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Urinary proteome and metabolome in dogs (*Canis lupus familiaris*): the effect of chronic kidney disease

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Abstract:

Chronic kidney disease (CKD) is a progressive and irreversible disease. Although urine is an ideal biological sample for proteomics and metabolomics studies, sensitive and specific biomarkers are currently lacking in dogs. This study characterised dog urine proteome and metabolome aiming to identify and possibly quantify putative biomarkers of CKD in dogs. Twenty-two healthy dogs and 28 dogs with spontaneous CKD were selected and urine samples were collected. Urinary proteome was separated by SDS-PAGE and analysed by mass spectrometry, while urinary metabolome was analysed in protein-depleted samples by 1D ^{1}H NMR spectra. The most abundant proteins in urine samples from healthy dogs were uromodulin, albumin and, in entire male dogs, arginine esterase. In urine samples from CKD dogs, the concentrations of uromodulin and albumin were significantly lower and higher, respectively, than in healthy dogs. In addition, these samples were characterised by a more complex protein pattern indicating mixed glomerular (protein bands ≥65kDa) and tubular (protein bands<65kDa) proteinuria. Urine spectra acquired by NMR allowed the identification of 86 metabolites in healthy dogs, belonging to 49 different pathways mainly involved in amino acid metabolism, purine and aminoacyl-tRNA biosynthesis or tricarboxylic acid cycle. Seventeen metabolites showed significantly different concentrations when comparing healthy and CKD dogs. In particular, carnosine, trigonelline, and cis-aconitate, might be suggested as putative biomarkers of CKD in dogs.

Significance: Urine is an ideal biological sample, however few proteomics and metabolomics studies investigated this fluid in dogs and in the context of CKD (chronic kidney disease). In this research, applying a multi-omics approach, new insights were gained regarding the molecular changes triggered by this disease in canine urinary proteome and metabolome. In particular, the involvement of the tubular component was highlighted, suggesting uromodulin, trigonelline and carnosine as possible biomarkers of CKD in dogs.

Introduction

Chronic kidney disease (CKD) is a progressive and irreversible disease characterised by the presence of structural or functional abnormalities in one or both kidneys over a period of three months or longer [1]. CKD is one of the most common renal diseases in dogs with an estimated prevalence varying from 0.5 to 3.64% depending on the inclusion criteria of the cases [1–3]. Early diagnosis of CKD may hinder the disease progression and improve patient quality of life. International Renal Interest Society (IRIS) guidelines for staging and treatment of CKD help clinicians to correctly classify patients and establish the best therapies [4]. Nonetheless, sensitive and specific biomarkers for early detection and monitoring of CKD in dogs are currently lacking. The gold standard to evaluate the renal function is the determination of the glomerular filtration rate (GFR); however, this value does not provide information on CKD aetiology and the available methods for its estimation are difficult to be applied in the routine clinical practice [5,6]. Renal biopsy is considered the gold standard for determining the type of renal damage, but it is an invasive procedure and not always feasible [7]. Therefore, the assessment of the kidney function is currently based on conventional blood (serum creatinine or urea) and urine (proteinuria and specific gravity) clinicopathological variables, whose alterations are usual findings of CKD but have limitations when used as early indicators of the disease [7]. For these reasons, other sensitive and specific biomarkers measurable in non- or minimally invasive biological samples are required in clinical practice to identify early renal damage in dogs.

Over the last years, significant efforts have been made in veterinary medicine to apply proteomics to search for new biomarkers or for validating detection methods for proteins already considered as potential early indicators of kidney disease in dogs and cats [8–17]. However, proteins are only some of the molecular species present in urine and a broader approach with the aid of metabolomics can offer additional clinical information.

Metabolomics enables the assessment of a broad range of endogenous and exogenous small molecular mass metabolites, potentially useful to investigate the physiologic status and the pathogenesis of the diseases, and to discover new biomarkers of altered biochemical pathways [18–21]. Metabolites are in general not specific for a single metabolic pathway and in most cases different biochemical reactions contribute to the production of the same metabolite; this peculiarity offers the opportunity to obtain a more comprehensive insight into the complexity of a biological sample. In human medicine, metabolomics was extensively applied to urine to analyse the healthy metabolome [22] and to search for small molecules as potential biomarkers of different diseases, such as immune-mediated inflammatory diseases [23], different cancers [24–26], and renal diseases [19,27–30]. However, in veterinary medicine, the application of metabolomics techniques to urine is still limited [31–35]. Owing to the metabolic and protein complexity of urine, the aim of this work was to combine the analytical power of proteomics and metabolomics to obtain a more comprehensive characterisation of the urine in healthy dogs and to compare it with the urine from CKD patients with our ultimate goal to suggest new biomarkers of CKD in the canine species.

Materials and Methods

Animal selection and sample collection

The present study was performed on urine samples collected at the Veterinary Teaching Hospital of the University of Bologna from owned dogs. The dogs were divided into two experimental groups and specimens considered as biological replicates. Upon arrival, all dogs were subjected to physical examination and routine laboratory tests, including complete blood count, serum chemistry and complete urinalysis with urine protein to creatinine ratio (UPC).

Blood samples were collected by venepuncture using a vacuum collection system (Vacutest Kima, Arzergrande, Italy) after at least a 12-hour fasting period. Blood samples were processed within one hour after collection. Serum samples were collected in tubes with clot activator (Vacutest Kima, Arzergrande, Italy), centrifuged at 3,000 *g* for 10 minutes and analysed in an automated chemistry analysed (AU 480, Olympus/Beckman Coulter, Atlanta, GE, USA).

Urine samples were collected by ultrasound-guided cystocentesis. All urine specimens were processed on a routine basis and evaluated in our laboratory within two hours after collection. In particular, urinalysis consisted in macroscopic examination, urine specific gravity (USG) measured by manual refractometer (American Optical, Buffalo, New York), urine dipstick test (Combur10Test, Roche Diagnostic, Mannheim, Germany) applied on an automated readers (Urisys 1100, Roche Diagnostic, Mannheim, Germany, respectively) and microscopic sediment evaluation. Urine sediment was obtained after centrifugation at 500 *g* for 10 minutes. Urine supernatants were immediately analysed (dipstick examination), divided in aliquots and stored at –80°C for the subsequent proteomics and metabolomics analysis. Urine chemistry was performed on a refrigerated (+4°C) aliquot if performed within 24 hours after the sample processing or on an aliquot kept frozen at -20°C for a maximum of 7 days.

Dogs were considered healthy or diseased on the basis of history, clinical signs and the results of the above-mentioned routine laboratory tests. The control group included 22 healthy dogs presented at the hospital as blood donors. The 22 healthy dogs were 10 males (3 castrated) and 12 females (7 spayed) with an average age of 37±20 months. The diseased group included 28 dogs affected by naturally occurring CKD. The 28 CKD dogs were 14 males (5 castrated) and 14 females (9 spayed) with a mean age of 111±61 months. The diagnosis of CKD based on history, clinical signs, clinicopathological and imaging results, according to the literature [3,4]. In particular, the presence of clinical findings, abdominal imaging results and (a) persistent pathologic renal proteinuria based on the UPC (UPC>0.5), assessed and confirmed over a one-month period, and/or (b) serum creatinine (sCrea) concentration ≥1.40 mg/dL and/or (c) urine specific gravity (USG) <1.030 were considered diagnostic. The IRIS CKD guidelines were used to subsequently stage CKD dogs [4]. Basing on serum creatinine, 8 dogs were classified with CKD stage 1, 6 with stage 2, 9 with stage 3 and 5 with stage 4. On the basis of UPC, 4 dogs were non-proteinuric (UPC<0.2), 6 dogs were borderline proteinuric (UPC 0.2-0.5) and 18 were proteinuric (UPC>0.5).

The study was conducted according to the EU Directive 2010/63/EU for animal experiments and approved by the Institutional Scientific Ethical Committee of the University of Bologna for animal testing.

Urine protein to creatinine ratio

Five mL of urine were collected from each animal by ultrasound-guided cystocentesis. After centrifugation at 500 *g* for ten minutes, urine total proteins and creatinine were measured using commercial kits (Urinary/CSF Protein, OSR6170, and Creatinine OSR6178, Olympus/Beckman Coulter, Atlanta, GE, USA) on an automated chemistry analyser (AU 480, Olympus/Beckman Coulter, Atlanta, GE, USA). The UPC was calculated with the following formula: UPC = urine protein (mg/dL)/urine creatinine (mg/dL).

SDS-PAGE and protein identification

Urine proteins were separated using an electrophoresis system (NuPAGE, Thermo Fisher Scientific, Waltham, MA, USA) as previously described [8,36]. Briefly, three to five μg of protein were loaded on 4-12% polyacrylamide gel in MOPS buffer with SDS (Thermo Fisher Scientific, Waltham, MA, USA). The gels were stained with Coomassie brilliant blue (PageBlu protein staining solution; Thermo Fisher Scientific, Waltham, MA, USA) compatible with mass spectrometry analysis. After staining, each gel was digitalized (ChemidocMP, BioRad, Hercules, California, USA) and the pherograms were obtained using a commercial software (ImageLab, BioRad, Hercules, California, USA). The bands at 100, 67 and 18 kDa were cut and identified by electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF/MS) as previously reported [8,36].

To quantify the bands at 100 kDa and 67 kDa, on each sample, one μg of protein, obtained from a solution containing 1 μg/μL of lactate dehydrogenase (LDH), (Sigma-Aldrich/Merck KGaA, Darmstadt, Germany) was added as internal standard of quantity. The ImageLab software estimated the volume of each protein band based on pixel density within the band boundaries in the digital image. The volume of the band of interest was then compared to the internal standard (LDH) of the corresponding lane and the concentration was calculated as follows:

$$X \text{ mg/dL} = (V_{band}/V_{LDH}) / \mu L_{sample} * 100.$$

X = concentration of the protein at 100 kDa or at 67 kDa

V_{band} = volume of the band at 100 kDa, or at 67 kDa determined by the software

V_{LDH} = volume of the band of the internal standard (LDH) determined by the software

μL_{sample} = µL of the sample loaded in the gel

Subsequently, the respective ratios with urine creatinine (uromodulin [mg]: creatinine [mg], UMC; albumin [mg]: creatinine [mg], UAC) were calculated.

NMR Sample preparation

Urine metabolites were extracted for NMR as follows: 500 µl of urine supernatants were mixed with 550 µl of chloroform and 550 µl of methanol, vortexed for 1 min, left to rest for 15 min at +4°C and centrifuged at 12,000 *g* for 15 min at room temperature. Nine hundred µl of the upper phase (urine/methanol) were dried in a vacuum centrifuge (SpeedVac, Thermo Fischer Scientific, Waltham, MA, USA) overnight at 30°C. The resulting pellets were suspended with 200 µl of phosphate buffer (PB, 240 mM pH 7.4 in D_2O with trimethylsilylpropanoic acid [TSP] and sodium azide [NaN_3]) and 400 µl of D_2O to a final concentration of 80 mM PB, 0.087 mM TSP and 0.022% (v/v) NaN_3. Samples were vortexed for 1 min, centrifuged at 12,000 *g* for 1 min and 560 µl transferred into a 5 mm NMR tubes.

NMR acquisition

NMR spectroscopy was conducted on an 800 MHz spectrometer with a triple resonance HCN Z-gradient probe, at 298 K (Bruker AvanceII+, Ettlingen, Germany). Acquisition and processing were carried out using standard software (Topsin 3.2, Bruker Biospin, Billerica, MA, USA). One dimensional ^{1}H NMR spectra with Carr-Purcell-Meiboom-Gill (CPMG) filter to attenuate signals from macromolecules were acquired using a standard vendor pulse sequence (cpmgpr1d). Spectra were acquired at 25˚C, with a 20 ppm spectral width, spin lock duration of 78.72 ms, presaturation for 4 s using 20 µW and acquisition time of 2 s. A total of 16 dummy scans and 128 scans were acquired for each sample. All spectra were processed with an exponential window function with 1

Hz line broadening and automated phasing and baseline correction. For the chemometric analysis, the processed data were further processed in the "nmrprocflow" platform [37]. Bins were obtained using manually curated, intelligent binning after referencing, baseline correction, water signal removal and peak alignment. For selected samples, additional homonuclear and heteronuclear spectra (^{1}H J-resolved, ^{1}H-^{1}H COSY, and ^{1}H-^{13}C HSQC) were also collected to assist with compound identification.

Metabolite annotation and identification

The bins obtained from the "nmrprocflow" platform [37] were annotated with the help of database assisted spectral decomposition using commercial software (Chenomx 8.2 NMR Suit, Edmonton, Alberta, Canada) and the internal reference library (Version 10) as well as the Biological Magnetic Resonance Data Bank (BMRB, http://www.bmrb.wisc.edu) reference spectra for compounds absent in the internal reference library. Buckets were attributed to multiple metabolites where peaks were found to overlap. Pathway analysis module of a free web-based analytical platform (Metaboanalyst 4.0, www.metaboanalyst.ca), that used the high-quality Kyoto encyclopaedia of genes and genomes (KEGG) metabolic pathways as the backend knowledgebase, was used to search for the metabolic pathways.

Statistical analysis

Serum and urine chemistry data were analysed with statistical software (R version 3.4.4). Normal distribution was tested graphically and by Shapiro-Wilk normality test, and data were expressed as mean ± standard deviation (SD) or median (range; minimum – maximum value) if normally or non-normally distributed, respectively. Variables were compared between healthy (N=22) and CKD (N=28) dogs using the Student t-test or the Mann-Whitney U test depending on their distribution, assuming $P < 0.05$ as a significant probability. The Kruskal-Wallis rank sum test was applied to evaluate differences among healthy and CKD stages (stages 1-4, basing on serum creatinine and according to the IRIS guidelines [4]) and adjusted *P*-values lower than 0.05 were considered statistically significant.

For metabolomics statistical analysis, processed spectra were aligned, baseline corrected and divided into 397 variable width spectral regions or 'buckets' with the intensity of each bucket divided by the bucket width. To identify the signals differentially present in the two groups, the buckets were loaded into a web-based platform (Metaboanalyst 4.0, www.metaboanalyst.ca) which uses the R package of statistical computing software [38]. For multivariate analysis, buckets were scaled by auto scaling (mean-centred and divided by the standard deviation of each variable) while, for univariate analysis, and in order to remove the influences attributed to muscle mass and urine concentration, the bucket intensities were normalised to the peak of creatinine (bucket 3.0360 ppm). Both univariate and multivariate statistics were employed. t-test and fold change analysis were used to identify the buckets with differential presence, while the list was supplemented with the use of unsupervised principal components analysis (PCA) and supervised partial least squares discriminant analysis (PLS-DA). Both PCA and PLS-DA can identify signals (buckets) whose importance becomes significant via correlated variance. In addition, PCA provides a global view of the differentiability between the two experimental conditions and the groups of observables that are mostly responsible. In contrast, PLS-DA, since it is a supervised method, highlights the variables most responsible for the differences between groups as previously used in other metabolomics approaches [39,40]. The small sample size that is typical in such studies and the inherent large number of variables obtained may affect the consistency of the multivariate analysis used. To evaluate the consistency of the results, the software performs a number of tests and reports the parameters Q^2 and R^2 as quality parameters of the models. Q^2 indicates the predictive ability of the model, while R^2 is the indicator of the suitability of the fit. For PLSDA $Q^2 > 0.6$ were selected as acceptable models. Variable importance in projection (VIP) scores greater than 1 and t-test with a P value <0.05 were used to identify metabolites as differentially expressed.

Results

Clinical data

Mean clinical data, serum and urine biochemistry of healthy and CKD dogs are reported in Table 1, while the results for each dog are reported in Supplement Table 1.

CKD dogs were significantly older ($P<0.0001$), had significantly higher concentration of serum creatinine ($P<0.0001$), urea ($P<0.0001$) and UPC ($P<0.0001$), while USG ($P<0.0001$) was significantly lower than in the healthy dogs. CKD patients were also staged according to serum creatinine concentration following IRIS guidelines [4] and the differences of UPC and USG were evaluated . USG was significantly lower in each CKD stage group than in the healthy dogs ($P<0.01$), and samples classified as CKD stage 1 had higher USG than those classified as Stage 3 ($P=0.016$) and 4 ($P=0.007$). UPC was significantly higher in each CKD stage groups than in healthy dogs ($P<0.05$), however, no significant differences were found among CKD stages.

SDS-PAGE Proteomics Analysis

Representative gels and pherograms from healthy and CKD dog urines are reported in Fig. 1.

Urine samples from the healthy group presented similar profiles characterised by the presence of three most abundant bands at apparent molecular mass (MM) of 103, 80 and 67 kDa, respectively. The bands at 103 and 67 kDa were identified by mass spectrometry as uromodulin and albumin respectively (Table 2). Moreover, most of the samples presented other three to five low abundance bands at apparent MM between 55 and 14 kDa and two bands at MM < 14 kDa. In addition, urine samples from entire males presented other two evident bands at apparent MM of 18 and 12 kDa. The band at 18 kDa was identified as arginine esterase (Table 2).

CKD samples presented different and more variable electrophoretic profiles. The disappearance of uromodulin and/or the increase of intensity of albumin and of the band at 80 kDa were clearly evident in all the analysed samples. The increase in number and intensity of the bands at high (>67 kDa) and low (<67 kDa) MM was also evidenced. Particularly, two samples presented an increase in number and intensity of the bands at high (>67 kDa) MM only, nine samples showed an increase in number and intensity of the bands at low (<67 kDa) MM only, while the remaining 17 samples presented an increase in number and intensity of the bands at both high and low MM. Additionally, in 12 samples (Fig. 1; Lanes 1, 3, 5, 8) was evidenced a band at 21 kDa that was not present in healthy samples. Concentrations of uromodulin and albumin and their ratio with creatinine (UMC and UAC) are reported in Table 3. Urine samples from healthy dogs presented a low amount of albumin (3.1 ± 1.4 mg/dL) and a high amount of uromodulin (11.9 ± 2.3 mg/dL). CKD dogs presented a significantly higher concentration of albumin (*P*=0.0025) and UAC value (*P*=0.0002) and a significantly lower concentration of uromodulin (*P*<0.0001) and UMC value (*P*=0.0044), compared to healthy animals.

Metabolites annotation and identification

Representative NMR spectra from healthy and CKD dog urine samples are reported in Fig. 2. An overview of the NMR spectra of samples from healthy dogs evidenced similar profiles, while the urine from CKD patients showed more variable spectra and differences in metabolite abundance. From the 397 buckets, 86 metabolites were identified in healthy samples, with different biological functions and belonging to different pathways. An entire spectrum of the urine of a healthy dog with the assigned metabolites is reported in Fig. 3. The five most abundant metabolites were creatinine, urea, taurine, lactate and 1-methylnicotinamide, while the list of all the identified metabolites is reported in Table 4. After MetaboAnalyst pathway analysis, metabolites were shown as belonging to 49 different pathways, and 23 of these pathways were represented by at least 3 different metabolites. The most represented pathways are mainly involved in amino acid metabolism, purine and aminoacyl-tRNA biosynthesis and tricarboxylic acid cycle (Table 5). In particular, 10 metabolites belonged to glycine, serine and threonine metabolism and aminoacyl-tRNA biosynthesis, while 8 metabolites were involved in phenylalanine metabolism and purine metabolism.

By univariate T-test, 83 buckets resulted significantly different between healthy and CKD dog urine samples. Unsupervised multivariate analysis (PCA) was able to distinguish between healthy and CKD dogs (Fig. 4). The supervised multivariate analysis using PLS-DA (Fig. 4, Table 6) indicated that the optimal model comprised 5 components (R^2=0.99, Q^2=0.74), but also the model with only one component had reasonable predictive value (R^2=0.73, Q^2=0.62). Both univariate and multivariate analysis were used to identify the differentially abundant metabolites. Of the 83 significantly different buckets, 21 were assigned to 17 metabolites (Table 6). The metabolites showing the highest increase in CKD samples were carnosine, 7-methylxanthine and cis-aconitic acid, while the metabolites showing the most evident decrease were trigonelline and urocanic acid.

Discussion

The aim of the present research was to characterise the urinary proteome and metabolome in healthy dogs and to compare it with that of urine collected from CKD patients to suggest biomarkers of the disease that would be useful in veterinary medicine.

In the present study, SDS-PAGE allowed the separation of the urinary proteins based on their molecular mass giving information about the localisation of the nephron damage. Most urine samples (17/28) of CKD dogs analysed in this study had protein bands at either high and low MM, indicating a mixed glomerular and tubular pattern. It is generally recognised that the renal proteinuria with an UPC>2 is strongly indicative of glomerular involvement [41,42]. Our data support this evidence, as the electrophoretic profiles of the seven urine samples with an UPC>2 were characterized by protein bands with high MM. However, in all these samples, bands with low MM were also present, suggesting a concomitant tubular damage. Other authors reported a tubular impairment in dogs with UPC>2 [42,43]. On the other hand, in our study, 7 of the 21 samples with UPC<2 indicated also a glomerular involvement and hence the evaluation of proteinuria by UPC could lead to misinterpretation regarding the nephronal origin of the proteinuria, as previously suggested by other authors [41,43–46].

In the present study, 8 dogs with early stages of CKD (I and II; serum creatinine <2.1 mg/dL), classified as non-proteinuric (UPC<0.2) or borderline proteinuric (UPC 0.2-0.5), showed altered electrophoretic profiles with the decrease of uromodulin and the increase in number and intensity of low MM bands. Chacar et al., [43] also reported the prevalence of tubular pattern in urine samples of dogs with early stages of CKD. On the other hand, out of 14 dogs affected by CKD at advanced IRIS stages (III and IV; serum creatinine >2.1 mg/dL), 10 patients had a mixed profile while 4 dogs presented a clear tubular pattern, with absent or mild glomerular involvement. Tubular epithelium seems to be more susceptible to ongoing stress and dysregulation promoting interstitial inflammation and fibrosis [47]. Therefore, it can be hypothesized that, in general, dogs with CKD in the initial phases (serum creatinine < 2.1 mg/dL and UPC < 0.5) might present a prevalent tubulointerstitial involvement followed by a gradual glomerular impairment leading to an increase of albumin and higher MM proteins in urine. In this complex scenario, the analysis of qualitative proteinuria could be essential to better characterise the kidney damage and the nephronal involvement.

In addition to the evaluation of the electrophoretic protein profiles, SDS-PAGE allowed the quantification of urinary uromodulin and albumin. In urine samples of healthy dogs, the abundance of uromodulin associated with the low concentration of albumin is confirmatory of data previously reported by other authors [43,48–50]. In our study, the quantification of these two proteins, followed by UMC and UAC calculation, represents an additional step for their clinical use. In fact, uromodulin and albumin are known markers of renal dysfunction or damage, in particular of CKD [51,52]. Raila et al., [50] reported a decrease of uromodulin in azotaemic and proteinuric dogs affected by renal disease and, despite the different method used for protein quantification (western blot), UMC values determined in the healthy dogs were comparable to our results. In addition, Chacar et al., [43] quantified uromodulin by western blotting and reported a decrease of uromodulin only in the late stages of CKD (IRIS 3-4), suggesting this protein as a marker of CKD progression rather than of early diagnosis. Differently, in the present study, the decrease of uromodulin was observed by SDS-PAGE already in stage 1 non-proteinuric CKD dogs, suggesting uromodulin as a promising and early biomarker of renal dysfunction in dogs.

Urinary albumin concentration is low in healthy dogs and an increase occurs in the presence of renal involvement [11,53]. Accordingly, in the present study, healthy dogs have low values of albumin and UAC, in the range of those reported by other authors [11,54–57]. Different authors determined albuminuria in dogs affected by a variety of diseases and conditions, including CKD [11,53–56,58–62]. However, despite the clinical importance of albumin quantification in urine, the reference intervals for albuminuria are still lacking for dogs and should be the aim of further research.

Finally, the presence of arginine esterase in urine of entire male dogs was also evidenced and needs to be considered to correctly interpret urine electrophoretic profile and to exclude false tubular involvement as previously reported [15,42].

The second part of the study focused on the application of NMR to characterise the urinary metabolome of healthy dogs and to evaluate the differences with CKD patients. As most CKD samples contained high protein concentrations, to avoid interferences on NMR spectra and possible false positives, a further step in sample preparation was added by precipitating the proteins. This step allowed the enrichment of urine metabolome, improving the quality of the spectra and the identification of a higher number of metabolites. Moreover, since the high repeatability of NMR metabolomics is well known [20,21], no technical replicates were analysed and only biological replicates were considered. From the corresponding spectra, 86 metabolites were identified in healthy samples, a number higher than those previously reported in dog urine by other authors [32,33,63,64] and producing, so far and to the best of our knowledge, one of the most complete dataset of canine urinary metabolome. Most of these metabolites are of endogenous origin, while others, like ferulic acid, are of exogenous or mixed origin. Most of the identified metabolites were previously reported in urine of healthy or diseased dogs [32,33,65], in human urine [19,22] and also in feline urine [31]. The majority of these metabolites is involved in amino acids metabolism, purine and pyrimidine metabolism, tricarboxylic acid cycle and methane metabolism. Nine metabolites were significantly increased in urine of CKD dogs. Carnosine, a dipeptide composed by alanine and histidine acting as an antioxidant scavenger, showed the most evident increase. This molecule is filtered by the glomerulus and then reabsorbed at the level of the proximal tubule by the proton-coupled oligotransporter PEPT2 [66]. It has been recently reported that the kidney has an intrinsic carnosine metabolism with carnosine synthase and carnosinase 1 activity in the glomeruli and tubular cells [67,68]. In CKD dogs, the increased urinary excretion of carnosine may reflect an oxidative stress suffered by the kidney, a condition hypothesised also by other authors in obese dogs [32,65]. Moreover, since carnosine is present at high concentrations in muscle tissues, and muscle weakness and atrophy are common findings in CKD patients, the increase of this molecule in urine may also reflect an increased muscle catabolism [65]. Finally, as a causative event, a damage of the epithelium of the proximal tubule might also be hypothesised, leading to impaired reabsorption of carnosine; this hypothesis is supported by the decrease of uromodulin evidenced by SDS-PAGE and by the increase of cis-aconitic acid in urine of CKD dogs. Cis-aconitic acid, an intermediate in the tricarboxylic acid cycle, was observed in the urine of type 2 diabetic human patients. In fact, increased excretion of this metabolite reflects local effects on tubular transport in the kidneys [69]. Therefore, a damage of the tubular epithelium might determine an inefficient reabsorption leading to an increased concentration of urinary cis-aconitic acid and carnosine.

Regarding other urinary metabolites increased in urine of CKD dogs, xanthosine, allantoin, and 7-methylxanthine are of interest. These metabolites belong to the complex pathways of purine metabolism; in particular, during purine catabolism, the nucleoside xanthosine is transformed into xanthine, which in turn is oxidized to uric acid by uricase. In humans, uric acid is the end product of purine catabolism, while in dogs an additional reaction transforms this metabolite into allantoin. In humans, some of these metabolites were suggested as possible markers of diabetic nephropathy [70], end stage renal disease [71] or other kidney disorders [72], while an increase of allantoin and xanthine to creatinine ratios were previously reported in urine of dogs affected by CKD [73]. Despite the possible influence of medications, such as allopurinol or diuretics received by two CKD dogs included in the present study, that could have affected purine metabolism, these data show evidence that CKD is associated with alterations in urinary concentrations of purine metabolites, and thus, this issue deserves more attention in further research.

Three additional metabolites increased in urine of CKD dogs. They were methylguanidine (MG), kynurenic acid (KnA) and dimethylamine (DA). These molecules are well known uremic toxins that accumulate in serum and urine due to the impairment of renal function [74,75]. MG derives from creatinine and is often detected in serum and urine of uremic human patients [76,77]. MG was detected also in serum of uremic dogs and was shown to increase in urine of dogs affected by transitional cell carcinoma [33,78]. In the present study, 14 samples were collected from dogs at advanced CKD stages (serum creatinine > 2.1 mg/dL; IRIS 3 and 4). Therefore, the increase of MG in urine of CKD dogs might be considered in further studies as a possible biomarker of advanced CKD stages. KnA is a key inflammatory metabolite of the tryptophan catabolic pathway: the degradation of tryptophan occurs through the formation of kynurenine, which in turn can be transformed into KnA and other related metabolites. Kidneys are involved in tryptophan metabolism either eliminating the catabolites or producing the enzymes involved in tryptophan metabolism. In case of renal failure, these metabolites, which are physiologically excreted in urine, accumulate in the blood, contributing to uremia. Accordingly, the study of Rhee et al., [79] reported that serum levels of KnA increased with CKD development and severity. Moreover, increased KnA urinary excretion was associated with adverse clinical outcomes in critically ill patients with acute kidney injury [80] and four tryptophan metabolites, including urinary KnA, were reported to be associated with an estimated glomerular filtration rate (eGFR) decline and with oxidative stress after eight years follow-up [81,82].

Eight metabolites were significantly reduced in urine of CKD patients and the most consistent decrease was evident for trigonelline, which can be obtained from the diet, or alternatively produced as a niacin-derived metabolite. Proximal tubule epithelia synthesize NAD from precursors taken up from urine and an excess of metabolites of the biosynthetic pathway, including trigonelline, is normally secreted in urine. In case of tubular damage, a reduced/absent absorption of nicotinamide or nicotinic acid occurs leading to a reduced/absent trigonelline secretion. Accordingly, in a mice model of acute kidney injury, trigonelline removal from urine was reported as a consequence of tubular damage [83–85].

Significant decrease was observed also for urocanic, indolelactic and trans-ferulic acids. The two first metabolites derive from hepatic histidine and tryptophan catabolism, respectively. In particular, histidine can be converted to histamine, 3-methylhistidine or urocanic acid by different pathways, while indolelactic acid is obtained through the reduction of indolepyruvic acid derived by oxidative deamination of tryptophan. Finally, trans-ferulic acid is a phenolic acid widely distributed in plants that can be absorbed by the small intestine and excreted through the urine. All these metabolites can be found in plasma and urine [22,31,86]. Serum indolelactic acid was recently associated to eGFR in human CKD patients [87], but, to the best of our knowledge, no information is available in the literature on the decrease of these metabolites in the urine of CKD patients. Further studies are therefore needed to clarify their role as possible biomarkers.

This study presents some limitations. The first one is related to the different age between healthy and diseased dogs. Since CKD is a disease of older animals, and adult/old dogs are usually presented to the Veterinary Teaching Hospital due to pathologic conditions, it was not possible to collect samples from age-matched controls. However, none of the different metabolites identified between healthy and CKD dog were reported by Wang et al., [88] as affected by age in healthy dogs. Therefore, despite a possible age effect on urine metabolome cannot be completely excluded, we hypothesise that the effect of CKD was more consistent than the effect of the age. Secondly, the limited number of CKD samples did not allow to highlight significant differences among CKD stages for both proteomics and metabolomics results and it was not possible to highlight clear trends in biomarkers as the disease worsen. The final limitation relates to the absence of technical replicates for the evaluation of the robustness of our data. The technical evaluation of the performance of NMR applied to the dog urine was out of the scope of the present research, especially since the high repeatability of NMR metabolomics is well-known [20,21].

Conclusions

The integrated application of proteomics and metabolomics on urine samples yielded new insight into the molecular complexity of urine in healthy dogs and highlighted biochemical changes in response to CKD. SDS-PAGE evidenced the involvement of the tubular compartment with the decrease of uromodulin and the presence of low MM bands also in non-proteinuric and non-azotaemic dogs and could be considered a useful and complementary diagnostic tool for clinical pathologists, clinicians and researchers working in veterinary nephrology and urology.

NMR metabolomics was successfully applied to canine urinary samples allowing the identification of 86 metabolites. Of these, 17 showed significant differences in CKD dogs. In particular, the increase of carnosine and cis-aconitic acid and the decrease of trigonelline are indicative of the tubular involvement, adding further evidence to the results of SDS-PAGE. Additional studies are needed to clarify the molecular mechanisms underlying the pathophysiology of CKD and to confirm the role of the discovered metabolites as biomarkers of this disease in dogs. In particular, increasing the number of urine samples collected from dogs affected by all stages of CKD should be the focus of future research to confirm early biomarkers and highlight trends as the disease worsen.

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Table 1. Clinical data for healthy and CKD dogs. Data are reported as mean ± SD or median (range) depending on normal or non-normal distribution, respectively.

Signalment	**Healthy (N=22)**	**CKD (N=28)**			***P***
Age in months	37±20	112±61			<0.0001
Female n (entire/neutered)	12 (5/7)	14 (5/9)			
Male n (entire/neutered)	10 (7/3)	14 (9/5)			
Serum biochemistry	**Healthy**	**CKD**	**N (%) CKD < / > RI**	**RI**	
Total Proteins (g/dL)	6.4±0.4	6.0±0.8	5 (17.9) < / 1 (3.6) >	5.6-7.3	0.109
Albumin (g/dL)	3.4±0.3	3.0 (1.1-3.8)	12 (42.9) <	2.8-3.9	<0.0001
Creatinine (mg/dL)	1.1±0.2	2.0 (0.6-9.8)	1 (3.6) < / 20 (71.4) >	0.8-1.4	<0.0001
Urea (mg/dL)	33±8	110 (17-519)	22 (78.6) >	17-48	<0.0001
Phosphorus (mg/dL)	4.6±0.9	4.9 (2.6-14.1)	11 (39.3) >	2.7-5.4	0.056
Urine biochemistry	**Healthy**	**CKD**	**N (%) CKD < / > RI**	**RI**	
UPC	0.07 (0.04-0.19)	0.78 (0.09-12.8)	18 (64.3) >	< 0.5	<0.0001
USG	1052 (1034-1064)	1014 (1006-1062)	27 (96.4) <	> 1.030[a]	<0.0001
IRIS Stage		**N (%)**		**RI**	
I		8 (28.6)		< 1.4	
II		6 (21.4)		1.4-2.0	
III		9 (32.1)		2.1-5.0	
IV		5 (17.8)		> 5.0	

RI, reference intervals; N, number of samples; UPC, urine protein to creatinine ratio; USG, urine specific gravity;

[a] Considered as adequate USG in dogs.

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

Protein name	**Protein entry name** [a]	**MM (kDa)** [b]	**Score** [c]	**Pept** [d]	**Sign Pept** [e]	**Seq** [f]	**Sign seq** [g]
Uromodulin	UROM_CANFA	73	2298	138	113	15	13
Albumin	ALBU_CANFA	69	5802	470	321	44	39
Arginine esterase	ESTA_CANFA	29	532	111	52	10	9

[a] Protein entry name from UniProt knowledge database.

[b] Theoretical protein molecular mass.

[c] The highest scores obtained with Mascot search engine.

[d] Peptides: total number of peptides matching the identified proteins.

[e] Significant peptides: total number of significant peptides matching the identified proteins.

[f] Sequence: total number of distinct sequences matching the identified proteins.

[g] Significant sequences: total number of significant distinct sequences matching the identified proteins.

Table 3. Data for albumin and uromodulin quantification by SDS-PAGE. Data are reported as mean±SD or median (range) depending on normal or non-normal distribution, respectively.

	HEALTHY	**CKD**	***P***
Albumin (mg/dl)	3.1±1.4	26.6 (1.4-228.9)	0.0025
UAC	0.010±0.007	0.213 (0.028-1.395)	0.0002
Uromodulin (mg/dl)	11.9±2.3	0 (0-5.1)	<0.0001
UMC	0.038±0.012	0 (0-0.044)	0.0044

Table 4. Assigned metabolites in the urine of healthy dog. Biological function, process and pathway are also indicated.

Query	HMDB	PubChem	KEGG
1,7-Dimethylxanthine	HMDB0001860	4687	C13747
1-Methyladenosine	HMDB0003331	27476	C02494
1-Methylguanine	HMDB0003282	70315	C04152
1-Methylhistidine	HMDB0000001	92105	C01152
1-Methylnicotinamide	HMDB0000699	457	C02918
2-Furoylglycine	HMDB0000439	21863	NA
2-Hydroxybutyric acid	HMDB0000008	11266	C05984
2-Hydroxyphenylacetic acid	HMDB0000669	11970	C05852
2-Ketobutyric acid	HMDB0000005	58	C00109
2-Methylglutaric acid	HMDB0000422	12046	NA
3-Aminoisobutyric acid	HMDB0003911	64956	C05145
3-Hydroxyphenylacetic acid	HMDB0000440	12122	C05593
3-Indoxylsulfic acid	HMDB0000682	10258	NA
3-Methyl-2-oxovaleric acid	HMDB0000491	47	C03465
3-Methylglutaric acid	HMDB0000752	12284	NA
3-Methylxanthine	HMDB0001886	70639	C16357
4-Aminohippuric acid	HMDB0001867	2148	NA
4-Hydroxybenzoic acid	HMDB0000500	135	C00156
4-Hydroxyphenylacetic acid	HMDB0000020	127	C00642
4-Pyridoxic acid	HMDB0000017	6723	C00847
7-Methyladenine	HMDB0011614	71593	C02241
7-Methylxanthine	HMDB0001991	68374	C16353
Acetic acid	HMDB0000042	176	C00033
Acetylcisteine	HMDB0001890	12035	C06809
Adenosine	HMDB0000050	60961	C00212
Alanine	HMDB0000161	5950	C00041
Allantoin	HMDB0000462	204	C01551
Arabinitol	HMDB0001851	439255	C00532
Ascorbic acid	HMDB0000044	54670067	C00072
Betaine	HMDB0000043	247	C00719
Carnitine	HMDB0000062	2724480	C00318
Choline	HMDB0000097	305	C00114
cis-Aconitic acid	HMDB0000072	643757	C00417
Citric acid	HMDB0000094	311	C00158
Creatine	HMDB0000064	586	C00300
Creatine phosphate	HMDB0001511	587	C02305
Creatinine	HMDB0000562	588	C00791
Cytosine	HMDB0000630	597	C00380
Dimethylamine	HMDB0000087	674	C00543
Ferulic acid	HMDB0000954	445858	C01494
Formic acid	HMDB0000142	284	C00058

Fucose	HMDB0000174	17106	C01019
Galactonic acid	HMDB0000565	128869	C00880
Galactose	HMDB0000143	439357	C00984
Glucaric acid	HMDB0000663	33037	C00818
Glucuronic acid	HMDB0000127	444791	C00191
Glycine	HMDB0000123	750	C00037
Glycolic acid	HMDB0000115	757	C00160
Glyoxylic acid	HMDB0000119	760	C00048
Hippuric acid	HMDB0000714	464	C01586
Histidine	HMDB0000177	6274	C00135
Hypoxanthine	HMDB0000157	790	C00262
3-Methylhistidine	HMDB0000479	64969	C01152
Indole-3-lactic acid	HMDB0000671	92904	C02043
Isobutyric acid	HMDB0001873	6590	C02632
Isoleucine	HMDB0000172	6306	C00407
Kynurenic acid	HMDB0000715	3845	C01717
Lactic acid	HMDB0000190	107689	C00186
Lysine	HMDB0000182	5962	C00047
Mannitol	HMDB0000765	6251	C00392
Methylguanidine	HMDB0001522	10111	C02294
N,N-Dimethylglycine	HMDB0000092	673	C01026
N6-Acetyllysine	HMDB0000206	92832	C02727
N-Acetylglycine	HMDB0000532	10972	NA
N-Phenylacetylglycine	HMDB0000821	68144	C05598
Oxoglutaric acid	HMDB0000208	51	C00026
Phosphorylcholine	HMDB0001565	1014	C00588
Pseudouridine	HMDB0000767	15047	C02067
Pyridoxamine	HMDB0001431	1052	C00534
Serine	HMDB0000187	5951	C00065
Succinic acid	HMDB0000254	1110	C00042
Taurine	HMDB0000251	1123	C00245
Threonine	HMDB0000167	6288	C00188
trans-Aconitic acid	HMDB0000958	444212	C02341
Trigonelline	HMDB0000875	5570	C01004
Trimethylamine	HMDB0000906	1146	C00565
Trimethylamine N-oxide	HMDB0000925	1145	C01104
Tryptophan	HMDB0000929	6305	C00078
Tyramine	HMDB0000306	5610	C00483
Tyrosine	HMDB0000158	6057	C00082
Uracil	HMDB0000300	1174	C00106
Urea	HMDB0000294	1176	C00086
Urocanic acid	HMDB0000301	736715	C00785
Valine	HMDB0000883	6287	C00183
Xanthine	HMDB0000292	1188	C00385
Xanthosine	HMDB0000299	64959	C01762
Xanthurenic acid	HMDB0000881	5699	C02470

Table 5. Significant pathways obtained by the pathway analysis module of MetaboAnalyst.

Pathway	Total [a]	Hits [b]	Raw p	Metabolites
Glycine, serine and threonine metabolism	48	10	1.94E-06	L-Serine; Choline; Betaine; Dimethylglycine; Glycine; L-Threonine; Creatine; 2-Ketobutyric acid; Glyoxylic acid; L-Tryptophan
Phenylalanine metabolism	45	8	7.59E-05	Hippuric acid; Phenylacetylglycine; Succinic acid; Ortho-Hydroxyphenylacetic acid; 4-Hydroxybenzoic acid; p-Hydroxyphenylacetic acid; L-Tyrosine; 3-Hydroxyphenylacetic acid
Aminoacyl-tRNA biosynthesis	75	10	0.00012	L-Histidine; Glycine; L-Serine; L-Valine; L-Alanine; L-Lysine; L-Isoleucine; L-Threonine; L-Tryptophan; L-Tyrosine
Caffeine metabolism	21	6	0.000452	Paraxanthine; 3-Methylxanthine; 7-Methylxanthine; Xanthosine; Xanthine; Glyoxylic acid
Methane metabolism	34	6	0.000666	Glycine; Formic acid; Trimethylamine; Trimethylamine N-oxide; Dimethylamine; L-Serine;
Glyoxylate and dicarboxylate metabolism	50	7	0.001004	cis-Aconitic acid; Glyoxylic acid; Oxoglutaric acid; Formic acid; Glycolic acid; Citric acid; Succinic acid;
Nitrogen metabolism	39	6	0.001418	L-Tyrosine; L-Tryptophan; Taurine; L-Histidine; Glycine; Formic acid;
Citrate cycle (TCA cycle)	20	4	0.00349	Succinic acid; Oxoglutaric acid; cis-Aconitic acid; Citric acid;
Propanoate metabolism	35	5	0.005027	2-Ketobutyric acid; Succinic acid; L-Lactic acid; 2-Hydroxybutyric acid; L-Valine;
Valine, leucine and isoleucine biosynthesis	27	4	0.010619	L-Threonine; L-Valine; L-Isoleucine; 2-Ketobutyric acid;
Taurine and hypotaurine metabolism	20	3	0.025942	Taurine; L-Alanine; Acetic acid;
Purine metabolism	92	8	0.029144	Xanthine; Adenosine; Xanthosine; Hypoxanthine; Urea; Glyoxylic acid; Glycine; Allantoin
Alanine, aspartate and glutamate metabolism	24	3	0.041947	L-Alanine; Oxoglutaric acid; Succinic acid;
Pyrimidine metabolism	60	5	0.044772	Cytosine; Uracil; Pseudouridine; Urea; 3-Aminoisobutanoic acid;

[a] Total metabolites belonging to the pathway as reported by the pathway analysis module of MetaboAnalyst.

[b] Metabolites assigned in urine of healthy dogs belonging to the pathway as obtained by the pathway analysis module of MetaboAnalyst.

Table 6. Metabolites showing significant differences

Bucket	**Metabolite**	**Fold change CKD/Healthy**	**VIP score [a]**	***P* value**
B6_9876	Carnosine	3.15	1.922	0.001
B3_9190	7-Methylxanthine	2.94	1.444	0.037
B5_6610	cis-Aconitic acid	2.67	1.754	0.014
B2_7085	Dimethylamine	1.86	1.512	0.017
B2_8135	Methylguanidine	1.80	1.415	0.025
B7_8490	Kynurenic acid	1.77	1.333	0.045
B5_8415	Xanthosine	1.72	2.054	0.002
B4_2825	Pseudouridine	1.70	1.886	0.002
B7_6681	Pseudouridine	1.59	2.294	0.000
B5_3745	Allantoin	1.47	2.051	0.007
B7_3740	Urocanic acid	0.49	1.323	0.032
B0_9355	2-Hydroxybutyrric acid	0.48	1.343	0.005
B1_0360	L-Valine	0.44	1.583	0.010
B7_7874	4-Hydroxybenzoic acid	0.40	1.278	0.042
B7_1303	trans-Ferulic acid	0.34	1.566	0.009
B8_1155	7-Methyladenine	0.32	1.360	0.030
B7_7217	Indolelactic acid	0.26	1.450	0.018
B6_3648	trans-Ferulic acid	0.26	1.590	0.009
B6_3739	Urocanic acid	0.21	1.713	0.005
B8_8262	Trigonelline	0.15	1.284	0.043
B9_1121	Trigonelline	0.10	1.345	0.034

[a] Variable Importance in Projection (VIP) scores

Fig. 1. Representative SDS-PAGE gels of urine samples from healthy and CKD dogs. Black continuous box indicates uromodulin (103 kDa); black dotted box indicates albumin (67 kDa); black dashed box indicates the internal standard of quantity (1 μg); black dashed and dotted box indicates arginine esterase (18 kDa). M, male; MC, male castrated; F, female; FS, female spayed. LMM, low molecular mass (kDa<67kDa); HMM high molecular mass (kDa>67kDa). S1-4 under each lane indicate the CKD stage of the patient according to IRIS guideline. NP (non-proteinuric, UPC < 0.2), BP (borderline proteinuric, UPC 0.2 - 0.5) or P (proteinuric, UPC>0.5) under each lane indicate the classification of proteinuria according to IRIS guideline.

Fig. 2. Representative NMR spectra of urine samples collected from healthy and CKD dogs.

Fig. 3. Representative spectrum of urine from CKD and healthy dog. For a better visualisation, the spectrum has been divided into four parts. a) From 0.0 to 2.8 ppm; b) from 2.4 to 4.9 ppm; c) from 4.6 to 7.0 ppm; d) from 7.0 to 10.0 ppm. The reported metabolites are: 1 valine; 2 fucose; 3 lactate; 4 alanine; 5 acetate; 6 N6-acetyllysine; 7 N-acetylgycine; 8 acetylcisteine; 9 succinate; 10 pyridoxamine; 11 citrate; 12 dimethylamine; 13 methylguanidine; 14 trimethylamine; 15 N,N-dimethylglycine; 16 creatine; 17 creatinine; 18 choline; 19 phosphorylcholine; 20 carnitine; 21 taurine, trimethylamine N-oxyde, betaine; 22 Taurine; 23 trans-aconitate; 24 3-hydroxyphenilacetate; 25 3-methylxantine; 26 2-hydroxyphenilacetate; 27 glycine; 28 N-phenyilacetylglicine; 29 7-methylxantine; 30 creatine, creatine phosphate, glycolate; 31 pseudouridine; 32 trigonelline; 33 1-methylnicotinamide; 34 allantoine; 35 cis-aconitate; 36 urea; 37 xanthosine; 38 cytosine; 39 urocanate; 40 tyramine, tyrosine; 41 1-methylhistidine; 42 histidine; 43 3-indoxylsulphate; 44 tyramine; 45 hippurate; 46 hypoxanthine.

Fig. 4. a) Principal component analysis (PCA) score plot of healthy (crosses and dark grey circle) and CKD (triangles and faint grey circle) urine samples. b) Partial Least Square – Discriminant Analysis (PLS-DA) distribution plot of healthy (crosses and dark grey circle) and CKD (triangles

and faint grey circle) urine samples. c) Variable Importance in Projection (VIP) scores for the 25 most influential buckets of PLS-DA.

Supplement Table 1. Clinical data, serum and urine biochemistry of each dog included in the study.