

RESEARCH ARTICLE

Multi-cohort profiling reveals elevated CSF levels of brain-enriched proteins in Alzheimer's disease

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Abstract

Objective: Decreased amyloid beta (A β) 42 together with increased tau and phospho-tau in cerebrospinal fluid (CSF) is indicative of Alzheimer's disease (AD). However, the molecular pathophysiology underlying the slowly progressive cognitive decline observed in AD is not fully understood and it is not known what other CSF biomarkers may be altered in early disease stages. **Methods:** We utilized an antibody-based suspension bead array to analyze levels of 216 proteins in CSF from AD patients, patients with mild cognitive impairment (MCI), and controls from two independent cohorts collected within the AETI-ONOMY consortium. Two additional cohorts from Sweden were used for biological verification. **Results:** Six proteins, amphipysin (AMPH), aquaporin 4 (AQP4), cAMP-regulated phosphoprotein 21 (ARPP21), growth-associated protein 43 (GAP43), neurofilament medium polypeptide (NEFM), and synuclein beta (SNCB) were found at increased levels in CSF from AD patients compared with controls. Next, we used CSF levels of A β 42 and tau for the stratification of the MCI patients and observed increased levels of AMPH, AQP4, ARPP21, GAP43, and SNCB in the MCI subgroups with abnormal tau levels compared with controls. Further characterization revealed strong to moderate correlations between these five proteins and tau concentrations. **Interpretation:** In conclusion, we report six extensively replicated candidate biomarkers with the potential to reflect disease development. Continued evaluation of these proteins will determine to what extent they can aid in the discrimination of MCI patients with and without an underlying AD etiology, and if they have the potential to contribute to a better understanding of the AD continuum.

Introduction

The Alzheimer's disease (AD) pathogenesis is believed to start up to 20 years before the onset of symptoms. The neuropathological hallmarks of AD include plaques composed of amyloid beta and tangles composed of hyperphosphorylated tau.¹ During the progression of the disease, a widespread loss of structure and function of neurons is observed throughout the brain, originating from the medial temporal lobe. An early diagnosis is already today of importance and will be even more crucial in the future, as it may enable the delivery of disease-modifying treatments before the neuronal damage has become widespread.

The diagnosis of AD is made based on the clinical evaluation of the individual and might be combined with measurements of three cerebrospinal fluid (CSF) protein markers.² These core AD markers are total tau (t-tau), phosphorylated tau (p-tau), and the 42 amino acid form of amyloid β (A β 42).^{3–5} However, the CSF proteome contains a large number of proteins with the potential to reflect several ongoing biological processes, such as synaptic dysfunction^{6–8} and additional biomarkers could contribute to an earlier and more precise AD diagnosis.

AD is a slowly progressive disorder, with all patients undergoing a phase of mild cognitive impairment (MCI) that comprises problems related to memory, language or judgment. These symptoms are greater than the changes noticed during normal aging and the condition is often referred to as an early stage of AD. To understand which MCI individuals will develop AD dementia, and therefore could be considered for future early treatment, this group needs to be further investigated on a molecular level. Concentrations of the core CSF AD markers show high sensitivity and specificity in predicting conversion from MCI to AD dementia,⁹ but additional markers are still needed to complement these measurements. Although previous efforts have been made to identify additional proteins with altered levels in the MCI group, further evaluation and identification of such potential markers is necessary.^{6,10–13}

Herein, we investigated alterations of CSF levels of brain-enriched proteins in patients with MCI and AD dementia with the aim to investigate alterations between protein levels in early disease stages. An antibody-based suspension bead array was initially used to profile 216 proteins in 354 CSF samples from two independent cohorts. Two additional cohorts consisting of 163 CSF samples were also analyzed for further verification of the obtained protein profiles.

Methods

In this study, an antibody-based suspension bead array was used for the analysis of CSF protein levels. The

method enables a high throughput analysis of samples and measurements of hundreds of proteins in parallel. The targeted discovery study included the analysis of 216 proteins in two independent cohorts with a total of 354 CSF samples followed by the analysis of two additional cohorts for biological verification.

Samples

The CSF samples analyzed in the screening phase of this study were from two independent cohorts collected as part of the AETIONOMY consortium. Cohort 1 was collected at the Hospital Clínic de Barcelona/ Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS) in Barcelona, Spain and cohort 2 was collected at Universitätsklinikum Bonn (UKB) in Bonn, Germany. The cohorts consisted of individuals diagnosed with AD dementia, MCI, and control subjects. The MCI group in cohorts 1 and 2 was divided into subgroups according to the local cut-off values of CSF concentrations of A β 42 and tau. Individuals with a concentration below 550 pg/ml were denoted A+ and individuals with a concentration of t-tau above 450 pg/ml or p-tau above 65 pg/ml were denoted T+ according to previous definitions.¹³ Sample demographics are presented in Table 1.

Two additional cohorts (cohort 3 and cohort 4) were also analyzed to verify the obtained protein profiles. Cohort 3 consisted of CSF samples collected by lumbar puncture at the Sahlgrenska University Hospital in Gothenburg, Sweden. The cohort included AD patients, preclinical AD, non-AD MCI, and controls. Cohort 4 included CSF samples collected at Uppsala University Hospital in Uppsala, Sweden, and included AD patients, MCIA, MCIN, and controls. Sample demographics for cohorts 3 and 4 are presented in Table 2. More information about the four cohorts are presented in supplementary materials.

Suspension bead array assay

The protein content of the CSF samples was directly labeled with biotin as described previously.^{14,15} The proteins ($n = 216$) were carefully selected either based on potential association to AD according to literature or by previously unpublished and published internal neuroproteomic efforts¹⁶ with a focus on proteins with brain-enriched mRNA levels.¹⁷ The used antibody set was polyclonal rabbit antibodies generated within the Human Protein Atlas project (www.proteinatlas.org). The antibodies were coupled onto carboxylated color-coded magnetic beads (MagPlex-C, Luminex Corporation) using EDC-NHS chemistry with one bead identity corresponding to a

Table 1. Sample demographics in cohorts 1 and 2.

Cohort 1: Hospital Clínic de Barcelona/ Institut d'Investigació Biomèdica August Pi i Sunyer				
	Total	AD	MCI	Control
Number of individuals [N]	134	67	44	23
Sex distribution [F/M]	81/53 ^a	40/27	24/20	17/6
Age	63 ^b (45-82)	62 (50-82)	68 (49-79)	56 (45-78)
[median years (range)]				
t-tau (pg/ml)	541 (98-2640)	627 (270-2640)	548 (98-1213)	205 (125-308)
[median (range)]				
p-tau (pg/ml)	79 (16-331)	86 (44-331)	86 (16-156)	46 (31-79)
[median (range)]				
Aβ42 (pg/ml)	429 (184-1303)	394 (184-803)	396 (185-1261)	892 (606-1303)
[median (range)]				
APOE ε4 alleles	57/51/12/14	28/27/3/9	13/17/9/5	16/7/0/0
[0/1/2/NA]				
MMSE	24 (7-30) 122	19 (7-30) 58	27 (21-30) 41	29 (23-30) 23
[median (range) N]				
Cohort 2: Universitätsklinikum Bonn				
	Total	AD	MCI	Control
Number of individuals [N]	220	103	77	40
Sex distribution [F/M]	89/131 ^a	51/52	26/51	12/28
Age	73 ^b (43-92)	74 (50-92)	72 (49-86)	68 (43-81)
[median years (range)]				
t-tau (pg/ml)	539 (94-2270)	661 ^c (142-2270)	450 (139-1254)	271 (94-1210)
[median (range)]				
p-tau (pg/ml)	66 (18-213)	79 ^c (23-213)	60 (24-158)	43 (18-157)
[median (range)]				
Aβ42 (pg/ml)	409 (104-1653)	343 ^c (104-1110)	457 (120-1326)	576 (176-1653)
[median (range)]				
APOE ε4 alleles	53/73/19/75	24/40/12/27	23/31/6/17	6/2/1/31
[0/1/2/NA]				
MMSE	25 (6-30) 197	22 (6-29) 99	27 (21-30) 70	30 (27-30) 28
[median (range) N]				

^aThe sex distribution was not significantly different (by Fisher's exact test) between the sample groups in cohort 1, but a significant difference was observed in cohort 2 ($p = 0.04$). Details in Figure S1.

^bThe age distribution was significantly different (by Wilcoxon rank-sum test) between a number of sample groups. Details in Figure S1.

^cTwo AD patients in cohort 2 had CSF levels of both Aβ42 and tau within the normal range.

certain antibody, as described previously.^{15,18,19} The different bead IDs were subsequently pooled together to form the suspension bead array.

The antibody-based suspension bead array assay procedure was performed as described previously.^{14,15} In short, the labeled samples were further diluted 1/8, heat-treated at 56°C for 30 min before incubation overnight with the antibody-coupled beads. A streptavidin-conjugated fluorophore (Streptavidin R-Phycoerythrin Conjugate, Invitrogen, diluted 1:750 in PBS with 0.05% Tween) was added to enable the detection of captured proteins. The readout was performed in a FLEXMAP 3D instrument (Luminex Corporation) where binding events were displayed as relative fluorescence intensity. Cohort 3 and cohort 4 analyses were performed in a study partly

reported previously.²⁰ All samples included in the same cohort were analyzed on the same assay plate.

More information about the sample processing and labeling and details about the development of sandwich assays can be found in supplementary materials.

Data analysis

The open-source software R (version 4.0) was used for data processing and visualizations,²¹ mainly using functions from the collection of packages within *tidyverse*.²² The data were processed by a position-based normalization to diminish the effects of delay time during read out using robust linear regression (*rlm*, MASS²³), where the median signal intensity per protein was added to the

Table 2. Sample demographics in cohorts 3 and 4.

Cohort 3: Sahlgrenska University Hospital					
	Total	AD	Preclinical AD	Non-AD MCI	Control
Number of Individuals [N]	90	43	14	10	23
Sex distribution [F/M]	58/32 ^a	28/15	10/4	8/2	12/11
Age [median (range)]	82 ^b (44-102)	81 (53-102)	85 (73-96)	85 (56-93)	79 (44-91)
t-tau (pg/ml) [median (range)]	625 (171-3178)	834 (490-3178)	821 (565-1092)	282 (172-367)	308 (171-399)
p-tau (pg/ml) [median (range)]	62 ^c (26-179)	86 ^c (59-179)	95 (78-131)	36 (26-46)	47 (29-60)
Aβ42 (pg/ml) [median (range)]	530 (244-1192)	453 (260-639)	416 (244-518)	754 (570-913)	706 (559-1192)
Cohort 4: Uppsala University Hospital					
	Total	AD	MCIA	MCIN	Control
Number of Individuals [N]	73	29	12	17	15
Sex distribution [F/M]	41/32 ^a	18/11	4/8	8/9	11/4
Age [median (range)]	70 ^b (40-85)	72 (51-85)	66 (51-78)	64 (40-84)	71 (44-77)
t-tau (pg/ml) [median (range)]	455 ^{d,e} (170-2430)	600 ^{e,f} (210-2430)	430 (170-950)	320 (200-910)	NA
p-tau (pg/ml) [median (range)]	64 ^{d,e} (27-282)	78 ^{e,f} (34-282)	65 (27-128)	51 (31-118)	NA
Aβ42 (pg/ml) [median (range)]	415 ^{d,e} (160-1130)	350 ^{e,f} (160-950)	340 (170-440)	720 (480-1130)	NA

^aThe sex distribution was not significantly different (by Fisher's exact test) between the sample groups. Details in Figure S1.

^bThe age distribution was significantly different (by Wilcoxon rank-sum test) between a number of sample groups. Details in Figure S1.

^cEight AD patients in cohort 3 were missing data on p-tau concentration

^dNo data available for the control group in cohort 4

^eTwo AD patients in cohort 4 were missing data on t-tau, p-tau, and Aβ42 concentration

^fThree AD patients in cohort 4 had CSF levels of both Aβ42 and tau within the normal range.

obtained residuals. Furthermore, a second normalization step was performed in order to reduce differences between different 96-well plates.²⁴ The differences of protein levels between diagnostic groups were evaluated by Wilcoxon rank-sum test (*wilcox.test*, *stats*) where a $p < 0.05$ was regarded as significant.

Correlations between clinically measured core AD markers and the herein measured relative protein levels were calculated using nonparametric Spearman's correlation coefficients (*cor*, *stats*). Correlations between the AD conversion time and protein levels as well as the correlation between and relative protein levels obtained using the single binder assay and the sandwich assay were calculated using Pearson correlation coefficients (*cor*, *stats*). Correlations $p < 0.05$ were regarded as significant.

Results

An antibody-based suspension bead array was used to obtain protein profiles from 216 proteins in CSF samples

from individuals with either AD dementia, MCI or controls with the aim to investigate alterations between protein levels in early disease stages.

Disease-associated proteins in cohorts 1 and 2

Six proteins, out of the 216 proteins studied, were identified with reproducible significant differences ($p < 0.05$) in CSF between AD dementia patients and controls in both cohorts 1 and 2 (Table 3). All six proteins, amphiphysin (AMPH), aquaporin 4 (AQP4), cAMP-regulated phosphoprotein 21 (ARPP21), growth-associated protein 43 (GAP43), neurofilament medium polypeptide (NEFM, also known as NfM), and synuclein beta (SNCB), were found at higher levels in AD dementia patients compared with controls (Fig. 1). Comparing individuals with MCI and controls revealed significantly increased levels of SNCB in CSF from MCI patients in both cohorts. In addition, the other five proteins with significantly

Table 3. Proteins present at altered levels in comparisons of Alzheimer's disease (AD), mild cognitive impairment (MCI), and controls.

HGNC ID	Antibody	Protein name	Uniprot ID	AD versus Control		MCI versus Control		AD versus MCI	
				Cohort 1	Cohort 2	Cohort 1	Cohort 2	Cohort 1	Cohort 2
AMPH	HPA019829	Amphiphysin	P49418	2E-02	1E-02	6E-03	ns	ns	ns
AQP4	HPA014784	Aquaporin 4	P55087	1E-03	3E-03	2E-03	ns	ns	ns
ARPP21	HPA017303	cAMP-regulated phosphoprotein 21	Q9UBL0	4E-04	2E-02	8E-03	ns	ns	ns
GAP43	HPA013603	Growth-associated protein 43	P17677	2E-03	5E-03	7E-04	ns	ns	ns
NEFM	HPA022845	Neurofilament medium	P07197	6E-07	2E-02	5E-06	ns	4E-02	2E-02
SNCB	HPA035876	Synuclein beta	Q16143	6E-03	2E-03	6E-03	3E-02	ns	ns

different CSF levels between the AD and control groups in both cohorts also showed a significant difference between MCI and control group in cohort 1 but not in cohort 2 (Table 3, Fig. 1). However, concordant trends were observed in cohort 2 for AMPH ($p = 0.069$), AQP4 ($p = 0.065$) and GAP43 ($p = 0.0501$) with higher levels in CSF from MCI individuals compared with controls. NEFM was the only protein with significantly higher levels in AD compared with MCI CSF (Fig. 1); the lowest levels were measured in CSF from the control group.

Stratification of the MCI group based on pathological AD biomarker levels

The MCI group in cohorts 1 and 2 was further divided into subgroups based on the CSF concentration of A β 42 and tau in order to further explore this heterogeneous group. Most MCI patients ($n = 40/44$) in cohort 1 had abnormal A β 42 levels and were therefore only divided based on tau levels. We identified 34 MCI individuals with levels of t-tau above 450 pg/ml or p-tau levels above 65 pg/ml (denoted MCI T+) and 10 individuals that displayed both t-tau and p-tau within the normal range (MCI T-). The MCI individuals were divided into four groups in cohort 2. We identified 27 individuals with abnormal A β 42 and tau levels (MCI A+T+), 14 individuals with high tau levels but A β 42 within the normal range (MCI A-T+), 19 individuals with abnormal A β 42 levels but normal tau levels (MCI A+T-) and finally 17 individuals with both A β 42 and tau within the normal range (MCI A-T-) (Table S1).

The p-values for group comparisons of AMPH, AQP4, ARPP21, GAP43, NEFM, and SNCB protein levels after stratifying the MCI group are visualized in a heatmap in Fig. 2 and listed in Table S2. The group of MCI patients with pathological tau concentrations (MCI T+) had significantly higher levels of all six proteins in cohort 1 compared with the controls. An illustrative example of GAP43 is presented in Fig. 2. The same pattern could be seen in cohort 2 where both MCI A+T+ and MCI A-T+ individuals had significantly higher levels of AMPH, AQP4,

ARPP21, GAP43, and SNCB compared with controls. However, we did not observe any differences between the MCI group and controls for NEFM in cohort 2, regardless of how the MCI group was stratified. Furthermore, we did not observe any significant differences between the MCI individuals with normal A β 42 levels and normal tau levels (MCI A-T-) and controls.

We next investigated the correlation between the six measured protein levels and CSF concentrations of t-tau, p-tau, and A β 42 in order to elucidate their potential association. We assessed the correlation both for all samples per cohort as well as per sample group. A strong correlation was observed between the levels of GAP43 in AD patients and the concentration of t-tau and p-tau. These results were identified in both cohorts (t-tau; $\rho = 0.86$ in both cohorts, p-tau; $\rho = 0.88$ in cohort 1, $\rho = 0.90$ in cohort 2). In addition to GAP43, strong to moderate correlations with t-tau and p-tau in the AD patients were identified in both cohorts 1 and 2 for SNCB, AMPH, AQP4, and ARPP21. However, protein levels of NEFM in AD patients showed weak correlations with both p-tau and t-tau compared to the other proteins ($\rho < 0.3$) (Table 4). The same pattern was observed in the MCI individuals but the correlation with t-tau and p-tau were in general slightly weaker compared to the correlations obtained from the AD patients, although not for all proteins. The correlations between GAP43 levels and the concentration of t-tau and p-tau in MCI individuals were $\rho = 0.73$ and $\rho = 0.78$, respectively, in cohort 1 and the same pattern was seen in cohort 2 (Fig. 2 and Table 4). The control group did also display moderate to strong correlations with t-tau and p-tau for most proteins (Table 4). The correlation between the six studied proteins and A β 42 was weaker than the correlation seen for both t-tau and p-tau. However, we observed a significant but weak to moderate correlation with A β 42 for the AD group for all six proteins in both cohorts, except for NEFM in cohort 2. Next, we investigated the correlation between the three core CSF AD biomarkers. A strong correlation was identified between t-tau and p-tau ($\rho > 0.90$ when including all samples), but the correlation

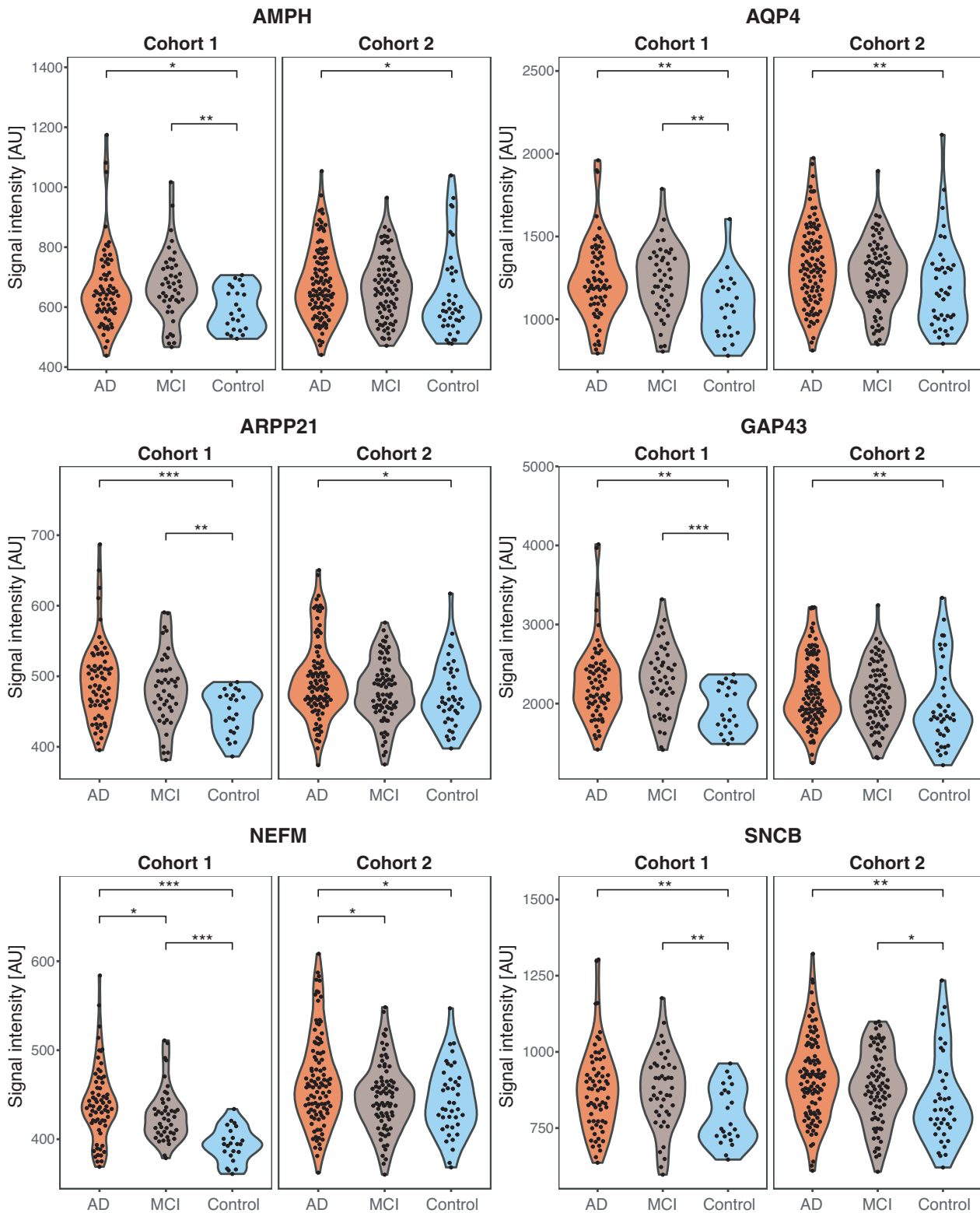


Figure 1. Protein profiles in CSF for the six proteins in cohorts 1 and 2. The protein profiles are visualized per sample group for all analyzed samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Wilcoxon rank-sum test.

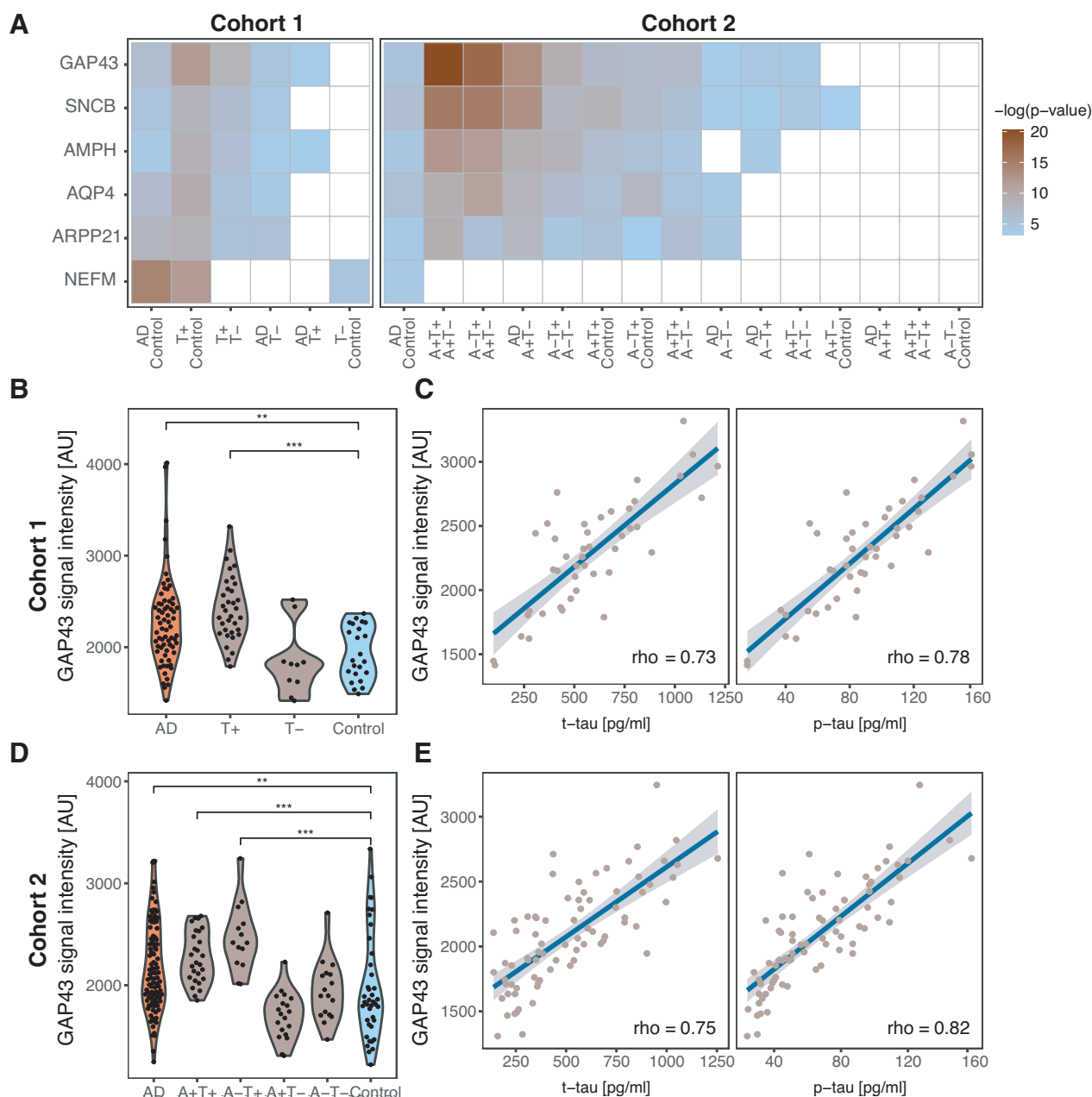


Figure 2. (A) Heatmap per cohort for p-values obtained per protein for the comparison between each sample group. White color indicates a p-value above 0.05. (B) Protein profiles of GAP43 in cohort 1 when the MCI group is divided based on the levels of t-tau and p-tau. (C) Correlations between the protein levels of GAP43 and t-tau or p-tau, respectively, for the MCI individuals in cohort 1. (D) Protein profiles of GAP43 in cohort 2 when the MCI group is divided based on the levels of t-tau, p-tau, and A β 42. (E) Correlations between the protein levels of GAP43 and t-tau or p-tau, respectively, for the MCI individuals in cohort 2. Significant differences between the control group and the remaining sample groups are indicated in (B) and (D) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Wilcoxon rank-sum test.

between both t-tau and p-tau with A β 42 was weak, as expected (data not shown). Furthermore, we investigated the potential association between protein levels and apolipoprotein E (APOE) ϵ 4 carrier status, results are presented in supplementary materials.

Biological verification in two additional cohorts

To further investigate the obtained protein profiles, we analyzed levels of the six proteins in two additional

Table 4. The correlation between the levels of AMPH, AQP4, ARPP21, GAP43, NEFM, and SNCB with t-tau, p-tau, and Aβ42. The correlations are presented per cohort for all samples, but in addition also per sample group. The shading corresponds to the correlation where a darker green indicates a stronger correlation. A correlation with a p-value above 0.05 was regarded as non-significant and denoted ns.

			AMPH	AQP4	ARPP21	GAP43	NEFM	SNCB
Cohort 1	t-tau	All	0.65	0.63	0.65	0.76	0.48	0.66
		AD	0.71	0.59	0.59	0.86	ns	0.71
		MCI	0.67	0.63	0.67	0.73	ns	0.67
		Control	0.75	0.70	0.54	0.84	ns	0.77
	p-tau	All	0.75	0.71	0.69	0.84	0.47	0.75
		AD	0.76	0.62	0.63	0.88	0.28	0.74
		MCI	0.73	0.70	0.70	0.78	0.35	0.76
		Control	0.84	0.76	0.59	0.89	ns	0.87
	Aβ42	All	ns	ns	ns	ns	ns	ns
		AD	0.48	0.56	0.38	0.49	0.28	0.48
		MCI	ns	ns	ns	ns	ns	ns
		Control	0.54	ns	ns	0.53	ns	0.52
Cohort 2	t-tau	All	0.71	0.67	0.59	0.78	0.29	0.77
		AD	0.77	0.72	0.62	0.86	0.29	0.80
		MCI	0.66	0.62	0.55	0.75	0.26	0.76
		Control	0.65	0.60	0.54	0.72	ns	0.73
	p-tau	All	0.79	0.70	0.64	0.86	0.25	0.83
		AD	0.81	0.72	0.63	0.90	0.26	0.83
		MCI	0.73	0.63	0.59	0.82	ns	0.80
		Control	0.84	0.61	0.68	0.90	ns	0.91
	Aβ42	All	0.15	ns	ns	ns	-0.16	ns
		AD	0.44	0.47	0.29	0.41	ns	0.39
		MCI	0.26	ns	ns	0.25	ns	0.26
		Control	ns	ns	ns	ns	-0.38	ns

Table 5. Significant p-values for comparison of sample groups in cohort 3 and cohort 4. All group comparisons not displayed were non-significant for all six proteins.

HGNC ID	Antibody	Cohort 3				Cohort 4		
		AD versus Control	AD versus non-AD MCI	preclinical AD versus non-AD MCI	preclinical AD versus Control	AD versus Control	AD versus MCIA	AD versus MCIN
AMPH	HPA019829	5E-04	1E-03	9E-04	4E-04	ns	ns	ns
AQP4	HPA014784	3E-04	3E-04	5E-05	1E-05	1E-02	4E-02	ns
ARPP21	HPA017303	3E-05	2E-03	2E-02	4E-03	3E-02	ns	ns
GAP43	HPA013603	1E-06	7E-05	5E-05	2E-05	2E-02	ns	ns
NEFM	HPA022845	3E-04	ns	ns	ns	2E-02	ns	1E-03
SNCB	HPA035876	3E-07	7E-05	2E-04	5E-06	ns	ns	ns

cohorts; cohorts 3 and 4. Results on GAP43 were presented in a previous publication²⁰ and an adaption of the results based on an updated data normalization method is shown here. Higher protein levels were identified in AD patients compared to controls for all six proteins in cohort 3. Furthermore, four proteins (AQP4, ARPP21, GAP43, and NEFM) had significantly increased levels in AD patients compared with controls in cohort 4 (Table 5).

Cohort 3 included individuals with preclinical AD and non-AD MCI, in addition to AD and control subjects.

Significantly higher levels of AMPH, AQP4, ARPP21, GAP43, and SNCB were observed in AD patients compared to non-AD MCI. In addition, the group of individuals with preclinical AD had significantly higher levels of the five proteins compared to both non-AD MCI as well as controls. NEFM, moreover, only had significant differences between AD individuals and controls. See Fig. 3 for the protein profile of AQP4 and NEFM, and Fig. S3 for AQP4, ARPP21, GAP43, and SNCB.

Cohort 4 included MCIA and MCIN individuals in addition to AD and control subjects. We observed

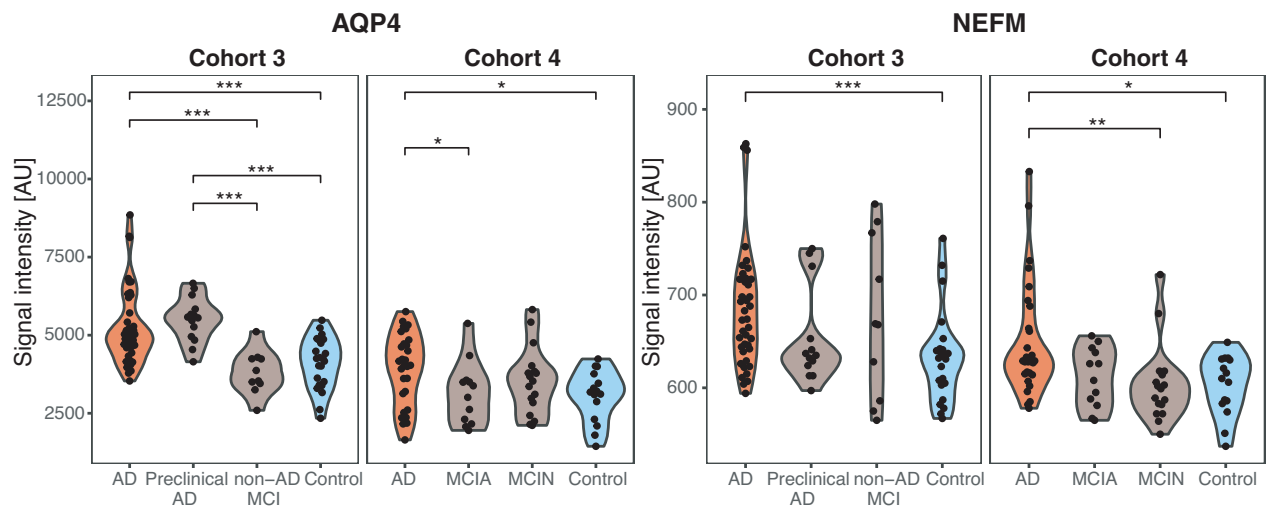


Figure 3. Protein profiles for AQP4 and NEFM in cohorts 3 and 4. Three samples in cohort 3 were removed for visualization purposes for NEFM. The signal intensity and group for those samples were: 969 (AD), 1120 (preclinical AD), and 1258 (AD).

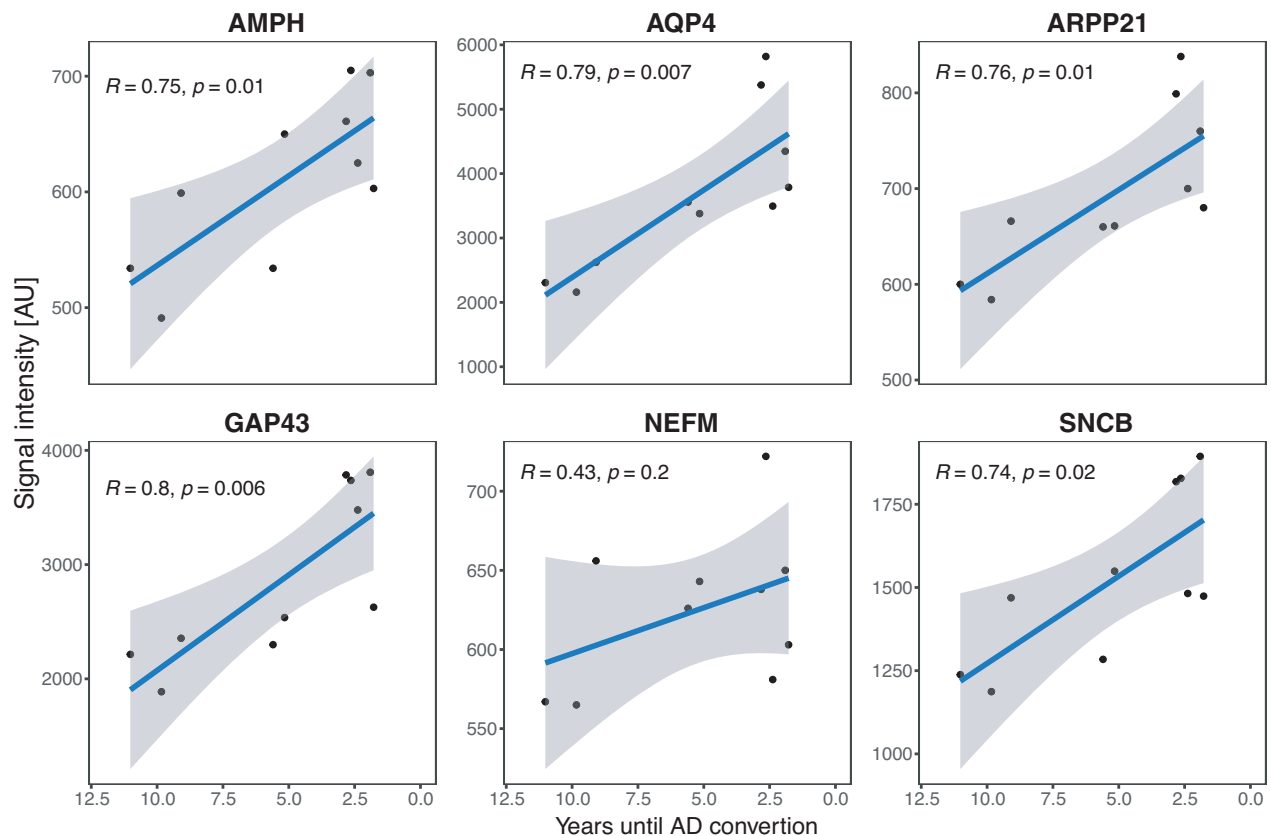


Figure 4. Correlation between protein levels for the six analyzed proteins and years until conversion in cohort 4.

significantly higher levels of AQP4 in the AD group compared to MCIA and higher levels of NEFM in the AD group compared to MCIN. Apart from these alterations

and the differences observed between AD and control, no significant differences were observed between the groups in cohort 4 (Fig. 3 and Table 5).

Next, the association between the obtained protein levels and time until conversion to AD was investigated for individuals in the MCIN and MCIA groups. This information was available for 18 of the 29 MCI individuals, and ten of them had converted to AD after sampling, with conversion times ranging from less than 2 years up to 11 years after the sample collection. We observed a correlation between the levels of AMPH, AQP4, ARPP21, GAP43, and SNCB and the number of years left until conversion (Fig. 4), with higher protein levels in the individuals closer to AD conversion. NEFM did not display a significant correlation with conversion time.

Technical validation using sandwich assays

Sandwich assays were developed for AMPH, AQP4, ARPP21, and SNCB using combinations of polyclonal antibodies (S Table 3). The correlation between the single binder assay and respective sandwich assay was $R = 0.87$ for AMPH, $R = 0.85$ for AQP4, $R = 0.53$ for ARPP21 and $R = 0.93$ for SNCB (Fig. S4).

Discussion

This study aimed to increase the knowledge about how brain-enriched proteins can reflect disease processes within the AD continuum. To accomplish this, an affinity-based proteomics approach was used to analyze 216 proteins in CSF samples from two independent screening cohorts (cohort 1 and 2). Six potential markers, AMPH, AQP4, ARPP21, GAP43, NEFM, and SNCB were found with increased CSF levels between AD dementia and controls. This pattern could be replicated in two verification cohorts (cohort 3 and 4), except for AMPH and SNCB with higher but not significantly higher levels in AD dementia in cohort 4. A stratification of the MCI group in cohorts 1 and 2 based on the concentrations of the core AD CSF markers revealed higher levels of AMPH, ARPP21, AQP4, GAP43, and SNCB already in the that the MCI individuals with abnormal tau levels compared to controls. In addition, we observed strong to moderate correlations between these five proteins and the levels of t-tau as well as p-tau.

According to the Human Protein Atlas, AMPH, GAP43, NEFM and SNCB are all proteins of which the corresponding genes have an enriched expression in the brain, compared to 31 other tissues, while AQP4 and ARPP21 have high expression in 2-3 tissues, including the brain.²⁵ According to the recently compiled Brain Atlas,²⁶ ARPP21 has a regionally enriched expression in basal ganglia. No regional brain specificity was identified for the other five proteins.

GAP43, SNCB, and AMPH are all presynaptic proteins and increased levels of synaptic markers in CSF likely reflect synaptic degradation in the brain. Synaptic degradation is widely recognized as an early feature of AD and has been associated with cognitive dysfunction in AD patients.²⁷ Markers reflecting synaptic dysfunction and loss would be useful to improve differential diagnosis and several synaptic markers, such as NRG1 and SNAP25, have previously been studied in the context of AD.^{20,28,29} GAP43 and SNCB have also been studied within AD before, and the patterns found in our study are in concordance with previous publications.^{30,31} GAP43 is important for neuronal growth and synaptic plasticity³² and was observed to be specifically increased in AD compared with several other neurodegenerative disorders by Sanderius *et al.*³⁰ Other neurodegenerative disorders, such as corticobasal syndrome and different types of primary progressive aphasia, were, however, not significantly different from AD in these studies. The lack of difference was not surprising since individuals suffering from these conditions often have underlying AD pathology.

The physiological function of SNCB still needs further investigations, as the protein has so far mainly been studied in the context of alpha-synucleinopathies. In our study, we observed an increase of SNCB in both AD and MCI CSF compared with controls. However, increased levels of SNCB do not seem to be a marker for general neurodegeneration as it has not been observed in other neurodegenerative disorders such as Parkinson's disease (PD),³³ similarly to what is known about other synaptic proteins, for example, NRG1.²⁸ In a recent study, Oeckl *et al.* investigated the levels of SNCB in an AD context using quantitative mass spectrometry in three different cohorts.³¹ They observed a significant increase of SNCB in AD patients in both CSF and plasma. The largest difference was, however, found between individuals with Creutzfeldt-Jakob disease and controls, whereas no significant differences were observed between the control group and individuals with frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS) or PD. In this context, it is noteworthy that also alpha-synuclein is increased in CSF from AD patients.³⁴

To our knowledge, AMPH has not previously been studied in CSF in the context of AD. However, AMPH (also known as AMPH1) forms homodimers and heterodimers with BIN1 (also known as AMPH2) and is highly concentrated in presynaptic terminals.³⁵ BIN1 polymorphisms have been suggested to mediate the risk of AD by the alteration of tau expression.³⁶ It has been indicated that AMPH is essential for sustaining synaptic transmission³⁷ and the altered levels of AMPH in CSF might be connected to the increased synaptic dysfunction in the brains of AD patients. Further studies are needed to

increase the knowledge about AMPH and its potential to reflect synaptic dysfunction or loss in order to elucidate the relation to AD pathology.

Although the other three analyzed proteins (ARPP21, AQP4 and NEFM) are all brain-enriched, they have various biological functions. AQP4 is the most abundant water channel in the brain and critical for maintaining brain water homeostasis. A change in the expression or localization of AQP4 has been reported as associated with several neurological conditions.³⁸ It has been suggested that impaired clearance of A β from the brain rather than increased A β production rate, underpins A β plaque deposits in sporadic AD³⁹ and those deficiencies in AQP4 may play a part in the reduction of A β clearance.³⁸ Zepfenfeld et al. observed an increased expression of AQP4 in the aging brain and a loss of perivascular localization of AQP4 was associated with increased neurofibrillary and A β pathology.⁴⁰ Recent studies furthermore suggest that AQP4 might play a role in the regulation of synaptic plasticity.⁴¹ We have herein identified increased levels of AQP4 in CSF from AD patients compared with controls in four independent cohorts. In contrast, Arighi et al.⁴² found lower levels of AQP4 in CSF in AD patients compared with controls, although in a small sample set (AD $n = 11$, controls $n = 9$). Further studies are needed to explain the potential role of CSF AQP4 in AD and the discordant results in our study compared with others.

NEFM was also found at higher levels in AD compared to control CSF. NEFM is one of the subunits of neurofilaments, together with neurofilament light polypeptide (NEFL, also known as NfL) and neurofilament heavy polypeptide (NEFH). These structural filaments are important for axonal caliber and neuronal morphology⁴³ and an increased CSF level is a marker of neuroaxonal damage. NEFL is the most well-studied subunit of neurofilaments and CSF levels of NEFL have been measured in several neurodegenerative disorders, such as FTD, ALS, AD, and PD.⁴⁴ Increased levels were observed with different magnitudes for FTD, ALS, and AD among others, while PD had levels of NEFL similar to healthy controls.⁴⁵ Furthermore, the increased sensitivity obtained by the recently developed single-molecule array method⁴⁶ has enabled measurements of NEFL in blood, resulting in a substantial number of publications on NEFL levels in blood within different neurodegenerative disorders.^{47–51} Neurofilaments are important for the stability and function of neurons and we have previously reported higher levels of the medium subunit, NEFM, in CSF from FTD patients.⁵² In another study, we identified increased levels in plasma from ALS patients compared with controls.⁵³ Further studies will elucidate the potential added value of NEFM in relation to NEFL and NEFH. Apart from being brain-enriched, another characteristic that several of the

studied proteins have in common is that they have calmodulin-binding properties. ARPP21 is a calmodulin-binding protein that regulates calmodulin signaling⁵⁴ and other calmodulin-binding proteins include GAP43,⁵⁵ AMPH,⁵⁶ and BIN1.⁵⁶

Elevated concentrations of t-tau and p-tau in CSF are biomarkers of tau secretion and phosphorylation, which can predict AD-type tangle formation and neurodegeneration.⁵⁷ Higher CSF concentrations of both proteins are associated with more rapid clinical disease progression and manifestation of more severe symptoms.^{58,59} However, increased CSF levels of p-tau have shown to be more specific to AD type dementia compared to increased CSF t-tau levels, which are observed in other neurodegenerative disorders as well.⁶⁰ As mentioned previously, the MCI group is heterogeneous and some of the individuals will remain stable and never develop dementia. In order to get a better understanding of the early phases of dementia and AD, individuals with MCI are a specifically interesting group to study. When stratifying the MCI group in cohorts 1 and 2 based on the core biomarkers in CSF for AD, we observed large differences in protein levels between the MCI subgroups connected to abnormal concentrations of t-tau and p-tau.

Furthermore, we observed strong to moderate correlations between AMPH, AQP4, ARPP21, GAP43, and SNCB and the levels of t-tau as well as p-tau. A weak correlation was identified for NEFM, which might indicate that the protein represents a different disease mechanism in AD, not reflected by the CSF levels of tau. Many proteins have been found to correlate with t-tau levels previously.^{7,61,62} Dayon et al. identified 790 proteins in CSF using MS-based shotgun proteomics, 50 of those proteins were associated with t-tau and 46 proteins were associated with p-tau.⁶² The proteins that display an association with t-tau or p-tau were mainly enriched in brain tissue, despite a large number of proteins in CSF originating from blood. We have here analyzed brain-enriched proteins and identified several proteins with strong to moderate correlations with t-tau and p-tau in concordance with Dayon et al. In a review, Wesenhagen et al. found enrichment of pathways associated with the immune system, gene expression, and signal transduction among the proteins that correlated with CSF levels of t-tau.⁶¹

The suspension bead array with its single binder assay format enables the detection of hundreds of proteins in hundreds of samples. However, to add support for on-target binding we developed sandwich assays with two antibodies targeting the same protein. A strong correlation ($R > 0.85$) was identified for AMPH, AQP4, and SNCB and a moderate correlation ($R = 0.53$) for ARPP21 between the single binder assay and the data obtained using the developed sandwich assay, validating the data

obtained using the suspension bead array. The performance of the GAP43 and NEFM antibodies was previously validated using an in-house developed PRM assay with a strong correlation to data obtained from the single binder assay setup (GAP43: $R = 0.76$, NEFM: $R = 0.71$).⁶³ In addition, a previously in-house developed NEFM sandwich assay showed a strong correlation to the single binder data ($\rho > 0.82$).⁵²

This is a cross-sectional study with some inevitable limitations. Longitudinal studies are needed in order to more precisely investigate the temporal dynamics of the biological processes connected to the development of AD. The individual variation of the concentration of these proteins could be diminished if we were able to follow the same individuals over time. Even though we observed significant differences between different sample groups, an overlap of protein levels between the groups could be seen for all six proteins. An important next step will be to try to identify subgroups of patients within the different sample groups. Future information about which individuals in cohorts 1 and 2 with MCI that developed AD would for this purpose be valuable. This information was available for 18 of the 29 individuals with MCI in cohort 4, where 10 individuals had converted after 11 years, and a correlation was observed between conversion time and protein levels for AMPH, AQP4, ARPP21, GAP43, and SNCB. Noteworthy, however, is that these proteins also showed a correlation with tau levels which previously have been identified to contribute with diagnostic relevant information already at early disease stages.² Different preanalytical sample handling procedures were used for the different cohorts. These types of factors might have an impact on the results and the ability to validate a potential biomarker. Furthermore, the subjects in the control group in cohorts 1 and 2 are significantly younger than the AD patients in the same cohort (Fig. S1). In addition, the controls in cohorts 1 and 3 have normal levels of A β 42, t-tau, and p-tau, while this was not analyzed in cohort 4. With a median age in the control group of 71 years, it is possible that several of these cognitively healthy individuals had decreased A β 42 levels. All controls in cohort 2 did not have normal levels of A β 42, t-tau, and p-tau which might contribute to the smaller differences seen in this cohort compared to cohorts 1 and 3 (Fig. S2). The use of several cohorts with different distributions regarding age and levels of core AD CSF markers reflects the clinical reality. Moving forward, it will likely be more and more important to divide individuals into distinct subgroups within the AD continuum. To be able to achieve this we would need larger cohorts to ensure that enough people are included in each subgroup. In the current study, we would probably have found more proteins to be altered, out of the 216 analyzed proteins, if the same

precise subgroups had been included in all cohorts. Despite this, we could see similar protein patterns of AMPH, AQP4, ARPP21, GAP43, NEFM, and SNCB in all four included cohorts, indicating that they could have the robustness required for a biomarker in clinical practice.

In conclusion, we have identified six proteins, namely AMPH, AQP4, ARPP21, GAP43, NEFM, and SNCB, with increased levels in CSF from patients with AD dementia compared to controls. Concordant trends were observed in four independent cohorts. A few of the proteins did also display altered levels between individuals with MCI and controls, as well as between AD dementia and MCI. The patterns we have identified indicate that these six proteins might reflect early disease-related changes in the brain of AD patients, but further studies are needed to explore their potential role in AD pathogenesis and their possibility to aid the clinical assessment of patients for the prediction of dementia.

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Authors' Contributions

SB performed the experimental work with support from JR and JO. JY performed the technical validation with support from SB, RSV, BB, MH, FB, MI, KK, ML, LK, KB, BN, and HZ coordinated sample collection. SB analyzed and interpreted the data with support from JR, JO, IM, AM, PN, JCC, and SC coordinated and managed all the logistics around the clinical work package of AETIONOMY. PN, AM, and PS supervised the project. SB wrote the manuscript with input from all co-authors.

Conflict of Interest

KB has served as a consultant or at advisory boards for Abcam, Axon, Biogen, Lilly, MagQu, Novartis, and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program. HZ has served at scientific advisory boards for Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics, and CogRx, has given lectures in symposia sponsored by Fujirebio, Alzecure, and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program

(outside submitted work). J.C.C. has served in advisory boards for Air Liquide, Biogen, Denali, Ever Pharma, Idorsia, Prevail Therapeutic, Theranexus, UCB; and received grants from Sanofi and the Michael J Fox Foundation. The other authors declare that they have no competing interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Material. Extended information about the cohorts, labeling of the proteins, and development of sandwich assays. Extended tables and figures with additional information.