UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

BIOMARCADORES CARDIACOS EN LEISHMANIOSIS CANINA

DOMINGO CASAMIÁN SORROSAL

PROGRAMA DE DOCTORADO EN BIOMARCADORES DE SALUD Y ESTADOS PATOLÓGICOS

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Esta tesis cuenta con la autorización del director/a y codirector/a de la misma y de la Comisión Académica del programa. Dichas autorizaciones constan en el Servicio de la Escuela Internacional de Doctorado de la Universidad de Extremadura.

2023

EXPERTS´ REPORTS

SMALL ANIMAL
MEDICAL CONSULTANCY

Bristol, 23 October 2023

To whom it may concern:

Domingo Casamián-Sorrosal has requested to review and provide feedback on the following four publications in relation to his eligibility for being awarded an International Doctorate degree.

1. Evaluation of heart fatty acid-binding protein as a biomarker for canine leishmaniosis. **Domingo Casamián-Sorrosal**, Rafael Barrera-Chacón, Sonja Fonfara, José Ignacio Cristobal-Verdejo, Jesús Talavera-López, Silvia Belinchón-Lorenzo, Guadalupe Miró-Corrales, Alicia Caro-Vadillo, Francisco Javier Duque. *Veterinary Record* **2023**;e2683. https://doi.org/10.1002/vetr.2683.

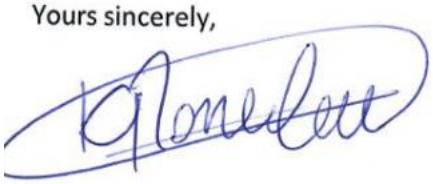
2. Association of myocardial parasitic load with cardiac biomarkers and other selected variables in 10 dogs with advanced Canine Leishmaniasis. **Domingo Casamián-Sorrosal**, Rafael Barrera-Chacón, Sonja Fonfara, Silvia Belinchón-Lorenzo, Luis Gómez-Gordo, Javier Galapero-Arroyo, Javier Fernández-Cotrina, Jose Ignacio Cristobal-Verdejo, Francisco Javier Duque. *Veterinary Record* **2021**;e198. https://doi.org/10.1002/vetr.198.

3. Comparison of N-terminal proB-type natriuretic peptide levels at different stages of visceral leishmaniosis and in patients with chronic kidney disease. **Domingo Casamian-Sorrosal**, Rafael Barrera-Chacon, Luis Gómez, Silvia Belinchón-Lorenzo, Javier Galapero Arroyo, Luis Carlos Gomez, Jose Ignacio Cristobal, Javier Duque. *Veterinary Record* **2019**; doi: 10.1136/vr.105205.

4. Comparison of myocardial damage among dogs at different stages of clinical leishmaniasis and dogs with idiopathic chronic kidney disease. L. Martínez-Hernández, **D. Casamian-Sorrosal**, R. Barrera-Chacón, J.M. Cuesta-Gerveno, S. Belinchón-Lorenzo, L.C. Gómez Nieto, F.J. Duque-Carrasco. *The Veterinary Journal* **2017**;221:1–5. http://dx.doi.org/10.1016/j.tvjl.2016.11.015.

These four articles have been published in high impact international veterinary journals and present original information regarding heart disease and canine leishmaniosis. All studies presented in these publications maintain a high scientific standard and make a significant contribution to knowledge. I have no doubt that they fulfil the requirements for the International Doctorate Mention. I strongly recommend that Domingo Casamián-Sorrosal proceed with defending his thesis in order to be awarded the International Doctorate degree.

Yours sincerely,



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Maisons-Alfort, the 31 October 2023

To whom may concern,

Recently, I was contacted by my colegue Domingo Casamián-Sorrosal to review and give my feedback on his four recent publications listed below in relation to his eligibility for being awarded with International Doctorate degree.

1. Evaluation of heart fatty acid-binding protein as a biomarker for canine leishmaniosis. **Domingo Casamián-Sorrosal**, Rafael Barrera-Chacón, Sonja Fonfara, José Ignacio Cristobal- Verdejo, Jesús Talavera-López, Silvia Belinchón-Lorenzo, Guadalupe Miró-Corrales, Alicia Caro-Vadillo, Francisco Javier Duque. *Veterinary Record* **2023**;e2683. https://doi.org/10.1002/vetr.2683.

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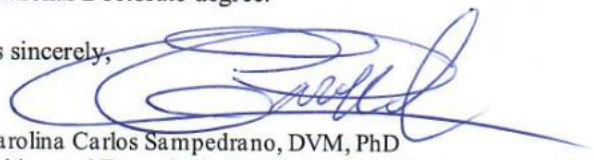
All articles listed above have been published in peer reviewed veterinary journals. They include relevant and original scientific information concerning canine Leishmaniosis and their impact in cardiac structure and function.
Domingo Casamián-Sorrosal work fulfill all the requirements for the International Doctorate Mention, and I strongly recommend that he proceed with defending his thesis in order to be awarded with the International Doctorate degree.

Yours sincerely,



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First, I would like to thank Rafa (Dr Rafael Barreda) for accepting me into his research group and for the trust he has placed in me. I thank him for his patience, perseverance, and wisdom, which have been fundamental for me to be able to complete this Thesis. I would like to also thank Javier (Dr Francisco Javier Duque), co-director of the Thesis and friend, who has also guided me with patience, joy, experience, and wisdom, until the end of all the studies and the completion of the Thesis. Both are professional and personal role models and a reference to our profession in many clinical and academic aspects. I hope that what they have taught me and passed on to me will remain with me, and that I will also be able to pass it on in the future. I would also like to thank my other co-director, Sonja (Dr Sonja Fonfara). I do not really know what I would be professionally without Sonja. Sonja was my supervisor and mentor during my specialisation as a cardiologist, she taught me to have critical thinking, to operate, to write scientific papers, to be very consistent, to (at least try to) ramble less and be more (german-way) concise and direct, and a thousand of other things. I admire her deeply. She has a mix of talent, wisdom and humility that are and will always be a reference for me. She is an extraordinary person. Someone who will always be there, and I am lucky to be able to consider myself a friend of hers.

I would also like to thank all my co-authors in the studies and my colleagues in the teams I have been involved with during this Thesis, at Dick White Referrals, Southfields Veterinary Specialists, The Veterinary Hospital of the Catholic University of Valencia, and at the Internal Medicine, LeishmanCeres Laboratory and Pathological Anatomy teams of The University of Extremadura. I would like to thank the following people for their silent help in some parts of the studies: Emi Barker, Sergio Almiñana, Patricia Sebastián, Maite Masia and Nacho Cristóbal.

Thank you to my parents and my sister. I love them and admire them deeply.

Thank you to Julia, for her smile where the world is a better place, for allowing me to hold her hand, which is carrying me even if She thinks I am the carrier.

And above all. Thank you to Elena. My other half (*mi otra mitad*). For understanding me, for loving me, and for letting me do all this from her galaxy.

INDEX

RESUMEN

La leishmaniosis canina (CanL) es una enfermedad multisistémica causada por el organismo protozoario *Leishmania infantum* que puede afectar a muchos órganos, tejidos o fluidos biológicos. El corazón y el pericardio pueden verse afectados por la enfermedad, causando miocarditis y pericarditis, aunque se desconoce la prevalencia de las mismas. La carga parasitaria miocárdica en los casos de miocarditis ha sido variable cuando se ha detectado mediante citología, histopatología o inmunohistoquímica, pero el uso de la reacción en cadena de la polimerasa cuantitativa (qPCR) no se ha investigado para este fin. La clasificación de la CanL por el grupo LeishVet estratifica la enfermedad en fase leve (estadio I), moderada (estadio II), grave (estadio III) o muy grave (estadio IV). Esta clasificación se estructura principalmente en función de la gravedad de los signos clínicos, biomarcadores de diagnóstico como la serología, y biomarcadores renales, como la ratio proteína-creatinina en orina, y los niveles de creatinina. Los marcadores de daño miocárdico troponina I (cTnI) y la proteína cardiaca de unión a ácidos grasos (HFABP), así como el biomarcador de estrés miocítico péptido natriurético N-terminal de proBNP (NT-proBNP), se utilizan con frecuencia con fines diagnósticos y/o pronósticos en muchas enfermedades cardíacas y sistémicas, pero rara vez se han explorado en la CanL. Los biomarcadores cardiacos podrían añadir información valiosa en CanL para identificar a los individuos con afectación cardiaca o como biomarcadores generales de la enfermedad. También es fundamental, que el clínico, conozca las posibles alteraciones debidas a CanL, en ecocardiografía, y en la concentración en sangre de los biomarcadores cardíacos, cuando se investiga una posible enfermedad cardíaca en un paciente con CanL, o en una zona endémica de *Leishmania infantum*.

Este trabajo de investigación se estructuró en cuatro estudios científicos con el objetivo de describir, y comparar, los niveles de cTnI, HFABP y NT-proBNP, en grupos de perros en

diferentes estadios (LeishVet) de CanL, y evaluar la relación de estos biomarcadores con un grupo de variables clínicas, diagnósticas y ecocardiográficas seleccionadas. Otros objetivos incluyeron el evaluar los hallazgos ecocardiográficos, la prevalencia, tipo y gravedad de la miocarditis, y la carga parasitaria miocárdica detectada por qPCR, en un grupo de perros eutanasiados debido a leishmaniosis canina grave (LeishVet III) o muy grave (LeishVet IV), y la evaluación de la relación de estos hallazgos patológicos, y la carga parasitaria miocárdica, con los biomarcadores cardíacos y otras variables seleccionadas.

Los resultados mostraron que la cTnI está consistente y marcadamente elevada en la mayoría de los perros con CanL grave (LeishVet III) y muy grave (LeishVet IV), y también en algunos perros con CanL moderada (LeishVet II). Los niveles de cTnI no se asociaron con factores extracardiacos frecuentes en CanL, como la presión arterial o el grado de anemia, y los perros con enfermedad renal crónica mostraron elevación de la troponina I cardiaca, pero esta alteración fue notablemente inferior a la de los perros con CanL en un estadio similar de enfermedad renal (estadio IRIS). Además, los niveles de cTnI se asociaron muy fuertemente con la carga parasitaria miocárdica.

En los estudios se observó que el biomarcador NT-proBNP se elevó, también de manera marcada como en el caso de cTnI, en la mayoría de los perros con CanL grave (LeishVet III) o muy grave (LeishVet IV). Los perros con enfermedad renal crónica en un estadio IRIS, similar a los perros con CanL, mostraron también menores elevaciones de NT-proBNP que estos. Sin embargo, al contrario que en el caso de cTnI, los niveles de NT-proBNP estuvieron fuertemente asociados con factores secundarios a CanL como la anemia y la presión arterial sistémica, no se asociaron con la carga parasitaria miocárdica, y no se aumentaron en estadios más leves de la enfermedad.

La HFABP aumentó en raras ocasiones, y de forma inconsistente, en perros con CanL, y no se observaron asociaciones de interés clínico, de este biomarcador, con otras variables clínicas o biomarcadores.

La mayoría de los perros con CanL grave (LeishVet III) o muy grave (LeishVet IV) mostraron miocarditis linfoplasmocítica en histopatología y aumento de la masa del ventrículo izquierdo en ecocardiografía. *Leishmania,* detectada mediane qPCR*,* estaba presente en el miocardio de todos los casos, en cantidad variable, pero en la mayoría de los casos considerada como una carga parasitaria elevada.

En conclusión, la mayoría de perros con CanL grave (LeishVet III) y muy grave (LeishVet IV), y algunos con enfermedad moderada (LeishVet II), tienen daño miocárdico secundario a miocarditis linfoplasmocítica, y la cTnI es un biomarcador excelente para evaluar esta enfermedad miocárdica asociada a CanL. El grado de daño miocárdico es a menudo muy grave, y se observa frecuentemente un aumento de la masa ventricular izquierda en ecocardiografía. *Leishmania*, detectada por qPCR, puede observarse con frecuencia en el miocardio de perros con enfermedad grave (LeishVet III) o muy grave (LeishVet IV), y la carga parasitaria miocárdica está muy fuertemente asociada a la concentración sanguínea de cTnI. NT-proBNP se considera un biomarcador menos adecuado para futuras investigaciones o uso clínico que cTnI, ya que las elevaciones sanguíneas de este marcador están asociadas a factores secundarios de CanL, es menos probable que detecten enfermedad miocárdica incipiente, y sus niveles no están asociados a la carga parasitaria miocárdica. La HFABP no se eleva de manera consistente en CanL. Los resultados llevan a desaconsejar el uso en CanL, en clínica o en investigación, de HFABP.

ABSTRACT

Abstract

Canine leishmaniosis (CanL) is a multisystemic disease caused by the protozoal organism *Leishmania infantum* which can affect many organs, tissues, or biological fluids. The heart and the pericardium can be affected by the disease, causing myocarditis and pericarditis, although the prevalence of myocarditis and myocardial damage in CanL is unknown. The myocardial parasitic load in cases of myocarditis has been variable when detected by cytology, histopathology or immunohistochemistry, but the use of quantitative polymerase chain reaction (qPCR) has not been investigated for this purpose. Classification of CanL by the LeishVet group stratifies the disease in mild (stage I), moderate (stage II), severe (stage III) or very severe (stage IV) stages. This classification is structured primarily based on severity of clinical signs, diagnostic biomarkers such as serology, and renal biomarkers such as urine protein-creatinine ratio and creatinine levels. Blood levels of myocardial damage troponin I (cTnI) and heart-type fatty acid binding protein (HFABP), and myocyte stress biomarker N-terminal proB-type natriuretic peptide (NT-proBNP), are frequently used for diagnostic and/or prognostic purposes in many cardiac and systemic diseases but have been rarely explored in CanL. Cardiac biomarkers may add valuable information to identify individuals with cardiac involvement or as a general disease biomarker tool. It is also important to be aware of the potential alterations in echocardiography and in cardiac biomarkers, due to CanL, when cardiac disease is investigated in a patient with the disease or living in an endemic area.

This research work was structured in four scientific studies aimed to describe and compare the blood levels of cTnI, HFABP and NT-proBNP in groups of dogs at different LeishVet stages of CanL, and to assess the relationship of these biomarkers with selected clinical, diagnostic and echocardiographic variables. Other aims were to evaluate the echocardiographic findings and the prevalence, type and severity of myocarditis, and myocardial parasitic load detected

by qPCR, in a cohort of dogs euthanised due to severe (LeishVet III) or very severe (LeishVet IV) CanL, and to assess the relationship of the myocardial histopathological findings and myocardial parasitic load with cardiac biomarkers and other selected variables.

Results showed that cTnI is consistently and often markedly elevated in most dogs with severe (LeishVet III) and very severe (LeishVet IV) CanL, and in some dogs with moderate (LeishVet II) disease. cTnI levels were not associated with extracardiac factors such as blood pressure or anaemia and dogs with chronic kidney disease showed elevated cTnI but this increase was markedly lower than the elevation observed in dogs with CanL in similar stage of renal disease (IRIS stage). cTnI was also very strongly associated with the myocardial parasitic load.

NT-proBNP natriuretic peptide was also consistently and markedly elevated in most dogs with severe (LeishVet III) or very severe (LeishVet IV) CanL. Dogs with chronic kidney disease, in similar IRIS stage than dogs with CanL, also showed lower NT-proBNP elevations. However, NT-proBNP levels were strongly associated with secondary factors such as anaemia and systemic arterial hypertension, were not associated with myocardial parasitic load, and were not elevated at milder disease stages.

HFABP was rarely and inconsistently increased in dogs with CanL. Associations of clinical interest of HFABP with other clinical variables or biomarkers were not observed.

Most dogs with severe (LeishVet III) or very severe (LeishVet IV) CanL showed lympho-plasmacytic myocarditis on histopathology and increase in left ventricular mass on echocardiography. *Leishmania*, as detected by qPCR, was present in the myocardium of all cases. The myocardial parasitic load was variable in quantity but most commonly high.

In conclusion, lymphoplasmacytic myocarditis and myocardial damage occur in most dogs with severe (LeishVet III) or very severe (Leishvet IV) CanL, and in some dogs with moderate

disease (LeishVet II) and cTnI is an excellent biomarker to evaluate this CanL associated myocardial disease. The degree of myocardial damage in advanced cases is very severe and increase in left ventricular mass is often observed on echocardiography. *Leishmania*, as detected by quantitative PCR, can be frequently observed in the myocardium of dogs with severe (LeishVet III) or very severe (LeishVet IV) disease and the myocardial parasitic load is very strongly associated with cTnI. NT-proBNP is considered a less adequate biomarker for future research or clinical use than cTnI, as elevations are associated with secondary factors of the disease, are less likely to detect early myocardial disease and are not associated with the individual myocardial parasitic load. HFABP is not consistently elevated in CanL and further clinical or research use of this biomarker in this disease is discouraged.

ABBREVIATIONS

Notes:

1.Canine leishmaniosis has been named throughout this document as leishmaniosis rather than leishmaniasis. Both terms have been used interchangeably in the literature and indeed leishmaniasis was used in some of the scientific studies part of this Thesis. Leishmaniosis was elected at the time of writing this Doctoral Thesis as it is considered the preferred term by the LeishVet scientific group.

2.Through the text CanL myocarditis is used to name the myocarditis associated with canine leishmaniosis. *Trypanosoma* myocarditis is used when referring to the myocarditis associated with *Trypanosoma cruzi* infection. Myocarditis (as an isolated disease) is used as a broad term for the disease, as it is the way used clinically and, in the literature, which mostly encompasses dogs without a known cause for the myocardial inflammation and necrosis observed, even if viral origin may be suspected. The term myocarditis is also used as part of histopathology nomenclature. I have tried that the meaning in which the term is used (disease vs histopathology diagnosis) is clear throughout the text by the context and associated words. Nevertheless, both concepts may sometimes overlap, as the gold standard diagnosis for myocarditis is indeed the finding of myocarditis on histopathology.

3. I have used the term advanced leishmaniosis to name severe or very severe disease which corresponds to LeishVet stage III and IV. I have used these terms interchangeably in the manuscript.

4. When using the word or abbreviation of cardiac biomarkers troponin I (cTnI), heart-type fatty acid binding protein (HFABP), and N-terminal proB-type natriuretic peptide (NT-proBNP), I most commonly refer to the blood concentration levels of these biomarkers to avoid repetitive redundant wording, but occasionally, the word may also refer to the protein itself. This is clear throughout the manuscript by the context within the text.

A:G: Albumin:globulin ratio.

ALT: Alanine aminotransferase.

APP: Acute phase proteins.

AV: Atrioventricular.

CanL: Canine leishmaniosis.

CLWG: Canine Leishmaniasis Working Group.

CNS: Central nervous system.

CKD: Chronic kidney disease.

CRP: C-reactive protein.

Ct: Threshold cycles (PCR).

CTnI: Cardiac troponin I.

DCM: Dilated cardiomyopathy.

DPP: Immunochromatographic dual path platform.

ELISA: Enzyme-linked immunosorbent assay.

Hb: Haemoglobin.

HCT: Haematocrit.

HFABP: Heart-type fatty acid binding protein.

IFAT: Immunofluorescence antibody test.

IRIS: International Renal Interest Society.

LVMi: Left ventricular mass index.

L. infantum: Leishmania infantum.

MVD: Mitral valve disease.

MPL: Myocardial parasitic load.

NT-ProBNP: N-terminal proB-type natriuretic peptide.

PCV: Packed cell volume.

PCR: Polymerase chain reaction.

PON-1: Paraoxanase.

RARI: Renal artery resistivity index (calculated by Doppler ultrasound).

SAA: Serum amyloid A.

SERCA: Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase.

sSMDA: Serum symmetric dimethylarginine.

uGGT: Urine gamma-glutamyl-transferase.

uGGT/uCR: Urinary gamma-glutamyl-transferase/urinary creatinine ratio.

UPC: Urine protein:creatinine ratio.

INTRODUCTION

Leishmania spp are the causative agents of human and animal infections in wide areas of America, Asia, Africa and Europe (1–4). Canine leishmaniosis (CanL) is the disease caused by Leishmania infantum (*L. infantum*) in the dog which is primarily transmitted by the bite of sand flies belonging to the genera *Phlebotomus* in Europe, and *Lutzomyia* in America (1–4). Canine infection by other *Leishmania* species such as *Leishmania donovani, Leishmania major* or *Leishmania tropica* have been reported but are considered sporadic (2,4,5). When *Leishmania* is used therefore in this manuscript, it refers therefore to *L. infantum*. Leishmaniosis in the dog is considered a zoonosis and the dog is considered the main reservoir for *L. infantum* (2,3). CanL is endemic in many areas of southern Europe with a very high prevalence reported in certain geographical locations (6). The parasite has a digenetic life cycle alternating between a mammalian and the insect vectors (1,3). When the sandfly bite infects a host, it ingests macrophages infected by rounded and non-motile amastigotes (1,3), and once within the insect, the parasite transforms into the flagellate promastigote state, multiplies, and migrate to the mouth parts (1,3). When the insect bites another host, deposition of infective *L. infantum* promastigotes takes place in the dermis along with salivary content vector (1,3,7). This process recruits phagocytic cells to site creating a proinflammatory environment and upon phagocytosis of the macrophage, the parasite evolves into the amastigote form (1,3). Within the macrophage the parasite spreads through the body and reproduces continuously until cell rupture, with subsequent macrophage and local tissue invasion, and overall parasite dissemination across many organs (1,7). Upon vector-host interaction three major clinical situations may occur: 1. positive serology, or positive parasitic detection in organ or blood by polymerase chain reaction (PCR), and clinical signs; 2. positive serology and PCR but no clinical signs; or 3. positive PCR with negative

serology and no clinical signs (3–5,5,8). Dogs may move one stage up or down over time depending on several factors and the disease has indeed a wide range of incubation periods (4,5). A complex genetic background is suspected to influence the susceptibility towards developing the disease and immunological factors are known to be a key factor (1,3,4,9–11). A balanced innate and acquired immunity with a dominant type IV immune reaction and activation of Th1 lymphocytes leads to an appropriate immunological response and dogs eluding the disease (3,4,10,11). However, a reduced T-cell mediated immunity and a marked humoral response leads to organ deposition of immune complexes (type III immune reaction) (3,5,10–12). Other pathomechanisms such as granulomatous inflammation and autoantibody production are also known to play a role in some tissues (5,10,11,13,14,14–16). Eventually all, or some of these mechanisms, may lead to organ and tissue inflammation, altered organ function and clinical signs (3,5,14).

CanL is a multisystemic disease and can affect any organ, tissue or biological fluid (3,5,17). The skin, lymphoid tissue, kidneys, visceral abdominal organs, musculoskeletal system and the eyes are commonly affected (3,5,17,18). Clinical signs in CanL are often non-specific or associated to specific organ damage, and several classical clinical signs include skin lesions, generalised lymphadenopathy, progressive weight loss, muscular atrophy, exercise intolerance, decrease appetite, lethargy, splenomegaly, polyuria/polydipsia, ocular lesions, epistaxis, lameness, vomiting and diarrhoea (3,5). Two major classifications exist for CanL: the LeishVet (3) and the Canine Leishmaniasis Working Group (CLWG) (19). In both classifications the disease is structured primarily based on severity of clinical signs, serology levels and renal function (urine protein:creatinine ratio -UPC- and creatinine levels) (3,19). The way both classifications are structured leads to UPC and creatinine levels, and therefore

renal function, to be the cornerstone of CanL classification (3,19). The use of biomarkers highlighting the involvement of other tissues or organs is not specifically considered.

The heart and the pericardium can be affected in CanL (20–26). Important evidence has indeed recently mounted, including the studies that will be presented in this Thesis, confirming the frequent presence of myocarditis in advanced cases of CanL, and the potential pathophysiological mechanisms for this CanL associated myocardial disease (13,14,26,27). Whether CanL myocarditis contributes to some of the non-specific clinical signs observed in CanL is possible, but currently unknown. The main clinical signs observed in most types of myocarditis (28,29) are non-specific, and similar to the general clinical signs observed in CanL (3,19). On the contrary, pericardial effusion due to CanL pericarditis has been reported and can lead to more evident direct clinical consequences such as cardiac tamponade, collapsing episodes and right sided heart failure (30–32). Similarly, visceral leishmaniosis in humans can lead to myocarditis and pericarditis (33–38) which, in severe cases, may be fatal (38). Previous studies have investigated ECG abnormalities and structural echocardiographic changes in CanL (21,35,39). Non-complex ECG abnormalities and arrhythmias have been reported with relative frequency including elevated T waves, ST segment elevation, sinus arrest, right bundle branch block and atrial premature complexes (35,39). Limited information exists on echocardiography, however, in one study left ventricular hypertrophy was reported in hypertensive CanL dogs (21) and pericardial effusion has been described sporadically in cases with CanL associated pericarditis (30–32).

A biomarker can be defined as a parameter that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (40,41). In the context of CanL, a biomarker would be expected

to add valuable information about some of the following aspects: subclinical disease, organs or tissues affected, severity, risk stratification, prognosis and/or treatment monitoring. Routine widespread biomarkers used in CanL include creatinine, UPC, serum electrophoresis, quantitative serology levels, globulin and γ-globulins levels, and blood or tissue PCR (3,5,17,19). Many other renal or inflammatory biomarkers have also been studied (17,42–49). Other organ-specific biomarkers have also been explored, and are anecdotally reported, including skeletal muscle creatinine kinase (50) and liver enzymes such as alanine aminotransferase (ALT) (49).

Considering the involvement of cardiac disease in CanL, cardiac biomarkers could become a specific tool to identify individuals with cardiac involvement in the clinical or research setting. They could also be valuable as a general disease biomarker for the identification of subclinical disease, and they could contribute to classification and risk stratification, prognosis and treatment guidance. CanL often presents in a silent form and has high prevalence in some geographical areas (3,19). Therefore, it would also be of clinical importance to recognise the effects of CanL on cardiac biomarkers that are used clinically in the diagnosis of dogs with cardiac disease. Three blood borne biomarkers that have been frequently used in dogs in different clinical situations for different heart diseases include: myocardial damage biomarkers cardiac troponin I (cTnI) (52–60) and heart-type fatty acid binding protein (HFBPA) (61), and the myocyte stress biomarker N-terminal proB-type natriuretic peptide (NT-proBNP) (58,60,62–66). Prior to, and during the time of research and completion of the studies of this Doctoral Thesis, cTnI and NT-proBNP were explored in CanL by other research groups, showing promising but variable results (22,24,67,68). Both biomarkers, cTnI and NT-proBNP, were reported in these previous studies to be elevated in many dogs with CanL with a

tendency to be higher in dogs with more advanced disease (22,24,67,68). Further characterisation of both biomarkers regarding their association with LeishVet staging, their relationship with other diseases and clinical markers, and the effects of extracardiac factors commonly found in CanL such as anaemia, systemic arterial hypertension or abnormal renal function would be of interest. It would be also of interest to further elucidate aspects of cardiac remodelling and histopathological findings in CanL, including the presence and severity of myocardial parasitic load (MPL) and the relationship between remodelling, histopathological alterations, and MPL, with cardiac biomarkers.

LITERATURE REVIEW

The disease and its classification

CanL is a multisystemic disease with a plethora of clinical signs. Different clinical classifications have emerged over time to stage the severity of the disease (3,19,69–72). Disease staging is important as it provides specific easily interchangeable and concise terminology among clinicians and researchers to group individuals with the disease (3,35,46–49). Ultimately, staging of any disease should be helpful to guide prognosis and treatment and should be an easy-to-use tool for ongoing research. The criteria for classification must be simple and clinical, with the use of uncomplicated biomarkers and diagnostic methods (44), and with the aim of being widely available and globally used (3,41,44).

Classification of disease in CanL in general has been based on the change, and degree of change, of specific biomarkers, the type and severity of clinical signs, and the type and number of organ or systems involved (3,19,69–72). The former two aspects have been key in most classifications. The two major classifications currently used are The LeishVet (3) (table 1) and the Canine Leishmaniasis Working Group (CLWG) (19) which are the only classifications providing therapeutic recommendations and prognostic information for each stage (3,19,44). The LeishVet classification uses four disease stages (table 1) with one substage for the moderate group (3), while the CLWG classification groups patients into four stages from exposed to severely sick. Prior to these classifications, clinical staging have divided dogs with CanL as asymptomatic, oligosymptomatic or symptomatic (69); clear, mild or severe (70); initial stage, established disease or advanced stage (71), and subpatient infections, asymptomatic infection and symptomatic active infection (72). When evaluating aspects of the disease such as new biomarkers, new therapies, or prognosis, using the most accepted classification is important in order to achieve standardisation.

Table 1. LeishVet clinical classification of CanL (3)

STAGE	CLINICAL SIGNS	LABORATORY FINDINGS	QUANTITATIVE SEROLOGY	THERAPY OR FOLLOW-UP	PROGNOSIS
STAGE I **Mild disease**	Mild clinical signs such as papular dermatitis or localized lymphadenomegaly	No clinicopathological abnormalities. Normal renal profile: creatinine < 1.4 mg/dl; UPC<0.5	Negative to low positive antibody levels	Scientific neglected	Good
STAGE II **Moderate disease**	Diffuse or symmetrical cutaneous lesions such as exfoliative dermatitis-onychogyrphosis, ulcerations (planum nasale, ears, footpads, bony prominences, mucocutaneous junctions), generalised lymphadenopathy, loss of appetite and weight loss	Clinicopathological abnormalities compatible with *L. infantum* such as mild non-regenerative anemia, hypergammaglobulinaemia and hypoalbuminaemia Substage a)Normal renal profile: creatinine < 1.4mg/dl; non-proteinuric UPC < 0.5 b)Proteinuric creatinine: <1.4 mg/dl; UPC=0.5-1	Low to high positive antibody levels	Meglumine antimoniate + allopurinol Miltefosine + allopurinol Substage b: Follow IRIS Guidelines for CKD	Good to guarded
STAGE III **Severe disease**	Dogs which apart from the signs listed in stages I and II, may present signs originating due to immune-complex deposition (e.g. glomerulonephritis, uveitis)	Clinicopathological abnormalities listed in stage II. CKD IRIS stage 1 with proteinuria UPC>1 or IRIS stage 2 (creatinine 1.4-28 mg/dl)	Medium to high positive antibody levels	Meglumine antimoniate + allopurinol Miltefosine + allopurinol Follow IRIS Guidelines for CKD	Guarded to poor
STAGE IV **Very severe disease**	Dogs with clinical signs listed in Stage III. Pulmonary thromboembolism, or nephrotic syndrome and end stage renal disease	Clinicopathological abnormalities listed in stages II and III. CKD IRIS stage 3 (creatinine 2.9-5 mg/dl) and CKD IRIS stage 4 (creatinine > 5 mg/dl) or Nephrotic syndrome or marked proteinuria UPC > 5	Medium to high positive antibody levels	Specific treatment should be instituted individually Follow IRIS Guidelines for CKD	Poor

When the LeishVet and CLWG are compared, clinical differences and similarities rapidly emerge (44,73). The LeishVet classification appears to be the most widely used. It is easy to apply, and the disease is staged into four groups with the main markers used being clinical signs (severity and type), semi-quantitative serology levels, creatinine levels and presence and

degree of proteinuria (table 1) (3). Stage 2 appears to be the most common clinical stage diagnosed in the Spanish region of Catalonia (74), and this is suspected to be the case also in other Spanish areas and in other countries (75). The CLWG, separates the disease into five groups (19). The main markers used in this classification are, similarly, type and severity of clinical signs, semi-quantitative serology titre, presence of parasites (by microscopy, culture or PCR), presence of proteinuria, creatinine levels and International Renal Interest Society (IRIS) stage (19). In both classifications renal function (creatinine levels and IRIS stage) is the cornerstone of CanL staging. The effects of CanL in other organs have a much lesser importance. In the LeishVet classification the presence of disease associated with immune-complex deposition such as arthritis or uveitis and pulmonary thromboembolism is considered in the clinical signs section in stages III and IV (3). In the CLWG classification the presence of clinical and clinicopathological abnormalities associated with a high titre or the presence of severe ophthalmic disease, severe joint disease or any other concomitant disease is also considered. Specific cardiac or pericardial involvement or the presence of elevated cardiac biomarkers is not considered, described, or discussed in these classifications (3,19). Classification systems are widely used in human medicine and tend to evolve over time as new outcome studies and new biomarkers arise, with the aim of aiding evaluation of the efficacy of different therapies, decisions on the most appropriate therapy for each patient, and prognostic evaluation (76–79). There are however few studies yet using the LeishVet or the CLWG system for clinical or research purposes in veterinary medicine (47,73,80).

Myocarditis in CanL

It has been shown that dogs with CanL frequently suffer from myocarditis associated with the disease (CanL myocarditis) (14,20,23,25,26,31). A total of 71 cases of dogs with CanL myocarditis are described in the literature (14,20,23,25,26,31,32). This information arises from histopathological studies looking specifically into the presence and nature of CanL myocarditis in cohorts of euthanised dogs with CanL, or in dogs dying naturally due to the diseases (14,20,26,31), and from single case reports (23,25,31,32). In the studies exploring the presence and prevalence of CanL myocarditis diagnosed by histopathology, in samples taken after euthanasia or natural death, the majority of individuals were affected (14,20,26). The prevalence in those studies was 15/15 (100%) (14), 27/30 (90%) (26) and 18/22 (81%) (20). Importantly, not every dog in these studies suffered from advanced CanL, and indeed, these dogs were euthanised for a variety of reasons. The study by Costagliola et al (14) was the only one in which dogs died naturally or where euthanised due to advanced clinical disease, but in the study by Rosa et al (26) and Alves et al (20), euthanasia was performed as a compulsory measure of stray CanL dogs in the region. Not every dog in the latter studies had advance disease, which raises the question of when myocarditis may occur during the course of the disease. Looking in depth into disease severity of these 67 dogs, and extrapolating the data provided to the LeishVet classification (3), most dogs appear to be in Leishvet stage II or III while some individuals were in stage IV. Interestingly, in the study by Alves et al (20), 7 asymptomatic dogs, which would be considered in LeishVet stage I, were included and mild to moderate myocarditis was observed in 6/7 (85%). Even within the single case reports, not all dogs had advance LeishVet staging. They were in LeishVet stage II in Font et al (32) and Lopez-Peña et al (25), and in LeishVet stage III in Silva et al (31) and Torrent et

al (23). A possible additional case of CanL myocarditis also exists in the literature in which a dog with advanced atrioventricular (AV) block and myocarditis (identified by endovascular myocardial biopsy at the time of pacemaker implantation) had a positive *L. infantum* PCR in myocardial tissue (81). This case presented to a cardiologist for diagnosis and treatment of the bradyarrhythmia. There is no description to whether the patient had positive serology or other clinical signs of systemic CanL associated disease, whether general disease developed afterwards, or whether the dog was specifically treated for CanL.

The most common reported type of myocarditis in CanL is lymphoplasmacytic although granulomatous myocarditis (diffuse or focal) has also been observed in some cases (11/71; 15%). The distribution of the inflammation is usually between cardiomyocytes, and it was observed, in one study, to be more severe in the subendocardial and subepicardial regions (26). A perivascular distribution of the inflammation or vasculitis is less commonly observed (6/71; 8%). Another frequent feature of CL myocarditis is the presence of myocyte degeneration and/or necrosis (46/71; 65% of cases). Other occasional histopathological findings reported in the myocardium of dogs with CanL include fibrosis (31/71; 44%) or haemorrhage (5/70; 7%). The severity of CanL myocarditis varies across the literature from mild to severe (14,20,23,25,26,31,32). The infiltration appears to be patchy or focal in some cases and diffuse in others (14,20,23,25,26,31,32). However, sampling site might play a role in the characterisation of the extent of infiltration. Sampling sites have varied from right ventricular to right atrial and interventricular, but have also included the left chambers (14,20,23,25,26,32). Higher prevalence and severity have been described for the right atrium and right ventricle (26). Factors associated with the presence and severity of CanL myocarditis have, in general, not been studied. However, in one study, the severity of the inflammatory

infiltrate correlated with the degree of parasite presence detected by anti-Leishmania immunohistochemistry (26).

The myocardium is not the only type of muscle affected in CanL. CanL myocarditis share many similarities to skeletal muscle myositis which has been well described in CanL (27,50). The inflammatory infiltrate in the skeletal muscle is multifocal and surrounds the muscle fibers in the endomysium, although it distributes perivascularly in the perimysium (27,50). Necrosis of muscle fibers is usually observed (27,50). In the chronic stages, endomysial and perimysial thickening due to fibrosis can be observed (27,50).

Pericarditis has also been reported in dogs with CanL. Pericarditis, and less commonly myocarditis, is also occasionally reported in humans with visceral leishmaniosis (34,38,82). There are five clinical cases reported in dogs in which CanL associated pericarditis led to pericardial effusion (30–32,83), and in three cases (30,32,83), to cardiac tamponade. In two of these cases concurrent myocarditis was also reported (31,32). However, specific studies describing the prevalence and severity of pericarditis are lacking. The studies evaluating myocarditis in dogs with CanL (14,20,26) did not include sampling pericardial tissue. However, considering the severity of pericarditis on histopathology reported in single case reports (30–32), the description in some CanL myocarditis studies of predominant subepicardial inflammation and epicarditis in conjunction with myocarditis (20,23,26), and the reported presence of inflammatory pericardial effusions with positive Leishmania PCR in some cases of CanL (83), it is likely that pericarditis is also a common, yet under-described, feature of CanL.

Leishmania in the myocardium

Detection of *Leishmania* organisms in the myocardium of dogs with CanL myocarditis has been inconsistent when both, direct observation on routine histopathological staining, and/or immunoperoxidase staining, are considered (14,20,23,25,26,31,32). *Leishmania* amastigotes were observed in 26/71 (37%) of cases in the literature (14,20,23,25,26,31,32). However, immunoperoxidase technique is known to be more sensitive for *Leishmania* detection and it was not performed in all cases. This type of immunohistochemistry is usually performed by using a mouse polyclonal anti-*Leishmania* antibody with subsequent sample assessment by a sensitive peroxidase system (20). When only cases in which immunoperoxidase technique was performed are scrutinised, *Leishmania* amastigotes were observed in a higher percentage of cases, 26/46 (56%), increasing to 32/46 (70%) when assessment of *Leishmania* antigens (without entire distinct parasitic presence) was also considered (20,23,25,26,31). The presence of *Leishmania* antigens has however been rarely explored in the literature (in only 12/71 of cases; 17%) (20). When organisms are observed in the myocardium of dogs with CanL myocarditis, they are in low to medium numbers. The presence of high number of organisms in the myocardium is rare (14,20,23,25,26,31,32). *Leishmania* amastigotes in the myocardium are observed within macrophages or in between muscle cells (20,26,31,32) as it is also observed in skeletal muscle (15,27,50,84). Amastigotes do not appear to be able to penetrate muscle cells (15,20,26,27,31,32,50,84) and consequently *Leishmania* DNA is not found in the sarcoplasm (15,27). In the paper by Rosa et al (26), the severity of myocarditis was found to be associated with the MPL detected by immunoperoxidase staining. This has also been the case in skeletal muscle studies of dogs with CanL, where the severity of

inflammation correlated with the parasitic load detected by direct visualization or by quantitative PCR techniques (15,84).

PCR is a molecular technique considered more sensitive and specific than direct parasitological examination for the detection of *Leishmania* organisms in tissues (5,85–90). This technique can therefore be also used to identify *Leishmania* in the myocardium. Real-time quantitative PCR (qPCR) not only detects the parasite but reliably detects quantity of it as it measures the products generated during each cycle of the PCR process, which are directly proportional to the amount of template prior to start of the PCR process (85–87,89,91). Blood, bone marrow or lymph node PCR is routinely used clinically for the diagnosis and monitoring of CanL (3–5,17,19,85–87). It can be a useful tool for the diagnosis of elusive cases in endemic areas where many individuals are exposed to the disease and as a marker of severity, as higher threshold cycles (Ct) are usually observed in more severe cases (3,5,17,85,86,92). Following treatment, qPCR shows a gradual decrease in these tissues and ultimately, and ideally, no organism is detected (17,19,85) which makes it also a tool for monitoring treatment response (19,85,89,92). *Leishmania* PCR has been specifically studied in other tissues or body locations such as hair (88), skin (87,93), urine (94) or conjunctival swab (95), but it can be applied to any affected organ or cavity potentially involved, such as liver (51) or lung (20), as the disease is multisystemic. There are many different protocols reported for PCR molecular testing that depend on factors such as DNA extraction method, primers, target copy numbers and technical conditions. Different DNA targets are used which include PCR using the variable part of the small sub-unit rRNA gene, the alpha and beta actin gen, the internal transcribed spacer 1 or kinetoplastid minicircle sequences (85,89,91,96,97). The latter is commonly used as a target (87,88,97). Kinetoplast DNA is located in the parasite

mitochondrion and comprise of two subunits, the maxicircles and the minicircles (present in a number of 30-50 copies/parasite, with 20-40kb in length) (88). The high number of copies of this minicircle and the existence of conserved regions between species allows using molecular probes based on it for diagnosis (88). PCR in general, and real-time qPCR in particular, have rarely been used for *Leishmania* detection in the studies evaluating CanL myocarditis. In only 1/71 cases (1-2%) PCR was used and was indeed positive for *Leishmania* detection (23). We also described in the previous section the case reported of myocarditis and third degree AV block (with unknown general clinical or laboratorial status of the disease described) associated with positive *Leishmania* PCR in the myocardium (81). To the author´s knowledge, prior to the studies of this Doctoral Thesis, *Leishmania* qPCR had not been studied in the myocardium of dogs with CanL. Interestingly, in a study evaluating PCR analysis of several organisms in the pericardial fluid and blood of dogs with idiopathic pericardial effusion or neoplastic effusions in Spain, positive *Leishmania* PCR was observed in 7/68 cases (10%) (83). Three of these dogs had idiopathic pericardial effusion (*Leishmania* PCR was positive in pericardial fluid of one case and in blood of two cases) and in four cases with neoplastic effusions (in two cases positive PCR using pericardial fluid and in the other two cases a positive PCR in a blood sample) (83). All three dogs with idiopathic pericardial effusion had also clinical signs consistent with CanL and positive results in serology and bone marrow and/or lymph node cytology (83). A diagnosis of *Leishmania* associated pericarditis in these cases appears appropriate. In this study, two of the four dogs with neoplastic effusions (one with a heart base mass and one with an unspecified cardiac mass) had also been diagnosed with recent or current CanL by appropriate testing (83).

Myocarditis in canine leishmaniosis: immune-mediated mechanisms

In dogs with CanL the innate immunity is evaded by *Leishmania* parasites by different mechanisms such as remodelling of phagosomal compartments and by interference with signalling pathways (11,98). The adaptive immune system is the principal actor in general and local disease control and the type and level of response depends upon a complex interaction between the parasite and the genetic and immunologic background of the host (11,99–101). These intriguing dynamic complex relationship leads to different individual susceptibility with a range of responses from markedly symptomatic dogs displaying a susceptible profile response, to asymptomatic dogs showing a resistance pattern (11,99–101). A resistance pattern is dominated by the regulatory effects of Th1 lymphocytes while a dominant Th2 response leads to large antibody production and a progressive non-healing disease (10,101). A higher level of T lymphocytes ($CD4^+$ and $CD8^+$) is associated with resistance pattern. $CD8^+$ lymphocytes have an important role, as they trigger a protective Th1 response during early stages of the infection and have direct cytolytic activity against *Leishmania*-infected macrophages. Reduced presence of regulatory $CD4^+$ T cells (Treg) and increased levels of regulatory (IgD^{hi}) B cells is also observed in symptomatic dogs when compared to asymptomatic dogs. These B cells produce IL-10 and suppress IFN-y from T cells favouring a non-protective humoral response versus a protective cellular response (11,99–102). Upregulation of microRNA-21 has been also implicated in the regulation of IL-10 expression in dogs with CanL (103). The extreme, ineffective, and ultimately deleterious, humoral response is known to encompass high levels of all IgG subclasses (104). The dissimilar immunopathological responses observed in different tissues and organs in CanL, further complicates the general immune-pathological mechanisms and the clinical picture observed

(3,11,12,14,19,99–101,105). In lymphoid organs, depletion of T lymphocytes is pseudo-compensated by an exuberant B-cell proliferation and activity which, together with increases in plasma cells, histiocytes and macrophages, explains the generalised lymphadenopathy, splenomegaly and hyperglobulinaemia (11). Other important organ-specific pathomechanisms include granulomatous inflammation, immune complex deposition and/or autoantibody production (3,11,19,51,99–101,106). The importance of each of these mechanisms is remarkably different across different organs and body tissues (11,12,14–16,18,51,84,99,105,107–109). For example, granulomatous inflammation and vasculitis are frequently observed in the liver or the kidney, but not in the myocardium (11,74,77,85,14,19,22,24,25,56,57).

Immunohistochemistry of the myocardium in dogs with CanL has shown predominantly CD3, CD8 and CD4 positive inflammatory cell leukocyte populations, in particular of CD8+T lymphocytes, together with macrophages and less commonly with CD79 cells (14). A mixed Th1/Th2 response is suspected (14,27). Intense staining for major histocompatibility complex (MHC) class I and II antigens has been seen, not only in the vascular adventitia, endothelial cells, and cellular infiltrates but also in the sarcolemma of many cardiomyocytes (14). These findings are very similar to those found in studies carried out in CanL skeletal muscle myositis (15,84). The presence of sarcolemmal expression of MHC I and MHC II antigens strongly suggest that an important mechanisms of CanL myocarditis and myositis is an antibody mediated autoimmune mechanism (14,110–112). Immunohistochemical detection of sarcolemmal MHC class I and II expression is considered a valid test for immune-mediated idiopathic inflammatory myositis in humans and dogs, and in this scenario, autoantibodies produced by dogs with CanL are directed against one or more proteins shared by skeletal and

cardiac muscle which triggers immune-mediated damage in both tissues (10,14,110,111). This has been further supported in CanL skeletal myositis studies by the observations of CD8+ T lymphocytes invading histologically normal muscle fibres expressing MHC class I antigens and forming CD8/MHC I complexes (15). This autoimmune mechanism is similar to what is observed in the myocardium and muscle of Syrian hamsters experimentally infected with *Leishmania (27,113)*, and in dogs with *Trypanosoma cruzi* myocarditis (114), an organism phylogenetically related to *Leishmania infantum (114,115)*. Furthermore, autoantibodies against the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) 1 have been identified in CanL (116). SERCA proteins are expressed in the sarcoplasmic reticulum and are indirectly in contact with the extracellular T-tubular system (117) which would facilitate antigenic extracellular exposure and subsequent autoantibody ligation (10,116). While cardiac myocytes do not have SERCA1, they have a similar protein, SERCA2a, which is also observed in slow-twitch skeletal muscle fibres (118). Cross reaction of SERCA2a autoantibodies with SERCA1 ones has been documented in dogs with CanL myositis (116) and SERCA 2a autoantibodies have been identified in experimental models of myocarditis (119,120). All these has led to believe that anti-SERCA1 autoantibodies partially cross-react with the other SERCA isoform in the myocardium, or that dogs with CanL have autoantibodies recognizing proteins shared by skeletal and cardiac muscle (16,119,120). Antigen mimicry is suspected to be behind this autoimmunity process, in which the proteins of the infectious agent have epitopes cross-reacting with the host-protein (16,119,120). Similar proteins may be, in the case of CanL myositis and myocarditis, the calcium-translocating P-type ATPase of *L. infantum* and the SERCA proteins of the host (116). Ultimately however, other cellular or humoral immune-mediated factors or other general factors may also play a role. Those may include

vasculitis, local inflammation directly triggered by parasitic migration, systemic inflammatory response state, anaemia, renal disease, myocardial hypoxia, and myocardial alterations due to the catabolic nature of the disease (14,24,27,38,116).

Cardiac specific clinical findings and cardiac diagnostic tests in CanL

While there is robust evidence that CanL myocarditis occurs in many dogs with the disease, it is less clear that this may have a clinical impact in affected individuals. A recent study describing the clinical signs (including findings on physical examination) in a large population of dogs with CanL found the following clinical findings (and the percentage of dogs affected) (44): cutaneous lesions (78%), lymphadenopathy (64%), weight loss (47%), ocular lesions (25%), fever (21%), vomiting and diarrhoea (19%), lameness (12%), polyuria and polydipsia (10%), epistaxis (8%), muscle atrophy (8%) and splenomegaly (6%). Other common clinical findings reported, possibly not included in the questionnaire of this latter study, are decreased appetite, lethargy, exercise intolerance and tachypnoea (4,5,18,70,92,121,122). Nevertheless, CanL has been described as a great pretender (4) due to its potential effects on almost any organ which leads to the presence of other, less common, clinical signs (4). Rarer reported clinical signs include swelling of the head and neck, abdomen and peripheral oedema (25,121), neurological signs due to ischaemia, vasculitis and/or thromboembolism (92,108), and clinical signs secondary to local vascular thromboembolism (3,90,108). An obvious, direct, and specific association of clinical signs and myocarditis may not, at first data evaluation, be apparent. However, dogs with myocarditis can show unspecific clinical findings at presentation including lethargy, hyporexia, weakness, gastrointestinal signs (vomiting and diarrhoea) and tachypnoea (29). Less commonly, they show exercise intolerance, fever,

cough, syncope, dyspnoea, presence of a heart murmur, femoral pulse absence, orthopaedic pain or polyuria/polydipsia (28,29). Congestive heart failure is less common in dogs with myocarditis (28,29) and has not been reported in dogs with CanL. Ultimately, when examining in detail the clinical findings of dogs with myocarditis, it clearly stands out that most of them are also unspecific, and similar to those with CanL. It is possible that CanL myocarditis may lead and contribute to some of the clinical signs observed in CanL, particularly in later stages of the disease.

In clinical practice, establishing an antemortem diagnosis of myocarditis in dogs is not always easy. It is usually supported by the presence of arrhythmias (28,29,81,123–125) and, most importantly, by the significant elevation of cardiac biomarkers, in particular cardiac troponin I (cTnI) (28,29,123). Myocardial biopsy, and cardiac functional and structural abnormalities and tissue characterisation by MRI, are two ancillary diagnostic tools often used in human medicine to improve diagnosis of myocarditis (112,126), but these are rarely used in clinical practice in dogs. As discussed in the previous section, a case of a dog with advanced AV block and myocarditis, diagnosed by myocardial biopsy and with a positive Leishmania PCR in the myocardium (81), has been reported. Also, recently, the MRI findings of *Trypanosoma cruzi* myocarditis in dogs, have been described (127). The MRI findings of CanL myocarditis have not yet been studied.

Echocardiography

In humans, structural or functional changes on echocardiography, although less consistent than MRI findings, may also be seen in cases of myocarditis (112,126). Increased wall thickening, chamber enlargement, and/or regional or global systolic or diastolic dysfunction, can be observed (112,126). Endocavitary trombi are also occasionally seen (126). Although

the echocardiographic findings have been less consistently characterised in dogs with myocarditis than in humans, functional or structural changes have been observed in 45-72% of cases (28,29) including global systolic dysfunction, left ventricular chamber enlargement, increased left ventricular wall thickening, ventricular wall heterogenicity and focal hypokinesis (28,29). More specifically, in cases of myocarditis caused by *Trypanosoma cruzi*, functional and structural echocardiographic abnormalities have been reported (128). In a recent study, 63% of dogs naturally infected with *Trypanosoma cruzi* had cardiac enlargement of at least one chamber (128). Right ventricular enlargement was most common (36% of cases) and was classified as mild to moderate eccentric hypertrophy (128). Moreover, the presence of RV enlargement was associated with a 3.6 greater risk of death in multivariate analysis (128). A decrease fractional shortening, suggesting a degree of left ventricular systolic dysfunction, was also found in 19% of dogs (128). When reviewing the possible echocardiographic findings of CanL myocarditis in the literature, there is only one study (21) which has evaluated the two-dimensional echocardiographic abnormalities in dogs with CanL. In this study, the echocardiographic findings of 76 dogs were evaluated as part of a research project investigating the prevalence and clinical consequences of systemic arterial hypertension in dogs with CanL (21). Extrapolating the results of this study to the Leishvet classification, most dogs in Leishvet stage I and II did not have cardiac alterations on echocardiography (21). It is possible that indeed all dogs from these two groups did not have any alteration, but this information cannot be fully obtained, as the data is depicted based on blood pressure groups and, a degree of overlapping among the Leishvet groups and the reported groups exists (21). Similarly, the study suggests that most dogs in LeishVet stages III and IV have structural abnormalities found on echocardiography, which again, it might

actually represent all of them, or nearly all of them (21). The main cardiac abnormality detected in this group of dogs was symmetrical or asymmetrical increase in left ventricular wall thickening (21). The severity of this change was not specified in the article (21). Transvalvular spectral Doppler was also evaluated in the dogs of this study and showed impaired relaxation pattern in 25.7% of dogs with increased left ventricular wall thickening (21). Dogs were however 5.3 ± 2.8years of age (range 1-13 years) and this may be an age-related change, at least in some individuals (21). Importantly, while most dogs with systemic arterial hypertension had increased left ventricular thickening, a cause and effect cannot be confirmed. Most dogs with advanced CanL have systemic arterial hypertension (21) but it is possible that the cardiac changes observed on echocardiography are indeed directly associated with CanL myocarditis due to myocardial infiltration and oedema, and occur, independently of hypertension, at least in more advance cases. Other factors such as potential pseudo thickening due to dehydration and hypovolemia (due to renal disease) at the time of echocardiography, may also play a role (129). To the best of the author knowledge, calculation of left ventricular mass index (LVMi) or left ventricular or atrial systolic and/or diastolic function assessed by advanced echocardiographic techniques, such as tissue Doppler or strain echocardiography, has not been carried out in dogs with CanL. Moreover, assessment of right sided (ventricular or atrial) myocardial function by conventional (or advanced) echocardiographic techniques, has not been carried out, with the exception of a small study in which the right ventricular Tei index, the acceleration time to ejection time ratio, and the tricuspid regurgitation peak velocity, when present, was assessed in 10 dogs with CanL (130). Most dogs in this study had early LeishVet stages of the disease. A peak tricuspid regurgitation velocity consistent with pulmonary hypertension was observed in the only dog with tricuspid

regurgitation (which had concurrent mitral valve disease (MVD)); and right ventricular myocardial dysfunction, diagnosed by Tei index, was observed in another dog (130). Evaluating right sided myocardial atrial and ventricular function in dogs with CanL, particularly in advanced stages, may be of interest because, as previously discussed the right side was shown to develop more severe myocarditis in one study (26) and dogs with *Trypanosoma cruzi* also show preference for the development of right sided cardiac abnormalities (128).

Electrocardiography

As discussed above, in addition to the presenting clinical signs, echocardiographic abnormalities, and the use of cardiac biomarkers (29), the presence of ECG abnormalities and/or arrhythmias can be important in the antemortem diagnosis of myocarditis in dogs (28,29). Ventricular and supraventricular premature complexes, ventricular tachycardia, supraventricular tachycardia, atrial fibrillation, atrioventricular blocks of different severity, sinus bradycardia, sinus tachycardia and sinus arrest have all been reported in dogs with myocarditis (28,29,123,125). In humans, the use of ECG or Holter recordings for the presence of newly developed bundle branch block, ST/T wave changes (ST-elevation, T wave inversion in 12 lead ECG), reduced R wave height, intraventricular conduction delay with widened QRS complex, or abnormal Q waves, together with the presence of arrhythmias, are considered in the diagnosis of myocarditis (112,126). Humans with visceral leishmaniosis can indeed show ECG alterations such as corrected QT prolongation (38,131). In dogs with *Trypanosoma cruzi* infection (128) supraventricular arrhythmias (supraventricular premature complexes, supraventricular tachycardia, atrial fibrillation) are observed in 16% of cases, conduction abnormalities (atrial standstill, AV block, bundle branch block) in 49% of cases, and ventricular arrhythmias (ventricular premature complexes or ventricular tachycardia) in 65% of cases

(128). Abnormal amplitude duration or interval duration are also reported with changes in P wave amplitude or duration in 88% of cases, prolongation of PR intervals in 58% of cases, prolongation of QRS complexes in 70% of cases and increased or decreased R wave amplitude in 63% of cases (128).

Several studies have looked into the ECG changes in CanL (23,25,35,39,81,132,133). Short ECG traces of 133 dogs with CanL were evaluated within two studies (35,39). Arrhythmia (including conduction abnormalities) were observed in 14-21% of dogs and included right bundle branch block, atrial premature beats and sinus arrest (39). Sinus tachycardia and bradycardia is also reported in some dogs. Severe or complex arrhythmia are however not apparent by looking at the data in these studies, although the severity of the sinus arrest episodes is not reported, the appropriateness of the bradycardia and tachycardia not explained, and the frequency of atrial premature complexes is not known. Abnormal amplitude duration and intervals were also not uncommonly observed in the largest of these studies. Increased P wave duration and QRS duration in 33% and 26.7% of cases respectively, and spiked T waves and ST segments shifts in 19% and 9.5% respectively were recorded (39). Unfortunately, the severity of CanL (i.e., their Leishvet staging) is not provided for most cases (105 dogs) and cannot be extracted from the limited clinical data provided. However, in the 28 dogs with known severity of the disease, and who were staged according to the Leishvet classification (35), nearly all dogs with severe (LeishVet III) or very severe (LeishVet IV) disease presented ECG abnormalities. Another study (22) assessed the ECG trace over longer period (24 hours) in 28 dogs with CanL. All dogs were classified as having mild ECG abnormalities (as opposed to moderate or severe) and normal corrected QT interval and heart rate (22). However, the ECG criteria for classification of findings as mild, moderate or severe, or the specific changes observed within

the mild group, are not described (22). An important limitation for this study (22) is that most dogs (93%) had mild to moderate disease (LeishVet I and II) and may not have developed myocarditis yet. Within the single clinical cases reports available the literature information on ECG findings is not always displayed. In a case of confirmed CanL myocarditis (23) antemortem ECG findings included first degree AV block and ST segment depression. Decreased amplitude of QRS complexes with normal heart rate and rhythm was shown in another case report (25), and one case with third degree AV block and positive myocardial *Leishmania* PCR has been reported (81). Recently, a case of a dog with very severe CanL (LeishVet IV), elevated cTnI, and ventricular tachycardia, was described (133). Lastly, a study investigated the presence of P wave dispersion in dogs with CanL (132). P-wave dispersion is defined as the difference between the maximum and the minimum P-wave duration recorded from different surface ECG leads (134). Increase in P-wave dispersion reflects prolongation of intraatrial or interatrial conduction time and has been linked with the risk of supraventricular arrhythmias and atrial fibrillation in a wide range of cardiac diseases that alter myocardial tissue structure and electrophysiological properties (134). In this study (132), dogs in LeishVet stage IV had a significantly longer P-wave dispersion than dogs in lower LeishVet stages or controls, suggesting more severe atrial myocardial remodelling (134).

Myocarditis in CanL: clinical and diagnostic implications

While there is robust evidence that myocarditis occurs in dogs with CanL, a consistent implication of this process in the clinical picture and outcome in some dogs with CanL is possible, albeit unclear at this stage. However, alterations in diagnostic tests and cardiac biomarkers can occur due to CanL myocarditis. We have described the echocardiographic and ECG changes observed in many dogs with CanL and clinicians should be aware of them. This

rationale will also apply, and is discussed later in this Thesis, in the clinical use of cardiac biomarkers in dogs with CanL. A situation may arise for example in which a clinician has diagnosed or suspects CanL in a patient, and ECG or echocardiographic alterations known to occur CanL are observed. The clinician should be aware that CanL can cause this alterations and the search for a different primary cardiac disease would not be required. Conversely, when increase left ventricular thickening on echocardiography is observed, or myocarditis is suspected, CanL should be included in the differential diagnosis in a patient living in an endemic area. Nevertheless, further studies characterising ECG, echocardiographic and MRI findings, particularly in severe or very severe cases (Leishvet III and IV) are certainly warranted. Also, it remains to be elucidated whether CanL myocarditis increases its severity as the rest of other current markers of the disease are increased (e.g., at higher LeishVet stage), and therefore other tissues are simultaneously also more severely affected, or if in certain individuals, it may become one of the predominant organs affected or one of the organs more severely affected. Cardiac biomarkers would certainly be of fundamental use for this purpose. This heterogenicity of disease distribution is not uncommonly observed in CanL, with some dogs showing, for example, marked involvement and associated clinical signs due to specific involvement in the liver or CNS, while others do not (3,51,108). Ultimately, another reflexion becomes apparent from the above discussion. Considering the consistent presence of myocarditis, and the lack of specificity of clinical signs and routinely available cardiac diagnostic tests, cardiac biomarkers appear as an indispensable tool for the evaluation of CanL myocarditis both for clinical and research purposes.

Clinical signs in pericarditis associated with CanL

As discussed above in this manuscript, pericarditis in CanL, albeit only occasionally reported (30–32,83), has led to some cases of severe clinical signs such as lethargy, exercise intolerance, ascites and collapse, due to pericardial effusion and cardiac tamponade (30,32,83). It is paramount that clinicians are aware of CanL being also a potential cause for pericarditis and pericardial effusion. Pericarditis and myocarditis would be suspected, as it has been reported in some cases (31,32), to often occur in combination, but this logical assumption has not been thoroughly investigated.

General biomarkers in CanL

When a framework is used to evaluate the general utility of biomarkers the following clinical applications have been suggested (135): detection of sub-clinical disease, diagnosis of acute or chronic syndromes, risk stratification of patients, monitoring disease progression, response to therapy and selection of therapy. Biomarkers may also be used in the investigation of pathological or pathophysiological mechanisms of a disease without any specific clinical purpose. When all this is extrapolated and specifically postulated for CanL several general aims may be established. Those include a biomarker which can contribute to the diagnosis of the disease (particularly of borderline or occult cases), to understand aspects about the disease and the involvement of a particular organ or system, to aid in the evaluation of response to treatment and/or disease relapse, and to help with disease prognostication and stratification or staging of the disease (41,43). As new biomarkers emerge, the first two aims may be evaluated with cross sectional studies to enable a marker to qualify as useful and once this is the case longitudinal studies are launched afterwards (41). The more studies available

and the longer a marker has been available the more likely it is that its value has been characterised (41).

The word biomarker often refers to blood borne factors or biochemical marker testing as they often fulfil several other characteristics which makes a biomarker useful and practical. Those are therefore the main field of study in this literature review and Doctoral Thesis. Urine biomarkers will also be discussed. A good biomarker should ideally be easily assessed, widely available and at a realistic cost, which would allow its widespread use. An easily assessed biomarker refers to the ability of collecting it in an easy and quick manner, such as a blood test, be routinely measure at accessible laboratory, or in house, and ideally be transported in a non-complex and inexpensive way.

Diagnostic biomarkers in CanL

Routine diagnostic tests used for disease or parasitic detection are also considered blood borne biomarkers. They fulfil several of the aims and ideal characteristics proposed above (3). Serology, detection of specific serum antibodies (IgG) in serum, is essential for the diagnosis, and for establishing a prognosis in CanL, although there is no detection of the actual *Leishmania* parasite and there is potential for cross-reactivity with *Trypanosome* (3,5). It is well known that high antibody titres correlate with high parasitism and disease (136) and most dogs show detectable concentrations of anti-*Leishmania* IgG in serum between 3 and 4 months after infection (137). The presence of anti-*Leishmania* IgG in serum above the cut-off is estimated to pre-date clinical signs by 2 to 5 months (137). Qualitative methods, such as the immunochromatographic Dual Path Platform (DPP), Kalazar Detect™, SNAP® *Leishmania*, Speed Leish K™ and WITNESS® *Leishmania*, have the advantage of being rapid in-clinic tests but provide only positive or negative results and have variable sensitivities and performance,

with risk of false negatives (3,4,138). Also, a positive result needs to be further evaluated by quantitative serology (3,5). Quantitative serology can be a definitive diagnostic test as the presence of high antibodies levels in the presence of compatible clinical signs and/or clinicopathological abnormalities are conclusive of clinical CanL (3). Immunofluorescence antibody test (IFAT), with an end-point titre, and enzyme-linked immunosorbent assay (ELISA), with optical density reading, can provide quantitative assessment of antibodies (3,5). Following quantitative serology a classification of antibodies as negative, doubtful, low, medium or high is provided (3,5). Quantitative serology is a key parameter in the LeishVet staging. Dogs with negative to low antibody levels are classified as stage I, those with low to high titres as stage II, and those with moderate to high titres as stage III or IV (3). However, interpretation of serology results should be carried out in conjunction with clinical signs and other tests (3,5). Performance, accuracy and cut off depend on the laboratory and differences between laboratories exist (3). The sensitivity of IFAT has been estimated as 90% in symptomatic patients and 31% in asymptomatic dogs (139). The specificity was 98% and 97% in non-endemic and endemic areas, respectively (139). IFAT is considered the reference method (43) although sensitivity and specificity for ELISA are also very high, especially when recombinant proteins are used as antigen (43). Different tests use different soluble, crude and/or total antigenic extracts as their target and, their sensitivity and specificity, although usually high, can vary (75,140). In recent years, immunoproteomics have been studied to develop new diagnostic targets and improve tests performance (46). Even if serology titres are usually elevated during infection, low levels in dogs with clinical suspicion of CanL can occur, and these patients may require further work up with other diagnostic methods such as cytology or PCR of lymph node or other tissues (3). Vaccination may pose a problem when

using serology to identify dogs with CanL infection (75,141). The Brazilian vaccines Leishmune® (Zoetis) and Leishtec® (Hertape Calier Saude Animal) and the European vaccine CaniLeish® (Virbac) may lead to detection of vaccinal antibodies with routine tests (75,141). Leishmune®, withdrawn from the market in 2014, led to detection of vaccinal antibodies by DPP, ELISA and IFAT during the initial 45 days after vaccination (75,141). Leishtec® leads to detection of vaccinal antibodies by ELISA (75,141). In the case of CaniLeish®, ELISA and IFAT may detect vaccinal antibodies but they are detected very rarely by the qualitative rapid test Speed Leish™ (75,141). The European vaccine Letifend®, on the contrary, does not appear to lead to detection of vaccinal antibodies by quantitative tests (IFAT and ELISA) or by rapid qualitative tests (75,141). Therefore, when using serology as a diagnostic biomarker it is fundamental to be aware of vaccine history. Moreover, the effect on vaccination in most biomarkers routinely used in CanL, or under investigation, has not been established. It would be warranted to document previous vaccination history in any research carried out in dogs with CanL in the field of biomarkers.

Serology is therefore an excellent biomarker for several of the potential general aims that we have previously discussed, such as a being a reliable diagnostic tool. Its use as a prognostic factor prior to treatment has not provided consistent results (142) although it is, together with clinical signs, creatinine, and UPC, an important semi-quantitative biomarker for Leishvet disease staging (3). There is also controversy about another potential use: serology as a tool for monitoring CanL treatment (3,4,19,74,86,143–145). A decrease in antibody titres certainly occurs over time and values consistent with simple exposure (<4-fold the threshold value of the laboratory) are expected over time (135,143). However, for dogs living in endemic areas a complete disappearance of anti-*Leishmania* antibodies is unlikely (74,135,143) which may

in some cases complicate treatment follow up. Moreover, serology is indeed not considered a reliable parameter to monitor treatment efficacy in the short or mid-term, as a distinguishable decrease in titres may only be observed 6-12 months after initiation of therapy (19,135,143) and may remain high for years in a minority of individuals (19). A recent study showed however that the use of an end point sera dilution ELISA method may allow earlier detection of reduction of *Leishmania infantum* antibodies after treatment (74). Monitoring serology in the long term is more widely advocated as an increase in titre should be considered to be indicative of a lack of response to treatment or, if therapy was interrupted, suggestive of recurrence of the disease (3,5,19). Monitoring serology every 3-6 months during the first year and every 6 months afterwards, has been advice (3). The relationship between IgG levels (serology titres) and CanL myocarditis or cardiac biomarkers has not been thoroughly investigated. An exception is a study in which cTnI correlated weakly with positive serology titres in a cohort of dogs with CanL (24).

PCR techniques to identify the presence of the organism in CanL have been discussed in depth in this manuscript in a previous section. In this context, detection of PCR in blood would also be considered a (molecular) blood-borne biomarker, and it is used clinically for the diagnosis and monitoring of CanL (92). However, its sensitivity is considered lower than PCR evaluated in tissues such as lymph node or bone marrow (3–5,17,19,85–87,146) and, its use as a clinical diagnostic marker is often not necessary in a dog with appropriate clinical signs, positive serology and detection of *Leishmania* organisms in tissue by cytology, histopathology or PCR (17,92,122). Sensitivity of blood PCR has been very variable among different studies (11-90%) (89,97,138,146–148) and dogs may show positive blood PCR with negative serology (93,138,147), highlighting the diagnosis conundrum that CanL may present in some cases. The

robustness of the test is ultimately not easy to assess in CanL considering the presence of long term asymptomatic or mildly symptomatic dogs with CanL, transient not clinical disease which may show a positive PCR in healthy dogs in endemic areas, and the variable results among different techniques and laboratories (3,87,89,92,93,95,138,146–148). Measurement of qPCR is however a useful routinely used biomarker for evaluation of treatment success, particularly in the mid and long term (17,85,92,149). If treatment has been successful, qPCR in blood should show a clear reduction of DNA copies after 3-6 months, with disappearance after 12 months (17,92,149). Monitoring treatment by evaluating parasitic load by qPCR in lymph node has also been explored and this technique yielded better results than serology in differentiating dogs with partial response vs dogs with total response (86). There are no studies investigating PCR techniques in the context of CanL myocarditis. The MPL identified by qPCR in dogs with CanL and CanL myocarditis, and the relationship between blood qPCR and CanL myocarditis would be of clinical and pathophysiological interest but have not yet been studied.

Further to biomarkers of disease diagnosis, other biomarkers in CanL could be grouped in three main categories: those of general inflammation, biomarkers of renal damage and/or renal function, and other type of biomarkers (including cardiac biomarkers).

Biomarkers of general inflammation

Within the group of inflammatory biomarkers, we have already reviewed the detection of specific *Leishmania* antibodies (serology). Other important biomarkers within this group include serum protein electrophoresis, acute phase proteins (APP), white blood cells alterations, and circulating immune complexes (3,17,19,43). The relationship between all these inflammatory biomarkers and CanL myocarditis, MPL or cardiac biomarkers has not

been studied. Serum protein analysis, and more specifically electrophoresis, may reveal changes early during the course of CanL, and is considered an extremely sensitive clinical test (3,17,19,43,90). Total proteins and globulins are frequently increased which has been shown to correlate with the severity of clinical score (3,19,44,70,150,151). Albumin is frequently decreased due to proteinuric nephropathy which leads to a decreased A/G ratio (3,17,19,44,70,151). The gammopathy in CanL is typically polyclonal but biclonal or monoclonal patterns may be occasionally observed (1,3,17,19,43). The typical profile displays hypoalbuminaemia, moderate increase of α_2-globulins and marked increase in γ-globulins (3,17,19). The α_2-globulins incorporates most APP while the γ-globulins consist of circulating antibodies, immune-complexes and other molecules with γ-globulin-like mass and charge (17). Electrophoresis changes, and in particular the decrease in albumin/globulin ratio, occurs early in the disease and is considered one of the most sensitive tests for CanL (17,70,151). Hypoalbuminaemia is a negative prognostic factor and is associated with advanced renal disease and Leishvet staging (3,17,142). Protein electrophoresis is also a powerful biomarker for evaluation of treatment response. It is likely the most widely used tool to guide therapy in the short term (17,19,143). Normalisation of α_2-globulins and γ-globulins may not occur at least until 3-4 months following treatment (17,43,143), however, changes start to become evident after 2-3 weeks for α_2-globulins and 4-6 weeks for γ-globulins (17,143,146,152). The type of therapy however may affect timings and should be taken into account when evaluating the expected short and mid-term improvements in electrophoresis (145,149). Globulins may take longer to decrease if miltefosine is used versus meglumine antimoniate (149). Ultimately, albumin and A/G ratio are a less reliable marker for treatment monitoring

as albumin may remain low, in spite of disease remission, due to persistent glomerular damage (17,19,43).

APP are an early non-specific defence mechanism to local or general disturbances in homeostasis attributable to infection, inflammation, trauma, neoplasia or immunological disorders (49). Therefore, when used as a biomarker in CanL, they are non-specific in terms of disease diagnosis. They are however valuable in CanL as they are more sensitive than globulins or total proteins for early disease detection (153), and for the assessment of disease severity, response to treatment, and prognosis (153,154). In an experimental study (155) APP rose 2 months post-infection and clinical signs were preceded by APP rise for approximately 2 months. As discussed above, collectively, α_2-globulins are currently the best short-term biomarker for response to treatment, and relapse episodes, monitoring (17,143,146,152). Some APP have also been studied individually in CanL (49,153–156). Those include C-reactive protein (CRP) (49,153,154,156,157), haptoglobin (153,156,157), ceruloplasmin (153,157), ferritin (49,156), transferrin (49) and serum amyloid A (SAA) (155,157). CRP is usually elevated in CanL. High concentration are observed at diagnosis and usually normalise within 1 month after treatment (152,155,157). Unlike most inflammatory biomarkers, CRP is elevated early in the disease and it appers to be also elevated in asymptomatic cases (49,153,155,157). CRP levels are however not increased in all dogs as some may show normal values at diagnosis (156). A trend towards decreased values immediately after beginning of treatment is usually highly suggestive of successful therapy (19). CRP has been shown to be associated with short term prognosis (49), to have negative correlation with iron and transferrin (49), and to have a positive correlation with ferritin (49). The latter supporting the rational of inflammation in CanL being a contributor to the commonly observed anaemia of chronic disease (49). Ferritin

has also been shown to be a prognostic factor for short term survival in CanL (49). Ceruloplasmin and haptoglobin have been studied individually and were shown to be markedly increased in dogs with CanL, although a difference between symptomatic and asymptomatic dogs was not observed (153). Ceruloplasmin was shown to decrease reliably early after treatment but less consistent results were observed for haptoglobin for this purpose (157). SAA may also be a less favourable biomarker, when used in isolation, as it has shown conflicting results (155,157). In one study SAA increased in some dogs with CanL but remained within normal values in other patients in which there was an elevation of other APP (157).

In summary, although APP are generally considered very valuable biomarkers in CanL, variable and sometimes conflicting results have been obtained among different studies. Further studies have been advocated (156). A biomarker related to APP is paraoxanase (PON-1). PON-1 is an enzyme involved in the metabolism of oxidised lipids and is considered an inflammatory and oxidative stress marker (154,156,158). In general, PON-1 activity decreases in dogs with inflammatory diseases and therefore is considered a fast negative APP (152). Mixed results for both diagnosis and treatment have been shown in dogs with CanL, likely due to individual variation and disease severity influencing the degree of oxidative stress (152,154,156,158). PON-1 does not appear to be altered, and/or fluctuate consistently, before or after treatment in CanL (152,154,156,158).

White blood cells alterations are common in CanL. Neutrophilia is frequent in dogs with CanL (3,17,19,44,159) and is associated with the inflammatory response resulting from the presence of parasites in many organs (17). Other less commonly observed changes include monocytosis, lymphopenia, eosinophenia and leukopenia (3,17,19,44). Lymphopenia at

presentation has been associated with worse prognosis (142). Peripheral blood flow cytometry of lymphocytes, and in particular the CD4/CD8 ratio, has been explored in dogs with CanL, with the hypothesis that a dog with a positive PCR and a low CD4/CD8 ratio may be expected to be more predisposed to clinical signs (160). However, great variability and overlap was observed (160–162). The CD4/CD8 ratio has nevertheless been advocated as a potential marker for disease monitoring (17,160,162). Its limited availability and its cost may however preclude the use of this biomarker.

Among inflammatory biomarkers, other less common or readily available biomarkers have also been studied. Direct measurement of circulating immune complexes assessed by ELISA assay have been investigated (48,106). Circulating immune complexes were increased in more advance stages of the disease and correlated with serology and blood qPCR levels (48,106). Reduced serum activity of adenosine deaminase and butyrylcholinesterase (163) and increase expression or activity of leptin (164), and matrix metalloproteinases (165), have also been reported. Considering the complex immunopathological mechanisms in CanL, it is not surprising that inflammatory cytokines in peripheral blood have also been used in research and evaluated clinically in dogs with CanL (43,149,166–170). Conflicting results have been observed when evaluating prognosis for most of cytokines including IFN- γ, IL-4, IL-10, IL-2 and TNF-α (17,43,149,166–171). However, tracking IFN-γ and IL-2, the latter also in the short term (172), may be useful for clinical assessment of response to treatment (17,43,173). Overall, most of these inflammatory biomarkers have been (and will likely be in the future) employed in research investigating the pathological and immunopathological mechanisms of CanL. The costs and availability of these tests will possibly limit their routine clinical use, which is an important aspect of a good clinical biomarker. Overall, to establish the potential

usefulness and characteristics of these non-specific inflammatory biomarkers for diagnostic or prognostic purposes in CanL require further studies. The association of these biomarkers with CanL myocarditis or cardiac biomarkers has not been explored.

Biomarkers of renal disease and renal function

The presence of renal disease is one of the most important features of CanL (3,19). Membranoproliferative, membranous, mesangial and focal segmental glomerulonephritis, and acute or chronic tubulointerstitial nephritis, can develop and, in advanced cases, will lead to severe or end-stage CKD (3,19,107,174). Advanced renal disease is the major cause of death or reason for euthanasia in dogs with CanL (19,69,175) . Biomarkers of renal damage and renal function are therefore the other core group of biomarkers (together with those of inflammation) used in CanL (3,19). Serum creatinine and UPC are routinely measured in CanL. These biomarkers are strong markers of survival and are currently the cornerstone for disease classification (3,19,142). The relationship between myocardial damage (cTnI levels) with creatinine and UPC, has been investigated(24). A positive moderate correlation between UPC and cTnI was observed but significant correlation with creatinine was not detected (24). Most dogs in this study appeared to have (extrapolating the information from the available data) mild to moderate (Leishvet I and II) rather than severe or very severe (LeishVet III and IV) disease (24). It would be of particular interest to determine whether the myocardial damage in CanL, in cases with azotaemia and/or elevated UPC, is due to renal disease (as it has been reported for idiopathic CKD (176–178)), or if this is of lesser or negligible importance in the setting of parasite-triggered myocarditis. Several hypotheses have been drawn for cTnI to be elevated in renal disease including subclinical ischaemia, left ventricular hypertrophy due to hypertension and uraemic myocarditis (176–178). Decreased renal clearance is not believed

to be a major factor (176). Further evaluation of CanL myocarditis with markers of myocardial damage and myocardial stress at different stages of renal disease, and therefore at different Leishvet staging, is warranted.

Other less frequently used blood or urine renal biomarkers have been evaluated in CanL. Those include serum cystatin C (42), serum symmetric dimethylarginine (sSDMA) (47,179,180), serum asymmetric dimethylarginine (ADMA) (180), plasma arginine (180), urine cystatin C (181), urine N-acetyl-beta-D-glucosaminidase (NAG) (181), urine gamma-glutamyltransferase (uGGT) (42), urinary GGT/urinary creatinine ratio (uGGT/uCR) (42) and renal artery resistivity index calculated by Doppler ultrasound (RARI) (42). Serum cystatin C, a marker of glomerular filtration rate was not found to be useful for the detection of early disease (42). In a study, sSDMA was found to be elevated in 83% of dogs with azotaemia and in 26% of dogs without azotaemia, and to have a moderate positive correlation with proteinuria (47). Two studies showed different results in terms of sSDMA elevation according to the LeishVet classification (47,180). Dogs in Leishvet stage II had elevated sSDMA in one study (47) but not in another (180), and differences in sSDMA concentration between dogs in Leishvet stage III and IV were found in one (180) but not in the other study (47). Plasma arginine was low and ADMA high in dogs with CanL but differences among Leishvet groups were not observed (180). The urinary marker uGGT and uGGT/uCR, a marker of tubular injury, was able to detect early renal disease in dogs with CanL prior to azotaemia, and was therefore able to identify tubulopathies before (due to the disease) and after leishmanicide therapy (due to the known potential drug related cytotoxic effects) (42). RARI may only be a marker of very severe disease. It was shown to be consistently elevated only at Leishvet stage IV (42).

As two robust markers of renal disease exist and they are the cornerstone of the existing classifications, it is not surprising that an association of new renal biomarkers and the existing ones, and with the LeishVet classification, exist. However, it remains to be determined whether they may add extra information to the current biomarkers in use. In this context, detecting earlier renal disease may be useful, for example, in further substaging dogs in LeishVet II stage and refining prognosis. Ultimately, they may be used as pure research biomarkers when investigating the pathophysiological mechanism of CanL. Further studies are required.

Other biomarkers

Beside inflammatory and renal biomarkers, other type of blood-borne biomarkers have been studied. Attention has been brought to some of the routinely measured parameters in haematology or biochemistry. Anaemia is commonly observed in CanL with the most likely type being a mild to moderate normocytic-normochromic anaemia as observed in anaemia of chronic disease. Thrombocytopenia is also seen due to immune-mediated peripheral destruction of circulating platelets or platelet consumption, and hypercoagulability may also be seen in cases with associated glomerulonephritis (3,17,44). Usually, these haemostatic markers are used in clinical situations as initial general tests which leads to including CanL in the differential diagnosis, or as part of treatment guidance. In the latter case resolution of the abnormal findings occurs in the mid to long term and it is a marker of response to therapy (3,17,19,44). Other biomarkers studied represent abnormal activity or function of other organs or systems affected in CanL (50). Cardiac biomarkers are included in this group and will be discussed in depth in the next section. An increase of the activity of skeletal muscle enzymes creatinine kinase (CK) and lactate dehydrogenase (LDH) has been documented in

CanL (50). Liver disease is known to develop in CanL (51) and can contribute to clinical signs. Dogs at different stages of clinical disease have different severity and type of histopathological hepatic lesions (51). Liver enzymes are a routine clinical marker to evaluate the degree of liver involvement, and in selected cases, the response to treatment, and remission of the liver damage, can be monitored (17,19,44,51). AST and ALT levels have indeed been shown to correlate with the degree of liver inflammation and clinical staging (51). Pancreatitis is frequently observed in humans with CanL after therapy (182) and canine pancreatic lipase (cPLI) was evaluated in one study. Contrary to what has been reported in humans, an increase in cPLI was not observed before or after antimonial therapy, indicating the absence of pancreatitis in CanL (109). Activity of the brain isoenzyme of CK has also been evaluated in dogs with CanL and neurological clinical signs, and was shown to be increased in this population of patients (183).

Cardiac biomarkers in CanL

Scientific evidence of the presence of myocardial damage and myocarditis in dogs with CanL has been presented in this review. However, many questions remain unanswered. Those would include the true prevalence, distribution and clinical effects of CanL myocarditis across Leishvet stages, its association with MPL, and the effects of factors such as uraemia, anaemia and systemic arterial hypertension in this myocardial alterations. If we consider how complex and invasive obtaining myocardial biopsies is, the exploration of cardiac biomarkers as a research or clinical tool to investigate all these aspects emerges as a logical and important endeavour. I have discussed above the use of many inflammatory and renal biomarkers in CanL. Cardiac biomarkers are a different type of biomarker, and therefore, may also add

valuable distinct information in CanL. They may act, for example, as research biomarkers for the specific investigation of myocardial damage at different disease scenarios. Those may include the identification of CanL myocarditis development, the detection of general disease progression or MPL, the investigation of whether some subpopulations may be more affected by CanL or CanL myocarditis, and the investigation of myocardial damage when different treatments with potential myocardial toxicity are applied. Ultimately, if cardiac biomarkers show consistency in identifying the nature and extent of CanL myocarditis, they may prove useful as an additional general disease biomarker to help tailoring prognosis, substaging the disease, or evaluating response to therapy. Cardiac biomarkers usually fulfil the characteristics described above for a good biomarker, such as being easily obtained by a simple blood sample, readily available, and at a reasonable price.

A wide range of cardiac biomarkers exist. They include markers of myocyte injury such as cTnI, cardiac troponin T and CK isoenzymes; markers of myocyte stress such as natriuretic peptides or adrenomedullin; markers of remodelling such as procollagen type III amino-terminal peptide or tissue inhibitors of metalloproteinases; markers of endothelial dysfunction such as nitric oxide metabolites or vascular adhesion protein 1; and neurohormonal markers such as endothelin 1 or big endothelin 1 (41). We focus our review and the research work of this Doctoral Thesis on the two most widely used and available cardiac biomarkers in dogs, cTnI and NT-proBNP, and in an emerging biomarker in canine cardiology, HFABP.

Cardiac troponin I

The myocyte consist of contractile bundles called myofibrils (117). Each myofibril is composed of numerous contractile units called sarcomeres which are joined end to end (118). The filamentous partitions in between sarcomeres are called Z-lines and between these Z-lines

two forms of filaments exist, the thick myosin and thin actin (117,118). When these two proteins bind, and form cross-bridges, contraction ensues (117,118). Troponins are regulatory proteins of the myosin-actin interactions and are therefore a fundamental part of the contractile apparatus of cardiac muscle, although they can also be found in skeletal muscle as an specific isoform (55,117,118,184). The troponin complex includes three proteins that, along with tropomyosin, are found in the thin filaments (118). Troponin I is called that way because of its inhibitory functions in the actin-myosin interaction (118). Troponin C belongs to the family of EF-hand proteins which includes the myosin light chains and calmodulin (118). It contains four peptide chains with two alpha-helical regions separated by a short non-helical sequence (118). Troponin C binds to calcium when the concentration of this ion in the sarcoplasm rises secondary to its release from the sarcoplasmic reticulum storage (117,118). This will be the trigger for the molecular conformational change that shifts tropomyosin-troponin complexes deeper into the F-actin groove, thereby exposing the myosin-binding sites of the F-actin and therefore initiating the myosin-actin crossbridges (117,118). Troponin T is the largest of the three components and mediates allosteric effects within the thin filament which influences, regulated by protein kinase A (PKA) and protein kinase C (PKC), calcium sensitivity and tension development (117,118). While troponin T has also been used as a marker of cardiac injury, the most widely used troponin in canine and feline medicine is cTnI (55,184) which is the troponin used as a biomarker in this research work.

There are three isoforms of troponin I (55,184). Two are present in skeletal muscle and one, cTnI, in the heart. cTnI is only present in the heart and with a weight of 24.000 Da is larger than the skeletal muscle isoforms (55,118,184). The full gene sequence of cTnI in dog and cats

has been determined and it is very similar to human cTnI genes (95% homology for both dogs and cats) (185). Foetal tissue does not express cTnI, unlike the skeletal isoforms (55,184). cTnI inhibits actomyosin AT-Pase and prevents the structural interaction of myosin with acting-binding sites (55,117,118,184).

Troponins in general, and cTnI in particular, are considered a leakage marker. Damage to the myocyte leads to membrane integrity disruption and leakage of troponins to the systemic circulation, where they can be measured taking a blood sample (55,184). Apoptosis (programmed death) and physiological cardiomyocyte turnover do not lead to loss of membrane integrity and do not appear to be major contributors to cTnI elevation (186,187). The majority of cTnI is bound to the contractile apparatus (structural pool) but a small percentage of the protein, approximately 2-4%, remains free in the cytosol (cytosolic pool) (55,184). The myocyte release and subsequent blood elevation of cTnI levels has two phases (55,184). When the cytosolic pool is released the surge of cTnI is of low blood concentration, rapid in increase and short lasting, while the release of the structural pool leads to a much higher cTnI serum concentration, with slower release and more sustained blood concentrations (55,184). Myocardial necrosis leads to cell death and consistent release of cTnI but other pathophysiological mechanisms such as increased permeability, intracellular proteolysis or vesicle formation may also lead to cTnI release and elevated values (55,184). The number of cells affected, and the severity of the insult, determines the concentration of cTnI (55,184). Cellular necrosis and irreversible cell damage leads to the highest increases in cTnI, as the structural pool is overwhelmingly exposed (55,184). Reversible cellular injuries on the contrary, such as loss of permeability, may expose primarily the cytosolic pool and lead to lower cTnI increases (55,184). Although variation depending on the nature and extent of the

insult exist, cTnI elevation is usually seen following an acute insult (myocardial infarction) after 2-3 hours, peaks at 24-48h and remains elevated for 10-28 days (188). Persistently increased cTnI blood concentration suggest active cardiomyocyte damage (184,189–191). The pathway of elimination of cTnI is not fully understood but it is suspected to be carried out by the reticulo-endothelial system (192,193), although renal clearance of smaller degradation products may also occur (194). However, increased concentrations of cTnI in humans and dogs with CKD have been robustly shown to occur due to cardiac release, and not due to decreased renal clearance (176,195–197).

Troponin levels are usually determined using ELISA. There are several assays available for cTnI from a variety of manufacturers which require validation in dogs prior to its use. The Siemens Immulite 2000 (198–200), the Biosite Triage meter (52), the Access AccuTnI^{TM} system (63) and the Stratus CS stat fluorometric analyzer (52,56), have been frequently used in dogs and cats. Normal values for these assays closely correlate but each assay must have its own range established. Older dogs and dogs of breeds Greyhound (201) and Boxer (53) have generally higher cTnI normal values. High intensity moderate duration of exercise however, did not raise cTnI levels relevantly in sledge dogs (202). Two high-sensitivity assays have also been frequently used in dogs: the Siemens ADVIA Centaur CP TnI-ultra, a 3-site assay for cTnI analysis that utilizes direct chemiluminometry (200,203), and the Beck-man Coulter Access hsTnI, also a two-step immunoenzymatic assay (53,58,204). Biological variability has been observed in healthy dogs and dogs with MVD with high-sensitivity assays but not with the standard assay, which lacks sensitivity to detect low concentrations (205). High sensitivity assays have an imprecision below 10% at the 99% percentile of a healthy population and are able to measure concentrations below the 99% percentile, but above the detection limit, in

more than 50% individuals (ideally more than 95%) (55,184). Serum and plasma values correlate but a tendency toward slightly lower serum concentrations has been reported (55,184). Troponin has long-term stability at -70 to -80°C but is not stable at room temperature, refrigerator temperature, or at -20°C (55,184). Short term storage up to up to 5 days at 4°C and up to 3 months at -20°C is however acceptable. It has also been shown to be stable in serum undergone up to 5 freeze-thaw cycles (55,184).

When cTnI is examined as a specific biomarker of primary cardiac disease in dogs, it is one of the most explored and used biomarker for both clinical and research purposes. Across a variety of cardiac diseases, cTnI above 1 ng/mL has been shown to be associated with poorer prognosis and shorter survival times in dogs (191). cTnI has been extensively studied in dogs with dilated cardiomyopathy (DCM) and has emerged as a very useful screening biomarker for asymptomatic dogs and a robust prognostic marker (58,59,199,200,204,206). Boxer dogs with arrhythmogenic right ventricular cardiomyopathy showed a significant increase in serum cTnI concentration compared to normal dogs and cTnI concentration correlated with the number of ventricular premature complexes in 24 hours and the grade of arrhythmia (53). In dogs with MVD cTnI has shown variable results, with some studies showing significant differences at different stages of the disease (199,204,207–209). Serum cTnI was also a prognostic factor for dogs in the preclinical and clinical stages of MVD (210–212). Serum cTnI has also been shown to be elevated in dogs with precapillary and postcapillary pulmonary hypertension (secondary to MVD) and to have modest positive correlation with estimated pulmonary artery pressures (208). Serum cTnI was however not a useful test, because of considerable overlapping, to differentiate between dogs having collapse due to cardiogenic syncope and those having seizures due to neurological disease (54).

When examining inflammatory cardiac diseases, a mild negative association of cTnI with survival was observed in a cohort of dogs with myocarditis and severe bradyarrhythmia requiring pacemaker implantation; dogs with higher cTnI had a higher risk of sudden death (213). In dogs with endocarditis, a cutoff of 0.625 ng/mL has been suggested, with a specificity of 100% and a sensitivity of 52%, to differentiate dogs with the disease vs dogs with MVD or immune-mediated diseases (214). Among infectious agents causing myocarditis and/or myocardial damage, either as a primary pathology or as a part of a multisystemic disease, cTnI has been useful for the diagnosis of myocarditis associated with agents such as *Bartonella spp (28,215) or Trypanosoma cruzi (128),* eosinophilic myocarditis (216) *,* and myocarditis in dogs with bradyarrhythmias (57). Serum cTnI was seen to be elevated in dogs with parvovirus. Dogs who died due to the disease had higher cTnI concentration than those who survived (217,218). In dogs with canine babesiosis cTnI was significantly higher in cases with complicated disease and in dogs that died, and correlated with histological changes (219). In dogs with leptospirosis, cTnI showed higher values when observed in dogs with more severe disease and emerged as a prognostic factor (220). Canine ehrlichiosis has been shown to lead to very high cTnI levels in some dogs, particularly in those with systemic inflammatory response syndrome, although it did not show prognostic value (221–223).

Dogs with systemic disease leading to myocardial ischaemia or myocardial inflammation may also show cTnI elevations acting as a biomarker of associated myocardial injury. This has been shown in cases of gastric dilation volvulus (224,225), pancreatitis (226), immune mediated haemolytic anaemia (227), blunt trauma (228), heat stroke (229) and adder bite (230,231). An increase in cTnI is also a negative prognostic indicator for death in critically ill dogs independent of the underlying disease (232–234). Dogs with higher cTnI following blunt chest

trauma or adder bite are at higher risk of clinically significant arrhythmias (228,230). In dogs receiving chemotherapy with doxorubicin, elevation of cTnI has been used to evaluate the degree of myocardial damage, which can be observed with early doses but was particularly consistent after cumulative doses of 120mg/mg^2 (235). In dogs with CKD, elevation of cardiac biomarkers in general, and cTnI in particular, is frequent (176,177). Contrary to NT-proBNP, plasma volumen and PCV (the degree of anaemia) were not independently associated with cTnI levels in dogs with CKD (176). Considering the frequent presence of azotaemia, systemic hypertension and anaemia in dogs with CanL (3,17,19,44), selecting the best cardiac biomarker for CanL requires further elucidation.

Prior to the beginning of this Doctoral Thesis two studies explored the use of cTnI in CanL (22,24). One study (24) evaluated cTnI for the first time in dogs with CanL. Dogs were not classified by the Leishvet (or any other) classification, but at least 22% of cases (9/40 cases) were likely in severe or very severe stages as they had increased in serum creatinine, and some dogs had hypoalbuminaemia (24). However, the population likely comprised mostly dogs in LeishVet stage I and II (24). A correlation (moderate) was observed between cTnI concentration and decreased albumin concentration and with increased UPC (24). This pointed towards myocardial damage occurring as the disease became more advanced. However, a correlation of cTnI with creatinine or a difference in cTnI concentrations between dogs with elevated or not elevated creatinine was not seen (24). In this study the median cTnI was not elevated 0.043 ug/L (ref <0.060) but marked elevations were present in some dogs as the results ranged from 0.00-3.47 ug/L and 40% of dogs (16/40) had elevation of cTnI (24). A second study investigated the possibility of myocardial injury secondary to CanL therapy with N-methyl-glucamine-antimoniate (22). Antimonial toxicity is reported in humans

undergoing treatment for leishmaniosis (236,237). ECG findings and cTnI were used as markers of myocardial injury in this study. We have already discussed the ECG findings of this study above in this literature review. The majority of dogs in this research (26/28; 93%) were dogs with mild (LeishVet I) or moderate (LeishVet II) stage of the disease, and only 2/28 dogs (7%) were in Leishvet III, with no dog within the LeishVet IV stage (22). Prior to therapy the median cTnI value was normal (0.1 ng/mL; range 0.1-0.35 ng/mL; reference <0.2 ng/mL) and only 1 case (the LeishVet group not specified) had elevated values (presuming 0.35 ng/mL by examination of the general data) (22). After therapy (60 days after the initial sample), no differences in median value of cTnI were seen (0.1ng/mL; range 0.1-1.5ng/mL) which suggested the absence of relevant myocardial injury caused by N-methyl-glucamine-antimoniate in dogs with mild to moderate CanL (22). However, the highest cTnI value of the range of the post-treatment result was markedly elevated, and different to the initial value, which indicated that at least one dog had a severe cTnI elevation (22). It was not reported in this study, how many dogs had abnormal cTnI results in the post-treatment sample (22). It remains possible that a subpopulation of dogs showed fluctuating cTnI levels due to the disease, or that post-treatment myocardial injury occurred in some dogs, particularly those at more advance stages of CanL and with pre-existing CanL myocarditis.

During the period of publication of the studies comprising this Doctoral Thesis an additional research paper was published(67). A population of 18 dogs with CanL was studied and grouped into asymptomatic (4/18), oligosymptomatic (6/18) and polysymptomatic (8/18) individuals (67). Serum cTnI elevation was reported for all dogs in this study, although moderate to severe increases were not apparent with a median of 0.220ng/mL (range 0.150-0.510 ng/mL). Serum cTnI was higher in polysymptomatic or oligosymptomatic dogs than in

asymptomatic dogs, contributing towards the hypothesis that myocardial damage occurs at more advanced general disease stages. In this study, there were likely more dogs in the severe group (Leishvet stage III) than in the previous studies, however, definitive classification cannot be made by the provided data (67).

NT-proBNP

B-type natriuretic peptide is a hormone initially elaborated in the atrial and ventricular myocardium as long peptides named pre-proBNP (41,118,238,239). Removal of a signal peptide yields a shorter peptide, proBNP (41,118,238,239). ProBNP, after adequate stimulus, undergoes proteolytic cleavage into the mature C-terminal fragment (active hormone) and N-terminal fragment (NT-proBNP) which are release together into the circulation (64,118,238,240). Natriuretic peptides decrease cardiac preload, supress renin and aldosterone secretion and exert natriuretic functions (117,118). The principal stimulus is an atrial or ventricular stretch, and they are considered biomarkers of increased myocyte stress, although can also become elevated due to myocardial injury (241). NT-proBNP, the nonactive amino-terminal portion of the BNP, is the most commonly used biomarker to evaluate BNP activity, as the C-terminal´s half´s life is 5 to 15 times shorter than that of NT-proBNP (64,242), and the C-terminal is particularly sensitive to collection and storage methods (240).

NT-proBNP testing has evolved over time. Several kits and different generations were used up to 2013 when the Cardiopet proBNP, from IDEXX laboratories, was launched and has become the principal test employed in dogs (62,64,238,240,241,243–245). Quantitative values from previous tests cannot be directly compared with results of the current test (64,203,212,246,247). The NT-proBNP molecule degrades when stored at room temperature and mishandling of serum or plasma samples can lead to inaccurately low NT-proBNP

measurements (243,248). Recommendations on how to keep and submit the sample evolved initially and were established and have been stable for the past decade. A EDTA plasma sample, which should be separated as soon as possible, is preferred and gross hemolysation should be avoided (64). The sample can remain up to 7 days at -2 to -8°C without altering results but should be kept frozen (ideally at -80°C) after this period (245). Biologic variability, such as week to week variation in the same individual (205,249), and breed variation (250), should be considered when interpreting results.

NT-proBNP has been used in dogs as a diagnostic and staging biomarker in dilated cardiomyopathy (DCM) (58,60,206,251–253) and MVD (65,66,205,211,212,247,254–258). It has been shown to be a useful ancillary tool for disease screening in DCM (58,206,251–253), to evaluate the risk of developing congestive heart failure in MVD (66,211,259), for overall prognosis in both diseases (58,60,65,206,211,251–254,260,261), and it has been evaluated with promising results for treatment monitoring and therapy adjustment in dogs with congestive heart failure (65,247,254). NT-proBNP assessment has also been suggested as a diagnostic test to be evaluated during exercise testing in dogs with cardiac disease (244). In dogs with pulmonary hypertension, NT-proBNP level has shown to correlate with the severity of precapillary pulmonary hypertension (62). In dogs with canine dirofilariosis, NT-proBNP was consistently elevated in dogs with severe disease and severe clinical signs (often in right sided heart failure) and in dogs with caval syndrome (262).

Many extracardiac factors and diseases may affect NT-proBNP (176,178,239,263,264). This influence may have stronger effects on the measurement of stress biomarkers than in myocardial damage markers, such as cTnI, which require a severe cardiac insult with loss of myocyte integrity to become elevated. Increase in cardiac volume load (in the absence of

underlying cardiac disease) leads to elevation of NT-proBNP (176,239,265). Anaemia may have different effects on the heart depending on its severity, its speed of onset (acute vs chronic) and the concurrent presence of factors leading to plasma volume alterations not due to the anaemia (e.g. loss of plasma through bleeding, renal disease or concurrent dehydration) (266–268). In this context, normal, decreased or increased preload may be observed (266,268,269). Concurrently, peripheral vasodilation, mediated by hypoxia and nitric oxide, and decreased blood viscosity, may lead to decreased afterload (266,270). A hyperkinetic state with an increased sympathetic tone and contractility and increased cardiac output is also often observed in many clinical situations (266). All these mechanisms may have an impact on myocyte stress and can therefore cause increased values of NT-proBNP in dogs with anaemia that are not due to primary cardiac disease (271,272). CKD in dogs leads to an increase in NT-proBNP (176,238,263,273). This increase is not believed to be primarily due to decreased renal clearance (176), as indeed an increase in urine NT-proBNP is observed in humans (274). It has been linked to increased myocardial release due to an increase in plasma volume, disease associated anaemia and potentially uraemic myocardial damage (176,271). Systemic arterial hypertension, often linked to chronic or acute renal disease (275), but also observed with other conditions or as idiopathic disease(275), leads to an increase in afterload and may lead to left ventricular hypertrophy (275,276) and elevations of NT-proBNP (255). Systemic arterial hypertension was however not independently associated with elevation of NT-proBNP in dogs with CKD (176). In a large study of dogs with systemic arterial hypertension (276), 47% of dogs with systemic arterial hypertension had a degree of symmetric or asymmetric increase in left ventricular wall thickening, although it was mild in most cases (276). Blood pressure level did not correlate with the degree of left ventricular wall thickening

(276). Abnormal systolic and diastolic left ventricular function was also observed independently of the presence or absence of hypertrophy (276). Other comorbidities including trauma, neurological disease, neoplasia, pancreatitis or metabolic conditions have also been shown to elevate natriuretic peptides and may complicate the results of this test in primary cardiac conditions (226,264).

Some of the systemic factors discussed and potentially involved in NT-proBNP elevation such as change in plasma volume, anaemia, CKD or systemic arterial hypertension, are often present in dogs with CanL (3,19,21,220) and may affect the use of this biomarker in the disease. No study had evaluated NT-proBNP in CanL prior to the beginning of this Doctoral Thesis. At the same time of the studies conducted in this Doctoral Thesis, two studies were published which evaluated NT-proBNP in dogs with CanL. In one of the studies (67) 18 dogs with CanL were tested, probably prior to any therapy, and all showed elevation of NT-proBNP above the reference given for the used test (800pmol/L; E90485CA, USCN Life Science Technology) with a median of 1160 pmol/L (IQR 986pmol/L-1494pmol/L; range 803pmol/L-2034pmol/L). The population in this study probably comprised dogs from LeishVet I to LeishVet III considering the overall clinical signs, UPC and creatinine reported. Differences in NT-proBNP levels among asymptomatic, oligosymptomatic and polysymptomatic CanL dogs, were not observed. The second study (68) found very different results. This study included 28 dogs with CanL, 7 dogs in each LeishVet group (from mild to very severe). Dogs from stage I to III had normal NT-proBNP values (LeishVet I 67.8±7.5 pmol/L; LeishVet II 140.1±53.7 pmol/L; LeishVet III 224.1±51.3 pmol/L; reference <900 pmol/L). For dogs in LeishVet IV, 4/7 dogs showed elevation of this biomarker, and there was a statistical difference (p=0.001) in NT-proBNP levels between LeishVet IV group and the rest of the groups (1355±791 pmol/L).

In neither of these studies, exploration of the relationship of this biomarker with other clinical variables or biomarkers was carried out.

HFABP

HFABP is a stable intracellular cytosolic myocardial protein with low molecular weight (14-15kDa) responsible for the transportation of lipids within the heart (277). Other types of fatty acid binding proteins are found in other tissues with high levels of lipid metabolism such as skeletal muscle and liver (277). HFABP is present in great abundance in the myocardium and serves as lipid chaperone reversibly interacting with hydrophobic ligands and mediating transport to sites of lipid metabolism (278). It is homogenously distributed within the myocardial regions (subepicardial, mid myocardial, subendocardial) (279). HFABP is rapidly released into the circulation in response to cell damage in myocardial injury (278,280). Its small size and water solubility facilitates rapid diffusion through the interstitial space appearing in the blood stream in humans early after myocardial injury peaking, approximately within 6 hours of the myocardial insult (281,282). It has also been shown to be excreted through the urine in humans. Abnormal renal function may alter renal clearance of serum HFABP and may lead to increased blood levels (282,283). Reference ranges have been established in humans and slight increases with age have been observed (284). HFABP has been used in human medicine as a diagnostic and/or prognostic cardiac biomarker in the diagnosis of early acute myocardial infarction (278), non-ischaemic dilated cardiomyopathy (280) and congestive heart failure (279,285). Most immunoassays used in human medicine are ELISA assays (282) and an ELISA assay has been validated for dogs (61). HFABP was used as a marker for myocardial injury in an experimental study in dogs which evaluated the use of a long-acting beta agonist in a population of healthy young beagles (286). In the clinical

setting, HFABP has shown to be significantly higher in dogs with MVD and dogs with DCM than healthy control dogs (61). Dogs with MVD in stage C showed higher levels than stage B and controls (61). HFABP was also shown to be an independent predictor of survival both in dogs with MVD and DCM (61). HFABP has not been investigated as a biomarker in dogs with myocarditis or with CanL.

HYPOTHESES

The hypotheses behind this research work are:

1. That CanL myocarditis as detected by histopathology, and/or myocardial damage biomarker cTnI, occurs in dogs with moderate CanL (LeishVet II) and it is very frequent in dogs with severe or very severe disease (LeishVet III and IV).
2. That CanL myocarditis, identified in severe and very severe CanL (LeishVet III and IV), consistently shows lymphoplasmacytic myocarditis on histopathology, and increase in left ventricular mass on echocardiography. That the latter is independent of the presence, absence or degree of systemic arterial hypertension.
3. That *Leishmania* can be detected by qPCR in the myocardium of dogs with CanL myocarditis, and that the prevalence of this presence is higher than previously detected with other techniques of organism detection. That in CanL myocarditis, Leishmania MPL evaluated by qPCR, is associated with the overall myocardial damage, as detected by cTnI evaluation, and with the severity of histopathology alterations.
4. That cTnI would be the best biomarker for future clinical use and research in CanL, as it is consistently elevated in CanL myocarditis and cTnI levels are minimally associated with secondary factors such as anaemia, azotaemia, or systemic arterial hypertension and are strongly associated with the severity of myocardial histopathology alterations and MPL.
5. That NT-proBNP would show weak results as a biomarker in CanL as consistent results will not be observed, and the changes in levels would be not specific, similar to those of dogs with CKD at similar IRIS stages or azotaemia, and associated with secondary factors such as anaemia or systemic arterial hypertension. Moreover, that NT-proBNP levels would not show association with MPL or myocardial histopathology score.

6. That HFABP would be a consistent and reliable biomarker in CanL and would show elevation in most dogs with severe and very severe disease (LeishVet III and IV). That HFABP levels would show association with other general, inflammatory, and renal biomarkers and with cardiac remodelling and increased left ventricular mass as detected by echocardiography.

AIMS

The aims of this research work are:

1. To describe and compare the levels of NT-proBNP, cTnI and HFABP in a group of dogs at different LeishVet stages of CanL, compared to healthy dogs (HFABP) and in dogs with idiopathic CKD (NT-proBNP, cTnI).
2. To assess the relationship of NT-proBNP, cTnI and HFABP with selected clinical, diagnostic and echocardiographic variables in groups of dogs with CanL.
3. To evaluate the echocardiographic findings in a large cohort of dogs with advanced CanL (LeishVet III and IV).
4. To study the prevalence, type and severity of myocarditis and the MPL detected by qPCR in a large cohort of dogs euthanised due to advanced CanL (Leishvet III and IV); and to assess the relationship of pathological findings and MPL with cardiac biomarkers and other selected clinical, diagnostic and echocardiographic variables.

RESULTS

Article 1

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Comparison of myocardial damage among dogs at different stages of clinical leishmaniasis and dogs with idiopathic chronic kidney disease

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ABSTRACT

Canine leishmaniasis (CanL) is a systemic disease caused by the protozoan parasite *Leishmania infantum*. Myocarditis in CanL has been described previously in CanL by histopathological analysis of post-mortem specimens and by evaluation of cardiac troponin I (cTnI) levels. However, the degree of myocardial damage at different stages of CanL and the role that concurrent azotaemia plays in this myocardial injury are unknown. The aim of this study was to prospectively evaluate and compare the presence of myocardial injury in dogs at different stages of clinical CanL and in dogs with severe idiopathic chronic kidney disease (CKD) by measuring cTnI. Forty-eight dogs were included in the study, divided into four groups: (1) group A (10 healthy dogs); (2) group B (17 dogs with CanL without renal azotaemia, classified as mild to severe in the LeishVet scheme); (3) group C (11 dogs with CanL and renal azotaemia, classified as very severe in the LeishVet scheme); and (4) group D (10 dogs with idiopathic CKD). Dogs in group C had significantly higher cTnI than dogs in groups B and D, although cTnI was also elevated in these groups. Dogs in group A had normal cTnI values. Dogs in groups D and C had similar renal IRIS classification scorers. Severe lymphoplasmocytic myocarditis and a positive real time PCR of *L. infantum* DNA were observed in all dogs in group C. Dogs with very severe CanL exhibit more myocardial injury than dogs with milder CanL or dogs with idiopathic CKD.

Introduction

Canine leishmaniasis (CanL) is caused by the protozoan parasite *Leishmania infantum*. CanL is a systemic disease with variable clinical manifestations (Paltrinieri et al., 2010; Solano Gallego et al., 2011). Cardiac involvement with myocardial damage has been shown by histopathological evaluation of post-mortem specimens (Torrent et al., 2005; López Peña et al., 2009; Rosa et al., 2014) and by demonstration of raised levels of cardiac troponin I (cTnI) in affected cases (Silvestrini et al., 2012).

CTnI is a sensitive and specific biomarker for myocardial damage and it is released into the circulation in proportion to the degree of cardiac injury (Spratt et al., 2005; Burguener et al., 2006; Fonfara et al., 2010). In a retrospective study, dogs with CanL had higher levels of cTnI than normal dogs (Silvestrini et al., 2012). However, the degree of myocardial damage at different stages of the disease is unknown. Concurrent chronic kidney disease (CKD) may potentially play a role in CanL associated myocardial injury because azotaemic dogs with idiopathic CKD have also been shown to have higher cTnI concentrations than normal dogs (Porciello et al., 2008; Sharkey et al., 2009).

The aims of this prospective study were: (1) to compare the degree of myocardial injury in dogs at different stages of CanL by measuring cTnI; (2) to compare the degree of myocardial injury in dogs with idiopathic CKD versus dogs with very severe CanL by measuring cTnI; and (3) to describe the myocardial histopathological findings and the percentage of dogs with molecular evidence of *L. infantum* DNA found by PCR analysis in the myocardium of dogs with very severe CanL.

Materials and methods

Study population and selection criteria

The study was carried out at the Veterinary Teaching Hospital of the University of Extremadura, Spain, from October 2012 to January 2014. The study was reviewed and approved by the Animal Ethics Committee of the Veterinary Teaching Hospital of the University of Extremadura (protocol number 13/H07/10; date of approval 9 October 2013) and was performed in compliance with Spanish and European guidelines for research on animals (RD1201/2005 and ETS 170, respectively). Thirty-eight adult dogs with CanL or with idiopathic CKD presented to the internal medicine clinic and 10 healthy dogs owned by university staff were included in the study. All dogs included in the study completed all tests in a defined protocol and owner informed consent was obtained. Dogs with any evidence of concurrent disease, previous history of cardiac disease or dogs receiving prior therapy for CanL before

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Table 1
Cardiac troponin I (cTnI), age, haematology and biochemistry of healthy dogs (A), dogs with canine leishmaniasis (CanL) without renal azotaemia (B), dogs with CanL and renal azotaemia (C) and dogs with idiopathic chronic kidney disease with renal azotaemia not associated with CanL (D).

	Reference interval	Group A ($n = 10$)	Group B ($n = 17$)	Group C ($n = 11$)	Group D ($n = 10$)	P value*
cTnI (ng/mL)	<0.06	0.02 (0.1–0.03)	0.23 (0.20–0.24)	12.19 (2.66–14.05)	0.61 (0.23–0.62)	0.001
Age (years)		3 (2–3)	5.7 (4–8)	4.91 (4–5.5)	10.20 (7–13)	0.002
PCV (%)	39–52	46.3 (42.8–49.1)	34.4 (27.5–39.2)	34.5 (26.9–37.1)	32 (23–43.4)	0.017
Creatinine (mg/dL)	0.7–1.2	0.8 (0.7–0.9)	0.8 (0.7–0.9)	6.4 (3.5–9.4)	5.5 (2.5–8.7)	0.001
TP (g/dL)	5.1–7.3	6 (5.5–6.3)	8 (7.4–8.7)	7.2 (6.1–7.7)	6.3 (5.6–7.1)	0.001
Globulin (g/dL)	1.5–3.5	2.4 (1.8–3.1)	4.9 (3.7–5.5)	3.9 (3.1–4.8)	2.9 (2.6–3.4)	0.001
Albumin (g/dL)	2.5–3.9	3.6 (3.3–3.8)	3 (2.7–3.2)	3.3 (3.1–3.6)	3 (3–3.9)	0.036
UPC	<0.5	0.1 (0.1–0.3)	0.2 (0.1–0.3)	9.5 (3.9–11.8)	5.5 (4.5–8.9)	0.001
BP (mm/Hg)	>150	113.2 (100–200)	128.3 (110–145)	188.4 (200–205)	183.3 (183–200)	0.001
A:G	0.7–1.9	1.6 (1.2–1.9)	0.8 (0.5–0.9)	0.9 (0.6–1.1)	1.2 (1.2–1.4)	0.001
ALT (IU/L)	18–77	30.5 (14–55)	35 (18–77)	40 (21–77)	50.5 (12–76)	0.275
Na (mmol/L)	137–163	149.4 (117.6–152)	148 (142–155)	146.6 (131.5–156.5)	148.9 (130.9–172.8)	0.800
K (mmol/L)	4.2–5.7	3.9 (3.8–5.3)	4.4 (3.1–5.6)	4.6 (3.7–5.4)	4.6 (4.1–5.7)	0.116
Ca (mg/L)	9.2–13.0	9.7 (6.1–12.0)	10.2 (7.3–12.2)	10 (7.1–11)	9.9 (6.9–10.9)	0.465
P (mmol/L)	0.7–2.1	1.7 (1.2–1.9)	1.5 (1.1–2.1)	2 (1.5–2.4)	2 (1.5–2.1)	0.399

PCV, paced cell volume; TP, total protein; UPC, urine protein–creatinine ratio; BP, blood pressure; A:G, albumin to globulin ratio; ALT, alanine aminotransferase.
Values are expressed as median (interquartile range). P values represent comparisons of variables between the four groups.
* Significance was set at $P < 0.05$.

presentation were excluded from the study. The study was not blinded; investigators and clinicians were aware of the groups the dogs belonged to.

A comprehensive physical examination was carried out in each dog. Tests performed on all dogs included a complete cell blood count (CBC), biochemistry (BC), urinalysis including protein–creatinine ratio (UPC), ELISA on serum for *L. infantum*, serum cTnI concentration, blood pressure (BP) measurement, electrocardiography (ECG), thoracic radiographs and echocardiographic examination. Dogs were divided into four groups: (1) group A (10 healthy control dogs); (2) group B (17 dogs with CanL without renal azotaemia); (3) group 3 (11 dogs with CanL and renal azotaemia); and (4) group D (10 dogs with idiopathic CKD). Dogs in group B were those classified as mild to severe disease in the LeishVet scheme (Solano Gallego et al., 2011). They had general, cutaneous or ocular clinical signs, such as lymphadenopathy, dermatitis or keratoconjunctivitis, but absence of azotaemia and were in general less sick than dogs in group D. Dogs in group C included those classified as very severe in the LeishVet scheme (Solano Gallego et al., 2011). They had renal azotaemia and in general had more severe clinical signs and laboratory abnormalities. The diagnosis of CanL was achieved on the basis of appropriate clinical signs and a high serology titre (at least three-fold increase to the laboratory reference cut-off). This was often complemented with a positive visualisation on cytology of *Leishmania* amastigotes in lymph node or bone marrow, or a positive PCR in any of these tissues. In dogs with clinically suspected leishmaniasis and an inconclusive titre on serology, the diagnosis was made by visualisation of the parasite in lymph nodes or bone marrow or by a positive real time PCR in lymph node or bone marrow using as described by Belinchón-Lorenzo et al. (2013). Azotaemic dogs were staged according to the guidelines of the International Renal Interest Society (IRIS[1]). There was a predominance of mixed breed dogs (13/48), Spanish greyhounds (5/48) and American pitbull terriers (4/48), along with 18 other breeds. There were 21 males and 27 females, comprising four males and six females in group A, eight males and nine females in group B, four males and seven females in group C and five males and five females in group D.

Serum cardiac troponin I

Serum cTnI concentration was measured using an enzyme-labelled chemiluminescent immunometric assay validated for dogs, with the Siemens Immunite 1000 Troponin I immunoanalyser. A goat polyclonal anti-troponin I antibody (Laborti Veterinària Laboratories, Spain) was used and cTnI concentrations <0.06 ng/mL were considered to be normal (Pelander et al., 2002).

Leishmania infantum *serology*

An ELISA for semi-quantitative detection of specific antibodies against the total soluble antigen of *L. infantum* (obtained from *L. infantum* promastigotes MCAN/ES/1996/BCN150, zymodeme) was carried out, as described by Belinchón-Lorenzo et al. (2013).

Blood pressure measurement and cardiac evaluation

Systolic BP measurements were determined by Döppler ultrasonography following the guidelines of the American College of Veterinary Internal Medicine consensus statement (Brown et al., 2007). In order to exclude primary cardiac disease, electrocardiography (ECG), thoracic radiography and standard transthoracic echocardiography (Thomas et al., 1993) were performed. Measurements of the left ventricular diastolic dimension (LVDd), left ventricular systolic dimension (LVDs), left ventricle wall thickness in diastole (LVWd) and interventricular septum diastolic thickness (IVS) were performed from the right parasternal short axis view using M mode. The fractional shortening (%FS) was calculated as the LVDd minus the LVDs divided by the LVDd and multiplied by 100. The aortic (Ao) and left atrial (LA) diameters were measured and LA:Ao ratio was calculated by a two-dimensional method from a right-sided short axis parasternal view. Colour flow Döppler ultrasonography was used for the evaluation of transvalvular flows and the detection of valvular regurgitation. Peak systolic aortic and pulmonic velocities and peak early (E) and late (A) diastolic mitral flow velocities were also evaluated by pulse wave Doppler.

Histopathological and parasitological analysis of myocardial tissue

Ten dogs from group C were euthanased at the owner's request and post-mortem examinations was performed. Myocardial samples for histopathological analysis were taken from the left and right ventricular free walls, interventricular septum and left and right atrium. Samples were tested for the presence of *L. infantum* by real time PCR (Belinchón-Lorenzo et al., 2013) by detection and quantification of Kinetoplast minicircle DNA after deparaffinisation (Müller et al., 2003).

Statistical analysis

SPSS Statistics 21 (IBM) was used for statistical analysis. Descriptive statistics were applied and reported. The normality and homoscedasticity of serum cTnI concentration, age, packed cell volume (PCV), concentrations of creatinine, total protein (TP), albumin, globulins, albumin to globulin ratio (A:G), UPC, IRIS stage and systolic BP were tested using the Shapiro–Wilk test and Levene tests, respectively. None of the variables followed a normal distribution and they were reported as medians with interquartile ranges (IQRs). The Kruskal–Wallis test and the Mann–Whitney U test were used to examine differences in the variables studied among the four groups. Correlations between serum cTnI concentration and each variable studied were evaluated using Spearman's rank correlation test (ρ). $P < 0.05$ was considered to be statistically significant.

Results

The median (IQR) ages were 3 (2–3) years in group A, 5.71 (4–8) years in group B, 4.91 (4–5.5) years in group C and 10.2 (7–13) years in group D (Table 1). The median age of dogs in group D was statistically higher than in other groups ($P = 0.015$).

Serum cTnI concentrations in group A (median 0.02, IQR 0.01–0.03, ng/mL) were normal (<0.06 ng/mL) in all dogs. Serum cTnI concentrations of all dogs in groups B (median 0.23, IQR 0.20–0.24, ng/mL), C (median 12.19, IQR 2.66–14.05, ng/mL) and D (median 0.61, IQR 0.23–0.62, ng/mL) were >0.06 ng/mL. Significant differences were observed in serum cTnI concentrations between group A and groups B, C and D ($P = 0.001$). The median serum cTnI

[1] http://www.iris-kidney.com/guidelines (accessed 9 February 2014).

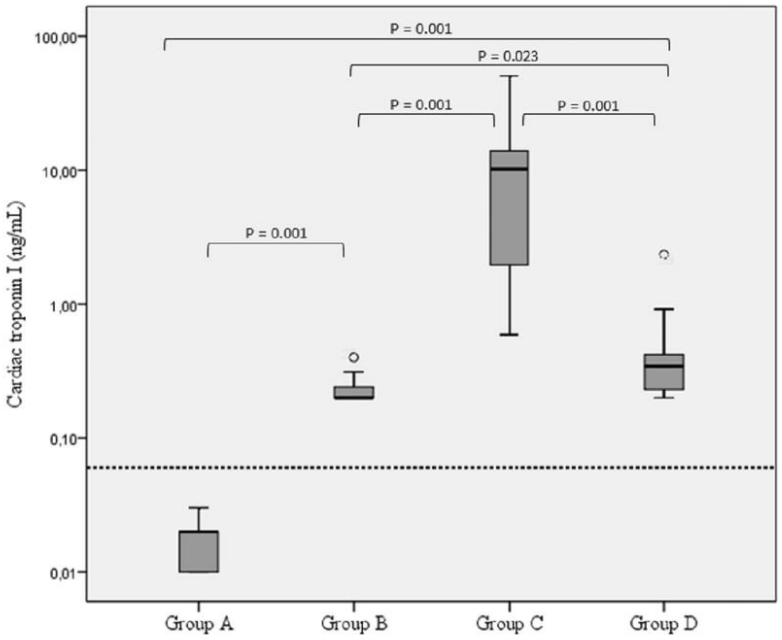

Fig. 1. Whiskers boxplot of cardiac troponin I (cTnI) in control dogs (A), dogs with canine leishmaniasis (CanL) without renal azotaemia (B), dogs with leishmaniasis and renal azotaemia (C) and dogs with idiopathic chronic kidney disease not associated with CanL (D). The values for the upper quartile, median and lower quartile are indicated in each box. The bars outside the box indicate the maximum and minimum values. Circles indicate outliers. Horizontal dotted line on *y*-axis indicates upper limit of normal cTnI concentration. Serum cTnI concentration in group C was significantly higher than in groups B and D ($P = 0.001$) and concentration in group D was also significantly higher than in group B ($P = 0.023$). Significance was set at $P < 0.05$.

concentration in group C was significantly higher than in groups A, B and D ($P = 0.001$) and the cTnI concentration in group D was significantly higher than in group B ($P = 0.023$) (Table 1; Fig. 1).

Echocardiographic abnormalities were detected in 10% of dogs in our study. Mitral valve regurgitation was detected in one dog from group B and one dog from group D. These valvular regurgitations were considered to be mild on the basis of colour flow Döppler and were not associated with left atrial enlargement. Two dogs from group C and one dog from group D had mild enlargement of LVDd and LVWd. These three dogs had systemic arterial hypertension (systolic pressures of 190 mmHg, 182 mmHg and 198 mmHg, respectively).

In the azotaemic groups (C and D), the median (IQR) plasma creatinine concentration was 6.40 (3.55–9.45) mg/dL for group C and 5.53 (2.50–8.70) mg/dL for group D. Five of 11 dogs in group C and 5/10 dogs in group D were in IRIS stage IV; 6/11 dogs in group C and 4/10 dogs in group D were in IRIS stage III and 1/10 dog in group D was in IRIS stage II. Elevated UPC ratios were present in groups C (median 9.51, IQR 3.90–11.80) and D (median 5.53, IQR 4.55–8.90; Table 1). There were no significant statistical differences between groups C and D in creatinine concentrations ($P = 0.438$), IRIS stage ($P = 0.968$) or UPC ($P = 0.360$) (Table 1).

Systemic arterial hypertension was evident in 8/11 dogs in group C and 6/10 dogs in group D. Median BP was 188.36 (IQR 200.00–205.00) mmHg in group C and 183.30 (IQR 183.00–200.00) mmHg in group D. There was no significant difference ($P = 0.434$) in blood pressures between these two groups.

Mild non-regenerative anaemia (PCV 30–35%) was evident in 6/17 dogs in group B (median 34%), 4/11 dogs in group C (median 34%) and 7/10 dogs in group D (median 32%), but no dogs in group A. Mild non-regenerative anaemia (30–35%) was evident in 6/17 dogs in group B (median 34%), 4/11 dogs in group C (median 34%) and 7/10 dogs in group D (median 32%); there were no statistical differences amongst the three groups. Anaemia was not observed in group A.

A significant correlation was found between cTnI concentration and creatinine ($\rho = 0.707$; $P = 0.001$), UPC ($\rho = 0.666$; $P = 0.001$), BP ($\rho = 0.676$; $P = 0.001$) and IRIS stage ($\rho = 0.855$; $P = 0.001$). However, no significant correlations were found between cTnI concentration and age ($\rho = 0.142$; $P = 0.333$), PCV ($\rho = -0.242$; $P = 0.097$), albumin concentration ($\rho = -0.003$; $P = 0.981$), PT ($\rho = -0.124$; $P = 0.397$), globulin ($\rho = -0.094$; $P = 0.524$) or A:G ($\rho = -0.098$; $P = 0.506$).

Myocardial lesions were detected in all dogs from group C that underwent post-mortem examination. The histopathological findings included lymphoplasmacytic myocarditis in all dogs (7/10 severe and 3/10 moderate), myonecrosis in three dogs (2 mild and 1 moderate) and lymphoplasmacytic perivasculitis in two dogs (both moderate). *L. infantum* DNA was detected in the myocardial tissue of all dogs (10/10).

Discussion

This study provides evidence that myocardial damage occurs in dogs with CanL and that dogs with severe CanL have more severe myocardial damage than dogs at an earlier stage of disease. This study also suggests that concurrent azotaemia in dogs with CanL is unlikely to be the major factor in CanL myocarditis, since dogs

with idiopathic CKD at a similar IRIS stage showed less myocardial damage than dogs with advanced CanL.

We observed evidence of myocarditis and myocardial damage in dogs with CanL by demonstration of markedly elevated cTnI and lesions of moderate to severe myocarditis on histopathology. Most importantly, we showed that the myocardial damage starts at an early stage and is more severe in dogs with more advanced disease. Mechanisms potentially involved in myocardial injury in CanL include direct damage by the organism, vasculitis, concurrent azotaemia, systemic arterial hypertension, immune-mediated disease or anaemia (Paltrinieri et al., 2010; Solano Gallego et al., 2011). Dogs that underwent histopathology in our study had very severe disease and had a similar prevalence of myocardial injury and similar histopathological appearance to cases reported by Rosa et al. (2014). A high percentage of myocarditis (100%) was evident in our study, similar to the value of 90% reported by Rosa et al. (2014), and there was a low level of vasculitis (10%), compared to 20% in the study by Rosa et al. (2014). There was molecular evidence of *L. infantum* in the myocardium of 100% of dogs in our study, whereas organisms could be visualised directly in the myocardium in 67% of dogs in the study by Rosa et al. (2014).

The relationship between azotaemia and elevated cTnI has been reported previously in dogs by Porciello et al. (2008) and Sharkey et al. (2009). Although the exact cause is unknown, many mechanisms have been proposed, including silent myocardial necrosis, ventricular hypertrophy, cardiac remodelling and altered renal clearance (Freda et al., 2002; Ronco et al., 2012). Renal disease and azotaemia occur in dogs with advanced CanL and have been attributed to immune-complex deposition and progressive glomerulonephritis (Solano Gallego et al., 2011). This had led to the hypothesis that this azotaemia may be the primary cause for the myocardial damage observed in advanced stages of CanL. We showed that it is unlikely that uraemic myocardial damage plays a primary role in CanL myocarditis, since high cTnI levels were found in dogs with CanL but without renal azotaemia, and there were markedly lower cTnI levels in dogs with idiopathic CKD compared to dogs with advanced CanL. The cardiac histopathological findings in experimental CKD (Bongartz et al., 2012) are markedly different to those observed in dogs with CanL in our study, as well as in the study by Rosa et al. (2014).

It has also been speculated that systemic arterial hypertension or chronic anaemia may play a role in the myocardial damage in dogs with CanL (Siciliano et al., 2000; Spotswood et al., 2006). Systemic arterial hypertension causes an increase in afterload, which can lead to myocardial remodelling, with changes such as myocyte hypertrophy (Siciliano et al., 2000). Systemic arterial hypertension was common in both groups of azotaemic dogs (groups C and D), but there was no statistical difference between the groups, despite the marked difference in cTnI levels. Mild anaemia was frequent in the dogs with CanL, but echocardiographic evidence of eccentric hypertrophy was not evident in any of the anaemic dogs. There was no correlation between PCV and cTnI levels. Furthermore, the degree of anaemia was not different between the two groups of dogs with CKD (groups C and D), despite their marked differences in cTnI. In light of the above findings, it appears unlikely that systemic arterial hypertension or anaemia plays a major role in the severe myocardial damage observed in dogs with CanL.

The cause of the mild left ventricular enlargement and mild left ventricular thickening observed in three dogs (two dogs in group C and one dog in D) is unknown. These changes may have been due to individual variation, systemic arterial hypertension or CanL myocarditis. Despite the marked myocardial changes on histopathology in most dogs, echocardiography did not identify major cardiac abnormalities in this study. It is unknown how much myocarditis observed in CanL plays a role in overall disease morbidity and mortality. Furthermore, myocardial histopathology of the non-azotaemic CanL and idiopathic CKD groups was not available.

Conclusions

This study provides further evidence that myocarditis occurs in dogs with CanL. Mild myocardial damage appears at an early stage of CanL and becomes severe in dogs with very severe CanL according to the LeishVet scheme. Our study also provides evidence that the concurrent azotaemic state, systemic arterial hypertension and anaemia are unlikely to be major factors in the myocardial injury that occurs in dogs with CanL.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Article 2

PAPER

Comparison of N-terminal proB-type natriuretic peptide levels at different stages of visceral leishmaniosis and in patients with chronic kidney disease

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Abstract

N-terminal proB-type natriuretic peptide (NT-proBNP) may be a useful marker in canine leishmaniosis (CanL). The aim was to compare NT-proBNP in dogs at different LeishVet stages of CanL and with idiopathic chronic kidney disease (CKD). Dogs diagnosed with CanL or CKD and a group of healthy dogs were included (group A, five normal dogs; group B, six dogs LeishVet 1–2; group C, 13 dogs LeishVet 3–4; group D, six dogs with CKD). NT-proBNP was higher (P<0.001) in group C (7.616 pmol/l, interquartile range (IQR) 3537–10,000 pmol/l) than in group A (293 pmol/l, IQR 257–373), group B (388.5 pmol/l, IQR 324–793) and group D (740 pmol/l, IQR 557–962 pmol/l). International Renal Interest Society (IRIS) kidney stage was not different between groups C and D or between groups A and B, but was different within all the rest of the group comparisons (P<0.001). In group C all dogs had echocardiographic increase in left ventricular mass index. NT-proBNP had negative correlation with haematocrit (P<0.001, r=0.749) and positive correlation with systemic blood pressure (P<0.001, r=0.728). NT-proBNP is consistently elevated in dogs with advanced CanL and is strongly correlated with the degree of systemic hypertension and anaemia. Moreover, dogs with advanced CanL exhibit increase in left ventricular mass. NT-proBNP may however be a less desirable cardiac marker as unlike cardiac troponin I it is often not elevated at earlier stages of CanL.

Introduction

Canine visceral leishmaniosis (CanL) is a disease caused by the protozoal organism *Leishmania infantum* and is endemic in many southern European and South-American countries.[1,2] The disease causes inflammation and damage in many organs and systems, including the kidneys, the skin, the reticuloendothelial system and the heart.[1] Recently, a classification of the disease has been established by the LeishVet group, which has proved useful in staging the disease.[2] Accurate classification of patients is important both for clinical and research purposes. The main markers currently used for disease classification under the LeishVet scheme include a subjective evaluation of clinical signs, serology levels and routine specific kidney markers (urine protein/creatinine ratio (UPC) and serum creatinine levels), and among these plasma creatinine level is the key cornerstone of the classification.[2] Creatinine is affected by factors such as volaemic status and muscle mass. It is also primarily a functional marker of one of the organs affected by the disease, but CanL has heterogeneous presentation in nature and can affect several systems with different severity.[1] In CanL, other biomarkers which may reflect the involvement of other body systems may add further independent information and help in disease classification. A good biomarker or a combination of biomarkers may not only substage the disease by its initial levels in order to act as a prognostic factor and

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guide therapy, it may also guide treatment adjustments based on its post-therapy fluctuations.[3] Cardiac biomarkers cardiac troponin I (cTnI) and N-terminal proB-type natriuretic peptide (NT-proBNP) are widely available, and considering the frequent presence of myocardial damage in dogs with CanL could potentially be used within this context. Indeed cTnI, a marker of myocardial damage, has been shown to be elevated in CanL and to be associated with disease severity[4 5] and with the degree of parasitic myocardial load calculated by real-time quantitative PCR.[6] Moreover, this marker has been shown to be altered at earlier stages of the disease before creatinine is elevated.[5] NT-proBNP is a cardiac stress marker which is elevated both in cardiac[7] and renal[8] disease and could also therefore be a potential good biomarker in CanL. It might potentially be added to other markers for disease classification and therapy and research purposes. NT-proBNP is derived from an intracellular 108 amino acid precursor protein (proBNP), which is cleaved into brain natriuretic peptide (BNP; the functional molecule) and NT-proBNP (the inactive part).[9 10] The precursor of proBNP (preproBNP) is synthesised by the cardiomyocytes under the effect of acute and chronic increments of ventricular stress.[10] NT-proBNP is often preferred as marker as it has a longer half-life than BNP.[10] NT-proBNP is a widely used tool for diagnosis, and for disease monitoring, in many types of cardiac diseases in human beings, dogs and cats.[7 10]

The aims of this study were (1) to evaluate and compare the levels of NT-proBNP in a group of dogs at different stages of clinical leishmaniosis and in dogs with idiopathic chronic kidney disease (CKD), and (2) to evaluate the relationship between NT-proBNP and several selected diagnostic variables.

Materials and methods

Animals and groups

Dogs attending the Veterinary Hospital of the University of Extremadura clinic were recruited. *Leishmania*-positive dogs were selected after being diagnosed with CanL by appropriate clinical signs and using ELISA for the quantitative detection of specific antibodies to the total soluble antigen of *L infantum*. Dogs diagnosed with non-neoplastic and non-infectious (idiopathic) CKD at the Veterinary Hospital were also included. Healthy dogs recruited from university staff dogs were used as controls. Cases were divided into healthy-not infected (group A), LeishVet 1 or 2 (group B) and LeishVet 3 or 4 (group C) according to the LeishVet classification,[2] and dogs with idiopathic CKD (group D).

Clinical analyses

After obtaining blood samples by jugular venepuncture, all dogs underwent a previously selected protocol of study tests. These include full haematology, biochemistry, blood pressure (BP) measurement, urinalysis including culture and sensitivity, NT-proBNP (blood samples were included in EDTA tubes and sent immediately to an external laboratory[i] for these analyses), UPC, abdominal ultrasonography and echocardiography. Dogs with any evidence of concurrent disease (eg, neoplastic) were excluded from the study. Dogs with trivial insufficiency of any of the valves (ie, colour flow Doppler map extending less than 1 cm from the valve[11]) were allowed in the study, but dogs with more severe regurgitations or with any other form of congenital cardiac disease were excluded from the study. As cardiac changes may potentially occur in *Leishmania*-infected dogs and idiopathic CKD dogs, dogs with systolic dysfunction, eccentric or concentric hypertrophy of the ventricles, and/or atrial enlargement were allowed in the study unless dilated cardiomyopathy had been previously diagnosed or they were suspected to be due to other primary cardiac disease.

Non-invasive systolic BP measurements were recorded in all groups by Doppler technique. Measurements were made by the same operator on the right or left thoracic limbs, and from every subject five values were recorded for the individual average BP determination as previously described according to the American College of Veterinary Internal Medicine consensus statement.[12]

A full echocardiographic examination was carried out by an experienced operator (JD) using standard right parasternal and left parasternal views. The following echocardiographic variables were obtained for the study: left ventricular dimensions in diastole and systole; left ventricular free wall thickness in diastole (LVFWd) and systole (LVFWs); and interventricular septal wall thickness in diastole (IVSd) and systole (IVSs). All these were calculated by M-mode from a right parasternal short-axis view at the level of the papillary muscles. From a two-dimensional image on a short-axis view at the level of the aortic valve, the left atrium to aorta ratio was also calculated. Left ventricular mass (LVM) was calculated as: LVM (g): 0.8 (1.04 ([LVIDD + PWTD + IVSTD] 3 - [LVIDD] 3))+ 0,6. LVM was then divided by the body surface area to calculate the left ventricular mass index (LVMI). Previous technique standardisation and reference intervals reported were used to establish the presence of left ventricular enlargement or thickening, atrial enlargement, and increased LVMI.[13–16]

All dogs that succumbed to the disease underwent a postmortem examination with histopathological assessment of the heart.

Statistical analysis

Statistical analysis was carried out with a commercial software (SPSS V.21). Normal variables were described as mean±sd, and those non-normally distributed were reported as median and interquartile range (IQR). Group

[i]Cardiopet® proBNP enzyme-linked immunosorbent assay (IDEXX Laboratories, Carrer Plom 2-8, 3ª 08038 Barcelona, Spain).

comparisons were performed by one-way analysis of variance (ANOVA) testing with post-hoc Tukey' s honest significant testor by Brown-Forsythe test if the ANOVA assumptions were not met. Correlations were carried out by Pearson's test if the data were normally distributed or by Spearman's rho if the data were not normally distributed. Statistical significance was set at P<0.05.

Results

A total of 30 dogs were recruited (group A, five dogs; group B, six dogs; group C, 13 dogs; group D, six dogs). The mean age was 5.1 years (±1.31) and age was not statistically different among the groups. There were 12 different breeds (crossbreed 19 per cent; labrador retriever 16 per cent; Yorkshire terrier 9 per cent; Spanish mastiff6.5 per cent; all others less than 5 per cent). Fifty-five per cent (16/30) were male and 45 per cent (14/30) were female, and sex distribution was not different among the groups.

The following were the results of the selected variables: haematocrit (HCT): 38.6 per cent (IQR 27.8–43.9); haemoglobin (Hb): 15.7 g/dl (IQR 9.3–14.1); creatinine: 2.9 mg/dl (IQR 0.9–7.2); NT-proBNP: 893 pmol/l (IQR 394–6946); UPC: 3.02 (IQR 0.5–8.25); and systolic BP (Doppler): 160 mmHg (IQR 110–182). International Renal Interest Society (IRIS) stages were as follows: group A: 100 per cent (5/5) in stage 0; group B: 100 per cent (6/6) in stage 1; group C: 38 per cent (5/13) in stage 3 and 61 per cent (8/13) in stage 4; and group D: 66 per cent (4/6) in stage 3 and 34 per cent (2/6) in stage 4.

Posterior reanalysis of the echocardiographic images could be carried out in nine cases in group C (previous images were not available in the other four cases) (table 1): 11 per cent (1/9) had left atrial enlargement, 11 per cent (1/9) had left ventricular enlargement. None of the dogs had overall systolic dysfunction (normal left ventricular dimension in systole, normal fractional shortening); 89 per cent (8/9) of dogs had increased left ventricular diastolic wall thickening, and 100 per cent (9/9) of dogs had increased LVMI (g/m^2) (56 per cent, 5/9 severe; 33 per cent, 3/9 moderate; 11 per cent, 1/9 mild).

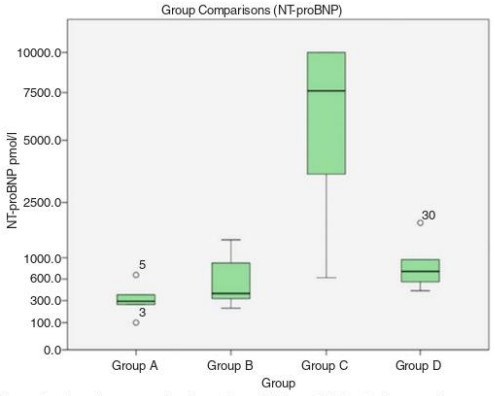

Figure 1 Box plot comparing the values of NT-proBNP by the four study groups (NT-proBNP was higher in group C compared with the rest of the groups; P<0.001). NT-proBNP, N-terminal proB-type natriuretic peptide.

All dogs in group C were ultimately euthanased as a consequence of CanL. All dogs underwent postmortem examination, and in all dogs moderate to severe lymphoplasmacytic myocarditis was observed.

When groups were compared for the selected variables, NT-proBNP was higher (P<0.001) in group C (7.616 pmol/l, IQR 3537–10,000) than in group A (293 pmol/l, IQR 257–373), group B (388.5 pmol/l, IQR 324–793) and group D (740 pmol/l, IQR 557–962) (figure 1). Arterial BP was also higher in group C (P<0.001; 183 mmHg, IQR 175–200 mmHg) than in the rest of the groups (A: 110 mmHg, 100–110; B: 110 mmHg, 100–117; C: 115 mmHg, 100–170). Creatinine was lower in group A (0.6 mg/dl, IQR 0.5–0.9) than in group C (5.8 mg/dl, IQR 3.8–9.9) (P<0.001) and in group D (3.1 mg/dl, IQR 2.2–7.1) (P<0.035). It was also lower in group B (0.8 mg/dl, IQR0.8–0.9) than in group C (P<0.001) and group D (P<0.023). IRIS stage was also different between groups A and C (P<0.001), A and D (P<0.001), B and C (P<0.001), and B and D (P<0.001). HCT and Hb were different between group

Table 1 Echocardiographic measurements of dogs in group C

Echocardiographic parameter	Value*†, n=9	Reference intervals‡§¶	Within normal (%)	Mildly elevated (%)	Moderately elevated (%)	Severely elevated (%)
LA/Ao	1.22 (1.1–1.3)†	<1.6†	89	0	11	0
LVddi	1.46±0.24*	1.27–1.85§	89	11	0	0
LVdsi	0.88±0.22*	0.71–1.26§	100	0	0	0
FS	39 (37.5–46.7)†	25–44§	100	0	0	0
LVFWdi	0.89±0.41*	0.29–0.60§	33	33	11	22
IVSWdi	0.70±0.20*	0.29–0.59§	33	11	22	22
LVMI	245.75±77.80*	90.76±63.4¶	0	11	33	56

*Normally distributed data shown as median±sd.
†Data not normally distributed shown as median (interquartile range).
‡From Rishniw and Erb.[15]
§From Cornell *et al*.[13]
¶From Takano *et al*.[16]
FS, fractional shortening; IVSWdi, interventricular free wall thickening in diastole; LA/Ao, left atrium to aorta ratio; LVddi, left ventricular internal dimension in diastole index; LVdsi, left ventricular internal dimension index; LVFWdi, left ventricular free wall thickening in diastole index; LVMI, left ventricular mass index.

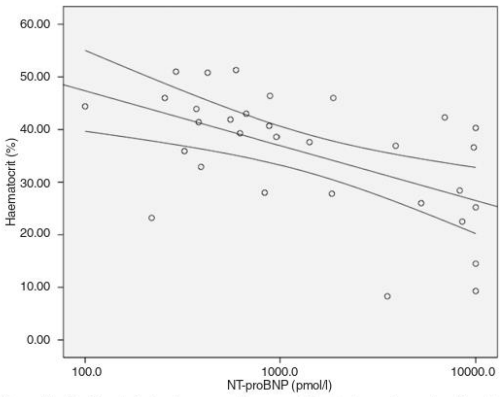

Figure 2 Scatter plot showing a negative correlation between haematocrit and NT-proBNP (r=0.749). The mean (straight line) and the 95 per cent confidence intervals (curved lines) of the mean are shown. NT-proBNP, N-terminal proB-type natriuretic peptide.

C (HCT 27.9 per cent, IQR 22.5–36.9; Hb 9.5 g/dl, IQR 7.8–12.6) and group A (HCT 44.4 per cent, IQR 43.9–46; Hb 15.6 g/dl, IQR 15.4–16 g/dl) (P<0.001); groups C and B (HCT 36.75 per cent, IQR 32.9–41.4; Hb 13.25 g/dl, IQR 10–15.8) (P<0.001); and groups C and D (HCT 43.95 per cent, IQR 40.7–50.8; Hb 15.4 g/dl, IQR 14.1–17.1) (P<0.001). NT-proBNP had a negative moderate correlation with Hb (P<003, r=–0.522), a strong negative correlation with HCT (P<0.001, r=0.749) (figure 2) and a strong positive correlation with creatinine (P<0.001, r=0.749) and BP (P<0.001, r=0.728) (figure 3).

Discussion

In this study NT-proBNP was markedly elevated in dogs with severe CanL but was normal in dogs with milder forms of the disease. This may make NT-proBNP a less desirable CanL cardiac marker than troponin I as the latter has been shown to be proportionally elevated at different stages of the disease and to be also

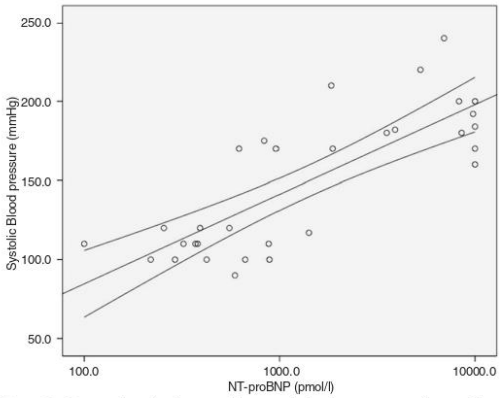

Figure 3 Scatter plot showing a positive correlation between systolic arterial blood pressure and NT-proBNP (r=0.728). The mean (straight line) and the 95 per cent confidence intervals (curved lines) of the mean are shown. NT-proBNP, N-terminal proB-type natriuretic peptide.

consistently elevated in all dogs at early stages.[4 5] This may also suggest that myocardial damage is present in this disease before the increase in myocardial wall stress. A marker which is not increased at earlier stages of the disease may be less useful in CanL as it may be less convenient for disease classification, because it may not provide more information than creatinine currently does. It would also not be the ideal marker to be investigated as an aid to guide therapy, at least at earlier stages. The currently used markers for decision making on those early disease situations when creatinine and protein/creatinine ratio may not be altered tend to include clinical signs (which may be subjective), and serology levels, protein electrophoresis and globulin levels (all of which may remain elevated over long periods of time in spite of the absence of active disease).[1 2] Further field studies are needed to further evaluate cardiac markers as a potential tool for clinical or research purposes or as a therapy guide. However, in light of the current information, troponin I may be preferred for this purpose.

Dogs in the CKD group showed evidence of higher NT-proBNP levels, although they did not reach statistical significance and were less pronounced than previously reported.[8] These elevations were markedly milder in the CKD group compared with the CanL group (in spite of both groups having a similar IRIS stage). Although this may suggest that CanL-specific factors driving the NT-proBNP elevation are different from those occurring in CKD (such as the direct effects of CanL-associated myocarditis), it is important to remark that hypertension and anaemia were in many cases not present in the CKD population of the present study as opposed to a previous recent study in which dogs at similar IRIS stage to the ones in the present study were evaluated.[8] The reason for this discrepancy is not clear, but different population characteristics and different timing of sampling within the disease or treatment course may play a role. Both systemic arterial hypertension and anaemia have been indeed suggested to play an important role in the increase in myocardial stress and the liberation of NT-proBNP in dogs with advanced CKD.[8] The results of this study, in particular the strong negative correlation with HCT and BP, suggest that this may also be the case in advanced stages of CanL.

The authors also observed cardiac remodelling with left ventricular hypertrophy and an increase in overall ventricular mass index in most dogs within the advanced CanL group. This is consistent with a previous study.[17] Although increase in ventricular mass index has not been previously described in CanL, left ventricular wall hypertrophy has been previously reported. Overall, factors involved in the observed cardiac remodelling and suspected myocardial wall stress leading to increase in NT-proBNP are likely to include a combination of secondary effects (such as haemodynamic alterations and systemic arterial hypertension) and primary effects

(such as the disease-associated myocarditis). Indeed, myocarditis is a common feature of CanL[5 18 19] and was consistently observed on postmortem examination in all the dogs in this study.

There are several limitations to this study. The number of cases is low and this may have affected some results. It is possible for example that higher elevation of NT-proBNP in group B may have been observed with higher number of cases (type II error). However, it is to note that most dogs in group B in this study had values within what is considered normal reference intervals, which would reinforce the concept that NT-proBNP may be a less desirable cardiac marker at early stages of CanL. Type I error was also possible as several statistical tests were carried out. The authors elected not to make statistical corrections but to evaluate and critically review the results if any of the major results did not appear robust. Most results however appear robust with P values below 0.01 and the majority at values of P<0.001. Another potential limitation is that dogs with valvular trivial insufficiencies were allowed in the study. This was permitted as this is often seen in normal dogs. It is very unlikely that these trivial insufficiencies would alter the results significantly. It is unlikely, for example, that they would have made a significant difference in group C as the values were very high or in group B in which NT-proBNP was not elevated and it was indeed within what is considered normal reference intervals for dogs.

In conclusion, NT-proBNP is elevated in dogs with advanced CanL, and this group of dogs exhibits increase in hypertrophy and ventricular mass. Moreover, NT-proBNP in dogs with CanL is strongly associated with the degree of systemic hypertension and anaemia. In spite of these findings NT-proBNP may be a less desirable marker for future research or clinical studies,as unlike cTnI, it is not elevated at earlier stages of CanL.

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Competing interests None declared.

Ethics approval The study was approved by the ethical committee at the University of Extremadura.

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Article 3

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Vet Record

ORIGINAL RESEARCH

Association of myocardial parasitic load with cardiac biomarkers and other selected variables in 10 dogs with advanced Canine Leishmaniasis

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Abstract

Background: The association between myocardial parasitic load (MPL) and cardiac biomarkers in Canine Leishmaniasis (CanL) has not been studied.

Methods: Dogs with advanced CanL were prospectively recruited and were included if they were euthanised. Prior to euthanasia these variables were assessed: hematocrit, globulin, creatinine, N-terminal-pro brain natriuretic peptide (NT-proBNP), cardiac troponin I (cTnI), blood pressure, urine protein/creatinine ratio and echocardiographic parameters. A left ventricular (LV) sample was taken for histopathology and MPL evaluation by quantitative PCR. Correlation of MPL with all variables was analysed. Dogs with lower and higher histopathology scores were compared.

Results: Ten dogs were included. NT-proBNP was 6946 pmol/ (interquartile range [IQR] 3751–9268 pmol/L) and cTnI 4.56 ng/mL (IQR 0.46–13.1 ng/mL). In all dogs, echocardiography showed an increase in LV thickening, and histopathology revealed moderate to severe lympho-plasmocytic myocarditis and/or myocardial cell degeneration. MPL was 215.53 parasites/gram (IQR 21.2–1372.63 parasites/gram). A strong correlation ($p < 0.001$; R = 0.90; $R^2$0.81) with cTnI was observed but correlation with any of the other variables or differences between the two histopathological scores, were not detected.

Conclusions: MPL in dogs with advanced CanL shows variable but generally high levels. A strong association between MPL and cTnI was observed, which encourages the exploration of cTnI as a marker in CanL.

INTRODUCTION

Canine visceral leishmaniasis (CanL) is a disease caused by the protozoal organism *Leishmania infantum*.[1,2] The disease causes inflammation and damage in several organs,[1–3] and there is evidence that myocardial damage occurs in many dogs[4–9] which becomes more severe as the disease progresses.[5] Lymphoplasmacytic myocarditis and necrosis are the most common pathological alterations observed in the myocardium of dogs with CanL, followed less frequently by fibrosis, focal granulomatous formation, fibrinoid vascular deposits and vasculitis.[4–9]

The cardiac involvement in CanL has led to research into the potential clinical or research use of cardiac biomarkers in this disease.[4,5,10,11] The main markers currently used for the classification of CanL comprise a subjective evaluation of clinical signs, serology and protein electrophoresis findings, and routine kidney markers such as urine protein/creatinine ratio (UPC) and serum creatinine levels.[3,12] In general, a clinically useful biomarker or a combination of biomarkers should be a tool for staging the disease, acting as a prognostic factor or helping to guide initial or subsequent therapy.[13,14] A specific cardiac biomarker may also function as a surrogate for the severity of the cardiac parasitic load and the degree of cardiac involvement in any particular individual with CanL. Several studies have evaluated the different aspects of cardiac biomarkers Troponin I (cTnI) and N-terminal proB-type natriuretic peptide (NT-proBNP) in CanL.[4,5,10,11] The cardiac biomarker cTnI, a marker of myocardial

damage[13] has been shown to be elevated in CanL from early stages and to increase gradually as the disease progresses.[4,5] The elevation of cTnI appears to be primarily linked to disease severity but not to secondary pathologies such as anaemia, chronic kidney disease or systemic arterial hypertension.[5] NT-proBNP, a marker of acute and chronic increments of ventricular wall stress,[13] has also been shown to be increased in advanced CanL, with values significantly higher than those of dogs with idiopathic chronic kidney disease at the same IRIS stage.[11] It is however not consistently elevated at early stages of the disease, and it is strongly correlated with the severity of systemic arterial hypertension and anaemia.[11]

The parasitic presence and load in dogs with CanL as detected on histopathology have been very variable among previous histopathological studies.[6,7,8] Detection of the parasite by PCR methods is more sensitive than histopathology, and quantitative PCR can provide specific information about the parasitic load.[12,15–17] The use of this technique in the myocardium of dogs with CanL and its association with cardiac biomarkers have not been studied to date.

The aims of this study were: 1. To describe the myocardial parasitic load (MPL) of dogs with advanced CanL and to evaluate the relationship of this parasitic load with the cardiac biomarkers cTnI and NT-proBNP. 2. To evaluate the relationship of MPL with other selected diagnostic and clinical variables.

MATERIAL AND METHODS

Ethical approval for the study was granted by the University of Caceres Ethical Committee. All owners were fully informed and gave their consent for their dogs to be included in the study. The study was prospective. Dogs diagnosed with advanced CanL based on appropriate clinical signs and a positive leishmania (ELISA or IFI) serology were preselected for the study. Any additional ancillary tests were recorded (e.g. cytological observation of amastigotes in lymph nodes). For the purpose of this study, advanced CanL was defined as dogs in Leishvet stages III or IV.[2] The study protocol comprised complete haematology and serum biochemistry, urinalysis including culture and sensitivity, UPC, abdominal ultrasonography and echocardiography. Any other diagnostic tests (e.g. thoracic radiographs) could also be performed but were not initially part of the study protocol. Dogs with evidence of concurrent disease (e.g. neoplasia) were excluded from the study. Dogs with trivial insufficiency of any of the valves (i.e., colour flow Doppler map extending less than 1 cm from the valve[18]) were allowed in the study, but dogs with more severe regurgitations or with any other form of congenital cardiac disease were excluded from the study. As cardiac remodelling may potentially occur in *Leishmania*-infected dogs, dogs with systolic dysfunction, eccentric or concentric hypertrophy of the ventricles, and/or atrial enlargement were allowed in the study unless dilated cardiomyopathy had been previously diagnosed or they were suspected to be due to other primary cardiac disease. From this pool of patients, dogs that underwent euthanasia due to a combination of severe CanL and owner wishes, were selected for the histopathological, as well as the other components of the study. Furthermore, blood samples for cTnI and NT-proBNP had to be available from these dogs within 24 hours prior to euthanasia. These blood samples were either collected for the study prior to euthanasia using the catheter placed for this purpose, or serum and EDTA samples from clinical samples collected that same day were used.

EDTA samples were used for NT-proBNP and serum tubes for cTnI. Samples were centrifuged, and serum and plasma were isolated within 30 minutes of blood collection and were sent immediately to an external laboratory for the analysis of NT-proBNP[a] and serum cTnI[b]. The latter was measured using an enzyme-labelled chemiluminescent immunometric assay validated for dogs using a goat polyclonal anti-troponin I antibody[b].

Non-invasive systolic blood pressure (BP) measurements were recorded from all dogs by Doppler technique[c]. Measurements were made on right or left thoracic limbs, and five values were recorded from each subject for the individual average BP determination according to the American College of Veterinary Internal Medicine consensus statement.[19]

A full echocardiographic examination was carried out by an experienced operator (Francisco Javier Duque) using standard right parasternal and left parasternal views.[20] The following echocardiographic variables were recorded for the study from a right parasternal short axis view at the level of the papillary muscles on M-mode: left ventricular (LV) dimensions in diastole (LVIDd) and systole (LVIDs); LV free wall thickening in diastole (LVFWd and systole LVFWs); interventricular septal wall thickening in diastole (IVSd) and systole (IVSs). From the same view but at the level of the aortic valve, the left atrium to aorta ratio (LA/Ao) was acquired as previously described.[21]

LV mass (LVM) was calculated as previously described[22,23]: LVM (g) ¼ 0.8*(1.04*(LVIDd+LVFWd+IVSd)^3 – (LVIDd)^3) + 0.6. LVM was then divided by the body surface area to calculate the LV mass index (LVMI). Previous technique standardisation and reference intervals reported were used to establish the presence of LV enlargement or thickening, atrial enlargement and increased LVMI.[21–25]

For histopathology the thorax was opened immediately following euthanasia, the heart was removed and opened routinely, and a full thickness myocardial specimen of 10 mm x 10 mm was collected from the middle LV free wall muscle for histopathology analysis and real time PCR. Specimens were fixed in 3.5% buffered formalin and were routinely processed to obtain sections of 6 um. The slides were stained using haematoxylin-eosin (H&E) and subsequently underwent light microscopic evaluation at 200 × magnification using a computer coupled to an optical Nikon Eclipse Ni microscope, equipped with a DS-Ri2 digital camera[d].

The specimens were analysed by one experienced histopathologist (Luis Gómez-Gordo) for the presence and intensity of changes of A. inflammatory cell infiltration, B. cellular degeneration (cellular shape alteration, nuclear loss, cellular striation and intercellular oedema) and C. other findings (i.e. fibrosis and haemorrhage). Inflammation was described as lympho-plasmacytic, granulomatous, neutrophilic or mixed (if one or more than one types were significantly present). Ten fields were evaluated for each section, and the average was recorded. The samples were scored for each of the final seven categories for intensity as follows: 0 (not detected), 1 (mild; 0–32% of the field), 2 (moderate; 33–66% of the field), 3 (severe; >66% of the field). A composite of the records of the seven categories (inflammatory cell infiltration, cellular shape alteration, nuclear loss, cellular striation, intercellular oedema, fibrosis and haemorrhage) provided the final histopathology score severity (maximum of 21). The median score for all dogs was calculated and used to classify dogs in groups A, with a total score ≤ 10 and B with a total score of > 10.

A portion of the same myocardial specimen used for histopathology underwent quantification of *Leishmania infantum* by real-time PCR (qPCR). DNA from all samples was obtained using the UltraClean BloodSpin kit according to the manufacturer´s instructions[e]. PCR reactions were carried out in 96 wells PCR plates in a final volume of 20 microlitres (4 microlitres of DNA + 16 microlitres of Reaction Mix), containing 20 microM of each primer, 10 microM of TaqMan Probe and the iTaq Universal Probes Supermix[f]. Primers, probe and the thermal cycling profile used are described elsewhere.[26] Each amplification run contained positive and negative controls. All qPCR analyses were performed in a Step One Plus Real Time PCR System[g]. A standard curve was carried out using DNA extracted from six quantities of *Leishamnia infantum* parasites (MCAN/ES/1996/BCN150, zymodeme MON-1) ranging from 50,000 to 0.5 (dilution factor x10). The threshold cycle (Ct) corresponding to the Y-intercept of each analysis (that is the expected Ct value for the estimated quantity of 1 parasite) was used as cut-off, being positive those samples whose Ct values were ≤Y-intercept value of each assay. Results are reported as number of parasites per gram of tissue.

Statistical analysis was carried out with a commercial software[h]. Variables with a normal distribution were described as mean ± standard deviation, and those non-normally distributed were reported as median and interquartile range (IQR). Correlations were carried out by Pearsons test if the data were normally distributed or by Spermans Rho if the data were not normally distributed. Correlations were performed between MPL and the following variables: cTnI, NT-proBNP, Globulin, Creatinine, haematocrit (HCT), BP, UPC and LVMI. Correlations between the two biomarkers studied (cTnI and NT-proBNP), and the other selected variables (Globulin, Creatinine, HCT, BP, UPC and LVMI) were also studied. Group comparison between histopathology group A and B in relation to the same selected variables (MPL, cTnI, NT-proBNP, globulin, creatinine, HCT, BP, UPC and LVMI) was performed by one-way ANOVA for normally distributed variables and by Mann-Whitney U test for variables with data not normally distributed. Statistical significance was set at $p < 0.05$.

RESULTS

Ten dogs fulfilled the criteria and were included in the study. Mean age was 4.8 years (±1.6). There were fourmixed breed dogs and one dog each of the following breeds: American Cocker Spaniel, Spanish hound, Brittany dog, Staffordshire Terrier, Spanish Mastiff and Dachshund. There were 80% (8/10) males and 20% (2/10) females.

All dogs were classified as having Leishvet stage IV disease[2]. Blood samples for all blood results variables used and BP values were from samples collected within the 24 hours prior to euthanasia. All urine tests and imaging tests were performed within 72 hours prior to euthanasia. Serum cTnI measured 4.56 ng/mL (IQR 0.46–13.1 ng/mL; reference < 0.05 ng/mL) and plasma NT-proBNP was 6946 pmol/L (IQR 3751–9268 pmol/L; reference < 900 pm/L) for all dogs. Results of clinical variables were: HCT 25.3 % (±10.3; reference 35–45%); Creatinine 6.2 mg/dL (IQR 4–11.5 mg/dL; reference 0.6-1.2); UPC 9.2 (±7.8; reference < 0.5) and BP 195 mm Hg (± 23.2; reference < 160 mmHg). On echocardiography (Table 1) all dogs had LV hypertrophy, either isolated to the LV free wall or the interventricular septal wall, or of both LV walls. Seven dogs had an increase in LV mass index, and in three dogs with normal values they were within the top 1/3 of the normal reference range. All dogs had thoracic radiographs performed, and none showed changes consistent with pulmonary oedema or pulmonary vein congestion or other clinically significant changes.

On histopathology all dogs had myocarditis and myocyte cell degeneration. The myocarditis was in all dogs lymphoplasmacytic and either diffuse (90% of cases; 9/10) or multifocal (10% of cases; 1/10). Mild inflammation was observed in 30% of cases (3/10), moderate in 30% (3/10) and severe in 40% (4/10). Cell degeneration was mild in 20% of cases (2/10), moderate in 40% of cases (4/10) and severe in 40% of cases (4/10). Mild haemorrhage was observed in 10% of cases (1/10), and moderate haemorrhage was present in 10% of cases (1/10). Fibrosis was also observed in two cases, in one of them (10%) mild and in another one (10%) moderate. Myocardial hypertrophy or disarray was not observed in any of the samples. The histopathology score in group A (5 dogs with less severe changes) was 5.8 (±2.8), and the score in group B (five dogs with more severe changes) was 12.4 (±1.5). PCR analysis detected parasitic presence in the myocardium of all dogs. The PCR MPL was 215.53 parasites/gram (IQR 21.2–1372.63 parasites/gram).

There was a very strong correlation between the MPL and cTnI ($p < 0.001$; R = 0.90; R^2 0.81;

TABLE 1 Table showing the echocardiographic measurements of all dogs (n = 10)

Echocardiographic parameter	Value[a,b] $n = 10$	Reference intervals[c,d,e]	Dogs with values within normal reference intervals
LA/Ao	1.14 ± 0.09[a]	<1.6[c]	10/10
LVddi	1.20 ± 0.15 [a]	1.27–1.85[d]	10/10
LVdsi	0.95 (34.10-42.40)[b]	0.71–1.26[d]	0/10
FS	38.25 (37.–46.7)[b]	25–44[d]	9/10[f]
LVFWdi	0.70 ± 0.18[a]	0.29–0.60[d]	2/10
IVSWdi	0.70 ± 0.14[a]	0.29–0.59[d]	0/10
LVMi	230.20 ± 97.28[a]	27.36–154.16[e]	3/10

[a]Normally distributed data shown as mean +/- standard deviation.
[b]Data not normally distributed shown as median (interquartile range).
[c]From Rishniw et al (JVIM 2000).
[d]From Cornell et al (JVIM 2004).
[e]From Takano et al (JVC 2015).
[f]In one dog FS was elevated at 62.70%.
Abbreviations: FS, fractional shortening; IVSWdi, interventricular free wall thickening in diastole index; LA/Ao, left atrium to aorta ratio; LVddi, left ventricular internal dimension in diastole index; LVdsi, Left ventricular internal dimension in systole index; LVFWdi, left ventricular free wall thickening in diastole index; LVMi, left ventricular mass index.

Figure 1) but absence of significant correlations between MPL and NT-proBNP or any of the other clinical variables investigated. Both biomarkers (cTnI and NT-proBNP) were also not correlated with any of the clinical variables. Statistical difference was not observed between groups A and B in relation to their MPL, cardiac biomarkers cTnI and NT-proBNP and the selected clinical variables (Table 2).

DISCUSSION

We describe for first time the MPL in dogs with advanced CanL. All dogs in this study had parasite presence in the myocardium associated with myocarditis and myocardial degeneration. Previous studies[6,7,8] have failed to consistently detect organisms in the myocardium of dogs euthanised due to CanL by routine histopathology or immunohistochemistry but PCR, as used in our study, is known to be a more sensitive test.[12,15,16,17] The cardiac biomarker cTnI levels were very robustly correlated with MPL. This indicates an association between the overall myocardial damage and the cardiac parasitic burden. It further supports the exploration for clinical and research purposes of cTnI as a surrogate for the degree of cardiac involvement in CanL. In this context, cTnI may be used as a non-renal marker adding relevant information to current renal and inflammatory markers. Although further clinical studies are warranted, it may for example be explored as an extra marker in the Leishmania classification in which the cornerstones are renal,[2] in a disease known to be multisystemic with myocardial damage occurring at early stages.[5] The short half-live of cTnI[27] may also support the exploration of this marker as an extra tool to guide response to therapy, which currently relies on resolution of clinical signs and markers such as globulin levels, which can remain elevated for a long period of time following treatment.[1,3,12]

An association between NT-proBNP and MPL was not observed. NT-proBNP has previously been observed to be consistently elevated in advance stages of CanL. Chronic kidney disease is known to be associated with increase NT-proBNP[28,29] but the values in CanL are significantly higher than those of dogs with idiopathic chronic kidney at similar IRIS stage,[11] which potentially might support its exploration as an independent marker in CanL. However, NT-proBNP has shown to be less consistently elevated than cTnI at early stages of the disease and to be strongly associated with secondary processes such as the degree of systemic arterial hypertension and anaemia.[11] The complex and variable interrelations in advanced CanL among several factors such as ventricular thickening, increase in peripheral vascular resistance and volume status, and the resultant ventricular stress, are likely to determine NT-proBNP release. Overall, the findings of this study and previous studies point towards NT-proBNP being a less adequate cardiac biomarker for the assessment of CanL.

Dogs in our study had classical clinical findings of advanced CanL including severely elevated creatinine, globulins and UPC, anaemia and systemic arterial hypertension.[1,3,12] None of the clinical variables studied were found to be associated with MPL. There was no association with creatinine or UPC levels for example, which are the cornerstone of Leishmania staging.[2] The population, however, is very uniform, and it is possible that an association may have been observed in a larger population of dogs including dogs with less advanced IRIS or Leishmania stage. Conversely, it may actually indicate that the relationship between the degree of renal disease and cardiac parasitic burden is indeed not consistent in a disease with variable and heterogenous multisystemic presentation in every patient.[2,12]

The echocardiographic evidence of ventricular wall thickening and increase in ventricular mass observed in this study is a feature consistently observed in dogs with advanced canine leishmaniasis throughout

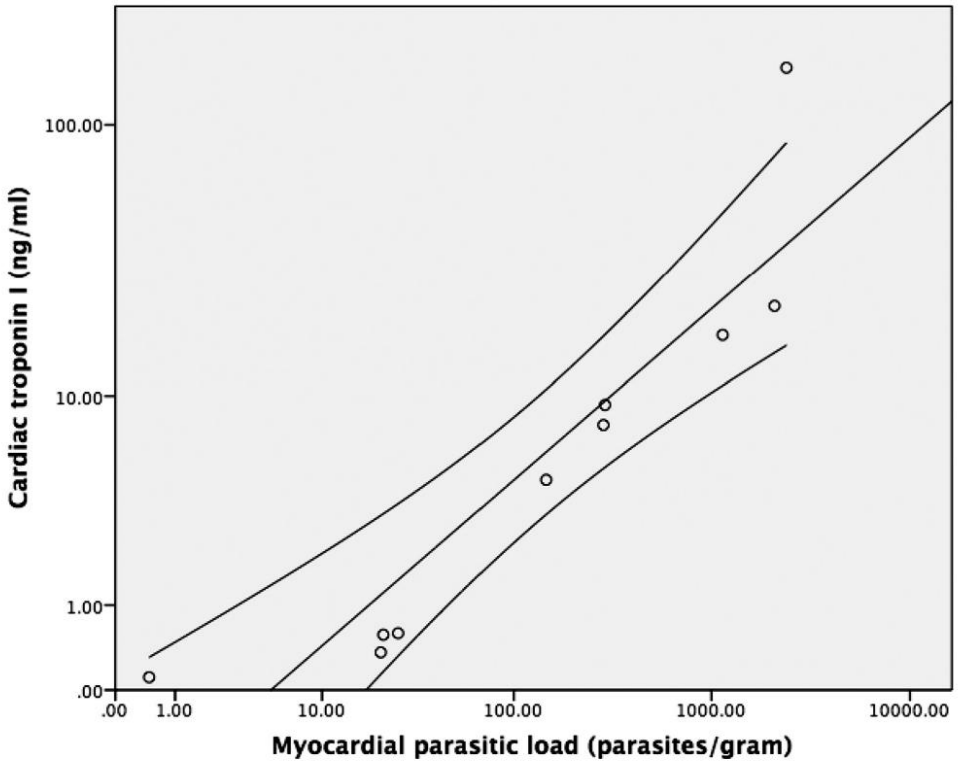

FIGURE 1 Correlation between cardiac troponin I and the myocardial parasitic load by quantitative PCR. There is a very strong correlation (R = 0.91; R^2 0.80). The mean (straight line) and the 95% confidence intervals of the mean (curved lines) are shown. Both variables are represented on logarithmic scale

TABLE 2 Table showing the results of the studied variables in both histopathology groups

Studied variable[a]	Histopathology score Group A[b]	Histopathology score Group B[b]	Significance (*p* value)[c]
cTnI (ng/mL)	0.59 (0.34–32.28)	9.23 (4.02–19.47)	0.347
NT-proBNP (pmol/L)	6923 (±3564)	4677 (±3435)	0.339
Creatinine (mg/dL)	6.9 (4.9–11.5)	6.2 (3.2–15.2)	0.754
UPC	5.6 ((±4.2)	13.7 (9.6)	0.134
BP (mm Hg)	200 (±24)	190 (±23)	0.528
MPL (parasites/gram)	25.70 (10.93–1266.71)	290 (152.80–1607.27)	0.427
LVMi	281.40 (±110.41)	179 (±50.50)	0.096

[a]Variables normally distributed are reported as mean ± standard deviation and variables not normally distributed as median ± interquartile range.
[b]Histopathology score in group A (cases with less severe changes) was 5.8 (±2.8), and it was 12.4 (±1.5) in group B (cases with more severe changes).
[c] Group comparisons were carried out by one way ANOVA for variables with normally distributed data and by Mann-Whitney U test for variables with not normally distributed data.
Abbreviations: BP, blood pressure; cTnI, cardiac troponin I; LVMi, left ventricular mass index; MPL, myocardial parasitic load NT-proBNP, N-terminal proB-type natriuretic peptide; UPC, urine protein/creatinine ratio.

the literature.[11,30] Processes such as inflammatory myocardial infiltration, myocardial oedema, myocardial hypertrophy secondary to systemic arterial hypertension[31,32] and pseudohypertrophy due to hypovolemia, could be potential explanations for these, often severe, echocardiographic findings. The consistent presence of inflammation in the myocardium with myocardial infiltration and oedema in our study and previous studies[6–9] would support this as the likely main cause. Conversely, it cannot be ruled out that hypertrophy secondary to systemic arterial hypertension and pseudohypertrophy may also play a role. Hypertrophy, as it is the case in previous histopathology studies,[6–9] was not observed in

histopathology, but the assessment was subjective (no specific myocyte measurements were carried out), and a degree of hypertrophy cannot be ruled out. Similarly, dogs with severe chronic kidney disease may suffer from hypovolemia at the time of the echocardiographic examination. Assessing hypovolemia is however complex, and it was not specifically evaluated or recorded at the time of echocardiography in this study.

All dogs in our study had moderate to severe myocardial histopathological changes, either associated with cell degeneration or inflammation. The type of inflammation was in all dogs lympho-plasmacytic, which is the most common type of inflammation (either patchy or diffuse) seen in CanL.[6–8] Dogs with higher histopathology score did not have, statistically, a higher MPL or cTnI. The reason for this is not clear as it might have been expected that dogs with higher MPL and higher cTnI exhibit more severe myocardial pathology. This may reflect a more consistent relationship between the parasitic load and myocardial cell damage, while the degree of subsequent inflammation and regeneration may vary among individuals. Myocardial inflammation might be less dependent on the initial local stimulus and/or strongly dependent on the general systemic inflammation. It is also possible that this is the result of a statistical error due to the subjective (non-numerical) nature of histopathology assessment, or due to type II error, in which the low number of cases may have failed to identify a true statistical association, as indeed both cTnI and MPL medians (and means) were much higher in the group with higher histopathology score. It is also possible that single site MPL (LV), obtained by PCR may be more sensitive and therefore represent the overall MPL, which is associated with overall myocardial damage (cTnI), whereas single site myocardial histopathology may miss lesions as these have been reported to be multifocal in nature.[7–9] If this hypothesis was true, an association between MPL and histopathology score might have been observed if samples from multiple cardiac sites had been collected.

There are several limitations to this study. Firstly, the number of cases is low, and Type II error for some of the statistical analysis performed is possible. Secondly, for this study we used dogs with very severe disease undergoing euthanasia as it would not be ethically appropriate to collect myocardial biopsies (by any technique) in dogs at earlier stages of the disease. It could therefore be argued that we cannot extrapolate the observed association of cTnI and MPL to earlier stages of the disease. However, dogs with CanL have been shown to have myocardial damage, detected by cTnI, at earlier stages of the disease[5] and considering the robust results and the wide range of MPL concentrations observed (which included low levels), it is likely that the observed association may also be found at earlier stages of the disease. Another limitation is the number of statistical tests performed. Many statistical tests were carried out, and there is always the possibility of type I error. However, the only statistically significant result was robust with a $p < 0.001$ and therefore unlikely to have occurred by chance. One last limitation would be that the human LVM and LVMI formulas used[23,33] have not been fully validated for dogs. They have however been used in previous research,[11,22] and the reference intervals previously reported[22] were used in this study. It is also not possible to fully rule out that some of the changes in echocardiography were present prior to CanL as prior echocardiography was not available.

In conclusion, all dogs with advanced CanL have parasite presence in the LV myocardium when evaluated by PCR analysis. The degree of MPL is variable, although most commonly high. The MPL is very strongly associated with the overall myocardial damage as detected by cTnI. These findings support further research into the use of cTnI as a clinical or laboratorial marker in CanL.

FOOTNOTE

[*]Cardiopet NT-proBNP, IDEXX laboratories, Spain
[†]Laborti Veterinaria Laboratories, Spain
[‡]Ultrasonic flow detector 811-B, Eickemeyer Veterinary, Surrey, UK
[§]Nikon, Japan.
[¶]MoBio Laboratorios, Carlsbad, CA, USA
[#]Biorad Laboratories, Hercules, CA, USA
[‖]Applied Byosystems Laboratories, Foster City, CA, USA
[**]SPSS 21, IBM, USA

AUTHOR CONTRIBUTIONS

Domingo Casamián-Sorrosal, Rafael Barrera-Chacón, Sonja Fonfara and Francisco Javier Duque conceived and planned the study. Domingo Casamián-Sorrosal, Rafael Barrera-Chacón, Silvia Belinchón-Lorenzo, Luis Gómez-Gordo, Javier Galapero-Arroyo, Javier Fernández-Cotrina, Jose Ignacio Cristobal-Verdejo and Francisco Javier Duque carried out the clinical investigations, contributed to sample preparation and/or performance of postclinical tests. All authors contributed in many ways to interpretation and presentation of results and/or subsequent statistical analysis. Domingo Casamián-Sorrosal took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Article 4

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Vet Record

ORIGINAL RESEARCH

Evaluation of heart fatty acid-binding protein as a biomarker for canine leishmaniosis

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Abstract

Background: Myocarditis frequently occurs in canine leishmaniosis (CanL). Heart fatty acid-binding protein (HFABP) is a biomarker of myocardial damage.

Methods: This study aimed to compare HFABP concentration (HFABPc) in healthy dogs and dogs at different stages of CanL and evaluate the correlation of this biomarker with several clinicopathological and echocardiographic variables. Thirty-one dogs diagnosed with CanL and 10 healthy dogs were included.

Results: HFABPc was not statistically different ($p > 0.05$) between groups of dogs at different LeishVet stages of CanL or between groups with high versus low to intermediate serology titres. In 70% of CanL dogs, HFABPc was within the 95% confidence interval limits of the mean of healthy dogs. A moderate negative correlation with globulin ($r = -0.519$; $p = 0.03$) and haematocrit (HCT) ($r = -0.538$; $p = 0.02$) was observed. No other significant correlation ($p > 0.05$) was observed with any other variable.

Limitations: Many statistical tests were performed, and therefore, type I error cannot be ruled out.

Conclusion: HFABPc is not consistently elevated in dogs with CanL and is not associated with the severity of the disease, or most echocardiographic or clinicopathological variables studied. The correlation with globulin and HCT was not strong and not considered clinically significant. HFABPc lacks sufficient predictive capacity in dogs with CanL, discouraging further research or clinical use of this biomarker in this disease.

INTRODUCTION

Canine leishmaniosis (CanL) is caused by the protozoal organism *Leishmania infantum*[1–3] and is endemic in many Mediterranean and South American countries.[1–3] CanL is a multisystemic disease causing inflammation and damage in many organs, such as the kidneys, liver, spleen, central nervous system and heart.[1–5] For clinical and research purposes, CanL is classified according to the LeishVet scheme,[2] which includes subjective evaluation of clinical signs, *L infantum*-specific antibody levels and kidney markers such as urine protein/creatinine ratio (UPC) and serum creatinine levels.[2] Among these markers, creatinine levels are the cornerstone of the classification.[2] No specific laboratory markers to evaluate the involvement of organs other than the kidneys are included in the LeishVet classification.[2]

Myocarditis and myocardial damage have been described in CanL,[4–10] particularly in patients with advanced disease,[5–10] and there has been interest in both human[11] and veterinary medicine[6,7,9,10,12] in identifying an adequate cardiac biomarker for CanL. The purpose of this biomarker[13] would be first to be used as an overall diagnostic, prognostic and/or therapy-guiding tool, and second as a specific biomarker to identify cardiac involvement in individual cases. Cardiac troponin I (cTnI) is a marker of myocardial damage[14] and has been shown to increase as the disease advances,[7,12] to be elevated in most dogs

with advanced cardiac disease[6,7,10,12] and to correlate very strongly with the degree of myocardial parasitic load.[10] It is a promising biomarker in CanL, and further clinical field research is required to best define its clinical role. N-terminal proB type natriuretic peptide (NT-proBNP), a marker of acute and chronic increments of ventricular stress,[13,15] has also been shown to be increased in CanL,[9,12] although it is less consistently elevated at early stages of the disease.[9,12] It is further strongly correlated with extracardiac variables such as systemic arterial hypertension and anaemia,[9] which makes it less cardiac specific in dogs with CanL.

Heart fatty acid-binding protein (HFABP) is a small, low molecular weight (15 kDa) cytoplasmic protein abundantly expressed in myocardial cells.[16] HFABP is involved in fatty acid transport and metabolism[16,17] and is rapidly released into the systemic circulation after myocardial damage.[16] HFABP is a novel biomarker used in human patients with cardiac disease,[16–20] and it has also been investigated in dogs with acquired cardiac diseases.[21] HFABP has been shown to be elevated and an independent predictor of survival in dogs with dilated cardiomyopathy (DCM) and degenerative mitral valve disease (MVD).[21] The principal cardiac alteration in CanL is myocarditis (mostly lymphoplasmacytic),[4,5,7,8,10] which leads to a variable degree of myocardial damage[4,5,7,8,10,12] and makes HFABP a potentially adequate biomarker in CanL. An increase in left ventricular thickening and increased left ventricular mass (LVM) have been consistently reported in dogs with advanced CanL.[9,10,22] This is suspected to be secondary to myocarditis (oedema and inflammatory cell infiltration), although systemic arterial hypertension may also play a role.[4,5,9,10,22]

We hypothesised that HFABP blood concentration (HFABPc) will be higher in dogs with CanL and HFABPc will correlate with severity of CanL (as per the LeishVet staging system) and with the degree of cardiac remodelling as observed by echocardiography. The aims of this study were therefore: (1) to evaluate and compare the levels of HFABPc in a group of dogs at different clinical stages of CanL and in a group of healthy control dogs. (2) To evaluate the relationship between HFABPc and selected clinicopathological and diagnostic variables.

MATERIALS AND METHODS

Dogs were recruited from the following centres: the veterinary hospital of the University of Extremadura (centre 1), the veterinary hospital of the Catholic University of Valencia (centre 2) and the veterinary hospital of the University of Murcia (centre 3). CanL diagnosis was confirmed by the presence of clinical signs, laboratory abnormalities compatible with the disease and a positive result to a *Leishmania* quantitative serology test (ELISA or indirect fluorescent antibody test [IFAT]). In some cases, additional positive tests included parasitological diagnosis by cytological observation of amastigotes infecting macrophages or positive PCR from lymph node or organ aspirates. Healthy dogs recruited from hospital staff (in centre 1) and from a nearby dog shelter (in centre 2) were included in the control group. The inclusion criteria for healthy controls included dogs being clinically healthy, with a normal full haematology (complete blood count [CBC]) and biochemistry profile, a negative serology and a normal full echocardiographic examination.

All dogs included in the study underwent the following tests as part of the study protocol (which are part of the routine work up): CBC, biochemical profile, urinalysis (including UPC), abdominal ultrasonography and echocardiography. After the completion of this protocol, dogs with any evidence of concurrent disease (e.g., neoplasia) were excluded from the study. Dogs with trivial valvular regurgitations (i.e., colour flow Doppler map extending less than 1 cm from the valve[23]) were included in the study, but dogs with degenerative MVD, other acquired cardiac diseases or any form of congenital cardiac diseases were excluded from the study. As cardiac changes may potentially occur in *Leishmania*-infected dogs,[9,10,22] dogs with systolic dysfunction, eccentric or concentric hypertrophy of the ventricles and/or atrial enlargement were allowed in the study unless DCM had been previously diagnosed or other primary cardiac disease was suspected.

The population of dogs was classified according to the LeishVet classification[2] as healthy (group A), mildly to severely affected (group B—LeishVet stages II and III) and very severely affected (group C—LeishVet stage IV). Within the dogs with CanL, a separate classification according to their quantitative serology results was also created: dogs with low to intermediate antibody levels (group 1) and dogs with high antibody levels (group 2). To classify the serology titre as low to intermediate versus high, cut-off values of 0.8 or higher and 0.0025 or higher were used for dogs tested by ELISA (centres 1 and 3) and IFAT (centre 2), respectively.

A minimum of 3 mL of blood was collected from the jugular or saphenous vein and placed into EDTA blood tubes for HFABPc quantification. Samples were centrifuged and plasma was isolated within 30 minutes of blood collection and stored at −80°C. The samples were shipped in batches on dry ice, and assays were performed upon sample arrival at the laboratory. HFABPc was measured in EDTA plasma using a dog-specific ELISA test (Kamiya Biomedical Company, Tukwila, WA, USA). Intra- and interassay coefficients of variation of the test had been previously determined using adult dog plasma samples and were 8.3% and 10.0%, respectively.[21] The range of the test was between 0.1 and 20 ng HFABP/mL.

Non-invasive systolic blood pressure (BP) measurements were recorded from all dogs by Doppler technique. Measurements were made on right or left thoracic limbs, and five values were recorded

from each subject for the individual average BP determination according to the American College of Veterinary Internal Medicine consensus statement.[24]

A full echocardiographic examination was carried out by experienced operators (F. J. D. in centre 1, D. C. S. in centre 2 and J. T. L. in centre 3) using standard right and left parasternal views. Sedation was not required for any of the dogs. The following echocardiographic variables were recorded for the study: left ventricular dimensions in diastole (LVIDd) and systole (LVIDs); left ventricular free wall thickening in diastole (LVFWd) and systole (LVFWs); interventricular septal wall thickening in diastole (IVSd). All these were obtained from M-mode from a right parasternal short-axis view at the level of the papillary muscles and were indexed to bodyweight.[25] From a two-dimensional short-axis view at the level of the aortic valve, the left atrium to aorta ratio (LA/Ao) was acquired. LVM was calculated as: LVM (g) = 0.8 × (1.04 × [LVIDd + LVFWd + IVSd]^3 − LVIDd)^3) + 0.6. LVM was then divided by the body surface area to calculate the left ventricular mass index (LVMI). Previous technique standardisation and reported reference intervals were used to establish the presence of left ventricular enlargement or thickening, atrial enlargement and increased LVMI.[25–28] Mild, moderate and severe elevations were considered for those values up to 20%, more than 20%–40% and more than 40% of the upper value of the reference interval, respectively. Mild, moderate and severe decreased values were considered for those below the lower limit of the reference interval down to 20%, less than 20%–40% and less than 40%, respectively.

Statistical analysis was performed with commercial software (SPSS 27, IBM, USA). For numerical variables, normality and homoscedasticity were tested using Shapiro–Wilk and Levene tests, respectively. Variables with a normal distribution were described as the mean ± standard deviation, and those non-normally distributed were reported as the median and interquartile range (IQR). Categorical variables were reported numerically and as percentages. Group comparisons for HFABPc, age and weight of dogs in groups A, B and C and between dogs in groups 1 and 2 were performed by one-way ANOVA for normally distributed variables (or Welch's test if one-way ANOVA assumptions were not met) and by Mann–Whitney *U*-test for variables with data that were not normally distributed. Games–Howell or Tukey's honestly significant difference post hoc tests were performed when needed. Group comparison of categorical variables was carried out with Fisher's exact test. Linear correlations were assessed by Pearson's test if the data were normally distributed or by Spearman's rho if the data were not normally distributed. Correlations were performed between HFABPc and the following clinicopathological variables: globulin concentration (globulin), creatinine concentration (creatinine), haematocrit (HCT), BP, UPC, LVFWd index (LVFWdi), IVSd index (IVSdi) and LVMI. Statistical significance was set at *p*-value less than 0.05. When a result is statistically significant, the exact *p*-value is provided in the text. The results not statistically significant are reported for simplicity as such in the text without a specific *p*-value.

TABLE 1 Breeds of the dogs with canine leishmaniosis.

Breed	Number of dogs (total of 31)	Percentage (approximation)
Crossbreed	4	12.5%
Labrador Retriever	4	12.5%
Brittany dogs	3	10%
English Setter	2	6.5%
American Staffordshire	2	6.5%
English Cocker	2	6.5%
German Pointer	2	6.5%
Spanish Alano	1	3%
English Bulldog	1	3%
French Bulldog	1	3%
Greyhound	1	3%
Belgium Shepherd	1	3%
Ratonero Valenciano	1	3%
Whippet	1	3%

RESULTS

A total of 41 dogs (10 healthy controls and 31 dogs with CanL) were included in the study. When classified using the LeishVet staging system, group A included 10 dogs, group B included 14 dogs (10 dogs LeishVet II and four dogs LeishVet III) and group C (LeishVet IV) included 17 dogs. When dogs with CanL were grouped by serology results, group 1 included 16 dogs and group 2 included 15 dogs. The average population age was 5.0 years (±2.4 years), and the average weight was 19.2 kg (±7.2 kg). The population consisted of 53% (22/41) males and 46.3% (19/41) females. Age, weight and sex were not statistically different between groups ($p > 0.05$). Breed distribution among the 31 dogs with CanL is depicted in Table 1.

Crossbreeds represented 60% (6/10) of the control group, and the remaining 4 controls were a German pointer, a Patterdale terrier, a Spanish Waterdog and an English Setter.

The HFABPc of dogs with CanL was 0.38 ng/mL (IQR 0.21–0.89 ng/mL) and was not significantly different to the HFABPc of the control dogs (0.22 ng/mL; IQR 0.19–0.51 ng/mL). Differences in HFABPc were also not observed between groups A (0.22 ng/mL; IQR 0.19–0.51), B (0.40 ng/mL; IQR 0.15–0.84 ng/mL) and C (0.38 ng/mL; IQR 0.22–0.78 ng/mL) or between groups 1 (0.27 ng/mL; IQR 0.17–1.64 ng/mL) and 2 (0.41 ng/mL; 0.21–0.85 ng/mL) (Figures 1 and 2). When the upper threshold of the 95% confidence interval of the mean HFABPc of the control dogs (0.64 ng/mL) was set as the upper normal limit, 70% of dogs with CanL (22/31) were below this threshold. The percentage and

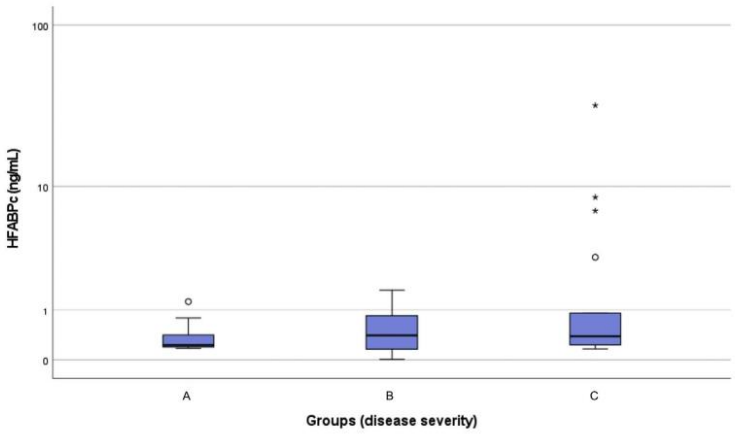

FIGURE 1 Box plot comparing the values of heart fatty acid-binding protein concentration (HFABPc) for the three disease severity groups. Group A (normal control dogs), group B (dogs with mild to severe canine leishmaniosis [CanL]—stages II and III LeishVet) and group C (dogs with very severe CanL—stage IV LeishVet). Statistically significant differences between groups were not observed

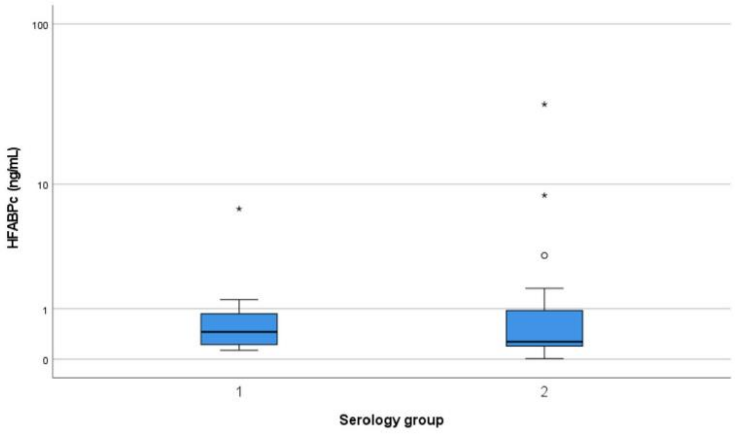

FIGURE 2 Box plot comparing the values of heart fatty acid-binding protein concentration (HFABPc) for the two serology groups within the population of dogs with canine leishmaniosis. Group 1 (dogs with low to moderate antibody levels) and group 2 (dogs with high antibody levels). Statistically significant differences between groups were not observed

number of dogs in each group with a mean HFABPc below this selected upper limit was as follows: 71% (10/14) in group B, 70% (12/17) in group C, 68% (11/16) in group 1 and 73% (11/15) in group 2. Markedly high values were observed in four cases in group C (32.45, 8.46, 6.86 and 3.15 ng/mL; Figure 1).

The results of selected echocardiographic and clinicopathological variables are depicted in Tables 2 and 3, respectively.

When the correlation between HFABPc and all the selected clinicopathological and echocardiographic variables was studied, a moderate negative correlation with globulin ($r = -0.519$; $p = 0.03$) and HCT ($r = -0.538$; $p = 0.02$; Figure 3) was observed. No further significant correlations were found.

DISCUSSION

The results of this study indicate that HFABP does not appear to be a useful biomarker of myocardial damage in CanL. HFABPc was not significantly different between healthy control dogs and dogs with leishmaniosis, and importantly, HFABPc was not elevated in dogs with very severe disease (stage IV of LeishVet). While it could be argued that dogs with milder forms of the disease might have variable myocardial involvement and therefore have less consistent results, it is well documented that most dogs at LeishVet stage IV have lymphoplasmacytic myocarditis[4,5,7–10] and parasitic presence in the myocardium as detected by myocardial PCR.[10] The absence of consistent

TABLE 2 Echocardiographic measurements of dogs with canine leishmaniosis.

Echocardiographic parameter	Value ($n = 31$)	Reference intervals	Within normal (%)	Mildly elevated (%)	Moderately elevated (%)	Severely elevated (%)
LA/Ao	1.23 (1.13–1.33)[a]	<1.6[b]	100%	0%	0%	0%
LVddi	1.47 ± 0.22[c]	1.27–1.85[d]	80%[e]	6%	0%	0%
LVdsi	0.83 ± 0.18[c]	0.71–1.26[d]	100%[f]	3%	0%	0%
FS	40 ± 7.56[c]	25–44[d]	100%	0%	0%	0%
LVFWdi	0.59 ± 0.13[c]	0.29–0.60[d]	58%	16%	22%	3%
IVSWdi	0.57 ± 0.13[c]	0.29–0.59[d]	52%	22%	22%	3%
LVMI	195.75 (139.60–235.06)[a]	90.76 ± 63.4[g]	39%	12%	29%	25%

Note: The value column shows the value of each echocardiographic parameter, while the reference column depicts the normal reference intervals for that parameter. Normally distributed data are shown as the median ± standard deviation, and data not normally distributed are shown as the median and interquartile range (IQR) (as indicated in the footnote). The four columns on the right of the table show the percentage of canine leishmaniosis dogs with normal, mildly elevated, moderately elevated or severely elevated values within each parameter. Mild, moderate and severe elevations were considered for those values up to 20%, more than 20%–40% and more than 40% of the upper value of the reference interval, respectively. Mild, moderate and severe decreased values were considered for those below the lower limit of the reference interval down to 20%, less than 20%–40% and less than 40%, respectively.

Abbreviations: FS, fractional shortening; IVSWdi, interventricular free wall thickening in diastole; LA/Ao, left atrium to aorta ratio; LVddi, left ventricular internal dimension in diastole index; LVdsi, left ventricular internal dimension index; LVFWdi, left ventricular free wall thickening in diastole index; LVMI, left ventricular mass index.

[a]Data not normally distributed are shown as the median (IQR).
[b]From Rishniw and Erb.[27]
[c]Normally distributed data are shown as the median ± standard deviation.
[d]From Cornell et al.[25]
[e]Note that 13% of cases (4/31) had a mild/moderate decrease in LVddi.
[f]Note that 13% of cases (4/31) had a mild/moderate decrease in LVdsi.
[g]From Takano et al.[28]

TABLE 3 Table showing the clinicopathological parameters and blood pressure of dogs with canine leishmaniosis.

Variable (unit)	Value	Reference intervals
Haematocrit (%)	30 ± 10.3[a]	35–55
Globulin concentration (g/dL)	4.84 ± 1.36[a]	2.7–4.4
Albumin/globulin ratio	0.49 (0.34–0.56)[b]	<0.9–2
Creatinine (mg/dL)	3.34 (0.9–5.4)[b]	<1.4
Urine protein/creatinine ratio	2.16 (0.91–5.3)[b]	<0.4
Systolic blood pressure (mmHg)	168.33 ± 25.07[a]	<160

Note: Normally distributed data are shown as the median ± standard deviation, and data not normally distributed are shown as the median and interquartile range (IQR) (as indicated in the footnote). The value column shows the value of each variable, while the reference column depicts the normal reference intervals.

[a]Normally distributed data are shown as the median ± standard deviation.
[b]Data not normally distributed are shown as the median (IQR).

elevation of HFABPc in these dogs with advanced disease, a group in which approximately 70% of dogs in this study had values below the elected reference threshold, discourages the exploration of this marker as a clinical tool for CanL.

When the CanL population was classified according to their antibody levels, no difference in HFABPc was found between dogs with low to intermediate titres and dogs with highly elevated titres.

Dogs with CanL, in particular those with advanced disease, often have evidence of cardiac remodelling on echocardiography, characterised by increased left ventricular wall thickening and increased LVM.[9,10,22] This type of remodelling was observed for most dogs in this study. The cardiac remodelling in these cases is thought to be primarily due to myocarditis, with the myocardium being altered by inflammation and myocardial infiltration of inflammatory cells.[9,10,22] However, the presence of increased afterload due to systemic arterial hypertension and/or pseudohypertrophy in hypovolaemic patients may also play a role.[9,10,22] We did not detect a correlation between cardiac remodelling or systemic arterial hypertension and increased HFABPc.

It is unclear why HFABP, unlike cTnI in previous studies,[6,7,10,12] was not associated with clinical results in CanL. HFABPc has been shown to be elevated in humans with myocardial infarction[16–19] and DCM[20] and in dogs with DCM[21] and MVD.[21] It is a biomarker characterised by rapid increase after myocardial injury,[16] a short half-life and a rapid decrease after the insult when compared with cTnI.[16–19] It is possible that the timing and severity of myocardial damage in CanL is better detected by a biomarker with a longer half-life, which allows a larger time window for detection after myocardial insult. It is also noteworthy to point out that the elevations of HFABPc in DCM and MVD dogs in previous studies[21] were much higher in dogs with congestive heart failure, which was not observed in dogs with CanL. Also, the cardiac remodelling in DCM and MVD is characterised by eccentric hypertrophy and/or systolic dysfunction, which is different from the remodelling observed in dogs with CanL. Ultimately, looking at individual cases, four out of 31 cases (group C—LeishVet IV) had markedly elevated HFABPc levels. Indeed, in one case, the HFABPc was 63 times higher than the upper threshold, and in another case, it was 13 times higher. It is possible that these may represent windows in which acute myocarditis with very recent severe myocardial damage had occurred and was detected in individual cases. A subjective evaluation of other clinicopathological or echocardiographic values of these cases was initially

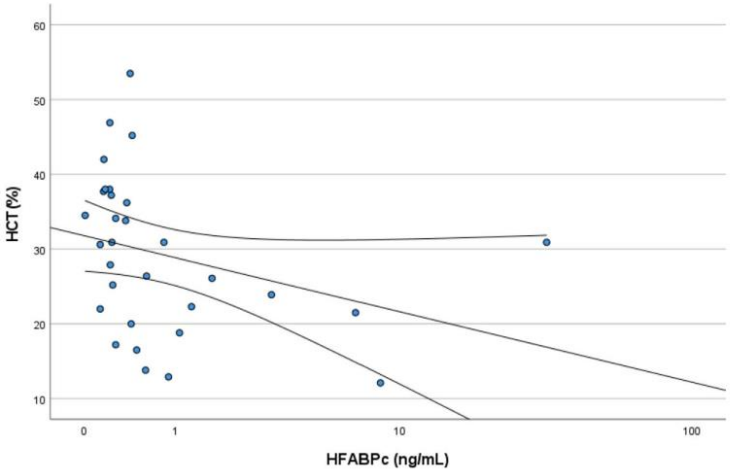

FIGURE 3 Scatter plot showing a moderate negative correlation between haematocrit (HCT) and heart fatty acid-binding protein concentration (HFABPc) ($r = -0.538$; $p = 0.02$). The mean (straight line) and the 95% confidence intervals (curved lines) of the mean are shown

performed to determine whether there was an obvious common characteristic in these four cases, but no variable was obviously different from the rest of the population. To avoid speculation solely based on these few outliers, further statistical analysis was not carried out. These findings, however, do not alter the overall poor results observed with HFABPc in CanL.

A negative correlation was present between HFABPc and HCT. This was also observed for NT-proBNP in a previous study and may suggest an association between HFABPc and more severe anaemia. Myocardial injury secondary to hypoxia is a possible cause for this association. The negative correlation of HFABPc with globulins is more difficult to explain, as a positive correlation would have been more reasonable as globulins are a good marker of disease. To the best of the authors' knowledge, there is no evidence of a possible interference of globulins with HFABPc analysis. Nevertheless, these two statistically significant correlations were not strong and are unlikely to have clinical relevance considering the overall results.

This study has several limitations. Firstly, the use of other myocarditis and/or myocardial damage markers, such as cTnI, endomyocardial biopsy or cardiac MRI, for comparison with HFABPc would have strengthened the results and provided further information. However, performance of HFABPc was poor in the population studied, which comprised dogs with severe or very severe disease, and this finding, we believe, strongly shows the inadequacy of HFABPc as a biomarker for clinical use or further research in CanL. ECG changes may have also been added to the data as a diagnostic variable to study and compare with HFABPc, as other types of myocarditis have been associated with arrhythmias.[29–32] This was not included in the initial diagnostic protocol and was therefore not available. However, major arrhythmias that required intervention on auscultation or simultaneous ECG during echocardiography were not recorded in the dogs included in the study. Indeed, CanL has not been associated with significant arrhythmias in previous studies.[33,34] However, it is important to note that many dogs in previous studies were not in very severe stages of the disease; therefore, future studies evaluating continuous 24–48-hour ECG recordings in this subpopulation would certainly be of interest.

It is possible that statistical differences might have been observed with a higher number of dogs, and this may also be considered a limitation. However, approximately 70% of CanL dogs were within the normal interval (and therefore overlapping with normal values), numerical differences were very small, and results for dogs at different stages of the disease were nearly identical. It is therefore unlikely that having a higher number of cases would have yielded results leading to a different clinical conclusion.

Many statistical tests were performed, and therefore, type I error cannot be ruled out. We elected not to obtain statistical numerical corrections but to evaluate and critically review the results for robustness and/or clinical relevance. The unexpected negative correlation of HFABPc and globulin might in this context represent a type I error.

Another limitation of our study may include the consideration of normal HFABPc intervals based on an approximation from study control dogs. The establishment of a reference interval requires a larger number of cases, and two or three standard deviations of the mean are generally used. Therefore, the true limits of normality may be lower or higher than the threshold used in this study. However, if we examine the HFABPc

values of a control population ($n = 21$) in a previous study,[21] they were very similar (indeed slightly higher) with an upper limit threshold at 0.80 ng/mL (0.64 ng/mL was used in our study). Therefore, the considerations and conclusions discussed in this article seem appropriate.

HFABPc is filtered by the kidney, and in humans, renal function affects HFABPc.[35,36] An increase of approximately 10% is observed when glomerular filtration rate is below 60 mL/min.[35] Age has also been shown to have a small effect on HFABPc.[35] It is likely that similar effects may also occur in dogs and could have an effect on the results. However, the studied population comprised a reasonably homogeneous population in terms of age, with a standard deviation of 2.4 years. Decreased renal function would have led to a bias towards an increase in HFABP at more advanced disease stages, which was not the case in our study. These limitations are thus unlikely to have a significant effect on the overall results and conclusion of the study.

A final limitation to consider may include the intrinsic variability in echocardiographic measurements, in particular when different operators are involved. However, the operators were experienced, and the echocardiographic parameters evaluated were basic and well established. We believe it is unlikely that this potential variability may affect the results of this study.

In conclusion, HFABPc is not consistently elevated in dogs with CanL. This lack of sensitivity is observed both in dogs with milder and more severe forms of the disease. A consistent association of HFABPc with echocardiographic or clinicopathological variables was also not observed. HFABP does not appear to be a good biomarker in CanL. Further research into or clinical use of this biomarker in CanL is discouraged by the results of this study.

AUTHOR CONTRIBUTIONS

Domingo Casamián-Sorrosal, Rafael Barrera-Chacón, Sonja Fonfara and Francisco Javier Duque conceived and planned the study. Domingo Casamián-Sorrosal, Rafael Barrera-Chacón, Silvia Belinchón-Lorenzo, Jose Ignacio Cristobal-Verdejo, Jesús Talavera-Lopez, Alicia Caro-Vadillo and Guadalupe Miró-Corrales carried out the clinical investigations and contributed to sample preparation and/or performance of postclinical tests. All authors contributed in many ways to interpretation and presentation of results and/or subsequent statistical analysis. Domingo Casamián-Sorrosal took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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DATA AVAILABILITY STATEMENT

The full data for further support of the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The study was approved by the Ethical Committee for Animal Experimentation of the University of Extremadura.

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DISCUSSION

The results of these studies indicate that cardiac biomarker cTnI emerges as the best biomarker for clinical use and further research in CanL. NT-proBNP showed, contrary to some aspects of our hypotheses, positive results, albeit many weaknesses were observed when compared to cTnI, which may make it a less adequate biomarker in CanL. HFABP was, in disagreement with our initial hypothesis, an inconsistent biomarker in CanL and further clinical use or research into this biomarker is not warranted.

The biomarker cTnI was consistently elevated in most dogs with advanced CanL and also in some dogs with milder disease. Elevations were indeed often marked in severe and very severe cases. The larger subpopulation in our study, dogs with severe and very severe disease in Leishvet stage III and IV, represent a group of dogs in which the degree of myocardial damage assessed by cTnI had not been reported before. The degree of elevations observed in many of these dogs with advanced CanL are rarely seen in primary myocardial disease such as DCM (58,59,200,206), or in most dogs with other non-cardiac specific systemic diseases (196,219,221,224,225,227,229,232,233). It is a degree of change seen in cases with severe and extensive myocardial damage, usually with necrosis, in cases of severe myocarditis (28,29) or myocardial infarction (55,184,287).

The studies showed, for first time in dogs with CanL, absence of association of cTnI levels with extracardiac factors such as blood pressure or anaemia, and a strong association with *Leishmania* MPL and LeishVet staging. Dogs with CKD showed elevated cTnI levels (as it has been previously reported (176)) but they were markedly lower than those of dogs with CanL at the same IRIS stage. These findings support our initial hypothesis and confirm cTnI as an excellent specific cardiac biomarker for clinical use and further research in CanL. Serum cTnI consistently detected myocardial damage due to CanL myocarditis in these patients and can therefore be used to assess the severity of CanL myocarditis in individual patients. The

consistency of these results also supports further studies into the potential use of cTnI as prognostic variable in CanL and its use as biomarker to aid guiding therapy. In this regard, cTnI is known to decrease rapidly after an insult (55,184,287) and may prove a good biomarker for short and mid-term monitoring of treatment.

Our studies added 20 cases of histologically confirmed myocarditis to the literature, increasing the total number of cases to 91. The prevalence was, as in previous studies, high (95%), and similar to the prevalence observed by Costagliola et al (14) which likely represented a similar population of dogs with most probably severe or very severe disease. In the studies by Rosa et al (26) and Silva et al (20), which included dogs with milder disease severity, the prevalence was lower, albeit still above 80%. Similar to what has been previously reported (14,20,26), lympho-plasmocytic infiltration and cellular degeneration and necrosis was the most common finding in our study population, with absence of granulomatous disease or perivascular distribution, which has been a rare finding in CanL myocarditis (14,20,23,26). We also report for first time the presence and quantity of *Leishmania* as detected by PCR in the myocardium. *Leishmania* was present in all cases, and it was variable in quantity but most commonly high. Variable MPL has been previously reported using other techniques. This discrepancy and the detection of *Leishmania* in all our cases is likely the result of PCR being a more sensitive technique. These results further support the persistent involvement of the myocardium and the presence of myocarditis in CanL. Contrary to our initial hypotheses however, MPL and cTnI were not higher in dogs with higher histopathology score. As discussed in depth in the discussion of the specific study, this may be the result of type II statistical error, be due to the subjective aspects of the histopathology scoring system, or be associated with the limitations of one site sampling in this particular study. In this

context, cTnI may quantify the severity of global myocardial damage with more consistency than single site myocardial histopathology.

Once established that myocarditis is a common feature in CanL and that cTnI emerges as the most appropriate biomarker to detect this myocardial pathology, a question arises: at what disease stage does CanL myocarditis occur? Merging previous data and the information of the studies comprising this Doctoral Thesis, it appears that most dogs with severe disease (Leishvet III) and all dogs with very severe disease (Leishvet IV) consistently suffer from CanL myocarditis. There is also a percentage of dogs with moderate disease (LeishVet II) which develop myocarditis and it is therefore within the II-III staging when myocardial involvement appears to develop. Knowledge of when myocarditis develops can support the selection of populations to investigate cTnI as a prognostic factor. In this context, establishing whether a further substage II (and potentially III) could be divided as cTnI + or cTnI –, for example, can be explored. Looking at our results, it also becomes clear that some patients in stages III and IV have remarkably high cTnI levels while in others the elevation is more modest. The quantity of cTnI elevation in this population may also have overall prognostic significance. A close examination of the data, together with the robust correlation of cTnI with MPL, suggests the disease may affect the myocardium with more virulence in some individuals. Being a heterogenous disease is indeed a characteristic of CanL, in which different degree of involvement is seen in other organs or systems such as the liver or the CNS (3,19,51,108,163).

A third important aspect also emerges within this time-event relationship in CanL. If cTnI is explored as a marker of therapy response, this may only be possible in LeishVet IV and III and in some cases of LeishVet stage II. Therefore, a baseline sample is mandatory because elevations vary and in some dogs with milder disease increased values will not be observed. Ultimately, the results of this thesis provide valuable information for a clinician investigating

cardiac disease in patients with CanL. A markedly elevated cTnI level would not be expected solely by CanL in moderate (LeishVet II) cases, while severe elevations of cTnI may be observed in patients with severe (LeishVet III) or very severe (LeishVet IV) disease and would not be due to other underlying cardiac disease.

NT-proBNP showed marked and consistent elevation in dogs with severe or very severe CanL (LeishVet III and IV). The elevation in moderate disease (Leishvet II) was rare (1 dog) and mild. This is similar to the results observed in a study conducted simultaneously (67) and suggests that, similarly to cTnI, NT-proBNP is expected to be consistently elevated in advanced stages. Contrary to cTnI however, it is rarely elevated in moderate disease, making it potentially less sensitive to early myocardial disease. The results of the studies comprising this Thesis and results from this previous study (67), are very different to another study in which NT-proBNP was rarely elevated, and only in a small number of cases with very severe disease (LeishVet IV) (68). Whether this reflects errors in the study, different populations, or highlights inconsistency of NT-proBNP as a biomarker in CanL, is not certain. Our studies showed NT-proBNP to be strongly associated with anaemia and systemic arterial hypertension but not with markers of CanL involvement in myocardial disease such as MPL or histopathological score. This does not rule out NT-proBNP as a marker of CanL for the purposes previously described but makes it less adequate than cTnI. Like for cTnI, the information described is important for clinicians and cardiologists investigating cardiac disease in patients with CanL. Marked elevations (markedly above the current threshold consistent with congestive heart failure) may be due to severe or very severe CanL (LeishVet III and IV) and not due to underlying cardiac disease or congestive heart failure. Conversely, an increase in NT-proBNP above reference ranges would very rarely occur at mild or moderate stages of the disease (LeishVet I and II).

Contrary to the other two biomarkers studied in the studies comprising this Thesis, HFABP was rarely and inconsistently increased in dogs with CanL. Associations of clinical interests with other clinical variables or general biomarkers were also not observed. This was unexpected as it was hypothesized that HFABP´s specificity for myocardial disease would make it an adequate candidate biomarker. Its short half-life, for example, could be considered an ideal characteristic for exploring a biomarker as a tool to guide therapy. The negative results however, preclude the use of HFABP for this purpose or, in general, as a biomarker for further research in CanL. It is possible that its negative performance may be indeed due to the intrinsic characteristics of this biomarker. HFABP is a cardiac damage biomarker characterised by a rapid increase and a very short half-life and may be less adequate to detect damage in CanL than a biomarker, such a cTnI, with a slightly longer slope of increase, longer time to peak values, and longer half-life which, may detect longer temporal windows of myocardial damage. While a small number of cases had marked increase in HFABP values and may have represented more severe myocardial disease or a peracute on chronic myocardial insult, this was very infrequent and does not support HFABP to be used as a CanL biomarker. In view of these negative results and considering HFABP availability is less widespread, when compared to cTnI, further use of this biomarker is not warranted.

On echocardiography, the majority of dogs with severe or very severe disease (LeishVet III and IV) showed an increase in left ventricular wall thickening and, consistent with our initial hypothesis, increase in left ventricular mass. The latter had not been reported before. These results provide additional data to complement the results previously reported in a study of dogs with CanL and systemic arterial hypertension(21). Although the studies were not specifically designed to evaluate the cause of this cardiac remodelling, the proven consistent presence of CanL myocarditis with inflammatory cell infiltration and oedema, and the

absence of features associated with systemic arterial hypertension such as myocyte hypertrophy (288), suggest that the myocarditis itself is likely the major factor of this remodelling. Nevertheless, as discussed in depth in the discussion of one of the studies, remodelling secondary to systemic arterial hypertension and pseudohypertrophy due to hypovolemia may also play a role. The consistent findings on echocardiography are also very useful for the cardiologist exploring a patient with CanL, or when increase LVMi or left ventricular thickening is observed during an individual examination. In a dog with CanL in severe or very severe disease, the left ventricular remodelling changes described would be expected and considered part of the disease, while others, like left atrial enlargement for example, would not. Conversely, in a dog in which a left ventricular hypertrophy phenotype is observed in an endemic area, CanL should be included in the differential diagnosis.

Ultimately, none of the dogs involved in the studies comprising this Thesis had been vaccinated for CanL. Vaccines are now becoming more frequently used in CanL and whether they may cause a degree of myocarditis, or they may affect cardiac biomarkers, is unknown. The evidence that myocarditis starts from moderate to severe stages likely makes the presence of CanL myocarditis, and subsequent biomarker alterations because of vaccination, unlikely. Further studies in the fields of biomarkers in CanL should nevertheless continue to specify CanL vaccination status.

CONCLUSIONS

1. Lymphoplasmacytic myocarditis occurs in dogs with CanL and cTnI is an excellent biomarker to evaluate this CanL associated myocardial disease.
2. CanL myocarditis occurs within the moderate to severe stages of the disease (Leishvet II or III) and is seen in nearly all dogs with very severe (LeishVet IV) disease.
3. In many advanced cases of CanL the degree of myocardial damage is very severe and remodelling on echocardiography is characterised by increase in LVMi and left ventricular thickening.
4. Myocarditis in CanL, and the associated cTnI elevation and cardiac remodelling, is likely the direct result of parasitic colonisation of cardiac tissue and autoimmune mechanisms. Secondary pathological processes such as renal disease, systemic arterial hypertension, and anaemia, appear to have a less important contribution to the myocardial damage and subsequent cTnI elevation occurring in patients with CanL myocarditis.
5. Leishmania organism, as detected by PCR, is observed in all dogs with CanL myocarditis in severe and very severe (LeishVet III and IV) stages. The MPL in these dogs is variable, but usually high, and it is very strongly associated with cTnI levels, while association is not seen with many other clinical variables and selected biomarkers including NT-proBNP.
6. NT-proBNP is considered a less adequate biomarker in CanL. Although it is severely elevated in dogs with severe and very severe CanL (LeishVet III and IV), NT-proBNP is strongly associated with extracardiac factors such as systemic arterial blood hypertension and anaemia, it does not appear to detect early CanL myocarditis and it is not associated with the degree of MPL or the severity of histopathology score.

7. HFABP is not consistently elevated in dogs with CanL, even at severe or very severe disease, and associations of clinical interest with clinicopathological biomarker or echocardiographic variables are not observed. Further clinical or research use of HFABP in CanL is discouraged.
8. Values of cTnI and NT-proBNP at different CanL staging is provided for clinical use to elude the misdiagnosis of cardiac disease and/or congestive heart failure in patients with CanL.

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