Brevican and Neurocan Peptides as Potential Cerebrospinal Fluid Biomarkers for Differentiation Between Vascular Dementia and Alzheimer's Disease

Running title: Profiling of brevican and neurocan peptides in human CSF

Karolina Minta[1*], Gunnar Brinkmalm[1,2], Erik Portelius[1,2], Per Johansson[3,4], Johan Svensson[4,5], Petronella Kettunen[1], Anders Wallin[1], Henrik Zetterberg[1,2,6,7], Kaj Blennow[1,2], Ulf Andreasson[1,2]

[1]Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, the Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden

[2]Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden

[3]Department of Neuropsychiatry, Skaraborg Hospital, Falköping, Sweden

[4]Department of Endocrinology, the Sahlgrenska Academy at the University of Gothenburg, Gothenburg and Mölndal, Sweden

[5]Department of Endocrinology, Skaraborg Hospital, Skövde, Sweden

[6]Department of Neurodegenerative Disease, UCL Institute of Neurology, London, UK

[7]UK Dementia Research Institute at UCL, London, UK

*Corresponding author:

Department of Psychiatry and Neurochemistry

Sahlgrenska University Hospital/Mölndal, S-431 80 Mölndal, Sweden

e-mail: karolina.minta@neuro.gu.se tel: +46735660741

Abstract

Background: Brevican and neurocan are central nervous system-specific extracellular matrix proteoglycans. They are degraded by extracellular enzymes, such as metalloproteinases. However, their degradation profile is largely unexplored in cerebrospinal fluid (CSF).

Objective: The study aim was to quantify proteolytic peptides derived from brevican and neurocan in human CSF of patients with Alzheimer's disease (AD) and vascular dementia (VaD) compared with controls.

Methods: The first cohort consisted of 75 individuals including 25 patients with AD, 7 with mild cognitive impairment (MCI) diagnosed with AD upon follow-up, 10 patients with VaD or MCI diagnosed with VaD upon follow-up, and 33 healthy controls and cognitively stable MCI patients. In the second cohort, 31 individuals were included (5 AD patients, 14 VaD patients and 12 healthy controls). Twenty proteolytic peptides derived from brevican (n = 9) and neurocan (n = 11) were quantified using high-resolution parallel reaction monitoring mass spectrometry.

Results: In the first cohort, the majority of CSF concentrations of brevican and neurocan peptides were significantly decreased inVaDas compared withADpatients (AUC = 0.83.0.93, p≤0.05) and as compared with the control group (AUC = 0.79.0.87, p ≤ 0.05). In the second cohort, CSF concentrations of two brevican peptides (B87, B156) were significantly decreased in VaD compared with AD (AUC = 0.86.0.91, p ≤ 0.05) and to controls (AUC = 0.80.0.82, p ≤ 0.05), while other brevican and neurocan peptides showed a clear trend to be decreased in VaD compared with AD (AUC =

0.64.80, $p > 0.05$). No peptides differed between AD and controls.

Conclusion: Brevican and neurocan peptides are potential diagnostic biomarkers for VaD, with ability to separate VaD from AD.

Keywords: Alzheimer's disease; brevican; cerebrospinal fluid; extracellular matrix; neurocan; vascular dementia

List of abbreviations: a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), Alzheimer's disease (AD), amyloid-β (Aβ), area under the curve (AUC), blood brain barrier (BBB), central nervous system (CNS), cerebrospinal fluid (CSF), coefficient of variation (CV), extracellular matrix (ECM), glial fibrillary acidic protein (GFAP), immunoprecipitation (IP), liquid chromatography (LC), mass spectrometry (MS), matrix metalloproteinase (MMP), mild cognitive impairment (MCI), mini-mental state examination (MMSE), neuron-specific enolase (NSE), parallel reaction monitoring (PRM), perineuronal nets (PNNs), phosphate-buffered saline (PBS), phosphorylated tau (p-tau), receiver characteristic curve (ROC), room temperature (RT), total tau (t-tau), vascular dementia (VaD).

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder, the most common form of dementia, characterized by memory impairment and cognitive decline [1].

The histopathological hallmarks of AD include extracellular senile plaques composed of amyloid-β (Aβ) and intracellular neurofibrillary tangles composed of hyperphosphorylated tau [1]. Even though treatments for AD symptoms are available

[2], there is currently no cure.

Mild cognitive impairment (MCI) is characterized by cognitive decline greater than that expected for age and education level, but does not interfere with everyday activities [3]. Often, it is an intermediate cognitive stage between healthy and dementia. However, not all MCI patients progress to dementia - they might remain clinically stable or revert to normal cognition [4].

Vascular dementia (VaD) is the second leading cause of dementia after AD [5]. It is a progressive disease caused by a reduced cerebral blood flow to the brain and it affects cognitive abilities [6]. The contribution of vascular pathology to dementia, makes VaD a heterogeneous group of brain disorders with different subtypes, including multi-infarct dementia, small vessel dementia, hypoperfusion dementia, haemorrhagic dementia and many more [7, 8]. Similarly to AD, there is no cure for VaD, but risk factors for cerebrovascular disease should be treated.

Since AD and VaD patients share many common pathological features and have clinically similar symptoms [9], there is a great need to develop biochemical markers to facilitate differentiation of VaD from AD and other dementias.

Cerebrospinal fluid (CSF) is a circulatory liquid found around and within the organs of the central nervous system (CNS). It can reflect the complex biochemical processes occurring in the brain, *e.g.,* low CSF levels of $A\beta_{42}$ and high CSF levels of total tau (t-tau) and phosphorylated tau (p-tau) proteins reflect plaque deposition, neuronal degeneration and tangle formation, respectively [10]. Since CSF contains less of non-brain derived proteins than blood [11], it is a better tool for exploring brain pathology and it shows an increasing diagnostic relevance for neurological diseases. Thus, CSF became an important source for the biomarker development to enable detection of pathological changes in the brain before symptoms are noticeable.

Even though there are three CSF protein biomarkers used for AD diagnosis: $A\beta_{42}$, t-tau and p-tau [12, 13], there is still a great need for novel biomarkers to detect differential pathological processes in AD. The fact that AD pathology is present in 40-80% of VaD patients [14], makes the pure VaD diagnosis very challenging. Even though the differentiation of VaD from AD was observed to be improved to 85% when combining all the three AD biomarkers in CSF [15], a better measurable indicator specific to either of the two most common dementia forms is needed.

Brevican and neurocan are CNS-specific chondroitin sulfate proteoglycans expressed by neuronal and glial cells [16, 17]. They are integral components of perineuronal nets (PNN), a specialized extracellular matrix (ECM) structure enwrapping CNS neurons, whose role is to regulate neuronal and synaptic functions in the brain [18]. PNNs keep the balance between structural stabilization and synaptic remodeling, which is crucial for development and normal tissue functioning [19]. This homeostasis is sensitive to altered expression of proteases and excessive ECM remodeling might lead to many pathologies [19].

Both brevican and neurocan are substrates for several matrix proteases. Brevican undergoes proteolytic cleavage resulting in N- and C-terminal fragments of ~55 kDa and ~80 kDa, respectively [20]. A family of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) are the proteases responsible for the generation of the lower molecular weight brevican fragments observed in cultures of neurons, astrocytes and rodent brain tissues [21-23]. Also, matrix metalloproteinases (MMPs) are able to cleave brevican resulting in a 53 kDa fragment observed in mouse brain extracts [23, 24]. Neurocan is processed into two fragments, 130 kDa and 150 kDa, observed in cultures of astrocytes and rat brain tissues [25, 26] and is a substrate for both ADAMTS [27] and MMPs [28]. Since brevican and neurocan are highly involved

in the creation of PNNs, it is believed that their processing may result in a loosing of PNNs and excessive ECM remodeling subsequently leading to many brain pathologies [29]. Since the proteolytic processing of brevican and neurocan in human brain tissues is largely understudied, the analysis of human CSF would provide an accessible window into the investigation of their degradation patterns in the brain.

Even though, brevican and neurocan were reported to not reflect AD-pathology in CSF when compared to the control group [30], their fragmentation patterns in CSF were not investigated in AD. Moreover, to our knowledge, there is no study reporting brevican or neurocan in relation to VaD.

The aim of this study was to investigate if CSF brevican and neurocan concentrations are associated with AD and VaD. To strengthen the study, it was carried out in two independent cohorts.

Material and methods

Patient samples

The demographic data of the subjects included in the study are shown in Table 1 and Table 2 for cohort 1 and 2, respectively.

The study was conducted in accordance with the Helsinki Declaration and approval by the ethical committee of Gothenburg University. Informed consent was obtained either from participants or next to kin.

The first cohort consisted of 75 individuals (37 men and 38 women), including patients with AD or MCI diagnosed with AD upon follow-up (n=32), patients with VaD or MCI diagnosed with VaD upon follow-up (n=10), patients with other dementias (n=8), and control group (n=33) consisting of healthy individuals and cognitively stable MCI

patients. The admission criteria of these patients and CSF sampling have been described in detail in a previous study [31].

The second cohort, consisted of 31 individuals (16 men and 15 women), including patients with AD (n=5), VaD (n=14) and healthy controls (n=12). CSF was obtained by lumbar puncture through the L3/L4 or L4/L5 interspace and collected in polypropylene tubes, followed by centrifugation at 2,000 × g at +4 °C for 10 min. The supernatant was stored at -80 °C pending analysis.

Healthy individuals showed no subjective symptoms of cognitive dysfunction. Patients with AD had to fulfil the criteria of dementia [32] and of probable AD defined by NINCDS-ADRA [33]; patients with MCI - criteria established by Petersen [34]; patients with VaD - criteria of dementia [32] and of VaD defined by NINDS-AIREN [35] or of subcortical VaD established by Erkinjuntti [36].

Validation

Intra-assay variability was determined by calculating the coefficient of variation (CV) for four replicates of CSF pool quality samples evenly spread out throughout the 96-well plate.

The stability of brevican and neurocan fragments during freeze-thaw cycles was tested by thawing and freezing CSF aliquots (n=5) one to five times.

To investigate the stability of brevican and neurocan peptides during storage, five CSF samples were stored under the following conditions: -80 °C for one month, -20 °C for a month, 5-8 °C for 24h, 5-8 °C for 7 days, and RT for 24 h.

To evaluate linearity of the method, two-fold serial dilutions of each IS peptide were spiked to CSF pool at five different occasions with subsequent digestion by trypsin/Lys-C and LC-MS/MS analysis. The curve fits were obtained using weighted sum of

squares ($1/Y^2$).

Explorative analysis

For the initial explorative analysis, immunoprecipitation (IP) followed by digestion by trypsin and subsequent analysis by a Dionex UltiMate 3000 nanoflow liquid chromatography (LC) (Thermo Fisher Scientific Inc.) coupled to electrospray ionization high-resolution hybrid quadrupole-orbitrap mass spectrometry (MS), Q Exactive (Thermo Fisher Scientific Inc.) to investigate the endogenous brevican fragment pattern in CSF.

Explorative analysis – IP followed by digestion by trypsin

Four µg of each monoclonal anti-brevican antibody (N-terminal B2739-70B and C-terminal B2739-70C, US Biological Life Science, Salem, MA, USA) were separately added to 25 µL magnetic Dynabeads M-280 Sheep Anti-Mouse IgG (Invitrogen, Carlsbad, CA, USA) and incubated for 1 h on a rocking platform at room temperature (RT). The remaining unbound antibody was removed by washing twice with phosphate-buffered saline (PBS, 10mM Na-phosphate, 0.15 M NaCl, pH 7.4). Each antibody-conjugated beads were added to 965 µL of CSF or 400 ng of full-length recombinant brevican standard (R&D, Minneapolis, MN, USA), and diluted to 1 mL in 0.025% Tween 20 in PBS. Sample incubation was carried for 1 h on a rocking platform at RT. Using a magnetic particle processor (KingFisher, Thermo Fisher Scientific, Waltham, MA, USA), each sample underwent several washing steps in 1 mL 0.025% Tween 20 in PBS, 1 mL PBS, and finally 1 mL 50mM ammonium bicarbonate (NH_4HCO_3, pH 8). Brevican fragments were eluted from the beads by adding 100 µL 0.5% formic acid in deionized water (v/v). The collected supernatant was dried down

in a vacuum centrifuge. Samples not digested by trypsin were stored at -20 °C pending LC-MS analysis. Samples intended for tryptic digestion were reconstituted in 10 µL NH_4HCO_3 and shaked for 30 min. They were then reduced (30 min, 60 °C, on a shaker at 1200 rpm) with 10 µL of 10 mM dithiothreitol dissolved in 50 mM NH_4HCO_3 followed by alkylation (30 min, RT, on a shaker at 600 rpm, in dark) with 5 µL of 10 mM iodoacetamide dissolved in 50 mM NH_4HCO_3. Next, the samples were digested (15 h, 37 °C, on a shaker at 1200 rpm) by adding 5 µL (0.025 µg) of trypsin (Sequencing Grade Modified Trypsin, Promega Corp., Madison, WI, USA). Digestion was stopped by adding 5 µL of 10% formic acid in deionized water (v/v) and the digests were dried down in a vacuum centrifuge and stored at -20 °C pending analysis.

Explorative analysis – LC-MS/MS

IP-purified samples were reconstituted in 7 µL of 8% formic acid/8% acetonitrile in deionized water (v/v/v), from which 6 µL was loaded onto an Acclaim PepMap C18 trap column (Thermo Fisher Scientific, Waltham, MA, USA). Sample loading buffer was 0.05% trifluoroacetic acid in deionized water (v/v). The separation was performed by reversed-phase Acclaim PepMap C18 analytical column (Thermo Fisher Scientific, Waltham, MA, USA) at a flow rate of 300 nL/min by applying a gradient of 0-40% B for 50 min (total cycle time 90 min). Mobile phases were A: 0.1% formic acid in deionized water (v/v) and B: 0.1% formic acid/ 84% acetonitrile in deionized water (v/v/v). The mass spectrometer was operated in data dependent mode using higher energy collision-induced dissociation (HCD) in a similar wat as previously published [37]. Both full mass (MS) and tandem (MS/MS) mass spectra were acquired at a resolution setting of 70,000, 1 microscan, target values 10^6, and maximum injection time 250 ms. Isolation width was 3 *m/z* units and the normalized collision energy was set to 25.

Database searches of the LC-MS/MS spectra were performed using Mascot Daemon (v2.6, Matrix Science, Boston, MA, USA) against a custom made ECM database with the following parameters: fixed modifications: Carboxymethyl, variable modifications: oxidation (M), peptide tolerance 20ppm, peptide charge 1+ and no missed cleavages.

Isotope-labelled peptides

Twenty isotope-labelled tryptic peptides (n=9 for brevican, n=11 for neurocan) were selected for monitoring (Supplementary table 1). Corresponding internal standard peptides purchased from JPT Peptide Technologies (Berlin, Germany) were labeled with both ^{13}C and ^{15}N at the C-terminal arginine or lysine (Δmass = +10 Da or +8 Da, respectively). Each peptide (10 nmol) was reconstituted in 1ml 10% acetonitrile in deionized water (v/v), aliquoted and stored at -20 °C pending analysis.

Brevican/neurocan panel

Twenty-five µL containing the 20 isotope-labelled peptides (n=9 for brevican, n=11 for neurocan) were spiked into 100 µL CSF in Micronic 0.75 mL tubes. The samples were reduced with 25 µL of 30 mM dithiothreitol dissolved in 50mM NH_4HCO_3 (30 min, 60 °C, on a shaker at 1200 rpm) and thereafter, alkylated with 25 µL of 70 mM iodoacetamide dissolved in 50mM NH_4HCO_3 (30 min, RT, on a shaker at 600 rpm, in dark). Samples were then digested with 25 µL trypsin/Lys-C mix (Promega Corp., Madison, WI, USA) dissolved in 50 mM NH_4HCO_3 to a concentration of 20 µg/mL (15 h, 37 °C, on a shaker at 1200 rpm) and this reaction was stopped by adding 25 µL of 10% trifluoroacetic acid (v/v). The digests were desalted by solid phase extraction using Oasis hydrophilic-lipophilic balance (HLB, 2 mg sorbent, 30 µm particle size, Waters Co., Milford, MA, USA) 96-well µElution plates. The samples were eluted using

100% methanol. Samples were then dried down in a vacuum centrifuge and stored at -20 °C.

Prior to LC-MS analysis, the samples were reconstituted in 100 µL 50 mM NH_4HCO_3. Each sample (50 µL) was loaded onto a Hypersil Gold reversed phase HPLC C18 column (Thermo Fisher Scientific Inc.). Separation was performed at a flow rate of 300 µL/min with a gradient from 0 to 40% B over 20 min using a Vanquish UHPLC (Thermo Fisher Scientific Inc.). The total cycle was 30 min. The parallel reaction monitoring (PRM) assay was performed using the Q Exactive hybrid quadrupole-orbitrap high resolution mass spectrometer (Thermo Fisher Scientific Inc.), with electrospray ionization, operated as described previously [38] with minor modifications. Briefly, fragment mass spectra were acquired by scheduled parallel reaction monitoring (PRM) with retention time windows of 30 s for each peptide, with the following acquisition parameters: resolution setting of 35,000, 1 microscan, target values 3×10^6, and maximum injection time 125 ms. Isolation width was 3 *m/z* units and normalized collision energies were optimized manually for each peptide (see Supplementary table 1 for values).

Statistical analyses

The differences between the groups were investigated using Kruskal-Wallis test with Dunn's multiple comparisons. Area under the curve (AUC) from receiver operating characteristic (ROC) analysis was used as a measure of the effect size. The correlation coefficients (rho) were calculated using the Spearman's rank correlation. The analyses were performed using SPSS software, version 25 for Windows (IBM Corp., Armonk, NY, USA) or GraphPad Prism, version 7 for Windows (GraphPad Inc., La Jolla, CA, USA). All tests were two-sided with a significance threshold set to $p \leq 0.05$.

Results

Validation

The repeatability of the brevican and neurocan peptides in quality control CSF samples ranged from 1-19% for both cohorts, except B834, N145 and N1242 peptides, which showed a high variability (CV>20%) and, thus, were excluded from further analysis.

The majority of brevican (except B87) and neurocan (except N582) peptides in CSF showed analytical stability for up to five freeze-thaw cycles (Supplementary fig. 1 and 2).

All brevican and neurocan peptides in CSF showed storage stability upon tested conditions (Supplementary fig. 3 and 4).

The relative error of the back-calculated concentrations were below 20% for all calibrators, except for N1195 (Supplementary fig. 5 and 6).

Exploratory analysis

Tryptic, but not intact endogenous brevican peptides, were detected in CSF. N-terminal brevican fragments (aa 23 - 429) were detected in IP-purified CSF using the N-terminal antibody, while C-terminal brevican fragments (aa 718 - 841) were detected in CSF using the C-terminal antibody (Fig. 1A).

IP of full-length brevican standard using either N-terminal or C-terminal antibody resulted in the detection of peptides spanning the whole brevican sequence (Fig. 1B).

Brevican/neurocan panel – cohort 1

For the CSF concentrations of brevican and neurocan peptides in cohort 1 see

Supplementary table 2.

In the first cohort, the CSF concentrations of all brevican (Fig. 2) and neurocan (Fig. 3) peptides were significantly decreased in VaD compared to AD patients (AUC=0.83-0.93, p≤0.05). Additionally, CSF concentrations of five brevican peptides (B156, B268, B313, B322, B741) and all neurocan peptides were significantly decreased in VaD when compared to control group (AUC=0.79-0.87, p≤0.05) (Fig. 2 and 3). There was no difference in CSF concentrations of neither brevican nor neurocan peptides between AD patients and control group (Figs. 2 and 3).

All the brevican and neurocan peptides significantly correlated to each other in control (rho=0.58-0.99, p≤0.05) and AD (rho=0.37-0.99, p≤0.05) groups (Fig. 6). In the VaD group, N-terminal brevican peptides were strongly correlated with each other (rho=0.69-0.99, p≤0.05), while C-terminal brevican peptides did not correlate neither to each other nor to N-terminal part of the protein (Fig. 6). Neurocan peptides also showed decreased correlation between each other in VaD group. The most N-terminal neurocan did not correlate with several other neurocan peptides (N257, N316, N582, N1155) (Fig. 6). Additionally, middle part of neurocan (N316, N582) did not correlate with the most C-terminal neurocan peptides (N1195, N1234) (Fig. 6).

There was no association between brevican or neurocan peptides and age, mini-mental state examination (MMSE) or CSF $A\beta_{42}$ (Supplementary table 3). All brevican and neurocan peptides significantly correlated to t-tau and p-tau (rho=0.42-0.64, p≤0.05) (Supplementary table 3). The majority of the peptides were associated with glial fibrillary acidic protein (GFAP) (except B718, B741), neuron-specific enolase (NSE) (except B879) and YKL40 (rho=-0.42-0.49) (Supplementary table 3). Two C-terminal peptides (B718, B741) correlated to CSF/serum albumin ratio (rho=0.29-0.30, p≤0.05) (Supplementary table 3).

Brevican/neurocan panel – cohort 2

For the CSF concentrations of brevican and neurocan peptides in cohort 2 see Supplementary table 4.

In the second cohort, CSF concentrations of two brevican peptides (B87 and B156) were significantly decreased in VaD compared to AD patients (AUC=0.86-0.91, $p \leq 0.05$) and controls (AUC=0.80-0.82, $p \leq 0.05$) (Fig. 4), while other brevican (Fig. 4) and neurocan (Fig. 5) peptides showed clear trend to be decreased in VaD group when compared to AD patients (AUC=0.64-80, $p > 0.05$). There was no difference in CSF concentrations for neither brevican nor neurocan peptides between AD patients and control group (Figs. 4 and 5).

All the brevican and neurocan peptides (except N89) significantly correlated to each other in the control group (rho=0.65-0.99, $p \leq 0.05$) (Fig. 6). In the AD group, N-terminal brevican peptides and all neurocan peptides were associated with each other (rho=0.60-0.99), even though most of these correlations were not significant due to the small sample size (n=5) (Fig. 6). The correlations between C-terminal brevican peptides (rho=0.30-0.90) were lower than in the N-terminal part of the protein (rho=0.90-0.99) (Fig. 6). In VaD group, the majority of brevican peptides were not associated with each other in contrast to neurocan peptides which, except N316, correlated with each other (rho=0.58-0.99, $p \leq 0.05$) (Fig. 6).

There was no or very weak association between brevican or neurocan peptides and age, MMSE or CSF $A\beta_{42}$ (rho=-0.16-0.43) (Supplementary table 5).

Most of the brevican peptides, except B87, correlated with p-tau (rho=0.38-0.78, $p \leq 0.05$), while only the most C-terminal brevican peptide (B879) showed association with t-tau (rho=0.70, $p \leq 0.05$) (Supplementary table 5). The neurocan peptides

correlated with both p-tau (all) and t-tau (except N316, N582) (rho=0.45-0.70) (Supplementary table 5). Some of the peptides (B87, B156, B313, B741, N316 and N582) were associated with CSF/serum albumin ratio (Supplementary table 5).

Discussion

Here, degradation profiles of brevican and neurocan proteins in human CSF are presented. The main findings of this study are that CSF brevican and neurocan peptide concentrations may represent potential novel diagnostic biomarkers to differentiate VaD from AD.

The detection of separate pools of N- and C-terminal brevican proteolytic peptides in CSF using N- and C-terminal brevican antibodies, respectively, indicate the presence of endogenous cleavage of brevican in CNS. The inability to detect non-tryptic brevican compounds in CSF samples is likely because the captured brevican variants in CSF were too long to be detected by MS at the present levels.

To date, there is no specific biochemical marker to distinguish between AD and VaD patients. The differentiation between AD and VaD is of a great importance since the treatment for these diseases will differ.

In this study, brevican and neurocan proteolytic peptides present a strong potential to provide a distinction between AD and VaD. This finding was observed in two independent cohorts. The first cohort showed significant differences between the AD and VaD groups for all the brevican and neurocan peptides, confirmed by the second cohort that showed a trend for these peptides to be decreased in the VaD group. Even though most of the differences in the second cohort were not statistically significant, the substantial effect size (AUC=0.64-0.91) confirms that brevican and neurocan peptides have discrimination capacity to distinguish between AD and VaD. The lack of

the significances in the second cohort is most probably caused by the low number of AD patients (n=5).

The decrease of CSF concentrations of brevican and neurocan peptides in the VaD group compared to controls and AD patients, indicate that brevican/neurocan degradation is enhanced in VaD. Increased levels of several MMPs in brain tissues from VaD patients have been previously observed [39], which might explain the decreased CSF concentrations of MMP's substrates, brevican and neurocan peptides, in VaD.

The lower correlations of brevican and neurocan peptides in VaD group compared to controls and AD patients, indicate that brevican/neurocan proteolytic processing is dysregulated in VaD. Different proteolytic processing along the brevican and neurocan protein cores in VaD might reveal imbalance between the synthesis and degradation of different parts of brevican and neurocan in this disease. It might be explained by possible differential activity of various extracellular enzymes along the brevican/neurocan protein core. For instance, CSF MMP-9, but not MMP-2, -3, -10, concentrations were previously observed to be increased in VaD compared to AD patients [40, 41]. However, even though one study showed that individual MMPs might cleave brevican at different sites [24], the proteolytic processing of neurocan is largely understudied. The fact that most of the brevican and neurocan peptides correlated to each other in the AD and control groups, but not in the VaD group, might also indicate that control and AD diagnosis are more homogenous, while VaD includes a number of different subtypes [7, 8]. Substantial decrease of correlations between C-terminal brevican peptides in the VaD group compared to control and AD groups indicate that the increased processing of brevican is mostly located in the C-terminal part of this protein. The decrease of correlations between neurocan peptides in VaD cohorts were

observed throughout the protein, indicating that there are several endogenous cleavages along the neurocan core in CNS.

Since none of the CSF brevican/neurocan peptide concentrations in AD differed compared to the control group, these proteolytic peptides do not reflect AD-pathology in CSF. High correlations between brevican/neurocan peptides in control and AD groups indicate that there is little AD pathology driven proteolytic processing of these proteins in CNS. In addition, brevican and neurocan are not linked to cognitive status as determined by MMSE test scores.

The correlation of the majority of brevican and neurocan peptides to CSF t-tau, p-tau and YKL40 suggest that they might be related to the neuronal injury, tangle pathology and neuroinflammation, respectively. The significant correlations of several brevican and neurocan peptides to CSF/serum albumin ratio indicate that they might be also related to blood brain barrier (BBB) dysfunction, which is a core feature of VaD pathophysiology [42]. MMPs, which cleave brevican and neurocan [23, 24, 28], degrade tight junctions between endothelial cells in BBB. This suggests that the brevican and neurocan processing might be implicated in BBB breakdown.

The lack of correlation of C-terminal brevican peptides to GFAP or NSE, in contrast to other brevican and neurocan peptides, suggest differential pathophysiological role of these peptides.

The strength of the study includes well-characterized, age and gender matched, populations in both cohorts. The multi-centre setting involving two independent cohorts allowed for demonstration of reproducibility of the findings.

However, there are some limitations to the study that should be considered, including lack of explorative IP-based data for neurocan protein, low number (n=5) of AD patients in the second cohort and lack of clinical information regarding the specific subtypes of

VaD.

In conclusion, this study demonstrates that CSF brevican and neurocan peptides concentrations have potential to become a complementary tool in the differentiation of VaD from AD.

Author's contributions

KM, GB, HZ, KB, and UA created the concept of the study. PJ, JS, AW and PK recruited subjects and acquired data. KM, GB and UA developed and evaluated the mass spectrometry method. KM carried out the experiments, statistical analysis and drafted the manuscript. HZ, KB, KM, GB, EP, UA, PJ, JS, AW and PK contributed to the interpretation of the results and provided critical feedback of the manuscript. All authors read and approved the final manuscript.

Funding

KM acknowledges funding from Stiftelsen för Gamla Tjänarinnor, Herbert och Karin Jacobssons Stiftelse and Gun och Bertil Stohnes Stiftelse. HZ is a Wallenberg Scholar supported by grants from the Swedish Research Council (#2018-02532), the European Research Council (#681712), Swedish State Support for Clinical Research (#ALFGBG-720931), the Alzheimer Drug Discovery Foundation (ADDF), USA (#201809-2016862), and the UK Dementia Research Institute at UCL. KB is supported by the Swedish Research Council (#2017-00915), the Alzheimer Drug Discovery Foundation (ADDF), USA (#RDAPB-201809-2016615), the Swedish Alzheimer Foundation (#AF-742881), Hjärnfonden, Sweden (#FO2017-0243), the Swedish state under the agreement between the Swedish government and the County Councils, the ALF-agreement (#ALFGBG-715986), and European Union Joint Program for

Neurodegenerative Disorders (JPND2019-466-236).

Conflicts of interest

HZ has served at scientific advisory boards for Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics and CogRx, has given lectures in symposia sponsored by Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work). KB has served as a consultant or at advisory boards for Abcam, Axon, Biogen, Lilly, MagQu, Novartis and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work). KM, GB, EP, PJ, JS, AW, PK and UA declare that they have no competing interests.

References

1. Blennow K, de Leon MJ, Zetterberg H. Alzheimer's disease. Lancet. 2006;368(9533):387-403.

2. Yiannopoulou KG, Papageorgiou SG. Current and future treatments for Alzheimer's disease. Ther Adv Neurol Disord. 2013;6(1):19-33.

3. Gauthier S, Reisberg B, Zaudig M, Petersen RC, Ritchie K, Broich K, et al. Mild cognitive impairment. Lancet. 2006;367(9518):1262-70.

4. Canevelli M, Grande G, Lacorte E, Quarchioni E, Cesari M, Mariani C, et al. Spontaneous Reversion of Mild Cognitive Impairment to Normal Cognition: A Systematic Review of Literature and Meta-Analysis. J Am Med Dir Assoc. 2016;17(10):943-8.

5. Jorm AF, Jolley D. The incidence of dementia: a meta-analysis.

Neurology. 1998;51(3):728-33.

6. Venkat P, Chopp M, Chen J. Models and mechanisms of vascular dementia. Exp Neurol. 2015;272:97-108.

7. O'Brien JT, Thomas A. Vascular dementia. Lancet. 2015;386(10004):1698-706.

8. Wallin A, Blennow K. Heterogeneity of vascular dementia: mechanisms and subgroups. J Geriatr Psychiatry Neurol. 1993;6(3):177-88.

9. Attems J, Jellinger KA. The overlap between vascular disease and Alzheimer's disease--lessons from pathology. BMC Med. 2014;12:206.

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

11. Reiber H. Proteins in cerebrospinal fluid and blood: barriers, CSF flow rate and source-related dynamics. Restor Neurol Neurosci. 2003;21(3-4):79-96.

12. Jack CR, Jr., Bennett DA, Blennow K, Carrillo MC, Dunn B, Haeberlein SB, et al. NIA-AA Research Framework: Toward a biological definition of Alzheimer's disease. Alzheimers Dement. 2018;14(4):535-62.

13. Dubois B, Feldman HH, Jacova C, Hampel H, Molinuevo JL, Blennow K, et al. Advancing research diagnostic criteria for Alzheimer's disease: the IWG-2 criteria. Lancet Neurol. 2014;13(6):614-29.

14. Kalaria RN, Ballard C. Overlap between pathology of Alzheimer disease and vascular dementia. Alzheimer Dis Assoc Disord. 1999;13 Suppl 3:S115-23.

15. Paraskevas GP, Kapaki E, Papageorgiou SG, Kalfakis N, Andreadou E, Zalonis I, et al. CSF biomarker profile and diagnostic value in vascular dementia. Eur J Neurol. 2009;16(2):205-11.

16. Yamaguchi Y. Lecticans: organizers of the brain extracellular matrix. Cell

Mol Life Sci. 2000;57(2):276-89.

17. Frischknecht R, Seidenbecher CI. Brevican: a key proteoglycan in the perisynaptic extracellular matrix of the brain. Int J Biochem Cell Biol. 2012;44(7):1051-4.

18. Carulli D, Rhodes KE, Brown DJ, Bonnert TP, Pollack SJ, Oliver K, et al. Composition of perineuronal nets in the adult rat cerebellum and the cellular origin of their components. J Comp Neurol. 2006;494(4):559-77.

19. Cox TR, Erler JT. Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. Dis Model Mech. 2011;4(2):165-78.

20. Yamada H, Watanabe K, Shimonaka M, Yamaguchi Y. Molecular cloning of brevican, a novel brain proteoglycan of the aggrecan/versican family. J Biol Chem. 1994;269(13):10119-26.

21. Matthews RT, Gary SC, Zerillo C, Pratta M, Solomon K, Arner EC, et al. Brain-enriched hyaluronan binding (BEHAB)/brevican cleavage in a glioma cell line is mediated by a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family member. J Biol Chem. 2000;275(30):22695-703.

22. Mayer J, Hamel MG, Gottschall PE. Evidence for proteolytic cleavage of brevican by the ADAMTSs in the dentate gyrus after excitotoxic lesion of the mouse entorhinal cortex. BMC Neurosci. 2005;6:52.

23. Gottschall PE, Howell MD. ADAMTS expression and function in central nervous system injury and disorders. Matrix Biol. 2015;44-46:70-6.

24. Nakamura H, Fujii Y, Inoki I, Sugimoto K, Tanzawa K, Matsuki H, et al. Brevican is degraded by matrix metalloproteinases and aggrecanase-1 (ADAMTS4) at different sites. J Biol Chem. 2000;275(49):38885-90.

25. Asher RA, Morgenstern DA, Fidler PS, Adcock KH, Oohira A, Braistead

JE, et al. Neurocan is upregulated in injured brain and in cytokine-treated astrocytes. J Neurosci. 2000;20(7):2427-38.

26. Matsui F, Watanabe E, Oohira A. Immunological identification of two proteoglycan fragments derived from neurocan, a brain-specific chondroitin sulfate proteoglycan. Neurochem Int. 1994;25(5):425-31.

27. Fontanil T, Mohamedi Y, Moncada-Pazos A, Cobo T, Vega JA, Cobo JL, et al. Neurocan is a New Substrate for the ADAMTS12 Metalloprotease: Potential Implications in Neuropathies. Cell Physiol Biochem. 2019;52(5):1003-16.

28. Turk BE, Huang LL, Piro ET, Cantley LC. Determination of protease cleavage site motifs using mixture-based oriented peptide libraries. Nat Biotechnol. 2001;19(7):661-7.

29. Miyata S, Kitagawa H. Formation and remodeling of the brain extracellular matrix in neural plasticity: Roles of chondroitin sulfate and hyaluronan. Biochim Biophys Acta Gen Subj. 2017;1861(10):2420-34.

30. Minta K, Portelius E, Janelidze S, Hansson O, Zetterberg H, Blennow K, et al. Cerebrospinal Fluid Concentrations of Extracellular Matrix Proteins in Alzheimer's Disease. J Alzheimers Dis. 2019;69(4):1213-20.

31. Johansson P, Mattsson N, Hansson O, Wallin A, Johansson JO, Andreasson U, et al. Cerebrospinal fluid biomarkers for Alzheimer's disease: diagnostic performance in a homogeneous mono-center population. J Alzheimers Dis. 2011;24(3):537-46.

32. Runeson BS, Rich CL. Diagnostic and statistical manual of mental disorders, 3rd ed. (DSM-III), adaptive functioning in young Swedish suicides. Ann Clin Psychiatry. 1994;6(3):181-3.

33. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM.

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):939-44.

34. Petersen RC. Mild cognitive impairment as a diagnostic entity. J Intern Med. 2004;256(3):183-94.

35. Roman GC, Tatemichi TK, Erkinjuntti T, Cummings JL, Masdeu JC, Garcia JH, et al. Vascular dementia: diagnostic criteria for research studies. Report of the NINDS-AIREN International Workshop. Neurology. 1993;43(2):250-60.

36. Erkinjuntti T, Inzitari D, Pantoni L, Wallin A, Scheltens P, Rockwood K, et al. Research criteria for subcortical vascular dementia in clinical trials. J Neural Transm Suppl. 2000;59:23-30.

37. Brinkmalm G, Portelius E, Ohrfelt A, Mattsson N, Persson R, Gustavsson MK, et al. An online nano-LC-ESI-FTICR-MS method for comprehensive characterization of endogenous fragments from amyloid beta and amyloid precursor protein in human and cat cerebrospinal fluid. J Mass Spectrom. 2012;47(5):591-603.

38. Brinkmalm G, Sjodin S, Simonsen AH, Hasselbalch SG, Zetterberg H, Brinkmalm A, et al. A Parallel Reaction Monitoring Mass Spectrometric Method for Analysis of Potential CSF Biomarkers for Alzheimer's Disease. Proteomics Clin Appl. 2018;12(1).

39. Rosenberg GA, Sullivan N, Esiri MM. White matter damage is associated with matrix metalloproteinases in vascular dementia. Stroke. 2001;32(5):1162-8.

40. Bjerke M, Zetterberg H, Edman A, Blennow K, Wallin A, Andreasson U. Cerebrospinal fluid matrix metalloproteinases and tissue inhibitor of metalloproteinases in combination with subcortical and cortical biomarkers in vascular dementia and Alzheimer's disease. J Alzheimers Dis. 2011;27(3):665-76.

41. Adair JC, Charlie J, Dencoff JE, Kaye JA, Quinn JF, Camicioli RM, et al. Measurement of gelatinase B (MMP-9) in the cerebrospinal fluid of patients with vascular dementia and Alzheimer disease. Stroke. 2004;35(6):e159-62.

42. Farrall AJ, Wardlaw JM. Blood-brain barrier: ageing and microvascular disease--systematic review and meta-analysis. Neurobiol Aging. 2009;30(3):337-52.

Table 1. Demographics of study population in cohort 1.

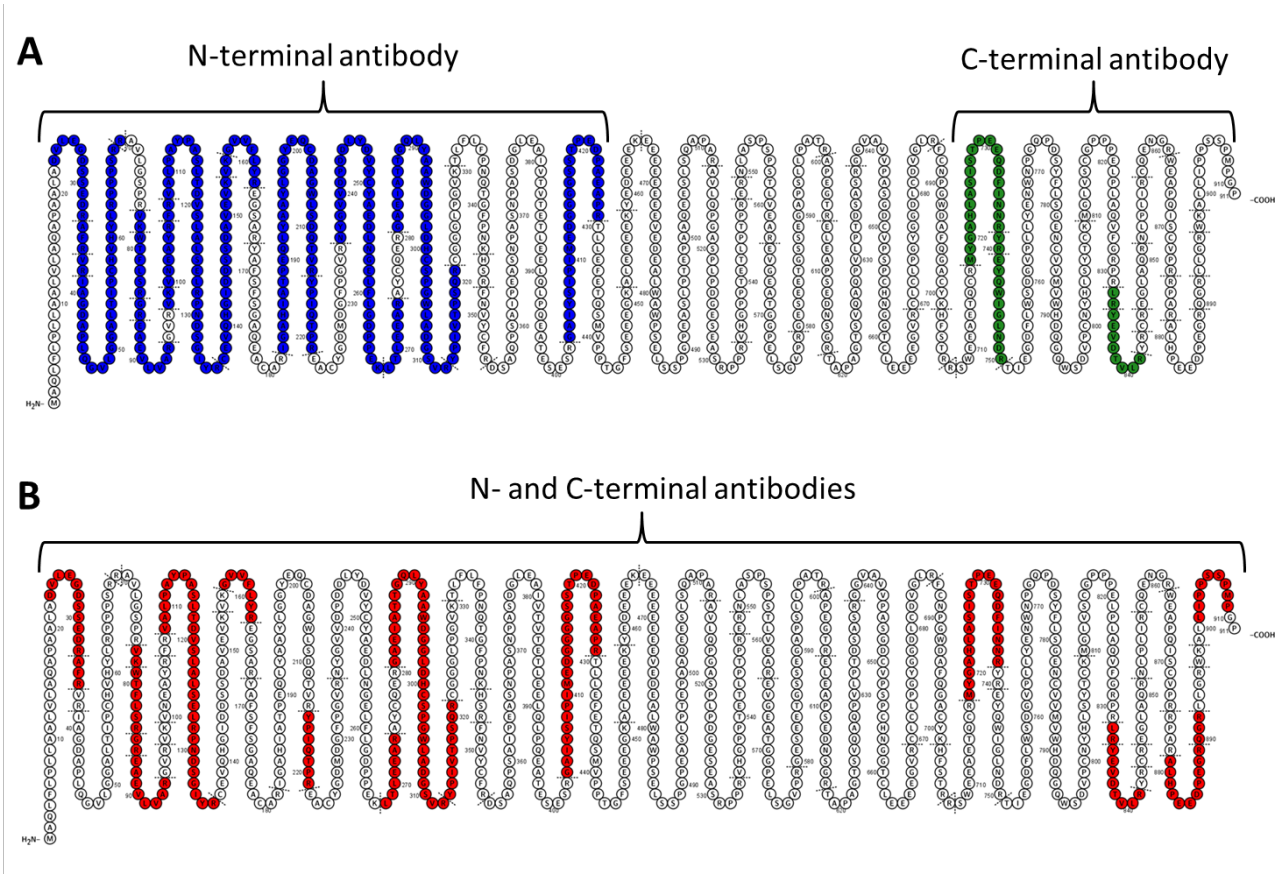
Group	**Diagnosis**	**n**	**Gender** M:F	**Age** median (quartile range)
AD	AD	18	8:10	73 (70-76)
	AD w vasc*	7	3:4	77 (77-78)
	MCI-AD	6	3:3	72 (71-74)
	MCI-AD w vasc*	1	1:0	71
		32	15:17	75 (71-77)
VaD	VaD	7	5:2	75 (72-77)
	MCI-VaD	3	2:1	74 (73-77)
		10	7:3	75 (72-77)
Controls	Controls	20	10:10	75 71-78)
	Stable MCI	13	5:8	72 (69-73)
		33	15:18	73 (70-76)
TOTAL		75	37:38	73 (71-77)

*AD with vascular components according to brain imaging

Table 2. Demographics of study population in cohort 2.

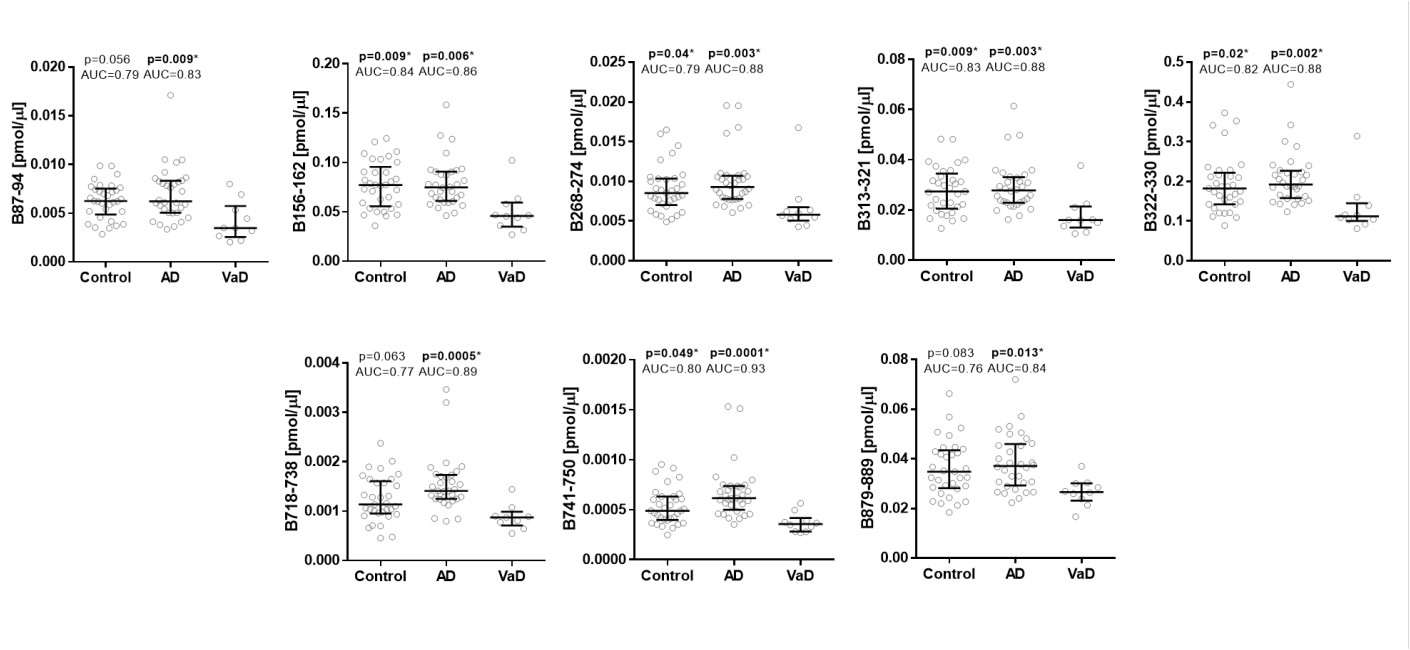
Diagnosis	**n**	**Gender** M:F	**Age** median (quartile range)
AD	5	2:3	71 (71-75)
VaD	14	9:5	70 (67-75)
Controls	12	5:7	68 (64-72)
TOTAL	31	14:17	70 (65-76)

Figure 1. Exploratory IP-based analysis of CSF (A) and full length standard protein (B).



In CSF (A), N-terminal brevican antibody captured only N-terminal brevican fragments (aa 23 - 429), while C-terminal brevican antibody only C-terminal brevican fragments (aa 718 - 841). For full-length brevican standard (B), N-terminal or C-terminal antibody captured peptides covering the whole brevican sequence.

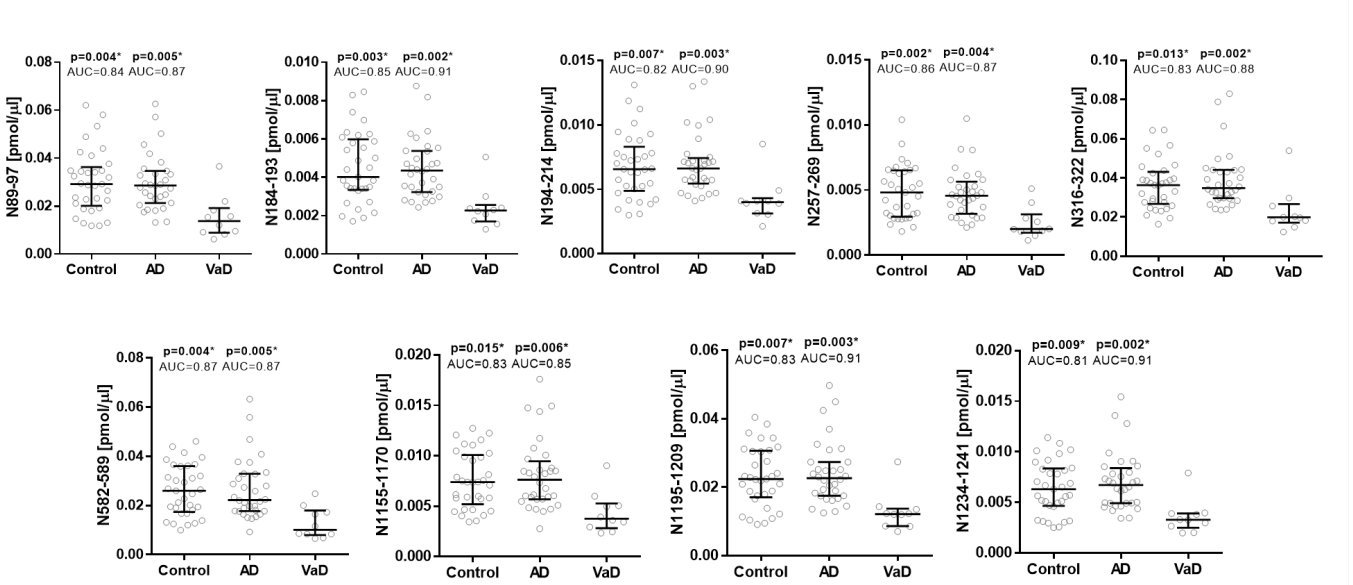
Figure 2. CSF concentrations in cohort 1 for the brevican N-terminal (upper five panels) and C-terminal (lower three panels) peptides in controls and Alzheimer's disease (AD) patients compared to vascular dementia (VaD) patients.



Statistical significance and effect size are in comparison to VaD group.

Abbreviations: Alzheimer's disease (AD), area under the curve (AUC), vascular dementia (VaD)

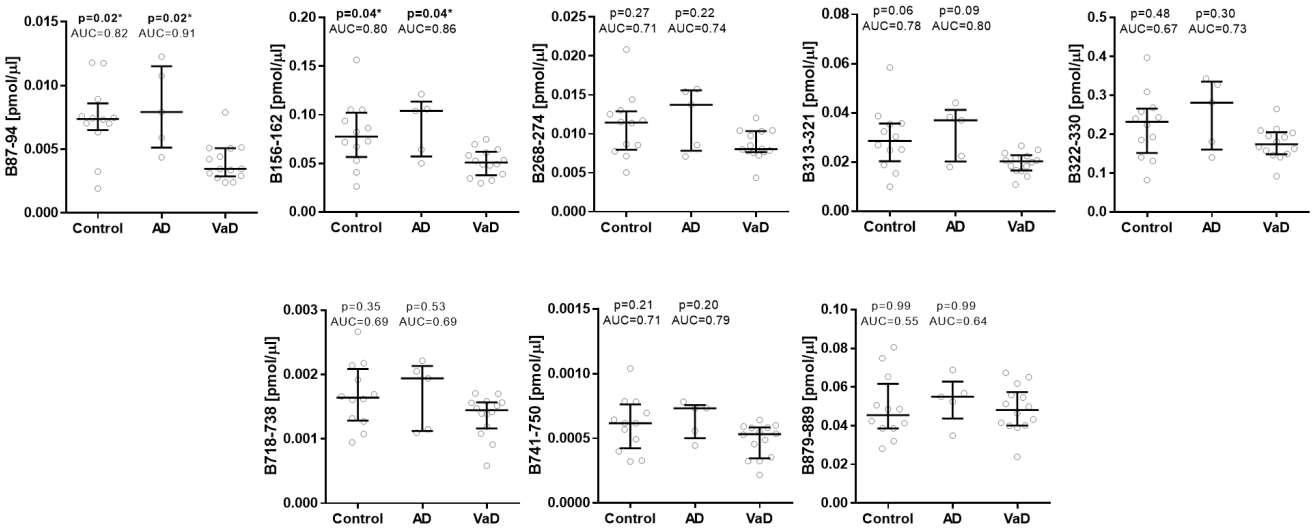
Figure 3. CSF concentrations in cohort 1 for the neurocan N-terminal (upper five panels) and C-terminal (lower four panels) peptides in controls and Alzheimer's disease (AD) patients compared to vascular dementia (VaD) patients.



Statistical significance and effect size are in comparison to VaD group.

Abbreviations: Alzheimer's disease (AD), area under the curve (AUC), vascular dementia (VaD)

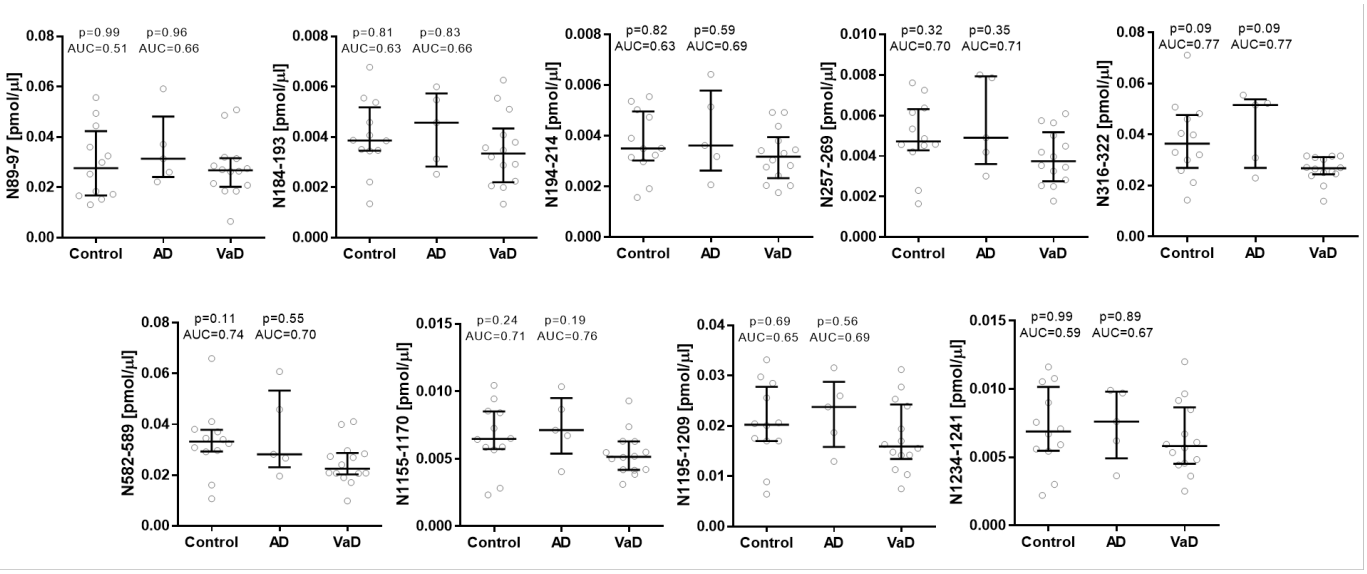
Figure 4. CSF concentrations in cohort 2 for the brevican N-terminal (upper five panel) and C-terminal (lower three panel) peptides in controls and Alzheimer's disease (AD) patients compared to vascular dementia (VaD) patients.



Statistical significance and effect size are in comparison to VaD group.

Abbreviations: Alzheimer's disease (AD), area under the curve (AUC), vascular dementia (VaD)

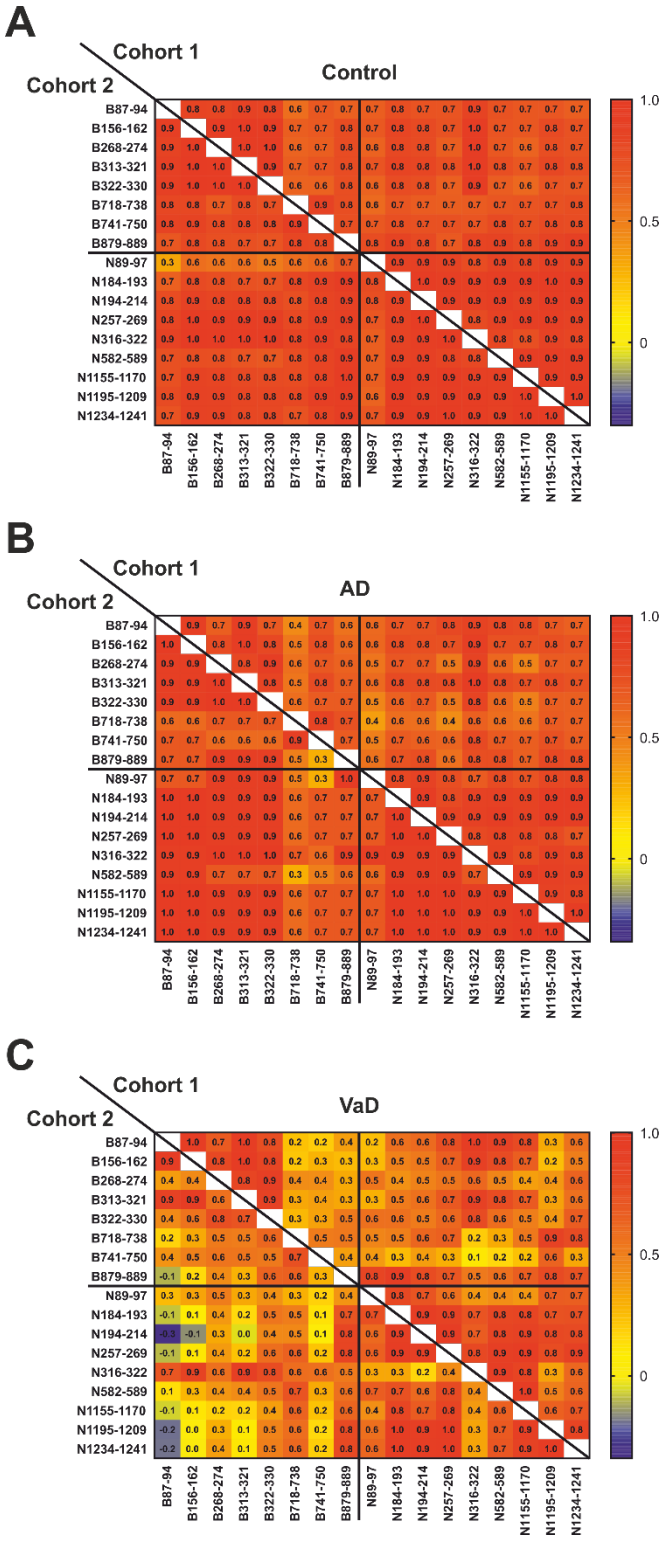
Figure 5. CSF concentrations in cohort 2 for the neurocan N-terminal (upper five panels) and C-terminal (lower four panel) peptides in controls and Alzheimer's disease (AD) patients compared to vascular dementia (VaD) patients.



Statistical significance and effect size are in comparison to VaD group.

Abbreviations: Alzheimer's disease (AD), area under the curve (AUC), vascular dementia (VaD)

Figure 6. Correlation matrix between CSF concentrations of brevican and neurocan peptides in control (A), AD (B) and VaD (C) groups in cohorts 1 and 2.



The correlation coefficients are presented as Spearman's rho.

Supplementary table 1. Brevican and neurocan peptides used in the PRM-MS assay with acquisition characteristics.

Name	Position	Sequence	m/z[a]	Charge, z	NCE[b]
B87	87-94	EAEVLVAR	443.75	2	15
B156	156-162	GVVFLYR	427.25	2	15
B268	268-274	LTLEEAR	416.23	2	15
B313	313-321	YPIVTPSQR	530.79	2	22
B322	322-330	CGGGGLPGVK	422.72	2	22
B718	718-738	MYGAHLASISTPEEQDFINNR	798.38	2	22
B741	741-750	EYQWIGLNDR	647.32	2	15
B834	834-841	YEVDTVLR	497.76	2	20
B879	879-889	ALHPEEDPEGR	625.29	2	24
N89	89-97	QDLPILVAK	498.81	2	18
N145	145-165	GIEDEQDLVPLEVTGVVFHYR	805.75	3	18
N184	184-193	LSSAIIAAPR	499.80	2	15
N194	194-214	HLQAAFEDGFDNCDAGWLSDR	808.68	3	15
N257	257-269	ELGGEVFYVGPAR	697.36	2	18
N316	316-322	YPIQTPR	437.74	2	22
N582	582-589	APVLELEK	449.77	2	18
N1155	1155-1170	DFQWTDNTGLQFENWR	1028.9	2	22
N1195	1195-1209	WNDVPCNYNLPYVCK	971.44	2	28
N1234	1234-1241	YNVHATVR	480.26	2	20
N1242	1242-1257	YQCNEGFAQHHVATIR	644.30	3	18

[a] – precursor m/z of the endogenous peptide
[b] – normalized collision energy

Supplementary table 2. The CSF concentrations of brevican and neurocan proteolytic peptides across the diagnostic groups in cohort 1.

Peptide [fmol/µL]	Diagnosis		
	AD	VaD	Controls
B87	6.22 (5.05-8.31)	3.48 (2.80-5.11)	6.24 (5.17-7.43)
B156	75.0 (62.0-90.6)	45.9 (38.1-55.3)	77.2 (57.6-90.7)
B268	9.27 (7.82-10.7)	5.82 (5.32-6.35)	8.51 (7.18-10.3)
B313	27.8 (23.0-32.9)	16.0 (13.9-19.8)	27.4 (21.3-22.5)
B322	192 (161-225)	112 (104-134)	162 (142-216)
B718	1.41 (1.25-1.72)	0.870 (0.776-0.898)	1.13 (0.960-1.57)
B741	0.615 (0.531-0.737)	0.358 (0.295-0.387)	0.490 0.402-0.632)
B879	37.1 (30.0-45.5)	26.6 (24.4-29.7)	34.8 (28.5-42.9)
N89	28.6 (22.2-34.1)	13.7 (9.21-17.6)	29.2 (21.1-34.8)
N184	4.36 (3.25-5.36)	2.27 (1.83-2.40)	4.01 (3.40-5.88)
N194	6.62 (5.58-7.30)	3.99 (3.33-4.09)	6.56 (5.03-7.80)
N257	4.55 (3.36-5.40)	2.01 (1.82-2.76)	4.81 (3.03-6.48)
N316	34.8 (29.7-43.9)	19.8 (18.0-24.3)	36.3 (27.6-42.4)
N582	22.3 (17.7-32.8)	10.1 (8.34-17.0)	25.9 (18.1-35.8)
N1155	7.63 (5.70-9.01)	3.77 (3.06-5.03)	7.39 (5.73-9.90)
N1195	22.6 (17.5-27.3)	12.1 (9.46-13.2)	22.4 (17.5-30.5)
N1234	6.71 (4.92-8.11)	3.28 (2.73-3.87)	6.30 (4.71-8.29)

Data are presented as median with interquartile ranges.

Supplementary table 3. Correlation matrix between CSF concentrations of brevican and neurocan peptides and other biomarkers in cohort 1.

The correlation coefficients are represented as Spearman's rho.

Abbreviations: amyloid-β (Aβ), cerebrospinal fluid (CSF), glial fibrillary acidic protein (GFAP), mini-mental state examination (MMSE), neuron-specific enolase (NSE), phosphorylated tau (p-tau), total tau (t-tau)

The significant rho followed by p-values are marked in bold.

		B87	B156	B268	B313	B322	B718	B741	B879	N89	N184	N194	N257	N316	N582	N1155	N1195	N1234
age	rho	-0.193	-0.149	-0.111	-0.150	-0.112	0.046	0.002	0.049	-0.083	-0.123	-0.101	-0.126	-0.118	-0.104	-0.130	-0.060	-0.044
	p	0.097	0.202	0.342	0.200	0.339	0.697	0.986	0.679	0.481	0.291	0.387	0.281	0.312	0.373	0.265	0.609	0.710
MMSE	rho	0.007	-0.018	-0.091	-0.020	-0.093	-0.241	-0.220	-0.072	0.078	0.032	0.039	0.119	-0.014	0.112	0.015	0.017	-0.014
	p	0.954	0.882	0.463	0.870	0.453	0.051	0.072	0.560	0.532	0.793	0.752	0.335	0.913	0.363	0.905	0.889	0.911
CSF/serum albumin ratio	rho	0.165	0.173	0.150	0.177	0.154	**0.290***	**0.298***	0.165	-0.023	0.042	0.057	0.100	0.145	0.095	0.066	0.114	0.115
	p	0.173	0.153	0.216	0.144	0.202	**0.017**	**0.012**	0.172	0.854	0.729	0.637	0.410	0.230	0.434	0.587	0.348	0.341
Aβ42	rho	0.121	0.215	0.103	0.175	0.139	-0.060	-0.113	0.135	0.100	0.110	0.077	0.125	0.137	0.155	0.086	0.098	0.077
	p	0.301	0.064	0.378	0.134	0.233	0.614	0.336	0.248	0.395	0.347	0.510	0.287	0.240	0.184	0.462	0.401	0.511
t-tau	rho	**0.439****	**0.421****	**0.481****	**0.433****	**0.475****	**0.568****	**0.610****	**0.551****	**0.490****	**0.518****	**0.517****	**0.474****	**0.484****	**0.456****	**0.498****	**0.540****	**0.552****
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
p-tau	rho	**0.519****	**0.453****	**0.499****	**0.481****	**0.518****	**0.567****	**0.626****	**0.605****	**0.617****	**0.612****	**0.620****	**0.584****	**0.536****	**0.594****	**0.603****	**0.623****	**0.639****
	p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
GFAP	rho	**-0.336****	**-0.424****	**-0.260***	**-0.401****	**-0.306****	0.052	-0.046	**-0.289***	**-0.270***	**-0.358****	**-0.332****	**-0.375****	**-0.373****	**-0.365****	**-0.360****	**-0.346****	**-0.318****
	p	**0.003**	**0.000**	**0.025**	**0.000**	**0.008**	0.664	0.694	**0.013**	**0.020**	**0.002**	**0.004**	**0.001**	**0.001**	**0.001**	**0.002**	**0.003**	**0.006**
NSE	rho	**0.367****	**0.383****	**0.329****	**0.361****	**0.355****	**0.322****	**0.368****	0.207	**0.324****	**0.335****	**0.330****	**0.414****	**0.309****	**0.290***	**0.373****	**0.343****	**0.362****
	p	**0.001**	**0.001**	**0.004**	**0.001**	**0.002**	**0.006**	**0.001**	0.074	**0.005**	**0.003**	**0.004**	**0.000**	**0.007**	**0.011**	**0.001**	**0.003**	**0.001**
YKL40	rho	**0.312****	**0.387****	**0.416****	**0.393****	**0.410****	**0.363****	**0.333****	**0.476****	**0.390****	**0.478****	**0.489****	**0.393****	**0.393****	**0.391****	**0.401****	**0.469****	**0.468****
	p	**0.008**	**0.001**	**0.000**	**0.001**	**0.000**	**0.002**	**0.004**	**0.000**	**0.001**	**0.000**	**0.000**	**0.001**	**0.001**	**0.001**	**0.000**	**0.000**	**0.000**

Supplementary table 4. The CSF concentrations of brevican and neurocan proteolytic peptides across the diagnostic groups in cohort 2.

Peptide [fmol/µL]	Diagnosis

	AD	VaD	Controls
B87	7.92 (5.90-10.8)	3.50 (2.97-4.92)	7.38 (6.86-7.92)
B156	104 (64.1-106)	50.8 (41.1-60.3)	77.6 (63.7-96.4)
B268	13.7 (8.58-15.4)	8.04 (7.70-10.2)	11.5 (8.36-12.6)
B313	37.0 (22.4-38.3)	20.3 (17.0-22.2)	28.6 (23.3-35.5)
B322	281 (181-328)	174 (151-201)	232 (174-261)
B718	1.94 (1.15-2.05)	1.45 (1.24-1.56)	1.64 (1.31-1.98)
B741	0.732 (0.558-0.734)	0.532 (0.378-0.582)	0.616 (0.469-0.718)
B879	54.9 (52.5-56.8)	48.1 (40.5-55.6)	45.5 (38.6-54.3)
N89	31.3 (26.0-37.1)	26.7 (21.0-30.7)	27.6 (17.1-38.1)
N184	4.57 (3.13-5.47)	3.34 (2.40-4.01)	3.86 (3.46-4.78)
N194	3.62 (3.18-5.15)	3.17 (2.51-3.71)	3.50 (3.11-4.81)
N257	4.91 (4.19-7.86)	3.75 (2.93-4.89)	4.72 (4.44-6.20)
N316	51.5 (30.9-52.2)	26.8 (24.7-30.8)	36.4 (29.0-46.6)
N582	28.2 (26.6-45.8)	22.5 (20.7-28.1)	33.2 (29.6-37.3)
N1155	7.13 (6.72-8.66)	5.15 (4.19-6.08)	6.48 (5.81-8.45)
N1195	23.8 (18.7-26.0)	15.9 (14.2-22.8)	20.3 (17.0-26.3)
N1234	7.61 (6.20-9.71)	5.82 (4.62-8.04)	6.88 (5.55-9.38)

Data are presented as median with interquartile ranges.

Supplementary table 5. Correlation matrix between CSF concentrations of brevican and neurocan peptides and other biomarkers in cohort 2.

	B87	B156	B268	B313	B322	B718	B741	B879	N89	N184	N194	N257	N316	N582	N1155	N1195	N1234

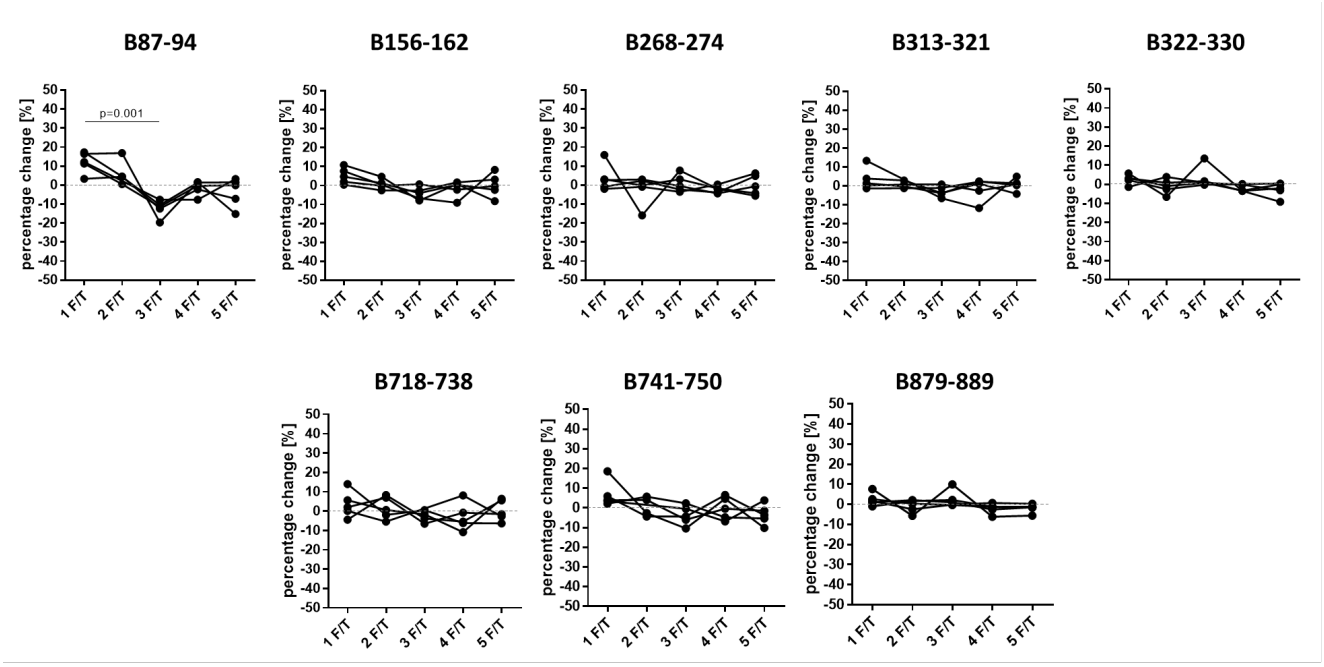
age	rho	-0.132	-0.063	0.047	-0.059	0.075	-0.085	-0.120	**0.432***	**0.417***	0.257	0.285	0.161	0.020	0.009	0.189	0.243	0.264
	p	0.480	0.738	0.802	0.751	0.689	0.649	0.520	**0.015**	**0.020**	0.164	0.120	0.387	0.916	0.961	0.309	0.187	0.152
MMSE	rho	0.117	0.085	0.124	0.107	0.051	0.084	0.058	-0.165	-0.126	0.025	0.053	0.068	0.020	0.189	-0.026	0.006	-0.021
	p	0.530	0.648	0.507	0.567	0.784	0.654	0.758	0.374	0.500	0.894	0.777	0.717	0.914	0.308	0.891	0.976	0.911
CSF/serum albumin ratio	rho	**-0.665****	**-0.565****	-0.316	**-0.501****	-0.304	-0.326	**-0.392***	-0.136	-0.121	-0.157	-0.155	-0.275	**-0.453***	**-0.381***	-0.340	-0.178	-0.124
	p	**0.000**	**0.001**	0.089	**0.005**	0.103	0.079	**0.032**	0.475	0.525	0.408	0.412	0.142	**0.012**	**0.038**	0.066	0.345	0.513
Aβ42	rho	0.279	0.239	0.192	0.279	0.122	0.299	0.183	0.013	0.095	0.015	-0.051	0.028	0.181	0.183	0.041	-0.020	-0.046
	p	0.136	0.204	0.309	0.136	0.522	0.108	0.333	0.944	0.619	0.938	0.790	0.884	0.340	0.334	0.828	0.918	0.811
t-tau	rho	0.133	0.187	0.340	0.188	0.355	0.277	0.275	**0.702****	**0.460***	**0.511****	**0.562****	**0.446***	0.277	0.320	**0.471****	**0.541****	**0.565****
	p	0.485	0.324	0.066	0.321	0.054	0.139	0.142	**0.000**	**0.011**	**0.004**	**0.001**	**0.014**	0.138	0.085	**0.009**	**0.002**	**0.001**
p-tau	rho	0.320	**0.380***	**0.513****	**0.398***	**0.541****	**0.507****	**0.507****	**0.775****	**0.538****	**0.643****	**0.667****	**0.586****	**0.460***	**0.436***	**0.556****	**0.664****	**0.690****
	p	0.090	**0.042**	**0.004**	**0.033**	**0.002**	**0.005**	**0.005**	**0.000**	**0.003**	**0.000**	**0.000**	**0.001**	**0.012**	**0.018**	**0.002**	**0.000**	**0.000**

The correlation coefficients are represented as Spearman's rho.

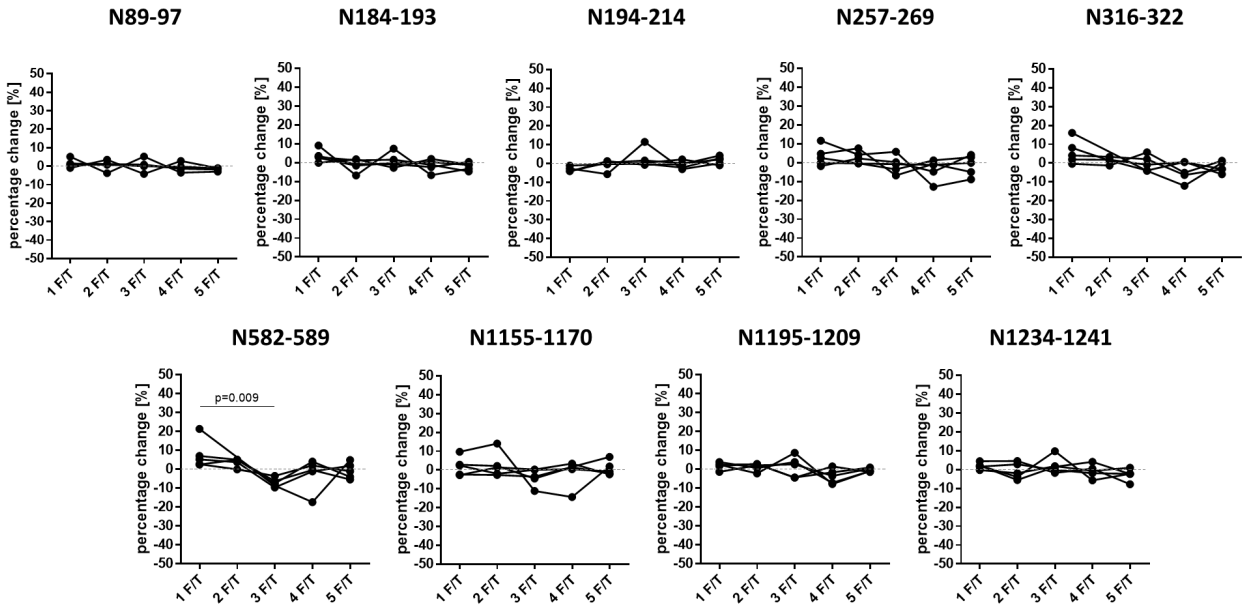
Abbreviations: amyloid-β (Aβ), cerebrospinal fluid (CSF), mini-mental state examination (MMSE), phosphorylated tau (p-tau), total tau (t-tau).

The significant rho followed by p-values are marked in bold.

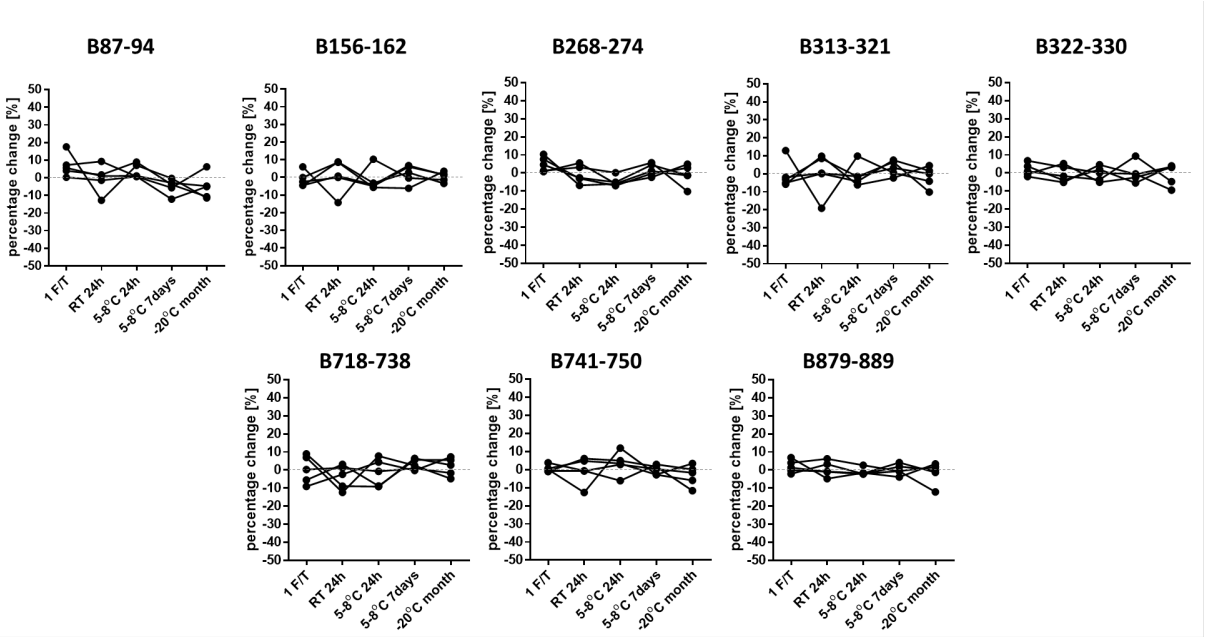
Supplementary figure 1. The effects of freeze/thaw cycles on the stability of brevican peptides in CSF.


B87-94
B156-162
B268-274
B313-321
B322-330
B718-738
B741-750
B879-889
p=0.001
percentage change [%]
50
40
30
20
10
0
-10
-20
-30
-40
-50
1 F/Γ
2 F/Γ
3 F/Γ
4 F/Γ
5 F/Γ

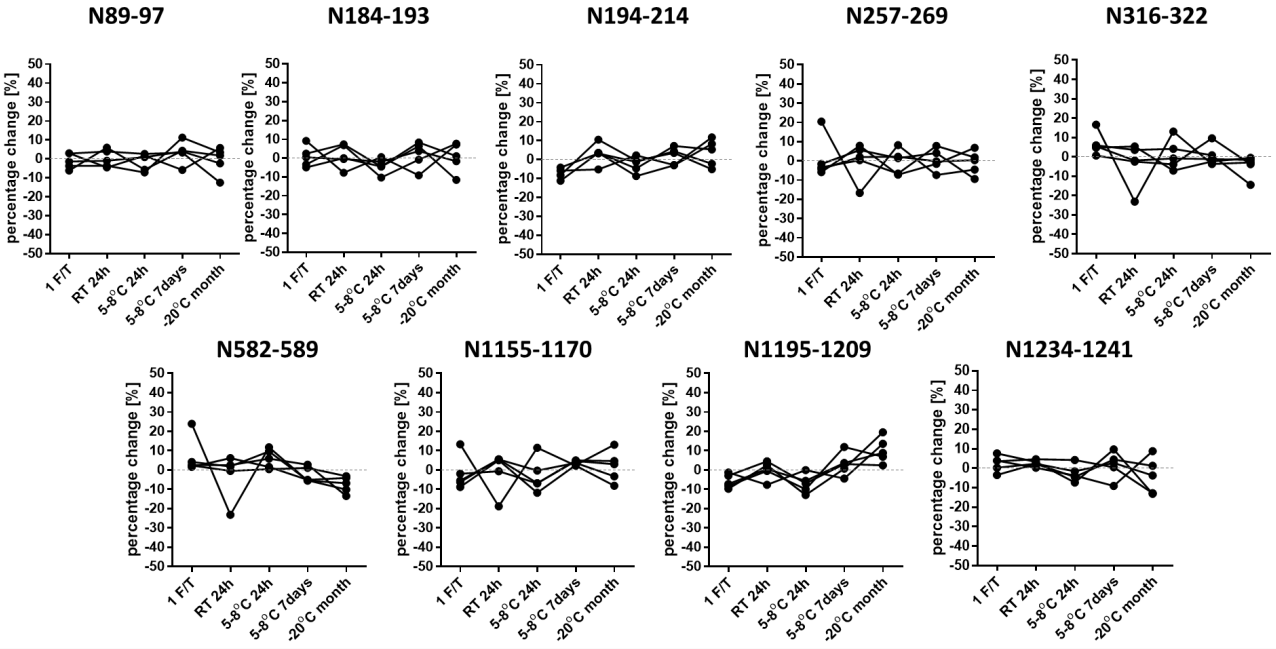
Supplementary figure 2. The effects of freeze/thaw cycles on the stability of neurocan peptides in CSF.



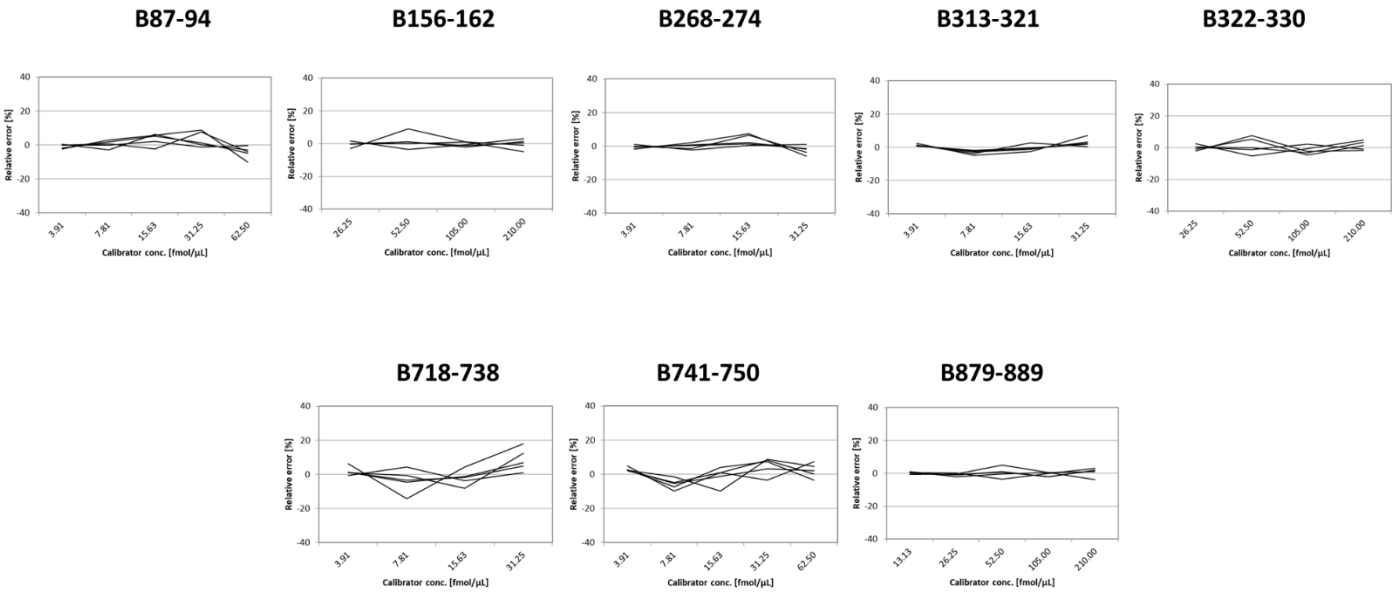
Supplementary figure 3. The effects of storage conditions on the stability of brevican peptides in CSF.



Supplementary figure 4. The effects of storage conditions on the stability of neurocan peptides in CSF.

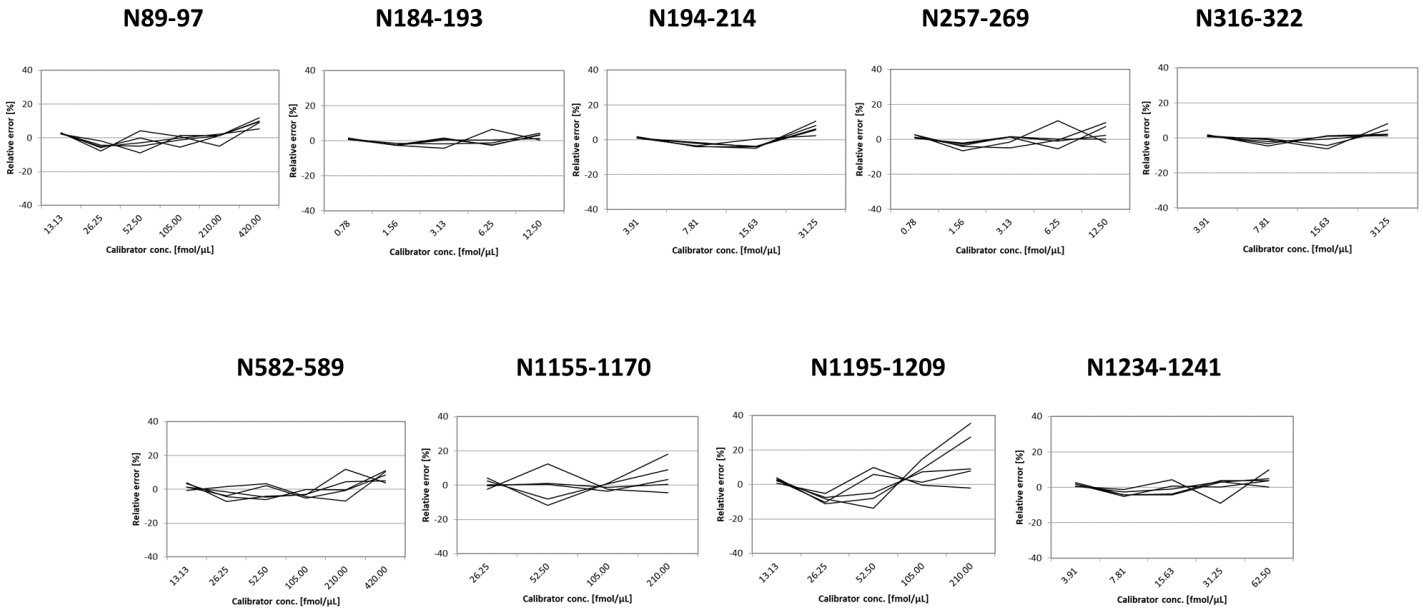


Supplementary figure 5. Weighted linear fit calibration curves for brevican IS peptides.



The curve fits were obtained using weighted sum of squares ($1/Y^2$).

Supplementary figure 6. Weighted linear fit calibration curves for neurocan IS peptides.



The curve fits were obtained using weighted sum of squares ($1/Y^2$).