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Abstract: Leishmaniases are zoonotic vector-borne diseases caused by a wide variety of *Leishmania* species with complex transmission cycles involving different reservoirs, potential new hosts and vectors. Similarly, to other eukaryotes, *Leishmania* use released extracellular vesicles (*L*EVs) to play important initial interactions that are crucial to modulate the subsequent systemic immune response on the establishment of infection in humans and other important hosts like dogs. Recent studies in endemic areas of Brazil concluded that canine infections were predominantly due to *L. amazonensis* and not restricted to *L. infantum* (syn. *Leishmania chagasi*). Under these premises, the diagnosis of canine leishmaniasis needs to be improved, including the identification of current etiological agent, the clinical differential diagnosis and the histopathologic features. In this way, the dual aim of that study is to register collected observations for the diagnosis of natural canine infections and to insert in vitro results in the field of *L*EVs that still research gaps to be filled to understand the mechanisms and biological aspects involving the parasite-host interactions. Therefore, the future studies of Parasitology research for both of these fields are very important for the interventions for the prevention, control, elimination and eradication worldwide.

Keywords: canine leishmaniasis; natural zoonotic infection; clinical and histopathologic features; cell communication by secreting extracellular vesicles; lipid profiles of cultured parasites

1. Introduction

Leishmania is the genus of a very successful group of parasitic protozoans of medical and veterinary importance divided into Old and New World species adapted to enormous diversity of hosts such as dogs (the main reservoirs to these parasites), rodents, and other vertebrates, being transiently infectious in humans (Figure 1) [1]. These hemoflagellates parasites have a high level of genetic variability in vivo and a propensity for rapid evolution *in vitro*, establishing infection by heterogeneous extracellular vesicles released containing a large number of molecules to modulate the host immune responses [1]. According WHO, their high ability to infect multiple hosts has facilitated the spread of *Leishmania* in 4 eco-epidemiological regions of the world: the Americas, East Africa, North Africa and West and South-east Asia [1,2]. Despite the development of some veterinary vaccines, the lack of effective vaccines and drug treatments for humans has allowed the increase of this major health problem worldwide [1–3]. However, there is increasing interest in the isolation and biological and functional characterization of the lipoproteic vesicles released by *Leishmania* (LEVs),

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given their apparent potential for the development of effective diagnostic and therapeutic approaches, including the prediction of the outcome of the interaction between cells [1–4]. Despite some advances, the mechanisms of the selective packaging of these extracellular vesicles are still poorly understood, and there is no consensus on the differential isolation and characterization of the extracellular vesicles or the ultrasensitive detection of specific extracellular vesicle subtypes, their specific biomarkers or their biogenesis [1–4]. Given these considerations, we present this work, with emphasis on the diagnosis of natural canine infections and to insert in vitro results of extracellular activity of *Leishmania*, complementing the set of studies we are developing during the last years and providing a reference base for future applied nanotechnological research towards in One Health.

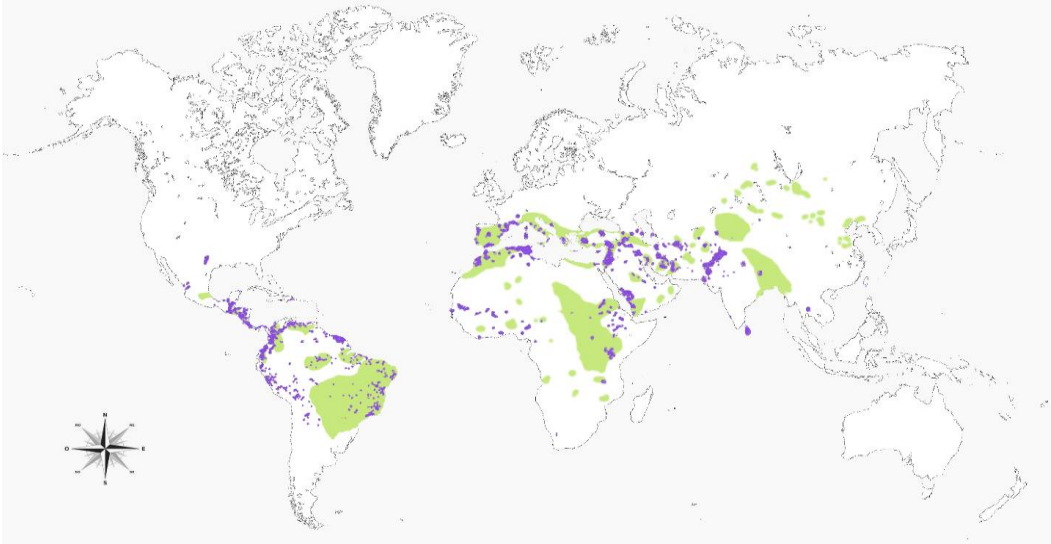

Figure 1. Tracking the global distribution of endemic areas in the tropics, subtropics, and southern Europe: human visceral leishmaniasis (green areas) and cutaneous leishmaniasis (purple dots) Both zoonotic forms present expanding geographic ranges and rapid adaptation influenced by risk factors, with new epidemiological scenarios emerging in previously disease-free areas [1].

2. Leishmaniotic dog: the infected animal, but healthy and the sick dog

Leishmania is an obligate intracellular parasite transmitted to dogs or humans via the bite of female sand flies to the genera *Phlebotomus* or *Lutzomyia*, that are most active in humid environment during the warmer months and at night from dusk to dawn [1–6]. The transmission may occur from domestic or wild animals to sand flies to human, or by non-sand fly transmission (infection through transfused blood products, from blood donors which are carriers of infection, transplacental transmission and venereal transmission) [3–8]. As well as also humans and mice can transmit the parasite between each other thought blood transfusion, vertical and sexual transmission of *Leishmania* [8–10]. However, there are hardly any case studies about direct dog-to-dog *Leishmania* transmission by wounds or dog bites [11,12]. After transmission or risk of transmission, is essential the veterinarian will can be able to identify the clinical signs and pathological abnormalities that can appear several months following *Leishmania* infection (it seems shorter for cutaneous forms), despite the incubation period is difficult to establish [3–13]. The clinical signs of leishmaniasis are directly related to the immune response of the infected dog, however it is estimated that more than half of infected dogs do not manifest clinical signs of the disease [14]. In susceptible animals, the organisms can spread from the skin to the local lymph node, spleen, and bone marrow within a few hours [15]. In resistant dogs, the parasite remains restricted to the skin and draining lymph node – either the animal remains healthy or develops a mild, self-limiting disease. In contrast, susceptible dogs mount a Th2 response characterized by high antibody levels but poor cell-mediated immunity [15]. These differences were attributed to the activities of IL-10-producing Treg lymphocytes. Furthermore, the parasite can actively suppress transcription of the IL-12 gene, ensuring that the Th2 response predominates [15]. Of course, all of these immune challenges affect the balance between progression to clinical disease

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and maintaining sub-clinical disease. Like this during a chronic infection, a progressive disease develops in susceptible dogs [15]. Vaccines and immunotherapies targeted at recovering or maintaining T and B cell function can be important factors in mending the immune balance required to survive canine leishmaniosis [16]. In the veterinary practice, animals with clinical leishmaniosis can present suggestive signs, but dogs with subclinical infection or infected but clinically healthy present neither clinical signs, nor clinicopathological abnormalities, however have a confirmed *Leishmania* infection [3]. In other side, is important to consider that the use of anti-*Leishmania* therapeutic protocols is known to reduce the parasite load and hence infectiousness by treated animals, however presenting only temporary efficacy [5].

3. Inserts of *Leishmania* infection during the activity of canine immune system (from newborns to seniors)

Dogs are an extraordinary heterogeneity in phenotype through the establishment of pure breeds; a change which has largely occurred over the past 200 years. With such selective inbreeding comes recognition that there is likely to be great diversity in the functioning of the immune system between breeds [17]. This has been clear for many years, based on the unique susceptibility of particular dog breeds to immune-mediated, infectious disease [16]. The immune response is crucial in the unfolding of the infectious process and in the establishment of the disease front the mechanisms of adaptive and innate immunity of dogs [18,19]. Understanding the mechanisms of the immune system of the hosts is an important factor to comparatively elucidate what happens to the individual's organism during the progression of the disease [20]. Following the knowledge of Immunology Veterinary, the Pattern Recognition Receptors (PRRs) are a class of receptors that can directly recognize the specific molecular structures on the surface of pathogens [15]. The most important of the soluble C-type lectins is mannose-binding lectin (MBL) present at high levels in serum and which has multiple carbohydrate-binding sites that bind to oligosaccharides such as N-acetylglucosamine, mannose, glucose, galactose and N-acetylgalactosamine [15]. Although the binding is relatively weak, the multiple binding sites confer high functional activity [15]. Thus, MBL binds very strongly to different pathogens including parasites such as *Leishmania*, playing an important role in the activation of the complement system [15]. The surface of phagocytic cells is also covered by many PRRs that can interact with their ligands on the surface of infectious agents [15]. Another important mechanism that promotes contact between pathogens and neutrophils suspended in plasma is the capture [15]. If, however, the pathogen is trapped by a neutrophil and another immune cell, and so not can get away, it can be quickly ingested by phagocytosis [15]. Thus, neutrophils can undergo a form of cell death called NETose as an alternative to apoptosis or necrosis [15]. After activation by CXCL8 or lipopolysaccharides, neutrophils can release the contents of their nuclei, with extrusion of large strands of decondensed nuclear ADN and associated proteins in the extracellular fluid [15]. This forms networks of extracellular fibers called networks extracellular neutrophils (NETs) [15]. The NETs They are abundant at sites of acute inflammation. These networks trap and kill several pathogens such as *L. amazonensis*) [15]. NETs can be very important in containing microbial invaders by acting as physical barriers, capturing large numbers of parasites and thus prevent its spread) [15]. When promastigote forms of this parasite are injected by sandflies in the skin of a dog, they are quickly phagocytosed by the neutrophils [15]. When neutrophils go into apoptosis, parasites are released and then engulfed by macrophages and dendritic cells, in which organisms become differentiate in amastigotes. *Leishmania* amastigotes are intracellular parasites obligators that divide in macrophages until the cells rupture, and when released into the body, they are phagocytosed by adjacent cells [15]. Depending on the degree of host immunity, parasites can be restricted to the skin (skin disease); alternatively, dendritic cells may migrate to the lymph nodes or enter the circulation and lodge in the internal organs, leading to visceral spread of the disease. Although the disease is widely spread in endemic areas, most dogs is resistant to Leishmania, and only 10% to 15% develop the visceral form of the disease [15]. Macrophages are the main host cell for *Leishmania* and effector cells for the death of the parasite. Parasites divide into infected macrophage phagolysosomes [15]. Its resistance to intracellular destruction is the result of multiple mechanisms, including genetic factors

- comparative studies of 245 macrophage genes demonstrated that 37% were suppressed by *Leishmania* infection [15]. *Leishmania* lipophosphoglicans delay the maturation of the phagosome, preventing the production of NO and inhibiting the response of macrophages to cytokines [15]. These parasites also reduce the presenting of macrophage antigen by suppressing the expression of the class II major histocompatibility (MHC), when the parasites stimulate chronic inflammation. Thus, initially will be characterized by granulocytic invasion, this is followed by macrophages, lymphocytes and NK cells that collectively form granulomas [15]. Additionally, one important factor that determine the success or failure of an infection is the availability of iron [15]. Innate resistance to many intracellular organisms such as *Leishmania* is controlled, in part, by a gene called Slc11a1 (short for solute carrier family 11), member 1a; formerly called Nramp1) [15]. By definition, parasites are able to evade the host's immune response by long enough for at least parasitic reproduction to occur. In general, antibody-mediated immune responses protect against extracellular protozoa, while cell-mediated one's control intracellular protozoa [15]. Parasitic protozoa employ some fairly sophisticated techniques to ensure its survival in the face of an animal's immune response [15]. The Th1-mediated responses that result in macrophage activation are important in many diseases caused by protozoa, in which organisms are resistant to intracellular destruction [15]. One of the most significant routes of destruction in the M1 cells is the production of nitric oxide (NO) [15]. The nitrogen radicals formed by interaction of NO with oxidants are lethal to many intracellular protozoa [15]. However, protozoa are also experts at surviving inside macrophages; for example, *Leishmania* and *Trypanosoma cruzi* can migrate to safe intracellular vacuoles by blocking the maturation of phagosome. *Leishmania* and *T. cruzi* can suppress the production of oxidants or cytokines [15].

The ontogeny of the canine immune organs was reviewed, it is known hematopoietic and immune cells arise from a common bone marrow stem cell. Thereafter, B cells undergo maturation in the fetal liver and bone marrow, which represent successive primary lymphoid organs. B cells maturation involves the acquisition of BCR and selection to ensure that only B cells that express functional BCR (positive selection) and do not ligate self-antigens (negative selection) survive. On the other hand, immature T cells are exported to the thymus for final maturation. Although the puppy was considered immunocompetent between 6–12 weeks of age, it is not possible to accurately predict the onset of immunocompetence, since it depends on the presence of MDA [21]. Increased life span allowed the recognition of age-related higher susceptibility to infectious, inflammatory, autoimmune, and neoplastic diseases. Age-related changes include impairment of the cell-mediated immune response, as demonstrated by the reduction of proliferative response of blood lymphocytes to mitogens and the reduction of cutaneous delayed type hypersensitivity [21]. Moreover, there is a decline in the humoral immune response probably related to the decreased functionality of Th cells. The ability to mount humoral immune responses seems to prevail, as demonstrated by the persistence of protective vaccine antibody titers, and respond to booster vaccination with elevation in titer [21]. Although the currently adopted triennial re-vaccination program, instead of the prior annual re-vaccination, offers adequate protection to young and adult dogs, this vaccination scheme may not confer protection to geriatric dogs [21]. Older dogs commonly present an impairment of immune responses to novel antigenic challenges, such as infections and vaccines, which probably is related to the reduction of the peripheral pool of naïve T cells and low diversity of the repertoire of T cell receptors [21]. The key genetic elements of immune responsiveness lie within the genes of the major histocompatibility complex (MHC); present as the dog leukocyte antigen (DLA) and feline leukocyte antigen (FLA) systems in the species under discussion. This would suggest that specific dog breeds have genetically determined immune function, and recent studies concern breed-specific serological response patterns to vaccination Such genetic background is also likely to impinge on maturation of the immune system in these species [17]. In dogs, C-reactive protein (CRP) is the main acute phase protein and its levels increase about a hundred times in infectious diseases such as babesiosis, leishmaniasis, parvovirus and colibacillosis. Acute phase protein levels increase moderately in canine inflammatory bowel disease. Concentrations of CRP, haptoglobin, and SAA are significantly elevated in the cerebrospinal fluid and serum of dogs with corticosteroid-responsive arthritis and meningitis. In pregnant dogs, the levels of haptoglobin, ceruloplasmin and fibrinogen

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are moderately higher [15]. Some studies showed, dogs in the asymptomatic and symptomatic groups with an outcome of heterogeneity in Cu, Zn, and Fe concentrations compared with the control group, emphasize the important roles of trace elements (TEs) in leishmaniasis [21]. Suggesting, TEs could be assessed as a prognosis factor in leishmaniasis, and/or an adjuvant for the treatment of leishmaniasis [22]. Is clear that, susceptible dogs mount a Th2 response characterized by high levels of antibodies, but showing a poor cell-mediated immunity [15]. These differences were attributed to the activities of IL-10-producing Treg lymphocytes [15]. In addition, also is consensus that the parasite can actively suppress IL-12 gene transcription, ensuring that the response Th2 predominates [15]. In that connection, a chronic and progressive disease develops in susceptible dogs as well as the macrophages loaded with parasites accumulate, with continuous multiplication in the organism, spreading throughout the body, and resulting in disseminated infection [15].

4. *Leishmania* diagnosis: some findings of natural canine infections

We present in that section the information's case report of "Jimmy", a healthy dog (Shih Tzu), male, 1 year old that had presented eye infection (Figure 2) during clinical examination.

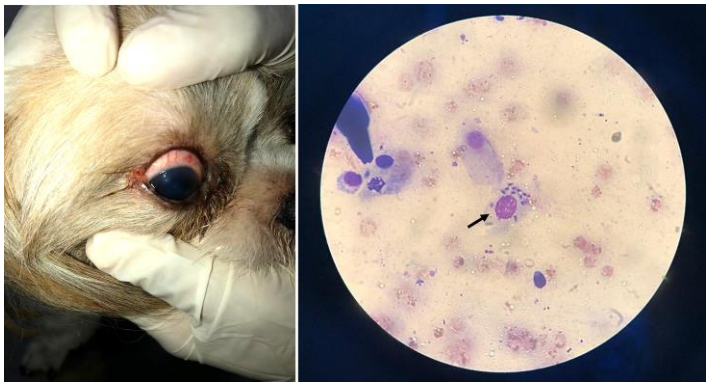

Figure 2. Ocular infection in canine leishmaniasis and light microscopy examination of a conjunctival cytological sample showing a macrophage containing multiple Leishmania amastigotes (arrow) (Authored by Dr. Gilvandro Rodrigues Galvão - UFRA).

3.1. Molecular results of clinical samples by Polymerase Chain Reaction PCR

PCR is based on the amplification of the number of copies of fragment(s) of Deoxyribonucleic Acid - DNA and/or Ribonucleic Acid of the researched microorganism (*Leishmania* and others). The POSITIVE result indicates the presence of the microorganism in the biological material analyzed, while the NEGATIVE result indicates its absence. - This test may present, although rarely, false-negative and false-positive results, which is a characteristic of the method. Animals with low parasitemia are more subject to false-negative results, especially when the test is performed from a peripheral blood sample. PCR test results that are at odds with the clinical status of the animal should be repeated with a new biological sample. Indirect immunofluorescence assay (IFAT) and quantitative real-time PCR (qPCR) are alternative methods that aid diagnosis.

3.1.1. Results of PCR for the biological samples collected from "Jimmy"

The biological samples were collected and analyzed according established protocols used in Molecular Biology of the Biomolecular Technology Laboratory of Institute of Biological Sciences of Federal University of Pará UFPA Brazil. The followed results were obtained:

- Auditive duct swabs: (PCR) *Leishmania* sp. NEGATIVE
- Mucous membrane of the anus swabs: (PCR) *Leishmania* sp. POSITIVE
- Oral Mucosa swabs: (PCR) *Leishmania* sp. POSITIVE
- Whole Blood*: (PCR) *Anaplasma platys* NEGATIVE
- Whole Blood*:(PCR) *Babesia vogeli* NEGATIVE
- Whole Blood*: (PCR) *Ehrlichia canis* NEGATIVE

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- Whole Blood*: (PCR) *Leishmania* sp. POSITIVE
- Whole Blood*: (PCR) *Mycoplasma* sp. NEGATIVE
- Whole Blood*: (PCR) *Rangelia vitalli* NEGATIVE
- Preputial secretion swabs: (PCR) *Leishmania* sp. POSITIVE
- Ocular Swab: (PCR) *Leishmania* sp. POSITIVE

*The whole blood sample was collected into Ethylenediaminetetraacetic acid (EDTA) tubes.

3.2. Hemogram and ELISA analyses

The results obtained from the samples of infected animal confirmed the *Leishmania* infection (Table 1).

Table 1. Hemogram blood and Serological results obtained from the samples of the dog "Jimmy" (Archive of LABPAT - UFRA).

COMPLETE BLOOD COUNT - VETERINARY
Material: Whole blood in EDTA

RESULTS	ABSOLUTE VALUE	REFERENCE VALUE
Erythrocytes: 3,38 millions		Dogs 5,5- 8,5 millions/mm3
Hemoglobin: 6,7 g/dl		Dogs 12,0- 18,0 g/dl
Haematocrit: 20%		Dogs 35-55%
Leukocytes: 10.400/mm3		Dogs 6000 a 17000 mm3
Mean corpuscular volume (MCV): 58,1 fl		Dogs 60-70% fl
Mean Corpuscolar Hemoglobin (MCH): 19,8 pg		Dogs 19-23 pg
Mean corpuscular hemoglobin concentration (MCHM): 33,5 g/dl		Dogs 32-36 g/dl
Eosinophils: 4%	416/mm3	Dogs 2-10%
Basophils: 0%	0/mm³	Dogs 0%
Lymphocytes: 13%	1.352/mm³	Dogs 12-30%
Monocytes: 4%	416/mm³	Dogs 3-10%
Myelocytes: 0%	0/mm³	
Metamyelocytes: 0%	0/mm3	
Bands: 0%	0/mm³	Dogs 0-3%
Segmented: 79%	8.216/mm3	Dogs s 60-70%
Platelets: 135.000%		Dogs 200 000 a 500 000 mm3

Method: Automated and Microscopic Analysis

Note: ANISOCYTOSIS ++; HYPOCHROMIA ++

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ALKALINE PHOSPHATASE - VETERINARY

Sample: SERUM
Result: 29,0 U/L

Reference value: Dogs 20 to 150 U/L;

Method: Dry Chemistry

URÉIA – VETERINÁRIO
Sample: SERUM
Result: 29,0 MG/DL

Reference value: Dogs 15 to 65 MG/DL;

Method: Dry Chemistry

TOTAL PROTEINS AND FRACTIONS – VETERINARY
Sample: SERUM

Total Proteins: 14,7 g/dl
Reference value: Dogs 5,8 to 7,9 g/dl

Serum Albumin: 1,1 g/dl
Reference value: Dogs 2,6 to 4 g/dl

Serum Globulin: 13,6 g/dl
Reference value: Dogs 2,3 to 5,2 g/dl

Method: Dry Chemistry

CREATININE - VETERINARY

Result: 0,6 mg/dl
Reference value: Dogs o 0,5 to 1,5 MG/DL
Sample: SERUM
Method: Dry Chemistry

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SERUM GLUTAMATE PYRUVATE TRANSAMINASE (SGPT)/ALANINE AMINOTRANSFERASE (ALT) - VETERINARY

Sample: SERUM
Result:28,0 U/L
Reference value: Dogs 10-88 U/L
Method: Dry Chemistry

CANINE LEISHMANIASIS (ELISA and IFAT)

METHOD: Enzyme-linked Immunosorbent Assays (ELISA)
Result: Reagent
CUT OFF: 0214
Value of OD*: 0,664

Kit with License in the Ministry of Agriculture of Brazil - MAPA

Number: 7.434/2000, Batch control 004/18, Validity: 02/2019

METHOD: Indirect Fluorescent Antibody Test (IFAT)

RESULT: REAGENT 1/80

Kit with License in the Ministry of Agriculture of Brazil - MAPA

Number: 9347/2007, Batch control 228, Validity: 07/2019

Reference value: ELISA: NON-REAGENT: Optical Density with value below the Cut off, INDETERMINATE: Result with intermediate values, corresponding to the Gray Zone, where the Tests were not able to determine whether it is Reagent or Non-Regent. To determine the amplitude, the cutoff point 0.03 is subtracted. A new test is recommended 30 days after the last test, as it may correspond to the onset of serum conversion, nonspecific reactions, or immune system failure. REAGENT: *Optical Density with value above the Cut off. RIFI: NON-REAGENT: Results without antibody titers. REAGENT: Result with a titre equal to or greater than dilution 1/40.

5. An emerging focus on lipids in *Leishmania* extracellular vesicles

Extracellular vesicles contain a lipid bilayer membrane that protects the encapsulated material, such as proteins, nucleic acids, lipids and metabolites, from the extracellular environment. These vesicles are released from cells via different mechanisms. During recent years extracellular vesicles have been studied as possible biomarkers for different diseases, as biological nanoparticles for drug delivery, and in basic studies as a tool to understand the structure of biological membranes and the mechanisms involved in vesicular trafficking. Lipids are essential molecular components of extracellular vesicles, but at the moment our knowledge about the lipid composition and the function of lipids in these vesicles is limited. However, the interest of the research community in these molecules is increasing as their role in extracellular vesicles

3.1. Quantification of lipid bodies CLs in promastigote of Leishmania (Leishmania) amazonensis and endocytosis and exocytosis process of Leishmania

The knowledge of specific cargo molecules identified within *L*EVs suggest their function as adjuvant-like in the immune responses, with a possible key advantage for the infection establishment and disease progression, inducing at the same time quantitative and qualitative changes in the protein content of infected host cells extracellular vesicles (Figures 3 and 4).

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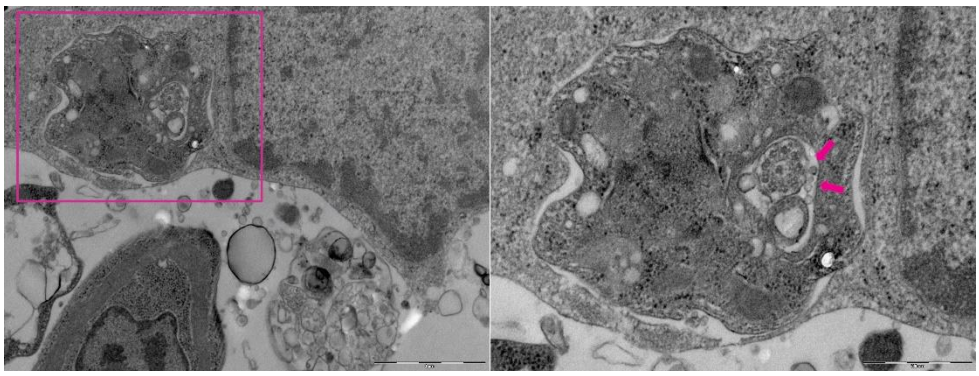

Figure 3. Tubular structures and vesicles (arrows) participating in the endocytosis and exocytosis process can be seen in the flagellar pocket (FP) of *L.* (*L.*) amazonensis promastigotes (MHOM/BR/2009/M26361 strain). F = flagellum; K = kinetoplast; M = mithocondria; N = nucleus. Scale-bar 1 μ and 500 nm.

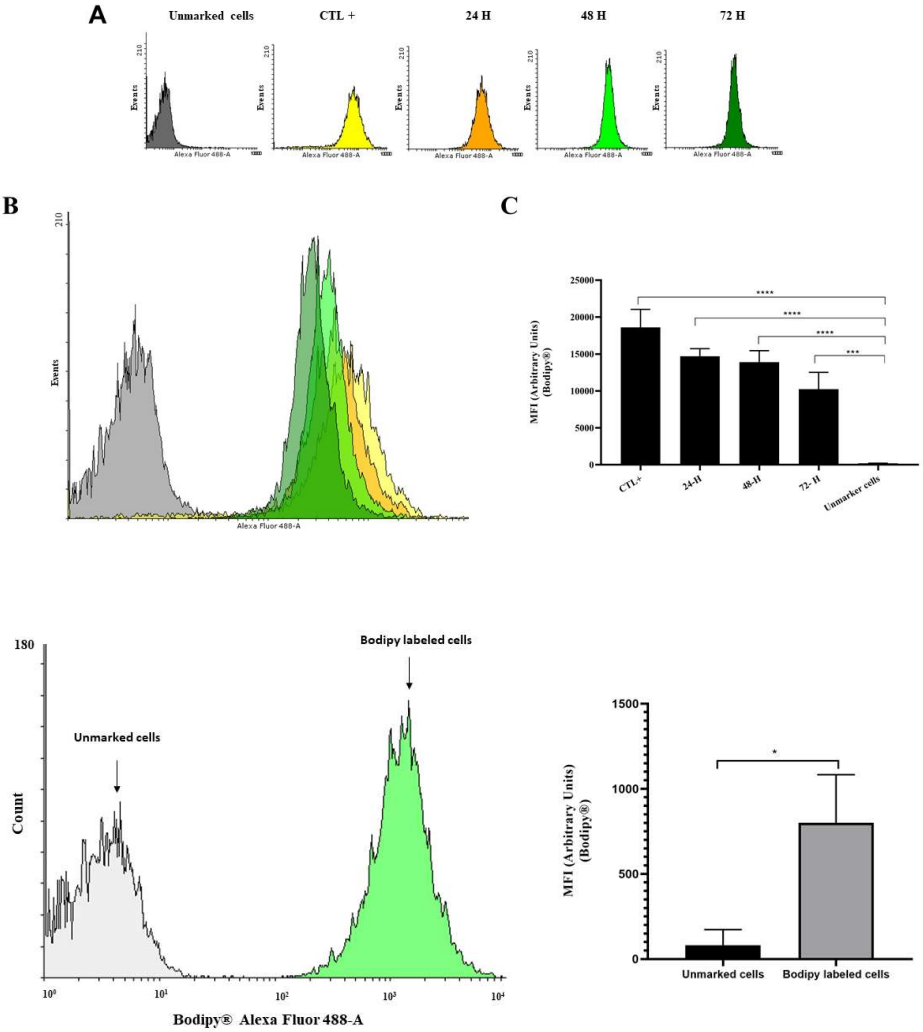

Figure 4. Quantification of lipid bodies in *Leishmania* (*L.*) *amazonensis* at different culture times; A) Histogram of each experimental group; B) Overlay histogram demonstrating increased fluorescence intensity of BODIPY™ 493/503-labeled cells at 24, 48 and 72 hours of culture and C) Graphic representation of BODIPY™ 493/503-labeling in *Leishmania* promastigotes ***$p < 0.001$ (ANOVA and Tukey post-hoc test).

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3.1.1. Methodology

Supplementary contents 1 and 2

3.2. Macrophages infected with Leishmania (Leishmania) amazonensis examined by Transmission Electronic Microscopy TEM

6. The immune expected effects of approved vaccines for canine leishmaniosis and some steps for the next-generation therapies following *L*EVs research advances

Effective vaccines are available against leishmaniasis. The most effective use purified fractions of *Leishmania*, including enriched fraction of glycoproteins, also called fucose-mannose ligands [15]. This vaccine not only prevents the development of the disease, but also serves as an immunotherapeutic agent, producing clinical improvement in dogs with disseminated disease [15]. An alternative vaccine containing excretory and secretory products of *L. infantum* promastigotes with an adjuvant Muramyl dipeptide also seems to work well. Experimental vaccines, including attenuated and ADN have shown promising results for Veterinary Medicine [15]. For additional *Leishmania* control mechanisms and tools are needed, including new drugs, vaccines, diagnostics, and vector control agents and strategies [23]. Considering these important points, for the future of prevention and treatment of leishmaniases we can include all advances resulting from the *L*EVs research in the world that represent variety of advantages over live biotherapeutics (next-generation therapies) [23]. Over the past years, isolation and analysis of extracellular vesicles from *Leishmania* is challenging. The protocols are not standardized yet, like we refer in our Guidelines for exosomal research published in 2021 [23], as many variants with potential effect on the outcome are used and published. Is important to refer that several protocols are labour intensive, requiring costly equipment, increase risks for loss of heterogeneous extracellular vesicles and do not discriminate well between exosomes and contaminating structures such as lager vesicles and protein/lipid aggregates [24]. We referred also that direct isolation with magnetic beads would can be used for *L*EVs research, requiring minimal hands-on provides highly pure exosomes with minimal loss and enables future automation opportunities in an immunoassay format and others lab experiments improved in the last years in the Ketil Winther Pedersen's Lab. To this end, multidisciplinary expertise cooperation is very important to further our understanding of the *Leishmania*-host-cell interactions, a broad-scale analysis of the cargo of their production of extracellular molecules and create very promising perspectives for the development of innovative applications following the *Leishmania* virulence factors that include lipophosphoglycan (LPG), surface acid proteinase (GP63), glycoinositolphospholipids (GIPLs), proteophosphoglycan (PPG), A2 protein, the kinetoplastid membrane protein (KMP-11), nucleotidases, heat-shock proteins (HSPs), and transmembrane transporters, which support the survival and propagation of the parasite in the host cell [1].

7. Discussion

The clinical signs of leishmaniasis are directly related to the immune response of the infected dog and we can account the disease into four stages based on serological status, clinical signs, laboratory findings and type of therapy and prognosis for each stage [3–15]. In susceptible animals, organisms can spread from skin to the local lymph node, spleen and bone marrow in a few hours [15]. In resistant dogs, the parasite remains restricted to the skin and draining lymph node [15]. According these considerations the first-choice samples should be used for PCR are: bone marrow, lymph node, spleen, skin and conjunctival swabs and others, however samples of blood, buffy coat and urine are considered less sensitive [3]. In parallel, the study of *L*EVs of prokaryotes and eukaryotes has aroused considerable interest in the scientific community, due to the possible potential for the development of diagnostic and therapeutic methodologies [1]. Despite advances in *Leishmania* studies, the selective mechanisms of *L*EVs are still poorly understood, there is no consensus on the differential characterization or ultrasensitive detection of their specific subtypes, biomarkers or their biogenesis and how this knowledge can be effective for faster diagnosis and

prevention [1]. Actually, the diagnostic methods for canine leishmaniasis include: parasitological (cytology/histology; immunohistochemistry and culture); molecular (conventional, nested and real-time PCR, considered the most sensitive technique) and serological quantitative (IFAT and ELISA) and quantitative (rapid tests) [3]. Increased transmission of the parasite and higher incidence of disease, as well as the emergence and re-emergence of this disease recorded in recent years may be related to many factors, among which we highlight the socio-economic conditions, climate and environmental change, closer contact between pets and wild ecosystems and the parasite resistance to drugs and insecticides vectors in use [1]. Concurrently with the spread of leishmaniasis recently studies reveal that dogs are at risk of acquiring coinfection with emerging zoonotic parasites [1]. In that sense, the most popular pet animals worldwide, such as dogs and cats can become infected by parasites [1]. Companion animals are important agents of different parasitic species, these infections can cause many complications with risk of high morbidity, including weight loss, anemia, and low immune resistance [25–27]. In turn, these conditions can lead to secondary infections and even death of these animals [27]. Many parasite species potentially threaten canine and feline health, while some dog and cat parasites are strictly associated to these animals, some of them may also infect humans causing zoonoses [27,28]. Protozoa and helminths are two major groups of organisms acting as etiologic agents of animal and human diseases with variable severity, especially for immunocompromised hosts resistance [28–31]. Parasitic infections caused by protozoa and helminths infect predominantly puppies, kittens, geriatric, chronically sick or immune-compromised animals and perhaps pregnant animals [32]. Older dogs and cats are mainly immune after previous infections and seldom show symptoms, however, may still be a source of transmission of infection [25–33]. Companion animals living in crowded conditions and poor sanitation or with access to the outdoors, may have a high risk of direct transmission of protozoan infections, for example the following: *Giardia* sp, *Trichomonas* sp, *Cryptosporidium* sp and *Cystoisospora* sp [25–35]. Dogs and cats may contact rodents or ingest raw meat are in risk to acquire infections caused by cyst-forming coccidia, i.e., *Neospora* sp, *Hammondia* sp, *Toxoplasma* sp and *Sarcocystis* sp [25–32]. Dogs and cats can also be infected with others gender and parasites species, like *Ancylostoma* sp *Angiostrongylus* sp [36–38]. Emerging infectious diseases may increase frequently in some regions, either due to increased importation of infected vertebrate hosts or by the establishment of pathogens and their vectors in previously non-endemic areas [39]. Cases of zoonotic infections such as leishmaniosis, babesiosis and dirofilariasis was detected in some non-endemic regions, mainly due to the expansion of the parasitic transmission area and their increasing occurrence in wildlife, which act as reservoir or hosts of their complex cycles [38–42]. Leishmaniasis affects humans and domestic companion animals and wild animals worldwide, involving reservoir and hosts such as rodents, marsupials, edentates, monkeys and wild canids [40–43]. More recent surveys reported from several countries in the world indicate that zoonotic parasites (*Leishmania* sp, *Babesia* sp, *Toxoplasma* sp, *Neospora* sp. and *Dirofilaria* sp.) are associated with infections and coinfections in companion animals [25–38]. Moreover, parasitic infections represent a serious public health threat, particularly in developing countries like Brazil where many unwanted animals are simply abandoned without clinical diagnosis to suffer and die on the streets of rural areas, but also in suburbs and large urban centers suffer deficient sanitation services [27–45] (Tables S1 and S2—Supplementary Contents 2) [1]. However individual case reports can add new parameters for the accuracy of diagnosis, to confirm the coinfection and the range of differential diagnoses – or if there the animal remains healthy or develops a mild, self-limiting illness [15]. Considering the canine leishmaniasis, these hardy dogs mount a weak antibody response, but a strong and effective Th1 response may have low antibody titers but produce IFN-γ in response to antigens parasitic, generate type I granuloma, mount a strong response of hypersensitivity of the late type, and eventually destroy the parasites [15]. The resistance to *Leishmania* has a strong genetic component; for example, dogs of the Podengo breed Ibicenco (Ibizan Hounds – antique hunter of rabbits) appear to be resistant to this parasite. There is also an association between resistance and certain MHC class II haplotypes, as well as certain Slc11a1 (Nramp) alleles in dogs [46]. Thus, the Ibizan Hounds may be an interesting canine model for the investigation of protective anti-*Leishmania* immune response [46]. Results of recent research show relevant differences between the cytokine

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serum profile and the data published for other canine breeds, and several genetic fixed variants in genes related to immune response, regulation of immune system, and genes encode cytokines and its receptors [47]. The most relevant genes that present such fixed polymorphisms were IFNG and IL6R [47]. Other variants with frequencies equal or above 0.7 were found in the genes ARHGAP18, DAPK1, GNAI2, MITF, IL12RB1, LTBP1, SCL28A3, SCL35D2, PTPN22, CIITA, THEMIS, CD180 [47]. Epigenetic regulatory genes as HEY2, L3MBTL3 show also intronic polymorphisms [47]. Future studies will can reinforce why the regulation of immune response is different in the Ibizan hound dogs compared to other breeds [47]. By other side, some dogs develop severe and generalized nodular dermatitis, lymphadenitis granulomatous, splenomegaly and hepatomegaly, exhibiting activation of polyclonal (occasionally monoclonal) B lymphocytes involving all four classes of IgG, as well as hypergammaglobulinemia, and develop lesions associated with hypersensitivity types II and III [15]. Additionally, excessive production of immunoglobulin can lead to the development of an immune-mediated hemolytic anemia, thrombocytopenia and the production of antinuclear antibodies [15]. The chronic deposition of Immune complexes can result in glomerulonephritis, uveitis, and synovitis, leading to failure renal and death [15]. The significant elevation of ant histone antibodies is a feature of some dogs with glomerulonephritis associated with leishmaniasis. There is a positive correlation between the levels of these ant histone autoantibodies and the protein/creatinine ratio once that antibodies increase the likelihood of the development of glomerulonephritis [15]. But, despite their antigenicity, parasitic protozoa manage to survive in their host using multiple evasion mechanisms acquired over many millions of years of co-evolution [1–15]. Following the reproducible results for lipid bodies of *Leishmania* labeled with BODIPY™ 493/503 we believe that the crucial 72 hours period has showed clear decrease of lipids released. This regression is in accord with Zhang (2021) in the way that amastigotes acquire most of their lipids from the host although they retain some capacity for de novo synthesis, differently of promastigotes that rely on de novo synthesis to produce the majority of their lipids including glycerophospholipids, sterols and sphingolipids [48].

8. Conclusions

Leishmania-infected dogs continue to be parasite reservoirs for sandfly vectors [1]. Therefore, new protocols are needed to achieve a better efficacy in the prevention and clinical treatment [1]. Different types of laboratory tests are available to diagnose parasitic infections, including conventional methods considered as gold standards and serological [43–50]. Several molecular diagnostic tools to detect parasites and new strains have been developed in the last decades [43–50]. Accurate diagnosis of zoonotic infections collaborates with the work of medical scientists, policy makers and public health officials planning to prevent the dissemination of these diseases, and establishing a world-wide network of surveillance for the coinfection of parasitic infections [49,50]. Actually, advanced techniques for the study and diagnosis of simultaneous infections are indicating that multi-parasitism is more common than single infections [51,52]. Evolutionarily, multi-parasite systems are ecologically dynamic, they involve key host species within multi-host parasite systems and their contribution to transmission [52]. The understanding of this complex relations is one of the highest priorities for biomedical sciences for the 21st century [53]. Clinically, coinfection of zoonotic parasites in companion animals may appear in its classical presentation, with acute aggressive evaluation or coming at long-standing infection, asymptomatic or sometimes non-specific, difficulting the clinical diagnosis [33–53]. Veterinarians and clinical researchers should consider the health status and background of the patients (animals or humans) to apply a better parasite management program [53]. Considering certain factors like resource-mediated processes most often influencing how, where and which co-infecting parasites interact, and may dictate more intensive monitoring for effective treatment, while others may suggest a less aggressive approach [32–53]. Furthermore, innovative strategies applying the knowledge about extracellular vesicles in their specific profiles, including proteic and lipidic data basis, on the studies of host-parasites mechanisms can be incorporated into immunotherapy to interfere with the dynamics of disease transmission and progression and the development of effective, safe and available vaccines against leishmaniasis in

helping to protect puppies and dogs of different ages. Actually 4 vaccines against canine leishmaniasis are available on the market, Leishmine® and Leish-Tec® in Brazil, CaniLeish® and LetiFend® in Europe (the first vaccine based on purified excreted//secreted antigens of *Leishmania* has been licensed in Europe since 2011) [1–54]. Challenges with these vaccines include current manufacturer recommendations which require the vaccination of seronegative dogs. In countries where disease is endemic in both dogs and people, identification of healthy uninfected animals is less than 100% accurate due to challenges with current diagnostics. Adverse events were mild and site specific, so use of vaccines in healthy sub-clinical dogs may warrant a change in current recommendations regarding vaccination/immunotherapy in infected healthy animals [16]. Despite the available studies on licensed vaccines for canine leishmaniasis, they are still considered insufficient, given the lack of standardization of the study design, methodological deficiencies and substantial differences in the characteristics of the study populations are some of the issues that impede comparative analysis between the available vaccines. In addition, research is needed on other aspects of vaccination: xenodiagnostic studies to assess the infectivity of vaccinated and infected dogs and an adequate assessment of the potential interference of vaccination in the diagnosis of *Leishmania* infection are some examples. In addition, long-term pharmacological surveillance should be maintained after licensing any vaccine to provide reliable information to relevant organizations and the general public [54]. In this way are expectable, like we had indicated in previous publication of Guidelines for Exosomal Research [1], that more advances in the techniques and protocols for accuracy of isolation and characterization of *L*EVs and their activity on the host immune responses, including their lipid bilayer membrane that protects the encapsulated material, such lipids (essential molecular components of extracellular vesicles), from the extracellular environment. Actually, the knowledge about the lipid composition and the function of lipids in *L*EVs is very limited. Changes in the lipid profile and metabolism in both parasite and host during development of the disease depend on the presence of lipid bodies. Further research is required to fully understand the relationship between the interactions between lipid metabolism of host and parasite, immune response, and the prognosis of the disease [55]. However, we propose with our findings to enlarge the interest of the research community in these molecules release by *Leishmania* as their role in host-parasites extracellular interactions.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Methodology 1 Methodology 2, Tables S1 and S2: (open field for future research).

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