Biochemical markers in dementia

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Chapter 4

Biomarkers in the clinic

4.1
Additional value of CSF Aβ40 levels in the differentiation between FTLD and control subjects

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Abstract

Background
To determine the additional value of CSF amyloid beta 1-40 (Aβ40) next to amyloid-beta 1-42 (Aβ42), total tau (Tau) and tau phosphorylated at threonine-181 (pTau) to distinguish patients with frontotemporal lobar degeneration (FTLD), Alzheimer's disease (AD), and controls.

Material and Methods
In 55 patients with FTLD, 60 with AD, and 40 control subjects, CSF levels of Aβ40, Aβ42, pTau and Tau were measured. Logistic regression was used to identify biomarkers that best distinguished the groups. Additionally, a decision tree (cost=test method; Matlab 7.7) was used to predict diagnosis selecting the best set of biomarkers with the optimal cut-off.

Results
Logistic regression showed that Aβ42 and pTau CSF levels provided optimal distinction between AD and FTLD. A combination of Aβ42, Tau and Aβ40 optimally discriminated FTLD from controls and AD from controls. The decision tree used Aβ42 (cut-off 578pg/ml) to identify AD (positive predictive value (PPV) 97%), followed by Tau (cut-off 336pg/ml) to identify FTLD (PPV 67%) and in the last step Aβ40 (cut-off 10 ng/ml) was used to differentiate controls (PPV 68%). Applying CSF Aβ40 levels in the model, the PPV of diagnosis increased to 75% as opposed to 70% when only Aβ42 and Tau were used.

Conclusions
CSF Aβ40 levels added to the conventional CSF biomarkers increases the potential to discriminate subjects with dementia from controls. Our findings favour the implementation of CSF Aβ40 CSF in differential diagnosis between FTLD, AD and controls.

Introduction

Frontotemporal lobar degeneration (FTLD) is a spectrum of neurodegenerative disorders affecting the frontal and/or temporal lobes. Its three prototypical clinical variants are the behavioural variant frontotemporal dementia (bvFTD), and the language variants semantic dementia (SD) and progressive non-fluent aphasia (PNFA) [93]. FTLD is pathologically heterogeneous, and can be grossly divided into tau-related pathologies and TAR DNA binding protein (TDP-43) related pathologies [160]. FTLD is commonly mistaken for Alzheimer's disease (AD) or primary psychiatric disorders [161].

Cerebrospinal fluid (CSF) biomarkers may serve as an aid in clinical diagnosis as they are thought to reflect pathological processes taking place in the brain. Until now, no satisfactory set of biomarkers to distinguish FTLD from both AD and controls, has been found [61, 64, 135, 162-170]. CSF biomarkers have been found to be most valuable in the distinction between AD and non-demented subjects. Lower CSF levels of amyloid-beta 1-42 (Aβ42) and higher CSF levels of Total Tau protein (Tau) and its phosphorylated form (pTau) can discriminate AD patients from controls at a high sensitivity [7, 60, 171]. However, the specificity of these biomarkers, is relatively lower, complicating their use in the differential diagnosis of dementia [7, 60, 171].

In a preliminary study we found that CSF Aβ40 (amyloid beta 1-40) levels were lower in FTLD patients compared with both AD patients and controls [61]. The goal of this study was to investigate whether measurement of CSF Aβ40 next to the conventional biomarkers Aβ42, pTau, and Tau has additional value in the discrimination between FTLD, AD and controls.

Materials and Methods

Patients

Fifty-five patients with FTLD (36 patients with bvFTD, 14 with SD, and 5 with PNFA), 60 patients with probable AD and 40 control subjects were included in this study. All patients underwent a standardized clinical investigation including medical history, physical and neurological examination, screening laboratory tests, neuropsychological assessment, EEG and brain MRI. Clinical diagnosis was made by consensus in a multidisciplinary meeting without knowledge of CSF results. The diagnosis of AD was made using the National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria [1]. For FTLD we used the international clinical diagnostic consensus criteria of Neary et al. [93] and the neuropsychological test used included the Visual Association Test (VAT) and the Rey auditory-verbal learning test for episodic memory, digit spans forwards and backwards for working memory and category fluency for semantic memory [172-174]. For executive dysfunction the following tests were used: the Trail Making Test (TMT) B, elements of the Behaviour Assessment of Dysexecutive Syndrome (BADS), the Stroop and letter fluency. The Boston naming test and VAT naming were used for naming and the Rey-Osterrieth Complex Figure Copying test for evaluating visuospatial function [175, 176]. The Mini Mental

State Examination (MMSE) was used to evaluate the degree of cognitive impairment and the Clinical Dementia Rate (CDR) was used to asses the clinical state of the patients [177].

The control group consisted of 40 patients who presented at our memory clinic with subjective complaints but who had normal clinical investigations and did not have significant cognitive deficits. These patients with subjective memory complaints have been followed up (mean 1.5 (1.4) year) and all control subjects remained stable at follow up, except one patient which was classified as MCI (follow up time 2.5 year). All Patients and control subjects were enrolled at the memory clinic of the VUMC (Amsterdam, The Netherlands) between 1998 and 2007. The local ethical review board approved the study protocol and all subjects gave written informed consent.

CSF analysis

CSF samples were obtained by lumbar puncture between the L3 and L4 or L4 and L5 intervertebral space, 12 mL was collected in polypropylene tubes and within two hours brought to the lab. Part of the CSF was used for routine analysis, including total cell and erythrocyte count, as well as total protein determination. The remaining CSF was then centrifuged at 1800 g for 10 minutes at 4°C. The CSF samples were aliquoted into polypropylene tubes and immediately stored at -80°C until analysis. CSF Aβ42, Tau and pTau were measured with Innotest® sandwich ELISA as described previously [64]. As the manufacturer does not supply controls, the performance of the assays was monitored with pools of surplus CSF specimens. In the study period multiple specimens with various concentrations which were included in 7-18 runs have been used for this purpose. The inter-assay coefficient of variation (mean±SD) was 11.3±4.9% for Aβ42, 9.3±1.5% for Tau and 9.4±2.5% for pTau. The team of the department of Clinical Chemistry (VUmc) involved in the CSF analysis was not aware of the clinical diagnoses.

Aβ40 was measured with two separate methods. One was a commercial ELISA (The Genetics Company, Zürich, Switzerland [99]) and the other was our in-house assay. The intra-assay coefficient of variation (CV) of the Genetics Company method was 3.5% and the inter-assay CV 10.2%. The in-house assay is a sandwich ELISA employing an in-house produced monoclonal antibody against the COOH terminus at residue 40 (VU-α-Aβ40) as catcher and biotinylated 6E10 (a commercially available monoclonal antibody against the NH2 terminus of the amyloid peptide; Signet laboratories, Dedham, MA, USA) as detecting antibody [178]. The intra-assay coefficient of variation (CV) was 1.4% and the inter-assay CV 7.3%.

Statistical analysis

Data were analyzed with the SPSS software package (version 15 for Windows SPSS, Chicago IL). In statistical analyses involving biomarker concentrations natural log-transformed concentrations were used, unless stated otherwise. Differences between groups were tested using Chi-squared test and analysis of variance (ANOVA) with sex and age as co-variates. Bonferroni tests were used to adjust for multiple comparisons and for correlations the Pearson test was used. To identify the best subset of biomarkers for pair wise discrimination (FTLD versus AD, FTLD versus controls and AD versus controls),

logistic regression analysis was used (forward likelihood; the entry of probability for stepwise analysis was set at $p<0.01$). Sensitivity and specificity were derived from the models.

In an additional analysis, we performed tree regression (cost=test method; Matlab 7.7). Several variables and outcomes can simultaneously be entered in this model (in our case four biomarkers and three groups) to predict the outcome score on the basis of dichotomized variables. The model starts with determining the variable and dichotomization threshold that give the best prediction of the outcome score. Then, either the subset above or the subset below the threshold is split into two smaller subsets. This process is repeated and a prediction tree is constructed. The number of subsets (or branches of the tree) will be determined by minimizing the cross-validation prediction error. As a result the model predicts several decision steps that have to be taken (each time by use of just one variable) to optimally discriminate the groups from each other. Additionally, this model delivers the best cut-off value for each biomarker that has to be used for the consecutive different decisions [179].

Results

The clinical characteristics and the biomarker levels by diagnostic groups are shown in Table 1. The FTLD, AD, and control groups did not differ in sex. However, the three groups differed in age, as controls were slightly younger than AD patients ($p<0.05$). The MMSE score was lower in FTLD and AD patients than in controls (for both comparisons $p<0.01$). Moreover, the MMSE score was lower in AD patients than in FTLD patients ($p<0.01$). ANOVAs revealed a significant group difference for all CSF biomarker levels (all $p \leq 0.01$). Post-hoc tests revealed higher levels of Tau and pTau, and lower levels of Aβ42 in AD compared with controls and FTLD patients (all $p<0.05$). Lower levels of Aβ40 (both methods) and higher Tau levels were found in FTLD compared with controls (both $p<0.05$).

We calculated the mean biomarker values for each FTLD subgroup. All subgroups had no different biomarker levels except the PFNA subgroup (N=5) where a lower Aβ42 (428pg/ml) was found in comparison with SD (803pg/ml) and bvFTD (830 pg/ml) (all $p<0.05$). Comparison of the main groups (AD-FTLD-controls) and the post-hoc results did not change.

In table 2 the results of logistic regression analysis are shown against the background of comparable CSF studies. Aβ42 and pTau were the best biomarkers subset to discriminate AD patients from FTLD patients (sensitivity 85% and specificity 87%).To discriminate FTLD from controls, levels of CSF Aβ42, Tau and the Aβ40 (in house method) were the best combination (sensitivity 86% and specificity 80%). Finally, to differentiate AD from controls Aβ42, Tau and Aβ40, were the best subset of biomarkers (sensitivity = 95% and specificity 95%). When Aβ40 was omitted from logistic regression analysis to discriminate FTLD from controls, Tau and pTau were the best subset of biomarkers and sensitivity and specificity dropped to 76% and 68%, respectively. When this was performed for AD and controls, Aβ42 and Tau were the best subset of biomarkers and only the specificity dropped to 92.5%.

Subsequently four variables (Aβ42, Aβ40, Tau and pTau) were entered in the regression tree model to predict the three diagnostic groups (Figure 1). In the first step, the model identified AD patients using CSF Aβ42 levels at a cut off value of 578 pg/ml (positive predictive value (PPV) 97%). Subsequently, the FTLD group was selected using CSF Tau levels (cut-off value 336 pg/ml). Finally, the control group (PPV 68 %) was separated from the FTLD group (PPV 67%) by use of CSF Aβ40 levels (cut-off value 10 ng/ml for the in house method, 6 ng/ml for the commercial test). If only Aβ42 and Tau were used, the overall PPV was 70%. The PPV increased to 75%, when CSF Aβ40 levels were included.

Since the PFNA group had significant lower CSF Aβ42, we omitted these patients from the FTLD group and performed again the logistic regression. Aβ42 and pTau remained the best biomarkers subset to discriminate AD patients from FTLD patients (sensitivity 86% and and specificity 92%). To discriminate FTLD from controls, levels of CSF Tau and the Aβ40 were the best combination (sensitivity 82% and specificity 75%). When we used the identified algorithm (=decision tree model), we yield for bvFTD a PPV of 75%.

Comparing the results of Aβ40 levels measured by both methods, a strong correlation was found ($r= 0.9$; $p<0.01$). The complete statistical analysis yielded the same results for both methods.

Discussion

In this study we investigated the additional value of CSF Aβ40 to the existing CSF biomarkers Aβ42, Tau and pTau in the differential diagnosis between FTLD, AD and controls. We found that CSF Aβ40 levels can aid in the discrimination between FTLD and controls and to a lesser extent in the discrimination between AD and controls. The PPV calculated by the decision tree model for Aβ40 was only 67%, however, together with the logistic regression analysis, this model confirmed that CSF Aβ40 levels help to distinguish FTLD form controls.

FTLD versus AD

No differences in CSF Aβ40 levels (measured with both systems) were found between AD and FTLD. This is in contrast with our previous study, investigating smaller patient groups [61]. The finding of higher CSF Aβ42 and lower CSF Tau and CSF pTau levels in FTLD than in AD is in line with other studies [61, 64, 135, 162-166, 170]. Moreover, a sensitivity of 87% and a specificity of 84% (to discriminate FTLD and AD) is similar to results of earlier studies from our group and others (see table 2), using various combinations of biomarkers [64, 72, 131, 135, 165, 170].

FTLD versus controls

With the present study we confirmed the presence of lower CSF Aβ40 levels in FTLD compared with controls using two separate methods [61]. In addition, higher levels of CSF Tau were found in FTLD patients in comparison with controls whereas no differences were found for Aβ42 and pTau. This is in line with several other studies yielding heterogeneous results, probably due to clinicopathological heterogeneity of the FTLD group itself (table 2) [61, 64, 135,

[162-170]. In this study, the best diagnostic discrimination between FTLD and controls was reached by using Aβ42 and Tau combined with Aβ40 (sensitivity and specificity of 86% and 80%), resulting in an essential higher diagnostic accuracy than the use of CSF Aβ42 and Tau alone. Till now, only one study applied Aβ40 in a model to discriminate FTLD form controls, showing a sensitivity of 87% and specificity of 90% in a model with CSF Aβ38 [72]. As that study used western blot measurements, we cannot compare our results to those for technical reasons.

AD versus controls

As expected, an increase of CSF Tau and pTau CSF levels and a decrease of CSF Aβ42 levels were observed in AD as compared with controls [7, 60, 171]. Similar levels of CSF Aβ40 in AD and controls were found, which is in line with our preliminary study [61] and with others [62, 66-73]. Yet, in this study, the best diagnostic potential, in terms of sensitivity and specificity (both 95%), was reached combining Aβ42 and Tau with Aβ40. This partially covers results found in other studies [62, 64, 66] (see table 2). Shoji et al. found [66], like in the present study, that the accuracy of neurochemical discrimination between AD and controls was improved when Aβ42 and Tau CSF levels were combined with Aβ40 CSF levels.

In this study, lower levels of Aβ40 have been found in FTLD patients compared to controls. This is quite difficult, since it is believed that in at least half of all FTLD cases a Tauopathy with little or no Aβ is involved [31, 180]. However, several colleagues published evidence which link the Tau abnormalities with Aβ [181-184]. In line with this, it has been hypothesized that common upstream drivers cause both elevation in Aβ and Tau hyperphosphorylation through independent but parallel mechanisms [183]. For example, one of the link-hypotheses concerns functioning of the Gsk3 enzyme. Gsk3 phosphorylates the Tau peptide to form P-Tau which eventually will cluster to filaments. Additionally, Gsk3 interacts with presenilin, which in turn is needed for the gamma-secretase cleavage of APP to create Aβ [181-184].

One of the strengths of the present study is that Aβ40 CSF levels were measured with two different ELISAs. Group analysis of these two different measurements gave the same results making our conclusions stronger. Overall, lower Aβ40 CSF levels were measured with the commercial ELISA. This is probably due to different sets of monoclonal antibodies used in the two tests, or to a different strategy used concerning the N- and C-terminal specificity of the coating and detecting antibodies. Furthermore, different standard preparations used in the two tests may have attributed to the outcome differences. In contrast to our preliminary study, larger numbers of patients have been used in this study increasing the statistical power. There is a statistical limitation, since the AD and FTLD are overrepresented in this study. The baseline probability to classify the different groups is not equal as in clinical practice. This may influence the results calculated by the decision tree. Additionally, post-mortem verification of the clinical diagnoses is lacking in the majority of cases, leaving the possibility of misdiagnosis. However, as we made the clinical diagnosis in a multidisciplinary team, and most patients were followed up for at least one year, it is unlikely that we have included a substantial number of misdiagnoses.

If the decision tree is being used in clinical practice, one has to take in consideration that there is a large inter-centre variability for the measurements of CSF biomarkers in AD (for Aβ42, Tau and pTau) [75, 142, 148]. This high variability results in differences in reference ranges and reference values, limiting the generalisability of our results. A simple interpretation algorithm to compare biomarker results between centres, as reported by Lewczuk et al., can be helpful in this perspective [185].

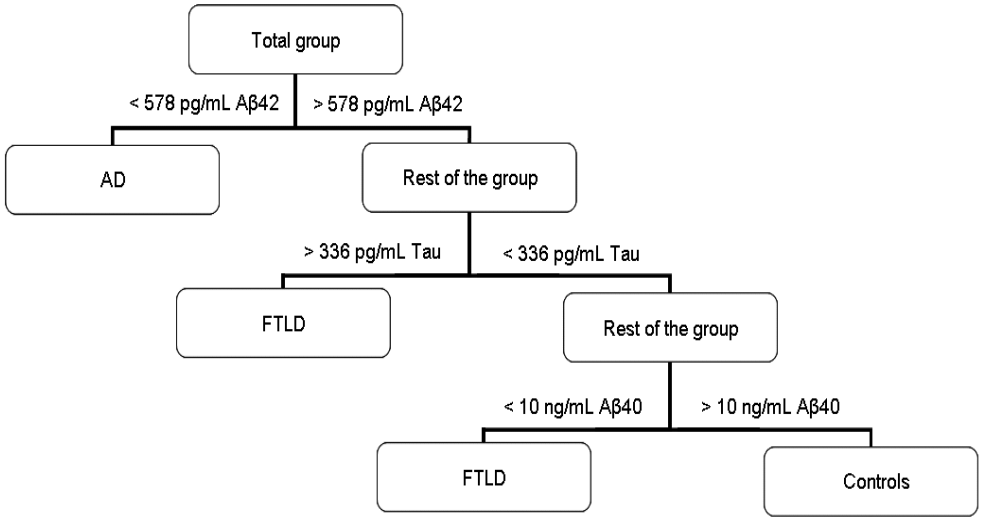
In summary, although the role of CSF Aβ40 appears to be limited in the distinction between FTLD and AD, our findings demonstrate an additional value of Aβ40 in the discrimination of FTLD patients from non demented subjects.

Table 1

	Controls	**FTLD**	**AD**	**p**
N	40	55	60	
Sex female (%)	19 (48%)	22 (40%)	33 (55%)	0.3
Age (years)	59 (10)	61 (7)	64 (8)	<0.05[b]
MMSE	29 (1)	24 (5)	21 (5)	<0.01[a, b, c]
CDR [median (min, max)]	0 (0, 0)	1 (0.5; 2)	1 (0.5; 2)	<0.01[a, b]
Aβ40, in-house method, ng/mL	12.3 (2.7)	10.6 (3.4)	11.0 (2.5)	0.01[a]
Aβ40 (genetics), ng/mL	9.4 (2.4)	7.9 (2.6)	8.4 (2.3)	0.01[a]
Aβ42 (Innogenetics), pg/mL	848 (200)	786 (296)	444 (139)	<0.01[b,c]
Tau (Innogenetics), pg/mL	274 (125)	422 (289)	777 (353)	<0.01[a,b,c]
pTau (Innogenetics), pg/mL	44 (15)	49 (25)	90 (31)	<0.01[b,c]

Data are shown as mean (SD) or n (%). Please note that raw biomarker levels are shown, whereas statistical analyses were performed using natural log-transformed concentrations. Group differences were analyzed using Chi-squared test (sex) or ANOVA, adjusted for age and sex, with post-hoc Bonferroni tests. MMSE score is known for 42 FTLD patients, 59 AD patients and 39 controls. CDR score is known for 53 FTLD patients, 57 AD patients and 40 controls. Post hoc results: p<0.05: [a] FTLD versus controls; [b] AD versus controls; [c] FTLD versus AD. AD=Alzheimer's Disease; FTLD=Frontotemporal lobar degeneration; MMSE=Mini Mental State Examination; Aβ40= amyloid beta 1-40; Aβ42= amyloid-beta 1-42, Tau= total tau; pTau = Tau phosphorylated at threonine-181.

Figure 1



The decision tree model identifies AD patients using CSF Aβ42 levels at a cut off value 578pg/ml (PPV 97%). Subsequently, the FTLD group was selected using CSF Tau levels (with a cut-off value 336pg/ml). Finally, the control group (PPV 88%) was separated from the FTLD group (PPV 60%) by use of CSF Aβ40 levels [no difference between the methods used to measure Aβ40; cut-off in-house method=10ng/ml; cut-off commercial ELISA 6ng/ml). If only Aβ42 and Tau were used, the overall PPV was 67% and this was increased by CSF Aβ40 levels to 73%. AD=Alzheimer's Disease; FTLD=Frontotemporal lobar degeneration.

Table 2

	N			Markers	AD versus FTLD			FTLD versus CON			AD versus CON		
Studies	**CON**	**FTLD**	**AD**		**AUC**	**sens**	**spec**	**AUC**	**sens**	**spec**	**AUC**	**sens**	**spec**
This study	40	55	60	Aβ42,Tau and Aβ40*				---	86%	80%	---	95%	95%
				Aβ42 and P-Tau	---	87 %	84%						
Bibl et al. [72]	30	30	30	Aβ38/Aβ40	---	87%	87%	---	87%	90%	---	100%	97%
Schoonenboom et al. [64]	21	28	47	Aβ42 and P-Tau	---	72%	93%						
	21	28	47	Aβ42 and Tau							---	81%	100%
Bian et al[165]	13	30	19	Tau/Aβ42	0.93	79%	97%						
Lewczuk et al.[62]	22		35	Aβ42/Aβ40							0.95	95%	91%
Riemenschneider et al. [135]	40	34	74	Tau and Aβ42	0.89	85%	85%	0.91	90%	77%	0.97	92%	95%
Kapaki et al. [170]	93	34	76	Tau/Aβ42	0.82	77%	81%	0.89	81%	87%			
	93	34	76	P-Tau/Tau							0.98	93%	100%
Shoij et al. [66]	34		55	Aβ40/Aβ42 and Tau							---	69%	88%

*Summary of studies that distinguish controls, AD, and FTLD with a combination of CSF biomarkers. AD=Alzheimer's Disease; FTLD=Frontotemporal lobar degeneration, CON=controls; Aβ40=amyloid beta 1-40; Aβ42=amyloid-beta 1-42, Tau=total Tau; P-Tau=Tau phosphorylated at threonine-181; Sens=sensitivity; Spec=specificity. * Model chooses in-house method. ---not reported.*

4.2
Serum amyloid P component as biomarker in MCI and AD

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Abstract

Background
Serum-Amyloid-P-component (SAP), present in amyloid-β (Aβ) plaques in Alzheimer's disease (AD), may protect Aβ-deposits against proteolysis, thereby promoting plaque formation. The aim was to investigate if CSF and serum SAP levels can be used to discriminate controls, AD and MCI patients, and to identify incipient AD among MCI patients.

Materials and Methods
CSF and serum SAP levels were determined in 30 controls, 67 MCI and 144 AD patients. At follow up 39 MCI patients had progressed to dementia, while 25 had remained stable (mean follow-up time: 2.6±1.0 and 2.1±0.8 year).

Results
Cross-sectionally no differences were found in SAP CSF and serum levels between groups. MCI patients that at follow up had progressed to dementia, had lower CSF SAP levels [13mg/L(range 3.3-199.3)] than MCI non-progressors [20.2mg/L(range 7.0-127.7); $p<0.05$]. Low CSF SAP was associated with a twofold increased risk of progression to AD (Hazard ratio=2.2; 95%CI=0.9, 5.4).

Conclusion
Our data suggest that measurement of CSF SAP levels can aid in the identification of incipient AD among MCI patients.

Introduction

Mild cognitive impairment (MCI) is characterized by an isolated memory deficit and a largely intact general cognitive functioning [5]. Patients with MCI are at a high risk to develop dementia of which Alzheimer's disease (AD) is the most common form [1, 6]. Early identification of MCI patients that progress to dementia is important in view of future therapeutic options. Diagnosing incipient or prodromal AD in MCI patients remains a difficult issue, however and thus there is need for biomarkers that discriminate stable MCI cases from MCI patients who will progress to AD. Neuropathologically AD is characterized by neurofibrillary tangles and beta-amyloid (Aβ) plaques [186]. A number of inflammation related factors, including serum amyloid P component (SAP), accumulate in Aβ plaques [187-191]. Human SAP is a glycoprotein that exists as a pentameric ring-like structure of non-covalently bound subunits in serum [192]. SAP binds a variety of ligands, including Aβ, in a Ca^{2+}-dependent manner [109, 110]. SAP binding to its ligands can, in turn, lead to binding of complement factor C1q and initiate activation of the classical pathway of the complement system. SAP binding to newly formed amyloid fibrils may protect the Aβ deposits from proteolysis, and as a consequence promote Aβ fibril and amyloid plaque formation in brain [109, 193-196]. Clustering of activated microglial cells in Aβ plaques was observed in SAP and C1q decorated Aβ plaques and to precede Tau-related neurodegenerative changes [52]. In vitro, SAP and C1q inhibit the microglial uptake of Aβ [197]. This inability to remove Aβ in the presence of SAP and C1q may explain the observed enhancement by SAP and C1q of the Aβ mediated activation of microglia in vitro [52, 53]. In addition, SAP can also have direct effect on neuronal cells, as SAP was shown to induce apoptosis in neuronal cells in vitro as well as in vivo [198]. These findings are indicative of a role for SAP in the initiation of the Aβ-mediated neuroinflammatory process in early stages of AD. It is therefore conceivable that SAP levels can be used as a biomarker for early diagnosis of AD. Cross-sectional studies in which CSF and/or serum SAP levels were measured in AD patients and non-demented elderly, showed contradictory results [137, 199-202]. Longitudinal studies in which the relation between CSF SAP levels and the clinical development of AD is investigated have not been reported yet. In this study, we investigated whether CSF and serum SAP levels can serve as a biomarker to discriminate AD patients, mild cognitive impaired (MCI) patients and controls. In addition, we followed our group of MCI patients over time, and investigated if SAP in CSF and serum can be used to identify incipient AD among mild cognitive impaired (MCI) patients.

Materials and methods

Patients

Sixty-seven patients with amnestic MCI, 144 patients with probable AD and 30 controls were included in this study. All patients underwent a standardized clinical investigation including medical history, physical and neurological examination, screening laboratory tests, and MRI. Clinical diagnosis was made by a team of neurologists, neuropsychologists, a radiologist, a clinical neurophysiologist and a psychiatrist, in a multidisciplinary meeting. The diagnosis of MCI was made according to the Petersen criteria [5]. The diagnosis of AD was made using the National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's

Disease and Related Disorders Association (NINCDS-ADRDA) criteria [1]. Only sporadic AD/MCI cases were included in this study. The control group consisted of 14 patients who presented at our memory clinic with subjective complaints but who had normal clinical investigations and did not have significant cognitive deficits (i.e. MCI criteria were not fulfilled). Additionally, we included 16 volunteers without cognitive complaints, who were willing to undergo the same diagnostic procedure as patients attending our memory clinic. The Mini Mental State Examination (MMSE) was used to evaluate the degree of cognitive impairment [177]. The local ethical review board approved the study protocol and all subjects gave written informed consent. Sixty-four of the 67 MCI cases were re-examined to obtain clinical follow-up information. Two patients refused follow-up and one patient died before the follow-up investigation had taken place. Thirty-nine MCI patients (average follow-up time: 2.6±1.0 year) progressed to dementia (progressors) [35 AD, 1 dementia with Lewy bodies [11], 2 Vascular dementia [203], 1 Frontotemporal lobe dementia [10]] and 25 MCI patients (average follow-up time: 2.1±0.8 year) remained clinically stable (non-progressors).

CSF analysis

CSF samples were obtained by lumbar puncture between the L3 and L4 or L4 and L5 intervertebral space, and 12 mL was collected in polypropylene tubes. A blood sample was obtained at the same time. Part of the CSF was used for routine analysis, including total cell and erythrocyte count, as well as total protein determination. The remaining CSF as well as serum samples were then centrifuged at 3,000 rpm for 10 min at 4°C. The CSF samples were aliquoted into polypropylene tubes and immediately stored at -80°C until analysis. All CSF and serum analyses were performed at the Department of Clinical Chemistry (VUMC). CSF amyloid 1-42 (Aβ42) and Tau were measured using commercially available sandwich ELISAs [Innogenetics, Ghent, Belgium] [154]. Serum and CSF levels of SAP were determined with an in-house developed sandwich ELISA, using commercially available antisera (DAKO A/S, Denmark). Polyclonal rabbit anti-human SAP (DAKO A/S, Denmark) was coated onto 96-well ELISA plates (Costar high binding, USA) in 50mM carbonate buffer (pH 9.6) at room temperature for 18 hours. After two washes with phosphate buffered saline (PBS), the residual binding sites were blocked with 2%, v/v, low fat milk in PBS (150microliter/well) for one hour at room temperature. This was followed by three times washing with PBT (PBS containing 0.05% Tween20). Serum-samples (1:40.000) and CSF-samples (1:50) were diluted with assay-buffer (PBS containing 0.1% Tween, 0.2% Gelatine and 10mM EDTA; pH 7.4), added to wells in duplicate and incubated for one hour. Bound SAP was detected after subsequent incubation with biotinylated anti-human SAP (DAKO A/S, Denmark) in assay-buffer for one hour and with peroxidase-labelled streptavidin (Amersham Biosciences, UK) for 30 min. All incubations were on a shaking platform (500 rpm) at room temp. Washing in between incubation steps was with PBT. Finally, 3,5,3',5'-tetramethylbenzidine (TMB; Sigma, Germany) at a concentration of 100µg/mL in 0.11M sodium acetate (pH 5.5) containing 0.003% H_2O_2 (100µL final volume) was used as a substrate. The substrate reaction was stopped by adding 2M H_2SO_4. Absorption at 450 nm was then determined in a Rainbow microtiter plate reader (SLT, Austria) and corrected for background (OD540). To make a standard curve we used commercially available SAP (Calbiochem cat nr. 565190), purified from human serum. The standard curve was plotted using different dilutions of SAP. The detection limit for SAP was 0.1µg/L. The intra-assay coefficients of variance was 3.2%

(N=14). The inter-assay coefficient of variance for three aliquoted pools (stored at -80°C and run each time as internal control) was 13.1% (N= 35; period from 2005 to 2008).

Statistical analysis

Data were analyzed with the SPSS software package (version 12.1 for Windows SPSS, Chicago IL). Differences between groups were tested using Fisher exact test, Chi-squared test, analysis of variance (ANOVA), independent t-test, Kruskal Wallis test and the Mann-Whitney U test. Spearman rank correlation coefficients (r_s) between MMSE, age, Aβ42, Tau, ApoE4, SAP levels in CSF and serum, were determined. Within the MCI patient group, a Cox proportional hazard model, which accounts for variability in length of follow-up, was used to assess the predictive value of CSF SAP levels within the MCI patient group. For this analysis, values of CSF SAP were dichotomized based on the median. Sex, age and MMSE were entered as co-variates. Main outcome was progression to dementia, second outcome, when four MCI cases that developed a different type of dementia were excluded, was progression to AD. The Hazard ratio (HR) is presented with 95% confidence interval (CI). Time to event curves were constructed with the Kaplan Meier method. Statistical significance was set at $p<0.05$.

Results

The clinical characteristics and the biomarker levels by diagnostic groups are shown in Table 1. The AD, MCI and control group did not differ in sex. Patients with MCI were on average older than AD patients and controls ($p<0.05$). ApoE4 genotype, CSF Aβ42 and Tau levels were significant different among the three different groups. ($p<0.01$). CSF and serum SAP levels did not differ among diagnostic groups. Table 2 shows the clinical characteristics and the biomarker levels within the MCI group. MCI progressors had lower baseline MMSE scores than MCI non-progressors ($p = 0.04$), but there was no difference in ApoE4 genotype, age and sex. MCI progressors had lower baseline levels of CSF Aβ42 and higher CSF Tau levels than MCI non-progressors ($p<0.01$). MCI patients who progressed to dementia had lower CSF SAP levels, than MCI non-progressors. (13 µg/L(3; 199); 20µg/L(7; 128), $p<0.05$). No differences in serum SAP levels were seen between MCI progressors and non-progressors. The Kaplan-Meyer curve for CSF SAP concentrations in MCI patients is presented in figure 1. After correction for sex, age and MMSE, the Cox proportional hazard model revealed that a low level of CSF SAP was associated with a twofold increased risk of progressing to dementia [HR=2.0, 95%CI(0.9; 4.6)]. Results remained essentially unchanged when the four MCI patients who converted to non-AD dementia were left out of the analysis [HR=2.2, 95%CI(0.9; 5.4)]. SAP levels in serum and CSF were found to correlate across groups ($r_s=0.48$, $p<0.01$). This was also observed within the AD, the MCI and the control group. ($r_s=0.48$, $p<0.01$; $r_s=0.43$, $p<0.01$; $r_s=0.56$, $p=0.01$). No correlation was found between CSF or serum SAP levels and CSF Aβ42, Tau, age, ApoE4 genotype or MMSE score (all $p>0.10$).

Discussion

The main finding of our study is twofold i.e. levels of SAP in CSF and serum are not discriminative across the three groups (AD patients, MCI subjects and controls), while within the MCI patient group low CSF SAP levels were associated with a twofold higher risk of progression to dementia, predominantly AD. When we cross-sectionally determined CSF SAP levels in AD patients, MCI patients and controls, no difference between the three groups was observed.

Despite the increased synthesis of SAP in AD brains as shown by McGeer et al. [204], we were unable to detect an increase in CSF SAP levels in AD and MCI cases compared to controls [204]. Possibly CSF SAP levels are not only dependent on the intra-parenchymal synthesis, but also on accumulation in Aβ plaques [52, 53] and diffusion or transport across the BBB; a subject for further study. Only three cross-sectional studies investigating CSF SAP levels in AD and controls have been reported (as summarized in Table 3). In two studies comparable CSF SAP levels were reported for AD and controls [137, 200], which is in line with the cross-sectional part of our study (comparison between AD, MCI and controls). In the third report increased levels of CSF SAP were found in AD patients in comparison to controls [202]. Differences in patient groups (size and age) or assay methods and antibodies used, may explain these contrasting results.

The use of serum SAP levels instead of CSF SAP levels as biomarker for progression to AD has several clear advantages. However, SAP concentrations in CSF are approximately 3000-fold lower than those in serum, which is mainly produced by the liver. This implies that brain-derived SAP very unlikely contributes to serum SAP levels. In this study no significant difference in serum SAP levels were found between the three groups. Serum SAP levels have also been measured in two other cross-sectional studies, one showing lower SAP levels in serum of AD patients in comparison with controls [199] whereas the other [201] showed increased serum concentrations (Table 3). The most likely reason for these differences in findings may be the group size and composition. Nishiyama et al. used relative small groups [199] and Nybo et al. [201] compared a group of cognitively impaired centenarians with age matched controls. In contrast we included relatively young controls, MCI and mild to moderate AD cases, which makes it difficult to compare our study with the two mentioned studies. Longitudinal studies in which the relation between CSF SAP levels and the clinical development of AD is investigated have not been reported yet. In this study we followed the MCI patient group for 2-3 years. The progression rate was 61% over a period of two and a half years, which is slightly higher than the rate described by Petersen [5], but comparable to that of other memory clinics [6]. The main reason for this is that because of the memory clinic setting patients suspected of having AD are referred to us, which results in an enrichment of cases prone to develop AD. We found significantly lower levels of CSF SAP in MCI patients who progressed to AD in comparison with patients who did not progress suggesting that SAP may have potential as a biomarker to predict the development of AD in patients with MCI.

Table 1

	AD	MCI	Controls	p
N	144	67	30	
Age, Mean (SD)	66 (9)	70 (9)	66 (10)	0.04
Sex, F (%)	78 (54%)	33 (51%)	17 (43%)	0.7
MMSE*, Mean (SD)	21 (5)	26 (3)	29 (1)	<0.01 [a, b, c]
ApoE4**				
Positive (%)	111 (77%)	35 (52%)	4 (13%)	<0.01 [a, b, c]
SAP CSF, µg/L				
Median (min, max)	14 (2 ; 170)	15 (3; 199)	15 (4; 109)	0.7
SAP-serum, mg/L				
Median (min, max)	50 (20 ; 147)	51 (25; 73)	51 (27; 87)	0.6
Aβ42, pg/ml				
Median (min, max)	384 (124 ; 1320)	441 (178; 1450)	812 (494; 1142)	<0.01 [a, b, c]
Tau, pg/ml***				
Median (min, max)	685 (75 ; 2615)	529 (92; 3515)	288 (129; 608)	<0.01 [a, b, c]

*Cross-sectional demographic and clinical information of the three groups. To analyze the differences within the three groups we used Chi-square in %; ANOVA in mean (SD); Kruskal-Wallis in median (max, min). Post-hoc results: a=AD versus MCI<0.01; b=AD versus controls p<0.01; c=Controls versus MCI p<0.01. * Only available for 228 subjects; ** Only available for 219 subjects; *** Only available for 235 subjects.*

Table 2

	MCI Non-progressors	MCI Progressors	p
N	25	39	
Age, Mean (SD)	68 (11)	70 (8)	0.6
Sex, F (%)	9 (36%)	23 (61%)	0.06
MMSE, Mean (SD)	27(2)	25 (3)	0.04
ApoE4 Positive (%)	10 (40%)	24 (62%)	0.08
SAP CSF, µg/L Median (min, max)	20 (7; 128)	13 (3 ; 199)	<0.05
SAP-serum, mg/L Median (min, max)	53 (25 ; 73)	49 (35 ; 70)	0.226
Aβ42, pg/ml Median (min, max)	570 (221 ; 1450)	408 (178 ; 1148)	<0.01
Tau, pg/ml* Median (min, max)	413 (92; 1156)	667 (165; 3515)	<0.01

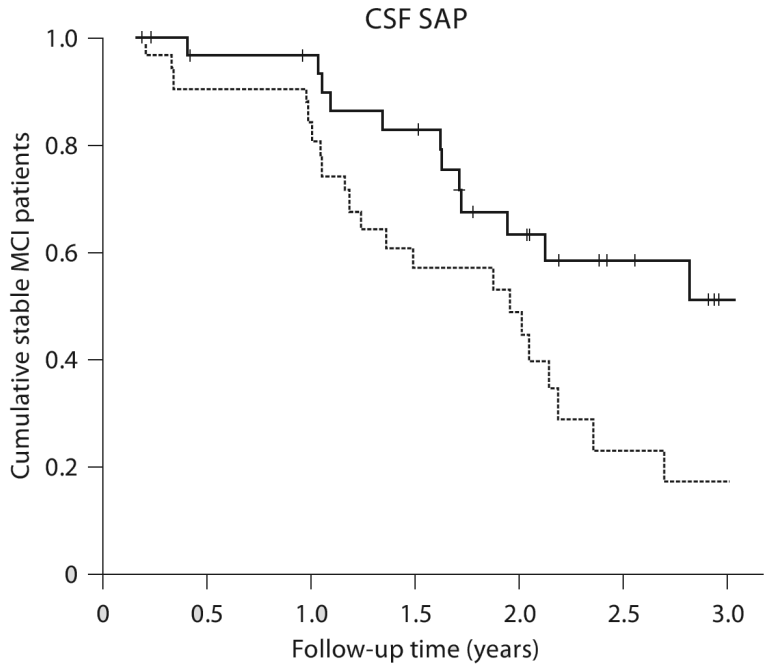
*Demographic and clinical information of MCI group after follow up. To analyze the differences within the two groups we used Fisher exact test in %; Independent t-test in mean (SD); Mann-Whitney test in median (max, min). *Only available for 63 subjects. SD=standard deviation; MMSE=mini mental state examination; Aβ42=amyloid-beta 1-42; SAP=serum amyloid P component; CSF=cerebrospinal fluid; MCI=mild cognitive impaired patients.*

Table 3

		AD	Controls	Cognitively impaired centenarians	MCI	Method	Antibodies	Results
Kimura et al. 1999	N CSF ng/ml	72 3.7 (3.5)	9 4.0 (4.0)			Fluid Phase RIA	Capture: Rabbit Anti-SAP antibody Detection: Anti-Rabbit IgG (Calbiochem)	No difference
Mulder et al. 2002	N CSF ng/ml	20 60 (41)	20 50 (34)			ELISA	Capture: monoclonal anti-hum. SAP (aSAP-14) Detection: rabbit anti-hum. SAP (PaSAP)	No difference
Hawkins et al.1994	N CSF ng/ml	51 12.8 (10.9)	50 8.5 (8.4)			Solid phase RIA	Sheep anti-SAP antiserum 125I- labelled Sheep anti-SAP antiserum	↑SAP in AD.
Nybo et al.1998	N Serum µg/ml		41 32.8 (11.4)	41 48.3 (16.9)		Rocket Electroph.	Rabbit anti-human SAP IgG (DAKO)	↓ SAP in Controls.
Nishiyama et al. 1996	N Serum µg/ml	16 22.4 (7.0)	16 34.4 (6.6)			ELISA	Capture: goat anti-rabbit IgG (Seikagaku) and rabbit anti AP antiserum (DAKO®) Detection: HRP-conjugated rabbit anti AP antiserum (DAKO®)	↑ SAP in Controls.
This study	N CSF ng/ml Serum µg/ml	144 19.0 (17.2) 50.9 (15.5)	30 21.0 (20.1) 53.0 (14.0)		67 25.0 (30.5) 50.9 (11.0)	ELISA	Capture: Polycl. Rabbit Anti-Hum. SAP antisera (DAKO®) Detection: Biotinylated Rabbit Anti-Hum. SAP antisera (DAKO®)	 No difference No difference

Summary of published cross-sectional studies in which CSF and serum SAP are measured in patients. CSF=cerebrospinal fluid; MCI=mild cognitively impaired patients; AD=Alzheimer's disease; SAP=Serum amyloid P component; CSF and serum SAP levels: mean (SD).

Figure 1



Kaplan Meyer curves for CSF SAP. CSF SAP levels were dichotomized using median split. Straight lines indicate patients with high levels of SAP. Dotted lines indicate low levels of SAP. The Cox proportional hazard model showed an HR=2.0 95%*CI(0.9; 4.6); CSF=cerebrospinal fluid; MCI=mild cognitively impaired patients; AD=Alzheimer's disease.*

4.3
Evaluation of plasma Aβ40 and Aβ42 as predictors of conversion to AD in MCI patients

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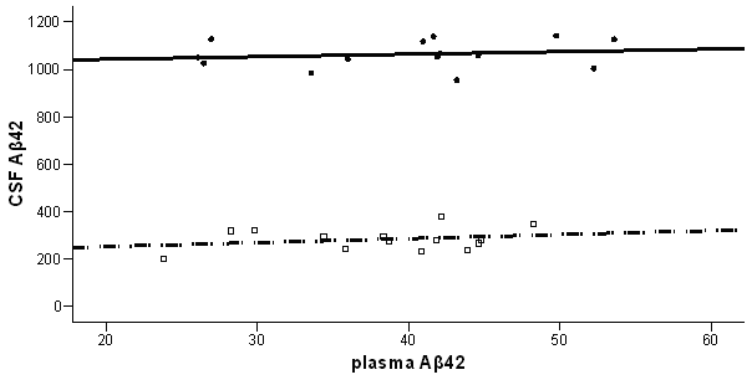
To the editor

We read with great interest the article of Hansson et al. in which a new plasma amyloid beta luminex test was used for evaluation of plasma amyloid beta 1-40 (Aβ40) and 1-42 (Aβ42) as predictors for conversion to Alzheimer's disease in patients with mild cognitive impairment [205]. In their study in MCI patients it was not possible to show a difference in plasma Aβ40 and Aβ42 levels between converting and stable patients. Furthermore, they found no correlation between amyloid beta levels in plasma and CSF. These findings are in line with our results. We have used the same test in a preliminary cross-sectional setting to differentiate controls from patients with Alzheimer's disease (AD) by determining Aβ40 and Aβ42 plasma levels. We used plasma samples from 14 AD patients (64F, age 66±8, MMSE 20±4) and 14 controls (42F, age 57±6, MMSE 28±2). Diagnosis was made according to NINCDS-ADRDA criteria for probable AD [1]. In all patients CSF Aβ42 and Tau were determined (Innotest® Aβ42, Innotest® hTau Ag, Innogenetics, Ghent, Belgium), as previously described [206]. Only AD patients with a pertinent AD CSF-profile (Aβ42<400 pg/ml, Tau >650pg/ml) and controls with a normal CSF-profile (Aβ42>900pg/ml, Tau <450 pg/ml) were selected. Plasma Aβ40 and Aβ42 levels were measured with Inno®-BIA plasma Aβ test, generously made available by Innogenetics. The plasma biomarker levels were compared between controls and AD patients with Mann-Whitney U tests. Correlations between plasma and CSF levels were analyzed with Spearman correlations. There was no difference in the values of plasma Aβ40 or Aβ42 or Aβ42/ Aβ40 ratio between controls and AD patients: Aβ40 192(124-231) vs 212(88-255)pg/ml; p=0.27, Aβ42 42(26-54) vs 40(24-48), p=0.65; Aβ42/ Aβ40 0.20(0.13-0.29) vs 0.19(0.11-0.32), p=0.43. There was no correlation between plasma and CSF levels of Aβ42 (Aβ42 for controls r=0.19, p=0.52 and for AD r=0.11, p=0.70; figure). Even in this sample of overt AD patients and controls, that show no overlap in CSF Aβ42, we could not observe any discriminating value in plasma Aβ42 or Aβ40. In analogy with the findings in MCI patients of Hansson et al., it seems that the clinical relevance of this plasma Aβ test is also limited for differentiation between patients with Alzheimer's disease and controls, especially compared to the discriminative power of the CSF biomarkers.

Figure 1



Plasma Aβ42 levels by CSF Aβ42 levels. Solid line and filled circles represent controls; broken line and open squares represent patients with AD.