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# Biomarker profiling beyond amyloid and tau: cerebrospinal fluid markers, hippocampal atrophy, and memory change in cognitively unimpaired older adults

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ABSTRACT

Brain changes occurring in aging can be indexed by biomarkers. We used cluster analysis to identify subgroups of cognitively unimpaired individuals (n = 99, 64–93 years) with different profiles of the cerebrospinal fluid biomarkers beta amyloid 1–42 (Aβ42), phosphorylated tau (P-tau), total tau, chitinase-3-like protein 1 (YKL-40), fatty acid binding protein 3 (FABP3), and neurofilament light (NFL). Hippocampal volume and memory were assessed across multiple follow-up examinations covering up to 6.8 years. Clustering revealed one group (39%) with more pathological concentrations of all biomarkers, which could further be divided into one group (20%) characterized by tauopathy and high FABP3 and one (19%) by brain β-amyloidosis, high NFL, and slightly higher YKL-40. The clustering approach clearly outperformed classification based on Aβ42 and P-tau alone in prediction of memory decline, with the individuals with most tauopathy and FABP3 showing more memory decline, but not more hippocampal volume change. The results demonstrate that older adults can be classified based on biomarkers beyond amyloid and tau, with improved prediction of memory decline.



## 1. Introduction

Biomarkers play an increasingly important role in research on age-related neurological conditions and diseases. Numerous studies have consistently shown a marked decrease in cerebrospinal fluid (CSF) concentration of beta amyloid 1–42 (Aβ42) together with increased total tau (T-tau) and phosphorylated tau (P-tau) in Alzheimer's disease (AD) dementia and mild cognitive impairment (MCI) cases showing progression to AD (Olsson et al., 2016). The National Institute on Aging-Alzheimer's Association's (NIA-AA) new research framework defines AD solely based on biomarkers reflecting the core pathologies of AD, while clinical symptoms only are used for staging of the disease (Jack et al., 2018). These criteria rely on the amyloid/tau/neurodegeneration (A/T/N) classification

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scheme for the core AD biomarkers (Jack et al., 2016a), wherein CSF Aβ42 reflects brain β-amyloidosis (Strozyk et al., 2003; Tapiola et al., 2009), P-tau reflects tau pathology/neurofibrillary tangles (Buerger et al., 2006; Tapiola et al., 2009), and T-tau reflects neurodegeneration (Hesse et al., 2001; Ost et al., 2006; Zetterberg et al., 2006). CSF concentrations of Aβ42, T-tau, and P-tau are also known to change even in cognitively well-functioning older adults (Jansen et al., 2015; Toledo et al., 2015). Accordingly, cognitively unimpaired older adults with abnormal amyloid and tau biomarkers are defined as having preclinical AD based on the NIA-AA criteria.

However, with higher age also come other changes such as neuroinflammation and different aspects of neurodegeneration (Fjell et al., 2014; Wyss-Coray, 2016). Chitinase-3-like protein 1 (YKL-40) is mainly expressed in astrocytes in the human brain (Bonneh-Barkay et al., 2010), and YKL-40 expressing astrocytes are found close to activated microglia (Bonneh-Barkay et al., 2010). Consequently, CSF YKL-40 is believed to be a biomarker of neuroinflammation (Baldacci et al., 2017; Dhiman et al., 2019). Fatty acid binding protein 3 (FABP3) is expressed in neurons of the brain (Pelsers et al., 2004), where it is involved in transport of fatty acids. FABP3 is found in the cytosol, and it is released following cellular injury; thus CSF FABP3 is considered a biomarker of neuronal damage (Dhiman et al., 2019; Pelsers et al., 2004). Neurofilament light chain protein (NFL) is a cytoskeletal component of neuronal axons (Khalil et al., 2018). NFL is released from neuronal axons in response to neuronal damage, and CSF NFL is believed to reflect axonal degeneration (Dhiman et al., 2019; Khalil et al., 2018). CSF YKL-40, FABP3, and NFL are not disease-specific biomarkers, and all of them have been found to be increased in both acute and chronic brain diseases (Baldacci et al., 2017; Bonneh-Barkay et al., 2010; Bridel et al., 2019; Olsson et al., 2016; Pelsers et al., 2004; Steinacker et al., 2004; Zetterberg et al., 2006). Although all the above mentioned CSF biomarkers reflect at least partly separate brain pathological processes, some processes may also be interrelated.

Relationships between the core AD CSF biomarkers and emerging CSF biomarkers like YKL-40, FABP3, and NFL in cognitively unimpaired older adults have mainly been studied by assessing correlations between the individual biomarkers. Our knowledge about how CSF biomarkers may cluster is, however, limited. Furthermore, acknowledging that most age-related brain changes are the result of a number of different processes that probably vary across individuals, it is a major task to be able to group older adults according to their brain states.

Clustering analyses can be used to identify subgroups with multiple co-occurring biomarker features. Unfortunately, beyond Aβ42 and tau (Nettiksimmons et al., 2010; Racine et al., 2016), we do not know whether clustering analyses can be used to classify cognitively unimpaired older adults in meaningful subgroups characterized by partly different and partly overlapping brain pathology.

To address these questions, we first performed correlation and clustering analyses to assess relationships between established and emerging CSF biomarkers (Table 1) in order to examine how biomarkers for different brain states are related at different superordinate levels. Second, we used the CSF biomarkers to identify participants with similar biomarker profiles using a blind, data-driven clustering approach across participants. The rationale was to test whether subgroups of older adults could be detected based on biomarker profiles. Third, we assessed whether these subgroups showed different trajectories of memory and hippocampal volume change across multiple follow-up examinations distributed over an interval up to 6.8 years. The performance of the clustering approach in prediction of memory decline and hippocampal volume change over time was compared to the NIA-AA classification based on amyloid and tau.

**Table 1**
The studied biomarkers and the pathologies they represent

| Biomarker | Related pathological process |
|---|---|
| CSF Aβ42 | Amyloid deposition |
| CSF FABP3 | Neuronal damage |
| CSF phosphorylated tau | Tau phosphorylation/tangle formation |
| CSF total tau | Altered tau metabolism/neurodegeneration |
| CSF YKL-40 | Neuroinflammation |
| CSF NFL | Axonal damage/neurodegeneration |

Key: Aβ42, beta amyloid 1–42; CSF, cerebrospinal fluid; FABP3, fatty acid binding protein 3; NFL, neurofilament light; YKL-40, chitinase-3-like protein 1.

## 2. Materials and methods

### 2.1. Participants

We recruited patients scheduled for elective gynecological (genital prolapse), urological (benign prostate hyperplasia, prostate cancer, or bladder tumor/cancer), or orthopedic (knee or hip replacement) surgery in spinal anesthesia turning 65 years or older the year of inclusion. Dementia, previous stroke with sequela, Parkinson's disease, and other neurodegenerative diseases likely to affect cognition were exclusion criteria at baseline. Participants were assessed with a multi-domain battery of cognitive tests before surgery, including the Mini Mental Status Examination (MMSE) (Folstein et al., 1975), Clock Drawing Test (Shulman, 2000), Word List Memory Task (Morris et al., 1989), Trail Making Test A (TMTA) and B (TMTB) (Reitan, 1955), and verbal fluency (FAS test and Animal Naming) (Spreen and Strauss, 1991). The median time from cognitive assessment to surgery was 6 days (interquartile range [IQR] 3–11). CSF samples were collected by the anesthesiologist in conjunction with the spinal anesthesia. The participants also underwent magnetic resonance imaging (MRI) after surgery. The mean time between CSF sampling and MRI at baseline was 8 weeks (standard deviation [SD] [range]: 6 [−20 to 24]). Participants were tested with the same battery of cognitive tests annually and with MRIs biennially for up to 6.8 years.

From all recruited participants, we selected only participants with CSF data available for all biomarkers (Aβ42, T-tau, P-tau, YKL-40, FABP3, and NFL). Furthermore, we performed a review of all neurological diagnoses and MRI findings at baseline or occurring though follow-up in the cohort. We excluded participants with diagnoses/lesions that we found likely to affect cognition or measures of hippocampal volume (details in Supplementary Material). As we wanted to study phenotypes in aging individuals without clinical symptoms of neurodegenerative diseases, we also excluded all participants who had received a diagnosis of dementia or MCI during follow-up, had a cognitive impairment according to hospital medical records, had developed other neurodegenerative diseases during follow-up, and participants who, based on a cognitive assessment in the study, had been offered referral to the hospital for further cognitive assessment. Finally, from the remaining sample, selection of participants cognitively unimpaired at baseline was based on the following procedure: (1) we included all participants with MMSE $\geq$ 28, if also Clock Drawing Test score was $\geq$4 and Word List Recall score was $\geq -1.5$ SD from the mean according to age, sex, and education adjusted norms, and (2) we included participants with MMSE $<$ 28, if Clock Drawing Test score was $\geq$4, and also test scores for Word List Recall score, TMTA, TMTB, FAS test, and Animal Naming were $\geq -1.5$ SD from the mean according to norms. Our selection resulted in 99 participants with CSF analyses available for all biomarkers, of which 99 had been cognitively assessed one or more times (median: 7 cognitive assessments; IQR 4–7, range 1–7), and 85 had one or more MRIs (median: 3 MRIs; IQR 2–4, range 1–4). Demographics, cognitive test results, and CSF biomarker

characteristics are shown in Table 2. Seventy-three participants were excluded (see reasons in Fig. S1 and Supplementary text). Excluded participants had poorer performance on MMSE and Word List Recall compared to the included participants (Table S1). Age, years of education, sex distribution, and CSF biomarker characteristics did not differ.

### 2.2. Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki and approved by the Regional Committee for Ethics in Medical Research in Norway (REK 2011/2052). All participants provided written informed consent.

### 2.3. Magnetic resonance imaging acquisition and processing

T1-weighted MPRAGE 3-dimensional images were acquired with a 1.5 T Siemens Avanto scanner using a 12-channel head coil [repetition time (TR) = 2400 ms, echo time (TE) = 3.79 ms, field of view = 240 mm, slice thickness = 1.20 mm, pixel size = 1.25 × 1.25 mm]. The same scanner was used at baseline and all follow-ups. Images were processed with FreeSurfer (version 6.0) and its specific longitudinal stream (https://surfer.nmr.mgh.harvard.edu). For each MRI, the FreeSurfer pipeline performs a set of automated procedures for the cortical reconstruction and volumetric segmentation, documented elsewhere (Dale et al., 1999; Fischl et al., 2002). More specifically, the segmentation algorithm assigns labels to all the brain regions of each individual scan, based on an available probabilistic atlas obtained from a training set of participants which has been accurately manually labeled (Fischl et al., 2002). The hippocampal volume is defined from this available atlas. The FreeSurfer longitudinal stream includes methods designed to minimize the bias to any time point in a participant and which lead to increased statistical power, better separation of groups based on atrophy, and higher reproducibility. These include the generation of a subject-specific intermediate template followed by a projection of each time point to this template (Jovicich et al., 2013; Reuter et al., 2012).

### 2.4. CSF collection and analyses

CSF was collected in polypropylene tubes, centrifuged at room temperature for 10 minutes, the supernatant aliquoted into polypropylene tubes, and frozen at −80 °C pending analyses. Samples were sent on dry ice to the Clinical Neurochemistry Laboratory at Sahlgrenska University Hospital, Mölndal, Sweden, for analyses. CSF Aβ42, T-tau, and P-tau concentrations were measured using INNOTEST enzyme-linked immunosorbent assay (ELISA; Fujirebio, Ghent, Belgium), CSF NFL concentrations using a commercial ELISA (UmanDiagnostics, Umeå, Sweden), YKL-40 concentrations using a commercial ELISA (R&D Systems, Minneapolis, MN), and FABP3 concentrations using an immunoassay with electrochemiluminescence detection (MSD Human FABP3 kit; Meso Scale Discovery, Gaithersburg, MD). Analyses were performed by board-certified laboratory technicians masked to clinical data. Intra-assay coefficients of variation were 9%–13%. All participants had detectable levels of all biomarkers.

**Table 2**
Demographics

| Demographics | Cognitively unimpaired older adults (n = 99) |
|---|---|
| Age at baseline (y) | 72 (68–78) |
| Sex: male | 50 (51) |
| Education (y) | 14 (12–17) |
| MMSE score, baseline | 29 (28–30) |
| CERAD, Word List Recall score | 6 (5–8) |
| CSF Aβ42 (pg/mL) | 731 (512–866) |
| CSF FABP3 (pg/mL) | 4.56 (3.36–5.93) |
| CSF P-tau (pg/mL) | 59 (46–75) |
| CSF T-tau (pg/mL) | 347 (272–486) |
| CSF YKL-40 (pg/mL) | 225,210 (175,208–280,877) |
| CSF NFL (pg/mL) | 1026 (794–1482) |

Values are represented as median (interquartile range) and n (%).
Key: Aβ42, beta amyloid 1–42; CERAD, Consortium to Establish a Registry for Alzheimer's disease; CSF, cerebrospinal fluid; FABP3, fatty acid binding protein 3; MMSE, Mini Mental Status Examination; NFL, neurofilament light; P-tau, phosphorylated tau; T-tau, total tau; YKL-40, chitinase-3-like protein 1.

### 2.5. Statistical analysis

We tested correlations between baseline age and CSF biomarkers and between the CSF biomarkers using bivariate and partial Spearman correlations in SPSS (version 25). These analyses were undertaken to describe the structure of the data, not to test specific hypotheses.

Clustering analyses were performed in MATLAB (MathWorks Inc). Clustering analysis is used to identify natural groupings of similar variables from a data set. First, we calculated the distance between variables using Spearman correlation, to account for non-normal distribution of the data. We then used the "ward" or inner squared distance as a linkage function to group the variables into clusters. The variables were reordered with the optimal leaf order and a hierarchical dendrogram was used to represent the clusters at the different levels. To remove the effect of age from all CSF biomarkers, we computed independent linear regressions of each biomarker against age, and the residuals of these regressions were used for the clustering analysis. The clustering approach was completely data-driven. As the relationships between several of the included CSF biomarkers are not known a priori, we did not impose restrictions of the clustering algorithms.

We performed 2 cluster analyses:

Cluster analysis 1: We used cluster analysis to establish clusters of CSF biomarkers with shared behavior across participants. In this analysis, all the available CSF biomarkers were used as variables and the participants as observations. The purpose of this was to see which CSF biomarkers tended to go together across different number of clusters.
Cluster analysis 2: In this analysis, we used the CSF biomarkers to identify subgroups of participants. Thus, we ran the cluster analysis to define groups of participants with the same profiles of CSF biomarkers. Here, participants were used as variables and the biomarker concentrations as observations. Thus, in cluster analysis 1, we tested which biomarkers clustered together (the CSF biomarkers were the variables), while in cluster analysis 2 we tested which participants clustered together (the participants were the variables).

The optimal number of clusters given by the Calinski-Harabasz algorithm was 2 (i.e., our first hierarchical partition). Visual inspection of the hierarchical distribution of the dendrogram indicated that each cluster could be further split into 2 clusters each, and this was confirmed by running the Calinski-Harabasz algorithm separately for each of the 2 main clusters. In order to characterize the biomarker profile for each clustering-based subgroup of participants, differences between the subgroups for each biomarker were quantified by calculating Cohen's *d* (the pairwise difference in mean biomarker values between groups divided by the pooled standard deviation weighted for group size). This was done to map the relative contributions of the different biomarkers in the grouping of participants. According to established rules of thumbs,

we considered effect sizes ≥0.80 as large, ≥0.50 as medium, and ≥0.20 as small.

As an alternative to the clustering approach, we classified participants into biomarker groups according to the NIA-AA criteria (Jack et al., 2016a), where A+T+ represents amyloid and tau positivity, A-T+ represents amyloid negativity and tau positivity, and A-T- represents amyloid and tau negativity. The criteria for amyloid positivity (A+) was Aβ42 < 530 pg/mL and for tau positivity (T+) P-tau > 60 pg/mL according to established cut-offs (Hansson et al., 2006). T-tau was not used for classification of neurodegeneration (N+), because of a very strong correlation between T-tau and P-tau ($r = 0.96$, $p < 0.001$).

Finally, we tested for intercept or slope differences in memory and hippocampal volume over time as a function of biomarker group by use of generalized additive mixed models (GAMM) run in R (https://www.r-project.org) using RStudio (www.rstudio.com) IDE. GAMM uses the package "mgcv" (Wood, 2006). Memory score from Word List Recall, for up to 7 time points covering up to 6.8 years, was used as outcome variable, biomarker group ("cluster") as factor, participant-specific time since baseline as covariate, and we included a time × biomarker group interaction term. Sex and baseline age were included as covariates of no interest. Random intercept was included. Separate analyses were run including number of memory test sessions completed as a proxy to control for practice effects. The same analyses were run for hippocampal volume across time, covering up to 6.81 years. The same variables and covariates as for the memory analyses were included. In addition, estimated total intracranial volume was included as an additional covariate of no interest. All analyses were run with age × time as an additional covariate, which in no instances had substantial impact on the reported results, and these analyses were thus not included in the manuscript. A major advantage of GAMM in the present setting is that relationships of any degree of complexity can be modeled without specification of the basic shape of the relationship, and GAMM is thus especially well-suited to map trajectories of neurocognitive variables which can be assumed to be non-linear and where the basic form of the curve is not known (Fjell et al., 2010). This means that if the trajectories of a given measure are compared across groups of participants, GAMM will detect possible slope differences around inflection points. GAMM fits are typically evaluated and inspected based on *p*- and *F*-values, edf (effective degrees of freedom) as a measure of the complexity of the curve, as well as by inspecting the plotted graphs. We also used the package "simr" in R to calculate how many annual examinations would be required to detect differences in memory change between the biomarker groups with 80% power for our sample with the given effect sizes (Green and MacLeod, 2016).

## 3. Results

### 3.1. CSF biomarker correlations

CSF Aβ42 did not correlate with age or any of the other CSF biomarkers. CSF T-tau, P-tau, YKL-40, FABP3, and NFL were all positively correlated with age (Table 3). Correlations between CSF biomarkers were therefore adjusted for age. T-tau, P-tau, YKL-40, NFL, and FABP3 were all positively correlated (Table 3). Such a correlation pattern between the biomarkers suggested that it could be possible to identify higher order structures in the data, that is, clusters of biomarkers.

### 3.2. Cluster analysis 1: clusters of CSF biomarkers

The cluster analysis yielded different levels of separation of the CSF biomarker clusters (Fig. 1). At level 1, one cluster was formed by Aβ42 and a second cluster by the remaining CSF biomarkers. At level 2, the second group from level 1 was further subdivided into one cluster consisting of FABP3, T-tau, P-tau, and YKL-40, and one cluster formed by NFL. At level 3, the cluster formed by FABP3, T-tau, P-tau, and YKL-40 was split into 2 clusters: 1 consisting of YKL-40 only and 1 with the remaining CSF biomarkers (FABP3, T-tau, and P-tau). At the final level, the tau biomarkers were separated from FABP3.

**Table 3**
CSF biomarker correlations

| Biomarkers | Age | Aβ42 | FABP3 | P-tau | T-tau | YKL-40 | NFL |
|---|---|---|---|---|---|---|---|
| Aβ42 | −0.01 | – | | | | | |
| | 0.96 | | | | | | |
| FABP3 | **0.26** | 0.15 | – | | | | |
| | **0.01** | 0.15 | | | | | |
| P-tau | **0.28** | 0.10 | **0.79** | – | | | |
| | **0.006** | 0.35 | **<0.001** | | | | |
| T-tau | **0.29** | 0.05 | **0.79** | **0.96** | – | | |
| | **0.003** | 0.65 | **<0.001** | **<0.001** | | | |
| YKL-40 | **0.36** | 0.01 | **0.44** | **0.62** | **0.67** | – | |
| | **<0.001** | 0.94 | **<0.001** | **<0.001** | **<0.001** | | |
| NFL | **0.47** | 0.02 | **0.52** | **0.32** | **0.33** | **0.35** | – |
| | **<0.001** | 0.87 | **<0.001** | **0.002** | **0.001** | **<0.001** | |

Numbers represent Spearman's rho and *p*-values for the first and second line, respectively. Correlations between the CSF biomarkers are adjusted for age by partial correlations. Bold indicates $p < 0.05$.
Key: Aβ42, beta amyloid 1–42; CSF, cerebrospinal fluid; FABP3, fatty acid binding protein 3; NFL, neurofilament light; P-tau, phosphorylated tau; T-tau, total tau; YKL-40, chitinase-3-like protein 1.

### 3.3. Cluster analysis 2: subgroups of participants identified by CSF biomarkers

We ran the cluster analysis to identify subgroups of participants with similar biomarker characteristics. We found that the participants could be divided in 2 main groups: group 1 (n = 60) and group 2 (n = 39), respectively (Fig. 2). To map out the relative contributions of each biomarker to the grouping, Cohen's *d* was calculated for each biomarker (Table 4). Group 2 participants were characterized by more pathological biomarker results for all biomarkers, with Cohen's $d > 0.80$—considered a large effect size—for all except NFL, where Cohen's *d* was >0.20 (small effect size). These differences should be interpreted as descriptions of the pattern of biomarker differences most contributing to the grouping. Mean biomarker concentrations in group 2 exceeded pathological thresholds for P-tau (>60 pg/mL) and T-tau (>350 pg/mL) (Hansson et al., 2006), and the mean Aβ42 concentration approached the pathological threshold of Aβ42 (<530 pg/mL) (Hansson et al., 2006). Accordingly, all A+T+ individuals were found in group 2 (Table 4). Group 1 had on average normal values for all core AD biomarkers (Aβ42, P-tau, T-tau). There were no differences in age, sex, years of education, or cancer morbidity between group 1 and 2 (Table S2).

In a second level analysis, each group was further divided in 2 smaller groups: group 1.1 (n = 24), group 1.2 (n = 36), group 2.1 (n = 20), and group 2.2 (n = 19). The group of participants with generally more pathological biomarker values (group 2) was split into one subgroup (group 2.2) with more pathological values of tau (T-tau, P-tau) and FABP3, and one (group 2.1) with more pathological values of Aβ42 and NFL and tendencies to higher levels of YKL-40 (Cohen's $d = 0.28$) (Table 5). Mean biomarker concentrations in both group 2.1 and group 2.2 exceeded the pathological thresholds for P-tau and T-tau referred above. Only group 2.1 had an average Aβ42 concentration satisfying usual criteria for amyloid

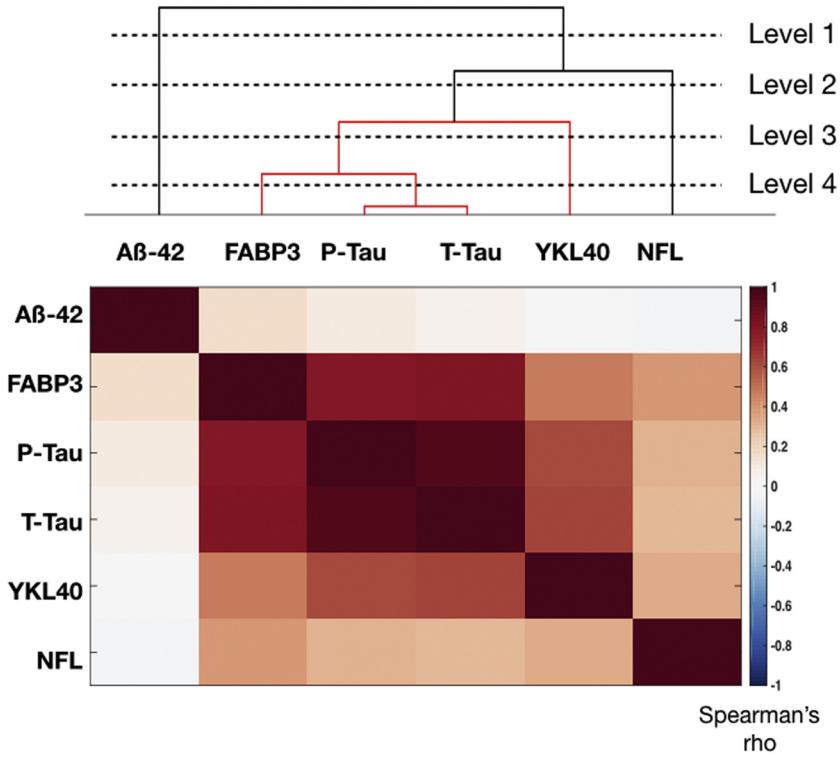


**Fig. 1.** Correlations and hierarchical clustering of CSF biomarkers. Abbreviations: Aβ42, beta amyloid 1–42; CSF, cerebrospinal fluid; FABP3, fatty acid binding protein 3; NFL, neurofilament light; P-tau, phosphorylated tau; T-tau, total tau; YKL-40, chitinase-3-like protein 1.

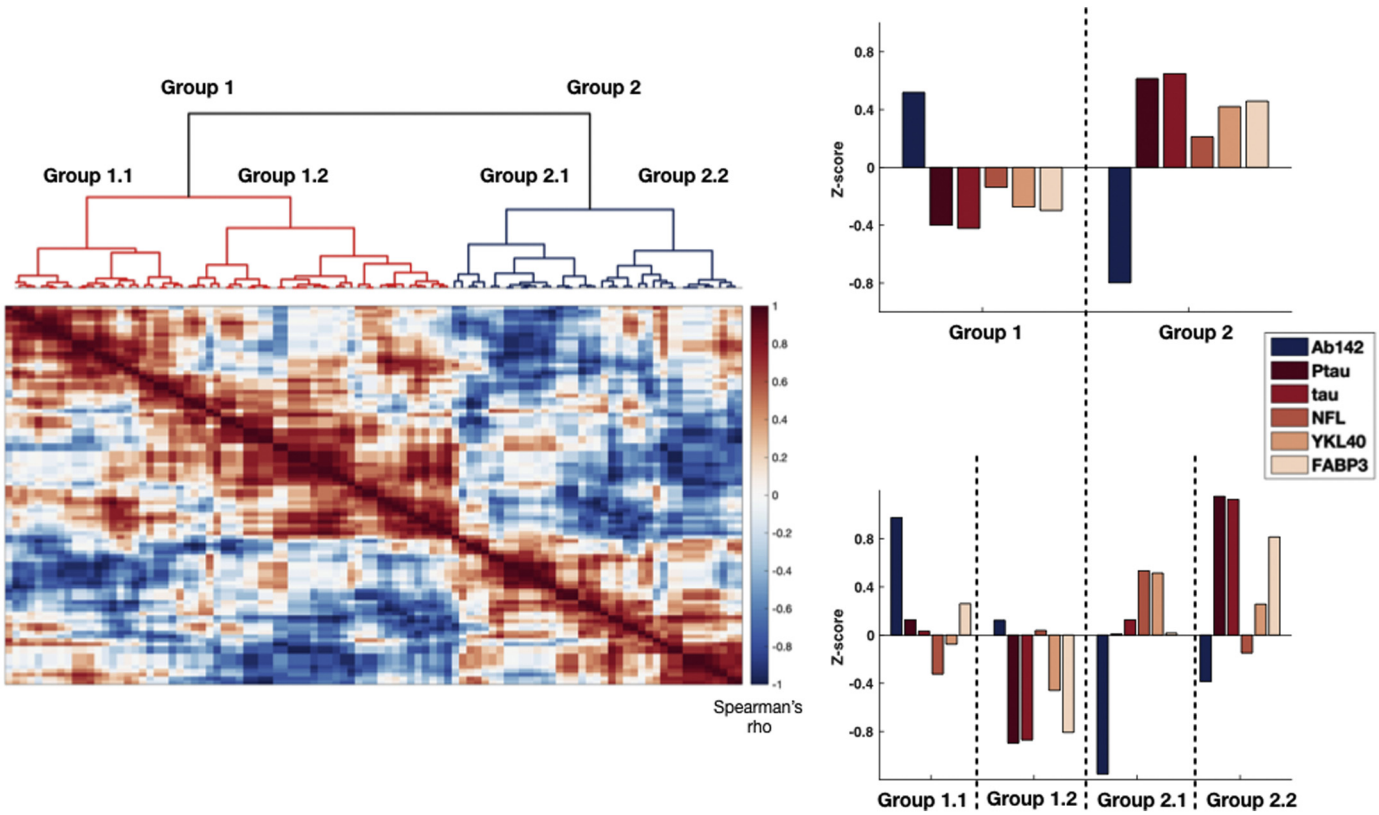


**Fig. 2.** Hierarchical clustering of participants. Left panel: Subject-wise correlation matrix and dendrogram of the groups at the different levels. Right panel: Mean *z*-scores of each variable within each group. The *z*-scores are calculated for the current sample, yielding a sample sum of 0 and a standard deviation of 1, thus the groups tend to approximately mirror each other around the $y = 0$ axis when the group sizes are similar. Abbreviations: Aβ42, beta amyloid 1–42; FABP3, fatty acid binding protein 3; NFL, neurofilament light; P-tau, phosphorylated tau; T-tau, total tau; YKL-40, chitinase-3-like protein 1.

**Table 4**
Differences in CSF biomarkers between 2 subgroups based on cluster analysis

| Biomarkers | Group 1 (n = 60) | | Group 2 (n = 39) | | Cohen's *d* |
|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | |
| Aβ42 (pg/mL) | 812 | 150 | 538 | 189 | **1.65** |
| FABP3 (pg/mL) | 4.31 | 1.57 | 5.93 | 2.23 | **0.88** |
| P-tau (pg/mL) | 53 | 15 | 76 | 22 | **1.27** |
| T-tau (pg/mL) | 317 | 104 | 499 | 179 | **1.32** |
| YKL-40 (pg/mL) | 209,512 | 67,132 | 277,101 | 86,599 | **0.90** |
| NFL (pg/mL) | 1155 | 678 | 1491 | 1083 | *0.39* |
| Biomarker status | n | % | n | % | *p*-value |
| A+ (Aβ42 < 530 pg/mL) | 4 | 7 | 25 | 64 | <0.001 |
| T+ (P-tau > 60 pg/mL) | 15 | 25 | 25 | 64 | <0.001 |
| A+T+ | 0 | 0 | 13 | 33 | <0.001 |

Means are based on raw data. Bold indicates Cohen's $d \geq 0.50$, italics indicates Cohen's $d \geq 0.20$. *p*-values were calculated using $\chi^2$ test.
Key: A+, amyloid positivity; Aβ42, beta amyloid 1–42; CSF, cerebrospinal fluid; FABP3, fatty acid binding protein 3; NFL, neurofilament light; P-tau, phosphorylated tau; SD, standard deviation; T+, tau positivity; T-tau, total tau; YKL-40, chitinase-3-like protein 1.

positivity, although the concentration in group 2.2 was also close to the pathological threshold. Group 2.1 participants also had mean NFL values of 1831 pg/mL, close to an established cut-off value of 1850 for this age-range (Yilmaz et al., 2017). The proportion of A+T+ participants was around 35% in both groups 2.1 and 2.2 (Table 5).

The participants in groups 1.1 and 1.2 had less pathological biomarker values than the participants in 2.1 and 2.2, but could still be differentiated. Group 1.1 had more pathological levels of FABP3 and tau, while group 1.2 had more pathological levels of Aβ42 and slightly higher levels of NFL (Cohen's $d = 0.37$) (Table 5). There were no differences in YKL-40 between group 1.1 and 1.2. Group 1.1 had mean tau levels above the pathological thresholds, and the proportion of T+ individuals was significantly higher than in group 1.2 (Table 5). These findings parallel the results from the comparison between group 2.1 and 2.2, with higher tau and FABP characterizing one group and more pathological levels of Aβ42 characterizing the other, although it must be noted that mean levels in group 1.2 were still not close to amyloid positivity.

Group 2.1 was significantly older than groups 1.1 and 2.2, otherwise there were no differences in age, sex, years of education, or cancer morbidity between the 4 clustering-based subgroups (Table S3).

To test whether our results were impacted by participant selection, we re-ran the clustering analyses including all participants that were classified as cognitively unimpaired at baseline (n = 116) (i.e., including also individuals who were later diagnosed with MCI or dementia). We found that the individuals could still be divided into 2 and 4 subgroups, and that these groups showed biomarker characteristics similar to the groups in our main analyses (Fig. S2 and Tables S4 and S5).

### 3.4. Differences in hippocampal atrophy between the biomarker subgroups

In the full sample, GAMM with time since baseline (interval) as predictor, including random effects for intercept, showed that hippocampal volume was significantly reduced over time in a slightly accelerated fashion (edf = 2.2, $F = 168.5$, $p < 2 \times 10^{-16}$). Significant atrophy was seen for both groups in the 2-cluster solution (group 1: edf = 1.8, $F = 109.7$, $p < 2 \times 10^{-16}$; group 2: edf = 1.1, $F = 132.8$, $p < 2 \times 10^{-16}$). Directly comparing hippocampal change over time between the groups from the 2-cluster solution, there were no significant differences in hippocampal volume loss over time ($F = 1.0$, $p = 0.32$), and no main group effect difference ($p = 0.63$) (Fig. 3). We repeated the analyses, comparing volume change pairwise between the groups from the 4-cluster solution, finding no significant effects (all *p*'s ≥ 0.20).

### 3.5. Memory differences between the biomarker subgroups

In the full sample, GAMM with time since baseline (interval) as predictor, including random effects for intercept, controlling for baseline age and sex, showed that memory scores, measured as number of words recalled, followed an inverted *U*-shaped trajectory over the 6.8-year interval (edf = 2.9, $F = 11.65$, $p = 7.22 \times 10^{-7}$; Fig. 4). The initial increase is likely due to practice effects. Thus, we re-ran the analyses, also controlling for practice effects using

**Table 5**
Differences between 4 subgroups based on clustering analysis

| Biomarkers | Group 1.1 (n = 24) | | Group 1.2 (n = 36) | | Cohen's *d* | Group 2.1 (n = 20) | | Group 2.2 (n = 19) | | Cohen's *d* |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | | Mean | SD | Mean | SD | |
| Aβ42 | 888 | 136 | 762 | 139 | **0.91** | 472 | 122 | 606 | 223 | **0.75** |
| FABP3 | 5.45 | 1.59 | 3.54 | 0.99 | **1.52** | 5.31 | 2.06 | 6.59 | 2.26 | **0.59** |
| P-tau | 63 | 14 | 47 | 13 | **1.21** | 67 | 19 | 87 | 20 | **1.02** |
| T-tau | 379 | 88 | 275 | 93 | **1.15** | 431 | 164 | 571 | 170 | **0.84** |
| YKL-40 | 205,901 | 80,065 | 211,920 | 58,047 | 0.09 | 289,103 | 88,997 | 264,468 | 84,517 | 0.28 |
| NFL | 1004 | 317 | 1256 | 827 | *0.37* | 1831 | 1380 | 1133 | 448 | **0.71** |
| Biomarker status | n | % | n | % | *p*-value | n | % | n | % | *p*-value |
| A+ | 0 | 0 | 4 | 11 | 0.14 | 15 | 75 | 10 | 53 | 0.15 |
| T+ | 12 | 50 | 3 | 8 | <0.001 | 10 | 50 | 15 | 79 | 0.06 |
| A+T+ | 0 | 0 | 0 | 0 | – | 7 | 35 | 6 | 32 | 0.82 |

Means are based on raw data. CSF concentrations of biomarkers are measured in pg/mL. Bold indicates Cohen's $d \geq 0.50$, italics indicates Cohen's $d \geq 0.20$. *p*-values were calculated using $\chi^2$ or Fisher's exact test.
Key: A+, amyloid positivity; Aβ42, beta amyloid 1–42; FABP3, fatty acid binding protein 3; NFL, neurofilament light; P-tau, phosphorylated tau; SD, standard deviation; T+, tau positivity; T-tau, total tau; YKL-40, chitinase-3-like protein 1.

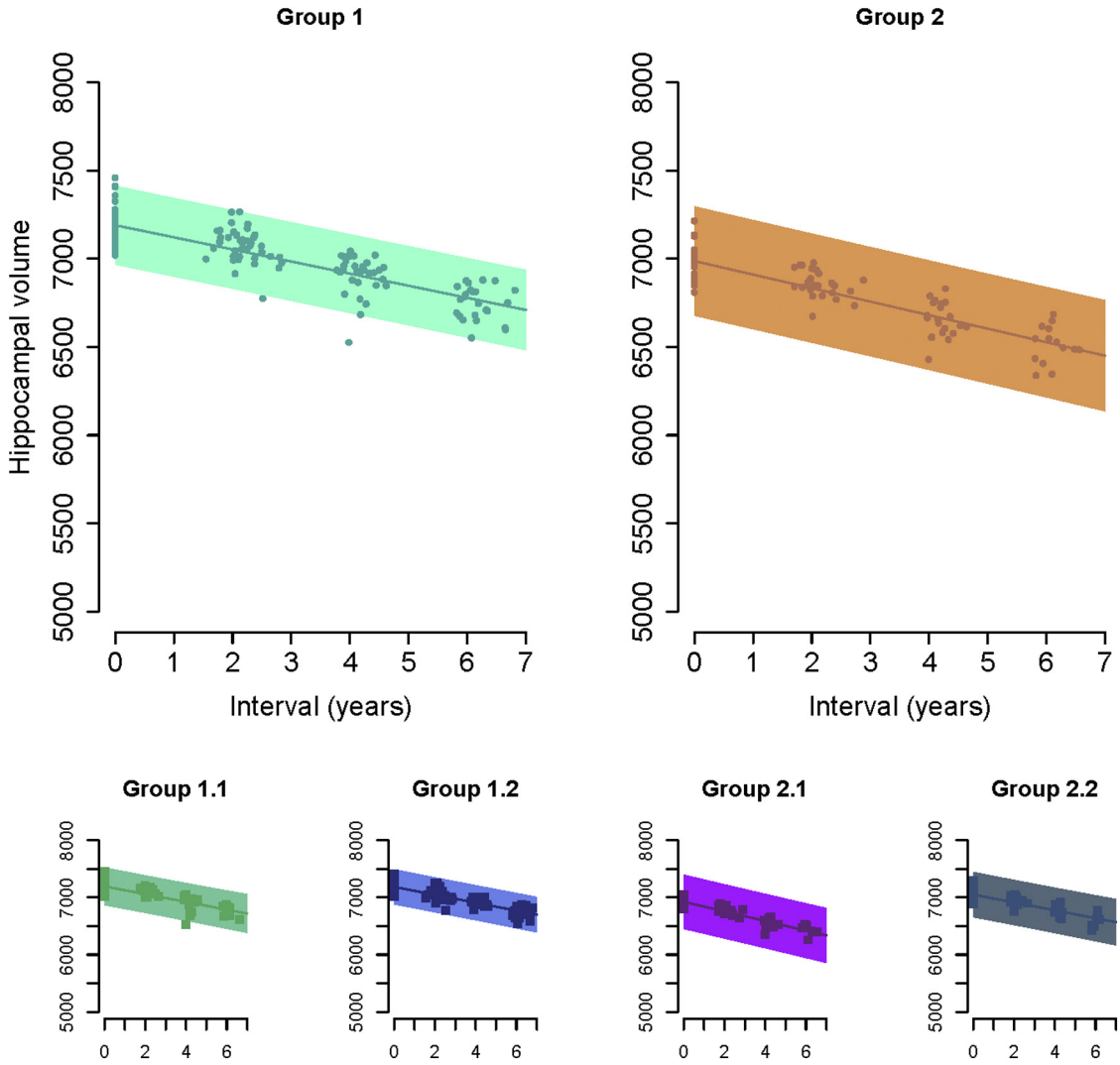


**Fig. 3.** Longitudinal change in hippocampal volume across biomarker groups. Upper panel: GAMM-fitted change slope in hippocampal volume for group 1 and group 2 across time. Lower panel: Hippocampal volume slopes for each of the groups in the 4-cluster solution. Abbreviations: GAMM, generalized additive mixed models.

number of test sessions completed. This showed a linear negative effect of interval on memory score (edf = 1.0, $F = 12.06$, $p = 0.0006$), and a positive but gradually reduced effect of number of test sessions as a proxy for practice effects (edf = 2.0, $F = 21.24$, $p = 6.55 \times 10^{-8}$).

After having established the trend for the change in memory scores over time in the total sample, we tested whether memory differed between biomarker groups in terms of intercept or slope, co-varying for baseline age and sex (Fig. 5, Table 6). Comparing group 1 and group 2, we found a significant difference in slope (edf = 1.0, $F = 4.7$, $p = 0.030$) if practice effects as indexed by number of follow-up test sessions were included as covariates, and a trend if not ($p = 0.098$). Plotting the results showed more memory decline in the group (group 2) with the more pathological biomarkers.

Pairwise comparisons between the memory trajectories from each of the groups in the 4-cluster solution showed significantly more memory decline for group 2.2 compared to group 1.2 ($p = 0.014$) (Fig. 6). Group 2.2 was the group with the highest levels of tau and FABP3, while group 1.2 was the group with the lowest levels of the same biomarkers. No other differences between groups in memory trajectories were seen. The proportion of participants followed up with cognitive assessment did not differ between the 4 cluster-based biomarker groups at any of the 7 time points ($p = 0.76$).

### 3.6. Memory and hippocampus changes in NIA-AA defined biomarker groups

As an alternative to the clustering approach, we classified participants as AD (A+/T+, n = 19), A−T+ (n = 27), and normal AD biomarkers (A−/T−, n = 32), based on cut-offs defined above. We tested if the groups A+T+ or A−T+ showed different changes in hippocampal volume or memory over 6.8 years compared to the A−T− group. For neither hippocampal volume (edf = 1.0, $F = 0.59$, $p = 0.44$, n = 130 observations) nor memory (edf = 1.0, $F = 0.04$, $p = 0.84$, n = 278 observations) were significant slope differences seen between A+T+ versus the A−T− group. The result was the same if number of test sessions was entered as a proxy for practice effects ($p = 0.63$). There was a tendency for A−T+ participants to show more memory decline over time compared to the A−T− group (edf = 1, $F = 2.9$, $p = 0.089$), with the $p$-value dropping just below 0.05 when number of test sessions was included ($p = 0.040$). No effect of A−T+ on hippocampal volume change was found ($p = 0.16$). To assess whether the lack of difference in memory trajectories between the AD group (A+/T+) based on biomarkers as described by NIA-AA and the normal AD biomarker group was due to too short follow-up interval or too small sample, we ran power simulations based on the observed effects (see Supplemental

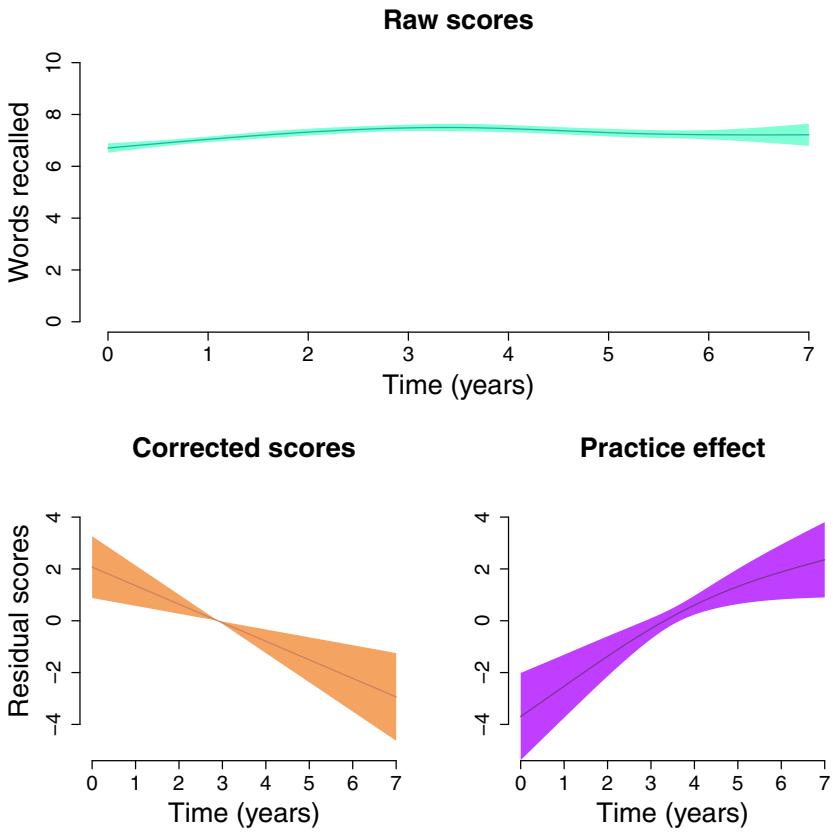


**Fig. 4.** Longitudinal change in memory function in the full sample. Upper panel: Memory scores over time in the full sample. Left bottom panel: Estimated memory performance over time corrected for practice effects. Bottom right panel: Estimated practice effects plotted over time.

Information for details). These simulations showed than even if we follow the participants over 15 years, we would not find a significant difference in memory slope between the AD group defined by the NIA-AA criteria versus the normal AD biomarker group (12% power at $\alpha = 0.05$ with 15 time points spanning 15 years) (Fig. 7). This shows that it is unlikely that the biomarker defined AD group and the normal AD biomarker group will experience different changes in memory function over the next 15 years. We also tested how increasing the number of participants included in the analyses would affect the power to detect slope differences between the groups. These simulations demonstrated that with a sample size of 1050 participants, power to detect an effect was no more than 13%. Thus, while the clustering approach was able to define subgroups of participants with different biomarker profiles showing significant differences in memory slope, using the NIA-AA criterion for AD, we were not able to detect differences, and this is highly unlikely to be due to short follow-up interval or a limited sample size.

## 4. Discussion

This study on biomarkers in cognitively unimpaired older adults has 3 key findings. First, clustering analysis of biomarkers showed that the novel CSF biomarkers NFL, FABP3, and YKL-40 clustered with T-tau and P-tau, whereas Aβ42 was separated out in an independent cluster. Second, clustering analyses of participants identified 2 main biomarker profiles, where one biomarker group had more abnormal levels of all biomarkers compared to the other biomarker group. At the 4-cluster level, the group with more pathological biomarkers was split into one group characterized by tauopathy and FABP3 and one group by brain β-amyloidosis, NFL, and YKL-40. Third, the group with tauopathy and FABP3 showed more memory decline over 6.8 years compared to a group with less pathological biomarker levels. The clustering-based classification of the participants yielded better predictions of memory decline across the subsequent 7 years than a canonical classification based on positivity for both amyloid and tau.

### 4.1. Relationships between CSF biomarkers

We assessed the relationship between the established AD biomarkers Aβ42, reflecting amyloid deposition, T-tau, reflecting neuroaxonal degeneration, and P-tau, reflecting phosphorylation state of tau and possibly neurofibrillary tangle pathology, and the novel biomarkers YKL-40, reflecting neuroinflammation, FABP3, reflecting neuronal damage, and NFL, reflecting axonal injury. Interestingly, the cluster analyses revealed a principal divide between Aβ42 and the rest of the biomarkers. Although T-tau, P-tau, YKL-40, FABP3, and NFL clustered together at the highest level, Aβ42 was separated in a single cluster. Thus, in cognitively unimpaired older adults, the less established CSF biomarkers clustered with P- and T-tau, while showing no relationships to Aβ42. Such a divide between Aβ42 and other CSF biomarkers including FABP3 and P-tau has also been shown using clustering analyses in a cohort with individuals from the entire AD cognitive continuum (Harari et al., 2014). Furthermore, lack of relationship between Aβ42 and the Tau-proteins in cognitively unimpaired adults has also been found in previous studies (Roe et al., 2013; Xiong et al., 2016), although weak to moderate negative correlations have also been reported (Bos et al., 2019; Pettigrew et al., 2015; Soldan et al., 2019). Non-existent relationships between Aβ42 and YKL-40 (Olsson et al.,

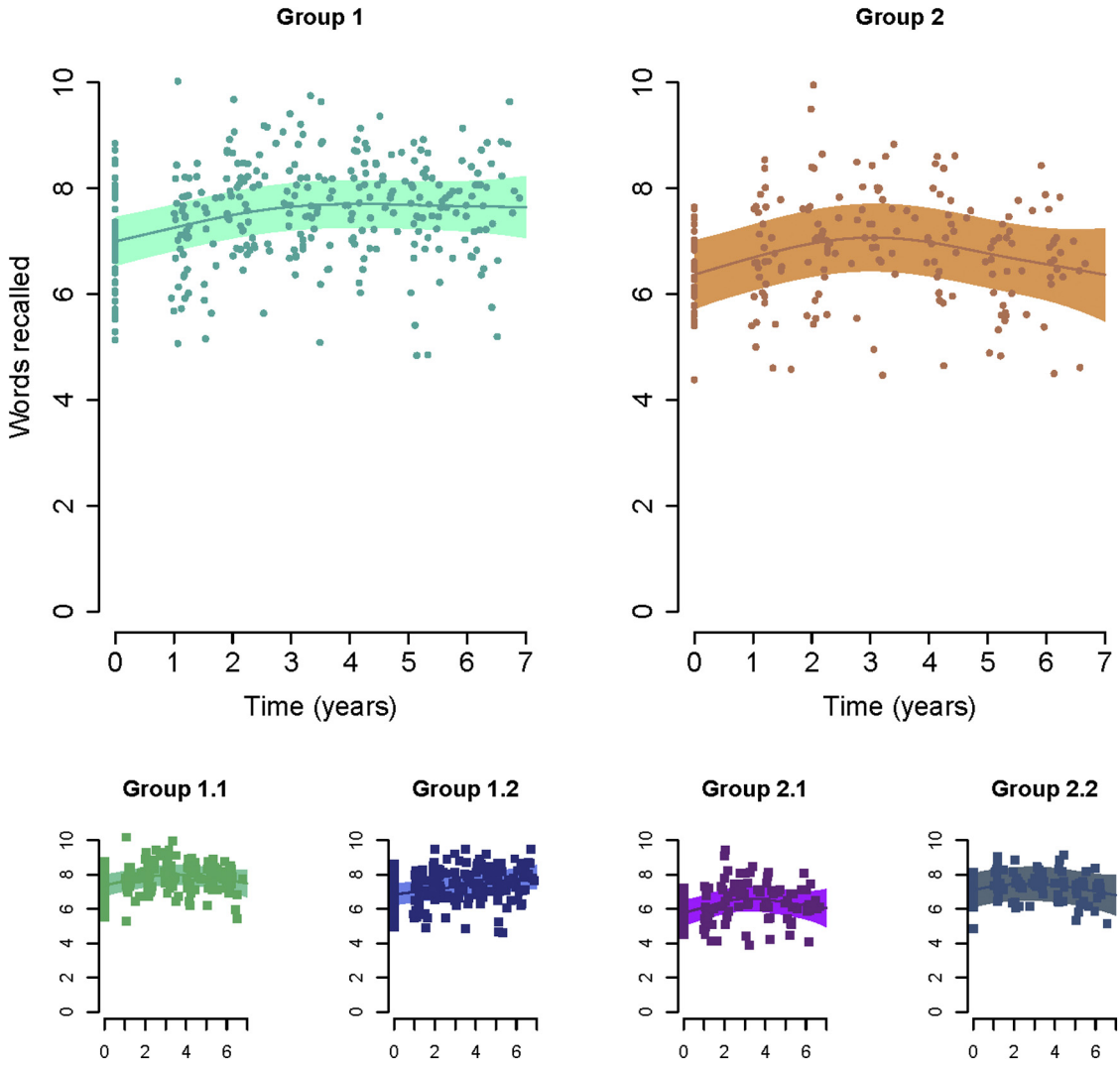


**Fig. 5.** Longitudinal change in memory function across biomarker groups. Upper panel: GAMM-fitted change slope in memory score for group 1 and group 2 across time. Lower panel: Memory slopes for each of the groups in the 4-cluster solution. Abbreviation: GAMM, generalized additive mixed models.

2013a), NFL (Bruno et al., 2012), and FABP3 (Olsson et al., 2013b) in cognitively unimpaired individuals are also in agreement with previous studies, but weak negative (Alcolea et al., 2015a; Bos et al., 2019) and weak positive (Alcolea et al., 2015a; Kern et al., 2019) correlations have also been shown in some larger studies. The cluster analysis results show that these less established biomarkers cluster with the Tau proteins, independently of amyloid pathology.

**Table 6**
Change in memory as a function of biomarker group

| Models | edf | *F* | *p* slope | *p* main effect |
|---|---|---|---|---|
| Two-group model | | | | |
| Time | 2.8 | 11.12 | $2.37 \times 10^{-6}$ | |
| Group 2 versus 1 | 1.3 | 3.65 | 0.098[a] | 0.13 |
| Four-group model | | | | |
| Group 1.1 versus 1.2 | 1.0 | 2.78 | 0.10 | 0.68 |
| Group 1.2 versus 2.1 | 1.0 | 2.20 | 0.14 | 0.57 |
| Group 1.2 versus 2.2 | 1.0 | β6.05 | 0.01 | 0.11 |
| Group 2.1 versus 2.2 | 1.0 | 0.76 | 0.38 | 0.32 |

GAMMs were run to test effects of biomarker group on changes in memory performance over time. Changes in memory performance were tested against time and then it was tested whether the effect of time on memory differed between biomarker groups. Baseline age and sex were used as covariates of no interest.
Key: edf, effective degrees of freedom (a measure of deviation from linearity); GAMM, generalized additive mixed models.
[a] $p < 0.030$ if practice effects were corrected for.

The very high correlation between T-tau and P-tau was expected, being consistent with previous studies of cognitively unimpaired adults (Blennow et al., 1995; Bos et al., 2019; Soldan et al., 2019). The strength of this correlation ($r = 0.96$) suggests that these 2 CSF tau-markers are statistically collinear in cognitively unimpaired older adults. The neuronal injury biomarkers, NFL and FABP3, were positively correlated both with each other and also with T-tau and P-tau, supporting their role as neurodegeneration biomarkers even in cognitively unimpaired older adults. This finding is in agreement with previous studies of cognitively unimpaired adults showing positive correlations between tau-proteins and NFL (Bos et al., 2019; Kern et al., 2019; Melah et al., 2016), and FABP3 (Olsson et al., 2013b), respectively. NFL was the first biomarker to separate from the cluster with tau biomarkers, supporting the hypothesis that CSF NFL provide information on neurodegeneration that is at least partly different from CSF T-tau (Mattsson et al., 2016). Interestingly, the neuroinflammation biomarker YKL-40 was also positively correlated with all the neurodegeneration biomarkers, indicating a link between neuroinflammation and neurodegeneration in aging. Previous data

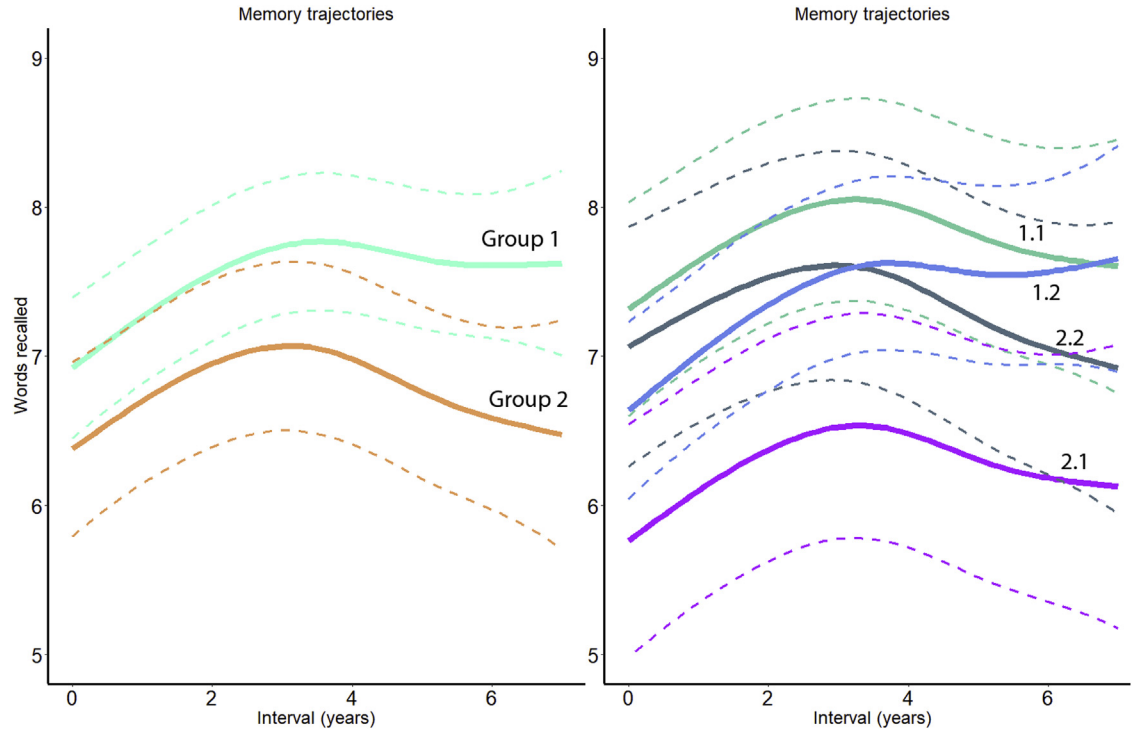


**Fig. 6.** Memory trajectories across biomarker groups. The memory trajectories from Fig. 5 plotted as a function of biomarker group overlaid in the same plot to allow inspection of differences. Dotted lines illustrate 95% confidence interval.

showing positive correlations between YKL-40 and T-tau and P-tau in cognitively unimpaired adults (Alcolea et al., 2015a; Bos et al., 2019; Melah et al., 2016; Olsson et al., 2013a), and in neurodegenerative diseases (Craig-Schapiro et al., 2010; Hall et al., 2018; Nordengen et al., 2019; Wennstrom et al., 2015), and studies finding associations between elevated YKL-40 and white matter degeneration (Racine et al., 2019), brain atrophy (Alcolea et al., 2015b, 2017; Janelidze et al., 2018; Swanson et al., 2016), and cognitive function (Bos et al., 2019; Janelidze et al., 2018; Sala-Llonch et al., 2017), provide further support for this link both in aging and neurodegenerative diseases. The association between YKL-40 and tau has been shown in both A+ and A− cognitively normal individuals (Alcolea et al., 2015a), suggesting that the link between neurodegeneration and neuroinflammation is independent of amyloid deposition. Positive correlations between YKL-40 and NFL (Bos et al., 2019; Melah et al., 2016) in cognitively normal adults have also been reported previously, whereas this is, to our knowledge, the first study to explore the relationship of FABP3 to YKL-40 and NFL in a cognitively unimpaired population. Positive correlations of FABP3 with YKL-40 and NFL have, however, previously been reported in populations including patients (Bjerke et al., 2011; Harari et al., 2014).

### 4.2. Grouping of participants based on biomarker profiles

Clustering analyses, based on objective biomarker measures and blind to any cognitive evaluation, revealed one group, consisting of 39% of the total sample, with more abnormal concentrations of all biomarkers. Although normative data are not available for all the biomarkers, this group had pathological mean levels of Aβ42, T-tau, and P-tau according to previously established criteria (Hansson et al., 2006) and all A+T+ individuals were found in this group.

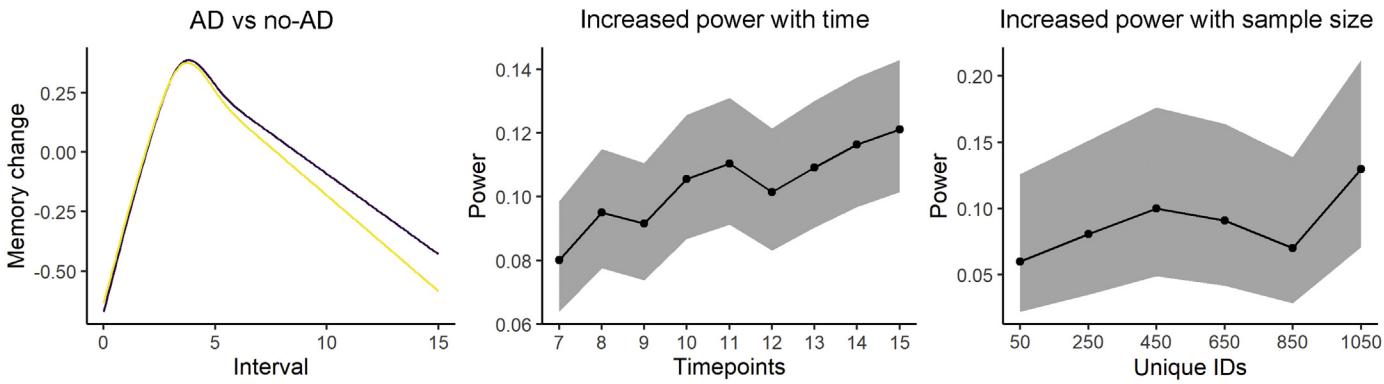


**Fig. 7.** Memory slope in AD versus no-AD. Participants were divided in an "AD" group based on amyloid and tau (A+/T+) and a no-AD group (A−/T−). Left panel shows the estimated differences in memory trajectories over 15 years. Middle panel shows how power to detect a slope difference between AD and no-AD increases as a function of number of follow-ups. Right panel shows how power increases as a function of sample size. Abbreviations: A+, amyloid positivity; A-, amyloid negativity; AD, Alzheimer's disease; T+, tau positivity; T-, tau negativity.

Further clustering analyses separated a group of participants with non-pathological mean biomarker concentrations (group 1.2), consisting of 36% of the sample. Studies show that there may be a proportion of older adults that never develops amyloid or neurodegenerative pathology (Jack et al., 2014; Khachaturian et al., 2004), and future studies on such groups of very low-risk older adults may give cues on how to prevent development of various brain pathologies (Vemuri, 2018). However, the proportion of individuals without pathology may be over-estimated in our sample, as we did not include cases with clinical diagnoses and those who progressed to MCI, dementia, and other neurodegenerative diseases during follow-up. Also, we cannot exclude that some of the participants with a non-pathological biomarker profile may develop pathological biomarkers later.

The 3 remaining groups represented different patterns of increased or pathological mean biomarker values. Group 1.1 was the least pathological of these, showing evidence for slight tauopathy. The most pathological group in the 2-cluster solution was divided into one group characterized by more pathological values of tau (T-tau, P-tau) and FABP3 (group 2.2), and one with more pathological values of Aβ42 and NFL, and tendencies to higher levels of neuroinflammation (YKL-40) (group 2.1). Although the mean tau levels in group 2.1 were clearly lower than in group 2.2, they still exceeded pathological thresholds. Accordingly, group 2.1 participants on average satisfied the NIA-AA criterion for AD (Jack et al., 2018). The participants in group 2.1 also had mean NFL values close to an established cut-off value of 1850, and slightly higher concentrations of YKL-40, suggesting ongoing neuroinflammation and axonal degeneration in addition to brain β-amyloidosis and tau pathology. Emerging evidence suggests that neuroinflammation in concert with AD neuropathology may contribute to the development of clinical symptoms (Craig-Schapiro et al., 2010; Heneka et al., 2015; Merluzzi et al., 2018), possibly through contributing to neurodegeneration (Alcolea et al., 2015b; Heneka et al., 2015; Janelidze et al., 2018).

The tauopathy found in 3 of the 4 identified groups may partly be age-related (Crary et al., 2014; Lowe et al., 2018), such as in primary age-related tauopathy, although preclinical phases of other tauopathies cannot be excluded (Arendt et al., 2016). As tau was measured in CSF, we could not assess the patho-anatomical location, that is, whether it is spread outside the medial temporal lobe. Group 2.2 was characterized by a neurodegeneration biomarker pattern with tauopathy and elevated FABP3, and was the group with highest mean levels of tau and FABP3. Such a pattern of neurodegeneration can represent normative, age-expected brain changes. Group 1.1 and 2.2 may also, according to some systems, be classified as suspected non-Alzheimer pathophysiology (SNAP) (Jack et al., 2016b), in which, for example, clinically silent cerebral microvascular disease, hippocampal sclerosis, or aging-expected processes could be responsible for the neurodegeneration. It should, however, be noted that group 2.2 on average had Aβ42 levels in a gray zone around the pathological threshold for amyloid positivity, and accordingly around 50% of the individuals in this group were amyloid positive, suggesting this group could also represent AD. Contrary, none of the individuals in group 1.1 were amyloid positive, supporting that this group represents SNAP.

The present results suggest that patients can be divided into subgroups based on their biomarker profiles also beyond amyloid and tau. This yields more extensive information about patients than what can be obtained by using biomarkers in isolation. Interestingly, all the 6 CSF biomarkers differed between at least 2 of the groups with a relatively large effect size, suggesting that all have contributed to the clustering results. We did not attempt to cluster the participants based on a subset of the CSF biomarker to test if any was redundant. Except P-tau and T-tau, which are statistically almost collinear, it seems that inclusion of all the biomarkers contributes to the different biomarker profiles of the subgroups. Although the use of biomarker clustering in a clinical setting would depend on the study of long-term clinical outcomes, such as progression to MCI or dementia, clustering may be a promising approach to identify patients with various biomarker profiles for clinical trials, intervention studies, and in the clinic to improve diagnosis and prognosis.

This biomarker-based grouping of the participants suggests that linear staging of CSF biomarkers, where the biomarkers become abnormal at different times in an ordered sequence, does not apply to the present data. It is possible that a well-defined clinical endpoint can follow a fixed chain of events in an orderly fashion. In cognitively unimpaired older adults, however, biomarkers do not seem to adhere to a fixed linear staging. Unfortunately, we do not have longitudinal data on the biomarkers in combination with different clinical endpoints, which would be necessary for proper staging of the biomarkers.

### 4.3. Biomarker profiles in prediction of hippocampal volume change and memory decline

The full sample showed an inverted *U*-shaped trajectory of memory scores over the 6.8-year interval since the baseline testing. As practice effects are well-known to increase performance on memory tests in longitudinal studies (Ronnlund et al., 2005), we attempted to tear apart real change in memory from practice-induced inflation of the scores. This analysis showed a linear decline in the corrected scores, accompanied by a decelerating increase due to repeated test exposure. Testing the difference in memory trajectories between the 2 main groups, the group with most pathological biomarkers showed slightly more memory decline over the examination interval than the group with the least pathological biomarkers. Examining this pattern in more detail in the 4-cluster solution, the group with most tauopathy and highest FABP3 showed significantly more memory decline compared to the group with normal biomarker levels. This indicates that a high degree of neuronal damage is the biomarker feature most predictive of memory decline in cognitively unimpaired older adults. Earlier clustering studies of cognitively normal adults have also found that subgroups with more neurodegeneration or tauopathy show greater rates of cognitive decline, for example, greater memory decline in a subgroup mainly characterized by lower Aβ42 and higher P-tau (Racine et al., 2016), and greater global cognitive decline in a subgroup characterized by more brain atrophy, more white matter hyperintensities, lower Aβ42, higher T-tau, and higher P-tau (Nettiksimmons et al., 2010). FABP3, YKL-40, or NFL has never been used for clustering of cognitively unimpaired adults. Yet, a recent study clustering individuals from the entire cognitive continuum using CSF NFL, YKL-40, and the core AD biomarkers reported that a subgroup characterized by high T-tau and P-tau, but not Aβ42 levels, included almost 50% of all patients with respectively MCI and AD dementia (Toschi et al., 2019). High levels of each of the 3 less established biomarkers have, however, been associated with cognitive decline or development of cognitive impairment in cognitively normal adults (Bos et al., 2019; Harari et al., 2014; Kern et al., 2019; Sala-Llonch et al., 2017).

Moreover, group 2.2 may have brain β-amyloidosis. The predictive value of brain β-amyloidosis for later cognitive or clinical symptoms is however controversial (Morris et al., 2018). Although brain β-amyloidosis may be a risk factor for cognitive decline (Hedden et al., 2013), up to 40% of cognitively unimpaired older adults have brain β-amyloidosis (Jansen et al., 2015), depending on the age of the participants. Likely, amyloidosis has to be accompanied with neurodegeneration or tau pathology in order to result in

dementia and cognitive decline (Burnham et al., 2016; Desikan et al., 2012; Merluzzi et al., 2018; Soldan et al., 2019). Therefore, we cannot exclude that the combination of gray zone amyloid positivity and neuronal damage is responsible for more memory decline in group 2.2. However, the A−T+ group showed a tendency toward more memory decline compared to the group with normal AD biomarkers—an effect that reached significance if correction for practice effects was done—suggesting that the association between neurodegeneration and memory decline in this sample is independent of amyloid pathology. Furthermore, group 2.1 with lowest Aβ42 (i.e., most amyloid pathology), and also tauopathy and axonal degeneration, and group 1.1 with slight tauopathy, showed only age-expected changes in memory performance over time. This finding also underscores the fact that older adults can have good cognitive function for their age, and show age-expected changes in memory function over several years, despite biomarker profiles indicating amyloid, tau, and/or neurodegeneration pathology.

Interestingly, comparing memory change between the group with AD according to the NIA-AA A+/T+ criterion with the group with normal AD biomarkers (A−/T−), we did not observe any difference. This finding differs from several previous studies reporting that cognitively normal individuals with both amyloid and tau pathology show accelerated cognitive decline compared to those with one or none of these pathologies (Desikan et al., 2012; Soldan et al., 2019). Actually, simulations showed that even if we follow the participants for 15 years after baseline, or increase the sample size to above 1000 participants, it is unlikely that we would see a difference in memory slope between the biomarker defined AD group and the non-AD group. Thus, while the clustering approach was able to define subgroups of participants with different biomarker profiles that showed more memory decline over time, no differences in memory outcome was seen using a simple AD versus non-AD dichotomy based on Aβ42 and P-tau alone. These results demonstrated that the clustering approach, taking advantage of multiple biomarkers beyond amyloid and tau, clearly outperformed the NIA-AA AD biomarker classification system in prediction of memory decline.

There were no differences between any of the clustering-based biomarker groups or the NIA-AA defined groups in hippocampal volume trajectories, suggesting that none of these biomarker profiles were associated with more than age-related hippocampal atrophy. Others have, however, reported that A+T+ cognitively normal individuals show accelerated hippocampal atrophy compared to A−T− and A−T+ individuals (Gordon et al., 2016). The relationship between novel biomarkers and medial temporal lobe atrophy is less studied, but higher NFL (Mattsson et al., 2016; Pereira et al., 2017), YKL-40 (Alcolea et al., 2015b; Swanson et al., 2016), and FABP3 (Desikan et al., 2013) have been associated with medial temporal lobe atrophy in populations including both cognitively unimpaired and impaired individuals. We have previously shown an association between higher NFL levels and higher hippocampal atrophy rates in individuals from the same cohort (Idland et al., 2017), and one study found no relationship between YKL-40 and hippocampal volume in cognitively unimpaired adults (Melah et al., 2016). However, the relationship between medial temporal lobe atrophy and FABP3 has never been assessed in a cognitively unimpaired population. Previous research has shown that cognitively normal individuals with amyloid pathology show steeper memory decline if they also have pathological hippocampal volumes (Bilgel et al., 2018; Burnham et al., 2016). Accordingly, we speculate that the key to understand why some biomarker groups with amyloid and/or tau positivity only showed age-expected memory decline is that these participants did not show higher than age-expected hippocampal atrophy. Although followed for up to 6.8 years, there were no differences between any of the biomarker groups in hippocampal volume trajectories. Differences would have been expected if the sample also had included participants showing cognitive impairment such as in Alzheimer's dementia. We propose that older adults may uphold age-expected cognitive function for many years, even when harboring pathological biomarker profiles, as long as hippocampal atrophy is within the age-expected range.

### 4.4. Strengths and limitations

The inclusion of cognitively unimpaired adults only, constitutes both a strength and a weakness, causing the sample probably to be more homogenous than the general population, which likely affected both the clustering of biomarkers and the clustering of participants. Thus, the conclusions drawn are valid for cognitively unimpaired older adults only—the clusters may be different if examined in populations of participants with MCI or dementia. Furthermore, it is possible that exclusion of the few individuals who progressed to MCI, dementia, and other neurodegenerative diseases during follow-up may in part have impacted the associations of clustering-based biomarker groups and NIA-AA defined biomarker groups to hippocampal volume and memory change, for example, the lack of associations between biomarker groups and hippocampal volume change and between NIA-AA defined groups and memory. Yet, if these individuals were included, the results would very much depend on the etiology and severity of decline of these individuals, and we believe the results we present will have a higher probability of being replicated. On the other hand, the sample consisted of surgical patients including some patients who had cancer surgery, which also may reduce the generalizability of the results, although the consequences of this is difficult to assess. Nevertheless, we did not find any differences in cancer morbidity between the biomarker groups. Strengths of our work include measurement of both established and novel biomarkers, and multiple longitudinal measures of hippocampal volume and memory over 6.8 years. We also used a data-driven method, rather than defined cut-off values, to assess relationships between biomarkers.

## 5. Conclusion

Here we show that CSF biomarkers of AD pathophysiology can be grouped in superordinate clusters, and that Aβ42 is the biomarker with the least connections to other established as well as more novel CSF biomarkers. Using a large collection of CSF biomarkers enabled us to identify subgroups of participants with different biomarker profiles. This clustering-based grouping of participants outperformed biomarker profiling based on the NIA-AA AD classification system in predicting memory change over 6.8 years. The analyses of changes in memory function further showed that older adults may uphold age-expected cognitive function and hippocampal integrity even when harboring abnormal biomarker profiles, such as tauopathy, underscoring the complex relationship between cognitive function, maintenance, resilience and brain health in aging (Stern et al., 2018). Understanding the conditions for maintained cognitive function in aging despite various types of brain changes will be a major task for future research.

## Disclosure statement

Dr Walhovd has given a lecture on lifespan changes in brain and cognition for Shire International GmbH (2015) and has served in an expert group for ILSI Europe, for both of which honoraria were paid. Dr Zetterberg has served at scientific advisory boards for Roche Diagnostics, CogRx, Samumed, and Wave and is a co-founder of

Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg. Dr Hansson has acquired research support (for the institution) from Roche, GE Healthcare, Biogen, AVID Radiopharmaceuticals, Fujirebio, and Euroimmun. In the past 2 years, he has received consultancy/speaker fees (paid to the institution) from Biogen, Roche, and Fujirebio. Dr Blennow has served as a consultant or at advisory boards for Alzheon, CogRx, Biogen, Lilly, Novartis, and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg. The other authors report no conflicts of interest.

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## Appendix A. Supplementary data

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