Cite This: J. Proteome Res. 2018, 17, 2282−2292

Article

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Targeted Mass Spectrometry-Based Assays for Relative Quantification of 30 Brain-Related Proteins and Their Clinical Applications

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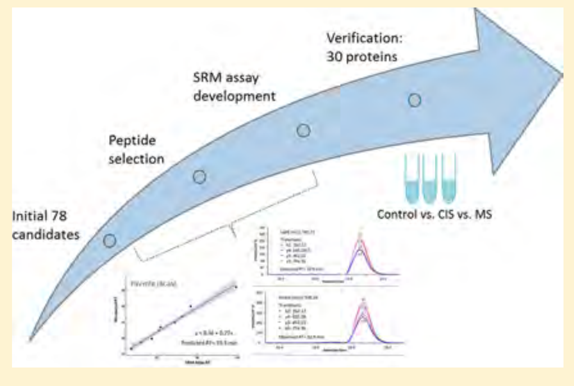
Supporting Information

ABSTRACT: Cerebrospinal fluid (CSF) is a promising clinical sample for identification of novel biomarkers for various neurological disorders. Considering its direct contact with brain tissue, CSF represents a valuable source of brain-related and brain-specific proteins. Multiple sclerosis is an inflammatory, demyelinating neurological disease affecting the central nervous system, and so far there are no diagnostic or prognostic disease specific biomarkers available in the clinic. The primary aim of the present study was to develop a targeted mass spectrometry assay for simultaneous quantification of 30 brain-related proteins in CSF and subsequently to demonstrate assay feasibility in neurological samples derived from multiple sclerosis patients. Our multiplex selected reaction monitoring assay had wide dynamic range (median fold range across peptides = 8.16×10^3) and high assay reproducibility (median across peptides CV = 4%). Candidate biomarkers were quantified in CSF samples from neurologically healthy individuals (n = 9) and patients diagnosed with clinically isolated syndrome (n = 29) or early multiple sclerosis (n = 15).



KEYWORDS: *cerebrospinal fluid, mass spectrometry, selected reaction monitoring, biomarkers, multiple sclerosis*

INTRODUCTION

Cerebrospinal fluid (CSF) has been the sample of choice in the quest of novel biomarkers of numerous neurological disorders. Given its direct contact with the brain parenchyma, CSF may reflect pathological changes in the brain and serve as a source of brain-related proteins. CSF matrix is relatively simpler than plasma with much lower total protein concentration; however, most of the abundant proteins found in plasma are also present in highest abundance in CSF (e.g., albumin).[1] Still, the concentration of brain-derived proteins is generally much higher in CSF compared to plasma, while plasma-derived proteins have lower concentration in CSF compared to blood.[2] Brain-derived proteins are not necessarily brain-specific, since they can be produced by other tissues. Yet, some of the brain-derived proteins have high specificity for cerebral tissue and certain cell types (e.g., neuronal and glial proteins: S100B, NSE, tau) and have been associated with several neurological pathologies.[2] For example, elevated CSF levels of proteins S100B and NSE were found in stroke patients and Creutzfeldt−Jacob disease, while tau protein (total and phosphorylated-tau) is known to be elevated in Alzheimer's disease.[2,3]

In order to seek disease-associated indicators, brain-specific proteins can be of significant interest as novel candidate biomarkers for various neurological disorders.[4] Previously, we have performed shotgun proteomic analysis of CSF and made selection of 78 highly specific brain proteins (predicted membrane and secreted origin) that can be reliably identified in the individual CSF samples.[5] These brain tissue-specific proteins were defined by the Human Protein Atlas (HPA) database based on their high mRNA expression in the brain, which makes HPA a valuable source of tissue-specific transcriptome and proteome suitable for biomarker research applications.[6] As these proteins are highly specific for the brain tissue, they may be relevant for the neurodegenerative disease pathology. Development of a quantitative, multiplex

Received: October 27, 2017
Published: April 30, 2018

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assay for these proteins could be therefore a valuable tool for testing their potential as biomarkers. Similar efforts have been made by our group for discovery of tissue-specific biomarkers using targeted proteomics for central nervous system (CNS) and other disorders.[4,7]

Biomarker discovery is dependent on application of high-throughput technologies for discovery and subsequent verification and validation of proteins abnormally regulated in disease. Selected reaction monitoring (SRM) is a targeted-mass spectrometry platform used in proteomics for relative and absolute quantification of proteins in complex biological samples. Peptide and protein quantification is based on the prior selection of the targeted precursor ion and its fragmentation pattern, suitable for an optimal assay with high specificity and sensitivity.[8] Despite the fact that the development of a high-quality targeted assay requires significant effort, it is still time- and cost-efficient, compared to development of an immunoassay such as enzyme-linked immunosorbent assay (ELISA).

Multiple sclerosis (MS) is a chronic, autoimmune disease that affects the CNS (white matter of brain and spinal cord). The typical hallmarks involve formation of multifocal plaques in the white matter which expand over time as a result of inflammatory, demyelinating, and degenerative processes.[9] According to the disease course, MS is classified into relapse-remitting multiple sclerosis (RRMS), primary progressive, with the gradual progression from the onset (without periods of remissions), and secondary progressive MS (SPMS), usually a continuum of the RRMS with further constant progression of the disease pathology. Clinically isolated syndrome (CIS) suggestive of MS is associated with acute or subacute neurological symptoms usually caused by a single lesion in white matter in patients that do not fulfill the diagnostic criteria of MS. About 85% of these patients later develop definite MS, indicating that CIS can be considered as an early, prestage of MS.[10]

Clinical course and prognosis of MS is largely unpredictable, and clinical manifestation is related to the affected CNS regions. Diagnosis of MS is based on a patient's history, clinical assessment, and additional imaging and/or laboratory examinations, including magnetic resonance imaging (MRI) and detection of oligoclonal IgG bands in CSF by isoelectric focusing.[11] Once there is evidence of CNS lesions disseminated in space and changing with time, after excluding alternative diseases, definitive diagnosis of MS can be made. Paraclinical tests (MRI and CSF oligoclonal IgG) have high diagnostic sensitivity; however, they are not specific to MS; for example, oligoclonal IgG can be found in other inflammatory neurological disorders (such as Guillain-Barre syndrome), while MS-like MRI lesions can be found in individuals without any clinical symptoms or in other diseases with neurological manifestation (such as in the chronic autoimmune disease systemic lupus erythematosus).[9,12]

Considering that there is no single biomarker or test specific for MS, there is a need for novel biomarkers that will allow accurate diagnosis of MS at an early stage of the disease. In addition, biomarkers that would discriminate between CIS and MS could indicate risk of progression of CIS patients into MS and ultimately lead to the better understanding of the early pathological mechanisms with a potential of development of new disease modifying treatments.

The principal aim of the present study is therefore to develop targeted, multiplex mass spectrometry-based SRM assays for 78 brain-related, highly specific proteins in the CSF. The second aim is to demonstrate the feasibility of the assay and evaluate its ability to differentiate between patients with MS, CIS, and neurologically healthy controls.

■ MATERIALS AND METHODS

Selection of Brain-Related Proteins As Biomarker Candidates

Brain-related proteins (n = 78) were selected using the HPA database (version 13) of tissue specific proteins (http://www.proteinatlas.org) and our comprehensive, in-house developed CSF proteome, as previously published.[5] Briefly, brain-enriched (n = 196) and group-enriched (n = 138) proteins, secreted and/or of membrane origin (as identified in HPA source) that were reproducibly detected in six individual CSF proteomes (present in at least 4 individuals) were selected as candidate proteins for SRM assay development (n = 78). According to the HPA, tissue-enriched are genes with at least five times higher mRNA expression in the particular tissue (i.e., brain) relative to other tissues, while group-enriched genes have mRNA expression at least five times higher in the group of 2−7 tissues (including brain), relative to all other tissues.

Selection of Peptides for Candidate Proteins

Peptides and transitions for 78 candidate proteins were initially selected from the SRM Atlas database (www.srmatlas.org) and from our previously developed and published SRM assays.[4] Initially, between 1 and 7 unique proteotypic peptides per protein were selected (7 to 25 amino acids in length), with a maximum of 7 transitions per precursor peptide (b- and y-ions). Peptides containing C-terminal cysteine or glutamine were excluded, and peptides with methionine in the sequence were preferentially avoided. In addition, peptides with +1 and +4 charge were omitted as well as peptides with possible tryptic misscleavage at N- and C-terminus. Peptides were searched against the Basic Logical Alignment Search Tool (BLAST, www.uniprot.org/blast) to confirm peptide uniqueness for the targeted proteins.[13]

Identification of Peptides and Selection of Transitions in CSF

Sets of peptide transitions for 78 candidate proteins were further evaluated to establish a single injection multiplex SRM assay. Retention times (RT) for each peptide were predicted using two prediction methods (Supporting Information Table S1 and Figure S1). In one of the approaches, RT was predicted using 9 endogenous CSF peptides with elution profile covering most of the 60 min liquid chromatography (LC) gradient. The experimentally observed RTs on TSQ Quantiva (Thermo Scientific, San Jose, USA) were correlated with the RTs of the same peptides obtained from the SRM Atlas database. In another approach, RT was predicted using the elution profile of a commercial heavy peptide set (15 synthetic heavy labeled peptides for lysine and arginine, Pierce Peptides Retention Time Calibration Mixture solution, Thermo Scientific). Briefly, 100 fmol of Pierce Peptide solution was spiked-in into the CSF pool digest. The experimentally observed RT (60 min LC gradient) was correlated with the hydrophobicity of the peptide sequence (using SSRCalc 3.0, 300 A calculator, Skyline software, version 3.1).

Predicted RTs were calculated for all peptides under evaluation, and scheduled SRM assays were prepared (avoiding peptide elution overlap). RT windows were set ±3 min from predicted elution times. Unscheduled methods were prepared

DOI: 10.1021/acs.jproteome.7b00768
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for peptides for which predicted RT was outside of the LC gradient.

For peptides positively identified in our in-house developed CSF proteome, RT observed with a discovery Q-Exactive Plus instrument (RT extracted with Proteome Discoverer software, Thermo Scientific) was compared with the RT observed with TSQ Quantiva (both 60 min LC gradient) and was used as one of the criteria to confirm peptides' identification.

Peptides were then selected over several rounds of peptide evaluation, based on the following criteria: (i) absence of interferences, (ii) observed coelution of transitions, (iii) transitions' pattern and order (compared to SRM Atlas data available), peptides containing proline were preferred (generate high intensity signal).

Peptide identity was further confirmed using heavy peptides. Lyophilized heavy labeled peptides (JPT Peptide Technology, Berlin, Germany) were reconstituted in 100 μL of 20% ACN (Fisher Scientific) in 0.1 M ammonium bicarbonate (Fisher Scientific), aliquoted and stored at −20 °C. Heavy labeled peptides were combined in equal amounts to create a heavy peptide pool for further analysis. Light (endogenous) peptides' identity was confirmed using heavy labeled peptides based on the same RT observed, identical order, and relative ratios of transitions in both light and heavy peptides. Transitions for the multiplexed SRM method were then selected; transitions with the highest intensities were preferred.

Mass Spectrometry Sample Preparation

For the SRM assay development phase, two different pools of nonpathological CSF samples were prepared (combining 5 to 6 individual samples), stored at −80 °C, and used for assay evaluation. Individual CSF samples were retrospectively collected as leftovers after routine biochemical examinations at Mount Sinai Hospital, Toronto. Ethics approval was obtained from the Mount Sinai Hospital Research Ethics Board (REB 15-0265-E).

Aliquots of CSF pools were thawed and centrifuged for 10 min at 17,000*g*. Volumes corresponding to 10−15 μg of total protein were denatured with 0.05% RapiGest (Waters, Milford, USA) and reduced with 5 mM dithiothreitol (Sigma-Aldrich, Oakville, Canada) at 60 °C for 40 min. Alkylation was achieved with 15 mM iodoacetamide (Sigma-Aldrich, Oakville, Canada) for 60 min in the dark at 22 °C. Protein digestion was carried out with trypsin (Sigma-Aldrich, Oakville, Canada) in 50 mM ammonium bicarbonate (1:30 trypsin to total protein ratio), for 24 h at 37 °C. Heavy peptides were then spiked into the digest followed by addition of 1% trifluoroacetic acid (TFA, Fisher Scientific). Samples were then centrifuged at 1,000*g* for 30 min, and supernatants were retained. Peptides were purified using OMIX C18 tips. During initial evaluation of light peptides in the CSF sample pool, we eluted peptides in 5 μL of acetonitrile solution followed by addition of 60 μL of water-formic acid solution. This was further optimized to 4.5 μL elution and 54 μL dilution in order to decrease the final peptide dilution; in all subsequent experiments we used the optimized elution/dilution volumes.

Liquid Chromatography-Tandem Mass Spectrometry (LC-*MS/MS*)

Peptides were analyzed using a triple quadrupole mass spectrometer, TSQ Quantiva. Each sample was injected (18 μL) into an in-house packed 3.3 cm precolumn (5 μm C18 particle, column inner diameter 150 μm) followed by a 15 cm analytical column (3 μm C18 particle, inner diameter 75 μm, tip diameter 8 μm). The liquid chromatography, EASY-nLC 1000 system (Thermo Fisher, Odense, Denmark) was coupled online to the TSQ Quantiva mass spectrometer with a nano-electrospray ionization source. The 60 min LC gradient was applied with increasing percentage of buffer B (0.1% formic acid in acetonitrile) for peptide elution at a flow rate of 300 nL/min (increasing percentage of buffer B over 60 min gradient: 1−5% over 2 min, 5−35% over 47 min, 35−65% over 3 min, 65−100% over 1 min, and hold for 7 min at 100% B at the flow rate of 450 nL/min (washing)). Collision energy (CE) for each precursor peptide was tested over the range of 8 V (2 steps, ± 2 V from nonoptimized CE). Optimized CE for each peptide was selected based on the highest peak area observed. Additionally, dwell time and RT windows were adjusted to ensure a minimum of 15 points per LC peak. The SRM assay parameters were thus set up as follows: positive-ion mode, optimized collision energy values, adjusted dwell time, 0.2/0.7 Th Q1 resolution of full width at half-maximum and 0.7 Th in Q3 resolution. Raw data were uploaded and analyzed with Skyline software (University of Washington).

Linearity Assay

A pool of CSF samples was prepared and the volume corresponding to 15 μg of total protein was aliquoted for each point of linearity. Digestion was carried out as previously described. Heavy labeled peptide pool solution was prepared as defined before and used for linearity analysis. Serial dilution of heavy labeled peptide pool was spiked into aliquots of CSF pool digests with constant concentration of endogenous (light) peptide (4000, 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98, 0.49, 0.24, 0.12, 0.06, and 0.03 fmol of heavy peptides per injection). All samples were analyzed in triplicate and run on the instrument from lowest to highest concentration. Coefficient of variation (CV) was calculated for all points.

Based on the linearity of the observed heavy to light ratios, heavy labeled peptide amount was optimized relative to the light peptide amount observed in the CSF pool so that the ratio of light and heavy peptide is close to 1. The heavy peptide pool with amounts close to the levels of endogenous peptide was prepared and used for reproducibility, freeze/thaw, and carry-over effect studies and clinical sample analysis.

Reproducibility

Four aliquots of the CSF pool were prepared for testing the reproducibility of the assay. Aliquots were digested independently, in the same way as clinical samples and simultaneously with the clinical samples. Aliquots were then distributed over multiple wells, ran in three replicates each (12 in total), and analyzed over several days (scheduled at the beginning, in the middle, and at the end of the clinical samples).

Freeze−Thaw Assay and Carry-over Effect

For freeze/thaw (F/T) study a pool of CSF samples (prepared from 9 individual samples, previously kept below −20 °C) was subjected to an additional four F/T cycles (−80 °C freeze and 20 °C thaw). After each cycle, an aliquot corresponding to 15 μg total protein was kept for mass spectrometry sample preparation. All cycles were processed at the same time and run on the instrument in duplicate. For each F/T cycle, the mean value of the L/H ratio was calculated and compared against the baseline (first F/T cycle).

The effect of carry-over was tested under the LC-*MS/MS* conditions. A CSF pool sample digest was prepared (as

DOI: 10.1021/acs.jproteome.7b00768
J. Proteome Res. 2018, 17, 2282−2292

described under mass spectrometry sample preparation) and heavy peptide mix was spiked into the digest. Carry-over effect for endogenous and heavy-labeled peptides was examined using pattern of injections as follows: two blank injections, three sample injections, followed by 3 blank injections (blank: BSA solution in MS Buffer A). An experiment was repeated three times. The carry-over ratio was estimated for endogenous and heavy-labeled peptide using following formula: AUC (first blank injection) − AUC (last blank injection)/AUC (last sample injection) − AUC (last blank injection), according to the published guidelines and recommendations (AUC = area under the curve).[14,15] The result was presented as percentage of carry-over effect.

Liquid Chromatography Gradient Adjustment

During the SRM development phase, 60 min LC gradients were used for the initial peptide/transition selection and the linearity assay. In the interest of reducing instrument run time for clinical sample analysis, the gradient was adjusted from 60 to 37 min; this gradient was used for further analysis, including the freeze−thaw assay, carry-over effect, and reproducibility. All LC peaks were manually inspected to ensure that sufficient points per LC peak were achieved with the modified gradient. Only transitions without present interferences (quantifiers) were used for quantifications, as indicated in the Supporting Information. Calibration curves were run with the shorter gradient and compared against the linearity assay (60 min LC gradient) to confirm the linearity (Supporting Information Figure S2).

Method Comparison: KLK6 SRM Assay and ELISA

For method comparison, clinical samples were analyzed with a KLK6 ELISA. The in-house sandwich-type KLK6 ELISA utilized is well-characterized, as described elsewhere.[16]

Clinical Samples

Fifty six CSF samples were prospectively collected at the Department of Neurology, University Hospital Clinical Center “Sestre Milosrdnice”, Zagreb, Croatia, from January 2015 to February 2016. Ethical approval for sample collection was obtained from the institution’ ethics committee (EP-1506/14-11). Patients admitted with the diagnosis of MS and CIS were eligible for inclusion (in addition to “control” group). Clinical diagnosis of patients with MS and CIS were made based on history, clinical assessment, clinical tests (such as MRI, visual evoked potential, VEP), and CSF laboratory analysis (such as oligoclonal IgG band analysis), according to the published guidelines and recommendations.[11,17] Patients admitted in the Department of Neurology with symptoms suspicious for neurological diseases but ultimately cleared (after clinical and CSF examination) for diagnosis of any neurological disorders (e.g., neurological infectious and neurodegenerative disease or stroke) were included in the “control” group. These individuals were diagnosed with headache, dysphagia, spontaneous intracranial pressure, and benign intracranial hypertension. Out of 12 control individuals included in the study, 3 were additionally excluded after examination of the CSF IgG oligoclonal bands, indicating abnormal CSF results. An exclusion criterion for all samples was the presence of blood contamination (based on visual inspection and erythrocyte count). The following clinical parameters were collected for all samples: age, sex, disease duration, and therapy.

All CSF samples were obtained with lumbar puncture and upon collection transferred at the Department of Chemistry, University Hospital Clinical Center “Sestre Milosrdnice”, Zagreb, Croatia for CSF laboratory analysis. After CSF cell count (Fuchs Rosenthal counting chamber), samples were centrifuged and biochemical analysis (glucose, lactate, and total protein concentration) was performed (Architect c 8000 analyzer, Abbott Laboratories, Abbott Park, Illinois, USA) as part of the routine CSF analysis before sample storage. Additional immunochemical CSF tests were performed such as oligoclonal IgG band analysis (Hydrasys analyzer, Sebia, Evry Cedex, France). Samples were stored at −20 °C.

CSF samples were shipped on dry ice to the Lunenfeld−Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, Canada, and stored at −80 °C until processing. Ethics approval was obtained from the institution’s Research Ethics Board for the usage of these samples (REB Amendment 15-0265-E). Samples were thawed and centrifuged at 17,000*g*, and volumes equal to 15 μg of total protein were aliquoted for processing as previously described.

The final SRM method for clinical samples included myelin basic protein (MBP) as positive control, using previously developed and published settings,[4] extracellular matrix protein 1 (ECM1) as negative control (settings for SRM-based quantification were developed together with the SRM assays for 30 candidate proteins), and apolipoprotein B (APOB) as a control for blood contamination using settings as described previously.[18] For methionine-containing peptides (SST protein), both oxidized and nonoxidized forms were monitored. The SRM raw mass spectrometry data were deposited to the Peptide Atlas repository (http://www.peptideatlas.org/PASS/PASS01082) with the data set identifier PASS01082 and following specifications: Username: PASS01082, Password: DH2545re.

Data Analysis

Clinical samples were randomized and run in duplicate. The raw files were uploaded to Skyline software (version 3.5.0.9319, University of Washington), which was used for peak integration and quantification of the AUC as well as light to heavy peptide ratios (AUC_{light}/AUC_{heavy}). For relative quantification, the avearge AUC_{light}/AUC_{heavy} was multiplied by the amount of the heavy labeled peptide spiked-in into the samples to calculate the relative amount of endogenous light peptide (fmoles per injection) taking into consideration the volume of CSF used. For peptides with methionine in the sequence, AUC_{light}/AUC_{heavy} was calculated as the sum of AUC for mono-oxidized (due to methionine oxidation during sample processing) and nonoxidized forms of the peptide, for both light and heavy labeled peptide as indicated: AUC $(\text{oxidized + nonoxidized})_{light}$/AUC $(\text{oxidized + nonoxidized})_{heavy}$. SRM data were manually evaluated, and samples with poor integration and not reliable quantification were excluded. Linearity was assessed (linear regression, sigmoidal curve, coefficient of variation profile) using R statistical software (www.Rproject.org).

Statistical analysis

Statistical analysis was performed with R statistical and graphics software. Means, medians, standard deviations, coefficients of variations, and interquartile ranges were calculated for descriptive and comparative purposes. Nonparametric, Kruskal−Wallis, and Dunn tests were used for comparison of age and protein concentration (not adjusted by age difference) between three and two groups of samples, respectively. For tests involving dichotomous variables, such as gender, the Fisher’s exact test was used. Correlation analysis between age and protein concentration was performed with Spearman’s rank

DOI: 10.1021/acs.jproteome.7b00768
J. Proteome Res. 2018, 17, 2282−2292

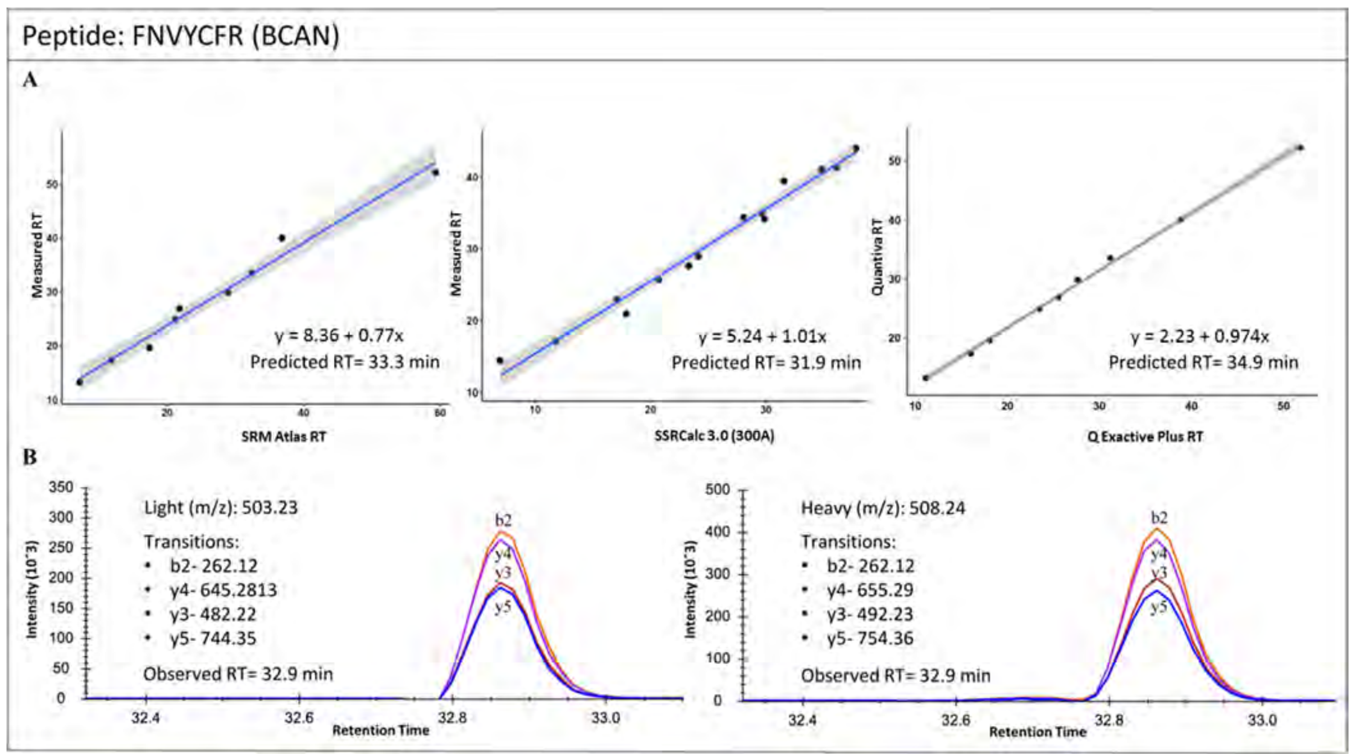

Figure 1. Identification of endogenous peptides for SRM assay development. (A) Predicted retention time (RT) based on RT of endogenous peptides vs their RT reported in the SRM Atlas (left graph), RT of Pierce synthetic peptides vs hydrophobicity index calculated using Skyline SSRCalc. 3.0 (middle graph), and RT of endogenous peptides from Q-Exactive Plus instrument (discovery phase) vs their RT from TSQ Quantiva instrument (SRM assay) (right graph). (B) Co-elution of SRM transitions from endogenous (light) and isotopically labeled standard (heavy) peptides.

correlation coefficient. To account for difference in age between groups, multivariate linear regression was performed to test the difference in protein concentration. Passing−Bablok regression analysis and Bland−Altman plots were used to compare the assays between SRM and ELISA. R package "mcr", version 1.2.1 was used for analysis. P-values for comparison between groups were indicated as nonadjusted ($p < 0.05$ was considered statistically significant) and adjusted by Bonferroni correction ($p < 0.0016$ was considered statistically significant).

■ RESULTS

Identification of Proteotypic Peptides in CSF for SRM Assay Development

Peptide identification in CSF for SRM assay development was a multistep process with several rounds of peptide evaluation, performed in two different CSF pools. RT prediction algorithms were used for initial peptide assessment (Supporting Information Table S1 and Figure S1). Experimentally observed peptides' RT was compared with predicted RT; peptides consistently detected around the specific RT within the scheduled RT window during the evaluation steps were selected. Additionally, if peptides were identified in our discovery phase, their observed RT (with SRM assay) was compared with discovery RT and was used as confirmation of peptides' identity. Figure 1A shows an example of predicted RTs calculated based on different algorithms and prediction based on the observed RTs during the discovery phase. The latter RT prediction was based on the correlation of RTs of selected endogenous peptides on Q-Exactive Plus mass spectrometer and SRM RTs observed on TSQ Quantiva mass spectrometer.

Apart from RT, the following criteria were applied for the final endogenous peptide selection: (i) absence of interference, (ii) transitions' coelution, (iii) transition's pattern and order.

If multiple peptides per protein were available after the evaluation phase, the peptides were analyzed in serially diluted CSF and the peptide with the higher intensity transitions observed at the lower points of the serial dilution was selected. In addition, for peptides with possible +3 charge, both charge forms of the peptides were evaluated (+2 and +3) and the peptide with the more intense signal for a given charge was selected.

Initially, 377 proteotypic peptides and 2591 transitions, representing 78 candidate proteins were selected from SRM Atlas for SRM assay development. In total, 48 scheduled (approximately 100 transitions per method) and 6 unscheduled (approximately 45 transitions per method) SRM methods were initially prepared.

After the light peptide evaluation phase, 47 peptides, with 3−5 transitions per peptide, were considered for final assessment based on heavy-labeled synthetic peptides.

Light peptides were then evaluated based on the observed coelution of transitions for both heavy and light peptides, identical order of transition intensities, equivalent transition intensity ratios, and RT alignment. An example of such evaluation is shown in Figure 1B. Out of 47 peptides, 17 endogenous peptides were not confirmed using heavy peptides in the CSF pool tested, likely due to low concentration of the corresponding protein and limited sensitivity of the SRM assay, while 30 peptides were identified and confirmed (Table 1, Supporting Information Table S2). To minimize sample preparation and manipulation, the method was intended for direct digestion, without additional protein/peptide enrichment.

DOI: 10.1021/acs.jproteome.7b00768
J. Proteome Res. 2018, 17, 2282−2292

Table 1. Protein and Peptides of the Developed SRM Assay

Accession UniProt	Gene Name	Protein Description	Peptide Sequence	Precursor m/z
P51693	APLP1	Amyloid-like protein 1	DELAPAGTGVSR	586.80
Q96GW7	BCAN	Brevican core protein	FNVYCFR	503.23
Q8N3J6	CADM2	Cell adhesion molecule 2	SDDGVAVICR	546.26
Q8IUK8	CBLN2	Cerebellin-2	VAFSATR	376.21
Q96KN2	CNDP1	Beta-Ala-His dipeptidase	ALEQDLPVNIK	620.35
Q02246	CNTN2	Contactin-2	VTVTPDGTLIIR	642.88
Q8NFT8	DNER	Delta and Notch-like epidermal growth factor-related receptor	VTATGFQQCSLIDGR	826.91
Q92876	KLK6	Kallikrein-6	LSELIQPLPLER	704.41
O14594	NCAN	Neurocan core protein	TGFPSPAER	481.24
O95502	NPTXR	Neuronal pentraxin receptor	VAQLPLSLK	484.81
Q92823	NRCAM	Neuronal cell adhesion molecule	VFNTPEGVPSAPSSLK	815.43
Q99784	OLFM1	Neolin1	LTGISDPVTVK	565.33
Q14982	OPCML	Opioid-binding protein/cell adhesion molecule	ITVNYPPYISK	647.86
P23471	PTPRZ1	Receptor-type tyrosine-protein phosphatase zeta	AIIDGVESVSR	573.31
P13521	SCG2	Secretogranin-2	ALEYIENLR	560.80
Q9BYH1	SEZ6L	Seizure 6-like protein	ETGTPIWTSR	574.29
P10451	SPP1	Osteopontin	AIPVAQDLNAPSDWDSR	927.95
O15240	VGF	Neurosecretory protein VGF	FGEGVSSPK	454.23
Q9NT99	LRRC4B	Leucine-rich repeat-containing protein 4B	DLAEVPASIPVNTR	741.40
Q8N126	CADM3	Cell adhesion molecule 3	LLLHCEGR	333.18
Q8WXD2	SCG3	Secretogranin-3	LLNLGLITESQAHTLEDEVAEVLQK	921.83
Q15818	NPTX1	Neuronal pentraxin-1	FQLTFPLR	511.30
Q9P0K9	FRRS1L	DOMON domain-containing protein FRRS1L	HDIDSPPASER	612.29
Q96PX8	SLITRK1	SLIT and NTRK-like protein 1	LSNVQELFLR	609.85
P61278	SST	Somatostatin	SANSNPAMAPR	558.27
Q16653	MOG	Myelin-oligodendrocyte glycoprotein	FSDEGGFTCFFR	735.31
P01303	NPY	Pro-neuropeptide Y	ESTENVPR	466.23
Q86UN3	RTN4RL2	Reticulon-4 receptor-like 2	LFLQNNLIR	565.84
Q99574	SERPINI1	Neuroserpin	ALGITEIFIK	552.84
O60241	BAI2	Brain-specific angiogenesis inhibitor 2	LLAPAALAFR	521.82

For the method, two to four transitions per peptide were selected.[13] The multiplex method contained in total 192 transitions for 64 peptides (endogenous and heavy-labeled), derived from 30 candidates (30 peptides plus one additional peptide with oxidized methionine for SST protein) and control protein ECM1. All mass spectrometry-related parameters and SRM methods for the candidates are summarized in Supporting Information Table S3. Total ion chromatogram and relative intensities of peptides (light and heavy) eluting over 37 min LC gradient are represented in Supporting Information Figure S3.

Linearity Assay

Assay linearity for all peptides was assessed using CSF samples spiked with different levels of heavy peptides covering the range of 5 orders of magnitude (0.03 to 4000 fmol/injection). Data was inspected manually, and outliers in three replicate measurements were excluded if an aberrant chromatogram was observed. The minimum of two transitions per protein was used for peptide quantification, for both endogenous and isotope-labeled peptides (Supporting Information Table S3). Developed assays showed a wide dynamic range with overall median fold span of 8.16×10^3. All SRM assays showed good linearity with coefficient of determination $r^2 > 0.987$ for most of the peptides; two lower abundance peptides representing OLFM1 and SLITRK1 proteins showed good, but somewhat lower r^2 value in comparison to other proteins ($r^2 = 0.961$ and 0.974, respectively).

Coefficients of variation (CV) were calculated for each L/H ratio across the established linear range for each peptide. All points within the linear range had CV $\leq$ 20% (for all peptides); 0.7% was the lowest (PTPRZ1 protein) and 10.5% the highest (OLFM1 protein) observed median CV. Linear range, correlation coefficients, and median CV values for all peptides are summarized in Supporting Information Table S4.

The amount of heavy-labeled peptide spiked into the CSF samples should result in a H/L ratio within the linear range, to allow reliable quantification. For all peptides the amount of spiked heavy was confirmed to be within the linear range. For some heavy labeled peptides higher spikes were selected, still within the linear range, to ensure best peak shape and optimal quantification.

Reproducibility

The reproducibility of the analytical process was tested over several days (n = 8), using aliquots of the same CSF pool. CVs for all peptides were below 20%. The precision for all analyzed replicates (total CV) derived from four reproducibility samples and for all peptides was below 10%, except for the protein FRRS1L with a total CV of 17%. Overall, CVs for all peptides ranged from 2% (SEZ6L) to 17% (FRRS1L), with a median CV of 4%. Higher CVs were mostly observed for the lower abundance proteins and for proteins represented by very hydrophobic peptides (derived from SCG3 protein). Total reproducibility for all peptides is shown in Supporting Information Table S5.

Freeze–Thaw Assay and Carry-over Effect

Repeated freeze–thaw cycles were performed using a CSF sample pool. Comparison of mean values of peptide abundance (L/H ratio) for all F/T cycles against the first F/T cycle as a

DOI: 10.1021/acs.jproteome.7b00768
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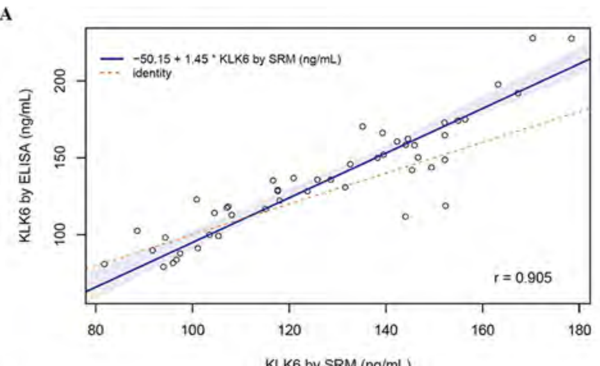
baseline showed that the levels of all peptides were not affected by 5 F/T cycles (Supporting Information Figure S4).

The carry-over effect on the LC-*MS/MS* instrument was tested using samples with optimized L/H ratios as described earlier. The carry-over for all candidate peptides, both endogenous and heavy-labeled peptides, was below 2.5% in all three experiments performed, apart from the peptide HDIDSPPASER (corresponding to protein FRRS1L) with observed carry-over of 4.0%, in one out of three experiments (experiment 2: −0.6% and experiment 3: −2.2%). Average carry-over for all peptides (endogenous and heavy-labeled) was below 2% (Supporting Information Table S6).

Method Comparison: KLK6 SRM Assay and ELISA

To assess the comparison between an SRM assay (multiplex method) and ELISA (singleplex method), KLK6 was measured with both methods, as KLK6 is one of the candidates in our multiplex assay and its abundance has been evaluated in our previous reports either in CSF or other fluids using both assays.[5,16,19,20] The two methods were compared using Passing−Bablok regression analysis and Bland−Altman plot. A good correlation was observed between the two methods (Pearson's correlation coefficient r = 0.90). Based on the Passing−Bablok regression analysis (shown in Figure 2A) the 95% confidence interval (CI) of the intercept was between −70.6 and −26.1, which indicates constant bias between methods (95% CI interval does not include 0). The 95% CI) of the slope was between 1.3 and 1.6, which indicates proportional bias between two methods (95% CI interval does not include 1). The Bland−Altman plot (Figure 2B) shows a difference in concentration between the two methods vs the average concentration of the two methods, indicating the mean (±2SD) of the difference as 8.3 (−2SD: −25.7, +2SD: 42.4) ng/mL.

A



B

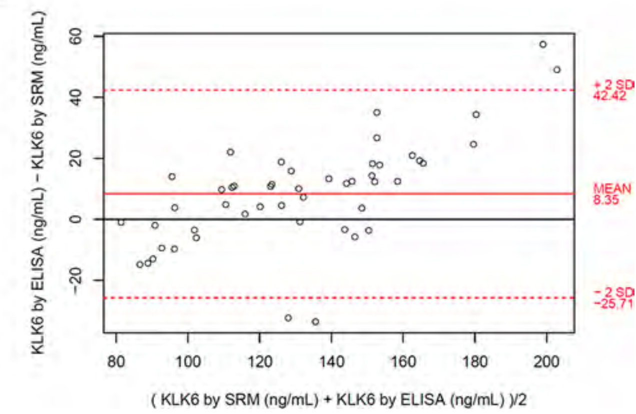


Figure 2. ELISA vs SRM KLK6 method comparison. (A) Passing−Bablok regression of the comparison between ELISA and SRM KLK6 methods. Pearson's correlation coefficient r and equation are shown in the inset. Dashed line indicates the line of identity. Shaded region indicates 95% confidence intervals. (B) ELISA vs SRM KLK6 Bland−Altman plot. The *y*-axis shows differences between the two methods, while the *x*-axis shows the average value of the two methods. The thick solid line shows the mean bias; dashed lines indicate mean bias ±2 standard deviations.

Clinical Samples

The multiplex SRM assay was applied to the measurement of brain-specific proteins in CSF samples from neurological patients. The final assay for clinical samples included peptides for 30 candidates (31 peptides) and peptides for control proteins (ECM1, MBP, and APOB), monitoring thus overall 67 peptides (heavy and light peptides) and 203 transitions (Supporting Information Table S7). In total, 53 samples were selected for SRM assay analysis, 9 controls (individuals without neurological diseases), and 29 CIS and 15 MS CSF samples. The mean duration of disease in CIS patients was 7.8 months, while in patients diagnosed with MS it was 9.4 months. The mean age in the control group was 26.7 years, and it was 39.1 and 40.9 years in the CIS and MS groups, respectively. There were 44% males in the control group, whereas 24% and 47% males were in CIS and MS, respectively. Patients' characteristics are shown in Table 2. All peptides were identified based on 2 to 4 transitions, and relative quantification was based on at least 2 transitions, except for the protein CNTN2 where only one ion-type transition was used (two transitions from the same ion fragment but with different charge). Transitions used for identification and relative quantification are indicated in Supporting Information Table S7. Overall, all protein candidates were detectable in CSF samples.

Table 2. Patients' Characteristics

Group	Control	Clinically isolated syndrome	Multiple sclerosis
Subjects, n	9	29	15
Age, median[a]	25 (23, 29)	37 (31, 47)	39 (36, 46)
Age, mean[b]	26.7 (5.3)	39.1 (9.5)	40.9 (8.6)
Sex-male, n	4	7	7
Disease duration, months[a]	3 (1, 3)	4 (3, 6)	12 (5, 12)
Disease duration, months[b]	2.3 (1.7)	7.8 (10.1)	9.4 (5.3)

[a]Expressed as median (25th, 75th percentile). [b]Expressed as mean (standard deviation).

Distribution of sex did not differ between the tested groups (p = 0.257); however, age distribution was significantly different between controls, CIS and MS ($p < 0.001$) (Supporting Information Table S8). When protein abundance was compared between groups, before correcting for age difference, only SPP1 protein showed a significant difference between controls and CIS and MS patients (p = 0.035, p-value unadjusted for multiple comparison) (Figure 3, Supporting Information Table S8). Increased SPP1 levels were observed between controls and CIS (p = 0.011) as well as between controls and MS patients (p = 0.007). We then further inspected the correlation between age and concentration of each protein. Twenty proteins showed significant correlation with age ($p < 0.05$, p-value unadjusted for multiple comparison, Supporting Information Table S8), and thus age was included as a covariate (using multivariate linear

DOI: 10.1021/acs.jproteome.7b00768
J. Proteome Res. 2018, 17, 2282−2292

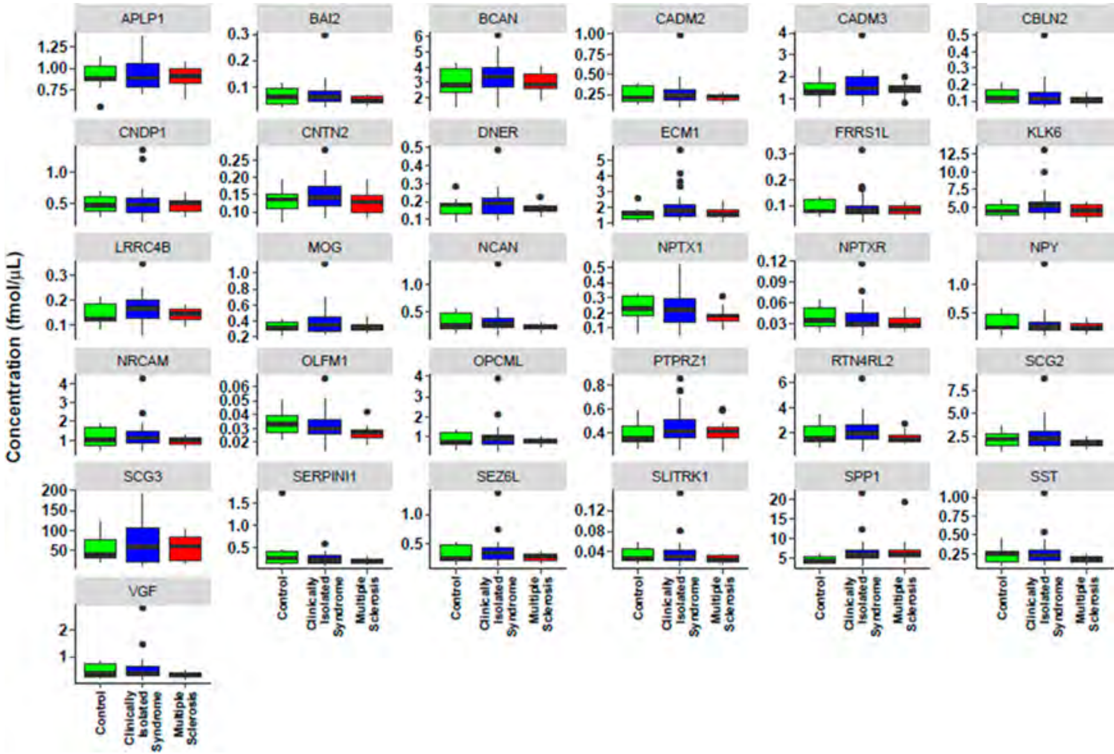


Figure 3. Candidate biomarkers in CSF samples from control ($n = 9$), clinically isolated syndrome ($n = 29$), and multiple sclerosis patients ($n = 15$). Proteins (30 candidates plus control proteins) were measured using SRM assay.

regression) for protein concentration comparison across disease groups for all proteins. When correcting by age, protein CNTN2 showed significant difference between the tested groups (p = 0.039, unadjusted by multiple comparison) with increased levels observed between controls and CIS (p = 0.015). When multiple correction testing was applied, no significance in SPP1 or CNTN2 remained ($p > 0.0016$). Supporting Information Table S8 shows all analyses and p-values of all proteins. There was no difference in protein ECM1 concentration between the three groups tested (p = 0.126). Protein MBP was not detected in most of the CSF samples; only one sample had detectable CSF levels. As confirmed by both erythrocyte count and presence of APOB there was no significant blood contamination in the CSF samples.

■ DISCUSSION

In this study we aimed at developing a multiplex SRM assay for a panel of 30 CSF brain-related and highly specific proteins, for relative quantification. The assay shows good linearity, shows wide dynamic range, and has acceptable reproducibility (CV < 20%). The SRM assay was applied to analysis of a small MS cohort in order to evaluate assay feasibility and candidates' biomarker potential. Proteins SPP1 and CNTN2 were discovered as potential proteins with increased levels in CIS and MS patients.

Targeted, multiplexed-proteomic platforms have been utilized for evaluation of CSF candidate biomarkers in different CNS disorders. The main advantages that make SRM the method of choice for verification of novel biomarkers are high specificity, relatively fast method development, and multiplex capacity. The sensitivity of SRM depends on the matrix (for example, it can reach high ng/mL or low μg/mL in plasma, without enrichment methods).[21] Limitations of SRM assays are often related to the poor sensitivity for very low abundance proteins in a complex matrix. From the initial 78 candidates for SRM assay development, we were able to develop assays for 30 proteins. This can be explained partially by the limited sensitivity for peptide detection in a complex protein background; initial identification of the 78 proteins was performed after decreasing the CSF complexity (SCX fractionation).[5] Some brain-related proteins may have a very low concentration in CSF and may not be consistently detected with SRM assays. Furthermore, it is possible that for some proteins, selected peptides were not optimal for development of a targeted assay. In general, sensitivity of an SRM assay can be improved with the immuno-SRM methodology (i.e., protein or peptide antibody enrichened prior to LC-*MS/MS* analysis). However, the utilization of such approach was not the prime goal of this study. Assays developed in this study do not require enrichment methods and are suitable for relatively fast sample preparation.

To our knowledge, the SRM assays for 19 of our candidates have been previously reported by other groups for quantification in CSF, where different peptides or the identical peptides reported here were used in the assays.[4,22−26] For 11 of our candidates, this study is the first report of the SRM assay developed for quantification in CSF (e.g., SST, SEZ6L, SLITRK1, RTN4RL2, BAI2).

Since we aimed to develop a targeted assay for proteins highly specific to the brain tissue, this multiplexed panel could be of interest for testing potential CSF protein biomarkers from patients with various CNS conditions. In this study we evaluated the diagnostic and prognostic potential of candidates in a small cohort of MS and CIS patients and neurologically healthy individuals. Protein SPP1 showed significant difference in CSF levels between CIS, MS, and healthy controls. However, since the tested groups differ in age and a substantial number of

DOI: 10.1021/acs.jproteome.7b00768
J. Proteome Res. 2018, 17, 2282−2292

proteins (n = 10) correlated with age, it was important to take the possible age effect into account. After adjustment for age, the observed difference in SPP1 disappeared. Nevertheless, using an age-adjusted linear model, protein CNTN2 showed a significant difference between the three groups; increased levels were found in CIS patients relative to the control group. Still, for both proteins, SPP1 and CNTN2, the observed change in abundance between groups was small; fold difference in SPP1 between CIS and MS versus controls was 1.37 and 1.47, respectively, while fold difference in CNTN2 between CIS versus controls was 1.14. Thus, these results should be interpreted with caution.

Although the statistical difference did not remain after adjustment for multiple comparisons testing, both proteins have been previously reported to be involved in MS pathology and should be further investigated. Protein SPP1 appears to be a promising candidate biomarker for MS; other studies have reported its relevance and significance for this disease.[27,28] Several studies reported higher SPP1 levels in MS patients with heterogeneous subtypes (in combined group of RRMS, PPMS, SPMS), RRMS, PPMS, and CIS patients, when compared to other neurological diseases.[27−29] Since some studies found elevated levels during the presence of active disease (relapses) in RRMS and CIS patients, SPP1 has been suggested as a biomarker of disease activity.[28,30] However, the trend for elevated SPP1 levels in MS patients is not specific, since higher levels were also found in patients with other neurological inflammatory conditions.[29]

SPP1 is a glycosylated phosphoprotein with several functions; it is involved as an extracellular matrix protein and as a cytokine with pro-inflammatory and anti-inflammatory actions.[31] SPP1 can enhance production of interferon-γ and interleukin-12 and decrease interleukin-10 production, acting as a T cell helper type-1 (Th-1) cytokine, and, in addition, it can influence survival of activated T cells.[31−33] SPP1 is highly expressed in human MS brains, and its expression has been allocated within active MS lesion (e.g., in macrophages and microvascular endothelial cells) and surrounding white matter.[32] SPP1 expression has also been found in an animal model of MS (experimental autoimmune encephalomyelitis, EAE) in perivascular inflammatory lesions, and its high expression has been associated with acute phase and relapses and severity of disease.[32,33]

CNTN2 is an adhesion molecule located at the juxtaparanodes, a part of nodes of Ranvier around myelin loops. In adults, CNTN2 is expressed by Schwann cells, oligodendrocytes, axons, as well as neurons in the hippocampus and spinal cord.[34] Although CNTN2 is not well studied as a CSF biomarker of MS, it has been connected with disease pathology as a potential autoantigen. Derfuss et al. observed increased CNTN2-reactive IgG response in CSF of MS patients compared to patients with other neurological inflammatory diseases.[35] The same study demonstrated CNTN2-associated increased proliferation of peripheral blood mononuclear cells, induced production of interferon-γ (Th-1 cells) and interleukin-17 (Th-17 cells) by CNTN2 and gray matter pathology, initiated in an EAE model by CNTN2-induced T-cells.

Some of our other candidates have not been previously evaluated in CSF from MS patients, such as PTPRZ1, DNER, and BAI2. In addition, some of the proteins from the multiplexed, 30-protein panel tested in this study have been previously evaluated as MS biomarkers, and differential expression in MS was reported;[36−38] however, for these proteins, we could not find significant differences among examined groups.

As mentioned, one of the modulators of CSF protein concentration is age. Since age was significantly different between our study groups, the statistical analysis was adjusted for this variable. In general, the effect of age has been associated with decreased CSF flow rate with increasing age, resulting in increase in protein abundance; however, this is also dependent on the origin of the proteins in CSF.[2] Indeed, age dependent variations on the concentration of several proteins have been previously reported.[39,40] In the study by Stoop and colleagues 3.2% of quantified CSF proteins were found to be affected by age (at $p < 0.01$).[40] Similar to our observation, the concentration of protein SPP1 in this study differed among certain age groups, however, with $p > 0.01$ but <0.05. In addition, a biomarker study by Bornsen et al. found weak positive correlation of SPP1 with age in one of the study groups.[28] Nevertheless, this indicates that age may introduce bias in biomarker studies and has to be examined properly before interpreting results.

Myelin basic protein is an indicator of an active CNS demyelination (not specific to MS) and was included in the multiplex panel as a positive control of our assay; however, MBP levels were undetectable in all samples apart from one with MS diagnosis. This observation can be partially due to the sensitivity limitations of SRM assay to measure low levels of MBP in CSF. The MBP SRM assay used in this study was previously published by our group where MBP was largely undetectable in control subjects (increased in stroke patients).[4] MBP was also not detectable with SRM assay in CSF from patients with Alzheimer's disease and healthy controls.[24] Generally, MBP levels are very low or not detectable in normal CSF but are elevated in an active phase of MS and are decreased in the remission phase.[41,42] This could also indicate that our patents were likely not in an acute exacerbation of the disease, when recruited.

CONCLUSION

Different sets of candidate proteins are being tested as potential biomarkers of various neurological disorders. Our approach was to focus on proteins that are highly specific to the brain, and we developed a multiplex SRM assay for their quantification in the CSF. We were able to develop highly reliable SRM assays for simultaneous quantification of 30 proteins in human CSF samples. The developed assay can be applied in the search for biomarkers of various neurological disorders. In this assay, the 30-plex panel was evaluated in a small cohort of MS, CIS, and neurologically healthy control patients.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00768.

> Table S1. Peptides used for prediction of retention time (RT). Figure S1. Correlation between RT of endogenous peptides and RT from SRM Atlas (A) and RT of Pierce peptides and hydrophobicity indexes. Figure S2. Linearity curves. Figure S3. Total ion chromatogram and relative intensities of peptides during the 37 min LC gradient. Figure S4. Freeze−thaw assay. (PDF)
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> Table S2. Endogenous peptides identified in CSF. Table S3. Mass spectrometry parameters of developed SRM assay. Table S4. Analytical characteristics of SRM assays for 30 proteins. Table S5. Reproducibility assay. Table S6.

DOI: 10.1021/acs.jproteome.7b00768
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Carry-over effect. Table S7. Multiplex SRM assay for clinical samples. Table S8. Statistical analysis. (XLSX)

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Author Contributions

IB performed acquisition of data, analysis, and preparation of the manuscript. DB performed statistical analysis and helped with interpretation of data and revision of the manuscript. LD, AMS, IZ, and VBK helped with the design of the cohort used and provided critical comments of the manuscript. EMM contributed to the method section, guidance for the data analysis, and manuscript revision. APD and EPD helped design the study. IB provided valuable comments for the data analysis. All authors read the manuscript and agreed with the publication of the study.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors would like to acknowledge Antoninus Soosaipillai for his help with performing KLK6 ELISA.

■ NONSTANDARD ABBREVIATIONS

CSF, cerebrospinal fluid; CIS, clinically isolated syndrome; RRMS, relapse-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; SRM, selected reaction monitoring; MS, multiple sclerosis; MRI, magnetic resonance imaging; HPA, human protein atlas; RT, retention time; LC, liquid chromatography; LC-*MS/MS*, liquid chromatography-tandem mass spectrometry; CE, collision energy; F/T, freeze/thaw; H/L, heavy/light; AUC, area under the curve; TFA, trifluoroacetic acid; MBP, myelin basic protein; ECM1, extracellular matrix protein 1

■ REFERENCES

(1) Kroksveen, A. C.; Opsahl, J. A.; Aye, T. T.; Ulvik, R. J.; Berven, F. S. Proteomics of human cerebrospinal fluid: discovery and verification of biomarker candidates in neurodegenerative diseases using quantitative proteomics. *J. Proteomics* **2011**, *74* (4), 371−88.

(2) Reiber, H. Dynamics of brain-derived proteins in cerebrospinal fluid. *Clin. Chim. Acta* **2001**, *310* (2), 173−86.

(3) Blennow, K.; Hampel, H.; Weiner, M.; Zetterberg, H. Cerebrospinal fluid and plasma biomarkers in Alzheimer disease. *Nat. Rev. Neurol.* **2010**, *6* (3), 131−44.

(4) Martinez-Morillo, E.; Garcia Hernandez, P.; Begcevic, I.; Kosanam, H.; Prieto Garcia, B.; Alvarez Menendez, F. V.; Diamandis, E. P. Identification of novel biomarkers of brain damage in patients with hemorrhagic stroke by integrating bioinformatics and mass spectrometry-based proteomics. *J. Proteome Res.* **2014**, *13* (2), 969−81.

(5) Begcevic, I.; Brinc, D.; Drabovich, A. P.; Batruch, I.; Diamandis, E. P. Identification of brain-enriched proteins in the cerebrospinal fluid proteome by LC-MS/MS profiling and mining of the Human Protein Atlas. *Clin. Proteomics* **2016**, *13*, 11.

(6) Uhlen, M.; Fagerberg, L.; Hallstrom, B. M.; Lindskog, C.; Oksvold, P.; Mardinoglu, A.; Sivertsson, A.; Kampf, C.; Sjostedt, E.; Asplund, A.; Olsson, I.; Edlund, K.; Lundberg, E.; Navani, S.; Szigyarto, C. A.; Odeberg, J.; Djureinovic, D.; Takanen, J. O.; Hober, S.; Alm, T.; Edqvist, P. H.; Berling, H.; Tegel, H.; Mulder, J.; Rockberg, J.; Nilsson, P.; Schwenk, J. M.; Hamsten, M.; von Feilitzen, K.; Forsberg, M.; Persson, L.; Johansson, F.; Zwahlen, M.; von Heijne, G.; Nielsen, J.; Ponten, F. Proteomics. Tissue-based map of the human proteome. *Science* **2015**, *347* (6220), 1260419.

(7) Drabovich, A. P.; Dimitromanolakis, A.; Saraon, P.; Soosaipillai, A.; Batruch, I.; Mullen, B.; Jarvi, K.; Diamandis, E. P. Differential diagnosis of azoospermia with proteomic biomarkers ECM1 and TEX101 quantified in seminal plasma. *Sci. Transl. Med.* **2013**, *5* (212), 212ra160.

(8) Picotti, P.; Aebersold, R. Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions. *Nat. Methods* **2012**, *9* (6), 555−66.

(9) Compston, A.; Coles, A. Multiple sclerosis. *Lancet* **2008**, *372* (9648), 1502−17.

(10) Miller, D.; Barkhof, F.; Montalban, X.; Thompson, A.; Filippi, M. Clinically isolated syndromes suggestive of multiple sclerosis, part I: natural history, pathogenesis, diagnosis, and prognosis. *Lancet Neurol.* **2005**, *4* (5), 281−8.

(11) Polman, C. H.; Reingold, S. C.; Banwell, B.; Clanet, M.; Cohen, J. A.; Filippi, M.; Fujihara, K.; Havrdova, E.; Hutchinson, M.; Kappos, L.; Lublin, F. D.; Montalban, X.; O'Connor, P.; Sandberg-Wollheim, M.; Thompson, A. J.; Waubant, E.; Weinshenker, B.; Wolinsky, J. S. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann. Neurol.* **2011**, *69* (2), 292−302.

(12) Andersson, M.; Alvarez-Cermeno, J.; Bernardi, G.; Cogato, I.; Fredman, P.; Frederiksen, J.; Fredrikson, S.; Gallo, P.; Grimaldi, L. M.; Gronning, M.; et al. Cerebrospinal fluid in the diagnosis of multiple sclerosis: a consensus report. *J. Neurol., Neurosurg. Psychiatry* **1994**, *57* (8), 897−902.

(13) Lange, V.; Picotti, P.; Domon, B.; Aebersold, R. Selected reaction monitoring for quantitative proteomics: a tutorial. *Mol. Syst. Biol.* **2008**, *4*, 222.

(14) Henry, P. The measurement of splashover and carryover in centrifugal analyzers. *J. Autom. Chem.* **1979**, *1* (4), 195−8.

(15) CHEMISTRY, I. U. O. P. A. A.. Proposals for the description effects in clinical chemistry and measurement of carry-over. *Pure Appl. Chem.* **1991**, *63* (2), 301−306.

(16) Diamandis, E. P.; Yousef, G. M.; Soosaipillai, A. R.; Grass, L.; Porter, A.; Little, S.; Sotiropoulou, G. Immunofluorometric assay of human kallikrein 6 (zyme/protease M/neurosin) and preliminary clinical applications. *Clin. Biochem.* **2000**, *33* (5), 369−75.

(17) Filippi, M.; Rocca, M. A.; Ciccarelli, O.; De Stefano, N.; Evangelou, N.; Kappos, L.; Rovira, A.; Sastre-Garriga, J.; Tintore, M.; Frederiksen, J. L.; Gasperini, C.; Palace, J.; Reich, D. S.; Banwell, B.; Montalban, X.; Barkhof, F.; Group, M. S.. MRI criteria for the diagnosis of multiple sclerosis: MAGNIMS consensus guidelines. *Lancet Neurol.* **2016**, *15* (3), 292−303.

(18) Martinez-Morillo, E.; Nielsen, H. M.; Batruch, I.; Drabovich, A. P.; Begcevic, I.; Lopez, M. F.; Minthon, L.; Bu, G.; Mattsson, N.; Portelius, E.; Hansson, O.; Diamandis, E. P. Assessment of Peptide chemical modifications on the development of an accurate and precise multiplex selected reaction monitoring assay for apolipoprotein e isoforms. *J. Proteome Res.* **2014**, *13* (2), 1077−87.

(19) Karakosta, T. D.; Soosaipillai, A.; Diamandis, E. P.; Batruch, I.; Drabovich, A. P. Quantification of Human Kallikrein-Related Peptidases in Biological Fluids by Multiplatform Targeted Mass Spectrometry Assays. *Mol. Cell. Proteomics* **2016**, *15* (9), 2863−76.

(20) Dukic, L.; Simundic, A. M.; Martinic-Popovic, I.; Kackov, S.; Diamandis, A.; Begcevic, I.; Diamandis, E. P. The role of human kallikrein 6, clusterin and adiponectin as potential blood biomarkers of dementia. *Clin. Biochem.* **2016**, *49* (3), 213−8.

(21) Addona, T. A.; Abbatiello, S. E.; Schilling, B.; Skates, S. J.; Mani, D. R.; Bunk, D. M.; Spiegelman, C. H.; Zimmerman, L. J.; Ham, A. J.; Keshishian, H.; Hall, S. C.; Allen, S.; Blackman, R. K.; Borchers, C. H.;

DOI: 10.1021/acs.jproteome.7b00768
J. Proteome Res. 2018, 17, 2282−2292

Buck, C.; Cardasis, H. L.; Cusack, M. P.; Dodder, N. G.; Gibson, B. W.; Held, J. M.; Hiltke, T.; Jackson, A.; Johansen, E. B.; Kinsinger, C. R.; Li, J.; Mesri, M.; Neubert, T. A.; Niles, R. K.; Pulsipher, T. C.; Ransohoff, D.; Rodriguez, H.; Rudnick, P. A.; Smith, D.; Tabb, D. L.; Tegeler, T. J.; Variyath, A. M.; Vega-Montoto, L. J.; Wahlander, A.; Waldemarson, S.; Wang, M.; Whiteaker, J. R.; Zhao, L.; Anderson, N. L.; Fisher, S. J.; Liebler, D. C.; Paulovich, A. G.; Regnier, F. E.; Tempst, P.; Carr, S. A. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat. Biotechnol.* **2009**, *27* (7), 633−41.

(22) Spellman, D. S.; Wildsmith, K. R.; Honigberg, L. A.; Tuefferd, M.; Baker, D.; Raghavan, N.; Nairn, A. C.; Croteau, P.; Schirm, M.; Allard, R.; Lamontagne, J.; Chelsky, D.; Hoffmann, S.; Potter, W. Z.; Alzheimer's Disease Neuroimaging, I.; Foundation for, N. I. H. B. C. C. S. F. P. P. T.. Development and evaluation of a multiplexed mass spectrometry based assay for measuring candidate peptide biomarkers in Alzheimer's Disease Neuroimaging Initiative (ADNI) CSF. *Proteomics: Clin. Appl.* **2015**, *9* (7−8), 715−31.

(23) Wildsmith, K. R.; Schauer, S. P.; Smith, A. M.; Arnott, D.; Zhu, Y.; Haznedar, J.; Kaur, S.; Mathews, W. R.; Honigberg, L. A. Identification of longitudinally dynamic biomarkers in Alzheimer's disease cerebrospinal fluid by targeted proteomics. *Mol. Neurodegener.* **2014**, *9*, 22.

(24) Paterson, R. W.; Heywood, W. E.; Heslegrave, A. J.; Magdalinou, N. K.; Andreasson, U.; Sirka, E.; Bliss, E.; Slattery, C. F.; Toombs, J.; Svensson, J.; Johansson, P.; Fox, N. C.; Zetterberg, H.; Mills, K.; Schott, J. M. A targeted proteomic multiplex CSF assay identifies increased malate dehydrogenase and other neurodegenerative biomarkers in individuals with Alzheimer's disease pathology. *Transl. Psychiatry* **2016**, *6* (11), e952.

(25) Shi, M.; Movius, J.; Dator, R.; Aro, P.; Zhao, Y.; Pan, C.; Lin, X.; Bammler, T. K.; Stewart, T.; Zabetian, C. P.; Peskind, E. R.; Hu, S. C.; Quinn, J. F.; Galasko, D. R.; Zhang, J. Cerebrospinal fluid peptides as potential Parkinson disease biomarkers: a staged pipeline for discovery and validation. *Mol. Cell. Proteomics* **2015**, *14* (3), 544−55.

(26) Kroksveen, A. C.; Jaffe, J. D.; Aasebo, E.; Barsnes, H.; Bjorlykke, Y.; Franciotta, D.; Keshishian, H.; Myhr, K. M.; Opsahl, J. A.; van Pesch, V.; Teunissen, C. E.; Torkildsen, O.; Ulvik, R. J.; Vethe, H.; Carr, S. A.; Berven, F. S. Quantitative proteomics suggests decrease in the secretogranin-1 cerebrospinal fluid levels during the disease course of multiple sclerosis. *Proteomics* **2015**, *15* (19), 3361−9.

(27) Wen, S. R.; Liu, G. J.; Feng, R. N.; Gong, F. C.; Zhong, H.; Duan, S. R.; Bi, S. Increased levels of IL-23 and osteopontin in serum and cerebrospinal fluid of multiple sclerosis patients. *J. Neuroimmunol.* **2012**, *244* (1−2), 94−6.

(28) Bornsen, L.; Khademi, M.; Olsson, T.; Sorensen, P. S.; Sellebjerg, F. Osteopontin concentrations are increased in cerebrospinal fluid during attacks of multiple sclerosis. *Mult Scler* **2011**, *17* (1), 32−42.

(29) Braitch, M.; Nunan, R.; Niepel, G.; Edwards, L. J.; Constantinescu, C. S. Increased osteopontin levels in the cerebrospinal fluid of patients with multiple sclerosis. *Arch. Neurol.* **2008**, *65* (5), 633−5.

(30) Comabella, M.; Montalban, X. Body fluid biomarkers in multiple sclerosis. *Lancet Neurol.* **2014**, *13* (1), 113−26.

(31) Denhardt, D. T.; Noda, M.; O'Regan, A. W.; Pavlin, D.; Berman, J. S. Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. *J. Clin. Invest.* **2001**, *107* (9), 1055−61.

(32) Chabas, D.; Baranzini, S. E.; Mitchell, D.; Bernard, C. C.; Rittling, S. R.; Denhardt, D. T.; Sobel, R. A.; Lock, C.; Karpuj, M.; Pedotti, R.; Heller, R.; Oksenberg, J. R.; Steinman, L. The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. *Science* **2001**, *294* (5547), 1731−5.

(33) Hur, E. M.; Youssef, S.; Haws, M. E.; Zhang, S. Y.; Sobel, R. A.; Steinman, L. Osteopontin-induced relapse and progression of autoimmune brain disease through enhanced survival of activated T cells. *Nat. Immunol.* **2007**, *8* (1), 74−83.

(34) Stathopoulos, P.; Alexopoulos, H.; Dalakas, M. C. Autoimmune antigenic targets at the node of Ranvier in demyelinating disorders. *Nat. Rev. Neurol.* **2015**, *11* (3), 143−56.

(35) Derfuss, T.; Parikh, K.; Velhin, S.; Braun, M.; Mathey, E.; Krumbholz, M.; Kumpfel, T.; Moldenhauer, A.; Rader, C.; Sonderegger, P.; Pollmann, W.; Tiefenthaller, C.; Bauer, J.; Lassmann, H.; Wekerle, H.; Karagogeos, D.; Hohlfeld, R.; Linington, C.; Meinl, E. Contactin-2/TAG-1-directed autoimmunity is identified in multiple sclerosis patients and mediates gray matter pathology in animals. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (20), 8302−7.

(36) Mattsson, N.; Ruetschi, U.; Podust, V. N.; Stridsberg, M.; Li, S.; Andersen, O.; Haghighi, S.; Blennow, K.; Zetterberg, H. Cerebrospinal fluid concentrations of peptides derived from chromogranin B and secretogranin II are decreased in multiple sclerosis. *J. Neurochem.* **2007**, *103* (5), 1932−9.

(37) Canto, E.; Tintore, M.; Villar, L. M.; Borras, E.; Alvarez-Cermeno, J. C.; Chiva, C.; Sabido, E.; Rovira, A.; Montalban, X.; Comabella, M. Validation of semaphorin 7A and ala-beta-his-dipeptidase as biomarkers associated with the conversion from clinically isolated syndrome to multiple sclerosis. *J. Neuroinflammation* **2014**, *11*, 181.

(38) Kroksveen, A. C.; Aasebo, E.; Vethe, H.; Van Pesch, V.; Franciotta, D.; Teunissen, C. E.; Ulvik, R. J.; Vedeler, C.; Myhr, K. M.; Barsnes, H.; Berven, F. S. Discovery and initial verification of differentially abundant proteins between multiple sclerosis patients and controls using iTRAQ and SID-SRM. *J. Proteomics* **2013**, *78*, 312−25.

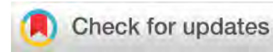
(39) Zhang, J.; Goodlett, D. R.; Peskind, E. R.; Quinn, J. F.; Zhou, Y.; Wang, Q.; Pan, C.; Yi, E.; Eng, J.; Aebersold, R. H.; Montine, T. J. Quantitative proteomic analysis of age-related changes in human cerebrospinal fluid. *Neurobiol. Aging* **2005**, *26* (2), 207−27.

(40) Stoop, M. P.; Coulier, L.; Rosenling, T.; Shi, S.; Smolinska, A. M.; Buydens, L.; Ampt, K.; Stingl, C.; Dane, A.; Muilwijk, B.; Luitwieler, R. L.; Sillevis Smitt, P. A.; Hintzen, R. Q.; Bischoff, R.; Wijmenga, S. S.; Hankemeier, T.; van Gool, A. J.; Luider, T. M. Quantitative proteomics and metabolomics analysis of normal human cerebrospinal fluid samples. *Mol. Cell. Proteomics* **2010**, *9* (9), 2063−75.

(41) Whitaker, J. N. Myelin basic protein in cerebrospinal fluid and other body fluids. *Mult Scler* **1998**, *4* (1), 16−21.

(42) Ohta, M.; Ohta, K.; Ma, J.; Takeuchi, J.; Saida, T.; Nishimura, M.; Itoh, N. Clinical and analytical evaluation of an enzyme immunoassay for myelin basic protein in cerebrospinal fluid. *Clin Chem.* **2000**, *46* (9), 1326−30.

DOI: 10.1021/acs.jproteome.7b00768
J. Proteome Res. 2018, 17, 2282−2292



RESEARCH ARTICLE

Neuronal pentraxin receptor-1 is a new cerebrospinal fluid biomarker of Alzheimer's disease progression [version 1; referees: 4 approved]

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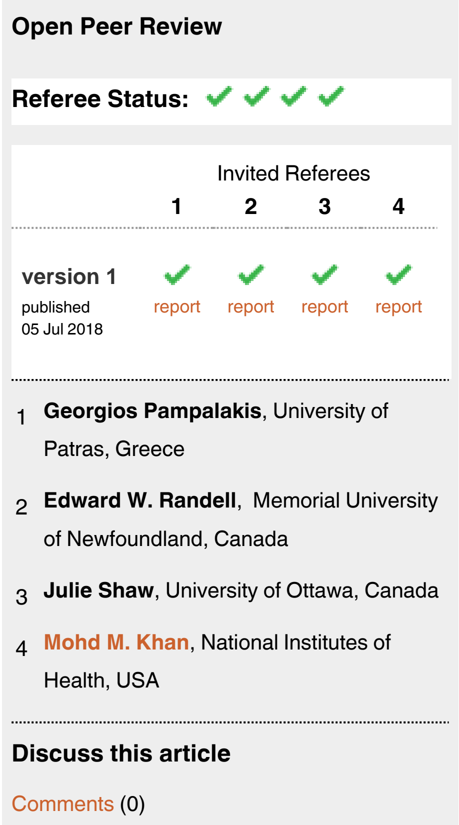
v1 **First published:** 05 Jul 2018, **7**:1012 (https://doi.org/10.12688/f1000research.15095.1)
Latest published: 05 Jul 2018, **7**:1012 (https://doi.org/10.12688/f1000research.15095.1)

Abstract

Background: Alzheimer's disease (AD) is the most common type of dementia, with progressive onset of clinical symptoms. The main pathological hallmarks are brain deposits of extracellular amyloid beta plaques and intracellular neurofibrillary tangles (NFT). Cerebrospinal fluid reflects pathological changes in the brain; amyloid beta 1-42 is a marker of amyloid plaques, while total and phosphorylated tau are markers of NFT formation. Additional biomarkers associated with disease pathogenesis are needed, for better prognosis, more specific diagnosis, prediction of disease severity and progression and for improved patient classification in clinical trials. The aim of the present study was to evaluate brain-specific proteins as potential biomarkers of progression of AD.
Methods: Overall, 30 candidate proteins were quantified in cerebrospinal fluid (CSF) samples from patients with mild cognitive impairment (MCI) and mild, moderate and severe AD dementia (n=101) using mass spectrometry-based selected reaction monitoring assays. ELISA was used for neuronal pentraxin receptor-1 (NPTXR) confirmation.
Results: The best discrimination between MCI and more advanced AD stages (moderate and severe dementia) was observed for protein NPTXR (area under the curve, AUC=0.799). A statistically different abundance of this protein was observed between the two groups, with severe AD patients having progressively lower levels ($p<0.05$). ELISA confirmed lower levels in AD, in a separate cohort that included controls, MCI and AD patients.



Conclusions: We conclude that NPTXR protein in CSF is a novel potential biomarker of AD progression and could have important utility in assessing treatment success in clinical trials.

Keywords
Alzheimer's disease, biomarkers, cerebrospinal fluid, mass spectrometry, selected reaction monitoring, neuronal pentraxin receptor-1, Alzheimer's disease progression, dementia

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Competing interests: Dr. Eleftherios P. Diamandis holds a consultant/advisory role with Abbott Diagnostics.

Grant information: This work was supported by Mount Sinai Hospital.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Begcevic I, Tsolaki M, Brinc D *et al.* **Neuronal pentraxin receptor-1 is a new cerebrospinal fluid biomarker of Alzheimer's disease progression [version 1; referees: 4 approved]** *F1000Research* 2018, **7**:1012 (https://doi.org/10.12688/f1000research.15095.1)

First published: 05 Jul 2018, **7**:1012 (https://doi.org/10.12688/f1000research.15095.1)

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive cognitive decline, behavioural problems and impairment of daily living activities. The main pathological hallmarks of AD are brain extracellular deposits known as amyloid β (Aβ) plaques, composed of aggregated Aβ fragments and intracellular neurofibrillary tangles (NFT), containing hyperphosphorylated protein tau (p-tau) fibrils[1]. The diagnosis of AD is currently made based on core clinical criteria, including medical history, mental status testing, and neurological and physical assessment[2]. With such criteria, only probable dementia due to AD can be diagnosed, while definitive diagnosis of AD can be made only post mortem, by neuropathological examination of different brain regions. More recently, a blood test for AD diagnosis has been suggested but it has not as yet been clinically validated[3]. Three stages of AD have been recognized by the National Institute on Aging (NIA) and the Alzheimer's Association newly revised diagnostic and research criteria: preclinical stage, mild cognitive impairment (MCI) due to AD, and dementia due to AD[4]. Preclinical stage describes asymptomatic individuals with existing early brain pathology, while MCI due to AD includes patients with prodromal, mild symptoms as a result of disease pathology. Patients with dementia due to AD have impaired memory, thinking and behavioural functions, accompanied by severe pathological brain changes. Clinical symptoms typically appear gradually, indicating different levels of dementia severity: mild dementia (or early stage), moderate dementia (or middle stage) and severe dementia (or late stage)[5].

Cerebrospinal fluid (CSF) is a proximal fluid of the central nervous system, residing in direct contact with the brain parenchyma and thus can reflect physical and pathological changes in the brain[6]. As such, CSF may be the most promising source of AD biomarkers; especially highly specific, brain-related protein biomarkers. The best evaluated AD biomarkers to date are CSF Aβ1-42, total tau (t-tau) and p-tau levels[7]. These core AD biomarkers reflect main pathological hallmarks: Aβ1-42 peptide is a marker of Aβ plaque formation, while t-tau and p-tau are biomarkers of neuronal injury. Decreased CSF levels of Aβ1-42 and increased levels of t-tau and p-tau have been observed in AD patients, compared with healthy controls[8]. Still, these extensively studied biomarkers are not widely used in the clinic, largely due to the lack of method standardization, and are mostly utilized in research settings, as also suggested by the new AD diagnostic guidelines[2]. In addition, current CSF biomarkers have been tested in clinical trials of different AD therapeutic approaches. The results were contradictory, questioning their usefulness as indicators of efficacy of new therapies[9]. It has also been previously reported that current AD biomarkers do not correlate well with cognitive decline in AD patients[10,11].

There is a clinical need for novel biomarkers of AD progression. Such biomarkers could accurately and proactively identify evolving cases of AD and could be invaluable in clinical trials for patient enrichment and/or as surrogate endpoints. Moreover, such biomarkers could contribute to the better understanding of the underlying pathological mechanisms of AD.

Differential expression of proteins specific to a particular tissue can have strong disease specificity, pinpointing to pathology unique to that tissue. Some of these tissue-specific proteins have already shown promise as potential biomarkers, such as in male infertility (testis-specific protein TEX101) and in cerebral hemorrhagic stroke (brain-specific proteins NFM, α-Inx and β-Syn)[12,13]. In our recent study, we identified a set of brain-specific proteins that are consistently detected in the normal CSF proteome[14]. The brain-specific proteins were retrieved from the Human Protein Atlas (HPA) tissue-specific database[15] and encompassed tissue-enriched (mRNA expression at least five times higher in the particular tissue (i.e. brain) relative to other tissues) and group-enriched proteins (mRNA expression at least five times higher in the group of 2–7 tissues (including brain), relative to all other tissues). These proteins were also secreted and/or were membrane-bound (as defined by HPA). We have further developed targeted mass spectrometry-based assays for quantification of 30 of these highly specific brain proteins in CSF[16]. The main objective of the present study is to evaluate these 30 brain-related proteins for their ability to differentiate various stages of AD severity, i.e. MCI, mild, moderate and severe AD dementia, by utilizing state-of-the-art mass spectrometry-based selected reaction monitoring (SRM) assays. Considering that the apolipoprotein E (APOE) ε4 allele is the strongest genetic risk factor for developing AD, associated with disease pathology[17], we have further evaluated if the abundance of our candidates was related to the APOE phenotypes.

Methods

Multiplex selected reaction monitoring

A multiplexed, scheduled, SRM assay was developed for 30 brain-related proteins and is described in detail elsewhere[16]. A protein previously found not to change in AD CSF (extracellular matrix protein 1, ECM1) was included as a negative control. Also included was a protein primarily related to demyelinating diseases (myelin basic protein, MBP). The SRM method for MBP has been described elsewhere[13]. A peptide corresponding to apolipoprotein B (APOB) protein (a plasma protein) was also monitored, to check for blood contamination[18]. For peptides containing methionine, both oxidized and non-oxidized forms of the peptide were monitored. Four peptides that represent different APOE phenotypes where additionally added to the assay, including an APOE peptide for total APOE, as a control. The APOE method was previously published[18].

Mass spectrometry sample preparation

CSF samples were thawed and volumes equivalent to 15 μg of total protein were denatured with 0.05% RapiGest detergent (Waters, Milford, USA) and reduced with 5 mM dithiothreitol (Sigma-Aldrich, Oakville, Canada) at 60°C for 40 min. Alkylation was achieved with 15 mM iodoacetamide (Sigma-Aldrich) for 60 min in the dark at 22°C. A mixture of APOE heavy peptides was spiked into samples prior to addition of trypsin, while a mixture of 32 heavy peptides (30 candidates, ECM1, MBP) plus a heavy peptide for total APOE were spiked into the mixture after digestion, followed by addition of 1% trifluoroacetic acid. Digestion was carried out for 24 hours at 37°C with 1:30 trypsin-to-total protein ratio. Samples were then

centrifuged at 1,000 g for 30 min and the supernatants retained. Peptides were purified using OMIX C18 tips, eluted in 4.5 µL of acetonitrile solution (65% acetonitrile, 0.1% formic acid) and finally diluted with 54 µL of water-formic acid mix (0.1% formic acid).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Samples were analysed with a triple quadrupole mass spectrometer, TSQ Quantiva: (Thermo Scientific, San Jose, USA). Each sample (18 µL) was injected into an in-house-packed 3.3 cm pre-column (5 µm C18 particle, column inner diameter 150 µm), followed by a 15 cm analytical column (3 µm C18 particle, inner diameter 75 µm, tip diameter 8 µm). The liquid chromatography, EASY-nLC 1000 system (Thermo Fisher, Odense, Denmark) was coupled online to the TSQ Quantiva mass spectrometer with a nano-electrospray ionization source. A 37-min LC gradient was applied, with an increasing percentage of buffer B (0.1% formic acid in acetonitrile) for peptide elution at a flow rate of 300 nL/min. The SRM assay parameters were set up as follows: positive-ion mode, optimized collision energy values, adjusted dwell time, 0.7 Th Q1 resolution of full width at half-maximum and 0.7 Th in Q3 resolution. LC peaks for all peptides were manually inspected to ensure acquisition of minimum 10 points per LC peak. Raw data were uploaded and analyzed with Skyline software (University of Washington, Seattle, USA).

Neuronal pentraxin receptor-1 (NPTXR) ELISA assay

We used the RayBio Human NPTXR ELISA kit, as recommended by the manufacturer (catalog # ELH-NPTXR, Ray Biotech, Norcross, GA, USA). All CSF samples were analyzed after a 25-fold dilution. For this independent validation, we used CSF samples from 12 AD patients, 21 patients with MCI and 23 control subjects. This cohort was used previously for mass spectrometric analyses, as outlined elsewhere[19]. The samples were obtained by lumbar puncture and stored at -80°C until use. The Institutional Review Board of the Technical University of Munich approved the study and all patients signed an informed consent form.

Clinical samples

Age and sex data were collected from all participants. In total, 101 CSF samples were retrospectively collected at the memory and dementia clinic of the 3rd Department of Neurology, "G. Papanikolaou", School of Medicine, Aristotle University of Thessaloniki, Greece and from the Day Centers of the Greek Association of Alzheimer's Disease and Related Disorders (GAARD), Thessaloniki, Greece. A summary of patient characteristics is shown in Table 1.

Patients suspected of having AD were examined by a specialist neuropsychiatrist and diagnosis was made based on the NINCDS/ADRDA criteria for probable AD[20]. Disease severity was determined based on the Mini-Mental State Examination (MMSE) and clinical dementia rating (CDR) scores and patients were categorized as having mild (MMSE=20–26, CDR=1), moderate (MMSE=10–19, CDR=2) and severe (MMSE=0–9, CDR=3) dementia. Diagnosis of MCI was based on the description by Petersen, which is almost equivalent to the NIH-AA criteria for MCI due to AD[21]. This study was approved by the GAARD scientific and ethics committees and by the Institutional Review Boards of Aristotle University and the University of Toronto. All participants signed an informed consent form.

Table 1. Patient characteristics.

Set 1	Mild cognitive impairment	Mild AD dementia	Moderate AD dementia	Severe AD dementia
Participants, n	8	11	24	15
Age[a]	75 (70.7, 80.5)	71 (68, 76.5)	76.5 (70.7, 78.25)	76 (69.5, 82)
Age[b]	74.5 (7.8)	71.4 (8.4)	75.7 (6.4)	74.4 (9.3)
Sex-female, n (%)	3 (38)	3 (27)	13 (54)	6 (40)
MMSE[a,c]	28 (26, 29)	24 (22, 25.5)	19 (16.8, 20)	8 (2.5, 10)
MMSE[b]	27.6 (1.8)	23.9 (1.7)	18.5 (2.0)	6.5 (4.6)
Set 2				
Participants, n	6	8	16	13
Age[a]	68 (60, 74)	76.5 (71.5, 80.7)	78.5 (74.7, 83.2)	75 (72, 76)
Age[b]	67.6 (9.2)	76.2 (8.8)	78.1 (6.9)	71.1 (9.0)
Sex-female, n (%)	5 (83)	3 (38)	6 (38)	2 (15%)
MMSE[a,c]	27.5 (26.2, 28.7)	24 (22.7, 24)	17.5 (16.7, 19)	7 (2, 10)
MMSE[b]	27.7 (1.6)	23.6 (1.3)	17.6 (2.2)	6.2 (4.4)

AD, Alzheimer's disease.
[a] Expressed as median (25th, 75th percentile)
[b] Expressed as mean (standard deviation)
[c] Mini-Mental State Examination

A fraction of CSF samples were analyzed for core AD biomarkers (Aβ1-42, t-tau, p-tau) using an Innotest ELISA kit (Fujirebio Europe)[22]. Overall, 54 participants were tested for Aβ1-42 (distributed by groups, MCI: n=10, mild: n=7, moderate: n=23, severe: n=14), 42 for t-tau (distributed by groups, MCI: n=9, mild: n=6, moderate: n=16, severe: n=11) and 43 for p-tau (distributed by groups, MCI: n=9, mild: n=5, moderate: n=21, severe: n=8).

All CSF samples were collected by lumbar puncture, inspected macroscopically for blood contamination, centrifuged and stored at -80°C in polypropylene tubes. Samples were shipped to the Lunenfeld–Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, Canada and stored at -80°C until processing. Ethics approval was obtained from the Mount Sinai Hospital Research Ethics Board for use of these samples.

Data analysis

Clinical samples were randomized and ran in duplicate. The raw files were uploaded to Skyline software (version 3.5.0.9319), which was used for peak integration and quantification of the area under the curve (AUC). Relative quantification was performed as previously described[16]. For peptides with amino acid methionine in the sequence, AUC_{light}/AUC_{heavy} was calculated as: AUC $(\text{oxidized + non-oxidized})_{light}$/AUC $(\text{oxidized + non-oxidized})_{heavy}$. SRM data were manually evaluated and samples with poor integration were excluded. Identification of APOE phenotype was determined as described in our previous report[18].

Statistical analysis

Statistical analysis was performed with R statistical and graphics software, version 3.5.0. Means, medians, standard deviations, interquartile ranges and coefficients of variations were calculated. Linear regression was used to test for differences in ages. For tests involving a dichotomous variable, such as sex, the Fisher's exact test was used. Tests for differences in candidate protein abundance, MMSE score, CSF Aβ1-42, t-tau and p-tau across disease stages were adjusted by age and sex using multivariate linear regression. Correlation analyses for MMSE score and protein abundance were performed using Spearman's rank correlation test. ROC curves were prepared for the most significant proteins and AUC values with 95% confidence intervals were calculated using the bootstrap method. AUC values were covariate-adjusted by age or sex when there was a significant association ($p<0.05$) between a marker and the covariates in controls[23]. P-values for comparison between groups were reported as non-adjusted and adjusted for multiple comparison by the Holm method and $p<0.05$ was considered statistically significant.

Results

Patients' characteristics

CSF samples from MCI and AD patients with different dementia severity (n=101) were randomized into two sets. The rationale for the randomization was to confirm the validity of our findings in separate assays, performed on different days. In the first set, 8 patients were diagnosed with MCI, 11 with mild, 24 with moderate and 15 with severe dementia, while in the second set, 6 patients had MCI, 8 mild, 16 moderate and 13 severe dementia (Table 1).

The MMSE cognitive test was significantly different ($p<0.001$) in both sets between the four groups (Figure 1, Table 1). As expected, MCI patients had the highest MMSE score, followed by mild, moderate, and severe AD. In the first set, the mean age (years) was 74.5 for MCI, 71.4 for mild, 75.7 for moderate and 74.4 for severe dementia. In the second set, the mean age (years) was 67.6 for MCI, 76.2 for mild, 78.2 for moderate and 71.1 for severe dementia. In set 1 there were 3 females each in the MCI and mild dementia groups, 13 in moderate and 6 in severe dementia groups, whereas in set 2, 5 females were in the MCI group, 3 in mild, 6 in moderate and 2 in severe dementia groups. Between groups, there was no difference in age ($p=0.514$) or sex ($p=0.504$) in set 1, while a small difference was found for age ($p=0.041$) and sex ($p=0.047$) between the four groups in set 2.

Current CSF biomarkers were tested in a fraction of MCI, mild, moderate and severe AD patients. A statistical difference was observed between disease groups for Aβ1-42 (decreasing with severity) and t-tau (increasing with severity) ($p<0.05$); Aβ1-42 levels were differentially expressed between MCI *vs.* moderate AD dementia and MCI *vs.* severe AD dementia (Supplementary Table 1). The distributions of Aβ1-42, t-tau and p-tau in the four groups are shown in Figure 2.

Novel candidate biomarkers of AD patients

For evaluation of the 30 biomarker candidates, 101 CSF samples from MCI and AD patients were randomized into two separate sets.

Overall, the majority of the proteins showed similar distribution patterns across AD stages, with a trend towards a decline in CSF concentration with disease progression. Among all proteins, only NPTXR showed a statistically significant difference between MCI *vs.* combined moderate and severe AD groups in both sets of patients (set 1: $p=0.004$, set 2: $p=0.039$). The concentration of NPTXR decreased in advanced stages. However, this significance did not remain after multiple comparison correction by the Holm's method.

Several other proteins also showed decreases in advanced stages of AD but did not consistently achieve statistical significance. In the first data set, proteins NPTXR, NPY and VGF were significantly different between the four groups (p=0.014, 0.033, 0.038, respectively), before correction for multiple comparison testing. After correction, the significance disappeared. Likewise, the findings observed in the first data set were not always-replicated in the second data set, but some proteins showed differential levels when comparing MCI *vs.* moderate and severe AD. These included BAI2, ECM1, FRRS1L, NPTXR, NPY, SLITRK1 and VGF (p=0.044, 0.033, 0.042, 0.004, 0.004, 0.048, 0.005 respectively). Proteins NPTXR, NPY and VGF were the most consistent, showing reductions in concentration with increasing AD severity.

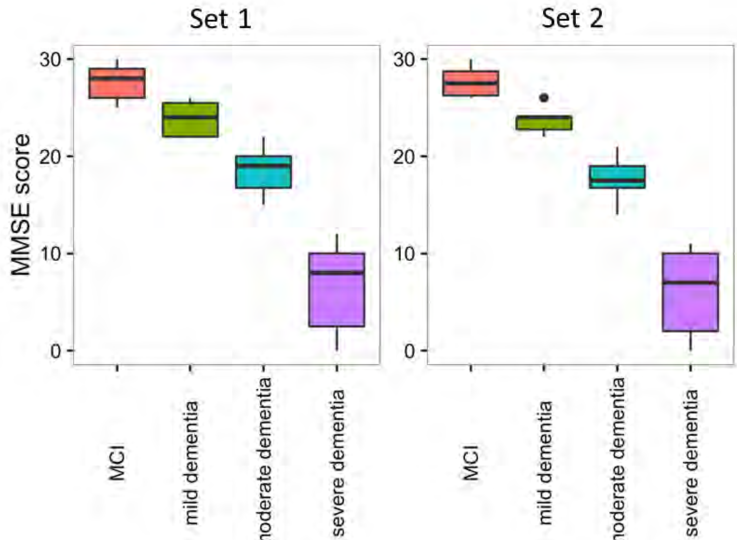

Figure 1. Distribution of cognitive test scores in mild cognitive impairment (MCI) and mild, moderate and severe Alzheimer's disease (AD) dementia groups. The cognitive test Mini-Mental State Examination (MMSE) was compared between MCI, mild, moderate and severe AD dementia patients. A statistically significant difference in cognitive performance was observed among the four groups, in both sets (p<0.001). Horizontal lines represent medians. The number of patients per group is mentioned in Table 1.

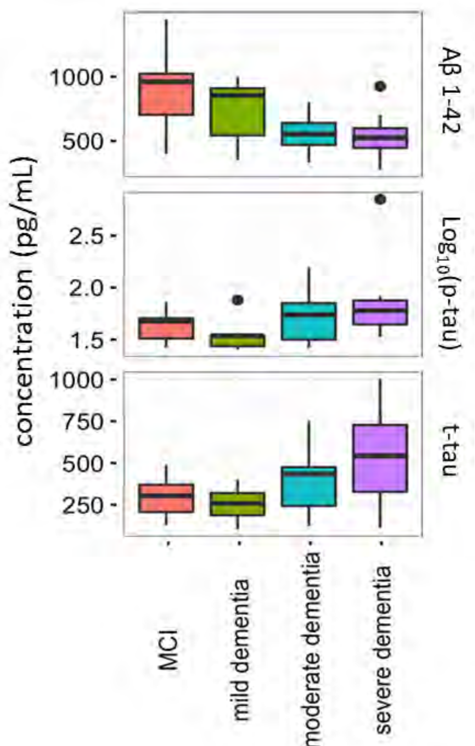

Figure 2. Distribution of core cerebrospinal fluid biomarkers Aβ1-42, t-tau and p-tau. The concentrations of these proteins were compared between mild cognitive impairment (MCI) (n=10) mild (n=7), moderate (n=23) and severe Alzheimer's disease (n=14). Aβ1-42 and t-tau were significantly different between the tested groups (p<0.05). Horizontal lines represent medians.

Control protein ECM1 did not differ among MCI, mild, moderate and severe AD patients (set 1 p=0.200, set 2 p=0.926) but differed between MCI vs. moderate and severe AD groups, only in set 1. However when multiple correction was applied, the difference disappeared.

Statistical analysis of all candidates between the four groups and between MCI *vs.* moderate and severe AD dementia is shown in Supplementary Table 2.

The reproducibility of the assays for control samples (pools of non-pathological CSFs) and clinical samples was <20% (data not shown). The distributions of all candidate proteins between the four disease groups in sets 1 and 2 are shown in Figure 3 and Figure 4.

Diagnostic performance

Diagnostic performance was evaluated by calculating the AUC for discriminating MCI *vs.* moderate and severe AD dementia. Based on the performance of candidates in both sets, only NPTXR protein showed a significant and reproducible separation between the two groups. In the first set, the AUC for NPTXR was 0.799 (95% CI: 0.628, 0.928) and in the second set was 0.799 (95% CI: 0.586, 0.960). Figure 5 shows ROC curves for this protein in both sets.

Correlation of candidate proteins with MMSE score

Pairwise Spearman's rank correlation was used to assess if there is a correlation between protein candidates and the cognitive test MMSE score. A few proteins showed a significant positive correlation with MMSE score (Supplementary Table 3), which

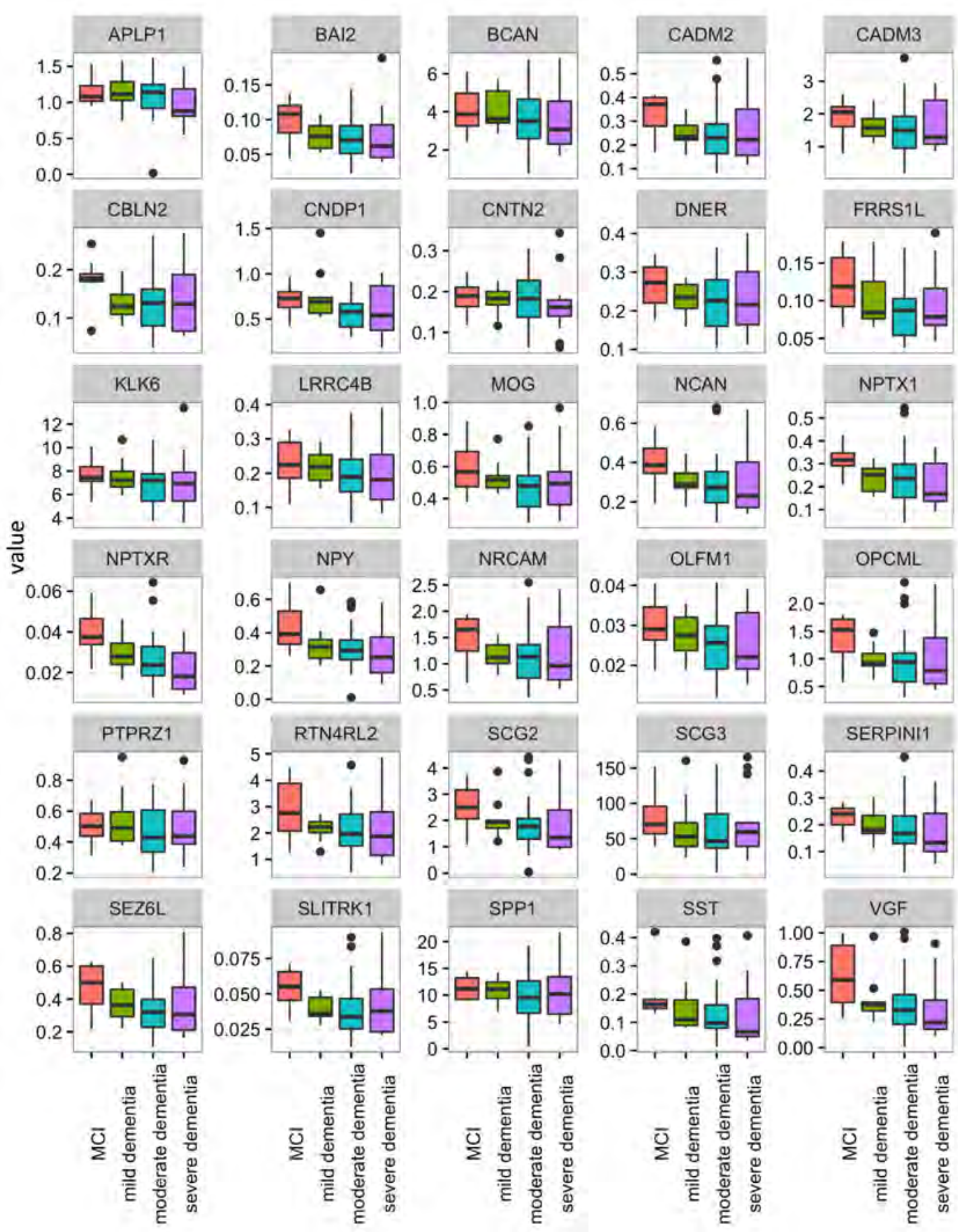

Figure 3. Distribution of candidate protein biomarkers in cerebrospinal fluid samples of set 1 (see text for definitions). Candidate proteins were measured with SRM assay and compared between mild cognitive impairment (MCI) (n=8), mild (n=11), moderate (n=24) and severe AD (n=15). Full gene names can be found in the website of the human gene nomenclature committee (https://www.genenames.org/).

means that a lower score was associated with a lower protein concentration in CSF. Spearman's rank correlation coefficients between level of these candidates and the cognitive test (for pairs significant at 0.05 level) was: 0.21 for BAI2, 0.23 for NCAN, 0.29 for NPY, 0.22 for OPCML, 0.29 for RTN4RL2, 0.26 for SCG2, 0.23 for SEZ6L, 0.25 for SST and 0.32 for VGF. The Spearman's coefficient for NPTXR was 0.20 (not significant).

Distribution of candidate proteins among APOE phenotypes

Overall, there was 31% APOE ε4 carriers among disease patients (14% among MCI, 21% among mild, 30% among moderate and 46% among severe AD dementia). APOE ε4 homozygous patients were present only in mild (n=2) and severe AD (n=2) groups. There was no significant difference in distribution of

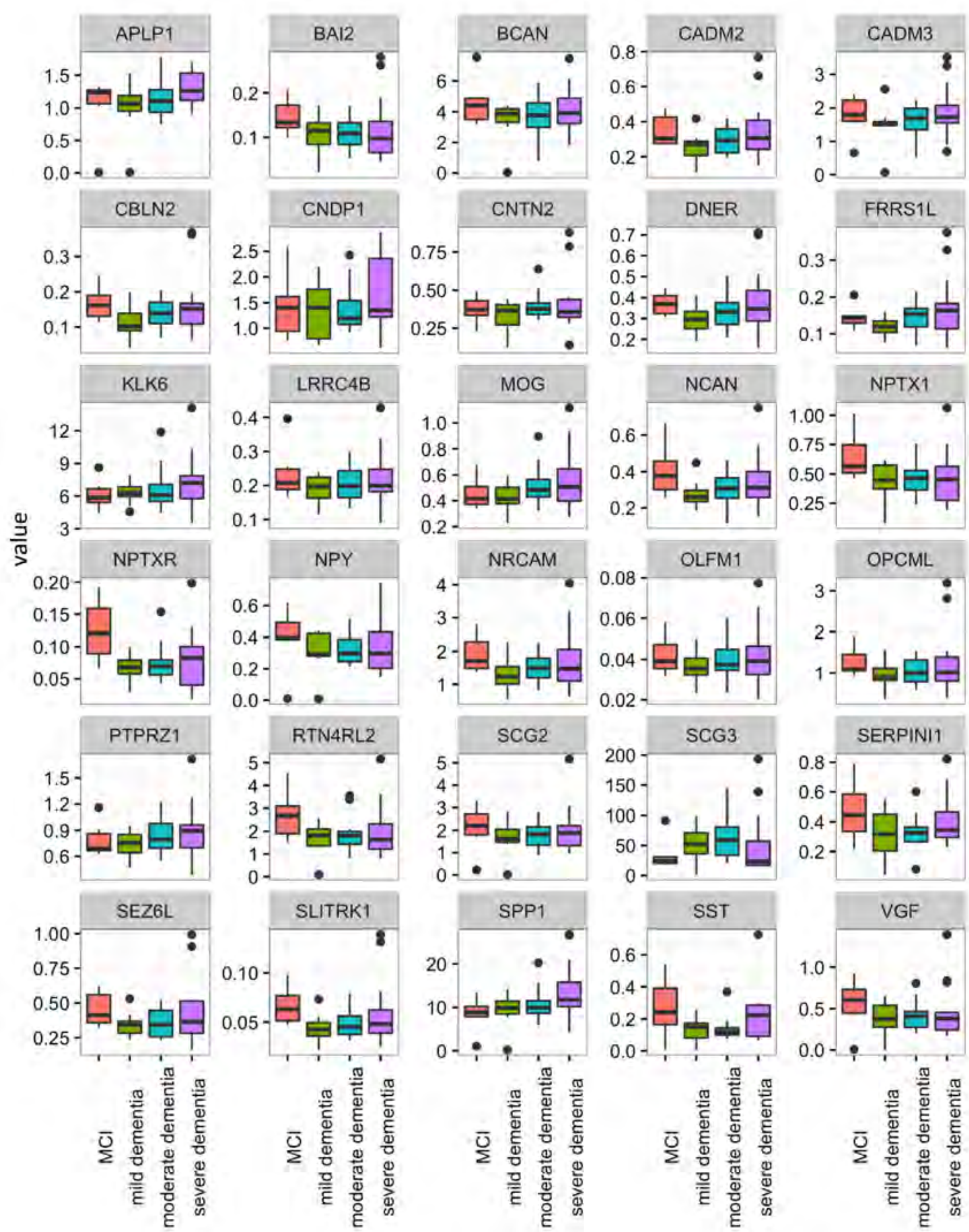

Figure 4. Distribution of candidate protein biomarkers in cerebrospinal fluid samples of set 2 (see text for definitions). Candidate proteins were measured with SRM assay and compared between mild cognitive impairment (MCI) (n=6), mild (n=8), moderate (n=16) and severe AD (n=13). Full gene names can be found in the website of the human gene nomenclature committee (https://www.genenames.org/).

ε4 carriers between disease patients with different severity (p=0.138). Overall, five APOE phenotypes were identified in all subjects, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4, with no difference in the APOE phenotype frequencies among tested groups (p=0.160). In set 1 all five APOE phenotypes were present, ε2/ε3 (n=2), ε2/ε4 (n=1), ε3/ε3 (n=38), ε3/ε4 (n=13) and ε4/ε4 (n=4), while in set 2 only three: ε2/ε3 (n=3), ε3/ε3 (n=27), ε3/ε4 (n=13). The frequencies of APOE phenotypes are shown in Table 2.

In both set of samples, none of the proteins showed a reproducible difference in abundance between APOE phenotypes

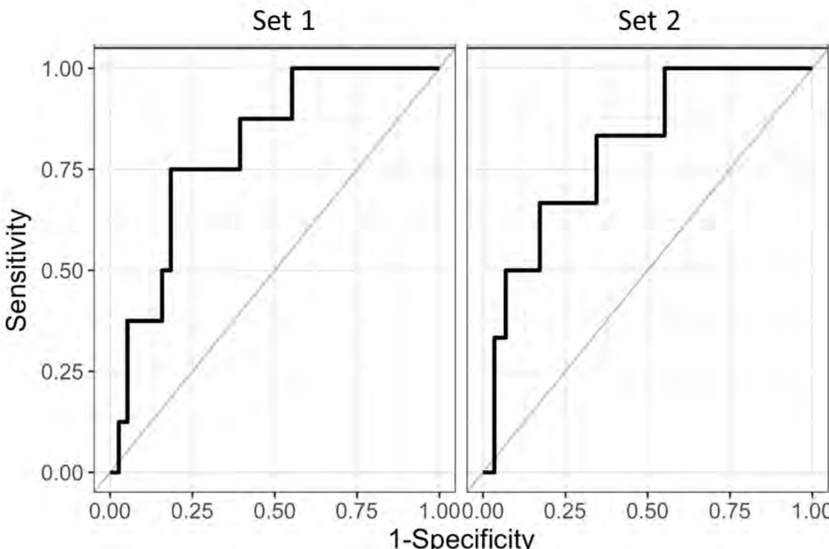

Figure 5. Receiver-operating characteristic (ROC) curves for the best performing candidate. ROC curve of NPTXR protein in set 1 and set 2; area under the curve value for set 1 was 0.799 (95% CI: 0.628, 0.928) and in set 2 was 0.799 (95% CI: 0.586, 0.960).

Table 2. APOE phenotype distribution.

APOE phenotype	Mild cognitive impairment	Mild AD dementia	Moderate AD dementia	Severe AD dementia	Total
ε4-carriers (%)	14	21	30	46	31
ε2/ε3	0	2	2	1	5
ε2/ε4	0	0	1	0	1
ε3/ε3	12	13	26	14	65
ε3/ε4	2	2	11	11	26
ε4/ε4	0	2	0	2	4
Grand total	**14**	**19**	**40**	**28**	**101**

APOE, apolipoprotein E; AD, Alzheimer's disease.

(data not shown). Only FRRS1L protein showed a modest significance and only in the first set (p=0.040, when not adjusted for multiple comparison). There was no difference in proteins in set 2 between different phenotypes (p>0.05).

Validation of NPTXR in CSF by ELISA

In order to validate our findings of decreased NPTXR in CSF of MCI and AD patients, we analyze CSF NPTXR by sandwich ELISA assay. For this independent validation, we used CSF samples from 12 AD patients, 21 patients with MCI and 23 control subjects. The results of CSF NPTXR concentration in the three groups of patients are shown in Figure 6. Controls had the highest level, followed by MCI and AD. The differences between controls and MCI were not statistically significant by the Mann-Whitney non-parametric test (p=0.52). Also, the differences were not significant between MCI and AD patients (p=0.10). However, the differences between controls and AD were highly significant (p=0.004). These results further support our hypothesis that NPTXR is a new CSF biomarker of AD, decreasing progressively with disease severity.

Dataset 1. Raw data for the results included in this study

http://dx.doi.org/10.5256/f1000research.15095.d208304

Discussion

The main objective of the present study was to evaluate 30 brain-related proteins as CSF biomarkers of AD severity and progression. These highly specific brain proteins were assessed in AD patients with different stages, including MCI, mild, moderate and severe AD dementia. Protein NPTXR showed potential as a biomarker of disease progression. Significant and consistent differences in CSF NPTXR levels were observed between MCI *vs.* combined moderate and severe AD dementia groups.

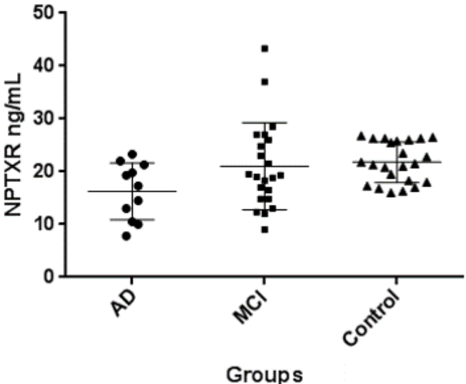

Figure 6. Distribution of CSF NPTXR concentrations, as measured by ELISA, in CSF of patients with Alzheimer's disease (AD; n=12), mild cognitive impairment (MCI; n=21) and controls (n=23). The differences were statistically significant only between controls and AD patients by Mann-Whitney test (p=0.004). Horizontal lines represent means and 25–75 percentiles. This cohort has been described elsewhere[19]. For more details see text.

NPTXR protein is a member of the neuronal pentraxin family, expressed predominately in the brain, with the highest expression observed in the hippocampus and cerebellum[24]. This transmembrane presynaptic protein was suggested to be involved in the activation of both excitatory and inhibitory neurons[25]. NPTXR has been suggested previously as a potential prognostic biomarker[26], more specific for AD, compared to Parkinson's disease[27]. Differential abundance of NPTXR has been observed in asymptomatic carriers of AD familial mutations, compared to non-carriers, with elevated levels observed in asymptomatic carriers[28]. Wildsmith *et al.* observed a similar trend of NPTXR abundance between MCI and AD groups, as found here; i.e. increased levels in the MCI group, with AD patients having lower levels[26]. A decreasing abundance of NPTXR was also observed in longitudinally followed AD patients. This data are in accord with our study, since NPTXR levels declined proportionally with advanced AD dementia stages. Additional accumulating evidence by Hendrickson *et al.* further suggests that NPTXR represents a new biomarker of AD disease progression, decreasing with the severity of AD[29]. The latter study identified VGF as an additional progression biomarker, as we also found here.

The observed pattern of decline in abundance with disease severity for a few proteins, in addition to NPTXR, could be partially explained by their brain-specificity. Since our candidates were selected to have high specificity for brain tissue expression, these proteins could be expressed throughout various brain regions and their decrease may represent the overall decline in cortical volume, thus serving as markers of brain atrophy. This suggestion could be confirmed in future studies, by comparing brain imaging data with abundance of specific proteins in CSF. The superiority of NPTXR over other proteins may be related to its high expression in the hippocampus.

APOE ε4 allele represents the main genetic risk factor for developing AD. Carriers for APOE ε4 have earlier age of disease onset and more pronounced amyloid pathology compared to non-carriers. For example, amyloid plaques are more abundant in ε4 carriers[30], with lower CSF concentration of Aβ1-42 in AD patients[31]. In addition to enhanced Aβ plaques in the brain, ε4 carriers exhibit vascular Aβ deposition[31]. Therefore, we aimed to evaluate if levels of our candidate biomarkers change with APOE phenotype. In this study, none of the candidates showed any reproducible difference in abundance between APOE phenotypes. However, this needs to be further investigated due to our limited sample size and considering that not all APOE phenotypes were identified in both set of samples.

Some limitations of our study are associated with the selection of the 30 brain-specific proteins. Our focus was on predicted secreted and membrane-bound proteins, since the majority of HPA brain-enriched proteins are membrane and/or secreted[15] and most of the CSF proteins are membrane-bound or secreted[32]. Intracellular proteins, which were excluded from this study, may have lower abundance in normal CSF but under pathological conditions they could be released into the CSF. Other limitations are related to the patients included in the study. Our cohorts did not include preclinical AD, which would allow assessment of the proposed candidates from the very early stage of developing AD. Cognitively healthy, age-matched controls were not included in this study since we aimed to test biomarkers in different stages of disease progression. Moreover, only a subset of patients had information on current AD biomarkers (Aβ1-42, t-tau, p-tau). Therefore, the diagnostic accuracy (ROC curve analysis) of NPTXR was not compared to the existing AD CSF biomarkers.

Our candidate biomarkers should be further tested in individuals encompassing the whole AD continuum, from preclinical to more advanced clinical stages. Preferentially, longitudinally followed patients should be monitored to assess the prognostic potential of the candidates, over sufficient period of time, allowing disease progression to the next stage. The approximate annual rate of progression of MCI to AD dementia is 10 to 15%[33].

In summary, in this study, we evaluated 30 brain-specific proteins as candidate CSF biomarkers of AD severity, utilizing multiplex mass spectrometry-based quantification. The protein NPTXR showed the most promise as a potential biomarker of disease progression. Interestingly, at least two other previous studies have also identified NPTXR as a highly promising biomarker of progression of AD. CSF NPTXR levels decline proportionally, as AD becomes more severe. This finding needs to be validated in a larger, longitudinally followed cohort. We suggest that NPTXR may have value as a CSF biomarker for assessing the efficacy of new therapies for AD.

Data availability

Dataset 1: Raw data for the results included in this study. DOI, http://dx.doi.org/10.5256/f1000research.15095.d208304[34].

Competing interests

Dr. Eleftherios P. Diamandis holds a consultant/advisory role with Abbott Diagnostics.

Grant information

This work was supported by Mount Sinai Hospital.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Supplementary material

Supplementary Table 1: Statistical analysis between four patient groups for Aβ1-42, t-tau and p-tau.
Click here to access the data.

Supplementary Table 2: Statistical analysis of candidates between four patient groups and MCI *vs.* moderate and severe AD dementia.
Click here to access the data.

Supplementary Table 3: Spearman's rank correlation between candidate levels and MMSE score.
Click here to access the data.

References

1. Ballard C, Gauthier S, Corbett A, *et al.*: **Alzheimer's disease.** *Lancet.* 2011; **377**(9770): 1019–1031.
PubMed Abstract | Publisher Full Text
2. McKhann GM, Knopman DS, Chertkow H, *et al.*: **The diagnosis of dementia due to Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease.** *Alzheimers Dement.* 2011; **7**(3): 263–269.
PubMed Abstract | Publisher Full Text | Free Full Text
3. Nakamura A, Kaneko N, Villemagne VL, *et al.*: **High performance plasma amyloid-β biomarkers for Alzheimer's disease.** *Nature.* 2018; **554**(4691): 249–54.
PubMed Abstract | Publisher Full Text
4. Jack CR Jr, Albert MS, Knopman DS, *et al.*: **Introduction to the recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease.** *Alzheimers Dement.* 2011; **7**(3): 257–262.
PubMed Abstract | Publisher Full Text | Free Full Text
5. WHO: **Dementia: a public health priority.** WHO. 2012.
Reference Source
6. Kroksveen AC, Opsahl JA, Aye TT, *et al.*: **Proteomics of human cerebrospinal fluid: discovery and verification of biomarker candidates in neurodegenerative diseases using quantitative proteomics.** *J Proteomics.* 2011; **74**(4): 371–388.
PubMed Abstract | Publisher Full Text
7. Blennow K, Hampel H, Weiner M, *et al.*: **Cerebrospinal fluid and plasma biomarkers in Alzheimer disease.** *Nat Rev Neurol.* 2010; **6**(3): 131–144.
PubMed Abstract | Publisher Full Text
8. Blennow K, de Leon MJ, Zetterberg H: **Alzheimer's disease.** *Lancet.* 2006; **368**(9533): 387–403.
PubMed Abstract | Publisher Full Text
9. Khan TK, Alkon DL: **Alzheimer's Disease Cerebrospinal Fluid and Neuroimaging Biomarkers: Diagnostic Accuracy and Relationship to Drug Efficacy.** *J Alzheimers Dis.* 2015; **46**(4): 817–836.
PubMed Abstract | Publisher Full Text
10. Sunderland T, Wolozin B, Galasko D, *et al.*: **Longitudinal stability of CSF tau levels in Alzheimer patients.** *Biol Psychiatry.* 1999; **46**(6): 750–755.
PubMed Abstract | Publisher Full Text
11. Williams JH, Wilcock GK, Seeburger J, *et al.*: **Non-linear relationships of cerebrospinal fluid biomarker levels with cognitive function: an observational study.** *Alzheimers Res Ther.* 2011; **3**(1): 5.
PubMed Abstract | Publisher Full Text | Free Full Text
12. Drabovich AP, Dimitromanolakis A, Saraon P, *et al.*: **Differential diagnosis of azoospermia with proteomic biomarkers ECM1 and TEX101 quantified in seminal plasma.** *Sci Transl Med.* 2013; **5**(212): 212ra160.
PubMed Abstract | Publisher Full Text
13. Martinez-Morillo E, Garcia Hernandez P, Begcevic I, *et al.*: **Identification of novel biomarkers of brain damage in patients with hemorrhagic stroke by integrating bioinformatics and mass spectrometry-based proteomics.** *J Proteome Res.* 2014; **13**(2): 969–981.
PubMed Abstract | Publisher Full Text
14. Begcevic I, Brinc D, Drabovich AP, *et al.*: **Identification of brain-enriched proteins in the cerebrospinal fluid proteome by LC-MS/MS profiling and mining of the Human Protein Atlas.** *Clin Proteomics.* 2016; **13**: 11.
PubMed Abstract | Publisher Full Text | Free Full Text
15. Uhlén M, Fagerberg L, Hallström BM, *et al.*: **Proteomics. Tissue-based map of the human proteome.** *Science.* 2015; **347**(6220): 1260419.
PubMed Abstract | Publisher Full Text
16. Begcevic I, Brinc D, Dukic L, *et al.*: **Targeted mass spectrometry-based assays for relative quantification of 30 brain-related proteins and their clinical applications.** *J Proteome Res.* 2018.
PubMed Abstract | Publisher Full Text
17. Liu CC, Liu CC, Kanekiyo T, *et al.*: **Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy.** *Nat Rev Neurol.* 2013; **9**(2): 106–118.
PubMed Abstract | Publisher Full Text | Free Full Text
18. Martínez-Morillo E, Nielsen HM, Batruch I, *et al.*: **Assessment of peptide chemical modifications on the development of an accurate and precise multiplex selected reaction monitoring assay for apolipoprotein e isoforms.** *J Proteome Res.* 2014; **13**(2): 1077–1087.
PubMed Abstract | Publisher Full Text
19. Begcevic I, Brinc D, Brown M, *et al.*: **Brain-related proteins as potential CSF biomarkers of Alzheimer's disease: A targeted mass spectrometry approach.** *J Proteomics.* 2018; **182**: 12–20.
PubMed Abstract | Publisher Full Text
20. McKhann G, Drachman D, Folstein M, *et al.*: **Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease.** *Neurology.* 1984; **34**(7): 939–944.
PubMed Abstract | Publisher Full Text
21. Petersen RC, Smith GE, Waring SC, *et al.*: **Mild cognitive impairment: clinical characterization and outcome.** *Arch Neurol.* 1999; **56**(3): 303–308.
PubMed Abstract | Publisher Full Text
22. Toledo JB, Zetterberg H, van Harten AC, *et al.*: **Alzheimer's disease cerebrospinal fluid biomarker in cognitively normal subjects.** *Brain.* 2015; **138**(Pt 9): 2701–15.
PubMed Abstract | Publisher Full Text | Free Full Text
23. Janes H, Longton G, Pepe M: **Accommodating Covariates in ROC Analysis.** *Stata J.* 2009; **9**(1): 17–39.
PubMed Abstract | Free Full Text
24. Dodds DC, Omeis IA, Cushman SJ, *et al.*: **Neuronal pentraxin receptor, a novel putative integral membrane pentraxin that interacts with neuronal pentraxin 1 and 2 and taipoxin-associated calcium-binding protein 49.** *J Biol Chem.* 1997; **272**(34): 21488–21494.
PubMed Abstract | Publisher Full Text
25. Lee SJ, Wei M, Zhang C, *et al.*: **Presynaptic Neuronal Pentraxin Receptor Organizes Excitatory and Inhibitory Synapses.** *J Neurosci.* 2017; **37**(5): 1062–1080.
PubMed Abstract | Publisher Full Text | Free Full Text

26. Wildsmith KR, Schauer SP, Smith AM, *et al.*: **Identification of longitudinally dynamic biomarkers in Alzheimer's disease cerebrospinal fluid by targeted proteomics.** *Mol Neurodegener.* 2014; **9**: 22.
PubMed Abstract | Publisher Full Text | Free Full Text

27. Yin GN, Lee HW, Cho JY, *et al.*: **Neuronal pentraxin receptor in cerebrospinal fluid as a potential biomarker for neurodegenerative diseases.** *Brain Res.* 2009; **1265**: 158–170.
PubMed Abstract | Publisher Full Text

28. Ringman JM, Schulman H, Becker C, *et al.*: **Proteomic changes in cerebrospinal fluid of presymptomatic and affected persons carrying familial Alzheimer disease mutations.** *Arch Neurol.* 2012; **69**(1): 96–104.
PubMed Abstract | Publisher Full Text | Free Full Text

29. Hendrickson RC, Lee AY, Song Q, *et al.*: **High Resolution Discovery Proteomics Reveals Candidate Disease Progression Markers of Alzheimer's Disease in Human Cerebrospinal Fluid.** *PLoS One.* 2015; **10**(8): e0135365.
PubMed Abstract | Publisher Full Text | Free Full Text

30. Schmechel DE, Saunders AM, Strittmatter WJ, *et al.*: **Increased amyloid beta-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease.** *Proc Natl Acad Sci U S A.* 1993; **90**(20): 9649–9653.
PubMed Abstract | Publisher Full Text | Free Full Text

31. Prince JA, Zetterberg H, Andreasen N, *et al.*: ***APOE* epsilon4 allele is associated with reduced cerebrospinal fluid levels of Abeta42.** *Neurology.* 2004; **62**(11): 2116–2118.
PubMed Abstract | Publisher Full Text

32. Zhang Y, Guo Z, Zou L, *et al.*: **A comprehensive map and functional annotation of the normal human cerebrospinal fluid proteome.** *J Proteomics.* 2015; **119**: 90–99.
PubMed Abstract | Publisher Full Text

33. Hansson O, Zetterberg H, Buchhave P, *et al.*: **Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study.** *Lancet Neurol.* 2006; **5**(3): 228–234.
PubMed Abstract | Publisher Full Text

34. Begcevic I, Tsolaki M, Brinc D, *et al.*: **Dataset 1 in: Neuronal pentraxin receptor-1 is a new cerebrospinal fluid biomarker of Alzheimer's disease progression.** *F1000Research.* 2018.
Data Source

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Current Referee Status:

Version 1

Referee Report 15 August 2018

https://doi.org/10.5256/f1000research.16440.r36772

Mohd M. Khan
National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, 20892, USA

In the US alone, Alzheimer's disease (AD) is the 6th leading cause of death. Unfortunately there are no clinical biomarkers of AD that could be used to efficiently and successfully diagnose the disease onset, monitor the AD progression, and evaluate the efficiency of therapeutic interventions against AD. According to Alzheimer's association, early and accurate AD diagnosis could save $7.9 trillion in hospital-associated costs. Hence to fill this diagnostic gap, present work aims to identify and validate AD biomarkers in cerebrospinal fluid (CSF) patient samples using mass spectrometry-based targeted proteomics. The manuscript is well written and methods are either sufficiently described and/or appropriate earlier works are cited (particularly for targeted method development and protein quantitation). However I suggest authors should consider submitting the raw MS data to ProteomeXchange if not done already.

Targeted proteomics was used to quantify 30 candidate proteins in CSF samples obtained from 101 patients that had mild to severe AD as well as patients with mild cognitive impairment (MCI). In both the sets, 28 severe AD, 40 moderate AD, 19 mild AD, and 14 MCI patient samples were quantified for candidate biomarkers using SRM method. One biomarker candidate of interest neuronal pentraxin receptor-1 (NPTXR), quantified by mass spectrometry and validated in a separate set by ELISA, was found useful in differentiating AD from control samples. Severe AD patients had lower levels of NPTXR in CSF than those in control CSF samples; in contrast, considerable variation in the levels of NPTXR levels in MCI patients was observed. However the rationale behind not segregating samples in 5 groups (control, MCI, mild-, moderate-, and severe-dementia) for ELISA validation is missing and could be added. For the NPTXR validation, authors could have done secondary validation of NPTXR levels in present cohort (large group; n=101) followed by an independent validation in a separate cohort as done in Figure 6 (n=56). Nonetheless this early work on AD biomarker discovery is interesting and a longitudinal study will be helpful in establishing NPTXR as a true biomarker of AD progression and disease severity.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 08 August 2018

https://doi.org/10.5256/f1000research.16440.r36375

Julie Shaw
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Currently there are no biomarkers for AD used in clinical practice. Current methods for evaluating patients only allow for diagnoses of probable AD while definitive diagnosis can only take place at the time of post-mortem examination. This study aims to identify much needed biomarkers for AD in CSF using mass spectrometry. The authors used tandem mass spectrometry to measure the concentrations of 30 CSF-specific proteins, which they previously identified, in patient specimens. The patient cohort included those with mild cognitive impairment, mild, moderate and severe AD, as determined by the MMSE and CDR ratings. The authors identified one candidate protein, neuronal pentraxin receptor -1 (NPTXR), as promising in its ability to differentiate MCI from moderate and severe AD. Independent assessment of NPTXR concentrations in patient specimens using an ELISA confirmed that concentrations of this protein are lower in CSF from patients with moderate and severe AD compared to control patients.

This manuscript is well written and I only have minor comments, which I will outline below.

In the results section, the authors state that the reproducibility of the assays for control samples and clinical samples was <20%. It is unclear to me what this refers to and it would be helpful for the authors to clarify, especially since the data are not shown.

How was cognitive level assessed for the patient specimens used for the ELISA measurements? It appears that these specimens came from a different institution than those used for the mass spectrometry analysis. If a different assessment scheme was employed for these specimens, this could be confounding. I would suggest that the authors comment on this.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 06 August 2018

https://doi.org/10.5256/f1000research.16440.r36373

Edward W. Randell
Department of Laboratory Medicine, Faculty of Medicine, Memorial University of Newfoundland, St. John's, NL, Canada

There are currently no blood based or CSF biomarkers commonly used on a routine basis for screening, diagnosis, or monitoring progression of Alzheimer's disease. This work is an effort to fill the gap as it examines 30 candidate proteins from CSF and their relationship with disease progression. This was done by examining levels of the CSF proteins in patients at various stages of disease ranging from mild cognitive impairment to severe dementia. Initial assay of the 30 proteins was carried out using tryptic digestion and analysis of signature peptides by LC-MS/MS selective reaction monitoring technique. An attempt to validate results was undertaken by examination in a second set of patients representing the four stages of disease, from mild cognitive impairment to mild, moderate, and severe dementia. The study shows that the biomarker, neuronal pentraxin receptor-1 best discriminates mild cognitive impairment from advanced stages of the disease. This finding was confirmed by ELISA assay of the same protein. The study concludes that this CSF protein in a potential biomarker of Alzheimer's disease progression with potential utility in monitoring treatment. The patient population was well described, and the experimental design sound. Findings of the study are well discussed with previous work and with limitations identified.

Although, not explicitly stated this study apparently measures the 30 candidate biomarkers in CSF from 101 different individuals at different disease stages. Future use of neuronal pentraxin receptor-1, or any biomarker, for disease monitoring purposes at least partially involves establishing a baseline for individual patients and possible serial measurement. Based on information in the box and whisker plots, results for

neuronal pentraxin receptor-1 in patients with mild cognitive impairment varied over a range that was about 3-fold and showed significant overlap with results of patients from the moderate and severe dementia groups. There were also noted outliers in the latter two groups (moderate and severe) showing results well within and even higher than in patients with mild cognitive impairment. Moreover, most of the biomarkers examined showed similar outliers in both sets of analyses. This presents many questions. It is not clear is these outliers for the different biomarkers are from the same patients (or samples) or if different patients and samples produce different biomarker outliers. Also, it is not clear if the neuronal pentraxin receptor-1 outliers, which represent 5 to 10% of the samples from patients with moderate and severe disease, are causes by pre-analytical issues concerning samples or confounding physiological/pathological processes in these patients. It is also not clear how variable levels of this protein is in CSF from an individual patient; and the imprecision of the two different assays (LC-MS/MS method and ELISA) for neuronal pentraxin receptor-1 used was not stated. Between-run assay imprecision and intra-individual biological variability will have bearing on the usefulness of a CSF neuronal pentraxin receptor-1 measurement in practice. It would also be interesting to see how levels change in individual patients as Alzheimer's disease progresses. But, the requirement of repeated lumbar puncture to assess disease course using this or any CSF biomarkers will be a difficult sell given the absence of effective disease modifying therapy. Nevertheless, this does not take away from the value of this early work in highlighting potential value of neuronal pentraxin receptor-1 for disease progression with potential future clinical value if effective disease modifying treatments become available. But as implied by the authors, what would be most useful is a biomarker, like neuronal pentraxin receptor-1, that not only correlates with disease severity but is also predictive of Alzheimer's disease progression and related outcomes.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 26 July 2018

https://doi.org/10.5256/f1000research.16440.r36054

Georgios Pampalakis
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AD is the most common neurodegenerative diseases. Unfortunately, there no molecular biomarkers for the disease yet. In the present study the authors used a well described cohort of AD patients to identify new protein-based biomarkers in CSF that can determine the progression of AD. The procedure was based on identifying potential protein biomarkers from Human Protein Atlas (30 brain-specific proteins were selected) and design analytical method based on MS for their determination in CSF. After careful examination of clinical specimens, the authors identified a new biomarker that was validated with ELISA, the NPTXR. The authors did not find any correlation of the potential biomarkers tested (including NPTXR) with the status of ApoE polymorphism. Finally, the limitations of their study are well-described. A major limitation as noted is the absence of normal samples for the analysis of the potential biomarkers. Although not entirely required for this study, analysis of normal CSF samples will help to identify biomarkers for AD diagnosis. However, the authors validated NPTXR in control samples with ELISA assay and found not statistically different levels between controls and patients with mild cognitive impairment. In conclusion, the present study is well-designed and adds new information on the development of new AD biomarker. It will worth in future studies to examine the levels of NPTXR in other neurodegerative diseases such as Parksinson to determine the specificity.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

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