Research Square

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Urinary Proteome Changes in Global Cerebral Ischemia-Reperfusion Injury Rat Model Using Proteomics

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Abstract

Background

Cerebral ischemia-reperfusion (I/R) injury is the leading cause of death in severe hypotension caused by cardiac arrest, drowning, and excessive blood loss. Urine can sensitively reflect pathophysiological changes in the brain even at an early stage.

Methods

In this study, a rat model of global cerebral I/R injury was established via Pulsinelli's four-vessel occlusion (4-VO) method. The proteomics techniques of data-independent acquisition (DIA) and parallel reaction monitoring (PRM) were applied to profile the urinary proteome. The differentially expressed proteins were subjected to Gene Ontology (GO) and protein-protein interaction (PPI) analysis.

Results

One hundred and sixty-four proteins significantly differed in the 4-VO rat urine samples compared to the control samples (1.5-fold change, $p<0.05$). GO analysis showed that the acute-phase response, the ERK1 and ERK2 cascade, endopeptidase activity, blood coagulation, and angiogenesis were overrepresented. After PRM validation, fifteen differentially expressed proteins were identified, and their expression was consistent with the DIA quantification. The abundance of FGG, COMP, TFF2, and HG2A was significantly changed only at 12 h after I/R injury. APOE, FAIM3, FZD1, IL1R2, UROK and CD48 were upregulated only at 48 h after I/R injury. KNG1, CATZ, PTGDS, PRVA and HEPC showed an overall trend of upregulation or downregulation at 12 and 48 h after I/R injury, reflecting the progression of cerebral I/R injury.

Conclusion

In this study, fifteen differentially expressed urinary proteins were identified and validated in a 4-VO rat model. Eight of these proteins were reported to be associated with cerebral I/R injury. These findings provide important clues to inform the monitoring of cerebral I/R injury and further the current understanding of its molecular biological mechanisms.

Introduction

Global cerebral ischemia-reperfusion (I/R) injury is the leading cause of death in severe hypotension caused by cardiac arrest, drowning, and excessive blood loss [1]. Because the brain is very sensitive to hypoxia, the brain tissue sustains irreversible damage 4 to 6 min after the loss of circulation, especially in highly metabolically active regions such as the hippocampus, thalamus, cerebral cortex, and striatum [2]. Global cerebral I/R injury is closely associated with neurological dysfunction, from mild cognitive

impairment to a minimally conscious state or even a persistent vegetative state [3]. According to reports, up to 80% of survivors are in a vegetative state, and patients who recover well often have serious psychological complications such as depression, anxiety, and posttraumatic stress disorder, which seriously affect their quality of life [4]. To date, the diagnosis and evaluation of global cerebral I/R injury have mainly relied on clinical symptoms, neuroimaging, electrophysiology, and biochemical examination of blood or cerebrospinal fluid. However, hemodynamic instability, sedation and hypothermia are common in these patients, limiting the application of these tests [5]. The main clinical treatment methods include supportive therapy, symptomatic treatment, mild hypothermia and hyperbaric oxygen therapy. There is still a lack of specific, effective neuroprotective strategies and drugs. Therefore, early and accurate assessment of the degree of brain injury and prognosis is critical for diagnosis and treatment.

Currently, blood and cerebrospinal fluid are the main sources of samples for the study of brain injury biomarkers. Although many candidate protein biomarkers have been reported in blood and cerebrospinal fluid, they have not been further applied [6, 7]. There are several drawbacks to focusing on these fluids: 1) An invasive sampling process is necessary to obtain blood or cerebrospinal fluid, and the latter is especially difficult to obtain. 2) Blood and cerebrospinal fluid are important components of the internal environment; therefore, they are strictly controlled by homeostatic mechanisms to maintain the relative stability of their components. When a certain change is introduced into the internal environment, the body will reduce this change as much as possible and eliminate it through various mechanisms to keep its composition relatively stable. Therefore, changes in biomarkers in blood and cerebrospinal fluid are not sufficiently sensitive. 3) Due to technical limitations, the sample size and the extent of protein identification in previous studies were relatively small, and the conclusions have not been extensively validated. Thus, simple, noninvasive, and sensitive biomarkers are needed.

According to the largest human urine proteome database including 6085 sequence of protein information, some of these proteins have been reported to be enriched in 32 tissues and organs, such as the brain, gastrointestinal tract, kidneys and so on [8]. Among these proteins, 1956 that were enriched in brain tissue were identified in urine; this number ranked first among the 32 tissues and organs evaluated. Several studies have shown that urine can reflect the pathophysiology of some neurological diseases, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and neuroendocrine tumors [9]. In addition, in a glioblastoma rat model and a Walker 256 lateral ventricle inoculation rat model, the urine proteome changed significantly before clinical symptoms and brain histopathological changes appeared [10, 11]. Overall, urine is a good source of specimens for the study of brain injury markers, and it can provide a sensitive reflection of brain pathophysiology at an early stage.

In this study, the proteomics technique of data-independent acquisition (DIA) was used to profile the urinary proteome in a global cerebral I/R rat model, and then the altered proteins were validated by using the parallel reaction monitoring (PRM) strategy. A summary of the overall experimental approach is presented in Figure 1. This study aims to explore urinary protein biomarkers of global cerebral I/R injury and provide clues to further understand its molecular biological mechanisms.

Results And Discussion

1. Histopathological Damage in the Hippocampus

To evaluate ischemia and histological damage, H&E staining was conducted on brain sections of the hippocampal CA1 region, which is most well known as being selectively vulnerable following ischemia. H&E staining indicated that no neuron morphology abnormalities were observed in the sham group (Fig. 2A). At reperfusion for 12 h, the number of neurons was reduced, the structure was complete, and no typical apoptotic cells were observed (Fig. 2B). With the reperfusion time prolonged to 48 h, the number of intact neurons decreased significantly, shrunken cell bodies and nuclear pyknosis (Fig. 2C).

2. Urine Proteome Changes

To preliminarily investigate how the urine proteome changes with I/R progression, twenty-one urine samples from the sham and I/R group (12 h and 48 h) were analyzed via a label-free DIA workflow.

To generate spectral library A, fractions separated with a spin column were analyzed by DDA-MS and then processed using Proteome Discoverer (version 2.3) and Spectronaut Pulsar X. The library included eight DDA analyses of fractions resulting in 1152 protein groups and 6260 peptides with at least one unique peptide and a Q value<0.01. Raw DIA-MS data files acquired with 30 refined isolation windows from the twenty-one individual urine samples were loaded into Spectronaut Pulsar X. Overall, a total of 866 (699 on average) protein groups were identified from twenty-one biological replicates. All identification and quantitation details are listed in supporting Table S3.

One hundred and sixty-four proteins significantly differed in the urine samples compared to the control samples (1.5-fold change, $p<0.05$). There were 59 and 123 altered urinary proteins at 12 and 48 h, respectively, after I/R (Table S4-5). Eighteen proteins changed significantly at both 12 and 48 h (Table 1). Among these proteins, 7 showed an overall upregulated or downregulated trend: T-kininogen 2, Prostaglandin-H2 D-isomerase, 14-3-3 protein theta, Ig gamma-2B chain C region, Cathepsin Z, Parvalbumin alpha and Hepcidin (highlighted in red in Table 1). This may suggest that these proteins have the potential to be used for the early detection of cerebral I/R injury.

Table 1. The urinary proteins that were consistently altered at 12 and 48 h after I/R injury.

UniProt ID	Protein name	12 h		48 h	
		FC	p-value	FC	p-value
P62260	14-3-3 protein epsilon	2.9	4.5E-02	2.3	2.1E-02
D3ZTV3	Leucine-rich repeat transmembrane protein FLRT2	2.8	3.1E-02	2.3	2.7E-03
Q6P9T8	Tubulin beta-4B chain	2.8	3.3E-02	2.3	6.8E-03
Q5ZQU0	Sushi, nidogen and EGF-like domain-containing protein 1	2.3	3.8E-02	2.3	1.9E-02
P08932	T-kininogen 2	1.6	3.6E-02	2.3	3.2E-03
P22057	Prostaglandin-H2 D-isomerase	-1.5	4.4E-02	-2.1	8.5E-03
Q9EQX6	Platelet-derived growth factor C	-1.6	3.8E-02	-1.6	1.5E-02
O89117	Beta-defensin 1	-1.6	3.0E-02	-1.5	3.4E-02
Q9WVH8	Fibulin-5	-1.7	8.1E-03	-1.7	2.2E-02
P68255	14-3-3 protein theta	-1.7	2.0E-02	-2.4	4.3E-03
P14046	Alpha-1-inhibitor 3	-2.1	1.6E-02	-1.9	2.4E-02
P20761	Ig gamma-2B chain C region	-2.2	2.9E-02	-2.4	2.1E-02
P05544	Serine protease inhibitor A3L	-2.3	5.2E-03	-1.6	4.6E-02
Q9R1T3	Cathepsin Z	-2.0	9.0E-03	-2.2	1.3E-02
O70534	Protein delta homolog 1	-2.3	2.6E-04	-1.9	3.3E-03
P02625	Parvalbumin alpha	-3.2	2.0E-02	-4.6	1.3E-02
Q99MH3	Hepcidin	-3.4	1.1E-03	-4.7	1.6E-02
P17559	Uteroglobin	-4.7	4.6E-02	-3.1	3.9E-02

“-” means a downward trend.

3. Function Annotation of Differentially Abundant Proteins

The functional annotation of differentially abundant proteins at 12 and 48 h consisted of sorting them into the “biological process”, “cellular component” and “molecular function” categories using DAVID (Figure 3). One hundred and sixty-four differentially abundant proteins were annotated. In the biological process category, cell-matrix adhesion, positive regulation of ERK1 and ERK2 cascade, cellular response to interleukin-6, negative regulation of endothelial cell apoptotic process, and acute-phase response were overrepresented at 12 h after 12 h I/R. At 48 h after I/R, negative regulation of endopeptidase activity, aging, negative regulation of blood coagulation, angiogenesis, and innate immune response were overrepresented (Fig. 3A).

In the molecular function category, receptor binding, serine-type endopeptidase inhibitor activity, cell adhesion molecule binding, and protein binding were overrepresented at both time points. Oxygen transporter activity was overrepresented at 12 h after I/R (Fig. 3B). In the cellular component category, most of these differentially abundant proteins were associated with extracellular exosomes, extracellular space, blood microparticles, and the external side of the plasma membrane (Fig. 3C).

4. Protein-Protein Interactions of Differentially Abundant Proteins

To better understand the pathogenic mechanisms in global cerebral I/R, a protein-protein interaction (PPI) network for 164 changed proteins was constructed using STRING (Fig. 4). The STRING PPI network analysis showed that the average node degree was 3.73, the average local clustering coefficient was 0.466, and the PPI enrichment p-value was less than 1.0E-16. The above results revealed that these proteins had more interactions among themselves than would be expected for a random set of proteins of similar size. Such an enrichment pattern indicates that the proteins are at least partially biologically connected as a group in I/R.

5. PRM Validation and Analysis of Differentially Abundant Proteins

In the validation phase, 255 peptides corresponding to seventy-one proteins were finally scheduled for PRM-MS analysis in another 21 urine samples. Overall, thirty-two proteins were significantly altered at multiple time points (1.5-fold change, $p<0.05$) (Table S6). After the p-value was adjusted by the Benjamini & Hochberg method, filtering yielded fifteen proteins with an adjusted p-value<0.05, including 8 increased and 7 decreased proteins (Table 2). The expression trends of the corresponding proteins were consistent with the results from the DIA discovery quantification.

Table 2. Differentially abundant urinary proteins validated by PRM quantification.

UniProt ID	Human homolog	Protein name	Trend	Related to brain I/R injury
P02680	P02679	Fibrinogen gamma chain	↑	Plasma [12, 13], brain [14]
P35444	P49747	Cartilage oligomeric matrix protein	↓	
Q09030	Q03403	Trefoil factor 2	↓	Plasma [15]
P10247	P04233	H-2 class II histocompatibility antigen gamma chain	↓	
P02650	P02649	Apolipoprotein E	↑	Brain [16], plasma [17]
Q5M871	O60667	Fas apoptotic inhibitory molecule 3	↑	
Q08463	Q9UP38	Frizzled-1	↑	Brain [18]
P43303	P27930	Interleukin-1 receptor type 2	↑	
P29598	P00749	Urokinase-type plasminogen activator	↑	
P10252	P09326	CD48 antigen	↑	
P08932	P01042	T-kininogen 1	↑	Blood [19]
Q9R1T3	Q9UBR2	Cathepsin Z	↓	Plasma [15]
P22057	P41222	Prostaglandin-H2 D-isomerase	↓	
P02625	P20472	Parvalbumin alpha	↓	Hippocampus [20]
Q99MH3	P81172	Hepcidin	↓	Plasma [21, 22], brain [23]

Four proteins were changed significantly only at 12 h after I/R, when no obvious histopathological changes had yet appeared; these proteins included Fibrinogen gamma chain (FGG), Cartilage oligomeric matrix protein, Trefoil factor 2, and H-2 class II histocompatibility antigen gamma chain. These differentially abundant proteins may provide important clues for the early diagnosis of cerebral I/R injury. Two of these differentially abundant proteins were associated with cerebral I/R injury. Plasma fibrinogen was independently associated with overall ischemic stroke and all subtypes, both in the acute stage ($p < 0.001$) and at the three-month follow-up ($p < 0.05$) [12]. In a case-control study, FGG was associated with a reduced risk of ischemic stroke [13]. FGG was also highly expressed in the ischemic penumbra of focal cerebral ischemia rats [14]. Trefoil factor 2 was upregulated in the plasma of atrial fibrillation patients with ischemic stroke [15].

Six proteins were upregulated only at 48 h after I/R injury, when there was certain histopathological damage in the hippocampus; these proteins included Apolipoprotein E, Fas apoptotic inhibitory molecule 3, Frizzled-1, Interleukin-1 receptor type 2, Urokinase-type plasminogen activator and CD48 antigen. These differentially abundant proteins may indicate the extent of cerebral I/R injury. Two of these differentially

abundant proteins were associated with cerebral I/R injury. Apolipoprotein E (APOE) is the primary apolipoprotein synthesized in the brain in response to ischemia-reperfusion injury with known neuroprotective effects exerted through antioxidant, anti-inflammatory, anti-excitotoxic, and neurotrophic mechanisms [16]. The APOE genotype showed a positive dose-response association with ischemic stroke in people of European ancestry [17]. Frz1 expression was significantly decreased in the brain tissue of middle cerebral artery occlusion rats [18].

Five proteins showed an overall upregulated or downregulated trend at 12 and 48 h after I/R injury, including T-kininogen 1, Cathepsin Z, Prostaglandin-H2 D-isomerase, Parvalbumin alpha and Hepcidin (Fig. 5). These differential proteins may reflect the progression of cerebral I/R injury. Three of these differential proteins were associated with cerebral I/R injury. The mRNA expression of T-kininogen 1 was upregulated in the blood of idiopathic thrombophilia [19]. Cathepsin Z was upregulated in the plasma of atrial fibrillation patients with ischemic stroke [15]. Parvalbumin alpha protein and mRNA were reduced in the hippocampal tissue of C57BL6 mice with unilateral right carotid ligation. Plasma/serum hepcidin levels were significantly higher in acute ischemic stroke patients than in the control group [21, 22]. The hepcidin mRNA levels and hepcidin/prohepcidin protein levels are upregulated in the ischemic brain [23].

Conclusion

In this study, fifteen differentially abundant urinary proteins were identified and validated in 4-VO rats. Eight of the differential proteins were reported to be associated with cerebral I/R injury. These findings may provide important clues for the monitoring of cerebral I/R injury and further understanding of its molecular biology mechanisms.

Methods

1. Animals

Forty-two male Wistar rats (200–250 g) were purchased from Charles River China (Beijing, China). All animals were maintained on a standard laboratory diet with a controlled indoor temperature (21±2℃), humidity (65–70%) and 12/12 h light–dark cycle conditions. The animal experiments were reviewed and approved by the Qingdao University Hospital Medical Ethics Committee (No.027-2019). All methods were carried out in accordance with relevant guidelines and regulations of the National Health Commission and the Ministry of Science and Technology and conformed to the guidelines for animal research.

The Wistar rats were randomly divided into two groups: a control group (n = 21) and an I/R group (n = 21). Ischemia was induced by Pulsinelli's four-vessel occlusion method [24]. Briefly, the bilateral common carotid arteries were surgically exposed and clamped shut with microclips for 10 min. The experiment was conducted in two phases; for details, see Figure 1. For the discovery phase, differentially abundant urinary proteins were identified by label-free DIA quantification in twenty-one independent samples from the control group (7 samples) and the I/R group at 12 and 48 h (7 samples per time point). For the

validation phase, the 21 remaining urine samples (7 from the control group and 7 per time point from the I/R group at 12 and 48 h) were evaluated by targeted quantification with PRM.

2. Histological Analysis

For histopathology, three rats in the I/R group and three rats in the control group were randomly sacrificed at 12 h and 48 h after I/R. The hippocampus was harvested and then quickly fixed in 10% neutral buffered formalin. The formalin-fixed tissues were embedded in paraffin, sectioned (4 mm) and stained with hematoxylin and eosin (H&E) to reveal histopathological lesions.

3. Urine Collection and Sample Preparation

Urine samples were collected from the control and I/R groups at 12 and 48 h after I/R. Rats were individually placed in metabolic cages for six hours. During urine collection, food was withheld from the rats to prevent the urine from being contaminated. After collection, the urine samples were immediately centrifuged at 2 000*g* for 30 min at 4°C and then stored at −80°C.

Urinary protein extraction: Urine samples were centrifuged at 12 000*g* for 30 min at 4°C. Six volumes of prechilled acetone were added after the pellets were removed, and the samples were precipitated at 4°C overnight. Then, lysis buffer (8 mol/L urea, 2 mol/L thiourea, 50 mmol/L Tris, and 25 mmol/L DTT) was used to dissolve the pellets. The protein concentration of each sample was measured by a Bradford protein assay.

Tryptic digestion: The proteins were digested with trypsin (Promega, USA) using filter-aided sample preparation methods [25]. Briefly, 100 μg of the protein sample was loaded onto a 10-kDa filter unit (Pall, USA). The protein solution was reduced with 4.5 mM DTT for 1 h at 37°C and then alkylated with 10 mM indoleacetic acid for 30 min at room temperature in the dark. The proteins were digested with trypsin (enzyme-to-protein ratio of 1:50) for 14 h at 37°C. The peptides were desalted on Oasis HLB cartridges (Waters, USA) and lyophilized for trap column fractionation and LC-MS/MS analysis.

4. Spin Column Separation

To generate a spectral library for DIA analysis, pooled peptide samples from all samples were fractionated using a high-pH reversed-phase peptide fractionation kit (Thermo Pierce, USA) according to the manufacturer's instructions. Briefly, 60 μg of a pooled peptide sample was loaded onto the spin column. A step gradient of increasing acetonitrile concentrations was applied to the column to elute the bound peptides. Ten different fractions were collected by centrifugation, including the flow-through fraction, the wash fraction and eight step gradient sample fractions (5, 7.5, 10, 12.5, 15, 17.5, 20 and 50% acetonitrile). The fractionated samples were dried completely and resuspended in 20 μL of 0.1% formic acid. Three microliters of each of the fractions was loaded for LC–data-dependent acquisition (DDA)–MS/MS analysis.

5. LC-MS/MS Setup for DDA and DIA

An Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific, Germany) was coupled with an EASY-nLC 1000 HPLC system (Thermo Scientific, Germany). For DDA-MS and data-independent acquisition (DIA)–MS modes, the same LC settings were used for retention time stability. The digested peptides were dissolved in 0.1% formic acid and loaded onto a trap column (75 μm × 2 cm, 3 μm, C18, 100 A°). The eluent was transferred to a reversed-phase analytical column (50 μm × 250 mm, 2 μm, C18, 100 A°). The eluted gradient was 5–30% buffer B (0.1% formic acid in 80% acetonitrile; flow rate of 0.8 μL/min) for 90 min. To enable fully automated and sensitive signal processing, the calibration kit (iRT kit from Biognosys, Switzerland) reagent was spiked at a concentration of 1:20 v/v in all samples. The iRT kit reagent was spiked into the urinary peptides for spectral library generation. Additionally, before the real DIA runs, the iRT kit reagent was also spiked into all urinary samples.

For the generation of the spectral library, the ten fractions from the spin column were analyzed in DDA-MS mode. The parameters were set as follows: the full scan was acquired from 350 to 1 550 m/z at 60 000, the cycle time was set to 3 s (top speed mode), the automatic gain control (AGC) was set to 1E6, and the maximum injection time was set to 50 ms. MS/MS scans were acquired in the Orbitrap at a resolution of 15,000 with an isolation window of 2 Da and collision energy of 32% (higher-energy collisional dissociation, HCD); the AGC target was set to 5E4, and the maximum injection time was 30 ms.

For the DIA-MS method, forty individual samples were analyzed in DIA mode. For MS acquisition, the variable isolation window DIA method with 26 windows was developed (Table S1). The full scan was set at a resolution of 60,000 over an m/z range of 350 to 1,200, followed by DIA scans with a resolution of 30,000, HCD collision energy of 32%, AGC target of 1E6 and maximal injection time of 50 ms.

6. LC-MS/MS Setup for PRM

In the discovery phase, seventy-one differentially abundant urinary proteins were identified by the label-free DIA proteomic method. All of these proteins were evaluated by the PRM-MS method in the remaining twenty-one urine samples. LC-PRM-MS/MS data were acquired in an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific, Germany) coupled with an EASY-nLC 1200 HPLC system (Thermo Scientific, Germany).

For the generation of the PRM spectral library, pooled peptide samples were analyzed in DDA-MS mode 6 times. The peptides were loaded on a reversed-phase trap column (75 μm × 2 cm, 3 μm, C18, 100 Å, Thermo Scientific, Germany), and the eluent was then transferred to a reversed-phase analytical column (50 μm × 250 mm, 2 μm, C18, 100 Å, Thermo Scientific, Germany). The elution gradient consisted of 5–35% buffer B (0.1% formic acid in 80% acetonitrile; flow rate 0.8 μL/min) for 90 min. The MS parameters were set as follows: the full scan was acquired from 350 to 1 550 m/z at 60 000, the cycle time was set to 3 s (top speed mode), the AGC was set to 1E6, and the maximum injection time was set to 50 ms. MS/MS scans were acquired using the Orbitrap at a resolution of 30 000 with an isolation window of 1.6 Da and collision energy at 30% (HCD), the AGC target was set to 5E4, and the maximum injection time was 60 ms.

For the PRM-MS method, thirty-two individual samples were analyzed in PRM mode. Ultimately, 255 peptides were scheduled, and the retention time (RT) segment was set to 8 min for each targeted peptide (Table S2). The normalized collision energy was fixed ats 30%, and the quadrupole isolation window was fixed at 1.6 Da. The other parameters were the same as described in the last paragraph.

7. Label-Free DIA Quantification Analysis

To generate the spectral library, the raw data files acquired for the ten fractions in DDA mode were processed using Proteome Discoverer (version 2.3; Thermo Scientific, Germany) with SEQUEST HT against the SwissProt *Rattus* database (released in May 2019, containing 8086 sequences) appended with the iRT peptide sequences. The search parameters consisted of a parent ion mass tolerance of 10 ppm; fragment ion mass tolerance of 0.02 Da; fixed modification of carbamidomethylated cysteine (+58.00 Da); and variable modifications of oxidized methionine (+15.995 Da) and deamidated glutamine and asparagine (+0.984 Da). For other settings, the default parameters were used. A false discovery rate (FDR) cutoff of 0.01 was applied at the protein level. The results were then imported to Spectronaut™ Pulsar (Biognosys, Switzerland) software to generate the spectral library [26].

The raw DIA-MS files were imported into Spectronaut Pulsar with the default settings. In brief, a dynamic window for the XIC extraction window and a nonlinear iRT calibration strategy were used. Mass calibration was set to local mass calibration. Cross-run normalization was enabled to correct for systematic variance in LC-MS performance, and a local normalization strategy was used [27]. Protein inference, which gave rise to the protein groups, was performed on the principle of parsimony using the ID picker algorithm as implemented in Spectronaut Pulsar [28]. All results were filtered by a Q value cutoff of 0.01 (corresponding to an FDR of 1%). Peptide intensity was calculated by summing the peak areas of the respective fragment ions for MS2. Student's t-test was applied with a significance criterion of <0.05. A minimum of two peptides matched to a protein and a fold change >1.5 were used as the criteria for the identification of differentially expressed proteins.

8. PRM-MS Quantification Analysis

Skyline (version 3.6.1 10279) [8] was used to build the spectrum library and filter peptides for PRM analysis. For each targeted protein, 2-6 associated peptides were selected using the following rules: (i) identification in the untargeted analysis with a q value <1%, (ii) complete digestion by trypsin, (iii) containing 8–18 amino acid residues, (iv) exclusion of the first 25 amino acids at the N-terminus of proteins, and (v) fixed carbamidomethylation of cysteine. Prior to individual sample analysis, pooled peptide samples were subjected to PRM experiments to refine the target list. Finally, forty-four proteins with 255 peptides (Table S2) were scheduled. The RT segment was set to 8 min for each targeted peptide with its expected RT in the center based on the pooled sample analysis.

All of the PRM-MS data were processed with Skyline. By comparing the same peptide across runs, the RT location and integration boundaries were adjusted manually to exclude interfering regions. Each protein's intensity was quantitated using the summation of intensities from its corresponding transitions. The

transition settings were as follows: precursor charges +2, +3; ion charge +1; ion type b, y, p; product ions from ion 3 to last ion -1; automatically select all matching transitions; ion match tolerance 0.02 m/z; select the 6 most intense product ions. The details of the transition are listed in supporting Table S2. Prior to the statistical analysis, the quantified protein intensities were normalized according to the summed intensity. The differentially abundant proteins were selected using one-way ANOVA, and p-values were adjusted by the Benjamini & Hochberg method. Significance was defined by a p-value of < 0.05 and a fold change of 1.5.

9. Bioinformatics Analysis

Bioinformatics analysis was carried out to better study the biological function of the dysregulated proteins. The Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.8 (https://david.ncifcrf.gov/) was used to perform the functional annotation of the differentially abundant urinary proteins identified at 12 and 48 h. In this study, significant GO enrichment was defined as $p<0.05$. Protein-protein interaction networks were constructed using the STRING database (http://www.string-db.org), which is a database of known and predicted protein interactions, including direct (physical) and indirect (functional) associations.

Declarations

Author Contributions

X.S., W.Q. and M.W. conceived and designed the experiments. X.S. and Q.L. performed the experiments. X.S. and W.Q. analyzed the data and wrote the manuscript. All authors approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

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References

1. Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, de Ferranti SD, Floyd J, Fornage M, Gillespie C, et al: **Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association.** *Circulation* 2017, **135:**e146-e603.
2. Sanganalmath SK, Gopal P, Parker JR, Downs RK, Parker JC, Jr., Dawn B: **Global cerebral ischemia due to circulatory arrest: insights into cellular pathophysiology and diagnostic modalities.** *Mol Cell*

Biochem 2017, **426:**111-127.

3. Bajan K: **Can we predict neurological outcome following out-of-hospital cardiac arrest (OHCA) and therapeutic hypothermia?** *Indian J Crit Care Med* 2015, **19:**301-303.
4. Shao F, Li CS, Liang LR, Li D, Ma SK: **Outcome of out-of-hospital cardiac arrests in Beijing, China.** *Resuscitation* 2014, **85:**1411-1417.
5. Nguyen PL, Alreshaid L, Poblete RA, Konye G, Marehbian J, Sung G: **Targeted Temperature Management and Multimodality Monitoring of Comatose Patients After Cardiac Arrest.** *Front Neurol* 2018, **9:**768.
6. Rech TH, Vieira SR, Nagel F, Brauner JS, Scalco R: **Serum neuron-specific enolase as early predictor of outcome after in-hospital cardiac arrest: a cohort study.** *Crit Care* 2006, **10:**R133.
7. Al-Mufti F, Amuluru K, Smith B, Damodara N, El-Ghanem M, Singh IP, Dangayach N, Gandhi CD: **Emerging Markers of Early Brain Injury and Delayed Cerebral Ischemia in Aneurysmal Subarachnoid Hemorrhage.** *World Neurosurg* 2017, **107:**148-159.
8. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC, MacCoss MJ: **Skyline: an open source document editor for creating and analyzing targeted proteomics experiments.** *Bioinformatics* 2010, **26:**966-968.
9. An M, Gao Y: **Urinary Biomarkers of Brain Diseases.** *Genomics Proteomics Bioinformatics* 2015, **13:**345-354.
10. Zhang L, Li Y, Meng W, Ni Y, Gao Y: **Dynamic urinary proteomic analysis in a Walker 256 intracerebral tumor model.** *Cancer Med* 2019, **8:**3553-3565.
11. Ni Y, Zhang F, An M, Yin W, Gao Y: **Early candidate biomarkers found from urine of glioblastoma multiforme rat before changes in MRI.** *Sci China Life Sci* 2018, **61:**982-987.
12. Jood K, Danielson J, Ladenvall C, Blomstrand C, Jern C: **Fibrinogen gene variation and ischemic stroke.** *J Thromb Haemost* 2008, **6:**897-904.
13. Cheung EY, Uitte de Willige S, Vos HL, Leebeek FW, Dippel DW, Bertina RM, de Maat MP: **Fibrinogen gamma' in ischemic stroke: a case-control study.** *Stroke* 2008, **39:**1033-1035.
14. Zou Y, Gong P, Zhao W, Zhang J, Wu X, Xin C, Xiong Z, Li Z, Wu X, Wan Q, et al: **Quantitative iTRAQ-based proteomic analysis of piperine protected cerebral ischemia/reperfusion injury in rat brain.** *Neurochem Int* 2019, **124:**51-61.
15. Hijazi Z, Wallentin L, Lindback J, Alexander JH, Connolly SJ, Eikelboom JW, Ezekowitz MD, Granger CB, Lopes RD, Pol T, et al: **Screening of Multiple Biomarkers Associated With Ischemic Stroke in Atrial Fibrillation.** *J Am Heart Assoc* 2020, **9:**e018984.
16. Tukhovskaya EA, Yukin AY, Khokhlova ON, Murashev AN, Vitek MP: **COG1410, a novel apolipoprotein-E mimetic, improves functional and morphological recovery in a rat model of focal brain ischemia.** *J Neurosci Res* 2009, **87:**677-682.
17. Khan TA, Shah T, Prieto D, Zhang W, Price J, Fowkes GR, Cooper J, Talmud PJ, Humphries SE, Sundstrom J, et al: **Apolipoprotein E genotype, cardiovascular biomarkers and risk of stroke:**

systematic review and meta-analysis of 14,015 stroke cases and pooled analysis of primary biomarker data from up to 60,883 individuals. *Int J Epidemiol* 2013, **42:**475-492.

18. Matei N, Camara J, McBride D, Camara R, Xu N, Tang J, Zhang JH: **Intranasal wnt3a Attenuates Neuronal Apoptosis through Frz1/PIWIL1a/FOXM1 Pathway in MCAO Rats.** *J Neurosci* 2018, **38:**6787-6801.
19. Sabater-Lleal M, Martinez-Perez A, Buil A, Folkersen L, Souto JC, Bruzelius M, Borrell M, Odeberg J, Silveira A, Eriksson P, et al: **A genome-wide association study identifies KNG1 as a genetic determinant of plasma factor XI Level and activated partial thromboplastin time.** *Arterioscler Thromb Vasc Biol* 2012, **32:**2008-2016.
20. Chavez-Valdez R, Emerson P, Goffigan-Holmes J, Kirkwood A, Martin LJ, Northington FJ: **Delayed injury of hippocampal interneurons after neonatal hypoxia-ischemia and therapeutic hypothermia in a murine model.** *Hippocampus* 2018, **28:**617-630.
21. Slomka A, Switonska M, Zekanowska E: **Hepcidin Levels Are Increased in Patients with Acute Ischemic Stroke: Preliminary Report.** *J Stroke Cerebrovasc Dis* 2015, **24:**1570-1576.
22. Petrova J, Manolov V, Vasilev V, Tzatchev K, Marinov B: **Ischemic stroke, inflammation, iron overload - Connection to a hepcidin.** *Int J Stroke* 2016, **11:**NP16-17.
23. Ding H, Yan CZ, Shi H, Zhao YS, Chang SY, Yu P, Wu WS, Zhao CY, Chang YZ, Duan XL: **Hepcidin is involved in iron regulation in the ischemic brain.** *PLoS One* 2011, **6:**e25324.
24. Pulsinelli WA, Buchan AM: **The four-vessel occlusion rat model: method for complete occlusion of vertebral arteries and control of collateral circulation.** *Stroke* 1988, **19:**913-914.
25. Wisniewski JR, Zougman A, Nagaraj N, Mann M: **Universal sample preparation method for proteome analysis.** *Nat Methods* 2009, **6:**359-362.
26. Bruderer R, Bernhardt OM, Gandhi T, Miladinovic SM, Cheng LY, Messner S, Ehrenberger T, Zanotelli V, Butscheid Y, Escher C, et al: **Extending the limits of quantitative proteome profiling with data-independent acquisition and application to acetaminophen-treated three-dimensional liver microtissues.** *Mol Cell Proteomics* 2015, **14:**1400-1410.
27. Callister SJ, Barry RC, Adkins JN, Johnson ET, Qian WJ, Webb-Robertson BJ, Smith RD, Lipton MS: **Normalization approaches for removing systematic biases associated with mass spectrometry and label-free proteomics.** *J Proteome Res* 2006, **5:**277-286.
28. Zhang B, Chambers MC, Tabb DL: **Proteomic parsimony through bipartite graph analysis improves accuracy and transparency.** *J Proteome Res* 2007, **6:**3549-3557.

Figures

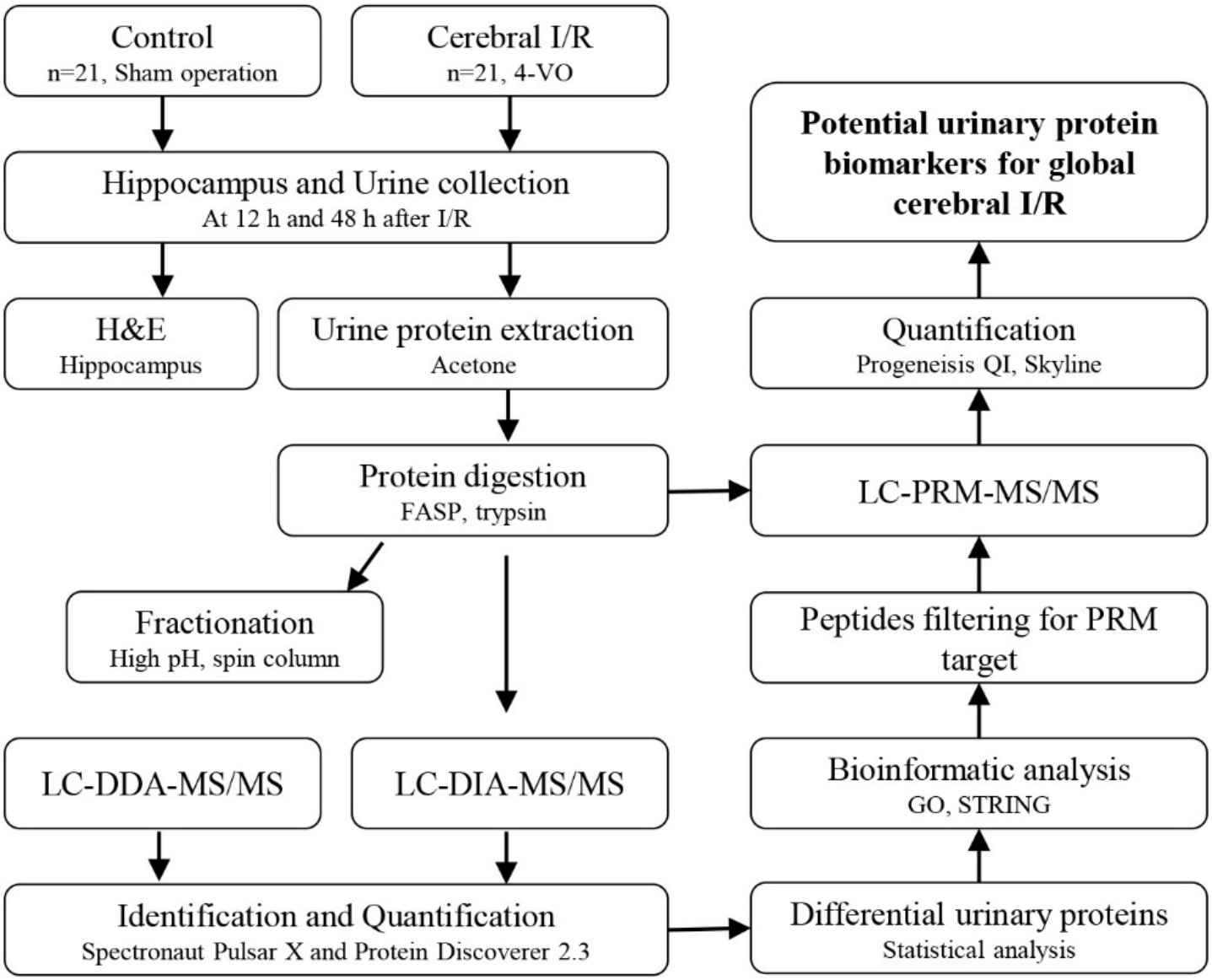

Figure 1

Workflow of this study.

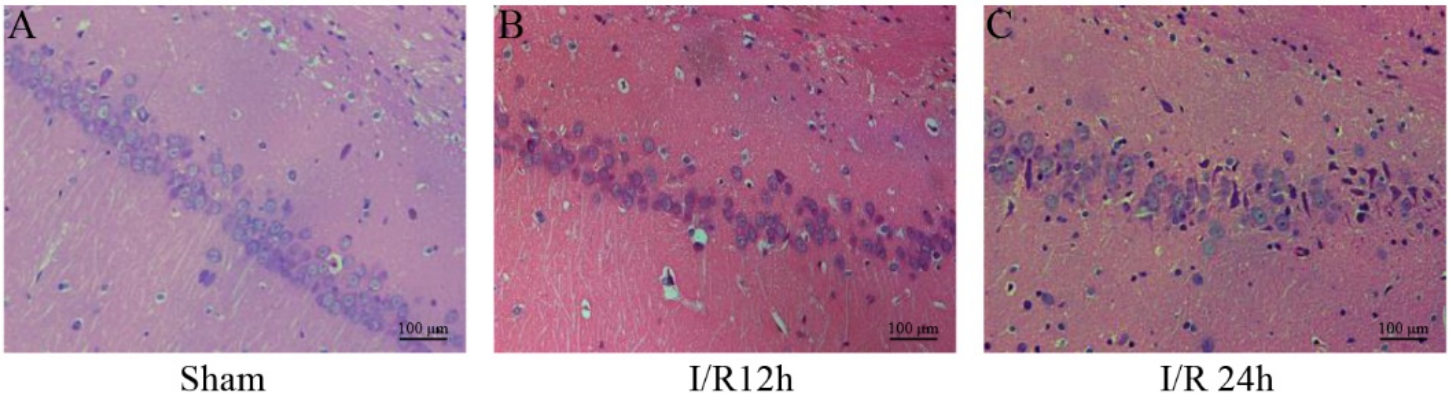

Figure 2

H&E staining assessment of neuronal morphology in the hippocampal CA1 area. A: The sham group showed an orderly arrangement of neurons with complete cell structure; B: The I/R 12 h group showed a reduced number of neurons, but their structure was complete, and there were no typical apoptotic cells; C: The I/R 48 h group showed a disorderly arrangement of cells, and the cytoplasm and nucleoli were stained deeply, indicating nuclear pyknosis. Scale bars=100 μm.

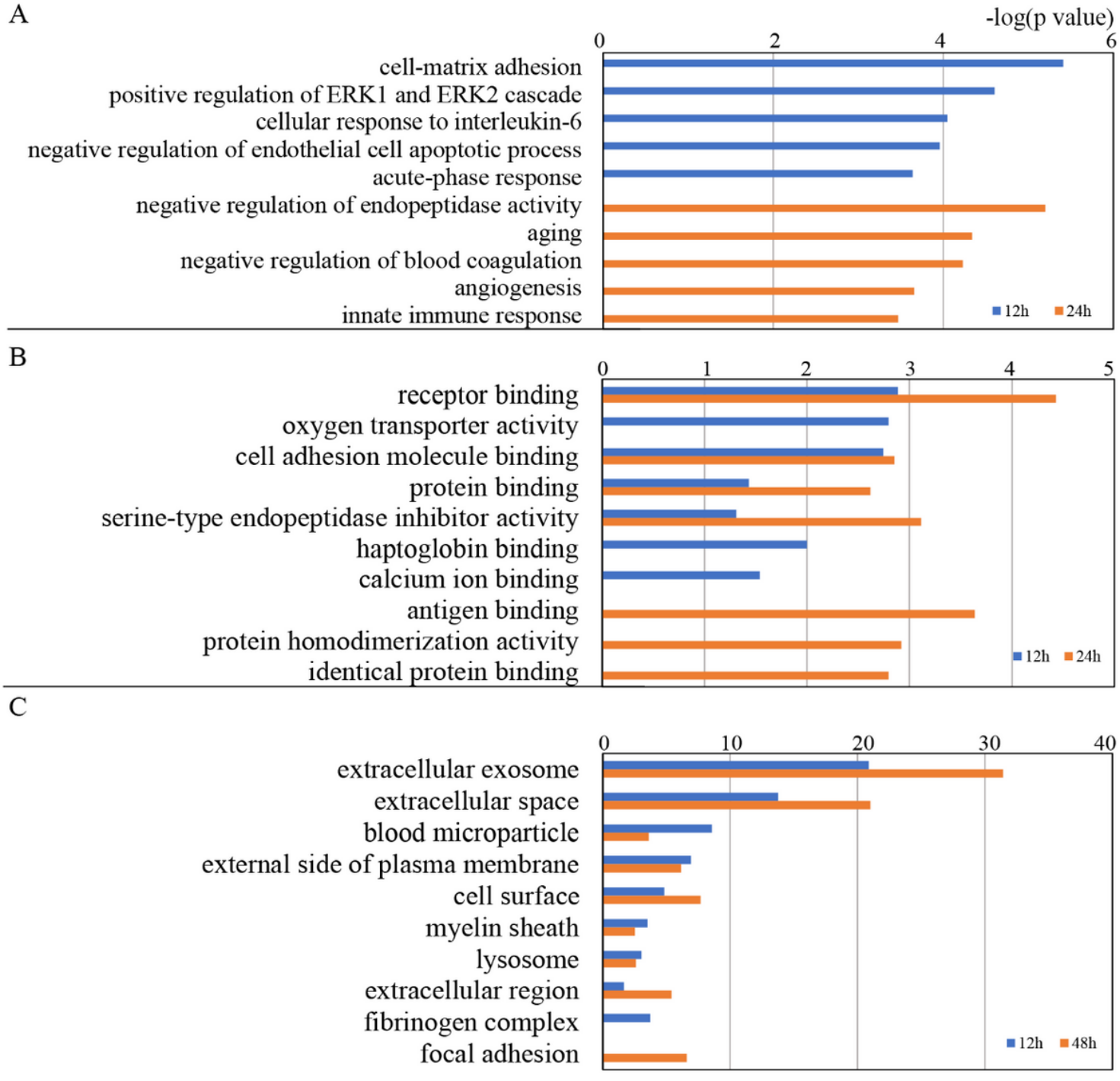

Figure 3

Functional analysis of differentially abundant proteins at 12 h and 48 h after I/R. A) Biological process category; B) Molecular function category; C) Cellular component category.

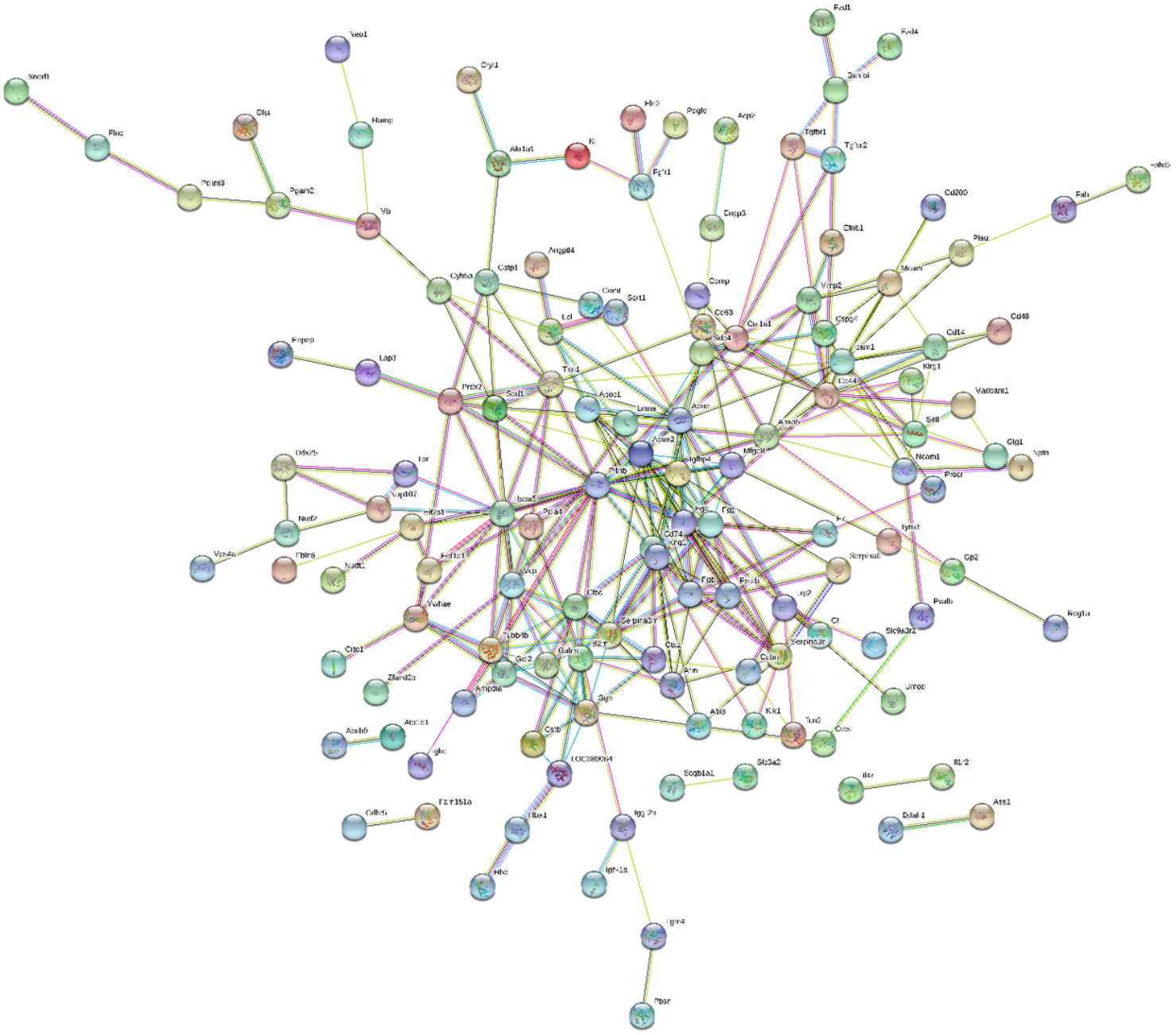

Figure 4

STRING PPI network analysis of the 164 differentially abundant proteins in I/R rats. The average node degree was 3.73, the average local clustering coefficient was 0.466, and the PPI enrichment p-value was less than 1.0E-16.

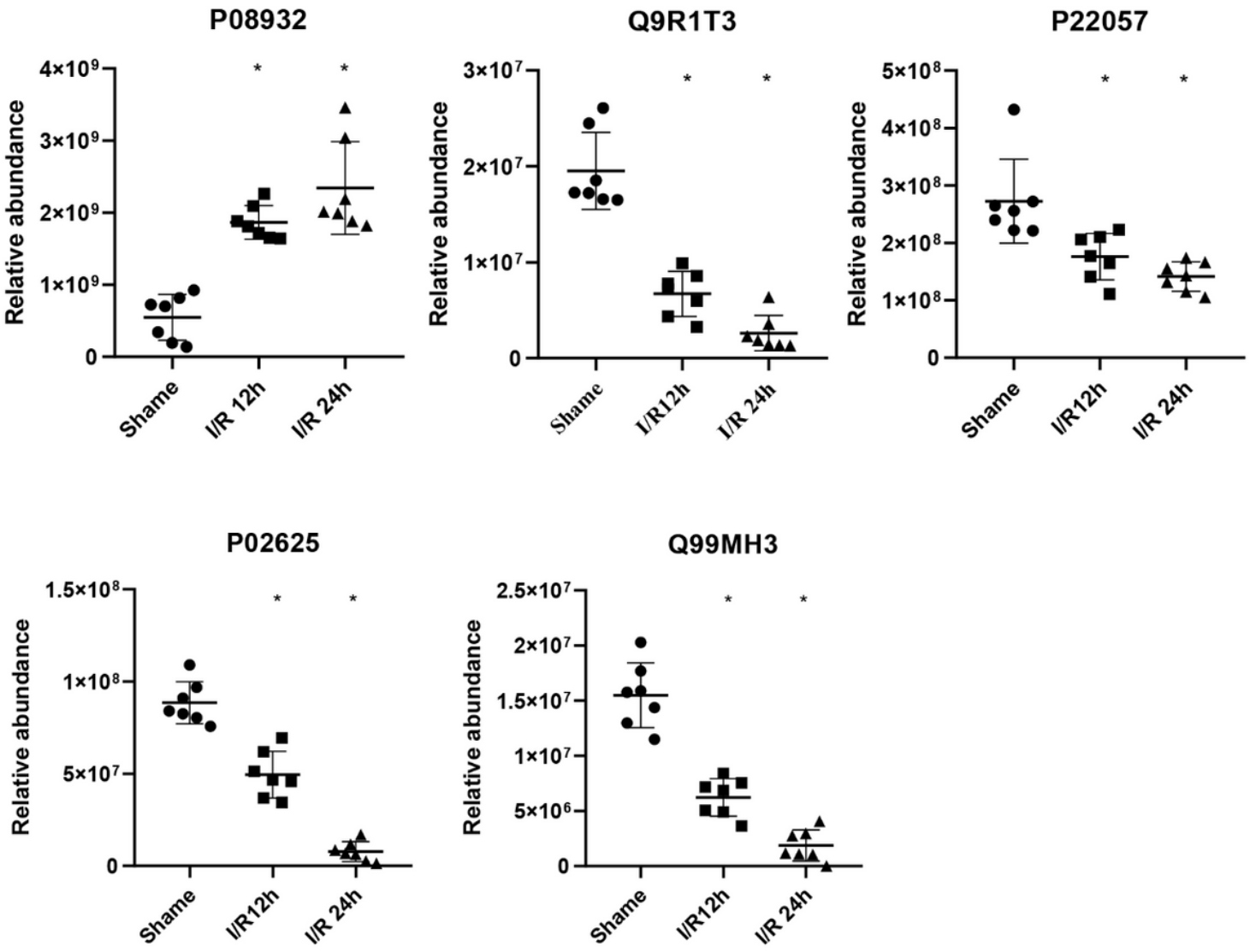

Figure 5

Abundance of candidate urine biomarkers in I/R rats by PRM quantification. The x-axis represents different groups, and the y-axis represents the area of intensity based on PRM quantification. * $p<0.05$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supportingtables.xlsx