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Archivio istituzionale della ricerca

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

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Published Version:

Availability:

This version is available at: <https://hdl.handle.net/11585/541121> since: 2020-02-27

Published:

DOI: <http://doi.org/10.1016/j.tvjl.2015.01.023>

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This is the final peer-reviewed accepted manuscript of:
Ferlizza E, Campos A, Neagu A, Cuoghi A, Bellei E, Monari E, Dondi F, Almeida AM, Isani G. Urine proteome in the domestic cat (*Felis catus*): the effect of chronic kidney disease. *The Veterinary Journal*. 2015, 204 (1), 73-81.

The final published version is available online at: [10.1016/j.tvjl.2015.01.023](https://doi.org/10.1016/j.tvjl.2015.01.023)

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16 **Original Article**

19
20 **Urine proteome in the domestic cat (*Felis catus*): the effect of chronic kidney disease**

21
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38 **Abstract**

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

47
48 1D-SDS-PAGE yielded an overall view of the protein profile and the separation of 32 ± 6 protein
49 bands in the urine of healthy cats, while CKD cats showed significantly fewer bands ($P<0.01$). 2-DE
50 was essential in fractionation of the complex urine proteome, producing a reference map that included
51 20 proteins. Cauxin was the most abundant protein in urine of healthy cats; we also identified several
52 protease inhibitors and transport proteins, e.g., alpha-2-macroglobulin, albumin, transferrin,
53 haemopexin and haptoglobin that all derive from plasma. We disclosed 27 spots differentially
54 expressed ($P<0.05$) in CKD cats, and 13 proteins were unambiguously identified. In particular, the
55 increase in retinol-binding protein, cystatin M and apolipoprotein-H associated with the decrease of
56 uromodulin and cauxin confirmed tubular damage in CKD cats and suggest these proteins are candidate
57 biomarkers.

58

59

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

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60

61 *Keywords:* Biomarkers; Cat; Nephropathy; Proteinuria; Urine proteome ~~Urine proteome~~ [Electrophoresis](#)

62

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63 **Introduction**

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

75
76 Sensitive and specific biomarkers for early prediction and monitoring of CKD in cats are
77 currently lacking. Quantitative methods for the detection of proteinuria, (urinary protein and urinary
78 albumin to creatinine ratios; UPC and UAC) are used to evaluate the severity of renal involvement but
79 offer no information on its aetiology or composition of the urine proteome (Tesch, 2010). In addition,
80 cauxin, a 70 kDa protein secreted physiologically by the tubule in cats, can interfere with the
81 assessment of proteinuria (Mischke, 2011; Miyazaki et al., 2011).

82
83 Urine is considered an ideal source of clinical biomarkers as it can be obtained repeatedly in
84 sufficient amounts and noninvasively. High-resolution electrophoresis coupled with mass spectrometry

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

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(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 \(Almeida et al., 2014\).](#)

94

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.

99

100 **Material and methods**

101 *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~~

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107 ~~International Renal Interest Society group (IRIS¹) CKD guidelines (Bartges, 2012)~~ In particular, Cats
108 had to have clinical findings of CKD and (a) persistent pathologic renal proteinuria based on the urine
109 protein to creatinine ratio (UPC), assessed and confirmed over a two-month period (UPC>0.2), and ~~or~~
110 (b) serum creatinine concentration ≥ 1.60 mg/dL and urine specific gravity (USG) <1.035. CKD cats
111 were staged according to the International Renal Interest Society (IRIS¹) CKD guidelines. Upon
112 arrival, all the animals were subjected to physical examination and routine laboratory tests, including
113 complete blood count, serum chemistry and complete urinalysis with UPC and urine culture. Five
114 millilitres of urine were collected from each animal by ultrasound-guided cystocentesis. After
115 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.

116
117 *Urine protein to creatinine ratio*

118 Urine total proteins and creatinine were determined using commercial kits (Urinary/CSF
119 Protein, OSR6170, and Creatinine OSR6178, Olympus/Beckman Coulter) on an automated chemistry
120 analyzer (AU 400, Olympus/Beckman Coulter). The UPC was calculated with the following formula:
121 $UPC = \text{urine protein (mg/dL)} / \text{urine creatinine (mg/dL)}$.

122
123 *One-dimensional gel electrophoresis (1D-SDS-PAGE)*

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

¹ See: <http://www.iris-kidney.com/guidelines/>

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128 digitalized and its pherogram was obtained using GelAnalyzer 2010 software². To evaluate differences
129 between genders, two pools were prepared by collecting and mixing 20 µg of proteins from each
130 healthy male ($n = 8$) and female ($n = 15$) sample. The pools were concentrated by Vivaspin500 spin
131 columns (Sartorius Stedim Biotech GmbH) with a molecular weight (MW) cut-off of 3 kDa and
132 separated by 1D-SDS-PAGE with the protocol reported above, with the exception of 3-(*N*-
133 morpholino)propanesulphonic acid buffer and Coomassie blue staining (PageBlu protein staining
134 solution; Thermo Fisher Scientific) compatible with mass spectrometry analysis.

135

136 *Two-dimensional gel electrophoresis (2-DE)*

137 Urine samples from four healthy and four CKD cats were selected for 2-DE. To concentrate and
138 desalt samples, 150 µg of protein for each sample were precipitated with trichloroacetic acid to a final
139 concentration of 10% in gentle shaking for one hour and then centrifuged at 15,000 *g* for 30 min at 4
140 °C. The protein pellets were washed three times with cold absolute acetone, air-dried and dissolved in a
141 rehydration buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-
142 1-propanesulfonate (CHAPS), 65 mM dithiothreitol (DTT) and 0.8% resolytes (pH 3-10) before
143 loading onto immobilized pH gradient (IPG) strips (non-linear pH gradient 3–10, 17 cm long)
144 (BioRad). IPG strips were rehydrated and equilibrated following Campos et al. (2013). The equilibrated
145 IPG strips were placed on top of 10% acrylamide gel, and protein separation was run at 24 mA per gel
146 for 6 h in Protean II XL (BioRad) in running buffer containing 25mM Tris, glycine 192 mM and SDS
147 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.gelalyzer.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).

150

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 $\geq 0.1\%$ 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 database³ 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>

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

171 ~~Protein identification peak lists were generated using the Mascot search engine against the~~
172 ~~UniProt database⁴ as previously described in full by Bertoldi et al. (2013) using Mammalian as~~
173 ~~taxonomy parameter. Proteins identified with at least two or more significant peptide sequences and the~~
174 ~~highest score hits were selected. “High scoring” corresponded to proteins above the significant~~
175 ~~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.

176
177 *Statistical analysis*

178 Data were analyzed with statistical software (MedCalc Statistical Software version 12.7.5) and
179 expressed as median and (range) or mean±standard deviation (SD). The different variables (UPC, age,
180 number of bands) were compared using the Kruskal-Wallis one-way analysis of variance assuming
181 $P<0.05$ as a significant probability.

182
183 *Animal experimentation disclosure*

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

187
188 **Results**

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189 *Animal selection and UPC*

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

195
196 ~~Eighteen~~ Seventeen cats (5 neutered females, 8 neutered males and 4 entire males) ~~13 males, 5~~
197 ~~females~~ were included in the CKD group. CKD cats were significantly older with a median age of 168
198 ~~163~~ months (2460-240; $P<0.01$) and had a significantly increased UPC value (median 0.9; 0.25-
199 6.513.3) than healthy cats ($P<0.01$). All urine samples presented had an inactive sediment and were
200 negative ~~to~~ on urine culture. Plasma biochemistry and urinalysis data are reported in Table 1.

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.

202 *1D-SDS-PAGE*

203 Representative gels and pherograms from healthy and CKD cats are reported in Fig. 1. We
204 separated 32±6 protein bands in the urine of healthy cats. The majority had a molecular weight (MW)
205 between 10 and 80 kDa. The CKD group presented a greater inter-individual variability and typical
206 tubular pattern, characterized by low MW protein bands. A significant decrease of the total number of
207 bands (2425±6) ($P<0.01$) was observed (Fig. 2a), particularly at MW higher than 100 kDa ($P<0.01$)
208 (Figs. 1b and 2b).

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

213

214 *2-DE and differential proteomics study*

215 Figure 4 reports representative 2-D gels obtained from healthy (Fig. 4a) and CKD ~~entire~~ cats
216 (Fig. 4b). **Plasma biochemistry and urinalysis data are reported in Table 2.** Out of the 66 spots detected,
217 27 showed differential expression ($P < 0.05$) between healthy and CKD samples; in particular, 18 spots
218 were overrepresented in the CKD group and nine spots were increased in healthy animals. The
219 remaining 39 spots were common and had similar expression levels. The nine most abundant common
220 spots and the 27 differentially expressed spots were excised from the gels for MS identification.

221

222 *Protein identification by mass spectrometry*

223 ~~Due to limited data on cat proteins in Mascot search engine, some of the proteins were~~
224 ~~identified in species other than cat.~~ From the 16 bands excised from 1-D gels, 14 proteins were
225 identified (Table ~~423~~). Out of the 36 2-DE spots analysed, ~~2420~~ yielded significant results by MS,
226 allowing the successful identification of 13 different proteins (Figs. 4a and b; Table ~~423~~). Albumin,
227 cauxin, haemopexin and alpha-1 microglobulin precursor/bikunin (AMBP) were identified in spots
228 characterized by different MW and/or isoelectric point. Seven proteins identified in 1-D gel were
229 confirmed by 2-DE, namely uromodulin, albumin, transferrin, cauxin, haptoglobin, retinol binding
230 protein (RBP) and immunoglobulin K light chain (IgK). Protein mass identification yielded a
231 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.

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

234
235 Cystatin M (CYSM), RBP, apolipoprotein-H (Apo-H), IgK and complement factor D (CFAD)
236 were overrepresented in CKD samples, while alpha-2-macroglobulin (A2M), uromodulin, cauxin,
237 inter-alpha-trypsin inhibitor heavy chain (ITIH4), pro-epidermal growth factor (EGF), angiotensin-
238 converting enzyme (ACE2) and perlecan were underrepresented (Table 324). Examples of
239 differentially expressed spots are reported in Fig. 4c. The other proteins did not show significant
240 differences between groups.

241
242 **Discussion**

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

250
251 The most abundant protein was cauxin, a serine esterase produced by healthy tubular cells,
252 specifically excreted in urine of cats and probably involved in the synthesis of feline pheromone
253 (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 324). 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).

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297 Among the underrepresented proteins, significant decreases were shown by uromodulin, cauxin
298 and perlecan. Uromodulin is a 95-kDa glycoprotein exclusively synthesized by the cells of the thick
299 ascending limb. Its exact molecular function is still unknown, but it is thought to be a potent immuno-
300 regulatory protein: recent studies hypothesized that uromodulin entering the renal interstitium through
301 the damaged tubuli can stimulate the cells of the immune system causing inflammation and CKD
302 progression. The decrease of uromodulin was previously observed also in dogs affected by
303 leishmaniasis (Buono et al., 2012), suggesting its use as a biomarker of renal damage in small animals.
304 2-DE was essential to obtain the separation of albumin from cauxin, demonstrating a significant
305 decrease of cauxin, though a possible influence of the entire/neutered status cannot be completely
306 excluded. Nevertheless, suggesting this protein could be considered is a promising biomarker for the
307 determination of tubular damage in CKD cats, particularly in entire male cats (Miyazaky et al., 2007,
308 Jepson et al., 2010). The decrease of perlecan in human urine is associated with damage in the
309 glomerular compartment (Ebefors et al., 2011) and could also suggest glomerular involvement in cats
310 affected by renal disease. The remaining underrepresented proteins are involved in protein metabolism
311 or cellular defence and communication. In particular, the decrease of the protease inhibitors A2M and
312 ITIH4 could have a role in the pathophysiology of CKD. In support of this mechanism, intensive
313 protein degradation has also been reported to occur in the urine of humans with CKD (Mullen et al.,
314 2011). This finding is in accordance with the increased protein fragmentation, especially of albumin,
315 found in our study.

316
317 Although the proteomic approach applied on cat urine proteome led to a preliminary map and to
318 the identification of new putative biomarkers of nephropathy, this study presented some limitations. To

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319 obtain samples with an adequate amount of proteins, we selected proteinuric cats with advanced stages
320 of CKD. Although we excluded patients with possible primary glomerular involvement, we cannot
321 state that all cats included in this study had the same underlying renal pathophysiologic condition.
322 Therefore, further studies are needed to confirm our results and to evaluate urine proteome also in non-
323 proteinuric CKD cats. Moreover, the differences of age and neuter status between healthy and CKD
324 cats could be considered minor limitations. In our study the age-related changes should be
325 ~~overwhelmed~~ minimized by the selection of proteinuric cats with advanced stages of CKD and the
326 neuter/entire influence should be reduced by the exclusion of borderline and proteinuric healthy male
327 entire cats.

328
329 **Conclusions**

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

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341

342 **Conflict of interest**

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

345

346 **Acknowledgements**

347 This study was supported by a grant from the University of Bologna (RFO) to G Isani. [Author](#)
348 [AM Almeida](#) is supported by grant SFRH/BPD/90916/2012 from Fundação para a Ciência e a
349 [Tecnologia](#) (Lisbon, Portugal) [A Campos](#) by the contract work [Ciência 2007](#) from FCT. Authors [AM](#)
350 [Almeida](#), [A Campos](#), [E Ferlizza](#) and [G Isani](#) are members of COST Action FA1002 – Proteomics in
351 [Farm Animals](#)⁵ funded by the European Science Foundation and to whom networking support is
352 acknowledged.

353

⁵ See: www.cost-FAProteomics.org

354 References

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356 more than 1500 proteins, including a large proportion of membrane proteins. *Genome Biology* 7,
357 R80.
- 358
359 Almeida, A.M., Bassols, A., Bendixen, E., Bhide, M., Cecilian, F., Cristobal, S., Hollung, K., Lisacek,
360 F., Mazzucchelli, G., McLaughlin M. et al., 2014. Invited review: advances in proteomics for
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503 **Table 1**
 504 Clinical data for cats affected by CKD (n=17).
 505

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

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)	

506 ^a RI = Reference Interval
 507 ^b UPC = urine protein to creatinine ratio
 508 ^c USG = urine specific gravity
 509 ^d Considered as adequate USG in cats
 510
 511

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Table 2

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

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

525 **Table 423**

526 Proteins identified in cat urine by mass spectrometry.

Band 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
	KV1_CANFA	Ig kappa chain V region GOM	12.1	6.41	91	3	2	1	71
4	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
	ACE2_FELCA	Angiotensin-converting enzyme 2	93.1	5.64	178	15	6	5	100
10	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
	EST5A_FELCA	Carboxylesterase 5A	60.9	5.58	254	30	12	6	100
14	TRFE_PIG	Serotransferrin	78.9	6.93	71	19	7	4	74
	ALBU_FELCA	Serum albumin	70.6	5.46	532	53	22	17	100
15	EST5A_FELCA	Carboxylesterase 5A	60.9	5.58	439	68	16	9	100
	ALBU_FELCA	Serum albumin	70.6	5.46	5932	346	51	42	100
16	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									

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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
19	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+	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.

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537 **Table 234**
538 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 communication; 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; 24	RET4_HUMAN	Retinol-binding protein 4	Up	Transporter	Transport

539
540 ^aNumber of the identified band or spot as marked in Fig 3 and 4 respectively.
541 ^b Protein entry name from UniProt knowledge database.
542 ^c Significantly (*P*<0.05) overrepresented (up) and underrepresented (down) proteins in CKD group respect to healthy.
543 ^d Molecular function according to Gene Ontology and Human Reference Proteome Database.
544 ^e Biological process according to Gene Ontology and Human Reference Proteome Database.
545

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

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

553 Fig. 3. 1D-SDS-PAGE of urine samples from healthy and CKD cats, stained with Coomassie Blue.
554 Lane 1, molecular weight marker; lanes 2-3, CKD urine samples; lanes 4-5, pools of urine from
555 healthy females and males respectively. Rectangles and numbers indicate the bands that have been cut
556 and identified by ESI-Q-TOF (Table 423).

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

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

563