Invited Review - LABORATORY TESTS FOR DIAGNOSING AND MONITORING CANINE LEISHMANIASIS

Running header: laboratory diagnosis of leishmaniasis

Authors: Saverio Paltrinieri[1], Luigi Gradoni[2], Xavier Roura[3], Andrea Zatelli[4], Eric Zini[5]

Affiliations: [1]Department of Veterinary Medicine – University of Milan, Italy; [2]Unit of Vector-borne Diseases & International Health, Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy, [3] Hospital Clínic Veterinari, Universitat Autònoma de Barcelona, Bellaterra, Spain; [4] Medical Consultancy Services, TàXbiex, Malta, [5]Clinic for Small Animal Internal Medicine, University of Zurich, Switzerland; [6]Department of Animal Medicine, Production and Health, University of Padova, Italy; Istituto Veterinario di Novara, Granozzo con Monticello (NO), Italy.

Correspondence:

Prof. Saverio Paltrinieri

Dipartimento di Medicina Veterinaria, Università di Milano

Via Celoria 10, 20133, Milano, Italy.

e-mail: saverio.paltrinieri@unimi.it

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Abstract

Although several reviews on canine leishmaniasis have been published, none thoroughly described clinico-pathologic abnormalities and their clinical usefulness. The aim of this review is to provide information concerning current diagnostic tests relevant for clinical pathologists and from a practical perspective. Specifically, in canine leishmaniasis non-regenerative normocytic normochromic anemia, thrombocytopenia or leukogram changes may be present. Clinical chemistry and urinalysis may indicate renal dysfunction (azotemia, decreased urine specific gravity, proteinuria) and inflammatory/immune response (increased acute phase proteins or α_2- and/or γ-globulins). Although a potential gammopathy by mechanism is usually polyclonal it may also appear oligo- or monoclonal, especially in dogs co-infected by other vector-borne pathogens. When lesions are accessible to fine needle aspiration (lymhpoadenomegaly, nodular lesions, joint swelling), cytology is strongly advised, as the presence of *Leishmania* amastigotes in a pattern of pyogranulmatous inflammation or lymphoplasmocytic hyperplasia is diagnostic. If the cytologic image is inconclusive, the parasite should be identified by histology/immunohistochemistry or PCR on surgical biopsies. Alternatively, cytology and PCR may be performed on bone marrow smears, where amastigotes, along with erythroid hypoplasia/myeloid hyperplasia, plasmocytosis, or secondary dysmyelopoiesis can be observed. Dogs with overt Leishmaniaisis generally have high antibody titers, while low titers predominate in immunologically resistant infected dogs, or in exposed dogs with no parasite confirmation. Quantitative serology is recommended in clinically suspect dogs as high-titer antibodies titers are conclusive. In confirmed and treated dogs, renal function and inflammatory/immune response variables should be periodically monitored.

Keywords: Dog; Leishmania infantum; clinical usefulness; diagnosis; follow-up

1. Introduction

Leishmaniasis is a frequent infectious disease of dogs living in endemic areas, associated with important morbidity and, despite appropriate treatment, potential lethal outcome Although several reviews have been published so far, none has fully described the diagnostic role of available laboratory tests that may be diagnostic or of values for monitoring dogs with leishmaniasis. Therefore, the aim of the present review is to provide information concerning typical laboratory abnormalities and current diagnostic tests that may be relevant for clinical pathologists, from a practical perspective.

2. Etiology and pathogenesis of canine leishmaniasis

Canine leishmaniasis is caused by the protozoan parasite *Leishmania infantum* or its New World synonym *Leishmania chagasi*.[1] Although non-vectorial transmission has been reported (e.g. transplacental, transfusional or venereal)[2-4], the parasite is usually transmitted by infected phlebotomine sand flies. Therefore, the geographic distribution and prevalence of the disease depends on the presence and abundance of competent vectors..[5] Blood-sucking females ingest the non-flagellated form (amastigote) during the bloodmeal on infected hosts. After multiplication, flagellated forms (promastigotes) transform into infectious metacyclic promastigotes that are inoculated into the host at the next blood meal. Parasites are phagocytosed by macrophages,[6] but the amastigotes interfere with the oxidative activity of these cells[7,8] and survive and replicate in macrophages, leading to cell destruction and infecting progressively more and more phagocytes. In longitudinal field studies on naïve dogs, *Leishmania* can be detected by PCR in bone marrow starting about 6 months from natural exposure to vectors.[9] Once bone marrow has been colonized it is generally accepted that the dog is persistently infected. However, a fraction of dogs with positive PCR in bone marrow may become negative in the following months without any treatment; it is unknown whether in these dogs the parasite density falls below the threshold limit of the test, the

infection persists in organs other than bone marrow, or the host defenses eradicate the infection.[9] Despite dogs can mount antibody responses shortly after the first contact with the parasites, resistance or susceptibility to progressive infection depends on the balance between Th1 (cell-mediated) and Th2 (humoral) immune responses: dogs with prevailing Th2 responses are likely prone to have parasite dissemination to all tissues and overt clinical signs.[10-13] Hence, the simple detection of circulating antibodies does not necessarily imply that the dog is actually clinically affected. Similarly, parasite detection in tissues does not mean that the infected dog is actually sick. Therefore, the guidelines for diagnosis and staging of canine leishmaniasis, released by the Canine Leishmaniasis Working Group (CLWG),[14] classify dogs as exposed, infected or sick based on a combination of clinical and laboratory findings, as follows:

- Infected dogs: dogs clinically unremarkable, without laboratory abnormalities, that test positive to PCR or cytology in bone marrow, lymph node, spleen, skin or peripheral blood;
- Sick dogs: infected dogs with typical clinical or clinicopathological changes.

The CLWG classification[14] includes 2 additional categories of dogs at the extremes of the spectrum:

- Exposed dogs: dogs clinically unremarkable with low-titer positive serology, in which PCR or cytology fail to demonstrate the presence of the parasite
- Severely sick dogs: sick dogs with a severe clinical condition (e.g. proteinuric nephropathy, chronic renal failure), with concurrent problems, related or not to leishmaniasis, (e.g. ocular disease causing functional loss, severe joint disease impairing motility), which require immunosuppressive treatment, with concomitant conditions such as coinfections or neoplastic, endocrine, or metabolic diseases, or that are unresponsive to repeated courses of anti-*Leishmania* drugs.

Conversely, the Leishvet guidelines classifies sick dogs in four stages according to the severity of clinical signs, clinicopathological findings and serological status.[15]

Clinical signs of canine leishmaniasis

The interpretation of clinicopathological, serological and molecular tests should be done in light of history (e.g. exposure to phlebotomine vectors), signalment (male dogs older than 2 years are at high risk) and clinical presentation: the spectrum of clinical presentations is wide and ranges from infections characterized by the absence of obvious clinical findings but detectable laboratory abnormalities, to overt clinical infections characterized by the presence of clinical and laboratory abnormalities that require or not hospitalization especially in the case of very severe life threatening disease.[14-16, 24-33]

Laboratory abnormalities that may support or confirm leishmaniasis

In addition to clinical findings, laboratory abnormalities detectable by routine hematology, clinical chemistry or urinalysis may further increase the clinical suspicion of canine leishmaniasis. Moreover, especially in the early phases of the disease, laboratory changes may occur in the absence of obvious abnormalities at physical examination. Thus, a basic panel of tests is mandatory when canine leishmaniasis is clinically suspected, or when a dog with positive result of tests for etiological diagnosis needs to be classified as "exposed", "infected" or "sick". Table 1 summarizes the clinicopathological changes that may be found in dogs with leishmaniasis (i.e. "sick" dogs).

1) Hematologic abnormalities

Hematological changes in canine leishmaniasis are non specific.[34] Neutrophilia, due to the systemic inflammatory response may be present and particularly prominent if ulcerative cutaneous lesions with secondary bacterial infection may occur, are present.[34,35] Conversely, numerical or morphological changes in the other leukocyte populations are less common, although lymphopenia, lymphocytosis or eosinophilia are occasionally described[35-37] Amastigotes may be rarely documented in circulating leukocytes of infected dogs (less than 0.5% of cases) within neutrophils but also in lymphocytes and monocytes.[36,38] The percentage of infected cells is so low that their

search is generally not rewarding. When a systemic disease and blood dissemination is suspected, more sensitive tests such as PCR or quantitative PCR should be preferred (see below).

The most common hematological changes in leishmaniotic dogs is anemia,[34,35,44] that is usually mild to moderate and has the normocytic normochromic non regenerative pattern typical of the anemia of inflammatory disease.[35,39] However the pathogenesis of anemia in leishmaniotic dogs include additional mechanisms such as renal failure leading to reduced erythropoietin synthesis. Moreover, it is very likely that anemia also has a hemolytic component as suggested by positive Coomb's test in a minority of cases.[17] This positivity may be associated with a "lupus-like" reaction along with other clinical or laboratory changes, such as positive ANA-test[40] or perinuclear antineutrophil cytoplasmic autoantibodies.[41]

Thrombocytopenia is fairly frequent in leishmaniotic dogs withouth concurrent infections. It is usually mild to moderate. If severe, co-infections with other vector-borne pathogens (e.g. *Ehrlichia canis*, *Anaplasma phagocytophilum* or *A. platys*) or other possible causes of reduced platelet concentration should be suspected. The most likely mechanism responsible for thrombocytopenia in leishmaniasis is a peripheral consumption of circulating platelets, possibly due to an immune-mediated mechanism, since anti-Plt antibodies has been demonstrated in leishmaniotic dogs.[42-44] Moreover, platelet loss may be associated to hypercoagulability caused by a decreased concentration of anti-thrombin III as in any other protein losing nephropathy[45] (see below) or to disseminated intravascular coagulation (DIC) that has been occasionally reported in leishmaniotic dogs.[46] However, the mechanism of thrombocytopenia in leishmaniotic dogs includes also a decreased production due to the depressed bone marrow activity cited above. Even in the absence of reduced platelet concentrations, however, platelets may be hypofunctional in dogs with leishmaniasis[47] although this reduced function is rarely responsible for hemostatic abnormalities.

One additional test that may be run in dogs with leishmaniasis is the flow-cytometric evaluation of the CD4/CD8 ratio. The rationale of this test is that as soon as Th1 responses decreases, thus increasing the susceptibility to the disease and favoring the shift from latent infection to overt

disease, the number of CD4+ lymphocytes decreases causing reduction of the CD4/CD8 ratio.[48-49] Therefore, a seropositive or PCR-positive dog with a low CD4/CD8 ratio is more predisposed to develop clinical signs than a similar dog with normal CD4/CD8 ratio. The practical applicability of this test, however, is limited by the high individual variability and by the difficulty to determine a cut-off for staging the disease Hence, this test may be used to monitor the post- treatment follow-up but not to stage a dog at first diagnosis of leishmaniasis. The authors do not recommend the use of this test for diagnostic purposes in dogs suspected to have leishmaniasis.

Finally, the hematological profile of leishmaniotic dogs may be completed by bone marrow cytology.[24,37,39,50] This analysis may be useful to confirm the infection through the detection of infected macrophages, as better specified below, but it may be also used to differentiate a simple infection from systemic disease (i.e. "infected" vs. "sick" dog).[14] Although some histological studies demonstrated that parasite density can be high despite few clinical signs,[51] generally the parasite load and the magnitude of cytological alterations increases as soon as the dogs show clinical sings.[52] Therefore, rare infected macrophages may be occasionally seen in the absence of other pathological findings in dogs that are simply infected, whereas "sick" dogs are characterized by a higher number of parasites detected cytologically and by a series of morphological changes. In the latter case cytology of the bone marrow usually reveals an erythroid hypoplasia,[35] without abnormalities in the ratio between maturative and proliferative pools of erythroid precursors, occasionally associated with myeloid hyperplasia (and thus with an increased M:E ratio). Moreover, bone marrow inflammation, generically defined by Stockham and Scott as "myelitis",[53] are usually found (figure 1). These include a proliferation of either infected or non-infected macrophages often with signs of erythrophagia or cytophagia, an increase of neutrophils, and a moderate to severe plasmocytosis characterized by a higher number of plasma cells, mott cells and lymphocytes.[35,39,54] Megakaryocyte hyperplasia may also be present, especially when peripheral consumption of platelets occurs.

Secondary dismyelopoiesis may be found, although less frequently (figure 2). This condition is characterized by multiple peripheral cytopenias (e.g. the anemia and thrombocytopenia cited above) associated with hypercellular bone marrow on which one or more cell lineages show dysplastic features. In canine leishmaniasis, these mostly include dyserythropoiesis (abnormal mitoses, asynchronous nucleo-cytoplasmic maturation, nuclear fragmentation, and/or late stage maturation arrest) and dysmegakariopoiesis (dwarf megakaryocytes emperipolesis), while dismyelopoiesis (abnormal maturation of granulocytes and ring forms) is only occasionally found.[35,54] The detection of secondary dysmyelopoiesis however, is not per se diagnostic for leishmaniasis, unless amastigotes are found. Therefore, the cause-effect association between secondary dysmyelopoiesis and seropositivity or PCR-positivity should be carefully considered. Ultimately, in this case the diagnosis of leishmaniasis should be based on the exclusion of other causes of secondary dysmyelopoiesis or of primary myelodysplastic syndromes.

In brief, bone marrow cytology may be useful for diagnostic purposes in some dogs, by detecting amastigotes and compatible cytological abnormalities, or to differentiate between infected dogs from those that are sick due to leishmaniasis.

2) Hemostatic abnormalities

Hemostatic abnormalities are uncommon in leishmaniotic dogs. Activated partial thromboplastin time (aPTT) and prothrombin time (PT) may be increased. In most cases, however, this is due to preanalytical factors since their prolongation may occur when the concentration of total globulin increases, which is frequent in dogs with leishmaniasis. Alternatively, prolonged coagulation times may result from DIC, although this complication is uncommon in leishmaniotic dogs.[46]

Conversely, hypercoagulability may be common in leishmaniotic dogs if affected by severe protein losing nephropathy. This is mostly due to glomerular loss of antithrombin III (ATIII), a protease inhibitor involved in the regulation of blood coagulation that prevents the conversion of fibrinogen into fibrin. The lack of this physiologic anticoagulant may induce hypercoagulability that in turn

promotes thrombosis and subsequent consumption coagulopathy.[55] Hypercoagulability is also favored by the hyperviscosity syndrome due to the increased circulating globulins. Hypercoagulability of leishmaniotic dogs was also demonstrated through a decreased clot formation time and an increased global clot strength using thromboelastography (TEG).[55] Differently, in another study the coagulation profile of leishmaniotic dogs assessed by thromboelastometry (TEM, a technique similar to TEG), was within normal limits.[56] However, it is worth noting that TEM and TEG are affected by the RBC mass,[57,58] possibly explaining the different results obtained by TEM and TEG.

In brief, to assess hypercoagulability in dogs with protein losing nephropathy associated with leishmaniasis the authors currently suggest including only ATIII measurement.

3) Biochemical abnormalities

Because the clinical presentation of dogs with leishmaniasis is variable, also the type of biochemical abnormalities varies accordingly Renal dysfunction and inflammation/immune reactions frequently observed and their presence should be evaluated in each dog with suspected or confirmed leishmaniasis. Biomarkers of hepatobiliary or pancreatic damage may be altered in case of pyogranulomatous infiltrates affecting these organs.[14,16] Muscular enzymes (LDH and CK), may increase in dogs with musculoskeletal lesion.[59] Nevertheless, increased CK may also be due to the increased CK-BB when neurological signs are present,[60] since *Leishmania* has been found in the brain of some affected dogs with cerebrovascular alterations,[61,62] or to CK-MB in cardiopathic dogs (increased.tropoinin I and cardiopulmonary lesions have been reported).[63,34] Biochemical abnormalities consistent with alteration of endocrine organs are rare, despite amastigotes and inflammatory lesions have been found in the adrenal cortex of leishmaniotic dogs.[65,66]

Assessment of renal function

229 The deposition of circulating immune complexes at the glomerular level induces inflammatory
230 changes detectable histologically and ultramicroscopically, [33,67-69] leading to a proteinuric
231 nephropathy.[69] The evolution of this condition is the development of a chronic kidney disease
232 (CKD) characterized by glomerulosclerosis, renal hypertension and tubulointerstitial nephritis[68,69]
233 In turn, advanced stages of CKD are characterized by hyperazotemia and may be associated with
234 systemic hypertension, both factors contributing to comorbidity in dogs with leishmaniasis.[69,70]
235 Therefore, the clinical and laboratory approach to leishmaniotic dogs with proteinuric nephropathy
236 is the same recommended by the International Renal Interest Society (IRIS)[71] for any type of CKD.
237 This approach is based on a thorough clinical evaluation, on the measurement of arterial pressure
238 and on the quantification of urinary proteins (described in the section of this article regarding
239 urinalysis) and of markers of renal function such as the urine specific gravity and the serum
240 concentration of creatinine.[71] This latter increases frequently in leishmaniotic dogs.[14-17,72]. However,
241 creatinine is not enough sensitive to detect the earliest stages of renal insufficiency.[73] Therefore, a
242 huge research activity is currently running to identify earlier markers of decreased glomerular
243 filtration rate (GFR), either in leishmaniotic dogs or in dogs affected by other types of CKD. The
244 direct measurement of GFR trough clearance tests would be the best method to assess in real time
245 the functionality of the kidneys.[74] Despite there is no evidence that serum Cystatin C (Cys C) is
246 more sensitive than creatinine in detecting early CKD,[74] the serum concentration of Cys C has been
247 assessed also in dogs with leishmaniasis.[75] Urinary Cys C seems to be a good marker of CKD[76] but
248 not in canine leishmaniasis.[77] Recently, symmetric dymethilarginine (SDMA) has been proposed as
249 an early biomarker for early diagnosis of CKD.[78,79] No studies on the use of SDMA in canine
250 leishmaniasis exists, but it is very likely that it will be used to assess renal function in leishmaniotic
251 dogs that are proteinuric but still have normal creatinine concentration.
252 Other blood markers may provide additional information in leishmaniotic patients with CKD. For
253 example in people the increased serum concentration of homocysteine (Hcy), endothelin-1 (ET-1)
254 or C-reactive protein (CRP) may predict, hypertension and/or inflammation associated with CKD.[80-

[83] Increases of Hcy and ET-1 have been reported in dogs with CKD, some of which affected by leishmaniasis.[84,85] However, further studies are needed before to recommend these markers as ancillary tests for the management of leishmaniotic dogs with CKD. Conversely, inflammatory markers such as CRP, ferritin and adiponectin may increase in the urine of leishmaniotic dogs, sometime in the absence of elevated serum creatinine.[77,86,87] Howeever, their increase depends on their high serum concentration due to the systemic inflammatory state, rather than to CKD. Finally, in leishmaniotic dogs, tubulointerstitial lesions may occur secondarily to proteinuria caused by glomerular damages. The presence of these lesions may be investigated using markers of tubular injury in urine and are described in the section on urinalysis.

It is also worth mentioning that some dogs with CKD may have acute deterioration of their renal dysnsfunction due to factors related or not to leishmaniasis (e.g. vomiting, diarrhea).

Assessment of inflammatory/immune reactions

Based on the pathophysiology above described, it is clear that leishmaniotic dogs with overt disease have an intense inflammatory reaction and produces high amount of molecules involved in the immune response, including antibodies. Both these phenomena may be investigated using tests such as serum protein electrophoresis or measurement of acute phase proteins (APPs).

Protein analysis and serum protein electrophoresis may reveal abnormalities very early during the course of the disease.[24] Total proteins and total globulin are frequently increased.[14,15,18,72,88] The increase of total protein has been shown to correlate with the severity of the clinical score.[89] Albumin decreases both because it is a negative APPs (see below) and due to the renal loss associated with proteinuric nephropathy, leading to decreased albumin:globulin (A/G) ratio.[72,88] The decrease of the A/G ratio is so frequent that it has been considered by some authors to be one of the more sensitive tests for canine leishmaniasis[88] and hypoalbuminemia is considered a negative prognostic factor in leishmaniotic dogs.[90] The typical electrophoretogram of leishmaniotic dogs

281 with overt clinical signs (figure 3) displays hypoalbuminemia, an increase of α_2-globulin, where
282 most of the positive APPs migrate, and a strong increase of γ-globulins, due to the huge amount of
283 circulating antibodies, immunecomplexes, and other molecules with γ motility. Occasionally, peaks
284 due to circulating antibodies are found in the β region, where IgM and some APPs migrates. The
285 gammopaty is typically polyclonal but sometime the peak may be narrower (oligoclonal), biclonal[91]
286 or definitely monoclonal,[92] especially using capillary zone electrophoresis.[93] (figure 4). However,
287 although monoclonal peaks associated exclusively with leishmaniasis have been described, the
288 detection of monoclonal peaks should suggest considering the possible presence of concurrent
289 diseases (e.g. other vector-borne diseases or multiple myeloma).[94,95]
290
291 Acute phase proteins are powerful indicators of inflammation: the pro-inflammatory cytokines
292 produced in inflammatory sites induce the so called "acute phase response", characterized by the
293 release of neutrophils from storage pools, by an activation of myelopoiesis (see above), and by a
294 modulation of protein synthesis in the liver.[82] This latter phenomenon leads to a decreased serum
295 concentration of the "negative APPs", and to an increased concentration of the "positive APPs" that
296 includes a series of immunomodulators, scavenger or transport proteins, antiproteases, and other
297 proteins involved in host defenses. Therefore it is not surprising that the serum concentration of
298 positive APPs in dogs with overt canine leishmaniasis is high. The list of APPs whose
299 concentration increases in serum of leishmaniotic dogs is long and includes CRP, Haptoglobin
300 (Hp), Ceruloplasmin (Cp) Serum Amyloid A (SAA) and ferritin.[96-101] Similarly, a decrease of
301 negative APPs other than albumin has also been reported; these are transferrin (total iron binding
302 capacity or TIBC), that induces also a reduction in the concentration of iron, and a decreased
303 activity of the enzyme paraoxonase (PON-1).[98,102,103] PON-1 is a negative APP that is bound to high
304 density lipoproteins (HDL) and represents a link between inflammation and oxidative stress.
305 Therefore its decrease is not constantly seen in leishmaniotic dogs but it may become evident when

oxidative stress is particularly severe.[102] Interestingly, in these cases also the concentration of HDL, that is converted into low density lipoprotein (LDL) after detachment of PON-1, decreases[103] and may be a cheap marker of inflammation and oxidative stress associated with leishmaniasis. Recently a reduced serum activity of adenosine deaminase (ADA) and butyrylcholinesterase (BChE), two enzymes involved in modulating immune responses, has also been reported in dogs with leishmaniasis.[104]

The APP changes summarized above are not diagnostic *per se* since mild increases of positive APPs have been reported also in infected dogs without clinical signs[99] and severely increased elevels may occur in diseases other than leishmaniasis.[82] In a dog in which leishmaniasis has been diagnosed by other clinical or laboratory findings, however, the magnitude of these changes may reflect the magnitude of inflammation and thus provide prognostic information. In particular, the decrease of PON-1 is evident in severe diseases and may therefore be a negative prognostic marker.

4) *Abnormalities at urinalysis*

As for any suspected proteinuric nephropathy, it is necessary to confirm the presence of CKD, of proteinuria, which is frequent in leishmaniotic dogs,[14-18] and of tubular damage, through the following steps:

Physico-chemical analysis

With a refractometer, the urine specific gravity (USG), that tends to decrease in dogs with tubulo-interstitial damage, should be assessed.[73] The supernatant should be tested with a dipstick, to assess:

- the pH that may be useful to correctly interpret other dipstick results: for example dipstick analysis may overestimate proteinuria in alkaline urine (pH>8);[73]
- the concentration of proteins, to be interpreted as described below;
- the presence of glucose, that, as specified below, may be an indicator of tubular damage.

Sediment analysis is another important step in leishmaniotic dogs: an active sediment (e.g. a sediment with high numbers of leukocytes, erythrocytes or bacteria) indicates a lower urinary tract infection superimposed on the primary disease (leishmaniasis) and may overestimate proteinuria;[105] conversely granular or cellular casts may be consistent with tubular damage.[73]

Evaluation of proteinuria

The evaluation of proteinuria is mandatory, since proteinuria is a risk factor for the progression of nephropathy.[106] According to the ACVIM guidelines,[107] proteinuria should be assessed in any dog with predisposing diseases, such as leishmaniasis. The ACVIM guidelines recommend to collect urines by cystocentesis, to avoid contamination from the lower urinary tract. However, a first evaluation may be done on voided samples, since results recorded with the two methods of collection overlap when the sediment is inactive.[108] Proteinuria may be first investigated using a dipstick, if the dipstick is negative the dogs is likely non proteinuric according to the IRIS classification[71] and any additional evaluation of proteinuria is not necessary.[109] Conversely, if the dipstick is weakly positive in dogs with low USG or strongly positive the dog is likely proteinuric and the protein to creatinine (UPC) ratio must be run to classify the dog as proteinuric (UPC >0.5), borderline proteinuric (UPC= 0.2-0.5) or non proteinuric (UPC <0.2) according to the IRIS classification, recently revised for the diagnosis of glomerular disease.[71,110] In the interpretation of data, particular attention should be paid to results close to these thresholds, that may be affected by several analytical factors.[111-113] Quantification of proteinuria must be repeatedly assessed (3 times in 2 weeks[107] or once on pooled urine[114]) because additional investigations or treatments should be performed only if proteinuria is persistent.[107-110] Finally, the origin of urinary protein should be assessed through a renal biopsy.[107] However, according to the recent IRIS guidelines[110] renal biopsy is recommended only in the case of rapid progression of CKD or in dogs not responding to conventional treatments. Alternatively, the origin of proteinuria can be argued on the basis of surrogate methods such as qualitative analysis of urinary proteins (see below).

Markers of tubular injury

In order to differentiate the dogs with a tubular component of proteinuria, that are in a more advanced stage of renal disease, urinary markers may be used.[115] Some rough markers such as granular or cellular casts and glycosuria in normoglycemic dogs are very specific indicators of tubular damage, but are not enough sensitive, do not detect dogs with early tubular damage and are rarely observed in leishmaniotic dogs. Early information about the presence of tubular damage may be achieved using sodium dodecylsulphate (SDS) electrophoresis of urinary proteins or using urinary markers of tubular damage. The SDS denaturates and charges negatively the urinary proteins. Therefore, after migration on polyacrylamide gel (SDS-PAGE) or agarose gel (SDS-AGE), proteins migrate according to their molecular mass.[116] This differentiates large proteins of glomerular origin, from small proteins of tubular origin. Results of SDS-PAGE or SDS-AGE well correlate with results of renal biopsies, especially for the identification of glomerular damage or of severe tubulo-interstitial damages.[117,118] However SDS-AGE may be not accurate in very concentrated or in diluted urine.[119] Using SDS-AGE it has been shown that leishmaniotic dogs have a mixed (glomerular and tubular) pattern. Only a minority of dogs, likely those with early CKD, have a pure glomerular proteinuria.[67,120] Occasionally, low molecular weight proteinuria with no signs of glomerular disease may be seen, possibly due to a free light chain proteinuria (pre-renal proteinuria associated with the intense antibody production) rather than to a tubular damage.[121] Enzymuria is considered a good marker of tubular damage: the enzymes of interest are located in the cytoplasms of tubular cells and may be found in urine when tubular cells are damaged. The two most popular urinary enzymes are γ-glutamyl transferase (GGT) and N-acetyl-β-N-glucosaminidase (NAG) that must be measured just after sampling since their activity decreases with storage.[122] Increases of these and other enzymes (e.g. alkaline phosphatase or β-glucuronidase), have been reported in dog with leishmaniasis[123] and the increase of GGT correlates with the presence of tubular bands in SDS.[120] On the contrary, no information is available on the utility in leishmaniotic

dogs of the measurement of other urinary analytes used to detect tubular damage in dogs with CKD non associated with leishmaniasis.[115,124,125]

Tests for etiological diagnosis that may support or confirm the diagnosis of leishmaniasis

Tests for etiological diagnosis are used to identify the presence of the parasite or its components (direct tests) or of the host's response to the parasite (indirect tests). As previously mentioned, positive indirect tests (i.e. serology) may or may not indicate a current infection. Conversely, positive direct tests (cytology, histology, immunohistochemistry, PCR, culture and xenodiagnosis) demonstrate that the dog is actually harboring *Leishmania* and it is therefore infected. However, as stated above, the relationship between infection and disease should be based on the evaluation of clinical findings and clinicopathologic tests. The most common tests for etiological diagnosis are described below.

Serology

Methods

Apart from some techniques such as Western blotting, that is highly accurate but not available in routine practice, or other methods that have been proposed but are not extensively used, such as latex agglutination test or detection of antibodies through immunosensors or flow cytometry,[126-129] the most common techniques used to detect antileishmanial antibodies are based on three analytical principles: immunofluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and immunochromatographic test (ICT). ICT is the basis of all-rapid "in clinic" assays, which have a major limitation being that they provide results in a qualitative manner (i.e. presence/absence of specific reactive bands).[130] Several commercial ICT kits are available, which employ single or multiple recombinant *Leishmania* antigens to be used on serum, plasma, whole blood or blood spots dried onto filter paper.[131] The specificity of these tests is quite acceptable, but sensitivity is usually low (in the approximate range of 30-70%) and largely depending on leishmaniasis stage.[132] Lowest

sensitivities are found in infected dogs without clinical signs, the highest ones in dogs with overt disease.[133] Therefore, ICT may be used as a first "in clinic" test to complete the laboratory evaluation of clinically suspected dogs and, in case of positivity, serology should be repeated by ELISA or IFAT, which provide quantitative results. However, due to its low sensitivity, a negative ICT result may be false and therefore, if the clinical suspicion persists, tests with higher sensitivity (IFAT or ELISA) should be performed. Recently, an ICT kit claiming detection of antibodies developed after natural infection but not those elicited by vaccination with the LiESP-based vaccine, has been proposed as a tool to differentiate vaccinated from infected dogs.[134] The principle of the test is sound, and the first studies reported a high sensitivity of this ICT format;[135] but other studies reported a low sensitivity also for this test.[136]

IFAT is recognized as the reference method to perform anti-*Leishmania* serology in dogs,[132-137] as it is very sensitive and also highly specific except in areas endemic for the New World parasite *Trypanosoma cruzi,* that may give false positive results; values approach 100% for both the parameters. ELISA is also very sensitive and specific when a combination of immunodominant, recombinant proteins are used as antigen, whereas it has slightly lower specificity when crude parasite lysates are employed instead.[130,136-138] Compared to IFAT, that is based on the evaluation of promastigote fluorescence at UV microscope and is therefore operator-dependent, ELISA is easier to standardize since results are read by an automated spectrophotometer. Both IFAT and ELISA have the advantage to provide quantitative results that are based on the final antibody titer (the last two-fold serial dilution of sample providing positive result) or, for ELISA only, on optical density values compared with reference titred samples. Owing to the unavoidable variability due to operator-dependent or analytical (antigen stability, antiserum or equipment performances) factors, reference sera with precise anti-*Leishmania* antibody titers are not universally available. Hence, a titer is considered "high" if it is 4 fold higher than the threshold value of the laboratory.[14] Similarly, 4 fold variations in titers of sequential samples of the same dog should be expected in

seroconversions, or in the outcome of therapy. Hence, sequential samples must be analyzed by the same method in the same laboratory.

Interpretation

Serological tests detect and quantify the presence of antibodies in serum or plasma. It should be noted that not every dogs will seroconvert after infection, and that it is difficult to measure precise times of seroconverion in naturally infected dogs. Antibodies can be found in blood as soon as 1 month after exposure to infected phlebotomines; the median time for seroconversion was estimated to be about 5 months in natural conditions and 3 months in experimental studies using artificial infection.[139] Therefore dogs living in highly endemic regions may seroconvert during the sand fly activity period (from late spring to early autumn in temperate zones, all over the year in tropical ones).[9] If the vector-transmitted parasites are efficiently controlled by the host's immune responses, the antibody titers, when present, tend to remain low and therefore these clinically-healthy dogs can be classified as exposed (when the infection is not confirmed by direct tests), or infected.[14] Conversely, the uncontrolled parasite dissemination is associated with an exaggerated humoral response and therefore antibody titers are high when the disease is evident. This condition is classified as "sick dog" or "severely sick dog" by CLWG classification,[14] and stage II, III or IV (mild, severe or very severe disease) by Leishvet classification.[15] Furthermore, a direct relationship between the clinical score and antibody titers exists.[89,140] However, low-medium antibody titers may also be detected in dogs with clinical signs . These have been classified as stage I or II (mild or moderate disease) according to the Leishvet classification.[15]

Therefore, quantitative serology should be always be performed when, despite strong clinical suspicion of leishmaniasis, lesions approachable by fine needle aspiration are not present or when cytological analysis of lesions, lymphoid organs and bone marrow fails to reveal the typical pattern associated with leishmaniasis, despite a possible PCR positivity. In this case a high antibody titer is often consistent with the disease, while, if the antibody titer is low, leishmaniasis should be

considered only if other diseases potentially responsible of the clinical presentation are ruled out.[14,15]

The increasing use in southern Europe of LiESP vaccination, known to elicit longstanding low-mid levels of antileishmanial antibodies, may complicate further the interpretation of serology in vaccinated dogs. Practical laboratory protocols aiming to discriminate between humoral responses in *Leishmania*- infected and LiESP-vaccinated dogs, are not yet available.

1) PCR

Methods

Several methods have been proposed to detect the presence of the parasite DNA in various biological samples. Some of these methods are not commonly used or recently validated, such as those based on the use of probes labelled with gold nanoparticles[141] or the loop-mediated isothermal amplification (LAMP).[142] Conversely, conventional PCR, nested PCR and quantitative (real time) PCR are widely used in routine practice.[14,15,132,137] PCR sensitivity and specificity varies according to the method and to the target DNA sequence. Most of the PCR tests currently used are targeting multicopy DNA sequences, such as the small subunit ribosomal RNA genes or the kinetoplast DNA minicircles, thus increasing the sensitivity of the test.[143] Compared with conventional and nested PCR, the quantitative PCR techniques offer two main advantages:[144] they may be run in close systems and are therefore less prone to contamination, and provide information about the copies of DNA that are present in the sample. This latter aspect may be relevant during the follow up to monitor the efficacy of leishmanicidal treatments and therefore it may advisable to use quantitative PCR at first diagnosis (before any treatment), in order to have a baseline value for further analyses during the follow up.[144,145] However, it does not seem that quantitative PCR techniques are more sensitive than conventional or nested PCR to diagnose leishmaniasis in dogs.[146] One additional limitation of quantitative PCR is that standardized methods to accurately quantify the DNA copies may not be offered by some laboratories.

Samples

PCR techniques may be applied virtually on any tissue or biological fluids. Theoretically, it may be superfluous to use molecular tests in affected tissues in which *Leishmania* amastigotes have been visualized by cytology or histology. However, these latter methods are less sensitive than PCR and therefore, a negative cytological result does not exclude that a low number of amastigotes is indeed present. Hence, when a fine needle aspirate or a tissue biopsy is performed, it may be advisable to prepare cytological or histological specimens and to store the remaining sample in the preservatives recommended by the laboratory to run PCR in case amastigotes are not visualized despite the cytological or histological pattern is highly consistent with leishmaniasis. If needed, PCR may also be performed on cytological material already fixed on glass slides[147] or on formalin fixed and paraffin embedded material.[148,149]

In routine practice PCR is rarely run on injured tissues, for which cytology and histology are preferred, but it may be done when cytology and histology do not demonstrate the parasite. When lesions are not present, or they are not approachable by fine needle aspiration or biopsy (for example when the prevalent clinical presentation is anemia or proteinuric nephropathy), bone marrow and/or lymph nodes and spleen provide the highest sensitivity in detecting *Leishmania* by PCR, especially in sick dogs,[15,150-154] pending that the quality of the sample is adequate. Recent studies demonstrated that conjunctival and, to a lesser extent, oral and nasal swabs are very sensitive for the detection of *Leishmania* DNA and, in addition, can provide positive results earlier than other tissues.[150,152,155-158] Buffy coat or whole blood may also be used for conventional or quantitative PCR analysis. Their sensitivity is lower than that the above tissues, but on the other hand blood collection is not much invasive and when positive it provides a diagnosis in a rapid and cheap way[14,15]

Interpretation

When interpreting PCR results it must be kept in mind the difference between infected and sick dogs. Ultimately, the detection of the parasite's DNA indicates that the dog is infected. The correlation between infection and disease should be based on the presence of clinical and laboratory abnormalities. From this perspective, the detection of *Leishmania* DNA in lesions with cytological or histological patterns highly consistent with leishmaniasis, or in blood or bone marrow of a dog with systemic signs of leishmaniasis supports the diagnosis of disease. Conversely, positive PCR results in dogs without signs clearly referable to leishmaniasis do not support the hypothesis that the infected dog is also affected by clinical leishmaniasis, unless any other possible disease is excluded. For example, a transient PCR-positivity in bone marrow may be found a few months since the natural exposure to sand fly bites, without necessarily meaning that the dogs is definitively infected, or even sick.[9] Similarly, PCR positivity in intact skin of dogs frequently exposed to vectors does not necessarily mean that dermal "contamination" by infectious bites will be followed by parasite dissemination throughout other body tissues.[10-13] Skin positive PCR results may in fact depend on the presence of recently-inoculated promastigotes, or of amastigotes phagocytosed by resident macrophages that, in resistant dogs, may efficiently control (or even eliminate) the agent at local level.[150,154,159]

2) Cytology

Samples and methods

Fine needle aspiration should be performed in all cases showing cutaneous papular or nodular lesions and/or lymph node enlargement.[14] Ulcerative cutaneous lesions can be sampled by scraping the lesion or using less invasive methods such as imprint smears. Additionally reports describing the presence of amastigotes and associated lesions in nodular masses with atypical localization, such as the tongue,[26,30] the testis,[160,161] and oral or nasal masses[162] have been reported and therefore any nodular lesion in dogs with clinical or laboratory signs potentially consistent with leishmaniasis (e.g. anemia, CKD, alterations of the electrophoretograms, positive serology) should be sampled by

fine needle aspiration. Nasal lesions may also be sampled using brush cytology[163] Similarly, when clinical or clinicopathological patterns are consistent with leishmaniasis, the possible presence of *Leishmania* should be investigated also in pathological body fluids such as joint fluids,[22,23] effusions,[36] or cerebrospinal fluid although in this latter sample, cellularity is usually so low that PCR may detect the parasite better than cytology.[61] When cutaneous lesions or nodular lesions in other organs, lymph node enlargement, abnormal accumulation of fluids are absent but the clinical suspicion of leishmaniasis is high, the presence of parasites should be investigated in organs rich of cells of the monocyte-macrophage system, such as bone marrow, lymph nodes or spleen[14,15,50]

Interpretation

Cytology aims to demonstrate the presence of *Leishmania* amastigotes within the macrophages or, when the parasite burden is high and cell lysis occurs, also on the background (figure 5). The detection of amastigotes may be difficult in cutaneous ulcerative lesions, where necrosis and cellular debris or contaminating bacteria may mask the presence of amastigotes. Attention should be paid to misinterpret as amastigotes cellular or granular debris that may be present in these lesions. Additionally, cytology may allow to detect the typical inflammatory patterns associated with leishmaniasis, that are usually characterized by granulocytic-macrophagic (pyogranulomatous) inflammation associated with a moderate to severe lymphoplasmocytic infiltration in skin or nodular lesions with atypical localization (figure 4) and, in lymph nodes, by a reactive hyperplasia of variable severity, characterized by lymphoplasmocytic and macrophagic infiltration, usually associated with numerous neutrophils.[50,164,165] Similarly, cytologic patterns typically associated with leishmaniasis may be found in the bone marrow, as described above. Neutrophils, lymphocytes and macrophages can be found also in body fluids of dogs affected by leishmaniasis.

The diagnosis of leishmaniasis is easy when amastigotes are detected in samples that show the cytologic patterns described above. However, when cytologic patterns consistent with leishmaniasis but no amastigotes are seen, leishmaniasis should not be ruled out, since it is known that the

564 diagnostic sensitivity of cytology is low.[132,137] In these cases, tests that have higher analytical and
565 diagnostic sensitivity, such as PCR, must be run. Alternatively, affected tissues can be biopsied to
566 perform histology and immunohistochemistry, as described below. Conversely, when amastigotes
567 are seen in the absence of cytological abnormalities, or cytology is done on bone marrow, lymph
568 node or spleen, positive results must be interpreted carefully, as systemic signs may be due to
569 diseases other than leishmaniasis.[14] Similarly, a diagnostic workup to differentiate "sick" from
570 "infected" dogs should be run when *Leishmania* is incidentally found in lesions that clearly have a
571 different origin. For example, several reports describe the association between the presence of
572 amastigotes and tumors such as lymphoma, transmissible veneral tumors and other types of
573 neoplasia.[166-171] On a practical standpoint in these cases it is important to understand if the dog is
574 affected by both diseases or affected by a neoplastic disease and simply infected with *Leishmania*.
575
576 3) *Histology*
577 Histology can demonstrate the presence of *Leishmania* in routinely hematoxylin and eosin stained
578 sections when cytology provides parasite-negative results in tissues having a cytological pattern
579 highly consistent with leishmaniasis. Compared with PCR, histology has two main disadvantages: it
580 is more laborious and time consuming, and the identification of amastigotes may be more difficult
581 than in cytological samples. As for the latter, amastigote presence can be confirmed by
582 immunohistochemistry (figure 6),[33,172] in situ hybridization[173,174] or PCR on formalin-fixed and
583 paraffin embedded samples.[148,149] On the other hand, histology has the advantage to provide
584 additional information on the cytoarchitectural pattern of the lesions. This is a great advantage since
585 it may allow to discriminate dogs in which the parasite is associated with typical lesions from those
586 in which the infection does not seem to be associated with the disease. Therefore, according to some
587 guidelines,[19] histology should always be performed. The interpretation of histological results is
588 facilitated by the elevated number of papers describing the distribution of parasites and the lesions
589 associated with active disease, mostly characterized by lymphoplasmacytic or granulomatous-

pyogranulomatous inflammations and/or by vasculitis either in organs usually affected by *Leishmania* (bone marrow, spleen, skin, lymph nodes, kidney, etc) but also in unusual sites such as heart, lung, adrenal gland, genital tract, central nervous system, skeletal muscle, gastrointestinal tract, nails, lacrimal glands and ocular muscles.[20,21,26-28,30,33,56,61,62,64,65,67,68,121,164,175-181]

4) Parasite culture and biological test for infectiousness (xenodiagnosis)

Conclusive diagnosis of active infection should be based on tissue cultures, which not only confirm whether dogs harbor parasites, but also demonstrate that the protozoa are viable. A diagnostic *Leishmania* culture requires biphasic blood-agar media that need fresh components.[132] A conclusive test for infectiousness (xenodiagnosis) requires that naive (laboratory-reared) sand flies are induced to feed on infected dogs and are examined thereafter for the presence of promastigotes in the gut.[182] However both tests are unpractical and restricted to specialized reference centers. Therefore these tests are mainly intended for research and cannot be recommended for routine practice.

Future perspectives

Several studies investigated the diagnostic potential of innovative markers in leishmaniotic dogs: for example, iron superoxide dismutase (Fe-SODe) secreted by the parasite has been evaluated as a possible marker of infection;[183] proteomic analysis revealed a series of proteins that are over- or under-represented in leishmaniotic dogs;[184] the expression level of cytokines or molecules such as leptin or inducible nitric oxide synthetase in blood or tissues is different in leishmaniotic dogs compared to controls[11,13,185-187] high levels of matrix metalloprotieinases have been reported in serum or CSF of leishmaniotic dogs.[188,189] Recently, the attention of researchers has been focused on markers of oxidative stress; inflammation is characterized by the release of reactive oxygen metabolites from phagocytes recruited in inflammatory sites and this leads to a consumption of antioxidant compounds.[190] Increases of oxidants or oxidized molecules (e.g. reactive oxygen metabolites, malonyldialdeide, lipoperoxides, thiobarbituric acid reacting substances) and decreases

of antioxidant compounds (total antioxidant capacity, trace elements, paraoxonase) have been reported in leishmaniotic dogs[99,102,103,191-194]

However, none of the studies cited above provided, to date, exhaustive information on the possible utility in practice of these markers. Nevertheless, preliminary results from these investigations are encouraging and useful to design future research to explore their potential clinical application.

Tests for monitoring the post-treatment follow up

Laboratory tests during the follow up should be focused in monitoring possible toxic effect of treatment as well as the clinical and the parasitological status of the patient following administration of drugs according to conventional treatments protocols. These mainly include the administration of antimonials or miltefosine, both in combination with allopurinol. Alternative drugs should be carefully considered only when conventional treatments are not effective.[195]

Monitoring the possible toxic effect of treatment

Theoretically, the possible toxic effects of treatment should be monitored. However, despite some studies reported possible nephrotoxicity of antimonials,[68,196] others did not confirm this finding,[197] and recent investigations demonstrated that no toxic effects on heart or pancreas are induced by these drug classes in dogs, differently from what is observed in humans.[198,199] Therefore, toxic effects should be monitored only in selected dogs, particularly when peculiar clinical findings are present or history might lead to hypothesize any drug adversity. The only possible adverse effect of allopurinol is the formation of xanthine crystals, and possibly urolithes, in urine.[200] These occur very frequently[201] and may be sometime abundant although associated clinical signs and urolith formation are not common and suspension of treatment is unusual. Therefore, the analysis of urine sediment should be always included in the laboratory workup when allopurinol is administered for a long time or when urine appears macroscopically turbid or forms an evident pellet after centrifugation (figure 7).

642

643 *Monitoring the clinical status*

644 Since the clinical presentation of leishmaniasis in dogs can be extremely variable, it is not possible
645 to define, *a priori*, a common and standardized laboratory procedure to be used during the follow
646 up. However, two main aspects must always be monitored, namely the presence of renal disease and
647 inflammation.

648 Renal function should be evaluated through the analysis of serum concentrations of creatinine and,
649 especially, through sequential quantification of proteinuria, due to its role as a risk factor for the
650 progression of CKD.[106] Proteinuria has been recently shown to be a negative prognostic factor in
651 leishmaniotic dogs.[90] After conventional leishmanicidal treatment, the degree of proteinuria
652 decreases in 4-8 weeks,[202] thus, additional pharmacological treatments for proteinuria should be
653 decided thereafter. The possibility to restore normal renal function depends on the severity of renal
654 damage at the time of first diagnosis. Therefore, creatinine and proteinuria should be repeatedly
655 assessed during the follow up. The frequency of testing depends on the severity of CKD: dogs in
656 IRIS stages 3 or 4[71] should be frequently tested also during the treatment period. Conversely, dogs
657 in IRIS stages 1 or 2[71] should be tested at the end of the first treatment cycle and then after 12
658 months in stage 1 dogs, every 6 months in dogs in stage 2, every 3 months in dogs in stage 3 and
659 every 6 weeks in dogs in stage 4.[203,204]

660 The inflammatory status may be monitored through sequential analysis of electrophoretograms and
661 of acute phase proteins, whereas the simple evaluation of total protein, albumin or A/G ratio, may
662 not be helpful because it is very likely that, despite treatment decreases globulin concentrations,
663 albumin concentrations remain low in dogs with persistent glomerular damage and proteinuria, in
664 turn leading to only minor changes in the A/G ratio. Differently, serum protein electrophoresis
665 allow to detect a progressive decrease of α-and γ-globulins. These decreases start to become evident
666 after 2-3 weeks and 4-6 weeks, respectively, following treatment with antimonials.[205] Therefore, the
667 first useful electrophoretogram to monitor the efficiency of treatment should be run not earlier than

668 one month after treatment begin.[203] The complete normalization of electrophoretograms, however,
669 requires at least 90-120 days.[200] If after 2-3 months the electrophoretograms still show abnormal
670 profiles, the possible presence of concurrent diseases such as other vector-borne diseases should be
671 considered, especially if the gammopathy tends to be characterized by narrower peaks (see figure
672 3). Treatments with miltefosine or with other drugs may require longer times to be beneficial (more
673 than 2 months to observe a decrease in γ-globulins) and are also characterized by more frequent
674 relapses after transient normalization of laboratory profiles.[206,207] Compared with serum protein
675 electrophoresis, monitoring the concentration of APPs provides earlier information regarding the
676 success of treatments with antimonials. CRP and SAA start to decrease in two weeks after treatment
677 and may return within the reference intervals in about one month.[100,101,205] The normalization of
678 PON-1 and HDL is even more rapid: significant increases may be observed 3-7 days after treatment
679 and values return within the reference intervals in two weeks.[82,205] Therefore, to assess the efficacy
680 of treatment, it may be advisable to measure the serum activity of PON-1 or the concentration of
681 HDLs or APPs 1-2 weeks after the first administration of drugs, when other clinical or
682 clinicopathological changes are likely still abnormal.
683
684 *Monitoring the parasitological status*
685 As at first diagnosis, the parasitological status can be monitored indirectly, through the assessment
686 of antibody titers, or by direct evaluation of the parasite presence.
687 In case of successful treatment, a decrease in antibody titers may be expected over time; hence,
688 serology should be repeated during the follow up.[203] Significant reduction in titers can be detected
689 already at 30 days post-treatment in sick or severely sick dogs with good clinical response to
690 therapy.[208,209] However, most of responders will show an evident decrease of titers around 6 months
691 from initiation of treatment,[200] With regard to serological results, it should be kept in mind that a
692 complete negativization of antileishmanial antibodies is unlikely, especially for dogs living in
693 endemic areas that may be repeatedly exposed to the parasite, boosting the antibody response.

694 Therefore, sequential serological tests during the follow up should aim to assess whether antibody
695 titers decrease to values consistent with the simple exposure (i.e. to less than 4 folds the threshold
696 value of the laboratory).[14]
697 In order to assess whether treatment completely eradicates the infection, ideally the presence of
698 parasites should be assessed in the tissues in which the parasite may establish a latent infection and
699 using very sensitive techniques. For this purpose the residual parasites burden should be evaluated
700 with repeated quantitative PCR analyses on bone marrow, spleen or lymph nodes, if still palpable.[14]
701 However this procedure is invasive and it is difficult that owners will accept the analysis, especially
702 if treatment has been successful and the dog looks clinically healthy. Therefore, in routine practice
703 the evaluation of treatment efficacy is usually assessed by serology or quantitative PCR analysis in
704 blood. If treatment has been successful, the latter test should show a clear decrease of the
705 *Leishmania* DNA copies after 3 to 6 months of therapy, with complete negativization between 6 and
706 12 months.[144]
707
708 ***Conclusive remarks and recommended protocols***
709 Diagnosing leishmaniasis in dogs may be difficult due to the complex pathogenesis and broad
710 spectrum of clinical and clinico-pathological findings. Hence,tests that need to be included in the
711 diagnostic protocol may vary according to case presentation or epidemiological scenario.[210]
712 In dogs with strong clinical suspicion of leishmaniasis, the use of quantitative serology is advisable,
713 as it can be conclusive for diagnosis when high-titer antibodies are detected. In clinically healthy
714 dogs living in or having travelled to an endemic area, again serology may be the test of choice to
715 assess any possible exposure to parasites. Based on the median time to achieve seroconversion,[139]
716 serology should be performed at least 6 months after exposure (e.g. in February-March where
717 transmission is seasonal, every 6-12 months where transmission is throughout the year). If serology
718 is positive, it is important to quantify the antibody response: a low antibody titer may be consistent
719 with exposure or an early phase of infection, while a high antibody titer can be suggestive of

720 infection or disease.[14,15] Therefore, the subsequent diagnostic steps should confirm the suspected
721 infection through cytological and PCR analysis of sensitive tissues, and/or on identification of
722 possible clinical or laboratory alterations, especially in dogs with high antibody titers. If serology or
723 PCR is positive and samplings have been performed during a non-transmission period, the
724 laboratory workup should aim to identify the most common abnormalities of dogs with
725 leishmaniasis in the absence of overt clinical signs (e.g. anemia, abnormal serum protein
726 electrophoresis, proteinuria). If changes are detected, additional clinical or laboratory tests must be
727 performed in order to stage the disease (e.g. tests recommended by the IRIS guidelines for CKD,[71]
728 tests to quantify the acute phase response or inflammation).
729 If the dog is examined because of clinical abnormalities, the veterinarian should try to sample any
730 accessible lesion to obtain cytological smears or biopsies.[15] If *Leishmania* amastigotes are
731 documented and the cytological or histological pattern is consistent with leishmaniasis the dog
732 should be considered sick. Thus, next diagnostic steps should clarify whether a systemic
733 involvement is also present (e.g. hematological disorders, inflammation, nephropathy) and the
734 antileishmanial antibodies and/or the parasite burden should be quantified with quantitative PCR to
735 obtain baseline values useful to treatment follow-up. Conversely, if amastigotes are not observed
736 but cytological patterns are consistent with leishmaniasis, the lesion can be further analyzed by
737 histology combined with immunohistochemistry, in situ hybridization or PCR.[14,15] A positive result
738 with one of these additional tests should lead to investigate the general health status of the sick dog.
739 Conversely, if these tests are negative, the presence of infection should be assessed in the bone
740 marrow through cytology and/or PCR and, in case of positive results, further clinco-pathological
741 tests should be performed as above.[14,15]
742
743 **Conflict of interest statement**

744 The authors are members of the Canine Leishmaniasis Working Group (CLWG). None of
745 the authors of this paper has a financial or personal relationship with other people or organisations
746 that could inappropriately influence or bias the content of the paper.
747
748 **Acknowledgements:**
749 The authors are grateful to Dr. Alessia Giordano for the collaboration in revising the
750 manuscript
751
752 **References**
753 1. Kuhls K, Alam MZ, Cupolillo E, Ferreira GE, et al. Comparative microsatellite typing of
754 New World *Leishmania infantum* reveals low heterogeneity among populations and its
755 recent Old World origin. *PLoS Negl Trop Dis.* 2011;5:e1155.
756 2. Rosypal AC, Troy GC, Zajac AM, Frank G, Lindsay DS. Transplacental transmission of a
757 North American isolate of *Leishmania infantum* in an experimentally infected beagle. *J*
758 *Parasitol.* 200;91:970-972.
759 3. De Freitas E, Melo MN, da Costa-Val AP, Michalick MSM. Transmission of *Leishmania*
760 *infantum* via blood transfusion in dogs: potential for infection and importance of clinical
761 factors. *Vet Parasitol.* 2006;137:159-167.
762 4. Silva FL, Oliveira RG, Silva TM, Xavier MN, Nascimento EF, Santos RL. Venereal
763 transmission of canine visceral leishmaniasis. *Vet Parasitol. 2009*;160:55-59.
764 5. Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, Gradoni L. Phlebotomine sandflies and
765 the spreading of leishmaniases and other diseases of public health concern. *Med Vet*
766 *Entomol.* 2013;27:123-147.
767 6. Bates PA. Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand
768 flies. *Int J Parasitol.* 2007;37:1097-1106.

7. Sacks D, Noben-Trauth N. The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nat Rev Immunol.* 2002;2:845–858;
8. Engwerda CR, Ato M, Kaye PM. Macrophages, pathology and parasite persistence in experimental visceral leishmaniasis. *Trends Parasitol.* 2004;20:524–530.
9. Oliva G, Scalone A, Foglia Manzillo V, et al. Incidence and time course of *Leishmania infantum* infections examined by parasitological, serologic, and nested-PCR techniques in a cohort of naive dogs exposed to three consecutive transmission seasons. *J Clin Microbiol.* 2006;44:1318-1322.
10. Pinelli E, Killick-Kendrick R, Wagenaar J, Bernadina W, del Real G, Ruitenberg J. Cellular and humoral immune responses in dogs experimentally and naturally infected with *Leishmania infantum*. *Infect Immun*. 1994;62:229-235.
11. Santos-Gomes GM, Rosa R, Leandro C, Cortes S, Romão P, Silveira H. Cytokine expression during the outcome of canine experimental infection by *Leishmania infantum*. *Vet Immunol Immunopathol.* 2002;88:21-30.
12. Brachelente C, Muller N, Doherr MG, Sattler U, Welle M. Cutaneous leishmaniasis in naturally infected dogs is associated with a T helper-2-biased immune response. *Vet Pathol.* 200;42:166-175.
13. Chamizo C, Moreno J, Alvar J. Semi-quantitative analysis of cytokine expression in asymptomatic canine leishmaniasis. *Vet Immunol Immunopathol.* 2005;103:67-75.
14. Paltrinieri S, Solano-Gallego L, Fondati A, et al. Guidelines for diagnosis and clinical classification of leishmaniasis in dogs. *J Am Vet Med Assoc.* 2010;236:1184-1191.
15. Solano-Gallego L, Koutinas A, Miró G, et al. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. *Vet Parasitol.* 2009;165:1-18.
16. Slappendel RJ: Canine leishmaniasis. A review based on 95 cases in The Netherlands. *Vet Q.* 1988;10:1-16.

794 17. Ciaramella P, Oliva G, Luna RD, et al. A retrospective clinical study of canine leishmaniasis
795 in 150 dogs naturally infected by *Leishmania infantum*. *Vet Rec.* 1997;141:539-543.
796 18. Koutinas AF, Polizopoulou ZS, Saridomichelakis MN, Argyriadis D, Fytianou A, Plevraki
797 KG. Clinical consideration on canine leishmaniasis in Greece: a retrospective study of 158
798 cases (1989-1996). *J Am Anim Hosp Assoc.* 1999;35:376-383.
799 19. Noli C, Saridomichelakis MN. An update on the diagnosis and treatment of canine
800 leishmaniosis caused by *Leishmania infantum* (syn. *L. chagasi*). *Vet J.* 2014;202:425-435.
801 20. Naranjo C, Fondevila D, Leiva M, Roura X, Peña T. Detection of *Leishmania spp.* and
802 associated inflammation in ocular-associated smooth and striated muscles in dogs with
803 patent leishmaniosis. *Vet Ophthalmol.* 2010;13:139-143.
804 21. Peña MT, Naranjo C, Klauss G, et al. Histopathological features of ocular leishmaniosis in
805 the dog. *J Comp Pathol.* 2008;138:32-39.
806 22. Sbrana S, Marchetti V, Mancianti F, Guidi G, Bennett D. Retrospective study of 14 cases of
807 canine arthritis secondary to *Leishmania* infection. *J Small Anim Pract.* 2014;55:309-13.
808 23. Santos M, Marcos R, Assuncao M, Matos AJ. Polyarthritis associated with visceral
809 leishmaniasis in a juvenile dog, *Vet Parasitol.* 2006;141:340-344.
810 24. Foglia Manzillo V, Di Muccio T, Cappiello S, et al. Prospective study on the incidence and
811 progression of clinical signs in naïve dogs naturally infected by *Leishmania infantum*. *PLoS*
812 *Negl Trop Dis.* 2013;;7:e2225.
813 25. Adamama-Moraitou KK, Rallis TS, Koytinas AF, Tontis D, Plevraki K, Kritsepi M.
814 Asymptomatic colitis in naturally infected dogs with *Leishmania infantum*: a prospective
815 study. *Am J Trop Med Hyg.* 2007;76:53-57.
816 26. Parpaglia ML, Vercelli A, Cocco R, Zobba R, Manunta ML. Nodular lesions of the tongue
817 in canine leishmaniosis. *J Vet Med A Physiol Pathol Clin Med.* 2007;54:414-417.
818 27. Silva FL, Rodrigues AA, Rego IO, et al. Genital lesions and distribution of amastigotes in
819 bitches naturally infected with *Leishmania chagasi*. *Vet Parasitol*. 2008;151:86-90.

820 28. Silva JF, Guimarães LB, Ribeiro LR, Moreira MV, Serakides R, Ocarino NM. Acute
821 oesophageal necrosis concurrent with *Leishmania chagasi* infection in a dog. *J Comp*
822 *Pathol.* 2014;;150:148-150.
823 29. Blavier A, Keroack S, Denerolle P, et al. Atypical forms of canine leishmaniosis. *Vet J.*
824 2001;162:108-120.
825 30. Viegas C, Requicha J, Albuquerque C, et al. Tongue nodules in canine leishmaniosis--a case
826 report. *Parasit Vectors.* 2012;5:120.
827 31. Mir F, Fontaine E, Reyes-Gomez E, Carlus M, Fontbonne A. Subclinical leishmaniasis
828 associated with infertility and chronic prostatitis in a dog. *J Small Anim Pract.* 2012;53:419-
829 422.
830 32. Ruiz G, Laloy E, Benchekroun G. Chronic gastritis and enterocolitis associated with
831 *Leishmania* infection in an 18-month-old, intact female dog. *Vet Q.* 2015; June 8:1-4 [Epub
832 ahead of print].
833 33. dos Santos JP, Alves LC, Ramos RA, et al. Histological changes and immunolabeling of
834 *Leishmania infantum* in kidneys and urinary bladder of dogs. *Rev Bras Parasitol Vet.*
835 2013;22:420-423.
836 34. Kiral, FK , Seyrek, K, Pasa, S, Ertabaklar, H, Unsal, C. Some haematological, biochemical
837 and electrophoretic findings in dogs with visceral leishmaniasis. *Revue Med Vet.*
838 2004;155:226-229.
839 35. Nicolato R de C, de Abreu RT, Roatt MB et al. Clinical forms of canine visceral
840 Leishmaniasis in naturally *Leishmania infantum*-infected dogs and related myelogram and
841 hemogram changes. *PLoS One.* 2013;8:e82947.
842 36. Ruiz de Gopegui, R, Espada Y. Peripheral Blood and Abdominal Fluid from a Dog with
843 Abdominal Distention. *Vet Clin Pathol.* 1998;27:64-67.
844 37. De Tommasi AS, Otranto D, Furlanello T, et al. Evaluation of blood and bone marrow in
845 selected canine vector-borne diseases. *Parasit Vectors*. 2014;7:534

846 38. Giudice E, Passantino A Detection of *Leishmania* amastigotes in peripheral blood from four
847 dogs. *Acta Vet Hung.* 2011;59:205-13.
848 39. Gavazza A , Lubas G , Gugliucci B , Pasquini A, Mancianti F. Hemogram and bone marrow
849 patterns in canine leishmaniasis. *Vet Clin Pathol.* 2002;31:198.
850 40. Smith BE, Tompkins MB, Breitschwerdt EB. Antinuclear antibodies can be detected in dog
851 sera reactive to *Bartonella vinsonii subsp. berkhoffii*, *Ehrlichia canis*, or *Leishmania*
852 *infantum* antigens. *J Vet Intern Med*. 2004;18:47-51.
853 41. Karagianni AE, Solano-Gallego L, Breitschwerdt EB, et al. Perinuclear antineutrophil
854 cytoplasmic autoantibodies in dogs infected with various vector-borne pathogens and in
855 dogs with immune-mediated hemolytic anemia. *Am J Vet Res.* 2012;73:1403-1409.
856 42. Terrazzano G, Cortese L, Piantedosi D, et al. Presence of anti - platelet IgM and IgG
857 antibodies in dogs naturally infected by *Leishmania infantum*. *Vet Immunol Immunopathol*.
858 2006;110:331-337.
859 43. Cortese L, Sica M, Piantedosi D, et al.: Secondary immune-mediated thrombocytopenia in
860 dogs naturally infected by *Leishmania infantum. Vet Rec.* 2009;164:778-782.
861 44. Cortese L, Terrazzano G, Piantedosi D, et al. Prevalence of anti-platelet antibodies in dogs
862 naturally co-infected by *Leishmania infantum* and *Ehrlichia canis*. *Vet J.* 2011;188:118-21.
863 45. Green RA, Kabal AL. Hypercoagulable state in three dogs with nephrotic syndrome: role of
864 acquired antithrombin III deficiency. *J Am Vet Med Assoc.* 1982;181:914-917.
865 46. Honse CO, Figueiredo FB, de Alencar NX, Madeira M de F, Gremião ID, Schubach TM.
866 Disseminated intravascular coagulation in a dog naturally infected by *Leishmania*
867 *(Leishmania) chagasi* from Rio de Janeiro - Brazil. *BMC Vet Res.* 2013;9:43.
868 47. Abid M, Kalbantner K, Mischke R. Platelet function in dogs with bacterial infections and
869 leishmaniasis. *Berl Munch Tierarztl Wochenschr.* 2015;128:289-296.

48. Papadogiannakis E, Andritsos G, Kontos V, Spanakos G, Koutis C, Velonakis E. Determination of CD4+ and CD8+ T cells in the peripheral blood of dogs with leishmaniosis before and after prolonged allopurinol monotherapy. *Vet J.* 2010;186:262-263.
49. Rosypal AC, Gogal RM Jr, Zajac AM, Troy GC, Lindsay DS. Flow cytometric analysis of cellular immune responses in dogs experimentally infected with a North American isolate of *Leishmania infantum*. *Vet Parasitol.* 2005;131:45-51.
50. Saridomichelakis MN, Mylonakis ME, Leontides LS, Koutinas AF, Billinis C, Kontos VI. Evaluation of lymph node and bone marrow cytology in the diagnosis of canine leishmaniasis (*Leishmania infantum*) in symptomatic and asymptomatic dogs. *Am J Trop Med Hyg.* 2005;73:82-86.
51. Momo C, Jacintho AP, Moreira PR, Munari DP, Machado GF, Vasconcelos Rde O. Morphological changes in the bone marrow of the dogs with visceral leishmaniasis. *Vet Med Int.* 2014;2014:150582.
52. Trópia de Abreu R, Carvalho Md, Carneiro CM, et al. Influence of clinical status and parasite load on erythropoiesis and leucopoiesis in dogs naturally infected with *leishmania (Leishmania) chagasi*. PLoS One. 2011;6:e18873.
53. Stockham SL, Scott MA. Bone marrow and lymph node. In: Stockham SL, Scott MA, eds. *Fundamentals of veterinary clinical pathologyy*, 2nd ed. Ames, IA: Blackwell Publishing; 2008:323-368.
54. Foglia Manzillo V , Restucci B , Pagano A , Gradoni L, Oliva G. Pathological changes in the bone marrow of dogs with leishmaniasis. *Vet Rec.* 2006;158:690-694.
55. Lennon EM, Hanel RM, Walker JM, Vaden SL. Hypercoagulability in dogs with protein-losing nephropathy as assessed by thromboelastography. *J Vet Intern Med.* 2013;27:462-468.
56. Bruno B, Maurella C, Falco S, et al. Thromboelastometric evaluation of hemostasis in dogs infected with *Leishmania infantum*. *J Vet Emerg Crit Care (San Antonio)*. 2015;25:502-511.

57. Brooks AC, Guillaumin J, Cooper ES, Couto CG. Effects of hematocrit and red blood cell-independent viscosity on canine thromboelastographic tracings. *Transfusion*. 2014;54:727-734.

58. McMichael MA, Smith SA, Galligan A, Swanson KS. In vitro hypercoagulability on whole blood thromboelastometry associated with in vivo reduction of circulating red cell mass in dogs. *Vet Clin Pathol.* 2014;43:154-163.

59. Vamvakidis CD1, Koutinas AF, Kanakoudis G, Georgiadis G, Saridomichelakis M. Masticatory and skeletal muscle myositis in canine leishmaniasis (*Leishmania infantum*). *Vet Rec.* 2000;146:698-703.

60. Paltrinieri S., Cazzaniga S., Pinto da Cunha N., Giordano A. Electrophoretic fractionation of creatine kinase isoenzymes and macroenzymes in clinically healthy dogs and cats and preliminary evaluation in central neurologic diseases. *Vet Clin Pathol.* 2010;39:329-333.

61. Márquez M1, Pedregosa JR, López J, Marco-Salazar P, Fondevila D, Pumarola M. *Leishmania* amastigotes in the central nervous system of a naturally infected dog. *J Vet Diagn Invest.* 2013;25:142-146.

62. José-López R1, la Fuente CD, Añor S. Presumed brain infarctions in two dogs with systemic leishmaniasis. *J Small Anim Pract.* 2012;53:554-557.

63. Silvestrini P, Piviani M, Alberola J, et al. Serum cardiac troponin I concentrations in dogs with leishmaniasis: correlation with age and clinicopathologic abnormalities. *Vet Clin Pathol*. 2012;41:568–574.

64. Rosa FA1, Leite JH, Braga ET, et al. Cardiac lesions in 30 dogs naturally infected with *Leishmania infantum chagasi*. *Vet Pathol.* 2014;51:603-606.

65. Momo C, Rocha NA, Moreira PR, et al. Morphological changes and parasite load of the adrenal from dogs with visceral leishmaniasis. *Rev Bras Parasitol Vet.* 2014;23:30-35.

66. Saridomichelakis MN, Xenoulis PG, Chatzis MK, et al. Thyroid function in 36 dogs with leishmaniosis due to *Leishmania infantum* before and during treatment with allopurinol with or without meglumine antimonate. *Vet Parasitol.* 2013;197:22-28.
67. Zatelli A, Borgarelli M, Santilli R, et al. Glomerular lesions in dogs infected with *Leishmania* organisms. *Am J Vet Res.* 2003;64:558-561.
68. Aresu L, Benali S, Ferro S, et al. Light and electron microscopic analysis of consecutive renal biopsy specimens from *leishmania*-seropositive dogs. *Vet Pathol*. 2013;50:753-760.
69. Koutinas AF, Koutinas CK. Pathologic mechanisms underlying the clinical findings in canine leishmaniasis due to *Leishmania infantum/chagasi*. *Vet Pathol.* 2014;51:527-538.
70. Braga ET, Leite JH, Rosa FA, et al. Hypertension and its correlation with renal lesions in dogs with leishmaniosis. *Rev Bras Parasitol Vet.* 2015;24:45-51.
71. International renal Interest Society (IRIS): Guidelines for staging chronic kidney disease (CKD). Available at: www.iris-kidney.com. Accessed, October 20, 2015
72. Ribeiro RR, da Silva SM, Fulgêncio Gde O, Michalick MS, Frézard FJ. Relationship between clinical and pathological signs and severity of canine leishmaniasis. *Rev Bras Parasitol Vet.* 2013;22:373-378.
73. Stockham SL, Scott MA. Urinary system. In: Stockham SL, Scott MA, eds. *Fundamentals of veterinary clinical pathologyy*, 2nd ed. Ames, IA: Blackwell Publishing; 2008:415-494.
74. Von Hendy-Willson VE, Pressler BM. An overview of glomerular filtration rate testing in dogs and cats. *Vet J.* 2011;188:156-165.
75. Pasa S, Bayramli G, Atasoy A, Karul A, Ertug S, Ozensoy Toz S. Evaluation of serum cystatin-C in dogs with visceral leishmaniasis. *Vet Res Commun.* 2009;33:529-534.
76. Monti P, Benchekroun G, Berlato D, Archer J. Initial evaluation of canine urinary cystatin C as a marker of renal tubular function. *J Small Anim Pract.* 2012;53:254-259.

77. García-Martínez JD, Martinez-Subiela S, Tvarijonaviciute A, Caldin M, Ceron JJ. Urinary ferritin and cystatin C concentrations at different stages of kidney disease in leishmaniotic dogs. *Res Vet Sci.* 2015;99:204-207.

78. Jepson RE, Syme HM, Vallance C, Elliott J., Plasma asymmetric dimethylarginine, symmetric dimethylarginine, l-arginine, and nitrite/nitrate concentrations in cats with chronic kidney disease and hypertension. *J Vet Intern Med.* 2008;22:317-324.

79. Nabity MB, Lees GE, Boggess MM, et al. Symmetric Dimethylarginine Assay Validation, Stability, and Evaluation as a Marker for the Early Detection of Chronic Kidney Disease in Dogs. *J Vet Intern Med.* 2015;29:1036-1044.

80. Stanger, O., Herrmann, W., Pietrzik, K., et al. DACH-LIGA Homocystein (German, Austrian and Swiss Homocysteine Society): Consensus paper on the rational clinical use of homocysteine, folic acid and B-vitamins in cardiovascular and thrombotic diseases: Guidelines and recommendations. *Clin Chem Lab Med.* 2003;41:1392-1403.

81. Barton M, Yanagisawa M. Endothelin: 20 years from discovery to therapy. *Can J Physiol Pharmacol.* 2008;86:485-498.

82. Ceron JJ, Eckersall PD, Martinez-Subiela S. Acute phase proteins in dogs and cats: Current knowledge and future perspectives. *Vet Clin Pathol.* 2005;34:85-99.

83. Raila J, Schweigert FJ, Kohn B. C-reactive protein concentrations in serum of dogs with naturally occurring renal disease. *J Vet Diagn Invest.* 2011;23:710-715.

84. Rossi S, Rossi G, Giordano A, Paltrinieri S. Homocysteine measurement by an enzymatic method and potential role of homocysteine as a biomarker in dogs. *J Vet Diagn Invest*. 2008;20:644-649.

85. Rossi G, Giordano A, Breda S, et al. Big-endothelin 1 (Big ET-1) and homocysteine in the serum of dogs with chronic kidney disease. *Vet J.* 2013;198:109-115.

968 86. Martínez-Subiela S, García-Martínez JD, Tvarijonaviciute A, et al. Urinary C reactive
969 protein levels in dogs with leishmaniasis at different stages of renal damage. *Res Vet Sci.*
970 2013;95:924-929.
971 87. Tvarijonaviciute A, Ceron JJ, Martinez-Subiela S, García-Martinez JD. Serum and urinary
972 adiponectin in dogs with renal disease from leishmaniasis. *Vet Rec.* 2012;171:297.
973 88. Almeida MA, Jesus EE, Sousa-Atta ML, Alves LC, Berne ME, Atta AM. Clinical and
974 serological aspects of visceral leishmaniasis in northeast Brazilian dogs naturally infected
975 with *Leishmania chagasi. Vet Parasitol.* 2005;127:227-232.
976 89. Proverbio D, Spada E, Bagnagatti de Giorgi G, Perego R, Valena E. Relationship between
977 *Leishmania* IFAT titer and clinicopathological manifestations (clinical score) in dogs.
978 *Biomed Res Int.* 2014;2014:412808.
979 90. Geisweid K, Mueller R, Sauter-Louis C, Hartmann K. Prognostic analytes in dogs with
980 *Leishmania infantum* infection living in a non-endemic area. *Vet Rec.* 2012;171:399.
981 91. Vailati Facchini R, Bertazzolo W, Zuliani D, et al. Detection of biclonal gammopathy by
982 capillary zone electrophoresis in a cat and a dog with plasma cell neoplasia. *Vet Clin Pathol*.
983 2010;39:440-446.
984 92. Font A , Closa JM , Mascort J . Monoclonal gammopathy in a dog with visceral
985 leishmaniasis. *J Vet Int Med.* 1994;8:233-235.
986 93. Giordano A, Paltrinieri S. Interpretation of capillary zone electrophoresis compared with
987 cellulose acetate and agarose gel electrophoresis: reference intervals and diagnostic
988 efficiency in dogs and cats. *Vet Clin Pathol.* 2010;39:464-473.
989 94. Antognoni MT, Birettoni F, Miglio A, Lalli P, Porciello F, Mangili Pecci V. Monoclonal
990 gammopathy associated with multiple myeloma and visceral leishmaniasis in the dog: a
991 comparison of two cases. *Vet Res Commun.* 2010;34 Suppl 1:S97-101.

95. Geigy C, Riond B, Bley CR, Grest P, Kircher P, Lutz H. Multiple myeloma in a dog with multiple concurrent infectious diseases and persistent polyclonal gammopathy. *Vet Clin Pathol.* 2013;42:47-54.

96. Martínez-Subiela S, Tecles F, Eckersall PD, Cerón JJ. Serum concentrations of acute phase proteins in dogs with leishmaniasis. *Vet Rec.* 2002;150:241-244.

97. Martinez-Subiela S, Strauss-Ayali D, Cerón JJ, Baneth G. Acute phase protein response in experimental canine leishmaniasis. *Vet Parasitol.* 2011;180:197-202.

98. Silvestrini P, Zoia A, Planellas M, et al. Iron status and C-reactive protein in canine leishmaniasis. *J Small Anim Pract.* 2014;55:95-101.

99. Martinez-Subiela S, Cerón JJ, Strauss-Ayali D, et al. Serum ferritin and paraoxonase-1 in canine leishmaniosis. *Comp Immunol Microbiol Infect Dis.* 2014;37:23-29.

100. Martínez-Subiela S, Bernal LJ, Cerón JJ. Serum concentrations of acute-phase proteins in dogs with leishmaniosis during short-term treatment. *Am J Vet Res.* 2003;64:1021-1026.

101. Sasanelli M, Paradies P, de Caprariis D, et al. Acute-phase proteins in dogs naturally infected with *Leishmania infantum* during and after long-term therapy with allopurinol. *Vet Res Commun.* 2007;31 Suppl 1:335-338.

102. Rossi G, Giordano A, Pezzia F, Kjelgaard-Hansen M, Paltrinieri S. Serum paraoxonase activity (PON1) in dogs: preanalytical and analytical factors and correlation with C-reactive protein and alpha-2-globulin. *Vet Clin Pathol.* 2013;42:329-341.

103. Ibba F, Rossi G, Meazzi S, Giordano A, Paltrinieri S. Serum concentration of high density lipoproteins (HDLs) in leishmaniotic dogs. *Res Vet Sci.* 2015;98:89-91.

104. Tonin AA, Calado AM, Bottari NB, et al. Novel markers of inflammatory response and hepatic dysfunction in canine leishmaniasis. *Comp Immunol Microbiol Infect Dis.* 2015 pii: S0147-9571(15)00064-8. doi: 10.1016/j.cimid.2015.09.004. [Epub ahead of print]

105. Vaden SL, Pressler BM, Lappin MR, Jensen WA. Effects of urinary tract inflammation and sample blood contamination on urine albumin and total protein concentrations in canine urine samples. *Vet Clin Pathol.* 2004;33:14-19.

106. Jacob F, Polzin DJ, Osborne CA, et al. Evaluation of the association between initial proteinuria and morbidity rate or death in dogs with naturally occurring chronic renal failure. *J Am Vet Med Assoc.* 2005;226:393-400.

107. Lees GE, Brown SA, Elliott J, Grauer GE, Vaden SL; American College of Veterinary Internal Medicine. Assessment and management of proteinuria in dogs and cats: 2004 ACVIM Forum Consensus Statement (small animal). *J Vet Intern Med.* 2005;19:377-385.

108. Beatrice L, Nizi F, Callegari D, et al. Comparison of urine protein-to-creatinine ratio in urine samples collected by cystocentesis versus free catch in dogs. *J Am Vet Med Assoc.* 2010;236:1221-1224.

109. Zatelli A, Paltrineiri S, Nizi F, Roura X, Zini E. Evaluation of a urine dipstick test for confirmation or exclusion of proteinuria in dogs. *Am J Vet Res.* 2010;71:235-240.

110. IRIS Canine GN Study Group Diagnosis Subgroup, Littman MP, Daminet S, Grauer GF, Lees GE, van Dongen AM. Consensus recommendations for the diagnostic investigation of dogs with suspected glomerular disease. *J Vet Intern Med.* 2013;;27 Suppl 1:S19-526.

111. Rossi G, Giori L, Campagnola S, Zatelli A, Zini E, Paltrinieri S. Evaluation of factors that affect analytic variability of urine protein-to-creatinine ratio determination in dogs. *Am J Vet Res.* 2012;73:779-788.

112. Rossi G, Bertazzolo W, Dondi F, et al. Inter-laboratory variability in protein:creatinine (UPC) ratio on canine urine. *Vet J.* 2015;204:66-72.

113. Rossi G, Bertazzolo W, Binnella M, Scarpa P, Paltrinieri S. Measurement of proteinuria in dogs: analytical and diagnostic differences using two laboratory methods *Vet Clin Pathol.* In press

114. LeVine DN, Zhang D, Harris T, Vaden SL. The use of pooled vs serial urine samples to measure urine protein:creatinine ratios. *Vet Clin Pathol.* 2010;39:53-56.

115. Smets PM, Meyer E, Maddens BE, Duchateau L, Daminet S. Urinary markers in healthy young and aged dogs and dogs with chronic kidney disease. *J Vet Intern Med.* 2010;24:65-72.

116. Schultze,AE, Jensen RK. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of canine urinary proteins for the analysis and differentiation of tubular and glomerular diseases. *Vet Clin Pathol.* 1998;18:93-97.

117. Brown JS, Nabity MB, Brock R, Cianciolo R, Lees GE. Comparison of urine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with renal histological findings and clinicopathologic data in dogs with renal diseases. *Vet Clin Pathol.* 2010;39:556.

118. Zini E, Bonfanti U, Zatelli A. Diagnostic relevance of qualitative proteinuria evaluated by use of sodium dodecyl sulfate-agarose renal histologic findings in dogs. *Am J Vet Res.* 2004;65:964–971.

119. Giori L, Tricomi FM, Zatelli A, Roura X, Paltrinieri S. High-resolution gel electrophoresis and sodium dodecyl sulphate–agarose gel electrophoresis on urine samples for qualitative analysis of proteinuria in dogs. *J Vet Diagn Invest.* 2011;23:682-690.

120. Ibba F, Mangiagalli G, Paltrinieri S. Urinary gamma-glutamyl transferase (GGT) as a marker of tubular damage in dogs with canine leishmaniasis, using sodium dodecylsulphate (SDS) electrophoresis as a reference method. *Vet J.* 2016;210:88-91.

121. Bonfanti U, Zini E, Minetti E, Zatelli A. Free light-chain proteinuria and normal renal histopathology and function in 11 dogs exposed to *Leishmania infantum, Ehrlichia canis*, and *Babesia canis*. *J Vet Intern Med.* 2004;18:618-624.

122. Brunker JD, Ponzio NM, Payton ME. Indices of urine N-acetyl-beta-D-glucosaminidase and gamma-glutamyl transpeptidase activities in clinically normal adult dogs. *Am J Vet Res.* 2009;70:297-301.

123. Palacio J, Liste F, Gascón M. Enzymuria as an index of renal damage in canine leishmaniasis. *Vet Rec.* 1997;140:477-480.

124. García-Martínez JD, Tvarijonaviciute A, Cerón JJ, Caldin M, Martínez-Subiela S. Urinary clusterin as a renal marker in dogs. *J Vet Diagn Invest.* 2012;24:301-306.

125. Nabity MB, Lees GE, Cianciolo R, Boggess MM, Steiner JM, Suchodolski JS. Urinary biomarkers of renal disease in dogs with X-linked hereditary nephropathy. *J Vet Intern Med.* 2012;26:282-293.

126. Sousa S, Cardoso L, Reed SG, et al. Development of a fluorescent based immunosensor for the serodiagnosis of canine leishmaniasis combining immunomagnetic separation and flow cytometry. *PLoS Negl Trop Dis.* 2013;7:e2371.

127. Ker HG, Coura-Vital W, Aguiar-Soares RD, et al. Evaluation of a prototype flow cytometry test for serodiagnosis of canine visceral leishmaniasis. *Clin Vaccine Immunol.* 2013;20:1792-1798.

128. Ramos-Jesus J, Carvalho KA, Fonseca RA, et al. A piezoelectric immunosensor for *Leishmania chagasi* antibodies in canine serum. *Anal Bioanal Chem.* 2011;401:917-925.

129. Akhoundi B, Mohebali M, Shojaee S, et al. Rapid detection of human and canine visceral leishmaniasis: assessment of a latex agglutination test based on the A2 antigen from amastigote forms of *Leishmania infantum*. *Exp Parasitol.* 2013;133:307-313.

130. Maia C, Campino L. Methods for diagnosis of canine leishmaniasis and immune response to infection. *Vet Parasitol.* 2008;158:274-287.

131. Rosypal AC, Pick LD, Hernandez JO, Lindsay DS. Evaluation of a novel dried blood spot collection device (HemaSpot™) to test blood samples collected from dogs for antibodies to *Leishmania infantum*. *Vet Parasitol*. 2014;205:338-342.

132. EFSA AHAW Panel (EFSA Panel on Animal Health and Welfare), 2015. Scientific Opinion on canine leishmaniosis. EFSA Journal 2015;13:4075, 77 pp. doi:10.2903/j.efsa.2015.4075

133. Grimaldi G Jr, Teva A, Ferreira AL, et al. Evaluation of a novel chromatographic immunoassay based on Dual-Path Platform technology (DPP® CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. *Trans R Soc Trop Med Hyg.* 2012;106:54-59.

134. Sagols E, Martin V, Claret E, McGahie D, Ciusinier AM, Gueguen S. Evaluation of the humoral immune response after vaccination with LiESP/QA21 (CaniLeish): interest of *Leishmania* specific anti-kinesin antibodies detection. *Proceedings of the 2012 BSAVA Congress*, Birmingham, United Kingdom, April 12th-15th, 2012

135. Ferroglio E, Zanet S, Mignone W, Poggi M, Trisciuoglio A, Bianciardi P. Evaluation of a rapid device for serological diagnosis of *Leishmania infantum* infection in dogs as an alternative to immunofluorescence assay and Western blotting. *Clin Vaccine Immunol.* 2013;20:657-659.

136. Solano-Gallego L, Villanueva-Saz S, Carbonell M, Trotta M, Furlanello T, Natale Aserological diagnosis of canine leishmaniosis: comparison of three commercial ELISA tests (Leiscan, ID Screen and *Leishmania* 96), a rapid test (Speed Leish K) and an in-house IFAT. . *Parasit Vectors.* 2014;7:111.

137. Gradoni L, Gramiccia M. Leishmaniosis. In *Manual of diagnostic tests and vaccines for terrestrial animals*. World Organisation for Animal Health (OIE); 2008:240-250. (2014 update available at: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.08_LEISHMANIOSIS.pdf; accessed, October 20, 2015)

138. Rodríguez-Cortés A, Ojeda A, Francino O, López-Fuertes L, Timón M, Alberola J. *Leishmania* infection: laboratory diagnosing in the absence of a "gold standard". *Am J Trop Med Hyg.* 2010;82:251-256.

139. Moreno J, Alvar J. Canine leishmaniasis: epidemiological risk and the experimental model. *Trends Parasitol*. 2002;18:399-405.

140. dos-Santos WL, Jesus EE, Paranhos-Silva M, et al. Associations among immunological, parasitological and clinical parameters in canine visceral leishmaniasis: emaciation, spleen parasitism, specific antibodies and leishmanin skin test reaction. *Vet Immunol Immunopathol.* 2008;5:3-4.

141. Andreadou M, Liandris E, Gazouli M, et al. A novel non-amplification assay for the detection of *Leishmania spp*. in clinical samples using gold nanoparticles. *J Microbiol Methods.* 2014;96:56-61.

142. Chaouch M, Mhadhbi M, Adams ER, et al. Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of *Leishmania infantum* in canine leishmaniasis based on cysteine protease B genes. *Vet Parasitol.* 2013;198:78-84.

143. Cortes S, Rolao N, Ramada J, Campino L. PCR as a rapid and sensitive tool in the diagnosis of human and canine leishmaniasis using *Leishmania donovani* s.l.-specific kinetoplastid primers. *Trans R Soc Trop Med Hyg.* 2004;98:12-17.

144. Francino O, Altet L, Sanchez-Robert E, et al.: Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis. *Vet Parasitol.* 2006;137:214-221.

145. Martínez V, Quilez J, Sanchez A, Roura X, Francino O, Altet L. Canine leishmaniasis: the key points for qPCR result interpretation. *Parasit Vectors.* 2011;4:57.

146. Solcà Mda S, Guedes CE, Nascimento EG, et al. Qualitative and quantitative polymerase chain reaction (PCR) for detection of *Leishmania* in spleen samples from naturally infected dogs. *Vet Parasitol.* 2012;184:133-140.

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

147. Santos TR, Carreira VS, Ferrari HF, Moreira MA, Luvizotto MC. Comparison of PCR with stained slides of bone marrow and lymph nodes aspirates with suspect diagnosis for leishmaniasis. *Acta Trop*. 2014;140:137-140.

148. Roura X, Fondevila D, Sanchez A, Ferrer L. Detection of *Leishmania* infection in paraffin-embedded skin biopsies of dogs using polymerase chain reaction. *J Vet Diagn Invest.* 1999;11:385-387.

149. Muller N, Zimmermann V, Forster U, Bienz M, Gottstein B, Welle M. PCR-based detection of canine *Leishmania* infections in formalin-fixed and paraffinembedded skin biopsies: elaboration of a protocol for quality assessment of the diagnostic amplification reaction. *Vet Parasitol.* 2003;114:223-229.

150. Gradoni, L. The diagnosis of canine leishmaniasis. In: Killick-Kendrick R, ed. *Canine leishmaniasis: moving towards a solution. Proceedings of the 2nd International leishmaniasis Forum, Sevilla, Spain.* Boxmeer, NL. Intervet International. 2002:7-14.

151. Ramos RA, Ramos CA, Santos EM, et al. Quantification of *Leishmania infantum* DNA in the bone marrow, lymph node and spleen of dogs. *Rev Bras Parasitol Vet.* 2013;22:346-350.

152. Carvalho Ferreira AL, Carregal VM, de Almeida Ferreira S, Leite RS, de Andrade AS. Detection of *Leishmania infantum* in 4 different dog samples by real-time PCR and ITS-1 nested PCR. *Diagn Microbiol Infect Dis.* 2014;78:418-421.

153. Almeida AB, Sousa VR, Gasparetto ND, et al. Canine visceral leishmaniasis: diagnostic approaches based on polymerase chain reaction employing different biological samples. *Diagn Microbiol Infect Dis*. 2013;76:321-324.

154. Reis LE, Coura-Vital W, Roatt BM, et al. Molecular diagnosis of canine visceral leishmaniasis: a comparative study of three methods using skin and spleen from dogs with natural *Leishmania infantum* infection. *Vet Parasitol.* 2013;197:498-503.

Formattato: Inglese (Stati Uniti)

155. Ferreira Sde A, Ituassu LT, de Melo MN, de Andrade AS: Evaluation of the conjunctival swab for canine visceral leishmaniasis diagnosis by PCR-hybridization in Minas Gerais State, Brazil. *Vet Parasitol.* 2008;152:257-263.

156. Gramiccia M, Di Muccio T, Fiorentino E, et al.: Longitudinal study on the detection of canine *Leishmania* infections by conjunctival swab analysis and correlation with entomological parameters. *Vet Parasitol.* 2010;171:223-228.

157. Di Muccio T, Veronesi F, Antognoni MT, Onofri A, Piergili Fioretti D, Gramiccia M. Diagnostic value of conjunctival swab sampling associated with nested PCR for different categories of dogs naturally exposed to *Leishmania infantum* infection. *J Clin Microbiol.* 2012;50:2651-2659.

158. Ferreira Sde A, Almeida GG, Silva Sde O, et al. Nasal, oral and ear swabs for canine visceral leishmaniasis diagnosis: new practical approaches for detection of *Leishmania infantum* DNA. *PLoS Negl Trop Dis.* 2013;7:e2150.

159. Madeira MF, Figueiredo FB, Pinto AG, et al.: Parasitological diagnosis of canine visceral leishmaniasis: is intact skin a good target? *Res Vet Sci.* 2009;87:260-262.

160. Diniz SA, Melo MS, Borges AM, et al. Genital lesions associated with visceral leishmaniasis and shedding of *Leishmania sp.* in the semen of naturally infected dogs. *Vet Pathol.* 2005;42:650-658

161. Manna L, Paciello O, Morte RD, Gravino AE. Detection of *Leishmania* parasites in the testis of a dog affected by orchitis: case report. *Parasit Vectors.* 2012;5:216.

162. Levy E, Mylonakis ME, Saridomichelakis MN, Polizopoulou ZS, Psychogios V, Koutinas AF. Nasal and oral masses in a dog. *Vet Clin Pathol.* 2006;35:115-118

163. Caniatti M, da Cunha NP, Avallone G, et al. Diagnostic accuracy of brush cytology in canine chronic intranasal disease. *Vet Clin Pathol.* 2012;41:133-140.

164. Costa MM, Lima WG, Figueiredo MM, Michalick MS, Tafuri WL, Tafuri WL. Cervical, mandibular, and parotid lymph nodes of dogs naturally infected with *Leishmania*

Formattato: Inglese (Stati Uniti)

infantum: a histopathologic and immunohistochemistry study and its correlation with facial skin lesions. *Vet Pathol.* 2008;45:613-616.

165. Mylonakis ME, Papaioannou N, Saridomichelakis MN, Koutinas AF, Billinis C, Kontos VI. Cytologic patterns of lymphadenopathy in dogs infected with *Leishmania infantum*. *Vet Clin Pathol.* 2005;34:243-247.

166. Ferro S, Palmieri C, Cavicchioli L, De Zan G, Aresu L, Benali SL. *Leishmania* amastigotes in neoplastic cells of 3 nonhistiocytic canine tumors. *Vet Pathol.* 2013;50:749-752.

167. Kegler K, Habierski A, Hahn K, Amarilla SP, Seehusen F, Baumgärtner W. Vaginal canine transmissible venereal tumour associated with intra-tumoural *Leishmania spp*. amastigotes in an asymptomatic female dog. *J Comp Pathol.* 2013;149:156-161.

168. Marino G, Gaglio G, Zanghì A. Clinicopathological study of canine transmissible venereal tumour in leishmaniotic dogs. *J Small Anim Pract*. 2012;53:323-327.

169. Foglia Manzillo V, Pagano A, Guglielmino R, Gradoni L, Restucci B, Oliva G. Extranodal gammadelta-T-cell lymphoma in a dog with leishmaniasis. *Vet Clin Pathol.* 2008;37:298-301.

170. Catone G, Marino G, Poglayen G, Gramiccia M, Ludovisi A, Zanghì A. Canine transmissible venereal tumour parasitized by *Leishmania infantum*. *Vet Res Commun*. 2003;27:549-553.

171. Albanese F, Poli A, Millanta F, Abramo F. Primary cutaneous extragenital canine transmissible venereal tumour with *Leishmania*-laden neoplastic cells: a further suggestion of histiocytic origin? *Vet Dermatol.* 2002;13:243-246.

172. Toplu N, Aydogan A. An immunohistochemical study in cases with usual and unusual clinicopathological findings of canine visceral leishmaniosis. *Parasitol Res.* 2011;109:1051-1057.

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

173. Menezes RC, Figueiredo FB, Wise AG, et al. Sensitivity and specificity of in situ hybridization for diagnosis of cutaneous infection by *Leishmania infantum* in dogs. *J Clin Microbiol.* 2013;51:206-211.

174. Dinhopl N, Mostegl MM, Richter B, et al. In situ hybridisation for the detection of *Leishmania* species in paraffin wax-embedded canine tissues using a digoxigenin-labelled oligonucleotide probe. *Vet Rec.* 2011;169:525.

175. Nieto CG, Navarrete I, Habela MA, Serrano F, Redondo E. Pathological changes in kidneys of dogs with natural *Leishmania* infection. *Vet Parasitol.* 1992;45:33-47.

176. Koutinas AF, Carlotti DN, Koutinas C, Papadogiannakis EI, Spanakos GK, Saridomichelakis MN. Claw histopathology and parasitic load in natural cases of canine leishmaniosis associated with *Leishmania infantum*. *Vet Dermatol.* 2010;21:572-577

177. Naranjo C, Fondevila D, Altet L, et al. Evaluation of the presence of *Leishmania spp.* by real-time PCR in the lacrimal glands of dogs with leishmaniosis. Vet J. 2012;193:168-173.

178. Figueiredo MM, Moura EP, Costa MM, et al. Histopathological and parasitological investigations of ear healthy skin of dogs naturally and experimentally infected with *Leishmania (Leishmania) chagasi*. Histol Histopathol. 2010;25:877-887.

179. Moreira PR, Vieira LM, de Andrade MM, et al. Immune response pattern of the popliteal lymph nodes of dogs with visceral leishmaniasis. *Parasitol Res.* 2010;107:605-613.

180. Pinto AJ, Figueiredo MM, Silva FL, et al. Histopathological and parasitological study of the gastrointestinal tract of dogs naturally infected with *Leishmania infantum*. *Acta Vet Scand.* 2011;53:67.

181. Bardagí M, Fondevila D, Zanna G, Ferrer L. Histopathological differences between canine idiopathic sebaceous adenitis and canine leishmaniosis with sebaceous adenitis. *Vet Dermatol.* 2010;21:159-165.

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

182. Bongiorno G, Paparcone R, Foglia Manzillo V, Oliva G, Cuisinier AM, Gradoni L. Vaccination with LiESP/QA-21 (CaniLeish®) reduces the intensity of infection in *Phlebotomus perniciosus* fed on *Leishmania infantum* infected dogs--a preliminary xenodiagnosis study. *Vet Parasitol.* 2013;197:691-695.

183. Longoni SS, Sánchez-Moreno M, López JE, Marín C. *Leishmania infantum* secreted iron superoxide dismutase purification and its application to the diagnosis of canine Leishmaniasis. *Comp Immunol Microbiol Infect Dis.* 2013;36:499-506.

184. Britti D, Gaspari M, Massimini G, Casalinuovo F, Morittu VM, Cuda G. Proteomic analysis in canine leishmaniasis. *Vet Res Commun.* 2010;34 Suppl 1:S91-596.

185. Menezes-Souza D, Corrêa-Oliveira R, Guerra-Sá R, et al. Cytokine and transcription factor profiles in the skin of dogs naturally infected by *Leishmania (Leishmania) chagasi* presenting distinct cutaneous parasite density and clinical status. *Vet Parasitol.* 2011;177:39-49.

186. Di Loria A, Squillacioti C, De Luca A, et al. Increased leptin mRNA expression in the blood of dogs naturally infected by *Leishmania infantum*. *Vet J.* 2014;202:634-636.

187. dos Santos FR, Vieira PM, Correa-Oliveira R, et al. Qualitative and quantitative immunohistochemical evaluation of iNOS expression in the spleen of dogs naturally infected with *Leishmania chagasi*. *Parasitol Res.* 2011;108:1397-1403.

188. Melo GD, Marangoni NR, Marcondes M, Lima VM, Machado GF. High levels of serum matrix metalloproteinases in dogs with natural visceral leishmaniosis: a preliminary report. *Vet J.* 2011;188:243-245.

189. Marangoni NR, Melo GD, Moraes OC, Souza MS, Perri SH, Machado GF. Levels of matrix metalloproteinase-2 and metalloproteinase-9 in the cerebrospinal fluid of dogs with visceral leishmaniasis. *Parasite Immunol.* 2011;33:330-334.

190. Lugrin J, Rosenblatt-Velin N, Parapanov R, Liaudet L. The role of oxidative stress during inflammatory processes. *Biol Chem.* 2014;395:203-230.

Formattato: Inglese (Stati Uniti)

191. Heidarpour M, Soltani S, Mohri M, Khoshnegah J. Canine visceral leishmaniasis: relationships between oxidative stress, liver and kidney variables, trace elements, and clinical status. *Parasitol Res.* 2012;111:1491-1496.

192. Almeida BF, Narciso LG, Melo LM, et al. Leishmaniasis causes oxidative stress and alteration of oxidative metabolism and viability of neutrophils in dogs. *Vet J.* 2013;198:599-605.

193. Souza CC, Barreto Tde O, da Silva SM, et al. A potential link among antioxidant enzymes, histopathology and trace elements in canine visceral leishmaniasis. *Int J Exp Pathol.* 2014;95:260-270.

194. Paltrinieri S, Ravicini S, Rossi G, Roura X Serum concentration of the derivative of reactive oxygen metabolites (d-ROMs) in dogs with leishmaniosis. *Vet J.* 2010;186:393-395.

195. Oliva G, Roura X, Crotti A, et al. Guidelines for treatment of leishmaniasis in dogs. J Am Vet Med Assoc. 2010;236:1192-1199.

196. Bianciardi P, Brovida C, Valente M, et al. Administration of miltefosine and meglumine antimoniate in healthy dogs: clinicopathological evaluation of the impact on the kidneys. *Toxicol Pathol.* 2009;37:770-775.

197. Miró G, Oliva G, Cruz I, et al. Multicentric, controlled clinical study to evaluate effectiveness and safety of miltefosine and allopurinol for canine leishmaniosis. *Vet Dermatol.* 2009;20:397-404.

198. Xenoulis PG, Saridomichelakis MN, Chatzis MK, et al. Prospective evaluation of serum pancreatic lipase immunoreactivity and troponin I concentrations in *Leishmania infantum*-infected dogs treated with meglumine antimonate. *Vet Parasitol.* 2014;203:326-330.

Formattato: Inglese (Stati Uniti)

199. Luciani A, Sconza S, Civitella C, Guglielmini C. Evaluation of the cardiac toxicity of N-methyl-glucamine antimoniate in dogs with naturally occurring leishmaniasis. *Vet J.* 2013;196:119-121.

200. Torres M, Bardagí M, Roura X, Zanna G, Ravera I, Ferrer L. Long term follow-up of dogs diagnosed with leishmaniosis (clinical stage II) and treated with meglumine antimoniate and allopurinol. *Vet J.* 2011;188:346-351.

201. Torres M, Pastor J, Roura X, et al. Adverse urinary effects of allopurinol in dogs with leishmaniasis. *J Small Anim Pract.* 2016 Apr 26. doi: 10.1111/jsap.12484. [Epub ahead of print]

202. Pierantozzi M, Roura X, Paltrinieri S, Poggi M, Zatelli A. Variation of proteinuria in dogs with leishmaniasis treated with meglumine antimoniate and allopurinol: 53 cases (2006-2010). *J Am Anim Hosp Assoc.* 2013;49:231-236.

203. Roura X, Fondati A, Lubas G, et al. Prognosis and monitoring of leishmaniasis in dogs: A working group report. Vet J. 2013;198:43-47.

204. Goldstein RE, Brovida C, Fernández-Del Palacio MJ, et al, Consensus recommendations for treatment for dogs with serology positive glomerular disease. J Vet Intern Med. 2013;27 Suppl 1:S60-6.

205. Rossi G, Ibba F, Meazzi S, Giordano A, Paltrinieri S. Paraoxonase activity as a tool for clinical monitoring of dogs treated for canine leishmanias. *Vet J.* 2014:199:143-149.

206. Rougier S, Hasseine L, Delaunay P, Michel G, Marty P. One-year clinical and parasitological follow-up of dogs treated with marbofloxacin for canine leishmaniosis. *Vet Parasitol.* 2012;186:245-253.

207. Manna L, Corso R, Galiero G, Cerrone A, Muzj P, Gravino AE. Long-term follow-up of dogs with leishmaniosis treated with meglumine antimoniate plus allopurinol versus miltefosine plus allopurinol. *Parasit Vectors.* 2015;8:289.

Formattato: Inglese (Stati Uniti)

208. Oliva G, Gradoni L, Cortese L, et al., Comparative efficacy of meglumine antimoniate and aminosidine sulphate, alone or in combination, in canine leishmaniasis. *Ann Trop Med Parasitol.* 1998;92:165-171.

209. Solano-Gallego L, Di Filippo L, Ordeix L, et al. Early reduction of *Leishmania* infantum-specific antibodies and blood parasitemia during treatment in dogs with moderate or severe disease. *Parasit Vectors* 2016;9:235.

210. Morales-Yuste M, Morillas-Márquez F, Díaz-Sáez V, Barón-López S, Acedo-Sánchez C, Martín-Sánchez J. Epidemiological implications of the use of various methods for the diagnosis of canine leishmaniasis in dogs with different characteristics and in differing prevalence scenarios. *Parasitol Res.* 2012;111:155-164.

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Table 1: summary of the laboratory findings detectable in canine leishmaniasis.

	Typical abnormalities	Frequent abnormalities	Occasional abnormalities
Routine CBC - leukogram		Neutrophilia[34,35]	Lymphopenia; Lymphocytosis; Eosinophilia[35-37]
Routine CBC – erythrogram	Normocytic normochromic non regenerative anemia		Positive Coombs test or anti.RBC antibodies[17]
Routine CBC – thrombogram			Thrombocytopenia (check for co-infections)[42,43]
Bone Marrow cytology	Erythroid hypoplasia; Myeloid hyperplasia; Macrophage proliferation-hyperplasia; Presence of intracytoplasmic amastigotes; Plasmocytosis[24,35,37,39,50,51,52,53,54]	Megakaryocyte hyperplasia; Secondary dysmyelopoiesis (dyserythropoiesis or dysegakaryopoiesis, occasionally dysgranulopoiesis)[35,54]	
Hemostasis		Decreased ATIII	Increased PT and aPTT[46] Hypercoagulability detected by thromboelastography or thromboelastometry[55]

Routine clinical chemistry	Increase of creatinine and/or urea;[14-17,24,72,74-77,87,85] Hyperproteinemia with hypoalbuminemia and inverted A:G ratio[14,15,18,72,88,89,90]		Abnormalities in other biochemical analytes (depending on the localization of lesions)[16,34,59-64]
Serum protein electrophoresis	Polyclonal gammopathy[14,15,18,72,88,89,90]	Oligoclonal gammopathy[93]	Mono- or bi-clonal gammopathy[91,92,94,95]
Acute phase proteins and other markers of inflammation	Increase of CRP, SAA, Hp, Cp, Ferritin; decreases of TIBC[96-101]	Decreased of PON1 and HDL[102-104]	
Urinalysis	Proteinuria; decreased USG;[14-18] mixed proteinuria at SDS-electrophoresis[117-120]	Increase of marker of tubular damage (GGT, NAG)[120,123]	

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Figure captions

Figure 1: Dog, bone marrow aspirates summarizing the main findings in canine leishmaniasis: A) several amastigotes are seen in the cytoplasms of infected macrophages; B) free amastigotes in the background; C) infected macrophage with signs of erytrhophagia; D) infected macrophage with signs of cytophagia. E) myeloid hyperplasia and erythroid hypoplasia in a microscopic field on which infected macrophages are also detectable; F) severe plasmocytosis, myeloid hyperplasia and a Mott cell. In D and F, free amastigotes are also visible in the background (arrows). May Grünwald-Giemsa stain. Bar: 15 µm in A, B, C, 20 µm in D, F, 70 µm in E

Figure 2: Dog, bone marrow smears, examples of secondary dysmyelopoiesis associated with leishmaniasis. A) atypical mitosis in a specimen with an infected macrophage; B) myeloid hyperplasia and plasmocytosis, and atypical mitosis of an erythroid precursor (arrowhead) with evident signs of asynchronous maturation; C) dwarf megacaryocytes; D) emeperiploesis in a megakaryocytes. In A and C free amastigotes are visible on the background (arrows). May Grünwald-Giemsa stain. Bar: 20 µm in A and B, 60 µm in C and D.

Figure 3: examples of electrophoretograms obtained from dogs with leishmaniasis using agarose gel electrophoresis: A) normal canine elettrophoretogram for comparison (a = albumin; α_1, α_2, β_1, β_2, γ = globulin fractions); B) Severe increase of α_2- and γ-globulin, with polyclonal gammopathy; C) mild increase of α_2-globulin (detectable only in the early phase of the disease); D) Severe hypoalbuminemia and polyclonal gammopathy. Also β_2- globulins are likely increased in this case; E) Severe increase of α_2-globulins and polyclonal gammopathy with a prominent peak in the β_2-region and a less evident polyclonal peak in the γ- region; F) Very severe hypoalbuminemia and severe oligoclonal gammopathy. This dog was co-infected with *E. canis.*

Figure 4: comparison of electrophoretograms obtained with agarose gel electrophoresis (AGE, A and C) or with capillary zone electrophoresis (CZE, B and D). The electrophoretograms in A and B are from the same sample of a dog with leishmaniasis. The electrophoretic profile is similar but in CZE hypoalbuminemia is more evident and the γ-globulin peak is narrower, possibly generating a false diagnosis of oligo- or monoclonal gammopathy. The electrophoretograms in C and D are from the same sample of a dog with leishmaniasis. In this case, the γ-globulin peak is higher in CZE than in AGE and evidences a biclonal origin, with a very narrow subpeak on the right side of the γ-globulin fraction, possibly indicating a monoclonal component.

Figure 5: A) imprint of an ulcerated skin lesion from a dog with leishmaniasis The cytological pattern is consistent with pyogranulomatous inflammation (degenerated and non degenerated neutrophils, macrophages, lymphocytes and plasma cells). Variably sized pigmented material, likely depending on cytophagia may be found in the macrophage and on the background. This material may also be confused with amastigotes; B) cytocentrifuged synovial fluid from a dog with leishmaniasis presenting joint swelling. Amastigotes are visible in a large mononuclear cells with signs of nuclear degeneration. Neutrophils and lymphocytes, indicating an inflammatory process, and erythrocytes are also visible, C) fine needle aspirate of a spleen on which intracytoplasmic amastigotes are visible, along with plasma cells and neutrophils; D: fine needle aspirate of a lymph node from a dog with leishmaniasis. No amastigotes are visible but in this case the diagnosis is supported by the presence of reactive hyperplasia, characterized by variably sized lymphocytes, neutrophils and plasma cells. May Grünwald-Giemsa; Bars: 20µm in A, C, D, 15 µm in B.

Figure 6: Dog, dermis, immunohistochemical detection of amastigotes (brown dots) within the cytoplasm of macrophages. Immunohistochemistry, avidin-biotin peroxidase (ABC) method;

chromogen: diaminobenzidine; counterstain: Mayer's hematoxylin. Bar = 20µm. (Courtesy of Prof. Eugenio Scanziani, MAPLab, Fondazione Filarete, Milan, and Dr. Raffaella Bergottini – Helab – Milan).

Figure 7: Urine from a dog with leishmaniasis treated with allopurinol. Xanthine crystals appear as roundish brown-yellow crystals of different size, single or forming small to medium clusters. Unstained sediment. Bar: 15 µm. (Courtesy of Dr. Tiziana Vitiello, DiMeVet, University of Milan).