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**URINE PROTEOME IN ANIMALS OF VETERINARY
INTEREST: SPECIES COMPARISON AND NEW
BIOMARKERS OF NEPHROPATHY**

Presentata da: Dott. Enea Ferlizza

Coordinatore Dottorato
Prof. Carlo Tamanini

Relatore
Prof.ssa Gloria Isani
Correlatore
Dott. Francesco Dondi

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INTRODUCTION

Urine is considered an ideal source of biomarkers, since it can be obtained non-invasively, in large amounts and it is rich in molecules important for clinical diagnostics. In human medicine the study of the urine proteome started in 1979 and in 2004 the first reference map of the normal urine proteome was reported. However, in veterinary medicine the literature is fragmentary and a complete study on the urine proteome in companion and farm animals is still lacking.

The aim of the present work is to produce a complete analysis of the urine proteome in animals of veterinary interest, dogs, cats, horses and cows including also some non-conventional species. In particular, the aims were to:

- validate high resolution electrophoresis (HRE) to quantify albuminuria in dogs and cats
- apply SDS-PAGE and 2DE coupled to mass spectrometry to produce a complete reference map of the urine proteome in healthy cats and compare it to cats affected by chronic kidney disease to identify putative biomarkers of nephropathy;
- apply SDS-PAGE coupled to mass spectrometry to characterize the urine proteome in healthy dogs and compare it to dogs affected by leishmaniasis to identify putative biomarkers of nephropathy;
- apply SDS-PAGE to characterize the urine proteome in dairy cows and evaluate changes during pregnancy;
- apply SDS-PAGE to characterize the urine proteome in healthy horses and compare it to diseased horses;

- quantify proteinuria and apply SDS-PAGE to characterize the urine proteome in non-conventional species (felids and giraffes) to evaluate kidney function and the general health status.

1. PART I: STATE OF THE ART

1.1. URINE PROTEOME IN HUMAN MEDICINE

Biomarkers are any measurable characteristics that reflect a particular physiological or pathophysiological state. More specifically, a clinical biomarker indicates a change in expression or state of a molecule that correlates with the risk or progression of a disease, or with the susceptibility of the disease to a given treatment. Ideally, a biomarker should be sensitive and specific, accurate and precise, non invasive and applicable to different population groups, due to variations related to age, gender or races.

Urine is considered one of the most easily accessible source of biomarkers, since it can be obtained in large quantities, in non-invasive ways and it is rich in molecules important for clinical diagnostics. The first studies on normal urine composition were performed in 1979, applying two-dimensional electrophoresis (2DE) at a time in which the term proteomics had not been coined yet (Anderson et al., 1979). The term *proteome*, which is a synthesis of the words protein and genome, was coined in 1996 by a PhD student trying to describe the set of all proteins that could be produced through the genome in a certain tissue (Wilkins et al., 1996). Thus, the term *urinary proteome* describes the entire set of proteins possibly present in the urine.

Urine, produced by healthy kidneys, normally contains only a small amount of proteins since large molecules are not able to pass the glomerular filter and most of the small molecular weight proteins and

protein fragments are reabsorbed along the tubular system. The abnormal presence of proteins in urine, which can derive either from plasma filtration or kidney damage, is known as *proteinuria*. Therefore, from a proteomic point of view, proteinuria can provide information on the systemic physiology (from serum proteins) as well as local physiology (from the proteins produced by kidneys).

The definition of the normal urine protein map is the starting point for the biomarker discovery. After the first study in 1979, proteomic techniques and in particular, 2DE coupled with mass spectrometry (MS), have been significantly improved and extensively applied for the purpose. Pieper et al., (2004) reached the observation of 1400 protein spots in urine, identifying the 30% of them. In the same year Oh et al., (2004) defined a preliminary urine proteome map (Figure 1).

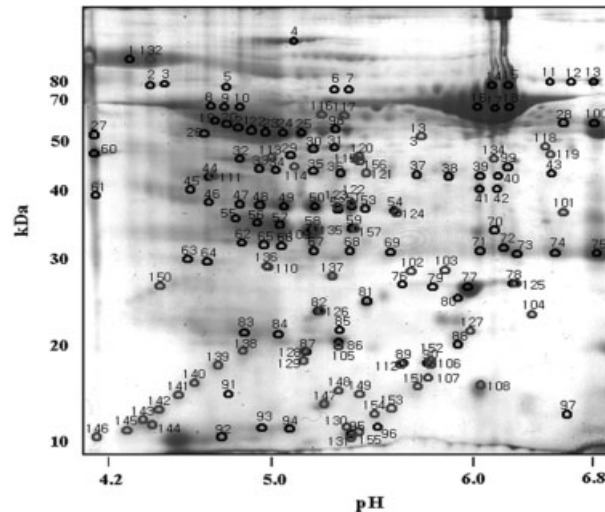


Figure 1. 2DE of the urine proteome (Oh et al., 2004).

Recently, a number of large-scale proteomics studies have been carried out to characterize the urinary proteome from healthy individuals. Marimuthu et al., (2011) compared the list of proteins identified in their study with two other large-scale studies, one by

Adachi et al., (2006) that reported 1543 proteins and another by Li et al., (2010) that reported 1310 proteins (Figure 2). Common proteins identified to all these three studies were 658 and represent the most commonly identifiable proteins in human urine. Combining all the data from the studies published to date, in human urine are present at least 2500 different proteins and peptides.

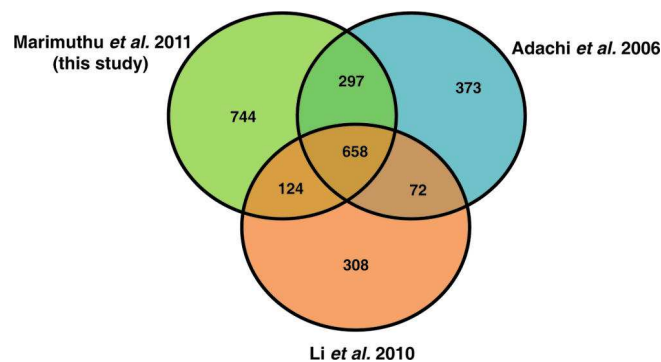


Figure 2. Venn diagram comparing large scale studies on urinary proteome (Marimuthu et al., 2011).

The proteins identified in normal urine can be classified in specific groups following a functional criteria. Kidney secretory and structural proteins, serum and transport proteins, coagulation and complement factors, immunoglobulins and other immune proteins, enzymes, metal binding proteins and lipoproteins are only some of the possible functional groups (Candiano et al., 2010).

Finally, the most challenging class of proteins of which urine is rich in is low abundance proteins. Most of the low abundance proteins in normal urine are still to be characterized. The most innovative techniques that are now developing are based on beads coated with hexameric peptide ligand libraries. The benefit of concentrating urine prior electrophoresis is that the most abundant proteins, such as albumin, are eliminated with a higher efficiency than low abundance

ones. Therefore, equalization offers a good chance for improving our knowledge on the composition of the normal urine proteome and move towards the biomarker discovery (Decramer et al., 2008; Candiano et al., 2010).

Many of the identified proteins in large-scale proteomics studies on normal urine are now under investigation in more specific and applied clinical trials to validate their use as sensitive and specific biomarkers of diseases. Chronic kidney disease (CKD), acute kidney injury (AKI) and diabetic nephropathy (DN) are only three of the diseases extensively studied in human medicine by the application of proteomic techniques. Lhotta, (2010) discussed the role of uromodulin during CKD. Good et al., (2010) identified an array of urinary biomarkers for accurate diagnosis of CKD using capillary electrophoresis coupled to mass spectrometry (CE-MS) and subsequently validated it. Devarajan, (2011) largely discussed some of the newly discovered biomarkers of AKI. Metzger et al., (2010) have identified twenty urinary peptides significantly associated with AKI, including fragments of albumin, α -1-antitrypsin and β -2-microglobulin proving that a proteomic classifier based on these 20 biomarkers would be of superior prognostic value in comparison to common single biomarkers of AKI. Regarding DN, Meier et al., (2005) applied CE-MS to urine samples of Type 1 diabetic patients revealing a specific clusters of 54 polypeptides. Also Andersen et al., (2010) applied CE-MS to study the low molecular weight (LMW) proteome in hypertensive type 2 diabetic patients with microalbuminuria to monitor the antihypertensive medication. They identified that the most prominent changes involved urinary collagen fragments associated with progression of diabetic nephropathy,

suggesting that a major benefit of treatment was the improvement of collagen turnover and the reduction of fibrosis.

The usefulness of the urine proteome as source of biomarker is important also for non-kidney related pathophysiology. A first example is the study of Zheng et al., (2013) on urine samples collected from pregnant and non-pregnant women. The proteome was analyzed by SDS-PAGE coupled with high resolution MS. In total, 2579 proteins were identified and 16 phosphoproteins were found to be significantly differentially expressed just before delivery. Other examples regards cardiovascular disease extensively reviewed by Delles et al., (2011) or hypertension (Carty et al., 2013).

1.2. URINE PROTEOME IN VETERINARY MEDICINE

In veterinary medicine, the application of proteomics techniques is still limited. Most of the studies focused on the identification and quantification by western blot or ELISA of select proteins, e.g. uromodulin or retinol binding protein, extensively studied in human medicine (Raila et al., 2007; Smets et al., 2010).

Recently, there have been significant efforts to study the urine proteome in dogs (Forterre et al., 2004; Nabity et al., 2011; Schaefer et al., 2011) and to a lesser extent in cats (Lemberger et al., 2011; Jepson et al., 2013; Ferlizza et al., 2015). In particular, Nabity et al., (2011) applied 2DIGE coupled to MS on urine samples to identify specific proteins related to tubulo-interstitial injury in a canine model of progressive glomerular disease. In another study, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) was used to perform protein-level profiling of urine from

dogs with and without renal disease (Forterre et al., 2004). Schaefer et al., (2011) applied SDS-PAGE to urine samples of dogs affected by severe inflammatory response syndrome (SIRS). Only in 2014 Miller et al., (2014) reviewed data on biological fluid in dogs and Brandt et al., (2014) published a complete characterization of the dog urine proteome.

Regarding the urine proteome in cats, the literature is even more fragmentary. Jepson et al., (2013) reported the application of SELDI-TOF to low molecular weight proteins in urine of cats at risk of developing azotemia. Another study with a proteomic approach referred to urine samples in cats affected by urinary tract disease, idiopathic cystitis (IdC), bacterial urinary tract infection (UTI), or urolithiasis (Lemberger et al., 2011). Recently, a preliminary characterization of the urine proteome in healthy cats and the comparison with cats affected by CKD has been published (Ferlizza et al., 2015).

The many applications of proteomics in farm animals has been recently reviewed (Almeida et al., 2015).

2. PART II: EXPERIMENTAL STUDY

2.1. VALIDATION AND PROTOCOL OPTIMIZATION

2.1.1. HIGH RESOLUTION ELECTROPHORESIS (HRE) TO QUANTIFY ALBUMINURIA

2.1.1.1. MATERIALS AND METHODS

Samples

This study was performed using urine samples of dogs and cats submitted to the Veterinary Clinical Pathology Service (SEPAC VET) of the Veterinary Teaching Hospital (Department of Veterinary Medical Sciences - University of Bologna) for different clinical conditions without selecting for age, sex, underlying disease or method of collection.

Urinalysis (USG, dipstick and sediment)

Each urine sample underwent complete routine urinalysis including the determination of the urine specific gravity (USG) by a refractometer, the semi-quantitative dipstick test (Combur10Test, Roche Diagnostic) and the microscopic sediment analysis at low (100x) and high (400x) power field.

Protein quantification

Urine total proteins were determined using commercial kits (Urinary/CSF Protein, OSR6170) on an automated chemistry analyzer (AU 400, Olympus/Beckman Coulter). Dog urines were also analyzed

by an immune-turbidimetric assay (ITA) developed for human albuminuria (Microalbumin OSR6167, Olympus/Beckman Coulter) and validated for dog urine (Gentilini et al., 2005).

High resolution electrophoresis (HRE)

For all urine samples were performed high resolution electrophoresis (HRE) on 0.8% agarose gel at pH 8.6 (HydraGel HR 15, SEBIA) in combination with the semi-automated system HYDRASYS (SEBIA), according to the manufacturer instruction. The gels were dried, stained by acid violet solution and acquired by the EPSON PERFECTION V700 PHOTO scanner/densitometer. The obtained pherograms were analyzed by the Phoresis software (version 6.1.2) and the albumin percentage calculated. The relative percentage and absolute concentration of albumin was calculated based on the density determined by the densitometer. As reported by the manufacturer, the limit of detection of this technique in serum is 1.5-2 mg/dL for albumin (0.15 to 0.2 micrograms per band), with dosages linear up to at least 5.8 g/dL and optimal protein concentration of 200 mg/dL. Urine samples with higher protein concentration were diluted in order to achieve the desired optimal concentration.

Validation

Within-assay variability was assessed on five cat urine samples (albumin concentrations 7.4; 13.4; 23.5; 38.8; 67.7 mg/dL) and on one dog urine sample (115.3 mg/dL). Samples were run five times on the same gel.

Between-assay variability was evaluated measuring four cat urine samples (7.4; 13.4; 23.5; 67.7 mg/dL) and one dog urine sample (137.2 mg/dL) three times in triplicate on three different days.

Linearity was tested by serial dilutions of one cat urine sample (79 mg/dL) and one dog urine sample (154.6 mg/dL) until the expected albumin concentration under the limit of detection (LOD) defined by the manufacturer (1.5 mg/dL) was reached. Each sample was made in duplicate. It was also possible to define the analytical sensitivity of HRE, evaluating both LOD (limit of detection, the lowest concentration with a visible albumin band on the gel) and LOQ (limit of quantification, the lowest concentration with a recognizable and quantifiable peak in the pherogram without interferences).

To evaluate *accuracy*, in the absence of a reference method for cat albuminuria, the recovery test was performed by adding to 100 μ L of a cat urine (alb 24.5 mg/dL) 100 μ L of two cat urines (alb 83.3 mg/dL; alb 41.7 mg/dL) and saline. The three solution high (H), medium (M) and low (L) had 53.75 mg/dL, 32.95 mg/dL e 12.1 mg/dL of expected albumin. Five measurement were made. In dog urine, albuminuria measured by HRE was compared to ITA validated for dog (Gentilini et al., 2005) and used in clinical practice.

Statistical analysis

Statistical analysis was performed with MedCalc [®] 11.3.3.0 and Excel 2007. Precision was evaluated by the calculation of the coefficient of variation (CV) as follows: $CV=(SD/mean) \times 100$. Linear regression analysis was used to evaluate the correlation between albumin expected and measured values and between HRE and ITA for dogs. Bland and Altman plot was also used to evaluate the accordance

between HRE and ITA. *P*-value was considered significant when less than 0.05.

2.1.1.2. RESULTS

Dog

HRE had within-assay CV of 1.37%, between-assay CV of 5.67% and a high linearity ($r=0.99$) (Table 1 and Figure 1). It was also possible to visually define LOD at 1.2 mg/dL of expected albumin and LOQ at 4.9 mg/dL. The comparison between HRE and ITA was performed by linear regression analysis and Bland and Altman plot (Figure 2). Data showed a highly significant correlation ($P<0.0001$). The Bland and Altman plot showed that HRE overestimated values lower than 50 mg/dL, while values higher than 100mg/dL were underestimated. In particular, some of the analyzed samples showed greater discrepancies between albumin values measured by ITA and HRE. Data are reported in Figure 3 and Table 2.

Table 1. Between-assay and within-assay CVs for dog albuminuria measured by HRE.

	Between-assay	Within-assay
Mean (mg/dL)	137.24	115.27
SD (mg/dL)	7.77	1.58
CV %	5.66	1.37

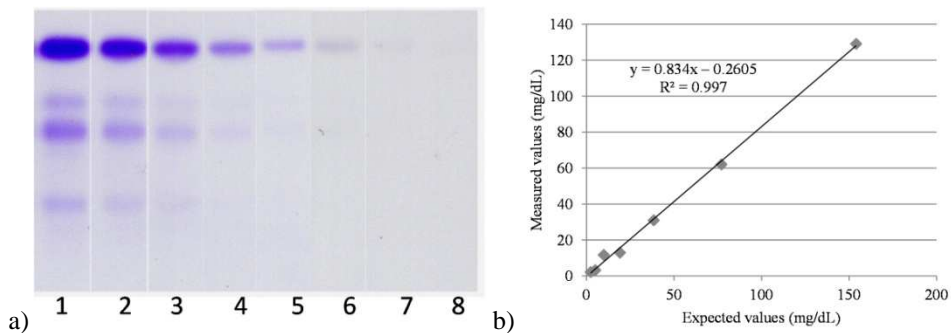


Figure 1. HRE (a) and correlation plot (b) showing linearity for dog albuminuria.

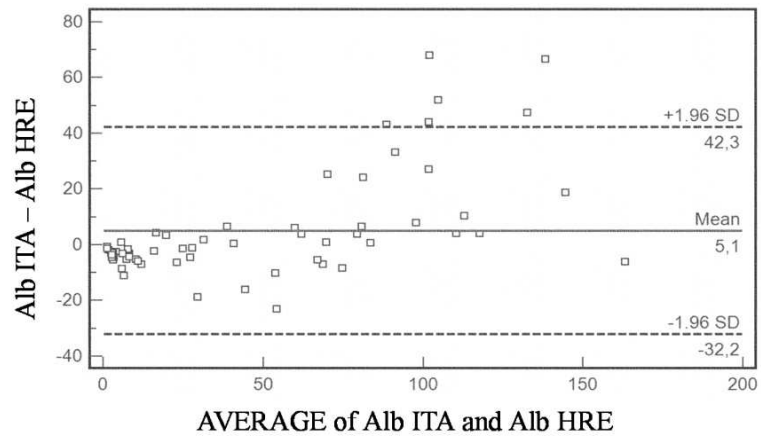


Figure 2. Bland and Altman plot for comparison between HRE and ITA.

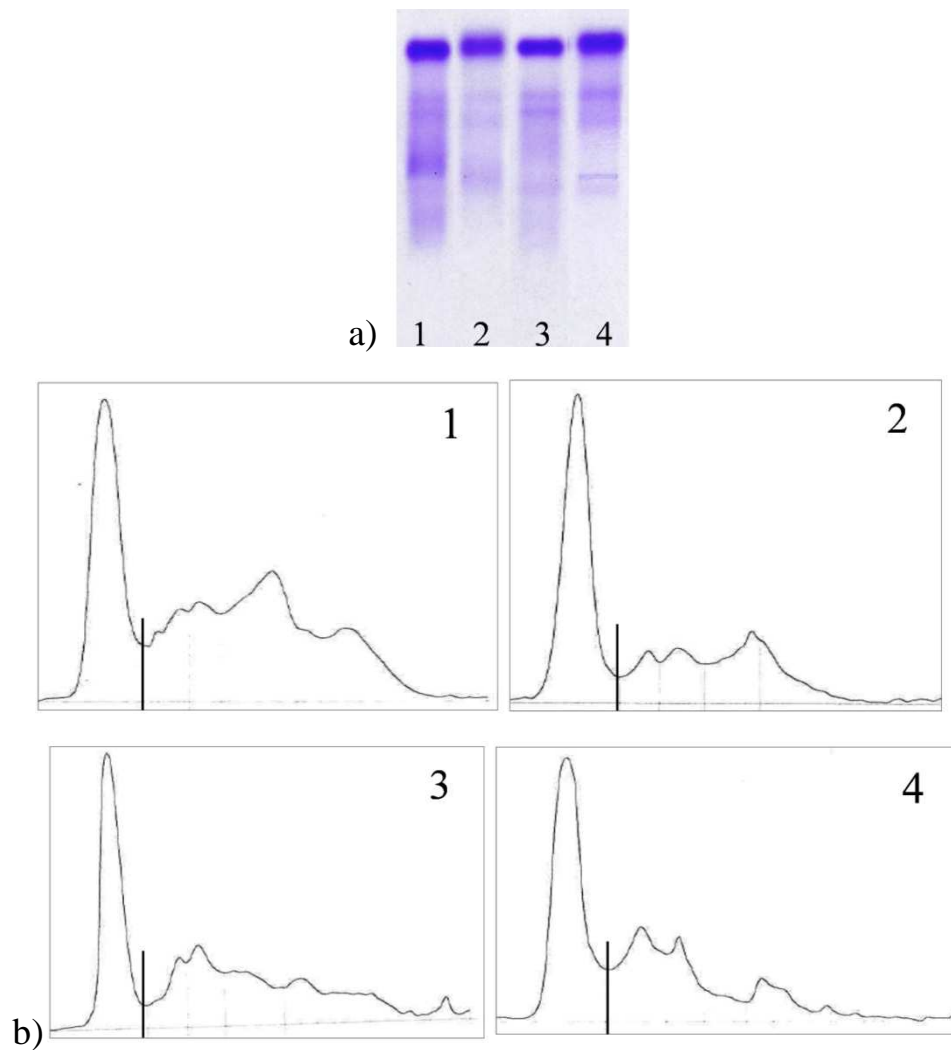


Figure 3. HRE (a) and pherograms (b) of urine samples that showed great discrepancies with ITA as reported in table 2.

Table 2. Data regarding urine samples (Figure 3) that reports discrepancies between ITA and HRE.

Lane and pherogram	Alb %		Alb (mg/dL)		UTP (mg/dL)
	HRE	ITA	HRE	ITA	
1	35	73.9	211	460	623
2	53	76.1	153	218	288
3	49	90.1	97	179	199
4	38.8	73.5	194	368	502

UTP: urine total portein

Cat

Table 3 reported data for within-assay and between-assay variability and recovery percentage. HRE had all CVs under 10% ranging from 2.58% to 7.6% for within-assay and from 1.2% to 9.1% for between-assay. Figure 4 reported some examples of gels. HRE showed high linearity ($r=0.99$; Figure 5) between expected and measured albumin values and the recovery percentage was of 97%, 93% and 109% for H, M and L concentration respectively. It was possible to define optically the LOD at 1.25 mg/dl of expected albumin and by densitometry the LOQ at 5 mg/dl (Figure 5).

Table 3. Between-assay and within-assay CVs and recovery percentage for albuminuria in cat measured by HRE.

WITHIN-ASSAY						
Mean (mg/dL)	67.73	38.83	23.50	13.37	7.40	
SD (mg/dL)	1.75	2.26	0.81	0.99	0.56	
CV %	2.59	5.81	3.44	7.40	7.60	
BETWEEN-ASSAY						
Mean (mg/dL)	67.25	24.53	13.23	7.10		
SD (mg/dL)	2.37	0.29	0.98	0.64		
CV %	3.52	1.20	7.45	9.06		
RECOVERY PERCENTAGE						
	H	M	L			
Expected albumin (mg/dL)	53.75	32.95	12.1			
Measured albumin (mg/dL)	51.9	30.6	13.2			
Recovery %	96.6	92.9	109.1			

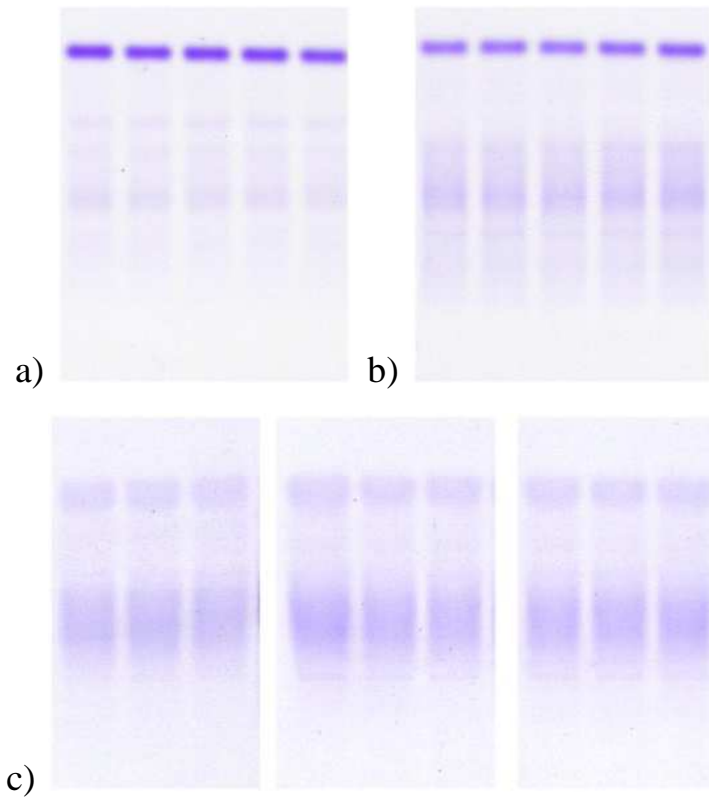


Figure 4. HRE of: a) within-assay, albumin 39mg/dL; b) within-assay, albumin 23.5 mg/dL; c) between-assay albumin, 7 mg/dL.

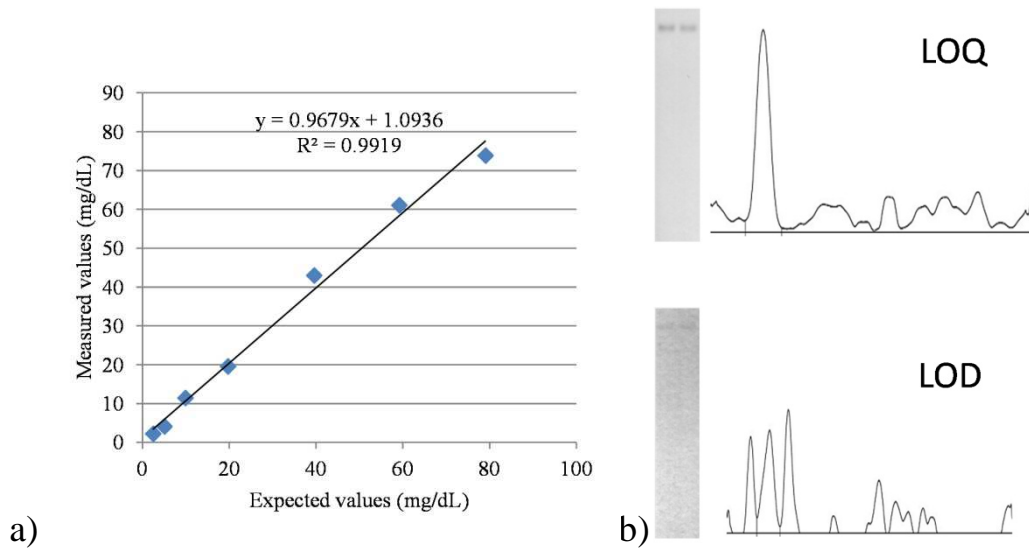


Figure 5. Linearity (a) and LOD and LOQ (b) for albuminuria measured by HRE.

2.1.1.3. DISCUSSION

In human patients proteinuria and albuminuria have been identified as independent risk factors for all-cause and cardiovascular mortality as well as adverse renal outcome in diabetic nephropathy and CKD (Wu et al., 2012). In veterinary medicine, albuminuria can be measured by a species-specific point-of-care, semiquantitative test (e.g., the Heska ERD-Health-Screen Urine test) and quantitative immunoassay at reference laboratories (Antech Diagnostics and Heska Corporation) (Grauer, 2011). A sensitive immuno-turbidimetric method for the quantification of albuminuria has been validated only for dogs (Gentilini et al., 2005). In the present study, HRE was validated to quantify albuminuria in dogs and cats. All the parameters evaluated (within and between-assay CVs <10%; recovery 97-109%; linearity, accuracy and correlation with ITA $P<0.05$; LOQ 5 mg/dL) can be considered satisfactory for a semiautomatic technique (Jensen and Kjølgaard-Hansen, 2006). However, the software of the densitometer was not able to perform an effective pherogram in sample with low amount of urine total protein (40 mg/dL) probably due to noise interferences, such as dust or gel irregularities as reported by Ferlizza et al., (2012) and Giori et al., (2011). Moreover, it was possible to compare data obtained by HRE in dog samples with adequate amount of proteins to data obtained by ITA showing a significant correlation. ITA validated in dog (Gentilini et al., 2005) is based on the high homology between human and dog albumin and on the cross reactivity between the anti-human albumin antibody and the dog albumin. The manufacturer reported that ITA is linear only in the range between 0.5 and 30 mg/dL, suggesting the dilution of samples with higher concentration. In contrast, our data showed that HRE is

linear for albumin in urine between 5 and 154 mg/dL and the manufacturer claimed HRE linear up to 5.8 g/dL for albumin in serum. Therefore data reported in the present study let us suggest that HRE could be more reliable than ITA for albumin quantification higher than 30 mg/dL, while ITA due to its higher sensitivity is more accurate for microalbuminuria.

2.1.2. TWO-DIMENSIONAL ELECTROPHORESIS (2DE)

2.1.2.1. MATERIAL AND METHODS

Samples

This study was performed using urine samples of pigs and cats submitted to the Veterinary Clinical Pathology Service (SEPA VET) of the Veterinary Teaching Hospital (Department of Veterinary Medical Sciences – University of Bologna) for different clinical conditions without selecting for age, sex, underlying disease or method of collection.

Urinalysis (USG, dipstick and sediment)

Each urine sample underwent complete routine urinalysis including the determination of the urine specific gravity (USG) by a refractometer, the semi-quantitative dipstick test (Combur10Test, Roche Diagnostic) and the microscopic sediment analysis at low (100x) and high (400x) power field.

Protein quantification

Urine total proteins were determined using a commercial kit (Urinary/CSF Protein, OSR6170) on an automated chemistry analyzer (AU 400, Olympus/Beckman Coulter).

Protocol optimization for sample preparation

To remove salts and concentrate proteins, 150 µg of proteins underwent three different protocols:

- a) Acetone precipitation: one volume of cold acetone 20% was added to one volume of pig urine to a final concentration of 10%, incubated at -20°C overnight and centrifuged at 15,000 g for 30 min;
- b) TCA/ethanol precipitation: one volume of trichloroacetic acid 20% was added to one volume of pig urine to a final concentration of 10% in gentle shaking for one hour at 4°C, centrifuged at 15,000 g for 30 min and washed three times with cold absolute ethanol;
- c) Ultrafiltration: 1.5 mL of pig urine were loaded in Vivaspin500 spin columns (Sartorius Stedim Biotech) with a molecular weight (MW) cut-off of 3 kDa and centrifuged at 15,000 g of 30 min; 500 µL of water was added to the retentate and centrifuged again up to reach a final volume of 100 µL.

The pellets were dissolved in a solubilisation buffer (SB) containing 7 M urea, 2 M thiourea, 4% CHAPS w/v, 65 mM DTT and 0.8% resolytes v/v (pH 4-7) before loading onto immobilized pH gradient (IPG) strips (pH gradient 4-7, 11 cm long) (BioRad). IPG strips were rehydrated, equilibrated and placed on top of 4-15% acrylamide gel.

2DE protocol optimization for animal urine proteome

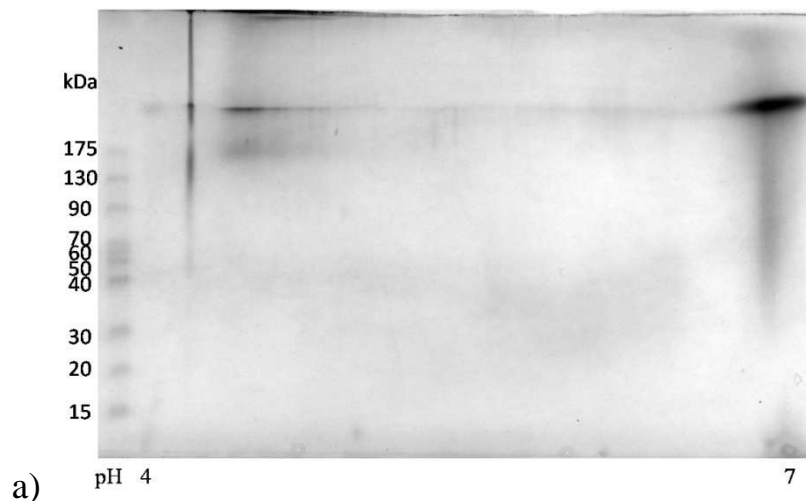
To optimize the 2DE protocol, two urine samples from healthy and CKD cats were analyzed with 3 different protocols:

- Protocol 1: 200 µg of protein, IPG strip 7 cm and pH 3-10, poliacrylamide 12%;
- Protocol 2: 100 µg of protein, IPG strip 7 cm and pH 4-7, poliacrylamide 10%;
- Protocol 3: 300 µg of protein, IPG strip 17 cm and pH 3-10, di poliacrylamide 10%

2.1.2.2. RESULTS

Protocol optimization for sample preparation

Figure 6 reports the 2DE of the three different methods used to prepare samples. The best resolution was obtained by the TCA/ethanol precipitation with the greatest number of visible spots. The acetone precipitation protocol did not allow the precipitation of proteins and none spots is visible. The protein concentration reached after ultrafiltration was not adequate and lower than that obtained by the TCA/ethanol precipitation.



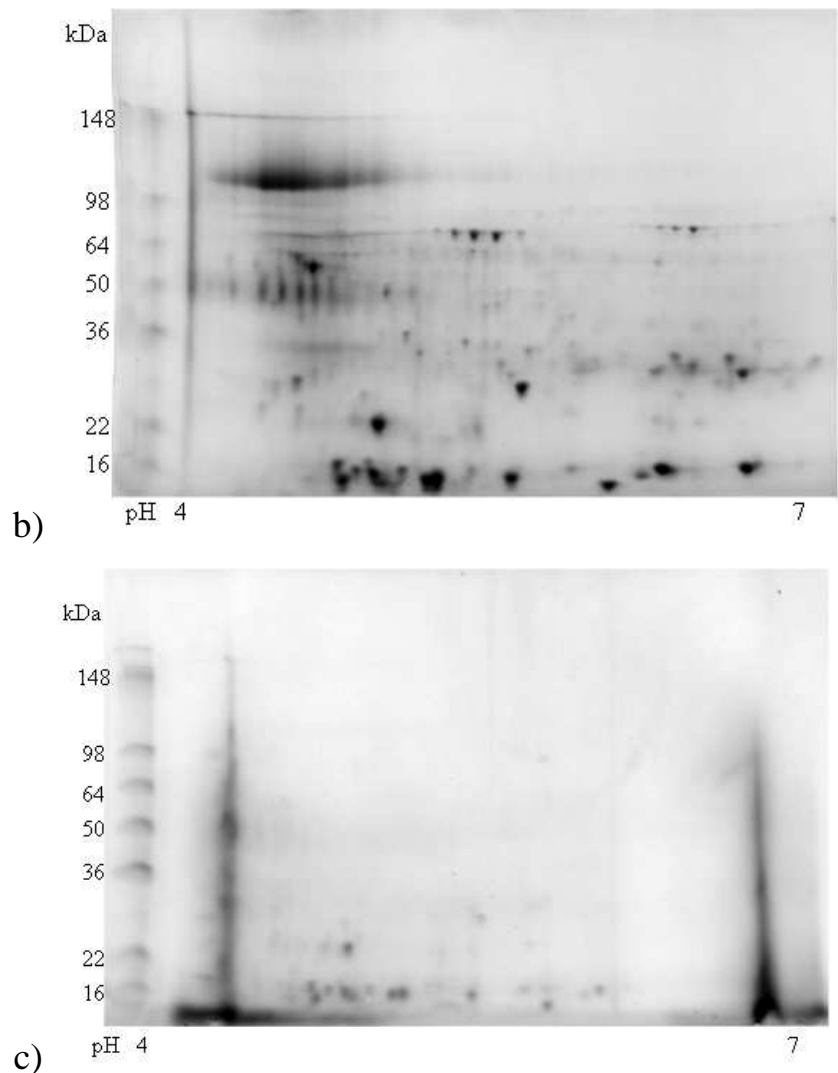
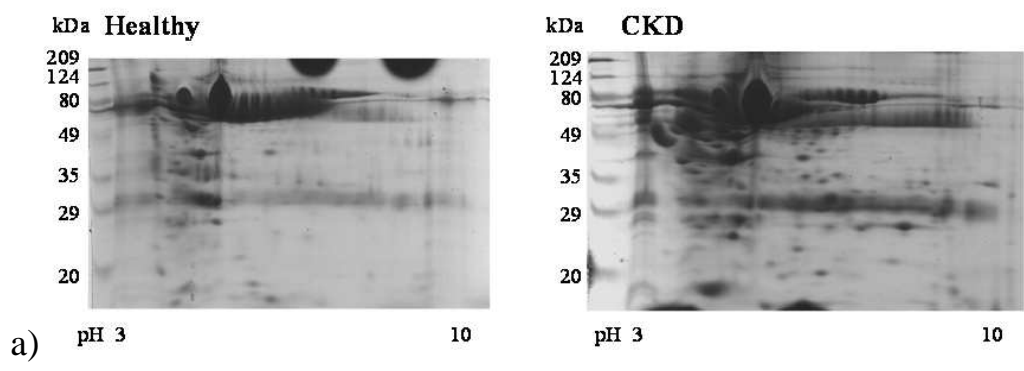


Figure 6. 2DE of pig urine after the three protocols tested to concentrate and desalt urine. a) Acetone precipitation; b) TCA/ethanol precipitation; c) ultrafiltration.

2DE protocol optimization for cat urine proteome

Figure 7 reported the 2DE of the three different protocol used to separate cat urine proteome.



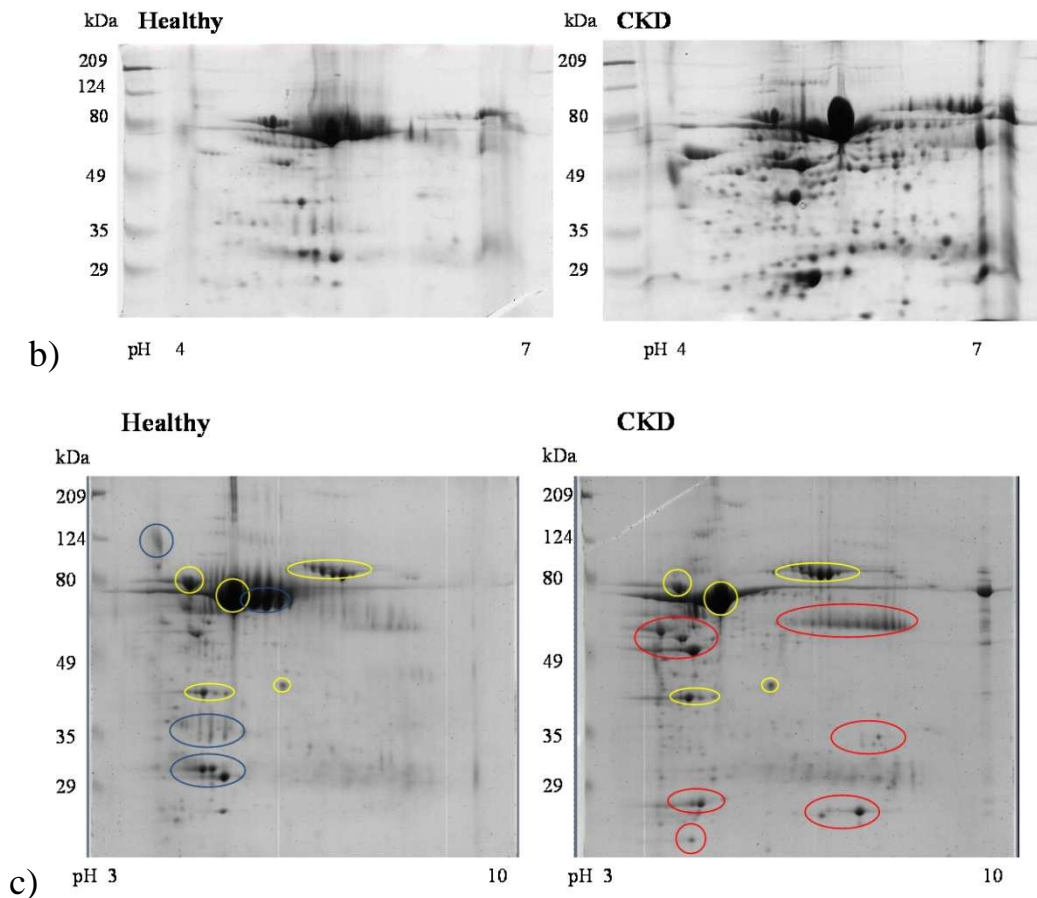


Figure 7. 2DE of healthy and CKD urine samples by means of (a) protocol 1, (b) protocol 2 and (c) protocol 3. Blue circles highlighted spots more evident in healthy; red circles highlighted spots more evident in CKD; yellow circles highlighted common spots.

The protocol 1 did not allow an optimal separation and visualization of the protein spots due to excessive protein amount. In particular, acidic and high MW proteins (>80 kDa) were not optimally visualized.

The protocol 2 allowed an optimal visualization of protein spots at intermediate MW and in the acidic pH range. Moreover, high MW proteins (>80 kDa) were also optimally visualized. However, basic and low MW proteins (<20 kDa) were excluded.

The protocol 3 allowed an optimal separation of urine protein covering a wide range of MW and pH. Differences between healthy and CKD cats were also highlighted.

2.1.2.3. DISCUSSION

The clinical interest of urine as an easily accessible biological fluid rich in potential markers of disease is largely approved. In human medicine many studies focused on urine proteome and applied 2DE for biomarker discovery (Candiano et al., 2010). However, urine matrix can be an important source of interferences due to the high amounts of salts and urea that can negatively affect sensitivity and spot resolution. In addition, due to low amount of proteins, particularly in samples from healthy animals, protein concentration is an essential step before electrophoretic separation. Therefore the sample pre-treatment procedure and the optimization of the 2DE protocol are essential (Martin-Lorenzo et al., 2014).

First, we tested the influence of three different methods to desalt and concentrate samples for 2DE profiling. Only the TCA/ethanol precipitation allowed the visualization of high number of spots, while the other methods did not permit an optimal spot visualization. Our data on pig and cat urine proteome differed from what reported for humans where all the pre-treatment procedures tested allowed a good resolution and high sensitivity (Martin-Lorenzo et al., 2014).

Secondly, we optimized the protocol for the better separation of the urine proteome trying different pH ranges and polyacrylamide gel concentration. In this case, all the protocol tested, except for the first where too much sample was loaded, allowed a satisfactory spot resolution. Therefore, depending on the pH and MW range of interest and the specific proteins to be investigated any of the protocols may provide satisfactory data in term of resolution.

2.2. COMPANION ANIMALS

2.2.1. DOG URINE PROTEOME

2.2.1.1. MATERIALS AND METHODS

Animals and samples

For the present study, healthy dogs and dogs affected by leishmaniasis (Leish) were selected. The healthy group included dogs presented to the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine - University of Extremadura, Spain, and to the Veterinary Teaching Hospital of the Department of Veterinary Medical Sciences - University of Bologna. Animals considered healthy on the basis of history and physical examination and with no history of urinary tract diseases were included. For the diseased group were selected dogs with leishmaniasis diagnosed on the basis of history, clinical signs and clinico-pathological results. The diagnosis of leishmaniasis was confirmed by the presence of amastigotes in cytology specimens or anti-leishmania antibody detection, given with Indirect ImmunoFluorescence assay (IIF) with the positivity higher than 1/80;

Upon arrival, all the animals were subjected to physical examination and routine laboratory tests, including complete blood count, serum chemistry and complete urinalysis with urine protein and urine albumin to creatinine ratios (UPC; UAC). Five millilitres of urine were collected from each animal by ultrasound-guided cystocentesis and analyzed with the following standardized protocol:

- urinalysis (USG and dipstick);

- centrifugation at 1,500 g for 10 minutes;
- microscopic analysis of urine sediment;
- protein quantification with UPC and UAC ratios calculation;

The supernatants were immediately stored at -80 °C for the subsequent analysis by high resolution electrophoresis (HRE) and SDS-PAGE.

Urinalysis (USG, dipstick and sediment)

Each urine sample underwent complete routine urinalysis including determination of the urine specific gravity (USG) by a refractometer, semi-quantitative dipstick test (Combur10Test, Roche Diagnostic) and microscopic sediment analysis at low (100x) and high (400x) power field.

Protein and albumin quantification

Urine total proteins (UTP) 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). Urine albumin was determined by an immuno-turbidimetric assay (ITA) developed for human albuminuria (Microalbumin OSR6167, Olympus/Beckman Coulter) and validated for dog urine (Gentilini et al., 2005). This method is linear from 0.5 mg/dL to 30 mg/dL and the limit of detection is 0.046 mg/dL. The UPC and UAC were calculated with the following formula: UPC or UAC = urine protein or albumin (mg/dL)/urine creatinine (mg/dL)

High resolution electrophoresis (HRE)

Urine samples were analyzed on precast 0.8 % agarose gel at pH 8.6 (HydraGel 15 HR, SEBIA) for quantification of albuminuria as previously described (Ferlizza et al., 2012). The kit was used in combination with the semi-automated HYDRASYS system (SEBIA). The gels were subsequently stained with an acid violet solution, dried and scanned by a densitometer (EPSON PERFECTION V700 PHOTO) yielding the pherogram (Phoresis 6.1.2 software). The relative percentage and absolute concentration of albumin were calculated based on the density determined by the densitometer. The limit of detection of this technique is 5 mg/dL for urine albumin with an optimal UTP of 200 mg/dL. Urine sample with UTP higher than 200 mg/dL were diluted in order to achieve the desired optimal concentration. The values obtained were compared with data obtained by the automated method.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Urine proteins were separated using the electrophoresis NuPAGE system (Thermo Fisher Scientific) on 12% polyacrylamide gel in 3-(*N*-morpholino) propanesulphonic acid (MOPS) buffer with sodium-dodecyl-sulphate (SDS) (Thermo Fisher Scientific). Two μg of proteins for each sample were loaded. The gels were stained with SilverQuest (Thermo Fisher Scientific). After staining, each gel was digitalized and its pherogram was obtained using GelAnalyzer 2010 software¹. To have a better visualization of low molecular weight (MW) protein bands and to prepare for mass identification, 10 μg of

¹ <http://www.gelanalyzer.com/>

proteins of eight urine samples were also analyzed using the Criterion system (BioRad) on a 16.5 % polyacrylamide gel in Tris-glycine buffer with SDS. The gel was stained with Coomassie blue staining (PageBlu protein staining solution; Thermo Fisher Scientific) compatible with mass spectrometry.

Protein identification by mass spectrometry

Protein identification was performed at the Department of Diagnostic, Clinical and Public Health Medicine, University of Modena and Reggio Emilia.

Protein bands 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).

Protein-identification peak lists were generated using the Mascot search engine against the UniProt database² as previously described (Bertoldi 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).

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

² www.uniprot.org

(albumin mg/dL, UPC, UAC, number of bands) were tested for normality with Shapiro-Wilk normality test and compared using the Kruskal-Wallis one-way analysis of variance assuming $P < 0.05$ as a significant probability. Samples measured by HRE with urine albumin lower than 5 mg/dL were considered as 4.9 and the UAC were calculated according to this value. Spearman's rank correlation test was used to establish correlation between HRE and ITA.

2.2.1.2. RESULTS

Animals and samples

Thirteen entire dogs (7 males, 6 females) were included in the study as the healthy group. The median age was 36 months (15-84) and median UPC and UAC were 0.07 (0.05-0.19) and 0.005 (0.001-0.015) respectively.

Twenty-six dogs affected by leishmaniasis (10 entire females, 14 entire males and 2 neutered males) were included in the Leishmania group (Leish). Leish dogs had a median age of 48 months (12-108) and had significantly increased UPC (median 2.1; 0.06-20.8) and UAC (0.29; 0.003-6.2) values than healthy dogs ($P < 0.01$) (Figure 1). Plasma biochemistry and urinalysis data are reported in Table 1.

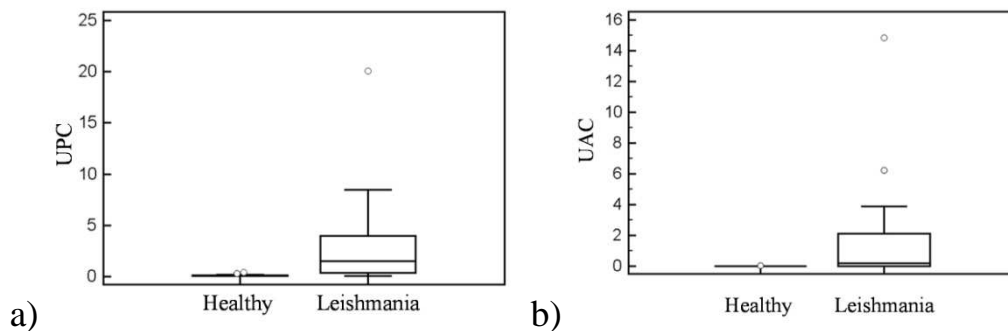


Figure 1. Graphs reporting (a) UPC and (b) UAC data for healthy and Leish dogs.

Table 1. Clinical data for dogs affected by leishmaniasis (n = 26).

Signalment	Mean±SD	n	
Age in months	48±27		
Female (entire/neutered)		10 (10/0)	
Male (entire/neutered)		16 (14/2)	
Plasma biochemistry	Mean±SD	n (%) < or >RI	RI
Total Proteins (g/dL)	8.2±2.2	1(4)< 16(62)>	5.3-7.6
Albumin (g/dL)	2.8±0.8	16(62)<	3.2-4.7
Creatinine (mg/dL)	1.8±2.6	10(37)< ; 8(31)>	0.5-1.4
Urea (mg/dL)	95±97	10(37)>	18-55
Phosphorus (mg/dL)	96.7±4.6	1(4)< 5(19)>	1.9-7.9
Urine biochemistry	Mean±SD	n (%) < or >RI	RI
UPC	3.2±4.3	15(58)>	<0.5
UAC	1.6±3.1	17(65)>	<0.03
USG	1.029±0.014	15(58)<	>1.030 ^a
Clinical signs		n (%)	
Lymphadenopathy		20(77)	
Dermatological lesions		17(65)	
Apathy / Depression		15(58)	
Weight loss		13(50)	
Anorexia		9(35)	
Onychogryphosis		8(31)	
Lameness / Joint pain		7(27)	
Nasal Hyperkeratosis		7(27)	
PU/PD		7(27)	
Diarrhea		6(23)	
Vomiting		6(23)	
Dehydration		5(19)	
Bloody Diarrhea		3(12)	
Epistaxis		3(12)	
Pale mucosas		3(12)	
Ocular lesions		2(8)	

RI, reference interval; UPC, urine protein to creatinine ratio; UAC, urine albumin to creatinine ratio; USG, urine specific gravity. ^a Considered as adequate USG in cats.

HRE and albuminuria

Representative gels and pherograms of urine samples of healthy and Leish dogs are reported in Figure 2. In samples with protein concentration higher than 40 mg/dL it was possible to qualitatively evaluate the protein content, separating different protein fractions,

showing different profiles between healthy and Leish dogs and quantifying albuminuria. UAC measured with HRE was significantly correlated with ITA ($P<0.01$) (Figure 2d).

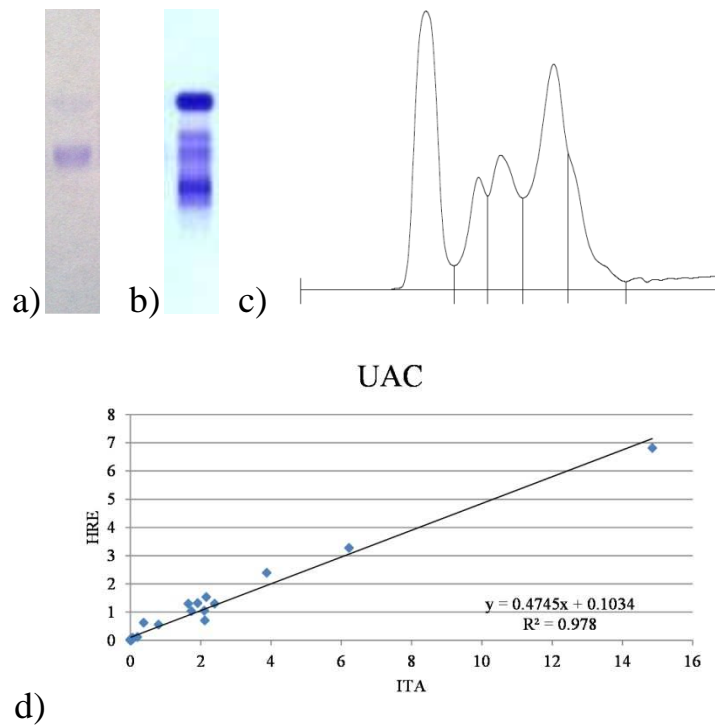


Figure 2. Representative HRE of urine samples of (a) healthy and (b) Leish dogs. c) Pherogram of a Leish sample. d) Correlation between ITA and HRE for albuminuria.

In particular, healthy samples showed a faint band of albumin and a diffused band between alpha and beta zone. Differently, Leish dogs showed more evident bands and in some cases the profile was similar to the serum electrophoretic profile (Figure 2b, 2c).

In Leish group it was also possible to highlight changes in the electrophoretic profile during the therapy. Figure 3 reported comparison among urine samples from the same patient at different time intervals. Lanes 2 and 3 showed the renal functionality improvement after one and two months post-treatment with

miltefosine; in these two lanes a decrease in albumin fraction and alfa zone is evident. Six months after the diagnosis, lane 4, albumin is still absent while the other fractions are slightly increased.

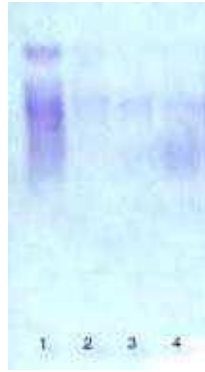


Figure 3. HRE of urine samples of the same patient at the moment of diagnosis T0 (1) and after the end of treatment with miltefosine (2,3,4) in the same patient. In lanes 2,3 and 4 the patient was treated with allopurinol.

Figure 4 reported electrophoretic profiles of two urine samples from the same dog during treatment (lane 1) with miltefosine and allopurinol and two months later after the end of treatment (lane 2). During the treatment the renal damage seems downgrading, but only two months later the electrophoretic pattern highlights a stronger damage with more albumin loss and an increased intensity of α , β and γ zones.

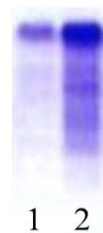


Figure 4. HRE of urine samples during (1) and after (2) treatment with miltefosine and allopurinol.

SDS-PAGE

Urine samples from the 13 healthy and 26 Leish dogs were analyzed by SDS-PAGE. Representative gels and pherograms are

reported in Figure 5. We separated 28 ± 5 protein bands in the urine of healthy dogs. Most protein bands had an apparent MW between 17 and 67 kDa. The Leish group presented a greater inter-individual variability and a greater number of bands at MW between 66 and 10 kDa. A significant increase of the total number of bands (37 ± 9) ($P < 0.05$) was observed, particularly at MW lower than 67 kDa ($P < 0.01$) (Figure 6).

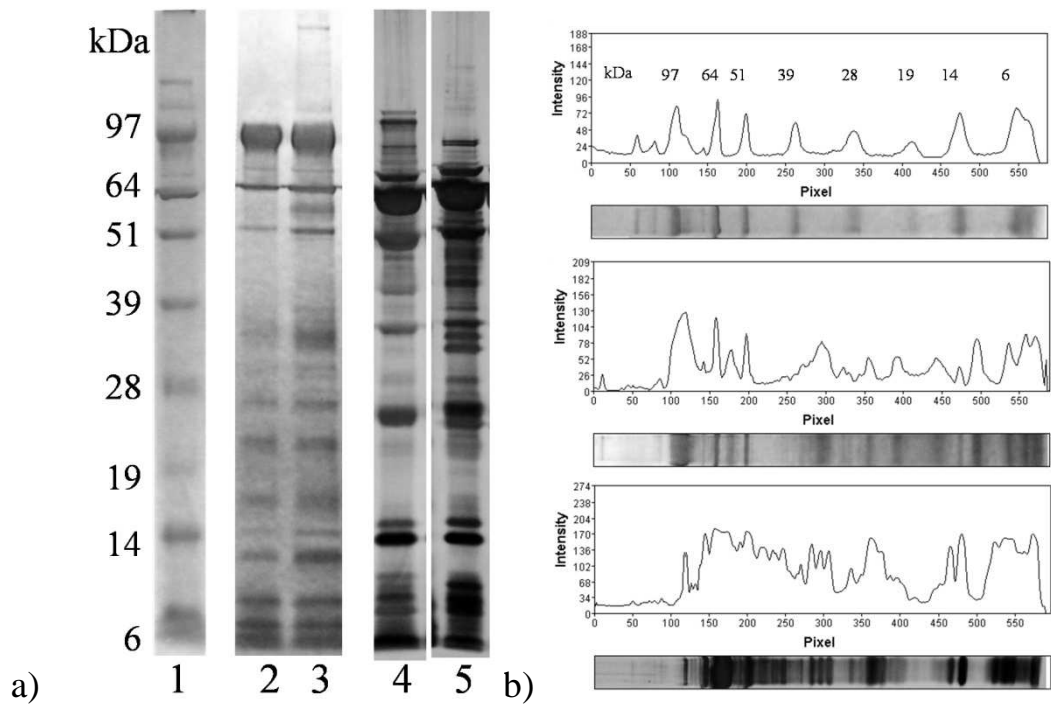


Figure 5. Representative (a) gels and (b) pherograms of urine samples of healthy (2-3) and Leish (4-5) dogs.

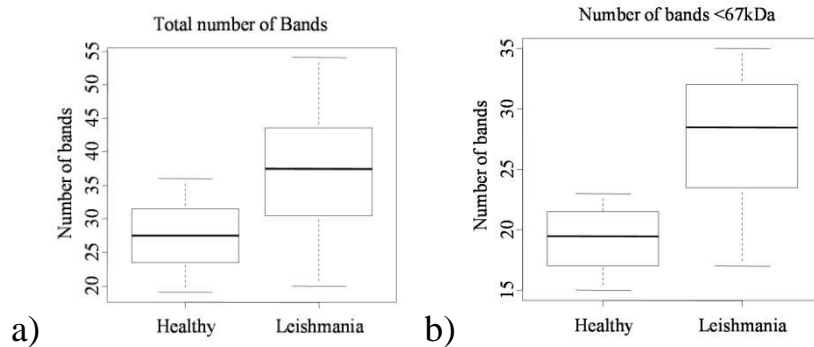


Figure 6. Comparison of the number of protein bands between healthy and Leish dogs. (a) Total number of bands. (b) Number of bands with MW < 67 kDa.

SDS-PAGE allowed to evaluate changes in the urine proteome during the treatment. Examples of specimen before and after treatment are reported in Figure 7 and 8. Figure 7 reported urine samples of the same patient before and after treatment with miltefosine and allopurinol. Increase of the 90 kDa band and the decrease of the bands of 67 kDa as well as the band in the zone between 64 and 14 kDa can be appreciated.

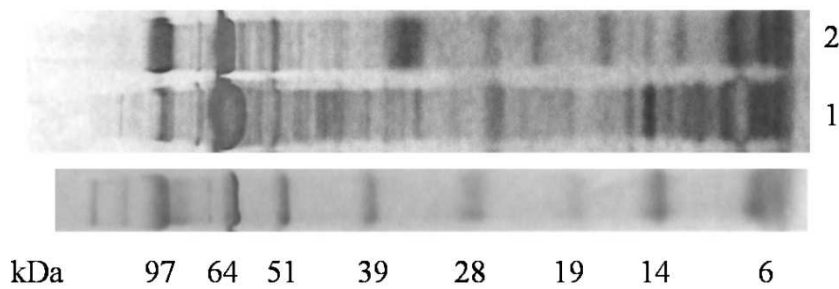


Figure 7. SDS-PAGE of a Leish dog (1) before and (2) after treatment.

Figure 8 reported urine samples of the same patient during and two months after treatment with miltefosine and allopurinol. During the treatment the renal damage is downgrading, but two months later the electrophoretic pattern highlighted a stronger damage.

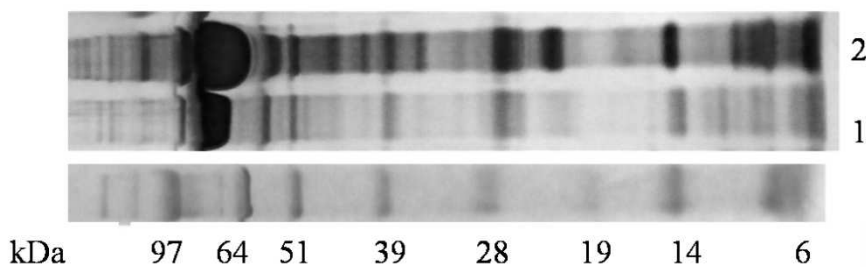


Figure 8. SDS-PAGE of a Leish dog (1) during and (2) after treatment.

Figure 9 reported SDS-PAGE of urine samples loaded with increasing UPC from healthy (lane 2) and Leish non proteinurics

(lanes 3) to borderline (lanes 4-9) and proteinurics (lanes 10-15). The decrease of the band at 90 kDa (green rectangle) and the increase of three bands at 76, 67 and in some samples at 15 kDa (red rectangles) are clearly evident.

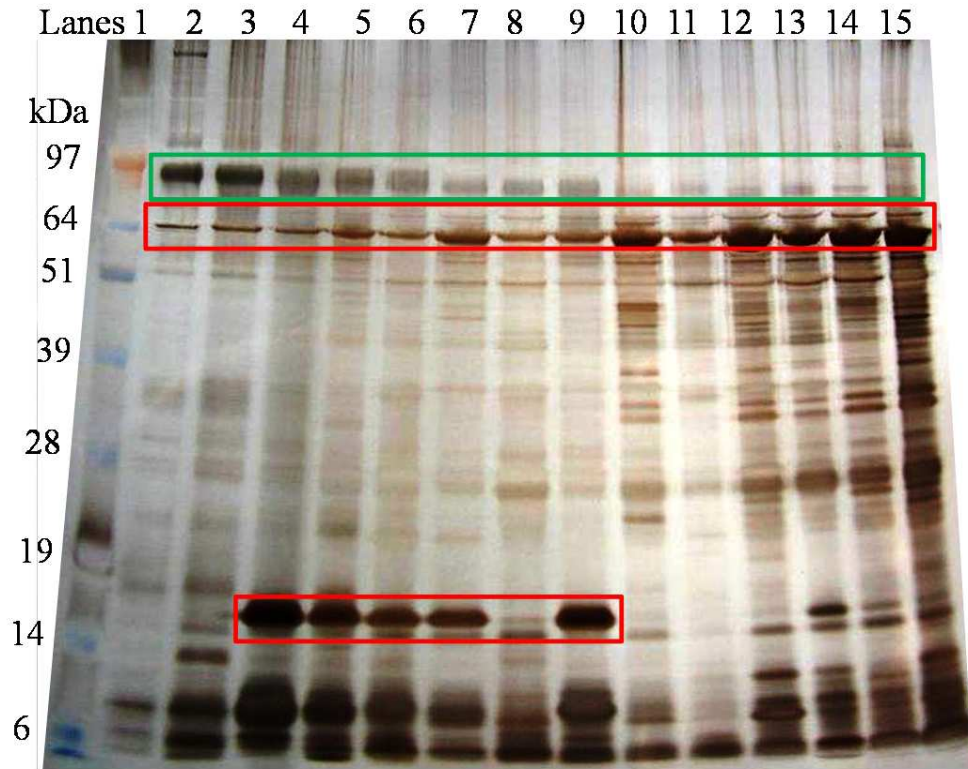


Figure 9. SDS-PAGE of urine samples loaded with increasing UPC. Lane 1, MW marker; 2, healthy; 3, non proteinuric Leish dog; 4-9 (UPC<0.2), borderline proteinuric Leish dog (UPC 0.2-0.5); 10-15, proteinuric Leish dogs (UPC>0.5). Green rectangle indicate the band at 90 kDa that showed decrease intensity in parallel to the increase of UPC; red rectangles indicate the bands at 67, 67 that showed increased intensity in parallel to the increase of UPC and the band at 15 kDa in borderline Leish dogs.

Protein identification by mass spectrometry

Eight urine samples from healthy and Leish dogs were separated on 16.5% SDS-PAGE in order to achieve a better separation of proteins at MW<67 kDa for subsequent MS identification. Figure 10 reported the protein bands (n=7) of healthy and Leish dogs excised from the gel for MS identification. Table 2 reported the identified proteins.

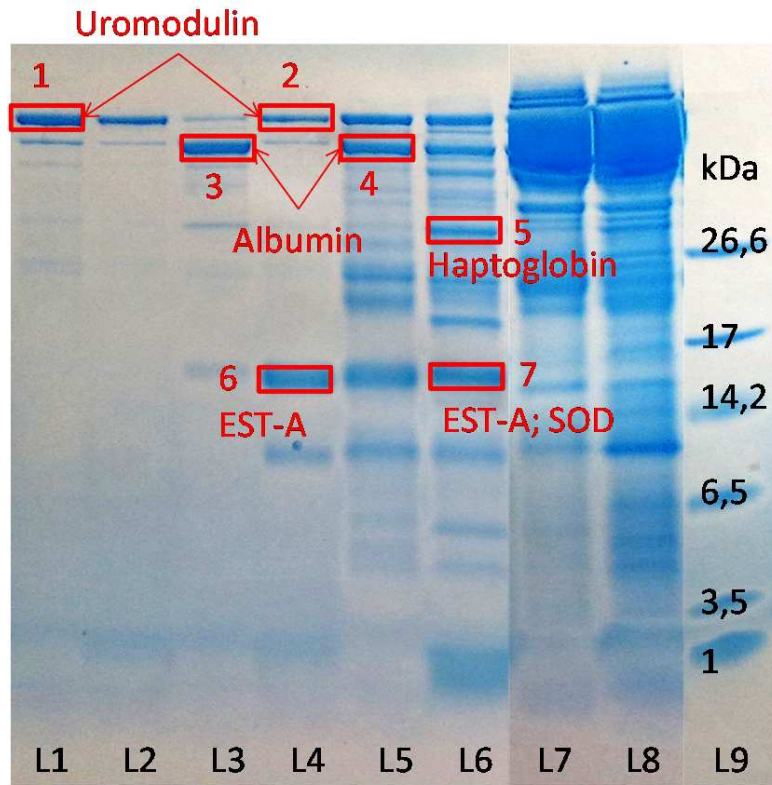


Figure 10. SDS-PAGE of urine samples from healthy and lesih dogs. Lanes 1-2, healthy samples; lanes 3-6, borderline proteinurics Leish dogs (UPC 0.2-0.5); lanes 7-8 proteinuric Lesih dogs (UPC>0.5); lane 9 MW marker. Rectangles and numbers indicate the bands that have been cut and identified by ESI-Q-TOF (Table 2).

Table 2. Proteins identified in dog urine by mass spectrometry.

Band ^a	Entry name ^b	Protein full name	MW ^c (kDa)	pI	Score ^d	Pept ^e	Seq ^f	Sign. Seq. ^g
1	UROM_CANFA	Uromodulin	73	4.94	2298	138	15	13
2	UROM_CANFA	Uromodulin	73	4.94	1546	114	16	13
3	ALBU_CANFA	Albumin	69	5.52	5802	470	44	39
4	ALBU_CANFA	Albumin	69	5.52	7408	453	54	44
5	HPT_CANFA	Haptoglobin	37	5.72	1526	198	18	14
6	ESTA_CANFA	Arginine esterase	29	8.03	532	111	10	9
	ESTA_CANFA	Arginine esterase	29	8.03	448	81	9	8
7	NPC2_CANFA	Epididymal secretory protein	16	8.25	419	99	8	6
	SODC_CANFA	Superoxide dismutase [Cu-Zn]	16	5.70	243	22	3	3

^a Number of the identified band or spot as marked in Figure 10.

^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.

From the seven bands, six proteins were identified (Table 2, Figure 10). In particular, uromodulin (90 kDa) was identified in both healthy and Leish dogs showing a progressive decrease parallel to the increase of UPC (Figure 9). Albumin, corresponding to the band at 67 kDa, showed increased intensity parallel to the increase of proteinuria. Band 6 and 7 contained 3 proteins, namely arginine esterase, epididymal secretory protein and superoxide dismutase. In our samples, this band showed a significant increase in 5 of the 6 borderline proteinuric Leish dogs. Haptoglobin was also identified in the band at 38 kDa.

2.2.1.3. DISCUSSIONS

The aim of this study was to evaluate the usefulness of HRE and SDS-PAGE in the separation of the urine proteome in dogs affected by leishmaniasis compared to healthy.

HRE allowed the separation of different pherograms between healthy and Leish dogs, highlighting the increase of albumin and gamma zone in Leish dogs. The increase of globulinemia is a significant feature of leishmaniasis, mostly due to the increase of IgG (Proverbio et al., 2014). Electrophoresis of serum samples from leishmaniotic patients pointed out a polyclonal gammopathy, due to the host response versus the parasite. In urine, the presence of high levels of all protein fractions, mostly of albumin, due to the renal damage, and gamma fractions, IgG (Solano-Gallego et al., 2003), due to host-response, can help during the follow-up of the leishmaniotic patient. A decrease of albuminuria could also indicate a downgrading of renal damage.

SDS-PAGE enabled the visualization of many protein bands due to the higher resolution power and the higher sensitivity of silver staining, particularly in healthy samples where low abundance proteins were completely undetectable by HRE. In this study we achieved an optimal separation of medium to low MW proteins in healthy samples as compared with data reported in literature where only uromodulin and albumin were reported (Figure 11) (Zaragoza et al., 2003; Raila et al., 2007). Accordingly, in our study the most represented protein in urine samples from healthy dogs was uromodulin, the most consistent band of high MW. Uromodulin is a glycoprotein produced in the thick ascending limb of Henle's loop and in the early distal convoluted tubules. Uromodulin is involved in different physiological, e.g. water reabsorption, immunosuppression, protective role against bacterial infection, and pathological, e.g. urolithiasis, tubulointerstitial nephritis, processes (Youhanna et al., 2014). The role of uromodulin in dog urine is still not completely known and some studies highlighted a decrease of this protein in dogs affected by renal disease (Forterre et al., 2004; Raila et al., 2007; Buono et al., 2012). In the present study, SDS-PAGE coupled with MS was essential to demonstrate the decrease of uromodulin in Leish dogs. Moreover, some patients showed an increase of uromodulin during treatment, indicating a downgrading of tubular damage. Therefore, this protein could be used during the follow-up of patients affected by leishmaniasis as a biomarker of positive response of the pharmacological treatment.

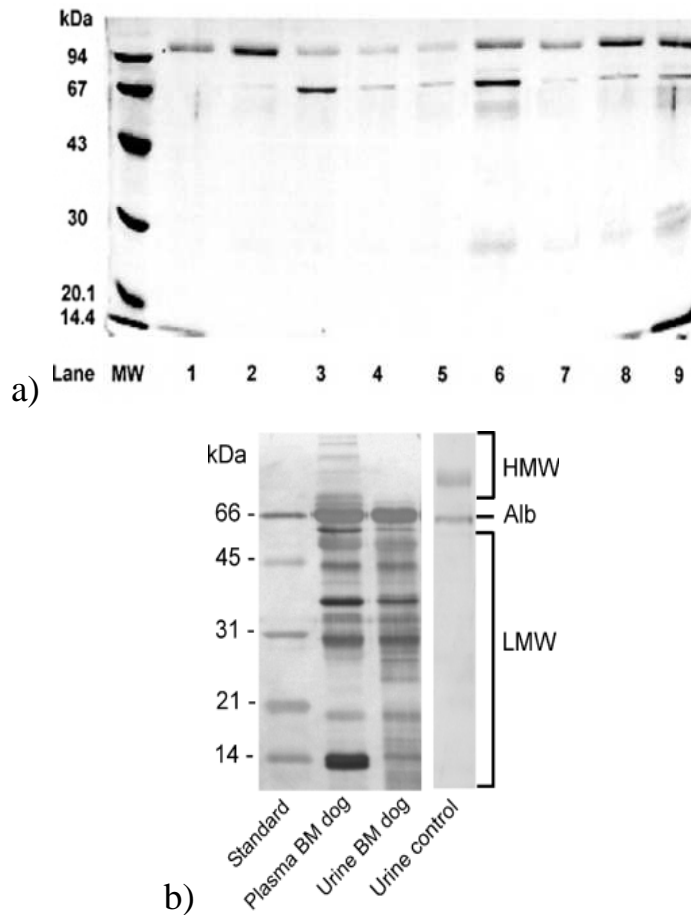


Figure 11. SDS-PAGE of dog urine samples. a) Zaragoza et al., (2003). b) Raila et al., (2007).

Among the proteins identified in Leish dogs was found arginine esterase (ESTA). ESTA is a member of the kallikrein gene family secreted by the canine prostate and has 58% homology with the human prostate specific antigen (PSA). PSA is a known marker of prostate carcinoma in men but its use in dogs is still controversial. ESTA is the major secretory product of the canine prostate and seems to be a diagnostic marker of non-neoplastic prostatic disorders (Gobello et al., 2002). Other authors found ESTA activity of normal canine bladder and in transitional cell carcinoma (LeRoy et al., 2004). Recently ESTA has been identified also in urine of male dogs (Schellenberg and Mettler, 2008; Hormaeche et al., 2014; Miller et al., 2014). We cannot exclude the presence of ESTA as a finding related

to the presence of entire male dog in the Leish group. Nevertheless, its role in the canine prostate disorders is not yet understood and additional studies are needed to unveil the role of ESTA also in dogs urine.

Among the other proteins evidenced by SDS-PAGE comparing to data reported in literature for humans, dogs and cats we could hypothesize the presence of different proteins that showed different intensities between healthy and Leish dogs. The band at 76 kDa (putative transferrin) present also in healthy dogs increased in Leish dogs as well as the bands at MW>100 kDa. The presence these proteins in urine samples could be an important finding to highlight the glomerular damage as reported in literature (D'amico and Bazzi, 2003).

Other bands of apparent MW of 55 and 26 kDa (putative heavy and light chains of IgG) were found in our patterns; the presence of these protein bands could be considered as markers of glomerular damage as previously described in the study conducted by Zaragoza et al. (2003) on patients affected by leishmaniasis.

In Leish dogs we found many bands in the MW range between 55 and 17 kDa. Among them the only one identified by MS was haptoglobin (41 kDa). Other proteins that could be present due to their MW and their possible role as biomarkers are alpha-1-microglobulin (A1mG) (33 kDa) and retinol binding protein (RBP) (22kDa). A1mG is a clinical marker for the detection and differentiation of proteinuria. Physiologically, A1mG passes the glomerulus and is subsequently reabsorbed by the tubule. High levels of A1MG in urine indicate a tubular dysfunction and has been highlighted in dogs with nephropathy (Yalçın and Çetin, 2004). RBP

is an interesting marker of tubular impairment recently studied in dogs (Maddens et al., 2010) and cats (van Hoek et al., 2009).

In addition, other proteins at low MW (<14kDa) were found in our samples. Woo et al., (1991) characterized human proteinuria with SDS-PAGE evidencing a correlation between the presence of low MW bands and a high incidence of chronic renal failure, tubular atrophy and tubulo-interstitial lesions. Bazzi et al., (1997) found an association between the presence of low MW proteins and high serum creatinine concentration, pathological proteinuria and severe tubulo-interstitial damage in patients with a primary glomerulonephritis. Furthermore in the same study they found a positive correlation between the presence of low MW bands and chronic renal failure progression and was also assessed that the response to pharmacological treatment was lower in patients with low MW proteins.

2.2.2. CAT URINE PROTEOME

2.2.2.1. MATERIALS AND METHODS

The present study on cat urine proteome is published on The Veterinary Journal and can be cited as: Ferlizza et al., 2015. The effect of chronic kidney disease on urine proteome in the domestic cat (*Felis catus*). Vet J. Doi: 10.1016/j.tvjl.2015.01.023

Animals and samples

For the present study healthy cats and cats affected by chronic kidney disease (CKD) were selected. The healthy group included entire cats presented to the Veterinary Teaching Hospital of the Department of Veterinary Medical Sciences – University of Bologna for neutering. Only animals considered healthy on the basis of history and physical examination and with no history of urinary tract diseases were included. For the diseased group were selected cats with CKD diagnosed on the basis of history, clinical signs, clinico-pathological and imaging results, according to Bartges, (2012). In particular, cats had to have (1) clinical findings of CKD, (2) persistent pathological renal proteinuria based on the urine protein to creatinine ratio, assessed and confirmed over a two-month period (UPC > 0.2), and (3) a serum creatinine concentration ≥ 1.60 mg/dL and urine specific gravity (USG) < 1.035. CKD cats were staged according to International Renal Interest Society (IRIS) guidelines³. 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

³ <http://www.iris-kidney.com/guidelines/>

urine were collected from each animal by ultrasound-guided cystocentesis and analyzed with the following standardized protocol:

- urinalysis (USG and dipstick);
- centrifugation at 1,500 g for 10 minutes;
- microscopic analysis of urine sediment;
- protein quantification with UPC ratio calculation;

The supernatants were immediately stored at -80 °C for the subsequent analysis by high resolution electrophoresis (HRE), SDS-PAGE and 2DE.

Urinalysis (USG, dipstick and sediment)

Each urine sample underwent complete routine urinalysis including determination of the urine specific gravity (USG) by a refractometer, semi-quantitative dipstick test (Combur10Test, Roche Diagnostic) and microscopic sediment analysis at low (100x) and high (400x) power field.

Protein quantification and urine protein to creatinine ratio (UPC)

Urine total proteins (UTP) 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: $UPC = \text{urine protein (mg/dL)} / \text{urine creatinine (mg/dL)}$.

High resolution electrophoresis (HRE)

Urine samples were analyzed on precast 0.8 % agarose gel at pH 8.6 (HydraGel 15 HR, SEBIA) for quantification of albuminuria as

previously described (Ferlizza et al., 2012). The kit was used in combination with the semi-automated HYDRASYS system (SEBIA). The gels were subsequently stained with an acid violet solution, dried and scanned by a densitometer (EPSON PERFECTION V700 PHOTO) yielding the pherogram (Phoresis 6.1.2 software). The relative percentage and absolute concentration of albumin were calculated based on the density determined by the densitometer. The limit of detection of this technique is 5 mg/dL for urine albumin with an optimal UTP of 200 mg/dL. Urine sample with UTP higher than 200 mg/dL were diluted in order to achieve the desired optimal concentration.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Urine proteins were separated using the NuPAGE system (Thermo Fisher Scientific) on 4-12% polyacrylamide gel in 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer with sodium-dodecyl-sulphate (SDS) (Thermo Fisher Scientific). Two µg of proteins for each sample were loaded. The gels were stained with SilverQuest (Thermo Fisher Scientific). After staining, each gel was digitalized and its pherogram was obtained using GelAnalyzer 2010 software⁴. 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 SDS-PAGE with the protocol reported above, with the exception of 3-(*N*-

⁴ <http://www.gelanalyzer.com/>

morpholino)propanesulphonic acid (MOPS) buffer and Coomassie blue staining (PageBlu protein staining solution; Thermo Fisher Scientific) compatible with mass spectrometry analysis.

Two-dimensional electrophoresis (2DE)

Two-dimensional electrophoresis was performed at the Interdisciplinary Centre of Marine and Environmental Research, University of Porto, and at the Chemical and Biological Technologies Institute of the New University of Lisbon.

Urine samples from four healthy and four CKD cats were selected for 2DE. 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 Coomassie Brilliant Blue (CBB). 2DE gels were digitalized in a GS-800 calibrated densitometer (Bio-Rad) and the images analyzed by Progenesis SameSpot software (Non-Linear

Dynamics) as described (Cruz DE Carvalho et al., 2014). All gels were aligned to the reference gel and the spots with an area less than or equal to 350 and an average normalized volume of less than or equal to 2000 were removed from the spot analysis. The volume of each spot over the volume of all spots in the gel was used for comparison of all groups by an analysis of variance (anova) test for n observations ($n=4$) to assess quantitative differences.

Protein identification by mass spectrometry

Protein identification was performed at the Department of Diagnostic, Clinical and Public Health Medicine, University of Modena and Reggio Emilia. 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 database⁵ 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 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).

Statistical analysis

Data were analyzed with statistical software (MedCalc Statistical Software version 12.7.5) and expressed as median and (range) or mean \pm standard deviation (SD). The different variables (albumin mg/dL, UPC, UAC, age, number of bands) tested for normality with the Shapiro-Wilk normality test and were compared using the Kruskal-Wallis one-way analysis of variance assuming $P<0.05$ as a significant probability. Samples with urine albumin lower than 5 mg/dL were considered as 4.9 and the UAC were calculated according to this value.

⁵ www.uniprot.org

2.2.2.2. RESULTS

Animal selection and UPC

Twenty-three entire 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).

Seventeen cats (5 neutered females, 8 neutered males and 4 entire males) were included in the CKD group. CKD cats were significantly older with a median age of 168 months (60-240; $P<0.01$) and had a significantly increased UPC value (median 0.9; 0.25-6.5) than healthy cats ($P<0.01$). Plasma biochemistry and urinalysis data are reported in Table 1.

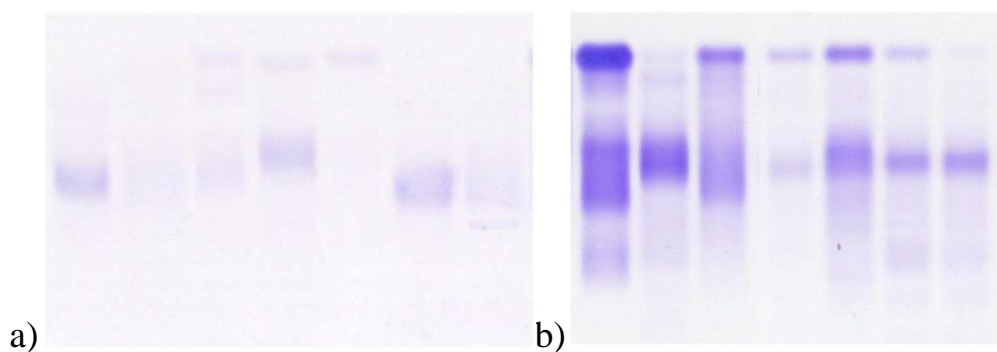
HRE and albuminuria

Figure 1 reports HRE of urine samples of healthy and CKD cats with the respective pherograms. In urine samples with total protein concentration higher than 40 mg/dL it was possible to separate different protein fractions. Different profiles between healthy and CKD cats were obtained. In particular, healthy urine samples showed a faint band of albumin and a diffused band between alpha and beta zone. Differently, CKD cats showed more evident bands and in some cases the pherogram was similar to the serum electrophoretic profile.

Table 1. 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
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	1.29±1.52	14(82)>	<0.4
USG	1.018±0.012	15(88)<	>1.035 ^a
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)	

RI, reference interval; UPC, urine protein to creatinine ratio, USG, urine specific gravity.^a considered as adequate USG in cats.



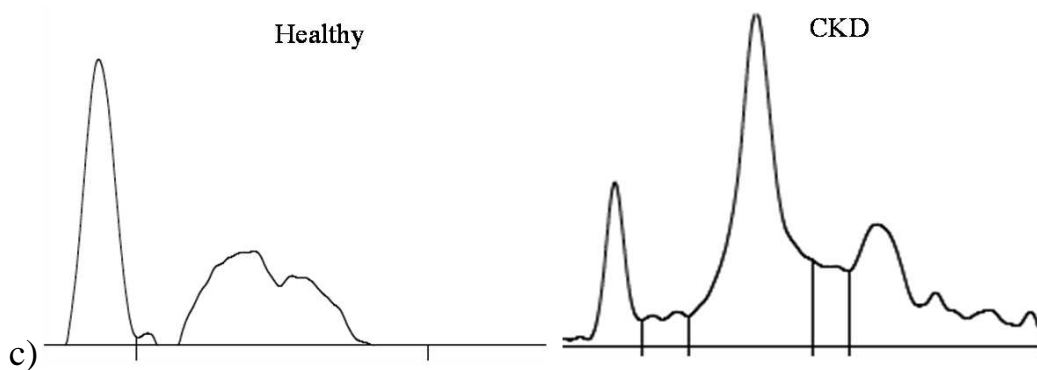


Figure 1. HRE of (a) healthy and (b) CKD urine samples with respective pherograms (c)

HRE allowed the quantification of albuminuria as previously reported (Ferlizza et al., 2012) and data for albumin concentration in mg/dL and UAC are reported in Table 2. Healthy group showed significant lower values of albumin mg/dL and UAC ($P>0.01$) than CKD (Figure 2).

Table 2. Data for albumin concentration in mg/dL and UAC for healthy and CKD cats.

	HEALTHY		CKD	
	Mean±SD	Median (range)	Mean±SD	Median (range)
ALB mg/dL	9.22±7.84	4.9 (4.9-33.1)	21.3±28.3	9.5 (4.9-116.9)
UAC	0.029±0.030	0.018 (0.008-0.12)	0.25±0.29	0.18 (0.05-1.3)

UAC, urine albumin to creatinine ratio

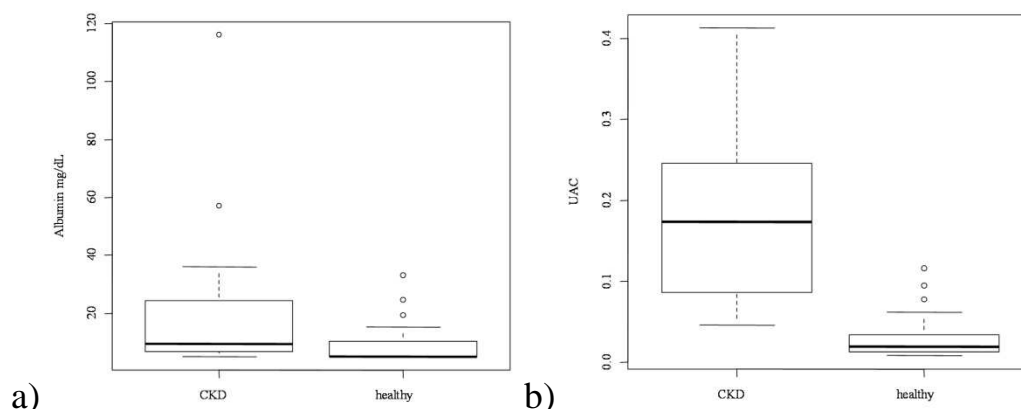
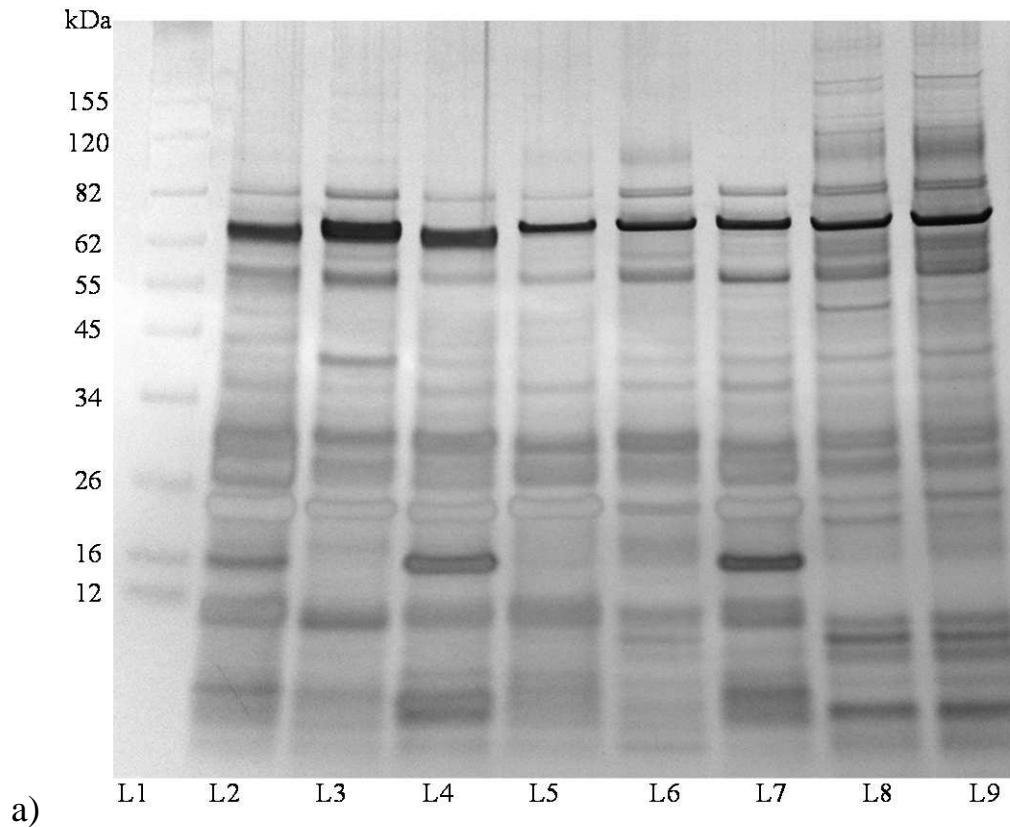
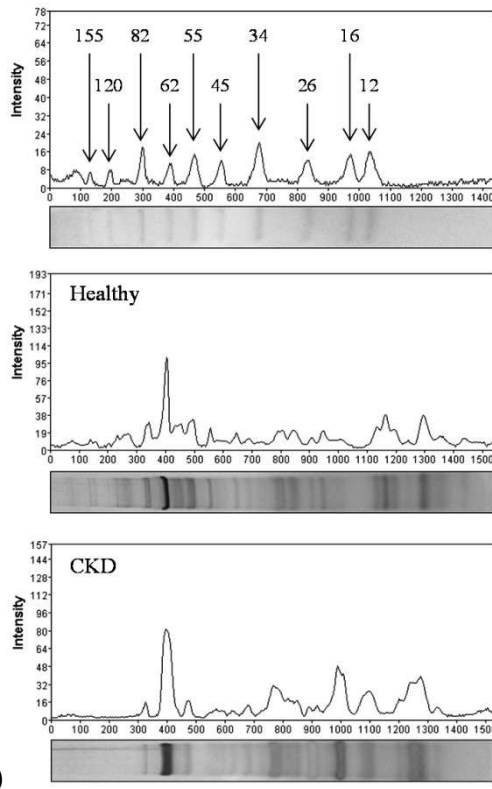


Figure 2. The plots reported data for albumin concentration in (a) mg/dL and (b) UAC for healthy and CKD cats. For a better visualization the highest UAC value for CKD (1.3) was excluded from the graph.

SDS-PAGE

Representative gels and pherograms from healthy and CKD cats are reported in Figure 3. 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 protein bands at $MW < 64$ kDa. A significant decrease of the total number of bands (25 ± 6) ($P < 0.01$) was observed (Figure 4a), particularly at MW higher than 100 kDa ($P < 0.01$) (Figure 4b and 5b).





b)

Figure 3. SDS-PAGE of cat urine samples. a) Representative gel (lane 1, MW marker; lanes 2–7, urine samples from CKD cats; lanes 8–9, healthy urine samples) and (b) pherograms are shown.

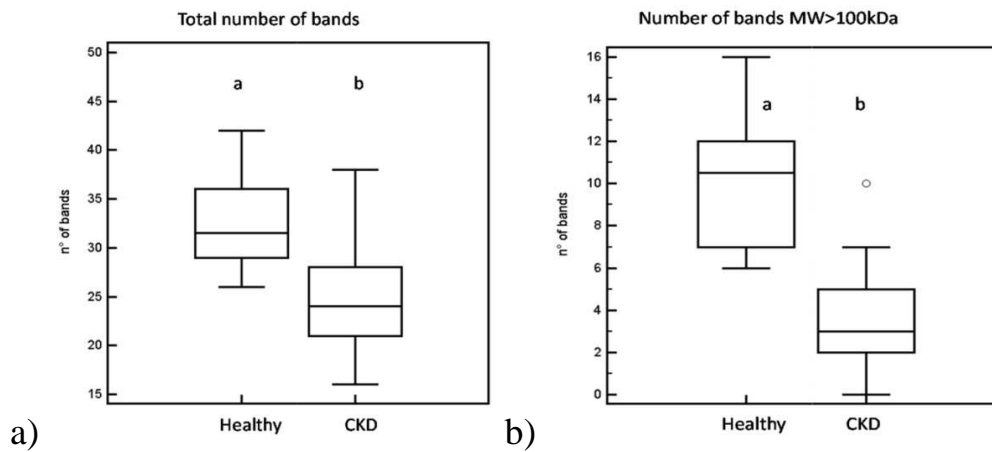


Figure 4. Comparison of the number of protein bands between healthy and CKD cats. (a) Total number of bands. (b) Number of bands with MW > 100 kDa. Different lower cases indicate significant differences ($P < 0.01$).

No significant differences were found between pooled urine samples collected from healthy males and females (Figure 3a). In Figure 5 is reported the gel used for the preparative separation of urines from two

CKD samples (lanes 2-3) and from male and female pooled urine (lanes 4-5 respectively). The most representative and reproducible protein bands (n=16) from healthy and CKD samples were excised from the gel for MS identification (Figure 5, Table 3).

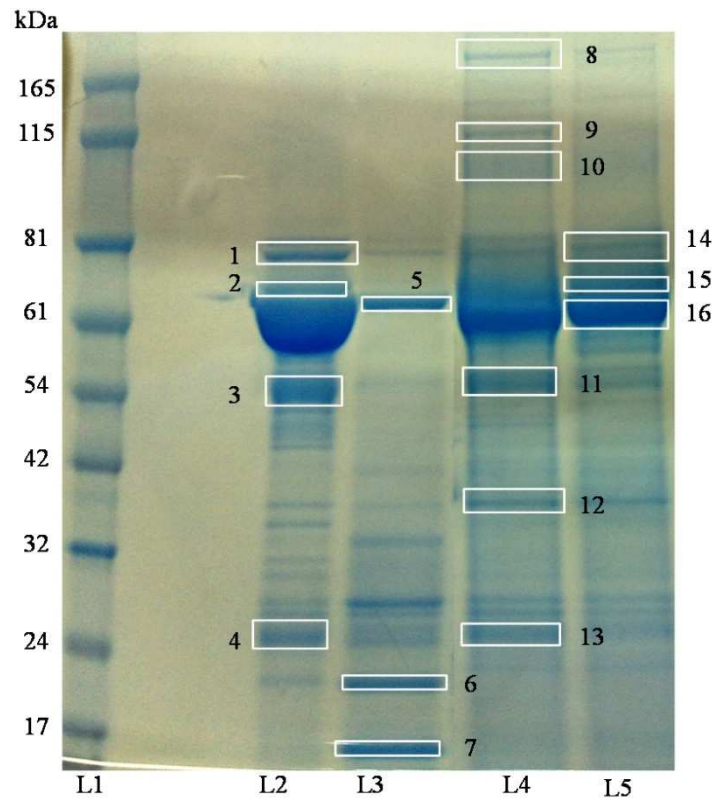
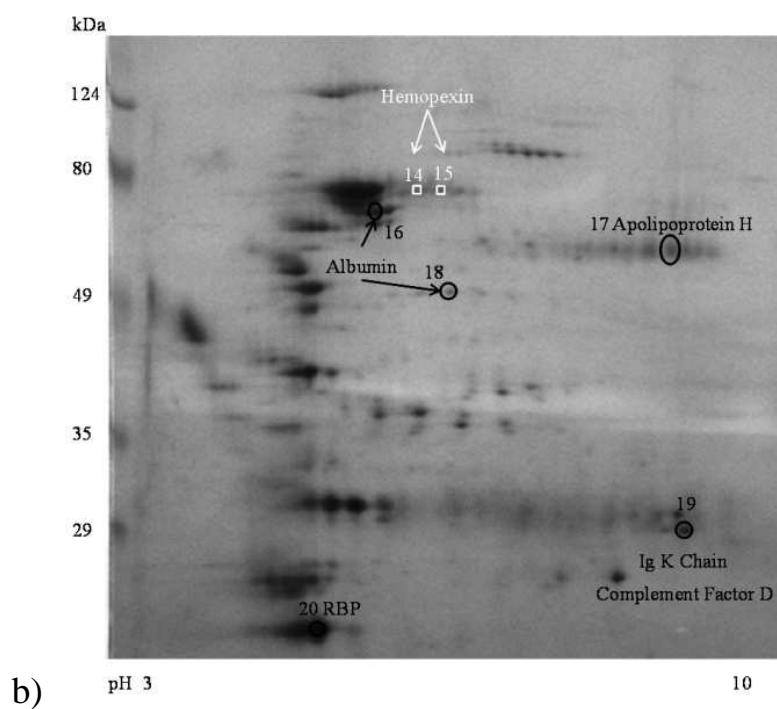
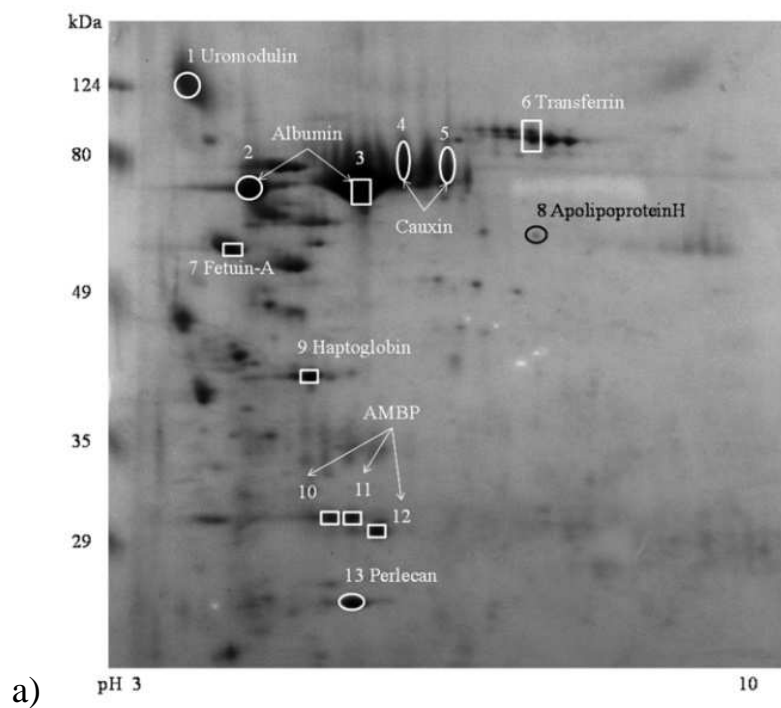


Figure 5. SDS-PAGE of urine samples from healthy and CKD cats. Lane 1, MW marker; lanes 2-3, CKD urine samples; lanes 4-5, pools of urine from healthy females and males respectively. Rectangles and numbers indicate the bands that have been cut and identified by ESI-Q-TOF.

2DE and differential proteomics study

Figure 6 reports representative 2DE obtained from healthy (Figure 6a) and CKD entire cats (Figure 6b). 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 more evident 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.



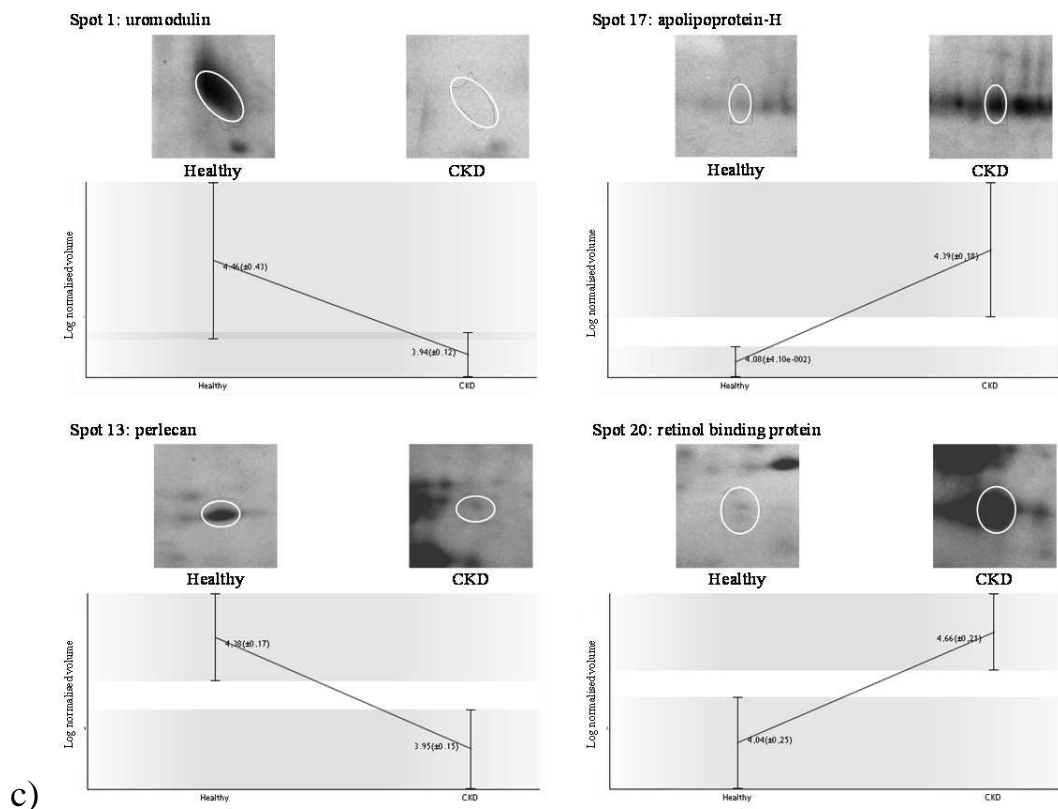


Figure 6.2DE of the urine proteome in (a) healthy and (b) CKD entire cats. White circles: spots with significantly greater intensity in healthy than in CKD; black circles: spots with significantly greater intensity in CKD; white rectangles: common spots without significant differences. (c) Examples of important differentially expressed proteins.

Protein identification by mass spectrometry

From the 16 bands excised from SDS-PAGE (Figure 5), 14 proteins were unambiguously identified (Table 3). Out of the 36 2DE spots analyzed, 20 yielded significant results by MS, allowing the successful identification of 13 different proteins (Figure 6a, b; Table 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 SDS-PAGE gel were confirmed by 2DE, namely uromodulin, albumin, transferrin, cauxin, haptoglobin, retinol binding protein (RBP) and immunoglobulin K light chain (IgK).

The identified proteins yielded a preliminary cat urine map, including 20 proteins that may be functionally classified as transport (25%), immune and cellular response (30%), protein metabolism (25%), and cellular communication and growth (15%) (Figure 7a). Most of the identified proteins were classified as extracellular (75%) (Figure 7b).

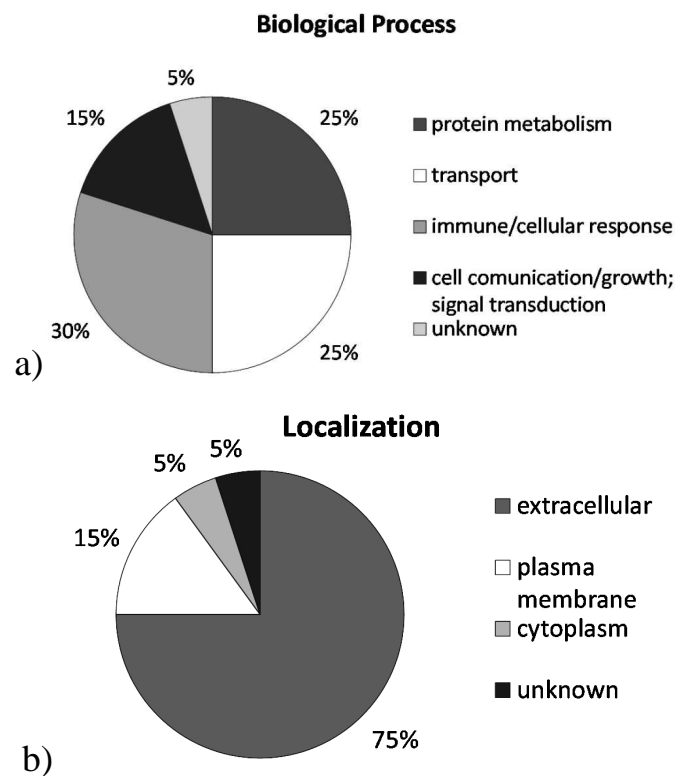


Figure 7. Classification of the proteins identified according to Gene Ontology and the Human Reference Proteome Database (HRPD). a) Biological process; b) localization.

Table 3. Proteins identified in cat urine by mass spectrometry.

Band 1DE ^a	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
	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
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
	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 2DE ^a	Entry name ^b	Protein full name	MW ^c (kDa)	pI	Score ^d	Pept. ^e	Seq. ^f	Sign. Seq. ^g	Identity ^h
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_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 Figure 5 and 6 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.

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 4). Examples of differentially expressed spots are reported in Figure 6c. The other proteins did not show significant differences between groups.

Table 4. Differentially expressed proteins identified by mass spectrometry (ESI-Q-TOF).

Band ^a SDS- PAGE	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-carboxypeptidase 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 2DE					
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	RET4_HUMAN	Retinol-binding protein 4	Up	Transporter	Transport

^a Number of the identified band or spot as marked in Fig 5 and 6 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.

2.2.2.3. DISCUSSIONS

The first aim of this work was the characterization of the urine proteome in healthy cat by three electrophoretic methods and the establishment of the urine proteome reference map in cats.

HRE allowed a first screening of the urine proteome in cats highlighting different pherograms between healthy and CKD and significant higher values of albuminuria in CKD cats. In human patients proteinuria and albuminuria have been identified as independent risk factors for cardiovascular mortality as well as diabetic nephropathy and CKD. Early detection of low levels of albuminuria (microalbuminuria) and the early setting of a therapy could therefore slow the progression of the renal damage (Wu et al., 2012). Some studies suggested the usefulness of microalbuminuria as an accurate marker of early renal disease also in dogs (Bacic et al., 2010; Smets et al., 2010) and cats (Syme et al., 2006; Hanzlicek et al., 2012). In addition to primary kidney disease, other conditions have been reported in dogs with albuminuria, including non-infectious inflammatory disease, hyperadrenocorticism and critically ill (Whittemore et al., 2006; Vaden et al., 2010; Smets et al., 2012). The studies on the prevalence of albuminuria in cats are fragmentary and are restricted to cats affected by diabetes mellitus or systemic disease (Whittemore et al., 2007; Al-Ghazlat et al., 2011). Due to its simple and fast protocol, HRE could be a useful tool to rapidly separate urine proteins and to quantify albuminuria particularly when UTP<40 mg/dL. More sensitive automatic analyzers and a sensitive method specific for cat albumin are needed to quantify low levels of albuminuria.

SDS-PAGE yielded an overall view of the protein profile highlighting 32 different bands in healthy samples, different profiles in CKD cats and no differences between males and females. In this point of view cats are different from dogs: in male dogs urine contains significant amounts of arginine esterase (Miller et al., 2014). When

coupled to MS, SDS-PAGE allowed the identification of 14 different urine proteins, e.g. uromodulin, largely studied in human and identified also in dogs (Nabity et al., 2011).

2DE 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 feline 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 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 hypothesized. However, most of these differentially expressed proteins are indicative of tubular dysfunction (e.g. RBP, CYSM, 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 tubules can stimulate the cells of the immune system causing inflammation and CKD progression (El-Achkar and Wu, 2012). 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. 2DE was essential to obtain the separation of albumin from cauxin, demonstrating a significant decrease of cauxin, however a possible influence of the

entire/neutered status cannot be completely excluded. Though Jepson et al., (2010) showed a weak correlation of cauxin with the future onset of azotemia, our data, according with Miyazaki et al., (2007), suggest this protein could be considered a promising biomarker for the determination of tubular damage in CKD cats particularly in entire male cats. 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.

2.3. FARM ANIMALS

2.3.1. BOVINE URINE PROTEOME

2.3.1.1. MATERIALS AND METHODS

Animals and samples

For the present part of the study dairy cows and heifer were selected. The animals were housed in University Dairy Farm of the Department of Veterinary Medical Sciences – University of Bologna. The animals were in good health status and periodically underwent to physical examination and routine analysis. Urine samples were collected in the early morning by manual stimulation of the vulva to elicit micturition and collected in clean urine container. Urine samples were collected monthly for one year from December 2012 to November 2013 and analyzed with the following standardized protocol:

- urinalysis (USG and dipstick);
- centrifugation at 1,500 g for 10 minutes;
- microscopic analysis of urine sediment;
- protein quantification with UPC ratio calculation;

The supernatants were immediately stored at -80 °C for the subsequent analysis by SDS-PAGE.

Urinalysis (USG, dipstick and sediment)

Each urine sample underwent complete routine urinalysis including determination of the urine specific gravity (USG) by a refractometer, semi-quantitative dipstick test (Combur10Test, Roche

Diagnostic) and microscopic sediment analysis at low (100x) and high (400x) power field.

Protein quantification and urine protein to creatinine ratio (UPC)

Urine total proteins (UTP) 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: $UPC = \text{urine protein (mg/dL)} / \text{urine creatinine (mg/dL)}$.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Urine proteins were separated using the electrophoresis NuPAGE system (Thermo Fisher Scientific) on 4-12% polyacrylamide gel in 3-(*N*-morpholino)ethanesulphonic acid (MES) 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 digitalized and the pherogram was obtained using GelAnalyzer 2010 software⁶.

Statistical analysis

Data were analyzed with statistical software (Excel 2007 and R version 2.15.1) and expressed as mean \pm standard deviation (SD). The different variables (UPC, USG and number of bands) were tested for normality with Shapiro-Wilk normality test and distribution was

⁶ <http://www.gelanalyzer.com/>

considered normal for $P>0.05$. Variables non normally distributed were normalized by exponential transformation. Variables with normal distribution were analyzed by t-test (difference between heifers and cows), one way ANOVA (difference between pregnancy trimesters) and two way ANOVA (trend analysis difference between heifers and cows for each variable).

2.3.1.2. RESULTS

Animals and samples

Fourteen dairy cows (8 heifers and 6 cows) were included in the study. From the 8 heifers 62 samples and from the 6 cows 35 samples were collected respectively. Heifers and cows both showed urine pH between 8 and 9 without any significant finding in the sediment analysis. Heifers showed mean USG of 1.032 ± 0.006 significantly higher ($P<0.01$) than cows (1.026 ± 0.004) (Figure 1a). The trend of USG was not significantly different between the two groups (Figure 1b), hence USG values of heifers and cows were analyzed together to evaluate the difference between the pregnancy trimesters (tr0, before pregnancy; tr1, first trimester; tr2, second trimester; tr3, third trimester; tr4, post-calving). USG during the second trimester (1.033 ± 0.005) was significantly higher ($P<0.05$) than before pregnancy (1.028 ± 0.003) and post-calving (1.026 ± 0.007) (Figure 1c).

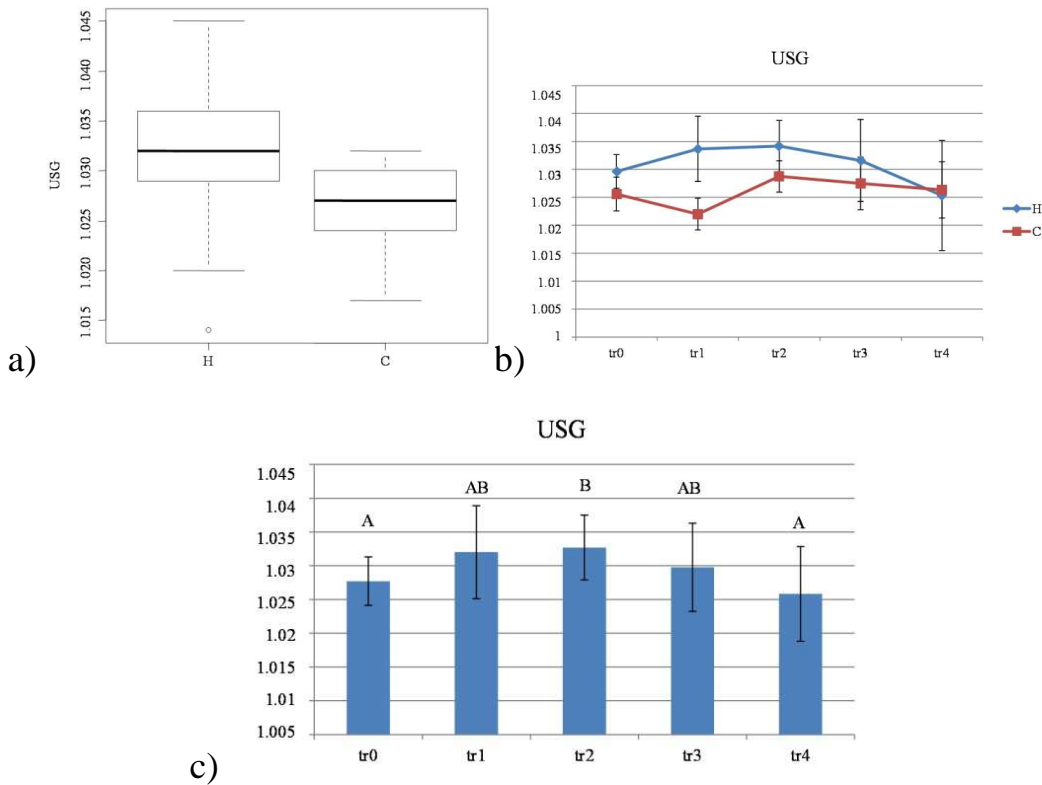


Figure 1. Graphs reporting USG data for heifers and cows. a) Comparison between USG data for heifers (H) and cows (C) ($P < 0.05$); b) Comparison of USG trends during pregnancy between heifers and cows. c) comparison between USG data during pregnancy trimesters. Tr0, before pregnancy; tr1, first trimester; tr2, second trimester; tr3, third trimester; tr4, post-calving. Different capital cases indicate significant differences ($P < 0.05$).

Mean UPC in heifers was 0.12 ± 0.03 , significantly lower than cows (0.17 ± 0.06) ($P < 0.01$) (Figure 2a), while the trend was not different between groups (Figure 2b). Therefore, considering together UPC values of heifers and cows to evaluate differences between pregnancy trimesters, values of the second (0.13 ± 0.05) and the third (0.11 ± 0.04) trimesters were significantly lower ($P < 0.05$) than values before pregnancy (0.18 ± 0.07) and post-calving (0.32 ± 0.4) (Figure 2c). Table 1 reported USG and UPC data for heifers and cows during pregnancy.

Table 1. USG and UPC data for heifers and cows during pregnancy.

	UPC		USG	
	Mean±SD (n)		Mean±SD (n)	
	Heifer	Cow	Heifer	Cow
tr0	0.14±0.02 (11)	0.22±0.08 (10)	1.030±0.003 (11)	1.026±0.003 (10)
tr1	0.12±0.02 (12)	0.23±0.11 (2)	1.034±0.006 (12)	1.022±0.003 (2)
tr2	0.11±0.01 (21)	0.19±0.07 (8)	1.034±0.005 (21)	1.029±0.003 (8)
tr3	0.10±0.03 (12)	0.13±0.04 (12)	1.032±0.007 (12)	1.028±0.005 (12)
tr4	0.50±0.56 (3)	0.15±0.03 (3)	1.025±0.010 (3)	1.026±0.005 (3)

Tr0, before pregnancy; tr1, first trimester; tr2, second trimester; tr3, third trimester; tr4, post-calving.

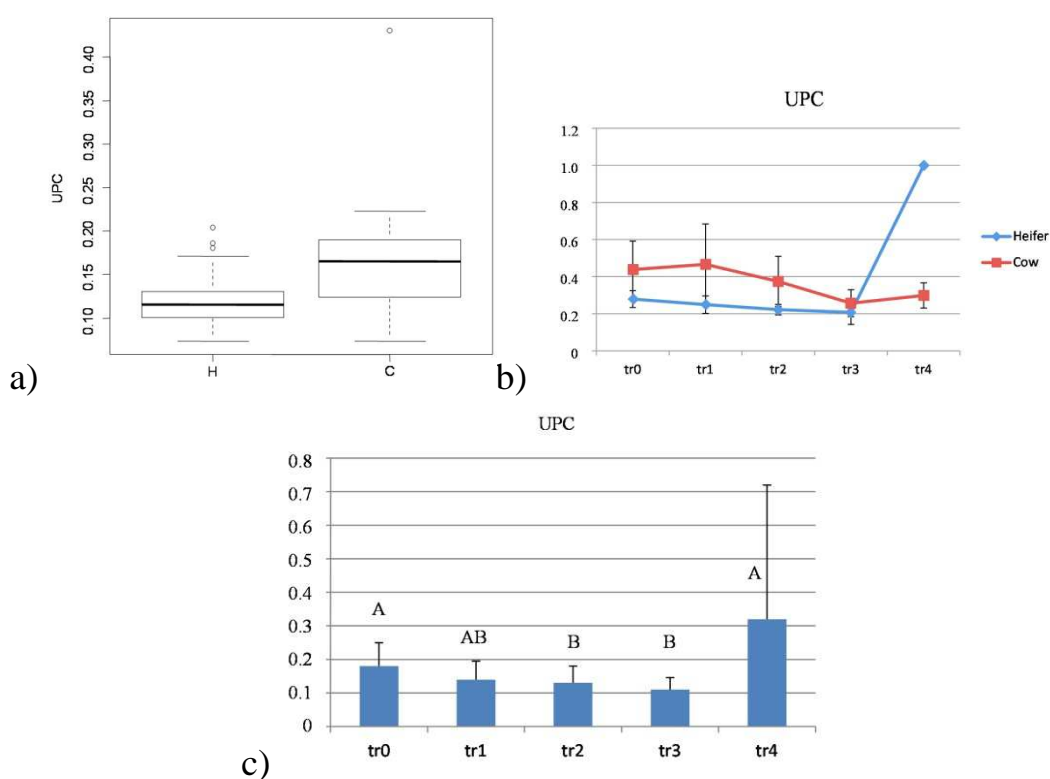


Figure 2. Graphs reporting UPC data for heifers and cows. a) Comparison between UPC data for heifers (H) and cows (C) ($P < 0.05$); b) Comparison of UPC trends during pregnancy between heifers and cows; c) comparison between UPC data during pregnancy trimesters. tr1, first trimester; tr2, second trimester; tr3, third trimester; tr4, post-calving. Different capital cases indicate significant differences ($P < 0.05$). For a better visualization, SD for heifers during tr4 (0.56) in graph (b), has been removed.

SDS-PAGE

Representative gels and pherograms of urine samples from heifers and cows are reported in Figure 3. We separated 17 ± 5 protein

bands in the urine of heifers. The majority had a molecular weight (MW) between 12 and 67 kDa (Figure 3). Cows presented a significant decrease of the total number of bands (13 ± 4) ($P < 0.05$) (Figure 4). Nevertheless, the electrophoretic profile was similar with some common bands (97, 86, 78, 70, 59, 38, 27, 23, 21 kDa and three proteins between 13 e 9 kDa).

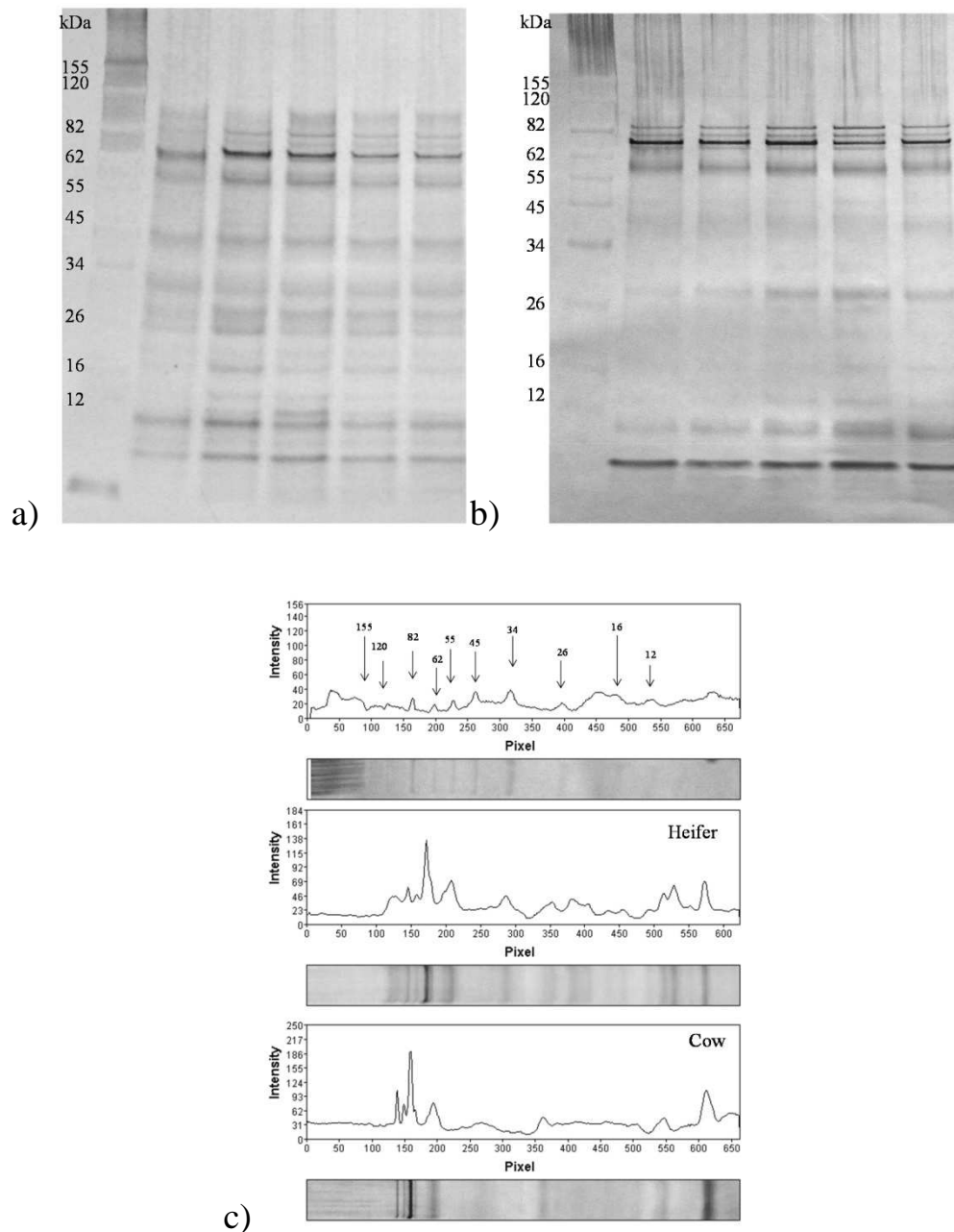


Figure 3. Representative SDS-PAGE of urine samples of (a) heifers and (b) cows. c) Representative pherograms.

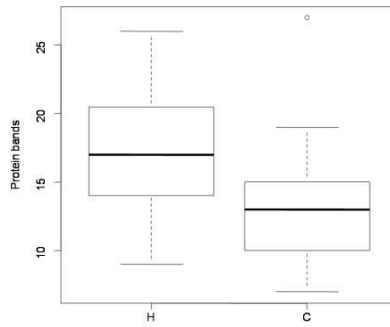


Figure 4. Comparison of total number of bands between heifers (H) and cows (C) ($P < 0.05$).

Regarding the possible proteome variation during pregnancy, non-significant differences were found in the total number of bands between trimesters. However, two bands at 67 and 31 kDa were absent in non-pregnant specimens (Figure 5).

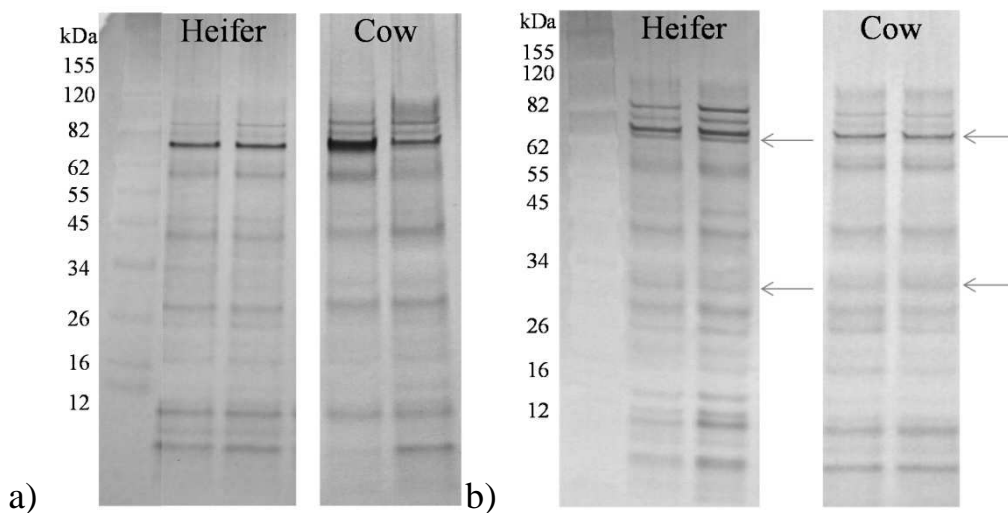


Figure 5. SDS-PAGE of urine samples of heifers and cows non-pregnant (a) and pregnant (b). Arrows indicate bands at 67 and 31 kDa absent in non-pregnant specimens.

2.3.1.3. DISCUSSIONS

Cows are an important source of income, hence many paper focused on the quality of meat and milk (Maulfair et al., 2010). However, data on the urinary proteins and their usefulness in clinical

practice are fragmentary (Adamu et al., 2007; Defontis et al., 2013; Parrah et al., 2013). Therefore, the aim of this study was to evaluate the main urine parameters (USG and UPC) and to evaluate the application of SDS-PAGE on the urine proteome of dairy cows.

Regarding USG, the range we measured resulted similar to what reported by Parrah et al., (2013) but wider than the range reported by The Merck Veterinary Manual (1.030-1.045). Furthermore, heifers presented USG values significantly higher than cows, according to the different water and food consumption between the two groups (Spek et al., 2012).

As regards UPC, only Defontis et al., (2013) tested two dipsticks to evaluate proteins in cows, but without selecting for age or clinical conditions. Data on urine total protein (UTP) in cows are scarce, however, Adamu et al., (2007) reported low values of UTP similar to our data. Our data on UPC as compared with IR for dogs and cats can be considered non-proteinurics ($UPC < 0.2$) (Lees et al., 2005).

SDS-PAGE enabled the visualization of many protein bands in urine from both heifers and cows and the profiles showed some common characteristics. First, the most represented bands were between 97 and 8-7 kDa according to Pyo et al., (2003) and different from what reported in humans (Zheng et al., 2013), rats (Calzada-García et al., 1996) and cats (Ferlizza et al., 2015). In humans, the urine proteome showed a wider distribution of MW range with protein at very high MW (>130 kDa), large quantity of small peptides with $MW < 3$ kDa, while the most represented proteins have MW included in the range of 20-50 kDa (Zheng et al., 2013) (Figure 6). Furthermore, the proteins present in bovine urine proteome are mainly

acidic with an isoelectric point (pI) between 3.5 and 5.5 (Pyo et al., 2003), different from humans that present proteins with a wider range of pI (4-12.5) (Zheng et al., 2013) (Figure 6).

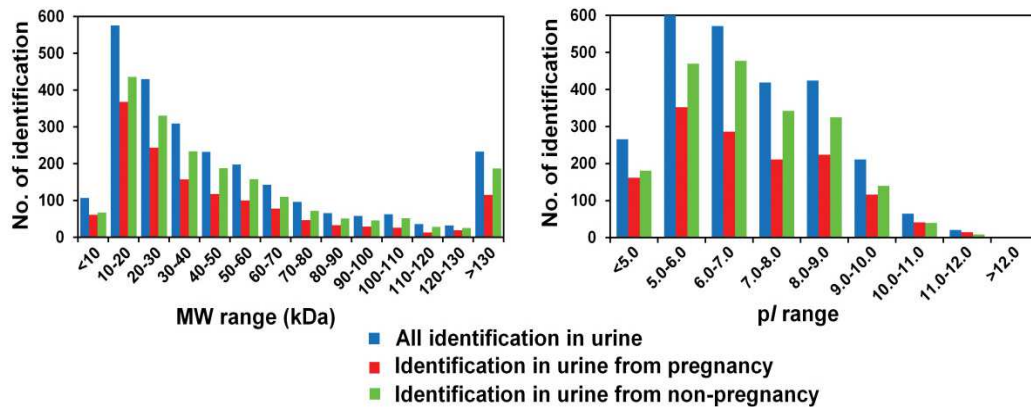


Figure 6. Proteins MW distribution and pI range in urine of pregnant and non-pregnant women (Zheng et al., 2013).

We could not identify the cow urine proteome by MS, as a consequence we made only hypothetical identification comparing the MW of the protein bands in the gels with data reported in literature for humans, dogs and cats. In particular in human medicine the most abundant proteins in urine are: uromodulin, albumin, immunoglobulins, alpha-1-antitrypsin, transferrin and haptoglobin. In addition, at least other 18 high abundance proteins are present, such as retinol-binding-protein (RBP) (He et al., 2012).

In the present study the band at 97 kDa could be identified as uromodulin (Tamm-Horsfall protein), a glycoprotein produced by the healthy tubular cells of the kidney which biological function is still under investigation (Youhanna et al., 2014). Uromodulin is the most abundant protein in urine of humans and dogs and its use as putative biomarker of nephropathy has been suggested (Lhotta, 2010; Buono et al., 2012). In women the role of uromodulin during pregnancy is still

controversial as for the increase of this protein during the second half of the pregnancy (Jeyabalan and Conrad, 2007). Differently, in the samples analyzed in the present study putative uromodulin is less evident than in dogs and humans.

The band at 70 kDa could be albumin, one of the most represented protein in urine of mammals. The MW of albumin is slightly different between species and can be present also with fragments at different MW due to proteolytic activity in urine (Mullen et al., 2011; Ferlizza et al., 2015). As widely discussed for dogs and cats, albumin has a MW near the threshold of the glomerular filtration barrier and is therefore studied as biomarker of glomerular damage.

From the comparison between pregnant and non-pregnant cows some qualitative difference has been highlighted. In particular two bands were absent in non-pregnant specimens at 67 and 31 kDa. Pregnancy induces qualitative and quantitative variations in urine and serum proteome. Zheng et al., (2013) reported that the urine proteome in pregnant women presented 594 unique proteins compared with the post-partum urine proteome (Figure 7). Some of them are used in human medicine as biomarkers of maternal disease or fetal syndrome, e.g. preeclampsia or Down syndrome. Regarding bovine urine proteome, only few proteins have been identified as putative early biomarkers of pregnancy. The most studied is bPAP (bovine pregnancy-associated protein), a 21 kDa protein homologous of the N-terminal of collagen subunit alpha (Pyo et al., 2003)

In the present study the band at 67 kDa was absent in samples from non-pregnant and present in all samples from pregnant specimens. This band could be identified as alpha-fetoprotein (AFP), a protein produced by fetal tissues and belonging to a gene family near

to albumin (Deutsch, 1991). In a previous study, AFP was detected in maternal serum, by means of radio-immunologic assay, to evaluate fetal suffering in pregnant cows (Baetz et al., 1981), while no data are reported for this protein in urine. The same authors suggest that the maternal-fetal transfer of AFP seemed to be minimal also in case of placental inflammation or necrosis. The presence of low amounts of the band at 67 kDa seemed to be in according with the data reported by Baetz 1981.

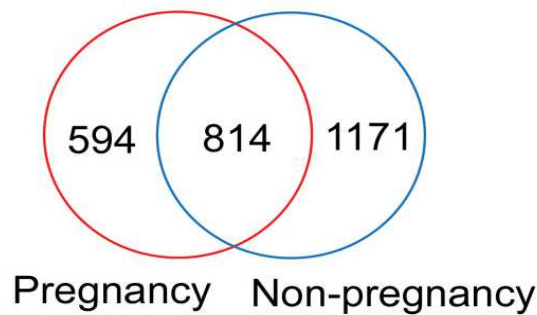


Figure 7. Venn diagram of urine proteomes in pregnant and non-pregnant women (Zheng et al., 2013).

2.3.2. HORSE URINE PROTEOME

2.3.2.1. MATERIALS AND METHODS

Animals and samples

For this study healthy and diseased horses were selected. The healthy group included privately owned horses near Forlì-Cesena. Only animals considered healthy on the basis of history and physical examination and with no history of urinary tract diseases were included. In some of the healthy horses urine samples were collected after exercise to evaluate possible influence on the urine proteome. The diseased group comprised horses presented to the Veterinary Teaching Hospital of the Department of Veterinary Medical Sciences-University of Bologna for different clinical conditions. All urine samples were collected by free catch in clean urine container and analyzed with the following standardized protocol:

- urinalysis (USG and dipstick);
- centrifugation at 1,500 g for 10 minutes;
- microscopic analysis of urine sediment;
- protein quantification with UPC ratio calculation;

The supernatants were immediately stored at -80 °C for the subsequent analysis by SDS-PAGE.

Urinalysis (USG, dipstick and sediment)

Each urine sample underwent complete routine urinalysis including determination of the urine specific gravity (USG) by a refractometer, semi-quantitative dipstick test (Combur10Test, Roche Diagnostic) and microscopic sediment analysis at low (100x) and high (400x) power field.

Protein quantification and urine protein to creatinine ratio (UPC)

Urine total proteins (UTP) 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: $UPC = \text{urine protein (mg/dL)} / \text{urine creatinine (mg/dL)}$.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Urine proteins were separated using the electrophoresis NuPAGE system (Thermo Fisher Scientific) on 4-12% polyacrylamide gel in 3-(*N*-morpholino)ethanesulphonic acid (MES) buffer with sodium-dodecyl-sulphate (SDS) (Thermo Fisher Scientific). Two μg of proteins for each sample were loaded. The gels were stained with SilverQuest (Thermo Fisher Scientific). After staining, each gel was digitalized and its pherogram was obtained using GelAnalyzer 2010 software⁷.

Statistical analysis

Data were analyzed with statistical software (Excel 2007 and R version 2.15.1) and expressed as mean \pm standard deviation (SD). The different variables (UPC, USG and number of bands) were tested for normality with Shapiro-Wilk normality test and distribution was considered normal for $P > 0.05$. The different variables (UPC, USG,

⁷ <http://www.gelanalyzer.com/>

number of bands) were compared using the Kruskal-Wallis one-way analysis of variance assuming $P<0.05$ as a significant probability.

2.3.2.2. RESULTS

Animals and samples

Sixty-two urine samples were collected from 37 healthy and 25 diseased horses. The healthy group comprised 17 males and 20 females, with a mean age of 142 ± 92 months. USG ranged from 1.009 to 1.048 with a mean of 1.030 ± 0.008 and a median of 1.032. UPC values ranged from 0.04 to 0.17 with a mean of 0.11 ± 0.03 and a median of 0.11. No difference was found between males and females and between before ($n=25$) and after ($n=12$) exercise for both USG and UPC.

The diseased group comprised 25 horses (19 males, 6 females) with a mean age of 155 ± 89 months. USG ranged from 1.005 to 1.048 with a mean of 1.025 ± 0.011 and a median of 1.028 significantly lower than healthy ($P<0.05$) (Figure 1a). UPC values ranged from 0.02 to 5.5 with a mean of 0.61 ± 1.2 and a median of 0.12, significantly higher than healthy ($P<0.05$) (Figure 1b). The diseased group was further divided in proteinurics and non proteinurics with cut-off at 0.2. The proteinurics were significantly ($P<0.05$) different from healthy and non-proteinurics for both UPC and USG. A summary table is reported (Table 1).

Table 1. USG and UPC values for healthy, non-proteinuric and proteinuric horses. Different lower cases indicate significant difference ($P<0.05$).

		Mean	Median	SD	Range
UPC	Healthy	0.11	0.11 ^a	0.03	0.04-0.17
	Non-proteinurics	0.10	0.10 ^a	0.02	0.07-0.15
	Proteinurics	1.39	0.60 ^b	1.82	0.2-5.5
USG	Healthy	1.031	1.032 ^a	0.008	1.009-1.048
	Non-proteinurics	1.031	1.032 ^a	0.005	1.023-1.040
	Proteinurics	1.017	1.014 ^b	0.014	1.005-1.048

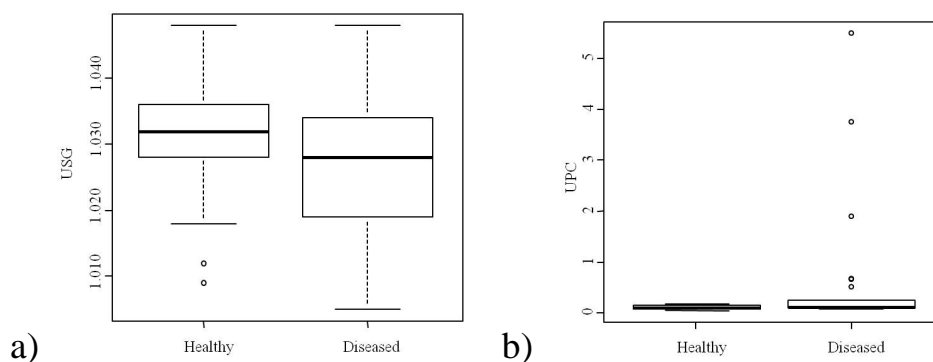


Figure 1. USG (a) and UPC (b) comparison between healthy and diseased horses.

SDS-PAGE

Representative gels and pherograms of urine samples from healthy and diseased horses are reported in Figure 2. We separated 13 ± 4 protein bands in the urine of healthy animals. The majority had an apparent molecular weight (MW) between 12 and 67 kDa. At 90 kDa an intense and diffused band was evident. Moreover, between 77 and 54 kDa 6 protein bands were evidenced in most of the healthy samples as well as in some of the non-proteinurics diseased horses.

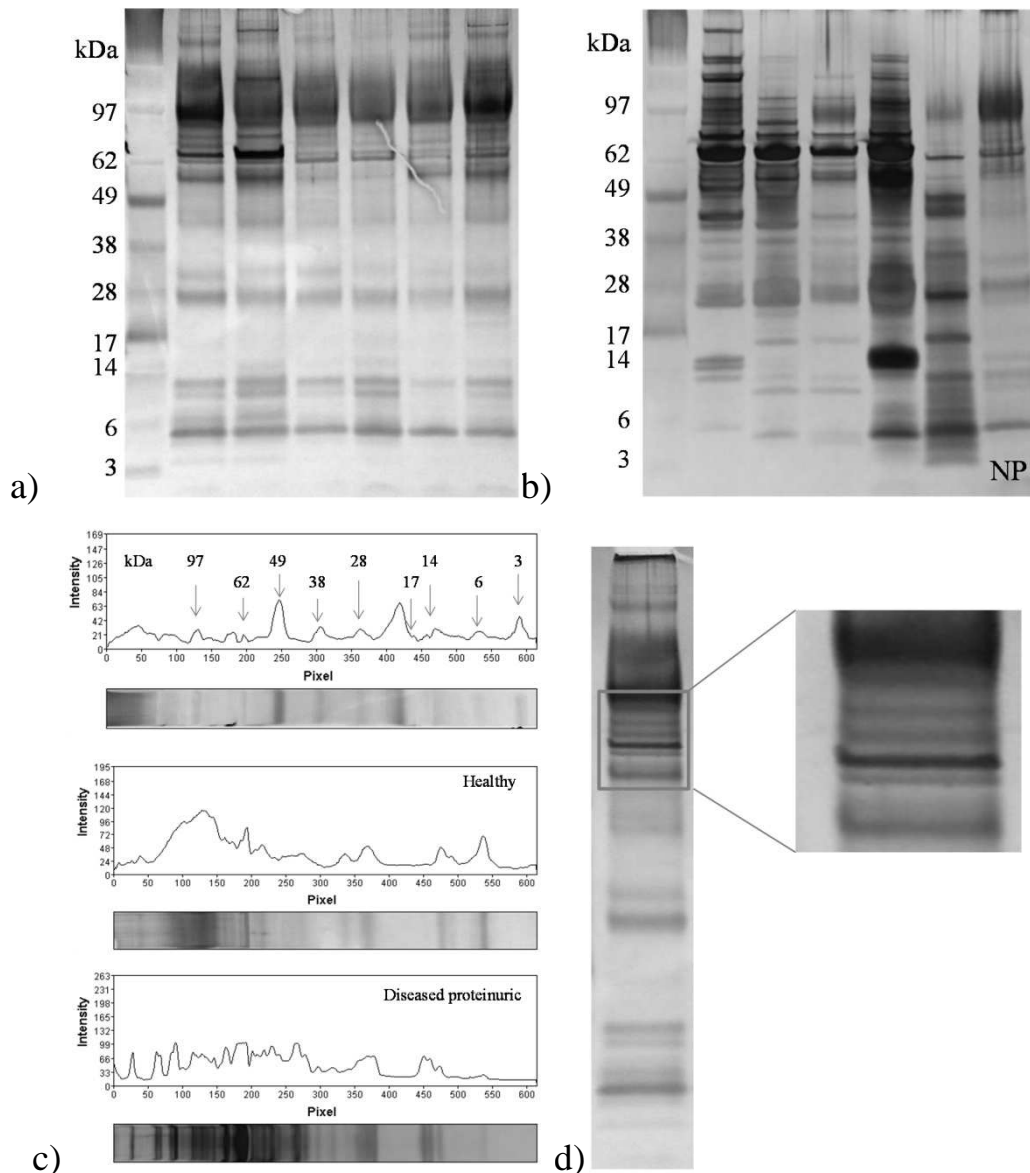


Figure 2. Representative gels of urine samples of (a) healthy and (b) diseased horses; c) representative pherograms; d) detail of the 6 protein bands between 77 and 54 kDa highlighted in healthy samples. NP=non-proteinuric diseased sample

Diseased animals presented a significant increase of the total number of bands (19 ± 7) ($P < 0.05$) (Figure 3) and a more variable electrophoretic profile particularly the proteinuric subgroup. The non-proteinuric group showed an electrophoretic profile similar to healthy group. Proteinuric horses showed a decrease of the band at 90 kDa and the appearance of 2 to 4 protein bands at MW between 90 and 150 kDa. Moreover proteinurics showed more bands at MW between 49

and 17 kDa and the increased intensity of the band at 27 kDa (Figure 2b).

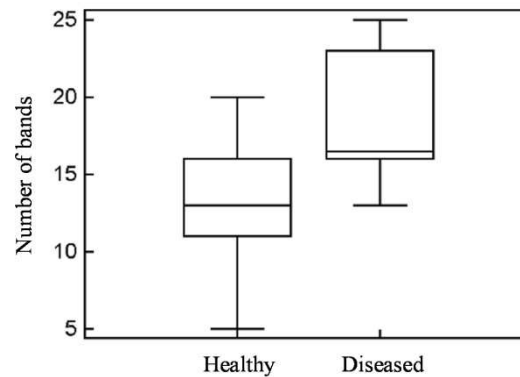


Figure 3. Comparison of total number of bands between healthy and diseased horses ($P<0.05$).

2.3.2.3. DISCUSSIONS

The aim of this research was to evaluate quantitative (UTP, UPC) and qualitative (SDS-PAGE) proteinuria in healthy and diseased horses.

Regarding quantitative proteinuria and in particular UTP, our data are in accordance with data previously reported in literature for healthy horses. Edwards et al., (1989) and Halbmayr and Schusser, (2002) reported mean UTP values lower than 20 mg/dL. To quantify proteinuria the parameter routinely used in clinical practice that need to be evaluated is UPC. Also for this parameter our data (0.17) are consistent with data reported by Uberti et al., (2009) and lower than the cut-off value used in clinical practice for horses (0.2).

SDS-PAGE enabled the visualization of many protein bands and a characteristic profile in urine of healthy horses according to literature (Halbmayr and Schusser, 2002). In particular, comparing the MW of the bands in our samples to literature we can hypothesize the identification of the most represented bands and their clinical relevance in healthy and diseased horses. Halbmayr and Schusser,

(2002) identified a band at 90 kDa as uromodulin and we could hypothesize the presence of this protein in the diffuse band at 90 kDa. According to data reported in humans (Lhotta, 2010) also in horses the putative uromodulin is the most abundant protein in urine of healthy specimens. Its biological function is still not completely known but is considered an important biomarker of tubular impairment. Diseased proteinuric horses showed a decrease of putative uromodulin band suggesting its usefulness as biomarker of tubular function also in horses.

The 6 thin bands at MW between 73 and 57 could be identified as albumin and/or transferrin isoforms. In human urine it has been reported that albumin undergo different post-translational modifications including fragmentation, glycosylation or phosphorylation (Candiano et al., 2008).

The bands at 55 and 27 kDa could correspond to immunoglobulin heavy and light chains identified by Halbmayr and Schusser, (2002). In general, the presence of these bands in urine is considered as indicative of glomerular damage. In our samples, the increase of these bands in proteinuric horses reinforced their identification and could suggest glomerular involvement. However, due to the low number of proteinuric horses and the great variety of diseases it was not possible to correlate other bands to specific disease. Nevertheless, the significant increase of total number of bands let us suggest that SDS-PAGE could be useful to classify proteinuria in glomerular or tubular, similar to what reported for humans, dogs and cats (Abate et al., 2005). Interestingly, one horse with laminitis, was classified as non proteinuric on the basis of UPC value, however the SDS-PAGE profile showed an increase in band of putative albumin (64 kDa) and

transferrin (77 kDa). On this basis, the patient could be considered affected by selective glomerular proteinuria, according to what reported by Uberti et al., (2009) that evidenced glomerular involvement during laminitis.

2.4. NON-CONVENTIONAL ANIMALS

2.4.1. INTRODUCTION

During the years, felids and giraffes underwent a critical reduction in the number of specimens leading to the threatened or extinction of some species. The main cause of this reduction is due to anthropogenic activities, e.g. the enlargement of urban areas and the poaching.

The International Union for Conservation of Nature (IUCN), in 1994 wrote up the Red List⁸ to provide information on the status, trend and threats in order to inform and catalyze actions for biodiversity conservation. In particular, different species of felids, e.g. *Panthera tigris*, *Acinonyx jubatus*, are considered as "Vulnerable" (VU) or "Near Threatened" (NT) as well as some sub-species of *Giraffa camelopardalis* e.g. *peralta* or *rothshildi*.

Recently, the increasing interest on welfare and conservation led to the constitution of specific societies and programs, e.g. *Cat Specialist Group*, *IUCN* or *Serengeti Giraffe Project*, for the defense and the protection of these species. Therefore, the evaluation of the health status of wild animals in natural areas or in zoos is vital for their conservation. The collection of biological samples in non-invasive manner, including fur, feces and urines are preferred, since they can be collected without causing stress conditions, that can interfere with the accuracy of the analysis and could occasionally lead the animal to death (Kelly et al., 2012).

⁸ <http://www.iucnredlist.org/>

2.4.2. FELIDS URINE PROTEOME

2.4.2.1. MATERIALS AND METHODS

Animals and samples

For the present study urine samples were collected from felids of different species, age and sex. The animals were housed in four different european zoos: Fondazione Bioparco (Rome, Italy), Givskud zoo (Give, Denmark), K benhavn zoo (Copenhagen, Denmark) and zoo de La Palmyre (Les Mathes, France). The animals included in the study underwent periodical clinical evaluations by the veterinarian staff of the hosting structure. Urine samples were collected in a non invasive manner without stressing animals. During the night animals were housed in cages with a logline to drain urines that were collected the following morning.

Urine samples were immediately centrifuged at 1,500 g for 10 minutes, stored at -20 C and sent to the Department of Veterinary Medical Sciences - University of Bologna for the subsequent analysis:

- protein quantification with UPC ratio calculation;
- SDS-PAGE.

Protein quantification and urine protein to creatinine ratio (UPC)

Urine total proteins (UTP) 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: $UPC = \text{urine protein (mg/dL)} / \text{urine creatinine (mg/dL)}$.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Urine proteins were separated using the electrophoresis NuPAGE system (Thermo Fisher Scientific) on 4-12% polyacrylamide gel in 3-(*N*-morpholino)ethanesulphonic acid (MES) 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 digitalized and its pherogram was obtained by GelAnalyzer 2010 software⁹.

Statistical analysis

Data were analyzed with statistical software (Excel 2007 and R version 2.15.1) and expressed as mean \pm standard deviation (SD). The different variables (UTP and UPC) were tested for normality with Shapiro-Wilk normality test and distribution was considered normal for $P > 0.05$. UTP and UPC, non normally distributed, were normalized by exponential transformation. Variables with normal distribution were analyzed by one way ANOVA to evaluate differences between species assuming $P < 0.05$ as a significant probability.

2.4.2.2. RESULTS

Fifty-five urine samples were collected from 34 felids of different species, age and sex: 10 lions (*Panthera leo*), 7 tigers (*Panthera tigris*), 8 leopards (*Panthera pardus*), 4 cheetah (*Acinonyx jubatus*) 3 caracals (*Caracal caracal*) and 2 jaguar (*Panthera onca*). Table 1 reported data for UTP and UPC for the different species. Only caracals showed significant differences compared to the other species.

⁹ <http://www.gelanalyzer.com/>

In particular, UTP values were significantly higher than the values measured in tigers ($P<0.01$) and leopards ($P<0.05$) (Figure 1a) and UPC was significantly higher in caracals than in lions ($P<0.01$), jaguars ($P<0.05$) and tigers ($P<0.05$) (Figure 1b). The other species did not show significant differences.

Table 1. UTP and UPC values for the felids species analyzed.

UTP (mg/dL)	Caracals (n=3)	Cheetahs (n=4)	Jaguars (n=2)	Leopards (n=8)	Lions (n=10)	Tigers (n=7)
Mean	313.5	47.9	149.3	88.7	81.9	38.3
Median	231.8	47.9	81.1	38.9	58.9	36.4
DS	166.1	20.5	151.1	167.7	61.2	18.7

UPC	Caracals	Cheetahs	Jaguars	Leopards	Lions	Tigers
Mean	1.16	0.33	0.07	0.25	0.14	0.17
Median	0.69	0.30	0.07	0.12	0.10	0.11
DS	0.96	0.23	0.01	0.27	0.13	0.16

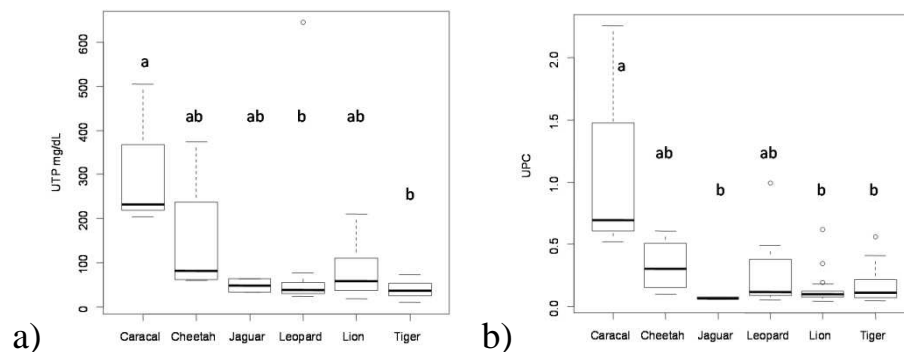


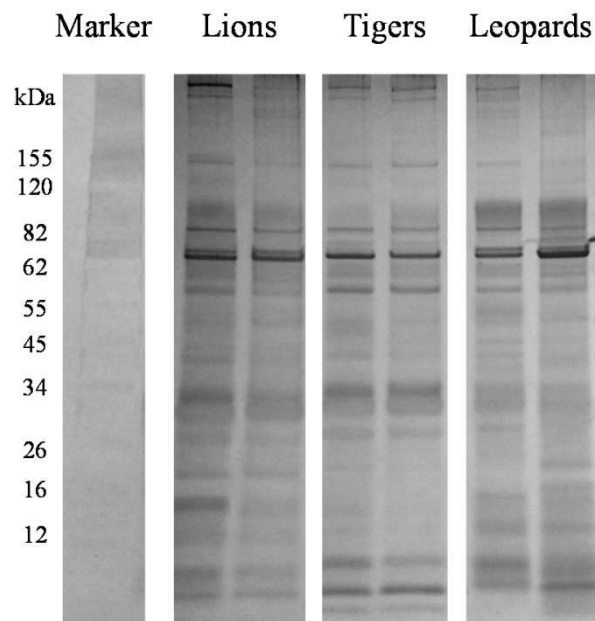
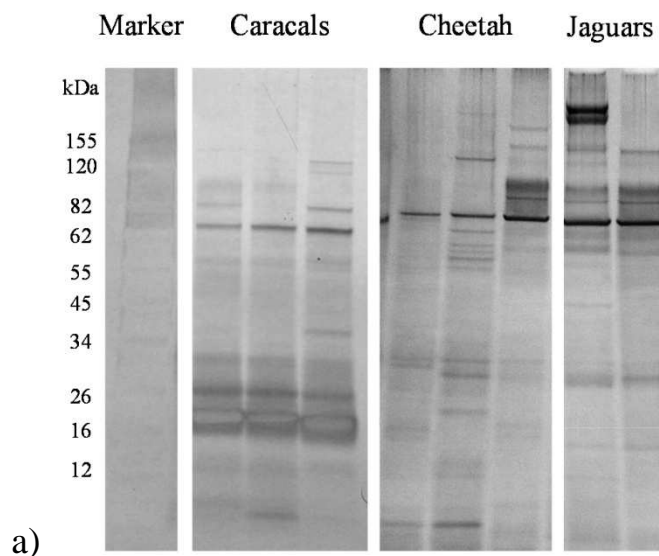
Figure 1. UTP (a) and UPC (b) comparison among species. Different lower cases indicate significant difference ($P<0.05$).

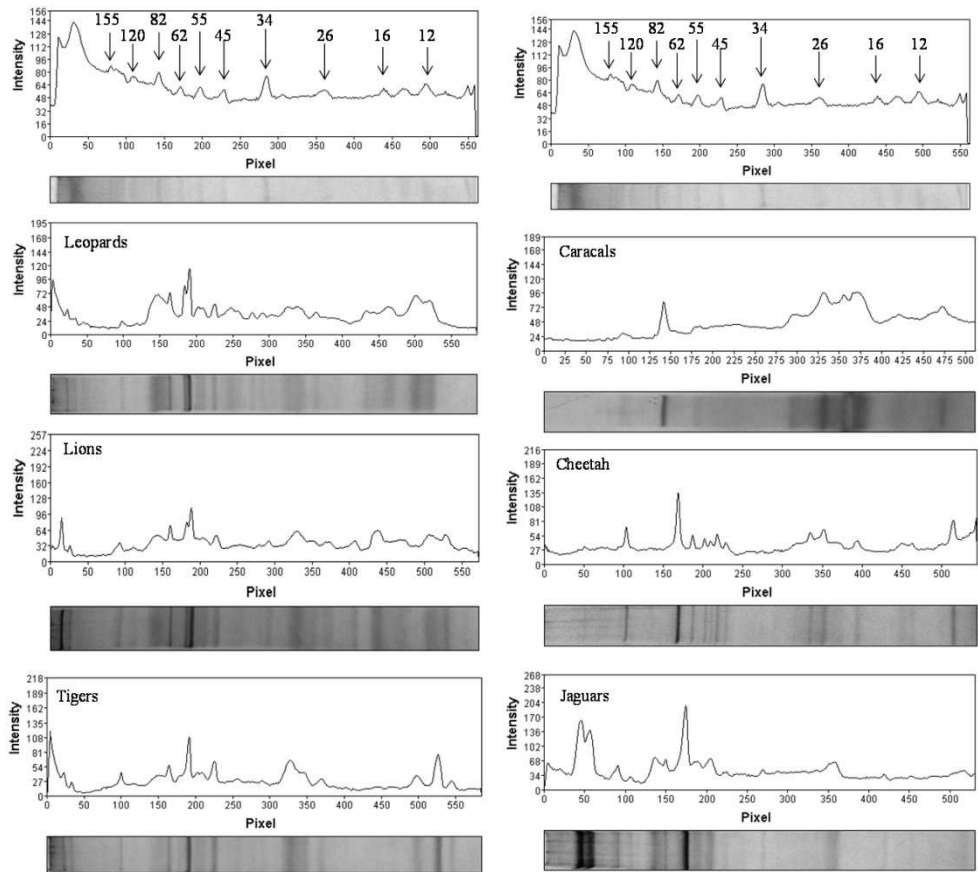
SDS-PAGE

SDS-PAGE allowed the separation of urine proteome resulting in different profiles for each species

with some common protein bands. Figure 2 reported examples of gels and pherograms for each species. Two common bands were present at 82 and 64 kDa, while the majority of bands with greater

variability among species was present at medium MW, between 62 and 120 kDa. All species showed also a common diffused band with different intensities at 90 kDa and 2 to 5 bands at low MW (<120 kDa). Lions, tigers, leopards and jaguars showed 3 to 5 protein bands at high MW (>100kDa), while caracals showed two intense bands at 23 and 17 kDa.





b)

Figure 2. Representative (a) gels and (b) pherograms of urine samples of felids.

2.4.2.3. DISCUSSIONS

For many years, studies on zoo felids focused only on anatomy and taxonomy, but now the interest on ethology, genetics, physiology and pathophysiology for the conservation of wild felids is increasing (Hosey et al., 2009).

Nationals laws ratify the obligation of clinical evaluation of the healthy status by veterinarians, focusing also on the welfare intended as reduced man-induced stress.

The health status evaluation in zoo animals is difficult, clinical signs of disease are not always evident and the collection of samples can be stressful for the animals, particularly if the restraint is needed. Therefore, non-invasive biological samples including fur, feces and

urines are preferred, since they can be collected in sufficient amounts, and repeatedly with less interferences. The sampling is easy to perform and can be standardized reducing the capture-induced stress (Kelly et al., 2012).

In particular, urine samples can be collected from the floor of the cages or extracted from snow or sand. Urine does not need particular treatments before storage, at least a short centrifugation, and can provide important information on endocrine and reproductive functions as well as on the general health status (Bechert, 2012). More specifically, qualitative (SDS-PAGE) and quantitative (UPC) proteinuria are essential for the assessment of kidney function and the progression of renal diseases (Grauer, 2011).

As regards UPC, for cats the cut-off values to classify patients as non-proteinurics, borderline proteinurics and proteinurics are 0.2 and 0.4 (Lees et al., 2005). However for wild felids no data are reported in literature. Therefore, assuming the values established for cats as reference, lions, tigers and jaguars can be considered non-proteinurics, leopards and cheetah borderline proteinurics and caracals proteinurics. To our knowledge, our data are the first UPC values reported in big cats and further studies are needed to evaluate the influence of sex and age on UPC and to establish the interval reference also in these species.

SDS-PAGE allowed the visualization of different profiles for each species. Comparing the MW of the protein bands to data reported in literature for cats we can hypothesize the identification of the most important urine proteins. In particular, as recently reported in cats, the band at 64 kDa should contain cauxin. Cauxin is a feline specific carboxyl-esterase produced by the epithelial cell of the of the tubule

and identified also in lions, tigers and leopards (McLean et al., 2007). The same authors reported that cauxin can be present also as polymers at high MW (Figure 3) similarly to what can be seen also in our samples.

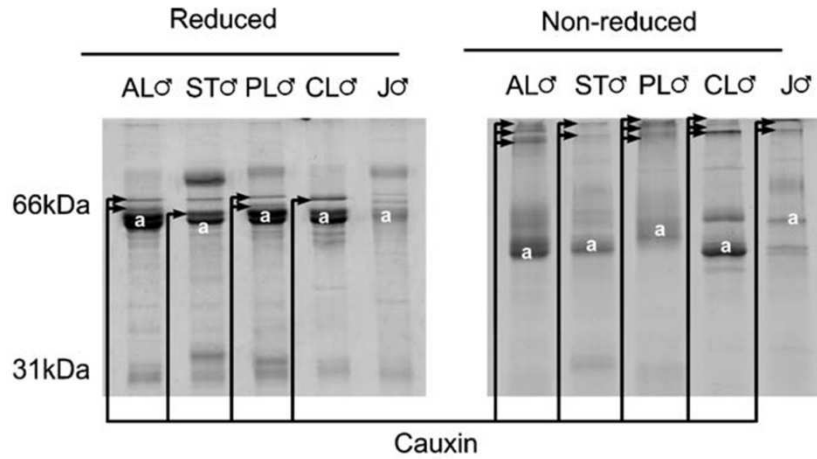


Figure 3. Electrophoresis of urine samples of Asiatic lion (AL), Sumatran tiger (ST), Persian leopard (PL), clouded leopard (CL), and jaguar (J) (McLean et al., 2007).

Regarding the band at 90 kDa, present in all species with some differences in intensities, could be uromodulin reported as the main urine component in healthy humans (Candiano et al., 2010) and dogs (Miller et al., 2014) and identified also in cats (Ferlizza et al., 2015). Finally, caracals showed an intense band at 17 kDa that, comparing to data reported in dogs, could be arginine esterase (Miller et al., 2014).

2.4.3. GIRAFFES

2.4.3.1. MATERIALS AND METHODS

Animals and samples

For the present study urine samples were collected from giraffes (*Giraffa camelopardalis*) of different sub-species (*camelopardalis*, *peralta* and *rothshildi*), age and sex. The animals were housed in two different italian zoos: group A) Parco Natura Viva (Verona) and group B) Parco Faunistico Le Cornelle (Bergamo). The animals included in the study underwent periodical clinical evaluations by the veterinarian staff of the respective zoo. Urine samples were collected from the ground with a syringe without stressing animals.

Urine samples were immediately centrifuged at 1,500 g for 10 minutes, stored at -20°C and sent to the Department of Veterinary Medical Sciences-University of Bologna for the subsequent analysis:

- protein quantification with UPC ratio calculation;
- SDS-PAGE.

Protein quantification and urine protein to creatinine ratio (UPC)

Urine total proteins (UTP) 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: $UPC = \text{urine protein (mg/dL)} / \text{urine creatinine (mg/dL)}$.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Urine proteins were separated using the electrophoresis NuPAGE system (Thermo Fisher Scientific) on 12% polyacrylamide gel in 3-(*N*-morpholino)ethanesulphonic acid (MES) 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 digitalized and its pherogram was obtained using GelAnalyzer 2010 software¹⁰.

Statistical analysis

Data were analyzed with statistical software (Excel 2007 and R version 2.15.1) and expressed as mean \pm standard deviation (SD). The different variables (UTP, UPC and number of bands) were tested for normality with Shapiro-Wilk normality test and distribution was considered normal for $P>0.05$. UTP (normally distributed) was analyzed by t-test, while UPC and number of bands (non-normally distributed) were analyzed by Wilcoxon test to evaluate differences between groups (A and B), age (young and adults) and gender (male and female) assuming $P<0.05$ as a significant probability.

2.4.3.2. RESULTS

Fifty-one urine samples were collected from 20 giraffes of different sub-species (*camelopardalis*, *peralta* and *rothshildi*), age and sex. Table 1 reports data for UTP and UPC for the two groups. Group B showed significantly higher values of both UTP ($P<0.05$) (Figure

¹⁰ <http://www.gelanalyzer.com/>

1a) and UPC ($P < 0.05$) (Figure 1b) than group A. No differences were found between males and females and between young and adults.

Table 1. UTP and UPC values for giraffes.

UTP (mg/dL)	Group A (n=29)	Group B (n=22)
Mean	12.1	19.5
Median	8.4	20.1
SD	9.4	6.5

UPC	Group A	Group B
Mean	0.062	0.096
Median	0.056	0.081
SD	0.012	0.038

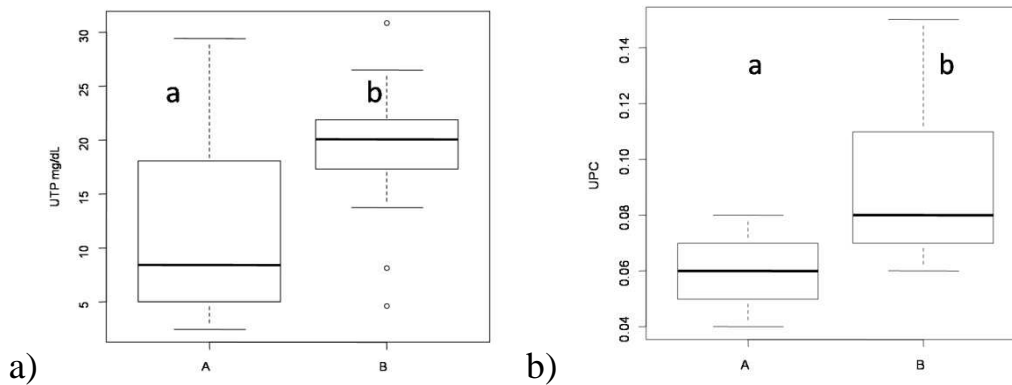


Figure 1. UTP (a) and UPC (b) comparison between groups. Different lower cases indicate significant differences ($P < 0.05$).

SDS-PAGE

Figure 2 reported representative gels and pherograms. SDS-PAGE allowed the separation of a typical urine pattern characterized by 7 ± 2 protein bands. The most evident bands present in all samples were at 90 and 64 kDa and 3 bands at $MW < 14$ kDa. Little inter-individual differences was found in bands between 62 and 14 kDa. No significant differences were found between the two groups, ages and genders.

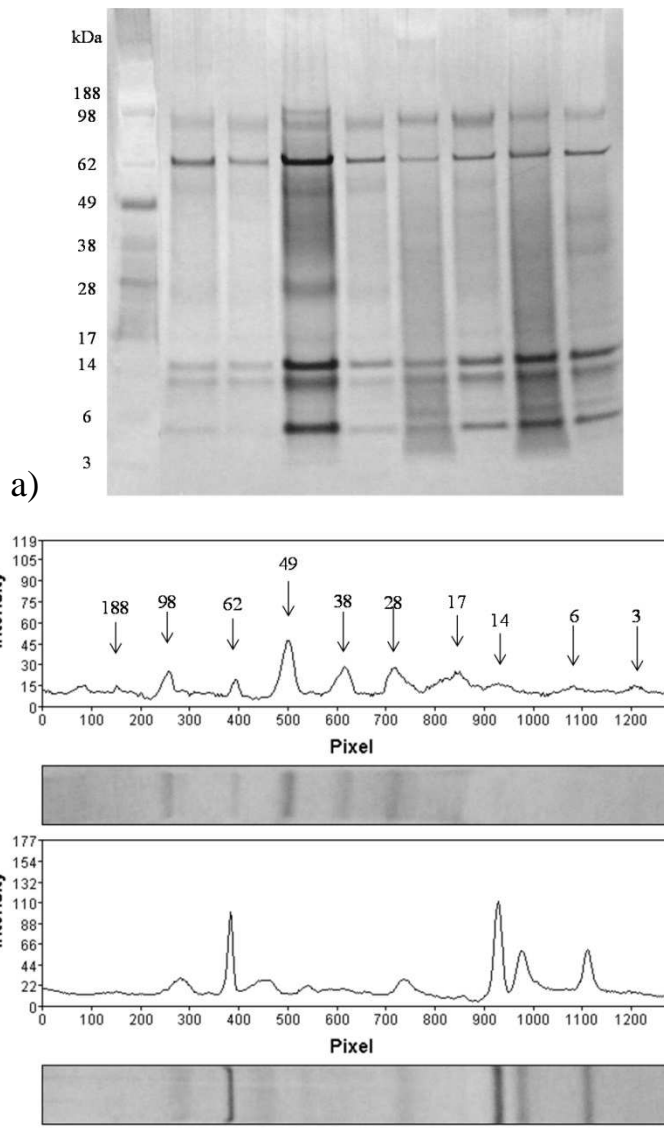


Figure 2. Representative gel (a) and pherograms (b) of urine samples of giraffes.

2.4.3.3. DISCUSSIONS

Wild animals kept in captivity encounter different sources of stress including artificial lights, loud noises, temperatures different from their natural habitat, reduced movements possibility, forced contact with humans and restrictions in their behaviour (Morgan and Tromborg, 2007). In these conditions, health status evaluation is difficult and indirect methods of health monitoring are now

developing (Hosey et al., 2009). As regards quantitative proteinuria (UTP and UPC), no data are reported in literature for giraffes. The only study on urines of giraffes correlated urine creatinine concentration to a diet rich in hay and concentrate. Our data for creatinine are lower than those reported by Sullivan et al., (2010) probably due to the different diet.

As regards UPC, since no data are present in literature for wild or domestic ruminants, comparing our data to reference values for dogs and cats (IRIS¹¹) we can classify our samples as non-proteinurics (UPC<0.2).

SDS-PAGE allowed the visualization of a typical profile with common bands to all animals. Differently from dogs, where the predominant urine proteins have medium MW (10-80 kDa) (Brandt et al., 2014) and from humans (30-69 kDa) (Oh et al., 2004), giraffes presented mainly low MW protein bands respect to high MW proteins and this profile is more similar to goat (Ozgo et al., 2009) and rat (Calzada-García et al., 1996).

Comparing our data to literature we can hypothesize the identity of some of the bands in giraffes urine proteome. In particular, the band at 90 kDa could be uromodulin, one of the main protein components in normal urine of mammals and already identified in humans, rats, dogs, cats and also camels (Calzada-García et al., 1996; Nagaraj and Mann, 2011; Alhaider et al., 2012; Miller et al., 2014; Ferlizza et al., 2015). Uromodulin is an important biomarker of the kidney health status. Moreover, since giraffes have hypertension as a para-physiological condition and Padmanabhan et al., (2014) showed a correlation of uromodulin to hypertension, the comprehension of its role in

¹¹ <http://www.iris-kidney.com/>

regulation of systemic and kidney blood pressure needs to be investigated further.

The band at 64 kDa should be albumin. Small amounts of this protein are physiological in urine of healthy animals and have been identified in urine also of camels (Alhaider et al., 2012) and cows (Pyo et al., 2003).

2.5. CONCLUSIONS

2.5.1. COMPANION ANIMALS

Our work has 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 confirmed tubulointerstitial damage in CKD cats and suggested that these proteins are candidate biomarkers to be investigated further.

Regarding the dog urine proteome, we highlighted a specific electrophoretic pattern in healthy animals showing important differences in patients affected by leishmaniasis. In particular, uromodulin could be a putative biomarker of tubular damage and albumin was confirmed as a biomarker of glomerular involvement. The role of arginine esterase and low MW proteins needs to be investigated further.

Finally, we also validated HRE for the quantification of albuminuria in dogs and cats. HRE and SDS-PAGE could be considered as useful tools that could help clinicians in the evaluation of the kidney function in dogs and cats. Our data on the proteins most represented in the cat and dog urine proteomes and their changes in pathological conditions could be useful in the advancement of research focused on the discovery of new biomarkers for later use in clinical practice.

2.5.2. FARM ANIMALS

This study on the horse urine proteome produced a reference electrophoretic pattern characterized by an intense band, presumably uromodulin, 5 to 6 bands between 77 and 56 kDa, possible isoforms of albumin and/or transferrin and other 6 to 8 bands at lower MW. We highlighted some differences in proteinuric horses showing the decrease of the putative uromodulin band and the appearance of 2 to 4 protein bands at higher MW and a greater variability in the range of MW between 49 and 17 kDa.

Regarding cows, we highlighted different electrophoretic patterns between heifers and cows showing also some interesting proteome changes during pregnancy. In particular, putative alpha-fetoprotein and b-PAP needs to be further investigated.

Finally, further studies are needed to identify by mass spectrometry the most represented bands to produce a reference urine profile of healthy horses and cows. The differentially represented bands in physiological and pathophysiological conditions needs to be investigated further, with particular attention to the better characterization of patients affected by kidney damage.

2.5.3. NON-CONVENTIONAL ANIMALS

Our work produced the first values for UTP and UPC in healthy felids and giraffes kept in captivity. Moreover, we highlighted species-specific electrophoretic patterns in big felids and giraffes. Further studies are needed to identify the most represented bands and to evaluate differences in the urine proteomes in animals kept in wild natural conditions.

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