UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

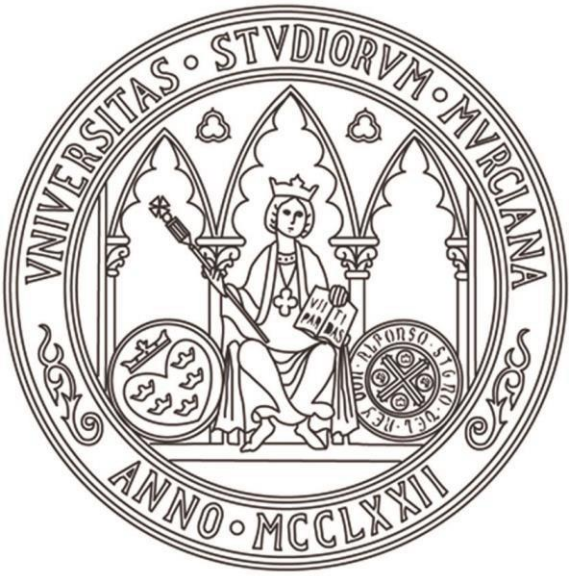
TESIS DOCTORAL

Advances in the study of biomarkers related to canine leishmaniosis

Avances en el estudio de biomarcadores relacionados con leishmaniosis canina

D. Luis Pardo Marín

2024



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Advances in the study of biomarkers related to canine leishmaniosis

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Autor: D. Luis Pardo Marín

Director/es: D. José Joaquín Cerón Madrigal.

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UNIVERSIDAD DE MURCIA

DECLARACIÓN DE AUTORÍA Y ORIGINALIDAD DE LA TESIS PRESENTADA EN MODALIDAD DE COMPENDIO O ARTÍCULOS PARA OBTENER EL TÍTULO DE DOCTOR

Aprobado por la Comisión General de Doctorado el 19-10-2022

D./Dña. Luis Pardo Marín

doctorando del Programa de Doctorado en

Ciencias Veterinarias

de la Escuela Internacional de Doctorado de la Universidad Murcia, como autor/a de la tesis presentada para la obtención del título de Doctor y titulada:

Advances in the study of biomarkers related to canine leishmaniosis / Avances en el estudio de biomarcadores relacionados con leishmaniosis canina

y dirigida por,

D./Dña. José Joaquín Cerón Madrigal

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Fdo.: Luis Pardo Marín

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Muchas gracias a todos.

INDEX

DOCTORAL THESIS AS COMPENDIUM OF PUBLICATIONS

This thesis, after the authorization of the directors of the PhD thesis and the Academic Commission responsible of the Veterinary Sciences PhD Program, is presented as compendium of eight studies previously published. Therefore this PhD thesis is integrated by the following articles' references:

A) ARTICLES PUBLISHED:

1. Use of acute phase proteins for the clinical assessment and management of canine leishmaniosis: general recommendations. Ceron JJ, **Pardo-Marin L**, Caldin M, Furlanello T, Solano-Gallego L, Tecles F, Bernal L, Baneth G, Martinez-Subiela S. BMC Vet Res. 2018 Jun 20;14(1):196.

2. Serum C-reactive protein and ferritin concentrations in dogs undergoing leishmaniosis treatment. Martinez-Subiela S, **Pardo-Marín L**, Tecles F, Baneth G, Cerón JJ. Res Vet Sci. 2016 Dec;109:17-20.

3. Comparison of acute phase proteins in different clinical classification systems for canine leishmaniosis. **Pardo-Marin L**, Ceron JJ, Tecles F, Baneth G, Martínez-Subiela S. Vet Immunol Immunopathol. 2020 Jan;219:109958.

4. Divergences between serum C-reactive protein and ferritin concentrations in canine pyometra. Ceron, J. J., **Pardo-Marin, L**., Wdowiak, A., Zoia, A., Wochnik, M., Szczubiał, M., Bochniarz, M., Tecles, F., Martinez-Subiela, S., Tvarijonaviciute, A., & Dąbrowski, R. BMC veterinary research 2023, 19(1), 78.

5. Ferritin in Obese Dogs: Changes and Comparison with Other Analytes. Franco-Martínez, L., **Pardo-Marín, L.**, Sánchez-Mateos,

L., Muñoz-Prieto, A., García-Martínez, J. D., Cerón, J. J., Martínez-Subiela S., P. Rubio, Camila, Tvarijonaviciute, A. Veterinary Sciences 10.7 (2023): 457.

6. Evaluation of various biomarkers for kidney monitoring during canine leishmaniosis treatment. **Pardo-Marín L**, Martínez-Subiela S, Pastor J, Tvarijonaviciute A, Garcia-Martinez JD, Segarra S, Cerón JJ. BMC Vet Res. 2017 Jan 23;13(1):31.

7. Measurement of urea and creatinine in saliva of dogs: a pilot study. Tvarijonaviciute A, **Pardo-Marin L**, Tecles F, Carrillo JD, Garcia-Martinez JD, Bernal L, Pastor J, Cerón JJ, Martinez-Subiela S. BMC Vet Res. 2018 Jul 20;14(1):223.

8. Identification of novel biomarkers for treatment monitoring in canine leishmaniosis by high-resolution quantitative proteomic analysis. Martinez-Subiela S, Horvatic A, Escribano D, **Pardo-Marin L**, Kocaturk M, Mrljak V, Burchmore R, Ceron JJ, Yilmaz Z. Vet Immunol Immunopathol. 2017 Sep; 191:60-67.

INTRODUCTION AND OBJECTIVES

Leishmaniosis caused by *Leishmania spp*. is a complex and zoonotic disease that affect to the dog. Canine leishmaniasis (CanL) can have an incubation period ranging from a few months to several years and its clinical presentation varies from subclinical or asymptomatic infection to severe disease. It is diagnosed by tests such as serology, polymerase chain reaction (PCR) or observation of the parasite in tissues such as cutaneous lesions, lymph nodes or bone marrow (Solano-Gallego et al., 2011a).

Acute phase proteins (APPs) are a series of molecules related to the innate immune response of the immune system in response to inflammatory processes and undergo variations in their concentration in serum in the course of the inflammatory process. They are considered as sensitive markers of the activation of immune system and therefore of the presence of an active inflammation (Cerón et al., 2005).

CanL is a disease very frequently associated to *kidney damage* due to the immune complex formation. Kidney damage can range from glomerular alteration, which can initially show proteinuria without azotemia, to tubulointerstitial lesions, and kidney failure. Serum creatinine (sCr), urinary protein to creatinine ratio (UPC) are the biomarkers traditionally recommended by the International Renal Interest Society (IRIS) to evaluate and monitor renal damage/dysfunction (Elliott et al., 2013). However, there are other biomarkers that can be measured in urine and are useful for kidney evaluation.

Saliva is a biological fluid that has varies advantages as a diagnostic medium compared with blood, since its collection is non-invasive and simple, and in case of the dogs can be made by owners. In addition, saliva can be sampled repeatedly without discomfort to the patient (Cerón, 2019).

This doctoral thesis aims to provide advances in the laboratory diagnosis of canine leishmaniosis mainly in the field of the three topics above indicated (APPs, kidney damage and saliva) and have the following main objectives:

1. To evaluate use of APPs as support in the classification and monitoring of the disease (**articles 1, 2, 3, 4 and 5**).

2. The application of new biomarkers of kidney damage in CanL that help monitor kidney during the treatment of canine leishmaniasis and that could provide information on the response to treatment (**article 6**).

3. The use of saliva, as a non-invasive technique, for the determination of urea and creatinine (**article 7**).

4. The use of proteomic techniques that are very sensitive to detect proteins in order to detect possible new biomarkers that could be used for monitoring leishmania treatment (**article 8**).

ARTICLES

Article 1

Use of acute phase proteins for the clinical assessment and management of canine leishmaniosis: general recommendations

Use of acute phase proteins for the clinical assessment and management of canine leishmaniosis: general recommendations

J. J. Ceron[1] L. Pardo-Marin[1], M. Caldin[2], T. Furlanello[2], L. Solano-Gallego[3], F. Tecles[1], L. Bernal[1], G. Baneth[4] and S. Martinez-Subiela[1]

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[2] San Marco Veterinary Clinic, Veggiano, Padova, Italy.

[3] Animal Medicine and Surgery Department, Universitat Autònoma de Barcelona, Cerdanyola del Valles, Spain.

[4] Koret School of Veterinary Medicine, Hebrew University, Rehovot, Israel.

Journal: BMC Veterinary Research

Abstract:

Background: Dogs with canine leishmaniosis (CanL) due to Leishmania infantum can show a wide spectrum of clinical and clinicopathological findings at the time of diagnosis. The aim of this paper is to describe the possible application of acute phase proteins (APPs) for the characterization and management of this disease, based on previously published information on the utility of APPs in CanL and the experience of the authors in using APPs as analytes in the profiling of canine diseases.

Main body: Dogs diagnosed with L. infantum infection by serology, polymerase chain reaction, cytological or histopathological identification, can be divided into three groups based on their clinical condition at physical examination and their APPs concentrations: Group 1: dogs with no clinical signs on physical examination and APPs in reference range; Group 2: dogs with changes in APPs but no clinical signs on physical examination; Group 3: dogs with clinical signs and changes in

APPs. This report describes the main characteristics of each group as well as its association with the clinical classification schemes of CanL.

Conclusion: APPs concentration can be a useful clinical tool to characterize and manage CanL.

URL: https://doi.org/10.1186/s12917-018-1524-y

Article 2

Serum C-reactive protein and ferritin concentrations in dogs undergoing: leishmaniosis treatment

Serum C-reactive protein and ferritin concentrations in dogs undergoing leishmaniosis treatment

Silvia Martinez-Subiela[1], Luis Pardo-Marín[1], Fernando Tecles[1], Gad Baneth[2], Jose J. Cerón [1]

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Journal: Research in Veterinary Science

Abstract: Monitoring of selected serum acute phase proteins like C-reactive protein (CRP) and ferritin could be useful for evaluation of the response to treatment in both naturally-occurring and experimentally-induced leishmaniosis. However studies until date have only been focused on dogs with an adequate response to the treatment and there is a lack of knowledge about the possible associations between the CRP and ferritin and the different clinicopathological conditions that can appear after treatment. Thus, the main objective of this retrospective study was to evaluate and compare the serum concentration of CRP and ferritin between three possible situations that dogs which undergo leishmaniosis treatment could have: responsive with total recovery, responsive with only clinical recovery but persistent abnormalities in biochemical analytes, and unresponsive with clinical and biochemical changes.

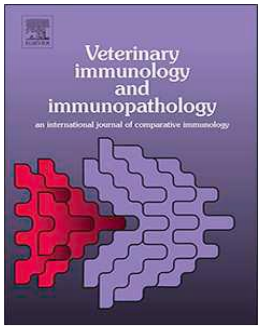
All dogswhich totally recovered after treatment showed CRP and ferritin values within reference ranges. Most of dogs classified as having only partial clinical remission had CRP and ferritinwithin the reference range values despite the presence of other biochemical

abnormalities such as hyperglobulinemia, hyperproteinemia, or proteinuria. On the other hand, most of dogs in the unresponsive group had increased CRP and ferritin. Although the study has limitations due to the variability in the protocols and time periods of treatments, it can be concluded that CRP and ferritin concentrations within the reference ranges are usually associated with the absence of clinical signs and adequate response to treatment and increased CRP and/or ferritin values could reflect a lack of appropriate response to treatment.

URL: https://doi.org/10.1016/j.rvsc.2016.09.003

Article 3

Comparison of acute phase proteins in different clinical classification systems for canine leishmaniosis



Comparison of acute phase proteins in different clinical classification systems for canine leishmaniosis

Luis Pardo-Marin[1], Jose J. Ceron[1], Fernando Tecles[1], Gad Baneth[2], Silvia Martínez-Subiela[1]

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[2] Koret School of Veterinary Medicine, Hebrew University, Rehovot, Israel

Journal: Veterinary Immunology and Immunopathology

Abstract: The main objective of the present study was to evaluate the acute phase protein (APP) concentrations that dogs naturally infected with Leishmania infantum show in different clinical stages of disease staged according to the Leishvet and CLWG classifications. In addition, these classifications are compared with the groups based on APP recommendations published recently.

Medical records of 458 dogs with leishmaniosis whose samples were submitted to our laboratory for clinical pathology evaluation were reviewed and 77 cases met the inclusion criteria. All dogs were classified according to the CLWG system and the majority of the dogs (33.8%) were classified in stage D. Although some dogs (41.6%) could not be classified by the Leishvet system since it includes only dogs with clinical disease, most of the classified dogs (27.3%) were at Leishvet stage II. According to the APP classification, the majority of dogs (32.5%) were classified in stage 3a. Dogs in the more advanced stages of Leishvet and CWLG classifications had significant increases in serum ferritin and C-

reactive protein (CRP) and decrease in Paraoxonase 1 (PON1). These findings indicate that APPs show more significant changes in the more advanced stages of Leishvet and CWLG classifications corresponding with more severe cases of canine leishmaniosis.

URL: https://doi.org/10.1016/j.vetimm.2019.109958

Article 4

Divergences between serum C-reactive protein and ferritin concentrations in canine pyometra

Divergences between serum C-reactive protein and ferritin concentrations in canine pyometra

Jose J Ceron[1], Luis Pardo-Marin[1], Anna Wdowiak[2], Andrea Zoia[3], Marco Wochnik[2], Marek Szczubiał[2], Mariola Bochniarz[2], Fernando Tecles[1], Silvia Martinez-Subiela[1], Asta Tvarijonaviciute[1] and Roman Dąbrowski[2]

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[3] Division of Internal Medicine, San Marco Veterinary Clinic, Viale dell'Industria 3, Veggiano, Padua, 35030, Italy.

Journal: BMC Veterinary Research

Abstract: The main aim of this report was to investigate and compare the response of serum C-reactive protein (CRP) and ferritin, two positive acute phase proteins (APPs) which usually show an increase in inflammatory processes, in dogs with pyometra. For this purpose, two different studies were made. In the first one , both proteins were measured together in an APPs profile in 25 dogs with pyometra, 25 dogs with pancreatitis (as an example of a positive inflammatory control group), and in 25 healthy dogs. In the second study, to advance the knowledge of the changes and evolution of serum ferritin and CRP in dogs with pyometra after treatment, the concentrations of both APPs were analyzed in 30 dogs with pyometra at diagnosis and after ovariohysterectomy and in 10 clinically healthy female dogs before and after elective spaying. In both studies, bitches with pyometra showed significant increases in serum CRP, indicating an inflammatory condition, but not in serum ferritin despite being a moderate positive APP. This divergence between the dynamics of these APPs could be a useful tool for the suspicion of cases of canine pyometra.

URL: https://doi.org/10.1186/s12917-023-03630-3

Article 5

Serum Ferritin in Obese Dogs: Changes and Comparison with Other Analytes

veterinary sciences

Serum Ferritin in Obese Dogs: Changes and Comparison with Other Analytes

Lorena Franco-Martínez[1,2], Luis Pardo-Marín[1], Laura Sánchez-Mateos[3], Alberto Muñoz-Prieto[1], Juan Diego García-Martínez[1], José J. Cerón[1], Silvia Martínez-Subiela[1], Camila P. Rubio[1] and Asta Tvarijonaviciute[1]

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[3] Clínica Veterinaria Dinos, Espinardo, 30100 Murcia, Spain.

Journal: Veterinary Sciences

Abstract: Canine obesity is the most common nutritional disorder and is associated with decreased quality of life and longevity as well as comorbidities including cardiorespiratory, endocrine, oncologic, or orthopaedic disorders. Ferritin is a major acute-phase protein in dogs, increasing during inflammation; however, it could also be affected by other conditions, including trauma, iron metabolism dysregulations, neoplasia, or hypoxia. Higher ferritin levels have been reported in obese humans, but ferritin has not been explored in canine obesity. To evaluate the possible changes in serum ferritin in canine obesity, ferritin levels from lean/normal weight (CG, n = 55) and overweight/obese dogs (OG, n = 37) were measured, together with complete hemogram and biochemical analyses. Statistically significant higher ferritin levels (1.2-fold) were found in OG (median, (interquartile range), 204 (166–227.5) g/L) in comparison to CG animals (172 (137–210) g/L)), with median levels of ferritin in OG dogs above the reference range for healthy animals in our laboratory (60–190 g/L). In addition, statistically significant higher mean corpuscular volume (MCV), mean cell haemoglobin concentration (MCHC), total proteins,

globulins, haptoglobin, total ferric fixation capacity (TIBC), alkaline phosphatase (ALP), butyrylcholinesterase (BChE), triglycerides, and calcium were observed in OG in comparison to CG. The higher levels in ferritin, together with higher TBIC, haematocrit, and MCV, could indicate tissue hypoxia in obese dogs.

URL: https://doi.org/10.3390/vetsci10070457

Article 6

Evaluation of various biomarkers for kidney monitoring during canine leishmaniosis treatment

Evaluation of various biomarkers for kidney monitoring during canine leishmaniosis treatment

Luis Pardo-Marín[1], Silvia Martínez-Subiela[1], Josep Pastor[2], Asta Tvarijonaviciute[1,2], Juan Diego Garcia-Martinez[1], Sergi Segarra[3] and José Joaquín Cerón[1]

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[3] R&D Bioiberica SA, Pça. Francesc Macià 7, 08029, Barcelona, Spain.

Journal: BMC Veterinary Research

Abstract:

Background: The objective of this study was to evaluate and compare the evolution of the profile currently recommended by the International Renal Interest Society (IRIS) (sCr, UPC and sSDMA) with a panel of other different kidney biomarkers during treatment for canine leishmaniosis. This panel included three urinary glomerular biomarkers (uIgG, uCRP and uferritin) and three urinary tubular biomarkers (uGGT, uNAG and uRBP).

These biomarkers were measured in two groups of dogs with canine leishmaniosis at IRIS stage I. Group 1: dogs showing proteinuria (UPC > 0.5) before treatment which did not decrease after treatment; Group 2: dogs showing proteinuria before treatment which decreased after treatment.

Results: Group 1 showed no significant changes in any biomarker after treatment. In group 2, among the biomarkers recommended by the IRIS, only UPC showed a significant decrease after treatment. However all biomarkers of glomerular damage showed a significant decrease after treatment, with uIgG/Cr and uCRP/Cr showing the greater decreases. In addition uRBP/Cr and uNAG/Cr showed significant decreases after treatment.

Conclusions: In dogs with leishmaniosis at IRIS stage I that reduced UPC after treatment, there were no significant changes in serum creatinine and sSDMA. However, all the urine biomarkers evaluated with exception of uGGT showed a significant decrease. These decreases were more evident in those markers related with glomerular function, being uIgG/Cr the biomarker more associated with UPC. Further studies involving a larger number of animals and histological analysis of the kidney would be recommended to confirm these findings and evaluate the routine practical use of these urine biomarkers in canine leishmaniosis.

URL: https://doi.org/10.1186/s12917-017-0956-0

Article 7

Measurement of urea and creatinine in saliva of dogs: a pilot study

Measurement of urea and creatinine in saliva of dogs: a pilot study

Asta Tvarijonaviciute[1], Luis Pardo-Marin[1], Fernando Tecles[1], Juana Dolores Carrillo[2], Juan Diego Garcia-Martinez[1], Luis Bernal[1], Josep Pastor[3], José J. Cerón[1] and Silvia Martinez-Subiela[1]

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Journal: BMC Veterinary Research

Abstract:

Background: Urea and creatinine in saliva have been reported to be possible markers of chronic kidney disease (CKD) in humans. The aim of this study was to assess if urea and creatinine could be measured in canine saliva, and to evaluate their possible changes in situations of CKD.

Results: The spectrophotometric assays for urea and creatinine measurements in saliva of dogs showed intra- and inter-assay imprecision lower than 12% and coefficients of correlation close to 1 in linearity under dilution tests. Healthy dogs showed median salivary concentrations of urea of 39.6 mg/dL and creatinine of 0.30 mg/dL, whereas dogs with CKD showed median salivary urea of 270.1 mg/dL and creatinine of 1.86 mg/dL. Positive high correlations were found between saliva and

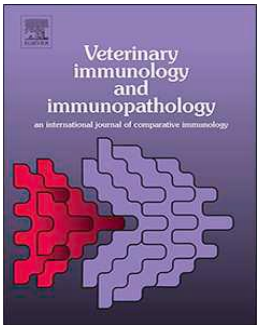
serum activities of the two analytes (urea, r = 0.909; P < 0.001; creatinine, r = 0.819; P < 0.001).

Conclusions: Urea and creatinine concentrations can be measured in canine saliva with commercially available spectrophotometric assays. Both analytes showed higher values in saliva of dogs with CKD compared with healthy dogs and their values were highly correlated with those in serum in our study conditions.

URL: https://doi.org/10.1186/s12917-018-1546-5

Article 8

Identification of novel biomarkers for treatment monitoring in canine leishmaniosis by high-resolution quantitative proteomic analysis



Identification of novel biomarkers for treatment monitoring in canine leishmaniosis by high-resolution quantitative proteomic analysis

Silvia Martinez-Subielaa,[1], Anita Horvatic[2], Damian Escribano[1], Luis Pardo-Marin[1], Meric Kocaturk[3], Vladimir Mrljak[2], Richard Burchmore[4], Jose J. Ceron[1], Zeki Yilmaz[3]

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[4] Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, Scotland, United Kingdom.

Journal: Veterinary Immunology and Immunopathology

Abstract: The objective of this study was to use the Tandem Mass Tag (TMT) isobaric label-based proteomic approach, in order to identify new potential biomarkers for the treatment monitoring of canine leishmaniosis that could not be identified by the use of gel-based techniques. For this purpose serum samples were obtained from 5 clinically diseased dogs before and one month after the treatment of canine leishmaniosis. The non-depleted

serum samples were subjected to reduction, alkylation and trypsin digestion, and the resulting peptides were labeled using 6-plex TMT reagents. To obtain information about protein identities and relative quantification, liquid chromatography–MS analysis of multiplexed TMT-labeled peptides was employed. This gel-free, label-based quantitative proteomic approach enabled identification of 117 canine proteins. Among these, 23 showed significant difference ($p < 0.05$) in expression (two downregulated and 21 upregulated ranging from 1.25 to 2.5 fold change). Comparison of gel-free TMT-based quantification and a gel-based approach previously applied to the same samples resulted in the identification of some common markers (Apo-A1, vitamin D binding protein and RBP4). However, 20 additional differentially represented proteins were highlighted by the gel-free approach, 13 of which have not been previously reported in canine leishmaniosis. In conclusion, the TMT-based proteomic approach allowed identification of new serum proteins that significantly change in concentration after canine leishmaniosis treatment. These proteins are involved in various physiopathological processes such as inflammatory, coagulation or defense mechanisms, and could potentially be suitable biomarkers for treatment monitoring of this parasitic disease.

URL: http://dx.doi.org/10.1016/j.vetimm.2017.08.004

GENERAL DISCUSSION

In this PhD Thesis, the following advances in the canine leishmaniosis from a laboratory point of view have been achieved:

1. *The establishment of the basis and applications of acute phase proteins in this disease.* APPs are important components of the innate immune system response that change in concentration when inflammation occurs (Gabay & Kushner, 1999). They are considered as sensitive markers of the activation of immune system and therefore of the presence of an active inflammation, (Cerón et al., 2008; Eckersall & Schmidt, 2014). Studies have demonstrated that there is an increase in positive APPs such as C-reactive protein (CRP) and ferritin (Martínez-Subiela et al., 2002). However, these APPs are still not applied in routine for the classification and management of the disease. This PhD provides the following advances in this field:

   In **article 1**, it is proposed that dogs diagnosed with *Leishmania Infantum* could be divided into three groups according to their clinical status on physical examination and their APPs concentrations. This classification allow to differentiate infected dogs with non-active leishmania, infected dogs with no external clinical signs and active leishmania, and infected dogs with clinical signs.

   In **article 2**, two of the main APPs used in the classification of article 1, CRP and ferritin levels were demonstrated to be of use for monitoring the treatment of this disease. Therefore in dogs with leishmaniosis under treatment, if both APPs are within the reference ranges, this is usually associated with the absence of clinical signs and adequate response to treatment. On the other hand, and elevated CRP and/or ferritin values could reflect a relapse or lack of adequate response to treatment.

   In the **article 3** the system described in the article 1 based on APP concentrations in dogs with CanL at different clinical stages was compared to the Leishvet and Canine Leishmaniosis Working Group (CLWG) classifications. It was observed that dogs in the most advanced stages of the Leishvet and CLWG classifications (Paltrinieri et al., 2010; Solano-Gallego et al., 2011b), had significant increases in serum ferritin and CRP. These

findings indicate that APPs show more significant changes at more advanced stages of the Leishvet and CLWG classifications corresponding to more severe cases of canine leishmaniasis.

In the **article 4**, we described how other diseases such as pyometra or pancreatitis have a totally different pattern of change in ferritin and CRP compared to canine leishmaniosis. In pyometra there is an increase in CRP much higher than in canine leishmaniosis, but ferritin does not show significant changes. In pancreatitis there is an increase in CRP much higher than in canine leishmaniosis and an increase in ferritin of lower magnitude.

In **article 5**, we studied serum ferritin, together with complete hemogram and biochemical analyses, in canine obesity as the most common nutritional disorder. Higher ferritin concentrations were observed in the overweight/obese group in comparison to the lean/normal weight group. The increases in ferritin, together with higher haematocrit and erythrocyte mean corpuscular volume, could indicate tissue hypoxia in obese dogs (Després & Lemieux, 2006; Lumeng & Saltiel, 2011).

2. *Development of new markers for the monitoring of Leishmania.* In this point, the following advances have been made:

In **article 6**, it was demonstrated that the measurement of urinary IgG/creatinine is together with UPC, the best biomarker to evaluate the kidney status in canine leismaniosis and its response to treatment. These analyse were more sensitive to others such as sCr or symmetric dimethylarginine (sSDMA) which are recommended by the IRIS to evaluate and monitor renal damage/dysfunction (Elliott et al., 2013). This disease can cause glomerulonephritis and chronic kidney disease (CKD) by immune complex deposition that may lead to end-stage renal failure (Paltrinieri et al., 2016; Solano-Gallego et al., 2007). Therefore, the use of adequate biomarkers for an early detection of kidney damage in CanL is of highly importance and can contribute to a much better management of the disease.

In **article 7,** we demonstrated that urea and creatinine can be measured in dog saliva and can be used to detect situations of CKD that occurs in

leishmaniosis. The assays validated were analytically precise and accurate and therefore can be used to measure urea and creatinine in saliva of the dog. Saliva is a biological fluid that has varies advantages as a diagnostic medium compared with blood, since its collection is non-invasive and simple, and in case of the dogs can be made by owners (Cerón, 2019). In addition, saliva can be sampled repeatedly without discomfort to the patient. Our results agreed with previous reports in humans that found that urea and creatinine can be measured in saliva and the salivary concentrations of both analytics are positively correlated to plasma levels (Venkatapathy et al., 2014; Zúñiga et al., 2012).

In **article 8**, we detected by Tandem Mass Tag (TMT) isobaric labels 23 proteins that showed significant difference ($p<0.05$) in expression (two downregulated and 21 upregulated ranging from 1.25 to 2.5 fold change) in dogs with leishmania before and after treatment. Comparison of gel-free TMT-based quantification and a gel-based approach previously applied to the same samples resulted in the identification of some common markers (Apo-A1, vitamin D binding protein and RBP4). However, 20 additional differentially represented proteins were highlighted by the gel-free approach, 13 of which have not been previously reported in canine leishmaniosis. These proteins are involved in various physiopathological processes such as inflammatory, coagulation or defense mechanisms, and could potentially be suitable biomarkers for treatment monitoring of this disease.

CONCLUSIONS

1. APPs concentration can be a useful clinical tool to characterize and manage CanL. The classification system based on the APP created in this PhD correlated with previous classifications systems based in clinical signs and other laboratory analysis.

2. Ferritin and CRP show a particular profile in canine leishmanisosis with a moderate increase in CRP and a higher increase in fold in ferritin that in CRP, which is different than other diseases such as pancreatitis, pyometra or obesity. In addition, ferritin and CRP were useful to monitor the treatment of leishmaniosis.

3. Urinary IgG/creatinine showed a higher accuracy to monitor the kidney disease during the treatment of canine leishmaniosis in dogs with proteinuria than sSDMA or sCre.

4. Urea and creatinine can be measured in saliva of dogs and are increased in chronic renal failure produced by leishmaniosis.

5. TMT isobaric labels could be used to identify new biomolecules that allow a better understanding of the physiopatological mechanism involved in canine leishmaniasis and also that could be potentially used as a biomarker of this disease.

RESUMEN

Esta Tesis Doctoral se ha centrado en el estudio de la leishmaniosis canina desde el punto de vista laboratorial, habiéndose logrado los siguientes avances:

1. ***Avances en el uso de proteínas de fase aguda (PFA) como apoyo en la clasificación y seguimiento de la enfermedad. (artículos 1, 2, 3, 4 y 5):***
   - *Se ha desarrollado un protocolo sobre el uso de PFA para la caracterización y manejo de esta enfermedad (**artículo 1**)*. En base a este protocolo, los perros diagnosticados con infección por *Leismania infantum* por serología, PCR, identificación citológica o histopatológica, se pueden dividir en tres grupos en función de su estado clínico al examen físico y sus concentraciones de PFA. Grupo 1: perros sin signos clínicos al examen físico y PFA en el rango de referencia; Grupo 2: perros con cambios en las PFA pero sin signos clínicos al examen físico; Grupo 3: perros con signos clínicos y cambios en PFA. En el artículo 1 de la Tesis describes las principales características de cada grupo, así como su asociación con los esquemas de clasificación clínica de CanL.
   - *Se ha descubierto que la CRP y la ferritina tienen una aplicación práctica directa para el seguimiento del tratamiento de la leishmania (**artículo 2**).* Para ello, se ha comparado la concentración sérica de CRP y ferritina entre tres posibles situaciones que podrían tener los perros sometidos a tratamiento contra la leishmaniosis: (1) animales que responden adecuadamente y tienen una recuperación total, (2) animales con sólo recuperación clínica, pero anomalías persistentes en los analitos bioquímicos, y (3) animales que no responden al tratamiento y que presentan cambios clínicos y bioquímicos.

     Todos los perros que se recuperaron por completo después del tratamiento mostraron valores de CRP y ferritina dentro de los rangos de referencia. La mayoría de los perros que presentaban remisión clínica parcial tenían CRP y ferritina dentro del rango de referencia a pesar de la presencia de otras anomalías bioquímicas como hiperglobulinemia, hiperproteinemia o proteinuria. Por otro lado, la mayoría de los perros en el grupo que no respondía habían aumentado la CRP y la ferritina. Aunque este estudio tiene limitaciones debido a la variabilidad en los protocolos y tiempos de los tratamientos, se puede concluir que las

concentraciones de CRP y ferritina dentro de los rangos de referencia suelen estar asociadas a la ausencia de signos clínicos y una adecuada respuesta al tratamiento, y el aumento de CRP y/o los valores de ferritina podrían reflejar una falta de respuesta adecuada al tratamiento.

- *Se ha demostrado que existe una relación entre el modelo de clasificación propuesto en nuestro estudio y las clasificaciones estándar previas de la leishmaniosis canina (**artículo 3**).* Para ello se evaluaron las concentraciones de PFA que muestran los perros infectados naturalmente con *Leishmania infantum* en diferentes estadios clínicos de la enfermedad según las clasificaciones Leishvet y Canine leishmaniosis working group (CLWG). Y estas clasificaciones se compararon con los grupos basados en las recomendaciones de las PFA del artículo 1.

  En concreto se revisaron las historias clínicas de 458 perros con leishmaniosis cuyas muestras fueron enviadas a nuestro laboratorio para evaluación de patología clínica y 77 casos cumplieron con los criterios de inclusión. Todos los perros se clasificaron según el sistema CLWG, la mayoría de los perros (33,8 %) se clasificaron en el estadio D. Aunque algunos perros (41,6 %) no pudieron ser clasificados por el sistema Leishvet, ya que incluye solo perros con enfermedad clínica, la mayoría de los perros incluidos (27,3%) se encontraban en el estadio II de Leishvet. Según la clasificación considerando las PFA, la mayoría de los perros (32,5%) se clasificaron en el estadio 3a. Los perros en las etapas más avanzadas de las clasificaciones Leishvet y CLWG tuvieron aumentos significativos en la ferritina sérica y la CRP y una disminución en la paraoxonasa 1 (PON1). Estos hallazgos indican que las PFA muestran cambios más significativos en las etapas más avanzadas de las clasificaciones de Leishvet y CLWG correspondientes a casos más graves de leishmaniosis canina.

- *Se ha descubierto que otras enfermedades como las piometras, las pancreatitis y la obesidad tienen un perfil de proteínas de fase aguda diferente al de la leishmania (artículo 4 y 5).* Para ello, se realizaron dos estudios diferentes (***artículo 4***). El primer estudio, se midieron ambas

proteínas en un perfil de PFA que se realiza de forma rutinaria en nuestra clínica, en 25 perros con piometra, 25 perros con pancreatitis (como ejemplo de una enfermedad inflamatoria) y en 25 perros sanos. En el segundo estudio, para evaluar cambios en las PFAs en piometra ctras el tratamiento, se analizaron las concentraciones de ambas PFA en 30 perras con piometra al diagnóstico y tras ovariohisterectomía y en 10 perras clínicamente sanas. En ambos estudios, las perras con piometra mostraron aumentos significativos de CRP sérica, lo que indica una condición inflamatoria, pero no en la ferritina sérica a pesar de ser una PFA positiva moderada que debería aumentar en la inflamación. Esta divergencia entre la dinámica de estás PFA podría ser una herramienta útil para la sospecha de casos de piometra.

En el ***artículo 5*** se revisaron las historias clínicas de 92 perros, de los cuales 37 tenían sobrepeso o estaban obesos y 55 estaban en su peso normal o delgados. Se encontró que la ferritina fue 1,2 veces mayor en perros con sobrepeso y obesos en comparación con animales delgados o de peso normal, y con niveles medios de ferritina en perros con sobrepeso y obesos por encima del rango de referencia para animales sanos en nuestro laboratorio (60-190 g/L); mientras que no se observaron cambios en la CRP. Adicionalmente, un perfil de PFA consistente CRP normal y aumento de haptoglobina, colinesterasa y ferritina podría sugerir la presencia de un estado de hipercortisolismo en esta enfermedad.

2. ***Avances en la aplicación de nuevos biomarcadores de daño renal en CanL que ayuden a monitorizar el daño renal durante el tratamiento de la leishmaniasis canina y que puedan aportar información sobre la respuesta al tratamiento (artículo 6):***
   - *Se descubrió que existen biomarcadores más útiles para evaluar el estado renal en la leishmaniosis canina que el sSDMA*. Para ello se realizión un estudio en el que se evaluó y comparó la evolución del perfil actualmente recomendado por la International Renal Interest Society (IRIS) (sCr, UPC y sSDMA) con un panel de otros biomarcadores

renales diferentes durante el tratamiento de la leishmaniosis canina. Este panel incluyó tres biomarcadores glomerulares urinarios (uIgG, uCRP y uferritina) y tres biomarcadores tubulares urinarios (uGGT, uNAG y uRBP). Estos biomarcadores se midieron en dos grupos de perros con leishmaniosis canina en estadio IRIS I. Grupo 1: perros que presentaban proteinuria (UPC > 0,5) antes del tratamiento que no disminuyó después del tratamiento; Grupo 2: perros que mostraban proteinuria antes del tratamiento que disminuyó después del tratamiento.

El grupo 1 no mostró cambios significativos en ningún biomarcador después del tratamiento. En el grupo 2, entre los biomarcadores recomendados por IRIS, solo la UPC mostró una disminución significativa después del tratamiento. Sin embargo, todos los biomarcadores de daño glomerular mostraron una disminución significativa después del tratamiento, con uIgG/Cr y uCRP/Cr mostrando las mayores disminuciones. Además, uRBP/Cr y uNAG/Cr mostraron disminuciones significativas después del tratamiento.

En perros con leishmaniosis en estadio I de IRIS que redujo la UPC después del tratamiento, no hubo cambios significativos en la creatinina sérica y el sSDMA. Sin embargo, todos los biomarcadores de orina evaluados con excepción de uGGT mostraron una disminución significativa. Estas disminuciones fueron más evidentes en aquellos marcadores relacionados con la función glomerular, siendo uIgG/Cr el biomarcador más asociado con la UPC.

3. ***Avances en el uso de la saliva, como técnica no invasiva, para la determinación de urea y creatinina (artículo 7):***
   - *Se ha descubierto que la urea y la creatinina se pueden medir en saliva canina, y que aumentan en la insuficiencia renal crónica (ERC).*

     Los ensayos espectrofotométricos probados para las mediciones de urea y creatinina en saliva de perros mostraron una imprecisión intraensayo e interensayo inferior al 12% y coeficientes de correlación cercanos a 1 en linealidad bajo pruebas de dilución. Los perros sanos mostraron unas

medianas en sus concentraciones salivales de urea de 39,6 mg/dL y creatinina de 0,30 mg/dL, mientras que los perros con ERC mostraron una mediana en su urea salival de 270,1 mg/dL y creatinina de 1,86 mg/dL. Se encontraron altas correlaciones positivas entre las actividades en saliva y suero de los dos analitos (urea, $r = 0,909$; $P < 0,001$; creatinina, $r = 0,819$; $P < 0,001$).

Por lo tanto, las concentraciones de urea y creatinina se pueden medir en la saliva canina con ensayos espectrofotométricos disponibles comercialmente. Ambos analitos mostraron valores más altos en la saliva de los perros con ERC en comparación con los perros sanos y sus valores estaban altamente correlacionados con los del suero en las condiciones de nuestro estudio.

4. ***Avances en el uso de técnicas proteómicas que proporcionan un análisis global detallado de las proteínas expresadas en el suero del perro antes y después del tratamiento para la leishmania (artículo 8):***

El objetivo de este estudio fue utilizar el enfoque proteómico basado en proteómica líquida para identificar nuevos biomarcadores potenciales para el seguimiento del tratamiento de la leishmaniosis canina que no pudieron identificarse mediante el uso de técnicas basadas en gel. Para ello se obtuvieron muestras de suero de 5 perros clínicamente enfermos antes y un mes después del tratamiento de la leishmaniosis canina. Las muestras de suero no empobrecido se sometieron a reducción, alquilación y digestión con tripsina, y los péptidos resultantes se marcaron utilizando reactivos de marcaje isobárico de masas en grupos (TMT) de 6 plex. Para obtener información sobre las identidades de proteínas y la cuantificación relativa, se empleó el análisis de cromatografía líquida-MS de péptidos multiplexados marcados con TMT. Este enfoque proteómico cuantitativo basado en marcadores y sin gel permitió la identificación de 117 proteínas caninas. Entre estos, 23 mostraron una diferencia significativa ($p < 0,05$) en la expresión (dos regulados a la baja y 21 regulados al alza con un cambio de 1,25 a 2,5 veces). La comparación de la cuantificación basada en TMT sin gel y un enfoque basado en gel aplicado previamente a las mismas

muestras dio como resultado la identificación de algunos marcadores comunes (Apo-A1, proteína de unión a vitamina D y RBP4). Sin embargo, el enfoque sin gel destacó 20 proteínas adicionales representadas diferencialmente, 13 de las cuales no se habían informado previamente en la leishmaniosis canina. En conclusión, el enfoque proteómico permitió la identificación de nuevas proteínas séricas cuya concentración cambia significativamente después del tratamiento de la leishmaniosis canina. Estas proteínas están involucradas en diversos procesos fisiopatológicos como mecanismos inflamatorios, de coagulación o de defensa, y podrían ser potencialmente biomarcadores adecuados para el seguimiento del tratamiento de esta enfermedad parasitaria.

REFERENCES

Cerón, J. J. (2019). Acute phase proteins, saliva and education in laboratory science: An update and some reflections. *BMC Veterinary Research*, *15*(1). https://doi.org/10.1186/s12917-019-1931-8

Cerón, J. J., Eckersall, P. D., & Martínez-Subiela, S. (2005). Acute phase proteins in dogs and cats: Current knowledge and future perspectives. In *Veterinary Clinical Pathology* (Vol. 34, Issue 2, pp. 85–99). Blackwell Publishing Ltd. https://doi.org/10.1111/j.1939-165X.2005.tb00019.x

Cerón, J. J., Martinez-Subiela, S., Ohno, K., & Caldin, M. (2008). A seven-point plan for acute phase protein interpretation in companion animals. In *Veterinary Journal* (Vol. 177, Issue 1, pp. 6–7). W.B. Saunders. https://doi.org/10.1016/j.tvjl.2007.12.001

Després, J. P., & Lemieux, I. (2006). Abdominal obesity and metabolic syndrome. In *Nature* (Vol. 444, Issue 7121). https://doi.org/10.1038/nature05488

Eckersall, P. D., & Schmidt, E. M. dos S. (2014). The final hurdles for acute phase protein analysis in small animal practice. In *Journal of Small Animal Practice* (Vol. 55, Issue 1, pp. 1–3). John Wiley & Sons, Ltd. https://doi.org/10.1111/jsap.12168

Elliott, J., Watson, A., Bonagura, J., & Twedt, D. (2013). Chronic kidney disease: IRIS staging and management. *Bonagura JD and Twedt DC, Kirk's Current Veterinary Therapy XV. Saunders Elsevier, St. Louis, MO*, 857–863.

Gabay, C., & Kushner, I. (1999). Acute-Phase Proteins and Other Systemic Responses to Inflammation. *New England Journal of Medicine*, *340*(6), 448–454. https://doi.org/10.1056/nejm199902113400607

Lumeng, C. N., & Saltiel, A. R. (2011). Inflammatory links between obesity and metabolic disease. In *Journal of Clinical Investigation* (Vol. 121, Issue 6, pp. 2111–2117). American Society for Clinical Investigation. https://doi.org/10.1172/JCI57132

Martínez-Subiela, S., Tecles, F., Eckersall, P. D., & Cerón, J. J. (2002). Serum concentrations of acute phase proteins in dogs with leishmaniasis. *Veterinary Record*, *150*(8), 241–244. https://doi.org/10.1136/vr.150.8.241

Paltrinieri, S., Gradoni, L., Roura, X., Zatelli, A., & Zini, E. (2016). Laboratory tests for diagnosing and monitoring canine leishmaniasis. In *Veterinary Clinical Pathology* (Vol. 45, Issue 4, pp. 552–578). https://doi.org/10.1111/vcp.12413

Paltrinieri, S., Solano-Gallego, L., Fondati, A., Lubas, G., Gradoni, L., Castagnaro, M., Crotti, A., Maroli, M., Oliva, G., Roura, X., Zatelli, A., & Zini, E. (2010). Guidelines for diagnosis and clinical classification of leishmaniasis in dogs. *Journal of the American Veterinary Medical Association*, *236*(11), 1184–1191. https://doi.org/10.2460/javma.236.11.1184

Solano-Gallego, L., Mirá, G., Koutinas, A., Cardoso, L., Pennisi, M. G., Ferrer, L., Bourdeau, P., Oliva, G., & Baneth, G. (2011a). LeishVet guidelines for the practical management of canine leishmaniosis. In *Parasites and Vectors* (Vol. 4, Issue 1). https://doi.org/10.1186/1756-3305-4-86

Solano-Gallego, L., Mirá, G., Koutinas, A., Cardoso, L., Pennisi, M. G., Ferrer, L., Bourdeau, P., Oliva, G., & Baneth, G. (2011b). LeishVet guidelines for the practical management of canine leishmaniosis. In *Parasites and Vectors* (Vol. 4, Issue 1). https://doi.org/10.1186/1756-3305-4-86

Solano-Gallego, L., Rodriguez-Cortes, A., Trotta, M., Zampieron, C., Razia, L., Furlanello, T., Caldin, M., Roura, X., & Alberola, J. (2007). Detection of Leishmania infantum DNA by fret-based real-time PCR in urine from dogs with natural clinical leishmaniosis. *Veterinary Parasitology*, *147*(3–4), 315–319. https://doi.org/10.1016/j.vetpar.2007.04.013

Venkatapathy, R., Govindarajan, V., Oza, N., Parameswaran, S., Pennagaram Dhanasekaran, B., & Prashad, K. V. (2014). Salivary creatinine estimation as an alternative to serum creatinine in chronic kidney disease patients. *International Journal of Nephrology*, *2014*, 1–6. https://doi.org/10.1155/2014/742724

Zúñiga, M. E., Estremadoyro, L. O., León, C. P., Huapaya, J. A., & Cieza, J. A. (2012). Validation of the salivary urea test as a method to diagnose chronic kidney disease. *Journal of Nephrology*, *25*(3), 431–436. https://doi.org/10.5301/jn.5000022