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Development of parallel reaction monitoring assays for cerebrospinal fluid proteins associated with Alzheimer's disease

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ABSTRACT

Detailed knowledge of protein changes in cerebrospinal fluid (CSF) across healthy and diseased individuals would provide a better understanding of the onset and progression of neurodegenerative disorders.

In this study, we selected 20 brain-enriched proteins previously identified in CSF by antibody suspension bead arrays (SBA) to be potentially biomarkers for Alzheimer's disease (AD) and verified these using an orthogonal approach. We examined the same set of 94 CSF samples from patients affected by AD (including preclinical and prodromal), mild cognitive impairment (MCI), non-AD dementia and healthy individuals, which had previously been analyzed by SBA. Twenty-eight parallel reaction monitoring (PRM) assays were developed and 13 of them could be validated for protein quantification.

Antibody profiles were verified by PRM. For seven proteins, the antibody profiles were highly correlated with the PRM results ($r > 0.7$) and GAP43, VCAM1 and PSAP were identified as potential markers of preclinical AD. In conclusion, we demonstrate the usefulness of targeted mass spectrometry as a tool for the orthogonal verification of antibody profiling data, suggesting that these complementary methods can be successfully applied for comprehensive exploration of CSF protein levels in neurodegenerative disorders.

Abbreviations: $A\beta_{1-42}$, Amyloid-β-peptide1–42; ACN, Acetonitrile; AD, Alzheimer's disease; AGC, Automatic gain control; ALDOC, Fructose-biphosphate aldolase C; APOA4, Apolipoprotein A-IV; BCA, Bicinchoninic acid; CCK, Cholecystokinin; CKB, Creatine kinase B-type; CSF, Cerebrospinal fluid; CTSD, Cathepsin D; CV, Coefficient of variation; C_{18}, C_{18} chains fused to silica particles; DKK, Dickkopf-related protein 3; DLB, Dementia with Lewy bodies; DTT, DL-dithiothreitol; ESI, Electrospray ionization; FA, Formic acid; FGA, Fibrinogen alpha chain; FTD, Frontotemporal dementia; GAP43, Neuromodulin; HLB, Hydrophilic-lipophilic balance; HPLC, High performance liquid chromatography; HSP90B1, Endoplasmin; IAA, Iodoacetamide; ISF, Interstitial fluid; IS, Internal standard; IT, Ion injection time; ITIH1, Inter-alpha-trypsin inhibitor heavy chain H1; LRG1, Leucine-rich alpha-2-glycoprotein; L/H, Light-to-heavy ratio; LOD, Limit of detection; LOQ, Limit of quantification; MAX, Mixed-mode (strong) anion exchanger; MCI, Mild cognitive impairment; MCX, Mixed-mode (strong) cation exchanger; MFI, Median Fluorescence Intensity; MS, Mass spectrometry; *m/z*, Mass-to-charge ratio; NBEA, Neurobeachin; NCE, Normalized collision energy; NEFM, Neurofilament medium polypeptide; ON, Overnight; PD, Parkinson's disease; PDD, Parkinson's disease with dementia; PLG, Plasminogen; PrEST, Protein Epitope Signature Tag; PRM, Parallel reaction monitoring; PSAP, Prosaposin; PSM, Peptide spectrum match; PSP, Progressive supranuclear palsy; QPrEST, Quantification Protein Epitope Signature Tag; QQQ, Triple quadrupole; *r*, Pearson's correlation coefficient; RF, Radio frequency; RT, Room temperature; R^2, Coefficient of determination; SD, Standard deviation; SDC, Sodium deoxycholate; SEM, Standard error of the mean; SERPINA1, Alpha-1-antitrypsin; SERPINA3, Alpha-1-antichymotrypsin; SPARCL1, SPARC-like protein 1; SPE, Solid phase extraction; SRM, Selected reaction monitoring; S100B, Calcium-binding protein B; TFA, Trifluoroacetic acid; VCAM1, Vascular cell adhesion protein 1; WAX, Weak anion exchanger

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1. Introduction

Neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), dementia with Lewy bodies (DLB) and frontotemporal dementia (FTD) predominantly affects older individuals. Although the underlying mechanisms are unclear, disease onset is most likely due to a concurrency of events including age related decline of biochemical processes together with other disease-promoting factors [1–4]. AD is the most common type of dementia in older adults (> 65 years), accounting for 60–70% of all dementia cases [5]. It is characterized by neuronal degeneration throughout the brain causing symptoms such as memory loss, difficulties in thinking, understanding and judging, personality changes, hallucinations and ultimately death [WHO, 2017].

Cerebrospinal fluid (CSF) covers an important role in central nervous system as mechanical protection for the brain but also in the regulation of brain interstitial fluid homeostasis and metabolism [6]. The protein concentration in CSF may change as a result of neuronal damage, altered neuronal functions or CSF flow rate. It therefore represents an exquisite source of information about the status of the central nervous system in physiological and pathological conditions [5,7]. In the past years, several efforts to build a comprehensive database of the human CSF proteome in healthy [8,9] and diseased [10] individuals have been initiated.

Although mass spectrometry (MS) is regarded as the workhorse of proteomic studies, antibody arrays, such as single binder immunoassays or suspension bead arrays (SBA) [11] have shown to be successful and advantageous for protein profiling of CSF samples. They have been used to discover candidate biomarkers in neurodegenerative disorders such as AD, PD, DLB [12] and multiple sclerosis [13,14].SBA assays allow for both high sample throughput and high analyte multiplexing, representing a very powerful approach for screening large cohorts of samples. However, the SBA technology has mainly been applied as an exploratory screening tool and subsequent evaluation of potential off-target interactions has, as for all multiplex protein profiling assays, been necessary.

Parallel reaction monitoring (PRM) is now emerging as an alternative to SRM [15–17] in the analysis of CSF and other bodily fluids [18,19].PRM is implemented on a quadrupole mass filter in line with a high resolution and accurate mass analyzer (Orbitrap), allowing for the analysis of all potential product ions of a precursor. For this workflow, less laborious method development is required compared to SRM, since product ions are not selected a priori [20,21], therefore making PRM an ideal targeted approach for qualification and verification of protein profiles.

The aim of our study was to investigate the use of PRM for the verification of SBA protein profiles in CSF. We selected 20 brain-enriched proteins previously profiled found to be interesting in the context of AD by Remnestål et al. [12] and we proceeded with development of PRM methods for their quantification in CSF. The assays implement a semi-automated approach for sample preparation and "single point calibration" quantification using QPrEST™ heavy labeled protein fragments as internal standards (Fig. 1) [22]. Despite a general agreement that assays in preclinical research do not need to follow the same standards as assays used in clinical applications, it is increasingly evident that the success of biomarker discovery depends on validated analytical assays [21,23,24]. We applied the PRM assays developed to the same cohort of 94 lumbar CSF samples previously analyzed using the antibody-based SBA with the aim of performing an orthogonal verification of protein profiles showing potential clinical interest.

2. Experimental section

2.1. Chemicals and reagents

Acetonitrile (ACN) HPLC Gradient grade was purchased from Fisher Chemical (Waltham, MA USA). Formic acid (FA) (LOT# 174566) was from Fisher Chemical (Waltham, MA USA). Ammonium bicarbonate (LOT# BCBN6056V, ≥99% purity), DL-dithiothreitol (DTT) (LOT# BCBR9439V, ≥99.5% purity), iodoacetamide (IAA) (LOT# SLBD7510V, ≥99% purity) and sodium deoxycholate (SDC) (LOT# 086K0045V, ≥97% purity) were purchased from Sigma Aldrich (St. Louis, MO, USA). Trypsin/Lys-C Mix, Mass Spec Grade (20 μg) was obtained from Promega (Madison, WI, USA). Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to obtain ultrapure water. Heavy labeled QPrESTs used as internal standards were obtained from the Human Protein Atlas and produced as described previously [22] (Table 1). The protein concentration of each QPrEST had previously been determined with the bicinchoninic acid (BCA) assay. The gene name displayed will be used to denote the QPrEST and the corresponding endogenous target in this report.

2.2. CSF samples

The cohort analyzed in this study consisted of CSF collected by lumbar puncture at the L3/L4 interspace from 94 living individuals with AD (43), prodromal AD (2), preclinical AD (14), non-AD dementia (2), non-AD mild cognitive impairment (MCI) (10) and healthy controls (23) at Sahlgrenska University Hospital in Gothenburg, Sweden. The study was approved by the regional ethics committee and all patients had given their informed consents. The diagnostic criteria applied was a combination of clinical diagnosis (cognitively normal, mild cognitive impairment or dementia) and the CSF biomarker profile of tau (total tau, t-tau and phospho-tau, p-tau) and $A\beta_{1-42}$ [25]. Patients diagnosed with prodromal AD (early-stage AD) had decreased CSF $A\beta_{1-42}$ and increased levels of either t-tau or p-tau, deteriorating memory but no functional impairments. Patients diagnosed with Preclinical AD had abnormally decreased CSF levels of $A\beta_{1-42}$. Control samples were collected from volunteers asymptomatic of dementia (cognitively normal) and CSF levels of tau and $A\beta_{1-42}$ within the normal ranges [12]. Table 2 shows the demographics of the cohort. After sampling, CSF was centrifuged at 2000 x g for 10 min at RT and stored at −80 °C. Prior to this study, the samples had undergone two freeze/thaw cycles. The samples were thawed on ice and aliquoted into a 96-well plate in a randomized layout. The plate was stored at −80 °C until analysis.

Commercial lumbar CSF pooled from healthy donors (LOT# W53211, Lee Biosolutions, Maryland Heights, MO, USA), was used for method development and parts of the method validation. Two pools of CSF, one with high and one with low levels of the proteins of interest, assembled from the clinical samples were also used for assays optimization and validation, where high and low protein levels estimations were based on the previous semi-quantitative measurements by SBA [12]. 10 μL from 26 samples showing the highest or lowest levels for most of the 20 targets were pooled to generate the "high" and the "low" pool, respectively.

2.3. Sample preparation for MS analysis

All 20 proteins, besides NBEA, were detected previously by MS analysis in CSF according to the PeptideAtlas database [26] with normalized peptide spectrum matches (PSMs) ranging from 0.3 (NEFM) to 1141 (SERPINA1).

2.3.1. Digestion

Samples required for method development were prepared from 10 μL of starting material in Eppendorf LoBind tubes (Eppendorf, Hauppauge, NY, USA). Samples were prepared in duplicates (shotgun-MS analysis/digestion test, spike-in determination of QPrESTs) or triplicates (peptide clean-up evaluation). Samples for comparison of starting material volume (10 vs. 20 μL) were prepared in triplicates and samples required for method validation were prepared in triplicate unless otherwise stated from 20 μL of starting material in low binding

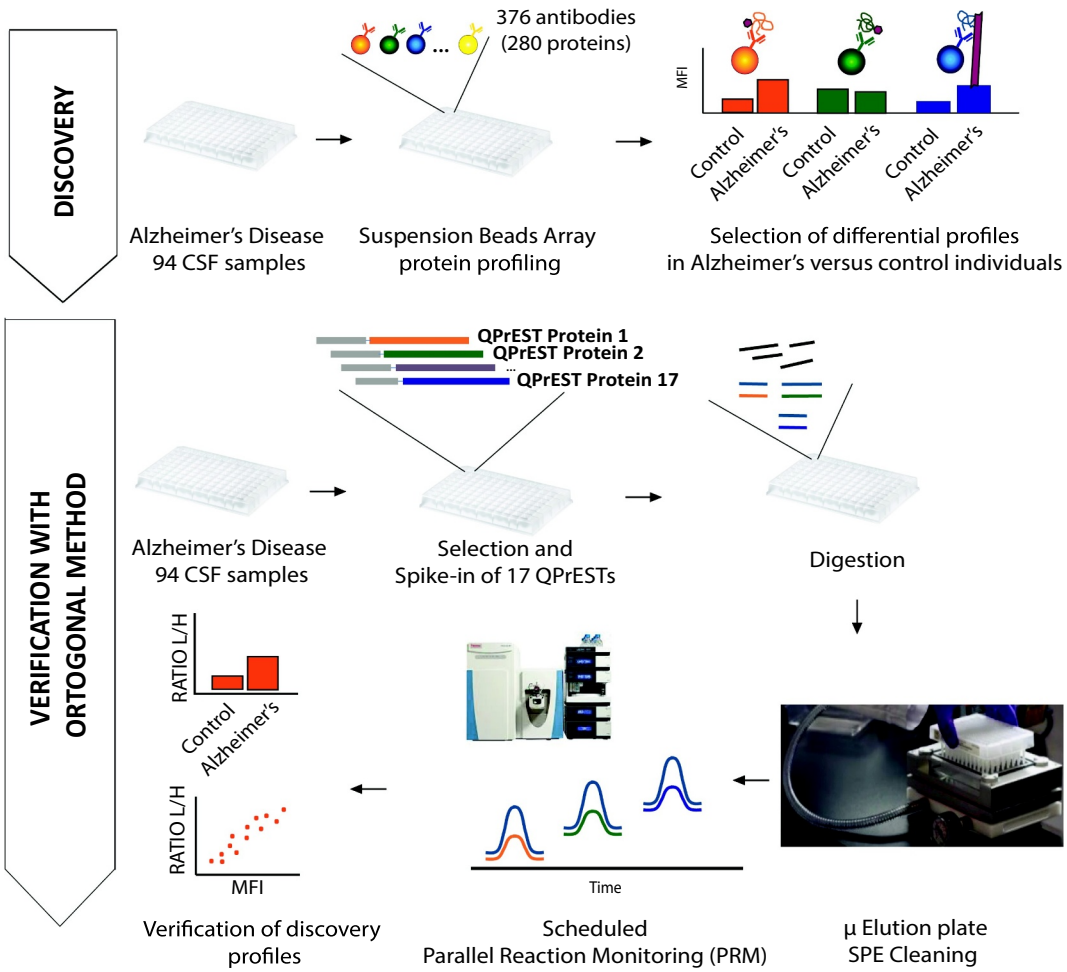


Fig. 1. Study design outline. 20 brain-enriched proteins previously profiled by antibody suspension bead arrays (SBA) were selected for a profile verification by targeted mass spectrometry. Parallel reaction monitoring (PRM) assays were developed for 17 out of 20 targets using heavy labeled protein fragments (QPrESTs) covering a region of the proteins of interest. Samples were spiked with QPrESTs for each target protein, and processed in a semi-automated protocol which exploit automated pipetting and on-plate clean up procedures. Previously obtained SBA results (MFI values) were compared with peptide ratio obtained by scheduled PRM.

96-well plates (KingFisher, Thermo Fisher Scientific). Patient samples (20 µL CSF) were aliquoted to low binding 96-well plates using the CyBio-SELMA system and reagents dispensed using an electronic stepper pipette (Eppendorf Multipipette Stream).

CSF samples were diluted 1:10 with ammonium bicarbonate (200 mM) and SDC (0.25%) and QPrEST internal standards were spiked into each sample followed by reduction (DTT 2.5 mM, 56 °C, 30 min) and alkylation (IAA 4.0 mM, room temperature (RT) in darkness, 30 min). The reaction was quenched with DTT at 2.5 mM and the volume adjusted to 95 µL with SDC (0.25%). Trypsin/Lys-C mix was added to the sample at a ratio of 1:25 to total protein and incubated at 37 °C overnight (ON). Incubation times of 10, 30, 60 and 180 min were compared to ON when selecting optimal digestion time. The sample was then acidified with 5 µl of 10% trifluoroacetic acid (TFA) and 5 µL of 5% acetic acid and incubated for 30 min at RT. By centrifugation, (1575 xg 30 min for samples in plates; 25,200 xg, 15 min for samples in tubes) the supernatant was separated from the precipitated SDC, collected and cleaned by solid phase extraction (SPE) as described below.

Appropriate QPrESTs spike-in concentrations were determined using a 5-point dilution series of QPrESTs spiked into constant level of commercial CSF. The linearity was determined using a 12-point dilution curve of the high pool in a blank matrix (artificial CSF), spiked with a constant concentration of QPrESTs. Artificial CSF was prepared with a mixture of electrolytes as described in Cold Spring Harbor Protocols, 2011 [27]. We supplemented the solution of electrolytes with rabbit immunoglobulin G (Bethyl Laboratories, P120–301) and bovine serum albumin (Sigma Aldrich, SLBR5497V, LOT# STBF7163V) to obtain a total protein concentration of 0.8 mg/ml. Briefly, the solution contained: 119 mM NaCl, 26 mM NaHCO3, 2.5 mM KCl, 1 mM NaH2PO4, 1.3 mM MgCl2, 10 mM glucose and 2.5 mM CaCl2, 0.67 mg/mL BSA and 0.13 mg/mL rabbit IgG. The solution was stored at 4 °C up to one week.

2.3.2. Clean-up strategies

ZipTip C_{18} tips with 0.6 µL bed of chromatography media (Millipore, Billerica, MA, USA) were used for the clean-up of the tryptic peptides in the first phases of development (shotgun-MS/digestion test, volume comparison). Selected SPE cartridges were evaluated for the clean-up of the tryptic peptides and compared to the performance of the C_{18} ZipTip. Tested SPE sorbents were mixed-mode strong cation exchange (MCX), mixed-mode strong and weak anion exchange (MAX and WAX, respectively) and hydrophilic-lipophilic balance (HLB) and HLBPrime (Oasis, 1 cc, Waters). Furthermore, a C_{18} cartridge (Sep-Pak, 130 mg sorbent, Waters) was also evaluated in comparison. The commercial CSF was used in all procedures. To avoid preparation artefacts not related to the SPE procedure, digests were pooled, thoroughly vortexed, and then divided into three aliquots for replicate comparison before SPE treatment. Protocols for each sorbent are found in Supplementary Excel Table 1. Three separate protocols were implemented for the HLB sorbent including acidified (HLBa), basified

Table 1
List of 20 proteins and QPrESTs.

Gene name	Uniprot ID	Protein name	nPSM in CSF	Aa coverage QPrEST	QPrEST spike concentration µg/ml	QPrEST spike concentration pmol	Peptides	m/z Light	m/z Heavy	Detectable QPrEST	Detectable Healthy CSF
NBEA	F5GXV7	Neurobeachin	–	1208–1308	0.97	0.67	IHTTSDGMSSISER	507.5718	510.9079		no
							GLEYAEMTATTLETESSSSK	1067.988	1071.995		
NEFM	P07197	Neurofilament medium polypeptide	0.319	115–238	0.20	0.12	EIEAEIQALR	586.3195	591.3236		no
							VQSLQDEVAFLR	702.8777	707.8818		
CKB	P12277	Creatine kinase B-type	3.909	237–381	0.31	0.19	F**C**TGLTQIETLFK	779.4027	783.4098		no
							GTGGVDTAAVGGVFDVSNADR	982.9691	987.9732		
							LGFSEVELVQMVVDGVK	924.9924	928.9995		
S100B	P04271	S100 Calcium Binding Protein B	6.596	4–92	–	–	AMVALIDVFHQYSGR	853.9378	858.9419	no	no
CCK	P06307	Cholecystokinin	9.382	33–99	1.2	0.96	AHLGALLAR	461.2851	466.2892		
GAP43	P17677	Neuromodulin	12.216	41–146	0.18	0.13	GEGDAATEQAAPQAPASSEEK	1022.459	1026.466		no
HSP90B1	P14625	Endoplasmin	15.985	282–392	–	–	TETVEEPMEEEEAAK	861.3747	865.3818	no	no
							EESDDEAAVEEEEEEK	933.8660	937.8731		
VCAM1	P19320	Vascular cell adhesion protein 1	17.800	327–413	0.54	0.40	GIQVELYSFPR	654.8510	659.8551		no
ALDOC	P09972	Fructose-bisphosphate aldolase C	61.034	239–359	0.56	0.40	YTPEEIAMATVTALR	833.4295	838.4336		no
							ALQASALNAWR	600.8278	605.8320		
							DNAGAATEEFIK	633.3040	637.3111		
PSAP	P07602	Prosaposin	88.344	214–356	1.8	1.1	LGPGMADI**C**K	531.2595	535.2666		
							EI**C**ALVGF**C**DEVK	770.3627	774.3698		
							NVIPALELVEPIK	717.9319	721.9391		
							SDVY**C**EV**C**EFLVK	824.3733	828.3804		
							EILDAFDK	475.7451	479.7522		
CTSD	P07339	Cathepsin D	95.073	103–250	0.67	0.40	LLDIACWIHHK	469.2518	471.9232		no
							FDGILGMAYPR	620.3132	625.3173		
							ISVNNVLPVFDNLMQQK	980.0220	984.0291		
							LVDQNIFSFYLSR	801.4197	806.4239		
ITIH1	P19827	Inter-alpha-trypsin inhibitor heavy chain H1	103.289	69–153	2.4	1.8	EVAFDLEIPK	580.8135	584.8206		
							AAISGENAGLVR	579.3173	584.3214		
FGA	P02671	Fibrinogen alpha chain	114.941	371–468	24	17	PNNPDWGTFEEVSGNVSPGTR	1130.5169	1135.521		
LRG1	P02750	Leucine-rich alpha-2-glycoprotein	118.814	193–326	3.3	2.0	DLLLPQPDLR	590.3402	595.3444		
							VAAGAFQGLR	495.2800	500.2841		
PLG	P00747	Plasminogen	159.208	251-312	2.4	2.0	WELCDIPR	544.7633	549.7674		
							CTTPPPSSGPTYQ**C**LK	897.4135	901.4206		
APOA4	P06727	Apolipoprotein A-IV	192.793	238–371	0.65	0.40	LAPLAEDVR	492.2796	497.2838		
							ALVQQMEQLR	608.3293	613.3335		
							VNSFFSTFK	538.7742	542.7813		
SPARCL1	Q14515	SPARC-like protein 1	313.867	215–309	3.3	2.3	QEEDNTQSDDILEESDQPTQVSK	879.0545	881.7259		
							TGLEAISNHK	535.2855	539.2926		
DKK3	Q9UBP4	Dickkopf-related protein 3	764.867	85–170	–	–	ITNNQTGQMVFSETVITSVGDEEGRR	956.7979	963.4701	no	
SERPINA3 (1)	P01011	Alpha-1-antichymotrypsin	1053.763	49–187	3.4	2.0	EQLSLLDR	487.2693	492.2734		
							LYGSEAFATDFQDSAAAK	946.4391	950.4462		
							EIGELYLPK	531.2975	535.3046		
SERPINA3 (2)	P01011	Alpha-1-antichymotrypsin	1053.763	281–423	3.4	2.0	AVLDVFEEGTEASAATAVK	954.4835	958.4906		
							ITLLSALVETR	608.3690	613.3731		
SERPINA1	P01009	Alpha-1-antitrypsin	1141.164	249–398	4.8	2.8	LSITGTYDLK	555.8051	559.8128		
							SVLGQLGITK	508.3109	512.3180		
							VFSNGADLSGVTEEAPLK	917.4651	921.4722		

Table 2
Cohort demographics.

Diagnosis	*N*	Age median (range)	Gender F/M
AD	43	81 (53–102)	28/15
Prodromal AD	2	90 (88–92)	2/0
Preclinical AD	14	85 (73–96)	10/4
Non-AD dementia	2	84 (82–86)	0/2
Non-AD mild cognitive impairment (MCI)	10	84.5 (56–93)	8/2
Healthy	23	79 (44–91)	12/11
Total	94		

(HLBb) and neutral sample (HLBg), as suggested by the provider [28] and described in Supplementary Excel Table 1.

Oasis® HLB was selected for method validation and cohort samples. In order to prepare the samples in a high-throughput fashion we chose the Oasis® HLB 96-well µElution plate format with 2 mg sorbent and 30 µm particle size (Waters, Milford, MA, USA). Solutions were drawn through the microcolumns by the use of a vacuum pump (KNF Laboport) and the Oasis® 96-well plate vacuum manifold kit (Waters, Milford, MA, USA). Columns were conditioned with 0.2 mL methanol and equilibrated with 0.2 mL Milli-Q. The samples were loaded (approx. 97 µL) and the flow through was discarded. The columns were washed with 0.2 mL 5% methanol and the samples were then eluted with 0.1 ml 2% FA in 60% ACN + 40% methanol. After elution, the solvent was evaporated (SpeedVac Concentrator Savant SPD111V and UVS400A, Thermo Fisher Scientific). Peptides were stored as dried pellets at −20 °C and re-suspended with buffer A (3% ACN + 0.1% FA) before injection. Injection volume was 20% of the total resuspension volume (2 of 10 µL or 5 of 25 µL).

2.4. Instrumental analysis

MS analysis was performed on a Q-Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled to a Dionex Ultimate 3000 RSLC nanosystem (Thermo Fisher Scientific) for reversed phase chromatography. Samples were automatically injected onto a C_{18} trap column followed by a C_{18} EASY-Spray analytical column. The system was controlled with the Xcalibur software (Thermo Fisher Scientific).

Shotgun analysis for peptide identification was performed using a C_{18} EASY-Spray analytical column, dimensions 50 cm × 75 µm ID, particle size 2 µm (Thermo Fisher Scientific). Chromatographic separations were achieved with a multistep gradient of buffer B (95% ACN + 0.1% FA) starting at 3% for 5 min: 3% to 8% for 3 min, 8% to 30% for 42 min, 30% to 43% for 5 min and 43% to 99% for 2 min. B was then held at 99% for 5 min before decreased to 3% to equilibrate the system. Total run time was 74 min with a flow rate of 250 nL/min. Electrospray ionization (ESI) source settings were positive ionization mode, spray voltage (kV) 3.50, capillary temperature (°C) 320, sheath gas flow rate 7, aux gas flow rate 2 and S-lens radio frequency (RF) level 60. The instrument was operating in data-dependent acquisition mode with a full MS1 scan in the range *m/z* 400–1600 with 60,000 resolving power at *m/z* 200, automatic gain control (AGC) target 3e6 and maximum ion injection time (IT) 100 ms. The five most abundant precursors were selected for MS2 and fragmented with normalized collision energy (NCE) 30. Single and unassigned charge states were excluded. Precursors selected for MS2 were put on a dynamic exclusion for 60 s. MS2 was performed with 30,000 resolving power at *m/z* 200, AGC target 1e5 and maximum IT 100 ms.

For PRM assays, peptide separation was optimized on a 25 cm C_{18} EASY-Spray analytical column (dimensions 25 cm × 75 µm ID, particle size 2 µm, Thermo Fisher Scientific) using a multistep gradient of B starting at 3% for 5 min: 3% to 32% for 35 min, 32% to 47% for 3 min and 47% to 99% for 2 min. B was then held at 99% for 7 min and then decreased to 3% to equilibrate the system. A flow rate of 400 nL/min was used and the total run time was 58 min. ESI source settings were maintained as described above. The PRM assays consisted of one full MS1 event followed by up to 25 targeted MS2 events. The full MS1 scan covered the mass range *m/z* 150–2000 with 60,000 resolving power at *m/z* 200, AGC target value 2e5 and maximum IT 55 ms. For MS2, precursors scheduled with an elution window of approximately 5 min (Supplementary Table 2) were fragmented with NCE 27. Scan settings were 30,000 resolving power at *m/z* 200, AGC target 1e6, maximum IT 250 ms and precursor isolation window was set to 2.0 Th. PRM parameters were optimized for up to eight points across the curve. For method development and optimization, PRM methods were run in unscheduled mode.

2.5. Suspension bead arrays (SBA)

The data generated by the SBA technology was previously reported by Remnestål et al. [12].

2.6. Data analysis

For peptide and protein identification in the shotgun-MS analysis, raw data was imported into MaxQuant version 1.5.3.30 [29]. Searches were performed with MaxQuant's search engine Andromeda against the human protein database from Uniprot (accessed on 03/17/2016, Canonical and Isoforms, 20,198 hits) customized with the QPrEST sequences. The following settings were used: trypsin and Lys-C cleavage after Arg and Lys residues but not when followed by Pro, fixed modifications: carbamidomethyl (Cys), variable modifications: oxidation of methionine residues, acetyl-group on the N-terminal, Arg-10, Lys-8, maximum missed cleavages: 5.

Skyline version 3.7.0.10940 [30] was used for PRM data analysis and peptide quantification. Selected peptides were verified to be unique to the human protein of interest. Identical heavy and light transitions and retention times confirmed peptide identity. A minimum of three transitions was required for reliable detection. All peaks were manually inspected to confirm correct detection and peak boundaries. Peak integration and calculation of the ratios between light endogenous and the heavy-labeled peptide (L/H) were done in Skyline and result reports exported from the software. Where applicable, an estimation of the endogenous protein concentration was made based on the L/H peptide ratio multiplied with the spiked-in QPrEST concentration for the corresponding protein ("single point calibration").

Linearity was evaluated by regression analysis using the three replicates for each point of dilution. The line of best fit with a 95% confidence interval was represented and used to calculate limits of detection and quantification (LOD and LOQ, respectively). LOD and LOQ were estimated as $3.3S_a/b$ and $10S_a/b$, respectively, where S_a is the SD of the y-intercepts and b is the slope of the dilution curves.

Four replicates of the commercial CSF were prepared on three different days to evaluate the intra-and inter-day variation of the sample preparation procedure for each peptide. The intra-day variation was defined as the mean CV (%) calculated from the CVs (%) obtained from the experiments that were performed on the same day. The inter-day variation was defined by two methods; 1) replicate 1 was compared on all days, replicate 2 was compared on all days, etc., as suggested in [33] and 2) the CV (%) calculated from the mean ratios from the intra-day replicates across the different days.

The accuracy was evaluated from all 12 replicates prepared (mean concentration ± SD) and defined as the closeness (expressed as %) to the mean concentration for one day of repeated experiments. Carry-over (expressed as %) was estimated by blank solvent injections after sample injection.

Peptide levels obtained by PRM were compared between clinical subgroups by pairwise Wilcoxon rank sum tests. False discovery rate

Table 3
Method validation.

		Calibration curve					Precision			Accuracy		Carry-over	
Gene	Peptide	Adj R^2	LOD (ng/mL)	LOQ (ng/mL)	Ratio at LOD	Ratio at LOQ	Intra-day mean CV% (n = 3)	Inter-day mean CV% (n = 4)[b]	Inter-day CV % (n = 3)[c]	Mean ± SD (μg/mL)	Closeness (%)	Light (%)	Heavy (%)
ALDOC	YTPEEIAMATVTALR	–	–	–	–	–	5.52	31.1	30.8	0.88 ± 0.24	1.80	0.00	0.00
	ALQASALNAWR	0.964	12.2	37.0	0.014	0.048	13.2	30.1	30.2	0.69 ± 0.20	4.01	0.00	0.00
	DNAGAATEEFIK	–	–	–	–	–	3.13	29.7	29.7	0.54 ± 0.14	0.84	0.00	0.00
APOA4	LAPLAEDVR	0.981	42.3	128	0.020	0.154	7.42	27.6	27.6	1.51 ± 0.37	6.84	0.00	0.00
	ALVQQMEQLR	0.957	67.1	203	0.023	0.214	10.5	32.3	31.7	2.30 ± 0.66	11.3	0.00	0.00
	VNSFFSTFK	0.975	48.6	147	0.063	0.220	13.1	23.4	21.5	2.20 ± 0.48	10.3	0.00	0.00
CCK	AHLGALLAR	–	–	–	–	–	38.5	47.5	32.2	0.03 ± 0.02	11.2	0.00	0.00
CKB	FCTGLTQIETLFK	–	–	–	–	–	19.3	34.3	35.6	0.01 ± 0.003	18.2	0.00	2.47
	LGFSEVELVQMVVDGVK	0.926	0.305	0.924	0.0003	0.002	12.6	14.6	12.8	0.01 ± 0.001	2.84	0.00	2.23
	GTGGVDTAAVGGVFDVSNADR	–	–	–	–	–	–	–	–	–	–	–	–
CTSD	LLDIACWIHHK	0.931	174	529	0.125	0.607	24.9	30.4	21.2	15.4 ± 4.75	16.6	0.00	0.00
	FDGILGMAYPR	0.973	148	450	0.191	0.543	10.5	15.9	14.5	8.79 ± 1.42	16.3	0.21	0.09
	ISVNNVLPVFDNLMQQK	0.960	137	414	0.294	0.665	4.81	14.8	14.7	8.57 ± 1.14	14.3	0.00	0.00
	LVDQNIFSFYLSR	0.986	80.2	243	0.070	0.260	5.85	12.9	12.6	7.53 ± 0.91	6.24	1.09	0.06
GAP43	GEGDAATEQAAPQAPASSEEK	0.905	1.79	5.41	0.006	0.009	11.9	31.2	32.8	0.02 ± 0.01	1.06	0.00	0.00
FGA	PNNPDWGTFEEVSGNVSPGTR	0.931	156	472	0.008	0.018	11.4	24.1	23.7	5.92 ± 1.4	3.12	0.00	0.00
ITIH1	EVAFDLEIPK	0.980	6.49	19.7	0.009	0.014	3.76	29.0	29.0	0.52 ± 0.13	9.25	0.42	0.26
	AAISGENAGLVR	0.984[a]	2.53	7.66	0.008	0.011	20.3	31.3	30.2	0.34 ± 0.11	11.2	0.00	0.00
LRG1	DLLLPQPDLR	0.982	20.0	60.7	0.006	0.018	4.25	19.5	19.4	1.05 ± 0.18	5.33	0.26	0.17
	VAAGAFQGLR	0.933	32.8	99.3	0.016	0.037	11.5	33.2	33.5	1.90 ± 0.60	12.6	0.00	0.00
NBEA	IHTTSDGMSSISER	–	–	–	–	–	–	–	–	–	–	–	–
	GLEYAEMTATTLETESSSSK	–	–	–	–	–	–	–	–	–	–	–	–
NEFM	EIEAEIQALR	–	–	–	–	–	–	–	–	–	–	–	–
	VQSLQDEVAFLR	–	–	–	–	–	22.5	23.4	19.7	–	–	0.00	0.61
PLG	WELCDIPR	0.963	228	692	0.150	0.320	4.22	41.4	41.5	10.8 ± 3.87	6.01	0.11	0.00
	CTTPPPSSGPTYQCLK	–	–	–	–	–	3.23	35.7	35.6	6.02 ± 1.83	3.16	0.00	0.00
PSAP	LGPGMADICK	0.877	74.1	225	0.064	0.139	9.62	29.1	28.5	1.16 ± 0.30	16.5	0.00	0.00
	EICALVGFCDEVK	0.889	9.92	30.1	0.004	0.017	–	–	–	–	–	–	–
	NVIPALELVEPIK	0.964	3.30	9.99	0.007	0.010	6.39	15.2	14.3	0.15 ± 0.02	4.08	0.48	0.97
	SDVYCEVCEFLVK	0.959	8.82	26.7	0.008	0.018	8.20	26.4	26.1	0.27 ± 0.06	11.1	2.00	1.67
	EILDAFDK	0.926	54.2	164	0.031	0.089	8.98	26.9	25.7	0.97 ± 0.23	18.9	0.00	0.00
SERPINA1	LSITGTYDLK	0.986	798	2417	0.128	0.475	11.0	25.4	24.3	81.5 ± 19.0	16.5	0.09	0.11
	SVLGQLGITK	0.958	1364	4132	0.319	0.902	5.32	28.5	28.2	66.1 ± 16.3	15.9	0.04	0.07
	VFSNGADLSGVTEEAPLK	0.982	992	3005	0.143	0.569	11.0	27.7	26.7	74.1 ± 18.3	28.2	0.68	0.80
SERPINA3	AVLDVFEEGTEASAATAVK	0.979	264	800	0.097	0.235	9.79	19.2	16.6	25.3 ± 4.32	5.51	0.46	0.46
	ITLLSALVETR	0.976	267	810	0.078	0.225	10.2	25.6	24.7	24.4 ± 5.62	4.47	0.14	0.26
SPARCL1	QEEDNTQSDDILEESDQPTQVSK	0.833	5.85	17.7	0.007	0.010	11.4	16.7	13.2	0.09 ± 0.01	10.6	0.00	0.37
	TGLEAISNHK	0.948	31.9	96.6	0.009	0.027	17.3	42.7	41.9	0.38 ± 0.15	33.4	0.00	0.00
VCAM1	GIQVELYSFPR	–	–	–	–	–	10.3	35.6	35.0	0.57 ± 0.18	3.50	0.00	0.00

[a] 8-point dilution curves from healthy pool, 10 μl CSF. Does not contain the two highest points.
[b] Inter-day precision CV calculated from replicate 1 on day 1–3, replicate 2 on day 1–3, replicate 3 on day 1–3, replicate 4 on day 1–3 [23]. "Method 1".
[c] Inter-day precision CV calculated from the mean ratios collected on the different days 1–3. "Method 2".

was adjusted using the Bonferroni method. Samples measuring below LOQ (if available) and small patient groups ($n = 2$) were excluded. Pearson's correlation coefficient (Pearson's r) was calculated to evaluate the concordance in the protein levels obtained by PRM (L/H peptide ratio) and the SBA technology (MFI values) and the strength of correlation evaluated based on elsewhere suggested strategies [31].

Plots were visualized and statistical analyses performed using R (RStudio version 1.0.153) [32] .

3. Results

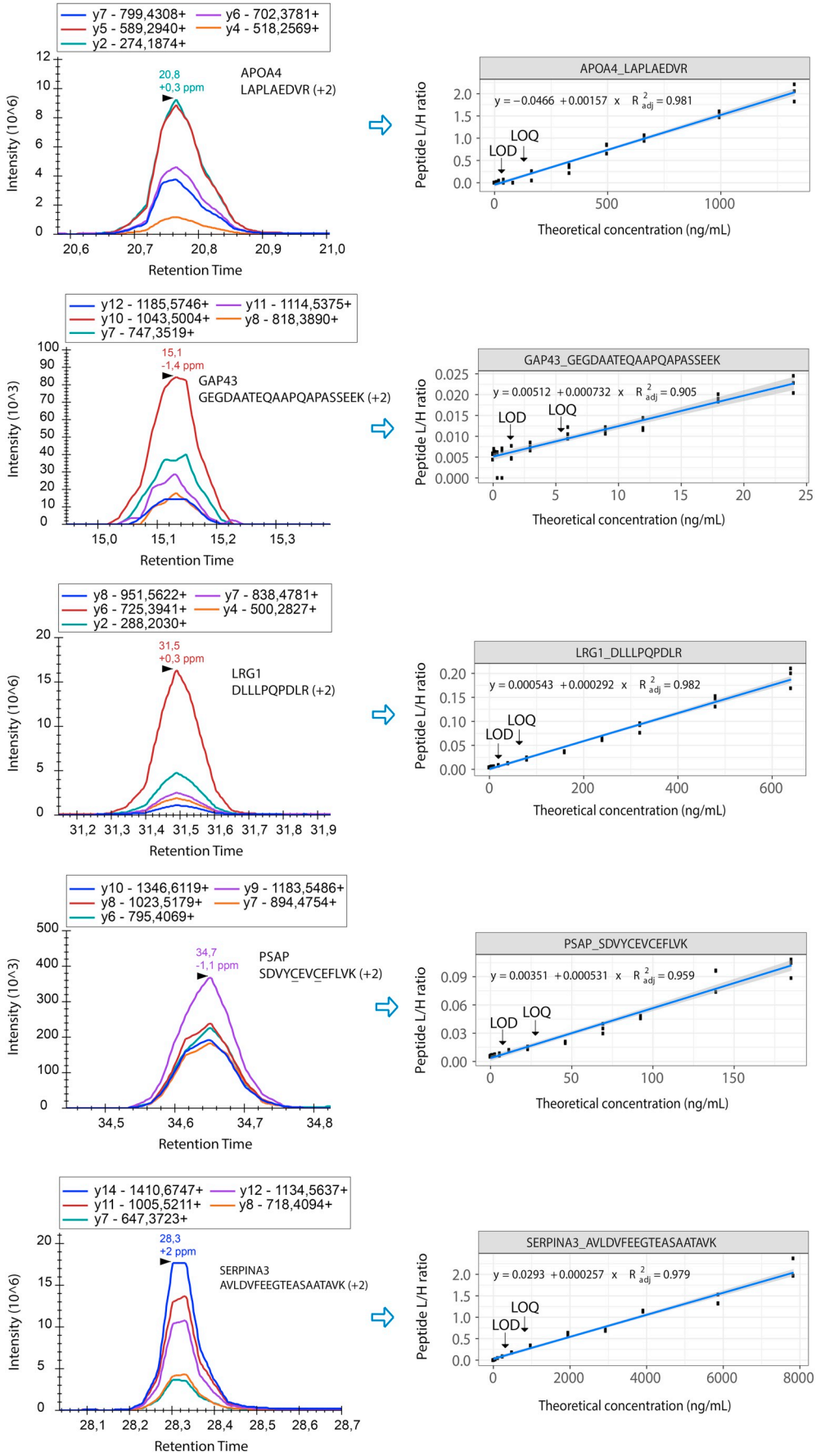
In this study, we have developed PRM assays for proteins in CSF with the aim to verify previously identified associations to AD as observed with antibody suspension bead arrays. In total, assay development for 20 brain-enriched proteins (Table 1) were performed and applied to 94 human CSF samples.

3.1. Development and validation of PRM assays

3.1.1. Selection of tryptic peptides and methods development

The initial list of targets of interest in the contest of Alzheimer's disease included 20 proteins. QPrESTs for each targets were selected and spiked into commercial CSF from healthy donors, digested and analyzed by shotgun-MS to select peptides appropriate for the intended analysis (Table 1). For the identified peptides (heavy and light), we prioritized the following: (1) unique; (2) no missed cleavages (3) not containing methionine; (4) no modifications; (5) detectable with the highest intensity. Peptides were subsequently evaluated in terms of giving reproducible signals between replicates and experiments to find the optimum target representatives of the proteins of interest. Although the shotgun-MS analysis of CSF served as a primary guide for peptide selection, we included in the final PRM inclusion list of 17 proteins, light and heavy peptides for the lower abundant proteins even when not detectable in CSF from healthy donors (Supplementary Excel Tables 2 and 6).

Method development for the 17 targets for which QPrEST peptides were detectable was performed in two steps.



(caption on next page)

Fig. 2. Selected peak identification and peptide linearity. Left: Chromatograms of PRM transitions. Right: Linearity plots from the peptide L/H ratio of the replicates (n = 3) with linear regression and 95% confidence interval. The x-axis shows the estimated concentration calculated by single point calibration based on the QPrEST spike-in concentration.

In a first pilot study, we considered eight targets: ALDOC, CTSD, FGA, GAP43, NEFM, PLG, SERPINA3 and VCAM1. For the analysis of these proteins, we applied a traditional sample preparation for proteomics involving ON trypsin digestion of 20 μl of CSF and peptide clean-up by C_{18} ZipTip. Subsequently for the remaining nine targets we decided to investigate the influence of experimental conditions including digestion time, volume of CSF and peptide post digestion clean-up procedure to obtain optimal recovery and assay performance for most of the peptides.

To investigate the effect of digestion time, we allowed digestion to proceed for 10, 30, 60, 180 min and ON (16 h) and the resulting peptides were analyzed by both shotgun-MS and PRM. Shotgun data analysis provided an overview of the digestion products, showing that the number of missed cleavages per peptide was reduced with longer digestions (Supplementary Fig. 1A). Even if, highly abundant proteins showed fully digested peptides already after a short digestion time (≤ 30 min) (Supplementary Fig. 1B), when evaluating the consistency between replicates (difference in percentage < 25%, Supplementary Fig. 1C), a clear improvement was found with ON digestion.

A selection of SPE cartridges with different sorbents was evaluated for peptide clean-up. The highest number of identified peptides and precision across all targets was found when using the neutral sorbent HLB using a generic protocol (HLBg, 17 endogenous and 24 heavy; HLBg; CV = 3.38%, $n = 3$) (Supplementary Fig. 1D, Supplementary Excel Tables 1 and 3).

Peptide recovery repeatability and robustness of the analytical method can increase increasing the amount of starting material analyzed. The use of 20 μL of CSF as the starting volume allowed for the detection of expected low abundant peptides such as GEGDAATEQAAPQAPASSEEK (GAP43) and LGFSEVELVQMVVDGVK (CKB) (Supplementary Fig. 2A). Moreover, the precision was higher for most of the peptides when starting from 20 μL of CSF (19 peptides, median CV = 9.5%) than from 10 μL of CSF (14 peptides, median CV = 15.4%), Supplementary Fig. 2B).

3.1.2. Validation of the methods

Based on the selected peptides and optimized parameters above, we continued by assessing the performance of the PRM method by evaluating linearity, LOQ, LOD, intra- and inter-day precision, accuracy and carry-over. First, L/H peptide ratio of 1 was extrapolated from reverse calibration curves constructed by a dilution series of the QPrESTs spiked into a constant level of commercial CSF (data not shown). The QPrESTs concentrations determined after spike-in by this experiment are stated in Table 1.

The linearity, LOQ and LOD were estimated with a 12-point dilution curve of the "high" pool (HLB clean-up) or commercial CSF (ZipTip clean-up) prepared in artificial analyte-free CSF. Linearity, LOD and LOQ were obtained for at least one peptide for each target protein (Table 3), excluding the five low abundant proteins CCK, CKB, NBEA, NEFM and VCAM1. These targets were detectable only in the high pool but not in all its dilutions and could therefore not be evaluated in terms of linearity. In summary, a validated method was developed for 13 out of the 17 target proteins.

Results from the dilution series showed linear ranges of 2 to 5 orders of magnitude depending on the target protein and specific peptides with adjusted coefficients of determination (Adj R^2) mean values of 0.945 (HLB method) and 0.964 (Ziptip method) (Fig. 2, Table 3 and Supplementary Fig. 4).

Precision was assessed as intraday (within-day variability, 4 assays), and interday repeatability (day-to-day variability, 3 days). Intraday repeatability was generally below 25% (median CV = 10.3%) with only one peptide over 30% (AHLGALLAR, CCK). For this peptide, low repeatability was expected since the concentration appears to be close to LOD. Inter-day repeatability calculated with method 2 (see Table 3) ranged between 12.6% and 41.9% (median CV = 26.7%). The peptides TGLEAISNHK (SPARCL1) and WELCDIPR (PLG) had the highest inter-day variation (41.9% and 41.5%, respectively) while all other measured peptides showed variation between 12 and 33%. In conclusion, interday precision was acceptable (CV < 20%) for at least one peptide per protein and the values obtained with method 1 and method 2 were comparable (Table 3).

For those proteins where standard reference material for human samples was not available, we estimated the accuracy using commercial CSF and considered the mean concentration ± SD from all the samples prepared ($n = 12$) as the reference value. Then, the closeness to this value was evaluated for one day of repeated experiments ($n = 4$) and accuracy ranged between 4.1% and 33%. Only the peptides VFSNGADLSGVTEEAPLK (SERPINA1) and TGLEAISNHK (SPARCL1) showed results > 20% and the rest of the peptides for those proteins had an accuracy of 11–20% to the mean estimated concentration in CSF. Carry-over (> 1%) was observed for 12 peptides (light and heavy) including SDVYCEVCEFLVK (PSAP, light and heavy), FCTGLTQIETLFK (CKB, heavy), LGFSEVELVQMVVDGVK (CKB, heavy) and LVDQNIFSFYLSR (CTSD, heavy) which displayed maximum values of 2.5%. All measured parameters describing the assay performance for each peptide are summarized in Table 3.

3.2. Protein levels in CSF from AD patients and healthy controls

The PRM assays developed were further applied to profile and estimate concentrations of all 17 proteins detectable in CSF (13 validated and 4 not fully validated methods) in 94 samples from both patients and neurologically healthy controls (Table 2). Peptide levels obtained by PRM were correlated with MFI data obtained by SBA analysis of the same samples and also compared between patient groups. When comparing the orthogonal methods used to analyze the CSF samples, Pearson correlations per protein between PRM (ratio L/H) and SBA (MFI) measurement revealed correlation coefficients between 0.31 and 0.97 with strong ($r > 0.60$) or very strong ($r > 0.80$) concordance between at least one peptide and antibody for GAP43, SERPINA3, APOA4, LRG1, CCK and VCAM1 (Fig. 3, Supplementary Fig. 5, Table 4 and Supplementary Excel Table 4). For the group comparisons, differences in peptide levels between groups were statistically significant (p-value < .05 after Bonferroni adjustment) for GAP43, VCAM1 CCK, CKB, and PSAP. GAP43, VCAM1 and PSAP were found higher in AD and preclinical samples with respect to non-AD MCI and controls (Fig. 4, Table 4 and Supplementary Excel Table 5).

GAP43 had an estimated concentration range between 14 and 217 ng/mL. The PRM assay was based on one peptide (GEGDAATEQAAPQAPASSEEK) with higher levels in AD and prodromal AD patients ($p \leq .01$). Strong correlations were observed between the peptide and antibody profiles obtained with HPA013392 ($r = 0.782$) and HPA013603 ($r = 0.764$) while only a moderate correlation was observed between the peptide and the third antibody HPA015600 ($r = 0.554$). The antibody HPA015600 correlated moderately also with the other two antibodies.

VCAM1 measurement was done using the peptide GIQVELYSFPR and concentration was found particularly higher in preclinical samples with respect to non-AD MCI and to the control group ($p < .01$), while the difference was not statistically meaningful after Bonferroni correction between AD patients and control groups. PRM results showed good correlation with one of the two anti-VCAM1 antibodies

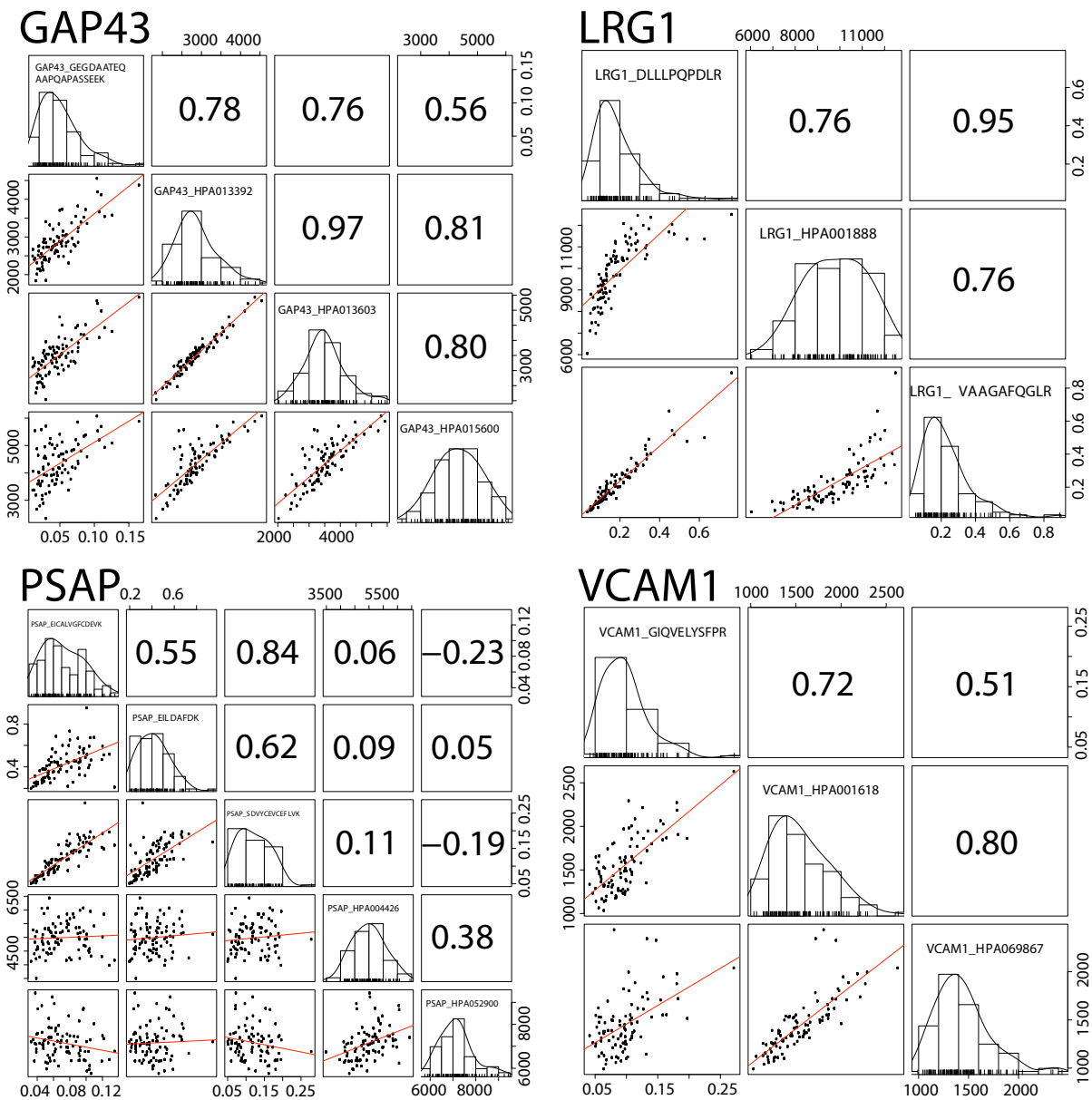


Fig. 3. Correlations between SBA and PRM measurement. Scatterplots and value of correlation (Pearson's *r*) between protein CSF levels measured by SBA (MFI value) and PRM (L/H ratio).

(HPA001618, $r = 0.723$) and lower with the second antibody, (HPA069867 $r = 0.551$).

For PSAP, all the three peptides revealed concordant profiles between sample groups, but the peptide EILDAFDK provided the highest statistical significance ($p \leq .05$). In this case, the same differences were not observed by antibody profiling (HPA004426, HPA052900). The two antibodies also showed poor correlation between each other.

For CCK and CKB, the differences between groups were statistically significant only between the AD patients and control groups (CCK, AHLGALLAR: AD vs. Control *p*-value = .02; CKB, LGFSEVELVQMVVDGVK: p-value = .009). The measurement of CKB performed with the second peptide (GTGGVDTAAVGGVFDVSNADR) showed good correlation ($r = 0.834$) highlighting that the same trend between sample groups was detected by the two peptides. Anyway the second peptide did not show a statistically significant difference (GTGGVDTAAVGGVFDVSNADR, AD-control p-value = .31). In is important to acknowledge that the quantitative method for GTGGVDTAAVGGVFDVSNADR was not validated (Table 3), therefore the accuracy and precision may not be good enough to detect correctly the differences between samples groups, while the method based on the first peptide is more reliable. LRG1 showed statistically increased levels in non-AD MCI compared to the control group (DLLLPQPDLR: non-AD MCI-control p-value = .035). Moreover, both peptides showed the same trend and there was a high correlation between peptides and antibody (HPA001888) ($r > 0.76$). All *p*-values obtained with and without correction and Pearson's coefficients for all the correlation are listed in Table 4 and Supplementary Excel Table 4.

Estimated concentration ranges based on the spiked in peptides for the remaining proteins were: AHLGALLAR (CCK) 0.84–221 ng/mL, GTGGVDTAAVGGVFDVSNADR (CKB) 0.87–24 ng/mL, LGFSEVELVQMVVDGVK (CKB) 0.90–20 ng/mL, DLLLPQPDLR (LRG1) 126–2530 ng/mL, VAAGAFQGLR (LRG1) 165–2954 ng/mL, EIEAEIQALR (NEFM) 0.10–12 ng/mL, EICALVGFCDEVK (PSAP) 61–250 ng/mL, EILDAFDK (PSAP) 371–1757 ng/mL, SDVYCEVCEFLVK (PSAP) 95–516 ng/mL and GIQVELYSFPR (VCAM1) 23–148 ng/mL (Table 4).

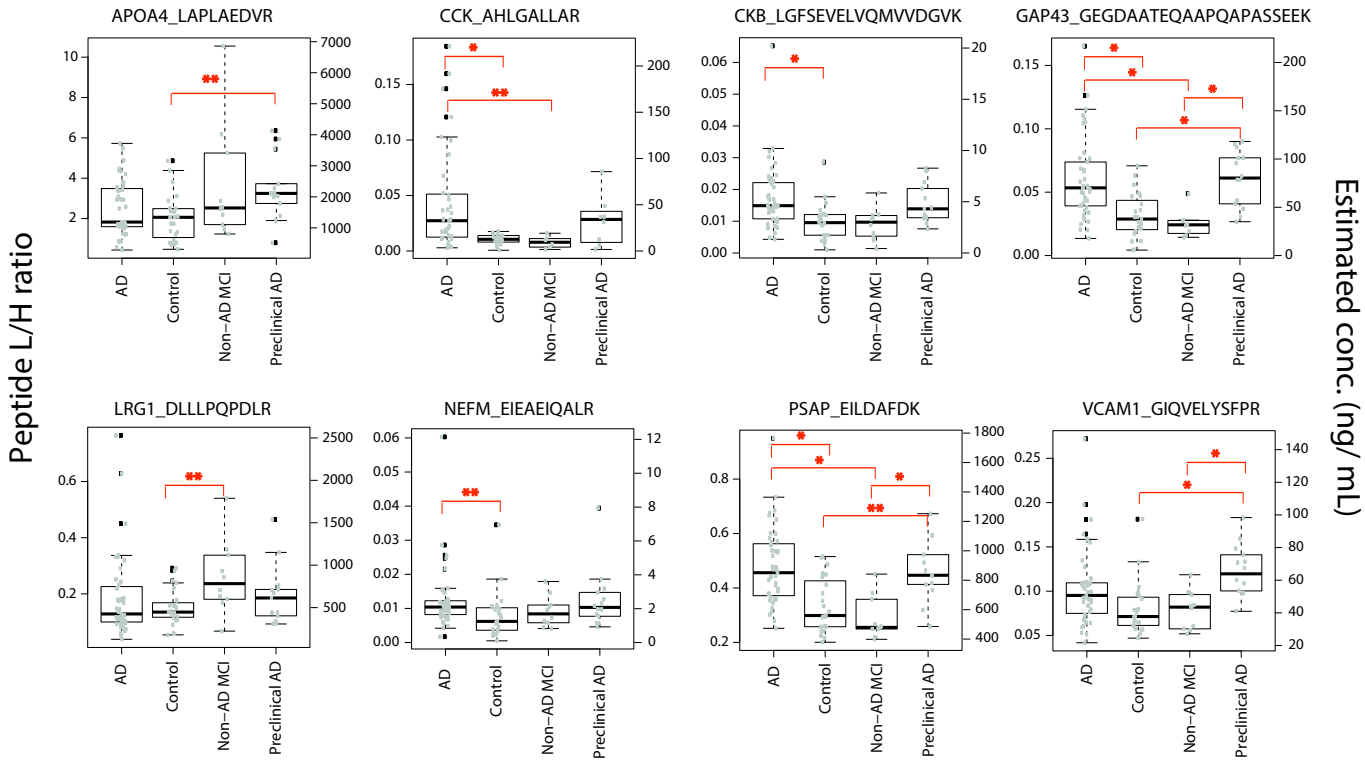


Fig. 4. Box-and-whisker plot visualization of protein profiles. Protein name and peptide are indicated on top of each plot. Left y-axis displays the peptide L/H ratio and Right y-axis displays the estimated concentration. Red lines and asterisks indicate significant differences between two groups. (*) p-value < .01;(**) p-value < .05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The aim of our study was to develop and validate PRM assays to perform an orthogonal verification of candidate biomarkers suggested by antibody-based protein profiling [12]. MS owns a great potential as an orthogonal method for validation of immunoassays data, featuring unsurpassed specificity and high theoretical multiplexing capacity. Nevertheless, a few aspects limit its application in respect to immunoassays: (i) the requirement of a laborious procedure for sample preparation, which affects throughput and robustness; (ii) higher sample consumption and (iii) lower sensitivity under the same conditions of sample processing. Sensitivity comparable to immunoassays is indeed achieved only upon protein/peptide pre-enrichment which implies a longer sample processing. Here we described a simple and straightforward analytical method that implies the digestion of un-depleted CSF, the purification of peptides with SPE cartridges with broad peptide affinity and the use of heavy labeled protein fragments as internal standards (QPrEST™). Starting from 20 targets of interest, 17 were detectable in CSF by PRM and analytical methods were developed and validated for 13 of these candidates. The limit was mostly imposed by the analytical sensitivity and by the lack of identification of proteotypic peptides.

4.1. Analytical aspects of data verification by an orthogonal method

In scientific discussions about the challenges in biomarker discovery and validation, there is general agreement that the quality of the analytical assays used for protein quantification is decisive for the success of a biomarker's verification and later validation (as long as the biomarker has a robust association to the process it is thought to reflect, which is a prerequisite). The extensive literature in analytical method development shows that optimal experimental conditions are often exquisitely peptide- and target-dependent. Therefore, an important point in the process of implementing novel biomarkers reside in the ability to translate a "proteomic assay", which applies the same experimental conditions to analyze hundreds of targets, into a "target-centric assay" passing gradually to a higher assay optimization. One of our goals was to evaluate a method that could support the verification of a large number of candidate biomarkers suggested by antibody-based protein profiling. Evaluating basic experimental parameters such as sample volume, time for digestion and SPE for purification, we concluded that a good approach to have sensitivity in the range of medium ng/ml included the use of ≥20 μL of CSF, ON digestion and HLB (Waters) solid phase peptide purification (Supplementary Figs. 1 and 2).

Assay validation in proteomic studies is typically hampered by the lack of standard references for the endogenous proteins. Indeed, even when the heavy label standard for quantification is available, the lack of endogenous protein references becomes the limiting factor. While it would be expensive and time-consuming to produce light standard protein for many dozens of analytes in the stage of verification, we found the suggestion to pool samples from healthy and disease patients (e.g. low and high pools) convenient (guidelines discussed during the workshop about mass spectrometry based assays held the National Institutes of Health in 2013) [21]. It is important to consider that low numbers of samples to pool and small volumes can become a limiting factor. In our case, the need for pooling many samples resulted in protein dilution with consequent flattening of protein levels between the pools (Supplementary Fig. 3). Therefore, we found it more convenient to generate a dilution curve of the high pool in artificial CSF. While linearity can be satisfactorily determined using this approach for the low range including LOQ and LOD, it becomes difficult to define the upper limit of the linearity, which is limited by the highest concentration of the analytes in the high pool. Using the single point standard approach, we obtained an estimated concentration for the proteins in each sample that ranged from 1 ng/ml to 1 mg/ml.

The correlations between PRM and SBA measurement for the targets ranged from null to very strong (> 0.79, Table 4, Supplementary

Table 4
Pairwise correlations between peptides and peptide-antibodies and disease significance.

Protein	Pairwise correlations (peptide-peptide, PRM; peptide-antibody, PRM-SBA)			Disease significance (Wilcoxon rank sum)				Estimated concentration range, samples (ng/mL)	
	Peptide 1	Peptide/antibody 2	Pearson correlation coefficient	Peptide	Patient groups	P-value (Bonferroni)	P-value (no correction)	Min	Max
APOA4	ALVQQMEQLR	HPA001352	0.717	ALVQQMEQLR	Preclinical AD vs. Control	0.19	0.031	343	7756
	ALVQQMEQLR	HPA005149	0.692	ALVQQMEQLR	Preclinical AD vs. AD	0.33	0.055		
	LAPLAEDVR	HPA001352	0.766	LAPLAEDVR	Preclinical AD vs. Control	0.027	0.0046	286	6858
	LAPLAEDVR	HPA005149	0.752	LAPLAEDVR	Preclinical AD vs. AD	0.36	0.061		
	VNSFFSTFK	HPA001352	0.680	VNSFFSTFK	Preclinical AD vs. Control	0.24	0.0041	264	5549
	VNSFFSTFK	HPA005149	0.648						
	ALVQQMEQLR	VNSFFSTFK	0.803						
	LAPLAEDVR	VNSFFSTFK	0.791						
	ALVQQMEQLR	LAPLAEDVR	0.908						
CCK	AHLGALLAR	HPA045039	0.312	AHLGALLAR	AD vs. Control	0.0070	0.0012	0.840	221
	AHLGALLAR	HPA069515	0.848	AHLGALLAR	AD vs. Non-AD MCI	0.040	0.0067		
CKB	LGFSEVELVQMVVDGVK	GTGGVDTAAVGGVFDVSNADR	0.834	LGFSEVELVQMVVDGVK	AD vs. Control	0.0036	0.00060	0.899	20.2
				LGFSEVELVQMVVDGVK	AD vs. Non-AD MCI	0.60	0.010		
				LGFSEVELVQMVVDGVK	Preclinical AD vs. Control	0.12	0.020		
				LGFSEVELVQMVVDGVK	Preclinical AD vs. Non-AD MCI	0.18	0.030		
				GTGGVDTAAVGGVFDVSNADR	AD vs. Control	0.12	0.021	0.868	24.4
				GTGGVDTAAVGGVFDVSNADR	Preclinical AD vs. Control	0.13	0.022		
GAP43	GEGDAATEQAAPQAPASSEEK	HPA013392	0.782	GEGDAATEQAAPQAPASSEEK	AD vs. Control	0.0020	0.00034	14.6	217
	GEGDAATEQAAPQAPASSEEK	HPA013603	0.764	GEGDAATEQAAPQAPASSEEK	AD vs. Non-AD MCI	0.0032	0.00054		
	GEGDAATEQAAPQAPASSEEK	HPA015600	0.558	GEGDAATEQAAPQAPASSEEK	Preclinical AD vs. Control	0.0051	0.00086		
				GEGDAATEQAAPQAPASSEEK	Preclinical AD vs. Non-AD MCI	0.0017	0.00028		
LRG1	DLLLPQPDLR	HPA001888	0.762	DLLLPQPDLR	AD vs. Non-AD MCI	0.16	0.027	126	2530
	VAAGAFQGLR	HPA001888	0.761	DLLLPQPDLR	Non-AD MCI vs. Control	0.035	0.0058		
	DLLLPQPDLR	VAAGAFQGLR	0.952	VAAGAFQGLR	AD vs. Non-AD MCI	0.19	0.032	165	2954
				VAAGAFQGLR	Non-AD MCI vs. Control	0.12	0.020		
NEFM	EIEAEIQALR	HPA022845	0.707	EIEAEIQALR	AD vs. Control	0.024	0.0040	0.100	12.1
	EIEAEIQALR	HPA022845.1	0.706	EIEAEIQALR	Preclinical AD vs. Control	0.14	0.023		
	EIEAEIQALR	HPA023138	0.598						

(*continued on next page*)

Table 4 (*continued*)

Protein	Pairwise correlations (peptide-peptide, PRM; peptide-antibody, PRM-SBA)			Disease significance (Wilcoxon rank sum)				Estimated concentration range, samples (ng/mL)	
	Peptide 1	Peptide/antibody 2	Pearson correlation coefficient	Peptide	Patient groups	P-value (Bonferroni)	P-value (no correction)	Min	Max
PSAP	EICALVGFCDEVK	EILDAFDK	0.551	EICALVGFCDEVK	AD vs. Control	0.19	0.045	60.9	250
	EICALVGFCDEVK	SDVYCEVCEFLVK	0.841	EICALVGFCDEVK	Preclinical AD vs. Control	0.034	0.0077		
	EILDAFDK	SDVYCEVCEFLVK	0.620	EICALVGFCDEVK	Preclinical AD vs. Non-AD MCI	0.30	0.049		
				EILDAFDK	AD vs. Control	0.00014	4.4E-05	371	1757
				EILDAFDK	AD vs. Non-AD MCI	0.00017	2.9E-05		
				EILDAFDK	Preclinical AD vs. Control	0.015	0.0034		
				EILDAFDK	Preclinical AD vs. Non-AD MCI	0.0028	0.00047		
				SDVYCEVCEFLVK	AD vs. Control	0.064	0.011	94.7	516
				SDVYCEVCEFLVK	AD vs. Non-AD MCI	0.13	0.022		
				SDVYCEVCEFLVK	Preclinical AD vs. Control	0.0053	0.00088		
				SDVYCEVCEFLVK	Preclinical AD vs. Non-AD MCI	0.031	0.0052		
SERPINA3	AVLDVFEEGTEASAATAVK	HPA000893	0.744	AVLDVFEEGTEASAATAVK	AD vs. Control	0.23	0.039	2341	22,267
	ITLLSALVETR	HPA000893	0.773	AVLDVFEEGTEASAATAVK	Preclinical AD vs. Control	0.27	0.046		
	ITLLSALVETR	AVLDVFEEGTEASAATAVK	0.917	ITLLSALVETR	AD vs. Control	0.20	0.034	2217	19,841
				ITLLSALVETR	Preclinical AD vs. Control	0.12	0.020		
VCAM1	GIQVELYSFPR	HPA001618	0.723	GIQVELYSFPR	AD vs. Control	0.053	0.0089	22.7	148
	GIQVELYSFPR	HPA069867	0.511	GIQVELYSFPR	Preclinical AD vs. AD	0.096	0.016		
				GIQVELYSFPR	Preclinical AD vs. Control	0.00016	2.7E-05		
				GIQVELYSFPR	Preclinical AD vs. Non-AD MCI	0.0030	0.00050		

Table 4). High correlations did not presuppose that the PrEST sequence used to generate the HPA-antibodies was the same as the QPrEST. Indeed, the highest peptide-antibody correlations were found when the antibody and QPrEST targeted different regions of the protein of interest (LRG1, GAP43, SERPINA3, Supplementary Excel Table 7).

There could be numerous reasons to why the results observed using antibodies and PRM differ. Exposure of the epitope is crucial for antibody functionality and the location of the epitope in the secondary structure could be the explanation as to why two antibodies to the same protein show discordant results. In addition, the presence of different proteoforms (isoform, fragments, and modifications) can typically lead to the differential detectability of the same protein by a peptide and an antibody., unless the assays are both developed to specifically targeted to the modified form [33,34]. Low correlations between two antibody measurements may also indicate off-target interactions while low peptide-to-peptide correlation can be ascribed differences in digestion or to the variable and unexpected modification.

4.2. CSF protein profiles associated with pre-clinical AD

In this study, we used PRM to verify the increased levels of GAP43 in CSF from early (preclinical-AD) and AD patients as previously observed by Remnestål et al [12]. The pre-synaptic protein GAP43 is central to neuronal development, axonal growth and plays an important role in memory and information storage and altered levels of GAP43 in CSF from AD and pre-clinical AD patients may reflect the synaptic degeneration in the early onset of the disease. Interestingly, on tissue level, expression of GAP-43 has been reported as lower in the frontal cortex but not in the hippocampus [36]. Another interesting protein that could be confirmed by PRM was VCAM1, a potential marker of microvascular cerebral damage. High levels of VCAM1 in pre-clinical AD patients would support the hypothesis that microvascular abnormalities within the brain may precede neurodegeneration in AD [37]. Plasma VCAM1 levels moreover correlate with the gravity of dementia in AD patients showing an association with white matter degradation [38].

The PRM and SBA measurements of the two proteins PSAP and CKB showed a poor or null correlation in our study. As mentioned above, there could be both biological and technical reasons why the data obtained using the two methods do not correlate. However, the strong statistically significant difference observed between AD and preclinical patients versus non-AD MCI and controls by PRM quantification highlights their possible role in the early phases of the disease.

PSAP (prosaposin) measurements were performed using three different peptides, all showing a similar trend. The peptide EILDAFDK provided the highest statistical significance in group comparison and good correlation with the second peptide SDVYCEVCEFLVK. The MS data in this case confirm previous studies, where PSAP was found to be significantly elevated in the CSF of AD and DLB but not in PD [14]. The two antibodies (HPA004426 and HPA052900) poorly correlate with each other and the lack of consistency between MS data and antibody-based data could in this case potentially be due to differential detection efficiency of the protein or alternatively events of cross-reactivity and off-target binding. PSAP is the precursor of saposins and is secreted in its full-length form into biological fluids such as CSF. PSAP is a neurotrophic factor secreted in response to cellular stress, which mediates pro-survival signaling pathways playing an important role in nerve rescue regeneration [50].

CKB is involved in the regeneration of chemical energy, ATP, in the brain. High consumptions of energy in the cells is associated with higher levels of CKB expression [39], while lower activity of CKB has been observed in neurodegenerative disorders such as AD and Pick's disease [40] and ascribed to the oxidative damage and cellular stress that occurs during AD and similar disorders [41].

CSF brain flow carries out several important functions including distribution of neurotrophic factors and clearance of neurotoxic products or solutes [35,36]. The high concentration of VCAM1, GAP43, PSAP and CKB found in CSF of preclinical AD patients provide further evidence to the involvement of cerebrovascular abnormalities [37], inflammation [38], and synapsis failure [39] at the onset of AD. One could also suggest that GAP43, VCAM1, PSAP and CKB might be present at higher concentrations in AD CSF due to a lower CSF flow in AD. However, the result that not all proteins are increased in a similar manner argues against this interpretation.

4.3. CSF profiles associated to late-stage AD and non-AD MCI

CCK was found higher in AD compared to controls but not in preclinical samples. This suggests that its presence in CSF may be more related to the consequences of the later stages of disease. CCK is a peptide hormone-synthetized from the 115-amino acid precursor, preprocholecystokinin which is further processed into pro-CCK (aa 21–115), and then into CCK-58, (aa 46–103) that can be cleaved to form nine different peptides. While the two anti-CCK antibodies (HPA045039, HPA069515) were raised against a sequence covering 9 out of 10 cholecystokinin peptides (aa 33–99, Supplementary Excel Table 7), using PRM, we targeted the full form of CCK preprotein, or specifically cholecystokinin-58 (CCK-58) (AHLGALLAR, aa 56–64, sequence not present in the other peptides). To best of our knowledge, this is the first report suggesting altered levels of CCK-58 in CSF in the context of AD, while decreased CSF concentrations of CCK-8 were found in PD patients in other studies [45]. CCK mediates the satiety signal in the peripheral digestive system, [46] and has a neuroprotective and proliferative role, affecting memory and learning processes in the hippocampus [42]. Interestingly while CCK levels physiologically increase in CSF after eating [47], an association between overeating and hippocampal impairment has been experimentally observed [43,44]. Little is known about the role of CCK-58 in CSF of late stage AD patients, but some elucidation might come in the future from studies about the communication between the gastrointestinal tract and the hippocampus through the vagus nerve [48]. LRG1 (Leucine-rich alpha-2-glycoprotein 1) levels correlated very well (Table 4), showing in agreement increased levels of LRG1 in MCI but not in AD and control groups. Our results are in line with those obtained by Miyajima et al. [45], where high levels of LRG1 were found in CSF from patients affected by Parkinson's disease with dementia (PDD) and progressive supranuclear palsy (PSP) but not in AD patients. LRG1 is known to be an inflammation-induced protein present at high levels in the CSF of ageing humans [46] and altered levels CSF may reflect specific molecular events involved in the onset of MCI and other neurodegenerative disease but not related specifically to the biological causes underpinning AD development.

4.4. From verification to validation

Tissue leakage, altered clearance process [47] and variation in the CSF flow are some of the known mechanism causing a change of protein concentration in CSF. The latter has been hypothesized to be a cause rather than a consequence of various forms of brain dysfunctions including neurodegenerative disorders [48,49]. In order to better understand the biological meaning of the proteins discussed here and to test their diagnostic value, further verification and validation studies should involve the analysis of CSF samples from patients with other neurodegenerative diseases such as PD and FTD, to assess similarities and dissimilarities between the different pathologies and to evaluate the specificity of the markers. Moreover in relation to the theory described by Reiber et al. [49] about CSF flow and the anatomical knowledge required to understand the meaning of variation in CSF protein concentration, parallel studies of protein expression in brain tissue should be performed.

5. Conclusions

Our study demonstrated that the application of an orthogonal method such as PRM for the verification of antibody-based experiments is a convenient approach to confirm the most robust protein profiles discovered. The comparison of data obtained by two different platforms is a very powerful approach but the information gained should be interpreted in the light of the fact that the two methods, based on different analytical principles, present peculiar limits in protein detection and should be regarded as complementary.

The PRM assays presented here were validated for 13 target proteins and can be useful in future for studies of larger sample cohorts. For the low abundant targets for which it was not possible to evaluate the linearity, it will be necessary to perform further analysis for method validation including for example the use of protein standards or to explore the possibilities of more sensitive assays such as immunocapture-MS or sandwich immunoassays.

The concordance between data obtained with two different methods, supported by literature on their biological role, strongly supports the analytical reliability of the markers identified associated to the early onset of AD. To establish their potential as disease-associated markers, further studies in independent sample collections are required.

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Competing financial interests

MU is a co-founder of Atlas Antibodies. PN and JS acknowledges formal links to Atlas Antibodies. The other authors declare that they have no conflict of interest.

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