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Urinary proteome of dogs with renal disease secondary to leishmaniosis

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ABSTRACT

Canine leishmaniosis is frequently associated with the development of renal disease. Its pathogenesis is complex and not fully understood. For this reason, this study aimed to describe the urinary proteome, and identify possible new biomarkers in dogs with kidney disease secondary to leishmaniosis. Urine samples were collected from 20 dogs, 5 from healthy dogs, and 15 from stages Leishvet III and IV. Urine samples were analyzed by UHPLC–MS/MS. The data are available via ProteomeXchange with identifier PXD029165. A total of 951 proteins were obtained. After bioinformatic analysis, 93 urinary proteins were altered in the study group. Enrichment analysis performed on these proteins showed an overrepresentation of the *complement activation* pathway, among others. Finally, 12 discriminant variables were found in dogs with renal disease secondary to leishmaniosis, highlighting *C4a anaphylatoxin, apolipoprotein A-I, haptoglobin, leucine-rich alpha-2-glycoprotein 1,* and *beta-2-microglobulin.* This study is the first to describe the urinary proteomics of dogs with renal disease caused by leishmaniosis, and it provides new possible biomarkers for the diagnosis and monitoring of this disease.

1. Introduction

Canine leishmaniosis (CanL) is a vector disease caused by the protozoan *Leishmania infantum*, which presents a complex pathogenesis. This disease is responsible for a wide variety of clinical presentations in dogs, highlighting among them the lessons in the kidney (Alvar et al., 2004; Costa et al., 2003; Koutinas and Koutinas, 2014; Roura et al., 2021).

CanL at the renal level produces glomerular damage mainly due to immunocomplex deposition, after which it can spread to the tubulointerstitial area to a greater or lesser degree, causing dysfunction (Benderitter et al., 1988; Costa et al., 2003; Koutinas and Koutinas, 2014; Roura et al., 2021; Zatelli et al., 2003). On many occasions, the disease culminates with the appearance of advanced glomerulonephritis (GN)(Alvar et al., 2004; Benderitter et al., 1988; Costa et al., 2003; Koutinas and Koutinas, 2014; Roura et al., 2021; Zatelli et al., 2003), and in some cases, dogs develop nephrotic syndrome (NS) in both scenarios with fatal consequences (Félix et al., 2008; Font and Closa, 1997; Koutinas and Koutinas, 2014). Despite advances in the knowledge of CanL and the high prevalence of the disease in some areas, due to the subclinical course of both leishmaniosis and the associated kidney disease, as well as the use of late biomarkers for the diagnosis and monitoring of kidney disease, many dogs are diagnosed in advanced stages of the disease. Frequently, these dogs are euthanized without a correct understanding of their kidney disease at the time of diagnosis. However, sometimes we may find a partially reversible condition compatible with

Abbreviations: CanL, Canine leishmaniosis; GN, Glomerulonephritis; NS, Nephrotic syndrome; CKD, Chronic kidney disease; CUP, Canine urinary proteome; HUP, Human urinary proteome; UP/C ratio, Urine protein/creatinine ratio; SLA, Total soluble antigen; DTT, Dithiothreitol; UHPLC, Ultra High-Performance Liquid Chromatography; Q-TOF, Quadrupole Time of Flight; MS, Mass Spectrometry; ESI, Electrospray Ionization; FDR, False discovery rate; PCA, Principal component analysis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genome; HP, Human Phenotype Ontology; CC, Cellular Component; MF, Molecular Function; BP, Biological Process; LC, Liquid Chromatography; CE-MS, Capillary Electrophoresis–Mass Spectrometry; LN, Lupus nephritis; AKI, Acute kidney injury; THP, Tamm-Horsfall protein; RBP, Retinol-binding protein; DN, Diabetic nephropathy; B2M, beta-2 microglobulin; XLHN, X-linked hereditary nephropathy; FB, Fetuin B; FSGS, Focal segmental glomerulosclerosis; HP, Haptoglobin; APOA-1, Apolipoprotein-A1; HDL, High-density lipoprotein; ABP, Amyloid-beta precursor; HPX, Hemopexin; LRG1, leucine-rich alpha-2-glycoprotein 1..

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an adequate quality of life (Roura et al., 2021).

In damage at the level of the nephron, the appearance of proteinuria stands out, which can be present in the initial stages and markedly worsening as the disease progresses (Roura et al., 2021, 2013). Therefore, urine becomes a valuable biofluid that contains a proteome of great potential for the diagnosis and monitoring of different pathologies that affect the kidney, even better than blood (Gao, 2015). However, routine analysis methods have great limitations in regard to providing information about the diagnosis and follow-up of renal patients with chronic kidney disease (CKD)(Fassett et al., 2011).

In recent decades, the evolution of molecular fluid analysis techniques has made possible, together with the development of bioinformatics, a large-scale evaluation of urine and other biological samples through proteomic methods. This allows us to qualitatively and quantitatively evaluate the concentration of proteins, identify their structure and function, and understand their clinical application (Laborde et al., 2011); in conclusion, providing a better knowledge of renal physiology and pathogenesis of its diseases. To date, the canine urinary proteome (CUP) has already been characterized (Brandt et al., 2014), and it has also described in dogs with CKD (Ferlizza et al., 2020; Pelander et al., 2019), as well as in infectious processes with renal repercussions such as babesiosis (Winiarczyk et al., 2019) and dirofilariasis (Hormaeche et al., 2014). These studies have revealed several biomarkers with great clinical potential. Furthermore, CUP has been compared with the human urinary proteome (HUP), considering dogs as a suitable model for the study of renal pathologies (Brandt et al., 2014). In the case of leishmaniosis, there are publications that have evaluated the proteome in various biological fluids of dogs, such as serum (Britti et al., 2010; Franco-Martínez et al., 2020) or saliva (Franco-Martínez et al., 2019), but none of them have evaluated the urinary proteome of dogs with CanL and renal disease.

Therefore, the objectives of this study were: 1) to characterize the urinary proteome of dogs with advanced stages of CanL, and 2) to identify possible biomarkers for the diagnosis and monitoring of renal disease in dogs with advanced stages of CanL.

2. Materials and methods

2.1. Animal handling

All handling protocols of the studied dogs and the collection of samples were reviewed and approved by the Ethics Committee of the Ministry of the Environment and Rural, Agrarian Policies and Territory, as well as Animal Health (Extremadura, Spain). In this study, dogs treated at the Veterinary Clinical Hospital of the University of Extremadura (Cáceres, Spain) between 2019 and 2020 were used, with a range of ages from 1 to 10 years, vaccinated and dewormed, that had not suffered from any other pathology, and that had not received any treatment before the beginning of the study. The dogs in this study were divided into two groups: a control group ($n = 5$; two females and three males) comprised of healthy dogs, and a study group ($n = 15$; five females and ten males) with dogs naturally infected by *Leishmania infantum* in advanced stages of the disease (stages III and IV), previously described in LeishVet guidelines in 2011 (Solano-Gallego et al., 2011). All dogs underwent blood hematology and biochemistry, urinalysis, urine protein/creatinine ratio (UP/C ratio), urine culture, abdominal ultrasound, and a 4DX SNAP® test (IDEXX Laboratories, Westbrook, Maine, USA) for the detection of vector diseases (antigen against *D. immitis* and antibodies against *E. canis*, *Anaplasma* spp. and *B. burgdorferi*), and they were tested for the detection of antibodies against *Leishamania infantum* with a commercial kit based on immunochromatography (Uranotest Leishmania, Urano®vet Inc.). The dog was considered healthy when all of the tests performed were within normal values, and the general physical examination and abdominal ultrasound did not show any alteration. In study group, all dogs showed clinicopathological alterations compatible with renal disease, and they were positive for *Leishmania infantum* by a commercial kit. This was confirmed by a semiquantitative ELISA technique for the determination of antibodies against the total soluble antigen (SLA) of *Leishmania infantum* (Belinchón-Lorenzo et al., 2013). Finally, dogs in the study group were sorted into stages III and IV of the Leishvet classification (Solano-Gallego et al., 2011).

2.2. Urine collection

All samples were collected from fasting dogs. Five milliliters of urine was collected from each dog by cystocentesis. It was immediately separated for urinalysis and urine culture. The remaining volume was centrifuged for 5 min at 200*g*. Later, the supernatant was frozen in 1 mL aliquots at −20 °C and then at −80 °C until subsequent analysis 12 months after collection.

2.3. Protein quantification

The pyrogallol red colorimetric method (gernon, RAL®, Barcelona, Spain) in the Saturno 100 automatic analyzer (Crony® instruments, Roma, Italy) was used for quantification of the protein concentration in the analyzed samples.

2.4. Tryptic digestion

Based on the quantification results, an aliquot containing 100 μg of protein was taken from each sample and adjusted with type I grade water to a final volume equal to the volume of the less concentrated urine sample. After that, 25 μL of 0.1 M of sodium bicarbonate ($NaHCO_3$) and 1.5 μL of 0.2 M of dithiothreitol (DTT) were added to each sample and incubated for 20′ at 56 °C to reduce the sample proteins. After cooling down to room temperature, 3 μL of 0.2 M iodoacetamide were added, and samples were incubated for 45′ at room temperature in the dark to achieve the alkylation of the proteins. Then, 1 μL of 0.2 M DTT was added to each sample and incubated for another 30′ at room temperature in the dark, neutralizing the possible excess of iodoacetamide. After verifying the pH (7–9) with indicator paper, 2 μL of porcine Trypsin Gold, Mass Spectrometry Grade (Promega) (trypsin solution: 1 μg/μL in 1 mM of hydrochloric acid, HCl) was added, and the samples were incubated overnight at 37 °C. To stop the digestion, 1 μL of 0.1% formic acid was added to the solution. Then, the samples were centrifuged at 6272*g* for 5′, and the supernatant was transferred to a new tube. After that, the tubes were placed in a centrifugal vacuum concentrator (HyperVAC-LITE, Gyrozen, Rep. of Korea) and left to evaporate at 100 mbar at 2000 rpm for 3 h at 37 °C. Once desiccated, the samples were reconstituted with 20 μL of a solution containing water, acetonitrile and formic acid (94.9/5/0.1).

2.5. UHPLC–MS/MS analysis

Once the urine samples were prepared in the previous stage, an Agilent 1290 Infinity II Series Ultra High-Performance Liquid Chromatography (UHPLC) (Agilent Technologies, Santa Clara, CA, USA) equipped with an automated multisampler module and a high-speed binary pump coupled to an Agilent 6550 Quadrupole Time of Flight (Q-TOF) Mass Spectrometry (MS) (Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual Electrospray Ionization (AJS-Dual ESI) interface was used to analyze them. Control of the UHPLC and Q-TOF was performed by MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.06.01). First, in an Agilent AdvanceBio Peptide Mapping UHPLC column (2.7 μm, 150 × 2.1 mm, Agilent Technologies) thermostatted at 55 °C, a urine sample was injected at a flow rate of 0.4 mL/min. A starting value of 2% B (buffer B: water/acetonitrile/formic acid, 10: 89.9: 0.1) was used in the gradient program; then, it remained in isocratic mode, then it increased linearly to 45% of B, then to 95% of B, and finally, it remained constant; the time

in each stage was 5 min, 40 min, 15 min and 5 min, respectively. Therefore, the analysis cycle of each sample was carried out in a total time of 65 min. In addition, 5 min were necessary between cycles to prepare and return the column to the initial conditions before the next analysis. Parameters established in the mass spectrometer operating in positive mode were as follows: a nebulizer gas pressure of 35 psi, a sheath gas flow of 12 l/min and a temperature of 300 °C, a drying gas flow of 10 l/min and a temperature of 250 °C. The set voltages were 3500 V for the capillary spray, 340 V for the fragmentor and 750 V for the octopole RF Vpp. Data acquisition was achieved with a dynamic extended range between 50 and 1700 *m/z* for both scans, MS and MS/MS scan; rate scans were 8 spectra/s and 3 spectra/s, respectively. Ion scans twice consecutively were turned down for subsequent evaluations. Data selection was performed using Auto MS/MS mode following these criteria: slope of 3.6 for the ramped collision energy and − 4.8 for the offset. Precursors were selected by abundance and 20 per cycle maximum. All samples were analyzed in duplicate.

2.6. Data processing

Relative quantification, and peptide and protein identification of the results obtained by MS were performed using Spectrum Mill MS Proteomics Workbench software (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA). For extracting the raw data, the following nonfixed modifications were preset for Agilent: [MH] + 50–10,000 m/z; maximum precursor charge +5; retention time and m/z tolerance ±60 s; and 12C signals were found with a minimum signal-to-noise MS (S/N) 25. UniProt (https://www.uniprot.org/), specifying the dog condition, was the protein database consulted to perform the MS/MS search. It was carried out using nonfixed modifications and carbamidomethylated cysteines, and 5 maximum missed cleavages for tryptic digestion were established as variable modifications.

The ESI-Q-TOF instrument had a minimum matched peak intensity of 50%, maximum ambiguous precursor charge +5, monoisotopic masses, peptide precursor mass tolerance 20 ppm, product ion mass tolerance 50 ppm, and the calculation of reversed database scores. An autothreshold was used for the autovalidation process, and its automatically optimized peptide score was used for a target protein % false discovery rate (FDR) (1.2%). After that, we used an FDR (0%) to enlarge the sequence coverage of the validated results.

2.7. Bioinformatics

2.7.1. Variance filtering and principal component analysis (PCA)

Bioinformatic analysis software Qlucore Omics Explorer version 3.6 Lund Sweden (https://qlucore.com) was used. After normalization and logarithmic conversion of the data, variables with a low variance were discarded, reducing the influence of noise. For a more objective visualization of these data, they were represented by a PCA in 3D. The projection score was used to establish the optimal filtering threshold (Bourgon et al., 2010; Fontes and Soneson, 2011). After that, for a better understanding of the relationship between dogs and their patterns of urinary protein expression, data were represented by a heatmap and hierarchical clustering (Eisen et al., 1998).

2.7.2. Identification of discriminating variables

The most significant differences between variables in the two groups (control and study groups) were evaluated by Qlucore Omics Explorer (https://qlucore.com) using the Benjamini–Hochberg method (Tamhane et al., 1996; Viskoper et al., 1989). Variables with adjusted *p* values <0.1 were considered statistically significant.

2.7.3. Enrichment analysis

This analysis was carried out using the g:Profiler web server (https://biit.cs.ut.ee/gprofiler/) (Raudvere et al., 2019) on the 93 altered proteins. Before the analysis, a series of preset data sources were selected as Gene Ontology (GO) (http://geneontology.org/) (Mi et al., 2019) annotations, pathways stored in the Kyoto Encyclopedia of Genes and Genome (KEGG) (https://www.genome.jp/kegg/) (Kanehisa, 2000) and Human Phenotype Ontology (HP) files (https://hpo.jax.org/app/) (Köhler et al., 2021). Only the annotations with strong evidence, using a threshold g:SCS $P < 0.01$, were considered valid.

2.8. Validation

Results obtained in the proteomic analysis were validated by measuring the urinary concentration of retinol-binding protein (RBP), and Tamm-Horsfall protein (THP). RBP concentration was quantified using a quantitative sandwich-type ELISA (Canine Retinol-binding protein 4 ELISA Kit; MyBioSource, Inc.; San Diego, California, U.S.A.; No MBS1602566). The same type of ELISA was employed to measure THP concentration (Canine T-H Glycoprotein ELISA Kit; MyBioSource, Inc.; San Diego, California, U.S.A; No MBS010151.). All samples were analyzed in duplicate, and following the manufacturer's instructions. The statistical analysis was performed using the Shapiro-Wilk test to study normality, and the results between groups were compared by a one-way analysis of variance in ranks using the Holm-Sidak method. A statistically significant difference was considered with $P < 0.001$.

3. Results

3.1. Influence of CKD by CanL on the urinary proteome

A total of 20 dogs were studied, and 40 urine samples were analyzed in this study; 10 belonged to the control group (5 dogs), and 30 belonged to the study group (15 dogs). After completing the analysis, 951 proteins were identified and quantified. The Mass Spectrometry proteomics data have been deposited at the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the assigned identifier PXD029165.

The initial bioinformatic analysis of the peptide patterns revealed a total of 752 proteins. These data were represented by a volcano plot (Fig. 1), and their distribution showed decreases and increases in various proteins in sick dogs with respect to the control group. To quantify the total number of proteins altered in disease relative to the condition of a healthy dog, the results were represented using a Venn diagram (Fig. 2), and within the total group of proteins, 93 proteins showed a marked difference in the group of dogs with renal disease secondary to leishmaniosis, while the remaining 659 proteins were not altered. A fold change of 3.8 was used for the analysis with $p = 0.047$ and $q = 0.019$.

Subsequently, the results were subjected to a comparative analysis between the two groups defined in the study: control group and study group; the final result was represented graphically for better visualization through a heatmap (Fig. 3). The heatmap shows the relationship between the results obtained for the samples of the different dogs. The colour assigned for the dogs in the control group is gray, all of which are represented in the right area of the image, and red for the study group, which is exclusively represented in the left zone. In this way, we can observe that most of the dogs who present with kidney disease secondary to leishmaniosis have an increase in almost all of the 93 proteins obtained, except in the levels of 3 of them; that is, they present very similar patterns in the urinary proteome. This is clearly observed when analyzing the vertical distribution of the table, which refers to the hierarchical grouping of the proteins analyzed. Certain dogs, especially in the study group, show an increase in some families, not being patent in others, despite presenting the disease. This finding may have pathophysiological connotations that may be useful in the diagnosis, classification and prognosis of the disease. The results obtained from proteins with a marked alteration in the disease are also presented in a 3D PCA (Fig. 4), with the aim of a clear and objective interpretation of the number of proteins that decreased or increased with respect to the control group. It is evident that there is a group of 3 proteins that

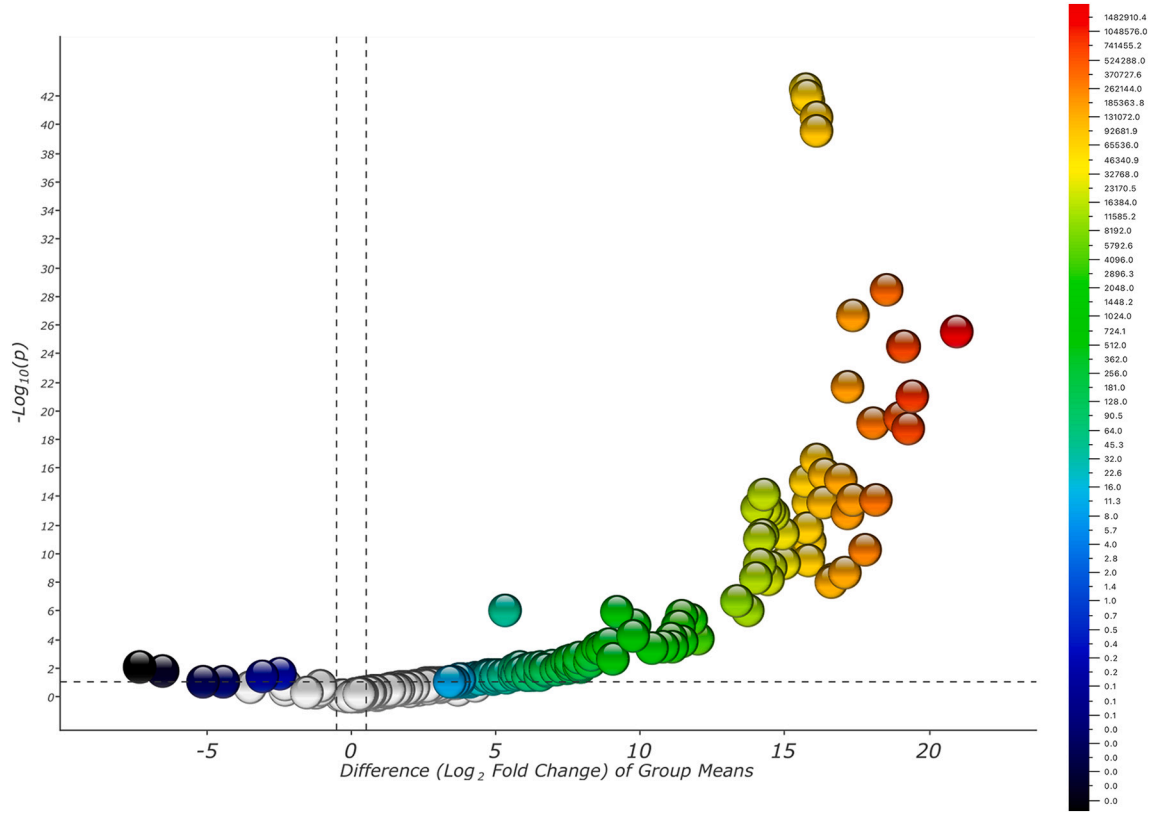


Fig. 1. Volcano plot representing changes in the canine urinary proteome secondary to renal disease caused by leishmaniosis. For sick dogs, less abundant proteins in urine samples are presented on the left-hand side of the graph, and the most abundant proteins are presented on the right-hand side of the volcano plot. The difference in protein content (log2-fold change) was depicted against the significance of the difference – log10(p) in this graph.

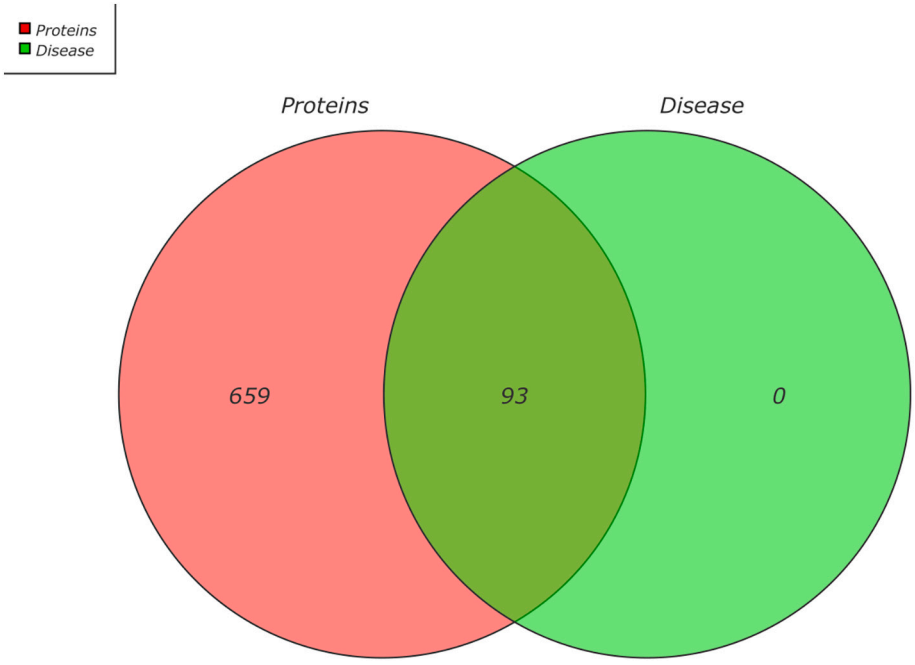


Fig. 2. Venn diagram showing changes in the canine urinary proteome between the control and study groups. This diagram depicts significantly different amounts of proteins, both decreased and increased. In the middle, the number of proteins with significantly different amounts in the study group compared to the control group.

decreased (*uromodulin, junctional adhesion molecule 1 and myelin protein zero like 1*) and a larger group of proteins that increased, among which we can find *cystatin domain-containing proteins, plasma retinol-binding protein, beta-2-microglobulin, immunoglobulin-like domain-containing proteins, fetuin-B, apolipoproteins I, II, IV and E, antithrombin III, haptoglobin or leucine rich alpha-2-glycoprotein 1* among others, already indicated by the

Fig. 3. Heatmap showing the influence of renal disease secondary to leishmaniosis in the canine urinary proteome. Proteins are classified following hierarchical clustering. The control group samples correspond to the gray marks, and the study group samples are indicated with red marks. The heatmap code is presented, with a lesser amount of protein in green areas and greater amounts of protein in red areas. Proteins were normalized and filtered by a fold change >3.8 with $p = 0.047$ and q = 0.019. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

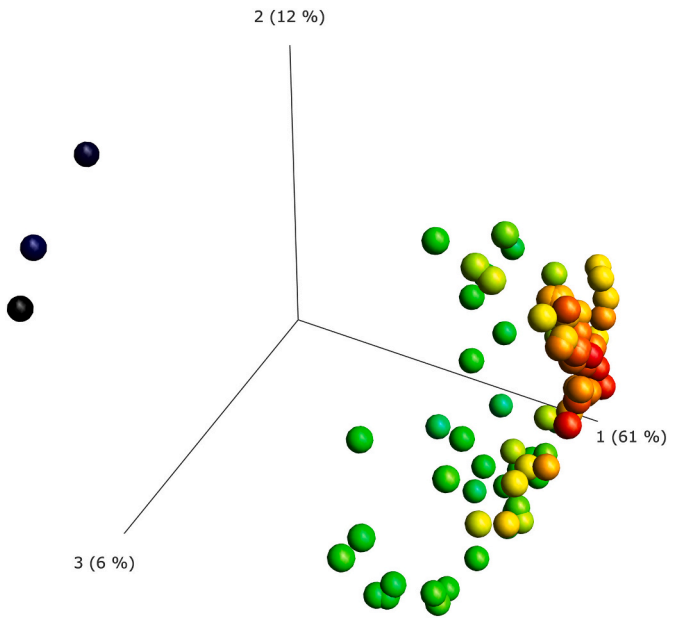


Fig. 4. 3D principal component analysis (PCA) of the variables influenced by renal disease secondary to leishmaniosis. Two groups are well differentiated: the black group shows proteins in which amounts decrease in renal disease, and the other group (green, yellow, orange, and red) shows proteins in which amounts increase. Variables were prefiltered by a standard deviation (S/Smax) of 0.71 and were then normalized and filtered by a fold change >3.8 with p = 0.047 and q = 0.019. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

heatmap. For this, the same statistical values established in the previous analysis were used (Figs. 3 and 4).

3.2. Validation of proteomic results

RBP and THP were measured in the urine of the 5 dogs in the control group and in 10 of the dogs with CKD secondary to CanL. The results were expressed as mean ± standard deviation. RBP (mg/L) showed a statistically significant increase ($P < 0.001$) when comparing the group of sick dogs concerning the control group (0.07 ± 0.02, and 0.03 ± 0.01, respectively). Differences were also observed in the case of THP (μg/mL) ($P < 0.001$), but in this case, its value in the sick group was lower than in the control group (34.87 ± 4.01, and 45.76 ± 2.99, respectively).

3.3. Enrichment analysis

For a better understanding of the biological processes and metabolic pathways in which the proteins obtained are involved, as well as their origin and hierarchical classification, an enrichment analysis was carried out using the g profiler data sources established by the platform and mentioned previously, namely GO, KEGG and HP ($P < 0.01$) (Fig. 5).

3.3.1. GO terms

The overrepresented GO pathways were classified according to their categorization criteria, that is, GO Cellular Component (GO:CC), GO Molecular Function (GO:MF), and GO Biological Process (GO:BP).

3.3.1.1. GO Cellular Component (GO:CC). Regarding the cellular components among which the origin of the identified proteins is located, three stand out: the first with a very abundant enrichment in the *extracellular region*, followed by the *extracellular space*, and finally, with a very high significance as well, but less than the above, the *circulating immunoglobulin complex* (Fig. 5).

3.3.1.2. GO Molecular Function (GO:MF). The molecular functions overrepresented in the CUP in renal disease secondary to leishmaniosis with the established level of significance were mainly *enzyme inhibitor activity, endopeptidase inhibitor and regulator activities, peptidase inhibitor and regulator activities*, and *immunoglobulin receptor binding* (Fig. 5).

3.3.1.3. GO Biological Process (GO:BP). Processes involved in a significant way are related to the *humoral immune response*, highlighting that *mediated by circulating immunoglobulins*, the *immune effector process, negative regulation of enzymatic activity* and finally, the most marked enrichment was shown by *activation of the complement* (Fig. 5).

3.3.2. Kyoto Encyclopedia of Genes and Genome pathways (KEGG)

The most abundant proteins in the urinary proteome analyzed are those related to the *complement activation* pathways and the *cascade of coagulation* (Fig. 5).

3.3.3. Human Phenotype Ontology (HP)

After a cross-species phenotype matching algorithm was applied, the canine phenotypes filtered out were related to different human diseases, such as *nephritis, altered hematopoiesis, amyloidosis* and *increased inflammatory response* (Fig. 5).

3.4. Analysis of discriminating variables

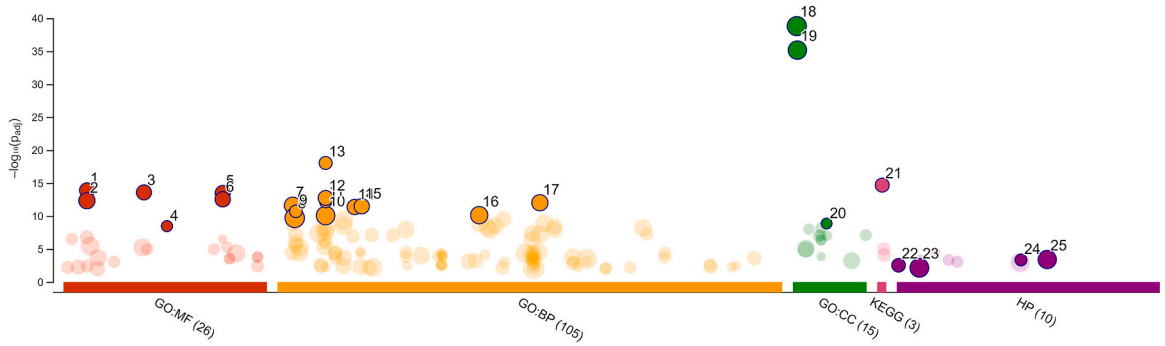
Finally, the proteins identified as altered in the study group (93 proteins) were subjected to a more exhaustive statistical analysis with a discriminative purpose, increasing the fold change to 710, with $p = 6 \times 10^{-15}$ and $q = 5 \times 10^{-14}$.

With these statistical settings, 12 urinary proteins stood out for their increase with respect to the control group dogs. Therefore, renal disease secondary to leishmaniosis induced a marked presence of proteins such as *C4a anaphylatoxin, apolipoprotein-A1, haptoglobin*, an *uncharacterized protein* similar to *immunoglobulin gamma-1 heavy chain* in humans (70,3% identity), an *immunoglobulin-like domain-containing protein*, *amyloid beta precursor protein binding family B member*, *fetuin B*, *leucine rich alpha-2-glycoprotein 1*, an *uncharacterized protein* similar to *immunoglobulin lambda fractions* in humans (86,7% identity), an *immunoglobulin-like domain-containing protein, beta-2-microglobulin* and *peptidase S1 domain-containing protein* (Fig. 6).

4. Discussion

In this study, a total of 951 proteins were identified in the CUP of dogs with kidney disease secondary to CanL. When we compare these results with literature of dogs with renal disease (Brandt et al., 2014; Ferlizza et al., 2020; Forterre et al., 2004; Hormaeche et al., 2014; Nabity et al., 2011; Palviainen et al., 2012; Pelander et al., 2019; Winiarczyk et al., 2019), this study has identified the highest number of proteins, due to protein separation by UHPLC coupled to ESI-TOF-MS analysis, one of the methods considered as the gold standard for the study of the urinary proteome (Sánchez-Juanes et al., 2013), in a complementary way to Capillary Electrophoresis–Mass Spectrometry (CE-MS) (Klein et al., 2014), which allows for a larger scale analysis of the urine (Klein et al., 2014; Sánchez-Juanes et al., 2013; Thongboonkerd, 2007). Moreover, this is the first study in which CUP was evaluated in natural CanL and renal disease by this method.

Regarding the origin of these proteins, when taking into account the results in the GO:CC category, as a result of its filtration or its local production, there was an overrepresentation of proteins of *extracellular origin*, which agrees with the previous literature on CKD in human medicine (Good et al., 2010; Kalantari et al., 2013), and dogs (Pelander et al., 2019), and proteins related with *circulating immunoglobulin complex*, fact already demonstrated in urine from gammopathies such as CanL (Solano-Gallego et al., 2003; Todolí et al., 2009), or human lupus



ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:MF	GO:0004866	endopeptidase inhibitor activity	1.278×10^{-14}
2	GO:MF	GO:0004857	enzyme inhibitor activity	4.834×10^{-13}
3	GO:MF	GO:0030414	peptidase inhibitor activity	2.654×10^{-14}
4	GO:MF	GO:0034987	immunoglobulin receptor binding	3.457×10^{-9}
5	GO:MF	GO:0061135	endopeptidase regulator activity	3.247×10^{-14}
6	GO:MF	GO:0061134	peptidase regulator activity	2.952×10^{-13}
7	GO:BP	GO:0002252	immune effector process	2.519×10^{-12}
8	GO:BP	GO:0002376	immune system process	1.944×10^{-10}
9	GO:BP	GO:0002455	humoral immune response mediated by circulatin...	1.985×10^{-11}
10	GO:BP	GO:0006955	immune response	8.581×10^{-11}
11	GO:BP	GO:0006958	complement activation, classical pathway	6.794×10^{-13}
12	GO:BP	GO:0006959	humoral immune response	1.845×10^{-13}
13	GO:BP	GO:0006956	complement activation	9.071×10^{-19}
14	GO:BP	GO:0010466	negative regulation of peptidase activity	4.317×10^{-12}
15	GO:BP	GO:0010951	negative regulation of endopeptidase activity	3.337×10^{-12}
16	GO:BP	GO:0043086	negative regulation of catalytic activity	7.479×10^{-11}
17	GO:BP	GO:0051346	negative regulation of hydrolase activity	9.770×10^{-13}
18	GO:CC	GO:0005576	extracellular region	1.578×10^{-39}
19	GO:CC	GO:0005615	extracellular space	6.870×10^{-36}
20	GO:CC	GO:0042571	immunoglobulin complex, circulating	1.396×10^{-9}
21	KEGG	KEGG:04610	Complement and coagulation cascades	2.102×10^{-15}
22	HP	HP:0000123	Nephritis	2.991×10^{-3}
23	HP	HP:0001871	Abnormality of blood and blood-forming tissues	7.305×10^{-3}
24	HP	HP:0011034	Amyloidosis	4.693×10^{-4}
25	HP	HP:0012649	Increased inflammatory response	3.992×10^{-4}

version	e104_eg51_p15_3922dba
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organism	clfamiliaris

g:Profiler

Fig. 5. g: GOST multiquery Manhattan plot representing enrichment analysis of proteins present in greater amounts in the study group. The urinary proteome was queried against the *Canis lupus familiaris* database. Gene Ontology (GO) terms for molecular function (MF) are shown in red, for biological process (BP) in orange, and for cellular component (CC) in green. KEGG path-ways are represented in pink, and Human Phenotype Ontology (HP) results are shown in purple. The *p* values are indicated on the left of the image on the y axis. Information about the highlighted results is provided in the table below the graph. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nephritis (LN) (Morell et al., 2021), among others.

In the GO:MF category, we found proteins with a great abundance within the *catalytic activity* family, being the most abundant those grouped as *endopeptidase inhibitor* and *regulator activities* and *peptidase inhibitor* and *regulator activities*. In human, this increasement has been related with progression of kidney disease, because inhibition or modulation of proteases promotes protein deposition in the renal extracellular matrix, and consequently, fibrosis (Catania et al., 2007; Genovese et al., 2014). Finally, there were abundant proteins related to *immunoglobulin receptor binding* term, fact probably associated with the production and regulation of immunoglobulins.

Regarding the biological processes of GO, we observed numerous terms with very high significance, some of which were related to immunological functions and were classified into the *immune effector process, immune system process* and *immune response, humoral immune response*, and *complement activation (classical pathway) groups,* a fact already published in the HUP of healthy patients (Adachi et al., 2006). For this reason, diseased urine is considered to have more immune activity than normal urine because the same results were not found in healthy dogs (Brandt et al., 2014). In addition, we also found proteins belonging to the *humoral immune response mediated by circulating immunoglobulin* terms only detected in this study, which can be justified by the information provided in the previous sections on circulating immunoglobulins and their origin. *Complement activation* was the most abundant process. This finding has also been associated with renal injury in human medicine in proteinuria (Morita et al., 2000), polycystic kidney disease (Bakun et al., 2012), and medullary sponge kidney disease (Bruschi et al., 2019). Until now, its increase has not been found in natural CUP (Brandt et al., 2014), unlike HUP (Adachi et al., 2006), and dogs with kidney disease (Nabity et al., 2011). The rest of the proteins belonging to prominent biological processes were defined as *negative regulators of catalytic, hydrolytic, endopeptidase*, and *peptidase activities*, proteins not present in CUP. However, they were observed in natural HUP (Adachi et al., 2006), as well as in CKD (Good et al., 2010).

In the KEGG, the most overrepresented group of molecules is associated with the biological pathway of *complement activation and coagulation cascades*. It was described in blood samples in a study of acute kidney injury (AKI) in a mouse model (Chen et al., 2020), and urine of patients with IgA nephropathy (Kalantari et al., 2013).

When comparing the results obtained with the HP database, coincidences have been found between the phenotypic pattern expressed by CUP studied in CanL, and those cataloged for certain pathological

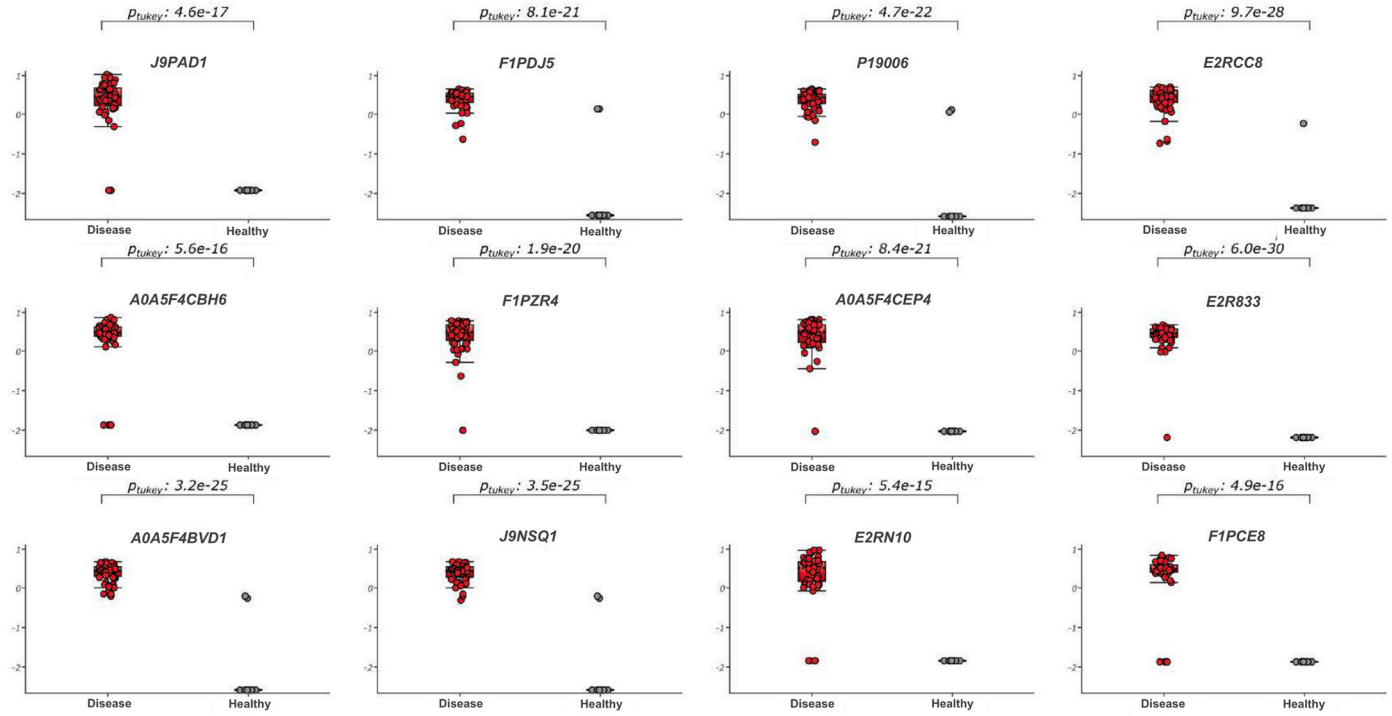

Fig. 6. More abundant proteins in the study group were filtered by a fold change >710 with $p = 6 \times 10^{-15}$ and $q = 5 \times 10^{-14}$. Qlucore Omics Explorer version 3.6 Lund Sweden (https://qlucore.com) was used to compare differences in the relative amounts of proteins based on the spectral counts between the control group (gray) and the study group (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

processes in humans, highlighting *nephritis, abnormality of blood and blood forming tissues, amyloidosis*, and *increased inflammatory response*, all of which are related to CKD. Therefore, dogs may be a good candidate for the study of kidney disease secondary to immunocomplex deposition by leishmaniosis, and other renal diseases with similar pathogenic mechanisms.

After bioinformatic analysis, among the proteins obtained as possible renal biomarkers for dogs with CKD secondary to CanL, we found some proteins previously studied, for example, *Tamm-Horsfall protein* (THP), and *retinol-binding protein* (RBP). The behavior of these two urinary biomarkers was previously evaluated in dogs with kidney disease (Chacar et al., 2017; Forterre et al., 2004), and CanL (Pardo-Marín et al., 2016), and their results coincide with the data obtained for the validated proteins in the urine of dogs with CanL and renal disease, exactly, urinary RBP increases and urinary THP decreases. These findings provide greater consistency to the results of this study.

After a discriminant analysis of the studied variables, those proteins that maintained a very high level of significance, concerning the control group, were selected. In this filtered set, 12 proteins were obtained, all of which were increased in dogs with kidney disease secondary to leishmaniosis. These are potential candidates to become biomarkers for the diagnosis, classification, and monitoring of this disease. First, among these biomarkers is *beta-2 microglobulin* (B2M), a glycoprotein present in all nucleated cells, which is filtered by kidney (Hall et al., 1982; Verroust et al., 2002). B2M has been found in HUP (Adachi et al., 2006), and, it has shown its usefulness as part of a pattern of biomarkers in AKI (Metzger et al., 2010), as well as in CKD (Good et al., 2010). Additionally, in dogs, the results obtained, suggests that B2M may be an earlier urinary biomarker in X-linked hereditary nephropathy (XLHN) as a model for CKD (Nabity et al., 2011). Furthermore, B2M was one of the increased proteins in the proteomic analysis of the urine of dogs with AKI secondary to *Vipera berus berus* bite (Palviainen et al., 2012), along with *fetuin B* (FB), a cysteine-type endopeptidase inhibitor increased in this study as well.

C4a or *anaphylatoxin* is another protein increased in dogs with CanL. It is involved in activation of the lectin pathway of complement system in response to parasites, among other agents (Sarma and Ward, 2011). In a study on human anti-glomerular basement membrane disease, an increase was shown in 100% of the patients studied, but it could not be clinically correlated (Ma et al., 2013). In addition, an increase in urinary levels of C4a, among other complement proteins, was found in patients with active LN (Mejia-Vilet et al., 2021) and focal segmental glomerulosclerosis (FSGS), where it is unknown whether its increase is due only to an increase in the activation of the complement system and its glomerular filtration or its activation in the lumen of the renal system (Thurman et al., 2015).

Haptoglobin (HP) is an acute-phase plasma protein synthesized in the liver, which participates in iron metabolism (Fagoonee et al., 2005; Ngai et al., 2007), and prevents the oxidative damage that hemoglobin causes at the tubular level (Schaer et al., 2014). A study suggested in situ production of urinary haptoglobin in a rat model of human membranous nephropathy for renoprotective purposes (Ngai et al., 2007), which was subsequently demonstrated in an AKI model in rats (Zager et al., 2012b). Moreover, its usefulness as a biomarker has also been demonstrated after the study of urinary proteomics in patients with LN (Aggarwal et al., 2017), and DN (Liao et al., 2018) in human medicine. In dogs, blood haptoglobin is increased with leishmaniosis according to proteomic analysis (Britti et al., 2010; Franco-Martínez et al., 2020), which promotes greater filtration in the context of a damaged filtration barrier. At the urinary level, proteomics has justified its use as a biomarker in dogs with AKI and babesiosis (Winiarczyk et al., 2019), and dogs with XLHN (Nabity et al., 2011).

Apolipoprotein-A1 (APOA-I) is the main high-density lipoprotein (HDL) (Clark et al., 2019; Goek et al., 2012). This is filtered at the glomerular level and mostly reabsorbed in the proximal tubule (Clark et al., 2019). APOA-I is present in the HUP (Adachi et al., 2006), but not in the CUP (Brandt et al., 2014) in a natural way. In the HUP, its increase has been associated with CKD, proteinuria and glomerular damage (Gomo, 1991; Good et al., 2010). Furthermore, APOA-I has demonstrated its utility as renal biomarker in IgA nephropathy (Kalantari et al.,

2013; Prikryl et al., 2017); and FSGS and in diseases of the proximal tubule in children (Clark et al., 2019), among others. In dogs, APOA-I was one of the urinary biomarkers increased in heartworm disease (Hormaeche et al., 2014), and male dogs with XLHN, in the latter, even before azotemia (Nabity et al., 2011).

Among the rest, we also found *amyloid-beta precursor* (ABP), which had an 80.1% of similarity with *hemopexin* (HPX). This has already involved as ABP in the fibrillogenesis of certain forms of amyloidosis (Ohta et al., 2018), something not previously reported in dogs. In addition, HPX precursor is present in the HUP (Adachi et al., 2006), but not in natural CUP (Brandt et al., 2014). However, HPX appear in natural CUP (Brandt et al., 2014), and dogs with XLHN, in azotemic stage (Nabity et al., 2011). HPX has a renoprotective function in proteinuric patients against oxidative damage caused by hemoglobin (Schaer et al., 2014; Zager et al., 2012a), which is observed in our study, however HPX is not among increased proteins. As a possible hypothesis, in the group of sick dogs, HPX, through an unknown mechanism, could lead to the production of ABP involved in the pathogenesis of renal damage.

Multiple corresponding *immunoglobulin-like domain-containing proteins* can be found, immunoglobulins or fractions of them, that may be present due to glomerular filtration or local production, among others. Furthermore, an uncharacterized protein stands out when compared to the human database, due to its similarity with *lambda-type immunoglobulins* (86,7%); specifically, *immunoglobulin lambda-1 light chain*, an immunoglobulin fraction related to CKD progression in human medicine (Fenton et al., 2018). Free light-chain proteinuria is also a recognized amyloid precursor in both, humans (Dember, 2006) and dogs (Kadota et al., 2020); in the latter, it has been associated with the appearance of other molecules, such as APOA-I, and apolipoproteins E and A-VI in CKD with amyloidosis (Kadota et al., 2020), precisely, these apolipoproteins were increased in this study as well. Nevertheless, in CanL, this kind of proteinuria has also been proven with no apparent association with kidney disease (Bonfanti et al., 2004).

Another of the most abundant proteins was a *peptidase S1 domain-containing protein*, which shares an identity of 98.8% with the *cationic trypsin* of the dog and it may be an isoform of this protein. When compared to humans, it is only 74% similar. Pigs have an identity of 80.5%, so they cannot be considered cross-contamination with the reagent used in tryptic digestion. Its presence had never before been described in the CUP.

Finally, a protein with greater significance was *leucine-rich alpha-2-glycoprotein 1* (LRG1), an acute phase plasma protein produced mainly in the liver and neutrophils, but it can also be induced in many other tissues as part of its inflammasome (Jiang et al., 2020; Lee et al., 2018). This glycoprotein has already been found to be increased in other diseases, such as those associated with tubular damage (Lee et al., 2018), IgA nephropathy (Kalantari et al., 2013), and DN in type II (Liu et al., 2017), acquiring an important role in the early diagnosis of kidney pathologies. This biomarker could have a great potential, especially in the evaluation at the tubular level, where it is believed that its synthesis is induced, as well as a possible therapeutic intervention point to slow the progression of the disease associated with inflammation.

Regarding the limitations of our study, they may be a consequence of the small sample size, as well as the possible variability associated with intra- and interindividual diversity that molecular tests may have (Thongboonkerd, 2007). To reduce its impact, the results were obtained from the comparison between a control group, healthy dogs, and a study group, dogs with kidney disease and CanL, exactly, Leishvet stages III and IV (Solano-Gallego et al., 2011), to find altered biomarkers as a consequence of kidney damage secondary to CanL as a whole, not being compared with a group with kidney disease to eliminate the variability present in its etiology, as has already been done in much of the previous literature consulted (Hormaeche et al., 2014; Palviainen et al., 2012; Winiarczyk et al., 2019), because this would lead to unreliable results. In addition, due to the way in which the results generated in MS are interpreted, and the kidney disease condition is shared between both groups, the unmodified variables would not stand out, and many of them would only do so due to their involvement in CanL, not being the objective of this study.

These twelve new biomarkers present marked differences, and when we look at the heatmap (Fig. 3), they distinguish very well between the healthy and sick conditions, even some, such as LRG1, FB, B2M, and C4a, completely differentiate these conditions. Something of great interest in kidney disease, in this case, secondary to CanL, because 26.7% of dogs (4/15) did not present azotemia. Therefore, these biomarkers may be useful in the early and they are in advanced diagnosis of CanL with kidney disease. Moreover, their involvement in such important mechanisms in kidney damage as complement activation and humoral response, may make them potential therapeutic targets in the future or biomarkers of therapeutic response as a reflection of the modification of the pathway in which they are involved. In addition, these biomarkers can also be useful in the study of kidney pathologies with the same pathogenesis. For all these reasons, studies in previous stages and post-treatment periods are necessary to clarify its implication in these contexts.

5. Conclusion

This study provides new and valuable information about the possible pathogenic mechanisms of this renal disease, revealing the important relationship with pathways associated with the *activation of complement* and the *circulating immunoglobulin complex*. In addition, the results propose several proteins that could be candidate biomarkers for future research; exactly, *leucine-rich alpha-2-glycoprotein 1* and *C4a or anaphylatoxin* that have not been described thus far, and others have scarcely been studied, such as *apolipoprotein A-1, fetuin B, beta-2 microglobulin*, and *haptoglobin,* all of them in dogs whit leishmaniosis and kidney damage. This provides greater knowledge about the diagnosis and possible points of therapeutic interventions in kidney disease due to CanL. Future studies will be necessary to determine the role of these potential candidates.

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References

Adachi, J., Kumar, C., Zhang, Y., Olsen, J.V., Mann, M., 2006. The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. Genome Biol. 7, R80. https://doi.org/10.1186/gb-2006-7-9-r80.

Aggarwal, A., Gupta, R., Negi, V.S., Rajasekhar, L., Misra, R., Singh, P., Chaturvedi, V., Sinha, S., 2017. Urinary haptoglobin, alpha-1 anti-chymotrypsin and retinol binding protein identified by proteomics as potential biomarkers for lupus nephritis: urinary biomarkers for lupus nephritis. Clin. Exp. Immunol. 188, 254–262. https://doi.org/10.1111/cei.12930.

Alvar, J., Cañavate, C., Molina, R., Moreno, J., Nieto, J., 2004. Canine Leishmaniasis. In: Advances in Parasitology. Elsevier, pp. 1–88. https://doi.org/10.1016/S0065-308X(04)57001-X.

Bakun, M., Niemczyk, M., Domanski, D., Jazwiec, R., Perzanowska, A., Niemczyk, S., Kistowski, M., Fabijanska, A., Borowiec, A., Paczek, L., Dadlez, M., 2012. Urine proteome of autosomal dominant polycystic kidney disease patients. Clin. Proteomics 9, 13. https://doi.org/10.1186/1559-0275-9-13.

Belinchón-Lorenzo, S., Iniesta, V., Parejo, J.C., Fernández-Cotrina, J., Muñoz-Madrid, R., Soto, M., Alonso, C., Gómez Nieto, L.C., 2013. Detection of Leishmania infantum kinetoplast minicircle DNA by real time PCR in hair of dogs with leishmaniosis. Vet. Parasitol. 192, 43–50. https://doi.org/10.1016/j.vetpar.2012.11.007.

Benderitter, Th., Casanova, P., Nashkidachvili, L., Quilici, M., 1988. Glomerulonephritis in dogs with canine leishmaniasis. Ann. Trop. Med. Parasitol. 82, 335–341. https://doi.org/10.1080/00034983.1988.11812255.

Bonfanti, U., Zini, E., Minetti, E., Zatelli, A., 2004. Free light-chain proteinuria and normal renal histopathology and function in 11 dogs exposed to Leishmania infantum, Ehrlichia canis, and Babesia canis. J. Vet. Intern. Med. 18, 618–624. https://doi.org/10.1111/j.1939-1676.2004.tb02596.x.

Bourgon, R., Gentleman, R., Huber, W., 2010. Independent filtering increases detection power for high-throughput experiments. Proc. Natl. Acad. Sci. 107, 9546–9551. https://doi.org/10.1073/pnas.0914005107.

Brandt, L.E., Ehrhart, E.J., Scherman, H., Olver, C.S., Bohn, A.A., Prenni, J.E., 2014. Characterization of the canine urinary proteome. Vet. Clin. Pathol. 43, 193–205. https://doi.org/10.1111/vcp.12147.

Britti, D., Gaspari, M., Massimini, G., Casalinuovo, F., Morittu, V.M., Cuda, G., 2010. Proteomic analysis in canine leishmaniasis. Vet. Res. Commun. 34, 91–96. https://doi.org/10.1007/s11259-010-9410-5.

Bruschi, Granata, Candiano, Fabris, Petretto, Ghiggeri, Gambaro, Zaza, 2019. Proteomic analysis of urinary extracellular vesicles reveals a role for the complement system in medullary sponge kidney disease. Int. J. Mol. Sci. 20, 5517. https://doi.org/10.3390/ijms20215517.

Catania, J.M., Chen, G., Parrish, A.R., 2007. Role of matrix metalloproteinases in renal pathophysiologies. Am. J. Physiol. Ren. Physiol. 292, F905–F911. https://doi.org/10.1152/ajprenal.00421.2006.

Chacar, F., Kogika, M., Sanches, T.R., Caragelasco, D., Martorelli, C., Rodrigues, C., Capcha, J.M.C., Chew, D., Andrade, L., 2017. Urinary Tamm-Horsfall protein, albumin, vitamin D-binding protein, and retinol-binding protein as early biomarkers of chronic kidney disease in dogs. Phys. Rep. 5 https://doi.org/10.14814/phy2.13262 e13262.

Chen, J., Chen, Y., Olivero, A., Chen, X., 2020. Identification and validation of potential biomarkers and their functions in acute kidney injury. Front. Genet. 11, 411. https://doi.org/10.3389/fgene.2020.00411.

Clark, A.J., Jabs, K., Hunley, T.E., Jones, D.P., VanDeVoorde, R.G., Anderson, C., Du, L., Zhong, J., Fogo, A.B., Yang, H., Kon, V., 2019. Urinary apolipoprotein AI in children with kidney disease. Pediatr. Nephrol. 34, 2351–2360. https://doi.org/10.1007/s00467-019-04289-5.

Costa, F.A.L., Goto, H., Saldanha, L.C.B., Silva, S.M.M.S., Sinhorini, I.L., Silva, T.C., Guerra, J.L., 2003. Histopathologic patterns of nephropathy in naturally acquired canine visceral Leishmaniasis. Vet. Pathol. 40, 677–684. https://doi.org/10.1354/vp.40-6-677.

Dember, L.M., 2006. Amyloidosis-associated kidney disease. J. Am. Soc. Nephrol. 17, 3458–3471. https://doi.org/10.1681/ASN.2006050460.

Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D., 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. 95, 14863–14868.

Fagoonee, S., Gburek, J., Hirsch, E., Marro, S., Moestrup, S.K., Laurberg, J.M., Christensen, E.I., Silengo, L., Altruda, F., Tolosano, E., 2005. Plasma protein haptoglobin modulates renal Iron loading. Am. J. Pathol. 166, 973–983. https://doi.org/10.1016/S0002-9440(10)62319-X.

Fassett, R.G., Venuthurupalli, S.K., Gobe, G.C., Coombes, J.S., Cooper, M.A., Hoy, W.E., 2011. Biomarkers in chronic kidney disease: a review. Kidney Int. 80, 806–821. https://doi.org/10.1038/ki.2011.198.

Félix, N., Mouro, S., Vilela, C.L., Peleteiro, M.C., Ferreira, A.J.A., Niza, M.M.R.E., 2008. Canine leishmaniasis with nephrotic syndrome and aortic and caudal vena cava thromboembolism: canine leishmaniasis and thromboembolism. J. Vet. Emerg. Crit. Care 18, 526–531. https://doi.org/10.1111/j.1476-4431.2008.00341.x.

Fenton, A., Jesky, M.D., Webster, R., Stringer, S.J., Yadav, P., Chapple, I., Dasgupta, I., Harding, S.J., Ferro, C.J., Cockwell, P., 2018. Association between urinary free light chains and progression to end stage renal disease in chronic kidney disease. PLoS One 13, e0197043. https://doi.org/10.1371/journal.pone.0197043.

Ferlizza, E., Isani, G., Dondi, F., Andreani, G., Vasylyeva, K., Bellei, E., Almeida, A.M., Matzapetakis, M., 2020. Urinary proteome and metabolome in dogs (Canis lupus familiaris): the effect of chronic kidney disease. J. Proteomics 222, 103795. https://doi.org/10.1016/j.jprot.2020.103795.

Font, A., Closa, J.M., 1997. Ultrasonographic localization of a caudal vena cava thrombus in a dog with Leishmaniasis. Vet. Radiol. Htmlent Glyphamp Asciiamp Ultrasound 38, 394–396. https://doi.org/10.1111/j.1740-8261.1997.tb02105.x.

Fontes, M., Soneson, C., 2011. The projection score - an evaluation criterion for variable subset selection in PCA visualization. BMC Bioinformatics 12, 307. https://doi.org/10.1186/1471-2105-12-307.

Forterre, S., Raila, J., Schweigert, F.J., 2004. Protein profiling of urine from dogs with renal disease using ProteinChip analysis. J. Vet. Diagn. Investig. 16, 271–277. https://doi.org/10.1177/104063870401600403.

Franco-Martínez, L., Tvarijonaviciute, A., Horvatić, A., Guillemin, N., Bernal, L.J., Barić Rafaj, R., Cerón, J.J., del Thomas, M.C., López, M.C., Tecles, F., Martínez-Subiela, S., Mrljak, V., 2019. Changes in saliva of dogs with canine leishmaniosis: a proteomic approach. Vet. Parasitol. 272, 44–52. https://doi.org/10.1016/j.vetpar.2019.06.014.

Franco-Martínez, L., Villar, M., Tvarijonaviciute, A., Escribano, D., Bernal, L.J., Cerón, J. J., del Thomas, M.C., Mateos-Hernández, L., Tecles, F., Fuente, J., López, M.C., Martínez-Subiela, S., 2020. Serum proteome of dogs at subclinical and clinical onset of canine leishmaniosis. Transbound. Emerg. Dis. 67, 318–327. https://doi.org/10.1111/tbed.13354.

Gao, Y., 2015. Urine is a better biomarker source than blood especially for kidney diseases. In: Gao, Y. (Ed.), Urine Proteomics in Kidney Disease Biomarker Discovery, Advances in Experimental Medicine and Biology. Springer Netherlands, Dordrecht, pp. 3–12. https://doi.org/10.1007/978-94-017-9523-4_1.

Genovese, F., Manresa, A.A., Leeming, D.J., Karsdal, M.A., Boor, P., 2014. The extracellular matrix in the kidney: a source of novel non-invasive biomarkers of kidney fibrosis? Fibrogenesis Tissue Repair 7, 4. https://doi.org/10.1186/1755-1536-7-4.

Goek, O.-N., Köttgen, A., Hoogeveen, R.C., Ballantyne, C.M., Coresh, J., Astor, B.C., 2012. Association of apolipoprotein A1 and B with kidney function and chronic kidney disease in two multiethnic population samples. Nephrol. Dial. Transplant. 27, 2839–2847. https://doi.org/10.1093/ndt/gfr795.

Gomo, Z., 1991. Urinary high density lipoprotein–a possible marker for glomerular proteinuria. Cent. Afr. J. Med. 37, 211–216.

Good, D.M., Zürbig, P., Argilés, À., Bauer, H.W., Behrens, G., Coon, J.J., Dakna, M., Decramer, S., Delles, C., Dominiczak, A.F., Ehrich, J.H.H., Eitner, F., Fliser, D., Frommberger, M., Ganser, A., Girolami, M.A., Golovko, I., Gwinner, W., Haubitz, M., Herget-Rosenthal, S., Jankowski, J., Jahn, H., Jerums, G., Julian, B.A., Kellmann, M., Kliem, V., Kolch, W., Krolewski, A.S., Luppi, M., Massy, Z., Melter, M., Neusüss, C., Novak, J., Peter, K., Rossing, K., Rupprecht, H., Schanstra, J.P., Schiffer, E., Stolzenburg, J.-U., Tarnow, L., Theodorescu, D., Thongboonkerd, V., Vanholder, R., Weissinger, E.M., Mischak, H., Schmitt-Kopplin, P., 2010. Naturally occurring human urinary peptides for use in diagnosis of chronic kidney disease. Mol. Cell. Proteomics 9, 2424–2437. https://doi.org/10.1074/mcp.M110.001917.

Hall, P.W., Chung-Park, M., Vacca, C.V., London, M., Crowley, A.Q., 1982. The renal handling of beta2-microglobulin in the dog. Kidney Int. 22, 156–161. https://doi.org/10.1038/ki.1982.147.

Hormaeche, M., Carretón, E., González-Miguel, J., Gussoni, S., Montoya-Alonso, J.A., Simón, F., Morchón, R., 2014. Proteomic analysis of the urine of Dirofilaria immitis infected dogs. Vet. Parasitol. 203, 241–246. https://doi.org/10.1016/j.vetpar.2014.01.025.

Jiang, W., Ma, Tai, Zhang, C., Tang, X., Xu, Q., Meng, X., Ma, Taotao, 2020. Identification of urinary candidate biomarkers of cisplatin-induced nephrotoxicity in patients with carcinoma. J. Proteome 210, 103533. https://doi.org/10.1016/j.jprot.2019.103533.

Kadota, A., Iwaide, S., Miyazaki, S., Mitsui, I., Machida, N., Murakami, T., 2020. Pathology and proteomics-based diagnosis of localized light-chain amyloidosis in dogs and cats. Vet. Pathol. 57, 658–665. https://doi.org/10.1177/0300985820934113.

Kalantari, S., Rutishauser, D., Samavat, S., Nafar, M., Mahmudieh, L., Rezaei-Tavirani, M., Zubarev, R.A., 2013. Urinary prognostic biomarkers and classification of IgA nephropathy by high resolution mass spectrometry coupled with liquid chromatography. PLoS One 8, e80830. https://doi.org/10.1371/journal.pone.0080830.

Kanehisa, M., 2000. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 28, 27–30. https://doi.org/10.1093/nar/28.1.27.

Klein, J., Papadopoulos, T., Mischak, H., Mullen, W., 2014. Comparison of CE-MS/MS and LC-MS/MS sequencing demonstrates significant complementarity in natural peptide identification in human urine: general. Electrophoresis 35, 1060–1064. https://doi.org/10.1002/elps.201300327.

Köhler, S., Gargano, M., Matentzoglu, N., Carmody, L.C., Lewis-Smith, D., Vasilevsky, N. A., Danis, D., Balagura, G., Baynam, G., Brower, A.M., Callahan, T.J., Chute, C.G., Est, J.L., Galer, P.D., Ganesan, S., Griese, M., Haimel, M., Pazmandi, J., Hanauer, M., Harris, N.L., Hartnett, M.J., Hastreiter, M., Hauck, F., He, Y., Jeske, T., Kearney, H., Kindle, G., Klein, C., Knoflach, K., Krause, R., Lagorce, D., McMurry, J.A., Miller, J. A., Munoz-Torres, M.C., Peters, R.L., Rapp, C.K., Rath, A.M., Rind, S.A., Rosenberg, A.Z., Segal, M.M., Seidel, M.G., Smedley, D., Talmy, T., Thomas, Y., Wiafe, S.A., Xian, J., Yüksel, Z., Helbig, I., Mungall, C.J., Haendel, M.A., Robinson, P. N., 2021. The human phenotype ontology in 2021. Nucleic Acids Res. 49, D1207–D1217. https://doi.org/10.1093/nar/gkaa1043.

Koutinas, A.F., Koutinas, C.K., 2014. Pathologic mechanisms underlying the clinical findings in canine leishmaniosis due to *Leishmania infantum/chagasi*. Vet. Pathol. 51, 527–538. https://doi.org/10.1177/0300985814521248.

Laborde, C.M., Zubiri, I., Alonso-Orgaz, S., Mourino-Alvarez, L., Álvarez-Llamas, G., Barderas, M.G., 2011. Aportaciones de la proteómica al laboratorio clínico. Rev. Lab. Clin. 4, 214–224. https://doi.org/10.1016/j.labcli.2011.06.007.

Lee, H., Fujimoto, M., Ohkawara, T., Honda, H., Serada, S., Terada, Y., Naka, T., 2018. Leucine rich α-2 glycoprotein is a potential urinary biomarker for renal tubular injury. Biochem. Biophys. Res. Commun. 498, 1045–1051. https://doi.org/10.1016/j.bbrc.2018.03.111.

Liao, W.-L., Chang, C.-T., Chen, C.-C., Lee, W.-J., Lin, S.-Y., Liao, H.-Y., Wu, C.-M., Chang, Y.-W., Chen, C.-J., Tsai, F.-J., 2018. Urinary proteomics for the early diagnosis of diabetic nephropathy in Taiwanese patients. J. Clin. Med. 7, 483. https://doi.org/10.3390/jcm7120483.

Liu, J.-J., Pek, S.L.T., Ang, K., Tavintharan, S., Lim, S.C., for SMART2D study, 2017. Plasma leucine-rich α-2-glycoprotein 1 predicts rapid eGFR decline and albuminuria progression in type 2 diabetes mellitus. J. Clin. Endocrinol. Metab. 102, 3683–3691. https://doi.org/10.1210/jc.2017-00930.

Ma, R., Cui, Z., Liao, Y., Zhao, M., 2013. Complement activation contributes to the injury and outcome of kidney in human anti-glomerular basement membrane disease. J. Clin. Immunol. 33, 172–178. https://doi.org/10.1007/s10875-012-9772-2.

Mejia-Vilet, J.M., Gómez-Ruiz, I.A., Cruz, C., Méndez-Pérez, R.A., Comunidad-Bonilla, R. A., Uribe-Uribe, N.O., Nuñez-Alvarez, C.A., Morales-Buenrostro, L.E., 2021. Alternative complement pathway activation in thrombotic microangiopathy associated with lupus nephritis. Clin. Rheumatol. 40, 2233–2242. https://doi.org/10.1007/s10067-020-05499-1.

Metzger, J., Kirsch, T., Schiffer, E., Ulger, P., Mentes, E., Brand, K., Weissinger, E.M., Haubitz, M., Mischak, H., Herget-Rosenthal, S., 2010. Urinary excretion of twenty peptides forms an early and accurate diagnostic pattern of acute kidney injury. Kidney Int. 78, 1252–1262. https://doi.org/10.1038/ki.2010.322.

Mi, H., Muruganujan, A., Ebert, D., Huang, X., Thomas, P.D., 2019. PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. Nucleic Acids Res. 47, D419–D426. https://doi.org/10.1093/nar/gky1038.

Morell, M., Pérez-Cózar, F., Marañón, C., 2021. Immune-related urine biomarkers for the diagnosis of lupus nephritis. Int. J. Mol. Sci. 22, 7143. https://doi.org/10.3390/ijms22137143.

Morita, Y., Ikeguchi, H., Nakamura, J., Hotta, N., Yuzawa, Y., Matsuo, S., 2000. Complement activation products in the urine from proteinuric patients. J. Am. Soc. Nephrol. 11, 700–707. https://doi.org/10.1681/ASN.V114700.

Nabity, M.B., Lees, G.E., Dangott, L.J., Cianciolo, R., Suchodolski, J.S., Steiner, J.M., 2011. Proteomic analysis of urine from male dogs during early stages of tubulointerstitial injury in a canine model of progressive glomerular disease: urine proteomics in canine renal disease. Vet. Clin. Pathol. 40, 222–236. https://doi.org/10.1111/j.1939-165X.2011.00307.x.

Ngai, H.H.-Y., Sit, W.-H., Jiang, P.-P., Thongboonkerd, V., Wan, J.M.-F., 2007. Markedly increased urinary preprohaptoglobin and haptoglobin in passive Heymann nephritis: a differential proteomics approach. J. Proteome Res. 6, 3313–3320. https://doi.org/10.1021/pr070245b.

Ohta, M., Sugano, A., Hatano, N., Sato, H., Shimada, H., Niwa, H., Sakaeda, T., Tei, H., Sakaki, Y., Yamamura, K., Takaoka, Y., 2018. Co-precipitation molecules hemopexin and transferrin may be key molecules for fibrillogenesis in TTR V30M amyloidogenesis. Transgenic Res. 27, 15–23. https://doi.org/10.1007/s11248-017-0054-x.

Palviainen, M., Raekallio, M., Vainionpää, M., Kosonen, S., Vainio, O., 2012. Proteomic profiling of dog urine after European adder (Vipera berus berus) envenomation by two-dimensional difference gel electrophoresis. Toxicon 60, 1228–1234. https://doi.org/10.1016/j.toxicon.2012.08.010.

Pardo-Marín, L., Martínez-Subiela, S., Pastor, J., Tvarijonaviciute, A., Garcia-Martinez, J. D., Segarra, S., Cerón, J.J., 2016. Evaluation of various biomarkers for kidney monitoring during canine leishmaniosis treatment. BMC Vet. Res. 13, 31. https://doi.org/10.1186/s12917-017-0956-0.

Pelander, L., Brunchault, V., Buffin-Meyer, B., Klein, J., Breuil, B., Zürbig, P., Magalhães, P., Mullen, W., Elliott, J., Syme, H., Schanstra, J.P., Häggström, J., Ljungvall, I., 2019. Urinary peptidome analyses for the diagnosis of chronic kidney disease in dogs. Vet. J. 249, 73–79. https://doi.org/10.1016/j.tvjl.2019.05.010.

Perez-Riverol, Y., Csordas, A., Bai, J., Bernal-Llinares, M., Hewapathirana, S., Kundu, D. J., Inuganti, A., Griss, J., Mayer, G., Eisenacher, M., Pérez, E., Uszkoreit, J., Pfeuffer, J., Sachsenberg, T., Yilmaz, S., Tiwary, S., Cox, J., Audain, E., Walzer, M., Jarnuczak, A.F., Ternent, T., Brazma, A., Vizcaíno, J.A., 2019. The PRIDE database and related tools and resources in 2019: improving support for quantification data. Nucleic Acids Res. 47, D442–D450. https://doi.org/10.1093/nar/gky1106.

Prikryl, P., Vojtova, L., Maixnerova, D., Vokurka, M., Neprasova, M., Zima, T., Tesar, V., 2017. Proteomic approach for identification of IgA nephropathy-related biomarkers in urine. Physiol. Res. 621–632. https://doi.org/10.33549/physiolres.933380.

Raudvere, U., Kolberg, L., Kuzmin, I., Arak, T., Adler, P., Peterson, H., Vilo, J., 2019. g: profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). Nucleic Acids Res. 47, W191–W198. https://doi.org/10.1093/nar/gkz369.

Roura, X., Fondati, A., Lubas, G., Gradoni, L., Maroli, M., Oliva, G., Paltrinieri, S., Zatelli, A., Zini, E., 2013. Prognosis and monitoring of leishmaniasis in dogs: a working group report. Vet. J. 198, 43–47. https://doi.org/10.1016/j.tvjl.2013.04.001.

Roura, X., Cortadellas, O., Day, M.J., Benali, S.L., Canine Leishmaniosis Working Group, D'Anna, N., Fondati, A., Gradoni, L., Lubas, G., Maroli, M., Paltrinieri, S., Zini, E., Zatelli, A., 2021. Canine leishmaniosis and kidney disease: Q&A for an overall management in clinical practice. J. Small Anim. Pract. 62 https://doi.org/10.1111/jsap.13237.

Sánchez-Juanes, F., Muñiz, M.C., Raposo, C., Rodríguez-Prieto, S., Paradela, A., Quiros, Y., López-Hernández, F., González-Buitrago, J.M., Ferreira, L., 2013. Unveiling the rat urinary proteome with three complementary proteomics approaches: proteomics and 2-DE. Electrophoresis 34, 2473–2483. https://doi.org/10.1002/elps.201200689.

Sarma, J.V., Ward, P.A., 2011. The complement system. Cell Tissue Res. 343, 227–235. https://doi.org/10.1007/s00441-010-1034-0.

Schaer, D.J., Vinchi, F., Ingoglia, G., Tolosano, E., Buehler, P.W., 2014. Haptoglobin, hemopexin, and related defense pathways-basic science, clinical perspectives, and drug development. Front. Physiol. 5 https://doi.org/10.3389/fphys.2014.00415.

Solano-Gallego, L., Rodríguez, A., Iniesta, L., Arboix, M., Portús, M., Alberola, J., 2003. Detection of anti- *Leishmania* immunoglobulin G antibodies in urine specimens of dogs with Leishmaniasis. Clin. Vaccine Immunol. 10, 849–855. https://doi.org/10.1128/CDLI.10.5.849-855.2003.

Solano-Gallego, L., Miró, G., Koutinas, A., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., 2011. LeishVet guidelines for the practical management of canine leishmaniosis. Parasit. Vectors 4, 86. https://doi.org/10.1186/1756-3305-4-86.

Tamhane, A.C., Hochberg, Y., Dunnett, C.W., 1996. Multiple test procedures for dose finding. Biometrics 52, 21–37.

Thongboonkerd, V., 2007. Practical points in urinary proteomics. J. Proteome Res. 6, 3881–3890. https://doi.org/10.1021/pr070328s.

Thurman, J.M., Wong, M., Renner, B., Frazer-Abel, A., Giclas, P.C., Joy, M.S., Jalal, D., Radeva, M.K., Gassman, J., Gipson, D.S., Kaskel, F., Friedman, A., Trachtman, H., 2015. Complement activation in patients with focal segmental glomerulosclerosis. PLoS One 10, e0136558. https://doi.org/10.1371/journal.pone.0136558.

Todolí, F., Solano-Gallego, L., Ojeda, A., Quintana, J., Lloret, A., Roura, X., Alberola, J., Rodríguez-Cortés, A., 2009. Anti-Leishmania IgA in urine samples from dogs with clinical leishmaniasis. Vet. Parasitol. 159, 17–23. https://doi.org/10.1016/j.vetpar.2008.10.010.

Verroust, P.J., Birn, H., Nielsen, R., Kozyraki, R., Christensen, E.I., 2002. The tandem endocytic receptors megalin and cubilin are important proteins in renal pathology. Kidney Int. 62, 745–756. https://doi.org/10.1046/j.1523-1755.2002.00501.x.

Viskoper, R.J., Laszt, A., Oren, S., Hochberg, Y., Villa, Y., Drexler, I., Bregman, L., Mishal, J., 1989. The antihypertensive effect of atenolol and bopindolol in the elderly. Neth. J. Med. 35, 185–191.

Winiarczyk, D., Michalak, K., Adaszek, L., Winiarczyk, M., Winiarczyk, S., 2019. Urinary proteome of dogs with kidney injury during babesiosis. BMC Vet. Res. 15, 439. https://doi.org/10.1186/s12917-019-2194-0.

Zager, R.A., Johnson, A.C.M., Becker, K., 2012a. Renal cortical hemopexin accumulation in response to acute kidney injury. Am. J. Physiol. Ren. Physiol. 303, F1460–F1472. https://doi.org/10.1152/ajprenal.00426.2012.

Zager, R.A., Vijayan, A., Johnson, A.C.M., 2012b. Proximal tubule haptoglobin gene activation is an integral component of the acute kidney injury "stress response.". Am. J. Physiol. Ren. Physiol. 303, F139–F148. https://doi.org/10.1152/ajprenal.00168.2012.

Zatelli, A., Borgarelli, M., Santilli, R., Bonfanti, U., Nigrisoli, E., Zanatta, R., Tarducci, A., Guarraci, A., 2003. Glomerular lesions in dogs infected with Leishmania organisms. Am. J. Vet. Res. 64, 558–561. https://doi.org/10.2460/ajvr.2003.64.558.