CSF Synaptic Biomarkers in the Preclinical Stage of Alzheimer Disease and Their Association With MRI and PET: A Cross-sectional Study

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Abstract

Objective: To determine whether CSF synaptic biomarkers are altered in the early preclinical stage of the Alzheimer’s continuum and associated with Alzheimer’s disease (AD) risk factors, primary pathology, and neurodegeneration markers.

Methods: Cross-sectional study in the ALFA+ cohort, comprising middle-aged cognitively unimpaired participants. CSF neurogranin and GAP-43 were measured using immunoassays and SNAP-25 and synaptotagmin-1 using immunoprecipitation mass spectrometry. AD CSF biomarkers Aβ42/40, p-tau and t-tau, and the neurodegeneration biomarker NfL were also measured. Participants underwent structural MRI, and fluorodeoxyglucose and Aβ PET imaging. General linear modeling was used to test the associations between CSF synaptic biomarkers and risk factors, Aβ pathology, tau pathology, and neurodegeneration markers.

Results: All CSF synaptic biomarkers increased with age. CSF neurogranin was higher in females, while CSF SNAP-25 was higher in APOE-ε4 carriers. All CSF synaptic biomarkers increased with higher Aβ load (as measured by CSF Aβ42/40 and Aβ PET Centiloid values) and, importantly, the synaptic biomarkers were increased even in individuals in the earliest stages of Aβ deposition. Higher CSF synaptic biomarkers were also associated with higher CSF p-tau and NfL. Higher CSF neurogranin and GAP-43 were significantly associated with higher brain metabolism, but lower cortical thickness in AD-related brain regions.

Conclusion: CSF synaptic biomarkers increase in early preclinical stages of the Alzheimer’s continuum even when a low burden of Aβ pathology is present, and they differ in their association with age, sex, APOE-ε4, and markers of neurodegeneration.
Introduction

Synaptic dysfunction is an early process in Alzheimer’s disease (AD) pathogenesis\(^1,2\), and pathological studies have shown that synapse loss is closely related to cognitive decline\(^3\). Therefore, it is crucial to further understand synaptic dysfunction occurring in early stages of AD.

Several synapse-specific proteins, involved in distinct synaptic pathways, can be measured in CSF. Among them, the most extensively studied include the postsynaptic protein neurogranin and presynaptic proteins synaptosomal-associated protein-25 (SNAP-25), growth-associated protein-43 (GAP-43) and synaptotagmin-1\(^4\). There is clear evidence of an increase of these CSF synaptic biomarkers in symptomatic AD patients (including prodromal AD and AD dementia)\(^5-14\). A recent study showed that these four CSF synaptic biomarkers accurately discriminate symptomatic AD from other dementias\(^6\). Nevertheless, less is known on the preclinical stage of the Alzheimer’s continuum.

Neurogranin is a postsynaptic protein highly expressed in the dendritic spines of hippocampus, amygdala, caudate and putamen, and it is involved in calcium signaling regulation and synaptic plasticity\(^15\). CSF neurogranin accurately differentiates AD, even at its prodromal phase, from healthy controls or other neurological diseases\(^5,8,16\). CSF neurogranin has also been investigated in the preclinical Alzheimer’s continuum, but with conflicting results. Whereas some studies demonstrated a significant increase in preclinical Alzheimer’s individuals compared with controls\(^17,18\), others did not observe this finding\(^19-21\).

CSF SNAP-25, GAP-43, and synaptotagmin-1 have been less investigated, but most studies show that they also increase in symptomatic AD\(^6,9-11,14\). SNAP-25 is a component of the SNAP receptor (SNARE) complex, which is located in the synaptic vesicles and is critical for the exocytosis process\(^22\). Two studies investigated CSF SNAP-25 in preclinical Alzheimer, and did not observe changes in this biomarker\(^19,23\). GAP-43 is a presynaptic protein mainly expressed in the hippocampus, entorhinal cortex, neocortex, cerebellum and olfactory bulb, and it is involved in synaptogenesis in the adult brain\(^24\). Two studies showed an increase of CSF GAP-43 in preclinical Alzheimer\(^25,26\). Finally, synaptotagmin-1 is a calcium sensor protein located in the presynaptic plasma membrane and participates in synaptic vesicles exocytosis and neurotransmitter release\(^27\). To our knowledge, synaptotagmin-1 has not been investigated in preclinical Alzheimer.

Overall, previous evidence on CSF synaptic biomarkers in preclinical Alzheimer is scarce and unclear. Studying preclinical Alzheimer is hampered by the difficulty of recruiting individuals at this early stage of the disease. The main aim of this study was to determine...
whether CSF synaptic biomarkers are altered in individuals at the preclinical stage of the Alzheimer’s continuum. We hypothesize that synaptic biomarkers levels will be altered in this early stage. Moreover, we sought to determine whether these synaptic biomarkers differ in their association with AD risk factors and neurodegeneration biomarkers.
Methods

Study participants

The ALFA (for ALzheimer’s and FAmilies) study\(^{28}\) (45-65/FPM2012 study) includes 2,743 middle-aged (45 - 74 years old) cognitively unimpaired individuals enriched for family history of AD (47.4%) and APOE-ε4 carriership (32.5%). The ALFA+ study is a nested longitudinal study that includes 450 participants that were invited to participate based on their specific AD risk profile, determined by an algorithm in which participants’ AD parental history and APOE status, verbal episodic memory score and CAIDE score were taken into consideration. A detailed phenotyping was performed in ALFA+ participants, including a lumbar puncture for the measurement of CSF biomarkers and imaging (MRI and PET) biomarkers acquisition\(^{28}\). ALFA+ inclusion criteria were: (i) individuals who had previously participated in the ALFA study; (ii) age between 45 and 65 years at the moment of the inclusion in ALFA; (iii) long-term commitment to the study: inclusion and follow-up visits and agreement to undergo all tests and study procedures (MRI, PET and lumbar puncture). ALFA+ exclusion criteria were: (i) cognitive impairment (Clinical Dementia Rating [CDR] > 0, Mini Mental State Examination [MMSE] < 27 or semantic fluency < 12); (ii) any significant systemic illness or unstable medical condition which could lead to difficulty complying with the protocol; (iii) any contraindication to any test or procedure; (iv) family history of monogenic AD. All the participants included in the study were visited and examined (lumbar puncture, MRI and PET imaging acquisition) between 2016 and 2019. At the moment of the present study, we included the 397 first consecutive ALFA+ participants with available CSF biomarkers.

CSF Aβ status was defined by the CSF Aβ42/40 ratio and participants were classified as CSF Aβ- Positive (A+) if CSF Aβ42/40 < 0.071. Participants were defined as CSF tau-positive (T+) if CSF p-tau > 24 pg/ml\(^{29}\). These cutoffs were previously derived using a two-Gaussian mixture modelling\(^{29}\). Each cutoff was defined as the mean plus 2 standard deviations (SD) of the non-pathologic Gaussian distribution.

In order to study very early changes in the continuum (when there are changes in soluble Aβ, as indicated by the CSF Aβ42/40 ratio, but not overt Aβ pathology, as indicated by Aβ PET), we defined the following three groups based on both CSF and PET Aβ status\(^{30}\), referred herein as CSF/PET Aβ status groups: (i) CSF/PET Aβ-negative (negative CSF Aβ42/40 and Aβ PET < 30 Centiloids), ii) group with low burden of Aβ pathology (positive CSF Aβ42/40 but Aβ PET < 30 Centiloids), and iii) CSF/PET Aβ-positive (positive CSF Aβ42/40 and Aβ PET ≥ 30 Centiloids).
CSF collection, processing, storage and biomarker measurements

CSF samples were obtained by lumbar puncture following standard procedures\textsuperscript{29,31}. Measurements of t-tau and p-tau were performed using the electrochemiluminescence Elecsys\textsuperscript{®} Total-tau CSF and Phospho-Tau(181P) CSF immunoassays on a fully automated cobas e 601 module (Roche Diagnostics International Ltd). CSF A\textsubscript{β}40, A\textsubscript{β}42, neurogranin, and neurofilament light chain (NFL) were measured with the exploratory Roche NeuroToolKit assays, a panel of automated robust prototype immunoassays (Roche Diagnostics International Ltd) on a cobas e 411 analyzer or cobas e 601 module\textsuperscript{29}.

CSF SNAP-25 and synaptotagmin-1 concentrations were measured by immunoprecipitation mass spectrometry following a previously established protocol\textsuperscript{6}. In particular, the longer soluble forms of SNAP-25 including at least amino acid 32-40 (SNAP-25aa40) were evaluated herein. CSF GAP-43 was measured by ELISA as previously described\textsuperscript{14}.

All the measurements were conducted at the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden, on coded and randomized samples. All analyses were performed using the same batch of reagents, using board-certified laboratory technicians (neurogranin and GAP-43) or by one of the co-authors, A. Brinkmalm (SNAP-25 and synaptotagmin-1).

\[^{18}F\]flutemetamol and \[^{18}F\]fluorodeoxyglucose PET acquisition and quantification

Participants underwent \[^{18}F\]flutemetamol (A\textsubscript{β}) and \[^{18}F\]fluorodeoxyglucose (FDG) PET scans following a cranial CT scan for attenuation correction on a Biograph mCT scanner (Siemens Healthcare, Erlangen, Germany) at Hospital Clínic, Barcelona. For A\textsubscript{β} PET scans, participants received an intravenous bolus dose of 185 MBq (range 104.25 – 218.3 MBq, mean ± SD: 191.75 ± 14.04) and, 90 min post-injection, PET data was acquired for 20 minutes (4 frames of 5 minutes each, mean ± SD: 90.15 ± 7.36 min). FDG PET scans were acquired for 20 minutes (4 frames of 5 minutes each) 45 min post-injection (mean ± SD: 45.69 ± 4.67) of 185 MBq (range 181.3 – 222 MBq, mean ± SD: 200.83 ± 12.83 MBq). PET images were reconstructed in 4 frames × 5 min using 3D Ordered Subset Expectation Maximization (OSEM) algorithm by incorporating time of flight (TOF) and point spread function (PSF) modelling.
Aβ PET processing was performed following a validated Centiloid pipeline using SPM12. Centiloid values were calculated from the mean values of the standard Centiloid target region using the transformation previously calibrated.

Quantification of FDG PET AD signature was performed by calculating the standard uptake value ratio (SUVr) within a meta-region of interest (meta-ROI composite). We used the same methodology as the one employed in a previous study, determined by identifying regions cited frequently in FDG PET studies of AD and MCI patients. This composite consists of five sub-regions including right and left angular gyri, middle/inferior temporal gyrus, and bilateral posterior cingulate gyrus. To this end, images were normalized into standard Montreal Neurological Institute (MNI) space using their corresponding MRI scans; the SPM12 algorithm and SUVr values were calculated as the ratio between the average FDG uptake in the meta-ROI voxels and those of the pons, as the reference region. SUVr values were calculated for the whole meta-ROI as well as for all of the individual regions.

MRI scans acquisition and quantification

MRI scans were obtained with a 3T scanner (Ingenia CX, Philips, Netherlands) at the neuroimaging unit at BarcelonàBeta Brain Research Center. The MRI protocol was identical for all participants and included a high-resolution 3D T1-weighted Turbo Field Echo (TFE) sequence (voxel size 0.75 × 0.75 × 0.75 mm³, TR/TE: 9.90/4.6 ms, flip angle = 8º).

T1-weighted images were automatically segmented and cortical thickness was measured in the regions from the Desikan-Killiany cortical atlas using Freesurfer version 6.0. Segmentation results were visually quality controlled by an expert. The cortical AD signature was then estimated for each subject based on the thickness of the following areas: entorhinal, inferior temporal, middle temporal, and fusiform. The signature was calculated as the mean thickness across these regions weighted by their surface area, as previously proposed. Cortical thickness was calculated for the whole signature, as well as for all the individual regions.

Quality control and visual assessment of MRI and PET scans

Quality controls of the T1-weighted MRI and Aβ and FDG PET images and ROI placements were carried out by specialists. A trained radiologist validated the image quality of MRI scans as well as incidental findings.
Statistical analysis

The normality of each biomarker distribution was assessed using visual inspection of the histogram and the Kolmogorov-Smirnov test. CSF p-tau, t-tau, NfL, neurogranin, SNAP-25, GAP-43, and synaptotagmin-1 did not follow a normal distribution and were log_{10}-transformed. The correlation between the four CSF synaptic biomarkers was analyzed using bivariate Spearman’s rank correlation coefficients test.

To assess the effect of demographic variables on CSF synaptic biomarkers, a linear regression model was performed with age, sex, years of education, and APOE-ε4 status as predictors variables. Associations between CSF synaptic biomarkers and age, Aβ pathology (CSF Aβ42/40 or Aβ PET Centiloid) or tau pathology (CSF p-tau) were tested using linear regression models adjusted by age and sex. The interaction terms ‘age × Aβ status’ or ‘Aβ biomarker × Aβ status’ were also added to each model. Moreover, we conducted multivariate analyses with both Aβ pathology and CSF p-tau as predictors in the model.

One-way analysis of covariance (ANCOVA) adjusting for the effect of age and sex was performed to compare CSF synaptic biomarker levels between CSF/PET Aβ-negative, low burden and CSF/PET Aβ-positive groups. Significant comparisons were followed by Dunnett-corrected post hoc pairwise comparisons, with the CSF/PET Aβ-negative group as the reference group.

Finally, we tested in a linear regression model the effect of CSF synaptic biomarkers on neurodegeneration biomarkers (CSF NfL and structural and functional neuroimaging variables), adjusting by age and sex. The interaction term between the neurodegeneration biomarkers and CSF Aβ status or, alternatively, CSF/PET Aβ status was also tested.

We performed stratified analyses within the Aβ status groups when significant interactions were identified. Yet, in the neuroimaging analyses, due to its exploratory nature and the fact that the expected effects were small, we performed stratified analyses regardless of the significance of interaction terms.

All tests were 2-tailed, with a significance level of \( \alpha = 0.05 \). A false discovery rate (FDR) multiple comparison correction was applied following the Benjamini-Hochberg procedure \(^{39}\) for all analyses. Statistical analyses were performed in SPSS IBM, version 20.0, statistical software and the open-source statistical software R. Figures were built using R.

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The ALFA+ study (ALFA-FPM-0311) was approved by the Independent Ethics Committee “Parc de Salut Mar”, Barcelona, and registered at Clinicaltrials.gov (Identifier: NCT02485730). All participating subjects signed the study’s informed consent form that had also been approved by the Independent Ethics Committee “Parc de Salut Mar”, Barcelona.

Data Availability

Due to participant’s privacy, individual level data cannot be made publicly available. Researchers who wish to use data of the ALFA study must obtain approval from the ALFA study Management Team.
Results

Participants’ characteristics and correlations between CSF synaptic biomarkers

397 ALFA+ participants with available CSF biomarkers were initially included in the study. Among the 397 subjects, 13 participants who were CSF Aβ-negative but tau-positive (i.e., non-AD pathologic change) and therefore not within the Alzheimer’s continuum, were excluded. Thus, 384 participants were finally included (Table 1). Among them, 327 (85.2%) participants had Aβ and FDG PET, and 365 (95.1%) had structural MRI with automatic segmentation available.

Participants’ characteristics and CSF biomarker levels are summarized in Table 1. CSF Aβ-positive participants were older and showed a higher prevalence of APOE-ε4 carriermship. As expected, Aβ PET Centiloid values and all the CSF biomarkers were significantly higher in the CSF Aβ-positive than in the CSF Aβ-negative group (Table 1). Still, the average Centiloid value for the CSF Aβ-positive group was of only 16.8 Centiloids, thus reinforcing the notion that Aβ-positive individuals in this cohort show minimal Aβ deposition in the brain.

CSF synaptic biomarkers were strongly correlated in the whole sample, as well as in CSF Aβ-negative and Aβ-positive participants when examined separately (Spearman rho: 0.66 – 0.92, P < 0.0001; eTable 1 and eFigure 1, https://doi.org/10.5061/dryad.vdncjsxv4).

Effect of demographic variables and main AD risk factors on CSF synaptic biomarkers

The effect of age, sex, APOE genotype, and years of education on the CSF synaptic biomarkers was tested. In the whole sample, CSF neurogranin, SNAP-25, GAP-43, and synaptotagmin-1 significantly increased with age (eTable 2, https://doi.org/10.5061/dryad.vdncjsxv4). This association was modified by CSF Aβ status for CSF neurogranin and a trend towards the same direction was seen for CSF GAP-43 (as shown by the ‘age x CSF Aβ status’ interaction term, Figure 1). In the stratified analyses, CSF neurogranin, GAP-43 and synaptotagmin-1 significantly increased with age only in CSF Aβ-positive participants. In contrast, CSF SNAP-25 showed a tendency to increase with age in the CSF Aβ-positive group but without reaching statistical significance (Figure 1).

CSF neurogranin was higher in females than in males (P = 0.021) and CSF SNAP-25 was higher in APOE-ε4 carriers than in non-carriers (P = 0.020). Education had a nominal
effect on CSF synaptotagmin-1 ($P = 0.035$), although it did not survive multiple comparisons correction (eTable 2, https://doi.org/10.5061/dryad.vdncjsxv4).

**Association with Aβ pathology and tau pathology**

Firstly, the CSF synaptic biomarkers association with Aβ pathology, as measured with CSF Aβ42/40, was analyzed. In the whole sample, higher levels of the four CSF synaptic biomarkers were significantly associated with higher Aβ pathology (i.e. lower CSF Aβ42/40; Figure 2). These associations were modified by CSF Aβ status (as shown by the significant ‘CSF Aβ42/40 × CSF Aβ status’ interaction term; Figure 2). When stratified by Aβ status according to CSF Aβ42/40 positivity, the four CSF synaptic biomarkers were positively associated with CSF Aβ42/40 in CSF Aβ-negative participants, and negatively associated with CSF Aβ42/40 in CSF Aβ-positive participants (Figure 2). The four CSF synaptic biomarkers also significantly increased as a function of Aβ PET Centiloids (eFigure 2).

To test whether the CSF biomarkers are altered early in the Alzheimer’s continuum, their levels were evaluated in the group of participants with low burden of Aβ pathology (eTable 3 for demographics of the groups compared, https://doi.org/10.5061/dryad.vdncjsxv4). This group was defined using a combination of CSF and PET Aβ (CSF/PET Aβ status), namely those participants with a positive CSF Aβ42/40 but less than 30 Centiloids in Aβ PET. Importantly, it was observed that CSF neurogranin, SNAP-25, GAP-43, and synaptotagmin-1 were all significantly increased in the low burden group (n = 89), and in the CSF/PET Aβ-positive group (n = 26), compared to the CSF/PET Aβ-negative one (n = 212; Figure 3).

Next, the association of CSF synaptic biomarkers with tau pathology, as measured by CSF p-tau, was analyzed. Similar to Aβ pathology results, higher levels of CSF synaptic biomarkers were significantly associated with increased CSF p-tau (CSF neurogranin: $\beta = +0.94$; CSF SNAP-25: $\beta = +0.79$; CSF GAP-43: $\beta = +0.96$; CSF synaptotagmin-1: $\beta = +0.93$; $P < 0.0001$ for all analyses). We additionally performed multivariate analyses including as predictors both Aβ pathology biomarkers (CSF Aβ42/40 or Aβ PET) and CSF p-tau. The main effect of CSF p-tau on the four CSF synaptic biomarkers remained significant when accounting by the effect of Aβ pathology biomarkers ($P < 0.0001$ for all analyses). In contrast, the significant increase of CSF synaptic biomarkers as a function of higher Aβ pathology was lost after accounting for CSF p-tau. These results suggest that the effect of Aβ pathology in the CSF synaptic biomarkers is dependent of tau pathology. Using CSF t-tau as a predictor instead of CSF p-tau yielded similar results.
Finally, associations between CSF synaptic biomarkers and neurodegeneration biomarkers, including CSF NfL and brain metabolism and cortical thickness in specific AD-related brain regions, were studied.

The four CSF synaptic biomarkers were positively associated with CSF NfL ($P < 0.0001$ for all associations), and these associations were not modified by CSF or CSF/PET Aβ status, as shown by the not statistically significant ‘CSF synaptic biomarker × CSF Aβ status’ or ‘CSF synaptic biomarker × CSF/PET Aβ status’ interaction terms (eTable 4, https://doi.org/10.5061/dryad.vdncjsxv4).

In relation to neuroimaging neurodegeneration outcomes, CSF neurogranin and GAP-43 were significantly and positively associated with brain metabolism in the FDG PET AD signature (Table 2 and Figure 4). These associations were not modified by CSF or CSF/PET Aβ status (Table 2 and eTable 5, https://doi.org/10.5061/dryad.vdncjsxv4). After stratifying by CSF Aβ status, a significant association for CSF neurogranin in the CSF Aβ-positive group and a trend to a significant result for GAP-43 was observed (Table 2). After stratifying by CSF/PET Aβ status, we observed the same result in the low burden group, but not in the CSF/PET Aβ-positive group (eTable 5 and eFigure 3, https://doi.org/10.5061/dryad.vdncjsxv4). However, the results of the stratified analyses did not survive FDR multiple comparison correction and should be interpreted cautiously.

Analyses of the individual regions that compose the FDG PET AD signature in the whole sample showed that the positive association of CSF neurogranin and GAP-43 with brain metabolism occurred mainly in angular and temporal regions (eTable 6, https://doi.org/10.5061/dryad.vdncjsxv4).

In the MRI AD signature analyses in the whole sample, higher CSF neurogranin and GAP-43 were associated with lower cortical thickness (Table 2 and Figure 5). Higher CSF synaptotagmin-1 was also nominally associated with lower cortical thickness ($P = 0.047$), but it did not survive FDR multiple comparison correction. Similar to the FDG PET analyses, these associations were not modified by CSF Aβ status or CSF/PET Aβ status (Table 2 and eTable 7, https://doi.org/10.5061/dryad.vdncjsxv4). In the stratified sample by CSF Aβ status, the negative association with cortical thickness was significant in CSF Aβ-positive participants for CSF GAP-43, while a trend in the same direction was observed for CSF neurogranin ($P = 0.060$; Table 2 and Figure 5). After stratifying by CSF/PET Aβ status, the
same result was observed in the CSF/PET Aβ-positive group, although it did not survive FDR correction (eTable7 and eFigure 4, https://doi.org/10.5061/dryad.vdncjsxv4).

Individual region analyses showed that these significant negative associations between CSF neurogranin and GAP-43 and cortical thickness occurred in entorhinal and middle-temporal areas (eTable 8, https://doi.org/10.5061/dryad.vdncjsxv4).
Discussion

The aim of our study was to investigate CSF synaptic biomarkers in the preclinical stage of the Alzheimer’s continuity. Firstly, synaptic biomarkers were found to be altered early in the Alzheimer’s continuum, as shown by their increase (i) as a function of higher Aβ pathology, and (ii) in individuals with low burden of Aβ pathology. Secondly, CSF neurogranin, SNAP-25, GAP-43, and synaptotagmin-1 had some specific features: (i) CSF neurogranin was higher in females, (ii) APOE-ε4 genotype had an effect on SNAP-25, (iii) CSF neurogranin and GAP-43 were associated with changes in brain metabolism and structure.

Despite the evidences of altered CSF synaptic biomarkers in symptomatic AD, studies in the preclinical stage of the Alzheimer’s continuum are sparse and generally include reduced numbers of participants. We demonstrate that CSF neurogranin, SNAP-25, GAP-43, and synaptotagmin-1 are increased during this stage. These results are consistent with those that previously showed an increased in CSF neurogranin and CSF GAP-43 in preclinical AD. Yet, other studies could not find a significant increase in CSF neurogranin or SNAP-25 at this stage. This may be explained by a lack of power since these studies included relatively few preclinical Alzheimer’s individuals (less than 50), whereas 135 individuals were included in this study. Recently, a study in BioFINDER showed that CSF neurogranin, SNAP-25, and GAP-43 significantly increased in A+T- individuals compared to A-T-, also suggesting an early increase of CSF synaptic biomarkers. The current study differs in the fact that all individuals included were CU and, therefore, the preclinical stage of the Alzheimer’s continuum could be specifically studied. Moreover, the CSF presynaptic protein synaptotagmin-1 was also analyzed.

CSF synaptic biomarkers are associated with Aβ pathology in symptomatic individuals. In preclinical AD, CSF neurogranin correlates with mean cortical binding potential on Aβ PET in Aβ-positive, cognitively normal controls. In the BioFINDER cohort, CSF neurogranin, but not CSF SNAP-25 or GAP-43, was associated with Aβ PET in A-T- and A+T- individuals. In the current study, the association between four different CSF synaptic biomarkers and Aβ pathology (as measured by CSF Aβ42/40 and Aβ PET) was confirmed in a larger cohort of preclinical Alzheimer’s individuals. This association appeared to be mainly driven by the presence of tau pathology, as the effect of Aβ was lost when accounting for CSF p-tau. Importantly, all CSF synaptic biomarkers were increased among participants with a low burden of Aβ pathology. In other words, as soon as there are changes in soluble Aβ and p-tau, and before there is overt Aβ pathology, there are...
observable changes in CSF synaptic biomarkers. These results suggest a very early role of synaptic dysfunction in AD pathogenesis.

An important open question is whether the different CSF synaptic biomarkers reflect different mechanistic aspects of synaptic dysfunction in AD and neurodegeneration. This is an important question because, if they all provide similar information on the mechanisms of the disease, it would be reasonable to choose only one as a routine CSF synaptic biomarker. It is beyond the scope of this observational study to investigate possible differences on the mechanistic information that each biomarker provides. Nonetheless, this study assessed whether these CSF synaptic biomarkers differ in their association with age, sex, and APOE-ε4 and neurodegeneration biomarkers. CSF neurogranin, GAP-43, and synaptotagmin-1, but not SNAP-25, significantly increased throughout age in the Aβ-positive group. Sex differences were observed only in CSF neurogranin, with females having higher levels than males. Higher levels of CSF neurogranin in females have been previously found\(^{17,20,43}\). Interestingly, CSF neurogranin was also found to be associated with AD-related atrophy only in females\(^{44}\). Whether these findings reflect a higher susceptibility to dendritic dysfunction in females need further investigation. CSF SNAP-25 was higher in APOE-ε4 carriers, a result that had previously been reported in MCI and dementia patients\(^{6,41}\), but not in cognitively normal individuals. Importantly, all CSF synaptic biomarkers were strongly associated with the neurodegeneration biomarker CSF NfL, regardless of Aβ status, which suggests that these CSF synaptic biomarkers may be also affected by other factors that need to be further studied (e.g. vascular factors, co-pathology, other sources of neuronal injury).

Finally, our study assessed whether CSF synaptic biomarkers were associated to imaging biomarkers of neurodegeneration, namely FDG PET uptake and cortical thickness in AD specific areas. Interestingly, both CSF neurogranin and GAP-43 were positively associated with brain glucose metabolism, whilst negatively associated with cortical thickness in the studied AD signatures. Previous studies have shown that baseline CSF neurogranin levels predict a longitudinal decrease in FDG PET brain metabolism in MCI and AD patients\(^{21}\) and, in a more recent study, in CU individuals\(^{17}\). In our cross-sectional study, the association between CSF neurogranin and GAP-43 and brain glucose metabolism may reflect synaptic dysfunction in very initial stages of the disease, when there may be brain metabolic increases in response to early Aβ pathology. In fact, there is a trend suggesting these associations are driven by Aβ-positive participants, although the analysis did not survive multiple comparison correction. Despite the fact that AD is typically characterized by glucose hypometabolism, previous studies have found an hypermetabolism in some brain areas in Aβ-positive individuals in early stages of the disease\(^{45-47}\). It may be argued that the
CSF neurogranin and GAP-43 association with glucose hypermetabolism found herein reflect this early stage. In contrast to FDG PET brain metabolism, higher levels CSF neurogranin and GAP-43 were associated with lower cortical thickness in the AD signature metacomposite. Previous literature showed that higher CSF neurogranin levels were associated with brain atrophy in symptomatic AD\textsuperscript{21,42}. In preclinical Alzheimer, CSF neurogranin levels were associated with cortical thinning in the left caudal middle frontal gyrus and right precuneus\textsuperscript{48}. As far as we know, no earlier studies have assessed the association of CSF GAP-43, SNAP-25, or synaptotagmin-1 with brain metabolism or structure in preclinical Alzheimer. The fact that CSF neurogranin and GAP-43 are the two biomarkers significantly associated with brain FDG PET and cortical thickness measures might suggest they reflect synaptic dysfunction more closely related to brain metabolic and structural changes.

Our results also have some practical implications in the design of therapeutic interventions in preclinical stages of the disease. The CSF synaptic biomarkers could potentially be used to measure target engagement in clinical trials testing drugs that target synaptic dysfunction, assess safety (whether drugs cause synaptic toxicity) or, considering their association with markers of neurodegeneration, as a secondary outcome measure. However, in order to determine whether CSF synaptic biomarkers are also useful to stage the disease, studies that include the whole \textit{continuum} of the disease are needed. Also, longitudinal studies are needed to determine whether they have prognostic value.

These findings should be considered in the light of some limitations. First, this is a cross-sectional study, and thus no statement can be made regarding biomarker trajectories over time. Second, there are other CSF synaptic biomarkers that were not included (e.g., SV2A, NPX2). Third, PET measures of tau pathology (tau PET) or synaptic density (e.g., SV2A PET) were not available.

Nevertheless, this study has significant strengths. First, four different CSF synaptic biomarkers were analyzed in the same cohort, while most reports include single biomarkers. Second, a very well characterized cohort of cognitively unimpaired individuals was studied, of whom 135 were Aβ-positive and therefore fall into in the preclinical stage of the Alzheimer’s \textit{continuum}. This is in striking contrast with previous studies, which include a lower number of preclinical Alzheimer’s individuals. Third, this is a multimodal study in which the association of CSF synaptic biomarkers with biomarkers of Aβ pathology and with FDG PET brain metabolism and structural MRI AD signatures were assessed.
Overall, these results suggest that the four CSF synaptic biomarkers studied are increased early in the preclinical stage of the Alzheimer’s continuum, and might reflect different aspects of synaptic dysfunction at this stage, differentially associating with AD risk factors, pathology, and neurodegeneration outcomes. These biomarkers should be considered in both observational and interventional future studies in preclinical Alzheimer.
<table>
<thead>
<tr>
<th>Name</th>
<th>Location</th>
<th>Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marta Milà-Alomà, MSc</td>
<td>BarcelonaBeta Brain Research Center, Barcelona, Spain</td>
<td>Contributed in the study design and conception of the presented idea. Analyzed and interpreted the data. Drafted the manuscript.</td>
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<td>PhD</td>
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<tr>
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<tr>
<td>Name</td>
<td>Affiliation</td>
<td>Contributions</td>
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</tr>
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</tr>
<tr>
<td>Name</td>
<td>Location</td>
<td>Role</td>
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</tr>
<tr>
<td>José Luis Molinuevo, MD, PhD</td>
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<td>Medical Advisor</td>
</tr>
<tr>
<td>Raffaele Cacciaglia, PhD</td>
<td>BarcelonaBeta Brain Research Center, Barcelona, Spain</td>
<td>Site investigator</td>
</tr>
</tbody>
</table>

**APPENDIX 2-Coinvestigators**
<table>
<thead>
<tr>
<th>Name</th>
<th>Affiliation</th>
<th>Role</th>
<th>Responsibilities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alba Cañas, Ms</td>
<td>Barcelona Brain Research Center, Barcelona, Spain</td>
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</tr>
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<td>Carme Deulofeu, PhD</td>
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<td>Senior Clinical Data Manager</td>
<td>Coordinated and supervised the data systems and networks</td>
</tr>
<tr>
<td>Irene Cumplido, MsC</td>
<td>Barcelona Brain Research Center, Barcelona, Spain</td>
<td>Site investigator</td>
<td>Performed imaging pipelines</td>
</tr>
<tr>
<td>Ruth Dominguez, Ms</td>
<td>Barcelona Brain Research Center, Barcelona, Spain</td>
<td>Clinical research nurse</td>
<td>Performed clinical visits</td>
</tr>
<tr>
<td>Maria Emilio, Ms</td>
<td>Barcelona Brain Research Center, Barcelona, Spain</td>
<td>Clinical research nurse</td>
<td>Performed clinical visits</td>
</tr>
<tr>
<td>Sherezade Fuentes, Ms</td>
<td>Barcelona Brain Research Center, Barcelona, Spain</td>
<td>Data manager</td>
<td>Performed quality control and data</td>
</tr>
<tr>
<td>Laura Hernandez, Ms</td>
<td>Barcelona Brain Research Center, Barcelona, Spain</td>
<td>Clinical research nurse</td>
<td>Performed clinical visits</td>
</tr>
<tr>
<td>Gema Huesa, PhD</td>
<td>Barcelona Brain Research Center, Barcelona, Spain</td>
<td>Clinical data manager</td>
<td>Performed and coordinated data entry, storage and organization</td>
</tr>
<tr>
<td>Jordi Huguet, PhD</td>
<td>Barcelona Brain Research Center, Barcelona, Spain</td>
<td>IT Neuroimaging specialist</td>
<td>Performed data entry, storage and organization, performed imaging pipelines</td>
</tr>
<tr>
<td>Paula Marne, Ms</td>
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<td>Neuropsychologist</td>
<td>Performed clinical visits</td>
</tr>
<tr>
<td>Tania Menchón, Ms</td>
<td>Barcelona Brain Research Center, Barcelona, Spain</td>
<td>Nurse Coordinator</td>
<td>Coordinated and performed clinical visits</td>
</tr>
<tr>
<td>Albina Polo, MD</td>
<td>Barcelona Brain Research Center, Barcelona, Spain</td>
<td>Medical Advisor</td>
<td>Advised/reviewed the clinical protocol, performed clinical visits</td>
</tr>
<tr>
<td>Sandra Pradas, Ms</td>
<td>Barcelona Brain Research Center, Barcelona, Spain</td>
<td>Nurse</td>
<td>Performed clinical visits</td>
</tr>
<tr>
<td>Anna Soteras, Ms</td>
<td>Barcelona Brain Research Center, Barcelona, Spain</td>
<td>Clinical Study Coordinator</td>
<td>Coordinated clinical visits</td>
</tr>
<tr>
<td>Marc Vilanova,</td>
<td>Barcelona Brain Research Center, Barcelona, Spain</td>
<td>MRI</td>
<td>Supervised and performed MRI</td>
</tr>
</tbody>
</table>
References


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### Table 1. Participants’ characteristics and CSF synaptic biomarkers in the whole sample and stratified by CSF Aβ status

<table>
<thead>
<tr>
<th></th>
<th>Total (n = 384)</th>
<th>CSF Aβ-negative (n = 249, 64.8%)</th>
<th>CSF Aβ-positive (n = 135, 35.2%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>61.1 (4.68)</td>
<td>60.5 (4.45)</td>
<td>62.2 (4.91)</td>
<td>0.0006</td>
</tr>
<tr>
<td>Females, n (%)</td>
<td>234 (60.9)</td>
<td>153 (61.4)</td>
<td>81 (60.0)</td>
<td>0.87</td>
</tr>
<tr>
<td>Education, years</td>
<td>13.5 (3.53)</td>
<td>13.6 (3.47)</td>
<td>13.3 (3.64)</td>
<td>0.49</td>
</tr>
<tr>
<td>APOE-ɛ4 carriers, n (%)</td>
<td>209 (54.4)</td>
<td>106 (42.6)</td>
<td>103 (76.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MMSE</td>
<td>29.2 (0.94)</td>
<td>29.1 (0.92)</td>
<td>29.1 (0.99)</td>
<td>0.93</td>
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<tr>
<td>Aβ PET Centiloids</td>
<td>2.95 (16.9)</td>
<td>-4.54 (6.59)</td>
<td>16.8 (21.1)</td>
<td>&lt;0.0001</td>
</tr>
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</table>

CSF biomarkers

<table>
<thead>
<tr>
<th></th>
<th>Total (n = 384)</th>
<th>CSF Aβ-negative (n = 249, 64.8%)</th>
<th>CSF Aβ-positive (n = 135, 35.2%)</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Aβ42/40</td>
<td>0.074 (0.019)</td>
<td>0.087 (0.009)</td>
<td>0.051 (0.012)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>p-tau (pg/ml)</td>
<td>15.4 (5.84)</td>
<td>13.9 (4.19)</td>
<td>18.4 (7.21)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>t-tau (pg/ml)</td>
<td>191 (63.8)</td>
<td>175 (48.0)</td>
<td>223 (76.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NFL (pg/ml)</td>
<td>80.8 (25.7)</td>
<td>76.3 (23.6)</td>
<td>89.2 (27.5)</td>
<td>0.0002</td>
</tr>
<tr>
<td>neurogranin (pg/ml)</td>
<td>773 (299)</td>
<td>715 (247)</td>
<td>882 (353)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SNAP-25 (pM)</td>
<td>21.5 (2.93)</td>
<td>20.9 (2.60)</td>
<td>22.5 (3.33)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GAP-43 (pg/ml)</td>
<td>2715 (1007)</td>
<td>2535 (912)</td>
<td>3053 (1090)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>synaptotagmin-1 (pM)</td>
<td>50.7 (11.5)</td>
<td>48.9 (10.4)</td>
<td>54.3 (12.7)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Data are expressed as mean (M) and standard deviation (SD) or number of participants (n) and percentage (%), as appropriate. CSF Aβ status was defined by the CSF Aβ42/40 ratio and participants were classified as CSF Aβ-positive if CSF Aβ42/40 < 0.071. P-values were computed with a t-test for age, education and MMSE; with a Chi square for sex and APOE genotype; and with an ANCOVA adjusted for age and sex for Centiloids and CSF biomarkers. Significant P-values are shown in bold. Abbreviations: Aβ, amyloid-β; GAP-43, growth-associated protein-43; MMSE, Mini Mental State Examination; NFL, neurofilament light; p-tau, phosphorylated tau; SNAP-25, synaptosomal-associated protein-25; t-tau, total tau.
Table 2. Effect of CSF synaptic biomarkers on FDG PET and MRI AD signatures in the whole sample and by CSF Aβ status

<table>
<thead>
<tr>
<th></th>
<th>FDG PET AD signature</th>
<th>MRI AD signature</th>
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<tr>
<td></td>
<td>Whole sample</td>
<td>CSF Aβ-negative</td>
</tr>
<tr>
<td></td>
<td>β (SE)</td>
<td>P-value</td>
</tr>
<tr>
<td>neurogranin</td>
<td>0.15 (0.055)</td>
<td>0.006</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>0.062 (0.056)</td>
<td>0.27</td>
</tr>
<tr>
<td>GAP-43</td>
<td>0.15 (0.056)</td>
<td>0.009</td>
</tr>
<tr>
<td>synaptotagmin-1</td>
<td>0.085 (0.056)</td>
<td>0.13</td>
</tr>
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</table>

The associations between each CSF synaptic biomarker and FDG PET uptake (SUVRr) or cortical thickness (mm) were tested in a linear model in the whole sample and stratified by CSF Aβ status. CSF Aβ status was defined by the CSF Aβ42/40 ratio and participants were classified as CSF Aβ-positive if CSF Aβ42/40 < 0.071. The interaction term between CSF Aβ status and each synaptic biomarker was also added in the model to test whether the regression slopes were significantly different between both groups. All analyses were adjusted for age and sex. The standardized regression coefficients (β) and standard errors (SE) are depicted. All P-values are corrected for multiple comparisons using the FDR approach. Significant P-values are shown in bold. Abbreviations: Adj, FDR-adjusted; GAP-43, growth-associated protein-43; SNAP-25, synaptosomal-associated protein-25.
Figure 1.

Title: Association of CSF synaptic biomarkers with age.

Scatter plots representing the association of each of the CSF synaptic biomarkers with age in the CSF Aβ-negative (A-; blue) and the Aβ-positive (A+; red) groups. CSF Aβ status was defined by the CSF Aβ42/40 ratio and participants were classified as CSF Aβ-positive if CSF Aβ42/40 < 0.071. Each point depicts the value of the CSF biomarker of an individual and the solid lines indicate the regression line for each of the groups. The standardized regression coefficients (β) and the P-values are shown and were computed using a linear model adjusting for sex. Additionally, the ‘age × CSF Aβ status’ interaction term was also computed. All P-values are corrected for multiple comparisons using the FDR approach.

Figure 2.

Title: Association of CSF synaptic biomarkers with CSF Aβ pathology.
Scatterplots representing the association of each CSF synaptic biomarker with CSF Aβ42/40 in the CSF Aβ-negative (A−; blue) and Aβ-positive (A+; red) groups. Each point depicts the value of the CSF biomarker of an individual and the solid lines indicate the regression line for each of the groups. The x-axis is reversed. The vertical dashed line indicates the cutoff for CSF Aβ42/40 positivity of 0.071. The standardized regression coefficients (β) and the P-values for the total and stratified analyses are shown and were computed using a linear model adjusting for age and sex. Additionally, the ‘CSF Aβ42/40 × CSF Aβ status’ interaction term was also computed. All P-values are corrected for multiple comparisons using the FDR approach. Abbreviations: Aβ, amyloid-β; GAP-43, growth-associated protein-43; SNAP-25, synaptosomal-associated protein-25.
Title: CSF synaptic biomarkers by CSF/PET Aβ status

Boxplots depicting the median (horizontal bar), interquartile range (IQR hinges) and 1.5 IQR (whiskers). Group differences were assessed by a one-way analysis of covariance (ANCOVA) adjusted by age and sex, followed by Dunnett-corrected *post hoc* pair-wise comparisons. The percentage of change between the low burden group and the CSF/PET Aβ-negative group is shown. eTable 3 in Supplementary data shows the demographics of the three groups compared. All *P*-values are corrected for multiple comparisons using the FDR approach. Abbreviations: Aβ, amyloid-β; GAP-43, growth-associated protein-43; SNAP-25, synaptosomal-associated protein-25.
Title: Association of CSF synaptic biomarkers with FDG PET AD signature.

Scatter plots representing the association of each of the CSF synaptic biomarker with FDG PET uptake (SUVRr) AD signature in the CSF Aβ-negative (A-; blue) and the CSF Aβ-positive (A+; red) groups. CSF Aβ status was defined by the CSF Aβ42/40 ratio and participants were classified as CSF Aβ-positive if CSF Aβ42/40 < 0.071. Each point depicts the value of the CSF biomarker of an individual and the solid lines indicate the regression line for each of the groups. The associations were computed using a linear model adjusting for age and sex.

Abbreviations: Aβ, amyloid-β; GAP-43, growth-associated protein-43; SNAP-25, synaptosomal-associated protein-25; SUVRr, Standardized Uptake Value Ratio.
Figure 5.

Title: Association of CSF synaptic biomarkers with cortical MRI AD signature.

Scatter plots representing the association of each of the CSF synaptic biomarker with the cortical thickness (mm) AD signature in the CSF Aβ-negative (A-; blue) and the CSF Aβ-positive (A+; red) groups. CSF Aβ status was defined by the CSF Aβ42/40 ratio and participants were classified as CSF Aβ-positive if CSF Aβ42/40 < 0.071. Each point depicts the value of the CSF biomarker of an individual and the solid lines indicate the regression line for each of the groups. The associations were computed using a linear model adjusting for age and sex.

Abbreviations: Aβ, amyloid-β; GAP-43, growth-associated protein-43; SNAP-25, synaptosomal-associated protein-25
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