Universitat Autònoma de Barcelona

New clinico-pathological findings and prognostic factors of canine leishmaniasis in endemic and nonendemic areas

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PhD Thesis

"New clinico-pathological findings and prognostic factors of canine leishmaniasis in endemic and non-endemic areas"

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In order to obtain the degree of Doctor in Veterinary Medicine

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Los Doctores JOSEP PASTOR MILÁN y MARTA PLANELLAS BACHS, Profesor Titular y Asociado del Departamento de Medicina y Cirugía Animales de la Facultad de Veterinaria de la Universitat Autònoma de Barcelona, respectivamente,

INFORMAN:

*Que la memoria titulada "***New clinico-pathological findings and prognostic factors of canine leishmaniasis in endemic and non-endemic areas***", presentada por el licenciado Paolo Silvestrini para la obtención del grado de Doctor en Veterinaria, se ha realizado bajo nuestra dirección y, considerándola satisfactoriamente finalizada, autorizamos su presentación para que sea evaluada por la comisión correspondiente.*

Y para que así conste a los efectos que sean oportunos, firmamos el presente informe en Bellaterra, 4 marzo 2016

Firmado: ***Josep Pastor Milán*** *Firmado:* ***Marta Planellas Bachs***

“…Nihil dificile volenti…”

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After all these years of studying hard, working long hours and travelling around the world for conferences and meetings, I find myself today sitting on the sofa of our lovely home in the UK thinking of all the people with whom I shared extraordinary experiences and who have helped me achieving my goals. They are so many, though, that I would need many pages to thank each one and the list below is not all-inclusive!

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1. SUMMARY

Canine Leishmaniasis (CanL) is due to *Leishmania infantum* (syn. *Leishmania chagasi*) and is endemic in Mediterranean countries, Portugal, Latin America and Southern Asia. In the last few decades, imported and even autochthonous cases have been recorded in traditionally non-endemic areas such as Central and Northen Europe and Northen America. This is possibly due to a wider spread of the vector and especially to a larger numbers of travelling dogs. Many studies about CanL have been published in the last years and have contributed in understanding different aspects of this disease, including the alternative ways of transmission and the pathologic mechanisms underlying the clinical findings. However, CanL still remains a very challenging disease to diagnose, treat and prevent. Moreover, it is still very difficult to predict the outcome given the low numbers of controlled studies evaluating markers of prognosis. So, the main aims of the present thesis were to investigate new clinico-pathological aspects of CanL and to possibly identify useful prognostic factors. The first study demonstrated that a significant proportion of dogs with leishmaniasis have increased serum cTnI concentration, suggesting that CanL can cause cardiac disease, mainly myo- and endocarditis. In the second study, the iron status and its relationship with C-reactive protein (CRP) was for the first time investigated in CanL. The results indicated that dogs with leishmaniasis have decreased serum iron, total iron-binding capacity (TIBC), unsaturated iron-binding capacity (UBIC) and percentage of transferrin saturation and increased concentrations of ferritin. Increased CRP and decreased TIBC are also risk factors for moratility. Finally, since the disease is progressively changing its geographical distribution, the last investigation was conducted in the United Kingdom (UK), currently considered a non-endemic country. The majority of dogs that were diagnosed of leishmaniasis have been adopted from an endemic area (especially from the Mediterranean countries) respect a minority that have travelled to those regions. No autochthonous cases were recognised. Pure-breed dogs and those that were classified in stage D according to the Canine Leishmaniasis Working Group guidelines were at higher risk of death. Differently to what has been reported in endemic countries, serology titre at

diagnosis and IRIS staging for chronic kidney disease did not influence the outcome.

2. INTRODUCTION

The Leishmaniases are a group of infectious diseases that affect humans and domestic and wild animals worldwide. The infection is principally transmitted by sandflies, causing a major diffusion of the diseases in warm temperate countries (40°N-40°S) where the vector is able to live.

Human leishmaniasis (HL) is endemic in 88 countries, including 66 in the Old World and 22 in the New World. No vaccine and/or completely effective prevention systems are currently available for humans, making the diffusion of the infection irrepressible. Approximately 12 million of humans are infected and around 350 millions are at risk of acquiring the disease. The world's poorest populations living in rural and suburban areas are at higher risk as well as immunocompromised people. HL is generally classified into two principal forms according to their clinical manifestations: cutaneous and visceral. A mucocutaneous form is also possible.

Canine Leishmaniasis (CanL) is due to *Leishmania infantum* (syn. *Leishmania chagasi*) and is endemic in Mediterranean countries, Portugal, Latin America and Southern Asia. At least 2.5 million dogs are infected in southerwestern Europe alone (Moreno *et al.* 2002). However, in the last few decades, imported and even autochthonous cases have been recorded with increased frequency in traditionally non-endemic areas such as Central and Northern Europe (Maia & Cardoso 2015; Shaw *et al.* 2009) and North America (Gaskin *et al.* 2002; Duprey *et al.* 2006). This is possibly due to a wider spread of the vector and especially to a larger numbers of travelling dogs. Moreover, the diffusion of the infection in non-endemic areas has increased the suspect of alternative ways of transmission, including in utero from dam to its offspring, by haematophagous arthropods, blood transfusion and possibly though direct dog-to-dog contact. CanL is essentially a chronic systemic disease that may potentially involve any organ and is manifested by very different clinical signs. Dogs usually have both visceral and cutaneous involvement. A delayed diagnosis and/or an ineffective therapy can cause very severe consequences such as renal failure, haemorrhagic diathesis and death. Recently, the Canine Leishmaniasis Working Group (CLWG) (Paltrinieri *et al.* 2010) and the LeishVet group (Solano-Gallego *et al.* 2011) have respectively created a staging system based on clinical signs, laboratory abnormalities and diagnostic test results. These systems have significantly helped to

standardise the diagnostic and therapeutic approach to the patients, clearly differentiating between “infected” and “diseased” animals. CanL is a disease in which infection does not equal clinical illness. Although the knowledge of the disease has significantly progressed in the last decades, the information and education of dog owners have improved and new and more effective preventive measures, such as insect repellents and vaccination, are now available, CanL still continues to cause concern to clinicians, mainly through the lack of a straightforward diagnosis, an uniformly effective, safe and permanent treatment and the paucity of prognostic factors. In particular, predicting the outcome of CanL is still very challenging, given the low numbers of controlled studies evaluating clinical and clinico-pathological markers of prognosis (Castagnaro *et al.* 2007; Roura *et al.* 2013).

3. AIMS

The major aims of this project were:

- Investigate new clinico-pathological aspects of CanL, primarily focusing on inflammatory markers

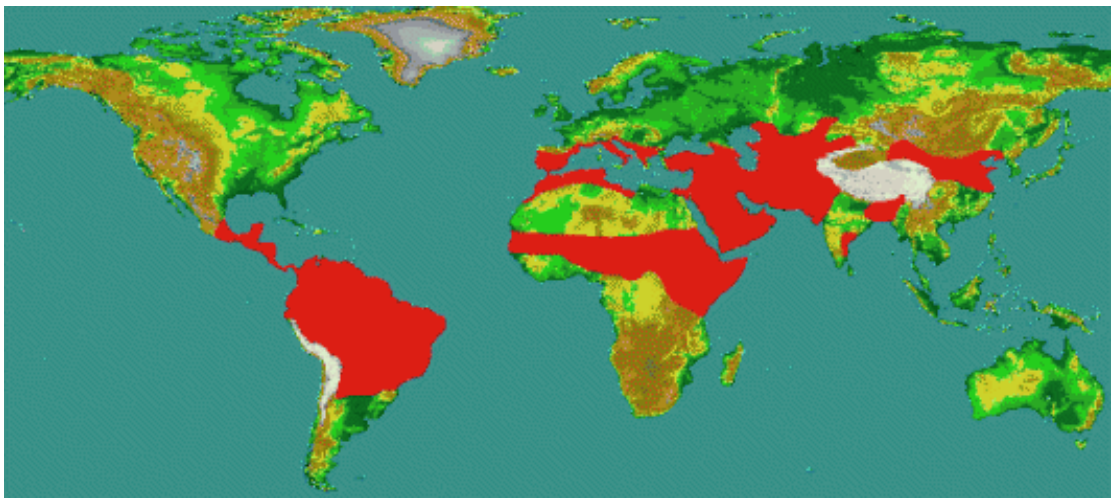
- Identify new possible prognostic factors in endemic areas

- Investigate clinical and clinico-pathological aspects of CanL in a non-endemic country as the United Kingdom (UK) and identify possible prognostic factors

4. LITTERATURE REVIEW

4.1 Aetiology and Epidemiology

The leishmanises are a group of zoonotic vector-borne diseases caused by various species of the protozoa *Leishmania* spp.*,* which belongs to the class Kinetoplastida and family Trypanosomatidae. Around 30 different leishmanial species are found in various parts of the Old and the New World. Most *Leishmania* spp. that infect humans are zoonotic, and only a few are principally anthroponotic (*Leishmania donovani* and *Leishmania tropica)*. Human and animal disease due to *Leishmania* spp. is distributed across five continents (Africa, Asia, Europe, and the Americas) and nearly 100 countries (Figure 1).



Human and canine leishmaniasis across the five continents

Infected dogs are an important reservoir of the parasite (Baneth *et al.* 2008) and play a significant role in the epidemiology of human visceral (HVL) and cutaneous (HCL) leishmaniasis. Approximately 12 million humans are infected with leishmaniasis, and around 350 millions are at risk of acquiring the disease. The yearly incidence is around 0.2 to 0.4 and 0.7 to 1.2 million HVL and HCL cases, respectively (Desjeux 1996; 2001). More than 90% of global HVL cases occur in the world's poorest populations living mainly in rural and suburban areas including India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil. HCL is more widely distributed with about one-third of cases occurring in each of three epidemiological regions, the Americas, the Mediterranean basin, and western Asia from the Middle East to Central Asia (Alvar *et al.* 2012).

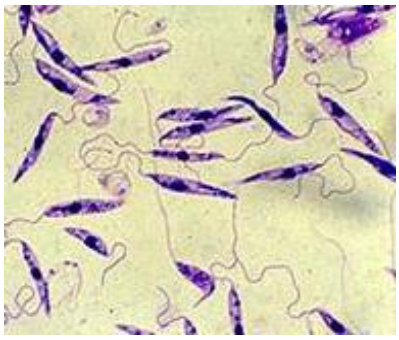
CanL is a severe systemic zoonotic disease caused by *Leishmania infantum* (*Leishmania chagasi* in neotropic ecozones) (Baneth *et al.* 2012). Reservoir hosts vary within different geographic areas and can include rodents, domestic and wild animals (mainly carnivores). CanL is endemic in more than 70 countries worldwide (Solano-Gallego *et al.* 2011) and especially in the Mediterranean areas of Europe (Cyprus, Greece, Albania, Croatia, Italy, Malta, France, and Spain) and Portugal, the Middle East and many tropical and subtropical areas of the world (Maia & Cardoso 2015). Canine infection rates approach 70% to 90%, as shown by polymerase-chain-reaction (PCR) and serology in highly endemic foci, such as Balearic Islands of Spain (Solano-Gallego *et al.* 2001), the Marseille area in France (Berrahal *et al.* 1996), throughout Greece (Leontidas *et al.* 2002), and the Naples area in Italy (Oliva *et al.* 2006). At least 2.5 million dogs are infected in southerwestern Europe alone (Moreno *et al.* 2002). However, only a low proportion of dogs succumbs to the infection and develops clinical signs, whereas the majority of them are resistant and harbour the pathogen subclinically.

Interestingly, the infection is spreading to non-endemic areas with an increasing number of cases reported in dogs living in North America (Gaskin *et al.* 2002; Duprey *et al.* 2006) and Northern Europe (Maia & Cardoso 2015; Shaw *et al.* 2009). Recent studies have documented the presence of the disease in Germany (Geisweid *et al.* 2012), United Kingdom (Shaw *et al.* 2009) and Netherlands (Teske *et al.* 2002). This is probably due to a wider spread of the vector in some of the above areas and especially to a larger numbers of dogs being imported from, or having visited, endemic countries.

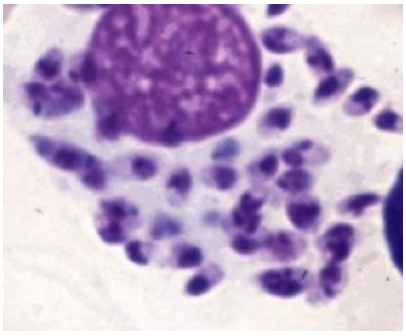
4.2 Life cycle and Transmission

Leishmania spp. is principally transmitted by female sandflies of the genus *Phlebotomus* in the Old World (Africa, Asia, and Europe) and *Lutzomyia* in the New World (the Americas). Sanflies breed in cracks in the walls of dwellings, rubble, and in rodent burrows, feed primarily at night, and fly a maximum of 2.5 km from their breeding sites.

The life cycle of *Leishmania* spp. alternates between two major forms: the *promastigote* and the *amastigote* (Figure 2).



Promastigotes



Amastigotes

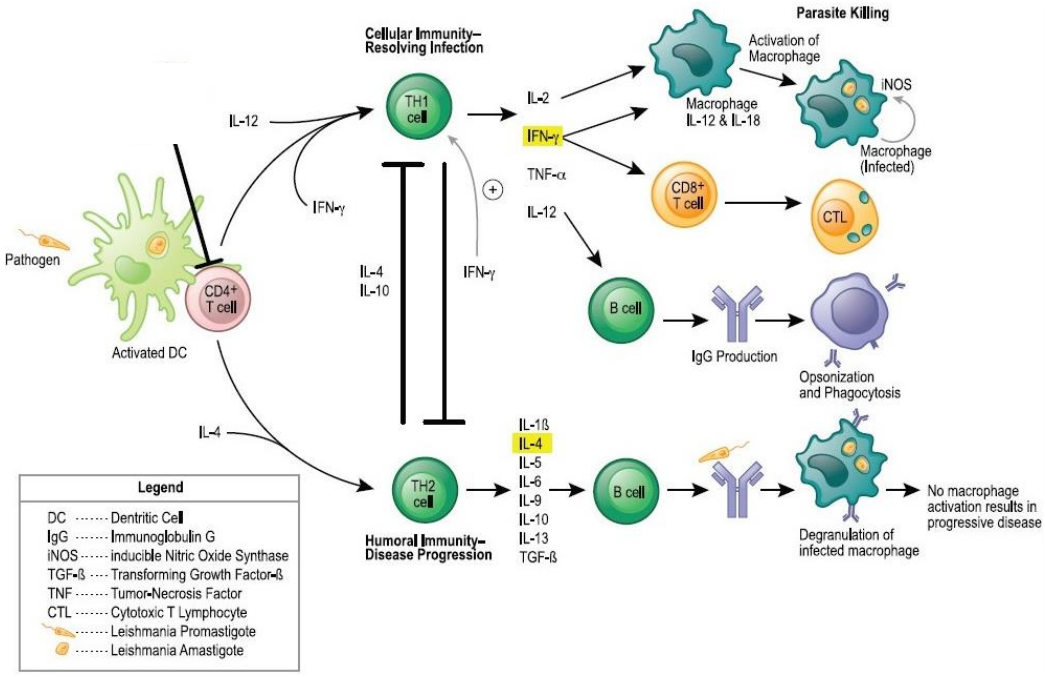
The promastigote resides in the gut of the sandfly vector and is an elongated, flagellated form. Promastigotes are inoculated into tissues when the sandfly feeds, where they are phagocytized by macrophages in the dermis and transform into intracellular, non-motile, ovoid, or round amastigotes. The amastigotes survive and replicate within macrophage phagolysosomes, and cause eventually the infected macrophages rupture. Released amastigotes then infect other macrophages that have been attracted to the site of infection. If the infected host fails to control the infection, amastigotes disseminate via regional lymphatics and the blood to infect the entire reticuloendothelial system. Intracellular amastigotes that are ingested by a female sandfly during feeding convert back into promastigotes over a period of about 1 week, and replication of promastigotes within the sandfly completes the life cycle.

Although *Leishmania* spp. is naturally and principally transmitted through the bites of sandflies, other modes of transmission are possible. In utero transmission from a

dam to its offspring has been documented in a few clinical reports (da Silva *et al.* 2009) and under experimental (Rosypal *et al.* 2005) and natural (Pangrazio *et al.* 2009) conditions. Venereal transmission from infected males to healthy bitches has been also documented (Silva *et al.* 2009). Transmission by haematophagus arthropods other than sandflies has been reported but still not proven to have epidemiologic significance. *Rhipicephalus sanguineous* ticks have been shown to acquire *Leishmania* spp. organisms in their guts after feeding on infected dogs (Coutinho *et al.* 2005). Transmission of *Leishmania* spp. by fleas is suspected but still not proven (Ferreira *et al.* 2009). Blood transfusion (Giger *et al.* 2002; Owens *et al.* 2001) has been reported as a possible way of transmission and is of special concern due to a significant risk of collecting blood from infected but clinically asymptomatic donors. A recent study (Tabar *et al.* 2008) showed that *Leishmania infantum* infection was quite common in canine blood donors and their blood products in an endemic area, despite a negative commercial serological screening for infectious diseases. Direct dog-to-dog transmission has been also suspected (Duprey *et al.* 2006) especially in areas where sandfly vectors are absent.

4.3 Pathogenesis and Clinical presentation

The evolution of *Leishmania* spp. infection is the result of an interaction among the vector (eg. repeated infectious bites), parasite (virulence), and host (eg. genetic background, immune response, coexisting diseases). As mentioned before, not every dog naturally or experimentally infected with *Leishmania* spp. develops diseases (Killick-Kendrick *et al.* 1994; Pinelli *et al.* 1995). The immune responses mounted by dogs at the time of infection and thereafter appear to be the most important factor in determining whether they develop a generalised infection and whether and when the infection will progress from a subclinical state into clinical disease. Infected dogs are typically allocated into *susceptible,* in which the unrestricted parasites multiplication leads to organ damage and dysfunction, and *resistant,* which eventually eliminate the parasite and remain clinically normal (Saridomichelakis *et al.* 2009). However, subclinical infection is not necessarily permanent, and factors such as immunosuppressive conditions or concomitant disease can break the equilibrium and lead to the progression of clinical disease. In the susceptible dog, the innate or non-specific immune system is evaded by *Leishmania* parasites via several mechanisms. One of these involves the ability of amastigotes to survive and replicate within macrophage phagolysosomes by producing compounds such as lipophosphoglycans that inhibit phagosome maturation (Sacks *et al.* 2002). The major role against the parasite is played by the specific immune response: the influence imposed by the balance between T-helper-type (Th1) and Th2 cellular immune response is crucial for the evolution and outcome of natural CanL (Figure 3).



Immune responses after *Leishmania spp.* infection *(Nature reviews: Immunology)*

Protective immunity is most likely mediated by the action of tumour necrosis factor (TNF)-α, interleukin (IL)-2 and interferon (IFN)-γ (typical of a Th1 response), that are secreted by activated T cells to upregulate the antileishmanial activity of macrophages through nitric oxide production that is responsible for the parasite killing by apoptosis (Holzmuller *et al.* 2006; Pinelli *et al.* 1994). In addition, *Leishmania infantum*-infected macrophages are lysed in a histocompatibility complex-restricted manner by CD8+ cytotoxic T cells. Increased levels of CD8+ T lymphocytes are a major feature of subclinical infection and low parasitism (Guerra *et al.* 2009; Reis *et al.* 2009). These processes are decreased or suppressed in sick dogs, in which T-cell proliferation and IFN-γ production are depressed and a marked IgG response to the parasite ensues (De Luna *et al.* 1999; Pinelli *et al.* 1994). Susceptible dogs mainly develop a Th2 reaction, characterised by secretion of cytokines such as IL-4, IL-10 and production of a significant antibody response. Severe clinical disease and high parasitism are accompanied by decreased numbers of CD4+ T, CD5+ T, CD8+ T, and CD21+ B lymphocytes and monocytes. A strong delayed-type hypersensitivity response with the leishmanin intradermal skin test (Montenegro test) indicative of a cell-mediated response to *Leishmania infantum* infection is found in resistant dogs that have been exposed to the parasites and is

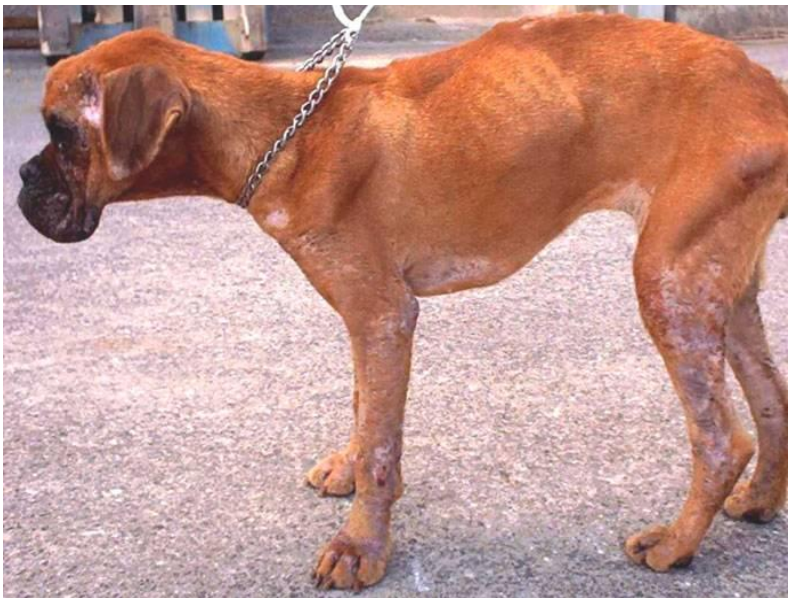
absent in severely ill dogs (Cardoso *et al.* 1998; Maia *et al.* 2008). Susceptibility and resistance to CanL appear to have also a genetic basis, as has been shown by the polymorphisms and mutations of the *SLc11c1* gene (solute carrier family 11, member a1), the association of its haplotype TAG-8-141 with Boxer breed predisposition to CanL, and the link between symptomatic CanL and the DLA-DRB1 genotype, considered a major histocompatibility complex class II allele in the dog (Altet *et al.* 2002; Quinnell *et al.* 2003; Sanchez-Robert *et al.* 2005; 2008). Apart from the Boxer, some other breeds (e.g. Cocker Spaniel, Rottweiler, and German Shepherd) are more susceptible to develop symptomatic leishmaniasis in contrast to the Ibizan Hound, in which the clinical disease is rather rare due to its predominant cellular-mediated Th1-type immune response (Franca-Silva *et al.* 2003; Sideris *et al.* 1999; Solano-Gallego *et al.* 2000). Age seems to be another important risk factor that influences the development of disease. The age distribution of clinical disease has two peaks, one of young dogs (2 to 4 years old) and another in older (more than 7 years old) dogs (Miranda *et al.* 2008).

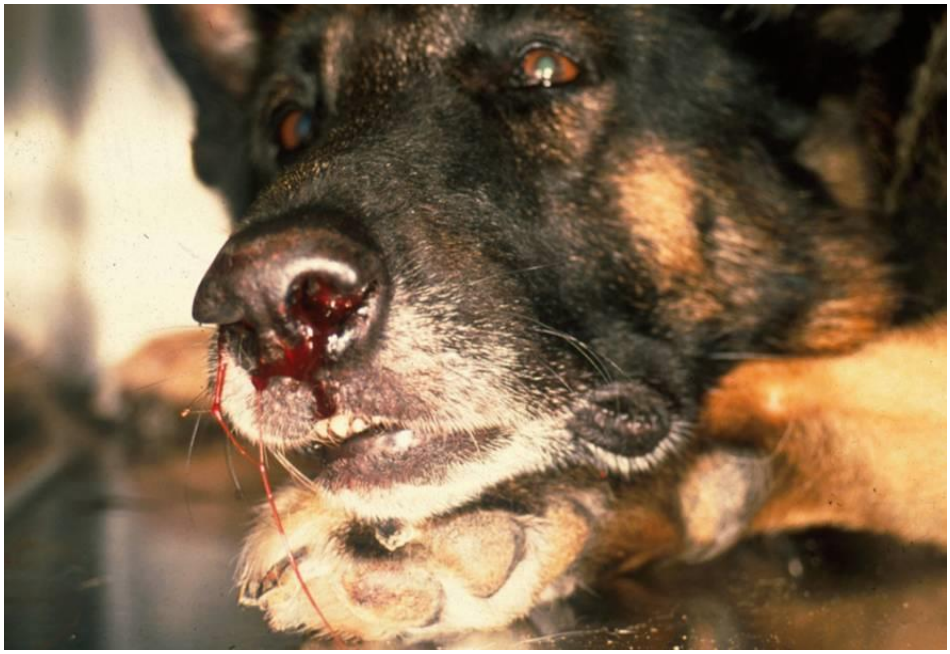
CanL is a chronic disease and clinical signs may develop 3 months to 7 years after infection. T-lymphocytes regions in the lymphoid organs become depleted, and antibody-producing B-cell regions proliferate. The proliferation of B lymphocytes, plasma cells, histiocytes, and macrophages results in generalised lymphadenomegaly, splenomegaly, and hyperglobulinaemia. The latter is not protective but detrimental, either directly or indirectly, via the generation of autoantibodies (e.g. immune-mediated thrombocytopenia), antihistone antibodies (e.g. glomerulonephritis), and/or circulating immune complexes (CICs) (Cortese *et al.* 2009; Ginel *et al.* 2008; Lopez *et al.* 1996). CIC deposition in the walls of blood vessels may cause vasculitis, polyarthritis, uveitis, glomerulonephritis (usually mesangioproliferative and membranoproliferative) and tubolinterstitial nephritis. Renal dysfunction can progress from mild proteinuria to nephrotic syndrome and end-stage kidney disease, which is considered the main cause of death of dogs with leishmaniasis (Planellas *et al.* 2009). In cold weather, cryoglobulins may also be generated, which precipitate in the blood vessels of the extremities and result in

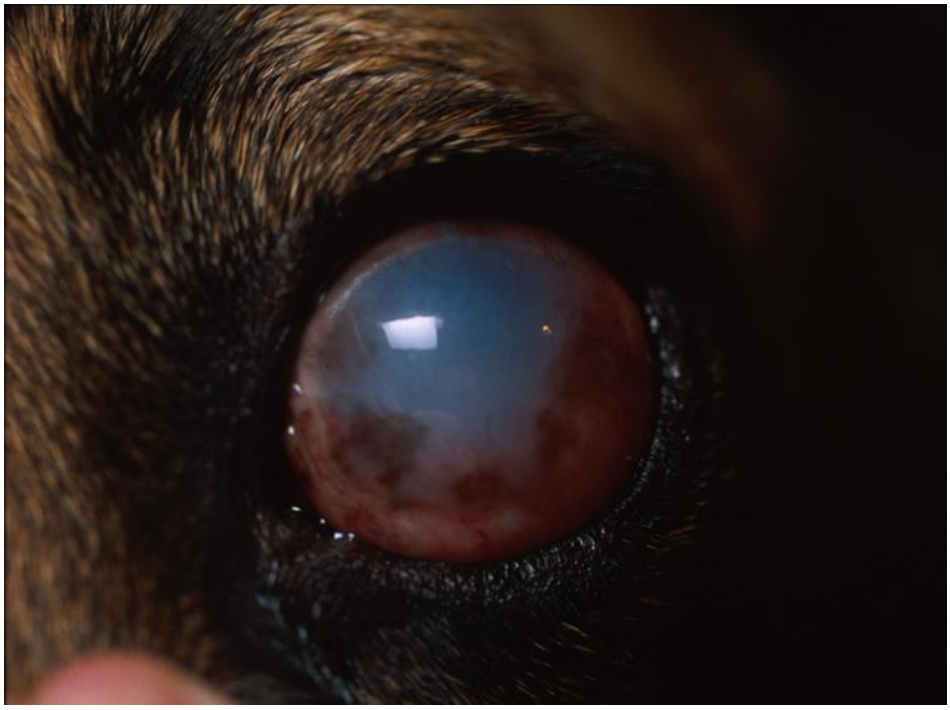
ischemic necrosis (Baneth *et al.* 2012). In the typical CanL case, history and physical examination may reveal reduced appetite or anorexia, lethargy, emaciation,

cachexia, peripheral lymphadenomegaly, exercise intolerance, skin lesions, temporal muscle atrophy, splenomegaly, polyuria/polydipsia, epistaxis, ocular lesions, onychogryphosis, lameness, and vomiting and diarrhoea, which appear alone or, more often, in various combinations (Figures 4,5,6,7; Table1).

(Photos from Dr Xavier Roura)







The variability and non-specificity of clinical signs in CanL make the list of differential quite extensive. The situation becomes more complicated because the clinical diversity of CanL may also be generated by other vector-borne organisms that flourish in the same geographic areas and may infect any leishmanial dog (Roura *et al.* 2005). This is far more common in dogs with an outdoor lifestyle and in those not routinely treated with ectoparasiticides (Solano-Gallego *et al.* 2004). Several epidemiologic and clinical studies have shown that leishmanial dogs can be

coinfected by some infectious or parasitic diseases, such as monocytic ehrlichiosis (*Ehrlichia canis*), granulocytic anaplasmosis, rickettsiosis, bartonellosis, babesiosis, hepatozoonosis, and dirofilariosis (Roura *et al.* 2005; Mekuzas *et al.* 2009; Tabar *et al.* 2009). Dogs with leishmaniasis may show signs of a haemorrhagic diathesis, manifested primarily as epistaxis, and less commonly as haematuria and haemorrhagic diarrhoea. The leading causes of epistaxis, which may be acute or chronic/recurrent, unilateral or bilateral, and sometimes severe enough to cause anaemia due to uncontrollable blood loss, are consistent with thrombocytopathy, increased serum viscosity due to hyperglobulinaemia, and rhinitis, ulcerative or not (Mylonakis *et al.* 2008; Petanides *et al.* 2008) . Vasculitis may also contribute in same cases causing bleeding ulcerations on the nasal philtrum and/or nostrils. Anaemia usually develops as a sequel to the decreased erythropoiesis of chronic disease or chronic kidney disease but may be aggravated but blood loss. Skin lesions are perhaps the most common clinical findings, occurring in 80% to 90% of cases (Koutinas *et al.* 1999; Ciaramella *et al.* 1997). They vary in character and extent and are rarely pruritic, including: 1) exfoliative dermatitis with focal or multifocal alopecia, generally localised on the face, ears, and limbs; 2) ulcerative dermatitis over bony prominences, and in muco-cutaneous junctions, paws, and the ear pinnae; 3) focal or multifocal nodular dermatitis; 4) mucocutaneous proliferative dermatitis; 5) popular dermatitis (Ordeix *et al.* 2005). Less common cutaneous manifestations include pustular dermatitis, nasal depigmentation, nasodigital hyperkeratosis, paronychia, panniculitis, acral lick dermatitis, alopecia areata or pemphigus foliaceous-like disease (Ginel *et al.* 1993), and erythema multiforme. In the endemic areas of CanL, all those dogs diagnosed of cutaneous sterile pyogranuloma/granuloma syndrome, kerion infection leproid granuloma, acral lick dermatitis, or reactive histiocytosis should be investigated for *Leishmania infantum* infection with aid of immunohistochemistry, immunofluorescence, or molecular techniques (Cornegliani *et al.* 2005; Santoro *et al.* 2008). Ocular lesions are also quite common in CanL and can include anterior uveitis, conjunctivitis, keratoconjunctivitis sicca, blepharitis, or a combination of these (Peña *et al.* 2000, 2008). Less common clinical conditions that have been linked or attributed to CanL include: 1) muscle weakness: generally associated with mononuclear myositis, neutrophilic vasculitis

and IgG immune complexes in muscle tissues in conjunction with serum anti-myofiber antibodies (Paciello *et al.* 2009; Vamvakidis *et al.* 2000); 2) joint disease: consistent with an erosive or non-erosive mono- or polyarthritis that results from the lymphoplasmacytic to granulomatous inflammation secondary to synovial membrane affliction by *Leishmania* amastigotes and neutrophilic inflammation that follows the deposition of CICs; 3) oral disease: tongue nodules or papules or mutifocal to diffuse ulcerative glossitis and stomatitis; 4) digestive disease is an uncommon to rare clinical presentation and is mainly expressed as chronic hepatitis and chronic colitis; 5) the relationship between cardiopulmonary disease and *Leishmania infantum* is rather unclear but some reports have described a possible link with non-suppurative myocarditis, fibrinous pericarditis, myocarditis and pneumonia (Font *et al.* 1993; Torrent *et al.* 2005); 6) CanL-associated meningo-encephalomyelitis would explain the various neurologic signs observed (e.g. seizures, painful and rigid neck, paraplegia), and is consistent with a granulomatous and/or neutrophilic meningitis, central nervous system granulomas, spinal haemorrhages, vasculitis, and/or brain infarcts (Font *et al.* 2004; Jose-Lopez *et al.* 2012; Vinuelas *et al.* 2001); 7) CanL has been also associated in male dogs with development of orchitis, epididymitis, chronic prostatitis causing haematospermia-teratozoospermia, penile granulomatous disease, and balanoposthitis (Diniz *et al.* 2005; Manna *et al.* 2012; Mir *et al.* 2012).

Finally, CanL can potentially involve any organ, tissue, and biological fluid and manifests by a plethora of clinical signs.

Table 1: Clinical findings in dogs with leishmaniasis *(Greene, C.E. 2012)*

Findings	% of Dogs
Clinical & Historical Findings	
Exercise intolerance	67.5
Weight loss	64
Lethargy	60
PU/PD	40
Anorexia	32.5
Diarrhoea	30
Vomiting	26
Epistaxis	6-15
Melaena	12.5
Sneezing	10
Coughing	6
Fainting	6
Physical examination abnormalities	
Lymphadenomegaly	62-90
Skin lesions	81-89
Cachexia	10-48
Abnormal locomotion	37.5
Hyperthermia	4-36
Ocular disease	16-81
Splenomegaly	10-53
Onychogryphosis	20-31
Rhinitis	10
Pneumonia	2.5
Icterus	2.5

4.4 Diagnosis

The purpose for which diagnosis of *Leishmania infantum* is carried out include: 1) confirm the disease in a symptomatic patient; 2) screen clinically healthy dogs living and/or travelling to endemic areas; 3) screen blood donor dogs. Diagnostic tests are also implemented to monitor response to treatment. Due to these different diagnostic indications, it is important to differentiate *Leishmania* infection from disease and choose different diagnostic techniques for each state. A clinically healthy infected dog does not show clinical signs and clinico-pathological abnormalities typical of the disease but the infection is confirmed by diagnostic tests. A sick dog shows compatible clinical signs and laboratory abnormalities and the diagnostic tests results confirmed the infection. More details are provided in the chapter 4.7 about "Clinical staging systems". The diagnosis of CanL is complex as the clinical spectrum is broad and the range of clinico-pathological abnormalities can be wide and non-specific. In addition, dogs with leishmaniasis can be co-infected with other vector-borne diseases or suffering from other concomitant infectious or non-infectious diseases. Consequently, the diagnosis should be always based on an integrated approach considering signalment, history, clinical findings, results of basic blood and urine analysis and of more specific diagnostic tests. Unfortunately all of them lack 100% sensitivity and specificity (Solano-Gallego *et al.* 2009).

Table 3 summarises the laboratory findings generally associated with CanL (Paltrinieri *et al.* 2010).

Table 3: Results of laboratory tests associated with CanL *(Paltrinieri, S. et al. 2010)*

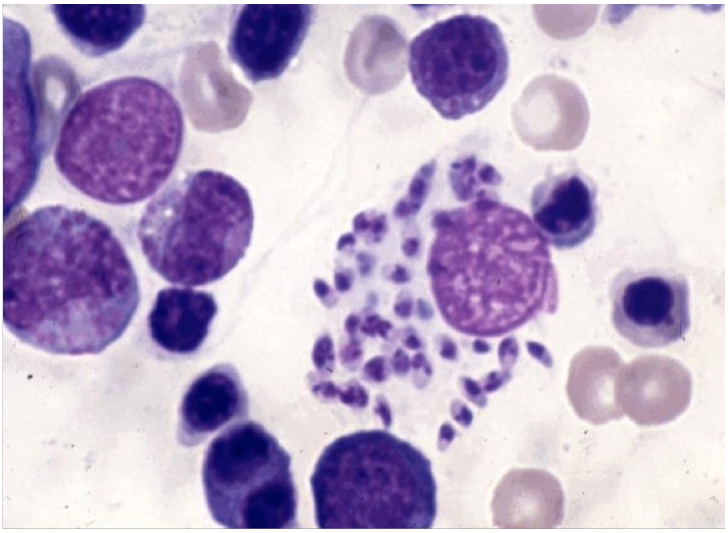
Basic test	Findings	Additional tests*
Haematology	Poorly regenerative or non-regenerative anaemia	-
	Possible regenerative anaemia (due to immune-mediated process)	Coombs' test or Flow cytometry to detect antibodies against RBCs
	Neutrophilia & monocytosis with lymphopaenia and eosinopaenia	-
	Leukopaenia	Bone marrow cytology
	Possible thrombocytopenia	aPTT, PT, FDPs, AT, D-dimers to rule in/out DIC; Tests for coinfection (eg. *Ehrlichia canis*); Flow Cytometry to detect antibodies against PLTs
Basic coagulation profile	Hyperfibrinogenaemia and increased PT and aPTT	Extended coagulation profile (FDPs, AT, D-dimers)
Serum biochemistry	Hyperproteinaemia, hypoalbuminaemia, hyperglobulinaemia, reduced albumin-to-globulin ratio	Acute-phase proteins (CRP, haptoglobin, SAA)
	Azotaemia (increased urea and/or creatinine)	Lipid concentrations (hypercholesterolaemia) Electrolytes concentrations (hypokalaemia) Mineral concentrations (hyperphosphataemia and hypermagnesaemia) Blood gas analysis (Metabolic acidosis)
	Increased hepatic enzymes	Liver function tests
Serum protein electrophoresis	Hypoalbuminaemia, increased α_2-globulin concentration, polycloncal or oligoclonal gammopathy	Acute-phase proteins (CRP, haptoglobin, SAA)
Urinalysis	Isosthenuria (s.g. 1.008 to 1.012) or poorly concentrated urine (< 1.030)	-
	Proteinuria (determined by dipstick test and UPC ratio)	SDS-AGE of urine to detect evidence of mixed or glomerular proteinuria

*To be performed for a more complete staging system, if findings of basic test are consistent with CanL.

aPTT = activated partial thromboplastin time; AT = antithrombin III; CRP = C-reactive protein; DIC = disseminated intravascular coagulation; FDPs = fibrin or fibrinogen degradation products; PLTs = platelets; PT = prothrombin time; RBCs = red blood cells; SAA = serum amyloid A; s.g. = specific gravity; UPC = urine protein-to-creatinine ratio; SDS-AGE = SDS-agarose gel electrophoresis.

To identify the parasite or a patient's responses to it, various methods of aetiologic diagnosis are available (Maia *et al.* 2008). These are generally classified as 1) parasitological, including cytology, histology, immunohistochemistry and culture; 2) serological, including quantitative tests such as immunofluorescence antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) and qualitative rapid tests; 3) molecular, including conventional, nested and real-time PCR. These diagnostic methods can be also grouped into two main categories: 1) direct (cytology, histology, culture, PCR and xenodiagnosis) that demonstrate the infection and presence of the protozoa and 2) indirect as serology, that demonstrates the production of specific antibodies and tests that evaluate the cellular immune response.

Cytologic evaluation allows microscopic detection of *Leishmania* amastigotes in macrophages within affected tissues (Figure 8).



***Leishmania* amastigotes in a macrophage**

Heavily infected cells may burst releasing parasites that can be found extracellularly. Microscopic evaluation of aspirates may also show cytologic changes consistent with CanL, such as lymphoplasmacytic or granulomatous-pyogranulomatous inflammation, lymph node reactive hyperplasia, myeloid hyperplasia, or erythroid hypoplasia. Cytology should involve fine-needle aspiration of the following tissues or

lesions: papular, nodular, and ulcerative skin lesions; bone marrow and lymph nodes; any biological fluids obtained from affected sites such as synovial fluid or cerebrospinal fluid (CSF) when arthritis/polyarthritis or central neurological signs are present, respectively. In the absence of clinical signs and/or involvement of any particular organ, samples should be obtain from tissues where the parasites are more likely to be detected as bone marrow, lymph node, spleen, and buffy coat, in descending order of diagnostic sensitivity (Pennisi *et al.* 2005).

Identification of the parasite by histopathological examination is not always possible and more challenging than with cytology due to the small size (shrinkage of amastigotes during formalin fixation) and the suboptimal staining properties of haematoxylin-eosin (Saridomichelakis *et al.* 2014). Visualisation of the parasite may be enhanced by additional stains, as Giemsa, but is typically based on immunohistochemistry or direct immunofluorescence (Peña *et al.* 2008; Ferrer *et al.* 1988; de Queiroz *et al.* 2010). Disadvantages of the histology include the increase cost, waiting time for the results, unknown specificity of immunohistochemistry and direct immunofluorescence and invasiveness of sampling (Roura *et al.* 1999).

Culture of *Leishmania* organisms is probably the most specific assay because of the development of viable promastigotes. However, the blood-agar-based media needed for diagnostic cultures is not available commercially, and thus this test can performed only at specialised laboratories. The long period required (up to 30 days) before results are obtained makes this test not useful for the clinical setting.

PCR allows amplification of specific sequences of the *Leishmania* genome. The method is very sensitive, particularly when small subunit rRNA genes or kinetoplast DNA (kDNA) minicircles, are targeted for amplification (Muller *et al.* 2003). The kDNA assays are considered the most sensitive due to the high copy number of this target (Lachaud *et al.* 2002). However, in endemic regions, the clinician should remember that a positive result simply means that the dog is affected and does not prove that the infection is the cause of the clinical signs (Saridomichelakis *et al.* 2009; Sellon *et al.* 2003). The three most commonly used techniques are conventional or traditional

PCR assay, nested PCR assay, and quantitative real-time PCR. In a conventional PCR assay, *Leishmania* DNA is amplified by use of specific primers (Muller *et al.* 2003; Cortes *et al.* 2004). In a nested PCR assay, which is a modification of the traditional PCR assay, two consecutive PCR assays with one or two internal primers are performed. This techniques is more sensitive that the conventional method but has lower specificity because more laboratory steps are used, increasing the risk of foreign DNA contamination (Roura *et al.* 1999; Fisa *et al.* 2001). In a quantitative PCR assay (real-time PCR assay), fluorescent probes are used to quantify the number of *Leishmania* DNA copies present in a biological samples. The assay is performed in a closed system and is less prone to false positive results. According to published findings (Francino *et al.* 2006), the quantitative PCR is also useful in monitoring the effectiveness of treatment. Samples with the highest chance of containing leishmanial DNA include, in descending order of sensitivity, bone marrow, lymph nodes, spleen, skin, conjunctiva, buffy coat, blood and urine (Maia & Campino 2008; Maia *et al.* 2009; Manna *et al.* 2008).

Xenodiagnosis consists of allowing laboratory-bred phlebotomine vectors to feed on a dog suspected of having leishmaniasis. The flies are then examined for the presence of promastigotes in the gut. The method is very specific and sensitive but it is not applicable for routine clinical practice.

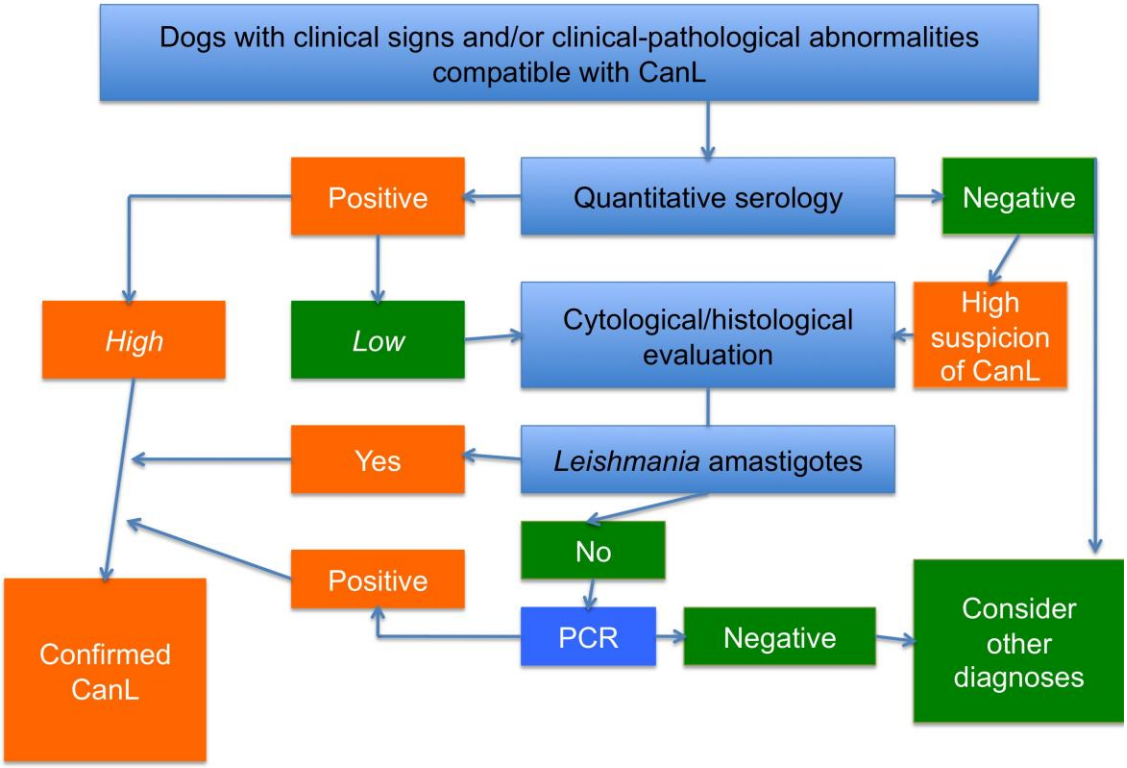
Various serologic methods have been used to detect serum anti-*Leishmania* specific IgG antibodies, including IFAT, ELISA, immunochromatography with rapid in-house devices, direct agglutination assays, and Western blotting. Generally, IFAT, ELISA and the rapid in-house kits are the most commonly employed (Bourdeau *et al.* 2014). In general, most of these methods have good sensitivity and specificity for the diagnosis of clinical CanL. Therefore, high antibody concentration in a dog with compatible clinical signs and clinico-pathological abnormalities are almost diagnostic of the disease. The clinical disease and high anti-*Leishmania* antibody levels are positively correlated with high parasite burdens (Manna *et al.* 2009). In cases with low antibody titers but presence of clinical signs, additional detection methods are

advised to exclude or confirm the disease. An IFAT is performed by placing serial serum dilutions onto slides coated with *Leishmania* promastigotes. Specific antibody binding and relative concentration (antibody titer) is revealed by use of fluorescent antibodies. Evaluation of fluorescence intensity by microscopy is prone to subjective interpretation. High titers are considered those values that are 2- to 4-fold higher than the threshold positive value indicated by the reference laboratory and low titers those that are equal to 1- to 2-fold higher than the threshold positive value. The IFAT is recommended by the World Organization for Animal Health (OIE) as the reference serological method (Gradoni *et al.* 2000). An ELISA is performed by placing diluted sera in *Leishmania* antigen-coated microplates. When a result is seropositive, a colorimetric reaction appears that can be quantified by spectrophotometry and therefore does not involve subjective evaluation. The ELISA is a specific test with a medium-high sensitivity that increases when multiple antigens are used (Reithinger *et al.* 2002). Various in-house serological tests are commercially available and are particularly attractive to practicing veterinarians because of immediate results (Bourdeau *et al.* 2014). Some of these tests have been evaluated and most shown adequate sensitivity and specificity (Athanasious *et al.* 2014; Rodriguez-Cortes *et al.* 2013; Solano-Gallego *et al.* 2014). However, the major disadvantage is that they are qualitative and thus the positive result needs to be followed by a quantitative test. An exception is the use of in-house tests that may differentiate truly seropositive dogs from those with post-vaccination antibody titers because they contain *Leishmania* antigens that are not included in the vaccine. An advantage of immunochromatographic strip tests that involve recombinant *Leishmania*-specific antigens is that they can discriminate serologic reactions to *Leishmania* spp.
from those to *Trypanosoma cruzi*, otherwise more frequent with the other serologic methods (da Costa *et al.* 2003).

Because resistance or susceptibility to *Leishmania* spp. infection is mediated by cellular immune responses, their evaluation is largely used in scientific research but still unavailable for clinical practice. Information on cell-mediated immunologic conditions can be obtained from some tests such as intradermal administration of leishmanin antigen (Montenegro test) or flow cytometry determination of the CD4-

to-CD8 ratio in T lymphocytes from peripherally obtained blood samples (Royspal *et al.* 2005).

Table 3 summarises the diagnostic approach for sick dogs living an endemic area (Solano-Gallego *et al.* 2011).



The management of clinically healthy infected dogs in areas where CanL is endemic is of great importance for practitioners. The most common reasons for investigating whether a healthy dog is infected with *Leishmania infantum* are: 1) epidemiological studies; 2) the use of dogs as blood donors; 3) the movement of dogs from endemic to non-endemic areas. For the above purposes, it is recommended using serology alone or in combination with PCR. The latter should be always used as a diagnostic screening of blood donor dogs.

The diagnostic approach for sick or healthy dogs living in a non-endemic area that have travelled to an endemic area, should include quantitative serology three months after the beginning of exposure in the endemic area.

4.5 Therapy

CanL is more resistant to therapy than HL, and only rarely are *Leishmania* organisms completely eliminated with available drugs (Baneth *et al.* 2002; Noli *et al.* 2005). Relapses necessitating retreatment are common, although dogs frequently become cured of the clinical disease. In addition, in endemic areas, reinfections occur and contribute to apparent treatment failure. The aim of the therapy is to control signs and clinico-pathological abnormalities, improve *Leishmania*-specific cell-mediated immunity, avoid relapses, and decrease parasitic load and competence to transmit the parasite. In order to prevent a recurrence of CanL, parasitostatic drugs, such as allopurinol, are usually combined with leishmanicidal therapy and continued for several months or years beyond clinical cure (Beneth *et al.* 2002; Noli *et al.* 2005). Allopurinol can be discontinued only when all the following conditions are met: 1) complete clinical recovery; 2) clinico-pathological normalisation; 3) antibody levels negative or below the test's cut-off level (Solano-Gallego *et al.* 2009; Martinez *et al.* 2011). Not every treated dog is able to reach this status enabling allopurinol treatment to be discontinued.

A variety of anti-*Leishmania* therapeutic interventions has been described since the late 1970s, but only some of them have shown a good evidence of recommendation in a recent systematic review (Noli *et al.* 2005).

N-methyl-glucamine (meglumine) antimoniate is the most used pentavalent antimony compound for treating leishmaniasis in dogs and humans. The drug selectively inhibits leishmanial glycolysis and fatty acid oxidation. In 94% to 95% of humans with leishmaniasis, a dose of 20 mg/kg/day for 28 days results in a parasitological and clinical cure (Gradoni *et al.* 1995, 2003). Meglumine antimoniate has a short half-life in dogs: 122 minutes when it is administered sub-cutaneously. By 6 to 9 hours after administration, 80% to 95% of the drug is eliminated through the kidneys (Tassi *et al.* 1994; Valladares *et al.* 1996)). Most studies in dogs have shown good clinical efficacy of this drug: during treatment, clinical amelioration is usually observed after a period of one or more weeks together with improvement in haematology and biochemistry profile. In addition, treatment with meglumine antimoniate induces a generalised reduction of the parasite load, together with a

temporary restoration of cell-mediated immunologic response. Pain and swelling of the injection site are the most common adverse effects. Fever, diarrhoea, and loss of appetite have been also reported (Denerolle *et al.* 1999). To date, there has been no evidence of renal damage induced by antimonials in dogs. The most commonly reported treatment regimen is 100 mg/kg once daily for 4 weeks. Because of the pharmacokinetic properties, the dosage might be better divided in 2 daily doses of 50 mg/kg. The development of *Leishmania infantum* strains that are resistant to pentavalent antimonials has been reported in France, Spain, and Italy and is a veterinary and public health concern (Gradoni *et al.* 2003; Lamothe *et al.* 2004).

Allopurinol has become an indispensable part of the treatment of CanL, frequently used in combination with leishmanicidal drugs. It is a hypoxanthine compound that is metabolised by *Leishmania* spp. to produce an analogue of inosine. The latter is incorporated into leishmanial RNA, causing faulty protein translation and inhibition of parasite multiplication. The tolerability of the drug is excellent and seems to slow the deterioration of renal function in dogs with proteinuria without renal insufficiency (Plevraki *et al.* 2006). Moreover, allopurinol has the potential to prevent or to decrease the episodes of clinical relapse (Torres *et al.* 2011). The most commonly prescribed dosages range between 5 and 20 mg/kg every 12 hours for 2 to 24 months. Use of allopurinol causes hyperxanthinuria, which may occasionally produce urolithiasis in about 12% of treated dogs (Torres *et al.* 2011).

Combined treatment with meglumine antimoniate and allopurinol is considered as the most effective therapy and constitutes the most frequently used protocol against the disease (Solano-Gallego *et al.* 2009, 2011). The combination is administered for 4 to 8 weeks, followed by continuation with allopurinol alone for at least 6 to 12 months. Dogs treated with this combination reportedly have a longer period of clinical remission than when treated with either drug alone (Denerolle *et al.* 1999) and degree of proteinuria is significantly reduced in a short period of time (Pierantozzi *et al.* 2013).

An alternative first line protocol is the administration of miltefosine (hexadecylphosphocholine) at the dose of 2 mg/kg once daily orally for 4 weeks,

combined with allopurinol. Miltefosine is an alkyphospholipid, originally developed as antineoplastic agent, which is able to kill parasites in vitro and in vivo by disturbing signalling pathways and cell membrane synthesis, thus leading to apoptosis (Farca *et al.* 2012; Verma *et al.* 2004). Furthermore, miltefosine is able to stimulate T-cell and macrophage activity and production of parasiticidal reactive nitrogen and oxygen species (Soto *et al.* 2007). When used alone, it greatly reduces the parasite load, but is not able to lead to a parasitological cure (Manna *et al.* 2008; Andrade *et al.* 2011). The side effects usually include vomiting, seen in about 11-23% of treated dogs (Andrade *et al.* 2011; Mateo *et al.* 2009; Woerly *et al.* 2009). Due to its low nephrotoxicity, miltefosine has been recommended instead of meglumine antimoniate for dogs with renal disease (Bianciardi *et al.* 2009). The combination miltefosine-allopurinol is clinically as effective as the standard protocol based on meglumine antimoniate and allopurinol (Miró *et al.* 2009). In a recent study (Miró *et al.* 2009), a significant reduction in clinical scores and parasite load was observed in both groups with no significant differences.

Aminosidine (also called paromomycin) belongs to the class of aminoglycosides and has antimicrobial and antiprotozoal activity. Aminosidine has been successfully used in the treatment of humans with leishmaniasis, 95% of whom are parasitologically cured when treated with a dosage of 11 mg/kg intra-muscular once daily for 21 days (Sundar *et al.* 2002). The drug has been used in dogs as a single agent and in combination with meglumine antimoniate but a severe limitation for more widespread use is related to its renal and vestibular toxic effects (Oliva *et al.* 1998).

Amphotericin B, a polyene macrolide primarily used as an antifungal drug, also has activity against some protozoa. It acts by binding to ergosterol and altering the cell membrane permeability. Amphotericin B has a good efficacy against leishmaniasis (Cortadellas *et al.* 2003; Lamothe *et al.* 2001), but its use is limited because it is administered intravenously and has a profound toxic effect on the canine kidney by causing vasoconstriction and reduction of the glomerular filtration rate and possibly acting directly on renal epithelial cells. Liposomal amphotericin is effective in the treatment of humans and has largely replaced therapy of human patients with antimonials in Italy and other European countries (Gradoni *et al.* 2003). To avoid the

occurrence of amphotericin B-resistant *Leishmania* strains, the World Health Organisation has discouraged its use in the treatment of dogs with leishmaniasis.

Domperidone is a gastric prokinetic and antiemetic drug acting as a dopamine D2 receptor antagonist that is able to stimulate the production of serotonin, which in turn increases prolactin secretion. Prolactin, besides its well-know role in milk production, is an important pro-inflammatory lymphocyte-derived cytokine that is able to stimulate cell-mediated Th1-lymphocyte driven immune response leading to the release of IL-2, IL-12, IFN-γ, and TNF-α and to the activation of macrophages and natural killer (NK) cells. Domperidone was administered orally twice daily, at the dosage of 1 mg/kg for 1 month to dogs naturally infected with leishmaniasis and clinical remission was seen in 94 of 98 dogs within 90 days (Gómez-Ochoa *et al.* 2009). In addition, activation of cellular immunity was confirmed by significant greater induration diameter of leishmanin skin tests after treatment. Domperidone has recently released on the veterinary market with an indication for the treatment and the prevention of CanL at the dose of 0.5 mg/kg twice daily orally, given every 4 months (Sabaté *et al.* 2014).

Finally, several other drugs including pentamidine, spyramicin-metronidazole, marbofloxacin, enrofloxacin, ketoconazole and buparvaquone has been used in CanL but have shown insufficient evidence of efficacy for recommending their use.

4.6 Prevention

To date three prevention strategies against CanL has been shown to be effective and are used more commonly: 1) the regular use of topical insecticides-repellents (pyrethroids); 2) oral treatment with domperidone and 3) vaccination. It is however necessary to remember that the protection of each single dog, although high, is not 100% guaranteed with any of these methods. The preventive efficacy of pyrethroids is of 84-98% in the individual dog and of 100% at population level (Maroli *et al.* 2010); the preventive efficacy of using domperidone (Sabaté *et al.* 2014) is of 77% and of vaccination (Oliva *et al.* 2014) of 70% in the individual dog. The various existing preventive strategies can be combined, in order to increase their efficacy; however, no data are available confirming that this approach increases the degree of protection compared to their use alone. Preventive measures are recommended in any healthy dog visiting endemic areas as well as in either infected or sick dogs, whether under treatment or not, as an effective strategy to reduce re-infection and the risk of infecting both humans and dogs.

Several studies have been carried out on synthetic pyrethroids to be used on dogs in topical formulations. These are classified as ectoparasiticides and act mainly in two ways: 1) after landing on treated dogs, sand flies may rest on the skin for a period sufficient to absorb a lethal dose of insecticide (effect: toxicity); 2) the flies may have only fleeting contact with insecticide-treated skin that is sufficient to cause irritation and disorientation, resulting in reduction of blood-feeding rate (effect: no-feeding) (Killick-Kendick *et al.* 1997). Synthetic pyrethroids used for application in dogs combine the properties of low to moderate mammalian toxic effects, low volatility, and high, fast insecticidal activity. Modes of application include slow-release protector band (dog collar), spot-on and/or spray. Safety test performed after application on dog skin have revealed rare and temporary skin reactions, including itchiness and erythema at the site of application (Maroli *et al.* 2001). The different starting periods of protections activity associated with various synthetic pyrethroids formulations should be carefully considered when choosing a product. In particular, dog owners that plan to take their pets from a non-endemic to an endemic area

during the sand fly activity period should be advised to take into account that periods for full protection may vary from immediate (spray) to 1 week (collar), depending on the product. Moreover, because of a shorter duration of activity, spot-on and spray formulations (3-4 weeks' duration) require more frequent applications, whereas slow-release collars do not need to be replaced more than twice a year in environments in which *Leishmania* vectors are active throughout the year.

As previously discussed, the European Medicines Agency has recently awarded marketing authorisation for the prophylaxis and early treatment of CanL to domperidone (Gómez-Ochoa *et al.* 2009). This can act as a proinflammatory cytokine able to skew immune response towards a Th1-like immune response, with cell-mediated immunity inducing natural killer cell induction and macrophage activation. A recent study (Sabaté *et al.* 2014) investigated the preventive efficacy of this drug in seronegative dogs living in a highly endemic area. Domperidone treated dogs had a seven-times lower risk to develop CanL compared to untreated subjects, with a prevention rate of 77% during the 21-month follow up period.

Among parasitic diseases, leishmaniasis is theoretically considered the most likely to be controlled by vaccination because resolution of the disease in humans results in strong resistance to reinfection. Thus, vaccines have been proposed as major cost-effective tools and have been established as a high priority by the World Health Organisation (resolution EB118.R3, Geneva 05/07). However, no vaccine against HVL has been marketed so far. Two vaccines have granted marketing authorisation against CanL in Brazil (Leishmune® and Leish-Tec®) (Boja-Cabrera *et al.* 2002; Fernandes *et al.* 2008), and another one has recently obtained a European registration (CanLeish®). The protocol for all the vaccines includes three administrations, each one three weeks apart, followed by an annual booster. Leishmune® is composed of a promastigote antigen (fucose-mannose ligand) and the adjuvant saponin (Parra *et al.* 2007; Nogueira *et al.* 2005). Efficacy in initial filed trials was approximately 80% and has correlated with a reduction of human leishmaniasis in the area. CanLeish® is composed of purified excreted-secreted proteins of

Leishmania infantum (LiESP) and is capable of stimulating an appropriate Th1-dominated cell-mediated immune response that is still present one year after the last injection of a primary vaccine course (Moreno *et al.* 2012, 2014; Martin *et al.* 2014). In a recent study (Oliva *et al.* 2014), the vaccine was well tolerated, and provided a significant reduction in the risk of progression to uncontrolled active infection or symptomatic disease, with an efficacy of 68.4% and a protection rate of 92.7%. The LiESP/QA-21 vaccine, when administered according to the recommended protocol, provides a significant reduction of the number of actively infected animals and of the probability of developing symptomatic disease. In those animals developing the disease despite vaccination, the progression is generally slower and the disease less severe.

Finally, indoor housing and/or use of fine-mesh netting around dogs and humans when sandflies feed at night (from dusk to dawn), and reducing microhabitats favourable to the vector such as piles of wood and stones and usage of indoor insecticide treatment are also recommended.

4.7 Clinical Staging Systems

Leishmania infantum infection may develop over a period of a few weeks to several months toward clinical conditions that can be highly variable, and therefore, it is not always easy to classify dogs within specific categories. Nevertheless, when CanL is diagnosed, veterinarians should be able to ascribe the infection or disease to a parasitological or clinical stage to determine adequate treatment or to predict progression toward more serious and irreversible stages. These were the objectives of two groups of expertise, the CLWG (Paltrinieri *et al.* 2010) and the LeishVet (Solano-Gallego *et al.* 2011), that respectively created a clinical stage system based on clinical signs, laboratory findings and serology titer. The great value of these systems is that for the first time a clear differentiation between infected and diseased animals was provided and treatment protocols and prognoses were suggested for each clinical stage.

In 2010, the CLWG proposed to classify dogs with positive results of serologic tests and/or in which the parasite is identified via direct diagnostic methods in the following four clinical stages:

- **Stage A (exposed dogs)** – includes dogs with negative cytologic, histologic, parasitological, and molecular diagnostic findings as well as low-titer anti-*Leishmania* antibodies. These dogs are clinically normal or have signs associated with other diseases and do not need any treatment. However, they should be serologically monitored for 2 to 4 months after the first finding of low-titer antibodies. If abnormal clinical findings develop, additional evaluation is indicated, including parasitological testing performed with a direct method (cytology, histology, PCR).
- **Stage B (infected dogs)** – includes dogs in which the presence of parasites is confirmed by direct (e.g. cytology, histology, culture, or PCR assay) methods and which have low-titer anti-*Leishmania* antibodies. Such dogs can be healthy or can have clinical or pathological signs associated with other diseases. In endemic areas, a positive finding of PCR assays of skin or peripheral blood in the absence of lesions and obtained during the infection transmission period may not be sufficient to consider a dog infected. Dogs need treatment if the direct detection

of parasites is associated with an increase in antibody titer a few weeks after the first serologic diagnosis. If the infected dogs do not seroconvert, treatment is not needed but a serological monitoring every 2 to 3 months is recommended. Dogs classified in this stage but living in non-endemic areas should receive a course of anti-*Leishmania* treatment especially if the competent vector is present. Moreover, treatment of infected dogs is suggested also in those non-endemic areas where established vectors species have not been detected, because of the potential for nonvectorial transmission of *Leishmania* spp.

- **Stage C (sick dogs with clinically evident leishmaniasis)** – includes dogs with positive cytologic results regardless of serologic tests and dogs with high-titer anti-*Leishmania* antibodies that show one or more clinical signs and/or laboratory abnormalities compatible with CanL. All these dogs need treatment with an appropriate anti-*Leishmania* drug regimen.

In dogs classified in stages B and C, a complete physical examination, haematology, serum biochemistry profile, serum protein electrophoresis and urinalysis should be repeated after treatment with meglumine antimoniate has been completed (30 days). If there has been a clinical improvement and results of laboratory tests are within reference limits, dogs should continue on allopurinol for at least five additional months. Dogs should be re-evaluated every six months after treatment concludes, including serologic titer. When a clinical relapse develops based on clinical signs and/or abnormal laboratory findings compatible with CanL, treatment should be reinitiated with the drug initially used or with an alternative treatment protocol.

- **Stage D (severely sick dogs)** – includes dogs with a severe clinical condition, showing evidence of proteinuric nephropathy and/or advanced chronic renal failure, ocular and severe joint disease and various coinfections or neoplastic, endocrine, or metabolic disorders. These dogs need both anti-*Leishmania* treatment and ancillary treatment, depending on the affected organs. Usually, it is necessary to perform both clinical and laboratory evaluation during the course of treatment, particularly if a dog has renal disease. At the end of treatment, follow-up should be performed at 1- to 2-month interval, with particular emphasis on evaluating affected organs.

For therapeutic purposes, the CLWG suggested an additional stage (Stage E), further classified in two sub-categories.

- **Stage E-a (sick dogs unresponsive to recommended treatment)** – dog's owner compliance in administration of medications should be verified and the adopted treatment regimen should be re-evaluated to ensure drug doses, frequencies of administration, and durations of treatment. Clinical and laboratory findings should be reassessed to verify whether a concomitant disease is present (e.g. other infectious diseases, neoplasia, or immune-mediated disorders). When diagnosis was made only on the basis of serologic titer, serologic testing should be repeated and PCR assay should be performed. If the aforementioned actions confirm CanL and therapy has been correctly administered, an alternative anti-*Leishmania* treatment regimen should be considered.
- **Stage E-b (sick dogs that relapse soon after recommended treatment ceases)** – as for dogs in stage E-a, the diagnostic scheme should be re-evaluated to rule out concomitant infectious, metabolic and neoplastic diseases. If CanL is confirmed and treatment has been administered as recommended, an alternative anti-*Leishmania* protocol should be considered.

In 2011, the LeishVet group proposed a similar clinical staging of CanL based on serological status, clinical signs and laboratory findings, also including the type of therapy and the prognosis for each stage. This classification is based on two important clinical aspects: 1) a high level of antibodies confirms the diagnosis of CanL in a dog with clinical signs and/or laboratory abnormalities compatible with the disease (Reis *et al.* 2006); 2) severity of clinical signs are positively correlated to anti-*Leishmania* antibody levels (Manna *et al.* 2009; Reis *et al.* 2006) and dogs with high titers at the time of diagnosis are generally more severely affected and more predisposed to developing immune-mediated complications (Torres *et al.* 2011).

- **Stage I (mild disease)** - includes dogs with negative to low-positive antibody titers, mild clinical signs such as papular dermatitis and peripheral lymphadenopathy and usually no clinico-pathological abnormalities. For these animals there is no consensus on the best intervention. However, it is advisable

to use topical insecticides, as even asymptomatic seropositive dogs are competent to transmit the parasites to sandflies (Laurenti *et al.* 2013).

- **Stage II (moderate disease)** – includes dogs with low- to high-positive antibody titers and more severe clinical signs, such as diffuse cutaneous lesions (exfoliative dermatitis, onychogryphosis, alopecia and ulcerations of the planum nasale, footpads, bony prominences and muco-cutaneous junctions), anorexia, weight loss, fever and epistaxis. A variety of clinico-pathological abnormalities are also present, including mild non-regenerative anaemia, hyperglobulinaemia and hypoalbuminaemia. Creatinine is generally within normal intervals (< 1.4 mg/dL; < 125 µmol/l) and dogs can be non proteinuric (**Substage IIa**: UPC < 0.5) or can present mildly elevated UPC ratio (**Substage IIb**: UPC = 0.5-1). For these dogs, the standard therapeutic protocols based on allopurinol and meglumine antimoniate or allopurinol and miltefosine are recommended.
- **Stage III (severe disease)** – includes dogs with medium- to high-positive antibody titers, clinical signs of stages I and II together with signs related to immune-complex deposition to the eye (uveitis), blood vessels (vasculitis), joints (polyarthritis) and kidneys (glomerulonephritis). Apart from the laboratory abnormalities listed under stage II, dogs have also CKD as International Renal Interest Society (IRIS; www.iris-kideny.com) stage I (creatinine < 1.4 mg/dl; , 125 µmol/l) but UPC ratio >1 or stage II (creatinine 1.4-2 mg/dl; 125-180 µmol/l). Therapy is based on the standard protocols, together with kidney support as recommended by the IRIS group.
- **Stage IV (very severe disease)** – includes dogs with medium-to high-positive antibody titers and, in addition to the clinical signs listed in stage III, pulmonary thromboembolism, nephrotic syndrome and end stage kidney disease. Clinico-pathological abnormalities includes those listed in stage II and dogs are generally classified in CKD IRIS stage III (creatinine 2.1-5 mg/dl; 181-440 µmol/l) and stage IV (creatinine > 5 mg/dl; > 440 µmol/l) with marked proteinuria (UPC > 5). These dogs should be treated only with allopurinol and renal therapy following the IRIS guidelines.

4.8 Prognosis and prognostic factors

Prognosis is the prediction of the probable course and outcome of a disease and typically depends on the severity of the disease and its response to treatment. For this reason, a complete clinical and laboratory-based assessment of each dog at the time of diagnosis, together with serological responses and parasites detection, are necessary to characterise the severity of the disease and assign the case to a clinical stage (Reis *et al.* 2009; Dos-Santos *et al.* 2009). Following the diagnosis, the patient should be periodically re-evaluated and re-classified in line with disease progression and/or regression (Solano-Gallego *et al.* 2009; Paltrinieri *et al.* 2010; Oliva *et al.* 2010).

As previously discussed, the CLWG and the LeishVet also provided general data regarding the prognosis.

Based on the CLWG staging system, dogs classified in **stage A (exposed dogs)** have a favourable prognosis and approximately a 25% of them can develop spontaneous sero-reversion within a few months, even in the absence of therapy (Roura *et al.* 2013). Similarly, for dogs in **stage B (infected dogs)**, the prognosis is favourable if the infection does not progress to overt disease. While this can occur over a period of time varying from weeks to years, dogs may also remain sub-clinically infected throughout their lives (Baneth *et al.* 2008). It is estimated that 30-70% of infected dogs living in endemic regions develop clinical disease within 2-3 years of diagnosis (Oliva *et al.* 2006; Manna *et al.* 2006; Otranto *et al.* 2009; Paradies *et al.* 2010), making their prognosis more guarded. Currently, there is no single laboratory test, or combination of tests, that can predict if an infected dog will develop overt disease.

For dogs classified in **stages C (sick dogs), D (severely diseased) and E (Ea: unresponsive to treatment; Eb: early relapse)**, the prognosis is generally guarded to poor and depends on the severity of clinical signs and of any clinico-pathological abnormalities presenting when therapy is initiated, the individual response to therapy, and particularly the severity of renal disease (Gradoni *et al.* 2003). Dogs with leishmaniasis at IRIS stages 3 or 4 have a worse prognosis than those at IRIS stages 1 or 2.

Similarly, in the LeishVet classification, dogs in **stage I (mild disease)** have a good prognosis as well as dogs in **stage II (moderate disease)** that do not present severe clinical signs and laboratory abnormalities at time of diagnosis. Those more severely diseased and especially those that do not respond favourably to the therapy have a guarded prognosis. **Stages III (severe disease)** and **IV (very severe disease)** are generally associated with a guarded to poor prognosis especially depending on the severity of renal disease.

Besides the recent clinical staging systems, three factors have been historically related to prognosis: 1) presence of renal disease and its severity; 2) the therapeutic protocol used; 3) the serology titer at time of diagnosis. Dogs with severe kidney disease (e.g. IRIS stage 3-4) have generally a guarded to poor prognosis and advanced stages as nephrotic and uremic syndromes are the most common causes of death, or the reasons for euthanasia in dogs with leishmaniasis (Planellas *et al.* 2009; Koutinas *et al.* 1999, 2014). The therapeutic protocol is recognised to influence prognosis: the combination of meglumine antimoniate and allopurinol is so far considered to be the most effective therapy for CanL (Noli *et al.* 2005; Miró *et al.* 2009) and the association of miltefosine and allopurinol has been recently demonstrated to have a similar efficacy (Solano-Gallego *et al.* 2009). In a recent study (Torres *et al.* 2011) on the long term follow of CanL, it was reported that, if treated adequately with the "gold standard therapeutic protocols" and monitored correctly, dogs with stage II disease can have long survival times (up to 9 years), a good quality of life and low risk of relapse. On the contrary, more than 50% of dogs had relapses after approximately 5 months if treated with other drugs and/or marbofloxacin alone (Rougier *et al.* 2012). Finally, it was demonstrated that the percentage rate of infected sandflies on a given dog increased with the presence and severity of its clinical signs, high anti-*Leishmania* antibody level and decrease in CD4+ T-cell count (da Costa-Val *et al.* 2007; Guarga *et al.* 2000; Michalsky *et al.* 2007). These findings corroborate that high anti-*Leishmania* antibody levels are positively correlated with high parasite burdens and with severity of clinical disease (Manna *et al.* 2009; Reis *et al.* 2006). Moreover, dogs with a high titre at the time of diagnosis are predisposed to developing immune-mediated complications during therapy and follow-up (Torres *et al.* 2011) and then carry a more guarded prognosis.

Although it remains somewhat guarded, the prognosis for cases of CanL has improved significantly in recent years, particularly in the absence of severe renal dysfunction, and where patients are effectively treated and monitored. The severity of clinico-pathological abnormalities, in particular those reflecting renal function, as well as the response to treatment, are probably at the moment the most useful prognostic indicators. Nonetheless, determining prognosis in the context of leishmaniasis remains challenging because there have been no controlled studies specifically assessing prognostic factors so far.

5. STUDIES

5.1 Serum cardiac troponin I concentrations in dogs with leishmaniasis: correlation with age and clinicopathologic abnormalities

ABSTRACT

Background: There is anecdotal evidence of myocardial injury in dogs with leishmaniasis due to generalised vasculitis and myocarditis.

Objective: The aims of this study were to evaluate serum concentration of cardiac troponin (CTnI) as an indicator of myocardial injury in dogs with leishmaniasis and to assess the relationship between cTnI concentration and age, serum antibody titer, and a variety of blood analytes.

Methods: In this retrospective study, serum cTnI concentration was measured in dogs with leishmaniasis and in age-matched healthy dogs. Diagnosis was based on clinical signs and moderate-to-high seropositivity for *Leishmania* as measured by ELISA. Correlations between cTnI concentration and ELISA seropositivity, PCV, concentrations of serum creatinine, total protein, albumin, and globulin, albumin:globulin ratio (A/G), and urine protein:creatinine ratio (UPC) were investigated. The Mann-Whitney test was used to compare analytes between dogs with normal and increased (>0.06 µg/L) cTnI concentration and to compare cTnI concentrations between dogs with and without anaemia, azotaemia, and proteinuria.

Results: In dogs with leishmaniasis (n=40), median cTnI concentration was higher than in controls dogs (*n*=11) (P=.011). Sixteen dogs (40%) with leishmaniasis had increased cTnI concentration; cTnI was moderately to weakly correlated with decreased albumin concentration, decreased A/G, increased UPC, decreased PCV, positive *Leishmania* titer, and increased age. Dogs with leishmaniasis had significantly higher total protein and globulin concentrations and lower PCV, albumin concentration, and A/G than control dogs. Haematologic and biochemical analytes did not differ significantly between dogs with cTnI concentration within the

reference interval and those with increased concentrations. Concentration of cTnI was higher in proteinuric dogs compared to nonproteinuric dogs (P=.017).

Conclusion: A proportion of dogs with CanL have increased cTnI concentration, indicative of some degree of cardiac injury. Additional studies are needed to investigate the relationship between leishmaniasis and possible myocardial injury.

INTRODUCTION

Canine leishmaniasis is a severe systemic disease caused by the protozoan *Leishmania infantum* (*L chagasi* in neotropic ecozones) (Baneth *et al.* 2012). The disease is enzootic in the Mediterranean area, where the prevalence of infection in dogs can be as high as 67% (Solano-Gallego *et al.* 2001). Clinical signs are variable and include pale mucous membranes, lymphadenomegaly, skin and ocular lesions, and epistaxis. Anaemia, hypoalbuminaemia, hyperglobulinaemia, azotaemia and proteinuria are the laboratory abnormalities most frequently associated with leishmaniasis (Solano-Gallego *et al.* 2001; Paltrinieri *et al.* 2010; Ciaramella *et al.* 1997). Myocardial injury due to *Leishmania* infection has been reported in human medicine (Mofredj *et al.* 2002), and recently, an unusual presentation of leishmaniasis in a dog with generalised vasculitis and myocarditis was described (Torrent *et al.* 2005). To our knowledge, however, the frequency of myocardial injury in canine leishmaniasis has not been reported.

Cardiac Troponin I (cTnI) has high sensitivity and specificity for myocardial ischaemia and necrosis and is considered a reliable biomarker of cardiac cellular injury in animals (Wells *et al.* 2008; O'Brien *et al.* 2006). CTnI is present only in cardiac muscle and is released into circulation in proportion to the degree of myocardial tissue injury and disruption of myocyte membranes (O'Brien *et al.* 2006). Numerous studies have highlighted the utility of this biomarker in detecting primary cardiac disease or in identifying cardiac injury secondary to other conditions such as pancreatitis, anaemia, or neoplasia (Serra *et al.* 2010). CTnI has been also evaluated in dogs with infectious diseases including babesiosis (Lobetti *et al.* 2002), ehrlichiosis (Diniz *et al.* 2008), Chagas disease (Barr *et al.* 2005), and leptospirosis (Mastrorilli *et al.* 2007) to detect occult myocardial injury. Serum cTnI concentration is also influenced by age and may be increased in case of marked anaemia and azotaemia, findings often associated with canine leishmaniasis (Serra *et al.* 2010; Ljungvall *et al.* 2010). The objectives of this study were to evaluate serum cTnI concentrations in dogs with leishmaniasis as a marker of potential myocardial injury and to investigate the relationship between cTnI concentration and age, serologic titers for *Leishmania*, and selected laboratory analytes, including PCV, serum creatinine, total protein (TP),

albumin, and globulin concentrations, albumin:globulin ratio (A/G), and urine protein:creatinine ratio (UPC).

MATERIALS AND METHODS

This retrospective study included dogs diagnosed with leishmaniasis at the Hospital Clínic Veterinari of the Universitat Autònoma de Barcelona from January 2007 to December 2008 for which nonhaemolysed serum specimens stored at -80°C were available. Following guidelines proposed by the Canine Leishmaniasis Working Group (Paltrinieri *et al.* 2010), medical records were reviewed to include only dogs that had been presented with at least one clinical sign compatible with leishmaniasis, e.g. systemic lymphadenomegaly, weight loss, dermatologic or ophthalmologic abnormalities, polyarthritis, and epistaxis, and that had moderate or high serum titers for *Leishmania*. Dogs with a previous history or clinical signs of cardiac disease, e.g. murmurs or arrhythmias, or that were currently receiving medication were excluded from the study. Results for PCV, serum creatinine, TP, albumin, and globulin concentrations, A/G, and UPC had to be available for dogs to be included in the study. These tests had been performed at admission as part of the diagnostic work-up for leishmaniasis. PCV was obtained within 12 hours of sample collection by microhaematocrit centrifugation. All biochemistry analytes were measured using an Olympus AU400 analyser and Olympus reagents (Olympus, Hamburg, Germany). The Jaffé method was used to measure creatinine in serum and urine, and the biuret method was used to measure total protein. Serum protein fractionation was performed by commercial agarose gel electrophoresis (Hydragel Protein (E), Sebia Hispania S.A., Barcelona, Spain) using a semi-automated Hydrasys system (Sebia Hispania S.A.) with manufacturer's reagents to determine the concentration of albumin and globulin fractions. For serologic testing, an ELISA for *Leishmania* was performed as previously described (Department of Pharmacology, Universitat Autònoma de Barcelona) (Riera *et al.* 1999). Results were quantified as units relative to positive serum used as a calibrator and arbitrarily set at 100 ELISA Units (EU). The positive threshold value previously established by the laboratory was 35 EU (mean + 4 SD for 32 dogs from nonendemic areas) (Riera *et al.* 1999). Results of

80-150 EU and > 150 EU were classified as moderate and high positive titers, respectively. Clinically healthy dogs presented for elective surgery or annual vaccination that had normal physical examinations, negative serologic tests for *Leishmania*, and CBCs, biochemical profiles, and urinalysis within reference intervals were included in the study as an age-matched control group.

Serum specimens from dogs with leishmaniasis and control dogs were stored in cryotubes (Deltalab, Barcelona, Spain) at -80°C for 6-18 months before being used to measure cTnI concentration; cTnI has been demonstrated to be stable in serum samples stored at - 80°C for at least one year (O'Brien *et al.* 2006; Tate *et al.* 2002). Serum cTnI concentrations were measured at the Advanced Diagnostics Laboratory, Nova UCD, University College Dublin, Belfield, Ireland, with an automated chemiluminescent assay previously validated in dogs (O'Brien *et al.* 2006), using the Advia Centaur immunoanalyser and manufacturer's reagents (Siemens Healthcare Diagnostic, Newbury, UK). The reported imprecision of the assay, as defined by the coefficient of variation (CV), varies from 6.4% at 0.52 µg/L to 3.7% at 36.90 µg/L (O'Brien *et al.* 2006). The reported functional sensitivity (lowest cTnI concentration determined at a CV of 20%) is 0.017 µg/L (Tate *et al.* 2002). Quality control procedures were performed daily with control materials from the manufacturer (Siemens Cardiac Markers 1, 2, 3), using the manufacturer's diluent (Siemens cTnI Diluent, Siemens Healthcare Diagnostics, Newbury, UK). The analyser was calibrated approximately each month using the manufacturer's calibrators (Siemens Calibrator, Siemens Healthcare Diagnostics).

Dogs were grouped according to cTnI concentrations, with the upper reference limit (URL) set at 0.06 µg/L as previously established by the laboratory (University of Dublin), and by creatinine concentrations, PCV, and UPC, based on laboratory reference intervals (Universitat Autònoma de Barcelona).

Statistical Analysis

Data were tested for normality using the Shapiro-Wilk test. Most data did not follow a normal distribution and nonparametric tests were used. Data were reported as median and minimum-maximum. Correlations between serum cTnI concentration

and age, PCV, concentrations of creatinine, TP, albumin, and globulins, A/G, UPC, and *Leishmania* titer were evaluated using Spearman's rank correlation test (ρ). A Mann-Whitney U-test was used to detect differences in cTnI concentrations and age, PCV, concentrations of creatinine, TP, albumin, and globulins, A/G, and UPC between control dogs and dogs with leishmaniasis. The same test was used to compare distributions of cTnI concentrations between dogs with and without anaemia, increased serum creatinine concentration, and proteinuria. Significance was set at $P < .05$. Statistical analysis was performed using SPSS software v. 17.0 (SPSS, Chicago, IL, USA).

RESULTS

Control dogs (*n*=15) had a median age of 7.5 years and comprised 8 mixed breed dogs, 2 Boxers, and 1 each of Weimaraner, Rottweiler, West Highland White Terrier, American Pit Bull Terrier, and Irish Setter. There were ten males (66%), two of which were neutered, and 5 females (33%), two of which were spayed. Two of 15 dogs (13%) had cTnI concentrations greater than the URL; one dog was an 8-year-old Boxer and the other was an 11-year-old mixed breed dog. In the disease group, forty dogs met inclusion criteria and had a median age of 5 years. Breed distribution was 14 mixed breed dogs, 4 German Shepherds, 3 English Bulldogs, 5 Labrador Retrievers, 3 Shar Peis, 2 Yorkshire Terriers, and 1 each of Boxer, Jack Russell Terrier, Siberian Husky, Bull Terrier, Golden Retriever, American Staffordshire Terrier, English Cocker Spaniel, English Pointer, and American Pit Bull Terrier. There were 22 males (55 %), two of which were neutered, and 18 females (45%), eight of which were spayed.

Twenty-eight dogs (70%) were anaemic, twenty-five (62.5%) had UPC > 0.5, and nine (22.5%) had increased creatinine concentration. Of these 40 dogs, 5 (12.5%) had a moderately positive and 35 (87.5%) a high positive titer for *Leishmania*. Sixteen dogs (40%) had cTnI concentrations above the URL. Of these 16 dogs, 13 (81.3 %) had UPC > 0.5, 13 (81.3 %) were anaemic, and 5 (31.3%) had increased creatinine concentrations; three dogs (18.8%) were not anaemic or did not have increased serum creatinine concentration, but had UPC > 0.5. CTnI had a significant

correlation with decreased albumin concentration (ρ=-0.620, P =.000), decreased A/G (ρ=-0.579, P =.000) increased UPC (ρ=0.493, P =.001), positive *Leishmania* titer (ρ=-0.386, P=.014), decreased PCV (ρ=-0.387, P=.014), and increased age (ρ=0.368, P=.019). A significant correlation was not found between cTnI concentration and increased creatinine (ρ=-0.102, P=.531), increased TP (ρ=-0.026, P=.875), and increased globulin (ρ=-0.286, P=.074) concentrations.

Dogs with leishmaniasis had significant higher concentrations of TP, globulins and cTnI, and significantly lower PCV, albumin concentration, and A/G than did control dogs; differences in serum creatinine concentration and UPC were not found (Table 1).

Table 1. Age, PCV, creatinine, total protein (TP), albumin, and globulin, albumin:globulin ratio (A/G), urine protein:creatinine ratio (UPC) and cardiac troponin I (cTnI) concentration in clinically healthy control dogs (*n*=15) and dogs with leishmaniasis (*n*=40).

	Reference Interval*	**Healthy controls** Median (Min-Max)	**Dogs with CanL** Median (Min-Max)	***P*-value^**
Age (years)		7.5 (1-12)	5.0 (1-12)	.174
PCV (%)	35-55	43 (37-49)	33 (20-53)	.000
Crea (mg/dL)	0.5-1.5	0.98 (0.77-1.32)	1.01 (0.46-5.28)	.670
TP (g/L)	54-80	70 (57-93)	88 (45-136)	.006
Albumin (g/L)	26.0-33.0	35.2 (24.4-35.4)	20.6 (6.0-36.9)	.000
Globulin (g/L)	27.0-44.0	39.2 (27.8-45.1)	62.2 (27.2-118.8)	.000
A/G ratio	0.5-1.1	0.8 (0.4-1.1)	0.3 (0.1-1.3)	.000
UPC ratio	<0.5	0.4 (0.1-0.5)	0.8 (0.1-16.8)	.164
cTnI (µg/L)	<0.060	0.021 (0.001-0.132)	0.043 (0.000-3.470)	.011

*Reference intervals were established by the University College Dublin for cTnI concentrations and by the Universitat Autònoma de Barcelona for other analytes.

^Significance was set at P-values < .05.

In the disease group, significant differences were not found for any of the measured analytes between dogs with increased cTnI concentration and those with cTnI concentration below the URL (Table 2).

Table 2. Age, serum *Leishmania* titer (ELISA), serum creatinine concentration, PCV, albumin and globulin concentrations, albumin:globulin ratio (A/G), and urinary protein to creatinine ratio (UPC) in dogs with serum cTnI concentration within the reference interval (<0.06 µg/L, *n*= 24) or > the upper reference limit (>0.06 µg/L, *n*= 16).

	cTnI	**Median (Min-Max)**	***P*-value**
AGE (years)	< 0.06	4.0 (0.9 - 8.0)	.061
	>0.06	6.5 (2.0-12.0)	
ELISA (EU)	< 0.06	294 (105-302)	.050
	>0.06	300 (191-387)	
Crea (mg/dL)	< 0.06	0.94 (0.54-3.03)	.615
	>0.06	1.13 (0.46-5.28)	
PCV (%)	< 0.06	33 (21-53)	.381
	>0.06	28 (20-42)	
TP (g/L)	< 0.06	81.6 (58.2-106.7)	.696
	>0.06	84.3 (45.2-135.9)	
Alb (g/L)	< 0.06	24.5 (8.2-36.9)	.086
	>0.06	16.8 (6.0-30.3)	
Glob (g/L)	< 0.06	57.3 (27.2-94.3)	.304
	>0.06	68.7 (34.4-118.8)	
A/G	< 0.06	0.38 (0.13-1.26)	.118
	>0.06	0.28 (0.08-0.52)	
UPC	< 0.06	0.50 (0.08-16.77)	.050
	>0.06	4.68 (0.20-14.83)	

For comparison of cTnI concentrations between groups, significance was set at $P < .05$.

However, 13 (81.25%) of 16 dogs with increased cTnI concentrations had UPC > 0.5 and two of the remaining three animals had UPC > 0.2. Of the 24 dogs with cTnI concentrations below the URL, 12 (50%) had UPC > 0.5, and one had UPC > 0.2. Dogs with proteinuria had significantly higher concentrations of cTnI compared to nonproteinuric dogs (Table 3). Significant differences in cTnI concentration were not found between dogs with and without anaemia or increased creatinine concentrations.

Table 3. Serum cTnI concentration in 40 dogs with leishmaniasis categorised by serum creatinine concentration, PCV, and urine protein:creatinine ratio (UPC).

			cTnI (µg/L)		
		n	Median	Min-Max	*P*-value
Creatinine (mg/dl)	<1.5	31	0.042	0.000-3.470	.483
	>1.5	9	0.081	0.011-1.167	
PCV (%)	>35	12	0.031	0.001-3.47	.236
	<35	28	0.053	0.000-1.167	
UPC	<0.5	15	0.014	0.000-0.177	.017
	>0.5	25	0.081	0.001-3.470	

For comparison of cTnI concentrations between groups, significance was set at $P < .05$.

DISCUSSION

In the current study, 40% of dogs with leishmaniasis had increased cTnI concentration, and cTnI concentration in diseased animals also was significantly higher than that of healthy control dogs. These findings support the hypothesis that myocardial injury can occur in dogs with leishmaniasis. Evidence of direct myocardial damage caused by *Leishmania* has been shown previously in dogs with parasites

presence in the myocardium as demonstrated by PCR or immunohistochemical analysis (Torrent *et al.* 2005; Pumarola *et al.* 1991). In another report, leishmaniasis was associated with cardiac tamponade in a dog, and *Leishmania* amastigotes were identified in the pericardium by immunohistochemical staining (Font *et al.* 1993). The occurrence of increased cTnI concentrations in two healthy control dogs suggested that the cut-off value established by the laboratory might include animals with no significant myocardial damage; however, the same cut-off value has been proposed by others (Sleeper *et al.* 2010). In addition, one of the dogs was an 8-year old Boxer, a breed predisposed to arrhythmogenic right ventricular cardiomyopathy, and even clinical healthy Boxers have been reported to have cTnI concentrations higher than healthy non-Boxer dogs (Baumwart *et al.* 2007). The other dog was an 11-year old dog with no history of heart disease, but early cardiac disease can be asymptomatic and is difficult to rule out completely based on physical examination alone.

Troponin I is considered a reliable serum biomarker for myocardial ischaemia and necrosis in human medicine and also has high sensitivity and specificity in animal patients with primary or secondary cardiac disease (Wells *et al.* 2009; O'Brien *et al.* 2006; Schober *et al.* 2002; Smith *et al.* 1997). After acute myocardial injury, cTnI is released from the cytosolic pool, resulting in increased blood concentrations within 2 hours, with a peak after 12–24 hours (Wells *et al.* 2008; O'Brien *et al.* 2006). Persistently increased cTnI blood concentration suggests irreversible and active ongoing damage to cardiomyocytes, (Wells *et al.* 2008; O'Brien *et al.* 2006; Stanton *et al.* 2005) and the degree of increase has been shown to be correlated with the extent of myocardial damage and with animal survival (Oyama & Sisson 2004; Fonfara *et al.* 2010; Ricchiuti *et al.* 1998).

The basis of myocardial injury in canine leishmaniasis could involve many pathogenetic mechanisms, such as vasculitis, an intense local or systemic inflammatory response, anaemia, renal disease, and possible myocardial hypoxia in addition to direct action of the parasite. Vasculitis is a process characterised by blood vessel inflammation and ischemic damage that can occur in any organ; in canine leishmaniasis, it typically results from immune-complex deposition in vessel walls (Torrent *et al.* 2005; Fauci e*t al.*1978). When systemic vasculitis develops,

multiple infarctions in different target tissues can produce progressive organ failure, and at the same time, the intense inflammatory response can promote inflammatory cell infiltration into the myocardium as previously demonstrated (Torrent *et al.* 2005; Pumarola *et al.* 1991).

Diseased dogs in this study had clinicopathologic abnormalities similar to those previously described in dogs with leishmaniasis (Ciaramella *et al.* 1997; Reis *et al.* 2006). Protein profiles indicated an inflammatory pattern suggestive of upregulated humoral immune responses characteristic of dogs with leishmaniasis.

Interestingly, proteinuria and hypoalbuminaemia were moderately correlated with increased cTnI concentrations in this study. Proteinuria and hypoalbuminaemia are hallmarks of canine leishmaniasis and typically occur secondary to glomerulonephritis from immune complex deposition (Ciaramella *et al.* 1997; Riera *et al.* 1999). The same pathogenic mechanism might be responsible for microvasculature damage in other tissues, such as the myocardium, potentially leading to cardiomyocyte damage and release of troponin I.

Increased cTnI concentrations can also result from noncardiac conditions, such as azotaemia and anaemia, which often occur in dogs with clinical leishmaniasis. In a recent study, cTnI concentrations were reported to be increased in 70% of dogs with azotaemic renal failure and in 70% of dogs with a variety of systemic noncardiac diseases; however, there was no correlation between cTnI concentration and degree of azotaemia, presence of murmurs, hypertension, or type of noncardiac illness (Porciello *et al.* 2008). Azotaemic renal failure may lead to altered elimination of cTnI. In addition, hypertension, myocardial remodeling, left ventricular hypertrophy, thromboembolism due to loss of antithrombin, and uraemic pericarditis or myocarditis may cause clinically unapparent myocardial injury. In our study, less than ¼ of dogs had azotaemia and the degree was usually mild; cTnI concentration did not different in azotaemic animals and healthy controls dogs, and was not correlated with serum creatinine concentration.

Mild-to-moderate non-regenerative anaemia occurs frequently in dogs with leishmaniasis and also is correlated with the severity of clinical signs (Reis *et al.* 2006). Severe anaemia can cause tissue hypoxia and can contribute to increased cTnI concentration secondary to myocyte damage (Serra *et al.* 2010). Dogs with primary

immune-mediated haemolytic anaemia have higher serum cTnI concentrations compared to both the healthy dogs and dogs with non-haematological diseases and secondary cardiac diseases (Gow *et al.* 2011). Almost ¾ of the diseased dogs in our study were anaemic, but the correlation between decreased PCV and cTnI concentration was weak; in addition, within the subgroup of dogs with increased cTnI concentration the proportion of anaemic dogs was only slightly higher and no difference in PCV was found between dogs with cTnI above or below the URL. Finally, in most cases, the degree of anaemia was mild and unlikely responsible for clinically relevant hypoxia. Thus, azotaemia and anaemia did not play a significant role in increased cTnI concentrations in this study.

The influence of age on serum cTnI concentration has been reported previously, and we found a weak correlation between age and cTnI concentration (O'Brien *et al.* 2006; Serra *et al.* 2010; Oyama *et al.* 2004). This association may be attributed to degenerative changes in the coronary vasculature, arteriosclerosis, and possible ischemic injury in aged dogs. Age was not significantly different between dogs with leishmaniasis and healthy control dogs or between dogs with cTnI concentrations above or below the URL; consequently, age was unlikely to be a factor in the difference in cTnI concentrations between these groups.

A limitation of our study was lack of a thorough cardiac evaluation, including electrocardiographic and echocardiographic evaluation, of all dogs; this limitation was due to the retrospective design of the study. Histologic examination of the heart would be required to evaluate the presence, severity, and type of myocardial injury and to demonstrate a definitive causal relationship between increased cTnI and leishmaniasis. However, antemortem myocardial biopsy is invasive, and postmortem examination would not permit evaluation of changes produced by the parasite acutely after infection, given that leishmaniasis is a chronic disease with a time course of months to several years.

Neither PCR analysis nor cytologic examination of tissues was performed to confirm the presence of *Leishmania* organisms in this study. Dogs exposed to *Leishmania* may be seropositive even after clearance of the infection, and asymptomatic infection can occur. However, in a symptomatic dog, a high antibody titer or titers that increase over time indicate that the parasitic infection is disseminated, and such

titers are considered diagnostic for leishmaniasis without the need of additional testing (Paltrinieri *et al.* 2010). In a recent study, all symptomatic dogs with mederate or high ELISA titers were reported to be positive by quantitative real-time PCR analysis of blood samples (Martinez *et al.* 2011).

In conclusion, a significant proportion of dogs with leishmaniasis had increased serum cTnI concentration, indicating some degree of myocardial damage. The increase was mild for most of the dogs and may have no clinical significance. Evaluation of cTnI as an early marker preceding overt cardiac disease or as a prognostic factor was beyond the aim of this preliminary study. Potential myocardial injury in canine leishmaniasis could involve many pathogenic mechanisms, including direct action of the parasite and other concurrent factors such as vasculitis, renal disease and anaemia. Prospective studies are warranted to further investigate the causal relationship between *Leishmania* infection and myocardial injury.

5.2 Iron status and C-reactive protein in canine leishmaniasis

ABSTRACT

Objective: To investigate iron status, its relationship with C-reactive protein and the prognostic value of both in canine leishmaniasis.

Method: Eighty-six dogs with leishmaniasis and two control groups (healthy dogs and dogs with diseases other than leishmaniasis) were selected. Iron status indicators and C-reactive protein were compared between the three groups. Correlations between C-reactive protein and iron, ferritin and total iron-binding capacity were evaluated in dogs with leishmaniasis. Iron, total iron-binding capacity and ferritin were compared between dogs stratified according to similar C- reactive protein concentrations. The mortality rate at 30 days post-diagnosis was compared between groups. Iron status indicators and C-reactive protein were compared between survivors and non-survivors.

Results: Dogs with leishmaniasis had lower iron and total iron-binding capacity and higher ferritin and C-reactive protein. There was a significant but low correlation of C-reactive protein with iron, ferritin and total iron-binding capacity. Dogs with leishmaniasis had decreased iron and total iron-binding capacity and increased ferritin compared to other ill patients with similar C-reactive protein concentrations. Mortality was not significantly different between groups but non-survivor dogs with leishmaniasis had higher C-reactive protein and lower total iron-binding capacity.

Clinical Significance: Inflammation contributes to the iron status alterations found in canine leishmaniasis but other mechanisms are likely involved. Low total iron-binding capacity and increased C-reactive protein are risk factors for outcome in canine leishmaniasis.

INTRODUCTION

CanL is endemic in more than 70 countries worldwide (Solano-Gallego *et al.* 2011) and the infection is spreading to non-endemic areas with an increasing number of cases reported in dogs living in Northern Europe (Shaw *et al.* 2009).

The disease is manifested by a broad spectrum of clinical signs and degree of severity. Sick dogs usually have generalised lymphadenopathy, weight loss, skin and ocular lesions, lameness, epistaxis, mucous membrane pallor, systemic vasculitis and renal dysfunction that can progress from mild proteinuria to nephrotic syndrome and end-stage kidney disease. Immune-complex formation and deposition and consequently an intense systemic inflammatory response are pivotal in developing clinical signs (Alvar *et al*. 2004). A marked increase in acute phase proteins including haptoglobin, C-reactive protein (CRP) and ceruloplasmin has been demonstrated in dogs infected with *Leishmania* (Martínez-Subiela *et al.* 2002). A recent study (Martínez-Subiela *et al.* 2011) has also suggested that acute phase proteins could be used as an early marker for CanL as well as for monitoring the response to treatment. One of the potential effects of inflammation is a relative iron deficiency in both the transport and functional pools, which limits availability of iron for erythropoiesis (McCown and Specht 2011). A mild to moderate non-regenerative anaemia is common in dogs with leishmaniasis. Only few studies have previously addressed iron status in dogs with leishmaniasis (Liste *et al.* 1994; Adamama-Moraitou *et al*. 2005) but none has investigated the prognostic value of such variables.

The objective of this study was to evaluate the iron status in CanL and its relationship with haematological variables and CRP, considered a sensitive marker of inflammation, as well as to assess the prognostic value of iron status and CRP in dogs with leishmaniasis.

MATERIALS AND METHODS

Dogs diagnosed with clinical leishmaniasis at the San Marco Veterinary Clinic of Padua, Italy, between November 2008 and December 2011 were retrospectively included in the study. Diagnosis of CanL was based on clinical signs, laboratory

findings, serological status and positive real time quantitative polymerase chain reaction (rtq-PCR) on bone marrow samples. These dogs were included in CanL group and classified as stage C and D according to the Canine Leishmaniasis Working Group guidelines (Paltrinieri *et al.* 2010). Two groups of control dogs with no clinical signs or clinicopathological findings compatible with leishmaniasis were included in the study. These dogs were individually matched to the CanL group for age (± six months), sex and breed. When a breed match of the same age and sex of a CanL dog was not found in the data base, a dog with similar weight (±5 kg) was chosen instead. These dogs were selected as closely as possible to the admission date of the corresponding dogs of the CanL group to reduce variations in results of laboratory analyses attributable to changes in performance of analysers. When two or more dogs met these criteria, the dog included in the group was selected via a randomisation procedure with a computer system. Control group 1 included healthy patients with unremarkable physical examination, presented for annual vaccination, blood donation or elective surgery. All blood donor dogs had negative serology for the following vector-borne pathogens: *Ehrlichia canis, Anaplasma phagocytophilum, Babesia species, Dirofilaria immitis* and *Leishmania infantum.* Control group 2 included sick dogs with a clinical diagnosis other than leishmaniasis. These dogs did not present with clinical signs and/or clinicopathological abnormalities suggestive of this disease and specific tests (serology, cytology of lymph nodes) were performed when leishmaniasis was still included in the differential diagnosis. All tested dogs of control group 2 had negative results.

Only dogs with a complete medical record, including history and results of physical examination, complete blood count, serum biochemistry analysis, including iron profile and CRP, and urinalysis were included in the study.

Owner consent was obtained for all the dogs included in the study.

For serology, an enzyme-linked immunosorbent assay (ELISA) test for *Leishmania* was performed as previously described (Riera *et al.* 1999). Results were quantified as units related to positive serum used as a calibrator and arbitrarily set at 100 ELISA Units (EU). The positive threshold value previously established by the laboratory was 35 EU (mean ± 4 sd of 32 dogs from non-endemic areas). Results classified as high positive corresponded to more than 150 EU.

Detection and quantification of *Leishmania* kinetoplastic DNA was performed on costochondral junction bone marrow samples by rtq-PCR as previously described (Caldin *et al.* 2004; Solano-Gallego *et al.* 2007).

The iron profile included serum iron, total iron-binding capacity (TIBC, which is an indirect measure of the total transferrin concentration), unsaturated iron-binding capacity (UIBC), percentage of transferrin saturation and serum ferritin. Serum iron (Iron OSR6186, Beckman Coulter) UIBC (UIBC OSR6124, Beckman Coulter) and ferritin (Tina-quant Ferritin Gen.4, Roche Diagnostic GmbH) concentrations were determined via quantitative assays with an automated analyser (Olympus AU 2700, Olympus diagnostics). TIBC was calculated by adding iron concentration to UIBC, and the percentage of transferrin saturation was calculated as the ratio between serum iron and TIBC. Ferritin assays in dogs have been previously validated for precision, linearity and recovery percentage (Caldin *et al.* 1999).

Serum CRP concentration was measured using an immunoturbidimetric assay for human (CRP OSR6147, Olympus Life and Material Science Europe GmbH) use for which results correlated well (r=0.98) with those of a previously validated canine-specific ELISA (Tridelta Phase range canine CRP kit, Tridelta Development Ltd.) (Martínez-Subiela *et al.* 2005; Caldin *et al.* 2009).

A full blood count was performed in every animal using an automated haematology analyser (ADVIA 120, Siemens Healthcare Diagnostics), and included data regarding calculated haematocrit (Hct), measured mean corpuscular volume (MCV), and calculated mean corpuscular haemoglobin concentration (MCHC). Anaemia, microcytosis and hypochromia were defined by values below the lower limit of the reference intervals established by the laboratory for Hct (37.0 to 59.2%), MCV (63.1 to 72.6 fL) and MCHC (33.3 to 36.8 g/dl).

Iron, TIBC, UIBC, percentage of transferrin saturation, ferritin and CRP were compared between the three groups of dogs. Correlations between CRP and iron, CRP and TIBC, and CRP and ferritin were calculated only in dogs with leishmaniasis to investigate if inflammation was potentially associated with iron profile alterations. In addition, to assess if abnormalities in serum iron, TIBC and ferritin concentrations were due only to inflammation, dogs were stratified according to increasing magnitude of CRP values. A first comparison was made between dogs of all three

groups with a CRP concentration between 0.1 and 22.2 mg/L (0-10 times increase above the upper limit of the laboratory reference interval: 0.1-2.2 mg/L). A second comparison was made considering only dogs in CanL group and group 2 with CRP concentrations between 22.3 and 66.9 mg/L (10-30 times increase above the upper limit of the laboratory reference interval). Group 1 was not included in this analysis because no healthy dogs had such values of serum CRP.

Hct, MCV, and MCHC, in addition to the proportion of anaemic, microcytic, and hypochromic dogs were compared between dogs in stage C and stage D of the CanL group and between this group and group 2.

The correlation between Hct and the iron status parameters was also calculated within the stages C and D of the CanL group. Iron status variables were compared between anaemic and non-anaemic dogs within the stages C and D of the CanL group.

Finally, the mortality rate at 30 days post-diagnosis was compared between the CanL group and control group 2, and in the CanL group iron, TIBC, ferritin and CRP were also compared between survivors and non-survivors.

Statistical analysis

Data were tested for normality using the Shapiro-Wilk test. Data that did not follow a normal distribution were analysed by nonparametric methods. Data are reported as median or mean and range (minimum to maximum). Kruskall-Wallis test with Bonferroni correction for post-hoc comparisons was used when comparing variables between more than two groups. When comparing only two independent groups, an unpaired *t*-test and a Mann-Whitney U test were used for data normally and non-normally distributed, respectively. Fisher's exact test was used to compare categorical data. Spearman's rho (ρ) or Kendall's tau (τ) rank correlation test was used to assess the statistical dependence between two variables non-parametrically distributed. Statistical significance was set at $P<0.05$ for all analyses. Statistical analysis was performed using the statistical softwares Analyse-it®(Software Ltd) and Stats Direct® (version 2.7.9, Stats Direct Ltd.).

RESULTS

Case material

The CanL group (n=86) included 48 males (56%) and 38 females (44%), with a median age of 6 years (1 to 13.5 years). The majority of dogs (30 of 86; 35%) were mixed breed dogs. According to the Canine Leishmaniasis Working Group guidelines (Paltrinieri *et al.* 2010), 57 (66%) dogs were classified as Stage C (sick dogs with clinically evident leishmaniasis) and 29 (34%) as Stage D (severely sick dogs).

The control group 2 included sick dogs diagnosed with different diseases including chronic valvular heart disease (n=6), chronic kidney disease (n=4), chronic hepatitis (n=7), neoplasia (multicentric lymphoma (n=7); multiple myeloma (n=3); osteosarcoma (n=1)), chronic diarrhoea (inflammatory bowel disease (n=16); antibiotic-responsive diarrhoea (n=9); alimentary lymphoma (n=2)), chronic regurgitation secondary to idiopathic megaoesophagus (n=2), endocrine diseases (diabetes mellitus (n=6); hyperadrenocorticism (n=9); hypothyroidism (n=6)) and primary immune-mediated haemolytic anaemia (IMHA) (n=8). Dogs with chronic kidney disease and IMHA were tested for *Leishmania infantum* as well as for other pathogens, including *Ehrlichia canis, Anaplasma phagocytophilum, Babesia species, Dirofilaria immitis* and *Leptospira species.* All these dogs had negative results for those tests.

Comparisons

Dogs with leishmaniasis showed significantly lower iron and TIBC serum concentrations, and significantly higher ferritin and CRP concentrations compared to both control groups. UIBC and percentage of transferrin saturation of dogs with leishmaniasis were significantly lower only compared to control group 1 (Table 1).

Table 1. Results of the comparison of iron status variables and C-reactive protein (CRP) between dogs with leishmaniasis (CanL group), healthy dogs (Control group 1) and dogs with diseases other than leishmaniasis (Control group 2).

		CanL group (n=86)	**Control group 1** (n=86)	**Control group 2** (n=86)	
Variable	RI	Median (range)	Median (range)	Median (range)	P value
Iron (µmol/L)	17-40	16*** (3-48)	24 (14-53)	22 (3-57)	< 0.0001
TIBC (µmol/L)	57-86	52*** (15-87)	67 (51-105)	63 (32-91)	< 0.0001
UIBC (µmol/L)	33-55	36* (3-68)	42 (20-80)	43 (3-70)	0.0181
% Saturation	28.2-56.8	29.1** (4.8-37.02)	36.55 (20.8-45.72)	32.45 (4.5-45.05)	0.0009
Ferritin (pmol/L)	191-643	1270*** (16-6262)	404 (175-512)	389 (92-5795)	< 0.0001
CRP (mg/L)	0.1-2.2	27.5*** (0.1-268.5)	0.6 (0.1-6.4)	4.9† (0.1-225.4)	< 0.0001

RI reference intervals, TIBC total iron-binding capacity, UIBC unsaturated iron-binding capacity.
Kruskall-Wallis test with Bonferroni correction for post-hoc comparisons. P values < 0.05 are considered statistically significant. RI established by the laboratory are also indicated.
* Significant difference only between CanL group and Control group 1, P=0.027
**Significant difference only between CanL group and Control group 1, P=0.0005
***Significant difference between CanL group and both Control groups, P<0.0001
† Significant difference between Control group 1 and Control group 2, †P<0.0001

Within the CanL group, serum CRP had a low negative correlation with iron (P<0.0001, ρ=- 0.46), a moderate negative correlation with TIBC (P<0.0001; ρ=-0.663) and a low positive correlation with ferritin (P=0.008, ρ=0.28). When serum iron, TIBC and ferritin were compared between the three groups only considering dogs with CRP concentrations between 0.1 and 22.2 mg/L (CanL group n=40, group 1 n=40, group 2 n=40), patients with CanL still showed significantly lower iron concentrations and higher ferritin serum concentrations compared to control groups. Lower concentrations of TIBC were found in the CanL group but without statistical significance. When serum iron, TIBC and ferritin were compared in dogs with CRP concentrations between 22.3 and 66.9 g/L (CanL group n=22, group 2 n=22), patients with leishmaniasis showed again significantly lower iron and TIBC, and higher ferritin concentrations compared to control group 2 (Tables 2 and 3).

Table 2. Results of the comparison of iron, total iron binding capacity (TIBC) and ferritin between dogs with leishmaniasis (CanL group), healthy dogs (Control group 1) and dogs with diseases other than leishmaniasis (Control group 2). All dogs had C-reactive protein (CRP) concentrations between 0.1-22.2 mg/L (0-10 times the upper reference limit).

		CanL group (n=40)	**Control group 1** (n=40)	**Control group 2** (n=40)	
Variable	RI	Median (range)	Median (range)	Median (range)	P value
Iron (µmol/L)	17-40	20*§ (3-48)	24 (14-51)	25 (15-53)	0.0001
TIBC (µmol/L)	57-86	64 (44-87)	66 (56-105)	70 (32-96)	0.0803
Ferritin (pmol/L)	191-643	1049**† (88-5689)	409 (187-863)	418 (92-2622)	< 0.0001

Kruskall-Wallis test with Bonferroni correction for post-hoc comparisons. P values < 0.05 are considered statistically significant. Reference intervals (RI) established by the laboratory are also indicated.

* Significant difference between CanL group and Control group 1 *P= 0.0008

§ Significant difference between CanL group and Control group 2 §P= 0.0003

** Significant difference between CanL group and Control group 1 **P< 0.0001

† Significant difference between CanL group and Control group 2 †P= 0.0004

Table 3. Results of the comparison of iron, total iron binding capacity (TIBC) and ferritin between dogs with leishmaniasis (CanL group) and dogs with diseases other than leishmaniasis (Control group 2). All dogs had C-reactive protein (CRP) concentrations between 22.3 and 66.9 mg/L (10-30 times the upper reference limit).

		CanL group (n=22)	**Control group 2** (n=22)	
	RI	Median (range)	Median (range)	P value
Iron (µmol/L)	17-40	13 (8-26)	22 (11-53)	< 0.0001
TIBC (µmol/L)	57-86	47 (15-87)	70 (36-96)	< 0.0001
Ferritin (pmol/L)	191-643	1766 (256-5224)	353 (117-533)	< 0.0001

Kruskall-Wallis test with Bonferroni correction for post-hoc comparisons. P values < 0.05 are considered statistically significant. Reference intervals (RI) established by the laboratory are also indicated.

Sixty % (52 of 86) of dogs with leishmaniasis and 80 % (16 of 86) of dogs of group 2 were anaemic. Dogs with leishmaniasis in both stages C and D had a significantly (P<0.001) lower Hct (stage C: median, 38.8%; range, 11.9-55.9%; Stage D: median, 31.35%; range, 15.6-60.3%) and higher proportion of anaemic dogs (stage C: 49%; stage D: 82%) compared to group 2 (Hct: median, 46.9%; range, 16.8-60.4%; anaemic dogs: 32%). Frequency of anaemia was higher and mean Hct was lower in dogs in stage D compared to dogs in stage C but there were no differences in the means of MCHC and MCV as well as in the number of dogs with microcytosis and/or hypochromia between the two stages (Table 4).

Table 4. Results of the comparison of haematological variables (Hct, MCV, MCHC) and frequency of anaemic, microcytic and hypochromic dogs between stage C and stage D.

		Stage C (n=57)	**Stage D** (n=29)	
Variable	RI			P value
Hct (%) Mean (range)	37.0-59.2	37.66 (11.9-55.9)	31.35 (15.6-60.3)	0.0127
MCV (fL) Mean (range)	63.1-72.6	67.18 (57.1-80.4)	67.43 (55.9-77.6)	0.8481
MCHC (g/dl) Mean (range)	33.3-36.8	32.89 (27.9-37.2)	32.51 (27.9-37)	0.4381
Frequency of anaemic (%)		28 (49%)	23 (82%)	0.0085
Frequency of microcytic (%)		8 (14%)	6 (20%)	0.406
Frequency of hypochromic (%)		28 (49%)	19 (65%)	0.252

Normality not rejected using Shapiro-Wilk test. Unpaired *t*-test used for comparisons.
P values < 0.05 are considered statistically significant. Reference intervals (RI) established by the laboratory are also indicated.

Within stage C, a statistically significant (P<0.05) positive correlation was found between Hct and TIBC (τ=0.41), UIBC (τ=0.26), iron (τ=0.40), and % of saturation (τ=0.18). A negative correlation was found between Hct and ferritin (τ=-0.26; P<0.005). No significant correlations were found between Hct and iron status variables within stage D. Anaemic dogs in stage C had significantly lower TIBC, UIBC

and total iron compared to non-anaemic dogs in the same stage (Table 5). Anaemic dogs in stage D had only significantly lower total iron concentration compared to non-anaemic dogs (Table 6).

Table 5. Iron status variables compared between anaemic and non-anaemic dogs in **stage C**.

		Anaemic (n=29)	**Non-anaemic** (n=28)	
Variable	RI	Median (range)	Median (range)	P value
Iron (µmol/L)	17-40	13 (3-48)	20 (10-44)	0.0007
TIBC (µmol/L)	57-86	45 (21-87)	67 (46-87)	<0.0001
UIBC (µmol/L)	33-55	33 (4-66)	46 (27-68)	0.0006
% Saturation	28.2-56.8	25.1 (4.8-82.4)	29.75 (17.8-60.8)	0.3152
Ferritin (pmol/L)	191-643	1270 (88-3797)	850 (303-5689)	0.0797

Normality rejected using Shapiro-Wilk test. Mann-Whitney U test used for comparisons. P values < 0.05 are considered statistically significant. Reference intervals (RI) established by the laboratory are also indicated.

Table 6. Iron status variables compared between anaemic and non-anaemic dogs in **stage D.**

		Anaemic (n=23)	**Non-anaemic** (n=5)	
Variable	RI	Median (range)	Median (range)	P value
Iron (µmol/L)	17-40	13 (3-22)	24 (13-32)	0.0126
TIBC (µmol/L)	57-86	40 (15-64)	45 (29-87)	0.4095
UIBC (µmol/L)	33-55	27 (3-53)	21 (13-55)	0.8232
% Saturation	28.2-56.8	29.1 (10.7-84.1)	53.5 (26.9-58.8)	0.15
Ferritin (pmol/L)	191-643	2110 (16-6262)	1894 (667-3278)	0.0694

Normality rejected using Shapiro-Wilk test. Mann-Whitney U test used for comparisons. P values < 0.05 are considered statistically significant. Reference intervals (RI) established by the laboratory are also indicated.

Finally, 6/86 (6.9%) dogs of the CanL group and 1 of 86 (1.2%) of the control group 2 died at 30 days post-diagnosis. The test failed to find a statistically significant difference in the number of survivors between the two groups (P=0.05). Reasons for death in dogs with leishmaniasis were generally correlated with worsening of renal function and severe azotaemia (data not shown). Non-survivor dogs with leishmaniasis had significantly higher CRP (P=0.0348) and lower TIBC (P=0.004) concentrations and a tendency to lower iron (P=0.2035) and higher ferritin (P=0.1031) concentrations compared to survivors (Table 7).

Table 7. Results of the comparison of iron, total iron binding capacity (TIBC), ferritin and C-reactive protein (CRP) between survivor and non-survivor dogs with leishmaniasis.

		Survivors (n=80)	**Non-survivors** (n=6)	
	RI	Median (range)	Median (range)	P value
Iron (µmol/L)	17-40	16 (3-48)	12 (8-20)	0.2035
TIBC (µmol/L)	57-86	54 (20-87)	27 (15-64)	0.004
Ferritin (pmol/L)	191-643	1222 (16-5689)	2090 (1067-6262)	0.1031
CRP (mg/L)	0.1-2.2	24.9 (0.1-268.5)	69.6 (45.4-99.6)	0.0348

Mann-Whitney U test. P values < 0.05 are considered statistically significant. Reference intervals (RI) established by the laboratory are also indicated.

DISCUSSION

To the authors' knowledge, this is the first study investigating iron status and its relationship with CRP in CanL. The results indicated that dogs with leishmaniasis have decreased serum iron, TIBC, UIBC and percentage of transferrin saturation and increased concentrations of ferritin. As suggested by Liste *et al.* (1994), this is typical of an inflammatory state. Moreover, these results are correlated with increased concentrations of CRP found in this study. CanL is a chronic and sometimes subclinical disease in which continuous antigenic stimulation and excess antibody production cause hypergammaglobulinaemia, resulting in formation and deposition

of immune complexes that may cause glomerulonephritis, vasculitis, polyarthritis, uveitis, and meningitis, in addition to the production of autoantibodies against platelets and red blood cells (Alvar *et al.* 2004). It has been demonstrated (Martínez-Subiela *et al.* 2002) that infection by *Leishmania infantum* is associated with increased concentrations of acute phase proteins.

The acute phase response is an early non-specific defense mechanism to local or general disturbances in homeostasis attributable to infection, inflammation, trauma, neoplasia or immunological disorders (Baumann and Gauldie 1994; Suffredini *et al.* 1999; Petersen *et al.* 2004). During this response there is an increased rate of synthesis and release of positive acute phase proteins such as haptoglobulin, CRP, ceruloplasmin, fibrinogen and ferritin concurrently with decreased concentrations of negative acute phase proteins such as albumin and transferrin (Eckersall and Conner 1988; Toussaint *et al.* 1995; Eckersall *et al.* 2000; McCown and Specht 2011).

During inflammation hepcidin expression is also increased especially when interleukin-6 and 1 are involved. Among acute phase proteins, hepcidin plays a pivotal role as an inhibitory regulator of iron metabolism (Ganz 2003; Nemeth *et al.* 2003; McCown and Specht 2011). Hepcidin exerts its effects by binding to the cell surface iron efflux protein, ferroportin, and inducing its internalisation and degradation. The effect of this interaction is to inhibit both absorption of dietary iron from intestinal epithelium and export of iron from macrophages and hepatocytes. Functional iron unavailability then ensues. Unfortunately, no commercial assays to measure hepcidin in dogs are currently available and only molecular analysis has been used for this purpose in previous studies (Fry 2004; Ganz 2003). Thus, hepcidin concentration could not be studied in this population.

In dogs with leishmaniasis, CRP had a negative correlation with iron and transferrin and a positive correlation with ferritin. These findings indicate that in CanL inflammation contributes to the status of iron unavailability, which may in turn contribute to the anaemia of chronic disease often described in this infection. In fact the frequency and degree of anaemia were greater in dogs with leishmaniasis then in dogs with other diseases and correlated with the clinical stage of the disease. In addition there was a significant correlation between the Hct and iron status variables. The presence of inflammation, as demonstrated by the iron profile and the

increased CRP concentration, may also explain other clinicopathological abnormalities commonly found in canine leishmaniasis, including leukocytosis, hypoalbuminaemia, and decreased albumin/globulin ratio (Liste *et al.* 1994; Paltrinieri *et al.* 2010).

When the iron profile was studied in dogs stratified according to CRP concentrations and so with similar degrees of inflammation, the iron status alterations were still more pronounced in patients with CanL compared to other dogs. Thus, inflammation, although an important contributor, does not completely explain the reason for the altered iron profile in CanL. It has been previously found (Chang and Chang 1985) that *Leishmania* is defective in the haeme biosynthetic pathway and requires iron for growth. Uptake of iron from transferrin or lactoferrin by promastigotes appears to be critical in the first hours after infection of a mammalian host (Wilson *et al.* 1994). In vitro studies have shown that an iron-deficient environment does not support the growth of promastigotes (Soteriadou *et al.* 1995), and the addition of iron salts to incubation fluids may prevent the killing of intracellular amastigotes by activated macrophages (Mauel *et al.* 1991). In a more recent study (Wilson *et al.* 2002) it was found that *Leishmania chagasi* is able to uptake iron in the reduced ferrous form and this finding was followed by the demonstration that *Leishmania chagasi* expresses a NADPH-dependent iron reductase, capable of converting oxidised, ferric Fe^{3+} into more soluble Fe^{2+}. Huynh and Andrews (2008) identified a plasma membrane *Leishmania* transporter, LIT1, that allows the uptake of ferrous iron and that plays a critical role in intracellular growth and virulence of the protozoa. Similar mechanisms have been described in infections with the bacteria *Salmonella* and *Mycobacteria* and with the protozoa *Trypanosoma chagasi* and *Plasmodium falciparum.* In the latter, asymptomatic malarial parasitaemia was found to be associated with increased ferritin concentrations, in the absence of a manifest acute phase response (deMast *et al.* 2010). These findings together with the current results may support the hypothesis that the iron depletion may be due to the consumption of the element by the parasite to allow its growth, and/or iron may be sequestered by the organism as a defense mechanism to decrease parasite survival. Alternatively, low iron concentrations in dogs with leishmaniasis can also be caused by chronic bleeding, as

in case of epistaxis, skin lesions and gastrointestinal ulceration secondary to renal azotaemia, which are quite frequent in CanL. Further investigations are needed to better understand the importance of these mechanisms in the pathophysiology of CanL.

Six of 86 dogs with leishmaniasis died 30 days post-diagnosis. All dogs of CanL group were on therapy with allopurinol (Zyloric; Teofarma) in association with meglumine antimoniate (Glucantime; Merial) or miltefosine (Milteforan; Virbac). Non-survivor dogs had significantly lower TIBC and increased concentrations of CRP. Previous studies (Singh *et al.* 1999; Martínez-Subiela *et al.* 2002) showed that measurement of CRP is of prognostic value in visceral leishmaniasis. Non-survivors also had a tendency to lower iron and higher ferritin concentrations, although no statistically significant differences were found. Further studies are needed to investigate the potential value of monitoring iron profile and CRP concentration during therapy and to investigate the potential correlation of these variables with the evolution of clinical signs and response to treatment. Iron profile variables might have a stronger prognostic value considering a longer follow-up.

The study has some limitations. CanL is a chronic disease and a too short follow-up may have influenced the results. It was decided to use a cut-off time of 30 days because this generally represents the first re-check for dogs on therapy for leishmaniasis. In fact, both meglumine antimoniate and miltefosine are usually administered for 28 days and then suspended, while therapy with allopurinol is continued for longer (Oliva *et al.* 2010, Solano-Gallego *et al.* 2011). Due to the small number of non-survivor dogs, a type I error likely limited the ability of the test to detect a statistically significance difference. Due to the retrospective nature of the study and the condition of a referral hospital, in which patients are generally sent back to the referring veterinarians after initial work-up, diagnosis and clinical stabilisation, a longer follow-up was not possible in the majority of the patients. Moreover, not all dogs included in control groups 1 and 2 had been tested for *Leishmania* but CanL was considered unlikely based on absence of clinical signs and clinicopathological abnormalities typical of this disease. However, it is possible that some of these dogs had been exposed to and infected by *Leishmania* without developing the clinical disease. Iron status and CRP were only investigated in dogs

with clinical leishmaniasis and at this point the effects that *Leishmania* can have on these parameters in infected but healthy dogs is not known.

In conclusion, dogs with leishmaniasis have lower iron and TIBC and higher ferritin and CRP concentrations compared to healthy dogs and dogs with other diseases. Inflammation contributes in part to the iron status alterations found in CanL. Dogs with leishmaniasis have lower iron and TIBC and higher ferritin compared to other ill dogs with similar CRP concentrations. For this reason, a decreased iron and increased ferritin in CanL are probably not only due to inflammation, but may reflect other specific mechanisms, as proposed in humans. The results of this study suggest that increased CRP and decreased TIBC are risk factors for mortality in CanL, while other iron status alterations at the time of diagnosis do not seem to impact the outcome of dogs with leishmaniasis.

5.3 Clinical leishmaniasis in dogs living in the UK

ABSTRACT

Objective: To investigate the prevalence of leishmaniasis in a canine population in the UK and to describe clinical presentation, clinicopathological abnormalities, therapeutic protocols and outcome. An additional aim was to determine the prognostic value of different clinical and laboratory parameters.

Materials and Methods: Medical records of dogs diagnosed with leishmaniasis at 7 referral centres in the UK were retrospectively reviewed. Simple and multiple logistic regressions were used to determine prognostic factors.

Results: The prevalence was between 0.007 and 0.04% with a higher number of cases in southern England. All dogs had a history of travel to or from an endemic country. Lethargy, dermatological signs, decreased appetite and lameness were the most common reasons for presentation. Allopurinol was used alone in the majority of cases. Pure breed dogs and those that received N-methylglucamine antimoniate had a poorer prognosis.

Clinical significance: CanL in the UK should be considered in patients showing compatible clinical signs and with a history of travel to or from endemic areas. Pure breed dogs, and those classified in stage D carry a guarded to poor prognosis.

INTRODUCTION

CanL is a systemic zoonotic disease caused by the protozoan *Leishmania infantum.* Infected dogs are the main reservoir of the parasite (Baneth *et al.* 2008) and play an important role in the epidemiology of human visceral (HVL) and cutaneous (HCL) leishmaniasis. Approximately 0.2 to 0.4 and 0.7 to 1.2 million HVL and HCL cases, respectively, occur each year. More than 90% of global HVL cases occur in six countries: India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil. HCL is more widely distributed with about one-third of cases occurring in each of three epidemiological regions, the Americas, the Mediterranean basin, and western Asia from the Middle East to Central Asia (Alavar *et al.* 2012). CanL is endemic in more than 70 countries worldwide (Solano-Gallego *et al.* 2011) and especially in the Mediterranean areas of Europe (Cyprus, Greece, Albania, Croatia, Italy, Malta, France, Spain), Portugal (Maia *et al.* 2015), the Middle East and many tropical and subtropical areas of the world. However, the infection is spreading to non-endemic areas with an increasing number of cases reported in dogs living in North America (Gaskin *et al.* 2002, Duprey *et al.* 2006) and Northern Europe (Shaw *et al.* 2009, Maia *et al.* 2015). Recent studies have documented the presence of the disease in Germany (Geisweid *et al.* 2012), United Kingdom (Shaw *et al.* 2009) and Netherlands (Teske *et al.* 2002). This is probably due to a wider spread of the vector in some of the above areas and especially to a larger numbers of dogs being imported from, or having visited, endemic countries. Since the introduction of the United Kingdom Pet Travel Scheme (PETS) in 2000, the number of dogs travelling into the UK has increased year after year with a total of 411,582 dogs recorded between 2000 and January 2008 (Mencke *et al.* 2011). As a result, the disease has gained importance in the UK, albeit largely limited to the dogs that travel. It is likely that only very little natural transmission occurs in the UK because environmental conditions prevent the viability of the vector (Shaw *et al.* 2009). However, other mechanisms of transmission are possible, including blood transfusion (de Freitas *et al.* 2006), vertical (Rosypal *et al.* 2005, Pangrazio *et al.* 2009, Boggiatto *et al.* 2011, Naucke *et al.* 2012, Turchetti *et al.* 2014) and venereal transmission (Diniz *et al.* 2006). Despite the increase in awareness, the prevalence of infected dogs entering the UK is

unknown, as no pre- or post-travel testing is required. Furthermore, clinically apparent cases represent the minority of infected dogs in endemic areas (Solano-Gallego *et al*. 2009; Schallig *et al.* 2013), so dogs with subclinical infection that appear healthy may unknowingly be imported.

CanL is manifested by a broad spectrum of clinical signs and laboratory alterations. Predicting the outcome is often challenging because there are only few controlled studies evaluating prognostic factors (Castagnaro *et al.* 2007). We have recently demonstrated that low total iron-binding capacity and increased C-reactive protein are risk factors for a negative outcome (Silvestrini *et al.* 2013). In previous studies (Pinelli *et al.* 1994; Solano-Gallego *et al.* 2001), there was evidence of a positive correlation between anti-*Leishmania* antibody titre and both disease severity and progression, for dogs living in endemic areas. A recent study conducted in the United States of America (USA), considered a non-endemic area, showed that increases in serologic titer were associated with disease progression and that the highest titers were observed in dogs displaying moderate to severe clinical signs (Boggiatto *et al.* 2010). However, Geisweid *et al.* (2012) found that antibody titre at diagnosis did not influence survival time and, therefore, serology was not considered to be a useful prognostic indicator in dogs living in Germany, a non-endemic region. A second possible prognostic indicator is renal dysfunction, which in CanL can progress from mild proteinuria to nephrotic syndrome and end-stage kidney disease, and is one of the most important causes of death or euthanasia (Planellas *et al.* 2009). Proteinuria and hypoalbuminaemia had a significant correlation with survival time also in the canine population of Geisweid *et al.*'s study (2009). A third possible prognostic factor is therapeutic protocol (Roura *et al*. 2013), with a combination of N-methylglucamine antimoniate (Glucantime; Merial) and allopurinol (Zyloric; Aspen) currently being considered to be the best treatment for CanL (Noli *et al.* 2005; Oliva *et al*. 2011), and the miltefosine (Miteforan; Virbac)-allopurinol combination also showing promising results (Miró *et al*. 2009). Finally, the Canine Leishmaniasis Working Group (CLWG) (Paltrinieri *et al.* 2010) and the LeishVet group (Solano-Gallego *et al*. 2011) have respectively created a staging system based on clinical signs, laboratory abnormalities and diagnostic test results. The clinical stage at

diagnosis is useful to guide selection of appropriate treatment and predict outcome (Oliva *et al.* 2008, 2010; Solano-Gallego *et al*. 2009; Paltrinieri *et al.* 2011).

Only one study (Shaw *et al.* 2009) has previously investigated dogs with positive diagnostic tests for CanL in the UK but none has investigated possible prognostic factors and provided information regarding therapy. In light of this, the objectives of this study were, firstly, to investigate the prevalence of leishmaniasis in a canine population attending referral centres in the UK and, secondly, to describe clinical presentation, most frequent clinicopathological abnormalities, diagnostic investigations and outcome of dogs diagnosed with CanL in the UK. Further aims included reporting the different therapeutic protocols used in the UK and correlating them with outcome, and determining the prognostic value of different clinical and laboratory parameters, including antibody titre at the time of diagnosis, the presence of concurrent renal disease and the clinical staging in a non-endemic country.

MATERIALS AND METHODS

Patients and eligibility

Medical records of dogs diagnosed of clinical leishmaniasis in seven different referral centres in the UK (University of Liverpool, University of Bristol, University of Edinburgh, University of Cambridge, Royal Veterinary College of London, Anderson Moores Veterinary Specialists, Animal Health Trust), between January 2005 and January 2014, were retrospectively reviewed. The database of each institution was searched by use of the following terms: leishmaniasis, leishmaniosis, allopurinol, N-methylglucamine antimoniate and mitelfosine. Dogs on therapy with allopurinol for other diseases than CanL were excluded. In this way only patients with a final diagnosis of clinical leishmaniasis were selected and then included. The prevalence of the disease was calculated as the ratio between the number of cases diagnosed of CanL in the study period at each referral centre and the total canine population that attended the respective centre in the same time period. The study was approved by the Veterinary Research Ethics Committee of the University of Liverpool.

Data collection

The diagnosis of CanL was made when there were compatible clinical signs and/or laboratory abnormalities together with detection of the parasites by polymerase chain reaction (PCR) or cytology (from lymph node, bone marrow, spleen or skin), and/or detection of antibodies using an immunofluorescence assay (IFAT) or an enzyme-linked immunosorbent assay (ELISA). Where available, information was reviewed regarding travel history (e.g. country to which the dog had travelled or from where it had been imported), reasons for presentation, physical examination findings, results of diagnostic investigations (e.g. haematology, biochemistry, urinalysis, cytology, serology, and PCR), therapeutic protocol used, and outcome. Dogs were tested for other vector-borne diseases (*Ehrlichia canis, Babesia canis)* if a co-infection was suspected.

Diagnostic investigations

All routine clinicopathological analyses, serology, and real-time quantitative PCR (qPCR) assays were conducted at the respective university or by commercial laboratories. Clinicopathological abnormalities such as anaemia, azotaemia, hypoalbuminaemia were defined when results were outside the reference intervals established by each corresponding laboratory. Proteinuria was diagnosed by an elevated urine protein:creatinine ratio (UPCR >0.5) with inactive urinary sediment. Renal azotaemia was defined as increased creatinine with concurrent isosthenuria (1.008-1.012). Dogs were also classified according to the International Renal Interest Society (IRIS) guidelines, based on measurement of serum creatinine concentration at the first two appointments.

For serological investigations, the upper reference interval for IFAT was either 1:80 or 1:128, depending on the laboratory, whilst the positive threshold value for ELISA used by all laboratories was 35 ELISA Units (EU). Serological results were classified as low, medium or high positive if IFAT titres were <2-fold, 2- to 4-fold, or >4-fold greater than the threshold positive value indicated by the reference laboratory. ELISA results were classified as mild when <80 EU, moderate when between 80 and 150 EU, and high when >150 EU. Detection and quantification of *Leishmania*

kinetoplastic DNA was performed on blood, bone marrow, and/or skin samples by qPCR as previously described (Caldin *et al*. 2004, Solano-Gallego *et al.* 2007, Maia *et al.* 2008).

Classification of cases

Dogs were classified at the time of diagnosis in different clinical stages according to the CLWG guidelines (Paltrinieri *et al.* 2010). Survival time was defined as time (in days) from first presentation to last re-check or to time of death. Finally, data regarding signalment, history, reasons for presentation, clinical signs, laboratory results (haematology, biochemistry profile, urinalysis), IRIS stage classification, serology titre at diagnosis, clinical stage according to the CLWG and therapeutic protocol were correlated with the outcome.

Statistical analysis:

Statistical analysis was performed using the statistical software Stats Direct® (version 2.7.9, Stats Direct Ltd., Altrincham, UK). Simple and multiple logistic regression was used to determine factors associated with mortality risk. Results are reported as odds ratios (OR), 95% confidence intervals (95% CI) and the associated *P*-value. The outcome variable was death, whereby dogs that died or were euthanased were assigned a score of 1, and those that survived were assigned a score of 0. A number of variables were tested including baseline characteristics (e.g. breed group [purebred vs. mixed breed], age, sex, neuter status, weight), clinical signs, clinicopathological findings, serology results, IRIS stage, CWLG stage, and the specific anti-*Leishmania* treatment used. Initially, all variables listed above were tested separately with simple logistic regression. A multiple logistic model was then built, which initially included the variables identified as $P<0.2$ in simple regression. The model was then refined over multiple rounds using backwards-stepwise elimination, of the least significant variable each time, and variables were only retained in the final model if they were significant ($P<0.05$).

RESULTS

Patient population

Thirty-eight dogs were included in the study: 14 were diagnosed at the Royal Veterinary College, 7 at the University of Liverpool, 7 at the University of Edinburgh, 3 at the University of Bristol, 3 at the University of Cambridge, 3 at the Animal Health Trust and 1 at Anderson Moores Veterinary Specialists. Thirty-two per cent (12/38) of patients were mixed breed dogs and Labrador retriever was the most represented pure-breed (4/38, 10%); the others were from 18 different breeds. Eighteen (18/38, 47%) were neutered males, 3 (3/38, 8%) entire males, 15 (15/38, 40%) neutered females and 2 (2/38, 5%) entire females. Neither of them was pregnant. Median age was 4.8 years (range 1.11 years-12.2 years) and median body weight was 26.3 kg (range 5.9-49 kg). The prevalence of the disease was between 0.007 and 0.04 per cent and higher incidences (0.04 per cent at the Royal Veterinary College of London, 0.03 per cent at the University of Bristol and 0.02 per cent at the Animal Health Trust) were found in southern England.

All dogs had a history of having travelled to or been imported from an endemic area for *Leishmania infantum.* In particular 16 (16/38, 42%) dogs were imported from Spain, 7 (7/38, 18%) from Greece, 3 (3/38, 8%) from Cyprus, 2 (2/38, 5%) from Italy, 2 (2/38, 5%) from Portugal, 1 from Hungary (1/38, 3%) (Tánczos B *et al*. 2012) and 1 from Brazil (1/38, 3%). Three (3/38, 8%) had travelled to Spain, 2 (2/38, 5%) to France and 1 (1/38, 3%) to Germany. No clinical or clinicopathological differences were noted between dogs imported from and dogs that have travelled to an endemic area. No autochthonous cases were found in this population.

Detection of *Leishmania infantum*

Leishmania infantum infection was demonstrated by serology and/or PCR and/or cytology. Details of the diagnostic tests are shown in Table 1. Only three dogs were tested for other vector-borne diseases, including two dogs tested by serology for *Ehrlichia canis* and one for *Babesia canis.* All three dogs were negative.

Table 1: Diagnostic tests used to indentify *L. infantum* infection

	Number of dogs (%)
Serology + PCR	10 (26%)
Serology + PCR + Cytology	8 (21%)
Serology	7 (18%)
PCR	7 (18%)
Serology + Cytology	3 (8%)
PCR + Cytology	2 (5%)
Cytology	1 (2%)
Serology	28 (74%)
ELISA	19 (68%)
IFAT	9 (32%)
Mild	*7 (25%)*
Moderate	*11 (39%)*
High	*10 (36%)*
PCR	27 (71%)
Blood	12 (44%)
Spleen	4 (15%)
Lymph node	3 (11%)
Bone marrow	2 (7%)
Blood + Bone marrow	2 (7%)
Blood + Spleen	1 (4%)
Spleen + Lymph node	1 (4%)
Blood + Conjunctiva + Skin	1 (4%)
Blood + Bone marrow + Joint fluid	1 (4%)
Cytology	14 (37%)
Lymph node	8 (57%)
Spleen	3 (22%)
Bone marrow	2 (14%)
Lymph node + Spleen	1 (7%)

Clinical signs

Reasons of presentation and physical examination findings are presented in detail in Table 2. All dogs had at least one clinical sign compatible with leishmaniasis. The most frequent reasons for presentation were lethargy (20/38, 53%), dermatological signs (17/38, 45%), decreased appetite and lameness (8/38, 21%). On physical examination the most common signs observed were dermatological signs (24/38, 63%, including localised or multifocal alopecia [10], and crusting dermatitis [8]) and

systemic lymphadenopathy (22/38, 58%). Twenty-four per cent (9/38) of dogs were diagnosed with polyarthritis.

Clinicopathological investigations

The results of all clinicopathological investigations are shown in Table 2. All dogs had at least one laboratory abnormality compatible with leishmaniasis. In total, 19/32 dogs (60%) were anaemic, with the anaemia being classified as mild (haematocrit [HCT] 30-36%) and moderate (HCT 18-29%) in 11 (58%) and 8 dogs (42%), respectively, and also classified as non-regenerative (reticulocytes < 60 x 10^9/L) in 4 of the 6 cases where reticulocyte count was available. Eight dogs (8/23, 35%) were thrombocytopenic and two (2/22,9%) were pancytopenic. Renal azotaemia was detected in 6 dogs (6/25, 24%) and 20 dogs (20/30, 67%) were classified as being in IRIS stage 1 CKD (creatinine < 125 µmol/l), 4 (4/30, 13%) in IRIS stage 2 (creatinine between 125-180 µmol/l) and 6 (6/30, 20%) in IRIS stage 3 (creatinine between 181-440 µmol/l). None of the dogs were classified as being in IRIS stage 4 CKD (creatinine > 440 µmol/l). Nineteen (19/28, 78%) of dogs were proteinuric based on increased UPCR (>0.5). Finally, 28 dogs (28/30, 93%) were hypoalbuminaemic, hyperglobulinaemic and had a low (<0.6) albumin/globulin ratio. Serum protein electrophoresis was rarely used in the diagnostic work-up and/or in the follow-up rechecks.

Table 2: Reasons of presentation, clinical signs at physical examination and laboratory abnormalities

	Number of dogs (%)
Reasons of presentation	
Lethargy	20/38 (53%)
Dermatological problems	17/38 (45%)
Lameness	8/38 (21%)
Decreased appetite	8/38 (21%)
Weight loss	7/38 (18%)
Gastro-intestinal problems	3/38 (8%)
Nasal discharge/epistaxis	2/38 (5%)
Urinary problems	2/38 (5%)
PU/PD	2/38 (5%)
Ophthalmological problems	1/38 (3%)

Generalised pain	1/38 (3%)
Seizures	1/38 (3%)
Abdominal enlargement	1/38 (3%)

Physical examination

Dermatological signs	24/38 (63%)
Alopecia	*10/24 (42%)*
Crusting	*8/24 (35%)*
Scaling	*3/24 (12%)*
Pododermatitis	*2/24 (8%)*
Pruritus	*1/24 (4%)*
Lymphadenopathy	22/38 (58%)
Polyarthritis	9/38 (24%)
Reduced body condition score	6/38 (16%)
Spleno-hepatomegaly	5/38 (13%)
Nasal discharge/epistaxis	3/38 (8%)
Pale mucous membranes	3/38 (8%)
Fever	3/38 (8%)
Ocular signs (conjunctivitis, uveitis, keratitis)	2/38 (5%)
Oral ulcer	2/38 (5%)
Ascites	2/38 (5%)
Heart murmur	1/38 (3%)
Cranial cruciate ligament rupture	1/38 (3%)
Onychogryphosis	1/38 (3%)

Laboratory abnormalities

Haematology

Anaemia	19/32 (60%)
Mild (Hct 30-36%)	*11/19 (58%)*
Moderate (Hct 18-29%)	*8/19 (42%)*
Non-regenerative	*4/6 (67%)*
Microcytic hypochromic	*6/20 (30%)*
Thrombocytopenia	8/23 (35%)
Leukopenia	7/22 (32%)
Neutropenia	*5/23 (22%)*
Lymphopenia	*5/24 (21%)*
Eosinopenia	*10/20 (50%)*
Pancytopenia	2/22 (9%)
Leukocytosis	1/22 (4%)
Neutrophilia	*4/23 (17%)*
Left shift	*4/11 (36%)*
Monocytosis	*1/21 (5%)*

Biochemistry profile

Azotaemia *(increased urea and/or creatinine)*	17/30 (57%)
Renal azotaemia *(increased creatinine and isosthenuria)*	6/25 (24%)
Hyperproteinaemia	20/30 (67%)
Hypoalbuminaemia	28/30 (93%)
Hyperglobulinaemia	28/30 (93%)
Decreased A/G ratio	28/30 (93%)
Hypecholesterolaemia	5/20 (25%)
Increased ALP	5/23 (22%)
Increased ALT	5/22 (23%)

Hypercalcaemia	5/22 (23%)
Hyperphosphataemia	10/23 (43%)
Hypernatraemia	1/21 (5%)
Hyponatraemia	1/21 (5%)
Hyperkalaemia	4/23 (17%)
Hyperchloraemia	2/20 (10%)
Urinalysis	
Isosthenuria (1.008-1.012)	16/26 (61%)
Proteinuria (UPC >0.5)	19/28 (68%)

USG: urine specific gravity; UPC: urine protein/urine creatinine ratio

Treatment

Of the 38 cases, 35 (92%) were given a specific treatment for CanL. In the majority of cases (17/35, 48%) allopurinol was used alone, followed by a combination of allopurinol and miltefosine (15/35,43%) or allopurinol and N-methylglucamine antimoniate (3/35, 9%). A variety of other drugs were used in addition to the anti-*Leishmania* therapy, depending upon the specific case and attending clinician's judgement. Treatments included ace-inhibitors (benazepril, enalapril), anti-hypertensive (amlodipine), platelets anti-aggregants (clopidogrel, aspirin), analgesics and anti-inflammatory (tramadol, meloxicam), gastro-protectants (sucralfate, famotidine), anti-emetics (maropitant, ondansetron, metoclopramide), immune-suppressive (prednisolone, azathioprine), diuretics (spironolactone), antibiotics (doxycycline, amoxicillin-clavulanate, enrofloxacin and marbofloxacin).

Staging and survival

Based on CLWG clinical staging, 32 dogs (32/38, 84%) were classified as stage C (sick dogs with clinically evident leishmaniasis), and 6 (6/38, 16%) as stage D (severely sick dogs often unresponsive to repeated courses of anti-*Leishmania* drugs). Twenty-eight (28/38, 74%) dogs were alive at the end of the study period and ten (10/38, 26%) had died or had been euthanased. Six of the ten non-surviving dogs (60%) were classified in stage D and 4 (4/10, 40%) in stage C. Median survival time was 400 days (range 2-2160 days). Reasons for death and/or euthanasia included worsening of kidney disease (3/10, 30%), lack of response to therapy (3/10, 30%), acute thrombo-

embolism (1/10, 10%), neurological signs due to myelomalacia likely secondary to severe systemic vasculitis (1/10, 10%) and developing of lymphoma (1/10, 10%) and osteosarcoma (1/10, 10%).

Prognostic factors

The results of logistic regression analysis, to determine factors associated with mortality, are shown in Tables 3 and 4. On simple logistic regression analysis, both breed and age were associated with mortality: compared with mixed breed dogs, pure-breed dogs had increased mortality risk (OR 8.33, 95%-CI 1.49-46.7, *P*=0.016) whilst, compared with dogs <5 years, dogs >10 years had an increased mortality risk (OR 9.5, 1.09-82.72, *P*=0.04). Five other factors were not significantly associated with mortality, but qualified (at *P*<0.2) for inclusion in the initial multiple regression model including: sex, neuter status, hyperproteinaemia, hypoalbuminaemia, and the type of anti-*Leishmania* therapy used (Table 3). None of the remaining factors tested qualified for inclusion in the multiple regression analysis (Table 3).

After the initial model was refined by backwards-stepwise elimination, the best-fit model was one that included two factors, breed and type of anti-*Leishmania* therapy used (Table 4). In this respect, being a pure-breed dog (OR 13.9, 95%-CI 1.2-154.8, *P*=0.033) and the combination of N-methylglucamine antimoniate and allopurinol as anti-*Leishmania* therapy were both positively associated with mortality risk (OR 25.3, 1.03-619.06, *P*=0.048).

Table 3: Results of the simple logistic regression to identify possible prognostic factors

	Odd Ratio (95%-CI)*	*P* value[γ]
Signalment & history:		
Breed (pure vs mixed)	8.33 (1.49-46.7)	0.016
Age (months)	1.02 (1.0-1.04)	0.07
Age groups (vs 1-5 years)		
Age 5-10 years	1.41 (0.2-9.96)	0.73
Age >10 years	9.5 (1.09-82.72)	0.04
Sex (male vs female)	8.00 (0.87-73.27)	0.06
Neuter (neut vs entire)	0.21 (0.02-1.83)	0.16
Weight (per kg)	0.99 (0.89-1.09)	0.80
Travel region (vs Spain/Portugal)		
Greece-Cyprus	1.71 (0.3-9.77)	0.54
Italy-France	0.80 (0.07-0.91)	0.86
Other	2.47×10^{-8}(---)	0.99
Reasons of presentation:		
Lethargy	0.37 (0.07-1.84)	0.22
Dermatological signs	1.64 (0.34-7.91)	0.54
Lameness	5.02×10^{-8} (---)	0.99
Weight loss	1.6 (0.25-10.36)	0.62
Decreased appetite	1.05 (0.17-6.41)	0.96
Gastro-intestinal signs	4.5 (0.52-38.6)	0.17
Nasal discharge/epistaxis	7.82×10^{7} (---)	0.99
Urinary signs/PUPD	2.89 (0.39-21.29)	0.30
Clinical signs at physical examination:		
Alopecia	0.88 (0.14-5.27)	0.88
Crusting/scaling	1.33 (0.26-6.83)	0.73
Lymphadenomegaly	0.45 (0.09-2.21)	0.33
Polyarthritis	5.84×10^{-9} (---)	0.99
Low body condition score	2.08 (0.31-14.17)	0.45
Spleno-hepatomegaly	-18.43 (---)	0.99
Nasal discharge/epistaxis	1.93 (0.15-24.46)	0.61
Pale mucous membranes	1.24 (0.11-13.81)	0.86
Pyrexia	1.55×10^{-8} (---)	0.99
Ocular signs	1.22×10^{-8} (---)	0.99
Laboratory abnormalities:		
Anaemia	1.5 (0.24-9.18)	0.66
Anaemia group (vs. no anaemia)		
Mild anaemia	2.22 (0.33-14.80)	0.41
Moderate anaemia	0.57 (0.04-7.74)	0.67
Thrombocytopenia	0.65 (0.06-7.31)	0.73
Leucocytosis	2.55×10^{8} (---)	0.99
Leucopenia	5.56×10^{-9} (---)	0.99
Neutropenia	1.09×10^{-8} (---)	0.99
Neutrophilia	1.25 (0.10-15.50)	0.86
Lymphopenia	0.67 (0.08-5.75)	0.71
Eosinopenia	7.08×10^{-9}(---)	0.99
Azotaemia	3.77×10^{7} (---)	0.99
Renal azotaemia	3.33 (0.23-49.09)	0.38
Hyperproteinaemia	0.17 (0.02-1.14)	0.07
Hypoalbuminaemia	0.19 (0.03-1.21)	0.08

Hyperglobulinaemia	0.27 (0.01-5.03)	0.38
Low A/G ratio	0.53 (0.04-7.49)	0.64
Hypercalcaemia	7.86×10^{-9} (---)	0.99
Hyperphosphataemia	0.65 (0.06-7.32)	0.73
Hypernatraemia	2.8 (0.14-53.7)	0.49
Hyperkalaemia	1.5 (0.11-20.30)	0.76
Hyperchloraemia	1.73×10^{-8} (---)	0.99
Increased ALP	0.65 (0.06-7.32)	0.73
Increased ALT	0.06 (0.05-6.79)	0.68
Inappropriately low USG	2.14 (0.25-18.50)	0.49
Proteinuria	0.56 (0.07-4.24)	0.58
IRIS stage (vs. stage 1)		
Stage 2	2.03×10^{-8} (---)	0.99
Stage 3	2.12 (0.28-15.97)	0.46
Serology group (vs. mild serology)		
Moderate serology	6.75 (0.52-86.56)	0.14
High serology	0.75 (0.04-13.68)	0.85
Clinical classification (vs. stage C)		
Stage D	0.46 (0.07-3.14)	0.43
Treatment:		
Allopurinol	2.15 (0.33-13.80)	0.42
Allopurinol and miltefosine	19.51 (---)	0.99
Allopurinol and N-M-antimoniate	13 (0.94-178.76)	0.06

[*] 95%-CI= 95% confidence interval, confidence intervals not reported when the range included infinity; [Y] Significance set at *P* <0.05; N-M-antimoniate= N-methylglucamine antimoniate; USG= urine specific gravity

Table 4: Final multiple logistic regression analysis to determine factors associated with mortality

	Odd Ratio (95%-CI)[*]	***P* value [Y]**
Pure vs mixed breed	13.9 (1.2-154.8)	0.033
Allopurinol and N-M-antimoniate vs other treatment	25.3 (1.03-619.06)	0.048

[*] 95%-CI= 95% confidence interval; [Y] significance set at *P* <0.05; N-M-antimoniate= N-methylglucamine antimoniate.

DISCUSSION

In this study data from dogs diagnosed with leishmaniasis in seven different referral centres across the UK are reported. This is the first time that clinical CanL has been described in all its aspects and prognostic factors investigated in a population living in the UK. The prevalence of the disease in this study was low demonstrating that leishmaniasis is relatively uncommon in dogs living in the UK. However, the real prevalence of the disease is likely higher than the current report suggests since no cases from primary practices were included. Furthermore, only dogs with clinical leishmaniasis were considered, with either exposed or infected animals (those having positive results to the diagnostic tests but not showing any clinical and clincopathological abnormalities of the disease) not being considered. It is unpredictable whether those dogs will develop clinical signs in the future.

Unfortunately, in many cases the time-frame between the travel from/to endemic areas and the development of clinical signs was not available. Anyway, it is well known that the time between the infection and the development of the clinical signs (incubation period) can be very variable and mainly dependent to the host's immunologic response (Fisa *et al.* 1999, Cardoso *et al.* 2007).

Similar to previous reports (Shaw *et al.* 2009), most cases were found in southern England. However, caution should be exercised when interpreting this because no all geographical regions across the UK were included in the present study. If cases from the south are genuinely overrepresented, it might be due to easier connections to Europe and warmer weather. With regard to the latter, the climate has recently changed enough to support the transmission and diffusion in these areas of other vector borne diseases (Medlock *et al.* 2007; Wilson *et al.* 2013). However, to date a vector of *L. infantum* has not been found in the UK and sand flies that are introduced in the country by car or plane likely die shortly due to a marked intolerance to temperature changes. In fact, the sand fly's range of activity is between 15° and 28°C in association with high relative humidity and absence of strong rain and winds (Bogdan *et al.* 2001, Killick-Kendrick, 1999, Maroli *et al.* 2013). This does not rule out possible future epidemiological changes due to the ongoing global warming. To date, there is little published information regarding the distribution of the competent sand

fly in Northern Europe and in the UK and how or if it is changing due to the increased dog travel and climate changes. Furthermore, other modes of transmission have been described including blood transfusion (de Freitas *et al.* 2006), vertical transmission from bitches to puppies (Rosypal *et al.* 2005, Pangrazio *et al.* 2009, Boggiatto *et al.* 2011, Naucke *et al.* 2012, Turchetti *et al.* 2014) and venereal transmission (Diniz *et al.* 2006). Dog-to-dog mechanisms have been also hypothesised to explain leishmaniasis outbreaks among foxhounds in the United States and Canada (Duprey *et al.* 2006).

All dogs included in the present study had a history of having travelled to or been imported from a region endemic for *Leishmania infantum*. The majority of dogs were imported (32/38, 84%) versus a minority that has traveled to an endemic country (6/38, 16%). This would suggest a higher risk in adopting a dog from an endemic area respect traveling with the dog to those countries. Travelling dogs usually stay for only a short period time and the overall risk they get infected with *Leishmania* is likely low (Hamel *et al.* 2011). However, veterinarians in non-endemic regions should be aware of CanL, including its non-vectorial transmission modes, and should advise dog owners on preventive measures (Shaw *et al.* 2009, Menn *et al.* 2010). The majority of dogs in the present study had been in Spain, which is compatible with the high prevalence of leishmaniasis in this country (Mattin *et al.* 2014) and its popularity as a destination for holidays. Imported shelter and stray dogs have higher risk to be infected because of decreased preventive measures (Manzillo *et al.* 2006) and greater exposure to sand flies during the evening period of peak of activity. No autochthonous cases were recognised in this study, which contrasts the findings of Shaw *et al.* (2009) who identified 3 positive dogs obtained from UK re-homing centres with no history of travel abroad. It remains questionable if transmission was due to vectors, transplacental or even by direct contact.

The spectrum of clinical signs in the study group of dogs was similar to that reported in endemic areas (Ciaramella *et al.* 1997, Koutinas *et al.* 1999, Paltrinieri *et al.* 2010, Solano-Gallego *et al.* 2011), with dermatological signs and lymphadenopathy being the most frequent findings. Polyarthritis was present in 9 dogs (24%), similarly to previously published work from the UK (Shaw *et al.* 2009) (17%). However, this finding might be biased by the fact that only cases from referral centres were

considered. That said, in the study from Geisweid *et al.* (2013), which included dogs diagnosed with leishmaniasis at a referral university in Germany, polyarthritis was recognised only in a 6% of the study population. Why dogs with leishmaniasis in the UK seem to develop polyarthritis more frequently than in other non-endemic regions is difficult to explain, and further studies may be required. Mild-to-moderate non-regenerative anaemia, thrombocytopenia, azotaemia, hypoalbuminaemia, hyperglobulinaemia and proteinuria were common clinicopathologic abnormalities, similar to what has previously been described in dogs in endemic areas (Ciaramella *et al*. 1997, Koutinas *et al.* 1999, Paltrinieri *et al.* 2010, Solano-Gallego *et al.* 2011). Given that non-pathognomonic clinical signs and laboratory abnormalities, as well as the low familiarity with the disease of the veterinary surgeons in the UK, more than one test was used to confirm the final diagnosis in the majority of cases. Furthermore, serum protein electrophoresis was included in the initial diagnostic investigation and in follow-up rechecks only in a very low number of cases. However, this test can provide important information, especially during reassessment, because improvement or normalisation of the protein electrophoresis trace generally happens before a negative serology titre occurs.

The majority of dogs were treated with allopurinol alone, most likely because N-methylglucamine antimoniate is not available in the UK and must be imported and miltefosine requires a "special treatment certificate" (STC). In light of this, it is noteworthy that the overall outcome was good with a reasonable survival time. Furthermore, it should be considered that only dogs with moderate-to-severe disease (stages C and D) were included in the study and that these animals are known to have a guarded-to-poor prognosis (Solano-Gallego *et al.* 2011; Roura *et al.* 2013). This finding is, perhaps, best explained by the minimal chance of re-infection given the geographical location, and low risk of having a concurrent vector-borne disease. The latter cannot be completely ruled out in this study population since only three dogs were tested for other vector-borne diseases. In this respect, response to CanL is known to be influenced by both concurrent disease and immunological stimulation or suppression by shifting the balance from a protective Th1 response to a Th2 immune response that favours the development of a non-protective and possibly detrimental humoral reaction (Koutinas *et al.* 2014). Moreover, it has been

recently demonstrated that “T cell exhaustion”, defined as antigen-specific effector T cell dysfunction, is associated with increasing symptomatology and reduction of the efficacy of vaccination and therapeutic strategies (Esch *et al.* 2013). Where additional anti-*Leishmania* drugs were used, miltefosine was more frequently used than N-methylglucamine antimoniate, probably because it is an oral solution and easier to administer. In contrast, N-methylglucamine antimoniate must be injected subcutaneously, and can often be associated with localised pain and inflammation. In the study population, the combination of allopurinol and N-methylglucamine antimoniate was associated with an increased mortality risk, which is probably because this option was selected for more severe cases, rather than because it was less effective. Moreover, only three dogs were treated with this therapeutic combination, making the correlation with mortality statistically weak. A recent study (Miró *et al. 2009)* compared the effectiveness and safety of miltefosine plus allopurinol to N-methylglucamine antimoniate plus allopurinol and found that both therapeutic associations were similarly effective in improving clinical signs and reducing the parasite load. Currently, both associations are recommended as standard therapy for CanL (Oliva *et al.* 2010, Solano-Gallego *et al.* 2011, Roura *et al.* 2013, Noli *et al.* 2014).

Some dogs also received other drugs according to the attending clinician’s decision. It is unlikely that these drugs interfered with the anti-*Leishmania* therapy and influenced outcome since they were mainly used as supportive and/or symptomatic therapies (gastro-protectants, anti-emetics, anti-hypertensive etc.).

In this study, mortality risk was positively associated with being a pure-breed dog. Previous work has demonstrated that susceptibility and resistance to CanL can be influenced by genetics, with Boxers, Cocker Spaniels, Rottweilers, and German shepherd dogs being more susceptible to CanL than Ibizan Hounds where clinical disease is rare. This is because the Ibizan Hound responds to infection predominantly with a cell-mediated immune response, and this makes it relatively resistant (Solano-Gallego *et al.* 2000).

Serology titre at diagnosis did not correlate with outcome in the current study. The prognostic value of the serology titer in non-endemic areas seems controversial at the moment since different results have been obtained so far. Geisweid *et al. (*2014)

did not found serology titer an useful prognostic factor in Germany while Bogiatto *et al.* (2010) showed that production of anti-*Leishmania* antibodies are key immunologic features of disease manifestation and progression in a canine population from the USA. It is possible that the lack of re-infection and super-infection in non-endemic regions cause less intense immune system stimulation and consequent lower serology titres despite presence of severe clinical signs.

IRIS staging for CKD did not influence outcome in the present study. However, there were only small numbers classified into each stage, and no dogs were classified as stage 4. Thus, this finding should be interpreted with caution given the possibility of a type II statistical error. Despite the lack of prognostic significance of kidney disease, most non-surviving dogs experienced a worsening of kidney disease. It is recognised that advanced renal failure is the major cause of death and/or euthanasia in CanL (Panellas *et al.* 2009). Further studies evaluating IRIS staging in a bigger population and also in patients already on therapy could be of higher prognostic value. Finally, all dogs in clinical stage D died or were euthanased. Currently clinical staging at time of diagnosis and periodic re-classification in line with disease progression and regression is considered a useful way to predict outcome (Solano-Gallego *et al.* 2009, Oliva *et al.* 2010, Paltrinieri *et al.* 2010).

In conclusion, although rare, veterinary surgeons in the UK should consider CanL in patients with a history of travel to or from endemic areas, where there are compatible clinical signs and clinicopathological abnormalities. As *Leishmania* infections are known to have a long incubation period, practitioners should inform the owners of imported dogs to retest them for *Leishmania* for at least two years after importation or in case of a clinical suspicion (Paltrinieri *et al.* 2010). Moreover, veterinarians should be aware of non-vectorial transmission ways, and should advice clients on preventive measures before travelling to endemic countries. An early diagnosis and appropriate therapy can be associated with a relatively good control of the disease. At the same time, clients should be informed of a possible more guarded to poor prognosis in case of pure-breed dogs and if classified in clinical stage D.

6. GENERAL DISCUSSION

CanL is a major zoonosis that potentially can cause severe or even fatal disease in humans and dogs. Infections caused by different *Leishmania* species are present in a variety of regions with different climatic conditions throughout the world. Millions of dogs are infected in endemic areas such as parts of South America and the Mediterranean basin. However, as documented in the third study of this thesis, CanL has become more apparent in northern latitudes within Europe, especially due to the increasing number of dogs travelling to or imported from endemic areas. In our study, the majority of dogs were imported versus a minority that had travelled to an endemic country, suggesting a higher risk in adopting a dog from an endemic area compared to travelling with the dog to those countries. However, veterinarians in non-endemic regions should advise dog owners on preventive measures before any travel to endemic regions. As the domestic dog is the main reservoir host of *Leishmania infantum*, increased movement of infected dogs across countries is contributing to the changing epidemiology of the disease. This highlights the importance for a deeper knowledge of the disease in endemic regions but also in those countries generally considered as non-endemic. In the last decade a considerable number of studies about CanL have been published and have contributed to our understanding of different aspects of this disease, including alternative methods of transmission and the pathologic mechanisms underlying the clinical findings. In particular, the establishment of the CLWG and the LeishVet has provided an evidence-based consensus and useful guidelines for a correct diagnostic, therapeutic and preventive approach to the disease. The clinical classification systems have clarified the difference between “infected” and “diseased” patients and standardised their respective management. However, CanL still remains a very challenging disease to diagnose, treat and prevent and will probably continue to cause concerns to clinicians. To date, in fact, there is still an important lack of an effective, safe and permanent treatment, of a universally recognised protocol for follow-up, of a totally effective preventive system and of prognostic factors for predicting the outcome. For this reason, the main aims of the present thesis were to investigate new clinico-pathological aspects of CanL and to possibly identify useful prognostic factors. Since the geographical distribution of the disease is progressively changing, the investigations were conducted in two Mediterranean countries, Spain

and Italy, and also in the UK, considered a non-endemic region. The first study interestingly demonstrated that a significant proportion of dogs with leishmaniasis have increased serum cTnI concentration, indicating some degree of myocardial damage. Before this study, only isolated case reports described the possible cardiac involvement in CanL. Mycocardial injury due to *Leishmania* infection is well recognised and described in human medicine. Since cTnI is a very sensitive and specific marker of myocardial injury, dogs with leishmaniasis should be considered at risk of developing cardiac diseases, mainly myo- and endocarditis. Our study represents the beginning of an interesting area of research that has recently culminated in the publication of a few papers describing the cardiac lesions caused by *Leishmania* infection. In particular, Rosa *et al.* (2013) evaluated histologically and immunohistochemically the hearts of 30 dogs naturally infected with *Leishmania infantum* and found that myocardial lesions were present in all dogs, including lymphoplasmacytic myocarditis, myonecrosis, increased interstitial collagen, lepromatous-type granulomatous myocarditis, fibrinoid vascular change, and vasculitis. The parasite was detected in the heart of 20 of 30 dogs and the number of parasitised cells correlated with the intensity of the inflammation and with the number of granulomas. These findings indicate that cardiac lesions are prevalent in dogs with leishmaniasis even in the absence of overt clinical signs of cardiac disease. According to these results, it is reasonable to consider that CanL can cause cardiac disease but its significance and contribution to the general clinical presentation of the patients need further studies. In particular, a prospective study including dogs with leishmaniasis that undergo a detailed cardiological examination (such as ECG, echocardiography and evaluation of cardiac biomarkers) at time of diagnosis and during the follow-up would be very helpful in clarifying the role of CanL in cardiac diseases, in considering additional diagnostic tests and procedures (e.g. ECG, echocardiography) in the routine management of the patients and in identifying possible new prognostic factors (e.g. cTnI). The latter was the main aim of the second study, in which, for the first time, iron status and its relationship with CRP was investigated in CanL. The results indicated that dogs with leishmaniasis have decreased serum iron, TIBC, UBIC and percentage of transferrin saturation and increased concentrations of ferritin, reflecting an intense inflammatory state.

Moreover, increased CRP and decreased TIBC are risk factors for mortality in CanL and future studies could also investigate the use of CRP as a marker of response to therapy during the follow-up of patients with leishmaniasis. The intense inflammatory state is likely the major cause of many of the clinical and clinico-pathological findings typical of the disease as non-regenerative anaemia. The negative correlation between CRP and iron and transferrin and its positive correlation with ferritin support that in CanL inflammation contributes to the status of iron unavailability, which may in turn contribute to the anaemia of inflammatory disease. During inflammation hepcidin expression is increased and plays a pivotal role as an inhibitory regulator of iron metabolism. Binding to the cell surface iron efflux protein, ferroportin, and inducing its internalisation and degradation, hepcidin inhibits absorption of dietary iron from intestinal epithelium and export of iron from macrophages and hepatocytes. A future study will evaluate the concentration and possibly the prognostic value of hepcidin in dogs with leishmaniasis. Interestingly, when the iron profile was studied in dogs stratified according to CRP concentrations and so with similar degrees of inflammation, the iron status alterations were still more pronounced in patients with CanL compared to those with diseases other than leishmaniasis. This suggests that inflammation alone does not completely explain the reason for the altered iron profile in CanL. Previous studies (Wilson *et al.* 1994; Soteriadou *et al.* 1995; Mauel *et al.* 1991) demonstrated that promastigotes in the first hours after infection of a mammalian host need the uptake of iron and that their growth is inhibited in an iron-deficient environment, and the addition of iron salts to incubation fluids may prevent the killing of intracellular amastigotes by activated macrophages. More recently Huynh & Andrews (2008) indentified a plasma membrane *Leishmania* transporter, LIT1, that allows the uptake of ferrous iron and that plays a critical role in intracellular growth and virulence of the protozoa. All these findings together with the results of our study support the hypothesis that iron depletion is in part due to the consumption of the element by the parasite for its growth, and iron is possibly sequestered by the organism as a defence mechanism against the parasite. Future studies will further evaluate this aspect of *Leishmania* infection especially focusing on its possible value in the management and therapy of the disease. Similarly, the main objective of the third

study was to identify useful prognostic factors in a population of dogs affected by leishmaniasis living in a non-endemic area such as the UK. Mortality risk was positively associated with being a pure-breed dog and being classified in stage D. Previous studies have demonstrated that susceptibility and resistance to CanL can be influenced by genetics, with Boxers, Cocker Spaniels, Rottweilers, and German shepherd dogs being more susceptible to CanL than Ibizan Hounds where clinical disease is rare due to their predominant cell-mediated immune response to the infection (Solano-Gallego *et al.* 2000). All dogs in clinical stage D died or were euthanased. Clinical staging at time of diagnosis and periodic re-classification are useful ways to choose the most appropriate therapy and especially to predict outcome. Serology titre at diagnosis and IRIS staging for CKD did not correlate with outcome. It is possible that the lack of re-infection and super-infection in non-endemic regions causes less intense immune system stimulation and consequently lower serology titres despite the presence of severe clinical signs. Despite the lack of prognostic significance of the IRIS classification, most non-surviving dogs experienced a worsening of kidney disease. It is recognised that advanced renal failure is the major cause of death and/or euthanasia in CanL (Panellas *et al.* 2009). Further studies evaluating IRIS staging at time of diagnosis and in patients already on therapy could provide more information on its prognostic value.

In conclusion, in this thesis interesting new aspects of *Leishmania* infection such as its effects on the myocardium and on iron status were for the first time investigated in dogs and useful prognostic factors as CRP and TIBC, and being a pure breed dog and being classified in stage D were identified in dogs with clinical leishmaniasis in endemic and non-endemic areas, respectively.

7. CONCLUSIONS

1. A significant proportion of dogs with leishmaniasis have increased serum cTnI concentration, indicative of some degree of cardiomyocyte injury.

2. Dogs with leishmaniasis have lower iron and TIBC and higher ferritin and CRP;

3. Inflammation contributes to the iron status alterations found in CanL; however, decreased iron and increased ferritin may also reflect other specific mechanisms implicated in intracellular infection.

4. Increased CRP and decreased TIBC are risk factors for a worse outcome in dogs with CanL.

5. Although rare, canine leishmaniasis should be considered in non-endemic areas such as the UK in dogs with compatible clinical signs and clinico-pathological abnormalities and with a history of travel from/to endemic areas.

6. Adopting a dog from an endemic area seems to be a higher risk than traveling with a dog to endemic countries.

7. Pure-breed dogs and those classified in stage D at time of diagnosis are at higher risk of death.

8. Clinical classification at diagnosis is a useful prognostic factor.

9. In a non-endemic area such as the UK, serology titre at diagnosis is not correlated with outcome.

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APPENDIX 1

Publications from work carried out during this thesis

APPENDIX 2

List of Abbreviations

A/G Albumin:globulin ratio

aPTT Activated partial thromboplastin

AT Antithrombin III

CanL Canine Leishmaniasis

CBC Complete blood cell count

CD Cluster of differentiation

CI Confidence interval

CICs Circulating immune complexes

CKD Chronic kidney disease

CLWG Canine Leishmaniasis Working Group

CSF Cerebrospinal fluid

CTnI Cardiac troponin I

CV Coefficient of variation

DIC Disseminated intravascular coagulation

ELISA Enzyme-linked immunosorbent assay

EU Elisa Units

FDPs Fibrin/fibrinogen degradation products

Hct Haematocrit

HL Human Leishmaniasis

HCL Human Cutaneousl Leishamanisis

HVL Human Visceral Leishmaniasis

IFAT Immunofluorescence antibody test

IFN Interferon

Ig Immunoglobulin

IL Interlukin

IMHA Immune-mediated haemolytic anaemia

IRIS International Renal Interest Society

MCHC Mean corpuscular heaemoglobin concentration

MCV Mean cell volume

NK Natural killer

OR Odds ratios

PCR Polymerase chain reaction

PCV Packed cell volume

PETS Pet travel scheme

PLTs Platelets

PT Prothrombin time

PU/PD Polyuria/polydipsia

RBCs Red blood cells

rtq-PCR Real time quantitative PCR

SAA Serum amyloid A

SDS-age SDS-agarose gel electrophoresis

S.G. Specific gravity

Th T-helper-type

TIBC Total iron-binging capacity

TNF Tumour necrosis factor

TP Total protein

UIBC Unsaturated iron-binding capacity

UPC Urine protein:creatinine ratio

URL Upper reference limit

UK United Kingdom

PAPER

Iron status and C-reactive protein in canine leishmaniasis

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Objective: To investigate the iron status, its relationship with C-reactive protein and the prognostic value of both in canine leishmaniasis.

Method: Eighty-six dogs with leishmaniasis and two control groups (healthy dogs and dogs with diseases other than leishmaniasis) were selected. Iron status indicators and C-reactive protein were compared between the three groups. Correlations between C-reactive protein and iron, ferritin and total iron-binding capacity were evaluated in dogs with leishmaniasis. Iron, total iron-binding capacity and ferritin were compared between dogs stratified according to similar C-reactive protein concentrations. The mortality rate at 30 days post-diagnosis was compared between groups. Iron status indicators and C-reactive protein were compared between survivors and non-survivors.

Results: Dogs with leishmaniasis had lower iron and total iron-binding capacity and higher ferritin and C-reactive protein. There was a significant but low correlation of C-reactive protein with iron, ferritin and total iron-binding capacity. Dogs with leishmaniasis had decreased iron and total iron-binding capacity and increased ferritin compared to other ill patients with similar C-reactive protein concentrations. Mortality was not significantly different between groups but non-survivor dogs with leishmaniasis had higher C-reactive protein and lower total iron-binding capacity.

Clinical Significance: Inflammation contributes to the iron status alterations found in canine leishmaniasis but other mechanisms are likely involved. Low total iron-binding capacity and increased C-reactive protein are risk factors for outcome in canine leishmaniasis.

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INTRODUCTION

Canine leishmaniasis (CanL) is endemic in more than 70 countries worldwide (Solano-Gallego *et al.* 2011) and the infection is spreading to non-endemic areas with an increasing number of cases reported in dogs living in Northern Europe (Shaw *et al.* 2009).

The disease is manifested by a broad spectrum of clinical signs and degree of severity. Sick dogs usually have generalised lymphadenopathy, weight loss, skin and ocular lesions, lameness, epistaxis, mucous membrane pallor, systemic vasculitis and renal dysfunction that can progress from mild proteinuria to nephrotic syndrome and end-stage kidney disease. Immune-complex formation and deposition and consequently an intense systemic inflammatory response are pivotal in developing clinical signs (Alvar *et al.* 2004). A marked increase in acute phase proteins including haptoglobin, C-reactive protein (CRP) and ceruloplasmin has been demonstrated in dogs infected with *Leishmania* (Martínez-Subiela *et al.* 2002). A recent study (Martínez-Subiela *et al.* 2011) has also suggested that acute phase proteins could be used as an early marker for CanL as well as for monitoring the response to treatment. One of the potential effects of inflammation is a relative iron deficiency in both the transport and functional pools, which limits availability of iron for erythropoiesis

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(McCown & Specht 2011). A mild to moderate non-regenerative anaemia is common in dogs with leishmaniasis. Only a few studies have previously addressed iron status in dogs with leishmaniasis (Liste *et al.* 1994, Adamama-Moraitou *et al.* 2005) but none has investigated the prognostic value of such variables.

The objective of this study was to evaluate the iron status in CanL and its relationship with haematological variables and CRP, considered a sensitive marker of inflammation, as well as to assess the prognostic value of iron status and CRP in dogs with leishmaniasis.

MATERIALS AND METHODS

Dogs diagnosed with clinical leishmaniasis at the San Marco Veterinary Clinic of Padua, Italy, between November 2008 and December 2011 were retrospectively included in the study. Diagnosis of CanL was based on clinical signs, laboratory findings, serological status and positive real time quantitative polymerase chain reaction (rtq-PCR) on bone marrow samples. These dogs were included in CanL group and classified as stages C and D according to the Canine Leishmaniasis Working Group guidelines (Paltrinieri *et al.* 2010). Two groups of control dogs with no clinical signs or clinicopathological findings compatible with leishmaniasis were included in the study. These dogs were individually matched to the CanL group for age (±six months), sex and breed. When a breed match of the same age and sex of a CanL dog was not found in the data base, a dog with similar weight (±5 kg) was chosen instead. These dogs were selected as closely as possible to the admission date of the corresponding dogs of the CanL group to reduce variations in results of laboratory analyses attributable to changes in performance of analysers. When two or more dogs met these criteria, the dog included in the group was selected via a randomisation procedure with a computer system. Control group 1 included healthy patients with unremarkable physical examination, presented for annual vaccination, blood donation or elective surgery. All blood donor dogs had negative serology for the following vector-borne pathogens: *Ehrlichia canis*, *Anaplasma phagocytophilum*, *Babesia species*, *Dirofilaria immitis* and *Leishmania infantum*. Control group 2 included sick dogs with a clinical diagnosis other than leishmaniasis. These dogs did not present with clinical signs and/or clinicopathological abnormalities suggestive of this disease and specific tests (serology, cytology of lymph nodes) were performed when leishmaniasis was still included in the differential diagnosis. All tested dogs of control group 2 had negative results.

Only dogs with a complete medical record, including history and results of physical examination, complete blood count, serum biochemistry analysis, including iron profile and CRP, and urinalysis were included in the study.

Owner consent was obtained for all the dogs included in the study.

For serology, an enzyme-linked immunosorbent assay (ELISA) test for *Leishmania* was performed as previously described (Riera *et al.* 1999). Results were quantified as units related to positive serum used as a calibrator and arbitrarily set at 100 ELISA Units (EU). The positive threshold value previously established by the laboratory was 35 EU (mean ±4 sd of 32 dogs from non-endemic areas). Results classified as high positive corresponded to more than 150 EU.

Detection and quantification of *Leishmania* kinetoplastic DNA was performed on costochondral junction bone marrow samples by rtq-PCR as previously described (Caldin *et al.* 2004, Solano-Gallego *et al.* 2007).

The iron profile included serum iron, total iron-binding capacity (TIBC, which is an indirect measure of the total transferrin concentration), unsaturated iron-binding capacity (UIBC), percentage of transferrin saturation and serum ferritin. Serum iron (Iron OSR6186, Beckman Coulter) UIBC (UIBC OSR6124, Beckman Coulter) and ferritin (Tina-quant Ferritin Gen.4, Roche Diagnostic GmbH) concentrations were determined via quantitative assays with an automated analyser (Olympus AU 2700, Olympus diagnostics). TIBC was calculated by adding iron concentration to UIBC, and the percentage of transferrin saturation was calculated as the ratio between serum iron and TIBC. Ferritin assays in dogs have been previously validated for precision, linearity and recovery percentage (Caldin *et al.* 1999).

Serum CRP concentration was measured using an immunoturbidimetric assay for human (CRP OSR6147, Olympus Life and Material Science Europe GmbH) use for which results correlated well (r=0·98) with those of a previously validated canine-specific ELISA (Tridelta Phase range canine CRP Kit, Tridelta Development Ltd.) (Martínez-Subiela & Cerón 2005, Caldin *et al.* 2009).

A full blood count was performed in every animal using an automated haematology analyser (ADVIA 120, Siemens Healthcare Diagnostics), and included data regarding calculated haematocrit (Hct), measured mean corpuscular volume (MCV), and calculated mean corpuscular haemoglobin concentration (MCHC). Anaemia, microcytosis and hypochromia were defined by values below the lower limit of the reference intervals established by the laboratory for Hct (37·0 to 59·2 %), MCV (63·1 to 72·6 fL) and MCHC (33·3 to 36·8 g/dL).

Iron, TIBC, UIBC, percentage of transferrin saturation, ferritin and CRP were compared between the three groups of dogs. Correlations between CRP and iron, CRP and TIBC, and CRP and ferritin were calculated only in dogs with leishmaniasis to investigate if inflammation was potentially associated with iron profile alterations. In addition, to assess if abnormalities in serum iron, TIBC and ferritin concentrations were due only to inflammation, dogs were stratified according to increasing magnitude of CRP values. A first comparison was made between dogs of all three groups with a CRP concentration between 0·1 and 22·2 mg/L (0 to 10 times increase above the upper limit of the laboratory reference interval: 0·1 to 2·2 mg/L). A second comparison was made considering only dogs in the CanL group and group 2 with CRP concentrations between 22·3 and 66·9 mg/L (10 to 30 times increase above the upper limit of the laboratory reference interval). Group 1 was not included in this analysis because no healthy dogs had such values of serum CRP.

Hct, MCV and MCHC, in addition to the proportion of anaemic, microcytic and hypochromic dogs were compared

between dogs in stages C and D of the CanL group and between this group and group 2.

The correlation between Hct and the iron status parameters was also calculated within the stages C and D of the CanL group. Iron status variables were compared between anaemic and non-anaemic dogs within the stages C and D of the CanL group.

Finally, the mortality rate at 30 days post-diagnosis was compared between the CanL group and control group 2, and in the CanL group iron, TIBC, ferritin and CRP were also compared between survivors and non-survivors.

Statistical analysis

Data were tested for normality using the Shapiro-Wilk test. Data that did not follow a normal distribution were analysed by nonparametric methods. Data are reported as median or mean and range (minimum to maximum). Kruskall-Wallis test with Bonferroni correction for post-hoc comparisons was used when comparing variables between more than two groups. When comparing only two independent groups, an unpaired *t*-test and a Mann-Whitney U test were used for data normally and non-normally distributed, respectively. Fisher's exact test was used to compare categorical data. Spearman's rho (ρ) or Kendall's tau (τ) rank correlation test was used to assess the statistical dependence between two variables nonparametrically distributed. Statistical significance was set at P<0·05 for all analyses. Statistical analysis was performed using the statistical softwares Analyse-it®(Software Ltd.) and Stats Direct® (version 2.7.9, Stats Direct Ltd.).

RESULTS

Case material

The CanL group (n=86) included 48 males (56%) and 38 females (44%), with a median age of six years (1 to 13·5 years). The majority of dogs (30 of 86; 35%) were mixed breed dogs. According to the Canine Leishmaniasis Working Group guidelines (Paltrinieri *et al.* 2010), 57 (66%) dogs were classified as Stage C (sick dogs with clinically evident leishmaniasis) and 29 (34%) as Stage D (severely sick dogs).

The control group 2 included sick dogs diagnosed with different diseases including chronic valvular heart disease (n=6), chronic kidney disease (n=4), chronic hepatitis (n=7), neoplasia [multicentric lymphoma (n=7); multiple myeloma (n=3); osteosarcoma (n=1)], chronic diarrhoea [inflammatory bowel disease (n=16); antibiotic-responsive diarrhoea (n=9); alimentary lymphoma (n=2)], chronic regurgitation secondary to idiopathic megaoesophagus (n=2), endocrine diseases [diabetes mellitus (n=6); hyperadrenocorticism (n=9); hypothyroidism (n=6)] and primary immune-mediated haemolytic anaemia (IMHA) (n=8). Dogs with chronic kidney disease and IMHA were tested for *L. infantum* as well as for other pathogens, including *E. canis, A. phagocytophilum, Babesia species, D. immitis* and *Leptospira species*. All these dogs had negative results for those tests.

Comparisons

Dogs with leishmaniasis showed significantly lower iron and TIBC serum concentrations, and significantly higher ferritin and CRP concentrations compared to both control groups. UIBC and percentage of transferrin saturation of dogs with leishmaniasis were significantly lower only compared to control group 1 (Table 1).

Within the CanL group, serum CRP had a low negative correlation with iron (P<0·0001, ρ=–0·46), a moderate negative correlation with TIBC (P<0·0001; ρ=–0·663) and a low positive correlation with ferritin (P=0·008, ρ=0·28). When serum iron, TIBC and ferritin were compared between the three groups only considering dogs with CRP concentrations between 0·1 and 22·2 mg/L (CanL group n=40, group 1 n=40, group 2 n=40), patients with CanL still showed significantly lower iron concentrations and higher ferritin serum concentrations compared to control groups. Lower concentrations of TIBC were found in the CanL group but without statistical significance. When serum iron, TIBC and ferritin were compared in dogs with CRP concentrations between 22·3 and 66·9 g/L (CanL group n=22, group 2 n=22), patients with leishmaniasis showed again significantly lower iron and TIBC, and higher ferritin concentrations compared to control group 2 (Tables 2 and 3).

Sixty percent (52 of 86) of dogs with leishmaniasis and 80% (16 of 86) of dogs of group 2 were anaemic. Dogs with leishmaniasis in both stages C and D had a significantly (P<0·001) lower Hct (stage C: median, 38·8%; range, 11·9 to 55·9%; stage D: median, 31·35%; range, 15·6 to 60·3%) and higher proportion of anaemic dogs (stage C: 49%; stage D: 82%) compared to group 2 (Hct: median, 46·9%; range, 16·8 to 60·4%; anaemic

Table 1. Results of the comparison of iron status variables and C-reactive protein (CRP) between dogs with leishmaniasis (CanL group), healthy dogs (Control group 1) and dogs with diseases other than leishmaniasis (Control group 2)

Variable	RI	CanL group (n=86) Median (range)	Control group 1 (n=86) Median (range)	Control group 2 (n=86) Median (range)	P value
Iron (µmol/L)	17 to 40	16*** (3 to 48)	24 (14 to 53)	22 (3 to 57)	<0·0001
TIBC (µmol/L)	57 to 86	52*** (15 to 87)	67 (51 to 105)	63 (32 to 91)	<0·0001
UIBC (µmol/L)	33 to 55	36* (3 to 68)	42 (20 to 80)	43 (3 to 70)	0·0181
% Saturation	28·2 to 56·8	29·1** (4·8 to 37·02)	36·55 (20·8 to 45·72)	32·45 (4·5 to 45·05)	0·0009
Ferritin (pmol/L)	191 to 643	1270*** (16 to 6262)	404 (175 to 512)	389 (92 to 5795)	<0·0001
CRP (mg/L)	0·1 to 2·2	27·5*** (0·1 to 268·5)	0·6 (0·1 to 6·4)	4·9† (0·1 to 225·4)	<0·0001

RI reference intervals, TIBC total iron-binding capacity, UIBC unsaturated iron binding capacity
Kruskall-Wallis test with Bonferroni correction for post-hoc comparisons. P values less than 0·05 are considered statistically significant. RI established by the laboratory are also indicated
*Significant difference only between CanL group and Control group 1, P=0·027
**Significant difference only between CanL group and Control group 1, P=0·0005
***Significant difference between CanL group and both Control groups, P<0·0001
†Significant difference between Control group 1 and Control group 2, P<0·0001

Table 2. Results of the comparison of iron, total iron binding capacity (TIBC) and ferritin between dogs with leishmaniasis (CanL group), healthy dogs (Control group 1) and dogs with diseases other than leishmaniasis (Control group 2)

Variable	RI	CanL group (n=40) Median (range)	Control group 1 (n=40) Median (range)	Control group 2 (n=40) Median (range)	P value
Iron (µmol/L)	17 to 40	20*,** (3 to 48)	24 (14 to 51)	25 (15 to 53)	0·0001
TIBC (µmol/L)	57 to 86	64 (44 to 87)	66 (56 to 105)	70 (32 to 96)	0·0803
Ferritin (pmol/L)	191 to 643	1049***,† (88 to 5689)	409 (187 to 863)	418 (92 to 2622)	<0·0001

Kruskall-Wallis test with Bonferroni correction for post-hoc comparisons. P values less than 0·05 are considered statistically significant. All dogs had C- reactive protein (CRP) concentrations between 0·1 and 22·2 mg/L (0 to 10 times the upper reference limit). Reference intervals (RI) established by the laboratory are also indicated
* Significant difference between CanL group and Control group 1 *P= 0·0008
** Significant difference between CanL group and Control group 2 §P= 0·0003
*** Significant difference between CanL group and Control group 1 **P< 0·0001
†Significant difference between CanL group and Control group 2 †P= 0·0004

Table 3. Results of the comparison of iron, total iron binding capacity (TIBC) and ferritin between dogs with leishmaniasis (CanL group) and dogs with diseases other than leishmaniasis (Control group 2)

	RI	CanL group (n=22) Median (range)	Control group 2 (n=22) Median (range)	P value
Iron (µmol/L)	17 to 40	13 (8 to 26)	22 (11 to 53)	<0·0001
TIBC (µmol/L)	57 to 86	47 (15 to 87)	70 (36 to 96)	<0·0001
Ferritin (pmol/L)	191 to 643	1766 (256 to 5224)	353 (117 to 533)	<0·0001

Kruskall-Wallis test with Bonferroni correction for post-hoc comparisons. P values less than 0·05 are considered statistically significant. All dogs had C-reactive protein (CRP) concentrations between 22·3 and 66·9 mg/L (10 to 30 times the upper reference limit). Reference intervals (RI) established by the laboratory are also indicated

Table 5. Iron status variables compared between anaemic and non-anaemic dogs in stage C

Variable	RI	Anaemic (n=29) Median (range)	Non-anaemic (n=28) Median (range)	P value
Iron (µmol/L)	17 to 40	13 (3 to 48)	20 (10 to 44)	0·0007
TIBC (µmol/L)	57 to 86	45 (21 to 87)	67 (46 to 87)	<0·0001
UIBC (µmol/L)	33 to 55	33 (4 to 66)	46 (27 to 68)	0·0006
% Saturation	28·2 to 56·8	25·1 (4·8 to 82·4)	29·75 (17·8 to 60·8)	0·3152
Ferritin (pmol/L)	191 to 643	1270 (88 to 3797)	850 (303 to 5689)	0·0797

Normality rejected using Shapiro-Wilk test. Mann-Whitney U test used for comparisons
P values less than 0·05 are considered statistically significant. Reference intervals (RI) established by the laboratory are also indicated

Table 6. Iron status variables compared between anaemic and non-anaemic dogs in stage D

Variable	RI	Anaemic (n=23) Median (range)	Non-anaemic (n=5) Median (range)	P value
Iron (µmol/L)	17 to 40	13 (3 to 22)	24 (13 to 32)	0·0126
TIBC (µmol/L)	57 to 86	40 (15 to 64)	45 (29 to 87)	0·4095
UIBC (µmol/L)	33 to 55	27 (3 to 53)	21 (13 to 55)	0·8232
% Saturation	28·2 to 56·8	29·1 (10·7 to 84·1)	53·5 (26·9 to 58·8)	0·15
Ferritin (pmol/L)	191 to 643	2110 (16 to 6262)	1894 (667 to 3278)	0·0694

Normality rejected using Shapiro-Wilk test. Mann-Whitney U test used for comparisons
P values less than 0·05 are considered statistically significant. Reference intervals (RI) established by the laboratory are also indicated

dogs: 32%). Frequency of anaemia was higher and mean Hct was lower in dogs in stage D compared to dogs in stage C but there were no differences in the means of MCHC and MCV as well as in the number of dogs with microcytosis and/or hypochromia between the two stages (Table 4).

Within stage C, a statistically significant (P<0·05) positive correlation was found between Hct and TIBC (τ=0·41), UIBC (τ=0·26), iron (τ=0·40), and % of saturation (τ=0·18). A negative correlation was found between Hct and ferritin (τ=–0·26; P<0·005). No significant correlations were found between Hct and iron status variables within stage D. Anaemic dogs in stage C had significantly lower TIBC, UIBC and total iron compared to non-anaemic dogs in the same stage (Table 5). Anaemic dogs in stage D had only significantly lower total iron concentration compared to non-anaemic dogs (Table 6).

Finally, 6 of 86 (6·9%) dogs of the CanL group and 1 of 86 (1·2%) of the control group 2 died at 30 days post-diagnosis. The test failed to find a statistically significant difference in the number of survivors between the two groups (P=0·05). Reasons for death in dogs with leishmaniasis were generally correlated with worsening of renal function and severe azotaemia (data not shown). Non-survivor dogs with leishmaniasis had significantly

Table 4. Results of the comparison of haematological variables (Hct, MCV, MCHC) and frequency of anaemic, microcytic and hypochromic dogs between stages C and D.

Variable	RI	Stage C (n=57)	Stage D (n=29)	P value
Hct (%) Mean (range)	37·0 to 59·2	37·66 (11·9 to 55·9)	31·35 (15·6 to 60·3)	0·0127
MCV (fL) Mean (range)	63·1 to 72·6	67·18 (57·1 to 80·4)	67·43 (55·9 to 77·6)	0·8481
MCHC (g/dL) Mean (range)	33·3 to 36·8	32·89 (27·9 to 37·2)	32·51 (27·9 to 37)	0·4381
Frequency of anaemic (%)		28 (49%)	23 (82%)	0·0085
Frequency of microcytic (%)		8 (14%)	6 (20%)	0·406
Frequency of hypochromic (%)		28 (49%)	19 (65%)	0·252

Normality not rejected using Shapiro-Wilk test. Unpaired *t*-test used for comparisons
P values < 0·05 are considered statistically significant. Reference intervals (RI) established by the laboratory are also indicated

Table 7. Results of the comparison of iron, total iron binding capacity (TIBC), ferritin and C-reactive protein (CRP) between survivor and non-survivor dogs with leishmaniasis

	RI	Survivors (n=80) Median (range)	Non-survivors (n=6) Median (range)	P value
Iron (µmol/L)	17 to 40	16 (3 to 48)	12 (8 to 20)	0·2035
TIBC (µmol/L)	57 to 86	54 (20 to 87)	27 (15 to 64)	0·004
Ferritin (pmol/L)	191 to 643	1222 (16 to 5689)	2090 (1067 to 6262)	0·1031
CRP (mg/L)	0·1 to 2·2	24·9 (0·1 to 268·5)	69·6 (45·4 to 99·6)	0·0348

Mann-Whitney U test. P values less than 0·05 are considered statistically significant
Reference intervals (RI) established by the laboratory are also indicated

higher CRP (P=0·0348) and lower TIBC (P=0·004) concentrations and a tendency to lower iron (P=0·2035) and higher ferritin (P=0·1031) concentrations compared to survivors (Table 7).

DISCUSSION

To the authors' knowledge, this is the first study investigating iron status and its relationship with CRP in CanL. The results indicated that dogs with leishmaniasis have decreased serum iron, TIBC, UIBC and percentage of transferrin saturation and increased concentrations of ferritin. As suggested by Liste *et al.* (1994), this is typical of an inflammatory state. Moreover, these results are correlated with increased concentrations of CRP found in this study. CanL is a chronic and sometimes subclinical disease in which continuous antigenic stimulation and excess antibody production cause hypergammaglobulinaemia, resulting in formation and deposition of immune complexes that may cause glomerulonephritis, vasculitis, polyarthritis, uveitis and meningitis, in addition to the production of autoantibodies against platelets and red blood cells (Alvar *et al.* 2004). It has been demonstrated (Martínez-Subiela *et al.* 2002) that infection by *L. infantum* is associated with increased concentrations of acute phase proteins.

The acute phase response is an early non-specific defense mechanism to local or general disturbances in homeostasis attributable to infection, inflammation, trauma, neoplasia or immunological disorders (Baumann & Gauldie 1994, Suffredini *et al.* 1999, Petersen *et al.* 2004). During this response there is an increased rate of synthesis and release of positive acute phase proteins such as haptoglobulin, CRP, ceruloplasmin, fibrinogen and ferritin concurrently with decreased concentrations of negative acute phase proteins such as albumin and transferrin (Eckersall & Conner 1988, Toussaint *et al.* 1995, Eckersall *et al.* 2000, McCown & Specht 2011).

During inflammation hepcidin expression is also increased especially when interleukin-6 and -1 are involved. Among acute phase proteins, hepcidin plays a pivotal role as an inhibitory regulator of iron metabolism (Ganz 2003, Nemeth *et al.* 2003, McCown & Specht 2011). Hepcidin exerts its effects by binding to the cell surface iron efflux protein, ferroportin, and inducing its internalisation and degradation. The effect of this interaction is to inhibit both absorption of dietary iron from intestinal epithelium and export of iron from macrophages and hepatocytes. Functional iron unavailability then ensues. Unfortunately, no commercial assays to measure hepcidin in dogs are currently available and only molecular analysis has been used for this purpose in previous studies (Ganz 2003, Fry 2004). Thus, hepcidin concentration could not be studied in this population.

In dogs with leishmaniasis, CRP had a negative correlation with iron and transferrin and a positive correlation with ferritin. These findings indicate that in CanL inflammation contributes to the status of iron unavailability, which may in turn contribute to the anaemia of chronic disease often described in this infection. In fact the frequency and degree of anaemia were greater in dogs with leishmaniasis then in dogs with other diseases and correlated with the clinical stage of the disease. In addition, there was a significant correlation between the Hct and iron status variables. The presence of inflammation, as demonstrated by the iron profile and the increased CRP concentration, may also explain other clinicopathological abnormalities commonly found in canine leishmaniasis, including leukocytosis, hypoalbuminaemia and decreased albumin/globulin ratio (Liste *et al.* 1994, Paltrinieri *et al.* 2010).

When the iron profile was studied in dogs stratified according to CRP concentrations and so with similar degrees of inflammation, the iron status alterations were still more pronounced in patients with CanL compared to other dogs. Thus, inflammation, although an important contributor, does not completely explain the reason for the altered iron profile in CanL. It has been previously found (Chang & Chang 1985) that *Leishmania* is defective in the haeme biosynthetic pathway and requires iron for growth. Uptake of iron from transferrin or lactoferrin by promastigotes appears to be critical in the first hours after infection of a mammalian host (Wilson *et al.* 1994). In vitro studies have shown that an iron-deficient environment does not support the growth of promastigotes (Soteriadou *et al.* 1995), and the addition of iron salts to incubation fluids may prevent the killing of intracellular amastigotes by activated macrophages (Mauel *et al.* 1991). In a more recent study (Wilson *et al.* 2002) it was found that *Leishmania chagasi* is able to uptake iron in the reduced ferrous form and this finding was followed by the demonstration that *L. chagasi* expresses a NADPH-dependent iron reductase, capable of converting oxidised, ferric Fe^{3+} into more soluble Fe^{2+}. Huynh & Andrews (2008) identified a plasma membrane *Leishmania* transporter, LIT1, that allows the uptake of ferrous iron and that plays a critical role in intracellular growth and virulence of the protozoa. Similar mechanisms have been described in infections with the bacteria *Salmonella* and *Mycobacteria* and with the protozoa *Trypanosoma chagasi* and *Plasmodium falciparum.* In the latter, asymptomatic malarial parasitaemia was found to be associated with increased ferritin concentrations, in the absence of a manifest acute phase response (deMast *et al.* 2010). These findings together with the current results may support the hypothesis that the iron depletion may be due to the consumption of the element by the parasite to allow its growth, and/or iron may be sequestered by the organism as a defense mechanism to decrease

parasite survival. Alternatively, low iron concentrations in dogs with leishmaniasis can also be caused by chronic bleeding, as in case of epistaxis, skin lesions and gastrointestinal ulceration secondary to renal azotaemia, which are quite frequent in CanL. Further investigations are needed to better understand the importance of these mechanisms in the pathophysiology of CanL.

Six of 86 dogs with leishmaniasis died within 30 days post-diagnosis. All dogs of CanL group were on therapy with allopurinol (Zyloric; Teofarma) in association with meglumine antimoniate (Glucantime; Merial) or miltefosine (Milteforan; Virbac). Non-survivor dogs had significantly lower TIBC and increased concentrations of CRP. Previous studies (Singh *et al.* 1999, Martínez-Subiela *et al.* 2002) showed that measurement of CRP is of prognostic value in visceral leishmaniasis. Non-survivors also had a tendency to lower iron and higher ferritin concentrations, although no statistically significant differences were found. Further studies are needed to investigate the potential value of monitoring iron profile and CRP concentration during therapy and to investigate the potential correlation of these variables with the evolution of clinical signs and response to treatment. Iron profile variables might have a stronger prognostic value considering a longer follow-up.

The study has some limitations. CanL is a chronic disease and a too short follow-up may have influenced the results. It was decided to use a cut-off time of 30 days because this generally represents the first re-check for dogs on therapy for leishmaniasis. In fact, both meglumine antimoniate and miltefosine are usually administered for 28 days and then suspended, while therapy with allopurinol is continued for longer (Oliva *et al.* 2010, Solano-Gallego *et al.* 2011). Due to the small number of non-survivor dogs, a type I error likely limited the ability of the test to detect a statistically significance difference. Due to the retrospective nature of the study and the condition of a referral hospital, in which patients are generally sent back to the referring veterinarians after initial work-up, diagnosis and clinical stabilisation, a longer follow-up was not possible in the majority of the patients. Moreover, not all dogs included in control groups 1 and 2 had been tested for *Leishmania* but CanL was considered unlikely based on absence of clinical signs and clinicopathological abnormalities typical of this disease. However, it is possible that some of these dogs had been exposed to and infected by *Leishmania* without developing the clinical disease. Iron status and CRP were only investigated in dogs with clinical leishmaniasis and at this point the effects that *Leishmania* can have on these parameters in infected but healthy dogs is not known.

In conclusion, dogs with leishmaniasis have lower iron and TIBC and higher ferritin and CRP concentrations compared to healthy dogs and dogs with other diseases. Inflammation contributes in part to the iron status alterations found in CanL. Dogs with leishmaniasis have lower iron and TIBC and higher ferritin compared to other ill dogs with similar CRP concentrations. For this reason, a decreased iron and increased ferritin in CanL are probably not only due to inflammation, but may reflect other specific mechanisms, as proposed in humans. The results of this study suggest that increased CRP and decreased TIBC are risk factors for mortality in CanL, while other iron status alterations at the time of diagnosis do not seem to impact the outcome of dogs with leishmaniasis.

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Conflict of interest

None of the authors of this article 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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ORIGINAL ARTICLE

Serum cardiac troponin I concentrations in dogs with leishmaniasis: correlation with age and clinicopathologic abnormalities

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Key Words
Canine, cTnI, *Leishmania*, myocardial injury

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Background: There is anecdotal evidence of myocardial injury in dogs with leishmaniasis due to generalized vasculitis and myocarditis.
Objective: The aims of this study were to evaluate serum concentration of cardiac troponin I (cTnI) as an indicator of myocardial injury in dogs with leishmaniasis and to assess the relationship between cTnI concentration and age, serum antibody titer, and a variety of blood analytes.
Methods: In this retrospective study, serum cTnI concentration was measured in dogs with leishmaniasis and in age-matched healthy dogs. Diagnosis was based on clinical signs and moderate-to-high seropositivity for *Leishmania* as measured by ELISA. Correlations between cTnI concentration and ELISA seropositivity, PCV, concentrations of serum creatinine, total protein, albumin, and globulin, albumin:globulin ratio (A/G), and urine protein:creatinine ratio (UPC) were investigated. The Mann–Whitney test was used to compare analytes between dogs with normal and increased (> 0.06 μg/L) cTnI concentration and to compare cTnI concentrations between dogs with and without anemia, azotemia, and proteinuria.
Results: In dogs with leishmaniasis (n = 40), median cTnI concentration was higher than in control dogs (n = 11) (P = .011). Sixteen dogs (40%) with leishmaniasis had increased cTnI concentration; cTnI was moderately to weakly correlated with decreased albumin concentration, decreased A/G, increased UPC, decreased PCV, positive *Leishmania* titer, and increased age. Dogs with leishmaniasis had significantly higher total protein and globulin concentrations and lower PCV, albumin concentration, and A/G than control dogs. Hematologic and biochemical analytes did not differ significantly between dogs with cTnI concentration within the reference interval and those with increased concentrations. Concentration of cTnI was higher in proteinuric dogs compared with nonproteinuric dogs (P = .017).
Conclusion: A proportion of dogs with leishmaniasis have increased serum cTnI concentration, indicative of some degree of cardiac injury. Additional studies are needed to investigate the relationship between leishmaniasis and possible myocardial injury.

Introduction

Canine leishmaniasis is a severe systemic disease caused by the protozoan *Leishmania infantum* (*L chagasi* in neotropic ecozones).[1] The disease is enzootic in the Mediterranean area, where the prevalence of infection in dogs can be as high as 67%.[2] Clinical signs are variable and include pale mucous membranes,

lymphadenomegaly, skin and ocular lesions, and epistaxis. Anemia, hypoalbuminemia, hyperglobulinemia, azotemia, and proteinuria are the laboratory abnormalities most frequently associated with leishmaniasis.[2–4] Myocardial injury due to *Leishmania* infection has been reported in human medicine,[5] and recently, an unusual presentation of leishmaniasis in a dog with generalized vasculitis and myocarditis was described.[6] To our knowledge, however, the frequency of myocardial injury in canine leishmaniasis has not been reported.

Cardiac troponin I (cTnI) has high sensitivity and specificity for myocardial ischemia and necrosis and is considered a reliable biomarker of cardiac cellular injury in animals.[7,8] CTnI is present only in cardiac muscle and is released into circulation in proportion to the degree of myocardial injury and disruption of myocyte membranes.[8] Numerous studies have highlighted the utility of this biomarker in detecting primary cardiac disease and in identifying cardiac injury secondary to other conditions, such as pancreatitis, anemia, and neoplasia.[9] CTnI also has been evaluated in dogs with infectious diseases, including babesiosis,[10] ehrlichiosis,[11] Chagas disease,[12] and leptospirosis,[13] to detect occult myocardial injury. Serum cTnI concentration is influenced by age and may be increased with marked anemia and azotemia, findings often associated with canine leishmaniasis.[9,14] The objectives of this study were to evaluate serum cTnI concentrations in dogs with leishmaniasis as a marker of potential myocardial injury and to investigate the relationship between cTnI concentration and age, serologic titers for *Leishmania*, and selected laboratory analytes, including PCV, serum creatinine, total protein (TP), albumin, and globulin concentrations, albumin:globulin ratio (A/G), and urine protein:creatinine ratio (UPC).

Materials and Methods

This retrospective study included dogs diagnosed with leishmaniasis at the Hospital Clínic Veterinari of the Universitat Autònoma de Barcelona from January 2007 to December 2008 for which nonhemolyzed serum specimens stored at −80°C were available. Following guidelines proposed by the Canine Leishmaniasis Working Group,[3] medical records were reviewed to include only dogs that had been presented with at least 1 clinical sign compatible with leishmaniasis, eg, systemic lymphadenomegaly, weight loss, dermatologic or ophthalmologic abnormalities, polyarthritis, and epistaxis, and that had moderate or high serum titers for *Leishmania*. Dogs with a previous history or clinical signs of cardiac disease, eg, murmurs or arrhythmias, or that were currently receiving medication were excluded from the study. Results for PCV, serum creatinine, TP, albumin, and globulin concentrations, A/G, and UPC had to be available for dogs to be included in the study. These tests had been performed at admission as part of the diagnostic work-up for leishmaniasis. PCV was obtained within 12 hours of sample collection by microhematocrit centrifugation. All serum biochemical analytes were measured using an Olympus AU400 analyzer and Olympus reagents (Olympus, Hamburg, Germany). The Jaffé method was used to measure creatinine in serum and urine, and the biuret method was used to measure total protein. Serum protein fractionation was performed by commercial agarose gel electrophoresis (Hydragel Protein [E]; Sebia Hispania S.A., Barcelona, Spain) using a semiautomated Hydrasys system (Sebia Hispania S.A.) with manufacturer's reagents to determine the concentration of albumin and globulin fractions. For serologic testing, an ELISA for *Leishmania* was performed as previously described (Department of Pharmacology, Universitat Autònoma de Barcelona).[15] Results were quantified as units relative to positive serum used as a calibrator and arbitrarily set at 100 ELISA units (EU). The positive threshold value previously established by the laboratory was 35 EU (mean + 4 SD for 32 dogs from nonendemic areas).[15] Results of 80–150 EU and > 150 EU were classified as moderate and high positive titers, respectively. Clinically healthy dogs presented for elective surgery or annual vaccination that had normal physical examinations, negative serologic tests for *Leishmania*, and CBCs, biochemical profiles, and urinalyses within reference intervals were included in the study as an age-matched control group.

Serum specimens from dogs with leishmaniasis and control dogs were stored in cryotubes (Deltalab, Barcelona, Spain) at −80°C for 6–18 months before being used to measure cTnI concentration; CTnI has been demonstrated to be stable in serum samples stored at −80°C for at least 1 year.[8,16] Serum cTnI concentrations were measured at the Advanced Diagnostics Laboratory, Nova UCD, University College Dublin, Belfield, Ireland with an automated chemiluminescent assay previously validated in dogs,[8] using the Advia Centaur immunoanalyzer and manufacturer's reagents (Siemens Healthcare Diagnostics, Newbury, UK). The reported imprecision of the assay, as defined by the coefficient of variation (CV), varies from 6.4% at 0.52 μg/L to 3.7% at 36.90 μg/L.[8] The reported functional sensitivity (lowest cTnI concentration determined at a CV of 20%) is 0.017 μg/L.[16] Quality

control procedures were performed daily with control materials from the manufacturer (Siemens Cardiac Markers 1, 2, 3), using the manufacturer's diluent (Siemens cTnI Diluent, Siemens Healthcare Diagnostics, Newbury, UK). The analyzer was calibrated approximately monthly using the manufacturer's calibrators (Siemens Calibrator, Siemens Healthcare Diagnostics).

Dogs were grouped according to cTnI concentrations, with the upper reference limit (URL) set at 0.06 μg/L as previously established by the laboratory (University College Dublin), and by creatinine concentrations, PCV, and UPC, based on laboratory reference intervals (Universitat Autònoma de Barcelona).

Statistical Analysis

Data were tested for normality using the Shapiro–Wilk test. Most data did not follow a normal distribution and nonparametric tests were used. Data were reported as median and minimum-maximum. Correlations between serum cTnI concentration and age, PCV, concentrations of creatinine, TP, albumin, and globulins, A/G, UPC, and *Leishmania* titer were evaluated using Spearman's rank correlation test (ρ). A Mann–Whitney *U*-test was used to detect differences in cTnI concentrations and age, PCV, concentrations of creatinine, TP, albumin, and globulins, A/G, and UPC between control dogs and dogs with leishmaniasis. The same test was used to compare distributions of cTnI concentrations between dogs with and without anemia, increased serum creatinine concentration, and proteinuria. Significance was set at $P < .05$. Statistical analysis was performed using SPSS software v. 17.0 (SPSS, Chicago, IL, USA).

Results

Control dogs ($n = 15$) had a median age of 7.5 years and comprised 8 mixed breed dogs, 2 Boxers, and 1 each of Weimaraner, Rottweiler, West Highland White Terrier, American Pit Bull Terrier, and Irish Setter. There were 10 males (66%), 2 of which were neutered, and 5 females (33%), 2 of which were spayed. Two of the 15 dogs (13%) had cTnI concentrations greater than the URL; 1 dog was an 8-year-old Boxer and the other was an 11-year-old mixed breed dog.

In the disease group, 40 dogs met inclusion criteria and had a median age of 5 years. Breed distribution was 14 mixed breed dogs, 4 German Shepherds, 3 English Bulldogs, 5 Labrador Retrievers, 3 Shar Peis, 2 Yorkshire Terriers, and 1 each of Boxer, Jack Russell Terrier, Siberian Husky, Bull Terrier, Golden Retriever, American Staffordshire Terrier, English Cocker Spaniel, English Pointer, and American Pit Bull Terrier. There were 22 males (55%), 2 of which were neutered, and 18 females (45%), 8 of which were spayed (Table 1). Twenty-eight dogs (70%) were anemic, 25 (62.5%) had UPC > 0.5, and 9 (22.5%) had increased serum creatinine concentration. Of these 40 dogs, 5 (12.5%) had a moderately positive and 35 (07.5%) a high positive titer for *Leishmania*. Sixteen dogs (40%) had cTnI concentrations above the URL. Of these 16 dogs, 13 (81.3%) had UPC > 0.5, 13 (81.3%) were anemic, and 5 (5/16; 31.3%) had increased serum creatinine concentration; 3 dogs (18.8%) were not

Table 1. Age, PCV, creatinine, total protein (TP), albumin, and globulin concentrations, albumin to globulin ratio (A/G), urine protein:creatinine ratio (UPC), and cardiac troponin I (cTnI) concentration in clinically healthy control dogs ($n = 15$) and dogs with leishmaniasis ($n = 40$).

	Reference Interval*	Healthy Control Dogs Median (Minimum –Maximum)	Dogs with Leishmaniasis Median (Minimum –Maximum)	*P*-value†
Age (years)		7.5 (1–12)	5.0 (1–12)	.174
PCV (%)	35–55[1]	43 (37–49)	33 (20–53)	.000
Creatinine (mg/dL)	0.5–1.5[1]	0.98 (0.77–1.32)	1.01 (0.46–5.28)	.670
TP (g/L)	54–80[1]	70 (57–93)	88 (45–136)	.006
Albumin (g/L)	26.0–33.0[1]	35.2 (24.4–35.4)	20.6 (6.0–36.9)	.000
Globulin (g/L)	27.0–44.0[1]	39.2 (27.8–45.1)	62.2 (27.2–118.8)	.000
Albumin/ globulin ratio	0.5–1.1[1]	0.8 (0.4–1.1)	0.3 (0.1–1.3)	.000
UPC	<0.5[1]	0.4 (0.1–0.5)	0.8 (0.1–16.8)	.164
cTnI (μg/L)	<0.060[2]	0.021 (0.001–0.132)	0.043 (0.000–3.470)	.011

*Reference intervals were established by the University College Dublin for cTnI concentrations and by the Universitat Autònoma de Barcelona for other analytes.

†Significance was set at $P < .05$.

anemic or did not have increased serum creatinine concentration, but had UPC > 0.5. CTnI had a significant correlation with decreased albumin concentration ($\rho = -0.620$, $P = .000$), decreased A/G ($\rho = -0.579$, $P = .000$), increased UPC ($\rho = 0.493$, $P = .001$), positive *Leishmania* titer ($\rho = -0.386$, $P = .014$), decreased PCV ($\rho = -0.387$, $P = .014$), and increased age ($\rho = 0.368$, $P = .019$). A significant correlation was not found between cTnI concentration and increased serum creatinine ($\rho = -0.102$, $P = .531$), increased TP ($\rho = -0.026$, $P = .875$), and increased globulin ($\rho = -0.286$, $P = .074$) concentrations.

Dogs with leishmaniasis had significantly higher concentrations of TP, globulins, and cTnI, and significantly lower PCV, albumin concentration, and A/G than did control dogs; differences in serum creatinine concentration and UPC were not found (Table 1). In the disease group, significant differences were not found for any of the measured analytes between dogs with increased cTnI concentration and those with cTnI concentrations below the URL (Table 2). However, 13 (81.25%) of the 16 dogs with increased cTnI concentrations had UPC > 0.5 and 2 of the remaining 3 animals had UPC > 0.2. Of the 24 dogs with cTnI concentrations below the URL, 12 (50%) had UPC > 0.5, and 1 had UPC > 0.2. Dogs with proteinuria had significantly higher concentrations of cTnI compared with nonproteinuric dogs (Table 3). Significant differences in cTnI concentration were not found between dogs with and without anemia or increased serum creatinine concentration.

Table 2. Age, serum *Leishmania* titer (ELISA), serum creatinine concentration, PCV, albumin and globulin concentrations, albumin:globulin ratio, and urinary protein:creatinine ratio (UPC) in dogs with serum cTnI concentration within the reference interval (< 0.06 μg/L, $n = 24$) or > the upper reference limit (> 0.06 μg/L, $n = 16$).

	cTnI	Median (Minimum–Maximum)	*P*-value
Age (years)	< 0.06	4.0 (0.9–8.0)	.061
	> 0.06	6.5 (2.0–12.0)	
Leishmania titer (EU)	< 0.06	294 (105–302)	.050
	> 0.06	300 (191–387)	
Creatinine (mg/dL)	< 0.06	0.94 (0.54–3.03)	.615
	> 0.06	1.13 (0.46–5.28)	
PCV (%)	< 0.06	33 (21–53)	.381
	> 0.06	28 (20–42)	
Total protein (g/L)	< 0.06	81.6 (58.2–106.7)	.696
	> 0.06	84.3 (45.2–135.9)	
Albumin (g/L)	< 0.06	24.5 (8.2–36.9)	.086
	> 0.06	16.8 (6.0–30.3)	
Globulin (g/L)	< 0.06	57.3 (27.2–94.3)	.304
	> 0.06	68.7 (34.4–118.8)	
Albumin/globulin ratio	< 0.06	0.38 (0.13–1.26)	.118
	> 0.06	0.28 (0.08–0.52)	
UPC	< 0.06	0.50 (0.08–16.77)	.050
	> 0.06	4.68 (0.20–14.83)	

For comparison of cTnI concentrations between groups, significance was set at $P < .05$.

Table 3. Serum cTnI concentration in 40 dogs with leishmaniasis categorized by serum creatinine concentration, PCV, and urine protein: creatinine ratio (UPC).

			cTnI (μg/L)		
		n	Median	Minimum–Maximum	*P*-value
Creatinine (mg/dL)	< 1.5	31	0.042	0.000–3.470	.483
	> 1.5	9	0.081	0.011–1.167	
PCV (%)	> 35	12	0.031	0.001–3.470	.236
	< 35	28	0.053	0.000–1.167	
UPC	< 0.5	15	0.014	0.000–0.177	.017
	> 0.5	25	0.081	0.001–3.470	

For comparison of cTnI concentrations between groups, significance was set at $P < .05$.

Discussion

In the current study, 40% of dogs with leishmaniasis had increased cTnI concentration, and cTnI concentration in diseased animals also was significantly higher than that of healthy control dog. These findings support the hypothesis that myocardial injury can occur in dogs with leishmaniasis. Evidence of direct myocardial damage caused by *Leishmania* has been shown previously in dogs with parasites present in the myocardium as demonstrated by PCR or immunohistochemical analysis.[6,32] In another report, leishmaniasis was associated with cardiac tamponade in a dog, and *Leishmania* amastigotes were identified in the pericardium by immunohistochemical staining.[17] The occurrence of increased cTnI concentrations in 2 healthy control dogs suggested that the cut-off value established by the laboratory might include animals with no significant myocardial damage; however, the same cut-off value has been proposed by others.[18] In addition, one of the dogs was an 8-year old Boxer, a breed predisposed to arrhythmogenic right ventricular cardiomyopathy, and even clinical healthy Boxers have been reported to have cTnI concentrations higher than healthy nonBoxer dogs.[19] The other dog was an 11-year-old dog with no history of heart disease, but early cardiac disease can be asymptomatic and is difficult to rule out completely based on physical examination alone.

Troponin I is considered a reliable serum biomarker for myocardial ischemia and necrosis in human

medicine and also has high sensitivity and specificity in animal patients with primary or secondary cardiac disease.[7,8,20–27] After acute myocardial injury, cTnI is released from the cytoplasmic pool, resulting in increased blood concentrations within 2 hours, with a peak after 12–24 hours.[7,8] Persistently increased cTnI blood concentration suggests irreversible and active ongoing damage to cardiomyocytes,[7,8,28] and the degree of increase has been shown to be correlated with the extent of myocardial damage and with animal survival.[22,29,30]

The basis of myocardial injury in canine leishmaniasis could involve many pathogenetic mechanisms, such as vasculitis, an intense local or systemic inflammatory response, anemia, renal disease, and possible myocardial hypoxia in addition to direct action of the parasite. Vasculitis is a process characterized by blood vessel inflammation and ischemic damage that can occur in any organ; in canine leishmaniasis, it typically results from immune-complex deposition in vessel walls.[6,31] When systemic vasculitis develops, multiple infarctions in different target tissues can produce progressive organ failure, and at the same time, the intense inflammatory response can promote inflammatory cell infiltration into the myocardium as previously demonstrated.[6,32]

Diseased dogs in this study had clinicopathologic abnormalities similar to those previously described in dogs with leishmaniasis.[4,33] Protein profiles indicated an inflammatory pattern suggestive of upregulated humoral immune responses characteristic of dogs with leishmaniasis. Interestingly, proteinuria and hypoalbuminemia were moderately correlated with increased cTnI concentrations in this study. Proteinuria and hypoalbuminemia are hallmarks of canine leishmaniasis and typically occur secondary to glomerulonephritis from immune complex deposition.[4,15] The same pathogenic mechanism might be responsible for microvasculature damage in other tissues, such as the myocardium, potentially leading to cardiomyocyte damage and release of troponin I.

Increased cTnI concentrations can also result from noncardiac conditions, such as azotemia and anemia, which often occur in dogs with leishmaniasis. In a recent study, cTnI concentrations were reported to be increased in 70% of dogs with azotemic renal failure and in 70% of dogs with a variety of systemic noncardiac diseases; however, there was no correlation between cTnI concentration and degree of azotemia, presence of murmurs, hypertension, or the type of noncardiac illness.[34] Azotemic renal failure may lead to altered elimination of cTnI. In addition, hypertension, myocardial remodeling, left ventricular hypertrophy, thromboembolism due to loss of antithrombin, and uremic pericarditis or myocarditis may cause clinically unapparent myocardial injury. In our study, less than ¼ of dogs had azotemia and the degree was usually mild; cTnI concentration did not differ in azotemic animals and healthy control dogs, and was not correlated with serum creatinine concentration.

Mild-to-moderate nonregenerative anemia occurs frequently in dogs with leishmaniasis and also is correlated with the severity of clinical signs.[33] Severe anemia can cause tissue hypoxia and contribute to increased cTnI concentration secondary to myocyte damage.[9] Dogs with primary immune-mediated hemolytic anemia have higher serum cTnI concentrations compared with both healthy dogs and dogs with nonhematologic diseases and secondary cardiac diseases.[35] Almost ¾ of the diseased dogs in our study were anemic, but the correlation between decreased PCV and cTnI concentration was weak; in addition, within the subgroup of dogs with increased cTnI concentration the proportion of anemic dogs was only slightly higher and no difference in PCV was found between dogs with cTnI above or below the URL. Finally, in most cases, the degree of anemia was mild and unlikely responsible for clinically relevant hypoxia. Thus, azotemia and anemia probably did not play a significant role in increased cTnI concentrations in this study.

The influence of age on serum cTnI concentration has been reported previously, and we found a weak correlation between age and cTnI concentration.[8,9,22] This association may be attributed to degenerative changes in the coronary vasculature, arteriosclerosis, and possible ischemic injury in aged dogs. Age was not significantly different between dogs with leishmaniasis and healthy control dogs or between dogs with cTnI concentrations above or below the URL; consequently, age was unlikely to be a factor in the difference in cTnI concentrations between these groups.

A limitation of our study was lack of a thorough cardiac evaluation, including electrocardiographic and echocardiographic evaluation, of all dogs; this limitation was due to the retrospective design of the study, Histologic examination of the heart would be required to evaluate the presence, severity, and type of myocardial injury and to demonstrate a definitive causal relationship between increased TnI concentrations and leishmaniasis. However, antemortem myocardial biopsy is invasive, and postmortem examination would not permit evaluation of changes produced by the parasite acutely after infection, given that leishmanisias is a chronic disease with a time course of months to several years.

Neither PCR analysis nor cytologic examination of tissues was performed to confirm the presence *Leishmania* organisms in this study. Dogs exposed to *Leishmania* may be seropositive even after clearance of the infection, and asymptomatic infection can occur. However, in a symptomatic dog, a high antibody titer or titers that increase over time indicate that parasitic infection is disseminated, and such titers are considered diagnostic for leishmaniasis without the need for additional testing.[3] In a recent study, all symptomatic dogs with moderate or high ELISA titers were reported to be positive by quantitative real-time PCR analysis of blood samples.[36]

In conclusion, a significant proportion of dogs with leishmaniasis had increased serum cTnI concentration, indicating some degree of myocardial damage. The increase was mild for most of the dogs and may have no clinical significance. Evaluation of cTnI as an early marker preceding overt cardiac disease or as a prognostic factor was beyond the aim of this study. Potential myocardial injury in canine leishmaniasis could involve many pathogenic mechanisms, including direct action of the parasite and other concurrent factors, such as vasculitis, renal disease, and anemia. Prospective studies are warranted to further investigate the causal relationship between *Leishmania* infection and myocardial injury.

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