The effect of chronic kidney disease on the urine proteome in the domestic cat (Felis catus)

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-+Original Article

Urine proteome in the domestic cat (*Felis catus*): the effect of chronic kidney disease

E. Ferlizza [a], A. Campos [b], A. Neagu [a], A. Cuoghi [c], E. Bellei [c], E. Monari [c] F. Dondi [a], A.M. Almeida[d], G. Isani [a,*]

[a] *Department of Veterinary Medical Sciences, Alma Mater Studiorum-University of Bologna, Ozzano, Bologna, Italy.*
[b] *Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Porto, Portugal*
[c] *Department of Diagnostic, Clinical and Public Health Medicine, University of Modena and Reggio Emilia, Modena, Italy*
[d] *CIISA – Interdisciplinary Research Centre of Animal Health, Faculty of Veterinary Medicine of the University of Lisbon, Lisbon, Portugal; IICT – Tropical Research Institute, Lisbon, Portugal, ITQB/UNL – Chemical and Biological Technologies Institute of the New University of Lisbon, Oeiras, Portugal and IBET – Technological and Experimental Biology Institute, Oeiras, Portugal.*

* Corresponding author. Tel.: +39 051 2097019.
E-mail address: gloria.isani@unibo.it (G. Isani)

Abstract

Chronic kidney disease (CKD) is a major cause of mortality in cats, but sensitive and specific biomarkers for early prediction and monitoring of CKD are currently lacking. The present study aimed to apply proteomic techniques to map the cat urine proteome and compare it with that in cats with CKD. Urine samples were collected by cystocentesis from 23 healthy young and ~~18~~17 CKD cats. One-dimensional sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (1D-SDS-PAGE) was ~~run~~ conducted on 4-12% gels. Two-dimensional electrophoresis (2DE) was applied to pooled urine from four healthy and four CKD urine samples. Sixteen protein bands and 36 spots were cut, trypsin-digested and identified by mass spectrometry.

Commentato [FdMV1]: We have excluded one CKD patient, as suggested by the reviewer 2

1D-SDS-PAGE yielded an overall view of the protein profile and the separation of 32±6 protein bands in the urine of healthy cats, while CKD cats showed significantly fewer bands (*P*<0.01). 2-DE was essential in fractionation of the complex urine proteome, producing a reference map that included 20 proteins. Cauxin was the most abundant protein in urine of healthy cats; we also identified several protease inhibitors and transport proteins, e.g., alpha-2-macroglobulin, albumin, transferrin, haemopexin and haptoglobin that all derive from plasma. We disclosed 27 spots differentially expressed (*P*<0.05) in CKD cats, and 13 proteins were unambiguously identified. In particular, the increase in retinol-binding protein, cystatin M and apolipoprotein-H associated with the decrease of uromodulin and cauxin confirmed tubular damage in CKD cats and suggest these proteins are candidate biomarkers.

Keywords: Biomarkers; Cat; Nephropathy; Proteinuria; ~~Urine proteome~~ Electrophoresis

Introduction

Chronic Kidney Disease (CKD) is the more frequent renal disease of cats and its prevalence is estimated at 1-3% in the general feline population reaching 50% in geriatric cats (Polzin, 2011; Bartges, 2012). ~~Although feline CKD is frequently sustained by chronic tubulointerstitial nephritis, many cats present a mild to moderate proteinuria even in the early stages of the disease.~~ . Feline CKD is frequently sustained by chronic tubulointerstitial nephritis with mild proteinuria, but the minority of cats, particularly with advanced CKD (IRIS stage 3 and 4), could be bordeline or proteinurics, due to a more severe tubular and glomerular involvement.~~, and some cats present secondary glomerular involvement with mild to moderate or even severe proteinuria, in particular at the later stages of disease.~~ It is well known that proteinuria itself could promote further renal damage and CKD progression; however the mechanism by which these excess proteins induce renal injury is still not entirely clarified (Bartges, 2012).

Commentato [FdMV2]: The sentence has been erased and modified as follows. (lines 51-52 previous version)

Sensitive and specific biomarkers for early prediction and monitoring of CKD in cats are currently lacking. Quantitative methods for the detection of proteinuria, (urinary protein and urinary albumin to creatinine ratios; UPC and UAC) are used to evaluate the severity of renal involvement but offer no information on its aetiology or composition of the urine proteome (Tesch, 2010). In addition, cauxin, a 70 kDa protein secreted physiologically by the tubule in cats, can interfere with the assessment of proteinuria (Mischke, 2011; Miyazaki et al., 2011).

Urine is considered an ideal source of clinical biomarkers as it can be obtained repeatedly in sufficient amounts and noninvasively. High-resolution electrophoresis coupled with mass spectrometry

(MS) allows fractionation and identification of the complex urine proteome and can therefore provide important information not only on kidney function but also on general health status. Over the last few years, large-scale proteomics has been extensively applied in human medicine first to define the protein urine map and then to search for novel biomarkers of pathologies, including CKD (Candiano et al., 2010; He et al., 2012). In veterinary medicine, the application of proteomics techniques is still limited, but recently there have been significant efforts to study the urine proteome in dogs (Nabity et al., 2011; Schaefer et al., 2011; Brandt et al., 2014; Miller et al., 2014) and to a lesser extent in cats (Lemberger et al., 2011; Jepson et al., 2013), although its applications are vast as recently reviewed (Almeida et al., 2014).

The aim of our work was to produce a comprehensive characterization of the urine proteome of the healthy cats (*Felis catus*) and to compare it with the proteome in CKD patients. Ultimately we aimed to identify putative biomarkers of nephropathy to be used for ~~early~~ detection of CKD or other renal diseases.

Material and methods

Animal selection, sample collection and preparation

This study was confined to privately owned cats divided into two experimental groups. The healthy group comprised by entire cats presented to a veterinary teaching hospital for neutering. Only animals considered healthy on the basis of history and physical examination and with no history of urinary tract diseases were included. The diseased group comprised cats with CKD diagnosed on the basis of history, clinical signs, clinicopathological and imaging results, according to Bartges (2012). ~~the~~

~~International Renal Interest Society group (IRIS[1]) CKD guidelines (Bartges, 2012) In particular,~~ Cats had to have clinical findings of CKD and (a) persistent pathologic renal proteinuria based on the urine protein to creatinine ratio (UPC), assessed and confirmed over a two-month period (UPC>0.2), and ~~or~~ (b) serum creatinine concentration ≥1.60 mg/dL and urine specific gravity (USG) <1.035. CKD cats were staged according to the International Renal Interest Society (IRIS[1]) CKD guidelines. Upon arrival, all the animals were subjected to physical examination and routine laboratory tests, including complete blood count, serum chemistry and complete urinalysis with UPC and urine culture. Five millilitres of urine were collected from each animal by ultrasound-guided cystocentesis. After centrifugation at 1,500 *g* for ten minutes, supernatants were immediately stored at -80 ˚C.

Commentato [FdMV3]: The sentence has been modified. Lines 85-87 previous version.

Commentato [FdMV4]: Following the suggestions of the Rev 2 about the clarity of this sentence, we have modified according to our clinical data (Table 1). Lines 87-89 previous version.

Urine protein to creatinine ratio

Urine total proteins and creatinine were determined using commercial kits (Urinary/CSF Protein, OSR6170, and Creatinine OSR6178, Olympus/Beckman Coulter) on an automated chemistry analyzer (AU 400, Olympus/Beckman Coulter). The UPC was calculated with the following formula: UPC = urine protein (mg/dL)/urine creatinine (mg/dL).

One-dimensional gel electrophoresis (1D-SDS-PAGE)

Urine proteins were separated using the electrophoresis NuPAGE system (Thermo Fisher Scientific) on 4-12% polyacrylamide gel in 2-(*N*-morpholino) ethanesulphonic acid buffer with sodium-dodecyl-sulphate (SDS) (Thermo Fisher Scientific). Two µg of protein for each sample were loaded. The gels were stained with SilverQuest (Thermo Fisher Scientific). After staining, each gel was

[1] See: http://www.iris-kidney.com/guidelines/

digitalized and its pherogram was obtained using GelAnalyzer 2010 software[2]. To evaluate differences between genders, two pools were prepared by collecting and mixing 20 µg of proteins from each healthy male (*n* = 8) and female (*n* = 15) sample. The pools were concentrated by Vivaspin500 spin columns (Sartorius Stedim Biotech GmbH) with a molecular weight (MW) cut-off of 3 kDa and separated by 1D-SDS-PAGE with the protocol reported above, with the exception of 3-(*N*-morpholino)propanesulphonic acid buffer and Coomassie blue staining (PageBlu protein staining solution; Thermo Fisher Scientific) compatible with mass spectrometry analysis.

Two-dimensional gel electrophoresis (2-DE)

Urine samples from four healthy and four CKD cats were selected for 2-DE. To concentrate and desalt samples, 150 µg of protein for each sample were precipitated with trichloroacetic acid to a final concentration of 10% in gentle shaking for one hour and then centrifuged at 15,000 *g* for 30 min at 4 °C. The protein pellets were washed three times with cold absolute acetone, air-dried and dissolved in a rehydration buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 65 mM dithiothreitol (DTT) and 0.8% resolytes (pH 3-10) before loading onto immobilized pH gradient (IPG) strips (non-linear pH gradient 3–10, 17 cm long) (BioRad). IPG strips were rehydrated and equilibrated following Campos et al. (2013). The equilibrated IPG strips were placed on top of 10% acrylamide gel, and protein separation was run at 24 mA per gel for 6 h in Protean II XL (BioRad) in running buffer containing 25mM Tris, glycine 192 mM and SDS 0.1%, pH 8.8 (Campos et al., 2013). At the end of each run, the gels were stained by CBB. 2-DE gels

[2] See: http://www.gelanalyzer.com/

were digitalized in a GS-800 calibrated densitometer (Bio-Rad) and the images analyzed by Progenesis SameSpot software (Non-Linear Dynamics) as described by Cruz De Carvalho et al. (2014).

Protein identification by mass spectrometry

Protein bands and spots were excised manually from the gels and subjected to in-gel tryptic digestion as previously described (Bellei et al., 2013). After digestion, the peptides were analyzed by a Nano LC-CHIP-MS system (ESI-Q-TOF 6520, Agilent Technologies). Data were acquired in data-dependent MS/MS mode in which, for each cycle, the three most abundant multiply charged peptides (2^+ to 4^+), above an absorbance threshold of 200 in the MS scan (*m*/*z* full scan acquisition range from 100 to 1700), were selected for MS/MS (*m*/*z* tandem mass spectrum acquisition range from 50 to 1700). Each peptide was selected twice and then dynamically excluded for 0.1 min. Raw mass spectrometry data were processed with MassHunter Qualitative Analysis B.05.00 software to obtain the Mascot generic files for database searching using the following parameters: deisotope, Absolute Height ≥10, Relative Height ≥ 0.1% of largest peak.

Since the domestic cat protein database is not well annotated, we chose to search a broader taxonomy, namely “all mammals”, to allow the identification on the basis of the sequence homology. Protein-identification peak lists were generated using the Mascot search engine against the UniProt database[3] specifying the following parameters: *Mammalian* taxonomy, parent ion tolerance ± 20 ppm, MS/MS error tolerance ± 0.12 Da, alkylated cysteine as fixed modification and oxidized methionine as variable modification, and two potential missed trypsin cleavages, as previously described by Bertoldi

[3] See: http://www.uniprot.org

et al. (2013). Proteins with a score hits >60 or identified with at least two or more significant peptide sequences were selected. The significant threshold in Mascot searches was set in order to obtain a False Discovery Rate <5% (5% probability of false match for each protein with a score above 60).

~~Protein identification peak lists were generated using the Mascot search engine against the UniProt database[4] as previously described in full by Bertoldi et al. (2013) using Mammalian as taxonomy parameter. Proteins identified with at least two or more significant peptide sequences and the highest score hits were selected. "High-scoring" corresponded to proteins above the significant threshold in Mascot searches (5% probability of false match for each protein above this score).~~

Commentato [FdMV5]: Following the suggestion of Rev 1 we have added more details. Lines 133-138 previous version.

Statistical analysis

Data were analyzed with statistical software (MedCalc Statistical Software version 12.7.5) and expressed as median and (range) or mean±standard deviation (SD). The different variables (UPC, age, number of bands) were compared using the Kruskal-Wallis one-way analysis of variance assuming $P<0.05$ as a significant probability.

Animal experimentation disclosure

The study was approved by our Institutional Scientific Ethical Committee for Animal Testing (approval number: 8-72-2012; date of approval 01/10/2012). Author AM Almeida holds a FELASA grade C certificate enabling the design and conduction of animal experimentation under EU law.

Results

Animal selection and UPC

Out of the 44 entire domestic shorthair cats selected for the healthy group, 21 were excluded due to inadequate USG (<1.035) or UPC>0.2 or any abnormality in their urinalysis results (glycosuria, haematuria, haemoglobinuria) or an active sediment (>5 white blood cells per high power field or bacteriuria). The remaining 23 cats (8 males, 15 females) were included in the study as the healthy group. The median age was 24 months (6-168) and median UPC was 0.11 (0.06-0.19).

~~Eighteen~~ Seventeen cats (5 neutered females, 8 neutered males and 4 entire males) ~~13 males, 5 females~~ were included in the CKD group. CKD cats were significantly older with a median age of 168 ~~163~~ months (~~24~~60-240; *P*<0.01) and had a significantly increased UPC value (median 0.9; 0.25-6.5~~13.3~~) than healthy cats (*P*<0.01). All urine samples ~~presented~~ had an inactive sediment and were negative ~~to~~ on urine culture. Plasma biochemistry and urinalysis data are reported in Table 1.

1D-SDS-PAGE

Representative gels and pherograms from healthy and CKD cats are reported in Fig. 1. We separated 32±6 protein bands in the urine of healthy cats. The majority had a molecular weight (MW) between 10 and 80 kDa. The CKD group presented a greater inter-individual variability and typical tubular pattern, characterized by low MW protein bands. A significant decrease of the total number of bands (~~24~~25±6) (*P*<0.01) was observed (Fig. 2a), particularly at MW higher than 100 kDa (*P*<0.01) (Figs. 1b and 2b).

Commentato [FdMV6]: We have modified the sentence by adding further information. Lines 159-161 previous version.

Commentato [FdMV7]: As suggested by the reviewer 2, we have excluded one cat from the CKD group, suspecting glomerular nephropathy and modified results accordingly.

No significant differences were found between pooled urine samples collected from healthy males and females. The most representative and reproducible protein bands from healthy and CKD samples (n = 16) were excised from the gel for MS identification (Fig. 3).

2-DE and differential proteomics study

Figure 4 reports representative 2-D gels obtained from healthy (Fig. 4a) and CKD entire cats (Fig. 4b). Plasma biochemistry and urinalysis data are reported in Table 2. Out of the 66 spots detected, 27 showed differential expression (P<0.05) between healthy and CKD samples; in particular, 18 spots were overrepresented in the CKD group and nine spots were increased in healthy animals. The remaining 39 spots were common and had similar expression levels. The nine most abundant common spots and the 27 differentially expressed spots were excised from the gels for MS identification.

Protein identification by mass spectrometry

~~Due to limited data on cat proteins in Mascot search engine, some of the proteins were identified in species other than cat.~~ From the 16 bands excised from 1-D gels, 14 proteins were identified (Table ~~1~~~~2~~3). Out of the 36 2-DE spots analysed, ~~21~~20 yielded significant results by MS, allowing the successful identification of 13 different proteins (Figs. 4a and b; Table ~~1~~~~2~~3). Albumin, cauxin, haemopexin and alpha-1 microglobulin precursor/bikunin (AMBP) were identified in spots characterized by different MW and/or isoelectric point. Seven proteins identified in 1-D gel were confirmed by 2-DE, namely uromodulin, albumin, transferrin, cauxin, haptoglobin, retinol binding protein (RBP) and immunoglobulin K light chain (IgK). Protein mass identification yielded a preliminary cat urine map, including 20 proteins that may be functionally classified as transport (25%),

Commentato [FdMV8]: Following the suggestion of reviewer 1 this sentence has been corrected and moved to M&M section. Lines 184-185 previous version.

Commentato [FdMV9]: One protein was excluded due to low score and significant sequence identification.

immune and cellular response (30%), protein metabolism (25%), and cellular communication and growth (15%) (Fig. 5a). Most of the identified proteins were classified as extracellular (75%) (Fig. 5b).

Cystatin M (CYSM), RBP, apolipoprotein-H (Apo-H), IgK and complement factor D (CFAD) were overrepresented in CKD samples, while alpha-2-macroglobulin (A2M), uromodulin, cauxin, inter-alpha-trypsin inhibitor heavy chain (ITIH4), pro-epidermal growth factor (EGF), angiotensin-converting enzyme (ACE2) and perlecan were underrepresented (Table ~~32~~4). Examples of differentially expressed spots are reported in Fig. 4c. The other proteins did not show significant differences between groups.

Discussion

The first aim of our research was the characterization of the urine proteome in healthy cat and the establishment of the proteome reference map. 1-D-SDS-PAGE yielded an overall view of the protein profile and resulted in a useful diagnostic tool that could help clinicians in qualitative evaluation of proteinuria. 2-DE was essential in fractionation of the complex urine proteome producing a reference map that included 20 proteins derived from either plasma ultrafiltration or kidney secretion, in accordance with data reported in humans (Adachi et al., 2006; Candiano et al., 2010; He et al., 2012) and dogs (Nabity et al., 2011; Brandt et al., 2014).

The most abundant protein was cauxin, a serine esterase produced by healthy tubular cells, specifically excreted in urine of cats and probably involved in the synthesis of felinine pheromone (Miyazaki et al., 2006). Most of the other proteins identified were involved in protein metabolism, immune response and transport. Regarding protein metabolism, we found several protease inhibitors (A2M, A1AT, ITIH4) that may play an important role in protecting the kidney from proteolytic damage. Among the proteins involved in immune and cellular defence response, we identified protein AMPB, IgK and uromodulin. Differently from dogs (Nabity et al., 2011; Brandt et al., 2014; Miller et al., 2014) and humans (Lhotta, 2010), uromodulin is not the most abundant urine-specific protein in cats. The transport proteins, albumin, transferrin, haemopexin and haptoglobin all derive from plasma and have been identified as common components of urine also from healthy humans (Candiano et al., 2010). The presence of high MW plasma proteins, e.g. transferrin and A2M, in cat urine could contradict the paradigm of glomerular selectivity that should be re-evaluated according to the findings of Candiano et al. (2010) and Brandt et al. (2014). However, a possible blood contamination of urine due to cystocentesis cannot be excluded. The remaining proteins, EGF, perlecan and fetuin-A, are involved in cell communication and growth. In particular, perlecan, a negatively charged proteoglycan of the glomerular filtration barrier, has also been identified in dog urine (Nabity et al., 2011).

Regarding the effect of CKD on the urine cat proteome, we identified 13 proteins differentially represented that could be studied as putative biomarkers of nephropathy (Table ~~32~~4). Our inclusion criteria led to the selection of proteinuric late stage CKD patients and based on UPC values a severe glomerular involvement could be hypothesised. However, ~~In particular,~~ most of these differentially expressed proteins ~~these proteins~~ are ~~can be~~ indicative of tubular dysfunction ~~when not reabsorbed~~ (e.g. RBP, ~~and~~ CYSM~~) or not secreted (e.g.~~ uromodulin and cauxin).

Among the overrepresented proteins, RBP is a low MW protein belonging to the family of lipocalins and is involved in plasma retinol transport. An increase in RBP is considered a biomarker of tubulointerstitial damage in humans and a significant correlation between urinary RBP and kidney interstitial fibrosis was recently demonstrated in CKD patients (Pallet et al., 2014). Elevated RBP in case of tubular damage has also been reported in dogs (Smets et al., 2010; Nabity et al., 2011). On the basis of our results, RBP can be considered an appealing marker to diagnose and monitor CKD in cats, as previously suggested by van Hoek et al. (2008). CYSM belongs to the cystatin family, a class of lysosomal cysteine protease inhibitors, and is considered a major regulator of epidermal cornification and desquamation (Brocklehurst and Philpott, 2013). To our knowledge, CYSM has never been found in urine, while an increase in the more widely studied cystatin C has been correlated with tubular dysfunction in humans, dogs (Monti et al., 2012) and cats (Ghys et al., 2014); further studies are needed to clarify the role of CYSM in urine. Apo-H (beta-2-glycoprotein 1) is a single chain multifunctional apolipoprotein also expressed in kidney tubular epithelium and involved in clotting mechanisms and lipid metabolism (Klaerke et al., 1997). The increase in urinary Apo-H in diabetic patients has been proposed as a marker of tubular dysfunction (Lapsley et al., 1993), and recent studies focused on the increased levels of IgA anti-Apo-H in CKD patients (Serrano et al., 2014); the role of this protein in cat urine is still unknown. The last two overrepresented proteins in CKD cats, namely CFAD and IgK, are involved in the immune response. CFAD is a serine protease synthesized mainly by adipocytes and macrophages belonging to the alternative complement pathway. The only report of this protein in urine regards a significant increase in human patients with preeclampsia (Wang et al., 2014).

Among the underrepresented proteins, significant decreases were shown by uromodulin, cauxin and perlecan. Uromodulin is a 95-kDa glycoprotein exclusively synthesized by the cells of the thick ascending limb. Its exact molecular function is still unknown, but it is thought to be a potent immuno-regulatory protein: recent studies hypothesized that uromodulin entering the renal interstitium through the damaged tubuli can stimulate the cells of the immune system causing inflammation and CKD progression. The decrease of uromodulin was previously observed also in dogs affected by leishmaniasis (Buono et al., 2012), suggesting its use as a biomarker of renal damage in small animals. 2-DE was essential to obtain the separation of albumin from cauxin, demonstrating a significant decrease of cauxin, though a possible influence of the entire/neutered status cannot be completely excluded. Nevertheless, ~~suggesting~~ this protein could be considered ~~is~~ a promising biomarker for the determination of tubular damage in CKD cats, particularly in entire male cats (Miyazaky et al., 2007, Jepson et al., 2010). The decrease of perlecan in human urine is associated with damage in the glomerular compartment (Ebefors et al., 2011) and could also suggest glomerular involvement in cats affected by renal disease. The remaining underrepresented proteins are involved in protein metabolism or cellular defence and communication. In particular, the decrease of the protease inhibitors A2M and ITIH4 could have a role in the pathophysiology of CKD. In support of this mechanism, intensive protein degradation has also been reported to occur in the urine of humans with CKD (Mullen et al., 2011). This finding is in accordance with the increased protein fragmentation, especially of albumin, found in our study.

Although the proteomic approach applied on cat urine proteome led to a preliminary map and to the identification of new putative biomarkers of nephropathy, this study presented some limitations. To obtain samples with an adequate amount of proteins, we selected proteinuric cats with advanced stages of CKD. Although we excluded patients with possible primary glomerular involvement, we cannot state that all cats included in this study had the same underlying renal pathophysiologic condition. Therefore, further studies are needed to confirm our results and to evaluate urine proteome also in non-proteinuric CKD cats. Moreover, the differences of age and neuter status between healthy and CKD cats could be considered minor limitations. In our study the age-related changes should be ~~overwhelmed~~ minimized by the selection of proteinuric cats with advanced stages of CKD and the neuter/entire influence should be reduced by the exclusion of borderline and proteinuric healthy male entire cats.

Conclusions

Our work produced a reference map of the normal urine proteome in cats and can be considered the starting point for future studies. Moreover, this is the first research linking of 13 differentially represented urine proteins with CKD in cats. The different amounts of uromodulin, cauxin, CFAD, Apo-H, RBP and CYSM confirm tubulointerstitial damage in CKD cats and suggest these proteins are candidate biomarkers to be investigated further. ~~These findings associated with the lack of differences in transferrin evidence a minor involvement of the glomerulus and a different pathogenesis of CKD in cats with respect to humans and dogs~~. The data reported in this paper on the most represented proteins in cat urine proteome and their changes in CKD could be useful ~~not only~~ for the advancement of research focused on the discovery of new biomarkers to be later applied ~~but also~~ in ~~routine~~ clinical practice. In particular, uromodulin, cauxin and perlecan, specifically secreted in urine, could help in the evaluation of renal function in cats.

Conflict of interest

None of the authors of this paper has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

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[5] See: www.cost-FAProteomics.org

References

Adachi, J., Kumar, C., Zhang, Y., Olsen, V.J., Mann, M., 2006. The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. Genome Biology 7, R80.

Almeida, A.M., Bassols, A., Bendixen, E., Bhide, M., Ceciliani, F., Cristobal, S., Hollung, K., Lisacek, F., Mazzucchelli, G., McLaughlin M. et al., 2014. Invited review: advances in proteomics for animal and food sciences. Animal, in press, doi:10.1017/S1751731114002602.

Bartges, J.W., 2012. Chronic kidney disease in dogs and cats. Veterinary Clinics of North America: Small Animal Practice 42, 669-692.

Bellei, E., Monari, E., Cuoghi, A., Bergamini, S., Guerzoni, S., Ciccarese, M., Ozben, T., Tomasi, A., Pini, L.A., 2013. Discovery by a proteomic approach of possible early biomarkers of drug-induced nephrotoxicity in medication-overuse headache. The Journal of Headache and Pain 14, 6.

Bertoldi, C., Bellei, E., Pellacani, C., Ferrari, D., Lucchi, A., Cuoghi, A., Bergamini, S., Cortellini, P., Tomasi, A., Zaffe, D.~~, Monari, E.,~~ et al., 2013. Non-bacterial protein expression in periodontal pockets by proteome analysis. Journal of Clinical Periodontology. 40, 573-582.

Brandt, L.E., Ehrhart, E.J., Scherman, H., Olver, C.S., Bohn, A.A., Prenni, J.E., 2014. Characterization of the canine urinary proteome. Veterinary Clinical Pathology 43, 193-205.

Brocklehurst, K., Philpott, M.P., 2013. Cysteine proteases: mode of action and role in epidermal differentiation. Cell and Tissue Research 351, 237-244.

Buono, A., Duque, J., Ferlizza, E., Zaragoza, C., Barrera, R., Isani, G., Dondi, F., 2012. Non-invasive diagnostic evaluation including qualitative proteinuria to detect an early renal damage in canine leishmaniasis. In: Proceedings of the European College of Veterinary Internal Medicine - Companion Animal Congress, Maastricht, NL. Journal of Veterinary Internal Medicine 26, 1537-1538.

Campos, A., Puerto, M., Prieto, A., Cameán, A., Almeida, A.M., Coelho, A.V., Vasconcelos, V., 2013. Protein extraction and two-dimensional gel electrophoresis of proteins in the marine mussel *Mytilus galloprovincialis*: an important tool for protein expression studies, food quality and safety assessment. Journal of the Science of Food and Agricolture 93, 1779-1787.

Candiano, G., Santucci, L., Petretto, A., Bruschi, M., Dimuccio, V., Urbani, A., Bagnasco, S., Ghiggeri, G.M., 2010. 2D-electrophoresis and the urine proteome map: where do we stand? Journal of Proteomics 73, 829-844.

Cruz De Carvalho, R., Bernardes Da Silva, A., Soares, R., Almeida, A.M., Coelho, A.V., Marques Da Silva, J., Branquinho, C., 2014. Differential proteomics of dehydration and rehydration in bryophytes: evidence towards a common desiccation tolerance mechanism. Plant, Cell and Environment 37, 1499-1515.

Ebefors, K., Granqvist, A., Ingelsten, M., Mölne, J., Haraldsson, B., Nyström, J., 2011. Role of glomerular proteoglycans in IgA nephropathy. PLoS ONE 6, e18575.

Ghys, L.F.E., Meyer, E., Paepe, D., Delanghe, J., Daminet, S., 2014. Analytical validation of a human particle-enhanced nephelometric assay for cystatin C measurement in feline serum and urine. Veterinary Clinical Pathology 43, 226-234.

He, W., Huang, C., Luo, G., Dal Prà, I., Feng, J., Chen, W., Ma, L., Wang, Y., Chen, X., Tan, J.~~,~~ ~~Zhang, X., Armato, U., Wu, J.,~~ et al., 2012. A stable panel comprising 18 urinary proteins in the human healthy population. Proteomics 12, 1059-1072.

~~Hernández-Castellano, L.E., Almeida, A.M., Ventosa, M., Coelho, A.V., Castro, N., Argüello, A., 2014. The effect of colostrum intake on blood plasma proteome profile in newborn lambs: low abundance proteins. BMC Veterinary Research 10, 85.~~

Jepson, R.E., Syme, H.M., Markwell, P., Miyazaki, M., Yamashita, T., Elliott, J., 2010. Measurement of urinary cauxin in geriatric cats with variable plasma creatinine concentrations and proteinuria and evaluation of urine cauxin-to-creatinine concentration ratio as a predictor of developing azotemia. American Journal of Veterinary Research 71, 982-987.

Jepson, R., Coulton, G., Cowan, M., Markwell, P., Syme, H.M., Elliott, J., 2013. Evaluation of mass spectrometry of urinary proteins and peptides as biomarkers for cats at risk of developing azotemia. American Journal of Veterinary Research 74, 333-342.

Klaerke, D.A., Røjkjaer, R., Christensen, L., Schousboe, I., 1997. Identification of β2-glycoprotein I as a membrane-associated protein in kidney: purification by calmodulin affinity chromatography. Biochimica et Biophysica Acta 1339, 203-216.

Lapsley, M., Flynn, F. V., Sansom, P.A., 1993. Beta 2-Glycoprotein-1 (apolipoprotein H) excretion and renal tubular malfunction in diabetic patients without clinical proteinuria. Journal of Clinical Pathology 46, 465-469.

Lemberger, S.I.K., Deeg, C.A., Hauck, S.M., Amann, B., Hirmer, S., Hartmann, K., Dorsch, R., 2011. Comparison of urine protein profiles in cats without urinary tract disease and cats with idiopathic cystitis, bacterial urinary tract infection, or urolithiasis. American Journal of Veterinary Research 72, 1407-1415.

Lhotta, K., 2010. Uromodulin and chronic kidney disease. Kidney and Blood Pressure Research 33, 393-398.

Miller, I., Preßlmayer-Hartler, A., Wait, R., Hummel, K., Sensi, C., Eberini, I., Razzazi-Fazeli, E., Gianazza, E., 2014. In between - Proteomics of dog biological fluids. Journal of Proteomics 106, 30-45.

Mischke, R., 2011. Assessing proteinuria in the cat: an innovative tip. The Veterinary Journal 189, 243-244.

Miyazaki, M., Yamashita, T., Hosokawa, M., Taira, H., Suzuki, A., 2006. Species-, sex-, and age-dependent urinary excretion of cauxin, a mammalian carboxylesterase. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 145, 270-277.

Miyazaki, M., Soeta, S., Yamagishi, N., Taira, H., Suzuki, A., Yamashita, T., 2007. Tubulointerstitial nephritis causes decreased renal expression and urinary excretion of cauxin, a major urinary protein of the domestic cat. Research in Veterinary Science 82, 76-79.

Miyazaki, M., Fujiwara, K., Suzuta, Y., Wakamatsu, N., Taira, H., Suzuki, A., Yamashita, T., 2011. Screening for proteinuria in cats using a conventional dipstick test after removal of cauxin from urine with a *Lens culinaris* agglutinin lectin tip. The Veterinary Journal 189, 312-317.

Monti, P., Benchekroun, G., Berlato, D., Archer, J., 2012. Initial evaluation of canine urinary cystatin C as a marker of renal tubular function. Journal of Small Animal Practice 53, 254-259.

Mullen, W., Delles, C., Mischak, H., 2011. Urinary proteomics in the assessment of chronic kidney disease. Current Opinion in Nephrology and Hypertension 20, 654-661.

Nabity, M.B., Lees, G.E., Dangott, L.J., Cianciolo, R., Suchodolski, J.S., Steiner, J.M., 2011. Proteomic analysis of urine from male dogs during early stages of tubulointerstitial injury in a canine model of progressive glomerular disease. Veterinary Clinical Pathology 40, 222-236.

Pallet, N., Chauvet, S., Chassé, J.-F., Vincent, M., Avillach, P., Levi, C., Meas-Yedid, V., Olivo-Marin, J.C., Nga-Matsogo, D., Beaune, P.~~, Thervet, E., Karras, A~~ et al., 2014. Urinary retinol binding protein is a marker of the extent of interstitial kidney fibrosis. PLoS ONE 9, e84708.

Polzin, D.J., 2011. Chronic kidney disease in small animals. Veterinary Clinics of North America: Small Animal Practice 41, 15-30.

Schaefer, H., Kohn, B., Schweigert, F.J., Raila, J., 2011. Quantitative and qualitative urine protein excretion in dogs with severe inflammatory response syndrome. Journal of Veterinary Internal Medicine 25, 1292-1297.

Serrano, M., Martínez-Flores, J.A., Castro, M.J., García, F., Lora, D., Pérez, D., Gonzalez, E., Paz-Artal, E., Morales, J.M., Serrano, A., 2014. Renal transplantation dramatically reduces IgA anti-beta-2-glycoprotein I antibodies in patients with endstage renal disease. Journal of Immunology Research 2014, 641962.

Smets, P.M.Y., Meyer, E., Maddens, B.E.J., Duchateau, L., Daminet, S., 2010. Urinary markers in healthy young and aged dogs and dogs with chronic kidney disease. Journal of Veterinary Internal Medicine 24, 65-72.

Tesch, G.H., 2010. Review: serum and urine biomarkers of kidney disease: a pathophysiological perspective. Nephrology 15, 609-616.

Van Hoek, I., Daminet, S., Notebaert, S., Janssens, I., Meyer, E., 2008. Immunoassay of urinary retinol binding protein as a putative renal marker in cats. Journal of Immunological Methods 329, 208-213.

Wang, T., Zhou, R., Gao, L., Wang, Y., Song, C., Gong, Y., Jia, J., Xiong, W., Dai, L., Zhang, L.~~, Hu, H.,~~ et al., 2014. Elevation of urinary adipsin in preeclampsia: correlation with urine protein concentration and the potential use for a rapid diagnostic test. Hypertension, in press, doi:10.1161/HYPERTENSIONAHA.113.02688.

Table 1

Commentato [FdMV10]: As suggested by the rev 2, we have added this table reporting biochemistry and urinalysis of CKD cats.

Clinical data for cats affected by CKD (n=17).

Signalment	Mean±SD	n	
Age in months	160±64		
Female (entire/neutered)		5 (0/5)	
Male (entire/neutered)		12 (4/8)	
Plasma biochemistry	Mean±SD	n (%) < or >RI	RI [a]
Total Proteins (g/dL)	7.9±0.8	6(35)>	6.0-8.0
Albumin (g/dL)	3±0.4	4(24)>	2.1-3.3
Creatinine (mg/dL)	5.9±3.6	17(100)>	0.8-1.6
Urea (mg/dL)	264±148	16(94)>	15-60
Phosphorus (mg/dL)	9.5±5.7	9(54)>	2.9-8.3
Urine biochemistry	Mean±SD	n (%) < or >RI	RI
UPC [b]	1.29±1.52	14(82)>	<0.4
USG [c]	1.018±0.012	15(88)<	>1.035 [d]
IRIS Stage		n (%)	
II		4(24)	
III		4(24)	
IV		9(53)	
Clinical signs		n (%)	
Disorexia/anorexia		15(88)	
Polyuria/polydipsia		11(65)	
Depression		7(41)	
Weight loss		4(24)	
Abnormal renal palpation		3(18)	
Oral lesions		3(18)	
Vomiting		2(12)	
Weakness		2(12)	
Dehydration		2(12)	
Diarrhoea		1(6)	
Blindness		1(6)	

[a] RI = Reference Interval
[b] UPC = urine protein to creatinine ratio
[c] USG = urine specific gravity
[d] Considered as adequate USG in cats

Table 2

Commentato [F11]: As suggested by the reviewer, we have added this table reporting biochemistry and urinalysis of healthy and CKD cats used for 2DE.

Clinical data for healthy and CKD cats selected for 2DE.

	Gender	Age (months)	TP [a] (mg/dL)	ALB [b] (mg/dL)	Creatinine (mg/dL)	Urea (mg/dL)	P [c] (mg/dL)	UPC [d]	USG [e]	IRIS stage
RI [f]			6.0-8.0	2.1-3.3	0.8-1.6	15-60	2.9-8.3	<0.4	>1.035 [g]	
CKD										
1	M [h]	96	6.35	2.35	1.76	97	4.9	0.50	1.020	II
2	C [i]	216	8	3	4.3	195	5.5	1.50	1.018	III
3	C	160	8.8	2.65	5.23	401	18.3	6.30	1.022	IV
4	M	170	9	2.8	8.9	474	17	3.50	1.014	IV
Healthy										
1	M	6	6.76	2.4	0.95	56	4.3	0.19	1048	
2	M	24	7.12	3	1.35	43	3.2	0.13	1056	
3	M	12	6.5	2.8	1.5	25	6.8	0.08	1072	
4	M	6	7.6	2.9	1.24	50	5.4	0.14	1044	

[a] TP = serum total protein
[b] ALB = serum albumin
[c] P = serum phosphorus
[d] UPC = urine protein to creatinine ratio
[e] USG = urine specific gravity
[f] RI = Reference Interval
[g] Considered as adequate USG in cats
[h] M = entire male
[i] C = neutered male

Table ~~12~~3

Proteins identified in cat urine by mass spectrometry.

Band [a] 1-DE	Entry name [b]	Protein full name	MW [c] (kDa)	pI	Score [d]	Pept. [e]	Seq. [f]	Sign. Seq. [g]	Identity [h]
1	TRFE_BOVIN	Serotransferrin	79.9	6.75	88	15	7	3	73
2	EST5A_FELCA	Carboxylesterase 5A	60.9	5.58	238	27	10	6	100
	ALBU_FELCA	Serum albumin	70.6	5.46	135	21	8	6	100
3	ALBU_FELCA	Serum albumin	70.6	5.46	346	37	16	10	100
	EST5A_FELCA	Carboxylesterase 5A	60.9	5.58	41	8	4	2	100
4	KV1_CANFA	Ig kappa chain V region GOM	12.1	6.41	91	3	2	1	71
	IGLL5_HUMAN	Immunoglobulin lambda-like polypeptide 5	23.4	9.08	66	11	1	1	79
	ALBU_FELCA	Serum albumin	70.6	5.46	59	9	6	2	100
5	ALBU_FELCA	Serum albumin	70.6	5.46	1340	115	34	25	100
6	RET4_HORSE	Retinol-binding protein 4	23.3	5.28	1121	42	6	4	93
7	CYTM_HUMAN	Cystatin-M	16.5	7.0	71	3	2	1	79
8	A2MG_BOVIN	Alpha-2-macroglobulin	168.9	5.71	121	9	4	1	75
	ALBU_FELCA	Serum albumin	70.6	5.46	115	18	9	4	100
9	ITIH4_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H4	103.5	6.51	70	9	2	2	73
10	ACE2_FELCA	Angiotensin-converting enzyme 2	93.1	5.64	178	15	6	5	100
	UROM_CANFA	Uromodulin	72.9	4.94	112	20	4	4	86
	EGF_FELCA	Pro-epidermal growth factor	137.3	5.8	83	13	7	4	100
11	ALBU_FELCA	Serum albumin	70.6	5.46	147	24	11	7	100
	EST5A_FELCA	Carboxylesterase 5A	60.9	5.58	145	20	8	2	100
12	HPT_CANFA	Haptoglobin	36.9	5.72	80	27	8	6	90
	EST5A_FELCA	Carboxylesterase 5A	60.9	5.58	102	16	7	3	100
13	IGLL5_HUMAN	Immunoglobulin lambda-like polypeptide 5	23.4	9.08	115	16	1	1	100
14	EST5A_FELCA	Carboxylesterase 5A	60.9	5.58	254	30	12	6	100
	TRFE_PIG	Serotransferrin	78.9	6.93	71	19	7	4	74
15	ALBU_FELCA	Serum albumin	70.6	5.46	532	53	22	17	100
	EST5A_FELCA	Carboxylesterase 5A	60.9	5.58	439	68	16	9	100
16	ALBU_FELCA	Serum albumin	70.6	5.46	5932	346	51	42	100
	EST5A_FELCA	Carboxylesterase 5A	60.9	5.58	1941	157	24	23	100
	A1AT_CHLAE	Alpha-1-antitrypsin	44.6	5.75	109	11	3	2	71
Spot [a] 2-DE									

1	UROM_CANFA	Uromodulin	72.9	4.94	130	36	6	3	86
2	ALBU_FELCA	Serum albumin	70.6	5.46	2383	196	39	28	100
3	ALBU_FELCA	Serum albumin	70.6	5.46	2133	208	35	29	100
4	EST5A_FELCA	Carboxylesterase 5A	60.9	5.58	524	66	14	10	100
5	EST5A_FELCA	Carboxylesterase 5A	60.9	5.58	447	89	14	10	100
6	TRFE_PIG	Serotransferrin	78.9	6.93	114	31	9	5	74
7	FETUA_HUMAN	Fetuin-A	40.1	5.43	141	34	6	4	70
8	APOH_CANFA	Apolipoprotein H	39.7	8.51	162	21	4	4	88
9	HPT_BOVIN	Haptoglobin	45.6	7.83	72	6	2	2	78
10	AMBP_BOVIN	Protein AMBP	40.1	7.81	141	5	1	1	78
11	AMBP_BOVIN	Protein AMBP	40.1	7.81	150	6	1	1	78
12	AMBP_BOVIN	Protein AMBP	40.1	7.81	274	11	1	1	78
13	PGBM_HUMAN	Perlecan	479.3	6.06	134	19	3	2	91
14	HEMO_PONAB	Hemopexin	52.3	6.44	73	25	3	1	83
15	HEMO_PONAB	Hemopexin	52.3	6.44	97	25	3	1	83
16	ALBU_FELCA	Serum albumin	70.6	5.46	1585	187	40	25	100
17	APOH_CANFA	Apolipoprotein H	39.7	8.51	119	16	5	4	88
18	ALBU_FELCA	Serum albumin	70.6	5.46	69	10	7	3	100
19	KV1_CANFA	Ig kappa chain V region GOM	12.1	6.41	111	4	2	2	71
	CFAD_PIG	Complement factor D	28.3	6.59	54	9	2	2	86
~~20~~	~~RET4_HORSE~~	~~Retinol-binding protein 4~~	~~23.3~~	~~5.28~~	~~60~~	~~4~~	~~2~~	~~1~~	~~93~~
20~~1~~	RET4_HUMAN	Retinol-binding protein 4	23.3	5.76	167	27	8	3	94

[a] Number of the identified band or spot as marked in Fig 3 and 4 respectively.
[b] Protein entry name from UniProt knowledge database.
[c] Theoretical protein molecular weight.
[d] The highest scores obtained with Mascot search engine.
[e] Peptides: total number of peptides matching the identified proteins.
[f] Sequence: total number of sequences matching the identified proteins.
[g] Significant Sequences: total number of significant sequences matching the identified proteins.
[h] Percentage of identical amino acids between the identified protein and the respective cat protein.

Table ~~23~~4

Differentially expressed proteins identified by mass spectrometry (ESI-Q-TOF).

Band [a] 1-DE	Entry name [b]	Protein full name	CKD vs healthy [c]	Molecular function [d]	Biological process [e]
6	RET4_HUMAN	Retinol-binding protein 4	Up	Transporter	Transport
7	CYTM_HUMAN	Cystatin-M	Up	Protease inhibitor	Protein metabolism
8	A2MG_BOVIN	Alpha-2-macroglobulin	Down	Protease inhibitor	Protein metabolism
9	ITIH4_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H4	Down	Protease inhibitor	Protein metabolism
10	ACE2_FELCA	Angiotensin-converting enzyme 2	Down	Protease-carboxylpeptidase activity	Protein metabolism
	UROM_CANFA	Uromodulin	Down	Unknown	Cellular defense response
	EGF_FELCA	Pro-epidermal growth factor	Down	Growth factor activity	Cell comunication; Signal transduction
Spot [a] 2-DE					
1	UROM_CANFA	Uromodulin	Down	Unknown	Cellular defense response
2	ALBU_FELCA	Albumin	Down	Transporter	Transport
4; 5	EST5A_FELCA	Carboxylesterase 5A	Down	Protease-hydrolase activity	Unknown
8; 17	APOH_CANFA	Apolipoprotein H	Up	Transporter	Transport
13	PGBM_HUMAN	Perlecan	Down	Extracellular matrix structural constituent	cell Growth/maintenance
16; 18	ALBU_FELCA	Albumin	Up	Transporter	Transport
19	KV1_CANFA	Ig kappa chain V region GOM	Up	Antigen binding	Immune response
	CFAD_PIG	Complement factor D	Up	Serine-type peptidase	Immune response
20~~; 21~~	RET4_HUMAN	Retinol-binding protein 4	Up	Transporter	Transport

[a] Number of the identified band or spot as marked in Fig 3 and 4 respectively.
[b] Protein entry name from UniProt knowledge database.
[c] Significantly (P<0.05) overrepresented (up) and underrepresented (down) proteins in CKD group respect to healthy.
[d] Molecular function according to Gene Ontology and Human Reference Proteome Database.
[e] Biological process according to Gene Ontology and Human Reference Proteome Database.

Figure legends

Fig. 1. 1D-SDS-PAGE of cat urine proteins. Two μg of proteins were loaded and stained with silver nitrate. Representative gel (lane 1, molecular weight marker; lanes 2-7, urine samples from CKD cats; lanes 8-9, healthy urine samples) (A) and pherograms (B) are reported.

Fig. 2. Comparison of the number of protein bands between healthy and CKD cats. (A) Total number of bands. (B) Number of bands with MW>100 kDa. Different lower cases indicate significant differences (P<0.01).

Fig. 3. 1D-SDS-PAGE of urine samples from healthy and CKD cats, stained with Coomassie Blue. Lane 1, molecular weight marker; lanes 2-3, CKD urine samples; lanes 4-5, pools of urine from healthy females and males respectively. Rectangles and numbers indicate the bands that have been cut and identified by ESI-Q-TOF (Table ~~12~~3).

Fig. 4. 2-DE of the urine proteome in healthy (A) and CKD (B) entire cats. White circles: spots with significantly greater intensity in healthy than in CKD; black circles: spots with significantly greater intensity in CKD; white rectangles: common spots without significant differences. (C) Examples of important differentially expressed proteins.

Fig. 5. Classification of the proteins identified according to Gene Ontology and the Human Reference Proteome Database (HRPD).