magnetic resonance imaging

NP = neuropsychological

p-tau = phosphorylated tau

RAVLT = Rey Auditory Verbal Learning Test

TMTB = Trailing Making Test B

WADRC = Wisconsin Alzheimer's Disease Research Center

WAIS-DS = Wechsler Adult Intelligence digit symbol

WM = white matter

WMH = white matter hyperintensity

WRAP = Wisconsin Registry for Alzheimer's Prevention

1 INTRODUCTION

Alzheimer’s disease pathology begins decades before clinical symptoms emerge (Sperling *et al.* , 2011, Morris, 2005) and cognitive impairment also begins many years before a diagnosis for mild cognitive impairment (MCI) or dementia due to Alzheimer’s disease (Rajan *et al.* , 2015, Howieson *et al.* , 2008). This long preclinical stage provides a critical window for intervention with disease-modifying pharmaceutical or behavioral therapies. However, a reliable, accurate, and unbiased way of detecting preclinical Alzheimer’s disease that does not rely on long-term trajectory has yet to be determined. Furthermore, although considerable research has been dedicated to identifying and validating neuroimaging, fluid, and other biomarkers for various stages of Alzheimer’s disease, the individual and combined power of these biomarkers to detect preclinical Alzheimer’s disease is, as of now, still not clearly established. Given that amyloid- β (A β) alone does not always cause cognitive impairment, it is most likely that a combination of these factors is responsible for the progressive cognitive decline in Alzheimer’s disease.

A potentially powerful tool is cluster analysis using Alzheimer’s disease biomarkers that can be detected during the preclinical stage. Cluster analysis can group individuals based on heterogeneity within a single biomarker, or an array of biomarkers; therefore, multiple co-occurring pathological features can simultaneously be captured in a single clustering, making it a particularly promising approach for detecting preclinical Alzheimer’s disease. Biomarker-based cluster analysis has been used in cognitively normal elderly and patients with MCI or dementia due to Alzheimer’s disease, but it has yet to be investigated for use in identifying preclinical Alzheimer’s disease in mid-life.

According to a theoretical model by Sperling *et al.* (2011), there are three stages of preclinical Alzheimer’s disease: 1) asymptomatic amyloidosis, 2) co-occurring amyloidosis and neurodegeneration, and 3) co-occurring amyloidosis, neurodegeneration, and subtle cognitive decline. However, as noted by Jack *et al.* (2013) and others, there is a paucity of empirical data regarding this important timeframe. The primary goal of this study, therefore, was to seek

empirical support for the Sperling *et al.* (2011) model in a late-midlife cohort. In addition to markers of amyloid pathology (A β 42/A β 40 levels in cerebrospinal fluid, CSF) and neurodegeneration (CSF-to-brain volume ratio and hippocampal volume), we additionally include a CSF marker of neurofibrillary tangles (phosphorylated tau 181, p-tau, levels) and a magnetic resonance imaging (MRI)-based marker of vascular risk, total white matter hyperintensity (WMH) lesion volume. Our hypothesis was that in addition to a healthy aging group (biomarker negative), a preclinical Alzheimer’s disease-like group would be identified based on an integrative biomarker profile (amyloidosis + neurofibrillary tangle pathology + neurodegeneration) and that this group would show evidence of early cognitive decline. Given the prevalence of mixed dementia, we further hypothesized that a group of participants would present with evidence suggestive of vascular pathology.

2 MATERIALS AND METHODS

2.1 Participants

Participants were selected for this study on the basis of participation in one of two large cohorts at the University of Wisconsin-Madison including the Wisconsin Registry for Alzheimer’s Prevention (WRAP) and the Wisconsin Alzheimer’s Disease Research Center (WADRC), and on the basis of having available biomarkers from magnetic resonance imaging and cerebrospinal fluid by lumbar puncture. The source cohorts are designed to identify biological and lifestyle risk factors associated with development of subsequent clinical Alzheimer’s disease in cohorts enriched for Alzheimer’s disease risk factors due to parental family history (FH) of Alzheimer’s disease (Koscik *et al.* , 2014, Sager *et al.* , 2005, Jonaitis *et al.* , 2013). The WRAP study consists of 1,545 participants (mean age=53.6 years, SD=6.6 at first cognitive assessment), of which 72.4% have a parental FH of Alzheimer’s disease. Recruitment for the WADRC cohort is ongoing. All subjects were between the ages of 45 and 64 at the time of enrollment. For the 300+ individuals currently being followed, mean age at enrollment was about 57 years, and approximately 2/3 of the cohort have a parental history of AD.

N=181 participants were selected from the two cohorts on the basis of having undergone both cerebrospinal fluid collection and magnetic resonance imaging. Of this sample, n=6 were excluded on the basis of an incomplete MRI sequence (n=1), incomplete CSF assays (n=1), having an interval from the MRI to lumbar puncture (LP) greater than one year (n=2, 374 and 780 days, interval range without these participants is -112 to 117 days), and two WMH outliers (n=1, >5 SD from mean) resulting in a sample of N=175. The University of Wisconsin Institutional Review Board approved all study procedures, each subject provided signed informed consent before participation, and all research was completed in accordance with the Helsinki Declaration.

2.2 MRI collection and calculation of neuroimaging variables

All participants were scanned on a GE 3.0 Tesla MR750 (Waukesha, WI) using an 8 channel head coil. T1-weighted, T2-weighted, and FLAIR anatomical scans were acquired as described previously (Johnson *et al.* , 2013, Racine *et al.* , 2014)(Berman et al 2015 pc vipr ref). T2-weighted and FLAIR anatomical scans were reviewed by a neuroradiologist (H.A.R.) for exclusionary abnormalities. The T1-weighted volume was segmented into tissue classes (CSF; gray matter, GM; and white matter, WM) using the segmentation tool in SPM12 (www.fil.ion.ucl.ac.uk/spm). CSF-to-brain volume ratio was calculated as the tissue volume ratio of CSF/(GM+WM). Hippocampal volume was calculated using FSL-FIRST, a model-based segmentation/registration tool (Patenaude *et al.* , 2011) and corrected for intracranial volume (ICV) calculated in SPM12. Total white matter hyperintensity lesion volume was measured using the SPSS Lesion Segmentation Tool (Schmidt *et al.* , 2012).

2.3 CSF collection and quantification

CSF was collected as described previously (Starks *et al.* , 2015) (Racine et al 2015 DADM; Berman et al 2015 pc vipr ref). CSF collection and processing methods were identical across studies. P-tau₁₈₁ was quantified with a sandwich ELISA (Phospho-Tau[181P], Fujirebio Europe, Ghent, Belgium). For the A β ₄₂/A β ₄₀ ratio, CSF levels of A β ₄₂ and A β ₄₀ (a less amyloidogenic A β fragment as compared to A β ₄₂) were quantified by electrochemiluminescence using an A β triplex assay (MSD Human A β peptide Ultra-Sensitive Kit, Meso Scale Discovery, Gaithersburg, MD).

2.4 Cognitive data collection

Longitudinal cognitive data is collected for both the WRAP and WADRC studies. WRAP study participants come in for follow-up cognitive testing approximately four years post their initial visit and every two years thereafter. WADRC participants come in for annual cognitive testing. At each wave of testing, participants complete a comprehensive neuropsychological (NP) battery consisting of measures that span traditional cognitive domains of memory, attention, executive function, language, and visuospatial ability.

We selected tests that were consistent across both studies that were sensitive to domains of episodic memory (total trials 1-5 and delayed recall for Rey Auditory Verbal Learning Test, RAVLT; immediate and delayed recall for Logical Memory, LM), semantic memory (Boston Naming, Animal Naming), executive function (Trail Making Test B, TMTB; Wechsler Adult Intelligence digit symbol, WAIS-DS), and global function (Mini Mental State Exam, MMSE). Table 1 summarizes the longitudinal cognitive data for each test across the five waves. RAVLT, Boston Naming, and TMTB, started at wave one for both WRAP and WADRC; LM, WAIS-DS, and MMSE started at wave two for WRAP and wave one for WADRC; and Animal Naming started at wave three for WRAP and wave one for WADRC. Therefore, for analyses and Table 1 for WRAP participants only, NP testing 2 or NP testing 3 is called NP testing 1 for tests initiated at waves two and three, respectively. WADRC participants received only story one for LM and the 30-item test for Boston Naming; their scores were doubled to maintain continuity between cohorts. Additionally, the protocol for the NP battery underwent revisions during the course of data collection for WADRC subjects. As a result, some WADRC subjects were not administered LM, Animal Naming, WAIS-DS, and MMSE for the second neuropsychological visit. The structurally missing data do not affect the validity of the statistical tests performed, as LME is robust to unbalanced designs, missing data, and unequally spaced data points.

Table 1. Summary of longitudinal cognitive data (n, mean (SD), range)

NP test	NP testing 1	NP testing 2	NP testing 3	NP testing 4	NP testing 5
Episodic memory					

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RAVLT	n=175	n=170	n=166	n=113	n=39
Total Trials	50.98 (8.15)	52.76 (8.99)	52.40 (8.91)	53.32 (8.46)	53.00 (8.89)
1-5	32-71	28-71	24-72	37-71	37-71
RAVLT	n=174	n=170	n=166	n=113	n=39
delayed	10.30 (2.86)	10.94 (2.84)	10.77 (2.90)	11.00 (2.49)	10.49 (3.03)
	2-15	0-15	0-15	4-15	4-15
*LM	n=174	n=110	n=146	n=67	n=14
immediate	29.17 (6.47)	28.77 (5.76)	29.52 (6.7)	31.76 (5.64)	31.86 (7.82)
	14-46	13-42	10-42	18-44	18-46
*LM	n=174	n=110	n=146	n=67	n=14
delayed	26.48 (7.27)	26.00 (6.93)	27.77 (6.77)	30.03 (6.56)	31.29 (7.87)
	1-46	0-43	4-42	18-48	18-46
Semantic memory					
*Animal	n=151	n=89	n=100	n=42	n=14
naming	23.19 (5.60)	23.06 (5.39)	23.36 (4.99)	25.33 (4.49)	24.21 (4.54)
	9-41	12-34	13-37	16-34	15-32
Boston	n=173	n=170	n=164	n=113	n=39
naming	57.20 (2.69)	57.30 (2.60)	57.70 (2.48)	57.91 (2.33)	57.54 (2.32)
	46-60	47-60	30-60	44-60	52-60
Executive function					
	n=174	n=170	n=166	n=113	n=39
TMT B	59.44 (19.62)	56.56 (18.46)	55.79 (18.86)	60.66 (24.43)	62.15 (23.27)
	23-138	14-144	26-125	23-182	38-152
*WAIS-DS	n=174	n=109	n=145	n=67	n=14
	58.57 (10.46)	58.24 (10.17)	58.34 (11.14)	57.79 (11.23)	62.71 (9.37)
	5-82	33-83	31-89	33-89	49-86
Global function					
	n=174	n=109	n=146	n=67	n=14
*MMSE	29.44 (.77)	29.28 (1.13)	29.44 (.86)	29.25 (1.11)	29.21 (.98)
	27-30	24-30	27-30	25-30	27-30

N, mean (SD), range; N=1 participants had a 6th NP visit (data not shown); *For WRAP participants only, NP testing 1 is Wave 2 for LM, WAIS-DS, and MMSE, and NP testing 1 is Wave 3 for Animal Naming; same NP tests were not administered to some of the WADRC participants at NP testing 2. RAVLT = Rey Auditory Verbal Learning Test; LM = Logical Memory; TMT B = Trail Making Test B; WAIS-DS = Wechsler Adult Intelligence Digit Symbol; MMSE = Mini Mental State Exam.

2.5 Clustering

Clustering broadly refers to the process of grouping a set of individuals into clusters based on how similar they are on certain criteria. Cluster analysis should classify individuals so that individuals within a cluster are as similar to each other as possible and clusters are as different from each other as possible.

Variables for clustering were CSF A β 42/A β 40, CSF p-tau, CSF-to-brain volume ratio, ICV-corrected hippocampal volume, and WMH total lesion volume. MRI data is also collected longitudinally in WRAP and WADRC, therefore, the three neuroimaging variables were selected for the MRI scan collected closest to the LP (mean=2.62 days, SD 20.56, range=-112 to 117). Because these variables are known to change with age, all variables were corrected for age by saving the unstandardized residual. Hippocampal volume was additionally corrected for ICV. The unstandardized residuals were then transformed into z-scores before being entered into the clustering algorithm. To ensure that there was not a high degree of collinearity among the clustering variables, which could lead to specific aspects being overrepresented in the clustering solution, bivariate correlations were run for all clustering variables using both Pearson’s correlation coefficient and Spearman’s rho to test for parametric and nonparametric relationships. A threshold of .7 was chosen to signify high collinearity.

Participants were grouped using agglomerative hierarchical clustering with Ward’s method of minimum variance and the squared Euclidean distance metric. Wards method joins two clusters to produce the smallest increases in the pooled within-cluster variation. The Euclidean distance is one of the most common and straightforward ways of computing distance between objects and

refers to the geometric distance in multidimensional space. Squaring the Euclidean distance places greater emphasis on objects that are further apart and is also commonly used.

2.6 Statistical Analyses

2.6.1 ANOVA: cluster characteristics

Clusters were compared by ANOVA with Statistical Package for the Social Sciences (SPSS) 22 on the five clustering variables, demographics, and clinical characteristics that may be related to dementia due to Alzheimer’s disease or vascular dementia. For clustering variables, raw values rather than the z-scores were compared, and no covariates were included with the exception of hippocampal volume for which ICV was included. Demographic variables assessed included age at LP, sex, years of education, parental FH, and whether a participant carries at least one copy of the apolipoprotein E4 allele (*APOE4*). Clinical characteristics included blood pressure (systolic and diastolic), total cholesterol, non-HDL cholesterol (total cholesterol minus LDL), fasting glucose, body mass index, and ASCVD risk score, a formula developed by the American College of Cardiology and the American Heart Association to estimate risk of an atherosclerotic cardiovascular disease event in the next ten years (Goff *et al.* , 2014). Because clinical characteristics are known to vary by age and sex, they were included as covariates for analyses of clinical data, in addition to the interval (months) between the LP and the medical examination/lab draw date.

2.6.2 Linear mixed effects: cognitive decline

Cognitive decline was measured by slope in linear mixed effects (LME) ANCOVAs in SPSS with cluster as the grouping variable and longitudinally measured NP scores as the dependent variables. Separate models were run for each NP test of interest (RAVLT total trials 1-5 and delayed, LM immediate and delayed, Boston Naming, Animal Naming, WAIS-DB, TMTB, and MMSE). First, unconditional means models adjusting for random effects were examined using unstructured covariance structure to determine significant random effects. Next, conditional ANCOVA models were run with cluster as the grouping variable which included significant random effects plus fixed effects of sex, years of education, *APOE4*, interval from the closest

MRI to the LP (days), interval from the LP to the first NP assessment (months), cohort (binary: WRAP or WADRC), time (age at each visit), cluster, and the interaction of time x cluster (slope).

Cluster 4, which was negative for all biomarkers (Fig 1), was used as the comparison group for LME analyses. In cases where one of the three biomarker-positive clusters was significantly different from cluster 4, post hoc comparisons were performed comparing that cluster to the other 2 clusters; results are reported only in cases where the post hoc tests were significant.

3 RESULTS

3.1 Clustering variables

No pairs of clustering variables exceeded either a Pearson’s correlation coefficient or Spearman’s rho of greater than .3. Four clusters emerged (Fig. 1 and supplementary Fig. 1). The first cluster (n=22) demonstrated elevated CSF p-tau and low CSF A β 42/A β 40, consistent with an Alzheimer’s-like profile. The second cluster (n=32) showed evidence of elevated CSF-to-brain volume ratio and WMH and low A β 42/A β 40, suggestive of vascular/mixed pre-dementia. The third cluster (n=45) had elevated atrophy and the smallest hippocampal volume, suggestive of a hippocampus-specific disease, lesion, or vulnerability. The fourth and largest cluster (n=76) was negative for all five biomarkers, suggestive of healthy aging.

Figure 1.

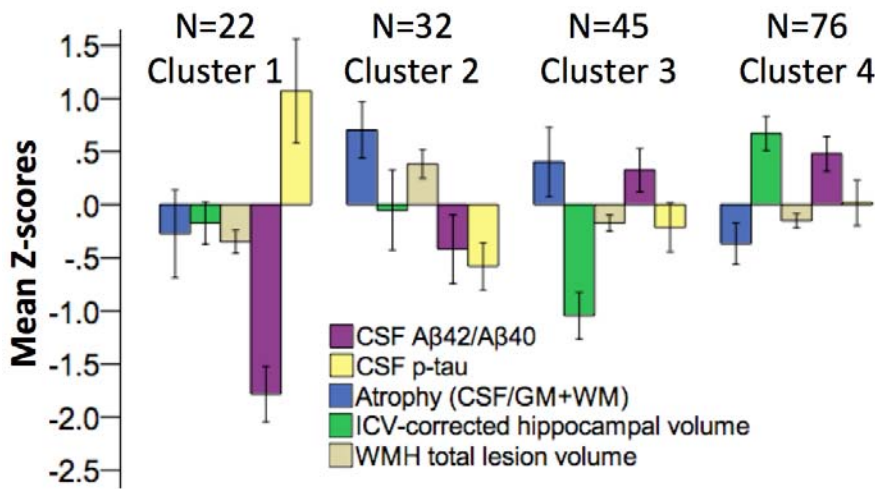
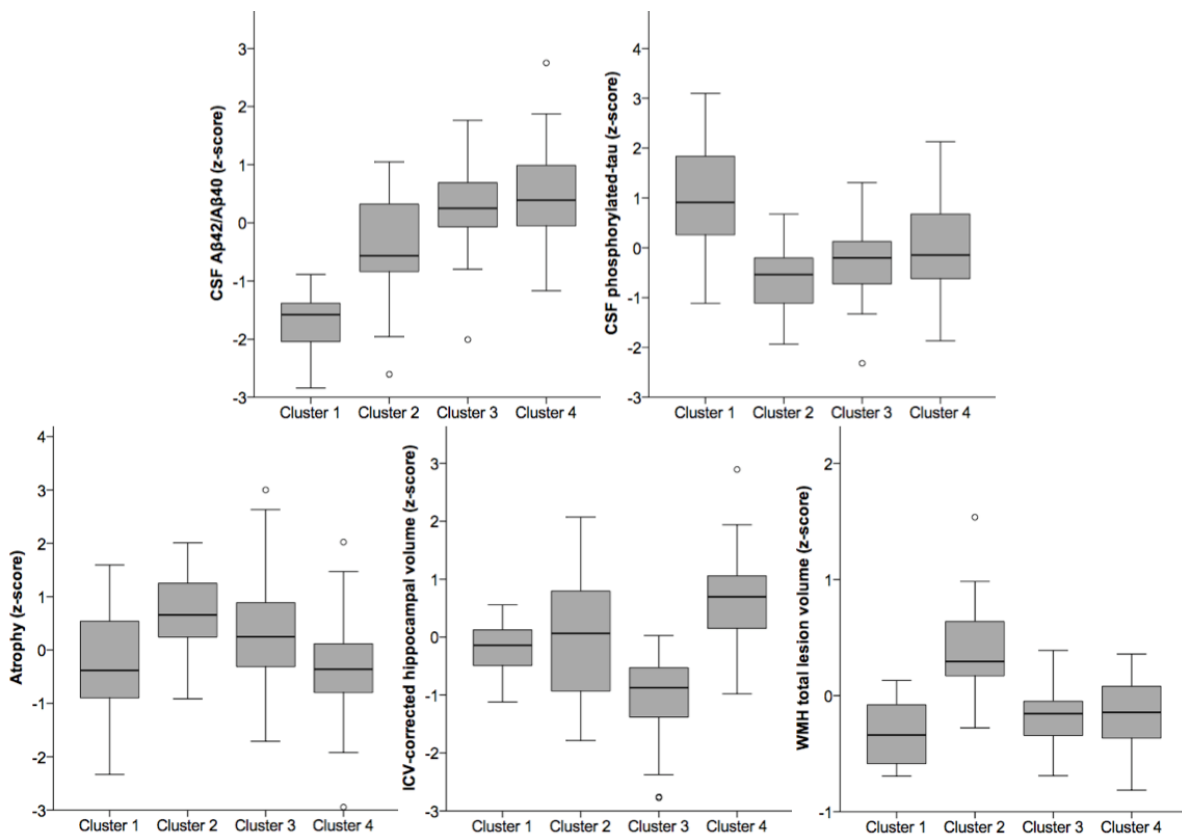


Figure 1. Results of hierarchical cluster analysis with Alzheimer’s disease biomarkers. Y-axis: z-scores of the five age-corrected clustering variables: CSF Aβ42/Aβ40 (purple), CSF p-tau (yellow), CSF-to-brain volume ratio [[need to change figure legend from “atrophy”]] defined as the tissue volumes of CSF/GM+WM (blue), ICV-corrected hippocampal volume (green), and WMH total lesion volume (beige). Error bars represent 95% confidence intervals.

Supplementary Figure 1.



Supplementary Figure 1. Box plots depict each clustering variable individually by cluster. The bar graph (bottom right) depicts all 5 clustering variables simultaneously by cluster. Error bars represent 95% confidence intervals.

ANOVA supported the visual characterization of the clusters. Cluster 1 had significantly elevated p-tau compared to all other clusters ($p < .001$) and cluster 2 had significantly lower p-tau compared to all other clusters ($p < .05$). Cluster 1 also had the lowest CSF A β 42/A β 40 compared to all other clusters ($p < .001$), and cluster 2 had significantly lower A β 42/A β 40 compared to clusters 3 and 4 ($p < .001$). Both clusters 2 and 3 had significantly greater CSF-to-brain volume ratio compared to cluster 4 ($p < .001$). Cluster 3 had significantly smaller hippocampal volume compared to all other clusters ($p < .05$), and cluster 4 had significantly greater hippocampal volume compared to all other clusters ($p < .05$). Cluster 2 had significantly greater WMH total lesion volume compared to all other clusters ($p < .001$).

Table X. Cluster comparisons on clustering, demographic, and clinical variables

Comparison variable	Cluster 1 n=22	Cluster 2 N=32	Cluster 3 N=45	Cluster 4 N=76	p- value
Clustering variable					
CSF A β 42/A β 40	.065 (.015)	.094 (.018)	.107 (.012)	.110 (.014)	$p < .001$
CSF p-tau	58.23 (15.3)	32.94 (9.8)	39.13 (10.7)	42.01 (13.1)	$p < .001$
CSF-to-brain volume ratio	.291 (.057)	.319 (.054)	.314 (.076)	.269 (.048)	$p < .001$
Hippocampal volume*	7646 (611)	7959 (873)	7134 (634)	8349 (732)	$p < .001$
WMH	13.58 (5.8)	25.76 (11.0)	14.61 (6.4)	15.35 (6.7)	$p < .001$
Demographic variable					
Age (years)	61.77 (4.9)	57.22 (7.3)	59.46 (5.4)	58.68 (5.3)	$p = .035$
Sex (% female)	.727 (.46)	.688 (.47)	.614 (.49)	.737 (.44)	$p = .554$
Education (years)	17.05 (1.9)	16.09 (2.7)	16.18 (2.8)	16.24 (2.4)	$p = .510$
<i>APOE4</i> carrier (%)	.682 (.48)	.500 (.51)	.364 (.49)	.329 (.47)	$p = .016$
Parental FH	.682 (.48)	.813 (.40)	.773 (.42)	.842 (.37)	$p = .399$
Clinical variable**					
ACVD (%)	6.39 (1.9)	5.71 (1.61)	6.47 (1.34)	6.09 (1.03)	$p = .595$
Systolic blood pressure	121.73	126.71	123.71	124.16	$p = .303$

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(mmHg)	(3.12)	(2.63)	(2.18)	(1.68)	
Diastolic blood pressure (mmHg)	71.05 (1.87)	76.29 (1.57)	73.98 (1.30)	72.93 (1.00)	p=.136
Total Cholesterol (mg/dL)	204.09 (7.61)	196.19 (6.41)	196.73 (5.32)	201.05 (4.09)	p=.839
4 Non-HDL Cholesterol (mg/dL)	143.05 (29.36)	138.32 (32.25)	135.44 (45.86)	139.09 (32.10)	p=.881
Fasting Glucose (mg/dL)	95.05 (3.10)	99.83 (2.70)	97.50 (2.19)	93.89 (1.73)	p=.186
Body mass index (kg/m ²)	27.03 (1.11)	29.18 (0.94)	27.52 (0.78)	28.88 (0.60)	p=.108

APOE4=apolipoprotein E4 allele; FH=family history. P-value is for omnibus ANOVA unless otherwise indicated; *ANCOVA controlled for ICV; **ANCOVA controlling for age, sex, interval between LP and medical examination

4.1 Demographics

Cluster 1 was significantly older than clusters 4 and 2 ($p<.05$) and had significantly more *APOE4* carriers than clusters 3 and 4 ($p<.05$). Clusters did not differ on sex, years of education, or parental FH.

4.2 Clinical characteristics

The ANOVA or ANCOVA omnibus tests were not significant for any clinical characteristics examined.

4.3 Cognitive decline

LME results of cognitive change over time are displayed in Figure 2. Cluster 1 had a steeper slope for Animal Naming ($p=.003$, $\beta= -0.57$); post hoc comparisons neared but did not reach significance compared to cluster 2 ($p=.053$) or cluster 3 ($p=.089$). Cluster 1 had a significantly steeper slope on WAIS-DS ($p=.016$, $\beta=-0.64$); post hoc comparisons were significant compared to cluster 3 ($p=.019$) but not cluster 2 ($p=.146$). Cluster 2 had a steeper slope for TMT B ($p=.005$, $\beta= 0.99$); post hoc comparisons were significantly different compared to cluster 1 ($p=.024$) and

cluster 3 ($p < .001$). Cluster 3 had a steeper slope than cluster 1 for LM immediate ($p = .018$, $\beta = -0.36$), LM delayed ($p = .017$, $\beta = -0.41$), and Boston Naming ($p = .004$, $\beta = -0.15$). Cluster 1 ($p = .008$, $\beta = -0.08$) and cluster 2 ($p = .016$, $\beta = -0.05$), but not cluster 3 ($p = .066$, $\beta = -0.04$) had significantly steeper slopes than cluster 1 on MMSE. Surprisingly, clusters 1-3 did not differ in slopes for RAVLT immediate or delayed compared to cluster 4, despite observable decline for clusters 1 and 2. Only results for Animal Naming, TMTB, and Boston Naming survive a significance threshold corrected for multiple comparisons using a Bonferroni corrected p-value for nine cognitive tests ($p = .05/9 = .006$).

Figure 2. Cognitive change over time by cluster

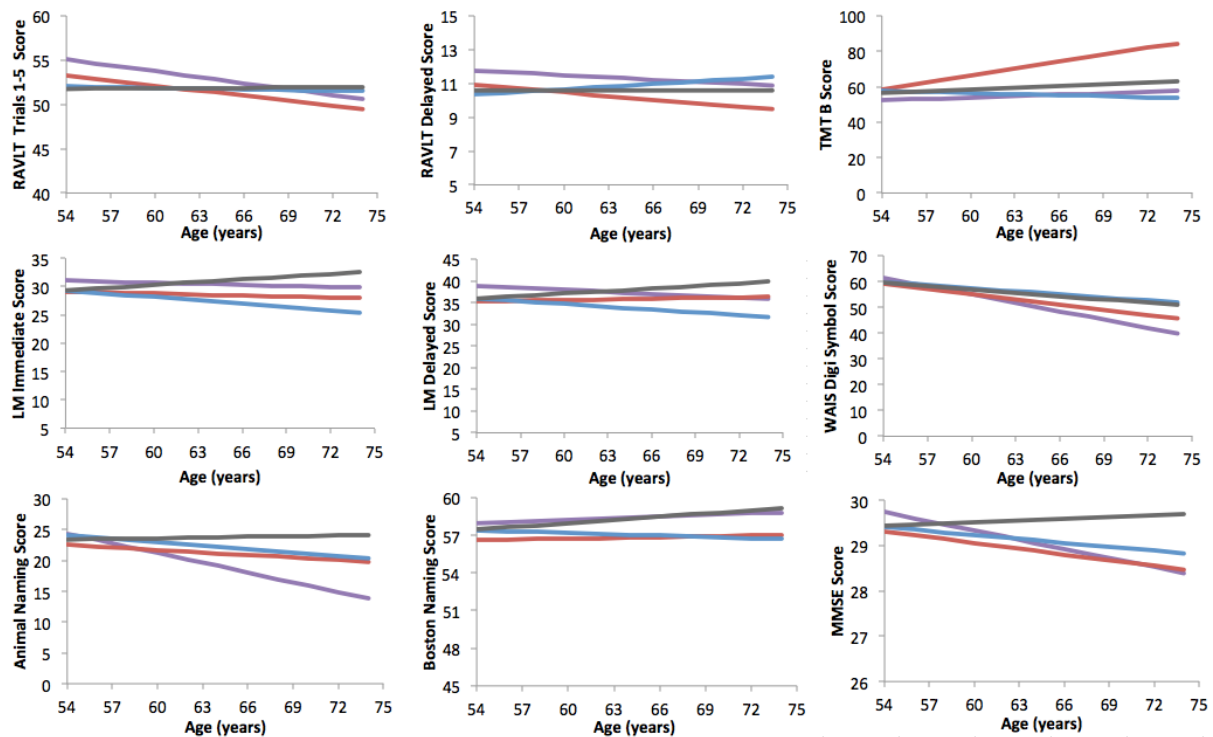


Figure 2. Cognitive change over time corrected for all other variables in the model for cluster 1 (purple, preclinical AD-like), cluster 2 (red, mixed pre-dementia), cluster 3 (blue, atrophy), and cluster 4 (gray, healthy aging). X-axis: age in years. Y-axis: raw cognitive test scores. RAVLT = Rey Auditory Verbal Learning Test; LM = Logical Memory; TMT B = Trail Making Test B; WAIS = Wechsler Adult Intelligence; MMSE = Mini Mental State Exam.

5 DISCUSSION

This study provided empirical support for the use of cluster analysis to detect heterogeneity and potentially identify a subgroup with preclinical Alzheimer’s disease in late mid-life. We identified four groups, two of which were consistent with clinical profiles for preclinical Alzheimer’s disease and preclinical mixed dementia. The third cluster showed evidence of hippocampal injury or intrinsic brain vulnerability, and the fourth cluster seems to represent normally aging individuals. All of the biomarker-positive clusters showed cognitive decline in at least one cognitive domain compared to the healthy cluster. The preclinical Alzheimer’s disease-like cluster (cluster 1) showed declining performance in semantic memory and executive function, and had the steepest declining slope on global functioning of the three biomarker-positive clusters; the preclinical mixed dementia-like cluster (cluster 2) showed decline in executive and global function; and the atrophy cluster (cluster 3) showed decline in memory broadly (delayed, immediate, and semantic) and global function. Consistent with an Alzheimer’s-like profile, cluster 1 was older and had greater incidence of *APOE4*, but clusters did not differ on any other demographic or clinical variables examined.

Preclinical Alzheimer’s disease is often simply characterized as “A β +” or “A β -” or by long-term clinical trajectories—both techniques have their limitations. Artificial cut-offs and dichotomization ignore potentially important variability in a continuous variable. Additionally, while A β is an important biomarker, it does not capture the multifaceted nature of Alzheimer’s disease, which is characterized by numerous pathological changes besides A β plaques. Long-term trajectories are helpful to researchers trying to understand preclinical Alzheimer’s disease, but are not pertinent to the ultimate goal of detecting preclinical Alzheimer’s disease during that stage so that interventions can be initiated. In contrast, cluster analysis provides an unbiased way to characterize multimodal data that captures multiple pathological characteristics simultaneously and can be used throughout the course of the Alzheimer’s disease continuum. Here we provided evidence for the use of cluster analysis to identify individuals who appear to be in a preclinical stage of Alzheimer’s disease in late mid-life, even preceding mild cognitive impairment.

Using biomarkers to cluster groups along the Alzheimer’s disease trajectory has been done previously in cohorts of normal elderly (Skillback *et al.* , 2013, Pike *et al.* , 2011, Nettiksimmons

et al. , 2013, Nettiksimmons *et al.* , 2010), patients with mild cognitive impairment (Nettiksimmons *et al.* , 2014, Escudero *et al.* , 2011), and patients with dementia (Wallin *et al.* , 2010, van der Vlies *et al.* , 2009, Noh *et al.* , 2014, Vemuri *et al.* , 2011). For instance, two studies in patients with mild cognitive impairment showed that clusters with biomarker profiles consistent with Alzheimer’s disease were more likely to progress to dementia due to Alzheimer’s disease, illustrating the potential utility for cluster analysis to predict disease progression (Nettiksimmons *et al.* , 2014, Escudero *et al.* , 2011). While clustering has demonstrated utility in elderly and cognitively impaired cohorts, it had yet to be validated during the preclinical Alzheimer’s disease stage in late mid-life. This study fills this gap by using cluster-based analysis with neuroimaging and CSF biomarkers in a late-middle aged risk-enriched cohort. Because this cohort is still relatively young, cognitive decline rather than clinical endpoints were used to evaluate trajectory.

Cluster 1 was most consistent with an Alzheimer’s disease-like biomarker profile (decreased CSF A β 42/A β 40, increased CSF p-tau). Significant neurodegeneration measured by gross CSF-to-brain volume ratio and hippocampal volume was notably absent, suggesting that this cluster is representative of an early stage of the Alzheimer’s disease continuum preceding frank neuronal death and atrophy. Although the cluster did not show expected declines on tests of episodic memory (RAVLT and LM), it showed significant declines on tests of semantic memory, executive function, and global function. Our results are largely consistent with a meta-analysis on 47 studies involving 9,097 controls and 1,207 preclinical Alzheimer’s disease cases that found that preclinical Alzheimer’s disease (including mild cognitive impairment) is characterized by marked deficits in global cognitive ability, episodic memory, perceptual speed, and executive functioning; smaller deficits in verbal ability, visuospatial skill, and attention; and no preclinical impairment in primary memory (Backman *et al.* , 2005). Furthermore, both Animal Naming and MMSE are commonly used clinical techniques that have high accuracy for diagnosing dementia. Animal Naming is a type of category fluency test where a cut-off of less than 15 is suggestive of cognitive impairment. Canning *et al.* (2004) found that Animal Naming has .88 sensitivity and .96 specificity in discriminating healthy controls from patients with dementia due to Alzheimer’s disease. Another study found that steeper declines on Animal Naming distinguished cognitively unimpaired individuals from those with preclinical Alzheimer’s disease (mean age 80.5 years),

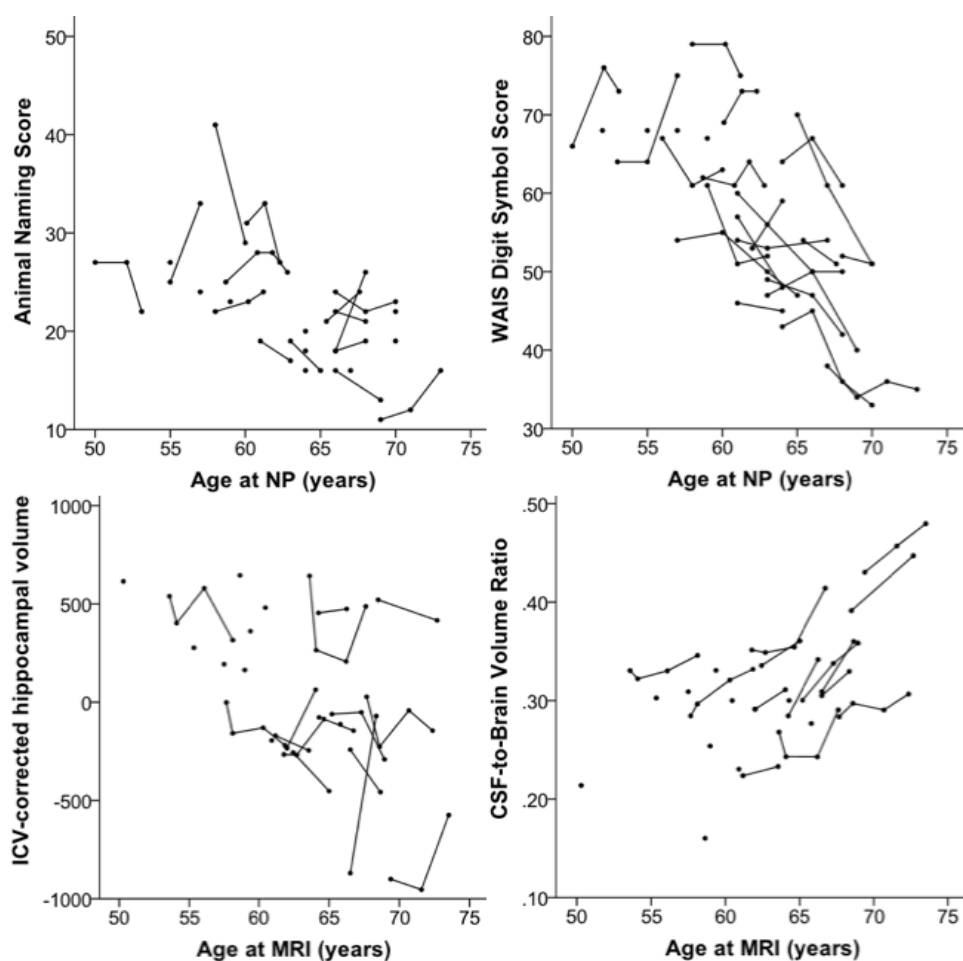
defined as initially having a CDR<1 at start of the study but having a dementia due to Alzheimer's disease diagnosis by follow-up (range 2.3-5.9 years) (Clark *et al.* , 2009). Similarly, a different longitudinal study found that individuals who developed Alzheimer's disease exhibited significant within-group decline in total MMSE score (Small *et al.* , 2000). Change in MMSE may not be as informative of disease progression once an individual has already developed dementia due to Alzheimer's disease (Clark *et al.* , 1999).

It is intriguing that the preclinical Alzheimer's disease-like cluster showed deficits in semantic but not episodic memory as deficits in episodic memory are consistently found in various stages of Alzheimer's disease (Backman *et al.* , 2001, Grober *et al.* , 2008) and is postulated to reflect pathological changes in the medial-temporal lobe, which occur early in the disease (Collie and Maruff, 2000). A potential explanation for our findings could lie in the fact that cluster 1 did not show marked hippocampal atrophy. Similar to our findings, a study using ADNI normal controls (mean age 76 years) found that a cluster with CSF and MRI biomarkers consistent with ADNI patients with MCI or Alzheimer's disease showed worsening performance on the Alzheimer's Disease Assessment Scale-cognitive subsection but not the RAVLT (Nettiksimmons *et al.* , 2010). Additionally, they found that hippocampal volume from this cluster was only .19 standard deviation units from the overall normal control mean while the MCI and Alzheimer's disease groups were 1.11 and 1.79 standard deviation units from the mean, respectively. It's been suggested that episodic memory primarily depends on the hippocampus while semantic memory predominantly depends on the perirhinal and entorhinal cortices (Vargha-Khadem *et al.* , 1997). Interestingly, the first cortical tau lesions also appear in the transentorhinal cortex (Braak and Del Tredici, 2015). One theory, therefore, is that tangle pathology could be associated with semantic memory deficits in the earliest stages of Alzheimer's disease. Our results showing semantic deficits in a late middle-aged group with tangle pathology (indicated by elevated CSF p-tau) but not hippocampal atrophy are consistent with this theory. It will be important to continue to follow this group longitudinally to see whether hippocampal atrophy and deficits in episodic memory emerge, and whether they are temporally related.

Based on the Sperling *et al.* (2011) model, it would be expected that individuals in the preclinical Alzheimer's disease-like cluster would represent individuals spanning the three stages of

preclinical disease. Therefore, to further qualitatively assess heterogeneity within this cluster, we made spaghetti plots for individuals in cluster 1 for two cognitive tests (Animal Naming and WAIS-DS) and the neurodegenerative clustering variables (CSF-to-brain volume ratio and ICV-corrected hippocampal volume) (Supplementary Fig. 1). Of the 22 participants in cluster 1, n=15 had at least two time points of MRI data, n=9 had three time points, and n=5 had four time points. The vast majority of individuals in this cluster appear to show declining performance on WAIS-DS and increasing CSF-to-brain volume ratio, and about half of the individuals with more than two time points show decline on Animal Naming; but the story for hippocampal volume is much less clear. There appears to be some evidence of multiple preclinical Alzheimer’s disease stages within this single cluster, but further studies with much larger sample sizes will be needed to quantitatively parse out this preclinical staging further.

Supplementary Figure 2.



Supplementary Figure 2. Spaghetti plots for cluster 1. For each panel, y-axis: cognitive or MRI variables; x-axis=age in years. WAIS= Wechsler Adult Intelligence; ICV=intracranial volume; CSF-to-brain volume ratio=tissue volume ratio of cerebrospinal fluid to gray matter plus white matter.

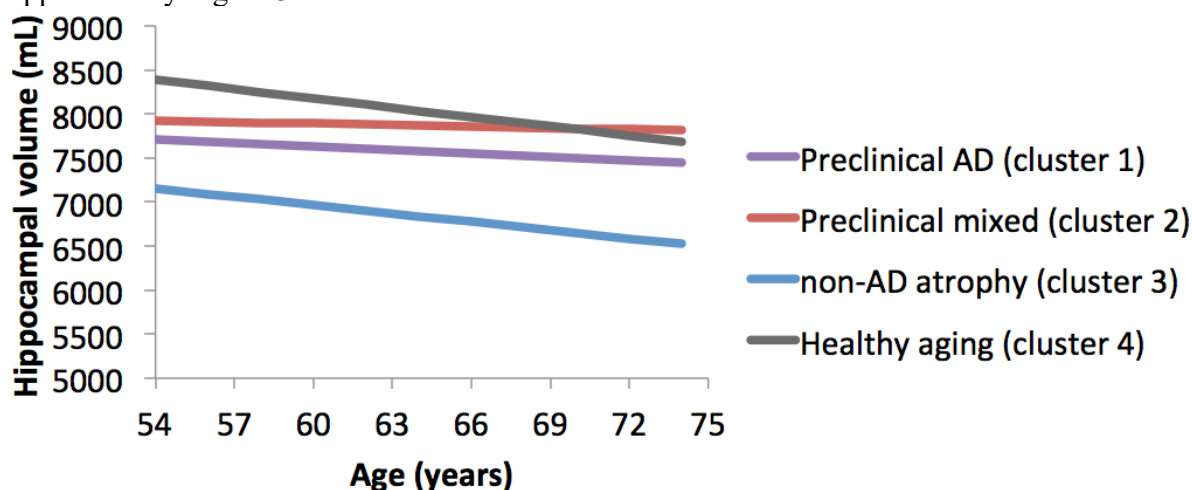
Cluster 2’s biomarker profile (elevated WMH total lesion volume and CSF-to-brain volume ratio, decreased CSF A β 42/A β 40) is consistent with preclinical mixed dementia due to vascular dementia and dementia due to Alzheimer’s disease. WMH are visible on T2-weighted fluid attenuated inversion recovery magnetic resonance imaging and are thought to have a vascular origin, increase with age, and are elevated in Alzheimer’s disease (Alosco *et al.* , 2013, Casado Naranjo *et al.* , 2015, Provenzano *et al.* , 2013, Wen *et al.* , 2009, Young *et al.* , 2008). A 2010 autopsy study found almost half of the brains of clinically diagnosed Alzheimer’s disease patients harbored mixed pathology, the most common of which were infarcts, and that infarcts increased the likelihood of cognitive impairment and dementia (Schneider and Bennett, 2010). White matter infarctions are postulated to be due to hypoperfusion of the white matter as they are

accompanied by vascular stenosis and signs of cardiovascular disease with hypotension (Brun and Englund, 1986). White matter lesions are also seen in non-demented controls (Scheltens *et al.*, 1995), suggesting that these lesions are more related to cardiovascular conditions than specific Alzheimer's disease pathology. However, it is unknown whether vascular brain lesions are co-occurring pathologies which contribute to cognitive impairment independent of amyloid and tau pathology, whether vascular lesions incur greater vulnerability which make the brain more susceptible to Alzheimer's disease pathology, or whether vascular and Alzheimer's disease pathology directly interact to accelerate the Alzheimer's disease cascade. Longitudinal evaluation of individuals in this cluster may provide greater understanding of early co-occurring vascular and amyloid pathology.

Cluster 2's cognitive profile is also consistent with preclinical mixed dementia. Previous studies have demonstrated a relationship between vascular pathology and executive function, particularly for attention/speed tasks (Moser *et al.*, 2001), even in non-demented patients compared to older healthy controls (Kramer *et al.*, 2002). Similarly, a study of 349 WRAP participants found that WMH were associated with lower cognitive speed and flexibility (Birdsill *et al.*, 2014). Another study found patients with greater WMH and atrophy also exhibit greater cognitive decline than subjects with less vascular pathology (Kooistra *et al.*, 2014) and that brain volume atrophy must be accompanied by infarct pathology to impair executive functioning (Muller *et al.*, 2011). Our finding that a group of subjects with elevated CSF-to-brain volume ratio, WMH, and decreased CSF A β 42 demonstrate worsening performance on TMTB is consistent with a link between vascular pathology and executive function, with greater decline associated with mixed pathology and atrophy. In contrast to our findings, a study of ADNI normal controls found that a cluster characterized by substantial brain atrophy and white matter hyperintensities (mean age 76.7 years) did not show decline on six tests of executive function, including Trails B, but did have significantly higher body mass index, Hachinski score, creatinine levels, triglycerides, and blood glucose (Nettiksimmons *et al.*, 2013). Differences between their findings and ours could be due to cohort age differences (cluster 2 is on average almost 20 years younger than their suspected vascular cluster) and unlike the Nettiksimmons *et al.* (2013) suspected vascular cluster, cluster 2 additionally demonstrated decreased CSF A β 42/A β 40.

Cluster 3 presented with increased CSF-to-brain volume ratio and the smallest ICV-corrected hippocampal volume. As would be hypothesized given the hippocampus’s involvement in memory (Collie and Maruff, 2000), this cluster showed impairments across the memory spectrum on tests of delayed, immediate, and semantic memory. The significantly smaller hippocampal volume in this cluster could be indicative of a hippocampus-specific impairment like hippocampal sclerosis or inherent brain vulnerability. In order to shed light on this question, we performed a supplementary LME analysis using longitudinal hippocampal volumes and fixed effects of sex, years of education, *APOE4*, cohort, ICV at each MRI, cluster, time (age at each MRI), and time*cluster, as well as an LME model for cluster 3 individuals only. Longitudinal MRI data was limited (n=14 had 2 time points of MRI data and n=12 at three time points), so these results should be interpreted with some caution. We found that cluster 3 had a significantly smaller intercept than the other three clusters and significant decline over time compared to a slope of 0, but did not differ in slopes compared to the other three clusters (Supplementary Fig. 3). These findings suggest the observed cognitive profile for cluster 3 is more likely due to hippocampal vulnerability and commensurate age-related atrophy rather than a detectable hippocampal volume loss. However, only longitudinal clinical outcomes can rule out the latter possibility.

Supplementary Figure 3.



Racine – Detecting preclinical Alzheimer’s disease

Supplementary Figure 3. Hippocampal volume change over time corrected for all other variables in the model for cluster 1 (purple, preclinical AD-like), cluster 2 (red, mixed pre-dementia), cluster 3 (blue, atrophy), and cluster 4 (gray, healthy aging). X-axis: age in years. Y-axis: hippocampal volume (mL).

There are several limitations of this study. First, CSF analyte levels are only an indirect measure of pathology, however, it is a common clinical and research technique and the CSF measures of interest ($A\beta_{42}/A\beta_{40}$ and p-tau) have been validated by neuroimaging and histological studies. Furthermore, it’s been suggested that CSF $A\beta_{42}$ and PET-PiB imaging provide partially independent information about a wide range of Alzheimer's measures, and that reduced cerebrospinal fluid amyloid- β may be more strongly related to early stage Alzheimer's disease (Mattsson *et al.* , 2015). Second, while biomarkers and cognitive data provide evidence of an Alzheimer’s disease-like trajectory, clinical endpoints are necessary to confirm the diagnostic accuracy of these clusters. Third, agglomerative hierarchical clustering is only one way to classify individuals on biomarkers. It will be important to investigate and compare other techniques like machine learning to the methods utilized in this study. Fourth, our study sample was largely white and well educated, and so is not be generalizable to all populations. It will be important to validate these results in more diverse cohorts.

Alzheimer’s disease is a growing epidemic for which there is currently no treatment. With clinical trials beginning in the preclinical time period (ex. A4 study), there is imperative need for techniques to identify individuals with preclinical Alzheimer’s disease they can be recruited for clinical trials and eventually treatment (Sperling *et al.* , 2013). Our results suggest that techniques which combine multimodal information about early pathological changes could inform detection of preclinical Alzheimer’s disease and that longitudinal slopes of decline provide further information about disease trajectory.

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10 FIGURE LEGENDS