Dissection of synaptic pathways through the CSF biomarkers for predicting Alzheimer's disease

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One Sentence Summary:

Multiple pre- and postsynaptic biomarkers were specifically increased in patients with Alzheimer's disease (AD) brain lesions including mild cognitive impairment (MCI) compared to controls and non-AD dementia. The levels of these synaptic biomarkers were affected by apolipoprotein E genotype. These new biomarkers could be useful for clinical differential diagnosis, for monitoring drug effects on synaptic functioning in clinical trials, and for understanding the link between the *APOE* gene and synaptic vulnerability.

Abstract

Objective: To assess the ability of a combination of synaptic CSF biomarkers to separate AD and non-AD disorders and to help in the differential diagnosis between neurocognitive diseases.

Methods: Retrospective cross-sectional monocentric study. All participants explored with CSF assessments for neurocognitive decline were invited to participate. After complete clinical and imaging evaluations, 243 patients were included. CSF synaptic (GAP-43, neurogranin, SNAP-25 total, SNAP-25 aa40, synaptotagmin-1) and AD biomarkers were blindly quantified using ELISA or mass spectrometry. Statistical analysis compared CSF levels between various groups AD dementias n=81, MCI-AD n=30, other MCI n=49, other dementias (OD) n=49, neurological controls n=35) as well as their discriminatory powers.

Results: All synaptic biomarkers were significantly increased in MCI-AD and AD -dementias patients compared to other groups. All synaptic biomarkers could efficiently discriminate AD dementias from OD (AUC ≥0.80). All but synaptotagmin were also able to discriminate MCI-AD from controls (AUC ≥0.85) and AD dementias from controls (AUC ≥0.80). Overall, CSF SNAP 25aa40 had the highest discriminative power (AUC=0.93) between AD dementias and controls or OD, and AUC=0.90 between MCI-AD and controls. Higher levels were associated with two alleles of apolipoprotein E (APOE) ε4.

Conclusion: All synaptic biomarkers tested had a good discriminatory power to distinguish patients with AD abnormal CSF from non-AD disorders. SNAP25aa40 demonstrated the highest power to discriminate AD CSF positive patients from non-AD patients and neurological controls in this cohort.

Classification of evidence: This retrospective study provides Class II evidence that CSF synaptic biomarkers discriminate patients with AD from non-AD patients.

Introduction

Alzheimer's disease (AD) is characterized pathologically by the accumulation of extracellular aggregation of amyloid-β (Aβ), intraneuronal hyperphosphorylated tau and synaptic and neuronal loss (*1*). According to the amyloid cascade hypothesis, the production of toxic Aβ could lead to altered kinase activities inducing tau phosphorylation, neuroinflammation and neurodegeneration with synaptic disintegration leading to cognitive decline (*2*). The abnormal accumulation of these proteins may occur several years before the first cognitive symptoms (*3*) and is accompanied by synaptic degeneration (*4-10*) (*11*). Several cerebrospinal fluid (CSF) biomarkers are now available, including total tau (Tau) and tau phosphorylated on threonine 181 (p-Tau181), reflecting neurodegeneration and tau pathology, respectively, and Aβ1-42 reflecting abnormal metabolism and deposition of the peptide into amyloid plaques (*12, 13*). Numerous studies have consistently shown a marked increase of CSF Tau and p-Tau181 accompanied by a reduction of Aβ1-42 levels in AD and mild cognitive impairment (MCI) due to AD (*12, 14*). These biomarkers help to carry out early AD diagnosis and are currently used in clinical research and also in daily clinical practice in a number of countries (*15*) (*16*). However, these three biomarkers do not give information on synaptic dysfunction or degeneration in the affected patients.

Pre- and post-synaptic protein expression levels are reduced in post mortem AD brains, and is an early neuropathological feature of the disease, and synaptic damage is increasingly recognized as a core feature of AD pathophysiology (*6-10, 17-19*). Synaptic proteins have been detected in the CSF of patients and controls (*20*). Therefore, synaptic biomarkers have been recently evaluated in MCI and AD, to assess synaptic dysfunction and degeneration (see below). Recent reports have explored CSF neurogranin (Ng), Synaptosomal-associated protein 25 (SNAP-25), growth-associated protein 43 (GAP-43) and synaptotagmin-1 levels in MCI and AD patients. The CSF concentrations of these proteins reflect pre-synaptic and post-synaptic dysfunction.

GAP-43, or neuromodulin, is a protein localized in pre-synaptic terminals and axons of cortical neurons. GAP-43 is involved in the maintenance of synapses and in neuritic regeneration (*21, 22*). In AD brains, GAP-43 levels are significantly reduced and correlate positively with senile plaque load and negatively with the duration of dementia (*11, 23, 24*). GAP-43 is also found in the dystrophic neurites surrounding plaques (*11, 25*) and is secreted into human CSF (*20*). Studies assessing GAP-43 levels in AD CSF have found correlations with tau levels, but studies so far have not settled whether there is a change in AD as compared to controls and to other

dementias (*23, 24*). Thus, further studies are needed to clarify the potential value of GAP43 as a diagnostic marker in AD.

SNAP-25 has 2 isoforms, with SNAP25A being expressed in embryonic tissues while SNAP25B is expressed in adult neural tissue (*26*). It is an essential pre-synaptic component of the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) complex and is a crucial protein for synaptic functions as these proteins initiate the fusion of synaptic vesicles (*27*). The formation of this protein scaffold is a fundamental step in neurotransmitter release and its modification could alter the exocytosis of neurotransmitters (*28-30*). In addition to the central function of SNAP-25 in the regulation of neurotransmitter release, new studies have suggested a postsynaptic role in receptor trafficking, spine morphogenesis and plasticity (*31*, *32*). We have previously shown that SNAP-25 is a promising AD CSF biomarker using an approach of affinity purification and mass spectrometry (*33*). With this approach all soluble forms of SNAP-25 (SNAP-25tot), as well as the longer soluble forms including at least amino acid 32-40 (SNAP-25aa40). The same approach has recently been used to investigate changes in CSF levels of both SNAP-25tot and SNAP-25aa40 in patients after radiotherapy (*34*)

Synaptotagmin-1, designated synaptotagmin in this paper, is a pre-synaptic calcium sensor indispensable for exocytosis of synaptic vesicles participating in neurotransmitter release in hippocampal neurons (*35-38*). Efficient sustained neurotransmitter release is also dependent on reformation of synaptic vesicles after stimulation by endocytosis in which synaptotagmin works as an essential vesicle cargo molecule (*38*). The major function of synaptotagmin is synaptic transmission (*38-40*) suggesting that it is a biomarker candidate reflecting synaptic decline in AD. Indeed, synaptotagmin, was detected in the CSF for the first time using a procedure based on affinity chromatography, reversed-phase chromatography, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and chemiluminescence immunoblotting (*41*). Several studies have found a marked reduction of brain synaptotagmin levels in cortical brain regions in AD (*41-43*) and a co-localization of synaptotagmin with neuritic plaques (*17*). We have previously shown that CSF synaptotagmin levels are increased in AD (*41*), but further studies are needed to validate this finding.

Ng is a post-synaptic protein highly expressed in the grey matter of brain regions that are affected in AD (cerebral cortex, hippocampus, amygdala) while it is almost absent in the thalamus, cerebellum, brainstem and spinal cord (*43, 44*). This synaptic protein is primarily found in

excitatory neurons and is concentrated in distal dendrites and dendritic spines (*45*). Ng is involved in synaptic plasticity and in memory consolidation (*46*) . In AD brains, a marked reduction of Ng levels was found in the hippocampus and the frontal cortex (*43*). Several studies have shown that CSF Ng levels are increased in AD compared to controls (*47-50*) (*51*). High CSF levels predict future rate of cognitive decline (*47, 52*) and are associated with brain atrophy (*50*). CSF Ng concentrations predict the presence of prodromal AD in MCI (*49, 50, 52-54*) and are also associated with CSF Tau levels. The correlation of CSF Ng and Aβ levels are inconsistent in various studies (*47, 48, 50, 51, 55-57*). In addition, these studies suggest that increased CSF Ng levels are early pathophysiological markers of AD related to synaptic loss.

Using a large cohort, we set out to investigate the usefulness of the described pre-synaptic and post-synaptic CSF biomarkers, evaluated simultaneously as combined biomarkers in AD dementia and in MCI due to AD. We have also performed an assessment of their concentrations on diagnostic performance, as well as a possible association with apolipoprotein E (*APOE*) genotype. Our synaptic approach may give a combination of new promising biomarkers that will help early detection of neurodegeneration and improve differential diagnosis of neurocognitive disorders.

Results

CSF concentrations in all groups

The characteristics of the study population are shown in Table 1. The results of all biomarkers are presented in Figure 1 and in table 2.

For all studied synaptic biomarkers, a clear significant difference was detected between the following groups: AD versus controls ($p<0.001$), AD versus other dementia (OD) ($p<0.001$), AD versus MCI not due to AD (Other MCI) ($p<0.001$), as well as between MCI due to AD (MCI-AD) and neurological controls (controls) ($p<0.001$), MCI-AD and other MCI ($p<0.001$), MCI-AD and OD ($p<0.001$). Only SNAP-25aa40 and GAP-43 CSF concentrations were significantly different between AD and MCI-AD ($p=0.0091$ and $p=0.041$, respectively, Fig. 1A and D), SNAP-25 total showed a similar trend for higher concentrations in the dementia than in the MCI stage of disease ($p=0.07$, Fig. 1C) but this trend was insignificant for Ng and synaptotagmin (respectively $p=0.2$, and $p=0.95$, Fig. 1B and E). Significant difference between OD and controls

was observed only for synaptotagmin (p= 0.04, Fig 1E). These differences were robust after adjustment for age and sex.

No significant difference in any synaptic biomarker concentration was found between other MCI and controls. There was no difference for Ng, SNAP-25aa40, SNAP25 total, GAP-43 concentrations between OD and controls.

Discriminatory power and sensibility/specificity of explored synaptic biomarkers

Table 3 and Figure 2 present the discriminatory power of CSF synaptic biomarkers. All synaptic biomarkers were found to efficiently discriminate AD from OD with an AUC higher than 0.80. With the exception of synaptotagmin, they were also able to discriminate MCI-AD from controls with AUC higher than 0.85 and AD from controls with AUC higher than 0.80. Overall, the highest discriminative power was found for CSF SNAP 25aa40 with an AUC=0.93 to discriminate AD from controls and AD from other dementia, and an AUC=0.90 to discriminate MCI-AD from controls.

Table 4 displays the sensitivity, the specificity and the optimum cut-off values of synaptic biomarkers between AD and controls. Overall, the sensitivity and specificity were higher than 70%. GAP-43, Total SNAP 25 and SNAP-25aa40 reach the required characteristics of validated biomarkers with a sensitivity and specificity higher than 80%. The optimum cut-off was 279 pg/ml for Ng, 2430 pg/ml for GAP-43, 78 pM for SNAP-25, 10.9 pM for SNAP-25aa40 and 362 pM for synaptotagmin:

Table 5 shows the results of the backward stepwise logistic regression carried out to classify AD from non-AD patients and includes all available CSF biomarkers, age, gender, and *APOE* ε4 status. The only parameters remaining statistically significant in the multivariate model are CSF CSF GAP 43 (p=0.05), and CSF SNAP 25 (P=0.02).

Correlation with classical AD CSF Biomarkers and association with APOE genotype

Table 6 presents the correlations between concentrations of Aβ, Tau, pTau181 and synaptic biomarker concentrations in various groups. In controls, MCI-AD and AD groups, all synaptic biomarkers are correlated with Aβ40, Tau and pTau181. In the overall population, all synaptic biomarkers are positively correlated between them ($p<0.0001$, r between 0.77 and 0.89, data not shown).

Aβ42 is correlated with all explored synaptic biomarkers in controls, MCI-AD groups but not in

the AD group. We did not find any other significant correlations between Aβ, Tau, pTau181 and synaptic biomarker concentrations in OD and other MCI groups.

A link between *APOE* genotypes and CSF synaptic biomarkers was found and is depicted in Fig 3. A significant increase of synaptic CSF biomarker concentrations was found in *APOE* ε4/4 patients. The findings were significant for Ng (p=0.004), GAP-43 (p=0.02) and SNAP-25aa40 (p=0.007) while no statistical significance was found for synaptotagmin and SNAP-25 total. We did not find any predictive value for cognitive decline evaluated over a one to eight years period, of any synaptic biomarker neither in the overall population nor in all subgroups (AD, MCI-AD, other MCI, OD and controls (data not shown).

Discussion

In this study, we show that all evaluated CSF synaptic biomarkers are significantly increased in patients with AD brain lesions (assessed by classical CSF biomarkers) compared to controls, and to other MCI or OD. We simultaneously evaluated several pre- and post-synaptic biomarkers in a large cohort of patients allowing a valid comparison of their concentrations in several groups of diseases, and show that these biomarkers are specifically elevated in AD and MCI-AD and not in other neurodegenerative disorders. Further, we demonstrate in living patients that synaptic dysfunction is an early event, measurable already in the MCI stage of the disease. Last, we show that there is a link between CSF synaptic biomarkers and the *APOE* ε4/4 genotype.

Using a comparison between several synaptic biomarkers, we have revealed that SNAP-25aa40 seems the best one to discriminate AD from controls and from OD. SNAP-25aa40 also displays the best sensitivity and specificity. In AD brain tissue, one study has found that presynaptic markers were more affected than post-synaptic markers (*58*), which matches our finding that presynaptic CSF biomarkers (SNAP-25, SNAP-25aa40 and GAP-43) broadly reveal a better sensitivity and specificity. However, the results of the discriminatory power between AD and controls as well as the sensitivity and specificity of synaptotagmin partially mitigate this hypothesis. It is interesting to notice that synaptotagmin is better to discriminate AD from OD than Ng. This divergence could be due to the difference between the insoluble form measured in the brain and the soluble form evaluated in the CSF. In order to clarify these comparative pathophysiological mechanisms, studies evaluating both neuropathological and CSF data are needed.

Among CSF synaptic biomarkers, Ng is the well-studied marker while much less data is available for the pre-synaptic GAP-43, SNAP-25 and synaptotagmin. Two previous studies evaluating GAP-43 in the CSF found somewhat discordant results. In the first paper, Sjogren et al. did not find any difference between AD and other neurodegenerative diseases, vascular dementia and controls while in the second paper the authors found a small but significant increase similar to that observed in our study (*23, 24*).. The discrepancy compared to earlier studies could be explained by the different methods used for quantification, relatively small cohort sizes in those studies, as well as the difference between the two recruitment methodologies in the cohorts including different diseases and more severe AD patients (*23, 24*). Also in earlier papers, there was a significant correlation between CSF levels of GAP-43 and Tau, which is consistent with

neuropathological findings describing GAP-43 in neuritic plaques mainly in frontal cortex and hippocampus (*11, 25*) and with our results.

CSF SNAP-25 was also poorly explored and a few papers have assessed this protein in various neurological diseases (*59, 60*). However, only our previous study has explored CSF SNAP-25 in AD patients (*33*). In this paper, we have shown in several cohorts that SNAP-25 is increased in AD CSF compared to controls and MCI-AD (prodromal in AD). There was no cohort effect and the best discriminative power was obtained for the longer form SNAP-25aa40 with an AUC at 0.90 (*33*). In the present cohort, SNAP-25aa40 displayed the best discriminatory power compared to other synaptic biomarkers. Altogether, these results confirm that SNAP-25 or specific forms of SNAP-25 could be a very good biomarker. However, future studies comparing all SNAP-25 forms in various populations are needed to evaluate their comparative value. Finally, the least examined synaptic CSF biomarker is synaptotagmin. Only two previous papers have demonstrated its detectability in the CSF and a difference between AD, MCI-AD and controls in two cohorts (*41, 61*). The biological profile was the same but the discriminatory power was better in the first study and no link with cognitive decline was found (*61*).

Concerning Ng, one recent study demonstrated in 116 patients a link between CSF Ng levels and neuropathological lesions including neuritic plaques and levels of neurofibrillary tangles with Braak stages highlighting the clinical interest of such a biomarker (*62*). Our study explored one of the largest cohort of synaptic biomarkers and has confirmed the increased CSF Ng levels in AD patients compared to controls (*50, 52, 53, 55, 56*) (*63-65*). More specifically, we demonstrate a good discriminatory power between AD and controls, AD and OD and MCI-AD and controls a result in line with a previous large study (*62*), and with findings in three smaller cohorts (*53, 56, 66*). The link between Ng and cognitive functions, the predictive value on cognitive decline as well as the correlation with Aβ vary somewhat across publications. On the one hand, four studies described that CSF Ng predicts the cognitive decline (*47, 50, 52, 64*), while we could not detect such an association, similar to some other studies (*51, 53*). This discrepancy could be explained by the variability of the cohorts, including the smaller cohort sizes in some studies, or by the difference of used Ng antibodies. On the other hand, results on the correlation between Ng and Aβ42 are variable. Two previous published reports did not find any correlation between these two biomarkers neither in AD nor in MCI and nor in controls (*47, 49, 52*) while one report found a correlation within the control group (*53*) and another one found correlation in AD and other neurodegenerative diseases (*62*). In our study, we show that the link is found in all groups but not in AD. In summary, our study has demonstrated that Ng CSF levels are significantly increased

only in patients with Aβ and tau lesions but without correlations with cognitive decline.

We have observed a link between the *APOE* ε4/4 genotype and the CSF levels of synaptic proteins. To our knowledge, this is the first time that a link is found between synaptic dysfunction or degeneration and *APOE* genotype in living patients. This finding is consistent with data from neuropathological and basic research. A large neuropathological study found an association between *APOE* genotype and the levels of brain synaptic proteins (*58*). Several studies using *APOE* ε4 human mouse models have found significant synaptic alterations at an early stage of the evolution prior to any neuropathological lesions (*19, 67*). Altogether, these results including our findings are in favor of a crucial role of the *APOE* ε4/4 genotype in synaptic fragility that may affect the response to injury (*58*).

Our results raise two questions: i/what is the meaning of the increased CSF synaptic biomarkers? Given that synapse degeneration occurs as part of a neurodegenerative process, why are synaptic CSF biomarkers specifically increased in AD? Recently, Portelius et al have shown that CSF Ng was correlated with a significant reduction in Ng brain levels. Furthermore, a recent PET study has demonstrated a significant decrease in SV2A (a synaptic protein) in AD compared to controls (*68*). Altogether, these data support the link between increased CSF synaptic biomarkers and synaptic degeneration or loss in the AD brain. However, these studies do not explain the specific increases in CSF in AD. One hypothesis could be a potential link between Aβ and tau spreading and synaptic loss. In contrast with other dementias, AD display the accumulation of two abnormal proteins that are synaptotoxic. Aβ oligomers are formed and transported to synapses and oligomeric tau may transmit from one neuron to another along the anatomical connected synapses. This double mechanism might explain why the release of synaptic proteins are more pronounced in AD than in other neurodegenerative diseases (*69*). The second hypothesis is the influence of ApoE4 on the synaptic trafficking and fragility that could contribute to the specifically increased levels of these biomarkers in AD groups. The last explanation is that the main brain regions affected in AD (parietal, frontal and temporal cortices amygdala and hippocampus) are also the regions with the highest expression of these proteins, contributing to the apparent AD specificity of CSF synaptic biomarkers.

This study has some limitations; this is a retrospective study rather than a prospective one which has limited the range of methodological approaches with the same follow-up of the evolution in all patients. In some group, the number of *APOE* genotype was too low to provide useful data for

statistical analysis per group. The *APOE* ε2/2 genotype is extremely rare and *APOE* ε4/4 is not frequent in non-AD group.

In order to be applied routinely in the clinic, these results need validation in large prospective confirmatory cohorts with a precise exploration of potential preanalytical factors. Using these biomarkers in practice, clinicians would have i/an early biomarker of the pathophysiological process while synaptic alteration is still reversible, ii/a very good differential diagnosis between various neurodegenerative diseases, iii/an indirect evaluation of the spreading of synaptic toxicity. Fundamentally, these biomarkers could bring about important information on the pathophysiological evolution of synapses in living patients and also provide important insights into selective neuronal vulnerability in neurodegenerative diseases.

Materials and Methods

This is a retrospective cross-sectional study. CSF samples from subjects with either dementia due to AD, MCI-AD, MCI non due to AD (designed as other MCI), and other dementia and from neurological Controls (designed as controls) were obtained from the Center of Cognitive Neurology at Lariboisière University Paris Diderot Hospital APHP. This department is highly experienced in the care management of patients with cognitive disorders and neurodegenerative diseases, and has used CSF biomarkers for a long period of time (*16, 70-90*) . Patients underwent a comprehensive clinical examination including personal medical and family histories, neurological examination, neuropsychological assessment, lumbar puncture (LP) with CSF biomarker analysis, a brain structural imaging study with MRI and a brain PET FDG imaging if needed. A consensus diagnosis was made by several clinicians (neurologists, geriatricians), neuropsychologists, and biologists who are experts in CSF biomarkers. For each patient, diagnosis was made considering CSF results and according to validated clinical diagnostic criteria according to the disease. These criteria was also applied to exclude other diseases and to specify the Controls. For all patients, diagnoses were validated in a second step by three neurologists (CP, EC, JH) and a biochemist (EB-A, KB) before selecting CSF samples. In the absence of consensus diagnosis and in cases of disagreement about the final diagnosis, patients were not included in the study. According to this method, CSF from patients suffering from AD, MCI-AD, other MCI, other dementia and controls were selected. The following cutoff values were used to define a biochemical AD signature as supportive criteria for AD (*91*): Aβ42 (<730 ng/L), Tau (>300 ng/L), and pTau181 (>58 ng/L).

Disease duration was recorded as the time in months from symptom onset to LP most patients underwent mini-Mental State Examination (MMSE) for grading of global cognitive ability.

CSF sampling

CSF was obtained by LP between the L3/L4 or L4/L5 intervertebral space, using an atraumatic 24-gauge needle, collected in 10-mL polypropylene tubes centrifuged at 1800 g for 10 min at +4◦C. The collected supernatant was aliquoted in 500µl polypropylene tubes and were stored at –80◦C pending biochemical analysis. Samples were frozen at −80 °C within 1 h after collection according to a standardized protocol described in a previous report (*16*). A small amount of CSF was used for routine analysis, including total cell count, bacteriologic examination, and total protein and glucose levels. Analysis of CSF biomarkers Aβ42, total tau, and tau phosphorylated

at threonine 181 (phosphorylated tau) protein measurements were performed using commercially available assays from Fujirebio (INNOTEST® Aβ(1-42), INNOTEST® hTAU Ag, and INNOTEST® Ptau181) according to the manufacturer's instructions. For the all sample set, the analysis of these biomarkers was performed in a single hospital laboratory (Lariboisère Hospital Paris) in two runs and averaged results were used for statistical analyses. The quality of CSF evaluations was validated by the Alzheimer's Association quality control program for CSF biomarkers (*92*)

Immunoassay for CSF Neurogranin (Ng)

All CSF Ng analyses were performed at the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital (Mölndal, Sweden) using a previously described analytical methodology (*49, 62*)). The plate was coated with the monoclonal antibody NG 36 (in-house developed), covered and put on a shaker at 4°C overnight. Wells were washed three times with PBST (10 mM phosphate buffered saline, pH 7.5, 0.05% Tween 20) on a Tecan plate washer, followed by incubation for 1 hour with blocking solution consisting in PBST with 1% BSA. Plates were washed before the addition in duplicates of samples or standard. The plate was covered and shaken for 3 hours at room temperature. After three washing steps, the detection antibody (biotinylated Ng 2, in-house developed), diluted in blocking solution, was added and the plate was shaken for 1 hour at room temperature. Wells were then washed followed by incubation with Streptavidin enhancer diluted in blocking solution for 30 min at room temperature. Wells were washed and 100 µL of tetramethylbenzidine substrate (TMB) was added, plates were incubated in dark for 20 min, 100 µL of H2SO4 was added and absorbance measured immediately at 450 nm, with a 650 nm reference, on a SpektraMax Plus384 microplate reader (Molecular Devices). The sigmoidal standard was evaluated with non-linear four-parameter fit using SoftMax Pro 5.2 software and sample amounts were obtained using the fitted standard curve. Each plate contained dilutions of pooled brain homogenates as internal control. The coefficient of variation was less than 20% for acceptance.

Immunoassay for CSF GAP-43

CSF GAP-43 level was determined by ELISA and were performed at the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital (Mölndal, Sweden) Plates were coated with 100uL of NM4 monoclonal antibody (FUJIRIBIO, Tokyo, Japan) diluted at 1:3500 in carbonate buffer (pH 9.6) and incubated up to 22 hours at 4°C. Plates were then

washed with PBST and blocked with a solution of PBST-Casein (10x Casein Blocking buffer B6429, Sigma-Aldrich, Missouri, USA) for 1 hour on a shaker at room temperature. After 3 more washes, the detection antibody (polyclonal ABB-135, Nordic Biosite, Täby Sweden)) diluted at 1:7000, samples and calibrators (recombinant GAP-43) diluted in PBST 1% BSA were co-incubated overnight at 4°C. Plates were washed three times and secondary antibody (anti-rabbit IgG HRP, Promega, Wisconsin, USA) diluted at 1:30000 was added and incubated on bench for 1.5 hours at room temperature.

Wells were washed and 100 µL of 3,3´,5,5´-tetramethylbenzidine (TMB, KemEnTech Diagnostics, Taastrup, Denmark) was added, plates were incubated in dark for 20 min, 100 µL of 0.2 M H2SO4 was added and absorbance measured immediately at 450 nm, with a 650 nm reference, on a SunriseTM microplate absorbance reader (Tecan group, Männedorf, Switzerland). The detection limit was 315 pg/ml.

In-house SNAP-25/synaptotagmin-1 IP-MS assay

CSF SNAP 25 and Synaptotagmin analyses were conducted at the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital (Mölndal, Sweden) following a previously established protocol (*33*, *61*) add fernström et al;Mouse monoclonal antibody 41.1 recognizing SNAP 25 and mouse monoclonal antibody SMI-81R recognizing Synaptotagmin were used to co-immunoprecipitate both proteins with a KingFisher TM Flex System (Thermo Fisher Scientific), which uses magnetic rods to move particles through the various binding, mixing, washing and elution phases in a 96-well plate format. Immunoprecipitated SNAP-25/synaptotagmin from CSF were subsequently digested and analysed on a quadrupole–orbitrap mass spectrometer Q Exactive (Thermo Fisher Scientific) coupled to an Ultimate 3000 chromatography system (Thermo Fisher Scientific). The samples (15 µL) were loaded directly onto a Hypersil Gold-C18 column, with 0.1% aqueous FA at 100 µL/min. After 2 min of loading, the peptides were eluted off the column using the following linear gradient steps: 0 min 0%B; 4 min 13%B; 30 min 17%B; 50 min 26%B; 52 min 90%B. The IonMax ion source settings were: spray voltage, +4100 V; capillary temperature, +320°C; sheath gas pressure, 25 arbitrary units; auxiliary gas pressure, 10 arbitrary units; and heater temperature, +300°C. The instrument was set to acquire scheduled pairs of PRM scans in profile mode allowing simultaneous detection of both the SNAP 25/Synaptotagmin peptides and the corresponding isotopically labeled peptide standards. Data acquisition and analysis were performed with Xcalibar software version 2.2 SP1.48 (Thermo Fisher Scientific) and Pinpoint 1.3.0.

Statistical analysis.

Patients' characteristics were described overall and according to their groups (AD, MCI-AD other MCI, other dementia and controls). We compared groups using analysis of variance for continuous measures and $\chi 2$ test for proportions (in adjusted and unadjusted models). We first analyzed the Spearman correlation coefficients of the synaptic biomarkers between them, and with the usual biomarkers (CSF Aβ42, CSF Tau) overall and stratified by groups of patients. Then we performed ROC curves analysis to investigate the ability of the various synaptic biomarkers to discriminate between groups of patients (AD vs controls, MCI-AD vs controls, and AD vs other dementia), and to establish the optimum cut-offs defined by the highest Youden index.

To investigate which parameters were independently predictive of the AD status among our population of patients, we ran a backward stepwise logistic regression models including all the CSF synaptic biomarkers, and other potential confounders: age, sex, *APOE* status. The cut-off for exclusions of the variable was fixed at p=0.10 in the stepwise procedure.

Finally, we investigated the association between CSF biomarkers and the longitudinal cognitive decline (repeated MMSE scores) among AD patients by using linear mixed models. The intercept and slope (time) were treated as random effects, allowing them to vary between individuals. Time in years from baseline was included as a continuous linear term after verification that a quadratic term did not improve model fit.

All P values were two tailed, and $P \leq 0.05$ was considered to be significant. All analyses were performed using SAS 9.3 (SAS Institute, Cary, North Carolina, USA).

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Author's contribution: Kaj Blennow and Claire Paquet designed the study, collected and analyzed the data and prepared the manuscript.

Jacques Hugon, Claire Paquet, Julien Dumurgier, Emmanuel Cognat have included the patients.

Marion Tible, Asa Persson, Kina Hoglund, Ann Brinkmalm have performed CSF analysis

All authors have read and validated the manuscript

Competing interests:

- Prof Kaj BLENNOW has served as a consultant or at advisory boards for Alzheon, BioArctic, Biogen, Eli Lilly, Fujirebio Europe, IBL International, Merck, Novartis, Pfizer, 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.

- Prof. PAQUET is member of the International Advisory Boards of Lilly, is consultant of Fujiribio, ALZOHIS, NEUROIMMUNE and GILEAD and is involved as investigator in several clinical trials for Roche, Esai, Lilly, Biogen, Astra-Zeneca, Lundbeck, Neuroimmune

- Prof. HUGON

- Drs COGNAT and Dr DUMURGIER are investigator in several passive anti-amyloid immunotherapies and other clinical trials for Roche, Eisai, Lilly, Biogen, Astra-Zeneca, Lundbeck.

Marion Tible, Åsa Sandelius, Kina Hoglund, Ann Brinkmalm declare that they have no conflict of interest.

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Figures:

Fig. 1. Comparison of synaptic CSF biomarker condentrations in control, MCI, AD and other dementia patients

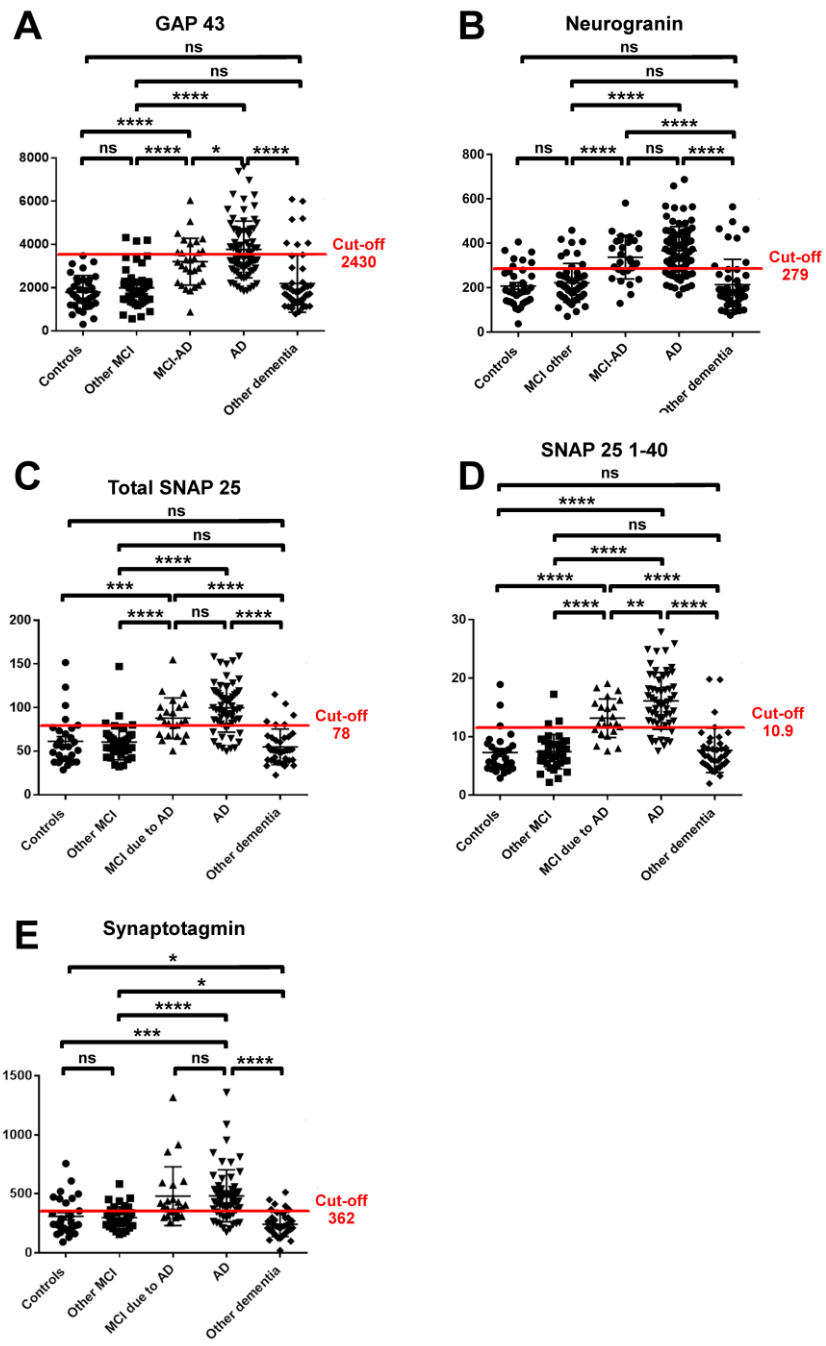

Figure 1 illustrate the quantification of the CSF synaptic biomarkers in Controls, Other MCI, MCI due to AD, AD and Other Dementia.

Fig. 2. Comparison of synaptic CSF biomarker levels in control, MCI, AD and other dementia patients

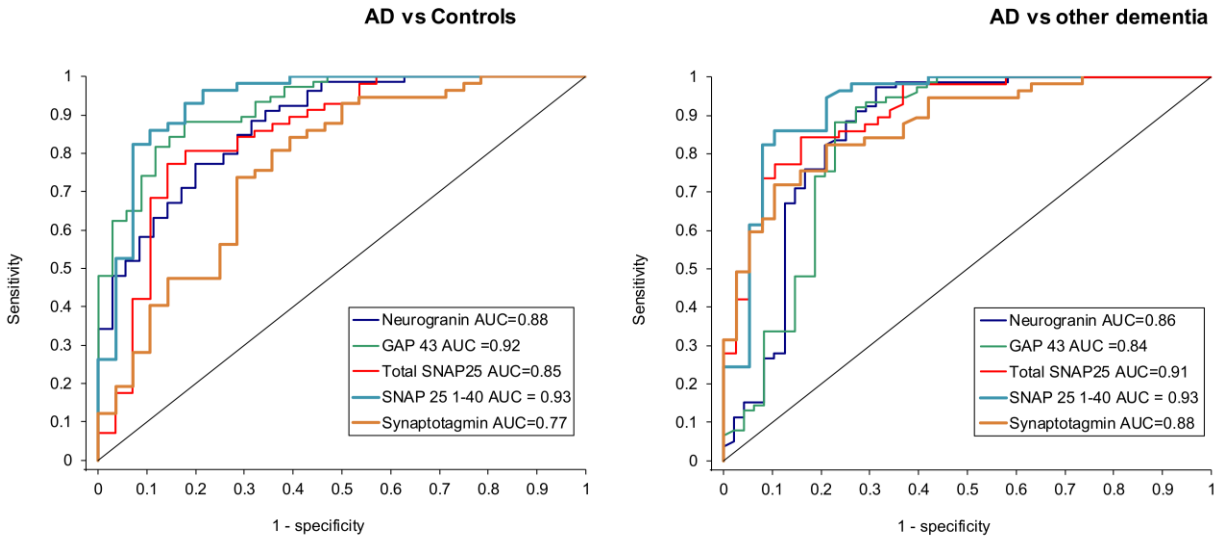

Figure 2 display the discriminatory power curves. On the left, the ROC for AD versus Controls, on the right, the ROC curve for AD versus Other Dementia. SNAP24 (1-40) display the best discriminatory power..

Fig. 3. Illustration of the link between Apolipoprotein genotype and synaptic CSF biomarker levels

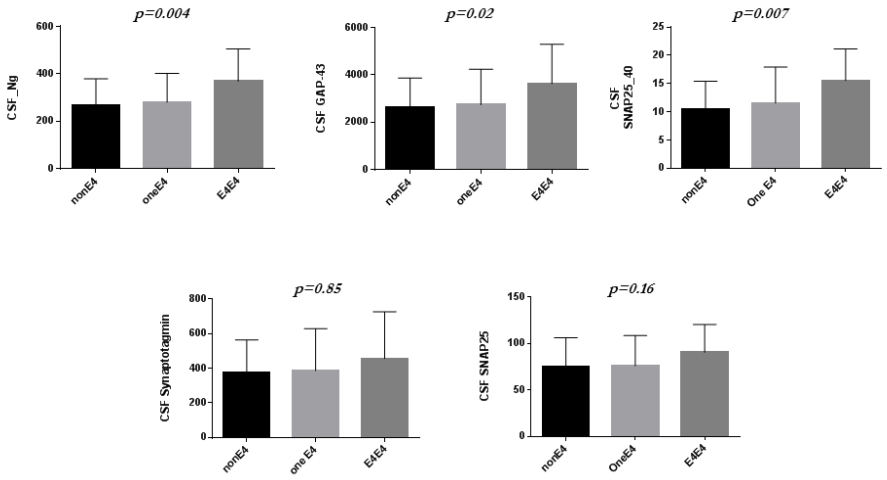

Figure 3: The CSF level of neurogranin, GAP-43 and SNAP25 (1-40) are significantly higher in the APOE ε4: ε4 *genotype group.*

Tables

Table 1. Characteristics of the population study.

Characteristics	Overall (N=246)	Controls (N=35)	AD (N=81)	MCI due to AD (N=30)	Other MCI (N=49)	Other dementia (N=51)	P v
Age, years, mean (SD)	67.1 (9.1)	60.7 (8.3)	69.3 (7.5)	70.6 (8.1)	63.3 (10.5)	69.5 (7.5)	<0
Women, n (%)	138 (56.1)	29 (82.9)	49 (60.5)	19 (63.3)	20 (40.8)	21 (41.2)	<0
MMSE, mean (SD)	23.7 (4.9)	26.6 (2.6)	20.4 (4.9)	27.0 (1.6)	25.3 (3.5)	22.9 (5.3)	<0
APOE ε4 carriers, n(%)	90 (40.9)	5 (15.6)	44 (57.9)	14 (51.9)	11 (27.5)	16 (35.6)	<0
CSF biomarkers, pg/mL, mean (SD)							
CSF Aβ42	751.3 (321.9)	981.3 (276.5)	530.4 (153.5)	609.7 (260.7)	921.1 (284.9)	864.6 (362.5)	<0
CSF Aβ40	12181 (5849)	11357 (5481)	14278 (6326)	14239 (6945)	11121 (4757)	9363 (3952)	<0
CSF Tau	400.8 (280.5)	199.3 (79.9)	667.9 (293.2)	458.1 (155.2)	214.9 (86.4)	259.7 (149.3)	<0
CSF p-Tau 181	61.8 (34.1)	36.0 (13.3)	93.6 (32.7)	75.6 (18.3)	40.5 (14.6)	41.2 (19.5)	<0

Table 2. Synaptic biomarkers according to patients' groups.

Synaptic biomarkers	Controls	AD	MCI due to AD	Other MCI	Other dementia	P value
CSF Neurogranin	207.2 (87.4)	366.4 (108.9)	337.3 (98.2)	221.5 (87.4)	212.7 (114.7)	<0.001
CSF GAP 43	1790.4 (770.8)	3767.2 (1310.0)	3202.8 (1080.4)	1990.6 (867.7)	2186.9 (1325.8)	<0.001
CSF total SNAP-25	61.3 (28.4)	99.7 (27.9)	87.9 (23.3)	60.5 (20.4)	55.0 (20.5)	<0.001
CSF SNAP-25aa40	7.3 (3.6)	16.1 (4.9)	13.2 (3.3)	7.4 (2.9)	7.7 (3.8)	<0.001
CSF synaptotagmin	309.9 (159.8)	483.7 (220.8)	480.5 (248.3)	297.5 (93.0)	242.4 (103.8)	<0.001

Table 3. Discriminatory power of CSF synaptic biomarkers. (Area under the curve)

CSF biomarkers	AD vs Controls	MCI due to AD vs Controls	AD vs other dementia
Neurogranin	0.88 (0.03)	0.84 (0.05)	0.86 (0.04)
GAP 43	0.92 (0.03)	0.86 (0.05)	0.84 (0.04)
Total SNAP-25	0.85 (0.05)	0.81 (0.06)	0.91 (0.03)
SNAP-25aa40	0.93 (0.03)	0.90 (0.05)	0.93 (0.03)
Synaptotagmin	0.77 (0.06)	0.75 (0.07)	0.88 (0.04)

Table 4. Optimum cut-offs to discriminate between AD patients and Controls

CSF biomarkers	Cut-off	Sensitivity	Specificity	Youden index
Neurogranin	279	0.77	0.8	0.57
GAP 43	2430	0.88	0.82	0.71
Total SNAP25	78	0.81	0.82	0.63
SNAP-25aa40	10.9	0.86	0.89	0.75
Synaptotagmin	362	0.74	0.71	0.45

Table 5. Outcome of backward stepwise logistic regression to classify AD from non-AD patients

Predictors	Estimates (SE)	P-value
CSF GAP 43	2.6 (1.4)	0.05
CSF SNAP-25aa40	2.2 (0.9)	0.02

Outcome of backward stepwise logistic regression, cut-off for exclusion p = 0.10.

Table 6. Correlation between synaptic biomarkers and Aβ/tau biomarkers

CSF synaptic biomarkers	Spearman correlation coefficients (P value)				
	Neurogranin	GAP 43	Total SNAP-25	SNAP-25aa40	Synaptotagmin
Controls					
Aβ42	0.52 (0.001)	0.56 (<0.001)	0.64 (<0.001)	0.65 (<0.001)	0.64 (<0.001)
Aβ40	0.46 (0.006)	0.59 (<0.001)	0.72 (<0.001)	0.66 (<0.001)	0.66 (<0.001)
Tau	0.78 (<0.001)	0.83 (<0.001)	0.70 (<0.001)	0.84 (<0.001)	0.64 (<0.001)
p-Tau 181	0.77 (<0.001)	0.78 (<0.001)	0.64 (<0.001)	0.72 (<0.001)	0.59 (<0.001)
MCI due to AD					
Aβ42	0.42 (0.02)	0.48 (0.008)	0.54 (0.007)	0.39 (0.07)	0.59 (0.003)
Aβ40	0.45 (0.02)	0.62 (<0.001)	0.76 (<0.001)	0.55 (0.01)	0.62 (0.004)
Tau	0.64 (<0.001)	0.75 (<0.001)	0.59 (0.003)	0.76 (<0.001)	0.35 (0.10)
p-Tau 181	0.84 (<0.001)	0.86 (<0.001)	0.74 (<0.001)	0.61 (0.002)	0.63 (0.001)
AD					
Aβ42	0.10 (0.40)	0.14 (0.22)	0.22 (0.10)	0.13 (0.35)	0.17 (0.21)
Aβ40	0.37 (0.002)	0.50 (<0.001)	0.54 (<0.001)	0.44 (0.002)	0.63 (<0.001)
Tau	0.75 (<0.001)	0.77 (<0.001)	0.74 (<0.001)	0.83 (<0.001)	0.53 (<0.001)
p-Tau 181	0.74 (<0.001)	0.79 (<0.001)	0.79 (<0.001)	0.83 (<0.001)	0.61 (<0.001)