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Urinary peptidome analyses for the diagnosis of chronic kidney disease in dogs

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ABSTRACT

Chronic kidney disease (CKD) is clinically important in canine medicine. Current diagnostic tools lack sensitivity for detection of subclinical CKD. The aim of the present study was to evaluate urinary peptidome analysis for diagnosis of CKD in dogs. Capillary electrophoresis coupled to mass spectrometry analysis demonstrated presence of approximately 5400 peptides in dog urine. Comparison of urinary peptide abundance of dogs with and without CKD led to the identification of 133 differentially excreted peptides (adjusted *P* for each peptide <0.05). Sequence information was obtained for 35 of these peptides. This 35 peptide subset and the total group of 133 peptides were used to construct two predictive models of CKD which were subsequently validated by researchers masked to results in an independent cohort of 20 dogs.

Both models diagnosed CKD with an area under the receiver operating characteristic (ROC) curve of 0.88 (95% confidence intervals [CI], 0.72–1.0). Most differentially excreted peptides represented fragments of collagen I, indicating possible association with fibrotic processes in CKD (similar to the equivalent human urinary peptide CKD model, CKD273). This first study of the urinary peptidome in dogs identified peptides that were associated with presence of CKD. Future studies are needed to validate the utility of this model for diagnosis and prediction of progression of canine CKD in a clinical setting.

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Introduction

Chronic kidney disease (CKD) is defined as structural or functional abnormalities of one or both kidneys, present for at least three months (Polzin, 2017). This heterogeneous disease is often not recognized until late in its course. Routine methods used for CKD diagnosis include serum creatinine concentration and urinalysis, evaluation of renal proteinuria, and diagnostic imaging. Current non-invasive diagnostic methods are insensitive for early detection of CKD, especially non-proteinuric disease (Bartges,

2012). Therefore, research with the aim of improving early diagnosis of CKD in dogs is needed. Circulating indirect biomarkers of decreased glomerular filtration rate (GFR) such as SDMA and cystatin C can be used to diagnose CKD (Wehner et al., 2008; Nabity et al., 2015). However, because of the immense compensatory capacity of the kidneys, reduction in GFR, even if measured by creatinine trending, renal clearance studies or by scintigraphy, does not ensue until this compensatory adaption fails (Brown et al., 1990) and therefore, these methods are not likely to represent a reliable way of detecting renal compromise at an early point in time. In recent years, several serum, plasma and urinary biomarkers have been evaluated in dogs with both acute kidney injury (AKI) and CKD for detection of kidney injury at an early point in time (Monti et al., 2012; Nabity et al., 2012; Segev et al., 2013; Hokamp et al., 2016).

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Urine is a potential source of peptide biomarkers for diagnostic use for diseases of the kidney and urinary tract (Decramer et al., 2008). It has some advantages over alternative biological fluids in that its peptide profile is relatively less complex, samples can be obtained non-invasively, and because of the stability of the samples following collection (minimal proteolysis; Mischak et al., 2010b). In human medicine, urine has been explored using 'omics' analyses for identification of biomarkers of disease (Suthanthiran et al., 2013; Lacroix et al., 2014; Boizard et al., 2016). Urinary peptidomics (i.e. analysis of the low molecular weight proteome), using capillary electrophoresis coupled to mass spectrometry (CE-MS), represents a particularly useful tool to diagnose and predict CKD in people (Schanstra and Mischak, 2015). The sample preparation is robust and several thousands of peptides that compose the human urinary peptidome can be identified by employing standard operating protocols and normalization procedures within approximately 1 h (Mischak et al., 2013). In this context, a model containing 273 urinary peptide biomarkers, CKD273, has been identified by CE-MS and shown to perform better than urinary albumin in early detection and prediction of progression of human CKD (Good et al., 2010; Schanstra et al., 2015). This model is currently used in the PRIORITY trial (NCT02040441) in Europe, in which diabetic patients are screened for presence of early renal lesions (Siwy et al., 2016).

Several studies have investigated the urinary proteome in healthy dogs or in dogs with kidney disease, but only two studies have explored proteomics for the diagnosis of CKD (Forterre et al., 2004; Nability et al., 2011). However, no attempt to validate study findings in a separate cohort of dogs was made in these studies. The aim of the present study was to evaluate the ability of CE-MS-based urinary peptidome analysis to discriminate between healthy dogs and those with CKD. A secondary aim was to identify the peptides included in the discriminating models.

Materials and methods

Study population

This observational study was performed at the Swedish University of Agricultural Sciences in Uppsala, after approval by Uppsala ethical committee (Approval Number C340/11, Approval date 16 December 2011; and Approval Number C119414/15, Approval date 1 April 2015). All experiments were performed in accordance with relevant guidelines and regulations and with informed consent from dog owners. Dogs with a previous diagnosis of CKD and healthy dogs, of any breed, bodyweight (BW) and age were prospectively recruited. Dogs were considered to have a conclusive diagnosis of CKD if they had multiple renal cysts, persistent (≥ 3 months) azotemia, persistent proteinuria, a persistently decreased measured glomerular filtration rate (mGFR), or a combination thereof. Exclusion criteria were the presence of other systemic or organ related disease. If receiving an angiotensin converting enzyme inhibitor, the drug was withdrawn a week before inclusion and reintroduced after study inclusion because of unknown effects of this drug on the canine urinary peptidome. Dogs chronically medicated with other drugs (except sodium pentosane polysulfate injections) were excluded, as were dogs with bacteriuria. Oral administration of glycosaminoglycan supplements and feeding a kidney diet was allowed. Healthy student-, client-, and staff-owned dogs of various breeds and ages were included as controls, as were six healthy beagles from a Swedish research institution. These beagles were considered free of kidney disease based on the absence of clinical signs, a creatinine concentration within the reference range and urinalysis, including semi-quantitative biochemical analysis, sediment examination, protein-to-creatinine ratio (UPC), and kidney histology without pathological lesions (light microscopy).

On the day of enrolment into the study, dogs (including all control dogs except the six research beagles) underwent a physical examination, collection of venous blood and urine, echocardiographic examination, abdominal ultrasound examination of the entire urinary tract, and a scintigraphic examination for calculation of individual kidney mGFR. Dogs had to be clinically stable and fasted for 12 h on the day of inclusion. Dogs with CKD were staged according to the International Renal Interest Society (IRIS) classification system,² based on stable serum creatinine

concentration. According to guidelines for human clinical proteomics (Mischak et al., 2007, 2010a) dogs were divided into two cohorts: a discovery cohort, in which the clinical status of each dog was known to those performing the analyses and an independent validation cohort, in which the clinical status of the dogs was unknown to those performing the analyses.

Examinations of blood and urine

Blood drawn from the cephalic vein was transferred to the laboratory at the University Animal Hospital for immediate hematological and biochemical analysis. For most dogs, urine was obtained by cystocentesis at the time of abdominal ultrasound examination. When cystocentesis was not possible ($n=8$), fresh spontaneously voided urine was obtained. No proteinase inhibitor was added to the urine (Havanapan and Thongboonkerd, 2009). Urine was divided into aliquots, and 5–10 mL was immediately used for analysis (dipstick and sediment examinations, specific gravity, UPC and aerobic culture). Remaining urine was stored immediately at -70°C . Urine from the six beagles had previously been obtained by cystocentesis, snap frozen and stored at -70°C . Remaining urine from the beagles had been cooled and analysed within 24 h (dipstick and sediment examinations, specific gravity, UPC and aerobic culture).

Abdominal ultrasound examination and renal scintigraphy

Complete upper and lower urinary tract ultrasound examinations were conducted according to a pre-defined protocol by radiologists (board certified or in residency training) at the university animal hospital diagnostic imaging clinic. Measurement of individual kidney GFR was performed by a board certified radiologist after renal scintigraphy, using the plasma volume method as previously described (Westgren et al., 2014). With the plasma volume method, the rate of glomerular filtration is indexed to an estimation of plasma volume (Westgren et al., 2014). A total (left + right kidney) mGFR <30.8 mL/min/L was considered subnormal (Kampa et al., 2003, 2007).

Sample preparation

Urine samples from all dogs were shipped on dry ice to Inserm U1048, Toulouse, France, and thawed immediately before use. A volume of 0.7 mL was diluted with 0.7 mL 2 M urea, 10 mM NH_4OH and 0.02% sodium dodecyl sulphate (SDS). In order to remove high molecular weight polypeptides, samples were filtered using Centriscart ultracentrifugation filter devices (20 kDa molecular weight cut-off; Sartorius) at 3000 g until 1.1 mL of filtrate was obtained. The filtrate was desalted with PD-10 column (GE Healthcare) equilibrated in 0.01% NH_4OH in HPLC-grade water. The prepared samples were lyophilized and stored at 4°C . Shortly before CE-MS analysis, lyophilised samples were resuspended in HPLC-grade water (Merck KGaA). The preparation method has previously been described in more detail (Theodorescu et al., 2006).

CE-MS analysis and data processing

CE-MS analysis was performed as previously described (Mischak et al., 2013). Briefly, CE-MS analyses were performed using a Beckman Coulter Proteome Lab PA800 capillary electrophoresis system (Beckman Coulter) on-line coupled to a micrOTOF II MS (Bruker Daltonic). The electro-ionization sprayer (Agilent Technologies) was grounded, and the ion spray interface potential was set to -4.5 kV. Data acquisition and MS acquisition methods were automatically controlled by the CE via contact-close-relays. Spectra were accumulated every 3 s, over a range of m/z 350–3000. In the next step the MosaiquesVisu software package was applied to deconvolute mass spectral ion peaks, because ionization produced ions at different charged states from the original urinary peptides. This deconvolution step groups these differently charged ions into single peptides with unique real mass. Only signals observed in a minimum of three consecutive spectra with a signal-to-noise ratio of at least 4 were considered. Signals with a calculated charge of $1+$ were automatically excluded to minimize interference with matrix compounds or drugs. Capillary electrophoresis migration time and MS-detected mass were normalized by the definition of 950 clusters of peptides covering a range of 17.23–47.74 min in CE migration time and 807–16,399 kDa in molecular mass. Samples were normalized by peptide abundance (intensity) calibration based on 141 endogenous internal urinary polypeptide standards displaying the highest frequency and stability in all analysed samples, to compensate for differences in hydration status and urine volume between dogs. This procedure has been described previously for people (Good et al., 2010; Dissard et al., 2013). Each polypeptide present in the list was defined by its normalized migration time (min), molecular mass (kDa), and signal intensity detected. Using a Microsoft Structured Query Language database, all detected polypeptides were deposited, matched, and annotated in order to allow for further comparison between groups. The criteria applied to consider a polypeptide identical was that within different samples, the mass deviation was lower than 50 ppm for masses <4 kDa, 150 ppm for masses >6 kDa, and between 50–150 ppm for masses between 4 and 6 kDa. Acceptable migration time deviation was between 1 and 2.5 min.

² See: International Renal Interest Society <http://www.iris-kidney.com/guidelines/staging.html> (Accessed 23 May 2019).

Sequencing of peptides

Candidate biomarkers and other native peptides from dog urine were sequenced using liquid chromatography (LC)–MS/MS and CE–MS/MS analysis (Klein et al., 2014). All LC–MS/MS analysis experiments were performed on a Dionex Ultimate 3000 RSLC nano flow system (Dionex). For CE–MS/MS, the samples were injected under constant flow and pressure conditions at a pH of 2.2 to ensure that all peptides are positively charged. Both CE and LC were directly interfaced with an LTQ–Orbitrap XL (Thermo Finnigan) using data-dependent high-energy collisional dissociation (HCD) MS/MS sequencing of a maximum of the top 20 ions. All resultant MS/MS data were analysed using Proteome Discoverer 1.3 (activation type: HCD; min-max precursor mass: 790–6000; precursor mass tolerance: 10 ppm; fragment mass tolerance: 0.05 Da; S/N threshold: 1) and were searched against the Uniprot canine non-redundant database without enzyme specificity. No fixed modifications were selected, oxidation of methionine and proline were selected as variable modifications. The peptide data were extracted using high confidence peptides, defined by an $X_{corr} \geq 1.9$, a delta mass between experimental and theoretical mass ± 5 ppm, absence of cysteine in the sequence (because cysteines without reduction and alkylation form disulphide bonds), absence of oxidized proline in protein precursors other than collagens or elastin, and top one peptide rank filters.

For further validation of peptide identification, the strict correlation between peptide charge at pH 2 and CE-migration time was utilized to minimize false-positive identification rates (Zurbig et al., 2006). Calculated CE-migration time of the sequence candidate based on its peptide sequence (number of basic amino acids) was compared to the charges of the peptides and the experimental CE-migration time. Peptides were accepted only if they had a mass deviation below ± 90 ppm and a CE-migration time deviation below ± 2 min.

Biomarker selection and modelling

For the identification of candidate urinary biomarkers, the reported P -values were calculated using the Wilcoxon rank-sum test (R software package, version 3.1.3) followed by adjustment for multiple testing (Benjamini and Hochberg, 1995). Peptides that were detectable in at least 75% of dogs in one of the two groups (healthy dogs vs. dogs with CKD) and reached an adjusted $P < 0.05$ were further considered as relevant. An R-based (version 3.1.3) support vector machine (SVM)-package and leave-one-out feature selection approach was used to generate biomarker models. Sensitivity and specificity were calculated based on the number of properly classified samples. The overall yield of the polypeptide pattern was evaluated by receiver operating characteristic (ROC) and area under curve (AUC) plots using the Prism 7.00 GraphPad software.

Results

Study design and canine data

In total, 50 dogs (25 dogs with CKD and 25 healthy dogs) were included in the study. Clinical data for the 25 dogs with CKD are summarized in Supplementary Table S1. The minimum number of individuals to include in a discovery cohort in a proteomic study is 24 (Dakna et al., 2010) and therefore, 30 dogs (15 healthy dogs and 15 dogs with CKD) were included in the discovery cohort. Thus, 20 dogs comprised the independent validation cohort (10 healthy dogs and 10 dogs with CKD). An attempt was made to accomplish an even distribution of dogs in different IRIS CKD stages in the discovery- and

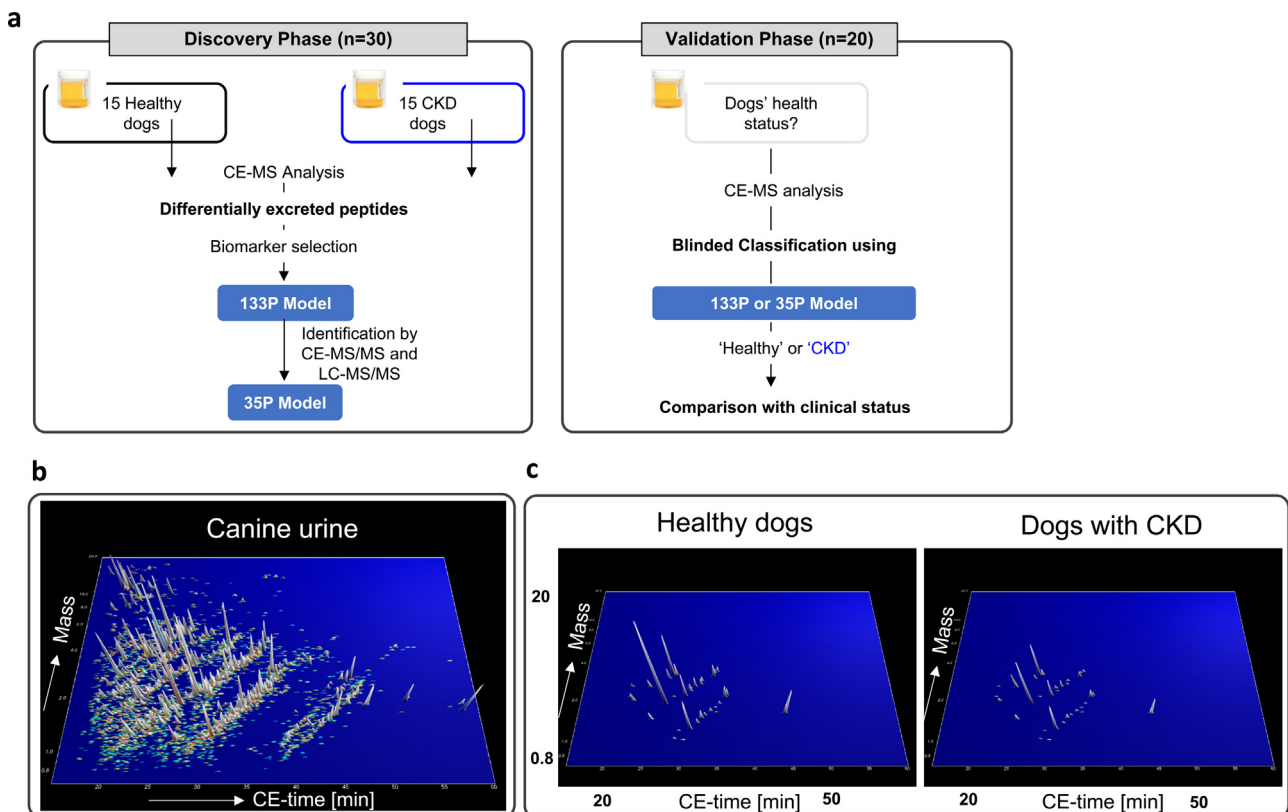


Fig. 1. Study design and capillary electrophoresis coupled to mass spectrometry (CE–MS) analysis of canine urine. (a) The analysis was performed in two separate phases: a discovery phase and a validation phase. Urine from 30 dogs (15 healthy dogs and 15 with chronic kidney disease [CKD]) was analysed, leading to the identification of 133 differentially secreted peptides (133P model). Of these peptides, 35 were identified by CE–MS/MS and liquid chromatography (LC)–MS/MS sequencing and included in the 35P model. In the validation phase, the two different models were tested on an independent, masked cohort of dogs ($n = 20$) to evaluate their predictive value. (b) Peptide pattern showing the compiled datasets of 30 canine urine samples. Each peptide ($n = 5398$) was identified based on CE migration time and specific mass (kD), with relative abundance represented by the peak height. (c) Sequenced peptide ($n = 35$) pattern distinguishing dogs with CKD from healthy dogs.

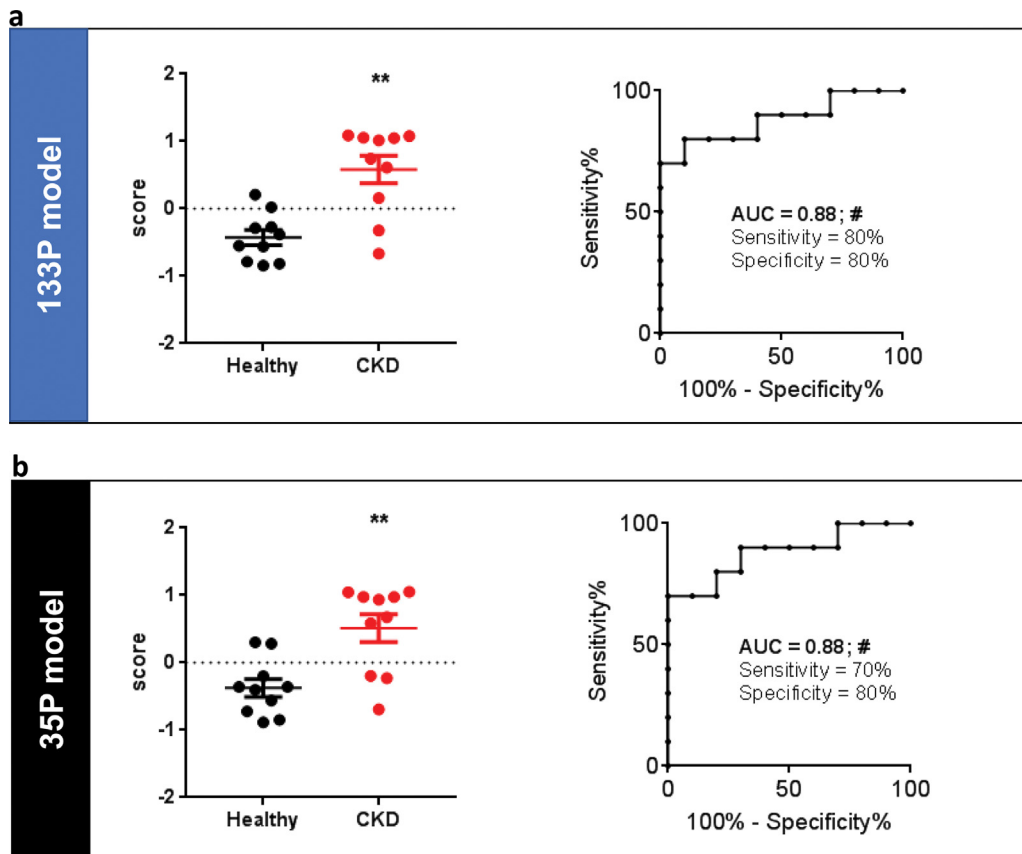


Fig. 3. Validation of urinary peptide models in an independent, masked cohort. Classification of healthy dogs and dogs with chronic kidney disease (CKD) in the validation cohort according to (a) 133P model scores and receiver operating characteristic (ROC) curve for the 133P model (area under the curve [AUC] 0.88; 95% confidence intervals [CI], 0.721–1.04); (b) 35P model scores and ROC curve for the 35P model (AUC 0.88; 95% CI, 0.723–1.04). **, $P < 0.01$ vs. healthy dogs, Mann–Whitney test for independent samples; #, $P < 0.005$.

Sequencing of peptides

Sequence information was obtained for 35 out of the 133 differentially excreted urinary peptides. Thirty-three of them were fragments of collagen (I and IV) and two of them were uromodulin fragments (Table 1). All 35 peptides were present in lesser (normalized) amounts in dogs with CKD (Fig. 1c). These 35 peptides were combined in a second SVM model (35P), which significantly separated healthy dogs and dogs with CKD in the discovery cohort (Fig. 2b).

Validation of 133P and 35P models

Masked validation was performed by scoring the dogs as 'healthy' or 'CKD' using both models. A positive score (>0) was used to predict CKD. After unmasking, these predictions were compared to the clinical status of the dog. The distribution of 133P and 35P scores showed significant separation of healthy dogs and dogs with CKD; the 133P model predicted CKD with a sensitivity of 80% (95% confidence interval [CI], 44–97), a specificity of 80% (CI: 44–97) and an AUC of 0.88 (CI: 0.72–1.0; Fig. 3a). The 35P model predicted CKD with a sensitivity of 70% (CI: 35–93), a specificity of 80% (CI: 44–97) and an AUC of 0.88 (CI: 0.72–1.0; Fig. 3b).

Discussion

In the present study, urinary peptide biomarkers of CKD in dogs were identified using a mass spectrometry-based approach. To our knowledge, this is the first time that canine urinary peptide markers have been identified for CKD. Combination of these

peptides in two different mathematical models composed of either all significant peptides, or only sequenced peptides, predicted CKD with an area under the ROC curve of 0.88 (95% CI, 0.72–1.0) in an independent validation cohort. Multiple marker-methods may identify complex processes (such as renal fibrosis) better than single-marker methods do (Fliser et al., 2007). It is therefore possible that this technology may represent a useful additional diagnostic tool for early detection of CKD in the dog, similar to the human counterpart model CKD273. An additional highly important clinical issue is whether CKD in an individual dog is progressive or not. There is initial evidence that CKD273, constructed under similar conditions as those in the present study, is associated with CKD progression (Schanstra et al., 2015). Therefore it is possible that the canine peptide models identified in the present study have similar characteristics. This will need to be confirmed in a cohort for which longitudinal clinical follow-up is available.

Most of the sequenced peptides associated with canine CKD were collagen I fragments, which were less abundant in the urine of dogs with CKD than in the urine of healthy dogs. This is in agreement with studies in human CKD (Rossing et al., 2008; Good et al., 2010). It is hypothesized that decreased urinary collagen fragments are associated with decreased collagenase activity and increased intrarenal extra-cellular matrix (ECM) deposition (Rossing et al., 2008). Intrarenal ECM deposition leading to fibrosis is the common final pathway in many kinds of CKD across species, including human (Rossing et al., 2008; Good et al., 2010), and therefore this reasoning most likely also holds true in dogs. Furthermore, a recent study showed a significant association between CKD273 score and degree of fibrosis in

renal biopsies (Magalhaes et al., 2017). If the peptidomic model scores are associated with ongoing fibrosis in CKD, this diagnostic modality would detect something entirely different than the current clinical standards (assessment of GFR by measurement of circulating indirect biomarkers such as serum creatinine, urinalysis and imaging) do, and consequently represent a completely new non-invasive diagnostic option. Therefore, this technique should be further validated in future studies.

Limitations of this study include the small validation group and lack of evaluation of biological variation of the peptide models. A further validation study is planned to investigate the performance of these models. This study will also allow evaluation of the potential impact of urine sampling technique (cystocentesis vs. free catch) on results of CE–MS analysis in canine urine and to further sequence all differentially excreted peptides identified in the present study.

In this study, 3 dogs with suspected glomerulopathy (UPC >3–4), were included. The resultant heterogeneity of the CKD groups may have influenced the results. However, because proteins >20 kDa are removed before analysis, this is probably not of major importance.

Because of the limited number of dogs within different stages of CKD in this study, dogs were not randomly allocated to discovery and validation cohorts. Instead, dogs were grouped with the intention to obtain similar average degree of severity of CKD in both groups. This way a large discrepancy regarding severity of CKD between the two cohorts was avoided.

Conclusions

Urinary peptide based models were able to discriminate healthy dogs from dogs with CKD in an independent cohort of 20 dogs. Thirty-five of the 133 peptides differentially expressed between healthy dogs and dogs with CKD were sequenced, and most of them were collagen fragments. Peptidome analysis by CE–MS is a promising future additional tool for diagnosis of canine CKD, but further investigations are necessary in order to validate the usefulness of these models for early CKD diagnosis and prediction of progression in a clinical setting.

Conflicts of interest statement

PZ and PM are employees of Mosaiques Diagnostics. None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.tvjl.2019.05.010>.

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