Early candidate urine biomarkers for detecting Alzheimer's disease before beta amyloid plaque deposition in an APP (swe)/PSEN1^{dE9} transgenic mouse model

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Abstract

Alzheimer's disease (AD) is an incurable age-associated neurodegenerative disorder that is characterized by irreversible progressive cognitive deficits and extensive brain damage. The identification of candidate biomarkers before beta amyloid plaque deposition occurs is therefore of great importance for the early intervention of AD. Urine, which is not regulated by homeostatic mechanisms, theoretically accumulates changes associated with AD earlier than cerebrospinal fluid and blood. In this study, an APP (swe)/PSEN1^{dE9} transgenic mouse model was used to identify candidate biomarkers for early AD. Urine samples were collected from 4-, 6-, and 8-month-old transgenic mouse models, and the urinary proteomes were profiled using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The levels of 33 proteins differed significantly between wild-type and 4-month-old mice, which had not started to deposit beta amyloid plaque. Among these proteins, 16 have been associated with the mechanisms of AD, while 9 have been suggested as AD biomarkers. Our

results indicated that urine proteins enable detecting AD before beta amyloid plaque deposition, which may present an opportunity for intervention.

Key words: Alzheimer's disease (AD), urine proteome, early diagnosis, APP (swe)/PSEN1^{dE9}

Introduction

Alzheimer's disease (AD) is a chronic age-associated neurodegenerative disorder associated with cognitive impairment and progressive dementia[1]. As the pathological process leading to AD begins decades before clinical symptoms, finding early clues in the early stages of AD, especially before beta amyloid plaque deposition, is urgent in today's AD research. Most candidate AD biomarkers came from cerebrospinal fluid (CSF) and blood studies; for example, tau levels increase in the CSF of most AD patients, and stable miRNAs in human serum are potentially valuable novel biomarkers for the diagnosis of AD. However, beta amyloid deposition can appear in AD patients, even in the stage 1 of preclinical AD, and can be detected by positron emission tomography (PET) amyloid imaging or CSF amyloid beta levels[1].

Biomarkers are measurable changes associated with disease. Unlike blood, which is stable because of homeostasis mechanisms, urine can accumulate many kinds of changes; some of these changes are associated with disease and will become biomarkers[2,3]. In addition, urine is a resource for biomarker discovery that easily and non-invasively collected from AD patients. As a sensitive biomarker source, urine therefore might reflect pathological changes, especially in the early stages of disease[4]. However, whether time-course analyses of urine proteins at different AD phases can reveal sensitive biomarker clues before beta amyloid deposition is unclear, as urinary proteins are easily affected by complicated factors such as medicine and diet, especially in human samples. Using animal models is therefore the most efficient way to establish an association with disease, as animal models can reduce the influence of genetic and environmental factors on the urine proteome to the greatest degree[2]. In addition, animal models can represent the early stages of disease without clinical symptoms as it is impossible to clinically collect such samples[5].

Several studies have applied proteomic[6] and metabolomic[7,8] analyses to characterize candidate biomarkers in mouse models. However, these studies used either plasma or brain tissues, and analyses of these materials were not sensitive enough for the early diagnosis of AD (before beta amyloid plaque deposition). Other studies have illustrated urine markers using samples from AD patients[9]. However, all these studies were conducted after beta amyloid deposition had appeared in brain tissue and cognitive impairment or movement disorder symptoms had occurred. CSF has a greater homeostatic priority than blood and changes less than blood. Urine may thus be a better choice for the detection of earlier biomarkers, even for brain diseases[10]. In addition, the limited studies about urine-based biomarkers of brain diseases suggest that urine is an ideal potential source for biomarkers in brain diseases[11]. For example, urine biomarkers in an astrocytoma rat model can reflect early astrocytoma changes before Magnetic Resonance Imaging (MRI) and thus gives potential clues for the early clinical diagnosis of astrocytoma patients[12].

Amyloid precursor protein (APP) (swe)/PSEN1^{dE9} transgenic mice, overexpressing mutant APP and PS1 (APP/PS1), have been widely used as a model of AD to elucidate the pathogenic processes of the disease and to investigate candidate biomarkers[13]. Deposition of beta amyloid in the hippocampus of 6-month-old mice occasionally occurred[14,15], and a mass of beta amyloid had been deposited in the hippocampus of 8-month-old mice [16,17]. Compared with wild-type mice, the 8-month-old transgenic mice were mainly impaired in the foreground fear conditioning test and in plasticity to acquire strategies to swim toward a cued escape platform[18]. The 12-month-old transgenic mice showed spatial learning deficits as well as long-term contextual memory deficits and large deposits of aggregated insoluble beta amyloid[19]. The cognitive abnormality of APP (swe)/PSEN1^{dE9} transgenic mice appeared at 4 months, when beta amyloid plaques had not yet deposited[20]. Potential candidate urinary biomarkers that appear before brain pathology can thus be identified by using 4-month-old APP (swe)/PSEN1^{dE9} transgenic mice. Figure 1 presents the characteristics of behavioral and pathological profiles of different stages of the APP (swe)/PSEN1^{dE9} transgenic mouse model.

Figure 1. Characteristics of behavioral and pathological profiles of different stages of APP (swe)/PSEN1^{ΔE9} transgenic mice

In this study, the APP (swe)/PSEN1^{ΔE9} transgenic mouse model was used to identify candidate early AD biomarkers. To identify changes in the urinary proteome during AD development, we collected and analyzed urine samples of APP (swe)/PSEN1^{ΔE9} transgenic mice at three growth stages (4-, 6- and 8-month-old). The workflow of the proteomic analysis in this study is shown in Figure 2. By using label-free and TMT-labeling proteomics analysis, we analyzed differential urine biomarkers from different stages of AD.

Figure 2. Workflow of urinary proteomics in APP (swe)/PSEN1^{ΔE9} transgenic mouse model. Urine samples were collected from the control and transgenic groups. The proteins were analyzed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) identification. Differential proteins were analyzed by DAVID and IPA.

Materials and methods

Experimental animals

APP (swe)/PSEN1^{ΔE9} transgenic mice and wild-type mice were used in this study. All animal protocols governing the experiments in this study were approved by the Institute of Basic Medical Sciences Animal Ethics Committee, Peking Union Medical College (Approved ID: ACUC-A02-2014-008). All animals were maintained with a standard laboratory diet at a controlled indoor temperature ($22 \pm 1^\circ\text{C}$) and humidity (65 ~ 70%) and with a 12-h light-dark cycle. The study was performed according to the guidelines developed by the Institutional Animal Care and Use Committee of Peking Union Medical College. All efforts were made to minimize suffering.

Urinary protein extraction

Urine samples from 4-month-, 6-month- and 8-month-old mice in transgenic and wide-type control groups were collected in metabolic cages. During urine collection, all rats were given free access to water without food to avoid contamination.

Urine was centrifuged at $2,000\times g$ for 15 min at 4°C . After the cell debris had been removed, the supernatant was centrifuged at $12,000\times g$ for 15 min at 4°C . Three volumes of acetone were added after the pellets had been removed, and precipitation was allowed to occur at 4°C . After the supernatant was removed, the pellets were resuspended in lysis buffer (8 M urea, 2 M thiourea, 25 mM dithiothreitol (DTT) and 50 mM Tris). Protein concentrations were measured using the Bradford method.

In-gel protein digestion

Proteins were digested with trypsin (Trypsin Gold, Mass Spec Grade, Promega, Fitchburg, Wisconsin, USA) using in-gel protein digestion[21]. Protein samples were separated by SDS-PAGE. The gel was rinsed three times, 5 minutes each, in pure water and stained for 30 minutes in Coomassie brilliant blue

at room temperature, after which the staining solution was discarded. The gel was destained for 1 hour in pure water at room temperature. When destaining was complete, the solution was discarded. A clean razor blade was used to cut the protein bands from the gel. The gel slices were then placed into a 0.5-ml microcentrifuge tube that had been prewashed twice with 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA). The gel slices were destained twice with 0.2 ml of 100 mM NH_4HCO_3 /50% ACN for 45 minutes each at 37°C to remove the Coomassie brilliant blue stain. The gel slices were dehydrated for 5 minutes at room temperature in 100 μl of 100% ACN. At this point, the gel slices were much smaller than their original size and whitish or opaque in appearance. The gel slices were dried in a Speed Vac® for 10 minutes at room temperature to remove the ACN. Trypsin Gold was resuspended at 1 $\mu\text{g}/\mu\text{l}$ in 50 mM acetic acid and then diluted in 40 mM NH_4HCO_3 /10% ACN to 20 $\mu\text{g}/\text{ml}$. The gel slices were preincubated in a minimal volume (10–20 μl) of the trypsin solution at room temperature (do not exceed 30°C) for 1 hour. The slices rehydrated during this time. If the gel slices appeared white or opaque after one hour, an additional 10–20 μl of trypsin was added, and they were incubated for another hour at room temperature. Sufficient digestion buffer (40 mM NH_4HCO_3 /10% ACN) to completely cover the gel slices was added, and the tubes were capped tightly to avoid evaporation. The samples were then incubated overnight at 37°C. Next, the gel slice digests were incubated with 150 μl of pure water for 10 minutes with frequent vortex mixing. The liquid was removed and saved in a new microcentrifuge tube. The gel slice digests were extracted twice with 50 μl of 50% ACN/5% TFA (with mixing) for 60 minutes each time at room temperature. All extracts were pooled and dried in a Speed Vac® at room temperature for 2–4 hours. The extracted peptides were purified and concentrated using ZipTip® pipette tips (Millipore Corporation, Cat# ZTC18S096, Darmstadt, Germany). The peptides eluted from the ZipTip® tips were then ready for mass spectrometric analysis.

Filter-aided protein digestion

Proteins were digested with trypsin (Trypsin Gold, Mass Spec Grade, Promega, Fitchburg, Wisconsin, USA) using filter-aided sample preparation methods [22]. After proteins were loaded into a 10-kDa filter unit (Pall, Port Washington, New York, USA), UA buffer (8 M urea in 0.1 M Tris-HCl, pH 8.5) and NH_4HCO_3 (25 mM) were added successively, and the tube was centrifuged at 12,000 $\times g$ for 30 min at 18°C. Proteins were denatured by incubation with 20 mM DTT at 37°C for 1 h and then alkylated

with 50 mM iodoacetamide (IAA) for 45 min in the dark. After the samples had been centrifuged with UA twice and NH_4HCO_3 four times, the proteins were redissolved in an NH_4HCO_3 solution and digested with trypsin (1:50) at 37°C overnight. The tryptic peptides were desalted using Oasis HLB cartridges (Waters, Milford, Massachusetts, USA), and the desalted peptides were dried by vacuum evaporation (Thermo Fisher Scientific, Bremen, Germany).

Peptide tandem mass tag (TMT) labeling

The filter-aided digested peptides were solubilized in 100 mM tetraethylammonium bromide (TEAB) and labeled with 6-plex Tandem Mass Tag Label Reagents provided by Thermo Fisher Scientific (Pierce, Rockford, IL, USA), which had been equilibrated to room temperature immediately before use. Then, 41 μl of anhydrous acetonitrile was added to each tube, and the reagent was allowed to dissolve for 5 min with occasional vortexing. The samples were briefly centrifuged to gather the solution, and 20 μl of the TMT Label Reagent was added. The reaction was then incubated for 2 h at room temperature. After the peptides were labeled with isobaric tags, they were mixed at a 1:1:1:1:1:1 ratio based on the amount of total peptide, which was determined by running equal volumes of labeled samples through liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and comparing the total signal intensities of all peptides. Finally, samples from the wild-type group (n=3) and AD group (n=3) were analyzed by two-dimensional LC-MS/MS (2DLC-MS/MS).

HPLC separation

The TMT-labeled samples were fractionated using a high-pH reversed-phase liquid chromatography (RPLC) column from Waters (4.6 mm $\times$ 250 mm, Xbridge C18, 3 μm) and loaded onto the column in buffer A1 (H_2O , pH=10). The elution gradient progressed from 5–25% buffer B1 (90% ACN, pH=10; flow rate=1 ml/min) over 60 min. The eluted peptides were collected at one fraction per minute. The 60 fractions were dried, resuspended in 0.1% formic acid and pooled into 30 samples by combining fractions 1 and 31, 2 and 32, and so on. The odd-numbered fractions were chosen for further analysis. A total of 15 fractions from urinary peptide mixtures were analyzed by LC-MS/MS.

LC-MS/MS analysis

The in-gel digested peptides and TMT-labeled peptides were analyzed with a reverse-phase C18 self-packed capillary LC column (75 $\mu\text{m} \times 100 \text{ mm}$). The elution gradient progressed from 5–30% buffer B2 (0.1% formic acid, 99.9% ACN; flow rate=0.3 $\mu\text{L}/\text{min}$) over 40 min. A Triple TOF 5600 mass spectrometer was used to analyze the peptides eluted from LC, and each fraction was run twice. MS data were acquired using the high-sensitivity mode with the following parameters: 30 data-dependent MS/MS scans per full scan, full scans acquired at a resolution of 40,000, MS/MS scans acquired at a resolution of 20,000, rolling collision energy, charge state screening (including precursors with a charge state of +2 to +4), dynamic exclusion (exclusion duration 15 s), an MS/MS scan range of 100–1800 m/z , and a scan time of 100 ms.

Data analysis

The MS/MS data were analyzed with Mascot software (version 2.4.1, Matrix Science, London, UK), and proteins were identified by comparing the peptide spectra against the Swissprot_2014_07 databases (taxonomy: mus). Trypsin was selected as the digestion enzyme, up to two missed cleavage sites were allowed, and carbamidomethylation of a cysteine was defined as a fixed modification. The precursor ion mass tolerance and the fragment ion mass tolerance were 0.05 Da. The TMT-labeled protein identification Mascot results were validated by using Scaffold Proteome Software (version Scaffold_4.3.3, Proteome Software Inc., Portland, OR). Peptide identification was accepted at a false discovery rate (FDR) of less than 1.0% at the protein level and if the sample produced at least 2 unique peptides. The Scaffold Q+ was used for Label-Based Quantification (TMT, iTRAQ, SILAC, etc.) of peptides and proteins. Normalized reporter ion intensities were used to calculate the relative protein abundance. The protein ratios were then quantified by the median of the transformed reporter ion intensity ratios [23]. Label-free quantification was performed using Progenesis LC-MS software (version 4.1, Nonlinear, UK) as described previously [24].

Statistical analysis

All statistical analysis was performed with the Statistical Package for Social Studies software (SPSS, version 16, IBM). Comparisons between independent groups were conducted using one-way ANOVA followed by post hoc analysis with the least significant difference (LSD) test, and P-values less than 0.05 were considered to indicate statistically significant differences.

Results and discussion

Urinary proteome changes in APP (swe)/PSEN1^{ΔE9} transgenic mice

The urinary proteins of 4-month-old mice were digested by an in-gel method and quantified by a label-free method. A total number of 443 protein groups were identified in the urinary proteome at an FDR < 1%, and each generated at least 2 unique peptides. The levels of 33 urinary proteins were significantly different between the AD and wild-type groups (Table 1); among these proteins, cadherin-16 levels were different in both the >85 kDa and 50-85 kDa lanes, sulphydryl oxidase 1 levels were different in both the 50-80 kDa and 30-50 kDa lanes, and deoxyribonuclease-1 levels were different in both the 15-30 kDa and <15 kDa lanes. The fold change of the significantly changed protein levels was more than 1.5. In these 33 differential proteins, 17 had been reported to be associated with AD. Specifically, 15 had been reported to be associated with the pathology mechanisms of AD, while 9 were identified as direct AD biomarkers (Table 1).

Seven proteins relate to the development of AD as well as biomarkers of AD. (1) Kallikrein-1 (KLK1) levels were different in 4-, 6- and 8-month-old groups than the wild-type group, and the kallikrein-kinin system mediates inflammation in AD in vivo[25]. Kallikrein-6, one member of the kallikrein family, was reported as a biomarker of AD and to have increased levels in CSF and serum and decreased levels in tissue [26]. (2) Galactocerebrosidase (GALC) levels were different in 4- and 8-month-old groups than the wild-type group, and a deficiency in galactosylceramidase potentially contributed to neurodegenerative disease [27]. Ceramide has been suggested to participate in the neuronal cell death that leads to AD. GALC, as a gene connected to ceramide metabolism, was upregulated in the brain tissue of AD patients, making it an attractive candidate for diagnostic purposes and for intervening in neurodegenerative processes [28]. (3) Ceruloplasmin (CERU) levels were higher in both 4- and 8-month-old groups than in the control group, with the same trend of increased levels found in the serum of AD patients[29]. The levels of CSF diagnostic markers, such as Aβ42, tau and phospho-tau, were correlated with lower^{[[1]]} plasma copper and CERU levels in patients with Alzheimer's disease[30]. The ratio of CERU concentrations measured by enzymatic methods (eCP) to those measured by immunological methods (iCP), eCP/iCP, reflects the high specificity of AD patients as well as a decreased risk of having AD[31]. CERU had less ferroxidase activity in AD patients than wild-type patients, which contributed to the development of AD[32]. (4) Fibronectin (FNC) levels

were different in 4- and 8-month-old groups than in the wild-type group and were reported to be a novel biomarker for AD from blood[33]. In addition, FINC levels were significantly lower in plasma in mild cognitive impairment ¹¹⁷_{SEP}(MCI) patients than healthy patients, which provides further insight into the biological pathways and processes that underpin the pathophysiology and progression of MCI and AD[34]. (5) Cathepsin B (CATB) levels were different in 4- and 8-month-old groups than the wild-type group. CATB has been previously reported to be upregulated in brain tissues from APP/PS1 transgenic mice, and its levels changed in the same direction relative to healthy mice as they changed in the serum of AD patients relative to control patients, making CATB a potential biomarker of AD[13]. In addition, CATB produces brain pyroglutamate amyloid beta, which represents a potential AD therapeutic[35]. (6) Angiotensinogen (ANGT) was upregulated in 4-month-old groups and has exhibited increased levels in the CSF of AD patients[36]. As a component of the renin-ANGT system (RAS), ANGT is helpful for AD processes[37]. (7) Annexin A11 (ANX11) was upregulated in 4-month-old groups. Annexin A1 was reported to be expressed strongly in the microglia of AD patients, which aids the surveillance of microglia and the maintenance of brain homeostasis by using Annexin A1-dependent mechanisms[38], and Annexin A5 was reported to be a biomarker of AD and present at increased levels in the plasma of AD patients[39]. The protein-normalized abundances and spectral counts of all seven differential proteins were greater in every mouse in the high-abundance group than those in the low-abundance group.

Two other differential proteins have been mentioned as potential AD biomarkers. (1) Ectonucleotide pyrophosphatase/phosphodiesterase family member 2 (ENPP2) levels were higher in the CSF of AD patients than the CSF of healthy controls. ENPP2 can be used to specifically discriminate AD from Lewy body dementia, making it a candidate AD biomarker[40]. (2) The Ig kappa chain C (IGKC) region was reported to be a potential serum biomarker of AD at the early stage[41].

Eight differential proteins are associated with the mechanism of AD pathology. (1) CDH1, the gene of cadherin-1, is an AD risk gene that has significant association in Caribbean Hispanic individuals[42]. (2) As glycosylated transmembrane proteins, cadherins such as cadherin-1 and cadherin-16 also directly interact with the presenilin-1/g-secretase complex, which contributes to the modulation of A β peptide formation and thus plays a critical role in AD etiology[43]. (3) Alpha-amylase 1 (AMY1) reflects M3 muscarinic agonist activity in AD[44]. (4) ASMase, the gene of sphingomyelin

phosphodiesterase, which is connected to ceramide metabolism, was upregulated in the brain tissue of AD patients, making it an attractive candidate for both diagnostic purposes and intervening in neurodegenerative processes[28]. Significant correlations were observed between brain sphingomyelin phosphodiesterase levels and the levels of amyloid beta peptide (A β) and phosphorylated tau protein, which contribute to AD disease pathogenesis[45]. (5) Platelet-activating factor acetylhydrolase (PAFA) was present in higher levels in the plasma of AD patients than that of healthy controls, and these higher levels were associated with oxidative damage of low-density lipoproteins (ox-LDL), which contributes to the inflammation and oxidative stress of plasma lipoproteins and is strongly associated with Alzheimer's disease[46]. (6) Plasma carbonic anhydrase 2 (CAH2) was elevated in AD patients and may thus play a role in the pathogenesis of AD[47]. (7) Acid ceramidase (ASAH1) levels were elevated in AD brain, suggesting that acid ceramidase might play a role in controlling neuronal apoptosis and that acid ceramidase-mediated signaling pathways might be involved in the molecular mechanism of AD[48]. (8) Lipoprotein lipase (LIPL) is associated with neurite pathology, and its levels were reduced in the dentate gyrus of AD brains[49]. Some differential proteins have not been reported in research papers and may provide clues to candidates involved in the AD pathology mechanism.

Figure 3. Urinary proteins with significantly different levels in the 4-, 6- and 8-month-old mice and wild-type mice

The urinary proteins of 6-month-old mice were digested by a filter-aided method and quantified by a TMT-labeling method. At the protein level, 310 protein groups were identified in the urinary proteome at an FDR < 1%, and each generated at least 2 unique peptides. The levels of 33 urinary proteins were significantly different between the AD and wild-type groups, and their fold changes were greater than

1.5. Eight of these 33 differential proteins had been reported to be associated with AD. To be specific, 6 were associated with the pathology mechanisms of AD, while 3 had been directly referred to be AD biomarkers (Table 2).

Three differential proteins (KLK1, IGKC, and RAB10) have been suggested as AD biomarkers. The target gene RAB10, which is regulated by miRNA-369-3p, miRNA-30e-5p, miRNA-30e-3p, and miRNA-655, may play key roles in the progression and development of AD and may be a biomarker of AD[50].

Six differential proteins are associated with the mechanism of AD pathology. (1) Neogenin (NEO1) expresses weak NEO1 immunoreactivity in a small subset of amyloid plaques of AD brains[51]. (2) Charged multivesicular body protein 2a (CHM2A) levels were elevated in the 6-month-old group. Mutations in charged multivesicular body protein 2B (CHMP2B) can give rise to FTL-UPS[52]. (3) Peroxisomal carnitine (OCTC) levels were higher in the 6-month-old group than the wild-type group, and new therapies acting on peroxisomal metabolism might be developed to prevent cognitive decline and other age-related neurological disorders[53]. (4) Heat shock cognate 71 kDa protein (HSP7C) was upregulated in 6-month-old groups, while its gene level was significantly lower across the three brain regions in AD patients than in controls patients, suggesting ^[1]_{SEP} their participation in AD pathogenesis[54]. (5) Cooperative expression of leukemia inhibitory factor (LIF) and its receptor (LIFR) in AD hippocampus may indicate a role for LIF in neuronal damage or repair at these sites[55].

The urinary proteins of 8-month-old mice were digested by an in-gel method and quantified by a label-free method. At the protein level, 273 proteins were identified in the urinary proteome at an FDR < 1%, and each generated at least 2 unique peptides. The levels of 89 urinary proteins were significantly different between the AD and wild-type groups. Thirty-three of these 89 differential proteins have been associated with AD. Specifically, 29 are associated with the mechanism of AD pathology, while 17 have been suggested as candidate AD biomarkers. (Table 3)

Thirteen proteins have been previously reported to be related to the development of AD as well as biomarkers of AD, while 5 of them (KLK1, GALC, CERU, FINC, and CATB) had been already noted in 4-month-old groups. For example, (1) Serum albumin (ALBU) was differentially expressed between brains of AD mouse models and those of healthy mice[56], and serum levels of albumin-amyloid beta

complexes were lower in AD patients than healthy controls, which is very useful to apply in monitoring the progression of AD[57]. (2) Clusterin (CLUS) levels were higher in AD patients than controls, especially in regions with most abundant in A β , facilitating the development of AD[58]. As a heterodimeric glycoprotein, clusterin was more abundant in the CSF of some neurodegenerative disease patients than that of healthy controls and could thus serve as a potential biomarker to differentiate Parkinson's disease (PD) from dementia with Lewy bodies (DLB)[59]. (3) CSF complement C3 (CO3) is a staging biomarker in AD[60]. In addition, the dysregulation of neuron-glia interactions through NF κ B/ C3/C3aR signaling may contribute to synaptic dysfunction in AD[61]. Seventeen differential proteins have been mentioned as potential AD biomarkers. For example, (1) sulfated glycoprotein 1 (SAP) is a novel CSF biomarker for neurodegeneration and has been validated by a high-throughput multiplexed targeted proteomic assay[40]. (2) Cystatin-C (CYTC) is a novel CSF biomarker for staging early AD[62]. Twenty-nine differential proteins are associated with the mechanism of AD pathology. For example, (1) CD44 antigen (CD44) gene expression was greater in lymphocytes derived from AD patients than in those from healthy controls, suggesting that it plays roles in the peripheral immune response during the development of AD[63]. (2) The rs2227564 polymorphism of the urokinase-type plasminogen activator (UROK) gene increased the risk of AD[64]. Other differential proteins are annotated in Table 3.

Functional analysis of differential urine proteins in APP (swe)/PSEN1^{dE9} transgenic mouse

Figure 4. Biological processes of differential proteins in different stages of AD development.

Functional annotation of differential urinary proteins was performed using DAVID[65]. The differential proteins at three time points were classified to be involved with certain biological processes (Figure 4), molecular components, and molecular functions (Supplemental Figures 1 and 2).

In 4-month-old mice, differential urine proteins involved in lipid metabolism and lipid catabolism were enriched. As lipid metabolism is a fundamental process for brain development and function, aberrant lipid metabolism was not surprisingly common in an animal model of AD that relates to AD pathogenesis[66-68]. For example, AD-induced perturbation of niche fatty acid metabolism can suppress the homeostatic and regenerative functions of neural stem cells, supporting the mechanism of AD pathogenesis[66]. Abnormal lipid metabolism influences A β metabolism and deposition in both brain parenchyma and vasculature, as well as tau hyperphosphorylation and aggregation, which then likely triggers a series of downstream catalytic events that eventually affect the progression of AD pathogenesis[67]. More importantly, lipid rafts from human cerebral cortex are associated with the pathogenesis of early AD, as β -secretase/A β PP (amyloid- β protein precursor) interactions and lipid raft microviscosity are strongly and positively correlated in AD frontal and entorhinal cortices, indicating that the aberrant lipid metabolism had already occurred in the early stage of AD[69]. Decidualization and calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules, which are not reported in AD studies, may play roles in the mechanism of AD pathology. Recent studies demonstrated that using a triple receptor agonist (TA), which activates GIP-1, GIP and glucagon receptors at the same time, reduces the total amount of beta amyloid in an APP/PS1 transgenic mouse model through a 2-month TA treatment [70]. However, this research used 6-month-old transgenic mice for a 2-month treatment, and beta amyloid had already appeared. We thus suppose that a more effective treatment can be achieved when the 4-month-old transgenic mice are used for therapy.

The differential proteins in 6-month-old mice were involved in transport processes, while axonal transport (AT) defects play an important role in the pathogenesis of AD[71]. Other biological processes such as microvillus assembly, the regulation of cell shape and protein localization to the plasma membrane may contribute to the formation of beta amyloid plaque deposits in the brain of AD patients, which provides potential clues for the studying the mechanism of AD.

A larger number of differential urine proteins were identified in 8-month-old mice and indicated other AD-related biological processes. Proteolysis processes were overrepresented and reported to be

associated with APP proteolysis, which is consistent with the pathology of AD[72]. In addition, the urinary proteins that were related to fibrinolysis may be factors contributing to AD[73]. What's more, lysosome organization accumulated at amyloid plaques in mouse models of AD, which gave rise to potential therapies of AD patients[74]. Lastly, angiogenesis processes were reported to contribute to the pathogenesis of AD, as they are a common feature of amyloid beta (A β) plaques[75, 76], and the levels of some angiogenesis factors were elevated in the plasma of AD patients[77].

In summary, in the biological process category, metabolic and carbohydrate metabolic processes were overrepresented in 4- and 8-month-old AD mice. The differential urine proteins in 4- and 8-month-old mice indicated an enrichment of metabolic process, while the three subtypes of AD can be distinguished by metabolic profiling[78]. Considering that the biological processes whose activities changed in this experiment did not extensively overlap at these three time points, this result indicates that only three different stages might occur, implying that different therapeutic strategies may be appropriate for different stages. If biomarkers can be found in human as early as was found for the 4-month-old in this model, we may have an early intervention window in which disease development can be stopped.

In the cellular component category (Supplemental Figure 1), most differential proteins came from the extracellular exosome, the extracellular space, the anchored component of membranes, and blood microparticles, whereas a small number of differential proteins were derived from organelles such as the lysosome and the Golgi apparatus. In the molecular function category (Supplemental Figure 2), hydrolase activity was overrepresented in 4- and 8-month-old mice; calcium ion binding was overrepresented in 4-month-old mice; protein complex binding, sodium ion transmembrane transporter activity and protein binding were overrepresented in 6-month-old mice; and peptidase, endopeptidase, serine-type peptidase activities were overrepresented in 8-month-old mice.

IPA of differential proteins in AD development

To identify the differential proteins with mostly affected cellular death and survival, ingenuity pathway analysis (IPA) was applied to cell death and survival networks (Figure 5). Cadherin-1, which was reported to be associated with AD[42], is a core component of these networks in 4-month-old mice. CDH16, CP, ENPP2, AGT, SMPD1, PLA2G7 were upregulated and play roles in AD[28, 29, 36, 40,

43, 46], whereas sialate O-acetyltransferase was downregulated in 4-month-old mice. In 6-month-old mice, NEO1, CROT, GP2, SEMA4A and LIFR were present in higher levels in transgenic mice than the control group, and NEO1 as well as CROT were associated with the pathology and mechanism of AD[51, 79]. In contrast, MEP1B, CLIC4, and PDZK1 levels were lower in transgenic mice than the control group. FINC was the core component of the network in 8-month-old mice and plays roles in AD[80]. In addition, AFM, MFGE8, C3, CD44 and PLA2 were upregulated in transgenic mice and are potential biomarkers or play roles in the development of AD[61, 63, 64, 81, 82], while VNN1, HEXB and CTSZ were downregulated in transgenic mice when compared with control groups.

Figure 5. IPA of differential proteins in AD development

Conclusion

Our results indicated that urine proteins enable AD early detection before beta amyloid plaque deposition, which may provide an opportunity for intervention.

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Table 1. Details of differential urinary proteins of 4-month-old mice

MW	Protein name	UniProt	Human UniProt	P value	Fold change	Confidence score	Normalized abundance						pathology and mechanism	biomarkers
							WT-1	WT-2	WT-3	AD-1	AD-2	AD-3		
>85 kDa	Cadherin-1	P09803	P12830	0.022	1.87	218.7	2619939	3821894	4545058	7387696	5702665	7448193	[42, 43]	-
	Cadherin-16	O88338	O75309	0.039	1.33	475.3	7842297	10683716	8044870	12305698	10097708	12879662	[43]	-
	Keratin, type II cytoskeletal 2 oral	Q3UV17	Q01546	0.041	0.56	181.9	95619	152124	160984	52841	108167	67233	-	-
50-85 kDa	Ceruloplasmin	Q61147	P00450	0.018	3.19	142.8	1405128	700799	1034296	4233275	2979048	2817111	[31, 32]	[30]
	Alpha-amylase1	P00687	P04745	0.037	2.98	1271.6	16862562	16394995	16747254	27733747	36333676	85064392	[44]	-
	Ectonucleotide pyrophosphatase	Q9R1E6	Q13822	0.038	2.89	438.4	6410477	4227746	3261439	14447745	15522911	10137661	-	[40]
	AnnexinA11	P97384	P50995	0.035	2.54	124.5	112847	385493	224516	851694	561465	420834	[38]	[39]
	Angiotensinogen	P11859	P01019	0.036	2.53	340.3	1832470	6008159	4047815	8130284	9777242	12155633	[37]	[36]
	Kallikrein-1	P15947	P06870	0.028	2.55	161.4	13993666	15481983	8200572	32915114	24952991	38216076	[25]	[26]
	Pantetheinase	Q9Z0K8	O95497	0.010	2.25	329.7	7264591	9291049	8941437	18429885	12901960	26056688	-	-
	Sulfhydryloxidase1	Q8BND5	O00391	0.029	1.93	444.1	4216654	7066885	5327850	12135722	10500098	9505950	-	-
	Sphingomyelin phosphodiesterase	Q04519	P17405	0.004	1.71	138.9	789408	934631	809095	1433994	1281043	1625436	[28, 45, 83]	-
	N-Acetylmuramoyl-L-alanine amidase	Q8VCS0	Q96PD5	0.001	1.63	237.3	4431595	4089554	4317879	6991718	6218791	7761766	-	-
	Galactocerebrosidase	P54818	P54803	0.002	1.51	296.1	23253378	21116228	25380610	39727782	31492109	33948684	[27]	[28]
	Cadherin-16	O88338	O75309	0.005	1.37	165.0	8088405	10041828	9001115	12089782	12239425	12726177	[43]	-
	Trehalase	Q9JLT2	O43280	0.036	1.38	176.5	1708628	1343343	1240247	2367601	1874206	1677882	-	-
	Platelet-activating factor acetylhydrolase	Q60963	Q13093	0.024	1.49	103.9	790161	1089782	1129603	1525671	1594208	1369456	[46]	-
	Carbonic anhydrase 2	P00920	P00918	0.050	7.64	139.9	1026487	137540	1196867	2483019	8183767	7363671	[47]	-

30-50 kDa	ATP-binding cassette sub-family A member 13	Q5SSE9	Q86UQ 4	0.003	3.97	186.0	162885	413064	239944	1326933	1045477	870531	-	-
	Protein LEG1 homolog	Q8C6C 9	Q6P5S2	0.046	3.17	331.5	617434	5666756	2910064	10253781	9689764	9179997	-	-
	Cathepsin B	P10605	P07858	0.029	3.13	338.5	7139961	1114522	1317986	9277618	8644600	12074430	[35]	[13]
	Acid ceramidase	Q9WV5 4	Q13510	0.031	0.42	264.6	39183681	22168743	30757070	11403664	10956829	16671159	[48]	-
	Sulfhydryl oxidase 1	Q8BND 5	O00391	0.005	0.40	271.4	6515045	6236645	3506111	2174726	1897719	2494845	-	-
	Eosinophil cationic protein 1	P97426	no	0.031	0.39	167.5	25123514 5	10362902 6	27345839 6	11217001 0	66736059	65435575	-	-
	Fibronectin	P11276	P02751	0.017	0.36	191.7	6815535	4157474	3089723	1594554	2037884	1485064	[34]	[33]
	Prostaglandin-H2 D-isomerase	O09114	P41222	0.018	0.27	196.1	11633843 5	11891642 2	35968640	25071521	28557837	19099588	-	-
	Dipeptidyl peptidase 2	Q9ET22	Q9UHL 4	0.017	0.23	329.3	36130176	10831853	12769425	4129725	4626310	4946023	-	-
	Lipoprotein lipase	P11152	P06858	0.025	0.18	157.9	12700373	4762033	4358911	1006139	706457	2137022	[49]	-
	Sialate O-acetylsterase	P70665	Q9HAT 2	0.041	0.17	150.5	5186756	1470459	629510	242721	323999	669184	-	-
	Alpha-1-antitrypsin 1-4	Q00897	no	0.029	0.09	386.0	47453084	7866154	10014171	1352876	1413734	3276721	-	-
15-30 kDa	Deoxyribonuclease-1	P49183	P24855	0.002	2.46	177.0	6661598	5715618	7105781	11646345	22351861	13973828	-	-
	Ig kappa chain C region	P01837	P01834	0.014	1.80	103.9	8822671	11713287	12329617	21590819	21713190	15916672	-	[41]
<15 kDa	Deoxyribonuclease-1	P49183	P24855	0.026	2.47	146.4	1360757	728821	1080584	1869443	3272391	2687073	-	-
	Secretoglobulin family 2B member 20	Q9JI02	no	0.026	0.56	323.6	15981747	29749962	27032579	15560357	9828085	15548114	-	-

Ig kappa chain V-V region L7 (Fragment)	P01642	no	0.021	0.20	130.7	893023	1868961	641920	208754	175548	309125	-	-
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Table 2. Details of differential urinary proteins of 6-month-old mice

Protein name	UniProt	Human	P	Fold	Spectral	MW	Normalized ratio						pathology and mechanism	biomarker
		UniProt	value	change	count		WT-1	WT-2	WT-3	AD-1	AD-2	AD-3		
Major urinary protein 1	P11588	NO	0.004	10.92	1657	21 kDa	0.45	0.95	1.6	8.45	13.9	10.4	-	-
Neogenin	P97798	Q92859	0.005	7.22	3	38 kDa	1	1.45	0.1	4.8	7.75	5.85	[51]	-
CMRF35-like molecule 2	Q8K249	Q496F6	0.004	5.31	3	22 kDa	0.5	3	3.4	12.45	9.7	14.5	-	-
Major urinary protein 5	P11591	no	0.011	3.79	326	21 kDa	1.05	4	3	12.45	7.55	10.5	-	-
WAP four-disulfide core domain protein 12	Q9JHY 3	Q8WWY7	0.029	2.94	5	10 kDa	0.9	0.95	0.65	3.25	2.5	1.6	-	-
Keratin, type I cytoskeletal 19	P19001	P08727	0.014	2.41	9	45 kDa	1	1.2	1.25	3.35	2.05	2.9	-	-
Cadherin-15	P33146	P55291	0.015	2.06	3	86 kDa	1	0.6	0.75	1.95	1.4	1.5	-	-
Charged multivesicular body protein 2a	Q9DB3 4	O43633	0.027	1.88	4	25 kDa	1	0.8	0.65	1.35	1.35	1.9	[52]	-
Cadherin-11	P55288	P55287	0.024	1.87	2	88 kDa	1	0.7	0.55	1.65	1.25	1.3	-	-
Peroxisomal carnitine	Q9DC5 0	Q9UKG9	0.030	1.86	2	70 kDa	1	1.05	0.85	2.25	1.75	1.4	[53]	-
Desmoplakin	E9Q557	P15924	0.041	1.80	27	333 kDa	0.8	0.85	0.8	1.3	1.2	1.9	-	-
Pancreatic secretory granule membrane major glycoprotein GP2	Q9D733	P55259	0.048	1.79	3	59 kDa	1	0.8	0.55	1.55	1.05	1.6	-	-

Alpha-1-antitrypsin 1-4	Q00897	no	0.022	1.75	6	46 kDa	0.9	0.8	0.9	1.45	1.85	1.25	-	-
Heat shock cognate 71 kDa protein	P63017	P11142	0.020	1.67	7	71 kDa	0.9	0.9	0.95	1.45	1.3	1.85	[54]	-
Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial	Q9WV M8	Q8N5Z0	0.002	1.63	2	48 kDa	1	1.15	0.85	1.65	1.6	1.65	-	-
Semaphorin-4A	Q62178	Q9H3S1	0.002	1.62	5	83 kDa	0.85	0.7	0.7	1.2	1.15	1.3	-	-
Serine protease inhibitor A3M	Q03734	no	0.006	1.53	3	47 kDa	0.9	0.95	0.8	1.2	1.45	1.4	-	-
Leukemia inhibitory factor receptor	P42703	P42702	0.044	1.51	6	122 kDa	0.9	0.9	1.05	1.45	1.15	1.7	[55]	-
Kallikrein-1	P15947	P06870	0.028	0.61	79	29 kDa	1	1.5	1.35	0.8	0.75	0.8	[25]	[26]
Prominin-1	O54990	O43490	0.027	0.60	2	97 kDa	1	0.75	0.75	0.55	0.4	0.55	-	-
Glycerophosphodiester phosphodiesterase domain-containing protein 3	Q99LY 2	Q7L5L3	0.039	0.59	7	38 kDa	0.9	1.35	1.4	0.65	0.7	0.8	-	-
Ig kappa chain C region	P01837	P01834	0.003	0.57	25	12 kDa	1	0.85	0.8	0.5	0.5	0.5	-	[41]
Solute carrier family 12 member 3	P59158	P55017	0.030	0.55	3	111 kDa	1	0.9	1.3	0.5	0.75	0.5	-	-
Meprin A subunit beta	Q61847	Q16820	0.029	0.54	6	79 kDa	1.1	1.05	0.9	0.3	0.7	0.65	-	-
Solute carrier family 23 member 1	Q9Z2J0	Q9UHI7	0.014	0.54	9	66 kDa	1	0.7	0.8	0.4	0.45	0.5	-	-
Sushi domain-containing protein 2	Q9DBX 3	no	0.001	0.54	3	90 kDa	0.9	1	0.9	0.5	0.45	0.55	-	-
Villin-1	Q62468	P09327	0.011	0.52	8	93 kDa	1	0.7	0.8	0.4	0.45	0.45	-	-
Ezrin	P26040	P15311	0.027	0.51	11	69 kDa	0.9	0.55	0.7	0.35	0.4	0.35	-	-

Chloride intracellular channel protein 4	Q9QYB1	Q9Y696	0.008	0.50	3	29 kDa	1	0.7	0.9	0.45	0.45	0.4	-	-
Na(+)/H(+) exchange regulatory cofactor NHE-RF3	Q9JIL4	Q5T2W1	0.013	0.48	4	56 kDa	1	0.8	0.7	0.3	0.45	0.45	-	-
Ras-related protein Rab-10	P61027	P61026	0.041	0.43	2	23 kDa	1	1.05	1.65	0.4	0.75	0.45	-	[50]
Na(+)/H(+) exchange regulatory cofactor NHE-RF1	P70441	O14745	0.011	0.42	13	22 kDa	1	0.65	0.75	0.3	0.35	0.35	-	-
Ig kappa chain V-V region L7 (Fragment)	P01642	no	0.005	0.38	10	13 kDa	1	0.8	0.7	0.35	0.3	0.3	-	-

Table 3. Details of differential urinary proteins of 8-month-old mice

MW range	Protein name	UniProt	Human UniProt	p value	MW	Spectral count						pathology and mechanism	biomarker
						WT T1	WT 2	WT 3	AD 1	AD 2	AD 3		
>100 kDa	Angiotensin-converting enzyme	P09470	P12821	0.0092	151 kDa	27	28	27	20	13	19	[84]	-
	Aminopeptidase N	P97449	P15144	0.025	110 kDa	6	4	8	15	10	14	[85]	-
	Uromodulin	Q91X17	P07911	0.027	71 kDa	95	98	165	186	208	227	-	-
	Lysosome-associated membrane glycoprotein 2	P17047	P13473	0.031	46 kDa	3	2	2	1	0	1	[86]	[87]
	Podocalyxin	Q9R0M4	O00592	0.042	53 kDa	0	0	0	2	1	4	-	-
65-100 kDa	Beta-glucuronidase	P12265	P08236	0.0001	74 kDa	5	4	6	0	0	0	[88]	-
	Carboxylesterase 1C	P23953	no	0.00029	61 kDa	17	18	17	12	12	11	-	-
	Biotinidase	Q8CIF4	P43251	0.0034	58 kDa	2	2	3	0	0	0	-	-
	Major urinary protein 20	Q5FW60	no	0.009	21 kDa	7	7	6	4	5	5	-	-

	Galactocerebrosidase	P54818	P54803	0.011	77 kDa	1	2	2	0	0	0	[27]	[28]
	Pancreatic alpha-amylase	P00688	no	0.016	57 kDa	0	0	0	6	4	10	-	-
	Uromodulin	Q91X17	P07911	0.016	71 kDa	39	33	43	75	81	115	-	-
	Complement factor D	P03953	P00746	0.016	28 kDa	1	0	2	8	4	5	-	-
	Tyrosine-protein kinase receptor UF	Q00993	P30530	0.018	98 kDa	0	0	1	2	2	2	-	-
	Serum albumin	P07724	P02768	0.02	69 kDa	21 5	187	174	153	137	127	[57]	[56]
	Isoform 2 of V-type proton ATPase catalytic subunit A	P50516	P38606	0.02	56 kDa	1	1	2	0	0	0	-	[56]
	Pantetheinase	Q9Z0K8	O95497	0.021	57 kDa	2	2	2	2	1	1	-	-
	Ceruloplasmin	Q61147	P00450	0.036	121 kDa	1	1	0	2	2	2	[31, 32]	[30]
	Alpha-N-acetylglucosaminidase	O88325	P54802	0.041	?	8	10	12	2	1	7	-	-
	Meprin A subunit alpha	P28825	Q16819	0.042	84 kDa	33	35	31	40	43	36	[89]	-
	Hemopexin	Q91X72	P02790	0.042	51 kDa	18	14	13	21	24	19	[90]	[91]
	Ig mu chain C region	P01872	no	0.049	50 kDa	0	0	0	3	2	7	-	-
50-65 kDa	Glycosylation-dependent cell adhesion molecule 1	Q02596	no	0.0002	16 kDa	0	1	0	8	10	10	-	-
	Afamin	O89020	P43652	0.0004 9	69 kDa	1	1	0	6	7	6	-	[81]
	Protein Fcgbp	E9Q0B5	no	0.0005	275 kDa	2	4	2	10	12	10	-	-
	Alpha-2-macroglobulin	Q61838	no	0.0007 6	166 kDa	11	8	9	0	0	0	-	-
	N-acetylgalactosamine-6-sulfatase	Q571E4	P34059	0.0009 6	58 kDa	11	15	11	0	0	0	-	-
	Lysosomal Pro-X carboxypeptidase	Q7TMR0	P42785	0.0013	55 kDa	8	7	5	0	0	0	-	-
	Beta-hexosaminidase subunit beta	P20060	P07686	0.0021	61 kDa	15	12	20	0	0	1	-	-
	Pancreatic alpha-amylase	P00688	no	0.0024	57 kDa	57	48	42	82	79	80	-	-
	Aminopeptidase N	P97449	P15144	0.0025	110 kDa	0	0	0	4	5	6	[85]	[92]
	Growth arrest-specific protein 1	Q01721	P54826	0.0026	36 kDa	1	0	0	4	4	5	[93]	-
	Desmocollin-2	P55292	Q02487	0.0027	100 kDa	0	0	0	2	3	2	-	-

Acidic mammalian chitinase	Q91XA9	Q9BZP6	0.0029	52 kDa	1	0	0	14	11	8	-	-
Beta-2-glycoprotein 1	Q01339	P02749	0.0038	39 kDa	1	0	0	5	4	6	-	-
Major urinary protein 20	Q5FW60	NO	0.0047	21 kDa	11	11	9	6	6	6	-	-
Serotransferrin	Q92111	P02787	0.0062	77 kDa	18	12	6	32	30	29	[94]	[95]
Acyloxyacyl hydrolase	O35298	P28039	0.0073	65 kDa	3	2	4	0	0	0	-	-
Alpha-N-acetylgalactosaminidase	Q9QWR8	P17050	0.0096	47 kDa	2	2	1	0	0	0	-	-
Lysosomal thioesterase PPT2	O35448	Q9UMR5	0.0098	34 kDa	1	2	1	0	0	0	-	-
Protein LEG1 homolog	Q8C6C9	Q6P5S2	0.011	38 kDa	7	12	10	1	3	1	-	-
Protein Ighg2b	A0A075B5 P3	no	0.011	36 kDa	0	0	0	7	3	6	-	-
Acid sphingomyelinase-like phosphodiesterase 3a	P70158	Q92484	0.011	50 kDa	7	5	5	3	1	2	-	-
Kallikrein-1	P15947	P06870	0.012	29 kDa	2	4	5	8	7	9	[25]	[26]
Lactadherin	P21956	Q08431	0.012	51 kDa	1	3	1	8	6	5	[82]	-
Alpha-1-antitrypsin 1-5	Q00898	P01009	0.015	46 kDa	22	17	14	7	8	7	-	[96]
Protein 9530053A07Rik	E9PVG8	Q9Y6R7	0.015	?	26	11	24	0	1	0	-	-
Sushi domain-containing protein 2	Q9DBX3	Q9UGT4	0.016	91 kDa	2	4	4	0	1	1	-	-
Polymeric immunoglobulin receptor	O70570	P01833	0.017	85 kDa	8	10	13	16	21	20	-	-
Protein Ighg2c	F6TQW2	no	0.018	?	0	1	2	4	5	3	-	-
Carboxylesterase 1C	P23953	no	0.018	61 kDa	1	2	0	4	3	3	-	-
Tyrosine-protein kinase receptor UF	Q00993	P30530	0.018	98 kDa	0	0	0	1	2	1	-	-
Hemopexin	Q91X72	P02790	0.019	51 kDa	4	5	0	11	11	18	[90]	[91]
Tripeptidyl-peptidase 1	O89023	O14773	0.019	61 kDa	1	3	3	0	0	0	-	-
Napsin-A	O09043	O96009	0.02	46 kDa	12	11	11	17	23	26	[97]	-
Complement C3	P01027	P01024	0.02	186 kDa	0	0	0	6	2	4	[61, 98]	[60]
Galactocerebrosidase	P54818	P54803	0.021	77 kDa	3	6	5	1	2	2	[27]	[28]-
Matrix-remodeling-associated protein 8	Q9DBV4	Q9BRK3	0.023	50 kDa	0	1	0	5	3	2	-	-
CD44 antigen	P15379	P16070	0.027	86 kDa	2	2	2	3	4	3	[63]	-
Major urinary protein 2	P11589	no	0.031	21 kDa	63	70	53	43	49	42	-	-
Actin, aortic smooth muscle	P62737	P62736	0.031	42 kDa	0	1	1	2	2	3	-	-

	Meprin A subunit alpha	P28825	Q16819	0.032	84 kDa	17	17	17	19	25	25	[89]	-
	Leucine-rich HEV glycoprotein	Q91XL1	P02750	0.034	?	15	13	11	8	9	9	-	-
	Pro-epidermal growth factor	P01132	P01133	0.036	133 kDa	55	56	59	68	72	62	-	-
	Fibronectin	P11276	P02751	0.038	273 kDa	3	2	0	6	7	4	[34]	[33]
	Knq2 protein	Q6S9I0	no	0.039	47 kDa	30	30	30	42	34	36	-	-
	Alpha-1-acid glycoprotein 1	Q60590	P02763	0.039	24 kDa	4	3	2	6	5	5	-	-
	MCG15829, isoform CRA_a	Q3KQQ2	no	0.04	?	8	11	9	5	4	7	-	-
	Ceruloplasmin	Q61147	P00450	0.044	121 kDa	0	1	0	2	2	4	[31, 32]	[29]
	N-acetylglucosamine-6-sulfatase	Q8BFR4	P15586	0.048	61 kDa	3	6	3	1	1	2	-	-
30-50 kDa	Mammalian ependymin-related protein 1	Q99M71	Q9UM22	0.0001	25 kDa	2	2	2	0	0	0	-	-
	N-acyl-aromatic-L-amino acid amidohydrolase (carboxylate-forming)	Q91XE4	Q96HD9	0.0001 7	35 kDa	7	6	6	0	0	0	-	-
	Pancreatic alpha-amylase	P00688	no	0.0002 2	57 kDa	7	2	2	28	29	27	-	-
	Protein Ighg2c	F6TQW2	no	0.0002 4	?	3	4	5	10	11	10	-	-
	Alpha-2-macroglobulin	Q61838	no	0.0002 6	166 kDa	21	21	17	1	1	1	-	-
	Glycosylation-dependent cell adhesion molecule 1	Q02596	no	0.0005	16 kDa	0	0	0	2	1	2	-	-
	Prothrombin	P19221	P00734	0.0007 3	70 kDa	4	4	5	7	8	7	[99-101]	-
	Cluster of Lysosomal thioesterase PPT2	O35448	Q9UMR5	0.0015	34 kDa	3	2	3	0	0	0	-	-
	Urokinase-type plasminogen activator	P06869	P00749	0.0023	48 kDa	6	8	7	17	13	16	[64]	-
	Nidogen-2	O88322	Q14112	0.0027	154 kDa	0	0	0	2	1	1	-	-
	Prostatic spermine-binding protein	P15501	no	0.0034	22 kDa	3	3	5	0	0	0	-	-
	dorant-binding protein 2a	Q8K1H9	no	0.0052	20 kDa	12	7	8	1	1	1	-	-
	Deoxyribonuclease-1	P49183	P24855	0.0053	32 kDa	22	24	24	14	17	12	-	-
	Complement factor D	P03953	P00746	0.0062	28 kDa	20	41	28	78	78	62	-	-
	UPF0762 protein C6orf58 homolog	Q8C6C9	Q6P5S2	0.0095	38 kDa	2	2	1	0	0	0	-	-

	Napsin-A	O09043	O96009	0.0099	46 kDa	12	11	10	14	13	14	[97]	
	Major urinary protein 20	Q5FW60	no	0.013	21 kDa	16	17	20	9	11	6	-	-
	L-lactate dehydrogenase B chain	P16125	P07195	0.016	37 kDa	1	2	1	0	0	0	-	-
	Procollagen C-endopeptidase enhancer 1	Q61398	Q15113	0.016	50 kDa	1	2	2	8	9	5	-	-
	Leucine-rich HEV glycoprotein	Q91XL1	P02750	0.017	?	6	5	7	4	3	2	-	-
	Hemoglobin subunit alpha	P01942	P69905	0.019	15 kDa	2	5	3	0	0	0	-	-
	Hemoglobin subunit beta-1	P02088	P68871	0.022	16 kDa	2	6	6	0	0	0	-	-
	Lysosomal protective protein	P16675	P10619	0.022	54 kDa	1	3	3	0	0	0	[102]	-
	Uromodulin	Q91X17	P07911	0.022	71 kDa	11	8	6	21	17	30	-	-
	Prostate stem cell antigen	P57096	O43653	0.028	13 kDa	0	0	1	2	1	3	[103]	-
	Cathepsin B	P10605	P07858	0.03	37 kDa	3	1	2	0	0	0	[35]	[13]
	Meprin A subunit alpha	P28825	Q16819	0.03	84 kDa	7	7	11	14	12	16	[89]	-
	Insulin-like growth factor-binding protein 7	Q61581	Q16270	0.032	29 kDa	5	5	5	6	7	8	[104]	-
	MCG140531	A2AV72	no	0.033	?	10	8	8	5	6	6	-	-
	Ig kappa chain V19-17	P01633	P06312	0.038	16 kDa	1	1	1	1	0	0	-	-
	Cathepsin Z	Q9WUU7	Q9UBR2	0.044	34 kDa	4	2	5	0	0	2	[105]	-
20-30 kDa	Odorant-binding protein 2a	Q8K1H9	no	0.004	20 kDa	2	2	1	0	0	0	-	-
	Lymphocyte antigen 6D	P35459	Q14210	0.0048	13 kDa	1	0	0	3	2	2	-	-
	Napsin-A	O09043	O96009	0.0068	46 kDa	1	1	0	3	3	2	[97]	-
	Complement factor D	P03953	P00746	0.0078	28 kDa	1	2	3	8	14	11	-	-
	Kallikrein-1	P15947	P06870	0.008	29 kDa	0	0	0	5	7	10	[25]	[26]
	Serum albumin	P07724	P02768	0.01	69 kDa	2	1	3	5	5	7	[57]	[56]
	Cluster of MCG15829, isoform CRA_a	Q3KQQ2	no	0.018	?	14 6	151	121	107	88	94	-	-
	Pro-epidermal growth factor	P01132	P01133	0.033	133 kDa	1	0	0	5	7	2	-	-
<20 kDa	Major urinary protein 20	Q5FW60	no	0.0012	21 kDa	20	15	17	5	2	5	-	-
	Sulfated glycoprotein 1	Q61207	P07602	0.003	61 kDa	16	16	13	8	6	6	-	[40]
	Clusterin	Q06890	P10909	0.0034	52 kDa	0	1	0	3	3	4	[58, 106]	[107]
	Lysozyme C-2	P08905	P61626	0.0049	17 kDa	0	0	0	4	3	2	-	-

Uromodulin	Q91X17	P07911	0.0083	71 kDa	1	2	1	5	7	7	-	-
Complement factor D	P03953	P00746	0.011	28 kDa	6	7	6	16	23	28	-	-
Cystatin-C	P21460	P01034	0.011	16 kDa	0	0	0	2	1	1	-	[62, 108]
Ubiquitin-60S ribosomal protein L40	P62984	P62987	0.011	15 kDa	0	0	0	2	1	1	-	-
Cluster of MCG116526	D3YYY1	no	0.029	?	10	6	5	2	2	1	-	-
Fibronectin	P11276	P02751	0.038	273 kDa	0	1	1	4	2	5	[34]	[33]
Protein Col6a3	E9PWQ3	P12111	0.038	?	0	0	0	3	1	1	-	-
Pancreatic alpha-amylase	P00688	no	0.046	57 kDa	0	1	1	5	2	3	-	-
Plasminogen	P20918	P00747	0.047	91 kDa	0	2	1	5	5	3	[109]	[110]

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