

Label Free Quantitative Comparison of Cerebrospinal Fluid Glycoproteins and Endogenous Peptides in Subjects with Alzheimer's disease, Mild Cognitive Impairment and Healthy Individuals

Wang J, Cunningham R, ZETTERBERG H, Asthana S, Carlsson C, Okonkwo O, Li L

¹Neuroscience Training Program, University of Wisconsin-Madison, Madison, WI, USA,

²Thermo Fisher Scientific, Rockford, IL

³Wisconsin Alzheimer's Disease Research Center, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA.

⁴Geriatric Research Education and Clinical Center, Wm. S. Middleton Veterans Hospital, Madison, WI, USA.

⁵Wisconsin Alzheimer's Institute, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA.

⁶School of Pharmacy, University of Wisconsin-Madison, Madison, WI, USA,

⁷Department of Chemistry, University of Wisconsin-Madison, Madison, WI, USA.

*Corresponding author

Tel.: +1 (608) 265-8491

Fax: +1 (608) 262-5345

E-mail: lingjun.li@wisc.edu

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Abbreviations

AD, Alzheimer's disease;

MCI, mild cognitive impairment

LAC, lectin affinity chromatography

MWCO, molecular weight cut-off

CSF, cerebrospinal fluid

ConA, concanavalin-A

WGA, wheat germ agglutinin

AUD, area under curve

TTR, transthyretin

ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia in the elderly. It is fundamentally necessary to discover and establish reliable and accurate biomarkers that are capable of predicting, diagnosing and monitoring disease progression. Here we report on a set of glycoproteins and endogenous peptides identified and compared in cerebrospinal fluid (CSF) from cognitively healthy individuals and patients with mild cognitive impairment or AD via mass spectrometry (MS)-based strategies. Using an optimized sub-microgram peptide separation with molecular weight cut-off filtration and an in house-constructed database, 645 peptides were identified. Glycoproteins were enriched by lectin affinity chromatography, resulting in 795 identified peptides. The discovery and alterations of ProSAAS-derived peptides and transthyretin are described and their roles in AD are discussed.

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia in the elderly population and the 6th leading cause of death in the United States. More than 36.5 million people were estimated to suffer from dementia in 2010, and there are 7.7 million new cases each year. Due to the aging baby boom generation, it is predicted that the number of people with Alzheimer's disease age 65 and older may triple by 2050, costing an estimated \$1.2 trillion.¹ AD is characterized by progressive accumulation of extracellular β -amyloid ($A\beta$) peptides and intracellular neurofibrillary tangles of protein tau, in addition to synaptic and neuronal loss in the brain.² Currently, the diagnosis of AD is mainly based on the history of a patient or an objective cognitive assessment and the exclusion of dementia due to other causes such as Lewy body dementia, vascular

dementia, frontotemporal dementia, Creutzfeldt-Jakob disease (CJD), etc.³ Three AD biomarkers: A β 1-42, total tau (t-tau) and phosphorylated tau (p-tau) in the cerebrospinal fluid (CSF) have been used to support a clinical diagnosis.^{4,5,6,7} These biomarkers may increase the certainty of AD pathophysiological process in patients who have already met the critical clinical criteria for probable AD dementia. However, additional markers are needed; one limitation of the current CSF tau and A β markers is that they do not change with clinical disease progression but are fully altered already in the mild cognitive impairment stage of the disease (ref: Blennow K et al., Nat Rev Neurol 2010). Moreover, they do not reflect pathophysiological processes, other than plaque and tangle pathology, that may be of importance. Specifically, the recently reported failure of a phase 3 clinical trial of humanized monoclonal antibody of soluble forms of amyloid^{8,9} necessitates the discovery and establishment of other biomarkers and targets for disease prevention and/or treatment.

CSF bathes the brain and spinal cord which makes it a valuable biological fluid for biomarker studies of neurodegenerative diseases.¹⁰ Although CSF sampling may be considered an invasive, the procedure is well tolerated by patients and the only complication, provided that standard contraindications are considered, is post-lumbar puncture headache, which is a rare event in the elderly (Blennow K et al., Nature Rev Neurol 2010). Approximately 70-80% of the CSF is generated in the choroid plexus as a plasma filtrate. The remaining 20-30% is derived from the brain interstitial fluid and there are no barriers between the CSF and the brain extracellular matrix. The total CSF volume is around 150 mL; the CSF production and clearance rates are 20 mL per hour

and in a regular lumbar puncture 10-20 mL is collected, which is then rapidly replenished.¹³ Blennow 2010 Nat Rev Neurol Despite the differences between CSF and plasma the proteome of both share the vast majority of abundant protein identifications, although it is estimated that around 20% of the CSF proteome are produced by brain itself.^{11, 12} CSF is reabsorbed into the blood four times per day and due to the constant turnover the dynamic changes of CSF composition is able to reflect the disease status of the central nervous system (CNS). In a clinical setting, the lumbar puncture is a routine procedure for the diagnosis of multiple sclerosis, Guillain-Barré syndrome and CJD etc. Previous large scale proteomic studies have greatly filled in the gap of our knowledge about CSF protein composition by two dimensional gel,^{14, 15, 16, 17} chromatography and mass spectrometry.^{18, 19, 20, 21, 22}

Glycosylation is one of the most complicated but common forms of post-translational modifications in proteins. It modulates cell-molecule, cell-matrix and cell-cell interactions and facilitates the assembly and development of complex organisms. The major functions of a glycan can be characterized into two categories: (1) structural and modular functions; and (2) recognition of glycans by other molecules.²³ There are two major forms of protein glycosylation: glycans linked to an asparagine (N-glycans) and glycans linked to a serine/threonine (O-glycans). N-glycosylation occurs in the endoplasmic reticulum (ER) while O-glycosylation is more dynamic occurring in nuclear and cytoplasmic proteins. Lectin affinity chromatography is a useful and practical separation technique in glycoproteomics. It is characterized by selectively binding a specific carbohydrate motif. Among them, concanavalin-A (ConA) and wheat germ

agglutinin (WGA) are most commonly used. Specifically, ConA has higher affinity for N-linked glycoproteins with high-mannose and terminal glucose carbohydrates while WGA has a higher affinity for terminal acetylglucosamine and sialic acid with O-linked glycosylation motif. In addition, ConA has a special binding site for excessively hydrophobic proteins while WGA is able to bind both hydrophobic and hydrophilic proteins.²⁴ Their functional properties are useful to utilize as a combination of both ConA and WGA which can obtain effective glycoprotein enrichment. Previous studies indicate that the global protein glycosylation pattern is altered in AD.^{25, 26, 24} Recently, the potential precursor of AD, mild cognitive impairment (MCI), has drawn increased attention since treatments could potentially be initiated before the damage of the neurodegenerative process becomes too extensive.^{27, 28} One can investigate patients with cognitive impairment that is worse than age-matched healthy individuals but not severe enough to fulfill the criteria for suspected AD. People with MCI display similar pathophysiologic symptoms as those with AD and have a 10%-15% relative risk of progressing to AD dementia annually.^{29, 30} Here we report a comparative glycoproteomic and peptidomic discovery study using CSF from healthy (control), MCI and AD individuals via mass spectrometry (MS). This study demonstrates the usefulness of glycoprotein enrichment in biomarker discovery which facilitates the isolation, identification and relative quantification of lectin affinity enriched glycoproteins from CSF samples via multidimensional separation and high resolution accurate mass tandem mass spectrometry.

MATERIALS AND METHODS

Participants

Twelve enrollees in the Wisconsin Alzheimer's Disease Research Center (ADRC) participated in this study. This research was conducted in March 5, 2010 to February 13, 2013. The samples comprised of four cognitively normal individuals who enrolled in the Wisconsin ARDC at late middle age without family history of AD and eight patients with cognitive impairment. The cognitively impairment group consisted of four people with amnesic MCI and four people with mild AD. All AD and MCI participants were diagnosed via applicable clinical criteria characterized by standardized, consensus, multidisciplinary consensus conferences,^{31, 3} while cognitive normalcy was determined based on intact cognitive performance by a comprehensive battery of neuropsychological tests, lack of functional impairment, and absence of neurological or psychiatric conditions that might impair cognition.^{32, 33} Women comprised seven out of a total of twelve participants and the mean (SD) age of the total sample was 75.24 (\pm 3.37) years. Cognitive reserve (CR) was indexed by years of education. Individuals with fewer than 16 years of education were considered as having low CR (n=2), while those with at least 16 years of education were considered as having high CR (n=10).³⁴ The University of Wisconsin Institutional Review Board approved all study procedures. Each enrollee provided a signed informed consent form before participation.

CSF Collection

A lumbar puncture for CSF sample collection was performed in the morning after a 12-hour fast. A Sprotte 24-gauge or 25-gauge spinal needle was inserted at L3/4 or L4/5 following proper local anesthesia. Each CSF sample was collected via syringes into polypropylene sample collection tubes. The total CSF sample (approximately 22 mL) was gently mixed to avoid gradient effects, centrifuged at 2000g for ten minutes and the supernatant was collected in 0.5 mL aliquots in polypropylene tubes, frozen and stored at -80°C. The samples were immunoassayed for A β 1-42, t-tau and p-tau by enzyme-linked immunosorbent assays (INNOTEST; Fujirebio) by board-certified technicians who were blind to clinical data and used protocols accredited by the Swedish Board for Accreditation and Conformity Assessment as described in a previous report.³⁵ Table 1 represents the demographic characteristics of the study participants. The mean age of all individuals was 75 with a SD of \pm 3.4, ranging from 70.3 to 82.5. The mean number of years of education was 16.4 with an SD of 2.5.

Materials

Iodoacetamide (IAA), N-acetyl-D-glucosamine, methyl-R-D-mannopyranoside, methyl-R-D-glucopyranoside, manganese chloride tetrahydrate and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). Ammonium bicarbonate, urea, formic acid (FA), Tris hydrochloride, acetonitrile, methanol, optima LC/MS grade water, sodium chloride (99.5%), calcium chloride, and sodium acetate were obtained from Thermo Fisher Scientific (Pittsburgh, PA). Agarose bound Concanavalin A (Con A, 6 mg lectin/mL gel) and Wheat Germ Agglutinin (WGA, 7 mg lectin/mL gel) were obtained from Vector Laboratories (Burlingame, CA). Sequencing grade modified trypsin and

dithiothreitol (DTT) were from Promega (Madison, WI). 660 nm protein assay kit was purchased from Pierce (Rockford, IL) and microplate reader was purchased from BioTek Instrument (Winooski, VT). Amicon Ultra 0.5 mL 10,000 molecular weight cut-off (MWCO) membrane-based centrifugal filters and C18 ZipTips were purchased from Millipore (Billerica, MA). 100 μ L Omix Tips packed with C18 reversed-phase resin were obtained from Agilent (Palo Alto, CA).

Peptide separation

Two aliquots of CSF samples (total 1 mL) of each participant were used for this study. Protease inhibitor cocktail was added to the CSF by the recommended ratio immediately before use. Endogenous peptide separations were performed using Amicon Ultra 0.5 mL 10,000 MWCO centrifugal filters. Before the MWCO separation, three washing steps were performed to remove the contaminants from the filter and achieve optimal peptide coverage. The three washes were 500 μ L water: methanol, 500 μ L of water, and 400 μ L 70:30 aqueous 1 M sodium chloride:methanol. This method yielded a better peptide coverage after separated from a complex protein mixture and was reported by our previous study.³⁶ The MWCO filter was subjected to centrifugation at 14,000 g for 5 min at 4°C via an Eppendorf 5415 D microcentrifuge (Brinkmann Instruments Inc., Westbury, NY). The flow-through was then concentrated by a Savant SC 110 SpeedVac concentrator (Thermo Electron Corporation, West Palm Beach, FL) and resuspended in 20 μ L 0.1% formic acid. The resulting sample was desalted by C18 ZipTips by the manufacturer's protocol. Specifically, the ZipTips were washed with 100% ACN and then pre-equilibrated with 0.1% formic acid in water for three times respectively. Next,

the endogenous peptides were loaded onto the C18 ZipTips repeatedly and gently. The loaded peptides were desalted using 0.1% formic acid in water three times and then eluted in 20 μ L of 50% ACN in 0.1% formic acid. Subsequently, the solution was dried down and resuspended in 10 μ L 0.1% formic acid in water and subjected to LC MS/MS analysis.

Lectin Affinity Chromatography

Lectin affinity columns were prepared in house by adding 75 μ L WGA and 150 μ L ConA slurry both bound to agarose beads into an empty Spin Columns-Screw Cap (Pierce, Rockford, IL). The columns were washed two times to remove contaminants with lectin affinity chromatography (LAC) binding buffer (0.15 M NaCl, 0.02 M Tris-HCl, 1 mM CaCl₂, pH=7.4). Each time the columns were centrifuged at 400 g for 30 seconds with the bottom plug removed. Subsequently, 500 μ L CSF was loaded into the lectin affinity column with the bottom plug placed back. After incubating with continuous mixing at room temperature for two hours, the sample was centrifuged at 400 g for 30 seconds and the unbound flow-through fraction was discarded. The lectin beads were washed with 300 μ L LAC binding buffer twice, centrifuged, and flow-through discarded. The lectin beads with captured glycoproteins were eluted after vortexing for 10 minutes with 300 μ L LAC eluting buffer (0.075 M NaCl, 0.01 M Tris-HCl, 0.2 M α -methyl mannoside, 0.2 M α -methyl glucoside, and 0.5 M acetyl-D-glucosamine) and the elution protocol was performed twice and combined for each participant. The protein concentration was determined by 660 nm protein assay and the final protein amount was normalized for all participant samples.

Protein digestion

Each sample consisted of 20 µg of protein and was denatured with 8 M urea in 50 mM ammonium bicarbonate buffer and reduced by DTT (final concentration as 20 mM) via incubation at 37 °C for 1 h. After incubation, the reduced sample was alkylated by IAA and allowed to react in the dark for 15 min, making final concentration of IAA as 60 mM. Afterwards, DTT was added to quench IAA and incubated for 10 min. The sample was diluted to 1 M urea by 50 mM ammonium bicarbonate (pH=8.5) followed by addition of trypsin (trypsin: protein= 1: 40). The digestion was incubated for 18 h at 37°C and subsequently quenched by 2.5 µL 10% formic acid. The solid phase extraction of the tryptic peptides were performed by Varian 100 µL C18 Omix Tips (Palo Alto, CA) by similar steps as described above. The peptides were sequentially eluted with 50% ACN in 0.1% formic acid, dried by SpeedVac and reconstituted with 20 µL 0.1% formic acid.

LC – ESI Orbitrap mass spectrometry data acquisitions

Online reversed-phase liquid chromatography separation of the tryptic peptides was performed on a nanoAcquity UPLC (Waters Corp., Milford, MA) and infused into a Q Exactive quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The chromatographic separation was carried out via mobile phase A that consisted of 0.1% formic acid in water and mobile phase B consisting of 0.1% formic acid in ACN. One µL of tryptic or endogenous peptides was loaded onto a 2 cm, 150 µm i.d. PLRP-S d_p 5 µm, pore size 1000 Å, trap column at a flow rate of 5 µL/min for 5 minutes at 95% A

and 5% B, and subsequently separated by a Waters BEH 130 Å C18 reversed-phase capillary column (150 mm × 75 µm, 1.7 µm). Emitter tips were pulled from capillary tubing 75 µm I.D. (Polymicro Technologies, Phoenix, AZ) using a model P-2000 laser puller (Sutter Instrument Co., Novato, CA). The LC gradient was 5-30% B over 120 minutes at a flow rate of 350 nL/min. Ions were generated under positive electrospray ionization (ESI) at a capillary voltage of 2.8 KV; 275°C capillary temperature; 30% collision energy via high energy collision dissociation (HCD). MS1 scans were acquired over 200–2000 *m/z* at 70 k resolution followed by data dependent selection of the top 10 most abundant precursor ions with an isolation window of 2.0 *m/z*. HCD fragmentation was then performed and analyzed at a resolution of 17,500. Other parameters include: automatic gain control 1e5; maximum ion injection time, 100 ms; dynamic exclusion enabled with unassigned, +1 and greater than +8 charges ignored for MS/MS selection. Each sample was injected three times to acquire technical triplicates.

Data analysis

Endogenous peptide discovery was performed via Peaks Studio 7 software (Bioinformatics Solutions Inc., Waterloo, ON, Canada). All raw LC-MS/MS data were processed by Peaks software for spectral interpretation. The database was in-house constructed based on Uniprot's reference database of *Homo sapiens* (release 06_2014) by predicting neuropeptides sequence based on *in silico* peptide cleavage principles.³⁷ There were 16690 entries in total. Data Refinement was applied as default to correct the precursor mass and charge states. For De Novo and Peaks Search the parameters were: non-enzyme; MS 1 and MS/MS mass tolerance as 10 ppm and 0.1 Da respectively;

amidation (C-terminal), acetylation (N-terminal), oxidation, pyroglutamination of glutamic acid and glutamine as variable PTMs; monoisotopic mass values. Estimation of false positive identification rate was determined by searching all spectra against a decoy database and with a false discovery rate (FDR) set at $\leq 1\%$, and only peptides with $-10 \log P$ score ≥ 15 were considered as confident identifications.

Tryptic peptide identification was carried out via Proteome Discoverer 1.4 (Thermo Scientific). A FASTA file was downloaded from Uniprot's reference database of *Homo sapiens* (release 06_2014). Other parameters include: allowed missed cleavage, 1; enzyme, trypsin; fixed modification, carbamidomethylation of cysteine (+57.0215 Da); variable modification, oxidation of methionine (+15.9949 Da); peptide mass tolerance, 10 ppm; fragment mass tolerance, 0.1 Da. q value was set to achieve 1% false discovery rate (FDR) via the Percolator node to verify the identified peptides, and the results were filtered by high confidence peptide identification. Only protein identifications that appeared in at least two technical replicates were considered. Label free quantification (LFQ) was conducted to compare control, MCI and AD glycoproteins. Protein concentration was estimated by the sum of normalized peak areas of its tryptic peptides. Area under curve of acquired data was automatically calculated by Proteome Discoverer 1.4 by an event detection node that was applied at a setting of 4 ppm along with the precursor ion peak detector node to extract ion chromatograms. Only unique peptides to one assigned protein were calculated and summed to achieve the cumulative peak area. One way ANOVA analysis was applied to detect the significant protein differential expression. Online software was applied and data was manually input into Statistica.

Protein peak area with significant changes in statistical analysis was subjected to post-hoc comparisons among means via Tukey's HSD test. $P < 0.05$ was considered to be statistically significant. Only proteins that were identified in three out of four individuals' CSF in each group were further analyzed by one way ANOVA.

RESULTS AND DISCUSSION

Endogenous Peptide Discovery

Figure 1 demonstrates the schematic illustration of the experimental design which enabled detection and analysis of large proteins and endogenous peptides simultaneously. It is worth noting that our previous work has provided evidence of significant sample loss when the amount of peptides were below micrograms using MWCO, and it also illustrated a solution to minimize the peptide loss and obtain an optimal peptide recovery.³⁶ Specifically, MeOH and NaCl were added to wash the MWCO membrane, which is usually comprised of regenerated cellulose, before separation. Numerous free hydroxyl groups of cellulose could potentially cause significant non-specific adsorption of endogenous peptides when separated by MWCO. The addition of MeOH and NaCl may disrupt the interaction and non-specific interactions between peptides and hydroxyl groups of the regenerated cellulose. This application has greatly facilitated our peptidomic studies of human body fluids and significantly increased the number of peptide identification.

Peptide identification from tandem MS data is a fundamental task in peptidomic studies. The sensitivity and accuracy greatly impacts the performance of peptide identification as well as downstream analysis. *De novo* sequencing and database searching are two of the most pivotal cornerstones in both proteomic and peptidomic research by MS. In this study, we have improved these two aspects and significantly increased the number of endogenous peptides identified expanding the peptidome of CSF. Our study has greatly surpassed the previously reported 563 peptides in the CSF peptidome by an extensive and combined strategy.³⁸ Specifically, our in house-constructed database was computed based on *in silico* peptide cleavage principles³⁷ of protein precursors and compiled of previous reported bioactive endogenous peptides of human. The rationale is functional peptides are produced from their precursor proteins by various processing enzymes through different pathways. Cysteine and subtilisin-like protease are involved in two major peptide processing pathways and they mainly cleave at the dibasic sites.^{39, 40} This database construction method was described in detail by a previous study.³⁷ With the exclusion of non-bioactive peptide precursors, potential novel neuropeptides could be identified more specifically and with improved sensitivity.⁴¹ *De novo* sequencing was mostly used when a protein database was unavailable and require spectra with higher mass accuracy. The Orbitrap mass spectrometer enables both high mass accuracy and increased speed of analysis for proteomics and peptidomics analysis. PEAKS DB was performed for *de novo* sequencing to assist in database searching due to its superior performance at sensitivity and accuracy.⁴² In addition, PEAKS provided a more robust result validation and precision in controlling the FDR. A total of 645 endogenous peptides (Supplementary

Table 1) were identified with the improved algorithm described above. Proteome Discoverer 1.4 (Thermo Scientific) was also used to search the same raw data file using the identical database and searching parameters, but only 53 peptides were identified among all the samples. The 645 endogenous peptides identified by Peaks Studio 7 were derived from 93 protein precursors. 391 confident identifications were discovered in cognitively healthy individuals, while 261 and 208 were found in MCI and AD patients, respectively, as illustrated by the Venn Diagrams (Figure 2c). The 645 unique peptides covered a wide variety of biological processes (shown in Figure 2d), molecular functions and pathways analyzed by PANTHER gene ontology database search. The top biological functional categories include cellular process (24.8%), biological regulation (13.5%), metabolic process (13.5%) and organismal process (12.8%). Secretory proteins comprised a large portion of the 93 protein precursors and their functions include binding (45.1%), catalytic activity (19.7%) and receptor activity (16.9%). The peptides consisted of several major neuropeptides families: proSAAS, neurosecretory protein VGF, Cholecystokinin (CCK), neuroendocrine protein 7B2, proenkephalin-A, neuroendocrine convertase 1, chromogranin-A, prepronociceptin, neurexophilin-3, neuropeptide Y, secretogranin1, 2, 3, tachykinin-3, proopioidmelanocortin, insulin-like growth factor-1, chromogranin-A, orexigenic neuropeptide QRFP, preprotachykinin B etc.

ProSAAS and AD

ProSAAS, a secretory protein, is expressed in neurons throughout the CNS.^{43, 44} It is proteolytically processed within the regulated secretory pathway and function as a potent and specific inhibitor of prohormone convertase 1/3 (PC1/3).^{45, 46} It is also expressed in

many non-PC1/3 cells, and therefore, many potential functions are still being unknown.⁴³

⁴⁷ Recently, proSAAS-derived peptides have drawn more and more attention and various studies have shown that they are involved in a significant number of physiologically behaviors, such as circadian rhythm,^{48, 49} food intake,⁵⁰ energy balance and fetal neuropeptide processing.⁴⁴ Intriguingly, proSAAS has been mentioned repeatedly in a number of neurodegenerative diseases. ProSAAS immunoreactivity has been observed in neuritic plaques and neurofibrillary tangles in patients with Pick's disease, parkinsonism-dementia complex and AD.^{51, 52} Specifically, the N-terminal of proSAAS was found to be co-localized with tau inclusions. Moreover, several proteomic studies have discovered reduced amount of proSAAS-derived peptides in AD and frontotemporal dementia, suggesting it being a potential biomarker.^{53, 54} More interestingly, a novel anti-aggregant chaperone function of proSAAS was reported recently and this anti-aggregation function of A β ₄₂ could be achieved sufficiently from residues 97-180.⁵⁵

In our study, forty two proSAAS-derived peptides were discovered in human CSF with peptide confidence level $-10 \log P$ score ≥ 15 by PEAKS DB, shown in Table 2. Intriguingly, all the observed peptides were either from N-terminus (SAAS₄₂₋₈₀) and/or C-terminus (SAAS₂₀₇₋₂₄₂, SAAS₂₄₅₋₂₆₀). It is worth pointing out that the intact forms of big LEN (SAAS₂₄₅₋₂₆₀) and little LEN (SAAS₂₄₅₋₂₅₄) were discovered, and their sequences are LETPAPQVPARRLLPP and LETPAPQVPA respectively. Various fragments of big SAAS (SAAS₃₄₋₅₉), little SAAS (SAAS₄₂₋₅₉), big PEN-LEN (SAAS₂₂₁₋₂₆₀), PEN (SAAS₂₂₁₋₂₄₂) and Big LEN (SAAS₂₄₅₋₂₆₀) were also identified. Figure 3a presents a representative example of MS/MS spectra of an identified fragment of PEN (SAAS₂₂₂₋₂₃₈),

DHDVGSELPPEGVLGA (m/z 796.3882), with a $-10 \log P$ score of 51.23. Similar with a tryptic peptide shown in Figure 3b, the endogenous peptide was identified with high confidence. The label free quantification using peak area was also performed on all the discovered endogenous peptides by Peaks Studio and Proteome Discoverer 1.4, but due to Peaks Studio not displaying any statistical functions nor the peak area for individual peptides of every single raw data file, the peptide quantification results should be discussed conservatively and cautiously. More than 80% of the discovered proSAAS-derived peptides showed a decrease in the MCI and AD groups compared with controls by Peaks Studio. More commonly, a decreasing trend of peak area was observed from healthy controls to MCI to AD. Using Proteome Discoverer two proSAAS-derived peptides were confidently identified and matched our criteria to be quantified, which was when a peptide was present in at least three out of four individuals' CSF within each group. Two peptides, DHDVGSELPPEGVLGA and DHDVGSELPPEGVLG, were present in all twelve participants and the peak area was highly abundant and was quantified by Proteome Discoverer. For peptide DHDVGSELPPEGVLGA, the mean peak areas among healthy control, MCI and AD were $4.37E+08$, $1.73E+08$ and $1.41E+08$, respectively, and the P value was 0.0206. Furthermore, Tukey's test suggested the concentration decrease of this peptide was significant in MCI ($P < 0.05$) and AD ($P < 0.05$), however the decrease in AD compared to MCI was insignificant. For peptide DHDVGSELPPEGVLG, the mean peak area among healthy control, MCI and AD were $1.87E+08$, $7.16E+07$ and $4.96E+07$ respectively, and the P value was 0.0297. Tukey's test indicated the concentration decrease of this peptide was significant in AD ($P < 0.05$) compared to control, however the decrease in MCI was not statistically significant.

Glycoprotein identification and quantification

For glycoprotein identification and quantification, Proteome Discoverer 1.4 was used exclusively. To broaden the identification of low abundant proteins in CSF, proteins with one or more unique peptides were considered with a false discovery rate of 0.05 at both the peptide and protein level. Proteins with only one unique peptide were manually examined by checking the peptide XCorr score and the alignment of amino acids. Each sample was subjected to technical triplicates and only proteins identified in at least two technical replicates were considered as a confident match. With the database searching algorithm and criteria discussed above, as shown in the Venn Diagram of Figure 2a, 502, 457 and 386 proteins were identified with high confidence, totaling 795 unique proteins among all 12 samples (Supplementary Table 2). The 795 proteins identified cover a wide variety of biological processes (shown in Figure 2b), molecular functions and pathways analyzed by the PANTHER gene ontology database search. The top biological functional categories include cellular process (23%), metabolic process (20%), development process (12%), and response to stimulus (10%). Secretory proteins comprised a large portion of all identified proteins and their functions include binding (28.3%), catalytic activity (28%) and receptor activity (17.5%) etc. Figure 3b displays a representative example of an MS/MS spectrum of a tryptic peptide of transthyretin YTIAALLSPYSYSTTAVVTNPKE (m/z 1245.1498).

Label free quantitation using AUC was used to provide relative quantitation among control, MCI and AD glycoproteins. Peak area for each protein was summed and normalized by its assigned tryptic peptides. Only peptide precursors with mass accuracy ≤ 4 ppm were subjected to extraction of their ion chromatograms. To avoid repeated use of a peptide for peak area of several proteins only unique peptides were calculated and summed to achieve a more accurate analysis. In order to be quantified and analyzed by ANOVA, proteins had to have been present in at least three out of four individuals' CSF within each group. Two example proteins were neural cell adhesion molecule L1 (L1CAM_HUMAN) and receptor-type tyrosine-protein phosphatase eta (PTPRJ_HUMAN) that were only discovered in cognitively healthy individuals. L1CAM was present in all four participants in the control group but was not detected in either the MCI or AD group. PTPRJ was identified in three out of four participants in the control group but none of the MCI or AD groups. Thirteen other proteins displayed significant changes ($P < 0.05$) via one way ANOVA analysis to compare the mean value of the peak area among these three groups. The differentially expressed proteins are shown in Figure 4 and the proteins were transthyretin (TTHY_HUMAN), apolipoprotein E (APOE_HUMAN), contactin-1 (CNTN1_HUMAN), alpha-2-HS-glycoprotein (FETUA_HUMAN), histidine-rich glycoprotein (HRG_HUMAN), leucine-rich alpha-2-glycoprotein (A2GL_HUMAN), cell surface glycoprotein MUC18 (MUC18_HUMAN), cell adhesion molecule 2 (CADM2_HUMAN), neuronal cell adhesion molecule (NRCAM_HUMAN), neural cell adhesion molecule L1-like protein (CHL1_HUMAN), neuronal pentraxin-1 (NPTX1_HUMAN), neuronal growth regulator 1 (NEGR1_HUMAN), and Dickkopf-related protein 3 (DKK3_HUMAN).

Protein Networks

The Mean peak area, one way ANOVA *P* value, post-hoc comparisons via Tukey's HSD test of the remaining differentially expressed proteins are shown in Table 3. Figure 5a illustrates the protein-protein interaction analysis generated by the database and web-tool STRING 10.0 (<http://string-db.org/>). The total fifteen significantly dysregulated proteins were subjected to this molecular interaction tool with parameters: (a) medium confidence (default); (b) no more than 10 interactors to show. Two functional modules formed tightly connected clusters in the network. Further molecular function analysis identified five categories: (1) Receptor activity (40%); (2) Catalytic activity (30%); (3) Binding capacity (10%); (4) Enzyme activity (10%); (5) Transporter activity (10%).

Transthyretin and AD

Intriguingly, transthyretin (TTR) was found to increase 2.42 times ($P < 0.05$) in MCI and decrease to 0.53 times in AD ($P < 0.01$) compared with controls (Table 3). The mean peak area in healthy control, ACI and AD were $5.55E+08$, $1.34E+09$, $2.96E+08$ respectively, with one way ANOVA *P* value 0.0074. TTR is a thyroid hormone carrier and plasma retinol transporter. It is encoded by a single gene copy on chromosome 18 in humans and is expressed in liver, kidney, pancreas, retinal epithelium, leptomeningeal epithelium, choroid plexus and potentially in neurons, which is most importantly related to AD.⁵⁶ Anti-TTR antibodies present in brain parenchyma indicate the neuronal endocytosis of TTR from choroid plexus is active and TTR mRNA is effectively

translated.^{57, 58} Human studies have shown that TTR co-localized in amyloid plaques and vessels in hippocampi of AD patients.^{57, 58} In addition, anti-TTR serum stained the majority of neuronal bodies in AD brains while only 10% of the neurons were stained in age-matched cognitively healthy individuals.⁵⁹ A large number of studies have reported a decreased level of TTR in AD patients consistent with our results.^{60, 61, 62} However, there is some inconsistency in the literature on TTR levels being increased in AD patients compared to controls in CSF proteomic studies.^{10, 63} Nevertheless, other CSF proteomics studies found a decrease of TTR in AD patients.^{64, 65, 66} The significance of the increased level of TTR in MCI and the decreased level of TTR in AD is not clear, but one potential hypothesis is that the increased level in MCI represents a microglial or neuronal reaction to early amyloid deposition, which then fails in later stages of AD, represented by the AD dementia patients in our study. Moreover, CSF TTR concentration may be determined partially by neuronal TTR synthesis, and the reduction in TTR could be explained by neuronal loss in AD.⁵⁹ An alternative hypothesis is that AD patients may have a genetic disposition or acquired low CSF TTR level independent of A β binding which could potentially put them at greater risk of developing plaque pathology, since TTR is an established A β -carrying protein.⁵⁶ The discovery that TTR bound A β and inhibited fibril tangle formation was proved both *in vitro* and *in vivo* as discussed below.

The first *in vitro* observation was made in *C. elegans*, where a phenotype of defective locomotion in A β -expressing worms were rescued by wild type human TTR expressed in muscle cells.⁶⁷ In addition, the transgenic AD mouse model Tg2576 showed up-regulation of TTR in the hippocampus and cerebral cortex and TTR immunoreactivity

was observed as co-localized with A β .^{68,69} Furthermore, increased A β deposition was seen in one ventricle that was injected with anti-TTR antibodies.⁵⁸ In another AD mouse model APP23, TTR and A β co-staining in the hippocampi, cortical regions and blood vessels were also observed.⁷⁰ The over-expression of wild type human TTR in the mouse strain (APP23/hTTR⁺) corrected the cognitive function and spatial learning as well as the diminished neuropathological changes and amount of A β deposition. *In vitro* studies reported that most recombinant TTR variants bound to A β and inhibited A β aggregation.⁷¹ Wild type human TTR binds to all forms of soluble A β , monomer, oligomer and fibrils, but has better affinity with A β aggregates than the monomer, and A β ₁₋₄₂ having higher affinity for binding than A β ₁₋₄₀.^{70, 72, 73} By mass spectrometry interaction analysis, it was reported that the human TTR monomer bound A β with higher affinity than its tetramer form; the binding occurred at the inner β -sheet and EF helix of the A strand of TTR.⁷⁴ Lastly, various groups have reported that TTR interrupted A β aggregation *in vivo*.^{75, 70, 72, 73}

More intriguingly, this interaction between TTR and A β was found to be mutual, more specifically, A β was found to dynamically regulate the expression of TTR as discussed below. The hippocampal slices of Tg2576 AD mice discussed above had more TTR mRNA and protein compared to wild type mice. The same phenomenon was found in the cortex and hippocampus of APP23 mice.^{70, 58, 68} It was assumed that this up-regulation of TTR also occurred in human brains since TTR was extensively stained in neurons of AD patients whereas there was minimal detectable neuronal staining in age-matched cognitively normal controls.⁵⁹ In addition, the TTR gene has been indicated to be a

specific downstream target of soluble amyloid precursor protein beta(sAPP β).⁷⁶ Thus, the quantitative result of TTR in this study could be better supported with these studies. During the early stages of AD such as preclinical AD or MCI, TTR is most likely sequesters and inhibits A β aggregation, and facilitated A β clearance. Simultaneously, the *TTR* gene is triggered for upregulation in response to the over-production of A β peptides and APP. This hypothesis explains why TTR was upregulated in MCI. Over time, the amount of pathogenic A β production exceeds the neuronal capacity to neutralize and remove them by TTR and other major A β binding proteins (ApoE and ApoJ) as disease progressed. The neuronal homeostasis network of the A β neutralizing capacity is finally disrupted and unable to control the disease. The negative feedback between A β and TTR was dysregulated and new TTR was not expressed due to A β over-production. Alternatively, neuronal TTR was consumed by A β which can cause a decline of TTR in advanced AD and neuronal loss in advanced AD led to reduced production of TTR. It was inferred that TTR was part of the conventional protein homeostasis network in the brain of AD, which includes unfolded protein response, heat shock induced chaperones and their co-chaperones, the proteasome ubiquitin system and autophagic responses.⁵⁶

CONCLUSION

In conclusion, with an optimized sub-microgram peptide separation method using MWCO and in house-constructed bioactive peptide-specific database 645 endogenous peptides in CSF were identified. Interestingly, 42 proSAAS peptides discovered were exclusively cleaved either from the N- or C-terminal of the proSAAS protein precursor, which represents similar sequences as the bioactive peptides big/little SAAS, PEN and

LEN. Among them, the intact form of big LEN and little LEN were identified, which indicates potential bioactivity. Glycoproteins were enriched via lectin affinity chromatography, digested, identified by Proteome Discoverer, and quantified by comparing area under curve between cognitively healthy, MCI and AD individuals. One way ANOVA was applied for statistical analysis, and 15 proteins were found to be differentially expressed among the three groups. The dynamic changes of transthyretin increasing in MCI and then declining in AD were reported for the first time.

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Table 1. Characteristics of Study Participants

Characteristic	Control	MCI	AD
Age, mean (SD)	74.0(3.1)	76.0(2.9)	75.7(4.1)
Education, mean (SD)	17.0(2)	16.3(3.1)	16.0(2)

MMSE score, mean (SD)	29.5(0.6)	28.8(0.9)	20.3(2)
Women, No. (%)	3 (75)	2 (50)	2 (50)
APOE4 positive, No. (%)	2 (50)	3 (75)	3 (75)
Family history of dementia, No. (%)	0 (0)	2 (50)	1 (25)

Abbreviations: MMSE, Mini-Mental State Examination; AD, Alzheimer's disease; MCI, mild cognitive impairment

Figure 1

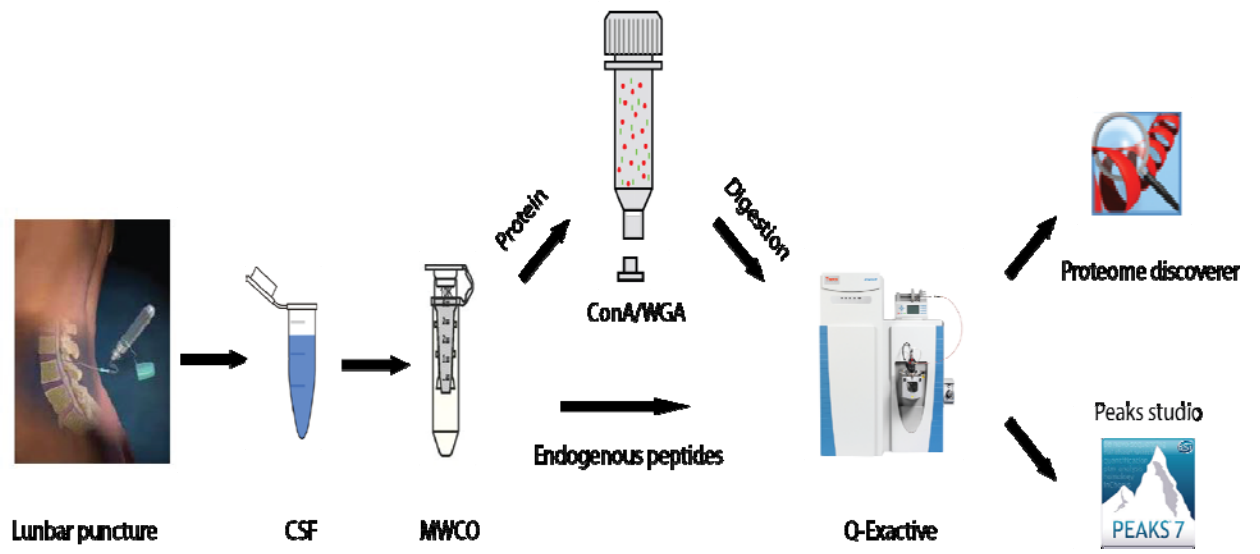
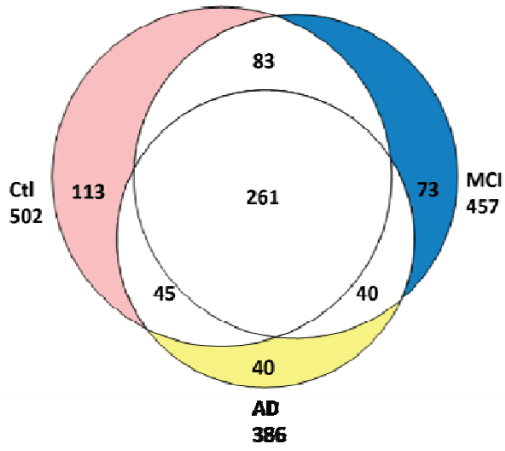
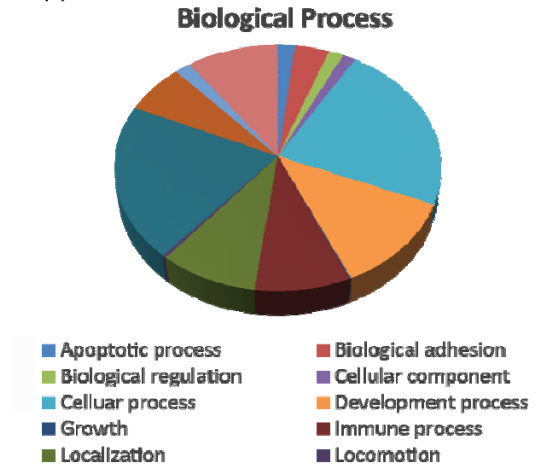


Figure 2

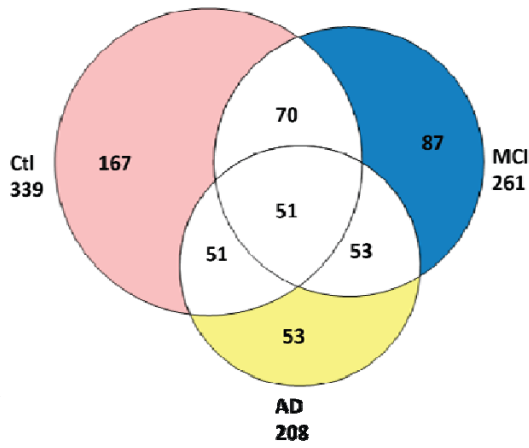
(a)



(b)



(c)



(d)

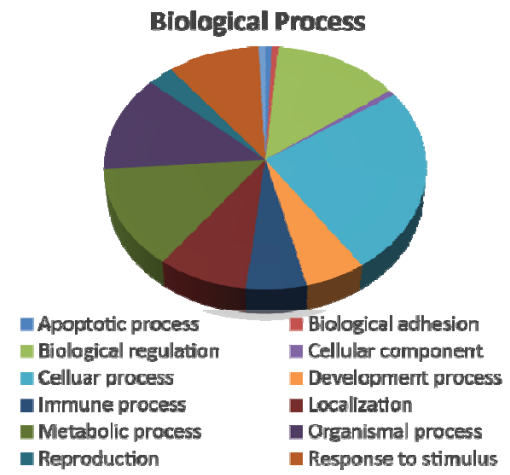


Table 2 ProSAAS peptides identified in CSF

Peptide	-10logP	Mass	PPM	m/z	RT	PTM
DHDVGSELPPPEGVLGA	51.23	1590.753	5.8	796.3882	35.2	
DHDVGSELPPPEGVLG	46.48	1519.716	-1.1	760.8641	33.67	
HDVGSELPPPEGVLGA	44.42	1475.7256	-0.9	738.8694	33.56	
DHDVGSELPPPEGVL	41.55	1462.694	-2.4	732.3525	36.4	
8						
SPPLAETGAPR	40.13	1094.572	-0.5	548.293	19.	
56						
SPPLAETG	38.93	770.381	-1.6	771.387	18.1	
AADHDVGSELPPPEGVLGA	38.49	1732.8268	-1.4	867.4194	34.41	
DHDVGSELPPPEG	37.35	1250.5415	-1.1	626.2773	22.56	
HDVGSELPPPEGVLG	37.08	1404.6885	-2	703.3501	32.33	
HDVGSELPPPEGVL	35.62	1347.667	-1	674.8401	33.62	
ADHDVGSELPPPEGVLGA	35.46	1661.7897	-1	831.9013	34.05	
TPAPQVPARRLLPP	34.97	1511.8936	-1.2	504.9712	30.02	
GLSAASPLAETGAPR	34.92	1493.7837	0.7	747.8997	27.91	
VGSELPPPEGVLGA	34.78	1223.6398	0.3	612.8273	34.71	
HDVGSELPPPEG	34.38	1135.5145	1.6	568.7654	21.13	
DHDVGSELPPPEGVLGAL	31.99	1703.8365	-0.5	852.9251	43.02	
DVGSELPPPEGVLGA	31.33	1338.6666	-0.7	670.3401	37.44	
SAASPLAETGAPR	30.54	1323.6782	1.5	662.8474	21.46	
SAASPLAETGAPRRF	28.46	1626.8478	-0.8	543.2894	26.92	
AADHDVGSELPPPEGVLG	27.04	1661.7897	1.5	831.9034	33.21	
ETPAPQVPARR	26.73	1220.6625	-1.2	407.8943	16.7	
SPPLAETGAPRRF	26.58	1397.7415	-0.6	466.9208	25.54	
ETPAPQVPARRLLPP	26.44	1640.9362	1.4	547.9868	31.03	
DVGSELPPPEG	26.23	998.4556	-1.4	500.2344	25.02	
DVGSELPPPEGVL	24.73	1210.608	0.2	606.3114	37.9	
DVGSELPPPEGVLG	24.32	1267.6295	1.7	634.8231	36.66	
ADHDVGSELPPPEGVLG	24.27	1590.7526	-3.3	796.3809	33.38	
DHDVGSELPPPEGVL	23.95	1461.71	-1.3	731.8613	33.14	
amidation						
LSAASPLAETGAPRRF	21.23	1739.9318	-0.3	580.9844	29.46	
DHDVGSELPPPEGVLGALL	21.18	1816.9207	1.1	909.4686	49.79	
DHDVGSELPPPEGVLGALLRV	21	2072.0901	-0.5	691.7036	51.84	
ADSEGVAAPRRLRRAADHDV-	20.34	2926.4756	-8.6	976.4908	30.44	
amidation						
-GSELPPPEG						
LPPEG	19.5	511.2642	-2.1	512.2704	14.01	
PPEGVLGAL	19.14	851.4752	-0.2	426.7448	31.34	
ETPAPQVPA	18	908.4603	-1.4	455.2368	22.48	
F(+42.01)RRSVP	17.92	802.4449	-3.8	402.2282	22.43	acetylation
SVPRGEAAGAVQELARA	17.31	1680.8906	1.6	561.3051	30.01	
LETPAPQVPARRLLPP	16.26	1754.0203	0	585.6807	32.7	
LSAASPLAETGAPR	16.22	1436.7623	-3.4	719.386	25.52	
LAETGAPR	15.75	813.4344	-4.7	407.7226	14.34	
DHDVGSELPPPEGV	15.13	1349.6099	-4.2	675.8094	27.96	
LETPAPQVPA	15.11	1021.5444	0	511.7795	26.94	

Figure 3

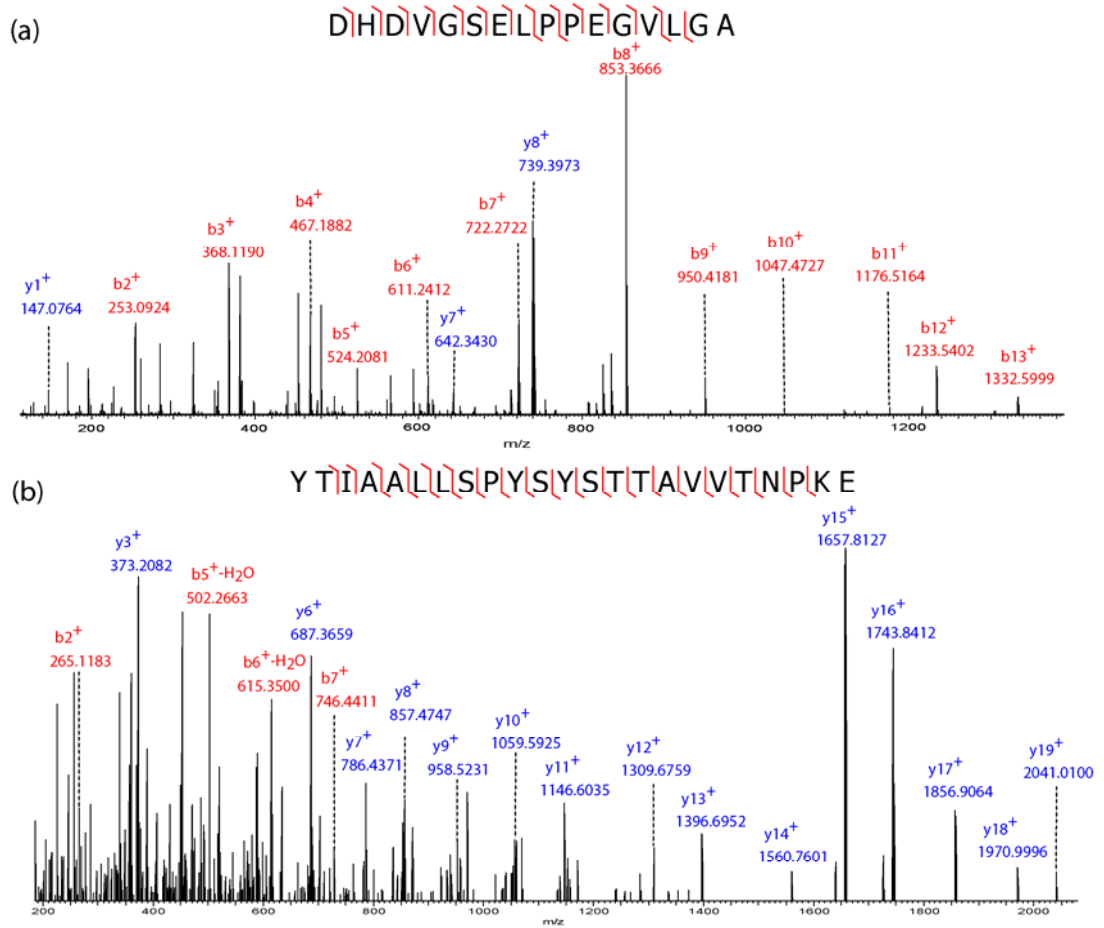
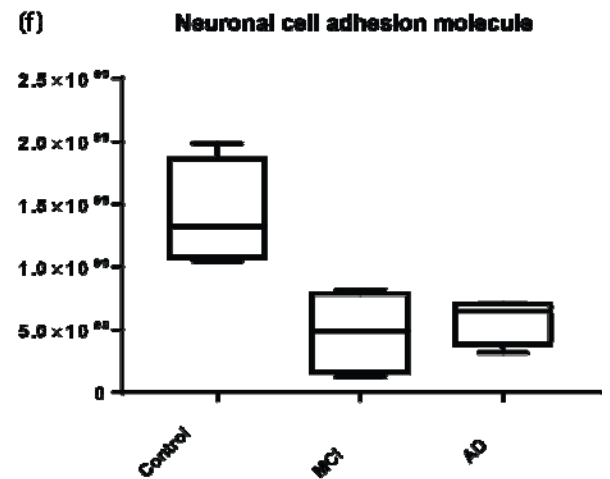
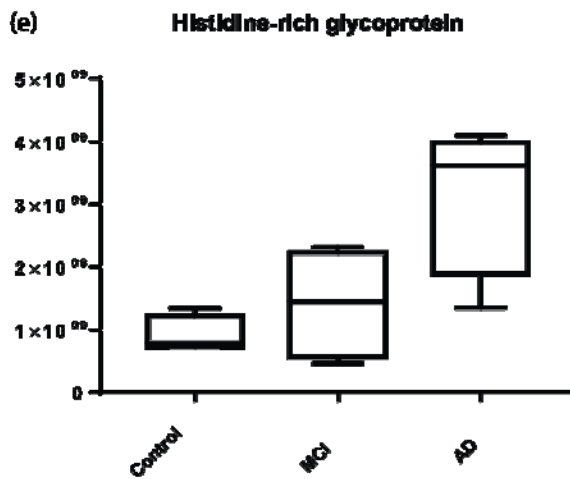
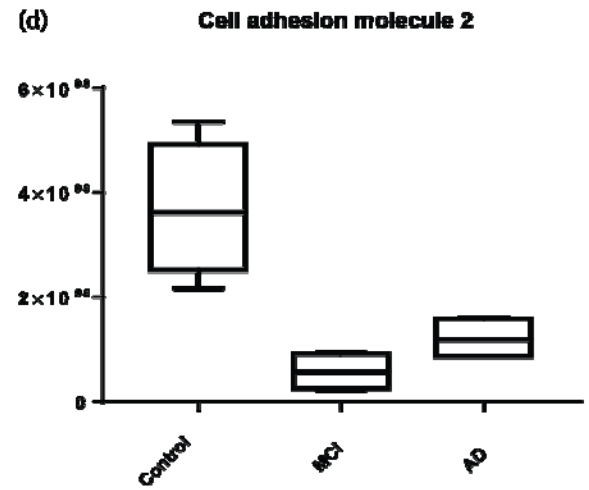
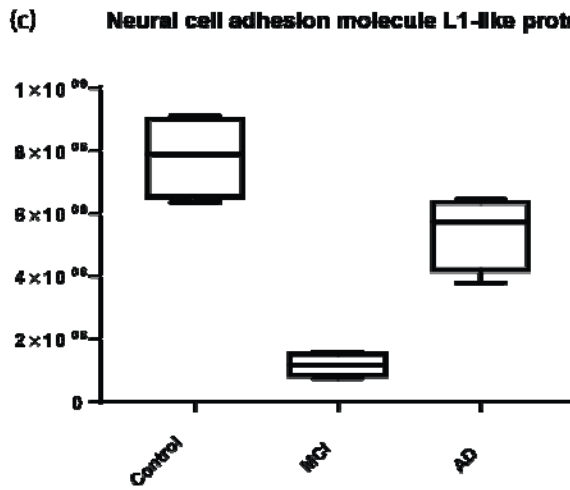
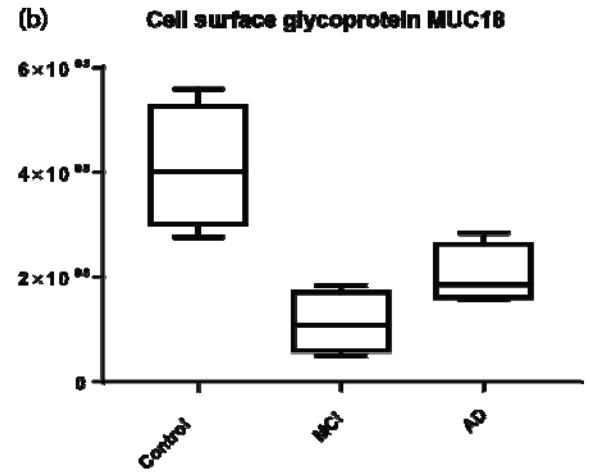
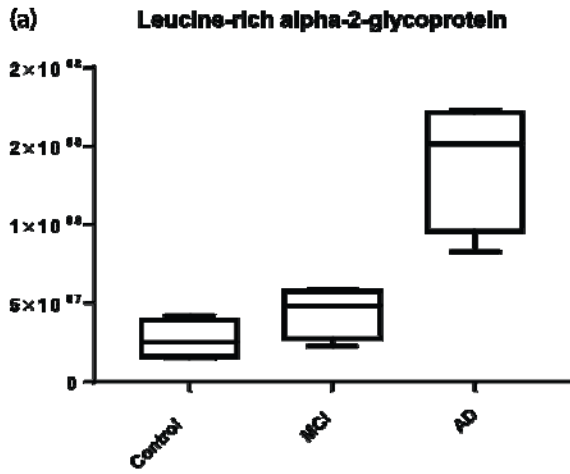
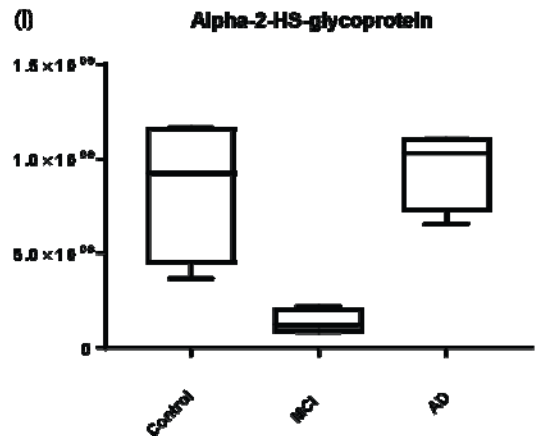
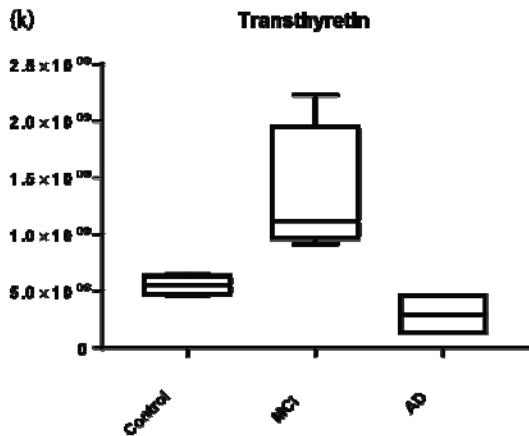
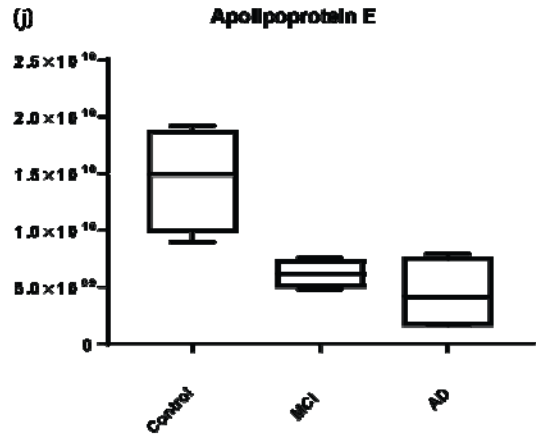
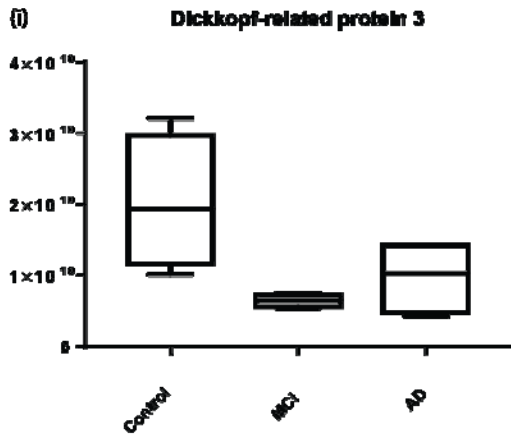
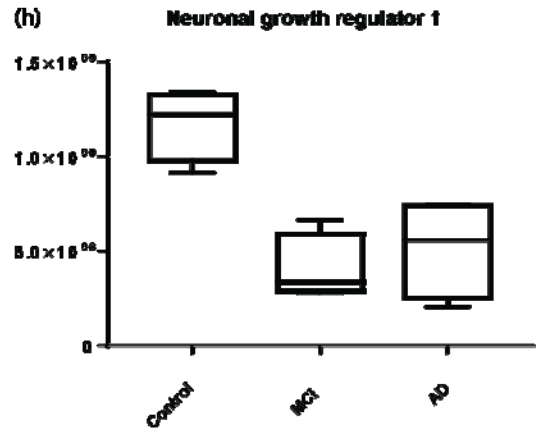
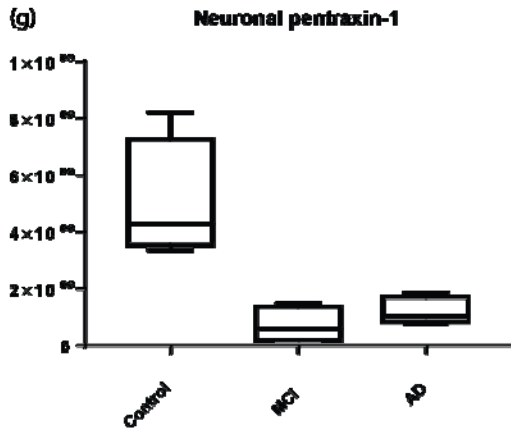


Figure 4





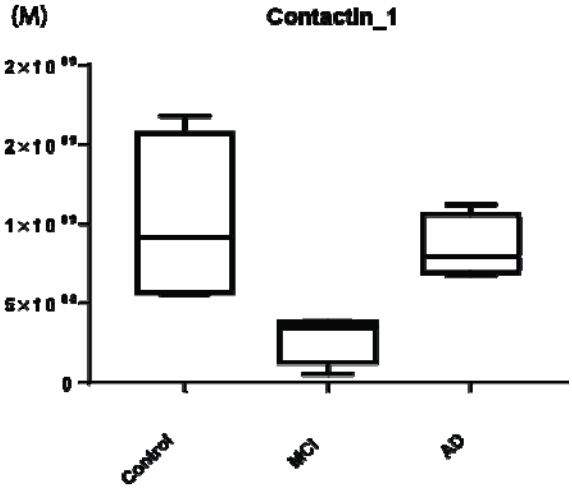
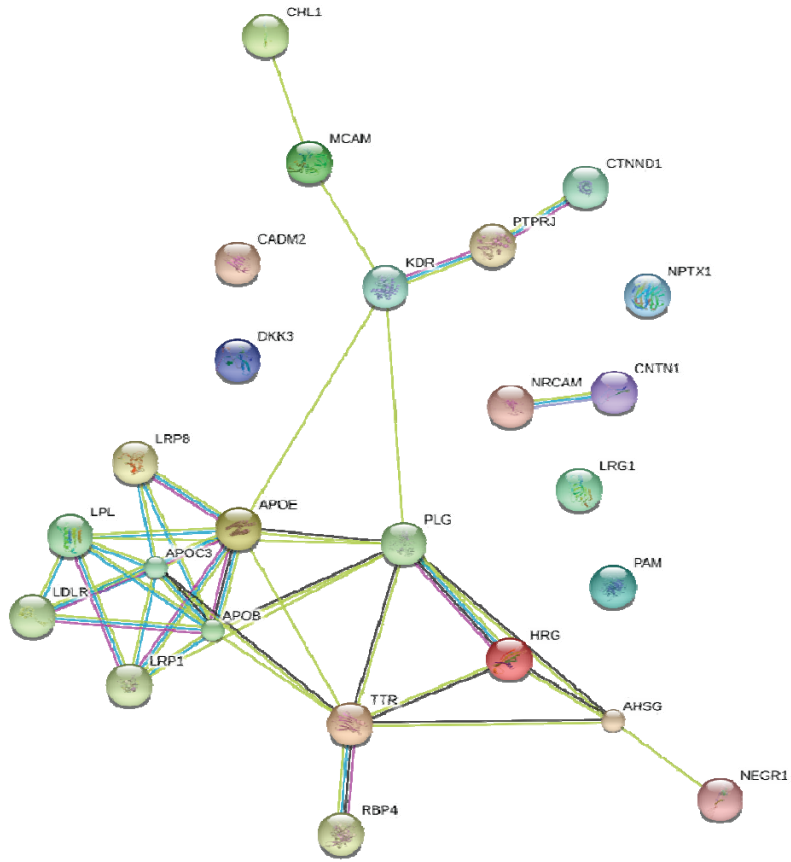


Table 3. Proteins showed differential expression in CSF among healthy control, MCI and AD

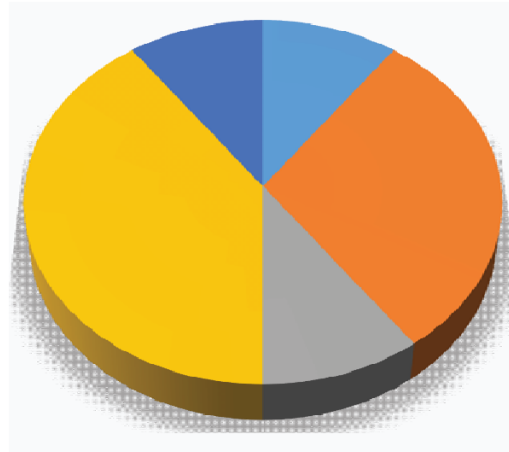
Figure 5

(a)



(b)

Molecular Function



■ Binding
■ Enzyme activity
■ Transporter activity
■ Catalytic activity
■ Receptor activity

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Figure Caption

Figure 1 Schematic illustration of the experimental design.

Figure 2 Number of proteins and endogenous peptides identified and their biological functions. (a) Venn Diagram displaying 502, 457 and 386 proteins identified with high confidence in control, MCI and AD participants biological process. (b) Biological

process of total identified 795 proteins by PANTHER gene ontology search. (c) Venn Diagram showing 391, 261 and 208 endogenous peptides found in control, MCI and AD individuals. (d) Biological process of total identified 93 protein precursors by PANTHER gene ontology search.

Figure 3 Tandem mass spectra of representative endogenous peptides and tryptic peptides (a) Endogenous peptide DHDVGSELPPGVLGA derived from proSAAS protein precursor (b) Tryptic peptide of transthyretin YTIAALLSPYSYSTTAVVTNPKE

Figure 4 One way ANOVA analysis results of differentially altered glycoproteins via bar chart. (a) leucine-rich alpha-2-glycoprotein (b) cell surface glycoprotein MUC18 (c) neural cell adhesion molecule L1-like protein (d) cell adhesion molecule 2 (e) histidine-rich glycoprotein (f) neuronal cell adhesion molecule (g) neuronal pentraxin-1 (h) neuronal growth regulator 1 (i) Dickkopf-related protein 3 (j) apolipoprotein E (k) transthyretin (l) alpha-2-HS-glycoprotein (M) contactin-1

Figure 5 (a) Protein-protein interaction analysis of fifteen significantly altered glycoproteins by STRING 10.0. (b) Molecular functions of significantly changed glycoproteins by PANTHER gene ontology search.