Effects of arginine vasopressin on the urine proteome in rats

Manxia An [1] , **Yanying Ni** [1] , **Xundou Li** [1] , **Youhe Gao** Corresp. [1, 2]

[1] Department of Pathophysiology, Institute of Basic Medical Sciences Chinese Academy of Medical Sciences, School of Basic Medicine Peking Union Medical College, Beijing, China

[2] Department of Biochemistry and Molecular Biology, Beijing Normal University,Gene Engineering and Biotechnology Beijing Key Laboratory, Beijing, China

Corresponding Author: Youhe Gao
Email address: gaoyouhe@bnu.edu.cn

Biomarkers are the measurable changes associated with a physiological or pathophysiological process. The content of urine frequently changes because it is not controlled by homeostatic mechanisms, and these alterations can be a source of biomarkers. However, urine is affected by many factors. In this study, vasoconstrictor and antidiuretic arginine vasopressin (AVP) were infused into rats using an osmotic pump. The rats' urinary proteome after one week of infusion was analyzed by label-free LC-MS/MS. A total of 420 proteins were identified; among these proteins, 8 and 10 proteins had significantly altered expression in the low- and high-dose groups, respectively, compared with the control group. Three differential proteins were described in prior studies as related to AVP physiological processes, and 9 differential proteins are known disease biomarkers. Sixteen of the 17 differential proteins have human orthologs. These results suggest that we should consider the effects of AVP on urinary proteins in future urinary disease biomarker research. The study data provide clues regarding underlying mechanisms associated with AVP for future physiological research on AVP. Additionally, large clinical samples must be examined to verify the differential proteins identified in this study before these proteins are used as biomarkers for pathological AVP increased diseases, such as syndrome of inappropriate antidiuretic hormone secretion (SIADH).

Effects of arginine vasopressin on the urine proteome in rats

Manxia An[1], Yanying Ni[1], Xundou Li[1], Youhe Gao[1, 2]
[1] Department of Pathophysiology, Institute of Basic Medical Sciences Chinese Academy of Medical Sciences, School of Basic Medicine Peking Union Medical College, Beijing, China
[2] Department of Biochemistry and Molecular Biology, Beijing Normal University, Gene Engineering and Biotechnology Beijing Key Laboratory, Beijing, China

Corresponding Author:
Youhe Gao
Email address: gaoyouhe@pumc.edu.cn; gaoyouhe@bnu.edu.cn

Abstract: Biomarkers are the measurable changes associated with a physiological or pathophysiological process. The content of urine frequently changes because it is not controlled by homeostatic mechanisms, and these alterations can be a source of biomarkers. However, urine is affected by many factors. In this study, vasoconstrictor and antidiuretic arginine vasopressin (AVP) were infused into rats using an osmotic pump. The rats' urinary proteome after one week of infusion was analyzed by label-free LC-MS/MS. A total of 420 proteins were identified; among these proteins, 8 and 10 proteins had significantly altered expression in the low and high dose groups, respectively, compared with the control group. Three differential proteins were described in prior studies as related to AVP physiological processes, and 9 differential proteins are known disease biomarkers. Sixteen of the 17 differential proteins have human orthologs. These results suggest that we should consider the effects of AVP on urinary proteins in future urinary disease biomarker researches. The study data provide clues regarding underlying mechanisms associated with AVP for future physiological researches on AVP. Additionally, large clinical samples must be examined to verify the differential proteins identified in this study before these proteins are used as biomarkers for pathological AVP increased diseases, such as syndrome of inappropriate antidiuretic hormone secretion (SIADH).

Key Words: Urine proteome; Arginine vasopressin; Biomarkers.

Introduction

Biomarkers are the measurable changes associated with a physiological or pathophysiological process (Gao 2013). Urine contains many changes because it is not controlled by homeostatic mechanisms of the body; therefore, urine can be a good source of biomarkers. Additionally, urine can be obtained easily and noninvasively. Therefore, urine has been used in many studies to detect disease biomarkers, such as kidney disease (Pejcic et al. 2010), cardiovascular disease (Delles et al. 2011) and liver disease (Trovato et al. 2015). However, many non-disease factors, including age, gender, lifestyle and medications, can affect changes in urine (Wu & Gao 2015). These factors can interfere or mask changes caused by the disease itself, increasing the difficulty of identifying reliable and disease-specific biomarkers. In clinical studies, age, gender and other factors can be balanced to a certain degree by the experimental design. The influence of medications is difficult to assess and balance between the experimental and control groups because only patients in the disease group usually receive medication treatment. It is possible that changes caused by medication are incorrectly considered to be disease biomarkers.

Therefore, the effect of medications should be separately studied to facilitate identifying disease-specific biomarkers.

AVP, which is also called antidiuretic hormone, is a polypeptide hormone consisting of 9 amino acids (Kounin & Bashir 2000). It is synthesized and secreted in the supraoptic and paraventricular nuclei of the hypothalamus and is released into the blood to participate in physical activities because of certain physiological stimuli. Its two most important physiological functions are as a vasoconstrictor and an antidiuretic (Kounin & Bashir 2000). AVP is also involved in a variety of physiological processes, including physiological stress (Coverdill et al. 2012; Zelena et al. 2009), memory (Nabe et al. 2007), thermoregulation (Bicego-Nahas et al. 2000) and pain regulation (Madrazo et al. 1987). In clinical applications, AVP is used for the treatment of many diseases, such as diabetes insipidus, hepatorenal syndrome, portal venous hypertension, bleeding disorders, septic shock and cardiopulmonary resuscitation (Treschan & Peters 2006).

SIADH has common clinical symptoms and can be complicated by diseases characterized by abnormal increases in AVP secretion (Frouget 2012). Excessive AVP secretion can promote the opening of water channels in the renal collecting duct and distal convoluted tubule (DCT), thereby increasing water reabsorption, reducing urine volume, increasing urine osmolality and decreasing serum sodium levels. Continuous AVP infusion can be used to establish a rat SIADH model for identification of SIADH urinary markers.

Studying the effects of AVP on urine can achieve the following goals: (1) providing some clues for understanding its physiological functions; (2) establishing a reference for urinary biomarker research when AVP is used as a medication in studies; and (3) identifying potential biomarkers for pathological increases in AVP in diseases such as SIADH.

In this study, AVP (10 ng/h and 50 ng/h) was infused into rats with an osmotic pump. The urine from the model rats and the controls was analyzed by label-free LC-MS/MS one week after AVP infusion. Using rat models can minimize the influence of other factors by strictly controlling the experimental conditions so that the AVP is the single influential factor. Another advantage of using rat models is that more reliable candidate biomarkers can be obtained from a small number of samples (Gao 2014).

Materials and methods

Animal experiments

Male Sprague-Dawley rats (160-180 g) were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Science (Beijing, China). The rats were fed a standard laboratory diet and had free access to water. They were housed in standard temperature (22±1°C) and humidity (65%-70%) conditions. The animal experiments were approved by the Institute of Basic Medical Sciences Animal Ethics Committee, Peking Union Medical College (Animal Welfare Assurance Number: ACUC-A02-2013-015).

The rats in control group were infused with normal saline (n=7), the rats in low dose group were infused with low dose AVP (10 ng/h, n=5), and the rats in high dose group were infused with high dose AVP (50 ng/h, n=6). The normal saline and two different concentrations of AVP (Sigma-Aldrich, St. Louis, MO, USA, dissolved in normal saline) were continuously infused to rats by osmotic pump respectively. The main process are descripted as follows. The rats were fasted with free access to water for 12 hours before the experiment. The rats were anesthetized by 2% pentobarbital sodium (40 mg/kg) injection. The dorsal skin of the rats' necks was disinfected with povidone-iodine and cut laterally around a 1 cm incision. The skin and subcutaneous tissue within the incision were separated with blunt tweezers. An ALZET (Cupertino, CA, USA) osmotic pump (model 2002; reservoir volume of 200 μL, and flow rate of 0.5 μL/h) containing 200 μL of 20 ng/μL AVP, 100 ng/μL AVP, or normal saline was implanted into the incision. The incisions were sutured and disinfected with povidone-iodine after the osmotic pump was confirmed as occupying a slack space in the subcutaneous layer.

Urine was collected in metabolic cages one week after the AVP infusion, and the volume of urine was recorded. Rats were fasted and allowed free access to water during urine collection. The body weights of the rats after the AVP infusion and one week after the AVP infusion were recorded. Physiological indicators of urine, including osmolality, total protein and creatinine, were measured at the Beijing Union Medical College Hospital. Due to the limited throughput of our mass spectrometry capacity in the laboratory, three rats were selected randomly from each group for urinary proteome profiling by LC-MS/MS.

Extraction of urinary protein

Urine was centrifuged at 2000 g for 30 min immediately after collection to remove the cell debris. The supernatant was centrifuged at 12,000 g for 30 min. Three volumes of acetone were added to the supernatant after removing the pellets, and precipitation was allowed to occur overnight at -20°C followed by centrifugation at 12000 g for 30 min. The pellets were

resuspended in lysis buffer (8 M urea, 2 M thiourea, 50 mM Tris, and 25 mM dithiothreitol (DTT)) for 2 h at 4 ℃. The solution was centrifuged at 12000 g for 30 min and the supernatant was collected. The protein concentrations were determined using the Bradford method.

SDS-PAGE analysis

Thirty micrograms of protein from each sample was mixed with sample buffer and incubated at 96°C for 10 min. The protein samples were then loaded onto 12% SDS-PAGE. The gel was stained using Coomassie Brilliant Blue.

Urine sample preparation and LC-MS/MS

Urinary proteins were digested with trypsin using the filter-aided sample preparation method (Wisniewski et al. 2009). One hundred micrograms of protein was deposited onto a 10 kD filter membrane (Pall, Port Washington, NY, USA). Then, urea buffer (UA; 8 M urea and 0.1 M Tris-HCl, pH 8.6) and ammonium bicarbonate (25 mM) were added to the membrane and centrifuged at 14000 g for 40 min at 18°C to wash the samples. The samples were reduced by incubation with 20 mM dithiothreitol at 37°C for 1 h and alkylated by 50 mM in iodoacetamide (IAA) in the dark for 40 minutes. UA and ammonium bicarbonate were added and centrifuged to remove the remaining DTT and IAA. Mass spec grade trypsin (Trypsin Gold, Mass Spec Grade, Promega, Fitchburg, WI, USA) was added to the filter membrane at an enzyme-to-protein ratio of 1:50 and incubated at 37°C for 13 h. The digested peptides were obtained by centrifugation, desalted with Oasis HLB cartridges (Waters, Milford, MA, USA) and dried by vacuum evaporation.

One microgram of peptides was loaded onto a reversed-phase microcapillary column by EASY-nLC 1200 UHPLC system and eluted with a gradient of 5-28% mobile phase B (0.1% formic acid and 99.9% acetonitrile; flow rate of 0.3 mL/min) for 60 min. The eluted peptides were analyzed by Thermo Orbitrap Fusion Lumos MS (Thermo Fisher Scientific, Bremen, Germany). Each peptide sample was analyzed three times as technical replicates.

MS data analysis

MS data were retrieved using the mascot software (version 2.5.1, Matrix Science, London, UK) and searched against the Swissprot_2014_07 database (taxonomy: Rattus, containing 7787 sequences). Trypsin was selected as the digestion enzyme, and two missed trypsin cleavage sites were allowed. Carbamidomethylation of cysteines was selected as a fixed modification. The

fragment mass tolerance was set to 0.6 Da, and the parent mass tolerance was set to 10 ppm. Mascot search results were screened and integrated using scaffold software (version 4.4.8, Proteome Software Inc., Portland, Oregon, USA). The peptide identification and protein identification false discovery rates were set to less than 1%, with each protein containing at least 2 identified peptides. Differential proteins between the control and AVP infusion groups were identified by quantitatively analyzing spectral counts. The Ensembl Compare database was searched to identify orthologous human proteins for these differential urinary proteins as reported (Vilella et al. 2009), and the human orthologs were then compared with the human core urinary proteome (Nagaraj & Mann 2011).

Hierarchical clustering of quantitative data

Hierarchical cluster analysis was used to understand the overall changes in proteins between groups and assess the parallelism and variations among technical replicates. An average-linkage hierarchical clustering of the top 280 proteins (after excluding proteins with low spectral count) was performed using Cluster 3.0 and visualized as a heatmap using Java TreeView (Stanford University, USA).

Biological function analysis of differential proteins

Biological functions of differential proteins, including molecular functions, biological processes and cellular components, were analyzed using the PANTHER classification system (http://www.pantherdb.org/).

Statistical analysis

The P value was analyzed by the Statistical Package for Social Studies (SPSS) 22.0. The differences in physiological indicators and urinary proteins were assessed using the SPSS software by one-way ANOVA followed by post hoc analysis with the least significant difference (LSD) test or Dunnett's T3 test. The difference between groups was considered significant when the P value equal to or less than 0.05.

Results

Weight gain and urine protein-to-creatinine ratio

There were no significant difference of weight gains in the normal, low-dose, and high-dose

194 groups by one week after AVP infusion (Figure 1 A). The urine protein-to-creatinine ratio was
195 slightly higher in the low-dose group and the high-dose group than in the control group, although
196 these differences were not statistically significant (Figure 1 B).
197
198 **Urine volume and urinary osmolality**
199 The 24 h urine volume of rats in the AVP infusion group was significantly less than that of those
200 in the control group, and the urine volume of the rats in the high-dose group was significantly
201 less than that of those in the low-dose group (Figure 1 C). The urinary osmolality of rats in the
202 AVP infusion group was significantly higher than that of those in the control group.
203 Additionally, urinary osmolality of rats was higher in the high-dose group than in the low-dose
204 group, although this difference was not statistically significant (Figure 1 D).
205
206 **SDS-PAGE analysis**
207 No bands with consistent differences between the control and AVP infusion groups were
208 observed in the SDS-PAGE analysis (Figure 2).
209
210 **Proteomics analysis**
211 Three rats each from the control, low-dose and high-dose groups were analyzed by LC-MS/MS.
212 Each sample was analyzed three times as technical replicates. Differential proteins were
213 identified by semi-quantitative analyses of spectral counts (Liu et al. 2004; Old et al. 2005;
214 Schmidt et al. 2014). The spectral count for each protein was calculated based on the mean
215 spectral count of the proteins from 3 replicates.
216 A total of 420 proteins were identified in the three groups. The identified proteins are shown
217 in Supplemental table 1. Forty-nine proteins significantly changed between control group and
218 AVP infusion groups (P by ANOVA$\leqslant$0.05). Compared to the control group, 21 differential
219 proteins in the low-dose group and 37 in high-dose group were significantly different. Nine
220 proteins were significantly changed in both groups.
221 To identify the most significantly differential protein, the following more stringent criteria
222 were chosen: (1) P by ANOVA$\leqslant$0.05, (2) fold change$\geqslant$1.5, and (3) spectral count for each
223 sample$\geqslant$4 in at least one group. Seventeen differential proteins were identified between control
224 group and AVP infusion groups by these criteria. Eight differential proteins in the low-dose
225 group and 10 in the high-dose group were identified relative to the control group (Table 1 and 2).

One protein was significantly changed in both groups.

Hierarchical clustering of quantitative data

As indicated in Figure 3, the high-dose group was easily distinguishable from the low-dose group and the control group. The three technical replicates for each sample can also be readily identified on the heatmap.

Biological function analysis of differential proteins

Biological functions of 49 differential proteins that significantly changed between control group and AVP infusion groups were analyzed. The major molecular functions of the differential proteins include catalytic activity, binding and receptor activity. The differential proteins' main biological processes include cellular processes, biological regulation and developmental processes. The differential proteins' main cellular components include cell parts, organelles and extracellular regions (Figure 4).

Differential proteins reported to be associated with AVP

Three differential proteins have been reported to be related to the functions of AVP. Increased expression of osteopontin in rat aortic adventitial fibroblasts can be induced by urotensin II (Zhang et al. 2011), which has vasoconstrictive functions similar to those of vasopressin. In this study, osteopontin in urine was 6-fold higher in the high-dose group than in the control group.

Calbindin, as Ca2+-buffer protein, can buffer Ca^{2+} concentrations within cells and thereby prevent cell injuries caused by high intracellular Ca^{2+} (Schwaller 2009). Calbindin localized to the DCT and the connecting tubule can regulate calcium transport and reabsorption (Hsin et al. 2006; Lambers et al. 2006).

It is well known that AVP can improve the permeability to water of the DCT and the connecting tubule and increase NaCl reabsorption. The $Na(^{+})/H(^{+})$ exchange regulatory cofactor NHE-RF3 plays an important role in regulating cell ion transport and membrane fluidity (Kato et al. 2005). NHE-RF3 also can mediate the formation of NHE3 (Yang et al. 2014), which is the major transporter in the proximal tubule that is involved in Na^{+} reabsorption (Schultheis et al. 1998).

Calbindin in urine was 5.17-fold higher in the high-dose group than in the control group. NHE-RF3 in urine was 1.58-fold higher in the high-dose group than in the control group.

Differential proteins as candidate biomarkers and their human orthologs

Nine of the 17 differential proteins have been identified as disease biomarkers in urine. Haptoglobin was identified as a biomarker for uranium nephrotoxicity (Malard et al. 2009), membranous nephropathy (Ngai et al. 2007), acute kidney injury (Zager et al. 2012), diabetic nephropathy (Rao et al. 2007), bladder carcinoma (Li et al. 2011), hepatic fibrosis (van Swelm et al. 2013) and acute phase response (Piras et al. 2014). Osteopontin can be used as a biomarker for ovarian cancer (Rainczuk et al. 2013), kidney stones (Bautista et al. 1996), bladder cancer (Yang et al. 2011) and drug-induced kidney injury (Phillips et al. 2016). Calbindin can serve as a biomarker for distal nephron segment injuries (Iida et al. 2014), nephrotoxicity (Hoffmann et al. 2010), acute kidney injury (Togashi et al. 2012)and drug-induced renal injury(Fuchs et al. 2014). Complement C3 can act as a biomarker of IgA nephropathy (Liu et al. 2014)and glomerulonephritis (Cumming et al. 1976). Pro-cathepsin H, regenerating islet-derived protein 3-gamma, glyceraldehyde-3-phosphate dehydrogenase, CD166 antigen and beta-2-glycoprotein 1 can be biomarkers of polycystic kidney disease (Schaefer et al. 1996), urinary tract infection (Spencer et al. 2015), ureteropelvic junction obstruction (Mesrobian et al. 2010), type 1 diabetes (Suh et al. 2015) and Dent's disease (Cutillas et al. 2004), respectively.

Sixteen of the 17 differential proteins have human orthologs. Twelve orthologs of these 16 proteins are part of the human core urinary proteome (Table 3).These results suggest that the effects of AVP on urinary proteins should be considered in future urinary disease biomarker researches.

Candidate biomarkers for SIADH

SIADH is often confused with cerebral salt wasting syndrome (CSWS) in the clinical setting, leading to difficulty in confirming its diagnosis. Therefore, identifying effective biomarkers can facilitate the differential diagnosis of SIADH and then contribute to the effective treatment of hyponatremia that connected with increased mortality (Corona et al. 2015).The differential proteins between the control and AVP infusion groups of this study may be used as candidate biomarkers to aid in diagnosing SIADH. Large clinical samples are needed to determine whether these proteins can actually be utilized in clinical testing.

Discussion

Urine is a good source of biomarkers since it contains many changes excluded from the body.

Urinary proteins are important bearer of information in urine. Many urinary protein biomarkers have been identified in various disease, such as renal disease (Schanstra & Mischak 2015), bladder cancer (Grossman et al. 2005), preeclampsia (Carty et al. 2011) and cardiovascular diseases (Delles et al. 2011). However some challenge exist in the urinary proteins biomarker studies such as standardization and normalization. The composition of the urinary proteins can be influenced by variation in sample handling process, including sample collection, protein extraction and protein digestion (Thongboonkerd et al. 2006). Standardized processes are considered to be beneficial for the integration analysis of the data from different sources. As for normalization, in this study, we actually compared the value of a specific protein/ total protein in disease group to its value in control group based on spectral count.

Among the most significant differential proteins, only one protein was identified in both the low and high dose AVP groups, indicating that different mechanisms may be involved in responses to low and high doses of AVP, although both types of doses decrease urine volume and increase urine osmolality. In addition, we compared the results from this study with those of previous studies that investigated the effects of diuretics on urine (Li et al. 2014). Osteopontin excretion in urine significantly decreased after the rats were given the oral diuretic furosemide. In this study, osteopontin concentration was significantly elevated in urine from the high-dose AVP group, a result consistent with the finding that diuretics influence urine. Interestingly, haptoglobin concentrations in urine were significantly increased in both the high-dose AVP group in our study and in rats that were given the oral diuretic furosemide. Thus, the anti-diuretic and diuretic share some of the same physiological processes and do not always exhibit opposing effects.

One challenge related to diagnosing SIADH is differentiating SIADH from CSWS because these two syndromes have similar clinical manifestations (hyponatraemia, high urine osmolality, and high natriuresis). It is important to distinguish SIADH from CSWS because these two syndromes differ with respect to pathogenesis and treatment (Adrogue & Madias 2012). Measuring effective arterial blood volume is the main approach used to differentiate between these two diseases (Palmer 2000). However, in clinical settings, it is difficult and expensive to determine effective arterial blood volume. Therefore, the efficiency of diagnosis and treatment would be improved by the identification of specific SIADH biomarkers. Differential proteins identified in this study can provide some clues for diagnose SIADH and future studies also be require to investigate biomarkers of CSWS and then aid the differential diagnosis.

Cancer is a common cause of SIADH. Various tumors, including lung cancer, pancreatic cancer, duodenal cancer, brain tumors and hematological malignancies, can cause SIADH(Keenan 1999). Additionally, 7%-12% of small cell lung cancers are complicated by SIADH (Berghmans et al. 2000). SIADH symptoms may occur before imaging existing tumors. Thus, SIADH may be used as an indicator for the early diagnosis of tumors, especially non-small cell lung cancer. Thus, the differential proteins identified in this study are helpful for the early detection of tumors and the diagnosis of SIADH after be validated in a larger blinded study with test specimens and validation specimens.

In this study, only the effect of AVP on male rats were analyzed. The previous study has demonstrated that vasopressin receptors gene AVPR2 is located on the X chromosome (Juul et al. 2014), suggesting that females may express more transcripts and receptors compared to males. Female rats should be included for future studies.

In conclusion, urinary proteins can be affected by AVP in vivo. Reports have indicated that several of the differential proteins identified in this study are associated with AVP; in addition, a number of the identified differential proteins have been recognized as disease biomarkers. These results suggest that several urinary biomarkers can be effected by AVP and thus we should consider the effects of AVP on urinary proteins in future urinary biomarker researches. The study data also provide clues regarding underlying mechanisms associated with AVP, and the identified differential proteins can be further studied to investigate their connections with AVP. Additionally, large clinical samples must be examined to verify that these differential proteins can actually be used as biomarkers of increases in pathological AVP in diseases such as SIADH.

354 References:

355

356 Adrogue HJ, and Madias NE. 2012. The challenge of hyponatremia. *J Am Soc Nephrol* 23:1140-1148.
357 10.1681/asn.2012020128
358 Bautista DS, Denstedt J, Chambers AF, and Harris JF. 1996. Low-molecular-weight variants of osteopontin generated
359 by serine proteinases in urine of patients with kidney stones. *J Cell Biochem* 61:402-409.
360 10.1002/(SICI)1097-4644(19960601)61:3<402::AID-JCB7>3.0.CO;2-X
361 Berghmans T, Paesmans M, and Body JJ. 2000. A prospective study on hyponatraemia in medical cancer patients:
362 epidemiology, aetiology and differential diagnosis. *Support Care Cancer* 8:192-197.
363 Bicego-Nahas KC, Steiner AA, Carnio EC, Antunes-Rodrigues J, and Branco LG. 2000. Antipyretic effect of arginine
364 vasotocin in toads. *Am J Physiol Regul Integr Comp Physiol* 278:R1408-1414.
365 Carty DM, Siwy J, Brennand JE, Zurbig P, Mullen W, Franke J, McCulloch JW, Roberts CT, North RA, Chappell LC,
366 Mischak H, Poston L, Dominiczak AF, and Delles C. 2011. Urinary proteomics for prediction of preeclampsia.
367 *Hypertension* 57:561-569. 10.1161/hypertensionaha.110.164285
368 Corona G, Giuliani C, Verbalis JG, Forti G, Maggi M, and Peri A. 2015. Hyponatremia improvement is associated with
369 a reduced risk of mortality: evidence from a meta-analysis. *PLoS One* 10:e0124105.
370 10.1371/journal.pone.0124105
371 Coverdill AJ, McCarthy M, Bridges RS, and Nephew BC. 2012. Effects of Chronic Central Arginine Vasopressin (AVP)
372 on Maternal Behavior in Chronically Stressed Rat Dams. *Brain Sci* 2. 10.3390/brainsci2040589
373 Cumming AD, Thomson D, Davidson AM, and Robson JS. 1976. Significance of urinary C3 excretion in
374 glomerulonephritis. *J Clin Pathol* 29:601-607.
375 Cutillas PR, Chalkley RJ, Hansen KC, Cramer R, Norden AG, Waterfield MD, Burlingame AL, and Unwin RJ. 2004. The
376 urinary proteome in Fanconi syndrome implies specificity in the reabsorption of proteins by renal proximal
377 tubule cells. *Am J Physiol Renal Physiol* 287:F353-364. 10.1152/ajprenal.00018.2004
378 Delles C, Diez J, and Dominiczak AF. 2011. Urinary proteomics in cardiovascular disease: Achievements, limits and
379 hopes. *Proteomics Clin Appl* 5:222-232. 10.1002/prca.201000125
380 Frouget T. 2012. [The syndrome of inappropriate antidiuresis]. *Rev Med Interne* 33:556-566.
381 10.1016/j.revmed.2012.07.005
382 Fuchs TC, Mally A, Wool A, Beiman M, and Hewitt P. 2014. An exploratory evaluation of the utility of transcriptional
383 and urinary kidney injury biomarkers for the prediction of aristolochic acid-induced renal injury in male rats.
384 *Vet Pathol* 51:680-694. 10.1177/0300985813498779
385 Gao Y. 2013. Urine-an untapped goldmine for biomarker discovery? *Sci China Life Sci* 56:1145-1146. 10.1007/s11427-
386 013-4574-1
387 Gao Y. 2014. Roadmap to the urine biomarker era. *MOJ Proteomics Bioinform* 1.
388 Grossman HB, Messing E, Soloway M, Tomera K, Katz G, Berger Y, and Shen Y. 2005. Detection of bladder cancer
389 using a point-of-care proteomic assay. *Jama* 293:810-816. 10.1001/jama.293.7.810
390 Hoffmann D, Fuchs TC, Henzler T, Matheis KA, Herget T, Dekant W, Hewitt P, and Mally A. 2010. Evaluation of a
391 urinary kidney biomarker panel in rat models of acute and subchronic nephrotoxicity. *Toxicology* 277:49-
392 58. 10.1016/j.tox.2010.08.013
393 Hsin YH, Cheng CH, Tzen JT, Wu MJ, Shu KH, and Chen HC. 2006. Effect of aristolochic acid on intracellular calcium
394 concentration and its links with apoptosis in renal tubular cells. *Apoptosis* 11:2167-2177. 10.1007/s10495-
395 006-0289-0
396 Iida T, Fujinaka H, Xu B, Zhang Y, Magdeldin S, Nameta M, Liu Z, Yoshida Y, Yaoita E, Tomizawa S, Saito A, and
397 Yamamoto T. 2014. Decreased urinary calbindin 1 levels in proteinuric rats and humans with distal nephron
398 segment injuries. *Clin Exp Nephrol* 18:432-443. 10.1007/s10157-013-0835-3
399 Juul KV, Bichet DG, Nielsen S, and Norgaard JP. 2014. The physiological and pathophysiological functions of renal
400 and extrarenal vasopressin V2 receptors. *Am J Physiol Renal Physiol* 306:F931-940.
401 10.1152/ajprenal.00604.2013
402 Kato Y, Sai Y, Yoshida K, Watanabe C, Hirata T, and Tsuji A. 2005. PDZK1 directly regulates the function of organic
403 cation/carnitine transporter OCTN2. *Mol Pharmacol* 67:734-743. 10.1124/mol.104.002212
404 Keenan AM. 1999. Syndrome of inappropriate secretion of antidiuretic hormone in malignancy. *Semin Oncol Nurs*

15:160-167.
Kounin G, and Bashir Q. 2000. Mechanism and role of antidiuretic hormone. *Surg Neurol* 53:508-510.
Lambers TT, Mahieu F, Oancea E, Hoofd L, de Lange F, Mensenkamp AR, Voets T, Nilius B, Clapham DE, Hoenderop JG, and Bindels RJ. 2006. Calbindin-D28K dynamically controls TRPV5-mediated Ca2+ transport. *Embo j* 25:2978-2988. 10.1038/sj.emboj.7601186
Li H, Li C, Wu H, Zhang T, Wang J, Wang S, and Chang J. 2011. Identification of Apo-A1 as a biomarker for early diagnosis of bladder transitional cell carcinoma. *Proteome Sci* 9:21. 10.1186/1477-5956-9-21
Li X, Zhao M, Li M, Jia L, and Gao Y. 2014. Effects of three commonly-used diuretics on the urinary proteome. *Genomics Proteomics Bioinformatics* 12:120-126. 10.1016/j.gpb.2013.12.002
Liu H, Sadygov RG, and Yates JR, 3rd. 2004. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal Chem* 76:4193-4201. 10.1021/ac0498563
Liu L, Zhang Y, Duan X, Peng Q, Liu Q, Zhou Y, Quan S, and Xing G. 2014. C3a, C5a renal expression and their receptors are correlated to severity of IgA nephropathy. *J Clin Immunol* 34:224-232. 10.1007/s10875-013-9970-6
Madrazo I, Franco-Bourland RE, Leon-Meza VM, and Mena I. 1987. Intraventricular somatostatin-14, arginine vasopressin, and oxytocin: analgesic effect in a patient with intractable cancer pain. *Appl Neurophysiol* 50:427-431.
Malard V, Gaillard JC, Berenguer F, Sage N, and Quemeneur E. 2009. Urine proteomic profiling of uranium nephrotoxicity. *Biochim Biophys Acta* 1794:882-891. 10.1016/j.bbapap.2009.01.010
Mesrobian HG, Mitchell ME, See WA, Halligan BD, Carlson BE, Greene AS, and Wakim BT. 2010. Candidate urinary biomarker discovery in ureteropelvic junction obstruction: a proteomic approach. *J Urol* 184:709-714. 10.1016/j.juro.2010.03.061
Nabe K, Honjo S, Ikeda H, Wada Y, Nomura K, Hamamoto Y, Koh T, Tatsuoka Y, and Koshiyama H. 2007. Diabetes insipidus and cognitive function. *Med Hypotheses* 69:764-766. 10.1016/j.mehy.2007.01.081
Nagaraj N, and Mann M. 2011. Quantitative analysis of the intra- and inter-individual variability of the normal urinary proteome. *J Proteome Res* 10:637-645. 10.1021/pr100835s
Ngai HH, Sit WH, Jiang PP, Thongboonkerd V, and Wan JM. 2007. Markedly increased urinary preprohaptoglobin and haptoglobin in passive Heymann nephritis: a differential proteomics approach. *J Proteome Res* 6:3313-3320. 10.1021/pr070245b
Old WM, Meyer-Arendt K, Aveline-Wolf L, Pierce KG, Mendoza A, Sevinsky JR, Resing KA, and Ahn NG. 2005. Comparison of label-free methods for quantifying human proteins by shotgun proteomics. *Mol Cell Proteomics* 4:1487-1502. 10.1074/mcp.M500084-MCP200
Palmer BF. 2000. Hyponatraemia in a neurosurgical patient: syndrome of inappropriate antidiuretic hormone secretion versus cerebral salt wasting. *Nephrol Dial Transplant* 15:262-268.
Pejcic M, Stojnev S, and Stefanovic V. 2010. Urinary proteomics--a tool for biomarker discovery. *Ren Fail* 32:259-268. 10.3109/08860221003599759
Phillips JA, Holder DJ, Ennulat D, Gautier JC, Sauer JM, Yang Y, McDuffie E, Sonee M, Gu YZ, Troth SP, Lynch K, Hamlin D, Peters DG, Brees D, and Walker EG. 2016. Rat Urinary Osteopontin and Neutrophil gelatinase-associated lipocalin Improve Certainty of Detecting Drug-Induced Kidney Injury. *Toxicol Sci*. 10.1093/toxsci/kfw038
Piras C, Soggiu A, Greco V, Cassinotti A, Maconi G, Ardizzone S, Amoresano A, Porro GB, Bonizzi L, and Roncada P. 2014. Serum protein profiling of early and advanced stage Crohn's disease. *EuPA Open Proteomics* 3:48-59.
Rainczuk A, Condina M, Pelzing M, Dolman S, Rao J, Fairweather N, Jobling T, and Stephens AN. 2013. The utility of isotope-coded protein labeling for prioritization of proteins found in ovarian cancer patient urine. *J Proteome Res* 12:4074-4088. 10.1021/pr400618v
Rao PV, Lu X, Standley M, Pattee P, Neelima G, Girisesh G, Dakshinamurthy KV, Roberts CT, Jr., and Nagalla SR. 2007. Proteomic identification of urinary biomarkers of diabetic nephropathy. *Diabetes Care* 30:629-637. 10.2337/dc06-2056
Schaefer L, Han X, Gretz N, and Schaefer RM. 1996. Alterations of cathepsins B, H and L in proximal tubules from polycystic kidneys of the Han:SPRD rat. *Kidney Int* 50:424-431.
Schanstra JP, and Mischak H. 2015. Proteomic urinary biomarker approach in renal disease: from discovery to implementation. *Pediatr Nephrol* 30:713-725. 10.1007/s00467-014-2790-y
Schmidt C, Gronborg M, Deckert J, Bessonov S, Conrad T, Luhrmann R, and Urlaub H. 2014. Mass spectrometry-based relative quantification of proteins in precatalytic and catalytically active spliceosomes by metabolic labeling (SILAC), chemical labeling (iTRAQ), and label-free spectral count. *Rna* 20:406-420.

10.1261/rna.041244.113
Schultheis PJ, Clarke LL, Meneton P, Miller ML, Soleimani M, Gawenis LR, Riddle TM, Duffy JJ, Doetschman T, Wang T, Giebisch G, Aronson PS, Lorenz JN, and Shull GE. 1998. Renal and intestinal absorptive defects in mice lacking the NHE3 Na+/H+ exchanger. *Nat Genet* 19:282-285. 10.1038/969
Schwaller B. 2009. The continuing disappearance of "pure" Ca2+ buffers. *Cell Mol Life Sci* 66:275-300. 10.1007/s00018-008-8564-6
Spencer JD, Jackson AR, Li B, Ching CB, Vonau M, Easterling RS, Schwaderer AL, McHugh KM, and Becknell B. 2015. Expression and Significance of the HIP/PAP and RegIIIgamma Antimicrobial Peptides during Mammalian Urinary Tract Infection. *PLoS One* 10:e0144024. 10.1371/journal.pone.0144024
Suh MJ, Tovchigrechko A, Thovarai V, Rolfe MA, Torralba MG, Wang J, Adkins JN, Webb-Robertson BJ, Osborne W, Cogen FR, Kaplowitz PB, Metz TO, Nelson KE, Madupu R, and Pieper R. 2015. Quantitative Differences in the Urinary Proteome of Siblings Discordant for Type 1 Diabetes Include Lysosomal Enzymes. *J Proteome Res* 14:3123-3135. 10.1021/acs.jproteome.5b00052
Thongboonkerd V, Chutipongtanate S, and Kanlaya R. 2006. Systematic evaluation of sample preparation methods for gel-based human urinary proteomics: quantity, quality, and variability. *J Proteome Res* 5:183-191. 10.1021/pr0502525
Togashi Y, Sakaguchi Y, Miyamoto M, and Miyamoto Y. 2012. Urinary cystatin C as a biomarker for acute kidney injury and its immunohistochemical localization in kidney in the CDDP-treated rats. *Exp Toxicol Pathol* 64:797-805. 10.1016/j.etp.2011.01.018
Treschan TA, and Peters J. 2006. The vasopressin system: physiology and clinical strategies. *Anesthesiology* 105:599-612; quiz 639-540.
Trovato FM, Tognarelli JM, Crossey MM, Catalano D, Taylor-Robinson SD, and Trovato GM. 2015. Challenges of liver cancer: Future emerging tools in imaging and urinary biomarkers. *World J Hepatol* 7:2664-2675. 10.4254/wjh.v7.i26.2664
van Swelm RP, Laarakkers CM, Kooijmans-Otero M, de Jong EM, Masereeuw R, and Russel FG. 2013. Biomarkers for methotrexate-induced liver injury: urinary protein profiling of psoriasis patients. *Toxicol Lett* 221:219-224. 10.1016/j.toxlet.2013.06.234
Vilella AJ, Severin J, Ureta-Vidal A, Heng L, Durbin R, and Birney E. 2009. EnsemblCompara GeneTrees: Complete, duplication-aware phylogenetic trees in vertebrates. *Genome Res* 19:327-335. 10.1101/gr.073585.107
Wisniewski JR, Zougman A, Nagaraj N, and Mann M. 2009. Universal sample preparation method for proteome analysis. *Nat Methods* 6:359-362. 10.1038/nmeth.1322
Wu J, and Gao Y. 2015. Physiological conditions can be reflected in human urine proteome and metabolome. *Expert Rev Proteomics* 12:623-636. 10.1586/14789450.2015.1094380
Yang J, Singh V, Chen TE, Sarker R, Xiong L, Cha B, Jin S, Li X, Tse CM, Zachos NC, and Donowitz M. 2014. NHERF2/NHERF3 protein heterodimerization and macrocomplex formation are required for the inhibition of NHE3 activity by carbachol. *J Biol Chem* 289:20039-20053. 10.1074/jbc.M114.562413
Yang N, Feng S, Shedden K, Xie X, Liu Y, Rosser CJ, Lubman DM, and Goodison S. 2011. Urinary glycoprotein biomarker discovery for bladder cancer detection using LC/MS-MS and label-free quantification. *Clin Cancer Res* 17:3349-3359. 10.1158/1078-0432.ccr-10-3121
Zager RA, Vijayan A, and Johnson AC. 2012. Proximal tubule haptoglobin gene activation is an integral component of the acute kidney injury "stress response". *Am J Physiol Renal Physiol* 303:F139-148. 10.1152/ajprenal.00168.2012
Zelena D, Domokos A, Jain SK, Jankord R, and Filaretova L. 2009. The stimuli-specific role of vasopressin in the hypothalamus-pituitary-adrenal axis response to stress. *J Endocrinol* 202:263-278. 10.1677/joe-09-0096
Zhang YG, Kuang ZJ, Mao YY, Wei RH, Bao SL, Wu LB, Li YG, and Tang CS. 2011. Osteopontin is involved in urotensin II-induced migration of rat aortic adventitial fibroblasts. *Peptides* 32:2452-2458. 10.1016/j.peptides.2011.10.018

Figure 1

Physiological indicators of rats in control group (n=7), low dose AVP group (n=5) and high dose AVP group (n=6).

A, weight gain of rats in three groups; B, urine protein-to-creatinine ratio of rats in three groups; C, 24 h urine volume of rats in three groups; D, urinary osmolality of rats in three groups.

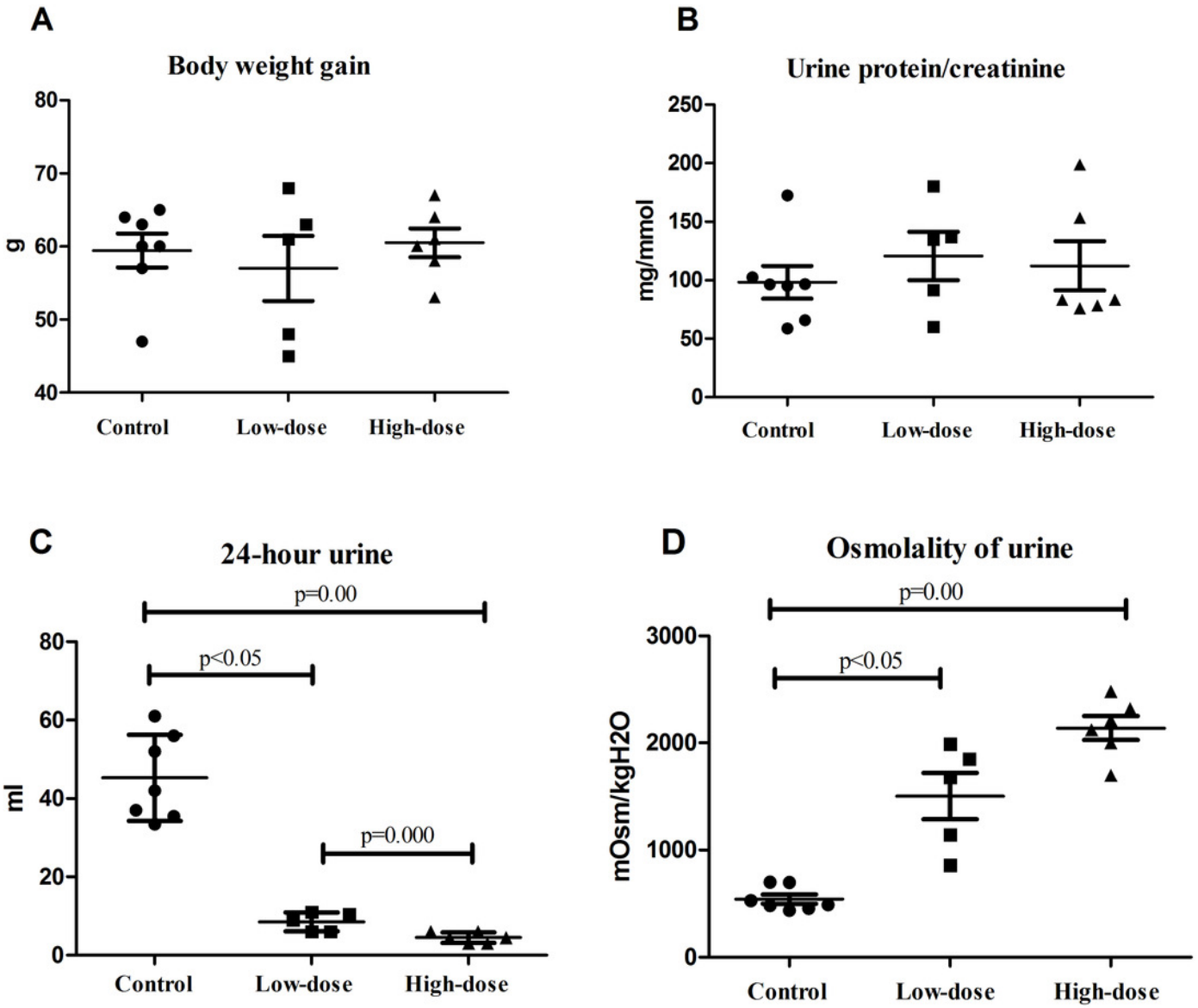

Figure 2

SDS-PAGE of urinary proteins in control group, low dose AVP group and high dose AVP group.

M, marker.

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

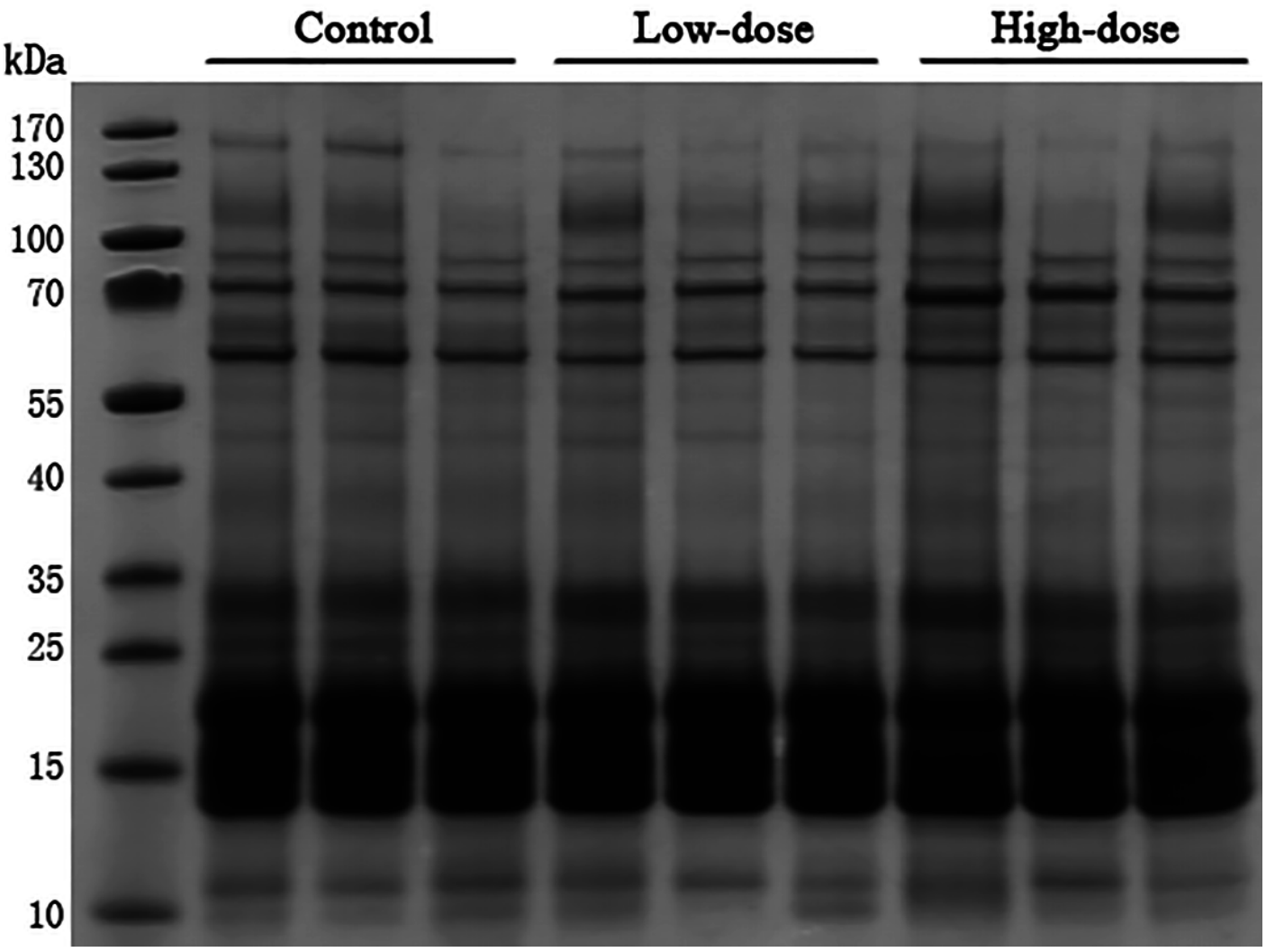

Figure 3

Heatmap of Hierarchical clustering.

1, 2, 3, control group; 4, 5, 6, low dose group; 7, 8, 9, high dose group; -1, -2, -3, three technical replicates of each sample.

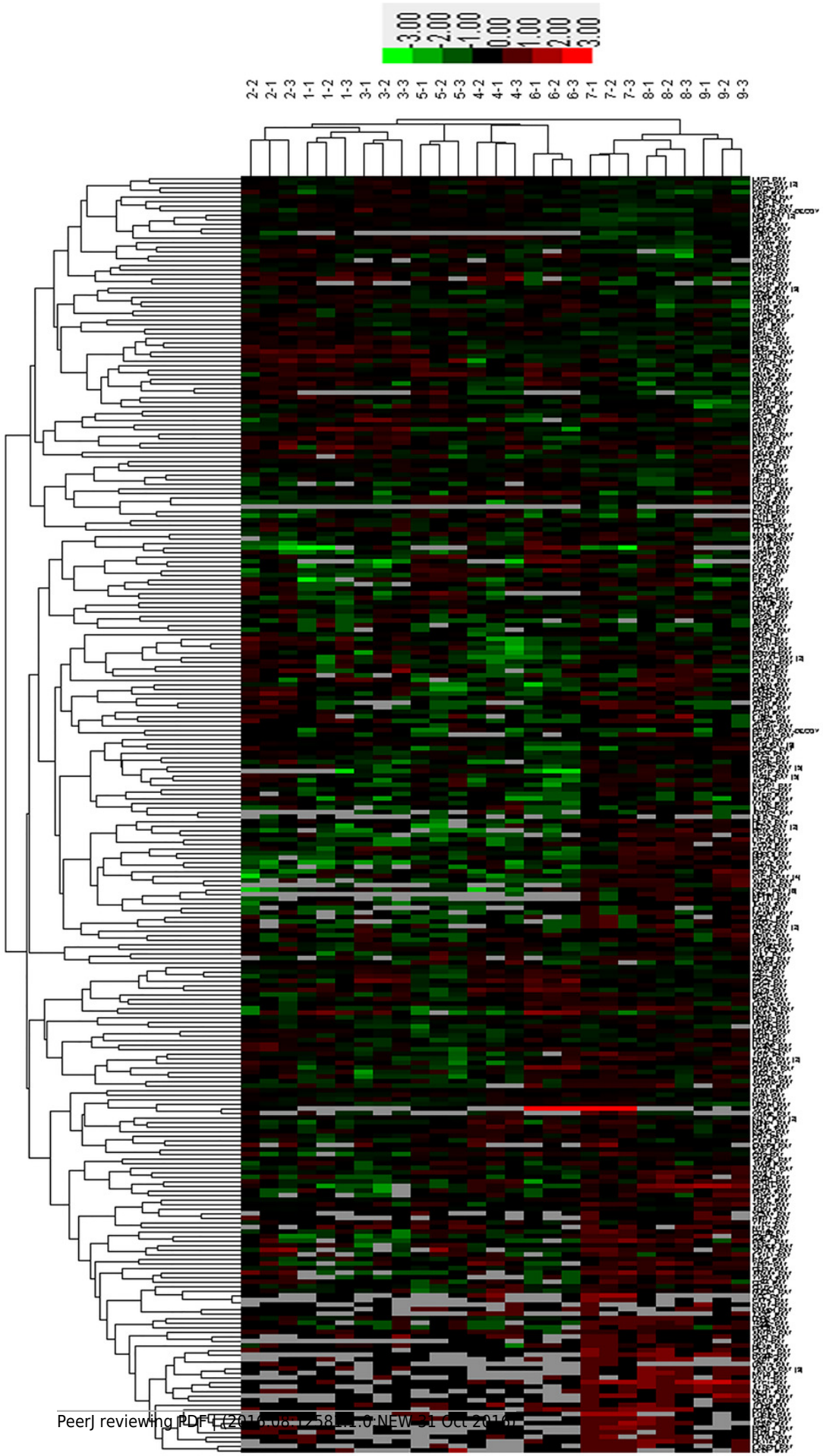
-3.00
-2.00
-1.00
0.00
1.00
2.00
3.00
2-2
2-1
2-3
1-1
1-2
1-3
3-1
3-2
3-3
5-1
5-2
5-3
4-2
4-1
4-3
6-1
6-2
6-3
7-1
7-2
7-3
8-1
8-2
8-3
9-1
9-2
9-3

Figure 4

Biological function analysis of differential proteins between control group and AVP infusion groups.

A, molecular functions of differential proteins; B, biological processes of differential proteins; C, cellular components of differential proteins.

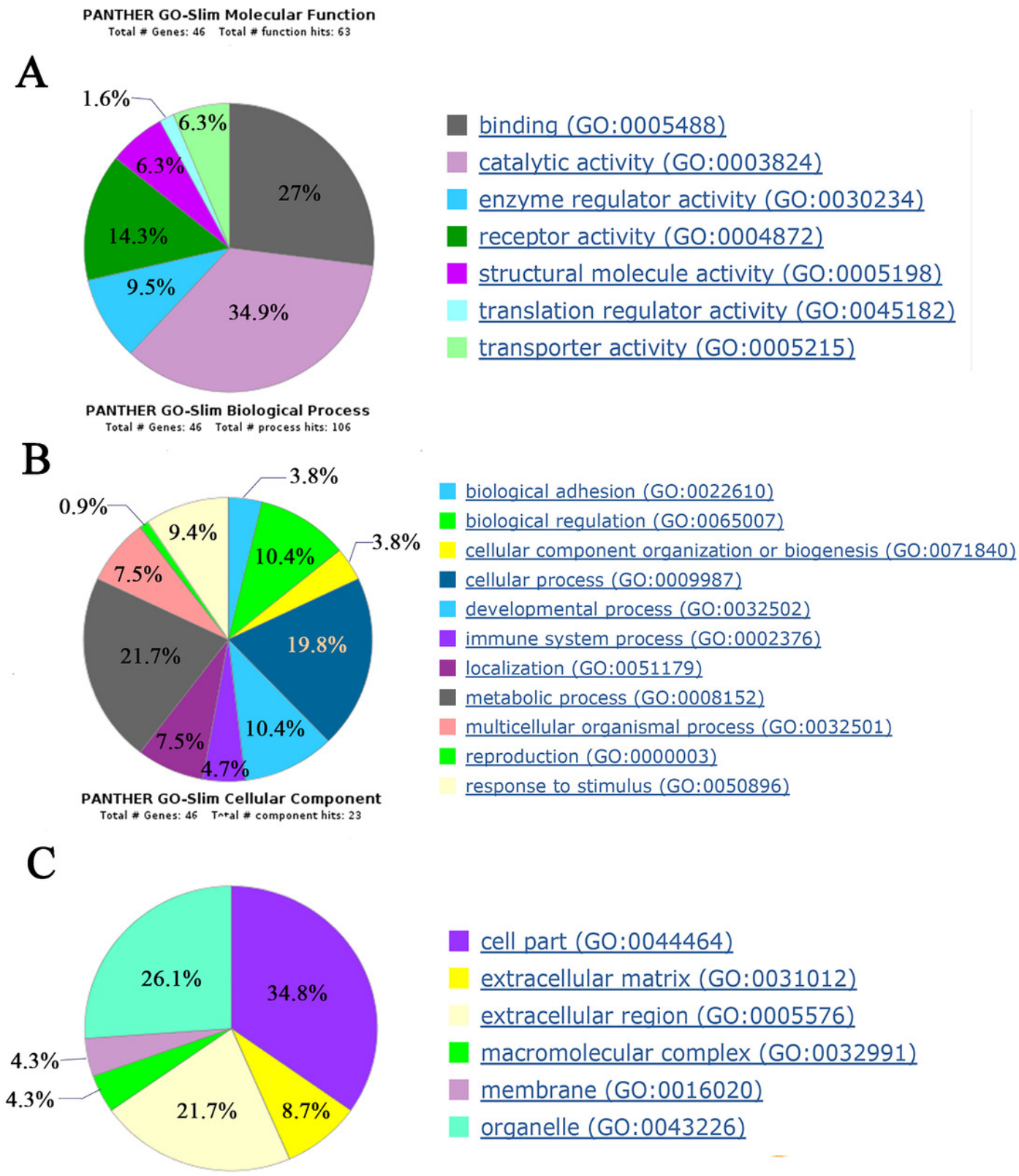
PANTHER GO-Slim Molecular Function
Total # Genes: 46 Total # function hits: 63
A
1.6%
6.3%
6.3%
27%
14.3%
9.5%
34.9%
binding (GO:0005488)
catalytic activity (GO:0003824)
enzyme regulator activity (GO:0030234)
receptor activity (GO:0004872)
structural molecule activity (GO:0005198)
translation regulator activity (GO:0045182)
transporter activity (GO:0005215)
PANTHER GO-Slim Biological Process
Total # Genes: 46 Total # process hits: 106
B
3.8%
0.9%
9.4%
3.8%
10.4%
7.5%
19.8%
21.7%
10.4%
7.5%
4.7%
biological adhesion (GO:0022610)
biological regulation (GO:0065007)
cellular component organization or biogenesis (GO:0071840)
cellular process (GO:0009987)
developmental process (GO:0032502)
immune system process (GO:0002376)
localization (GO:0051179)
metabolic process (GO:0008152)
multicellular organismal process (GO:0032501)
reproduction (GO:0000003)
response to stimulus (GO:0050896)
PANTHER GO-Slim Cellular Component
Total # Genes: 46 Total # component hits: 23
C
26.1%
34.8%
4.3%
4.3%
21.7%
8.7%
cell part (GO:0044464)
extracellular matrix (GO:0031012)
extracellular region (GO:0005576)
macromolecular complex (GO:0032991)
membrane (GO:0016020)
organelle (GO:0043226)

Table 1(on next page)

The significant differential proteins between control group and low dose group.

Rat 1, 2, 3, control group; rat 4, 5, 6, low-dose group; fold change is low dose-to-control group ratio.

Table 1 The significant differential proteins between control group and low dose group.
Rat 1, 2, 3, control group; rat 4, 5, 6, low-dose group; fold change is low dose-to-control group ratio.

Protein name	Accession number	Fold change	P value	Spectral counts						Candidate biomarker
				Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	
Haptoglobin	HPT_RAT	2.33	0.022	6	3	3	10	8	10	Yes
Prolactin-inducible protein homolog	PIP_RAT	1.88	0.009	2	3	3	5	6	4	No
Pro-cathepsin H	CATH_RAT	1.88	0.041	2	3	3	5	5	5	Yes
Junctional adhesion molecule A	JAM1_RAT	0.66	0.005	8	9	7	5	6	5	No
Regenerating islet-derived protein 3-gamma	REG3G_RAT	0.60	0.011	18	16	14	8	13	8	Yes
Glutamate--cysteine ligase catalytic subunit	GSH1_RAT	0.60	0.020	11	11	8	4	8	6	No
Attractin	ATRN_RAT	0.56	0.041	5	7	4	2	4	3	No
Malate dehydrogenase, cytoplasmic	MDHC_RAT	0.46	0.005	4	6	5	3	2	2	No

Table 2(on next page)

The significant differential proteins between control group and high dose group.

Rat 1, 2, 3, control group; rat 7, 8, 9, high-dose group; fold change is high dose-to-control group ratio.

Table 2 The significant differential proteins between control group and high dose group.
Rat 1, 2, 3, control group; rat 7, 8, 9, high-dose group; fold change is high dose-to-control group ratio.

Protein name	Accession number	Fold change	P value	Spectral counts						Candidate biomarkers
				Rat 1	Rat 2	Rat 3	Rat 7	Rat 8	Rat 9	
Osteopontin	OSTP_RAT	6.00	0.038	0	0	4	7	9	8	Yes
Calbindin	CALB1_RAT	5.17	0.019	2	4	0	5	12	14	Yes
Putative phospholipase B-like 2	PLBL2_RAT	3.50	0	1	2	1	5	5	4	No
Cluster of Glyceraldehyde-3-phosphate dehydrogenase	G3P_RAT	2.17	0.018	2	3	1	5	4	4	Yes
CD166 antigen	CD166_RAT	1.75	0.050	3	3	2	6	4	4	Yes
Complement C3	CO3_RAT	1.71	0.045	9	8	7	10	17	14	Yes
Beta-2-glycoprotein 1	APOH_RAT	1.70	0.048	3	4	3	5	5	7	Yes
Na(+)/H(+) exchange regulatory cofactor NHE-RF3	NHRF3_RAT	1.58	0.026	7	6	11	12	14	12	No
Copper transport protein ATOX1	ATOX1_RAT	1.53	0.013	6	4	5	7	7	9	No
Regenerating islet-derived protein 3-gamma	REG3G_RAT	0.46	0.003	18	16	14	8	8	6	Yes

Table 3(on next page)

Human orthologs of the significant differential proteins between control group and AVP infusion groups.

Table 3 Human orthologs of the significant differential proteins between control group and AVP infusion groups.

Protein name	Rat protein accession number	Human ensembl gene ID	Human protein accession number	Human core urinary proteome
Haptoglobin	HPT_RAT	ENSG00000257017	HPT_HUMAN	Yes
Prolactin-inducible protein homolog	PIP_RAT	ENSG00000159763	PIP_HUMAN	Yes
Pro-cathepsin H	CATH_RAT	ENSG00000103811	CATH_HUMAN	Yes
Junctional adhesion molecule A	JAM1_RAT	ENSG00000158769	JAM1_HUMAN	Yes
Regenerating islet-derived protein 3-gamma	REG3G_RAT	ENSG00000172016	REG3A_HUMAN	No
Glutamate--cysteine ligase catalytic subunit	GSH1_RAT	ENSG00000001084	GSH1_HUMAN	No
Attractin	ATRN_RAT	ENSG00000088812	ATRN_HUMAN	Yes
Malate dehydrogenase, cytoplasmic	MDHC_RAT	ENSG00000014641	MDHC_HUMAN	Yes
Osteopontin	OSTP_RAT	ENSG00000118785	OSTP_HUMAN	Yes
Calbindin	CALB1_RAT	ENSG00000104327	CALB1_HUMAN	Yes
Putative phospholipase B-like 2	PLBL2_RAT	ENSG00000151176	PLBL2_HUMAN	Yes
Cluster of Glyceraldehyde-3-phosphate dehydrogenase	G3P_RAT	ENSG00000111640	G3P_HUMAN	Yes
CD166 antigen	CD166_RAT	ENSG00000170017	CD166_HUMAN	No
Beta-2-glycoprotein 1	APOH_RAT	ENSG00000091583	APOH_HUMAN	Yes
Na(+)/H(+) exchange regulatory cofactor NHE-RF3	NHRF3_RAT	ENSG00000174827	NHRF3_HUMAN	No
Copper transport protein ATOX1	ATOX1_RAT	ENSG00000177556	ATOX1_HUMAN	Yes