Effects of three commonly used diuretics on the urinary proteome

Xundou Li, Mindi Zhao, Menglin Li, Lulu Jia, Youhe Gao

Biomarker is the measurable change associated with a physiological or pathophysiological process. Unlike blood which has mechanisms to keep the internal environment homeostatic, urine is more likely to reflect changes of the body. In other words, urine is likely to be a better biomarker source than blood. However, the urinary proteome are affected by many factors. In this study, the effects of three commonly used diuretics (furosemide, hydrochlorothiazide and spirolactone) on the urinary proteome were analyzed in rats. Urine samples were collected before and after the intragastric administration of diuretics at therapeutic doses and analyzed using LC-MS/MS. Based on quantification by Progenesis LC-MS software, there are 7, 5 and 2 proteins with the p value ≤0.05, a fold change ≥2, a spectral count ≥5 and FDR ≤1%, respectively. Most their human orthologs were considered to be stable in the healthy human urinary proteome. 10 of the 14 proteins have been reported as disease biomarkers in previous studies. So the effects of diuretics should be given more attention in future urinary protein biomarkers studies. The effects of diuretics on urinary proteome are different which can provide clues to elucidate the mechanisms of the diuretics.

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Introduction

Biomarker is the measurable change associated with a physiological or pathophysiological process. Unlike blood which has mechanisms to keep the internal environment homeostatic, urine is more likely to reflect changes of the body. In other words, urine is likely to be a better biomarker source than blood. However, the urinary proteome are affected by many factors. In this study, the effects of three commonly used diuretics (furosemide, hydrochlorothiazide and spirolactone) on the urinary proteome were analyzed in rats. Urine samples were collected before and after the intragastric administration of diuretics at therapeutic doses and analyzed using LC-MS/MS. Based on quantification by Progenesis LC-MS software, there are 7, 5 and 2 proteins with the p value $\leqslant$0.05, a fold change $\geqslant$2, a spectral count $\geqslant$5 and FDR $\leqslant$1%, respectively. Most their human orthologs were considered to be stable in the healthy human urinary proteome. 10 of the 14 proteins have been reported as disease biomarkers in previous studies. So the effects of diuretics should be given more attention in future urinary protein biomarkers studies. The effects of diuretics on urinary proteome are different which can provide clues to elucidate the mechanisms of the diuretics.

Keywords: urinary proteome; diuretics; biomarkers.

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Introduction

Biomarker is the measurable change associated with a physiological or pathophysiological process (Youhe 2013). Unlike blood which has mechanisms to keep the internal environment homeostatic, urine is more likely to reflect changes of the body. In other words, urine is likely to be a better biomarker source than blood (Gao 2013). Saving more urinary protein samples on the membrane can help to speed up the biomarker research in urine proteome (Jia et al. 2013). Furthermore, compared to plasma, urine has some unique advantages that make it a suitable source for disease biomarker discovery. First, urine can be collected continuously and noninvasively. Second, the urinary proteome directly reflects the condition of the urinary system. As a result, urine is not only a good source for the study of urological diseases but can also reflect the status of the whole body.

Many urinary biomarkers have been reported currently in various types of diseases (Shao et al. 2011), such as various chronic and acute renal injuries (Rosner 2009), bladder cancer (Vrooman & Witjes 2008), prostate cancer (Montagut et al. 2008) and coronary artery disease (Zimmerli et al. 2008). The results of this study can be found in the urinary protein biomarker database (Shao et al. 2011).

Studies focused on urinary protein biomarker discovery face some challenges. The major one is that the urinary proteome pattern of an individual may be affected by a number of factors (Zimmerli et al. 2008), including gender, age, oral diet (Mullen et al. 2011), medication, daily activities, exercises (Kohler et al. 2009; Kohler et al. 2010), smoking (Airoldi et al. 2009), stress, menstrual cycle and other physiological variations, as well as environmental factors such as

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temperature and humidity. Therefore, during the discovery of urinary biomarkers, these factors should be taken into consideration.

Some of these factors have been considered in previous studies (Doi et al. 2012; Jin et al. 2012; Li et al. 2012), such as gender, age, daily activity and environmental factors. However, some factors, especially medication, are difficult to control because during the collection of urine, the regular therapeutic process of patients should not be disturbed. As a result, when analyzing the research results, the medication's influences on the urinary proteome should be taken into account.

Diuretics are among the most commonly used medications. They can increase the rates of urine flow and sodium excretion and are used to induce negative fluid and sodium balances in a variety of clinical situations, including hypertension, heart failure, renal failure, nephritic syndrome, and cirrhosis (Reddy & Mooradian 2009). Therefore, during the discovery of urinary biomarkers for such diseases, the effects of diuretics on the urinary proteome should be given more attention. However, how diuretics affect the urinary proteome remains unclear.

In this study, the effects on the urinary proteome of three commonly used types of diuretics, thiazide diuretics, loop diuretics, and potassium-sparing diuretics, were analyzed using label-free quantitative proteomics (Nahnsen et al. 2013). The mechanisms of the three types of diuretics are different (Wile 2012), so we selected one representative medication from each type, furosemide, hydrochlorothiazide and spirolactone, respectively. The rat urine samples were collected before and after the diuretics were administered, digested using the FASP (filter aided proteome preparation) method (Wisniewski et al. 2009b) and analyzed using a high-speed TripleTOF™

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5600 system. Then, the Progenesis LC-MS software was used to quantify the urinary proteins.

Materials & Methods

Animals and Ethics Statement

This study was approved by the Institute of Basic Medical Sciences Animal Ethics Committee, Peking Union Medical College (Animal Welfare Assurance Number: # A5518). Specific pathogen-free male Sprague-Dawley rats (150-160 g) were purchased from the Institute Of Laboratory Animal Science, Chinese Academy of Medical Science. They were given a standard laboratory diet and free access to tap water and were maintained in a room with controlled temperature (22 ± 1 ℃) and humidity (65–70 %) and a 12:12 hours light:dark cycle. The study was performed after the rats had been allowed to acclimate for one week. All rats received humane care in compliance with the institutional animal care guidelines approved by the Institutional Animal Care and Use Committee of the Peking Union Medical College.

Intragastric Administration of Diuretics and Rat Urine Collection

First, rat urine samples were collected after given 1 ml saline by intragastric administration for 24 hours (three consecutive eight hours) using metabolic cages, which were used as controls. Then, the fifteen rats were divided into three groups randomly with five rats in each group. They were given furosemide and spirolactone at doses of 20 mg/kg.d as well as 25 mg/kg.d of hydrochlorothiazide, respectively. The dosing volumes of diuretics were adjusted to 1 ml. All rats were given diuretics by intragastric administration for 5 days, and the rat urine samples were collected on days 1, 3 and 5 after gavage as described above. The samples were acidified immediately with hydrochloric acid and then cooled to 4 ℃ to prevent bacterial growth and

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proteolysis.

Acetone Precipitation

Samples were centrifuged at 5000 × g for 30 min, and the pellets were removed. The supernatants were precipitated with 75% v/v acetone for 12 h followed by centrifugation at 12 000 × g for 30 min. After removing the acetone from the loose pellets, they were thoroughly air-dried, resuspended in lysis buffer (8 M urea, 2 M thiourea, 50 mM Tris, and 25 mM DTT) and subjected to protein quantitation by the Bradford method.

SDS-PAGE Analysis

For each sample, 30 μg of proteins was dissolved in PAGE sample buffer (50 mM Tris-HCl, pH 6.8, containing 50 mM DTT, 0.5% SDS, and 10% glycerol) and incubated at 97 ℃ for 5 min. The solution was then resolved by SDS-PAGE. After electrophoresis, the samples were stained by Coomassie brilliant blue.

FASP Cleanup and Overnight Digestion

FASP cleanup was carried out using NANOSPE 10 K OMEGA centrifugal devices (PALL, NY, Washington, USA) following previously described procedures (Wisniewski et al. 2009a). Briefly, 100 μg of urinary proteins was mixed with 0.2 mL of 8 M urea in 0.1 M Tris/HCl, pH 8.5, loaded into the membrane filter and centrifuged at 14 000 × g for 35 min. Then the samples were reduced and alkylated. Finally, sequencing grade modified trypsin was added at a protein-to-enzyme ratio of 50:1, followed by incubation overnight at 37 ℃. The digested peptides were eluted from the filters using two times 0.1 mL 50 mM ammonium bicarbonate and then desalted by solid-phase extraction (Oasis HLB Extraction Cartridge; Waters, Inc., Milford,

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Massachusetts, USA), dried in a SpeedVac, resuspended with 20 μL of 0.1% formic acid and stored at -80 ℃ until use.

Liquid Chromatography− Tandem Mass Spectrometry (LC − MS/MS)

Urine samples from 3 rats in each group were analyzed using an AB SCIEX (Framingham, MA, US) Triple-TOF 5600 mass spectrometer, and each sample was analyzed once. Briefly, the tryptic peptides were analyzed using a RP C18 capillary LC column from Michrom Bioresources (100 μm×150 mm, 3 μm). The eluted gradient was 5–30% buffer B (0.1% formic acid, 99.9% ACN; flow rate, 0.5 μL/min) for 100 min. The MS data were acquired in Triple-TOF MS using an ion spray voltage of 3 kV, curtain gas of 20 PSI, nebulizer gas of 30 PSI, and an interface heater temperature of 150 ℃. The precursors were acquired in 500 ms ranging from 350 to 1250 Da, and the product ion scans were acquired in 50 ms ranging from 250 to 1800 Da. A rolling collision energy setting was used. A total of 30 product ion scans were collected if exceeding a threshold of 125 counts per second (counts/s) and with a +2 to +5 charge-state for each cycle.

Database Searching and Protein Identification

The Mascot Daemon software (version 2.4.0, Matrix Science, London) was used to search the MS/MS data against the SwissProt_rat database (release 2012_07; taxonomy: *Rattus;* containing 7,787 sequences). The carbamidomethylation of cysteines was set as a fixed modification; the oxidation of methionine and protein N-terminal acetylation were set as variable modifications. The specificity of trypsin digestion was set for cleavage after K or R, and two missed trypsin cleavage sites were allowed. The mass tolerances in MS and MS/MS were all set to 0.05 Da. After the Mascot search, the significance threshold and ion score cut-off were set to

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0.05 using MudPIT protein scoring. The False Discovery Rate (FDR) was adjusted to less than 1% when the search result was exported.

Label-Free Quantification

For label-free quantification, the acquired raw data files corresponding to the different samples were imported into the Progenesis LC-MS software (Nonlinear Dynamics, Newcastle upon Tyne, UK) for feature detection, alignment, and quantification. All sample features were aligned according to retention times by automatic alignment to maximally overlay all the two-dimensional (m/z and retention time) feature maps. Then, the single-charged peptides and the peptides with charge states higher than three were excluded from the analysis. After alignment, the samples were divided into the appropriate groups (furosemide before (normal urine samples) and after (urine samples collected after gavage); hydrochlorothiazide before and after; and spirolactone before and after). Urine samples before and after gavage form the same rats were self-control. The peak lists generated by the Progenesis LC−MS software were used for protein identification as described above and then reimported into the software. For quantification, only unique peptides were included, and the total cumulative abundance was calculated by summing the individual abundances of all peptides assigned to each protein (Stoop et al. 2013).

Statistical Analysis

Percentages of variances were calculated from the median coefficient of variation (cv), which is the standard deviation divided by the mean of a measurement.

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Results and Discussion

The effects of diuretics on rat urine volumes

Rat urine samples were collected before the diuretics were administered and 1, 3, 5 days afterward, and no rats died during the experiments. As shown in Supplementary Table 1, the rat urinary volumes increased significantly after the intragastric administration of furosemide and hydrochlorothiazide ($p \leqslant 0.05$), especially within the first 8 hours after lavage with an increase of approximately two to three times. This period of time is the effective time of the diuretics. However, after the rats were administered spirolactone, the urine output had no significant increase ($p > 0.05$), which may be because it is an inefficient diuretic and usually applied in combination with others.

SDS-PAGE analysis of the urine samples

The urine samples collected on different days were separated by SDS-PAGE. As shown in Figure-1, the protein patterns of the urine samples in the hydrochlorothiazide group had slight differences before and 1, 3, and 5 days after the diuretics were administered. However, in the other two groups, there were some significant changes among the different days, especially the 3rd day after gavage in the furosemide group (Figure-2) and the 1st day in the spirolactone group (Figure-3). As a result, normal urine samples, the 3rd day's urine after the gavage of furosemide and hydrochlorothiazide and the 1st day's urine samples after intragastric administration of spirolactone were analyzed by 1D-LC-MS/MS.

The changes of the rat urine proteome before and after administration of diuretics

To investigate the changes of the urine proteome before and after rats were given diuretics,

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analyses were performed on a total of 18 LC−MS/MS runs of urine samples from three different rats in each diuretic group. The 18 files were analyzed with Progenesis LC−MS software and Mascot Daemon software using common criteria. The false positive rate for identifications was less than 1%. These analyses resulted in the identification of 331, 302, 325 proteins (Supplemental Table 2-4) with at least two peptides in furosemide, spirolactone and hydrochlorothiazide group, respectively. All the supplemental materials can be found in the urinary protein biomarker database (Shao et al. 2011) (*the website: http://122.70.220.102/biomarker*).

The coefficients of variation (cv) for each of the three levels of sample variation, before gavage, after gavage and between this two conditions were calculated. As shown in Figure-4, the CV values of the samples after gavage were a little higher than before (median cv: 0.25 vs 0.34; 0.35 vs 0.39: 0.28 vs 0.31), which may due to that rats respond differently to diuretics. The CV values of the samples between before and after gavage (median cv of F-diuretics is 0.45; median cv of S-diuretics is 0.55) are significantly higher, implying furosemide and spirolactone have great effects on urine proteome. However, CV values of H-diuretics (median cv is 0.33) was not changed significantly which indicates that hydrochlorothiazide has slightly effects on rat urine proteome at this dosage.

Effects of different diuretics on the urinary proteome

Based on label-free quantification by the Progenesis LC-MS software, there were 7 (5 up and 2 down), 5 (2 up and 4 down) and 2 (1 up and 1 down) proteins significantly changed in all the 3 rats with the p value $\leqslant$0.05, a fold change $\geqslant$2 and a spectral count $\geqslant$5 in the furosemide,

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spirolactone and hydrochlorothiazide group, respectively. As shown in Table 1 and 2, 5 of 7 proteins in furosemide group and all the 5 proteins in spirolactone group proteins have been reported to be disease biomarkers in previous studies. Haptoglobin has been reported in patients with bladder cancers, acute kidney injury and diabetic nephropathy. However, neither of the two significantly changed proteins (beta-microseminoprotein and EGF-containing fibulin-like extracellular matrix protein 1) has been reported as biomarkers in hydrochlorothiazide group.

There is no significantly changed protein shared between any two groups. Therefore, the effects of diuretics on the urinary proteome are different, and hydrochlorothiazide appears to have a smaller impact than furosemide and spirolactone at this dosages.

Most human orthologs of changed proteins were considered to be stable in the healthy human urinary proteome.

As it is typically assumed that orthologs (co-orthologs) retain similar functions between species (Koonin 2005; Remm et al. 2001), therefore, we transformed the significantly changed proteins after intragastric administration of diuretics to human orthologs. Based on the 122.R_norvegicus.orthologues database and Ensembl Compare (Shaye & Greenwald 2011), 8 of the 14 rat urinary protein were transformed to human orthologs (Table 3). Then, we compared the human orthologs with the human core urinary proteome (Nagaraj & Mann 2011; Sun et al. 2009) and 7 of the 8 proteins were found. Therefore, most of the significantly changed proteins are relatively stable proteins in normal human urinary proteome. Any significant qualitative or quantitative changes in these stable proteins may mean that such proteins could serve as potential urinary biomarkers (Sun et al. 2009). So in future human urinary protein biomarker studies these

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significantly changed proteins should be paid more attentions.

Conclusions

In this study, the effects of diuretics on urinary proteome were analyzed in rats, however, there were some limitations. First, as we used rats in this study their effects on humans need to be verified. Second, three rats in each groups were used for LC-MS/MS analysis (18 runs) due to the workload and MS machine time and in future it is necessary to validate in large scales, especially in humans. Furthermore, the effects of the doses and duration of diuretics on the urinary proteome should also be studied in future.

We have shown for the first time through a proteomic approach that some candidate biomarkers may be affected by diuretics, so the effects of diuretics should be given more attention in future urinary protein biomarker studies. The changing patterns caused by diuretics could help to eliminate their influences on urinary proteome in future urinary biomarker studies. And the significantly changed proteins can provide clues to elucidate the mechanisms of the diuretics and can also help to investigate the mechanisms for renal clearance of proteins. Other commonly used medications, such as glucocorticoids and ACEI, may likewise affect the urinary proteome and should also be further studied.

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Figure legends

Figure-1: SDS-PAGE of the urine samples from the hydrochlorothiazide group. M, markers; lanes 1 and 5, normal urine samples from two rats; lanes 2, 3, 4 and 6, 7, 8, urine samples obtained 1, 3, 5 days after the diuretics were administered. Unit: kDa

Figure-2: SDS-PAGE of the urine samples from the furosemide group. M, markers; lanes 1 and 5, normal urine samples from two rats; lanes 2, 3, 4 and 6, 7, 8, urine samples obtained 1, 3, 5 days after the diuretics were administered. Unit: kDa

Figure-3: SDS-PAGE of the urine samples from the spirolactone group. M, markers; lanes 1 and 5, normal urine samples from two rats; lanes 2, 3, 4 and 6, 7, 8, urine samples obtained 1, 3, 5 days after the diuretics were administered. Unit: kDa

Figure-4: The coefficients of variation (cv) for each of the three levels of sample variation, before gavage, after gavage and between this two conditions. F, furosemide; S, spirolactone and H, hydrochlorothiazide.

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Figures

Figure-1: SDS-PAGE of the urine samples from the hydrochlorothiazide group.

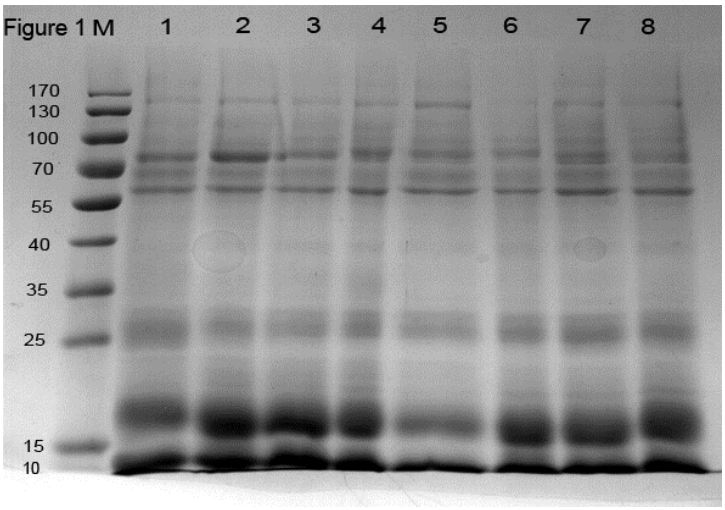

Figure-2: SDS-PAGE of the urine samples from the furosemide group.

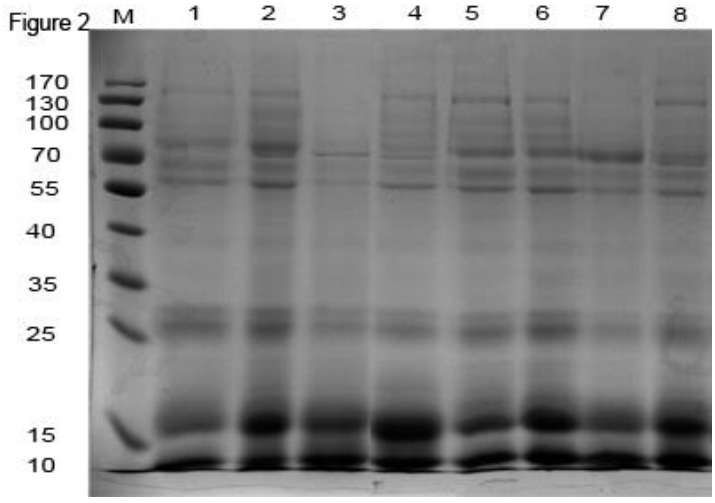

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Figure-3: SDS-PAGE of the urine samples from the spirolactone group.

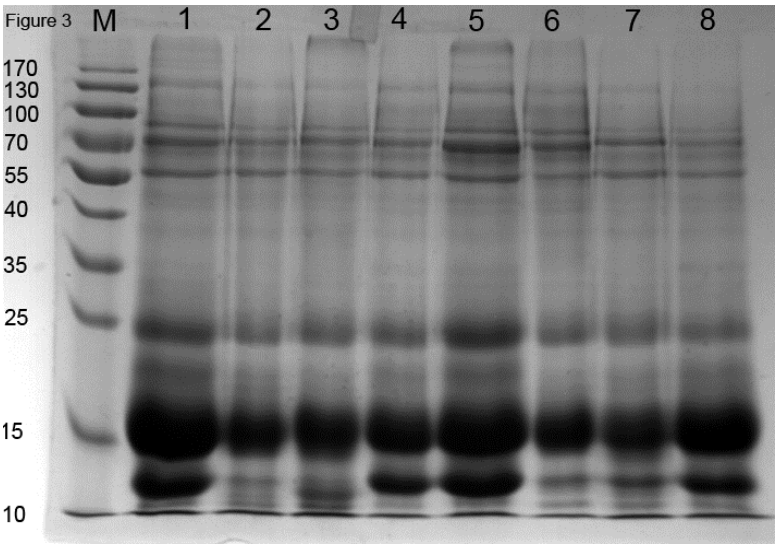
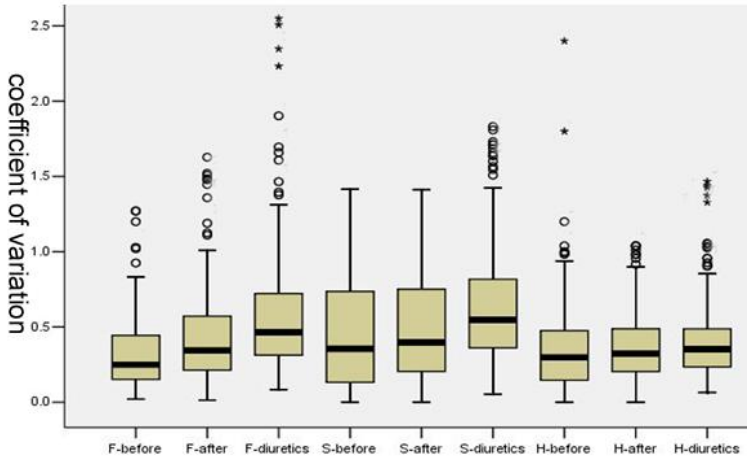


Figure-4: The coefficients of variation (cv) for each of the three levels of sample variation, before gavage, after gavage and between this two conditions.



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Tables

Table 1 Proteins changed after intragastric administration of furosemide

Accession	Protein name	Fold change			Candidate biomarkers
		Rat 1	Rat 2	Rat 3	
P02781	Prostatic steroid-binding protein C2	8.2 ↑	6.3 ↑	4.3 ↑	No
P07647	Submandibular glandular kallikrein-9	3.5 ↑	6.2 ↑	5.2 ↑	Yes (Wang et al. 2008)
P02782	Prostatic steroid-binding protein C1	7.6 ↑	5.7 ↑	5.6 ↑	No
P02780	Secretoglobin family 2A member 2	9.6 ↑	5.0 ↑	6.2 ↑	Yes (Wang et al. 2008)
P22283	Cystatin-related protein 2	4.7 ↑	3.7 ↑	4.3 ↑	Yes (Wang et al. 2008)
P08721	Osteopontin	7.3 ↓	7.4 ↓	5.9 ↓	Yes (Fuchs et al. 2012; Hoffmann et al. 2010; Ozer et al. 2010; Rouse et al. 2011)
Q01177	Plasminogen	2.1 ↓	2.1 ↓	3.0 ↓	Yes (Kentsis et al. 2010)

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Table 2 Proteins changed after intragastric administration of spirolactone

Accession	Protein name	Fold change			Candidate biomarkers
		Rat 1	Rat 2	Rat 3	
P06866	Haptoglobin	5.0 ↑	2.1 ↑	2.2 ↑	Yes (Bhensdadia et al. 2013; Jiang et al. 2009; Li et al. 2011; Malard et al. 2009; Riaz et al. 2010; Zager et al. 2012)
P81828	Urinary protein 2	3.6 ↓	3.3 ↓	3.9 ↓	Yes (Wang et al. 2008)
P81827	Urinary protein 1	7.3 ↓	4.3 ↓	4.4 ↓	Yes (Cutillas et al. 2004; Wang et al. 2008)
P10960	Sulfated glycoprotein 1	4.0 ↓	3.1 ↓	2.4 ↓	Yes (Wang et al. 2008)
Q09030	Trefoil factor 2	132 ↓	4.7 ↓	4.2 ↓	Yes (Lemberger et al. 2011)

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Table 3 Human orthologs of significantly changed proteins after administration of diuretics.

Rat Protein ID	Rat protein name	Human protein ID	Human protein name	Human core urinary proteome
Q01177	Plasminogen	P00747[a]	Plasminogen	yes
Q09030	Trefoil factor 2	Q03403 [a]	Trefoil factor 2	yes
P08721	Osteopontin	P10451 [a]	Osteopontin	yes
O35568	EGF-containing fibulin-like extracellular matrix protein 1	Q12805 [a]	EGF-containing fibulin-like extracellular matrix protein 1	yes
P10960	Sulfated glycoprotein 1	P07602 [a]	Sulfated glycoprotein 1	no
P06866	Haptoglobin	P00738 [a]	Haptoglobin	yes
P02781	Prostatic steroid-binding protein C2	P11684[b]	Secretoglobin family 1A member 1	yes
P07647	Submandibular glandular kallikrein-9	P06870 [b]	Kallikrein-1	yes

[a] presents they were found in the 122.R_norvegicus.orthologues database, [b] presents they were found in the Ensembl Compare.

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