

## Original article

## Correlations between peripheral parasite load and common clinical and laboratory alterations in dogs with visceral leishmaniasis



R.B.P. Torrecilha<sup>a,\*</sup>, Y.T. Utsunomiya<sup>b</sup>, A.M. Bosco<sup>a</sup>, B.F. Almeida<sup>a</sup>, P.P. Pereira<sup>a</sup>,  
L.G. Narciso<sup>a</sup>, D.C.M. Pereira<sup>c</sup>, L. Baptistioli<sup>a</sup>, L. Calvo-Bado<sup>d</sup>, O. Courtenay<sup>d</sup>, C.M. Nunes<sup>c</sup>,  
P.C. Ciarlini<sup>a,\*</sup>

<sup>a</sup> Departamento de Clínica, Cirurgia e Reprodução Animal, Faculdade de Medicina Veterinária de Araçatuba, UNESP-Univ Estadual Paulista, Rua Clóvis Pestana 793, Dona Amélia, Araçatuba, SP, 16050-680, Brazil

<sup>b</sup> Departamento de Medicina Veterinária Preventiva e Reprodução Animal, Faculdade de Ciências Agrárias e Veterinárias, UNESP- Univ Estadual Paulista, Via de Acesso Prof. Paulo Donato Castellane s/n, Jaboticabal, SP, 14884-900, Brazil

<sup>c</sup> Departamento de Apoio, Produção e Saúde Animal, Faculdade de Medicina Veterinária de Araçatuba, UNESP- Univ Estadual Paulista, Rua Clóvis Pestana 793, Dona Amélia, Araçatuba, SP, 16050-680, Brazil

<sup>d</sup> School of Life Sciences and Warwick Infectious Disease and Epidemiology Research Group (WIDER), Gibbett Hill Road, CV47AL, Coventry, England, UK

## ARTICLE INFO

## Article history:

Received 20 June 2016

Received in revised form 22 August 2016

Accepted 23 August 2016

## Keywords:

*Canis lupus familiaris*

*Leishmania* spp

Hematology

Biochemistry

Oxidative stress

Principal components analysis

## ABSTRACT

Intensity of peripheral parasite infection has an important role in the transmission of *Leishmania* spp. from one host to another. As parasite load quantification is still an expensive procedure to be used routinely in epidemiological surveillance, the use of surrogate predictors may be an important asset in the identification of dogs with high transmitting ability. The present study examined whether common clinical and laboratory alterations can serve as predictors of peripheral parasitism in dogs naturally infected with *Leishmania* spp. Thirty-seven dogs were examined in order to establish correlations between parasite load (PL) in multiple peripheral tissues and common clinical and laboratory findings in canine visceral leishmaniasis (CVL). Quantitative polymerase chain reaction was employed to determine PL in conjunctival swabs, ear skin, peripheral blood and buffy coat. Additionally, a series of hematological, biochemical and oxidative stress markers were quantified. Correlations between net peripheral infection and severity of clinical alterations and variation in laboratory parameters were assessed through a new analytical approach, namely Compressed Parasite Load Data (CPLD), which uses dimension reduction techniques from multivariate statistics to summarize PL across tissues into a single variable. The analysis revealed that elevation in PL is positively correlated with severity of clinical sings commonly observed in CVL, such as skin lesions, ophthalmic alterations, onychogriphosis, popliteal lymphadenomegaly and low body mass. Furthermore, increase in PL was found to be followed by intensification of non-regenerative anemia, neutrophilia, eosinopenia, hepatic injury and oxidative imbalance. These results suggest that routinely used clinical and laboratory exams can be predictive of intensity of peripheral parasite infection, which has an important implication in the identification of dogs with high transmitting ability.

© 2016 Elsevier B.V. All rights reserved.

## 1. Introduction

Visceral leishmaniasis is an anthroponosis caused by parasites of the *Leishmania* genus (Solano-Gallego et al., 2011). As the parasite is transmitted from one host to another by the bites of *Lutzomyia longipalpis* sandflies, the intensity of peripheral parasitism is an important contributor to the transmitting ability of the host.

The main urban reservoir of the disease is the domestic dog (*Canis lupus familiaris*), and the presence of infected dogs in the vicinity of humans has been incriminated as a risk factor for human leishmaniasis (Solano-Gallego et al., 2009).

Clinical staging in canine visceral leishmaniasis (CVL) has been shown to be associated with parasite density in specific tissue compartments, such as skin (de Almeida Ferreira et al., 2012), lymph nodes (Reale et al., 1999), spleen (Solcà et al., 2012) and bone marrow (Reis et al., 2006). *Leishmania* spp. infection has also been found to alter markers of hematological, biochemical and oxidative homeostasis in dogs (Almeida et al., 2013b; Nicolato et al., 2013; Reis et al., 2006). However, it remains unclear at what extent the

\* Corresponding authors.

E-mail addresses: [rafaelatorrecilha@gmail.com](mailto:rafaelatorrecilha@gmail.com) (R.B.P. Torrecilha), [ciarlini@fmva.unesp.br](mailto:ciarlini@fmva.unesp.br) (P.C. Ciarlini).

occurrence of clinical signs and variation in laboratory parameters translates into peripheral parasite load and consequently to transmission risk. This information is a key first step in the identification of highly infected reservoirs in endemic areas, as peripheral parasite load (PL) quantification techniques are still costly to be routinely used in epidemiological surveillance.

Here, we aimed at contributing to the further elucidation of correlations between parasite load and laboratory/clinical alterations in dogs naturally infected with *Leishmania* spp. Additionally, as the independent analysis of PL in specific compartments can be misleading, we developed a strategy to compile PL quantified in multiple tissues to have a general picture of the intensity of peripheral infection of an animal.

## 2. Material and methods

### 2.1. Ethics statement

The study was carried out in strict accordance with the recommendations in the Ethical Principles of the Brazilian College of Animal Experimentation (COBEA – <http://www.cobea.org.br>). The protocol was approved by the UNESP Ethics Committee on Animal Experimentation (CEUA-FOA permit number: FOA- 01984/12).

### 2.2. Data collection

Thirty seven adult mixed-breed dogs from the Center for Zoonosis Control of Araçatuba (Sao Paulo, Brazil) were evaluated to determine the severity of common clinical alterations found in leishmaniasis, especially tegumentary (including dermatitis, body alopecia, periocular alopecia or lunettes, hyperkeratosis, ulcerative lesions and onychogryphosis) and ophthalmic alterations (including uveitis and conjunctivitis), lymphadenomegaly, emaciation (evaluated by visual inspection of prominent ribs and lumbar vertebrae, as well as atrophy of the temporal muscle) and mucosa paleness (as a proxy for anemia). All dogs had infection status pre-determined by the detection of antibodies anti-*Leishmania* spp. with the enzyme-linked immunosorbent assay technique (Lima et al., 2003). Severity of clinical alterations was assigned in a discrete ordered scale and based on consensus scoring by two veterinary experts.

Blood samples were obtained via jugular venipuncture and used for PL estimation, complete blood cell count, biochemical analysis and quantification of oxidative stress markers, following protocols described elsewhere (Almeida et al., 2013b; Aycicek et al., 2005; Erel, 2005, 2004; Francino et al., 2006; Hunter et al., 1985; Jain, 1986; Kaneko et al., 1997). Bilateral conjunctiva swabs and skin biopsies (i.e., ear punching) were also collected for PL quantification. For details concerning the procedures used here, please see the *Extended Methods* section in the Supplementary information file.

### 2.3. Statistical analyses

Parasite load data was transformed to  $\log_{10}(\text{PL} + 1)$  prior to the statistical analyses. Additionally, in order to facilitate the multivariate analysis of PL across multiple tissues, we developed a new approach based on Principal Components Analysis to summarize all data into a single representative synthetic variable, namely Compressed Parasite Load Data (CPLD). Briefly, a singular value decomposition of the input matrix of PL across tissues was performed in order to obtain the leading principal component (i.e., PC1), which was used as a proxy for the intensity of net peripheral infection. This simplified our statistical assessment and allowed for obtaining insights about the correlations between peripheral parasitism and routinely collected exams.

Correlations between clinical and laboratory parameters and PL across tissues were tested using a permutation test for Spearman's correlations with 10,000 randomizations each. For all analyses, we considered significant  $P < 0.05$ , whereas  $0.05 \leq P < 0.10$  were considered suggestive correlations. For details concerning the statistical analyses, as well as R v3.2.1 (available at: <http://www.r-project.org/>) scripts for the computation of CPLD and permutation tests, please see the *Extended Methods* section in the Supplementary information file.

## 3. Results

### 3.1. Clinical findings

Clinical examinations revealed presence of skin lesions in 75.7% of the animals (28 out of 37 dogs), which included dermatitis (71.4%, 20 dogs), body alopecia (64.3%, 18 dogs), lunettes (32.1%, 9 dogs), hyperkeratosis (39.3%, 11 dogs) and ulcerative lesions (78.6%, 22 dogs). Among the ophthalmic alterations (67.6%), we observed 20 dogs with conjunctivitis (80.0%) and five with uveitis (20.0%). Other frequent alterations included onychogryphosis (73%, 27 dogs), popliteal lymphadenomegaly (70.3%, 26 dogs) and low body mass (59.5%, 22 dogs). All animals (100%, 37 dogs) had at least one clinical sign.

### 3.2. Descriptive statistics of hematological, biochemical and oxidative stress markers

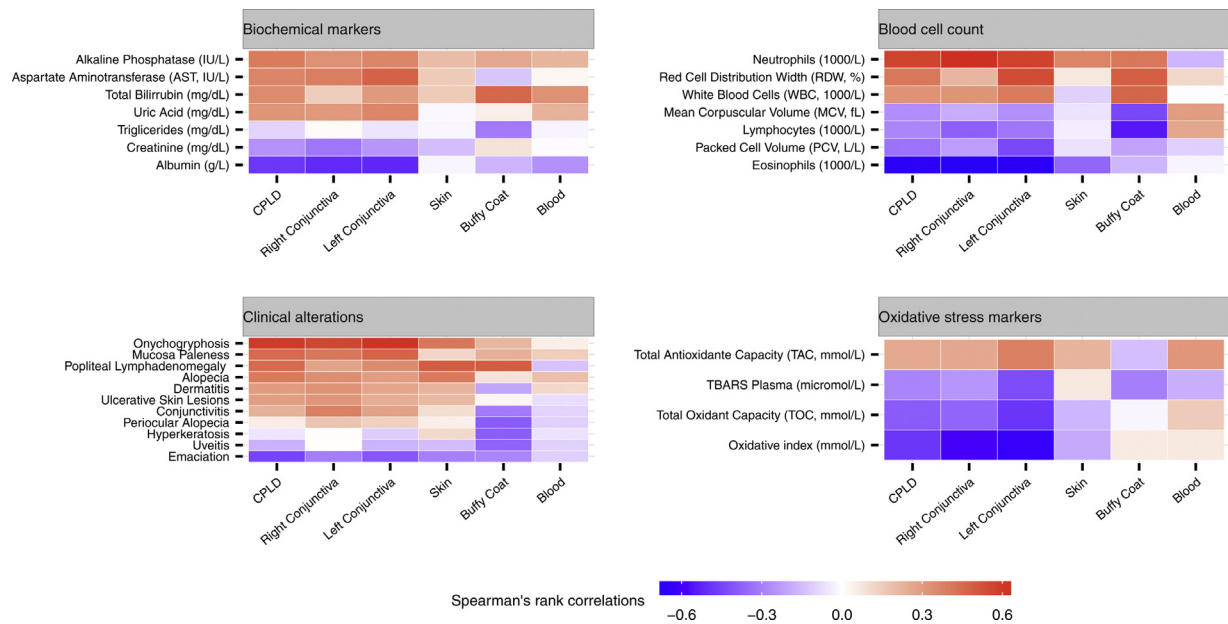
A complete summary of the clinical, hematological, biochemical and oxidative stress markers is provided in Supplementary Table 1. All samples exhibited at least one hematological alteration compared to reference values. All examined dogs had non-regenerative normocytic hypochromic anemia, except for a single dog that presented macrocytic hypochromic anemia. All animals presented leukogram abnormalities. Two animals with leukocytosis, neutrophilia and lymphopenia had suppurative skin lesions. Only one dog presented eosinophilia, and the other dogs had at least one of the following alterations: monocytopenia, lymphopenia or eosinopenia.

The biochemical analysis showed that all dogs had hypoalbuminemia. Hyperglobulinemia and hyperproteinemia were observed in 31 (86.5%) and 21 (56.75%) dogs, respectively. Biochemical profiles compatible with hepatic injury (considered here as alterations in two or more of the following biochemical markers: ALT, AST, alkaline phosphatase, GGT and albumin) was found in 31 (83.8%) dogs. Evidences of kidney alterations (considered here as increased creatinine and/or urea) were found in 13 dogs (35.1%). Importantly, no dog presented signs of dehydration such as bilateral enophthalmos and decreased skin turgor, which could affect the interpretation of the biochemical markers.

We observed reduced oxidant and antioxidant status in 73.3% and 82.6% of the examined samples, respectively. Accordingly, a large number of samples presented decreased values for other endogenous antioxidants, including albumin (100%) and total bilirubin (54.5%) in comparison to reference values. Only three and one sample presented increased TAC and TOC, respectively. Except for one animal, all dogs presented decreased lipid peroxidation in the plasma.

### 3.3. Descriptive statistics of parasite load

Supplementary information (Supplementary Table 2) presents summary statistics for the log-transformed PL across different tissues. The highest average PL was observed in the skin (29,511 parasites/mL). Absence of parasites was found in 54.9%, 24.32%, 13.51% and 12.5% of the peripheral blood, buffy coat, conjunctivas



**Fig. 1.** Correlogram of Spearman's rank correlation coefficients between *Leishmania* spp. density (horizontal axis) in different tissues and clinical alterations, blood cell count, biochemical and oxidative stress markers (vertical axis). Warm colors (towards red) represent positive correlations ( $r > 0$ ), whereas cool colors (towards blue) represent negative correlations ( $r < 0$ ). Only markers presenting at least one suggestive ( $0.05 \leq P < 0.10$ ) or significant ( $P < 0.05$ ) correlation in a randomization test with 10,000 permutations are shown. For a complete summary of correlations see the Supplementary information (Supplementary Table 3 and 4). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and skin samples, respectively. However, all dogs presented parasite infection in at least one tissue, confirming that all dogs were infected. Parasite load across tissues were moderately to highly correlated, with an average correlation of  $r = 0.400$  (Supplementary information). An exception was observed for blood, which was poorly correlated with all tissues (average correlation of  $r = 0.069$ ).

In order to have a representation of all tissues simultaneously, we created a new compressed variable, namely CPLD, based on a principal component analysis of the PL matrix. For that matter, we required complete observations of PL across tissues, which reduced the data from 37 to 31 samples. The first leading principal component explained 49.1% of the total variance contained in the original matrix, and therefore was adopted as the CPLD. The relative contributions of each tissue to the CPLD were 22.7% for skin, 28.5% for right conjunctiva, 29.6% for left conjunctiva, 12.3% for buffy coat and 6.9% for blood.

### 3.4. Correlations for peripheral parasite load

As the permutation test is conservative, we decided to discuss suggestive ( $0.05 \leq P < 0.10$ ) and significant ( $P < 0.05$ ) correlations. All clinical alterations were positively correlated ( $P < 0.05$ ) with parasite load in two or more tissues (Fig. 1). Ophthalmic alterations were only associated with PL in the conjunctivas. Parasite load in the blood did not present associations with clinical alterations. The CPLD variable successfully captured the correlations of the individual tissues, and only failed to predict ophthalmic alterations.

The following laboratory markers presented correlations with parasite load in at least one tissue (Fig. 1): WBC (positive), PCV (negative), MCV (negative), RDW (positive), neutrophil (positive), lymphocytes (negative), eosinophil (negative), triglycerides (negative), uric acid (positive), albumin (negative), total bilirubin (positive), AST (positive), creatinine (negative), alkaline phosphatase (positive), TAC (positive), TOC (negative), oxidative index (negative) and TBARS plasma (negative). Parasite load in total blood was not correlated with any given analyzed marker (see Supplementary Table 3).

Correlations between results for CPLD and individual tissues were 0.972, 0.936, 0.773, 0.543 and 0.251 for left conjunctiva, right conjunctiva, skin, buffy coat and blood, respectively. The compressed variable was able to capture the majority of the significant and suggestive correlations exhibited by the individual tissues, and properly conserved the magnitude and direction of the correlation coefficients.

## 4. Discussion

To date, no existing report in the literature has yet assessed the simultaneous correlation of peripheral parasite load in multiple tissues with severity of clinical signs or variation in biochemical, hematological and, especially, oxidative stress markers in dogs naturally infected with *Leishmania* spp. Elucidation of these correlations may allow for accurate identification of animals with high transmitting ability based on routine clinical examination and laboratory analysis. Here, we present such an investigation, which was facilitated by the development of a new approach, namely CPLD, which summarizes PL data across tissues into a single variable that serves as a proxy for net parasite infection. For the sake of clarity, we use PL, CPLD and net peripheral parasite infection interchangeably throughout the discussion, as CPLD was shown to efficiently capture the majority of the tissue-specific PL correlations.

The main clinical alterations observed in our study are consistent with those commonly found in CVL (Manna et al., 2009). We found that elevation of PL increases severity of clinical signs in infected dogs, consistent with previous reports suggesting that increased parasite density is associated with the severity of CVL (Manna et al., 2009; Reis et al., 2006). Interestingly, ophthalmic alterations were associated only with PL in conjunctival tissues and buffy coat, suggesting that this clinical sign is a specific indicator of local parasitism. Altogether, these results indicate that high peripheral parasitism can be inferred in dogs presenting clinical alterations such as alopecia, onychogryphosis, popliteal lymphadenomegaly, mucosa paleness and emaciation. Although these relationships are often speculated by veterinary

practitioners and health agents, we believe our study is the first to formally demonstrate them empirically.

The vast majority of the dogs in our study presented non-regenerative anemia, which is compatible with bone marrow injuries or impaired hormonal stimulation (Grimes and Fry, 2014). Although non-regenerative anemia in CVL is often described as normocytic normochromic (Lafuse et al., 2013; Solano-Gallego et al., 2009), our samples presented normocytic hypochromic anemia. Decreased MCHC paired with normal MCV is usually indicative of iron deficiency (Jain, 1986). *Leishmania* spp. amastigotes require iron from the host in order to survive and exert their pathogenicity (Flannery et al., 2013), and this could be related to the low MCHC in our sample.

As we found no variation in the type of anemia across dogs, our data suggests that the presence of non-regenerative anemia is independent of intensity of peripheral infection. However, by performing the analysis of each blood parameter individually, we found that elevation in PL leads to increased RDW and decreased PCV and MCV, which implies progression and worsening of iron deficiency anemia (Jain, 1986) with higher peripheral parasite density. This hypothesis is also supported by the positive correlation between PL and mucosa paleness.

The strongest positive correlation ( $r=0.563$ ) with PL across tissues was found for neutrophils. Neutrophilia may be attributed to the inflammatory response resulting from parasitism in multiple organs (Amusatogui et al., 2003; Mogami, 2008; Tryphonas et al., 1977). Neutrophils are deemed to be used as 'Trojan horses' by *Leishmania* spp. in order to invade the host for later multiplication inside macrophages (Zandbergen et al., 2004). Progression of CVL has been shown to lead to increased apoptosis (Almeida et al., 2013a) and reduced oxidation status (Almeida et al., 2013b) in neutrophils. We also observed that PL is positively correlated with TAC and negatively correlated with TOC and oxidative index. Moreover, *Leishmania* spp. has been found to also produce antioxidants as a defense mechanism against the respiratory burst (Assche et al., 2011). These findings point to parasitism-induced neutrophilia coupled with inhibition or over-consumption of reactive oxygen species (ROS) as a key mechanism used by the parasite to evade the immune system and multiply inside the host.

At the other extreme, we found the strongest negative correlation of PL with eosinophil ( $r=-0.651$ ). Anemia, neutrophilia and eosinopenia have been described as hallmarks of hematological dysfunction in CVL, and decreased eosinophil precursors in the bone marrow has been implicated in reduction of the number of peripheral blood eosinophils (Nicolato et al., 2013). As eosinophils are also a lineage of phagocytes actively producing ROS, lower TOC in highly infected animals could also be partly explained by eosinopenia.

The liver is one of the main affected organs in CVL and liver damage has been found to be intensified by increased parasitism (Giunchetti et al., 2008) and by the progression of the disease (Giunchetti et al., 2008; Melo et al., 2009). Here, increase in peripheral PL was followed by decrease in serum albumin and increase in AST, alkaline phosphatase, total bilirubin and uric acid, indicating intensification of liver injury (Afzali et al., 2010; Giunchetti et al., 2008; Wolf, 1999) as consequence of increased parasitism. Additionally, liver damage can lead to impaired lipid metabolism, which could justify the negative correlation between PL and triglycerides. One obvious limitation of the present study is the absence of PL measurements in internal organs such as the liver, as the scope here was the investigation of peripheral parasitism. However, other tissues could capture the association between PL and markers of liver function, suggesting some degree of correlation between parasitism in the liver and the analyzed tissues. Although examination of PL in internal organs was out of the scope of the present study,

future investigations regarding the interplay of PL in peripheral and internal organs are required.

Albumin, uric acid and bilirubin are not only important markers of liver function (Afzali et al., 2010; Wolf, 1999), but they are also important endogenous antioxidants underlying the TAC marker (Erel, 2004). This leaves us with two distinct hypotheses to explain the observed data: 1) TAC increases in response to PL as a compensatory mechanism to react against systemic ROS spilled by damaged tissues; or 2) elevation of TAC is a false positive oxidative imbalance, because higher levels of serum uric acid and bilirubin are in fact caused by liver damage. As tissue damage followed by spilling of ROS in the plasma is expected to produce lipid peroxidation (Hunter et al., 1985; Niki, 2009), the first hypothesis seems to be inconsistent with the non-significant correlation between PL and TBARS in the plasma and in erythrocytes in our study. Only TBARS in plasma showed suggestive correlation with one tissue. This also indicates that anemia is unlikely to be caused by lipid peroxidation of red blood cells in CVL. Nevertheless, our data did not allow for distinguishing the source of increase in TAC, and these two hypotheses should be addressed by future studies.

In spite of being lowly invasive and easy to measure, PL in peripheral blood did not present any association with clinical alterations or laboratory parameters in our study, and was poorly correlated with parasitism in other tissues. On the other hand, in comparison to other tissues, conjunctiva swabs are the least invasive sampling method for PL quantification and presented the largest number of associations here, indicating high sensitivity. This is in agreement with previous studies showing that conjunctiva PL is a reliable, sensitive proxy for parasite burden (de Almeida Ferreira et al., 2012; Lombardo et al., 2012).

A clear limitation of the present study is the absence of historical records for the studied dogs, thus not allowing us to account for potential confounding effects in our analysis, such as a putative nutritional causes for the observed iron deficiency anemia and the decreased levels of triglycerides. Moreover, as 59.5% of the dogs had low body mass, lower creatinine levels could be also explained by loss of muscle mass. Therefore, more studies are required in order to better elucidate the dynamics among parasite burden, anemia and nutrition.

## 5. Conclusions

We presented novel empirical evidence supporting the hypothesis that severity of clinical manifestations of the disease, as well as non-regenerative anemia, oxidative imbalance and liver and bone marrow damage are correlated with increased peripheral parasitism in dogs naturally infected with *Leishmania* spp. These results have direct impact in the identification of dogs with high transmitting ability in endemic areas. Additionally, these correlations may also serve as a tool to help veterinarians take decisions regarding prognosis and therapy based on routine clinical and laboratory exams. Finally, the new statistical tool presented here can be used to summarize parasite load data in future studies including quantification in multiple tissues, substantially simplifying the multivariate analysis of net parasite infection.

## Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Mention of trade name proprietary product or specified equipment in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the authors or their respective institutions.



## Acknowledgements

We thank the Center for Zoonosis Control of Araçatuba for technical assistance in sample acquisition. We are also grateful to Laine Margareth Gabas for her valuable laboratory assistance. This research was supported by: Coordination for the Improvement of Higher Education Personnel (CAPES) and Sao Paulo Research Foundation (FAPESP – <http://www.fapesp.br/>) (process 2014/01095-8. OC and LC-B were supported by the Wellcome Trust (Strategic Translation Award ([WT091689MF])). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.prevetmed.2016.08.006>.

## References

- Afzali, A., Weiss, N.S., Boyko, E.J., Ioannou, G.N., 2010. Association between serum uric acid level and chronic liver disease in the United States. *Hepatology* 52, 578–589, <http://dx.doi.org/10.1002/hep.23717>.
- Almeida, B.F.M., Narciso, L.G., Bosco, A. M., Pereira, P.P., Braga, E.T., Avanço, S.V., Marcondes, M., Ciarlini, P.C., 2013a. Neutrophil dysfunction varies with the stage of canine visceral leishmaniasis. *Vet. Parasitol.* 196, 6–12, <http://dx.doi.org/10.1016/j.vetpar.2013.02.016>.
- Almeida, B.F.M., Narciso, L.G., Melo, L.M., Preve, P.P., Bosco, A.M., Lima, V.M.F., Ciarlini, P.C., 2013b. Leishmaniasis causes oxidative stress and alteration of oxidative metabolism and viability of neutrophils in dogs. *Vet. J.* 198, 599–605, <http://dx.doi.org/10.1016/j.tvjl.2013.08.024>.
- Amusategui, I., Sainz, A., Rodríguez, F., Tesouro, M.A., 2003. Distribution and relationships between clinical and biopathological parameters in. *Eur. J. Epidemiol.*, 147–156.
- Assche, T., Van Deschacht, M., Inocência, R.A., Maes, L., Cos, P., 2011. Leishmania?macrophage interactions: insights into the redox biology. *Free Radic. Biol. Med.* 51, 337–351, <http://dx.doi.org/10.1016/j.freeradbiomed.2011.05.011>.
- Aycicek, A., Erel, O., Kocyigit, A., 2005. Decreased total antioxidant capacity and increased oxidative stress in passive smoker infants and their mothers. *Pediatr. Int.* 47, 635–639, <http://dx.doi.org/10.1111/j.1442-200x.2005.02137.x>.
- de Almeida Ferreira, S., Leite, R.S., Ituassu, L.T., Almeida, G.G., Souza, D.M., Fujiwara, R.T., de Andrade, A.S.R., Melo, M.N., 2012. Canine skin and conjunctival swab samples for the detection and quantification of Leishmania infantum DNA in an endemic urban area in Brazil. *PLoS Negl. Trop. Dis.* 6, e1596, <http://dx.doi.org/10.1371/journal.pntd.0001596>.
- Erel, O., 2004. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin. Biochem.* 37, 277–285, <http://dx.doi.org/10.1016/j.clinbiochem.2003.11.015>.
- Erel, O., 2005. A new automated colorimetric method for measuring total oxidant status. *Clin. Biochem.* 38, 1103–1111, <http://dx.doi.org/10.1016/j.clinbiochem.2005.08.008>.
- Flannery, A.R., Renberg, R.L., Andrews, N.W., 2013. Pathways of iron acquisition and utilization in Leishmania. *Curr. Opin. Microbiol.*, <http://dx.doi.org/10.1016/j.mib.2013.07.018>.
- Francino, O., Altet, L., Sánchez-Robert, E., Rodriguez, A., Solano-Gallego, L., Alberola, J., Ferrer, L., Sánchez, A., Roura, X., 2006. Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniasis. *Vet. Parasitol.* 137, 214–221, <http://dx.doi.org/10.1016/j.vetpar.2006.01.011>.
- Giunchetti R.C., Mayrink W., Martins-filho O.A., Jose M., Corre R., Tafuri W.L., Reis A.B., 2008. Histopathological and immunohistochemical investigations of the hepatic compartment associated with parasitism and serum biochemical changes in canine visceral leishmaniasis, *í* 84, 269–277. <http://dx.doi.org/10.1016/j.rvsc.2007.04.020>.
- Grimes, C.N., Fry, M.M., 2014. Nonregenerative anemia: mechanisms of decreased or ineffective erythropoiesis. *Vet. Pathol.* 52, <http://dx.doi.org/10.1177/0300985814529315> (0300985814529315-).
- Hunter, M.I., Nlemadim, B.C., Davidson, D.L., 1985. Lipid peroxidation products and antioxidant proteins in plasma and cerebrospinal fluid from multiple sclerosis patients. *Neurochem. Res.* 10, 1645–1652.
- Jain, N.C., 1986. *Schalm's Veterinary Hematology*, 4th ed. Lea & Febiger, Philadelphia.
- Kaneko, J.J., Harvey, J.W., Bruss, M.L., 1997. *Clinical Biochemistry of Domestic Animals*, 5th ed. Academic Press, California.
- Lafuse, W.P., Story, R., Mahylis, J., Gupta, G., Varikuti, S., Steinkamp, H., Oghumu, S., Satoskar, A.R., 2013. Leishmania donovani infection induces anemia in hamsters by differentially altering erythropoiesis in bone marrow and spleen. *PLoS One* 8, e59509, <http://dx.doi.org/10.1371/journal.pone.0059509>.
- Lima, V.M.F., Gonçalves, M.E., Ikeda, F.A., Luvizotto, M.C.R., Feitosa, M.M., 2003. Anti-Leishmania antibodies in cerebrospinal fluid from dogs with visceral leishmaniasis. *Med. Biol.*, 36, <http://dx.doi.org/10.1590/S0100-879X2003000400010>.
- Lombardo, G., Pennisi, M.G., Lupo, T., Miglizzo, A., Capri, A., Solano-Gallego, L., 2012. Detection of Leishmania infantum DNA by real-time PCR in canine oral and conjunctival swabs and comparison with other diagnostic techniques. *Vet. Parasitol.* 184, 10–17, <http://dx.doi.org/10.1016/j.vetpar.2011.08.010>.
- Manna, L., Reale, S., Vitale, F., Gravino, A.E., 2009. Evidence for a relationship between Leishmania load and clinical manifestations. *Res. Vet. Sci.* 87, 76–78, <http://dx.doi.org/10.1016/j.rvsc.2008.12.009>.
- Melo, F.A., Moura, E.P., Ribeiro, R.R., Alves, C.F., Caliani, M.V., Tafuri, W.L., Calabrese, K.D.S., Tafuri, W.L., 2009. Hepatic extracellular matrix alterations in dogs naturally infected with Leishmania (Leishmania) chagasi. *Int. J. Exp. Pathol.* 90, 538–548, <http://dx.doi.org/10.1111/j.1365-2613.2009.00681.x>.
- Mogami, S.R., 2008. Hematological evaluation of dogs naturally infected by 68–74.
- Nicolato, R.D.C., De Abreu, R.T., Roatt, B.M., Aguiar-Soares, R.D.O., Reis, L.E.S., Carvalho, M.D.G., Carneiro, C.M., Giunchetti, R.C., Bouillet, L.E.M., Lemos, D.S., Coura-Vital, W., Barbosa Reis, A., 2013. Clinical forms of canine visceral leishmaniasis in naturally Leishmania infantum-infected dogs and related myelogram and hemogram changes. *PLoS One* 8, <http://dx.doi.org/10.1371/journal.pone.0082947>.
- Niki, E., 2009. Lipid peroxidation: physiological levels and dual biological effects. *Free Radic. Biol. Med.* 47, 469–484, <http://dx.doi.org/10.1016/j.freeradbiomed.2009.05.032>.
- Reale, S., Maxia, L., Vitale, F., Glorioso, N.S., Caraccappa, S., Vesco, G., 1999. Detection of Leishmania infantum in dogs by PCR with lymph node aspirates and blood. *J. Clin. Pathol.* 37, 2931.
- Reis, A.B., Martins-Filho, O.A., Teixeira-Carvalho, A., Carvalho, M.G., Mayrink, W., França-Silva, J.C., Giunchetti, R.C., Genaro, O., Corrêa-Oliveira, R., 2006. Parasite density and impaired biochemical/hematological status are associated with severe clinical aspects of canine visceral leishmaniasis. *Res. Vet. Sci.* 81, 68–75, <http://dx.doi.org/10.1016/j.rvsc.2005.09.011>.
- Solano-Gallego, L., Koutinas, A., Miro, G., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., 2009. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniasis. *Vet. Parasitol.* 165, 1–18, <http://dx.doi.org/10.1016/j.vetpar.2009.05.022>.
- Solano-Gallego, L., Miró, G., Koutinas, A., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., 2011. LeishVet guidelines for the practical management of canine leishmaniasis. *Parasite & Vectors*, 4, <http://dx.doi.org/10.1186/1756-3305-4-86>.
- Solcà, M.D.S., Guedes, C.E.S., Nascimento, E.G., Oliveira, G.G.D.S., dos Santos, W.L.C., Fraga, D.B.M., Veras, P.S.T., 2012. Qualitative and quantitative polymerase chain reaction (PCR) for detection of Leishmania in spleen samples from naturally infected dogs. *Vet. Parasitol.* 184, 133–140, <http://dx.doi.org/10.1016/j.vetpar.2011.08.026>.
- Tryphonas, L., Zawadzka, Z., Bernard, M.A., Janzen, E.A., 1977. Visceral Leishmaniasis in a Dog: Clinical Hematological and Pathological Observations, 41 1–12.
- Wolf, P.L., 1999. Biochemical diagnosis of liver disease. *Indian J. Clin. Biochem.* 14, 59–90, <http://dx.doi.org/10.1007/BF02869152>.
- Zandbergen, G., Van Klinger, M., Mueller, A., Gebert, A., Solbach, W., Alerts, E., 2004. Cutting edge: neutrophil granulocyte serves as a vector for leishmania entry into macrophages. *J. Immunol.* 173, 6521–6525, <http://dx.doi.org/10.4049/jimmunol.173.11.6521>.