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# A nuclear magnetic resonance spectroscopy metabolomic approach to renal dysfunction in canine leishmaniasis

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ABSTRACT

Chronic kidney disease (CKD) is a major complication and the leading cause of mortality in canine leishmaniasis (CanL). The kidneys are essential for numerous metabolic processes, and specific metabolites may serve as predictive biomarkers of kidney function. Nuclear Magnetic Resonance (NMR) spectroscopy is a prominent analytical tool in metabolomics, capable of identifying metabolites in urine. This study aim to identify distinct patterns in the NMR spectra of urine samples from dogs with CKD in CanL, reflecting the underlying metabolic profiles

Fifty-five dogs were divided into three groups: 14 healthy control dogs (CG), 33 dogs with CKD secondary to leishmaniasis, and 8 dogs with CKD unrelated to leishmaniasis. CanL dogs were classified according to the International Renal Interest Society (IRIS) staging system: stage 1 (15 dogs), stage 2 (10 dogs), stage 3 (6 dogs), and stage 4 (2 dogs); and by LeishVet guidelines: stage I (5 dogs), stage II (4 dogs), stage III (14 dogs), and stage IV (10 dogs). Among dogs with CKD alone, one dog was in IRIS stage 1, two in stage 2, one in stage 3, and four in stage 4.

Low-field proton nuclear magnetic resonance ($^1$H NMR) spectroscopy and multivariate analysis were used to classify urine samples. Statistical analysis was conducted on hematology, urine and plasma samples from studied dogs.

Using $^1$H NMR spectroscopy to classify urine samples from dogs with CKD, both with and without leishmaniasis, revealed distinct spectral patterns between the different groups.

In conclusion, low-field $^1$H NMR spectroscopy demonstrated that CKD presents a distinct metabolic profile compared to kidney damage secondary to leishmaniasis.

## 1. Introduction

Chronic kidney disease (CKD) is a progressive and irreversible condition characterized by abnormalities in renal function and/or structure, associated with high mortality rates (Brunetto et al., 2021; Ferlizza et al., 2020; Gervasini et al., 2023). Multiple factors contribute to CKD development, including glomerular diseases, infections, ischemic events, and prior acute kidney injury. However, the cause often remains

*Abbreviations:* CKD, chronic kidney disease; CanL, canine leishmaniasis; SDMA, symmetric dimethyl arginine assay; NMR, nuclear magnetic resonance; C group, control group; IRIS, International Renal Interest Society; ALP, alkaline phosphatase; ALT, alanine aminotransferase; USG, urine specific gravity; UPC, urine protein-to-creatinine; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; HMDB, Human Metabolome Database; LI, stage I LeishVet; LII, stage II LeishVet; LIII, stage III LeishVet; LIV, stage IV LeishVet; uGGTcr, gamma-glutamyl transferase/creatinine urinary; 1H NMR, proton nuclear magnetic resonance; GFR, glomerular filtration rate.

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unknown at presentation and throughout the disease course (Dunaevich et al., 2020). CKD represents one of the severe complications and the leading cause of mortality in canine leishmaniasis (CanL), a zoonotic disease caused by the protozoan *Leishmania spp* (Maxfield & Crane, 2021). In CanL, CKD can progress from mild proteinuria to nephrotic syndrome or end-stage renal disease (González et al., 2022; Solano-Gallego et al., 2011).

The kidneys are crucial for various metabolic processes, including the secretion of cytokines and hormones, the excretion of waste metabolites, and maintaining electrolyte homeostasis (Brunetto et al., 2021; Ottka et al., 2021). Thus, specific metabolites hold potential as predictive biomarkers of kidney function (Gervasini et al., 2023). However, traditional biomarkers (plasma creatinine and urea, urinary proteinuria and specific gravity) become prominent only in the advanced stages of CKD (Ferlizza et al., 2020; Gervasini et al., 2023). Metabolomics, the comprehensive analysis of metabolites in biological samples, has emerged as a powerful strategy in medical sciences (Carlos et al., 2020; Emwas et al., 2015). In human CKD, metabolomics profiling at different disease stages has revealed metabolic changes correlated with disease progression. However, research utilizing metabolomics in canine CKD remains limited (Brunetto et al., 2021; Carlos et al., 2020; Ferlizza et al., 2020; Ottka et al., 2021), especially in CKD secondary to leishmaniasis.

Metabolomics could identify novel biomarkers to help detection of CKD, monitor its progression and identify early stages of the disease (Hocher & Adamski, 2017; Posada-Ayala et al., 2014). Nuclear Magnetic Resonance (NMR) spectroscopy has become a prominent tool in metabolomics, offering non-destructive analysis, minimal sample preparation, and comprehensive quantitative profiling of the metabolome. Urine is an ideal biofluid for metabolomic studies due to its non-invasive collection, large volume, and lower protein and lipid content (Beckonert et al., 2007; Bouatra et al., 2013; Emwas et al., 2015). Previous studies have demonstrated the effectiveness of NMR spectroscopy in examining urine metabolic profiles associated with various diseases, including CKD in humans and animals (Emwas et al., 2015; Ferlizza et al., 2020; Hunter et al., 2021). However, traditional high-field NMR spectrometers are expensive due their reliance on cryogenic technology and require specialized personnel to operate. Recent technical advancements have introduced benchtop NMR spectrometers, which operate at lower magnetic field strengths. These spectrometers offer excellent reproducibility, low maintenance, robustness, and ease of use, enabling wider application of NMR spectroscopy. However, their use in metabolomic studies is still relatively unexplored (Finch et al., 2021).

This study aims to utilize low-field NMR spectroscopy to identify differences CKD secondary to CanL compared to CKD without CanL and healthy dogs. Our objective is to establish a metabolomic signature associated with this specific disease that can complement existing diagnostic techniques. By leveraging the advantages of benchtop NMR, we envision routine metabolomic analyses becoming feasible in healthcare settings, enabling point-of-care patient assessments in both human and veterinary medicine.

## 2. Material and methods

The present prospective study was conducted at the Veterinary Teaching Hospital of the University of Extremadura from October 1, 2022, to December 1, 2023. The study included dogs that were either referred or presented as primary patients at the time of diagnosis. Informed consent was obtained from all owners for the collection of samples from their animals.

Approval from the Animal Experimentation Ethics Committee of the University of Extremadura (Spain) was deemed unnecessary, as the Bioethics Committee concluded that no animal experimentation was involved (Ref. 86/2023).

### 2.1. Study population

Fifty-five dogs were studied and divided into three groups: healthy control dogs (CG) consisting of 14 animals, dogs with CKD due to leishmaniasis consisting of 33 animals and 8 dogs with CKD without leishmaniasis. Dogs with CKD secondary to leishmaniasis were classified according to the staging system established by the International Renal Interest Society (IRIS) (*IRIS Kidney - Guidelines*, n.d.) as follows: stage 1 (15 dogs), stage 2 (10 dogs), stage 3 (6 dogs) and stage 4 (2 dogs). Additionally, they were classified according to the LeishVet expert group (Solano-Gallego et al., 2011) staging system as follows: stage I (5 dogs), stage II (4 dogs), stage III (14 dogs) and stage IV (10 dogs). Regarding dogs with CKD without leishmaniasis, one dog was in IRIS stage 1, two in IRIS stage 2, one in IRIS stage 3, and four in IRIS stage 4. All dogs with CKD without leishmaniasis were proteinuric with UPC $> 0.5$.

The control group comprised clinically healthy dogs. Of these, 6 dogs were presented to the hospital's Internal Medicine Service for routine clinical check-ups, and 9 dogs for elective sterilization programs. All of them adhered to the recommended deworming and vaccination schedules. Physical examinations, including systolic blood pressure measurement, were normal and blood and urine tests results were within reference ranges.

### 2.2. Laboratory analysis

Blood samples were obtained by puncture of the cephalic, saphenous, or jugular veins and placed in tubes containing EDTA for hematologic determinations and tubes containing heparin for biochemistry analysis. Plasma was prepared by centrifuging the blood samples at 540 g for 10 min. Hematologic analyses were performed with an automated analyser (ProCyte DX®, IDEXX Laboratories, USA). The biochemical variables determined included urea, creatinine, SDMA, alkaline phosphatase (ALP), total protein, albumin, cholesterol, calcium, alanine aminotransferase (ALT) and phosphorus. Globulin concentration was calculated by subtracting the albumin value from the total protein value. All biochemical analysis were performed using an automated biochemical analyser (Spin200E, Spinreact®, Barcelona, Spain) with commercial kits (Spinreact® Laboratory Barcelona, Barcelona, Spain), according to the manufacturer's instructions. Symmetric dimethylarginine (SDMA) was determined using a Catalyst Dx Chemistry Analyzer (IDEXX® Laboratories, Inc, Westbrook, USA).

Urine samples were collected by ultrasound-guided cystocentesis. One millilitre was used for urinary culture (dogs with positive cultures were excluded) and three millilitres for routine urinalysis (Multistix Reagent Strips®, Bayer Corporation, Madrid, Spain). The remaining urine was centrifuged at 130 g for 5 min to obtain urinary sediment, which was examined under an optical microscope (40x objective). The sediment was considered inactive if it met the following conditions: $< 5$ erythrocytes/hpf; $< 5$ leukocytes/hpf; occasional epithelial cells; absence of bacteria. Urine specific gravity (USG) was measured using a refractometer. The urine protein-to-creatinine ratio (UPC) was determined using the pyrogallol-molybdate red technique, while creatinine was measured using the Jaffé reaction with an automated blood chemistry analyser (RAL Diagnostics®, SA, Spain). Finally, the urinary concentration of GGT was determined using the automated blood chemistry analyser (Spin200E, Spinreact®, Barcelona, Spain) with commercial kits (Spinreact® Laboratory Barcelona, Barcelona, Spain), and the results were expressed as the ratio to creatinine (uGGTcr). The supernatant, free of any cellular debris and solid components, was frozen at $-80$ °C until NMR analysis.

A semi-quantitative ELISA was conducted to detect specific antibodies against the total soluble antigen of L. *infantum*, derived from promastigotes (strain MCAN/ES/1996/BCN150, zymodeme MON-1) (Belinchón-Lorenzo et al., 2013). The diagnosis of CanL was based on clinical signs and serology titres, requiring a three-fold increase above the laboratory's reference cut-off. Diagnosis was corroborated, when

possible, by visualization of *Leishmania* amastigotes in lymph node or bone marrow aspirates or a positive PCR (Laboklin Veterinary Laboratory SL, Alcobendas, Spain).

Additionally, all dogs included in the study (control, CKD with CanL and CKD without CanL) underwent testing for canine heartworm disease, *Anaplasma phagocytophylum, Borrelia burgdorferi*, and *Ehrlichia canis* antibodies using the Canine SNAP 4Dx from IDEXX® Laboratories, USA, ensuring a comprehensive assessment of potential canine infections.

### 2.3. Chemometric analysis

To classify urine samples, this study employed low field proton nuclear magnetic resonance ($^1$H NMR) spectroscopic analysis. This technique operates on the principle that hydrogen nuclei in different chemical environments resonate at distinct frequencies when exposed to a magnetic field. Consequently, each metabolite in the sample exhibits characteristic resonance frequencies, collectively generating a unique metabolic fingerprint. Centrifuged urine samples (300 µL) were mixed with 200 µL of 177 mM phosphate buffer (pH 7.4) containing 0.09 % $NaN_3$ and 25 mM formic acid. The resulting samples were loaded in 5 mm NMR tubes. Spectra were recorded in an 80 MHz Magritek Spinsolve Ultra spectrometer (Magritek, Philipsstraße 8, 52,068 Aachen, Germany) using a solvent suppression sequence (WET). Data acquisition parameters included a spectral width of 2500 Hz, 65,536 data points and 128 scans. The spectra were processed using MestreNova software (*Mestrelab Research Analytical Chemistry Software*, n.d.). Gaussian apodization of 0.6 MHz was applied to FID signals before Fourier transform, and automatic phase and baseline correction algorithms were employed. To further investigate the potential contribution of proteins and lipids to the observed NMR spectra, control NMR experiments were performed using different T2 filters strengths. T2 filtering is known to improve the spectral quality reducing broad, unstructured background by attenuating the signals from molecules with short T2 s (like proteins and lipids), while preserving signals from smaller metabolites with longer T2s. We observed no significant impact on the spectra, suggesting the concentration of proteins and lipids contributing to signal interference in these samples was negligible. Therefore, spectra acquired without T2 filters were used in this study. Spectra were referenced to the formic acid signal (8.44 ppm), cut between 0.5 and 4.20 and 5.15 and 8.35 ppm to exclude water and formic acid signals and normalized to the peak of creatinine at 3.02 ppm. Then, the spectra were divided into 0.04 ppm segments, which were integrated generating 173 variables per sample. Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were conducted using the mixOmics package (Rohart et al., 2017) in R (*R: The R Project for Statistical Computing*, n.d.).

For PLS-DA, the spectral integrals dataset was randomly divided into training (70 %) and test (30 %) sets, stratified to ensure a representative distribution of dog groups within each subset. This approach facilitated model construction, validation on the training set, and assessment of generalizability on unseen data from the test set. The PLS-DA models' performance was evaluated using leave-one-out cross-validation (LOO—CV). In this resampling technique, each data point is sequentially excluded for testing, while the remaining data serves for model training. This approach provides an unbiased estimate of model generalizability. To assess this generalizability, relevant classification metrics were calculated, including balanced accuracy (measures the overall performance of the model), kappa (measure model accuracy and recognition), sensitivity and specificity (measure how well a model can distinguish different classes).

### 2.4. Animal classification

While the International Renal Interest Society (IRIS) staging system aids in leishmaniasis diagnosis based on kidney function, it remains unclear whether the altered urinary metabolic profile observed in leishmaniasis solely reflects renal dysfunction or includes specific metabolic signatures related to the parasitic infection itself. To address this, we compared $^1$H NMR spectroscopic data of urine samples from CanL dogs and dogs diagnosed with non-leishmanial CKD. This comparison is essential to determine if the metabolic profile of leishmaniasis is distinct from that of other forms of kidney disease.

### 2.5. Statistical study

The statistical analyses corresponding to the analytical data obtained from blood and urine were performed using SPSS Statistics software, version 27 (IBM Corp., NY, USA) (*IBM SPSS Statistics 27.0.1.0*, n.d.). All data were reported as mean ± standard deviation to describe the distribution of continuous variables. All parameters were assessed for normality using the Shapiro-Wilk test. Variables with non-normal distribution were compared using the Mann-Whitney U test, followed by Dunn's post-hoc test. Differences with a p-value $< 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Demographic and classification data

The CG consisted of dogs of varying ages (4.98 ± 2.1 years old) and sex (9 males and 5 females). The dogs with CKD and leishmaniasis were classified according to the LeishVet expert group criteria (Solano-Gallego et al., 2011) as follows: stage I (LI; 5 dogs; 6.3 ± 2.1 years old; 3 males and 2 females), stage II (LII; 4 dogs; 5.9 ± 2.5 years old; 2 males and 2 females), stage III (LIII; 14 dogs; 6.2 ± 2.2 years old; 6 males and 8 females) and stage IV (LIV; 10 dogs; 6.8 ± 3 years old; 6 males and 4 females). These same dogs were also staged according to IRIS (*IRIS Kidney - Guidelines*, n.d.) as follows: stage 1 (15 dogs; 6.5 ± 2.1 years old; 8 males and 7 females), stage 2 (10 dogs; 5.8 ± 2 years old; 5 males and 5 females), stage 3 (6 dogs; 6.3 ± 2.1 years old; 3 males and 3 females) and stage 4 (2 dogs; 6 and 8 years old; 2 females). Dogs with CKD were classified according to IRIS (*IRIS Kidney - Guidelines*, n.d.) as follow: stage 1 (1 dog; 5 years old female), stage 2 (2 dogs; 6 years old; 1 male and 1 female), stage 3 (1 dog; 10 years old female) and stage 4 (4 dogs; 8.1 ± 6.9 years old; 3 males and 1 female). All dogs were different breeds.

### 3.2. Hematological analysis

The hematological study reveals a progressive decrease in hematocrit and red blood cell count as the leishmaniasis CKD stages advance, with statistically significant differences ($p < 0.05$) observed in all groups compared to the CG. This anaemia was characterized as normocytic, normochromic, and non-regenerative (Table 3).

### 3.3. Blood biochemistry

Blood biochemistry showed a statistically significant decrease ($p < 0.05$) in plasma albumin concentration in LII, LIII, and LIV compared with the CG, along with a concurrent increase in globulins levels. Additionally, elevated levels of phosphorus are observed in LIV, with statistically significant differences ($p < 0.05$) compared to the other groups. There is a progressive increase in creatinine and urea values as the disease progresses. In creatinine, significant differences ($p < 0.05$) were observed between LIV and CG, LII and LIII. In the case of urea, significant differences ($p < 0.05$) were observed between LIV and all groups and between LIII and CG. On the other hand, an increase in cholesterol was observed mainly in LIII and LIV with significant differences with respect to LI and LII and also with CG in the case of LIV (Table 1).

**Table 1**
Biochemical parameters of the control group and dogs with CKD with leishmaniasis classified according to LeishVet staging and CKD without leishmaniasis. Data are presented as median ± standard deviation.

| | Control group | LI | LII | LIII | LIV | CKD no CanL |
|---|---|---|---|---|---|---|
| Albumin (g/dl) | 3.22 ± 0.19[b,c,d] | 2.85 ± 0.55[d] | 2.28 ± 1.14* | 2.40 ± 1.02* | 2.28 ± 0.73*[a] | 2,73 ± ,49 |
| Globulin (g/dl) | 2.96 ± 0.51[a,c,d] | 3.86 ± 2.36*[d] | 4.70 ± 3.44 | 3.82 ± 2.72* | 5.50 ± 3.03*[a] | 3,63 ± 2,18 |
| Creatinine (mg/dl) | 1.16 ± 0,16[d] | 1.05 ± 0,3 | 1.13 ± 0.4[d] | 1.40 ± 1.03[d] | 3.40 ± 2.05*[b,c] | 4,25 ± 3,85* |
| Urea (mg/dl) | 36.05 ± 19.65[c,d] | 36 ± 21[d] | 37.50 ± 25[d] | 61 ± 77*[d] | 165 ± 92.6*[a,b,c] | 233 ± 112,3* |
| Phosphorus (mg/dl) | 4.25 ± 1.21[d] | 4.40 ± 2.35[d] | 3.78 ± 1.4[d] | 4.90 ± 1.55[d] | 7.30 ± 4.35*[a,b,c] | 10,29 ± 9,23* |
| Cholesterol (mg/dl) | 213 ± 69.5[a,b,d] | 150 ± 58*[c,d] | 128 ± 12*[c,d] | 236 ± 81.75[a,b] | 347 ± 178.25*[a,b] | 227,37 ± 49,64 |

LI: stage I LeishVet; LII: stage II LeishVet; LIII: stage III LeishVet; LIV: stage IV LeishVet. CKD no CanL: chronic kidney disease without leishmaniasis. * Statical differences respect control group; [a] statical differences respect LI; [b] statical differences respect LII; [c] statical differences respect LIII; [d] statical differences respect LIV.

### 3.4. Urine analysis

A progressive increase in the uGGTcr ratio was observed as the stages of the leishmaniasis disease advanced with significant differences with respect CG in LIV, along with a decrease in urine specific gravity. A progressive increase in the UPC was also observed (Table 2).

### 3.5. Chemometric analysis of the NMR data

Fig. 1 presents stacked $^{1}$H NMR spectra of urine samples representative from each dog group. The spectra exhibit distinct variations among individual samples, potentially reflecting biological variability or differences in health status. However, inherent variability hinders the immediate identification of significant intergroup differences. While limitations in spectral resolution preclude exhaustive metabolite identification, each spectrum captures a complete outline information on metabolite concentrations contributing to its profile (Izquierdo-Garcia et al., 2020), providing valuable information on the metabolic state of the animal.

Multivariate statistical techniques, such as Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA), are useful for extracting hidden patterns and meaningful information from complex datasets like NMR data (Galvan et al., 2023). A chemometric analysis employing these techniques can reveal underlying patterns and classify samples based on their metabolic profiles, offering insights into disease status and potentially identifying unique metabolic signatures associated with different conditions. To investigate the potential of NMR data for discriminating between healthy and leishmaniasis-infected dogs, urine samples were subjected to PCA for initial data exploration and visualization (Fig. 2A). The first three components accounted for 80.39 % of the total variance (PC1 57.32 %, PC2 17.12 % and PC3 5.95 %), amply exceeding the recommended threshold for meaningful analysis (Gervasini et al., 2023). A 3D PCA score plot revealed a preliminary separation between leishmaniasis-infected and control groups, suggesting distinct metabolic differences. This initial separation, while encouraging, highlighted the need for more powerful discriminatory models. Therefore, we further investigated the ability of PLS-DA models to enhance group discrimination. To this end, the NMR variables dataset was randomly divided into training (70 %) and test (30 %) sets using stratification by disease status (healthy, *Leishmania*-infected) to ensure that each fold contained a representative proportion of samples from each group and prevent bias. Leave-one-out cross-validation performed on the training subset, optimising for the max.dist metric in mixOmics (which maximises the separation between classes), was used to determine the optimal number of latent variables. The resulting PLS-DA model with four latent variables demonstrated excellent classification performance on the training set, achieving a balanced accuracy of 0.86, sensitivity of 0.92, specificity of 0.80, and a kappa value of 0.76. These high-performance metrics indicate that the PLS-DA model effectively captured the metabolic differences between healthy and *Leishmania*-infected dogs.

This model was then used to classify the samples from the test subset. Successfully, the PLS-DA scores plot representing the first three latent variables (Fig. 3) demonstrated a clear separation of the groups, both for the train and the test subsets, confirming the model's predictive power. The predicted classification for the test samples shows satisfactory metrics, with a balanced accuracy of 0.88, sensitivity of 1, specificity of 0.75 and kappa of 0.81.

As CKD is a common and challenging clinical manifestation of canine leishmaniasis, we decided to employ a similar PLS-DA approach to compare the $^{1}$H NMR spectroscopic data of urine samples from CanL dogs and dogs diagnosed with non-leishmanial CKD. This analysis aimed to determine if the metabolic changes observed in leishmaniasis-infected dogs were specific to the parasitic infection or simply a consequence of general kidney dysfunction. The model constructed with 3 latent variables exhibited acceptable discriminatory power on the train set, with a balanced accuracy of 0.71, sensitivity of 0.75, specificity of 0.67, and a kappa coefficient of 0.47. Importantly, this same model exhibited excellent performance when classifying the unseen test samples. As shown in Fig. 3, a clear separation between the two groups was observed in the PLS-DA scores plot, with dogs having non-leishmanial CKD positioned in the upper right quadrant and those with leishmanial CKD in the lower left quadrant. Classification of the test samples with this model achieved particularly good metrics, with a balanced accuracy of 0.94, sensitivity of 1, specificity of 0.88 and a kappa coefficient of 0.74. The substantial improvement in performance on the test set suggests that the model is robust and not overfit to the training data. The perfect sensitivity indicates that the model correctly identified all dogs with leishmanial CKD in the test set, a critical finding for accurate diagnosis. The high specificity further strengthens the model's ability to correctly classify dogs with non-leishmanial CKD. These findings imply that metabolic alterations associated with leishmaniasis extend beyond those caused by general kidney dysfunction.

Building upon the successful differentiation of healthy from *Leishmania*-infected dogs, the study further investigated the potential of $^{1}$H NMR spectroscopy for classifying different stages of leishmaniasis severity. Fig. 4A presents a 2D PCA score plot depicting the distribution

**Table 2**
Urinary parameters of the control group and dogs with leishmaniasis, classified according to LeishVet staging. Data are presented as median ± standard deviation.

| | Control group | LI | LII | LIII | LIV | CKD no CanL |
|---|---|---|---|---|---|---|
| Density | 1045 ± 8.77[c,d] | 1043 ± 7.23*[c,d] | 1042 ± 11.7*[d] | 1023 ± 13.7*[a] | 1017.50 ± 4.41*[a,b] | 1014,37 ± 7,98* |
| UPC | 0.1 ± 0.1[b,c,d] | 0.11 ± 0.08*[b,c,d] | 0.65 ± 0.61*[a,d] | 2.72 ± 2.62*[a,d] | 5.52 ± 8.49*[a,b,c] | 4,33 ± 8,88* |
| uGGTcr | 4.44 ± 6.85[d] | 20.57 ± 3.2 | – | 43.66 ± 13.14 | 78.44 ± 82.59 * | 78,52 ± 114,51* |

LI: stage I LeishVet; LII: stage II LeishVet; LIII: stage III LeishVet; LIV: stage IV LeishVet. CKD no CanL: chronic kidney disease without leishmaniasis. UPC: urine protein-to-creatinine. GGTcr: gamma-glutamyl transferase/creatinine urinary. * Statical differences respect control group; [a] statical differences respect LI; [b] statical differences respect LII; [c] statical differences respect LIII; [d] statical differences respect LIV.

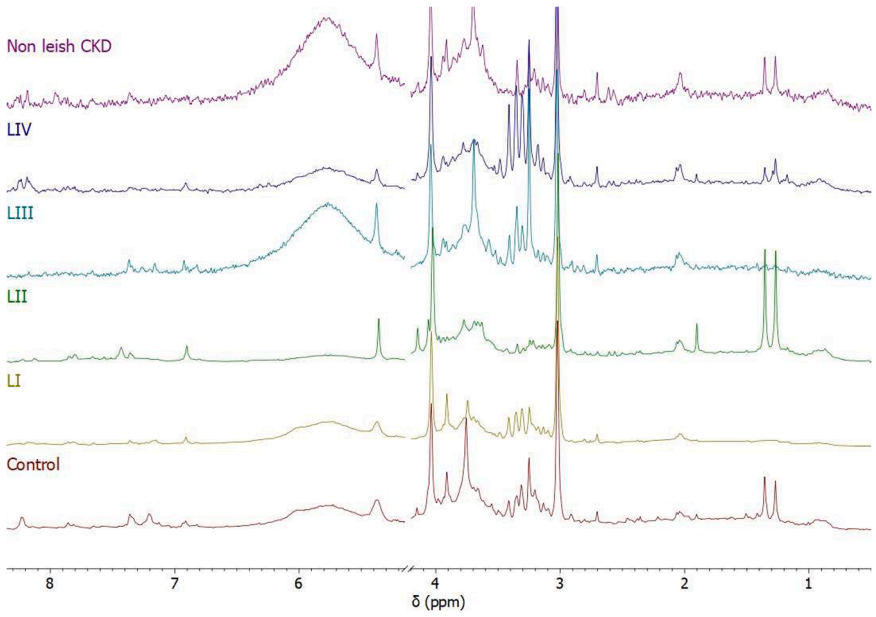


**Fig. 1.** Representative 1H NMR spectra of urine samples from healthy dogs, leishmania-infected dogs in different stages according to Leish-Vet criteria, and dogs with CKD non due to leishmaniasis.

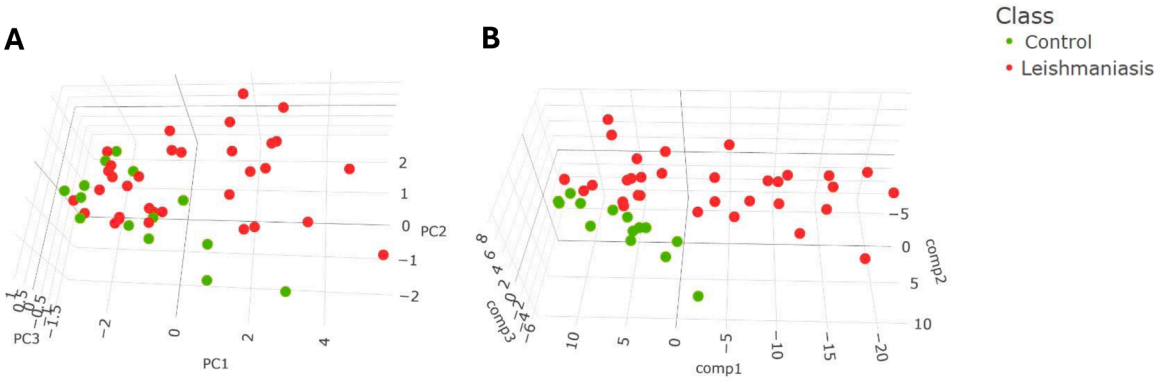


**Fig. 2.** A) PCA and B) PLS-DA of urine samples distinguishing the group of control and a group of all leishmania-infected dogs.

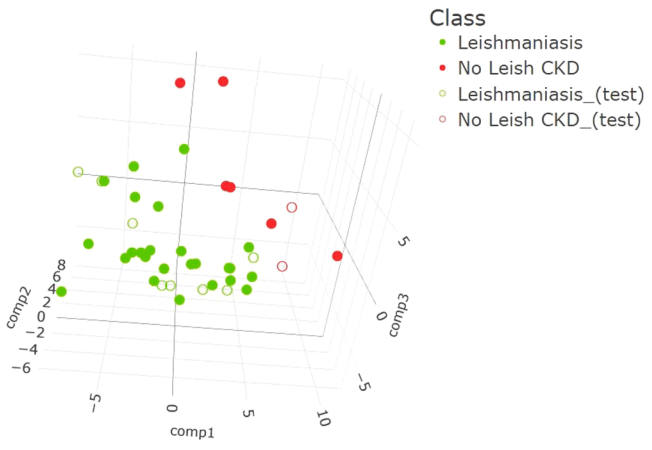


**Fig. 3.** PLS-DA of urine samples discriminating leishmania-infected dogs and dogs with CKD no derived from leishmaniasis.

of leishmaniasis samples classified by LeishVet expert group criteria. Notably, some clustering is evident between samples from healthy dogs and dogs with early stage leishmaniasis (L1 and L2), compared to those from dogs with severe leishmaniasis (L3 and L4). In contrast, Fig. 4B shows the same plot with samples classified according to the IRIS system for CKD staging, where clustering is less apparent. Healthy and IRIS stage 1 samples primarily group in the lower left quadrant, while IRIS stages 2–4 samples exhibit greater dispersion throughout the plot. These contrasting results highlight the potential utility of $^{1}$H NMR spectroscopy for classifying leishmaniasis severity according to the LeishVet criteria. The weaker clustering pattern observed with IRIS staging, a system not specific to leishmaniasis, suggests that the metabolic alterations associated with leishmaniasis progression may be more distinctive than those solely with CKD severity.

## 4. Discussion

CanL is a zoonotic disease that causes a multisystemic and chronic inflammatory condition. It can lead to symptomatic or asymptomatic infection, with the kidney being one of the primary organs affected (Ferreira et al., 2021; Freitas et al., 2012; Ruiz et al., 2023).

The hematological results obtained in dogs with CKD with leishmaniasis were consistent with those expected in CanL. Non-regenerative normocytic and normochromic anaemia is one of the most common laboratory findings in CanL, reported in up to 60 % of affected dogs (Table 3). This type of anaemia can result from various factors including

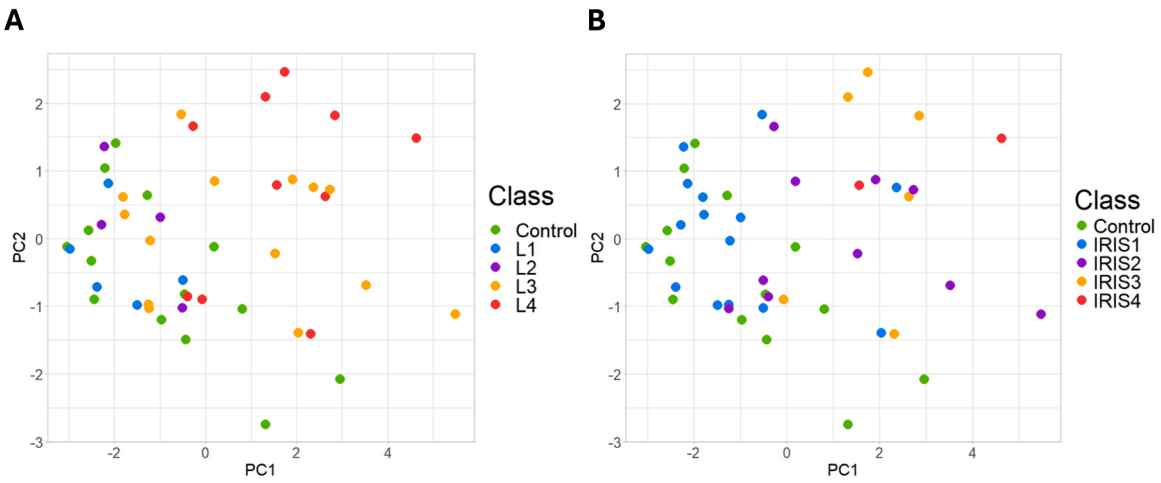


**Fig. 4.** PCA of urine samples of healthy and leishmania-infected dogs. A) Different degrees of CKD due to leishmaniasis are labelled according to LEISHVET criteria. B) Samples are labelled according to IRIS classification.

**Table 3**
Hematology parameters of the control group and dogs with CKD with leishmaniasis classified according to LeishVet staging and CKD without leishmaniasis. Data are presented as median ± standard deviation.

| | Control group | LI | LII | LIII | LIV | CKD no CanL |
|---|---|---|---|---|---|---|
| Hematocrit (%) | 50 ± 10.33$^{a,c,d}$ | 42.60 ± 10.95 *$^{d}$ | 40.35 ± 19.3$^{d}$ | 32.80 ± 16.05*$^{d}$ | 21.55 ± 10.55*$^{a,b,c}$ | 22,55 ± 23,95* |
| MCV (fl) | 68.30 ± 3.07$^{c,d}$ | 66.60 ± 9.2 | 64.60 ± 14.5 | 65.25 ± 9.9* | 59.95 ± 10.6* | 67,04 ± 4,22 |
| MCHC (g/dl) | 34.85 ± 0.5$^{b}$ | 34.70 ± 2 | 34.15 ± 0.78* | 34.30 ± 2.7 | 34.20 ± 2.7 | 36,05 ± 2,35 |
| Reticulocytes/µl | 39,350 ± 47,000 | 37,050 ± 25,000 | 65,200 ± 45,000 | 39,500 ± 49,650 | 23,000 ± 21,825 | 42,500 ± 38,800 |
| Leukocytes (x10$^{3}$/µl) | 9.29 ± 3.02$^{a}$ | 13.75 ± 7.36* | 8.79 ± 8.36 | 10.68 ± 13.49 | 11.33 ± 20.03 | 11,82 ± 18,78 |
| Platelets (x10$^{3}$/µl) | 251.79 ± 81.23 | 199.20 ± 78.35 | 223.25 ± 92.13 | 207.64 ± 94.27 | 221 ± 95.26 | 241,5 ± 384,5 |

LI: stage I LeishVet; LII: stage II LeishVet; LIII: stage III LeishVet; LIV: stage IV LeishVet. CKD no CanL: chronic kidney disease without leishmaniasis. MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration. * Statical differences respect control group; $^{a}$ statical differences respect LI; $^{b}$ statical differences respect LII; $^{c}$ statical differences respect LIII; $^{d}$ statical differences respect LIV.

the infection, disturbances in the erythroid bone marrow compartment, haemolysis, renal failure, and bleeding, among others (Freitas et al., 2012; Meléndez-Lazo et al., 2018; Reis et al., 2009; Solano-Gallego et al., 2009).

The most significant findings obtained in the plasma biochemistry were progressive increase in plasma creatinine and urea levels as the disease progresses, while albumin values decreased and globulin values increased, consistent with previous literature (Ferreira et al., 2021; Meléndez-Lazo et al., 2018; Paltrinieri et al., 2016). Hypoalbuminemia may result from albumin's role as a negative acute-phase protein, renal loss in glomerular damage, or the disease's chronic nature (Ruiz et al., 2023; Vieira Neto et al., 2011). The increase in globulins is primarily attributed to elevated gamma globulins with limited protective capacity. This excessive production contributes to the unfavourable progression of the disease (Meléndez-Lazo et al., 2018; Soares et al., 2014).

Elevated plasma phosphorus levels correlates with the worsening of CKD, directly linked to the decline in glomerular filtration rate (GFR), accelerating disease progression and reducing survival (Martiarena et al., 2014). Phosphorus levels were elevated in all affected groups, with the highest increase in stage IV (LIV). Additionally, increased cholesterol levels, characteristic of nephrotic syndrome, were observed in advanced CKD stages (González et al., 2022; Solano-Gallego et al., 2011).

Proteinuria, due to a disorder in glomerular filtration attributed to the presence of immune complexes in the glomeruli, can progress to nephrotic syndrome or lead to the development of CKD. Therefore, the UPC is used to classify the severity of the disease according to LeishVet and IRIS (*IRIS Kidney - Guidelines*, n.d.; Solano-Gallego et al., 2011). Statistically significant differences can be observed in LII and LIII with respect to CG, and in LIV compared to all other groups (Table 2). Similarly, an increase in the GGTcr ratio in urine, indicative of additional tubular damage, was observed (Ibba et al., 2016).

Chronic kidney damage causes restructuring of the nephrons and leads to a reduced response to antidiuretic hormone, resulting in an inability to concentrate urine, with lower density observed in the final stages of disease (Table 2).

Due to the kidneys' crucial role in metabolism, including the secretion of cytokines and hormones and the excretion of waste metabolites (Brunetto et al., 2021; Ottka et al., 2021), impaired kidney function leads to significant changes in the metabolite composition of urine. The study of metabolome holds promise in veterinary medicine, particularly as a diagnostic tool and for identifying biomarkers of kidney disease. Metabolic studies have explored various canine diseases, including obesity, heart disease, diabetes mellitus, and cancer (Carlos et al., 2020). However, there is a scarcity of research on metabolomics applications in the context of CanL in veterinary medicine. This study addresses this gap by investigating the urinary metabolic profile of dogs with leishmaniasis, comparing them to healthy controls and dogs with non-leishmanial CKD.

While stacked spectra (Fig. 1) reveal visual differences among individual samples, reflecting potentially biological variability or disease state, the complexity of urine and limitations in spectral resolution necessitate the use of multivariate statistical methods to discern meaningful patterns. Although exhaustive metabolite identification was not the primary goal of this study, each spectrum provides a comprehensive snapshot of the metabolite concentrations contributing to the overall metabolic profile, offering valuable insights into the metabolic status of the animal. Further targeted studies would be necessary for a precise identification of the characteristic individual metabolites.

Our findings, revealed through PCA and PLS-DA (Fig. 2),

demonstrate distinct differences in the urinary metabolic profiles of healthy dogs and those naturally infected with *Leishmania*. This aligns with previous research conducted on golden hamsters experimentally infected with the *Leishmania* parasites using ultra-performance liquid chromatography coupled with high-resolution mass spectrometry (UPLC—HRMS), which also reported differences in urine metabolome compared to controls (Yuan et al., 2023). However, our study extends these findings by examining naturally infected dogs, a more clinically relevant model, and by utilizing a more readily accessible and less expensive technique (benchtop NMR) than UPLC—HRMS. This makes our approach potentially more translatable to routine veterinary practice.

Ferlizza and coworkers (Ferlizza et al., 2020) demonstrated distinct discrimination in urine samples of dogs with kidney disease from various causes compared to healthy control dogs using high field $^{1}$H NMR. However, to our knowledge, no group discrimination based on the aetiology of kidney disease has been reported until now. The clear separation we observed in the PLS-DA of dogs with leishmanial CKD and those with non-leishmanial CKD (Fig. 3) is a key finding. This demonstrates that the metabolic changes associated with leishmaniasis are not simply a consequence of general kidney dysfunction, suggesting that *Leishmania* infection induces specific metabolic alterations beyond those attributable to kidney damage, such as liver or bone marrow among others (Freitas et al., 2012). Further research is needed to elucidate the precise mechanisms underlying these metabolic differences.

Our findings also reveal differences in metabolic profiles across different stages of leishmaniasis (Fig. 4A). The clustering observed between samples from healthy dogs and dogs with early-stage leishmaniasis (LI and LII), in contrast to those from dogs with severe leishmaniasis (LIII and LIV), suggests a progression of metabolic changes with disease severity. This could reflect potential variations in the parasite's nutrient requirements and/or the host's immune response throughout the infection course. Importantly, the LeishVet criteria provided superior discrimination of NMR samples compared to the IRIS staging system (Fig. 4B). These results demonstrate the potential of $^{1}$H NMR urinary metabolic profiles for the chemometric classification of the severity of leishmaniasis. This superior discrimination offered by the LeishVet criteria suggests that metabolic profiling may offer a more sensitive and comprehensive assessment of disease severity than traditional clinical staging methods, potentially allowing for earlier detection of disease progression and more tailored treatment strategies.

## 5. Conclusion

Globally, our results demonstrate distinct urine metabolic profiles between healthy dogs and those infected with *Leishmania*, as revealed by low-field $^{1}$H NMR spectroscopy. Furthermore, these profiles differed significantly between early and late disease stages, suggesting that the parasite's nutrient needs and/or the host's immune response may change over the course of the infection. These findings align with previously studies such as those of Yuan et al. (202)3 and Qin et al. (2022), who observed distinct metabolic signatures in urine and serum of *Leishmania*-infected golden hamsters. We can also conclude that chronic kidney damage presents a different metabolic profile than kidney damage secondary to leishmaniasis. This is consistent with previous publications demonstrating that leishmaniasis causes multiple alterations in the organism (Freitas et al., 2012; Meléndez-Lazo et al., 2018; Reis et al., 2009). The ability to distinguish between leishmanial CKD and other forms of CKD could improve diagnostic accuracy and guide treatment decisions. These considerations have significant implications for diagnosis and disease management. While traditional diagnostic methods, such as serology and clinical assessment, remain essential, metabolomics offers a complementary approach that can provide a more nuanced understanding of the disease process.

Ongoing studies with larger, more stratified cohorts, particularly according to IRIS classification, are warranted to validate these findings and explore the clinical utility of NMR-based metabolomics in canine leishmaniasis. Furthermore, a non-targeted metabolomic study using high field NMR spectroscopy will help us to identify specific metabolic biomarkers associated with disease progression. This would enable their future identification with benchtop NMR spectroscopy, potentially facilitating the development of novel therapeutic targets.

## CRediT authorship contribution statement

**Ángela Durán-Galea:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **José-Luis Ramiro-Alcobendas:** Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **FrancisoJavier Duque-Carrasco:** Writing – review & editing, Visualization, Validation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Paloma Nicolás-Barceló:** Writing – original draft, Project administration, Methodology, Investigation, Data curation. **José-Ignacio Cristóbal-Verdejo:** Writing – original draft, Visualization, Project administration, Methodology, Investigation. **Patricia Ruíz-Tapia:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Formal analysis. **Rafael Barrera-Chacón:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Carlos F. Marcos:** Writing – review & editing, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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