

## Cerebrospinal Fluid Biomarkers

# Can agrin cerebrospinal fluid concentration be used as an early biomarker for Alzheimer's disease?

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### Abstract

The need for effective treatments halting Alzheimer's disease (AD) urges the discovery of the earliest possible biomarkers. Agrin is increased in the early stages of AD and is involved in amyloid- $\beta$  (A $\beta$ ) fibrillation and synaptogenesis. We investigated the potential of agrin as an early AD cerebrospinal fluid (CSF) biomarker. We analyzed the agrin CSF concentration in nondemented controls (n = 20) and those with mild (n = 20) and severe (n = 20) AD. The levels of agrin CSF were not significantly divergent among the different patient groups and did not correlate with the concentration of A $\beta$ <sub>42</sub>, total tau, phosphorylated tau, or the Mini Mental State Examination scores. However, agrin strongly correlated with age in those with dementia. The results indicate that agrin cannot be used as an early AD CSF biomarker using the current immunoassay. However, our population was relatively young; thus, the correlation between agrin and age suggests that stronger differences in agrin concentrations might be found in older groups with more heterogeneous AD pathologic features.

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### Keywords:

Agrin; CSF; Biomarker; Diagnosis; Aging

## 1. Introduction

Alzheimer's disease (AD) is the most common form of dementia with an unknown etiology. It is pathologically characterized by the accumulation of amyloid  $\beta$  (A $\beta$ ) in senile plaques and phosphorylated tau (p-Tau) in neurofibrillary tangles (NFTs). In addition, a remarkable loss of neurons and synapses is also observed in AD [1,2]. It is widely accepted that interventions to slow down or halt the disease should be administered at the earliest possible stage, before neuronal damage has occurred. Hence, a strong need exists for the discovery of novel biomarkers that can better detect this critical phase.

The current core cerebrospinal fluid (CSF) biomarkers for AD diagnosis reflect the classic hallmarks of AD pathology: a decrease in CSF A $\beta$ <sub>42</sub> levels reflects senile plaque pathology and increased total tau (t-Tau) and p-Tau levels reflect axonal degeneration and NFT formation [3,4]. Their sensitivity and specificity for AD is high, and they can reasonably predict the transition of mild cognitive impairment (MCI) to AD [5]. Nevertheless, AD CSF biomarker patterns are often present in cognitively normal subjects and can be influenced by the patient's age, resulting in a loss of sensitivity with older age [6,7]. Thus, the quest for the earliest and most sensitive marker is ongoing. Interest in biomarkers reflecting synaptic dysfunction has been increasing because synaptic damage occurs at very early pathologic stages, preceding neuronal loss, and strongly correlating with cognitive symptoms [8,9]. In a previous hypothesis-free proteomics study, several potential

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CSF markers for early AD diagnosis were identified, including one of the major heparin sulfate proteoglycans, named agrin (Fig. 1A).

Agrin is expressed in neurons and glia cells [10], and its suppression in mature rat hippocampal cultures severely reduced the number of synapses formed, indicating a role for agrin in synaptogenesis [11,12]. In addition, agrin is an important component of the basal lamina, where it can regulate the expression of other proteins and influence the permeability of the blood-brain barrier [13,14]. Several studies have already implicated agrin in the AD etiology [10,15], because it is the predominant heparin sulfate proteoglycan associated with fibrillar and nonfibrillar senile plaques, cerebral amyloid angiopathy, and neurofibrillary tangles in postmortem AD tissue [16–18]. Agrin is an amyloid-associated protein able to enhance A $\beta$  fibril formation and to halt A $\beta$  degradation [19], suggesting that agrin might not only be a structural component of senile plaques but may also contribute to A $\beta$  fibrillation. Importantly, increased soluble agrin levels were found in the postmortem hippocampus and prefrontal cortex of patients with AD compared with that in controls [14], with significant hippocampal changes observed in the very early stages of AD (Braak III-IV for NFTs) [20,21].

Given the early changes found in human AD tissue and our recent proteomics data, we hypothesized that agrin is a potential early synaptic biomarker for AD. Thus, we analyzed the levels of agrin in the CSF from patients with AD and controls using a specific enzyme-linked immunosorbent assay (ELISA).

## 2. Methods

### 2.1. Human CSF samples

CSF material ( $n = 60$ ) was obtained from the Alzheimer Center Memory Cohort, NeuroUnit Biomarkers for Inflammation and Neurodegeneration VU Medical Center Biobank (Amsterdam, The Netherlands). For the initial pilot proteomic study, we selected age-matched patients with AD ( $n = 5$ ; Mini Mental State Examination [MMSE] score  $20.8 \pm 5.6$ ), subjects without dementia with subjective memory complaints ( $n = 4$ ; MMSE score  $29.5 \pm 1$ ), and patients with MCI who, after 2 years of follow-up, had developed AD ( $n = 5$ ; MMSE score  $25 \pm 2.5$ ) or had remained stable ( $n = 4$ ; MMSE score  $28.4 \pm 2$ ). For the analysis of agrin in a larger cohort using a specific immunoassay, the patients without dementia but with subjective memory complaints ( $n = 20$ ; MMSE score  $27 \pm 1.78$ ), mild AD ( $n = 20$ ; MMSE score  $21.1 \pm 1.0$ ), and severe AD ( $n = 20$ ; MMSE score  $15.3 \pm 1.9$ ) were selected. The diagnoses were defined in a multidisciplinary meeting, as described previously [22]. The diagnostic accuracy of specialized centers such as the Alzheimer Center Amsterdam has been previously reported [23,24]. Patients were matched for age and sex. Collection, storage, and analysis of the CSF biomarkers (A $\beta$ 1-42, t-Tau, and p-Tau) were

performed as previously described [5]. The CSF samples were stored in agreement with the JPND-BIOMARKAPD guidelines [16]. Patient age, sex, biomarker levels, and MMSE scores of all cases are listed in Table 1. The ethical review board of the VU Medical Center approved the study, and all subjects gave written informed consent.

### 2.2. Mass spectrometry analysis of CSF

CSF samples were analyzed by label-free gel electrophoresis liquid chromatography-tandem mass spectrometry-based proteomics and normalized spectral counting, as previously described [25]. The data obtained were processed and analyzed, as described previously [26]. The global protein profiling results of the CSF proteomics screen will be reported subsequently (Chiasserini et al, manuscript submitted). Agrin was one of the identified proteins.

### 2.3. Agrin CSF analyses

The concentration of agrin was determined using the sandwich human agrin ELISA kit (Wuxi Donglin Sci & Tech Development Co., Ltd., Jiangsu, China), in accordance with the manufacturer's instructions. Performance of the assay was evaluated using CSF pools as internal controls. The coefficient of variation (CV) was calculated for each sample in duplicate as the standard deviation divided by the mean. The mean CV of all the samples was calculated to establish the intra-assay CV, which was 8.6%. Samples with an intra-assay CV greater than 15% were excluded from additional analysis ( $n = 11$ ). Interassay CVs were calculated using two internal quality control CSF samples in two plates from one batch, which was 11.4%. For the analysis of clinical samples, different lots of the agrin ELISA kit were used in which a larger interlot variation was found for two internal controls (16.5%). Thus, the agrin concentration for the samples was corrected for the internal controls used in that assay.

### 2.4. Statistical analysis

Statistical analyses were performed using SPSS, version 20 (Chicago, IL, USA). Because the data were non-normally distributed, the initial correlation analyses were performed using nonparametric Spearman's correlations. For subsequent analyses, the data were log-transformed and analysis of covariance was used (with age as the covariate) to analyze the differences between the groups. The assumption of homogeneity of regression was tested ( $P > .05$ ). Partial correlation analyses were also conducted to analyze the associations between the different variables.

## 3. Results

In the present study, we investigated the potential of agrin as a CSF biomarker for the early diagnosis of AD using a specific ELISA immunoassay. The demographic data for the 60 patients are presented in Table 1. The

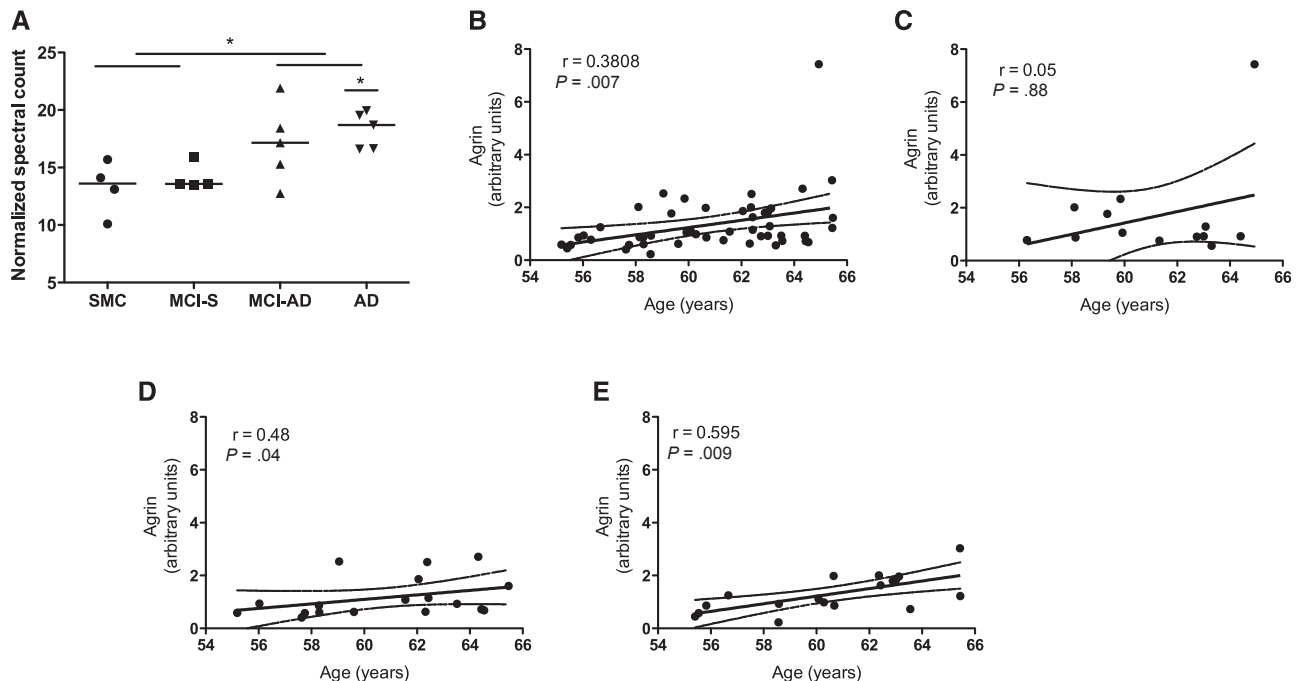


Fig. 1. Agrin cerebrospinal fluid (CSF) concentration correlates with patient age only in patients with Alzheimer's disease (AD). (A) Proteomic analysis of CSF samples from those with subjective memory complaints (SMCs) ( $n = 4$ ), stable mild cognitive impairment (MCI-S) ( $n = 4$ ), MCI converting to AD (MCI-AD) ( $n = 5$ ), and AD ( $n = 5$ ). The spectral counts of agrin peptides in CSF increased with disease progression. (B) Correlation analysis showed a significant association between agrin and patient age. When the results were divided according to the different clinical groups, the CSF agrin concentration did not correlate with age in the SMC cases ( $n = 13$ ) (C) but it did in those with mild ( $n = 18$ ) (D) and severe ( $n = 18$ ) (E) AD. Correlation coefficients and  $P$  values are presented in the inserts. Regression line is shown, and dot lines represent the 95% confidence intervals.  $*P \leq .05$ .

groups differed significantly in MMSE scores and CSF concentrations of  $A\beta_{42}$ , t-Tau, and p-Tau, but not in age or sex, a reflection of the selection criteria. Correlation analysis revealed a significant interaction between the agrin CSF concentration and patient age (Fig. 1B). The correlation analyses run within each diagnostic group revealed that the interaction between patient age and agrin levels was specific to, and strengthened in, the patients with dementia compared with controls (Fig. 1C–E). Consequently, patient age was introduced as a covariate in subsequent statistical models.

Our data revealed no significant differences in agrin CSF concentration between the different clinical groups (Fig. 2A). No significant associations were found between the agrin CSF concentration and MMSE scores or the CSF concentration of t-Tau, p-Tau, and  $A\beta_{42}$ , although a positive tendency was observed in the latter (Fig. 2B–E). In summary, the data indicate that the agrin CSF concentration cannot be used as a biomarker for the early diagnosis of AD. However, our findings did reveal an interesting association between the agrin level and patient age in the patients with dementia and a tendency for a correlation between agrin and  $A\beta_{42}$ .

#### 4. Discussion

In a previous hypothesis-free pilot proteomics study, we found that the levels of agrin were significantly increased in the AD CSF compared with the control CSF (Fig. 1A,

$n = 5$  per group). The discrepancy between our results and the proteomics data is likely explained by the epitope recognized by the antibody used in the current ELISA system. This antibody detects a sequence within the N-terminal 110-kDa fragment, and the vast majority of the peptides found in the proteomics study reside in the C-terminal region. In addition, the peptides sequences detected in this ELISA lie within the laminin epidermal growth factor-like and Kazal-like domains of the agrin protein, which can form disulfide bonds [27,28]. Those properties might prevent the detection of relevant peptides in the CSF and consequently weaken the possibility of finding differences between the clinical groups. Similarly, the present results challenged those from another study that found increased levels of soluble agrin in the postmortem hippocampus of patients with AD in early stages of the disease [14]. The discrepancy could again be attributable to the antibodies used in our ELISA system, because the previous investigation used an in-house ELISA kit that detected the 50-kDa C-terminal fragment of agrin. Taken together, we can conclude that the N-terminal 110-kDa fragment of agrin is not changed in AD CSF; however, whether the concentration of the 50-kDa C-terminal fragment is modified between the different patient groups requires additional investigation.

We observed a tendency for a positive correlation between the CSF levels of agrin and  $A\beta_{42}$ , suggesting an association of agrin with AD pathologic features and, more specifically, with  $A\beta$  plaque formation. This agrees

Table 1  
Demographic data of CSF sample

Variable	Proteomics analysis				ELISA		
	SMC	MCI-S	MCI-AD	AD	SMC	AD-M	AD-S
Mean age (y)	60.3 ± 4.5	62.1 ± 3.2	66.2 ± 6.4	63.9 ± 6.6	60.2 ± 2.61	60.9 ± 3.00	60.4 ± 3.21
Sex	4	4	5	5	20	20	20
Male	2	1	2	2	7	8	10
Female	2	3	3	3	13	12	10
Mean MMSE							
Baseline	29.5 ± 1.0	27.4 ± 2.2	27.0 ± 1.4	21.4 ± 6.3	27 ± 1.78	21.1 ± 1.0*	15.3 ± 1.9* <sup>†</sup>
Follow-up	—	28.4 ± 2.0	25.0 ± 2.5	20.8 ± 5.6	NA	NA	NA
Aβ <sub>42</sub> (pg/mL)	838 ± 133	875 ± 201	499 ± 78	384 ± 146* <sup>†</sup>	888 ± 211.5	491.5 ± 185*	390.5 ± 149.5* <sup>†</sup>
t-Tau (pg/mL)	200 ± 76	421 ± 347	1071 ± 248*	526 ± 120	228 ± 161	729 ± 599*	679.5 ± 856.25*
p-Tau (pg/mL)	47 ± 16	73 ± 48	138 ± 37*	102 ± 40	48 ± 18.5	98.5 ± 48.5*	95.5 ± 81.25*

Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; ELISA, enzyme-linked immunosorbent assay; MMSE, Mini Mental State Examination; MCI-S, stable mild cognitive impairment; MCI-AD, MCI converting to AD; NA, not applicable; SMC, subjective memory complaints; AD-M, mild AD; AD-S, severe AD; t-Tau, total Tau; p-Tau, phosphorylated Tau.

NOTE. Data reported as median and interquartile range or mean ± standard deviation.

\*At least  $P < .05$  from SMC.

<sup>†</sup>At least  $P < .05$  from MCI-S or AD-M.

with previous studies showing that agrin was associated with senile plaques in human AD brain tissue [16,18,19]. Moreover, agrin was able to bind Aβ, accelerating the fibrillation process [19] and, thus, suggesting a role in plaque formation. Taken together, previous data have indicated that agrin expression is changed in AD tissue and could play an important role in the development of this disorder [10,15]. However, according to the present results, those changes are not clearly reflected in CSF.

Although we did not observe a difference in the CSF levels of N-terminal agrin between those with and without AD, the strong correlation with patient age only in those with dementia was striking. These results suggest that age might produce greater and more detectable changes in the levels of agrin CSF between those with and without AD, which might be prompted by the greater heterogeneity of AD pathologic features in older patients. The influence of age on the agrin CSF concentration can be a relevant concern, because the incidence of AD increases in older

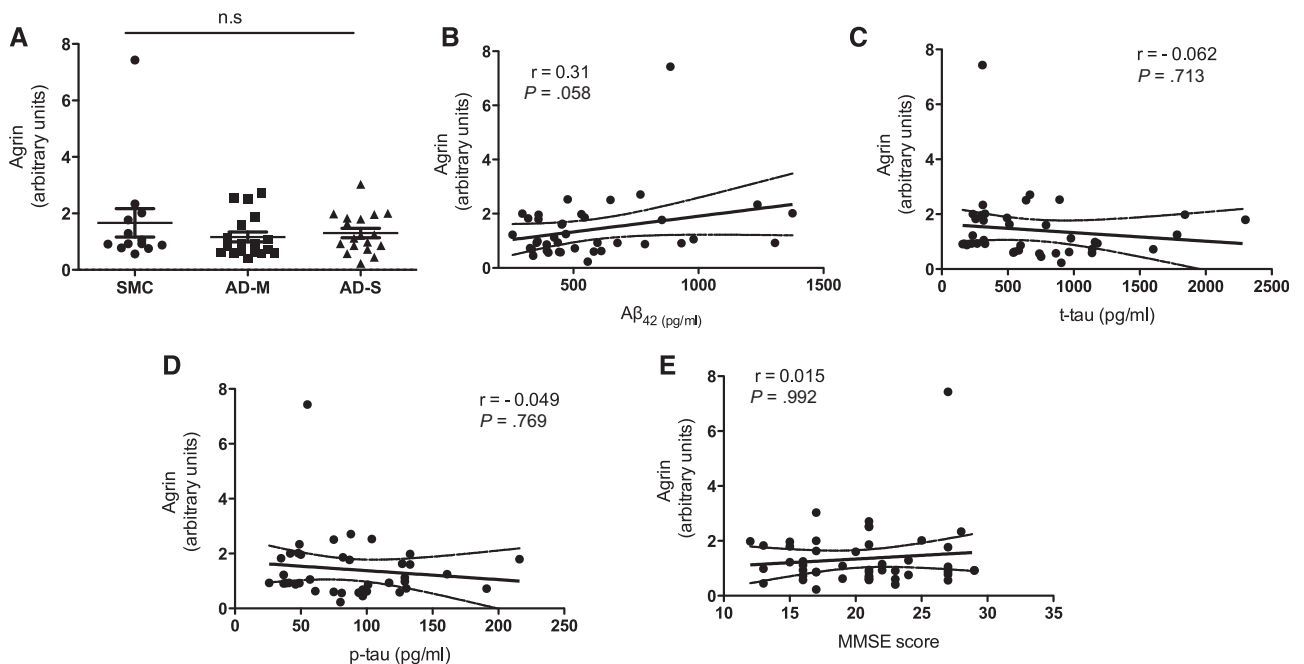


Fig. 2. The agrin cerebrospinal fluid (CSF) concentration was not changed in those with Alzheimer's disease (AD) and did not correlate with the classic AD markers. (A) Agrin concentration in CSF from those with subjective memory complaints (SMCs) (n = 13), mild AD (AD-M) (n = 18), and severe AD (AD-S) (n = 18). No significant difference was observed among the groups. Partial correlation analysis results of CSF concentrations of agrin with amyloid-β (Aβ<sub>42</sub>) (B), total Tau (t-Tau) (C), phosphorylated tau (p-Tau) (D), and Mini Mental State Examination (MMSE) scores (E). Correlation coefficients and P value are presented in the inserts. Regression line is shown, and dot lines represent the 95% confident intervals. ns, nonsignificant.

populations, with the prevalence reaching 19% in patients aged 75 to 84 years and 44% in patients older than 84 years [29]. Thus, it would be interesting to investigate agrin's potential as a CSF biomarker in older groups. Whether the correlation between patient age and agrin CSF concentration reflects an underlying pathologic process of aging and dementia remains to be investigated.

In conclusion, the results of the present study showed no difference in the concentration of agrin in CSF between controls and patients with AD, indicating that agrin cannot be used as an early biomarker for AD diagnosis using the current assay. However, it remains to be investigated whether changes in the agrin CSF concentrations would be detectable with a different ELISA. In addition, the positive correlation of agrin and patient age in patients with dementia suggests that changes in the CSF agrin concentration might be visible in older patients with more heterogeneous pathologic features.

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### RESEARCH IN CONTEXT

1. Systematic review: We used a hypothesis-free proteomics approach to identify potential CSF AD biomarkers. One of the candidates identified was agrin, which might reflect plaque formation or synaptic dysfunction, because it is involved in A $\beta$  fibrillation and synaptogenesis. Increased levels of agrin were previously found in early stages of AD.
2. Interpretation: Although agrin has been extensively analyzed in AD postmortem tissue, its role in CSF has never been investigated. Our results have revealed that the changes found in human tissue cannot be detected in CSF using the current immunoassay. However, the results revealed that agrin correlates with patient age only in the those with dementia.
3. Future directions: The levels of agrin in CSF should be measured using a different immunoassay recognizing a different agrin epitope. The association of agrin and patient age indicates that the future analysis should also include older cohorts in whom the incidence of AD is greater. Those analyses will further clarify whether the agrin level is changed in AD CSF.

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