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1 **Original Article**

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3

4 **Correlations between peripheral parasite load and common clinical and laboratory alterations in**
5 **dogs with visceral leishmaniasis**

6

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24 **Abstract**

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

45

46 **Keywords:** *Canis lupus familiaris*; *Leishmania spp.*; Hematology; Biochemistry; Oxidative stress;

47 Principal components analysis

48 **1. Introduction**

49 Visceral leishmaniasis is an anthroponosis caused by parasites of the *Leishmania* genus
50 ([WHO, 2010](#))□ ([Solano-Gallego et al., 2011](#)). As the parasite is transmitted from one host to another
51 by the bites of *Lutzomyia longipalpis* sandflies, the intensity of peripheral parasitism is an important
52 contributor to the transmitting ability of the host. The main urban reservoir of the disease is the
53 domestic dog (*Canis lupus familiaris*), and the presence of infected dogs in the vicinity of humans has
54 been incriminated as a risk factor for human leishmaniasis (Solano-Gallego et al., 2009; Werneck et al.,
55 2007)□.

56
57 Clinical staging in canine visceral leishmaniasis (CVL) has been shown to be associated with
58 parasite density in specific tissue compartments, such as skin (de Almeida Ferreira et al., 2012)□,
59 lymph nodes (Reale et al., 1999)□, spleen (Solcà et al., 2012)□ and bone marrow (Reis et al., 2006)□.
60 *Leishmania spp.* infection has also been found to alter markers of hematological, biochemical and
61 oxidative homeostasis in dogs (Almeida et al., 2013b; Nicolato et al., 2013; Reis et al., 2006)□□.
62 However, it remains unclear at what extent the occurrence of clinical signs and variation in laboratory
63 parameters translates into peripheral parasite load and consequently to transmission risk. This
64 information is a key first step in the identification of highly infected reservoirs in endemic areas, as
65 peripheral parasite load (PL) quantification techniques are still costly to be routinely used in
66 epidemiological surveillance.

67
68 Here, we aimed at contributing to the further elucidation of correlations between parasite load
69 and laboratory/clinical alterations in dogs naturally infected with *Leishmania spp.* Additionally, as the
70 independent analysis of PL in specific compartments can be misleading, we developed a strategy to
71 compile PL quantified in multiple tissues to have a general picture of the intensity of peripheral

72 infection of an animal.

73

74 **2. Material and Methods**

75 2.1. Ethics statement

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

80

81 2.2. Data collection

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

92

93 Blood samples were obtained via jugular venipuncture and used for PL estimation, complete
94 blood cell count, biochemical analysis and quantification of oxidative stress markers, following
95 protocols described elsewhere (Almeida et al., 2013b; Aycicek et al., 2005; Erel, 2005, 2004; Francino

96 et al., 2006; Hunter et al., 1985; Jain, 1986; Kaneko et al., 1997)□. Bilateral conjunctiva swabs and
97 skin biopsies (i.e., ear punching) were also collected for PL quantification. For details concerning the
98 procedures used here, please see the *Extended Methods* section in the Supplementary Information file.
99

100 2.3 Statistical analyses

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

109
110 Correlations between clinical and laboratory parameters and PL across tissues were tested using
111 a permutation test for Spearman's correlations with 10,000 randomizations each. For all analyses, we
112 considered significant $P < 0.05$, whereas $0.05 \leq P < 0.10$ were considered suggestive correlations. For
113 details concerning the statistical analyses, as well as *R* v3.2.1 (available at: <http://www.r-project.org/>)
114 scripts for the computation of CPLD and permutation tests, please see the *Extended Methods* section in
115 the Supplementary Information file.

116

117

118 **3. Results**

119 3.1. Clinical findings

120 Clinical examinations revealed presence of skin lesions in 75.7% of the animals (28 out of 37
121 dogs), which included ~~variable extension and severity of~~ dermatitis (71.4%, 20 dogs), body alopecia
122 (64.3%, 18 dogs), lunettes (32.1%, 9 dogs), hyperkeratosis (39.3%, 11 dogs) and ulcerative lesions
123 (78.6%, 22 dogs). Among the ophthalmic alterations (67.6%), we observed 20 dogs with conjunctivitis
124 (80.0%) and five with uveitis (20.0%). Other frequent alterations included onycogriphosis (73%, 27
125 dogs), popliteal lymphadenomegaly (70.3%, 26 dogs) and low body mass (59.5%, 22 dogs). All
126 animals (100%, 37 dogs) had at least one clinical sign.

128 3.2. Descriptive statistics of hematological, biochemical and oxidative stress markers

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

136
137 The biochemical analysis showed that all dogs had hypoalbuminemia. Hyperglobulinemia and
138 hyperproteinemia were observed in 31 (86.5%) and 21 (56.75%) dogs, respectively. Biochemical
139 profiles compatible with hepatic injury (considered here as alterations in two or more of the following
140 biochemical markers: ALT, AST, alkaline phosphatase, GGT and albumin) was found in 31 (83.8%)
141 dogs. Evidences of kidney alterations (considered here as increased creatinine and/or urea) were found
142 in 13 dogs (35.1%). Importantly, no dog presented signs of dehydration such as bilateral enophthalmos
143 and decreased skin turgor, which could affect the interpretation of the biochemical markers.

144

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

150

151 3.3. Descriptive statistics of parasite load

152 Supplementary Information (Supplementary Table 2) presents summary statistics for the log-
153 transformed PL across different tissues. The highest average PL was observed in the skin (29,511
154 parasites/mL). Absence of parasites was found in 54.9%, 24.32%, 13.51% and 12.5% of the peripheral
155 blood, buffy coat, conjunctivas and skin samples, respectively. However, all dogs presented parasite
156 infection in at least one tissue, confirming that all dogs were infected. Parasite load across tissues were
157 moderately to highly correlated, with an average correlation of $r = 0.400$ (Supplementary Information).
158 An exception was observed for blood, which was poorly correlated with all tissues (average correlation
159 of $r = 0.069$).

160

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

168

169 3.4. Correlations for peripheral parasite load

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

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

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

188

189 **4. Discussion**

190 To date, no existing report in the literature has yet assessed the simultaneous correlation of
191 peripheral parasite load in multiple tissues with severity of clinical signs or variation in biochemical,

192 hematological and, especially, oxidative stress markers in dogs naturally infected with *Leishmania spp.*
193 Elucidation of these correlations may allow for accurate identification of animals with high
194 transmitting ability based on routine clinical examination and laboratory analysis. Here, we present
195 such an investigation, which was facilitated by the development of a new approach, namely CPLD,
196 which summarizes PL data across tissues into a single variable that serves as a proxy for net parasite
197 infection. For the sake of clarity, we use PL, CPLD and net peripheral parasite infection
198 interchangeably throughout the discussion, as CPLD was shown to efficiently capture the majority of
199 the tissue-specific PL correlations.

200

201 The main clinical alterations observed in our study are consistent with those commonly found in
202 CVL (Manna et al., 2009)□. We found that elevation of PL increases severity of clinical signs in
203 infected dogs, consistent with previous reports suggesting that increased parasite density is associated
204 with the severity of CVL (Manna et al., 2009; Reis et al., 2006)□. Interestingly, ophthalmic alterations
205 were associated only with PL in conjunctival tissues and buffy coat, suggesting that this clinical sign is
206 a specific indicator of local parasitism. Altogether, these results indicate that high peripheral parasitism
207 can be inferred in dogs presenting clinical alterations such as alopecia, onychogryphosis, popliteal
208 lymphadenomegaly, mucosa paleness and emaciation. Although these relationships are often speculated
209 by veterinary practitioners and health agents, we believe our study is the first to formally demonstrate
210 them empirically.

211

212 The vast majority of the dogs in our study presented non-regenerative anemia, which is
213 compatible with bone marrow injuries or impaired hormonal stimulation (Grimes and Fry, 2014)□.
214 Although non-regenerative anemia in CVL is often described as normocytic normochromic (Lafuse et
215 al., 2013; Solano-Gallego et al., 2009)□, our samples presented normocytic hypochromic anemia.

216 Decreased MCHC paired with normal MCV is usually indicative of iron deficiency (Jain, 1986)□.
217 *Leishmania spp.* amastigotes require iron from the host in order to survive and exert their pathogenicity
218 (Flannery et al., 2013)□, and this could be related to the low MCHC in our sample.

219

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

226

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

239

240 At the other extreme, we found the strongest negative correlation of PL with eosinophil ($r = -$
241 0.651). Anemia, neutrophilia and eosinopenia have been described as hallmarks of hematological
242 dysfunction in CVL, and decreased eosinophil precursors in the bone marrow has been implicated in
243 reduction of the number of peripheral blood eosinophils (Nicolato et al., 2013)□. As eosinophils are
244 also a lineage of phagocytes actively producing ROS, lower TOC in highly infected animals could also
245 be partly explained by eosinopenia.

246

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

260

261 Albumin, uric acid and bilirubin are not only important markers of liver function (Afzali et al.,
262 2010; Wolf, 1999)□, but they are also important endogenous antioxidants underlying the TAC marker
263 (Erel, 2004)□. This leaves us with two distinct hypotheses to explain the observed data: 1) TAC

264 increases in response to PL as a compensatory mechanism to react against systemic ROS spilled by
265 damaged tissues; or 2) elevation of TAC is a false positive oxidative imbalance, because higher levels
266 of serum uric acid and bilirubin are in fact caused by liver damage. As tissue damage followed by
267 spilling of ROS in the plasma is expected to produce lipid peroxidation (Hunter et al., 1985; Niki,
268 2009)□, the first hypothesis seems to be inconsistent with the non-significant correlation between PL
269 and TBARS in the plasma and in erythrocytes in our study. Only TBARS in plasma showed suggestive
270 correlation with one tissue. This also indicates that anemia is unlikely to be caused by lipid
271 peroxidation of red blood cells in CVL. Nevertheless, our data did not allow for distinguishing the
272 source of increase in TAC, and these two hypotheses should be addressed by future studies.

273

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

281

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

288

289 **5. Conclusions**

290

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

301

302 **6. Conflict of interest statement**

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

308

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317

318 **8. Appendix A: Supplementary Information**

319 Supplementary data associated with this article can be found, in online version, at doi:

320

321 **9.**

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415 **10. Figure legends**

416

417 Fig 1. Correlogram of Spearman's rank correlation coefficients between *Leishmania spp.* density
418 (horizontal axis) in different tissues and clinical alterations, blood cell count, biochemical and oxidative
419 stress markers (vertical axis). Warm colors (towards red) represent positive correlations ($r > 0$),
420 whereas cool colors (towards blue) represent negative correlations ($r < 0$). Only markers presenting at
421 least one suggestive ($0.05 \leq P < 0.10$) or significant ($P < 0.05$) correlation in a randomization test with
422 10,000 permutations are shown. For a complete summary of correlations see the Supplementary
423 Information (Supplementary Table 3 and 4).