A Parallel Reaction Monitoring Mass Spectrometric Method for Analysis of Potential CSF Biomarkers for Alzheimer's Disease

Brinkmalm G1,2, Sjödin S1,2, Simonsen AH3, Hasselbalch SG3, Zetterberg H1,2,4,5, Brinkmalm A1,2, Blennow K1,2.

1Institute of Neuroscience and Physiology, Department of Psychiatry and Neurochemistry, The Sahlgrenska Academy at University of Gothenburg, Mölndal, Sweden.2Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden.3Danish Dementia Research Centre, Rigshospitalet, Copenhagen University, Copenhagen, Denmark.4Department of Molecular Neuroscience, University College London Institute of Neurology, Queen Square, London, UK.5UK Dementia Research Institute, London, UK.

ABSTRACT

SCOPE: The aim of this study was to develop and evaluate a parallel reaction monitoring mass spectrometry (PRM-MS) assay consisting of a panel of potential protein biomarkers in cerebrospinal fluid (CSF).

EXPERIMENTAL DESIGN: Thirteen proteins were selected based on their association with neurodegenerative diseases and involvement in synaptic function, secretory vesicle function, or innate immune system. CSF samples were digested and two to three peptides per protein were quantified using stable isotope-labeled peptide standards.

RESULTS: Coefficients of variation were generally below 15%. Clinical evaluation was performed on a cohort of 10 patients with Alzheimer's disease (AD) and 15 healthy subjects. Investigated proteins of the granin family exhibited the largest difference between the patient groups. Secretogranin-2 ($p<0.005$) and neurosecretory protein VGF ($p<0.001$) concentrations were lowered in AD. For chromogranin A, two of three peptides had significantly lowered AD concentrations ($p<0.01$). The concentrations of the synaptic proteins neurexin-1 and neuronal pentraxin-1, as well as neurofascin were also significantly lowered in AD ($p<0.05$). The other investigated proteins, β2-microglobulin, cystatin C, amyloid precursor protein, lysozyme C, neurexin-2, neurexin-3, and neurocan core protein, were not significantly altered.

CONCLUSION AND CLINICAL RELEVANCE: PRM-MS of protein panels is a valuable tool to evaluate biomarker candidates for neurodegenerative disorders.

KEYWORDS

Alzheimer’s disease; cerebrospinal fluid; biomarker; mass spectrometry; parallel reaction monitoring

INTRODUCTION

Alzheimer's disease (AD) is characterized by accumulation of aggregated hyperphosphorylated tau protein in neurofibrillary tangles and amyloid β (Aβ) peptides in plaques.[1-4] Knowledge on AD pathogenesis has resulted in several disease-modifying drug candidates that are being evaluated in clinical trials.[5] However, many trials have suffered from the low diagnostic accuracy of pure clinical assessment of patients, meaning that a high proportion of patients that do not have AD pathology have been enrolled.[6] Thus, there is a great need for biomarkers, both to improve diagnostics and to monitor treatment effects.[7]

The most validated cerebrospinal fluid (CSF) biomarkers for AD are $A\beta_{42}$, total tau (T-tau) and tau phosphorylated at threonine 181 (P-tau), which consistently have shown a marked change in AD dementia and also in the early prodromal phase of the disease.[8] Based on their high diagnostic performance, these core AD CSF biomarkers have been included in the diagnostic criteria for AD.[9,10] Even though these CSF biomarkers discriminate AD cases well from healthy subjects,[11] additional understanding of the disease mechanisms could be obtained by new biomarkers reflecting other aspects of pathophysiology. Moreover, it is frequently difficult with differential diagnosis between AD and other forms of dementia. Early loss of synaptic function is believed to play an important role in AD and recently the CSF levels of peptide products from both presynaptic[12,13] and postsynaptic[14] proteins have been shown to be altered in AD. Moreover, inflammatory processes[15] and oxidative stress[16] may be involved in AD pathogenesis. To better understand the complicated biochemistry leading to AD it is important to investigate complementary potential biomarkers. These may also be useful to improve the current diagnosis efficiency, in differential diagnoses of AD and other neurodegenerative diseases, and to monitor therapeutic effects.

While the immunoassay is presently the workhorse in biomarker analysis, in the search for novel biomarker candidates, mass spectrometry offers possibilities when no suitable antibodies exist.[17] Even if there is still a limited number of clinical studies performed, targeted mass spectrometric analysis of protein compounds is presently a fast growing research field.[18] An advantage is the possibility for multiplexing, allowing for analysis of a number of compounds in one analysis. However, complex samples, such as CSF, require instrumentation that can provide the necessary selectAivity. The Q Exactive is a high-resolution instrument capable of parallel reaction monitoring (PRM), a method related to selected reaction monitoring (SRM) but with the advantage of acquiring full fragment spectra instead of a choice of preselected fragments. This feature, together with the high mass resolution, considerably increases the possibility to avoid interfering signals that compromises the obtained data.[19,20] For the method to be robust and capable of handling large sample numbers without interruption it is, however, necessary to operate with a liquid chromatography (LC) system using larger columns and higher flow rates than typically employed in discovery work. Sensitivity is a key parameter for analysis of clinically relevant samples where available sample amounts are low. When operating in PRM mode the sensitivity is still very high and low femtomole levels can be quantified.[21,22]

Based on exploratory mass spectrometry studies, we selected novel biomarker candidates, which were associated with neurodegenerative diseases, involved in synaptic function, secretory vesicle function, and in the

innate immune system. Neurosecretory protein VGF, secretogranin-2, chromogranin A all three belong to the granin family: Granins are expressed in endocrine cells and peptidergic neurons, are present in large dense core vesicles, and have been associated with neurodegenerative diseases such as AD, multiple sclerosis, schizophrenia, and depression.[23] Secretogranin-2 and chromogranin A have been shown to colocalize and exhibited significantly lower immunoreactivity in AD brains.[24] Both proteins have been found in amyloid plaques[25] and high CSF chromogranin A levels are associated with longitudinal $A\beta_{42}$ reduction.[26] CSF biomarker studies on chromogranin A have yielded different results depending on the precise type of AD that was studied.[27-29] Cystatin C is a cysteine protease inhibitor affecting, for example, cathepsin B[30] and BACE1; in the latter case directly leading to lower $A\beta_{40}$ levels and increased sAPPα levels as measured in human brain microvascular endothelial cells.[31] Cystatin C has been shown to bind to Aβ and thus diminishing Aβ deposits in mouse brain.[32] CSF β2-microglobulin levels are increased as a result of immune system activation and are found to be elevated in, for example, purulent meningitis, viral meningitis/encephalitis, and neuroborreliosis.[33] For soluble amyloid precursor protein (sAPP) there is a number of studies conducted for total sAPP as well as for sAPPα and -β with different results concerning changes in level for AD compared to control patients.[34-42] CSF lysozyme C levels are increased in bacterial and fungal meningitis and acute inflammatory conditions.[43] Lysozyme C is shown to co-localize with Aβ, to prevent Aβ aggregation *in vitro* as well as in *Drosophila melanogaster*, and to be increased in AD CSF.[44] Neurexin-1, -2, and -3 are transmembrane proteins found in presynaptic terminals. Neurexins act as neuronal cell-surface receptors but the precise function and localization of the different variants is yet to be elucidated.[45] Recently, Schreiner *et al.*[46] performed a thorough investigation of neurexin profiling and relative distribution in the brain and concluded that neurexins are relatively abundant synaptic proteins and that the alpha isoforms were more abundant than the beta isoforms. Neuronal pentraxin-1 is a member of the pentraxin family, which members are involved in neurodegeneration,[47] and is found in pre- and post-synaptic compartments of excitatory synapses.[48] Neuronal pentraxin-1 colocalizes with both SNAP-25 and tau in dystrophic neurites surrounding amyloid deposits in human brain.[49] Neurofascin occurs in several isoforms, which are expressed in immature neurons and involved in neurite outgrowth and control of postsynaptic structures; expressed in mature neurons and involved in synaptic stabilization; and expressed in glia and involved in in stabilization of paranodes.[50,51] Neurocan core protein has been shown to be expressed by reactive astrocytes in mice subjected to cortical brain injury and by astrocytes in primary cell culture.[52] Neurocan core protein has also been shown to increase in astrocytes incubated with Aβ, possibly via Sox9 regulation.[53]

For the thirteen selected proteins we aimed to develop two biomarker panels for a sufficient sensitive work-load-efficient method robust enough for large sample sets. This was accomplished by adding of internal standards to the CSF, tryptic digestion in solution followed by SPE desalting and concentration step with subsequent drying of the samples. Reconstituted samples were then analysed by LC-PRM-MS. These biomarker panels were evaluated in CSF samples from a cohort of AD patients and controls.

MATERIALS AND METHODS

General

Quantitative mass spectrometric analysis of intact proteins is presently not feasible. By digestion, a number of peptides suitable for MS analysis are obtained. From previous nanoflow LC-MS analyses two or three suitable peptides were selected for each protein chosen to investigate. For PRM analysis, corresponding stable-isotope-labeled peptides are used as reference peptides in the LC-MS analyses. In addition, human albumin, which is not produced in the central nervous system, was monitored and bovine serum albumin (BSA) protein was added together with one corresponding stable isotope-labeled peptide. BSA, having the same level in all samples, was used to monitor the general performance, e.g., digestion efficiency. To ensure good quality data the mass spectrometer was operated in a relatively slow mode with long injection times per acquisition. This put limits on the maximum number of peptides that could be handled simultaneously and therefore the analysis was divided into two panels, each analysing 17 or 18 peptides. The time between injections was 72 min, of which 7 min was sample loading time. To monitor intra- and inter-day variations a CSF pool was aliquoted and used for quality control. A schematic of the workflow is shown in Fig. 1.

CSF samples

Samples from ten patients (6 males, 4 females) diagnosed with AD and fifteen healthy controls (9 males, 6 females) from the Danish Dementia Research Centre, Rigshospitalet, Copenhagen, Denmark, were analysed. Age for the AD group was 64.9 ± 7.6 years (mean ± standard deviation) and 62.7 ± 7.0 years for the control group. The project was approved by the ethical committee of the Copenhagen Capital Region and all patients gave informed consent.

CSF samples (10-12 mL) were obtained by lumbar puncture, collected in polypropylene tubes and gently mixed. The samples were centrifuged at 2000×g for 10 min at +4 °C to remove cells and other insoluble material and stored in polypropylene tubes at –80 °C pending analysis.

Samples were thawed, and divided into 100-µL-aliquots in Micronic 0.75 mL tubes with screw caps (Micronic, Lelystad; The Netherlands), refrozen and stored at –80 °C pending further preparation. The CSF pool was a mixture of CSF obtained from the Neurochemistry Laboratory at Sahlgrenska University Hospital, Mölndal, Sweden. It was divided and stored in the same way as the individual CSF samples.

Patients and AD biomarker analysis

Patients were divided into AD and control groups based on clinical evaluation as well as on the result of a computer tomography (CT) scan. The samples included in the control group all came from volunteers that were deemed not having any mental disorder. For the AD group an additional criterion for inclusion was a low CSF $A\beta_{42}$ level, to assure patients having brain amyloid deposition. The CSF $A\beta_{42}$ levels were determined at Statens Serum Institute, Copenhagen, Denmark, using Fujirebio immunoassay, and were 275.4 ± 106.8 pg/mL (mean ± standard deviation) for the AD group and 877.2 ± 206.3 pg/mL for the control group; P-value $<10^{-4}$ (Student's t-test).

Standards

Peptides to be analysed were identified previously by analysing trypsin digested CSF using nanoflow LC-MS/MS. Peptides providing a good enough signal and identification score were further investigated and verified to represent only the proteins of interest. Heavy-isotope-labeled standards were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA; FasTrack 1, usable for relative measurements). BSA protein was from Sigma-Aldrich Co. (Saint Louis, MO, USA). Peptides and BSA protein were dissolved in water or water/acetonitrile/formic acid 100/30/1 (v/v/v) and diluted individually in 50 mM NH_4HCO_3 to match protein levels in CSF samples. Diluted labeled peptides and BSA protein was pooled, aliquoted, and stored in solution at –20 °C pending further preparation. A parallel set of more concentrated standards (also diluted in 50 mM NH_4HCO_3) was prepared from the same stock solutions of heavy-isotope-labeled standards and BSA protein. These were then used to generate reverse calibration curves to evaluate the linearity and limits of quantification for each peptide in the assay.

Reverse calibration samples

Calibration samples were prepared by starting with the more concentrated standard solution and perform a two-step dilution in 50mM NH_4HCO_3 producing a 10 000-fold concentration range of standards. These standard mixes were then added to CSF pool samples to generate reverse calibration curves, which were used to determine linearity and limits of quantification. The quantification limit was defined so that the CV at a particular point should be less than 25%.

CSF protein digestion

Frozen 100-µL CSF samples (individual and pool) were thawed and 20 µL of the thawed standard cocktail was added to each sample. For calibration samples the respective calibration standard cocktails were added to CSF pool samples. Reduction of sulphur bridges was performed by adding 25 µL of 30 mM 1,4-dithiothreitol (DTT, 5 mM end conc., Sigma-Aldrich) in 50 mM NH_4HCO_3. Samples were then incubated at +60 °C for 30 min on gentle shaking using a Thermomixer Comfort (Eppendorf). After a cooling period of 30 min and subsequent spin-down, blocking of sulphur bridges was performed by adding 25 µL of 14 mM iodoacetamide (IAA, 2 mM end conc., Sigma-Aldrich) in 50 mM NH_4HCO_3 with subsequent incubation for 30 min at room temperature in darkness on gentle shaking. Digestion was performed by adding 25 µL of 0.8 µg/µL sequencing grade modified trypsin (Promega) in 50 mM NH_4HCO_3 and incubate over night (approximately 18 h) at +37 °C on gentle shaking. After a spin-down incubation was ended by adding 25 µL of 10% trifluoroacetic acid.

Desalting and reconstitution

Digested samples were desalted using Oasis 30 µm HLB 96-well µElution Plates (Waters). Wells were conditioned using 2×300 µL MeOH and equilibrated with 2×300 µL water using a rotary pump for controlled suction. Samples were the loaded onto the plate and the wells washed with 2×300 µL water. Samples were eluted with 2×100 µL MeOH into Micronic 0.75-mL tubes, which were dried in a speedvac and stored at –80 °C pending PRM analysis. Prior to PRM analysis samples were reconstituted in 50 mM NH_4HCO_3 and split as desired for each analysis set. The equivalent of 25 µL CSF was used for panel 1 and 50 µL for panel 2 (see Table 1). The two analyses were performed at two occasions, separated by two days.

LC-MS/MS analysis

Mass spectrometric analysis was performed using a Dionex 3000 system (SRD-3600 degasser, WPS-3000TRS autosampler, HPG-3400RS pump, Thermo Fisher Scientific) coupled to a Q Exactive (Thermo Fisher Scientific) electrospray ionization hybrid quadrupole–orbitrap high resolution mass spectrometer. Separation was performed with a Hypersil Gold reversed phase column (id 2.1 mm, length 100 mm, Thermo Fisher Scientific) operated at a flowrate of 100 µL/min. For each assay the gradient was tailored to maximize separation of the peptides to be analysed. For both panels the mobile phase A was 0.1% formic acid, mobile phase B was 84% ACN/0.1% formic acid (v/v), the gradient was 50 min., and the sample cycle time was 75 min. MS analysis was performed as scheduled PRM with retention time windows no shorter than 4 min for each peptide. At most four different peptide pairs were toggled between, see Fig. S1. For general acquisition, including the study, isolation window was set to 8 m/z units enabling simultaneous acquisition of both "native" and labeled peptides. For comparison, in a limited number of analyses, isolation window was instead set to 3 m/z units with separate acquisitions of unlabeled and labeled peptides. The automatic gain control target value was set to $3x10^6$ and maximum injection time to 300 ms for both precursor and fragment ion spectra. Acquisitions were made at a resolution setting of 70 000 (to match the maximum injection time) toggling between intact peptide and fragment mass spectra.

Data processing

Data processing was performed with PinPoint v1.3 (Thermo Fisher Scientific), which was used to generate peak areas of the "native" and labeled peptides. Extracted ion chromatograms for all transitions were inspected visually and those with peak shape differing from the internal standard were removed from the data set. Data was subsequently exported and further analysed using in-house developed software (PinPointEvaluator), which facilitated quality control and further refinement. Again, non-conformative transitions (e.g., transistions with light-to-heavy ratio different than the majority's), likely affected by interferences, as well as those with low intensity, were removed. The CSF pool samples were utilized to evaluate the stability of the method.

Statistical analysis

Statistical analysis and plot generation was performed using GraphPad Prism 7.02. For the evaluation two-tailed Mann Whitney U test was used and P-values <0.05 were considered significant. Because the experimental approach was of a screening type rather than testing a full model hypothesis no further correction of significance values was required.

RESULTS

Evaluation of the PRM acquisition procedure

The standard approach to set up parameters for SRM or PRM is to acquire unlabeled "native" and labeled compounds in separate acquisitions. To obtain good signals for the majority of the peptides analysed relatively long C-trap injection times (300 ms) were required. This meant that the number of data point acquired over the

chromatographic peak was on the low side. Because the limiting factor was the sensitivity therefore requiring long filling times, the instrument's trapping multiplexing possibility would give no advantage (this operating mode is useful when the ion signal is relatively high, but a higher resolution is required for peak separation). Since the m/z difference between the compounds used was relatively low (5 m/z units or less) we investigated the simultaneous isolation of both unlabeled "native" and labeled using an 8 m/z unit isolation window. Since the instrument should have a dynamic range of $>10^4$ this should be no problem provided that the abundance difference between unlabeled "native" and labeled was well within this range. Apart from obtaining twice the number of data points, effects from variation in the electrospray current would be minimized. Refinement of the acquired data was required to remove transitions affected by interferences, see Table S1 for a list of the transitions used for quantification and Fig. S2 for extracted ion chromatograms of transitions used for quantification as well as tandem mass spectrum examples.

The two approaches were evaluated using pool CSF and were found to give very similar results (Fig. 2a). The coefficient of variation (CV) was more or less the same. The average CV for the standard approach with separate isolation/acquisitions was 5.4% while it was 4.9% with simultaneous isolation. One difference was noted, however; the unlabeled "native"-to-labeled peptide ratio was different for the two approaches and was between 1.2 and 1.8 times higher (peptide dependent) when using separate acquisitions (Fig. 2a).

Acquisitions in SIM mode were also evaluated to evaluate the possibility to attain better sensitivity and utilizing the high resolution (together with MS/MS data for identification) to retain selectivity. However, it turned out that due to interferences it was for many of the peptides not possible to to obtain useful quantitative data.

Reverse calibration and quality control samples

The evaluation of the assay was performed by using pooled CSF. Reverse calibration curves spanning 4 orders of magnitudes were generated by spiking different amounts of the isotope labeled standards and BSA protein to 100 µL of CSF. Depending on peptide, the 1-3 lowest concentration points did not produce quantifiable signals; see Table S2 and Fig. S3 for details. Pool CSF samples were prepared and analysed as QC samples at two separate occasions, see Table S3 and Fig. 2b, c. One of these occasions was at the time of patient sample analysis to monitor the robustness and the digestion efficiency also for the study sample set preparation. In all samples the added BSA labeled peptide and unlabeled protein was also used to monitor the stability and digestion quality. Generally the CVs for the peptide concentrations obtained for the two panels were below 15%. During the acquisitions every eight sample was a blank to monitor carry-over, which was negligible during the whole acquisition period (≤0.1%).

Study samples

The assay was evaluated using a cohort of samples from the Danish Dementia Research Centre. All samples were prepared at the same day but each PRM panel was analysed at different occasions separated by four days. The results from the first panel are presented in Fig. 3. Of the proteins analysed it is clear that for β2-microglubulin and APP there is no significant difference between the groups. The best separation between the AD and control groups was obtained for neurosecretory protein VGF ($P<0.001$) and for secretogranin-2 ($P<0.005$) which were

both lowered in AD. For cystatin C and chromogranin A the situation is less clear since one peptide for each protein is not significantly altered while the others are lowered. In Fig. 4 the results from the second panel are presented. Lysozyme C, neurexin-2, and neurocan core protein exhibit no significant difference between the patient groups. The peptides representing neurexin-1, neuronal pentraxin-1, and neurofascin were all significantly lowered ($P<0.05$) while only one peptide showed decreased level for neurexin-3. Notable also is that for all peptides exhibiting difference the levels are lower in AD. The control peptides from human albumin and BSA were analysed in both panels and produced very similar results at the two analysis time points. (Fig. S4). As can be seen in panels c and f, the sample-to-sample variation for BSA was low.

DISCUSSION

Evaluation of the PRM acquisition procedure

The use of a high-resolution mass spectrometer capable of PRM provides both the advantage not to have to select the transitions in advance as well has having a very high degree of selectivity. Using high resolution MS we were able to investigate and evaluate possible interferences. We examined the possibility to use SIM acquisitions to monitor peptide precursor ion signals for the assay but that proved to be impossible because the limited specificity in this complex type of sample. However, we could use the SIM scans for trouble shooting. In the future there should be no need to collect SIM scans. With the additional selectivity step of monitoring product ions these interferences affected the quantification to a substantially lower degree and could be handled succesfully. Collection of signals from all product ions in a full MS/MS acquisition enabled us to refine the data by examining the unlabeled “native”-to-labeled transition intensity ratios. We could afford to remove transitions which ratios were different than that of the majority. Since LC conditions vary between analysis occasions a transition that was good at one occasion might not be useful due to an interfering signal at another.

Since there was no apparent disadvantage with the simultaneous isolation approach we decided to conduct the study accordingly. The main advantage, as stated above, was the doubled acquisition speed allowing for a more efficient analysis. Some caution is however required, which is reflected in the difference in unlabeled “native”-to-labeled peptide ratio which had a relative discrepancy of a factor about 1.6 between the acquisition approaches. The reason for this is that the isolation potential in the quadrupole is not entirely symmetric and may be further skewed with accumulation of deposits on the quadrupole [54]. This causes a non-symmetric but reproducible isotope mass distribution (the distribution will slowly shift with deposit increase over a period of weeks or months depending on usage; quadrupole cleaning is required at regular intervals to keep the sensitivity up). The shift was experimentally supported by the positive correlation of the effect with peptide m/z difference, where the ratio differed least for the peptides pairs with heavy isotope labeled lysine ($\Delta m = 8$ Da; $\Delta m/z = 4$) and most for those with heavy isotope labeled arginine ($\Delta m = 10$ Da; $\Delta m/z = 5$), see Fig. 2a. With an isolation window of 8 m/z units peptide pairs with larger m/z difference will be closer to the edge of the non-symmetric isolation potential well and thus more affected. Note that this would not affect the measured peptide levels since an accurate abundance level has to be determined using an abundance characterized unlabeled peptide anyway.

The approach has been successfully implemented previously both by our laboratory[55] and others.[56] When attempting this approach with an abundance difference of 10^3 or higher the low intensity peak reproducibility was reduced (data not shown) and the standard approach with separate isolations should be used. Gallien, *et al.* has investigated different acquisition approaches and suggest that a broader isolation window, e.g., 8 m/z units, would lead to increased background and also reduced signal intensity[57] but this was no problem with our current assays. There was a fair amount of co-isolation but not to the extent that the filling time was reduced. Care should, however, be taken if trap filling times get shorter than anticipated. This could mean that other compound eluting at the same time and having similar m/z to the compound of interest. If possible then a more narrow isolation window should be used.

Reverse calibration and quality control samples

From the standard curves it can be seen that the levels of the measured peptides were within the linear range; see Table S2 for quantification ranges. The overall reproducibility was deemed sufficient; CVs of about 15% is normal for these types of measurements. The column carry-over was carefully examined. It was 0.1% or lower depending on peptide, where carry-over correlated with hydrophobicity. By injecting a blank after every eight samples we could monitor the carry-over and verify that there was no visible build-up with consecutive injections.

Study samples

The first panel contained a number of proteins that have previously been implicated in AD and other neurological disorders. β2-microglobulin has been previously shown to have both increased CSF levels in AD[58,59] and decreased or non-altered levels in mild dementia depending on analysis method.[29] In the present study β2-microglobulin was clearly not significantly altered in AD. Although CSF levels for soluble α and β variants of sAPP have been assayed relatively thoroughly with variable results,[34-42] fewer studies have been conducted for the three splice variants of APP (APP695, APP751, and APP770). Splice variants containing the Kunitz protease inhibitory (KPI) encoded region (i.e., APP751 and APP770) have been shown to be increased relative to the non-KPI containing APP695 in AD brains compared to control brains, see for example.[60-62] In the present study, however, neither the peptide representing total APP nor the peptide representing APP containing the KPI domain were significantly altered in AD. CSF cystatin C levels have previously been reported to be decreased in AD,[29,63] a result that is partially confirmed in the present study where both peptides appeared to be lowered in AD, but only one at a significant level.

The most significantly altered proteins, secretogranin-2 and neurosecretory protein VGF, both belong to the granin family and both had lowered CSF levels in AD. For the third granin family member investigated, chromogranin A two peptides of three were also significantly down-regulated. These results are in line with previous reports. Endogenous secretogranin-2 peptides have previously been shown to have decreased CSF levels in AD[64] which is the case also for endogenous VGF peptides.[59,64-66] Endogenous chromogranin A peptide levels in CSF have been reported decreased in AD[64,65] The third chromogranin A peptide, CHGA_194-213, did not correlate well with the other two and exhibited no significant difference between the AD and control groups.

The reason for this remains elusive and would need further investigations to explain. Proteins in CSF, including chromogranin A, are to a large extent present in various processed forms.[67]

The proteins in panel 2 are much less investigated. In the present study CSF lysozyme level was not significantly altered in AD, a result contradicting the one previously published study on lysozyme C by Helmfors *et al.*[44] where increased CSF levels were reported for AD. Neurexin-1 has previously been reported to be lowered in AD CSF,[64] and our results corroborate this finding. For the remaining proteins in panel 2 this is to our knowledge the first investigation on AD CSF levels that has been reported. Two of the other proteins in panel 2, neuronal pentraxin-1 and neurofascin, also exhibited lowered AD CSF levels (also just below the 0.05 significance level). Analysis of a larger cohort preferably including other disease types would be required to evaluate the usefulness of these proteins as biomarkers for AD or other neurodegenerative diseases.

CONCLUSIONS

The two-panel assay is sufficiently robust with CVs generally below 15% and can be applied to larger study sets. The addition of BSA protein and labeled peptide proved to be very useful for keeping track of tryptic digestion efficiency and general method robustness monitoring. Using a slightly wider isolation window of 8 m/z units proved to be advantageous with a doubling of measured data points and limiting spray variability effects. Thirteen proteins were investigated in the pilot study; of these, secretogranin-2, neurosecretory protein VGF, and two out of three chromogranin A peptide had markedly lowered levels in the AD patient group, confirming earlier studies; the reason for the third chromogranin A peptide's discrepancy is presently not clear. A similar result was obtained for cystatin C where one peptide was significantly lowered in AD and the other not. Neurexin-1, neuronal pentraxin-1, and neurofascin were also lowered in AD; at a confidence level of 0.05. The remaining proteins exhibited a moderate significant difference for only one out of two peptides or for none of them. These findings for AD have to be validated with further studies and it would be very interesting to apply the method to other neurodegenerative diseases to investigate the potential for differential diagnosis.

ACKNOWLEDGEMENTS

We thank the patients and controls who contributed to this research.

This work was funded by the Swedish Research Council (projects 8266, 14002 and 2013-2546), Swedish State Support for Clinical Research (ALFGBG), the European Research Council and is part of the BIOMARKAPD Project in the frame of JPND. Further support was received from Åhlén-stiftelsen, Stiftelsen Gamla Tjänarinnor, Gun and Bertil Stohnes Stiftelse, and Magn. Bergvalls Stiftelse.

REFERENCES

Tables:

Table 1. Internal standard peptide characteristics.

Protein	Protein mass [kDa]	Peptide sequence[a]	Amino acid positions	Estimated conc. for ratio=1 [nmol/L]
Cystatin C[b]	13.3	LVGGPMDASVEEEGV[R]	35-50	140
		ALDFAVGEYN[K]	52-62	360
β2-microglobulin[b]	11.7	VEHSDLSFS[K]	69-78	84
		VNHVTLSQP[K]	102-111	90
Neurosecretory protein VGF[b]	65.0	NSEPQDEGELFQGVDP[R]	64-80	120
		AYQGVAAPFP[K]	268-278	1.9
Chromogranin A[b]	48.9	YPGPQAEGDSEGLSQGLVD[R]	194-213	33
		GLSAEPGWQA[K]	216-226	2.8
		EDSLEAGLPLQV[R]	400-412	2.9
Secretogranin-2[b]	67.8	ALEYIENL[R]	58-66	1.1
		VLEYLNQE[K]	593-601	2.4
APP (751/770 isoform) [b]	83.0	EV(C)SEQAETGP(C)[R]	289-301	5.0
APP[b]	85.2	VESLEQEAANE[R]	439-450	5.0
Lysozyme C[c]	14.7	WESGYNT[R]	52-59	9.8
		STDYGIFQINS[R]	69-80	4.4
Neurexin-1[c]	158.8	LTVDDQQAMTGQMAGDHT[R]	823-841	26
		VDSSSGLGDYLELHIHQG[K]	1168-1186	9.7
Neurexin-2[c]	182.0	TALAVDGEA[R]	124-133	4.3
		VDLPLPPEVWTAAL[R]	637-651	3.9
Neurexin-3[c]	177.8	FI(C)D(C)TGTGYWG[R]	665-677	4.9
		LTVDDDVAEGTMVGDHT[R]	790-807	1.2
Neuronal pentraxin-1[c]	45.0	LENLEQYS[R]	144-152	9.6
		LTPGEVYNLAT(C)ST[K]	386-400	7.7
Neurofascin[c]	147.5	GNPAPSFHWT[R]	67-77	9.8
		VIAINEVGSSHPSLPSE[R]	702-719	2.5
Neurocan CP[c]	140.7	ELGGEVFYVGPA[R]	257-269	1.6
		DFQWTDNTGLQFENW[R]	1155-1170	13
Albumin[b, c]	66.4	L(C)TVATL[R]	098-105	850
		AEFAEVS[K]	250-257	900
Bovine albumin[b, c]	66.4	LGEYGFQNALIV[R]	421-433	130

[a] [X] indicates stable isotope labeled amino acid and (C) indicates carbamidomethylated cysteine; [b] Included in panel 1; [c] Included in panel 2.

Figures:

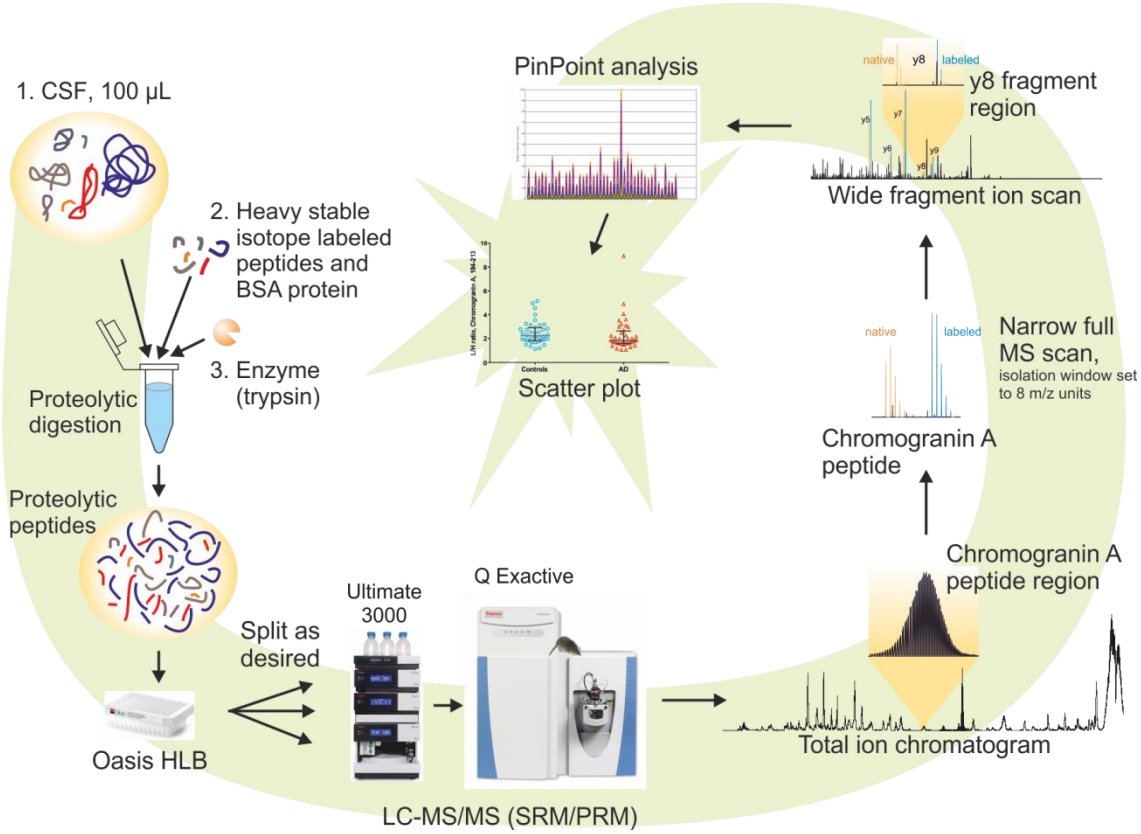


Figure 1. Schematic of the methodological workflow.

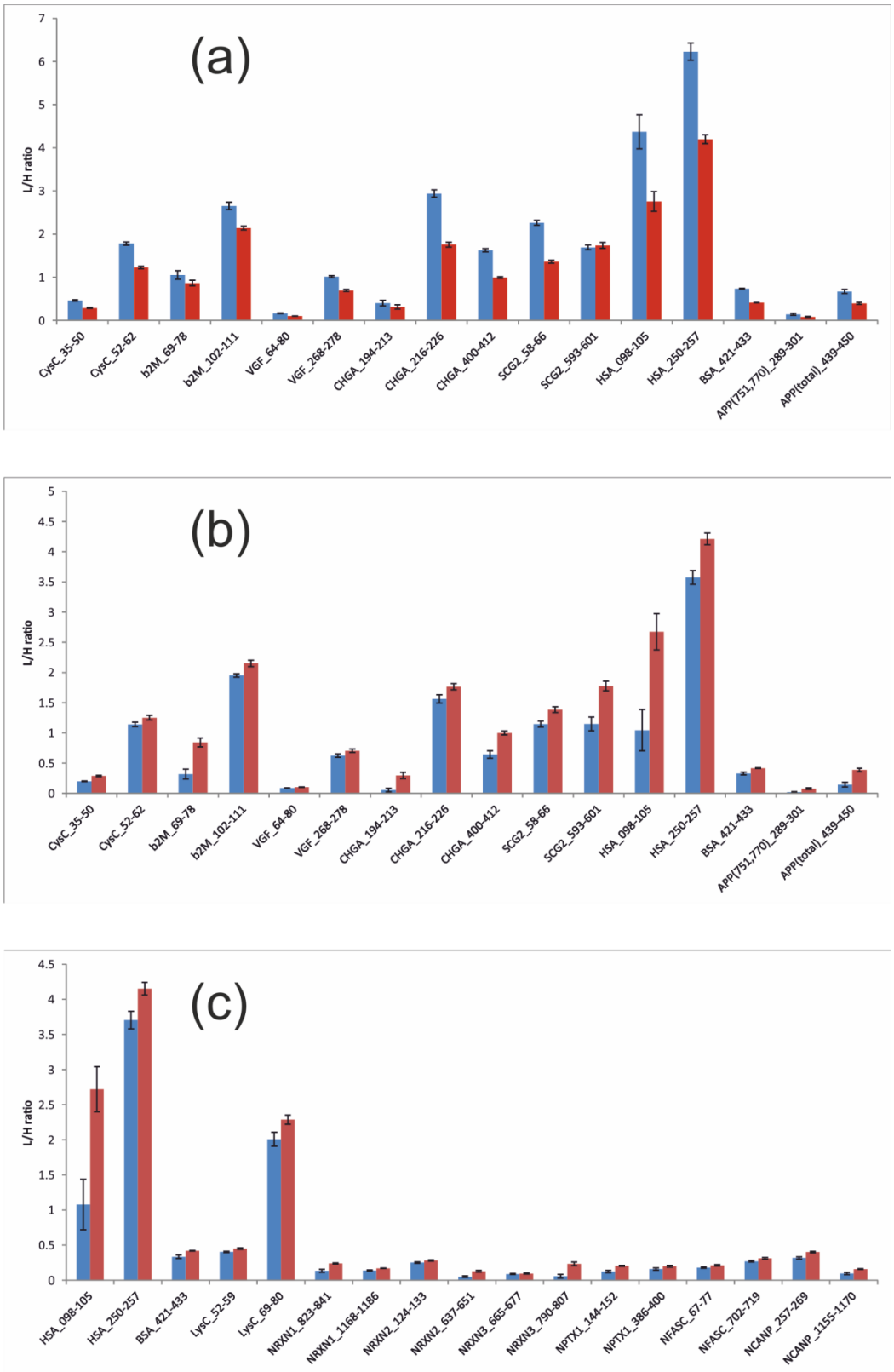


Figure 2. Mean ratios for QC pool CSF samples when using separate acquisitions for the unlabeled "native" peptide and the internal standard peptide (blue, n = 8) and acquisitions where both peptides are acquired simultaneously (red, n = 8) (a). Mean ratios for QC pool CSF samples at two different preparation and acquisition occasions for peptides included in (b) panel 1 (n = 13 + 14) and (c) panel 2 (n = 13 + 14). L/H ratio denotes light-to-heavy peptide ratio. Error bars indicate standard deviation.

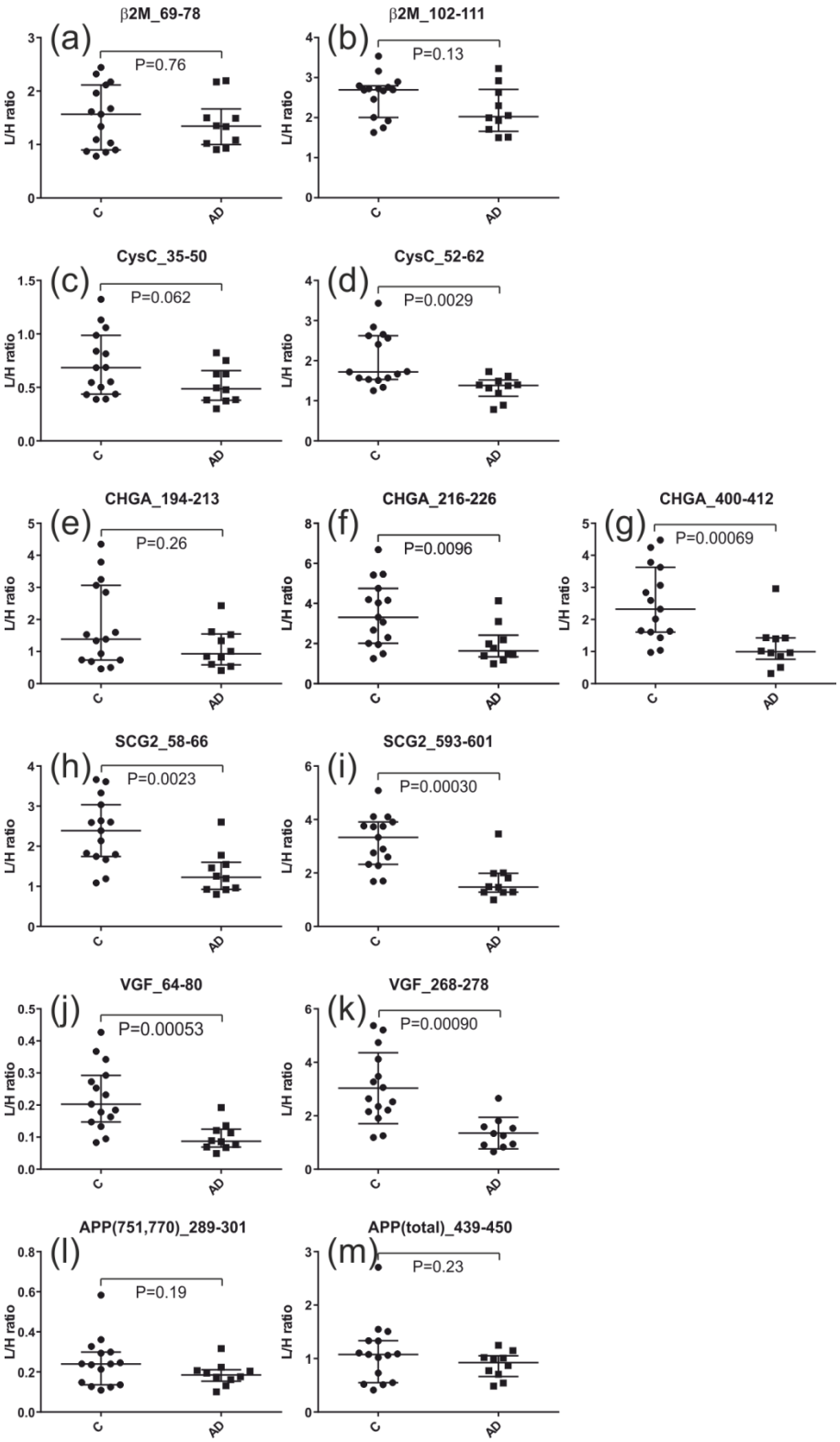


Figure 3. CSF level difference in control (C) vs AD groups for the peptides included in panel 1. For β2-microglubulin (a, b) and APP (l, m) none of the peptides exhibited a significant difference. For cystatin C (c, d) one out of two peptides was significantly lowered in AD and for chromgranin A (e, f, g) two out of three peptides were significantly lowered in AD. The most confident differences were obtained for secretogranin-2 (h, i) and neurosecretory protein VGF (j, k) for which both peptides were significantly lowered in AD. Control n = 15 and AD n = 10. L/H ratio denotes light-to-heavy peptide ratio.

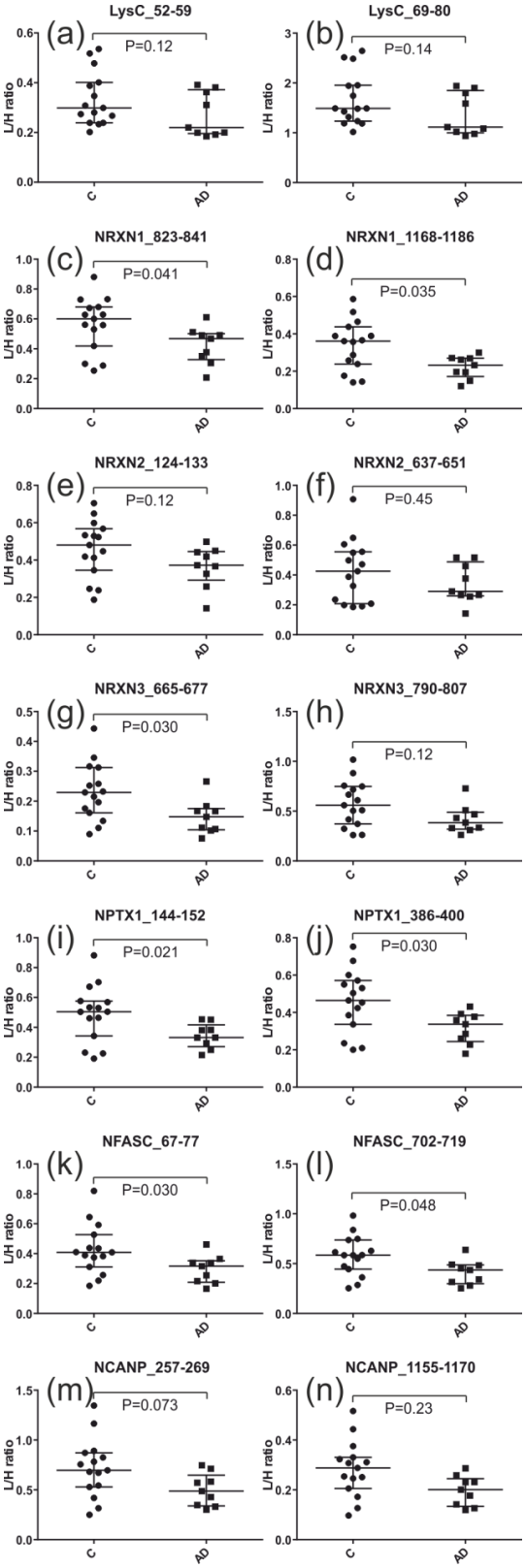


Figure 4. CSF level difference in control (C) vs AD groups for the peptides included in panel 2. For lysozyme C (a, b), neurexin-2 (e, f), and neurocan core protein (m, n) none of the peptides exhibited a significant difference. For neurexin-3 (g, h) one out of two peptides was significantly lowered in AD. For neurexin-1 (c, d), neuronal pentraxin-1 (i, j), and neurofascin (k, l) both peptides were significantly ($P<0.05$) lowered in AD. Control n = 15 and AD n = 9. L/H ratio denotes light-to-heavy peptide ratio.

Supporting Information:

Table S1. PRM transition characteristics.

Protein	Peptide sequence[a]	Amino acid positions	Precursor ion charge	Product ions used for quantification
Cystatin C[b]	LVGGPMDASVEEEGVR	35-50	2+	y6+, y7+, y8+, y9+, y10+, y11+, y12+, y14+
	ALDFAVGEYNK	52-62	2+	y5+, y6+, y7+, y8+, y9+
β2-microglobulin[b]	VEHSDLSFSK	69-78	3+	y3+, y4+, y5+, y7+
	VNHVTLSQPK	102-111	3+	y4+, y5+, y6+, y7+
Chromogranin A[b]	YPGPQAEGDSEGLSQGLVDR	194-213	3+	y3+, y4+, y5+, y6+, y7+, y8+, y9+
	GLSAEPGWQAK	216-226	2+	y6+, y7+, y8+, y9+
	EDSLEAGLPLQVR	400-412	2+	y3+, y5+, y6+, y7+, y8+, y9+, y10+
Secretogranin-2[b]	ALEYIENLR	58-66	2+	y4+, y5+, y6+, y7+
	VLEYLNQEK	593-601	2+	y4+, y5+, y6+, y7+
Neurosecretory protein VGF[b]	NSEPQDEGELFQGVDPR	64-80	2+	y3+, y5+, y6+, y7+, y8+, y10+, y11+, y12+
	AYQGVAAPFPK	268-278	2+	y4+, y5+, y6+, y7+, y8+, y9+
APP (751/770 isoform) [b]	EV(C)SEQAETGP(C)R	289-301	2+	y4+, y5+, y6+, y7+, y8+
APP[b]	VESLEQEAANER	439-450	2+	y3+, y4+, y5+, y6+, y7+, y8+, y10+
Lysozyme C[c]	WESGYNTR	52-59	2+	y3+, y4+, y5+, y7+
	STDYGIFQINSR	69-80	2+	y3+, y4+, y5+, y6+, y7+, y8+, y9+, y10+
Neurexin-1[c]	LTVDDQQAMTGQMAGDHTR	823-841	3+	y3+, y5+, y6+, y7+, y9+, y10+, y11+, y12+
	VDSSSGLGDYLELHIHQGK	1168-1186	4+	y3+, y4+, y5+, y6+, y7+, y8+
Neurexin-2[c]	TALAVDGEAR	124-133	2+	y5+, y6+, y7+, y8+
	VDLPLPPEVWTAALR	637-651	2+	y9+, y10+, y11+
Neurexin-3[c]	FI(C)D(C)TGTGYWGR	665-677	2+	y5+, y6+, y7+, y8+, y9+, y10+, y11+
	LTVDDDVAEGTMVGDHTR	790-807	3+	y5+, y6+, y9+, y10+, y11+
Neuronal pentraxin-1[c]	LENLEQYSR	144-152	2+	y3+, y6+, y7+, y8+
	LTPGEVYNLAT(C)STK	386-400	2+	y3+, y4+, y5+, y6+, y7+, y8+, y9+, y10+, y12+, y13+
Neurofascin[c]	GNPAPSFHWTR	67-77	3+	y4+, y5+, y6+
	VIAINEVGSSHPSLPSER	702-719	3+	y5+, y6+, y7+, y8+, y10+, y11+, y12+
Neurocan CP[c]	ELGGEVFYVGPAR	257-269	2+	y3+, y4+, y5+, y6+, y7+, y8+, y9+, y10+
	DFQWTDNTGLQFENWR	1155-1170	3+	y3+,y4+, y5+, y6+, y8+
Albumin[b, c]	L(C)TVATLR	098-105	2+	y3+, y4+, y5+, y6+, y7+
	AEFAEVSK[d]	250-257	2+	y3+, y4+, y5+, y6+, y7+
Bovine albumin[b, c]	LGEYGFQNALIVR	421-433	2+	y3+, y4+, y5+, y6+, y7+, y8+, y9+, y10+, y11+, y12+

[a] (C) indicates carbamidomethylated cysteine; [b] Included in panel 1; [c] Included in panel 2, [d] y3+ included only in panel 2.

Table S2. Reverse calibration dilution data. Estimated internal standard peptide concentrations for highest and lowest quantifiable points. Limits were set so that a CV below 25% was required for acceptance.

Protein	Amino acid positions	Sequence[a]	Highest [nmol/L]	Lowest [nmol/L]
Cystatin C	35-50	LVGGPMDASVEEEGV[R]	1400	0.47
	52-62	ALDFAVGEYN[K]	1800	6.0
β2-microglobulin	69-78	VEHSDLSFS[K]	2500	25
	102-111	VNHVTLSQP[K]	900	3.0
Chromogranin A	194-213	YPGPQAEGDSEGLSQGLVD[R]	990	9.9
	216-226	GLSAEPGWQA[K]	83	0.28
	400-412	EDSLEAGLPLQV[R]	88	0.29
Secretogranin-2	58-66	ALEYIENL[R]	33	0.11
	593-601	VLEYLNQE[K]	71	0.24
Neurosecretory protein VGF	64-80	NSEPQDEGELFQGVDP[R]	590	5.9
	268-278	AYQGVAAPFP[K]	58	0.19
APP 751/770	289-301	EV(C)SEQAETGP(C)[R]	25	0.25
APP	439-450	VESLEQEAANE[R]	25	0.25
Lysozyme C	52-59	WESGYNT[R]	290	0.97
	69-80	STDYGIFQINS[R]	130	0.43
Neurexin-1	823-841	LTVDDQQAMTGQMAGDHT[R]	780	2.6
	1168-1186	VDSSSGLGDYLELHIHQG[K]	290	0.97
Neurexin-2	124-133	TALAVDGEA[R]	130	0.43
	637-651	VDLPLPPEVWTAAL[R]	120	0.40
Neurexin-3	665-677	FI(C)D(C)TGTGYWG[R]	150	0.50
	790-807	LTVDDDVAEGTMVGDHT[R]	35	0.35
Neuronal pentraxin-1	144-152	LENLEQYS[R]	290	0.97
	386-400	LTPGEVYNLAT(C)ST[K]	230	0.77
Neurofascin	67-77	GNPAPSFHWT[R]	290	0.97
	702-719	VIAINEVGSSHPSLPSE[R]	74	0.25
Neurocan CP	257-269	ELGGEVFYVGPA[R]	49	0.16
	1155-1170	DFQWTDNTGLQFENW[R]	390	1.3
Albumin	098-105	L(C)TVATL[R]	2500	25
	250-257	AEFAEVS[K]	2700	9.0
Bovine albumin	421-433	LGEYGFQNALIV[R]	400	1.3

[a] [X] indicates stable isotope labeled amino acid and (C) indicates carbamidomethylated cysteine

Table S3. Quality control sample data. Values are light-to-heavy peptide ratios.

	Compound			Day 1 (n = 13)			Day 2 (n = 14)		
	Protein	Amino acid positions	Sequence[a]	Mean	St.dev.	CV [%]	Mean	St.dev.	CV [%]
Panel 1	Cystatin C	35-50	LVGGPMDASVEEEGVR	0.29	0.011	3.8	0.31	0.013	4.2
		52-62	ALDFAVGEYNK	1.3	0.039	3.1	1.4	0.13	9.3
	β2-microglubulin	69-78	VEHSDLSFSK	0.84	0.074	8.7	0.87	0.1	12
		102-111	VNHVTLSQPK	2.2	0.053	2.5	2.3	0.13	5.6
	Chromogranin A	194-213	YPGPQAEGDSEGLSQGLVDR	0.3	0.051	17	0.29	0.08	27
		216-226	GLSAEPGWQAK	1.8	0.053	3	1.9	0.07	3.6
		400-412	EDSLEAGLPLQVR	1	0.031	3.1	1.1	0.058	5.4
	Secretogranin-2	58-66	ALEYIENLR	1.4	0.047	3.4	1.6	0.18	12
		593-601	VLEYLNQEK	1.8	0.08	4.5	1.8	0.11	6.1
	Neurosecretory protein VGF	64-80	NSEPQDEGELFQGVDPR	0.1	0.0035	3.4	0.11	0.0033	3
		268-278	AYQGVAAPFPK	0.71	0.028	4	0.79	0.11	14
	APP (751/770 isoform)	289-301	EV(C)SEQAETGP(C)R	0.078	0.012	15	0.086	0.011	13
	APP	439-450	VESLEQEAANER	0.39	0.029	7.6	0.41	0.043	10
	Albumin	098-105	L(C)TVATLR	2.7	0.3	11	3	0.35	11
		250-257	AEFAEVSK	4.2	0.098	2.3	4.5	0.14	3.1
	Bovine albumin	421-433	LGEYGFQNALIVR	0.42	0.0055	1.3	0.56	0.044	7.9
Panel 2	Lysozyme C	52-59	WESGYNTR	0.45	0.0092	2	0.51	0.017	3.3
		69-80	STDYGIFQINSR	2.3	0.066	2.9	2.8	0.34	12
	Neurexin-1	823-841	LTVDDQQAMTGQMAGDHTR	0.24	0.0059	2.5	0.26	0.034	13
		1168-1186	VDSSSGLGDYLELHIHQGK	0.17	0.0038	2.2	0.23	0.013	5.7
	Neurexin-2	124-133	TALAVDGEAR	0.28	0.0097	3.4	0.28	0.009	3.2
		637-651	VDLPLPPEVWTAALR	0.13	0.013	9.9	0.22	0.069	31
	Neurexin-3	665-677	FI(C)D(C)TGTGYWGR	0.096	0.0079	8.3	0.15	0.0092	6.2
		790-807	LTVDDDVAEGTMVGDHTR	0.23	0.025	11	0.26	0.035	14
	Neuronal pentraxin-1	144-152	LENLEQYSR	0.21	0.0061	3	0.24	0.0069	2.9
		386-400	LTPGEVYNLAT(C)STK	0.2	0.013	6.3	0.32	0.045	14
	Neurofascin	67-77	GNPAPSFHWTR	0.21	0.0093	4.4	0.26	0.029	11
		702-719	VIAINEVGSSHPSLPSER	0.31	0.012	3.9	0.36	0.038	11
	Neurocan core protein	257-269	ELGGEVFYVGPAR	0.4	0.0098	2.4	0.43	0.041	9.5
		1155-1170	DFQWTDNTGLQFENWR	0.16	0.0051	3.2	0.22	0.026	12
	Albumin	098-105	L(C)TVATLR	2.7	0.32	12	3.2	0.39	12
		250-257	AEFAEVSK	4.2	0.09	2.2	4.6	0.13	2.8
	Bovine albumin	421-433	LGEYGFQNALIVR	0.42	0.0041	0.99	0.59	0.045	7.6

[a] (C) indicates carbamidomethylated cysteine.

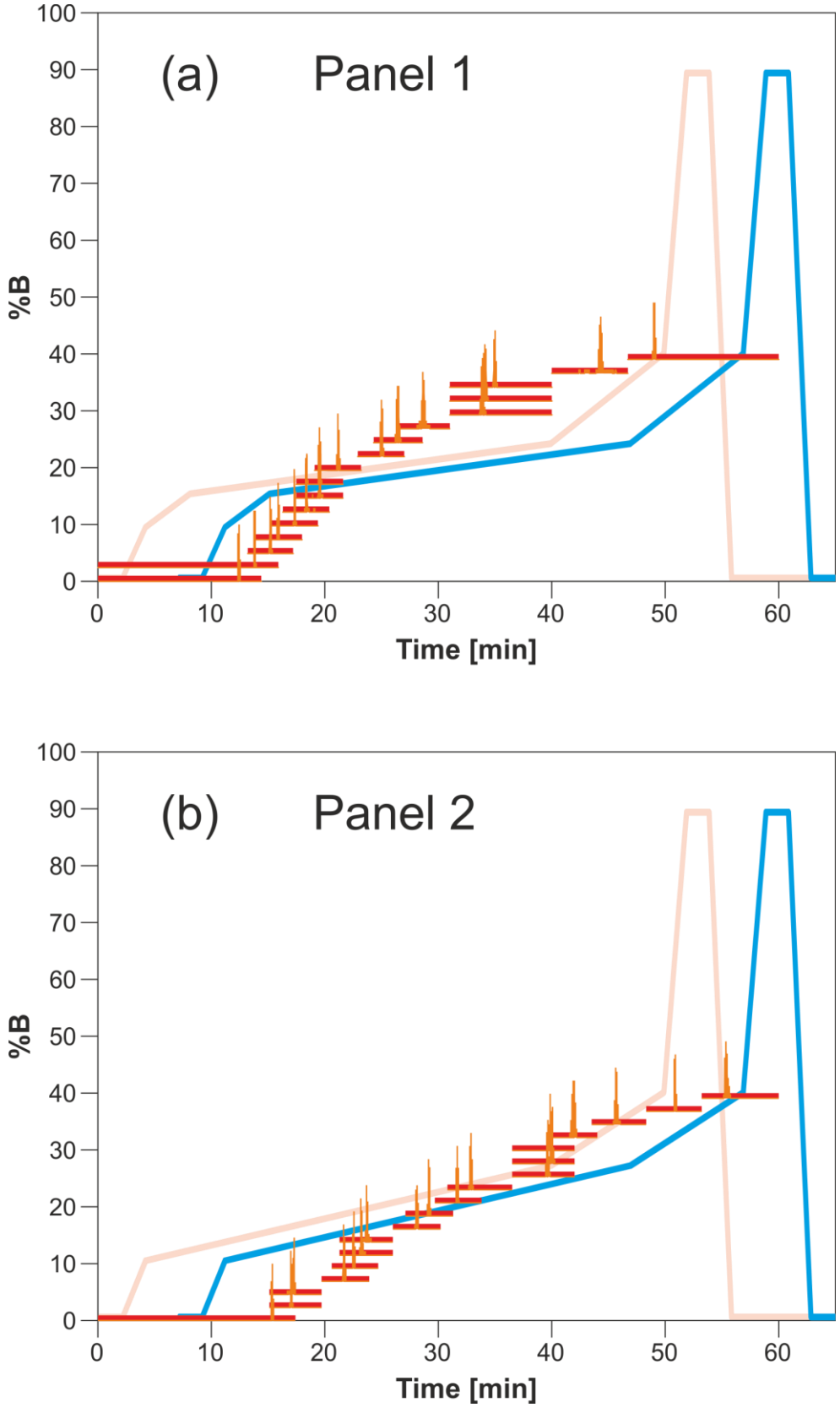
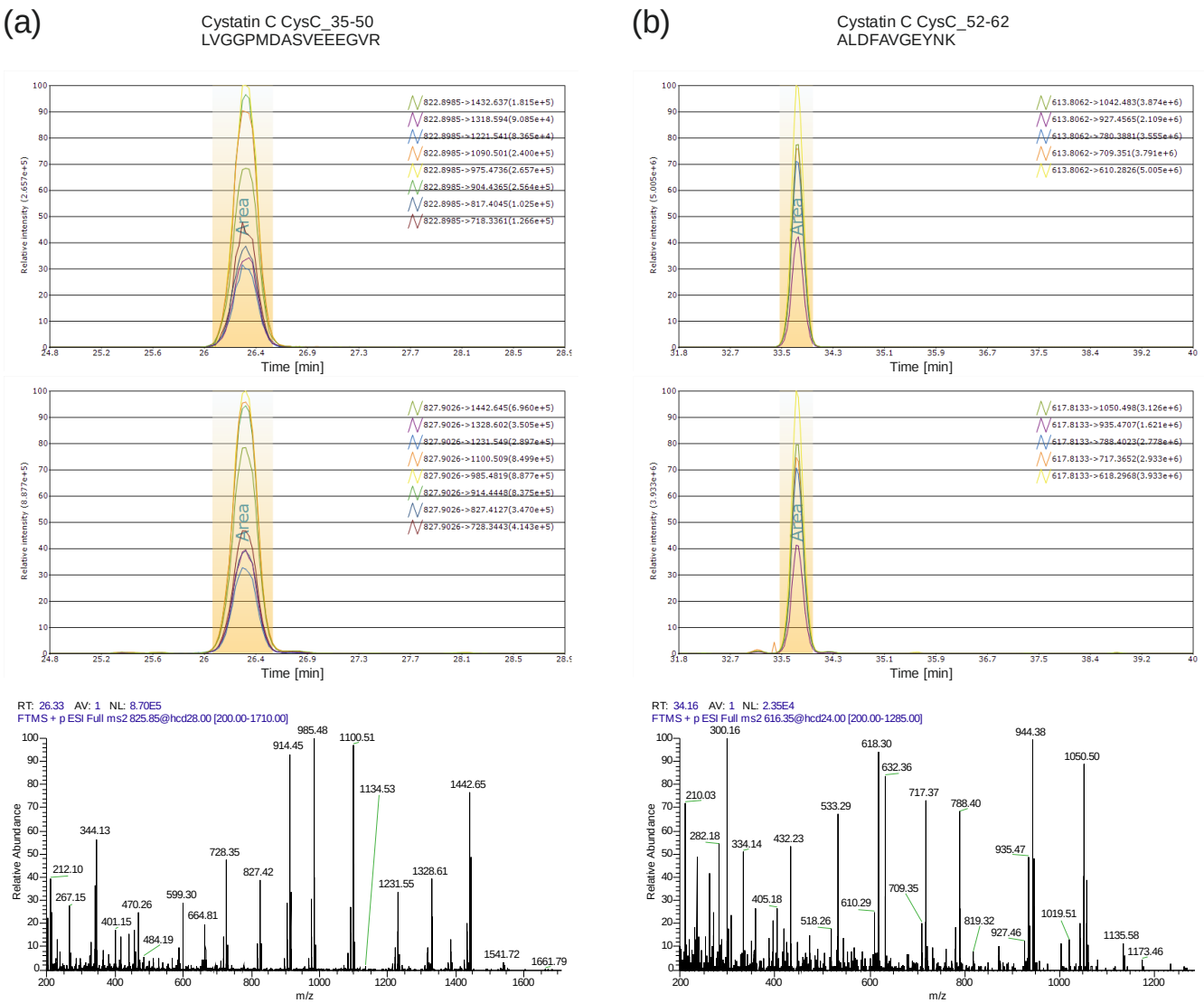
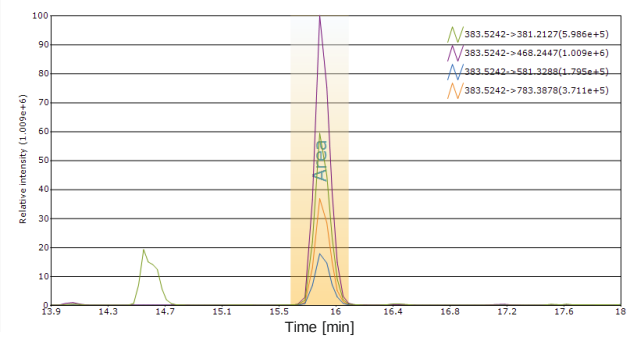


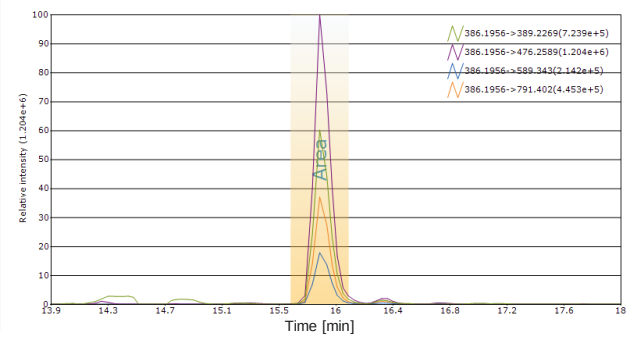
Figure S1. Acquisition schematics for (a) panel 1 and (b) panel 2. The red bars indicate the acquisition windows (at least 4 min) for each peptide (traces in orange). At most 4 peptides were acquired at the same time. The set gradients are shown in pink, while in blue is shown the actual conditions at time of spraying (the time delay due to the dead volume of the LC system was about 7 min).

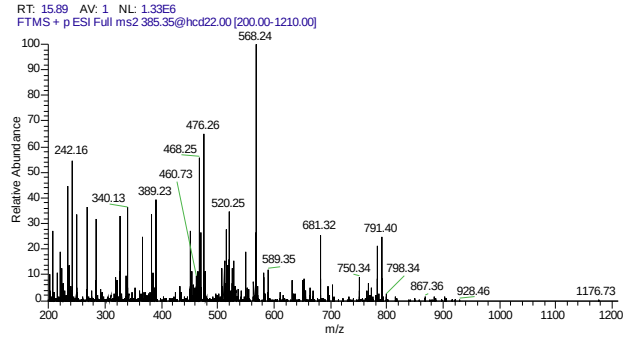
Figure S2. Extracted ion chromatograms for transitions used and example MS/MS acquisitions for all peptides analysed. (a)-(p) panel 1 and (q) - (ag) panel 2.



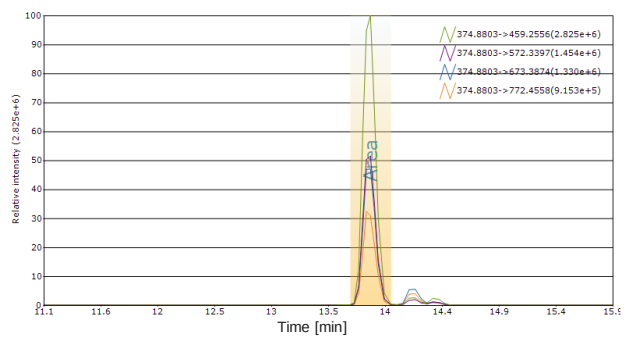
(c) β2-microglubulin b2M_69-78
VEHSDLSFSK

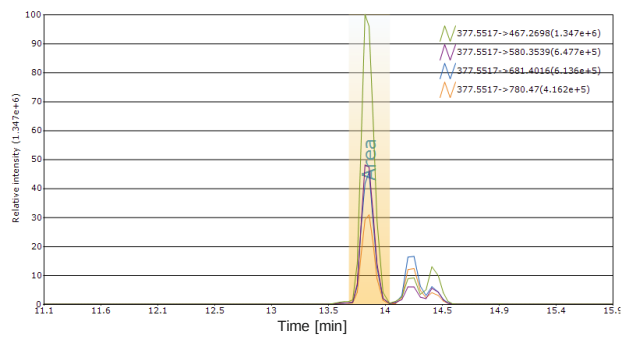


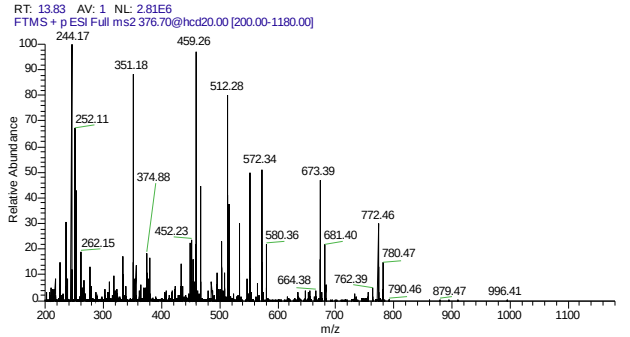




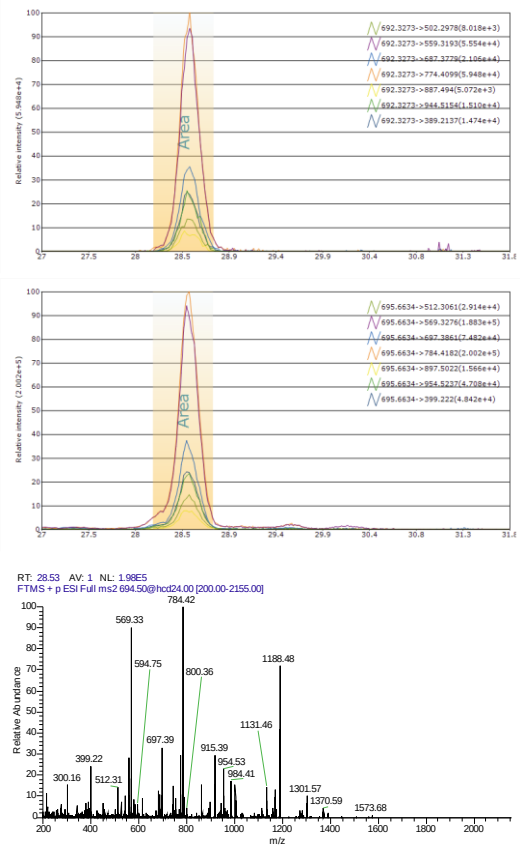
(d) β2-microglubulin b2M_102-111
VNHVTLSQPK



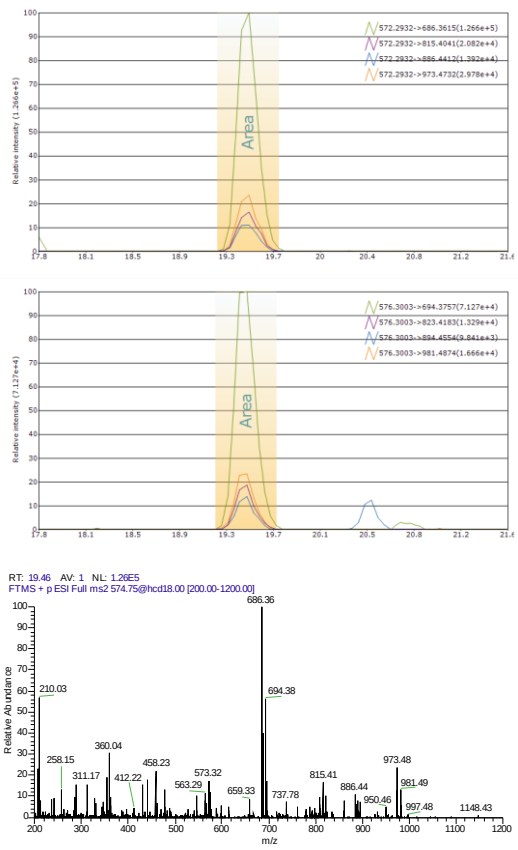




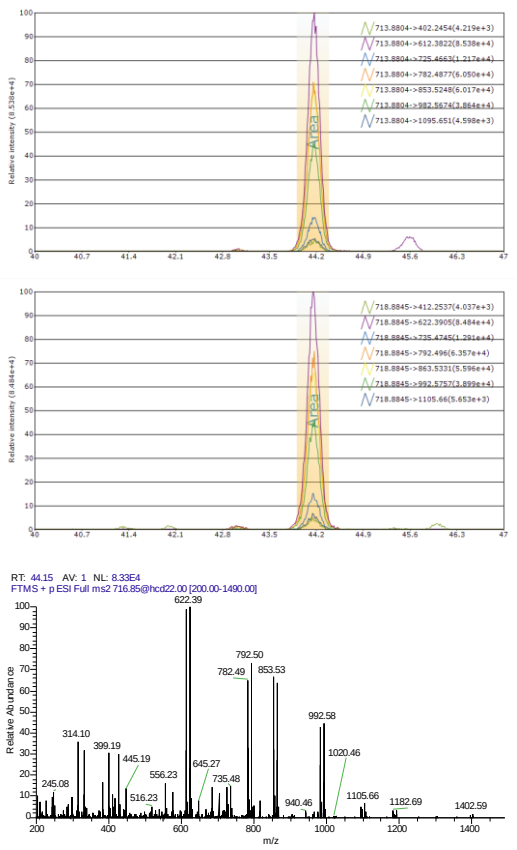
(e) Chromogranin A CHGA_194-213
YPGPQAEGDSEGLSQGLVDR



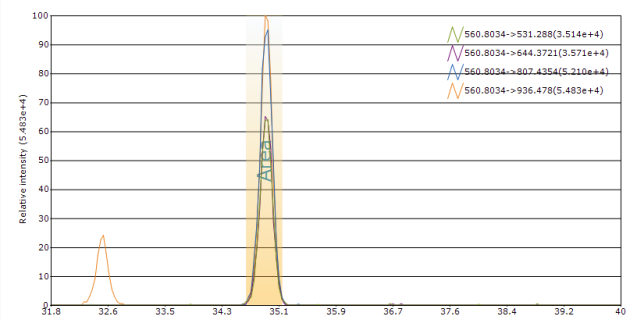
(f) Chromogranin A CHGA_216-226
GLSAEPGWQAK

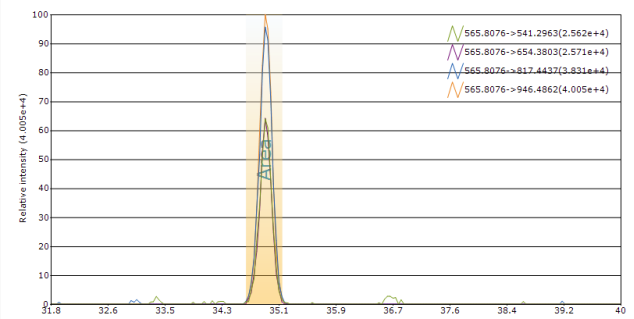


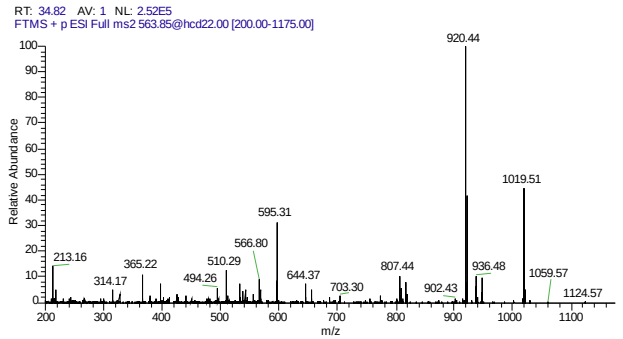
(g) Chromogranin A CHGA_400-412
EDSLEAGLPLQVR



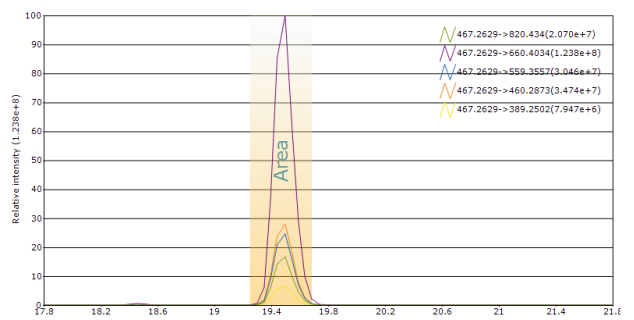
(h) Secretogranin-2 SCG2_58-66
ALEYIENLR

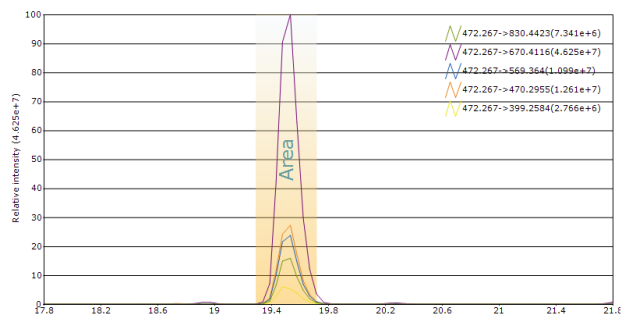


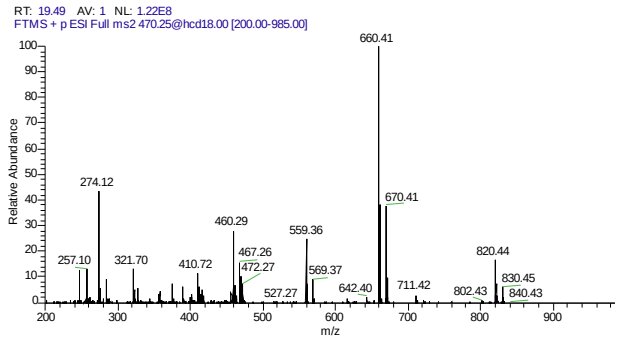




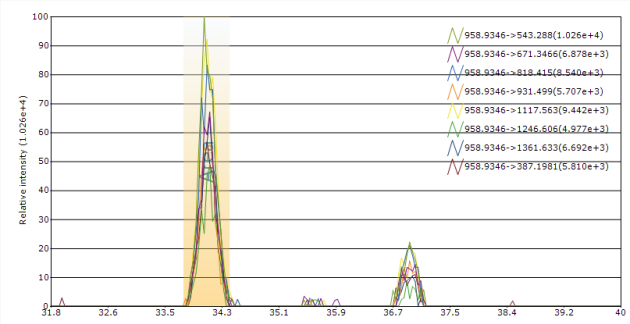
(n) Albumin HSA_098-105
LcTVATLR

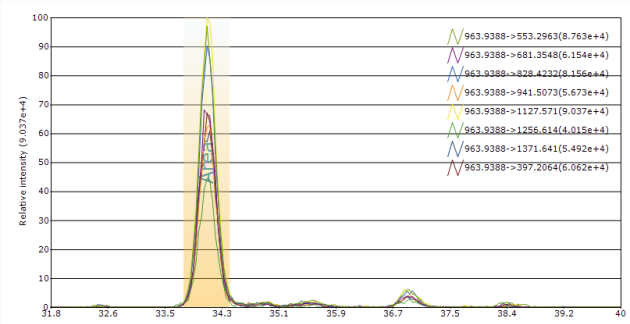


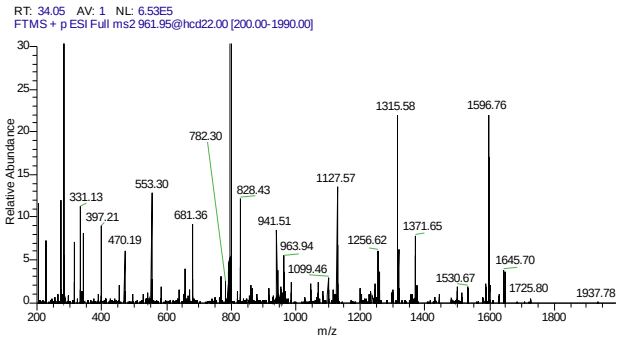




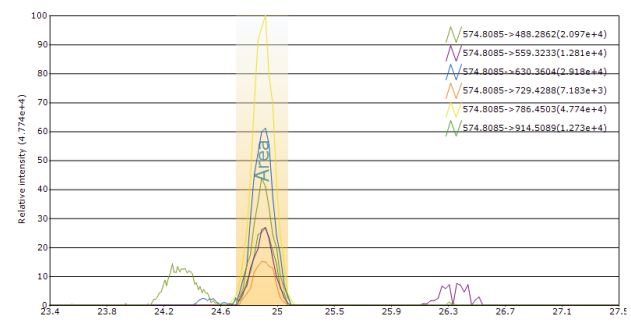
(j) Neurosecretory protein VGF VGF_64-80
NSEPQDEGELFQGVDPR

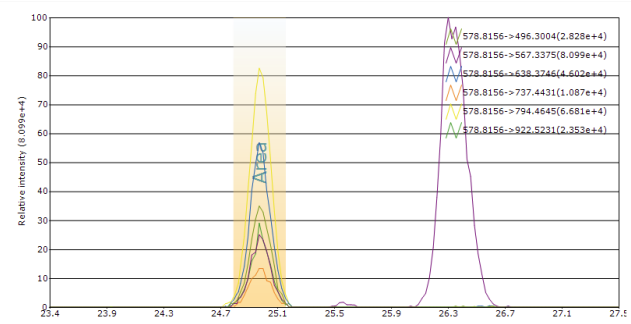


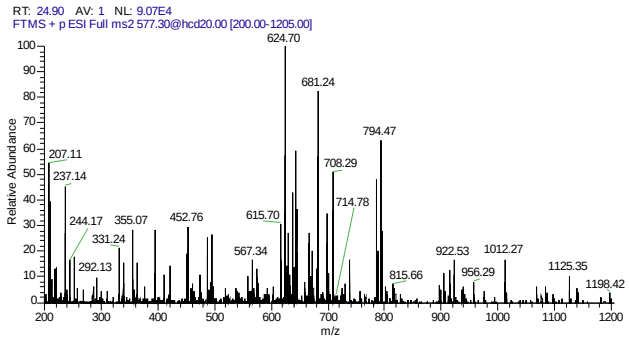




(k) Neurosecretory protein VGF VGF_268-278
AYQGVAAPFPK

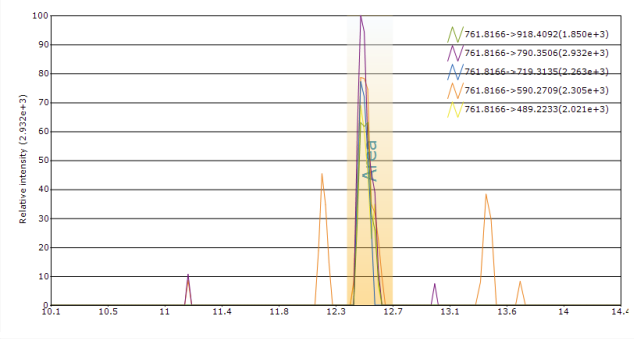


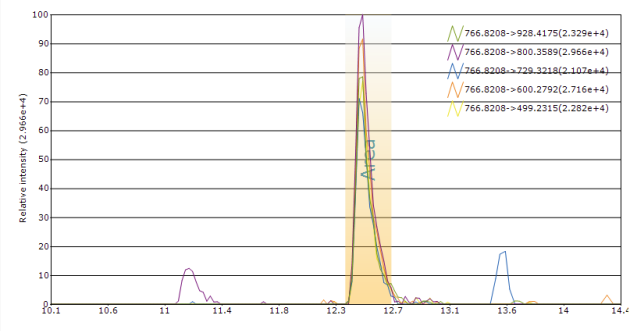


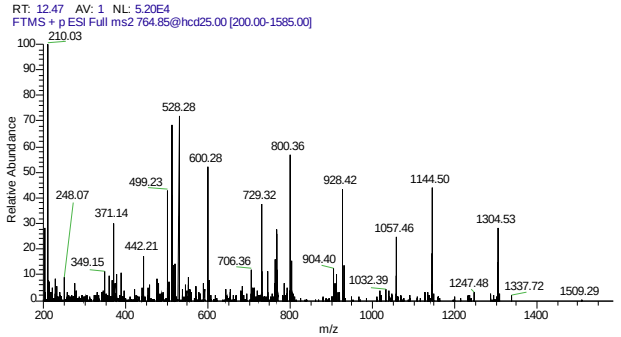


(l)

Amyloid precursor protein APP(751,770)_289-301
EVcSEQAETGPcR

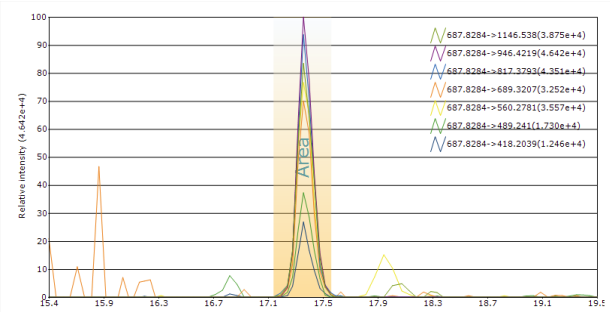


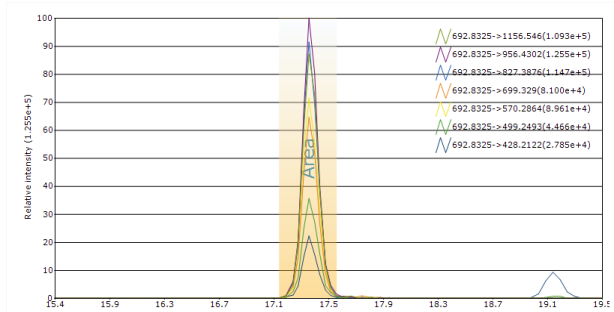


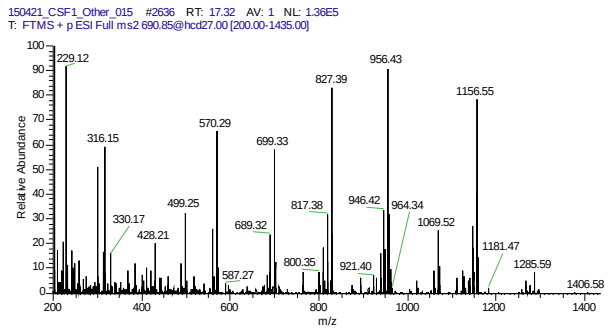


(m)

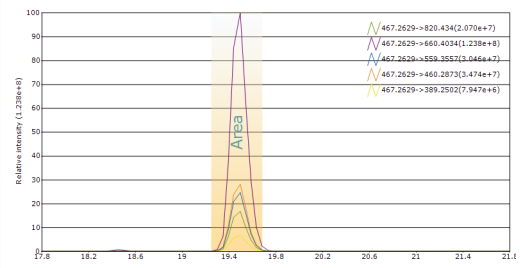
Amyloid precursor protein APP(total)_439-450
VESLEQEAANER

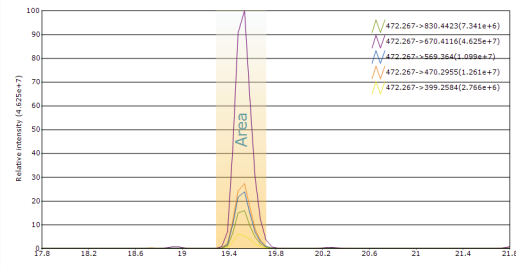


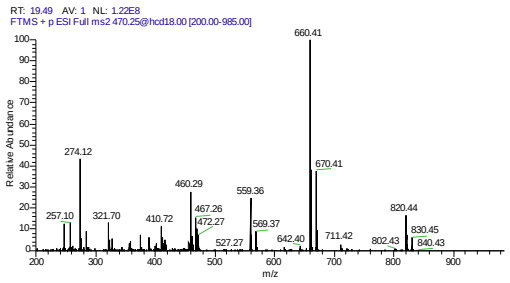




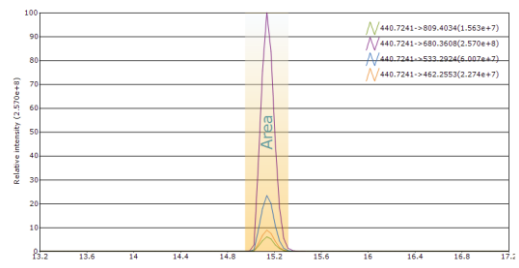
(n) Albumin HSA_098-105
LcTVATLR

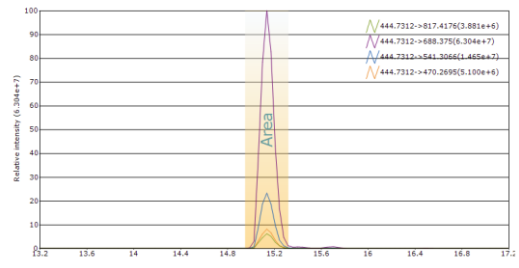


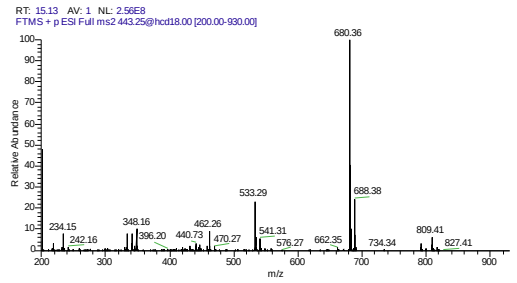




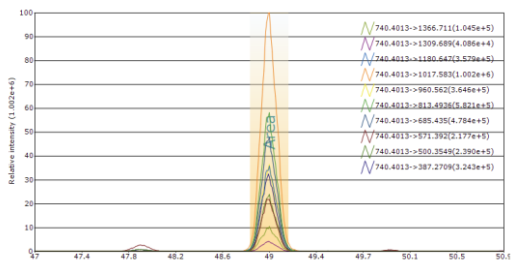
(o) Albumin HSA_250-257
AEFAEVSK

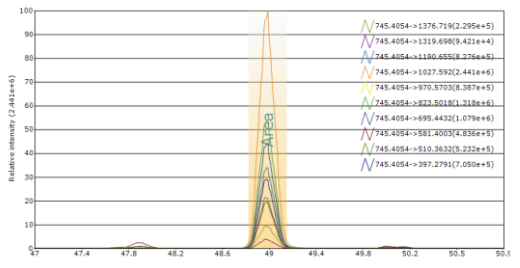


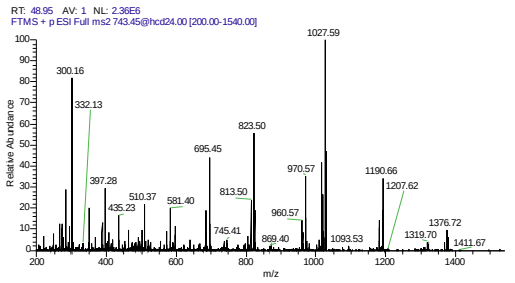




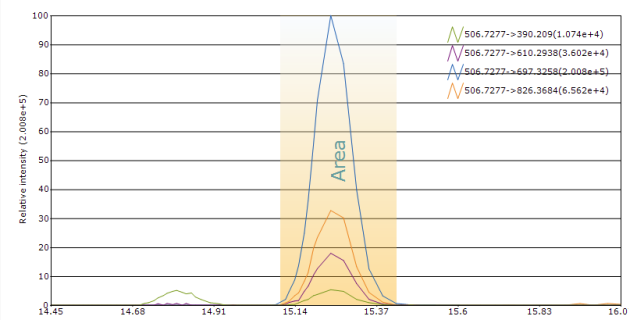
(p) Bovine serum albumin BSA_421-433
LGEYGFQNALIVR

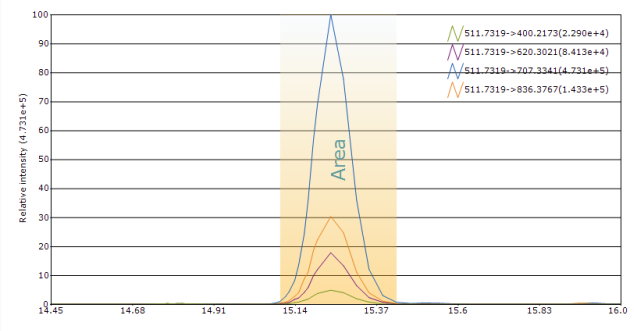


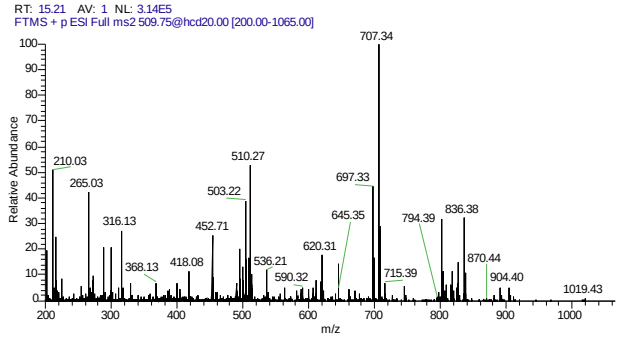




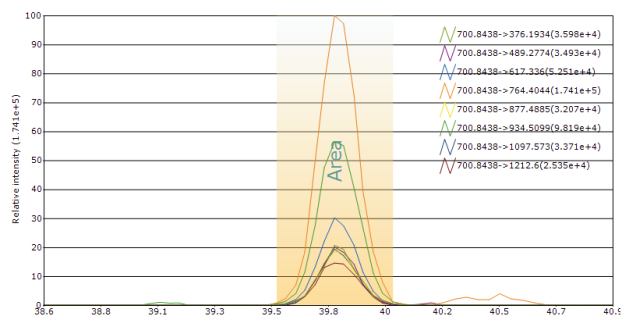
(q) Lysozyme C LysC_52-59
WESGYNTR

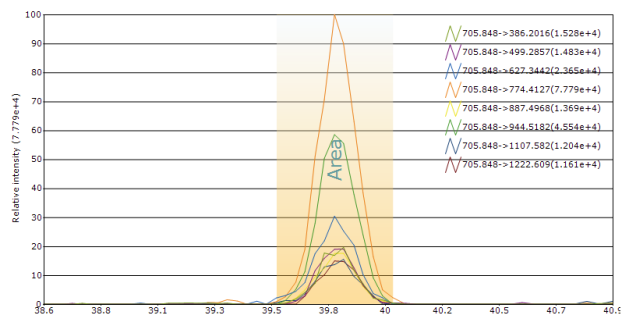


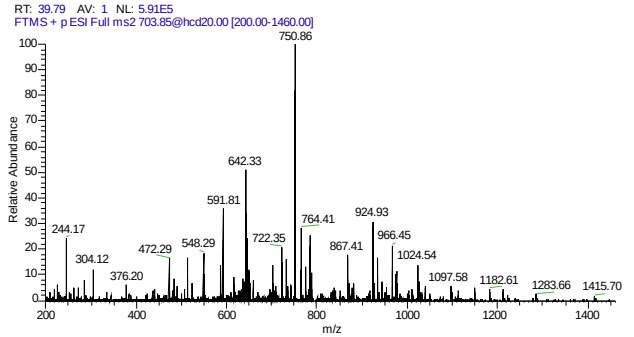




(r) Lysozyme C LysC_69-80
STDYGIFQINSR

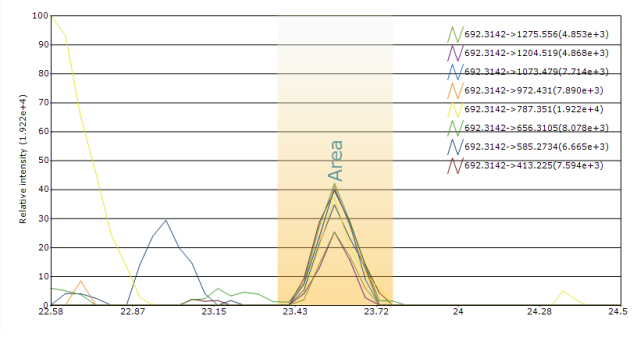


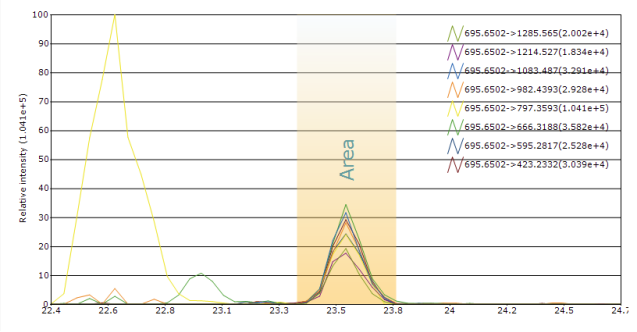


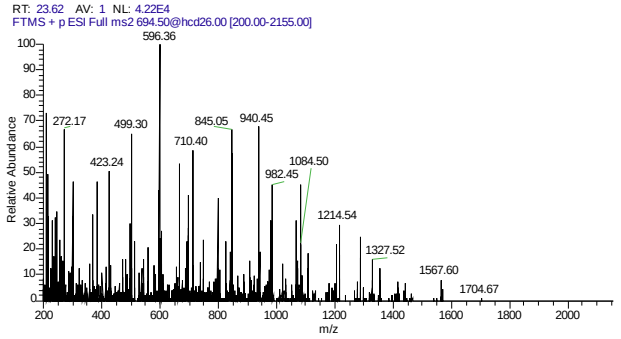


(s)

Neurexin-1 NRXN1_823-841
LTVDDQQAMTGQMAGDHTR

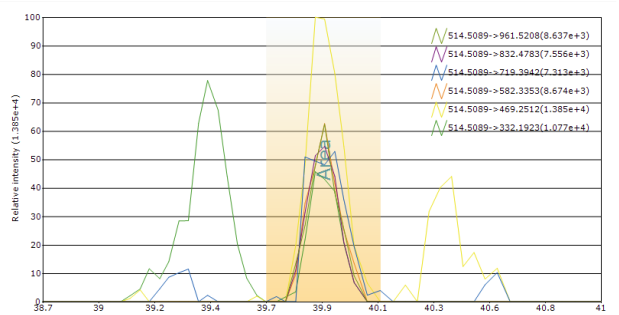


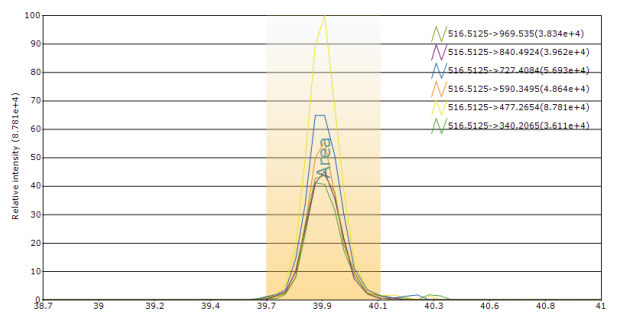


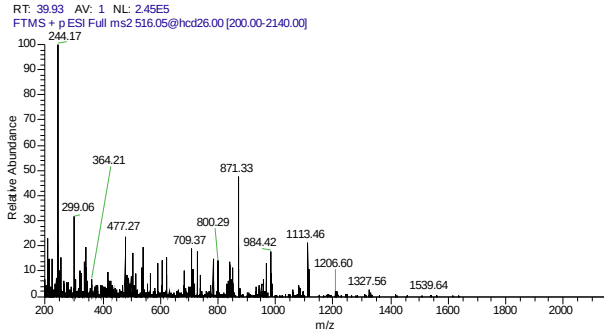


(t)

Neurexin-1 NRXN1_1168-1186
VDSSSGLGDYLELHIHQGK

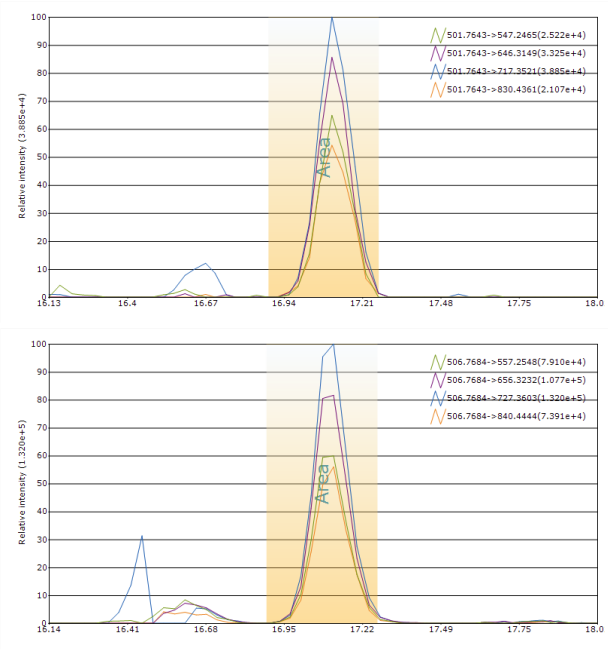


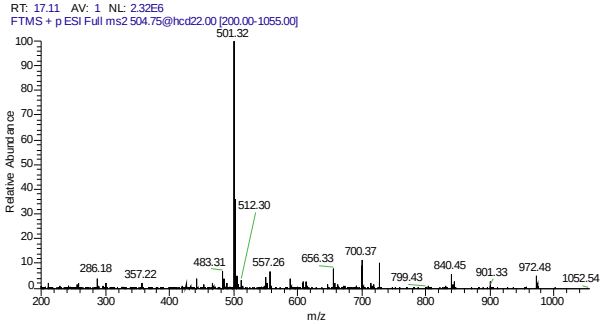




(u)

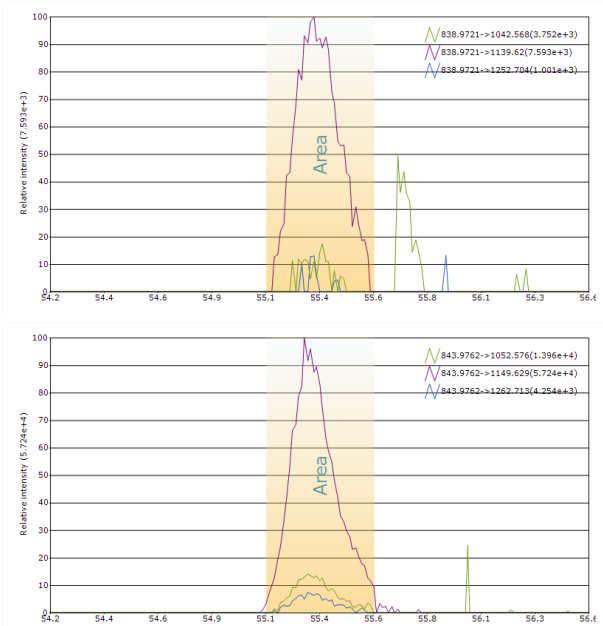
Neurexin-2 NRXN2_124-133
TALAVDGEAR

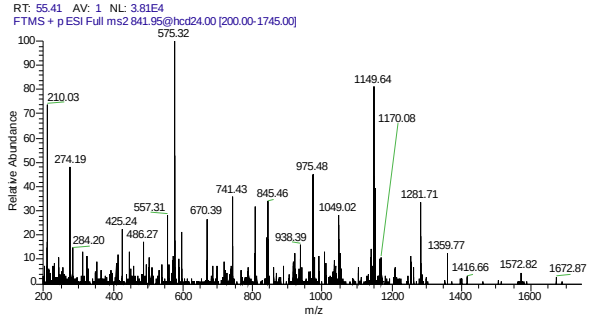




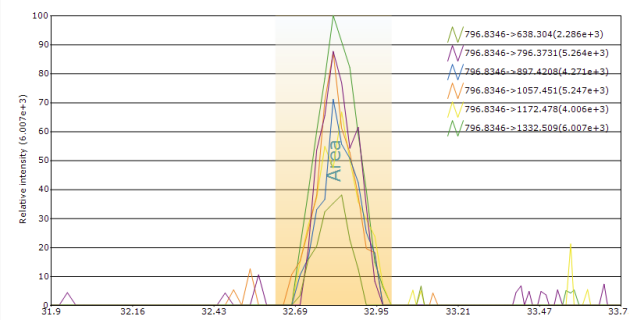
(v)

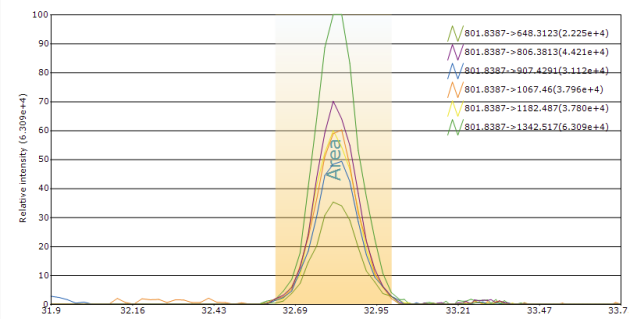
Neurexin-2 NRXN2_637-651
VDLPLPPEVWTAALR

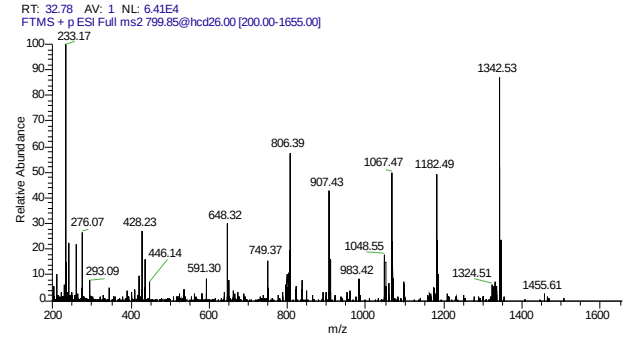




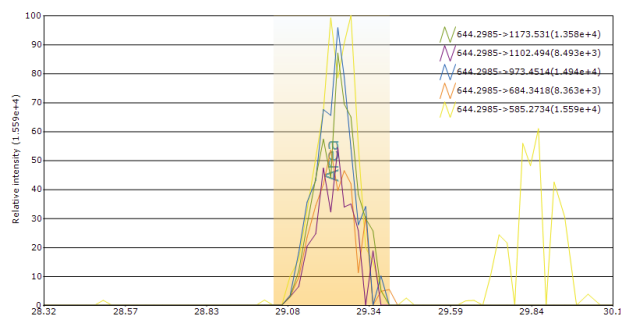
(w) Neurexin-3 NRXN3_665-677
FIcDcTGTGYWGR

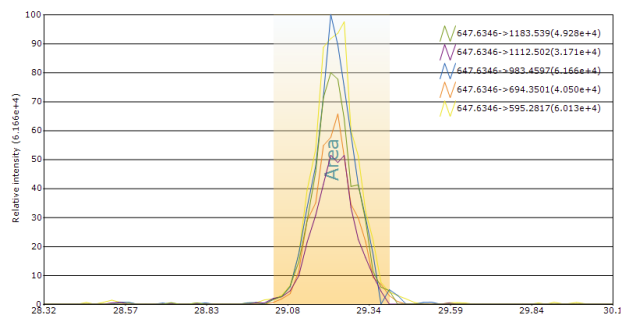


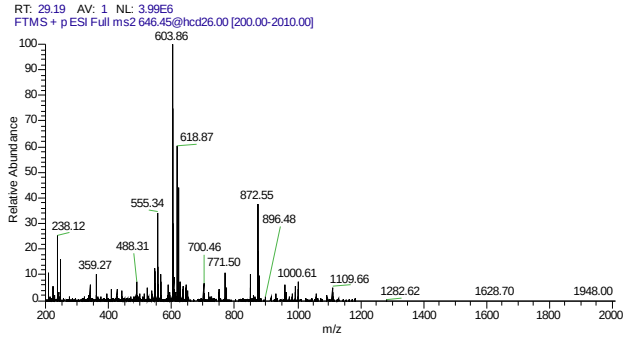




(x) Neurexin-3 NRXN3_790-807
LTVDDDVAEGTMVGDHTR

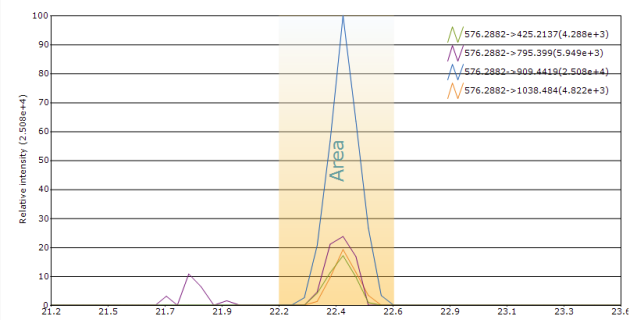


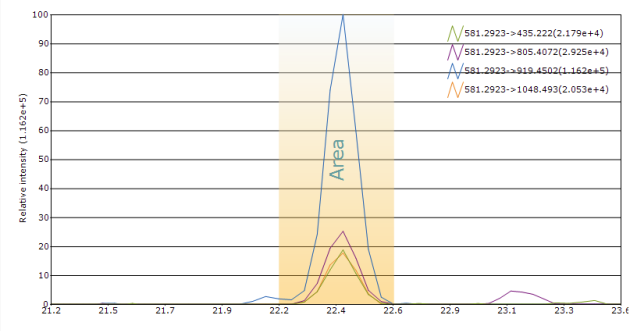


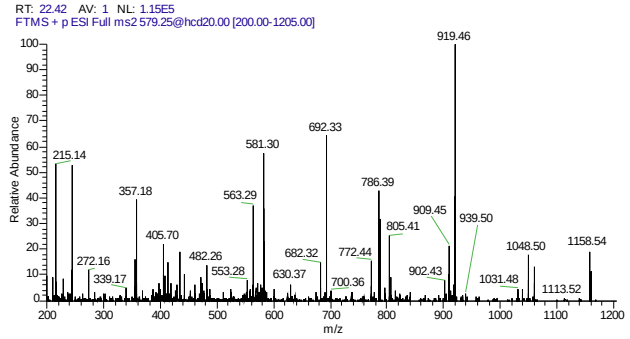


(y)

Neuronal pentraxin-1 NPTX1_144-152
LENLEQYSR

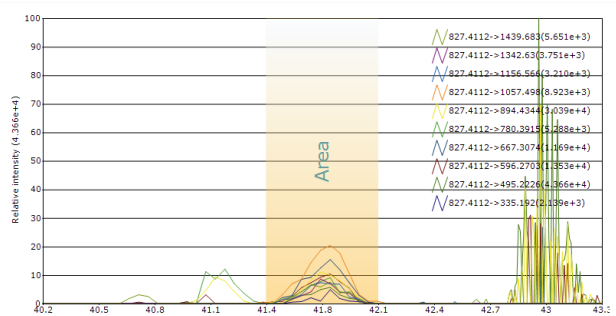


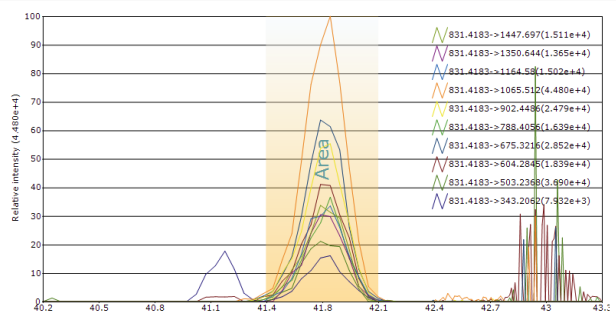


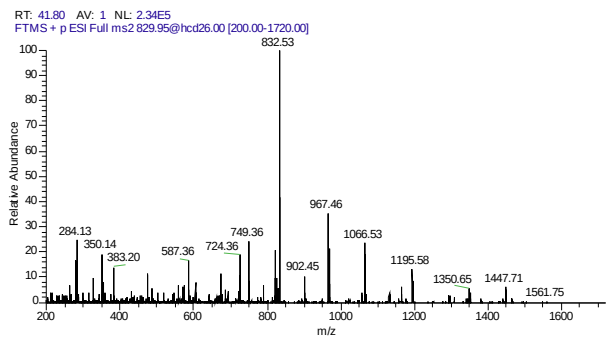


(z)

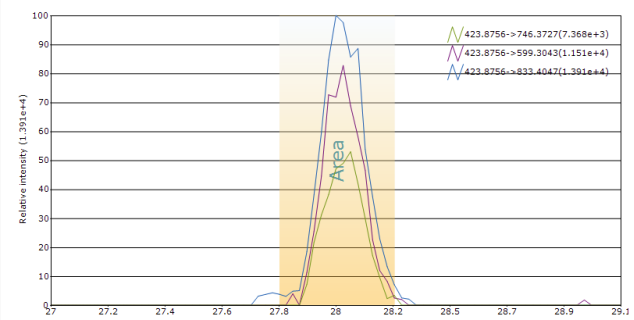
Neuronal pentraxin-1 NPTX1_386-400
LTPGEVYNLATcSTK

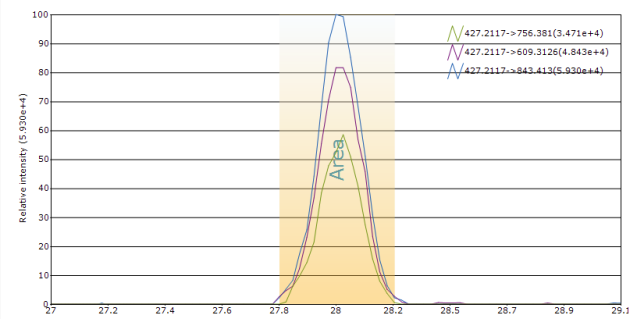


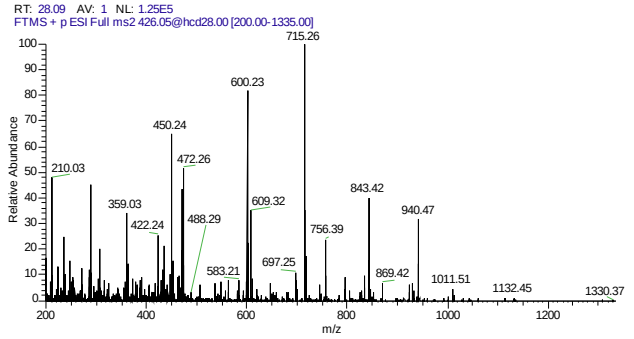




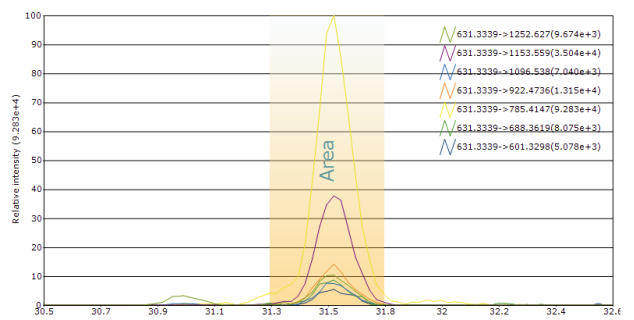
(aa) Neurofascin NFASC_67-77
GNPAPSFHWTR

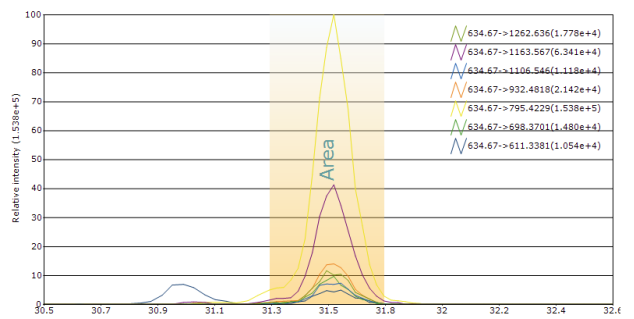


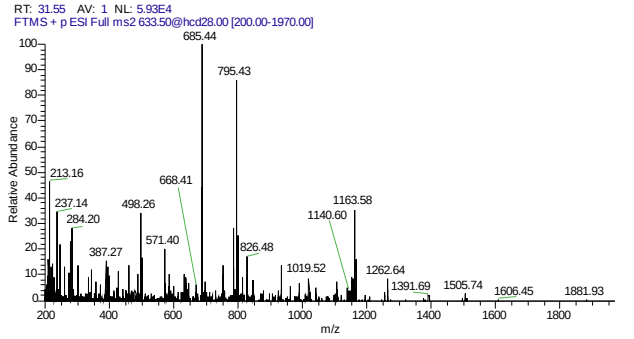




(ab) Neurofascin NFASC_702-719
VIAINEVGSSHPSLPSER

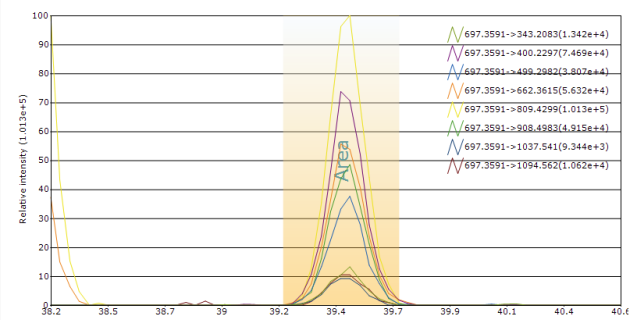


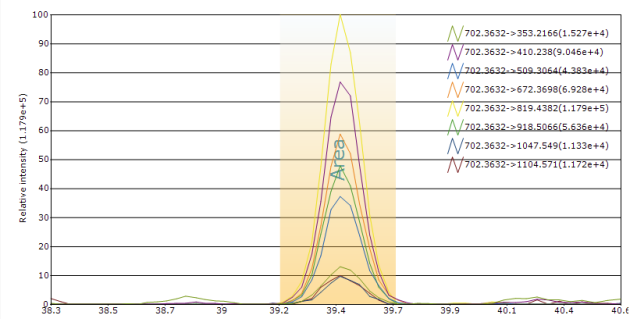


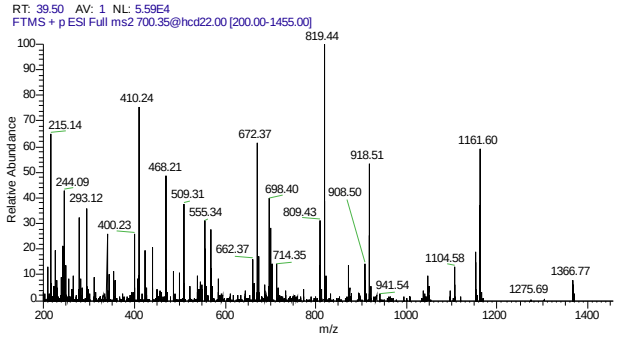


(ac)

Neurocan core protein NCANP_257-269
ELGGEVFYVGPAR

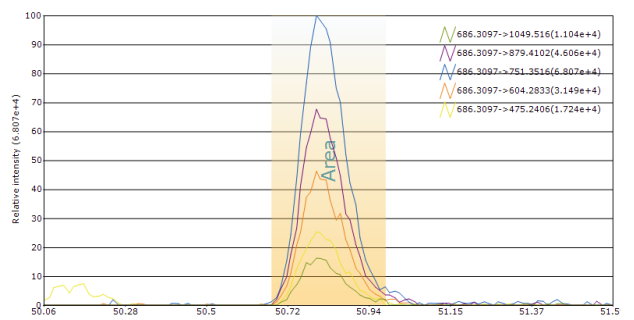


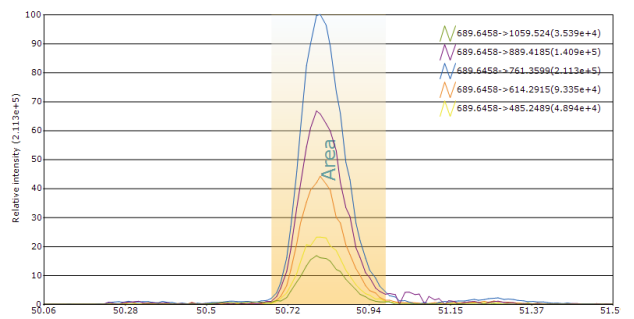


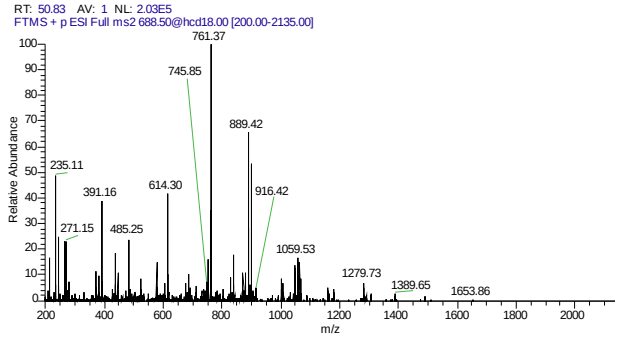


(ad)

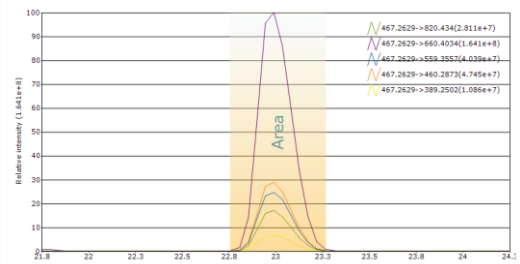
Neurocan core protein NCANP_257-269
ELGGEVFYVGPAR

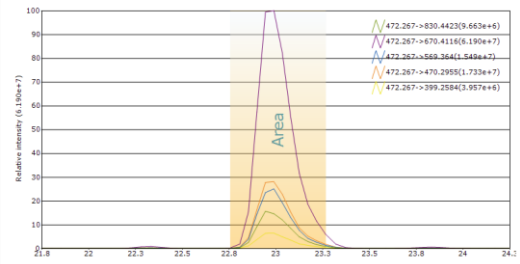


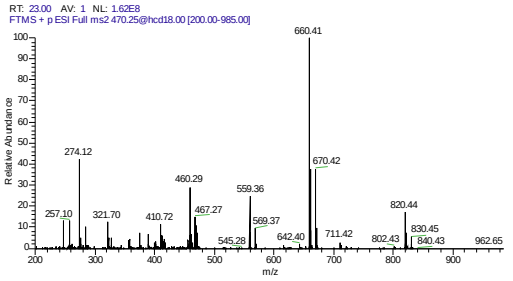




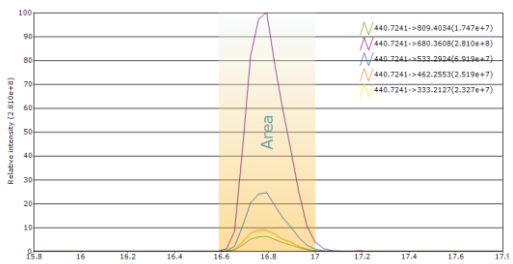
(ae) Albumin HSA_098-105
LcTVATLR

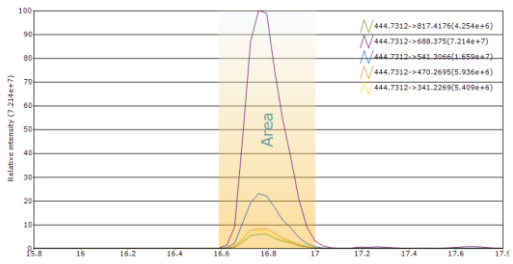


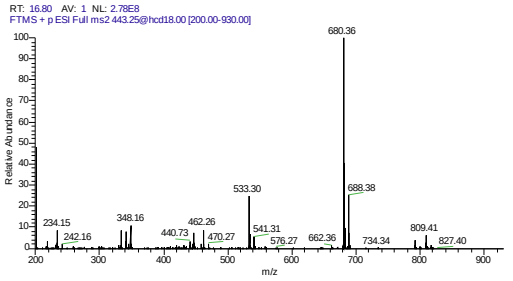




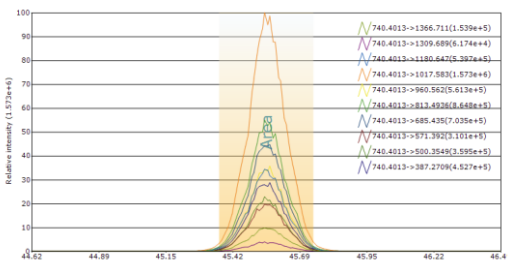
(af) Albumin HSA_250-257
AEFAEVSK

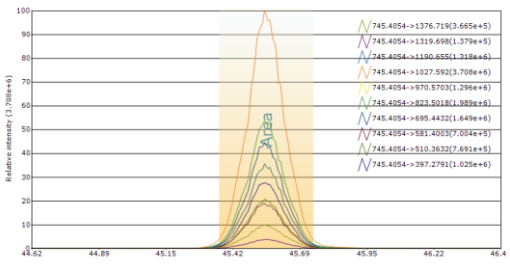


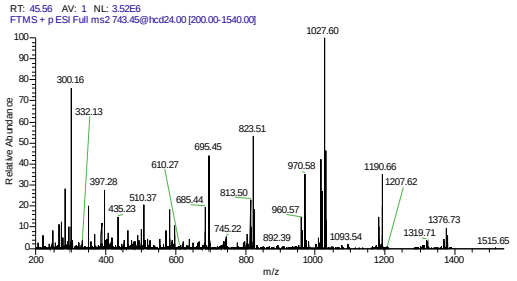




(ag) Bovine serum albumin BSA_421-433
LGEYGFQNALIVR







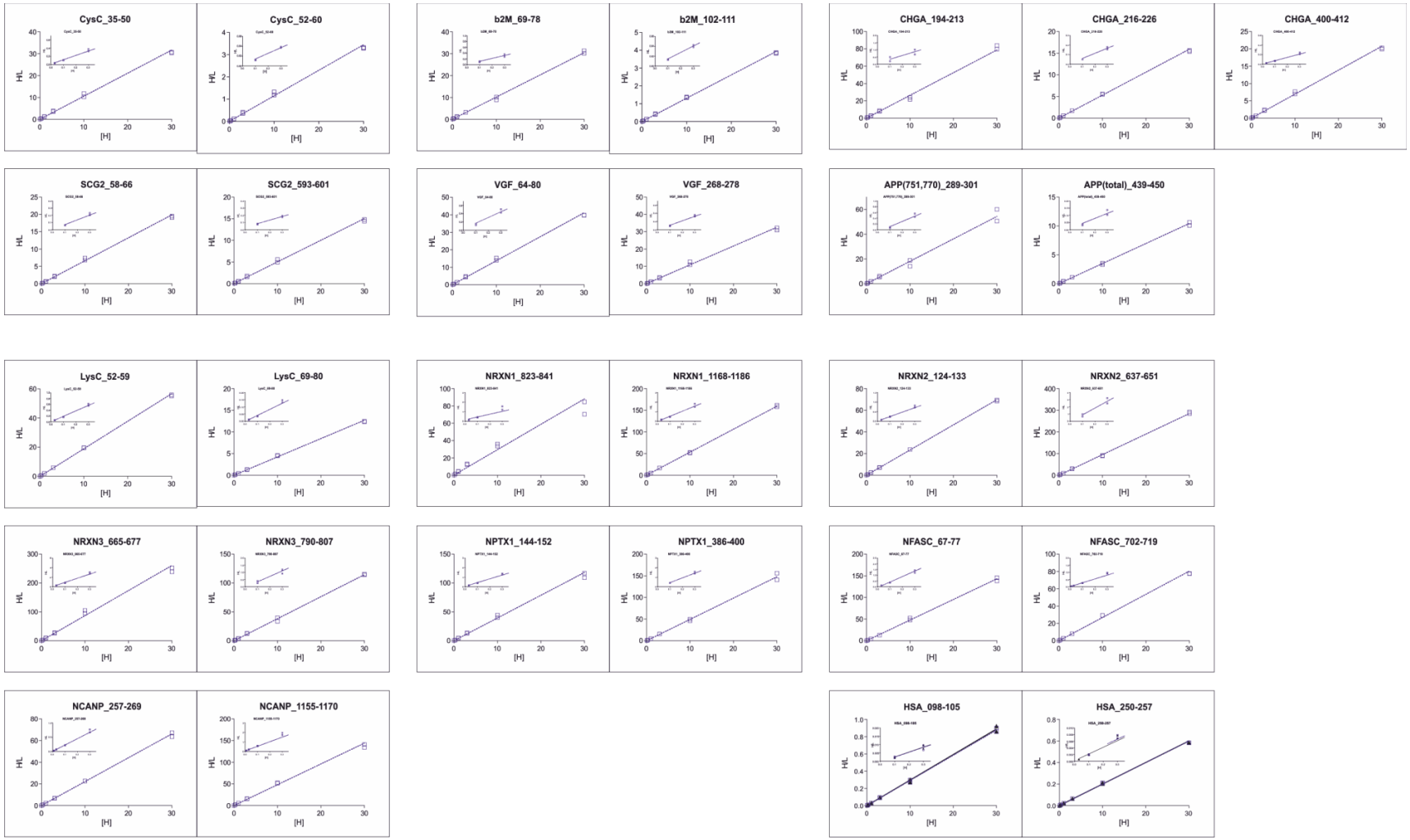


Figure S3. Individual peptide calibration curves for both panels. The graphs show the heavy-to-light peptide ratios (H/L) plotted vs the amount of spiked in heavy peptide (H). The human albumin peptides, which were included in both panels are presented as overlays.

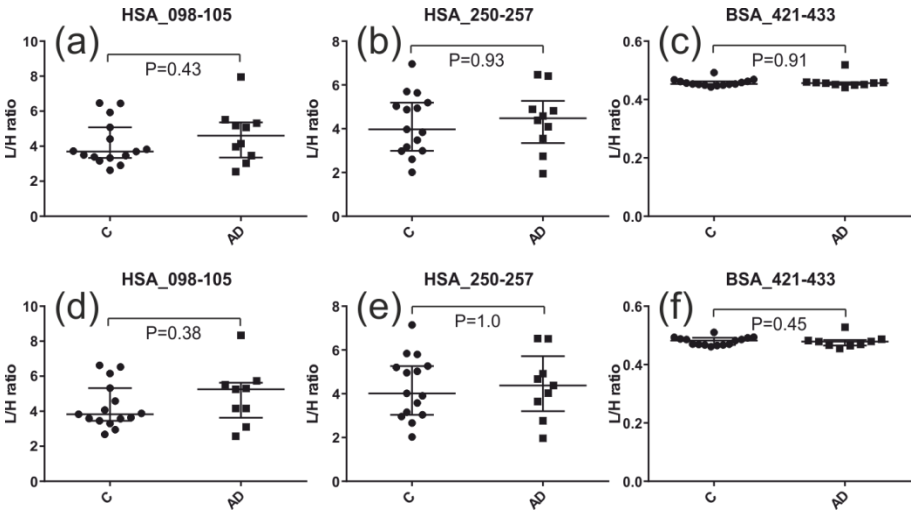


Figure S4. Difference in AD vs control groups for the human serum albumin peptides included in both panel 1 (a, b) and panel 2 (d, e). There was no significant difference between the groups for these peptides. For both panel 1 (c) and panel 2 (f) it can be concluded from the small variation of the bovine serum albumin control peptide used to monitor the tryptic digestion that the overall reproducibility was good. L/H ratio denotes light-to-heavy peptide ratio.

(1) Glenner, G. G.; Wong, C. W. Alzheimer's Disease: Initial Report of the Purification and Characterization of a Novel Cerebrovascular Amyloid Protein. *Biochem. Biophys. Res. Commun.* **1984**, *120*, 885-890.

(2) Iwatsubo, T.; Odaka, A.; Suzuki, N.; Mizusawa, H.; Nukina, N.; Ihara, Y. Visualization of Aβ42(43) and Aβ40 in Senile Plaques with End-specific Aβ Monoclonals: Evidence that an Initially Deposited Species is Aβ42(43). *Neuron* **1994**, *13*, 45-53.

(3) Tamaoka, A.; Kondo, T.; Odaka, A.; Sahara, N.; Sawamura, N.; Ozawa, K.; Suzuki, N.; Shoji, S.; Mori, H. Biochemical Evidence for the Long-Tail Form (Aβ1-42/43) of Amyloid β Protein as a Seed Molecule in Cerebral Deposits of Alzheimer′ s Disease. *Biochem. Biophys. Res. Commun.* **1994**, *205*, 834-842.

(4) Blennow, K.; de Leon, M. J.; Zetterberg, H. Alzheimer's Disease. *Lancet* **2006**, *368*, 387-403.

(5) Scheltens, P.; Blennow, K.; Breteler, M. M. B.; de Strooper, B.; Frisoni, G. B.; Salloway, S.; Van der Flier, W. M. Alzheimer's disease. *The Lancet* **2016**, *388*, 505-517.

(6) Salloway, S.; Sperling, R.; Fox, N. C.; Blennow, K.; Klunk, W.; Raskind, M.; Sabbagh, M.; Honig, L. S.; Porsteinsson, A. P.; Ferris, S.; Reichert, M.; Ketter, N.; Nejadnik, B.; Guenzler, V.; Miloslavsky, M.; Wang, D.; Lu, Y.; Lull, J.; Tudor, I. C.; Liu, E.; Grundman, M.; Yuen, E.; Black, R.; Brashear, H. R. Two Phase 3 Trials of Bapineuzumab in Mild-to-Moderate Alzheimer's Disease. *New England Journal of Medicine* **2014**, *370*, 322-333.

(7) Blennow, K.; Hampel, H.; Weiner, M.; Zetterberg, H. Cerebrospinal Fluid and Plasma Biomarkers in Alzheimer Disease. *Nature Reviews Neurology* **2010**, *6*, 131-144.

(8) Olsson, B.; Lautner, R.; Andreasson, U.; Öhrfelt, A.; Portelius, E.; Bjerke, M.; Hölttä, M.; Rosén, C.; Olsson, C.; Strobel, G.; Wu, E.; Dakin, K.; Petzold, M.; Blennow, K.; Zetterberg, H. CSF and blood biomarkers for the diagnosis of Alzheimer's disease: a systematic review and meta-analysis. *The Lancet Neurology* **2016**, *15*, 673-684.

(9) McKhann, G. M.; Knopman, D. S.; Chertkow, H.; Hyman, B. T.; Jack Jr, C. R.; Kawas, C. H.; Klunk, W. E.; Koroshetz, W. J.; Manly, J. J.; Mayeux, R.; Mohs, R. C.; Morris, J. C.; Rossor, M. N.; Scheltens, P.; Carrillo, M. C.; Thies, B.; Weintraub, S.; Phelps, C. H. 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. *Alzheimer's & Dementia* **2011**, *7*, 263-269.

(10) Dubois, B.; Feldman, H. H.; Jacova, C.; Hampel, H.; Molinuevo, J. L.; Blennow, K.; DeKosky, S. T.; Gauthier, S.; Selkoe, D.; Bateman, R.; Cappa, S.; Crutch, S.; Engelborghs, S.; Frisoni, G. B.; Fox, N. C.; Galasko, D.; Habert, M.-O.; Jicha, G. A.; Nordberg, A.; Pasquier, F.; Rabinovici, G.; Robert, P.; Rowe, C.; Salloway, S.; Sarazin, M.; Epelbaum, S.; de Souza, L. C.; Vellas, B.; Visser, P. J.; Schneider, L.; Stern, Y.; Scheltens, P.; Cummings, J. L. Advancing research diagnostic criteria for Alzheimer's disease: the IWG-2 criteria. *The Lancet Neurology* **2014**, *13*, 614-629.

(11) Shaw, L. M.; Vanderstichele, H.; Knapik-Czajka, M.; Clark, C. M.; Aisen, P. S.; Petersen, R. C.; Blennow, K.; Soares, H.; Simon, A.; Lewczuk, P.; Dean, R.; Siemers, E.; Potter, W.; Lee, V. M. Y.; Trojanowski, J. Q. Cerebrospinal fluid biomarker signature in Alzheimer's disease neuroimaging initiative subjects. *Annals of Neurology* **2009**, *65*, 403-413.

(12) Brinkmalm, A.; Brinkmalm, G.; Honer, W. G.; Moreno, J. A.; Jakobsson, J.; Mallucci, G. R.; Zetterberg, H.; Blennow, K.; Öhrfelt, A. Targeting Synaptic Pathology with a Novel Affinity Mass Spectrometry Approach. *Mol. Cell. Proteomics* **2014**, *Online manuscript*.

(13) Brinkmalm, A.; Brinkmalm, G.; Honer, W. G.; Frölich, L.; Hausner, L.; Minthon, L.; Hansson, O.; Wallin, A.; Zetterberg, H.; Blennow, K.; Öhrfelt, A. SNAP-25 is a promising novel cerebrospinal fluid biomarker for synapse degeneration in Alzheimer's disease. *Molecular Degeneration* **2014**, *9:53*.

(14) Kvartsberg, H.; Duits, F. H.; Ingelsson, M.; Andreasen, N.; Öhrfelt, A.; Andersson, K.; Brinkmalm, G.; Lannfelt, L.; Minthon, L.; Hansson, O.; Andreasson, U.; Teunissen, C. E.; Scheltens, P.; Van der Flier, W. M.; Zetterberg, H.; Portelius, E.; Blennow, K. Cerebrospinal fluid levels of the synaptic protein neurogranin correlates with cognitive decline in prodromal Alzheimer's disease. *Alzheimer's & Dementia* **2015**, *11*, 1180-1190.

(15) Wyss-Coray, T.; Rogers, J. Inflammation in Alzheimer Disease—A Brief Review of the Basic Science and Clinical Literature. *Cold Spring Harbor Perspectives in Medicine* **2012**, *2*.

(16) Wang, X.; Wang, W.; Li, L.; Perry, G.; Lee, H.-g.; Zhu, X. Oxidative stress and mitochondrial dysfunction in Alzheimer's disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **2014**, *1842*, 1240-1247.

(17) Zetterberg, H.; Rüetschi, U.; Portelius, E.; Brinkmalm, G.; Andreasson, U.; Blennow, K.; Brinkmalm, A. Clinical Proteomics for Biomarker Discovery in Neurodegenerative Disorders. *Acta Neurologica Scandinavica* **2008**, *118*, 1-11.

(18) Brinkmalm, A.; Portelius, E.; Öhrfelt, A.; Brinkmalm, G.; Andreasson, U.; Gobom, J.; Blennow, K.; Zetterberg, H. Explorative and targeted neuroproteomics in Alzheimer's disease. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **2015**, *1854*, 769-778.

(19) Peterson, A. C.; Russell, J. D.; Bailey, D. J.; Westphall, M. S.; Coon, J. J. Parallel Reaction Monitoring for High Resolution and High Mass Accuracy Quantitative, Targeted Proteomics. *Mol. Cell. Proteomics* **2012**, *11*, 1475-1488.

(20) Gallien, S.; Duriez, E.; Crone, C.; Kellmann, M.; Moehring, T.; Domon, B. Targeted Proteomic Quantification on Quadrupole-Orbitrap Mass Spectrometer. *Mol. Cell. Proteomics* **2012**, *11*, 1709-1723.

(21) Pannee, J.; Portelius, E.; Oppermann, M.; Atkins, A.; Hornshaw, M.; Zegers, I.; Højrup, P.; Minthon, L.; Hansson, O.; Zetterberg, H.; Blennow, K.; Gobom, J. A Selected Reaction Monitoring (SRM)-Based Method for Absolute Quantification of $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ in Cerebrospinal Fluid of Alzheimer's Disease Patients and Healthy Controls. *Journal of Alzheimer's Disease* **2013**, *33*, 1021-1032.

(22) Pannee, J.; Törnqvist, U.; Westerlund, A.; Ingelsson, M.; Lannfelt, L.; Brinkmalm, G.; Persson, R.; Gobom, J.; Svensson, J.; Johansson, P.; Zetterberg, H.; Blennow, K.; Portelius, E. The amyloid-β degradation pattern in plasma—A possible tool for clinical trials in Alzheimer's disease. *Neuroscience Letters* **2014**, *573*, 7-12.

(23) Bartolomucci, A.; Possenti, R.; Mahata, S. K.; Fischer-Colbrie, R.; Loh, Y. P.; Salton, S. R. J. The Extended Granin Family: Structure, Function, and Biomedical Implications. *Endocrine Reviews* **2011**, *32*, 755-797.

(24) Marksteiner, J.; Kaufmann, W. A.; Gurka, P.; Humpel, C. Synaptic proteins in Alzheimer's disease. *Journal of Molecular Neuroscience* **2002**, *18*, 55-63.

(25) Willis, M.; Leitner, I.; Jellinger, K. A.; Marksteiner, J. Chromogranin Peptides in Brain Diseases. *Journal of Neuronal Transmission* **2011**, *118*, 727-735.

(26) Mattsson, N.; Insel, P.; Nosheny, R.; Zetterberg, H.; Trojanowski, J. Q.; Shaw, L. M.; Tosun, D.; Weiner, M. CSF protein biomarkers predicting longitudinal reduction of CSF β-amyloid42 in cognitively healthy elders. *Transl Psychiatry* **2013**, *3*, e293.

(27) Blennow, K.; Davidsson, P.; Wallin, A.; Ekman, R. Chromogranin A in Cerebrospinal Fluid: A Biochemical Marker for Synaptic Degeneration in Alzheimer's Disease? *Dementia and Geriatric Cognitive Disorders* **1995**, *6*, 306-311.

(28) O'Connor, D. T.; Kailasam, M. T.; Thal, L. J. Cerebrospinal fluid chromogranin A is unchanged in Alzheimer dementia. *Neurobiology of Aging* **1993**, *14*, 267-269.

(29) Perrin, R. J.; Craig-Schapiro, R.; Malone, J. P.; Shah, A. R.; Gilmore, P.; Davis, A. E.; Roe, C. M.; Peskind, E. R.; Li, G.; Galasko, D. R.; Clark, C. M.; Quinn, J. F.; Kaye, J. A.; Morris, J. C.; Holtzman, D. M.; Townsend, R. R.; Fagan, A. M. Identification and Validation of Novel Cerebrospinal Fluid Biomarkers for Staging Early Alzheimer's Disease. *PLOS ONE* **2011**, *6*, e16032.

(30) Sun, B.; Zhou, Y.; Halabisky, B.; Lo, I.; Cho, S.-H.; Mueller-Steiner, S.; Devidze, N.; Wang, X.; Grubb, A.; Gan, L. Cystatin C-Cathepsin B Axis Regulates Amyloid Beta Levels and Associated Neuronal Deficits in an Animal Model of Alzheimer's Disease. *Neuron* **2008**, *60*, 247-257.

(31) Wang, X.-F.; Liu, D.-X.; Liang, Y.; Xing, L.-L.; Zhao, W.-H.; Qin, X.-X.; Shang, D.-S.; Li, B.; Fang, W.-G.; Cao, L.; Zhao, W.-D.; Chen, Y.-H. Cystatin C Shifts APP Processing from Amyloid-β Production towards Non-Amyloidgenic Pathway in Brain Endothelial Cells. *PLOS ONE* **2016**, *11*, e0161093.

(32) Mi, W.; Pawlik, M.; Sastre, M.; Jung, S. S.; Radvinsky, D. S.; Klein, A. M.; Sommer, J.; Schmidt, S. D.; Nixon, R. A.; Mathews, P. M.; Levy, E. Cystatin C inhibits amyloid-[beta] deposition in Alzheimer's disease mouse models. *Nat Genet* **2007**, *39*, 1440-1442.

(33) Svatoňová, J.; Bořecká, K.; Adam, P.; Lánská, V. Beta2-Microglobulin as a Diagnostic Marker in Cerebrospinal Fluid: A Follow-Up Study. *Disease Markers* **2014**, *2014*, 6.

(34) Palmert, M. R.; Usiak, M.; Mayeux, R.; Raskind, M.; Tourtellotte, W. W.; Younkin, S. G. Soluble Derivatives of the β Amyloid Protein Precursor in Cerebrospinal Fluid: Alterations in Normal Aging and in Alzheimer's Disease. *Neurology* **1990**, *40*, 1028-1034.

(35) Hock, C.; Golombowski, S.; Muller-Spahn, F.; Naser, W.; Beyreuther, K.; Monning, U.; Schenk, D.; Vigo-Pelfrey, C.; Bush, A. M.; Moir, R.; Tanzi, R. E.; Growdon, J. H.; Nitsch, R. M. Cerebrospinal Fluid Levels of Amyloid Precursor Protein and Amyloid β-Peptide in Alzheimer's Disease and Major Depression - Inverse Correlation with Dementia Severity. *European Neurology* **1998**, *39*, 111-118.

(36) Olsson, A.; Höglund, K.; Sjögren, M.; Andreasen, N.; Minthon, L.; Lannfelt, L.; Buerger, K.; Möller, H.-J.; Hampel, H.; Davidsson, P.; Blennow, K. Measurement of α- and β-Secretase Cleaved Amyloid Precursor Protein in Cerebrospinal Fluid from Alzheimer Patients. *Exp. Neurol.* **2003**, *183*, 74-80.

(37) Zetterberg, H.; Andreasson, U.; Hansson, O.; Wu, G.; Sankaranarayanan, S.; Andersson, M. E.; Buchhave, P.; Londos, E.; Umek, R. M.; Minthon, L.; Simon, A. J.; Blennow, K. Elevated Cerebrospinal Fluid BACE1 Activity in Incipient Alzheimer Disease. *Archives of Neurology* **2008**, *65*, 1102-1107.

(38) Prior, R.; Monning, U.; Schreiter-Gasser, U.; Weidemann, A.; Blennow, K.; Gottfries, C. G.; Masters, C. L.; Beyreuther, K. Quantitative Changes in the Amyloid βA4 Precursor Protein in Alzheimer Cerebrospinal Fluid. *Neuroscience Letters* **1991**, *124*, 69-73.

(39) Van Nostrand, W. E.; Wagner, S. L.; Shankle, W. R.; Farrow, J. S.; Dick, M.; Rozemuller, J. M.; Kuiper, M. A.; Wolters, E. C.; Zimmerman, J.; Cotman, C. W. Decreased Levels of Soluble Amyloid Beta-protein Precursor in Cerebrospinal Fluid of Live Alzheimer Disease Patients. *Proceedings of the National Academy of Sciences of the United States of America* **1992**, *89*, 2551-2555.

(40) Sennvik, K.; Fastbom, J.; Blomberg, M.; Wahlund, L.-O.; Winblad, B.; Benedikz, E. Levels of α- and β-Secretase Cleaved Amyloid Precursor Protein in the Cerebrospinal Fluid of Alzheimer's Disease Patients. *Neuroscience Letters* **2000**, *278*, 169-172.

(41) Perneczky, R.; Tsolakidou, A.; Arnold, A.; Diehl-Schmid, J.; Grimmer, T.; Förstl, H.; Kurz, A.; Alexopoulos, P. CSF Soluble Amyloid Precursor Proteins in the Diagnosis of Incipient Alzheimer Disease. *Neurology* **2011**, *77*, 35-38.

(42) Brinkmalm, G.; Brinkmalm, A.; Bourgeois, P.; Persson, R.; Hansson, O.; Portelius, E.; Mercken, M.; Andreasson, U.; Parent, S.; Lipari, F.; Öhrfelt, A.; Bjerke, M.; Minthon, L.; Zetterberg, H.; Blennow, K.; Nutu, M. Soluble Amyloid Precursor Protein α and β in CSF in Alzheimer's Disease. *Brain Research* **2013**, *1513*, 117-126.

(43) Firth, G.; Rees, J.; McKeran, R. O. The value of the measurement of cerebrospinal fluid levels of lysozyme in the diagnosis of neurological disease. *Journal of Neurology, Neurosurgery & Psychiatry* **1985**, *48*, 709-712.

(44) Helmfors, L.; Boman, A.; Civitelli, L.; Nath, S.; Sandin, L.; Janefjord, C.; McCann, H.; Zetterberg, H.; Blennow, K.; Halliday, G.; Brorsson, A.-C.; Kågedal, K. Protective properties of lysozyme on β-amyloid pathology: implications for Alzheimer disease. *Neurobiology of Disease* **2015**, *83*, 122-133.

(45) Dean, C.; Scholl, F. G.; Choih, J.; DeMaria, S.; Berger, J.; Isacoff, E.; Scheiffele, P. Neurexin mediates the assembly of presynaptic terminals. *Nat Neurosci* **2003**, *6*, 708-716.

(46) Schreiner, D.; Simicevic, J.; Ahrné, E.; Schmidt, A.; Scheiffele, P. Quantitative isoform-profiling of highly diversified recognition molecules. *eLife* **2015**, *4*, e07794.

(47) Osera, C.; Pascale, A.; Amadio, M.; Venturini, L.; Govoni, S.; Ricevuti, G. Pentraxins and Alzheimer's disease: At the interface between biomarkers and pharmacological targets. *Ageing Research Reviews* **2012**, *11*, 189-198.

(48) Xu, D.; Hopf, C.; Reddy, R.; Cho, R. W.; Guo, L.; Lanahan, A.; Petralia, R. S.; Wenthold, R. J.; O'Brien, R. J.; Worley, P. Narp and NP1 Form Heterocomplexes that Function in Developmental and Activity-Dependent Synaptic Plasticity. *Neuron* **2003**, *39*, 513-528.

(49) Abad, M. A.; Enguita, M.; DeGregorio-Rocasolano, N.; Ferrer, I.; Trullas, R. Neuronal Pentraxin 1 Contributes to the Neuronal Damage Evoked by Amyloid-β and Is Overexpressed in Dystrophic Neurites in Alzheimer's Brain. *The Journal of Neuroscience* **2006**, *26*, 12735-12747.

(50) Zonta, B.; Desmazieres, A.; Rinaldi, A.; Tait, S.; Sherman, Diane L.; Nolan, Matthew F.; Brophy, Peter J. A Critical Role for Neurofascin in Regulating Action Potential Initiation through Maintenance of the Axon Initial Segment. *Neuron* **2011**, *69*, 945-956.

(51) Kriebel, M.; Wuchter, J.; Trinks, S.; Volkmer, H. Neurofascin: A switch between neuronal plasticity and stability. *The International Journal of Biochemistry & Cell Biology* **2012**, *44*, 694-697.

(52) McKeon, R. J.; Jurynec, M. J.; Buck, C. R. The Chondroitin Sulfate Proteoglycans Neurocan and Phosphacan Are Expressed by Reactive Astrocytes in the Chronic CNS Glial Scar. *The Journal of Neuroscience* **1999**, *19*, 10778-10788.

(53) Yan, H.; Zhu, X.; Xie, J.; Zhao, Y.; Liu, X. β-amyloid increases neurocan expression through regulating Sox9 in astrocytes: A potential relationship between Sox9 and chondroitin sulfate proteoglycans in Alzheimer's disease. *Brain Research* **2016**, *1646*, 377-383.

(54) Makarov, A. Personal Communication, 2014.

(55) Sjödin, S.; Öhrfelt, A.; Brinkmalm, G.; Zetterberg, H.; Blennow, K.; Brinkmalm, A. Targeting LAMP2 in Human Cerebrospinal Fluid with a Combination of Immunopurification and High Resolution Parallel Reaction Monitoring Mass Spectrometry. *Clinical Proteomics* **2016**, *13*.

(56) Barthélemy, N. R.; Fenaille, F.; Hirtz, C.; Sergeant, N.; Schraen-Maschke, S.; Vialaret, J.; Buée, L.; Gabelle, A.; Junot, C.; Lehmann, S.; Becher, F. Tau Protein Quantification in Human Cerebrospinal Fluid by Targeted Mass Spectrometry at High Sequence Coverage Provides Insights into Its Primary Structure Heterogeneity. *Journal of Proteome Research* **2016**, *15*, 667-676.

(57) Gallien, S.; Bourmaud, A.; Kim, S. Y.; Domon, B. Technical considerations for large-scale parallel reaction monitoring analysis. *Journal of Proteomics* **2014**, *100*, 147-159.

(58) Davidsson, P.; Westman-Brinkmalm, A.; Nilsson, C. L.; Lindbjer, M.; Paulson, L.; Andreasen, N.; Sjögren, M.; Blennow, K. Proteome analysis of cerebrospinal fluid proteins in Alzheimer patients. *NeuroReport* **2002**, *13*, 611-615.

(59) Carrette, O.; Demalte, I.; Scherl, A.; Yalkinoglu, O.; Corthals, G.; Burkhard, P.; Hochstrasser, D. F.; Sanchez, J.-C. A panel of cerebrospinal fluid potential biomarkers for the diagnosis of Alzheimer's disease. *PROTEOMICS* **2003**, *3*, 1486-1494.

(60) Zhang, Y.; Thompson, R.; Zhang, H.; Xu, H. APP Processing in Alzheimer's Disease. *Molecular Brain* **2011**, *4*, 3.

(61) Menéndez-González, M.; Pérez-Pinera, P.; Martínez-Rivera, M.; Calatayud, M. T.; Blázquez Menes, B. APP Processing and the APP-KPI Domain Involvement in the Amyloid Cascade. *Neurodegenerative Diseases* **2005**, *2*, 277-283.

(62) Moir, R. D.; Lynch, T.; Bush, A. I.; Whyte, S.; Henry, A.; Portbury, S.; Multhaup, G.; Small, D. H.; Tanzi, R. E.; Beyreuther, K.; Masters, C. L. Relative Increase in Alzheimer's Disease of Soluble Forms of Cerebral Aβ Amyloid Protein Precursor Containing the Kunitz Protease Inhibitory Domain. *J. Biol. Chem.* **1998**, *273*, 5013-5019.

(63) Hansson, S. F.; Andréasson, U.; Wall, M.; Skoog, I.; Andreasen, N.; Wallin, A.; Zetterberg, H.; Blennow, K. Reduced Levels of Amyloid-β-Binding Proteins in Cerebrospinal Fluid from Alzheimer's Disease Patients *Journal of Alzheimer's Disease* **2009**, *16*, 389-397.

(64) Hölttä, M.; Minthon, L.; Hansson, O.; Holmén-Larsson, J.; Pike, I.; Ward, M.; Kuhn, K.; Rüetschi, U.; Zetterberg, H.; Blennow, K.; Gobom, J. An Integrated Workflow for Multiplex CSF Proteomics and Peptidomics—Identification of Candidate Cerebrospinal Fluid Biomarkers of Alzheimer's Disease. *Journal of Proteome Research* **2015**, *14*, 654-663.

(65) Simonsen, A. H.; McGuire, J.; Podust, V. N.; Davies, H.; Minthon, L.; Skoog, I.; Andreasen, N.; Wallin, A.; Waldemar, G.; Blennow, K. Identification of a novel panel of cerebrospinal fluid biomarkers for Alzheimer's disease. *Neurobiology of Aging* **2008**, *29*, 961-968.

(66) Jahn, H.; Wittke, S.; Zürbig, P.; Raedler, T. J.; Arlt, S.; Kellmann, M.; Mullen, W.; Eichenlaub, M.; Mischak, H.; Wiedemann, K. Peptide Fingerprinting of Alzheimer's Disease in Cerebrospinal Fluid: Identification and Prospective Evaluation of New Synaptic Biomarkers. *PLOS ONE* **2011**, *6*, e26540.

(67) Hansson, K. T.; Skillbäck, T.; Pernevik, E.; Kern, S.; Portelius, E.; Höglund, K.; Brinkmalm, G.; Holmén-Larsson, J.; Blennow, K.; Zetterberg, H.; Gobom, J. Expanding the cerebrospinal fluid endopeptidome. *Proteomics* **2017**, *Online 170103, accepted manuscript*.