 doi:10.20944/preprints202105.0754.v1

Review

Leishmania in 360°: guidelines for exosomal research

Áurea M. Gabriel [1,4] *, Adan Galué-Parra [4], Washington Luiz A. Pereira [2], Ketil W. Pedersen [3] and Edilene O. Silva [4,5]

[1] Global Health and Tropical Medicine, GHTM, Institute of Hygiene and Tropical Medicine of NOVA University of Lisbon, IHMT-UNL 1349-008 Lisbon Portugal.
[2] Laboratory Animal Pathology of Federal Rural University of Amazon, UFRA, 66077-830 Belém PA Brazil;
[3] Thermo Fisher Scientific Fisher Scientific AS Postboks 114, Smestad 0309 Oslo Norway.
[4] Institute of Biological Sciences of Federal University of Pará, Av. Augusto Correa 01 66075-110 Belém - PA Brazil.
[5] National Institute of Science and Technology in Structural Biology and Bioimaging, Rio de Janeiro, RJ, Brazil

* Correspondence: aurea.gabriel@ihmt.unl.pt, aureamartins@ufpa.br (Á. G.)

Received: date; Accepted: date; Published: date

Abstract: *Leishmania* parasites are a group of kinetoplastid pathogens that cause a variety of clinical forms while maintaining cell communication by secreting extracellular vesicles. Emerging technologies have been adapted for the studies of *Leishmania*-host-cell interactions to enable broad scale analysis of parasite extracellular vesicles. *Leishmania* extracellular vesicles (*L*EVS) are naturally released spheroidal nanoparticles of polydispersed suspensions surrounded by a lipid layer of membrane. Although *L*EVs have increasingly gained in importance, much is still unexplained, including bioavailability and function in the complex molecular mechanisms of pathogenesis. Considering the importance of *L*EVs in the parasite-host interaction and in the parasite-parasite relationships emerged during evolution, the current review aims at giving an overview of *Leishmania* summarizing knowledge and formulating guidelines for *L*EVs research. In the end, we report, direct methods for specific isolation of *L*EVs from promastigotes and amastigotes culture supernatant suitable for a range of different downstream applications increasing the compatibility and reproducibility to establish optimal and comparable isolation conditions and full *L*EVs characterization, and crucial immunomodulatory events triggered by this important group of parasites.

Keywords: Exosome isolation and characterization; Exosomal research guidelines; Intercellular communication and host manipulation; *Leishmania* extracellular vesicles cargo; Leishmaniases.

1. Introduction

Leishmaniases are zoonotic vector borne diseases that are found in parts of the tropics, subtropics and southern Europe, with new epidemiological scenarios emerging in endemic areas and the diseases spread to previously free areas because of human population displacement, continuing to pose major public health problems worldwide [1–2–3–4–5] [Figure 1]. In 2018, according to the World Health Organization (WHO), more than 95% of new cases reported to WHO occurred in 10 countries: Brazil, China, Ethiopia, India, Iraq, Kenya, Nepal, Somalia, South Sudan and Sudan, affecting mostly people living in the most impoverished areas of low-income countries furthering their economic stress [2–6–7–8–9]. Current estimates show an annual global incidence of 700 000 to 1 million new cases of these diseases and disability-adjusted life years (DALYs) lost due to leishmaniasis closer of 2.4 million, affecting approximately 12 million people in 98 countries with 350 million people at risk of infection [2–3–10–11–12]. The infection in humans, as well as other vertebrates, are caused by clinically significant kinetoplastid hemoflagellates and intracellular parasites from over twenty species of the genus *Leishmania* belonging to the two subgenera *Leishmania* and *Viannia* [4–10–11] [Table 1]. Over ninety sand fly species (Diptera, Psychodidae, Phlebotominae) are known to direct

transmit metacyclic promastigotes forms of *Leishmania* parasites to vertebrate hosts by the bite of naturally infected female during its opportunistic and eclectic feeding [2–3–4–5–8]. In the New World, *Leishmania* infected sand flies belongs to the genus *Lutzomyia*, which have evolved in isolation of the Old World vector genus *Phlebotomus* (from ca. 200 000 000 years) [2–3–4–5–8]. Leishmaniasis comprises a variety of clinical syndromes reported in three main ways: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL), depending greatly on the parasite subspecies and the genetic and immunologic factors of the host under complex evolutionary interactions [2–3–4–12]. VL, also known as kala-azar, is typically fatal if left untreated in over 95% of advanced cases, around 20 000 to 30 000 deaths per year [2–3–4–12-13]. Findings of different studies identified those children under 5 years old are at higher risk of premature death being relevant for planning interventions aimed at reduction of VL lethality in countries like Brazil [2–3–4–12]. Annually, estimated 200 000 to 400 000 VL cases and estimated numbers of new cases worldwide at 50 000 to 90 000 are reported, however only 58 000 effectively communicated to WHO [2–3–4–12]. Moreover, VL emerging pattern in human immunodeficiency virus (HIV) infected individuals [2–3–12]. Currently, under the context of the COVID-19 pandemic impacts and its consequences, several studies become to consider the aggravating potential risk for vector borne diseases like visceral and cutaneous Leishmaniasis, following the serious global health crisis and unprecedented socioeconomic crisis caused by the viral spreading worldwide [14–15–16]. In addition, in countries like Afghanistan, one amongst the poorest countries in the world, increasing economic impact for CL patients, mostly affect women, certainly related to the gender social roles leading to productivity loss, work absences and inability to perform housework [14–15–17]. Estimated 5 to 10% of VL cases, a potential source of *Leishmania* infection, develop post-kala-azar dermal leishmaniasis (PKDL), that emerges as a new disease entity representing more than 50% successfully VL recovered patients in some African countries, where pentavalent antimonial compounds such as meglumine antimoniate (Glucantime®) and sodium stibogluconate (Pentostam®) are prescribed routinely for leishmaniasis treatment [2–18–19–20]. CL is the more common form of the Leishmaniases affecting approximately 1 000 000 to 1 500 000 human beings, where 66 941 CL cases per year reported in the Americas, presenting high incidence mainly in areas with lack of economic resources, insufficient trained health professionals and low awareness for the health issue of leishmaniasis [2–3–4–21-22]. Roughly, 3–5% of CL infections can progress to the mutilating MCL form of the disease that has a significant negative effect on the decreased quality of life (QoL) and mental health [1–2–10–13-21]. Over 90% of MCL infections occur in Ethiopia, Bolivia, Brazil and Peru, where studies are being advanced, but the pathogenesis of MCL is still unclear [2–3–4–13–21]. Results based on epidemiological surveillance have shown that the incidence of CL diseases continues to increase worldwide, this is largely due to the rise of several risk factors, e.g., poverty, poor nutrition, population mobility (behavioural), climate changes (climate-sensitive infections) and environmental changes such as deforestation, intrusion into primary forest, building of dams, irrigation schemes, and urbanization [1–2–4–22]. *Leishmania* parasites exhibit rapid adaptation and epidemic expansion after environmental changes, so besides the risk factors, the increase of positive cases can be mainly attributed for the no effective and efficient vaccines or drugs to prevent *Leishmania* infections [1–2–18–19–23]. According to these premises, in this review we present an approach to action representation that is based on the importance of the study of *Leishmania* extracellular production in the general context of Leishmaniases and which may serve as a basis for future researches.

2. *Leishmania* (Kinetoplastida: Trypanosomatidae)

Leishmania is the genus of a very successful group of parasitic protozoans divided into Old and New World species adapted to different reservoir hosts such as dogs, rodents, and other vertebrates, presenting a large host range that could often be infected by more than one parasite species [Figure 2] [4–10–24–25–26]. In this context, progress of models studies parameterized at geographical concepts may be more sensitive to project substantial future changes on global extent of Leishmaniases under perspective of human socio-economic status and of the increase in risk factors, considering *Leishmania* phenotypic diversity distribution as well as facilitate vector and geographic

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 May 2021 doi:10.20944/preprints202105.0754.v1

range expansion for diseases control [27–28–29–30–31]. Different species of *Leishmania* are multi-host pathogens that cause parasitic disease in humans and other mammals like dogs, being associated to specific pathology and respective histopathological and clinical features [Figure 3 and Figure 4] [Table 1] [4–32–33–34–35]. *Leishmania* parasites developed efficient strategies to overcome protective mechanisms, modulating the physiological condition of different host immune responses by using genes, signaling pathways and mechanisms for the secretion of virulence factors [32–33–34–35–36]. The *Leishmania* virulence factors (lipophosphoglycan LPG, surface acid proteinase GP63, glycoinositolphospholipids GIPLs, proteophosphoglycan PPG, A2 protein, kinetoplastid membrane protein KMP-11, nucleotidases, heat-shock proteins HSPs, transmembrane transporters and other proteins), promote parasites survival and propagation into the host cells [32–33–34–35–36]. *Leishmania* parasites exhibit on external surface of the plasma membrane a dense glycocalyx composed of several molecules LPGs, PPGs glycoproteins 63 and glycosylinositolphospholipids (GIPLS), anchored by means of a glycosylphosphatidylinositol (GPI) [32–33–34–35–36]. Specifically, biochemistry and cell biology of *Leishmania* are similar to that of other kinetoplastids, presenting distinctive phases of genetic population proliferation and differentiation associated with each proliferative cell types and transmission cell types [37–38–39–40–41]. These parasites exhibit a digenetic life cycle involving a variety of cellular morphologies adapted to the hosts and vectors and a number of developmental forms defined by cross-linked sub-pellicular corset microtubules, so cell division relies on the insertion and elongation of these microtubules into the existing array [11–41–42–43–44]. *Leishmania* have two main forms during their development: extracellular promastigote morphology in the sand fly (length 16±40 μm and wide 1.5±3 μm) and intracellular amastigote morphology in the mammalian host (1.5-3 μm x 3-6.5 μm diameter) [41–42–43–44–45]. The amastigotes are smaller and spherical cell body with a short immotile flagellum for sensory functions, and promastigotes are highly motile elongated ovoid cells body with a long flagellum to provide propulsive force to traverse through the invertebrate vector digestive tract [41–42–43–44–45]. After sand fly ingestion, following the combination of the change in temperature and pH akin and adaptations to the intracellular or extracellular lifestyle in different hosts, amastigote *Leishmania* released from the macrophage begins to differentiate into a motile promastigote form with potential sensory functions to escape the peritrophic matrix and migrate to the invertebrate foregut [41–42–43–44–45]. In other way, studies *in vitro* differentiation of *Leishmania (Leishmania) amazonensis* amastigotes into promastigotes has been detailed by electron microscopy, gaining a greater knowledge of these important parasite mechanisms [44]. Currently, for high care and caution when defining cell types solely based on cell morphology, exist few molecular markers to identify life cycle forms [36–39–45–46]. Specifically, shape and cell form of *Leishmania* are intricately linked to their pathogenicity and ecological niches, needing to be accurately transmitted from one generation to the next during subsequent mitotic divisions, however recent studies has shown that sexual strategy as meiosis-like in *Leishmania*, and other sexual processes are possible [8–47–48–49–50]. Both evolutive forms conserve basic cellular architecture and their ultrastructural features during the life cycle, presenting the kinetoplast anterior to the nucleus [43–44–46–49–50]. These cells present an enlarged portion of the single branched mitochondrion containing mitochondrial DNA (kDNA) that is directly connected to the basal body from which a single flagellum extends from the Flagelar Pocket (FP) attached by a cytoskeletal structure called flagellum attachment zone (FAZ) [Figure 5] [41–42–43–44–45]. Until now, *Leishmania* FP is identified as the only site of endocytosis and exocytosis [Figure 5], thus a critical interface on inter-specific parasite interactions between parasites and parasite intra-host interactions, playing an important role in the resolution of cell membrane organization and morphogenetic resolution during successful *Leishmania* cell division from two flagella initially occupying the same zone [42–43–45]. In addition, morphological parameters *in vitro* highlight *Leishmania* developmental forms definition overarching different promastigote and amastigote morphologies play specific cell-extracellular matrix (ECM) interactions to keep vital functions and success in host infections [41–42–43–44–45]. Therefore, these variations emphasizes the need for new independent *Leishmania* markers for the different life cycle cell types such as the procyclic and metacyclic promastigotes identified in the sand flies based on the length/ width of the cell body and

 doi:10.20944/preprints202105.0754.v1

flagellum [42–43–45]. Morphologically, the following promastigotes classification can be applied to different studies of the *Leishmania* cultures: procyclic, nectomonad, leptomonad and metacyclic promastigote forms [40–42]. *In vivo*, leptomonad promastigotes live in mammals' phagocytic cells, replicating within membrane-enclosed compartments and establishing a persistent infection by inducing macrophage dysfunction through direct manipulation of macrophage signaling, suggesting it occurs both via flagellum first and predominantly via cell body [40–42–43–45]. Into the infected macrophages, the promastigotes differentiate to amastigote forms, changing the cell shape and minimizing cell surface to volume ratio, reducing the area exposed to the harsh environment of distinct types of parasitophorous vacuole, according the specific requirements of *Leishmania* species [51]. Further studies are necessary in order to elucidate the role of cellular components organization and its relation with molecules cargo and surface receptors used in the target cell recognition in *Leishmania* life cycle [52–53]. Therefore, the forms of development of *Leishmania* are distinguished by their morphology, environmental and nutritional requirements and stimulus *in vitro*, its metabolism, motility, growth rate and multiplication in the host or in medium cultures and, its molecular expression regulated by extracellular vesicles [54]. Increasing evidence has shown that parasite persistence seems to be very frequent in *Leishmania* infected patients, with the development of immunocompromised and in some cases the immunosuppression, affecting mechanisms of innate and adaptive immune systems, being highly related to inefficient and polarized immune responses [4–32–35–42–55]. Classically, CL is described in four categories, presenting different clinical forms: localized cutaneous leishmaniasis (LCL); mucocutaneous leishmaniasis (MCL), disseminated leishmaniasis (DL) and anergic diffuse cutaneous leishmaniasis (DCL) [2–4–56–57–58]. In CL epidemic regions of western Asia, *L. tropica* infections reported cases advanced to aggressive and prolonged disease courses, with lesions possibly affecting vital sensory organs function, including olfactory perception and vision [2–4–59]. Atypical CL, also known as nodular or tuberculoid form of the leishmaniasis, present diffused papules and nodules in uncovered areas of the body [2–4–13–59]. Conserved parasite genes expression may contribute to the diffuse nature of CL, acrossing multiple species and immunological conditions of underlying causes less well-understood [2–4–13–56–59]. DCL is a disabling and rare form of leishmaniasis caused by the parasite *L. (L.) amazonensis* that grows uncontrollably in diffuse lesions across the skin developed in an anergic host with poor immune response, being reported DCL patients typically refractory to chemotherapy lacking a DTH response [2–4–13–56–60]. The majority of CL cases present skin injuries on exposed parts of the body, mainly severe ulcers, leaving life-long scars and serious disability or unaesthetic stigma by multiple lesions [56–58–61]. Despite many advances in clinical studies and greater knowledge of leishmaniases, the spread of these parasitic diseases and the specific host-parasites interactions are still not fully known and, consequently, making it difficult to entirely understand the dynamics in thgoncalvese course and severity of *Leishmania* infection and persistence of host-pathogen relationships throughout the course of disease [4–6–56–59]. Hence, there the studies in this field of *Leishmania*-host interactions are considered excellent models for virulence factors, survival and transmission success of life cycle forms able to interact with the physiology and function of complex cellular and molecular mechanisms, modifying the cellular environment establishing niches and modulating with the histopathological and immune response of different hosts [4–39–62–63]. The knowledge of specific cargo molecules identified within *L*EVs suggest that 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 [32]. Thus, both host proteinases (matrix metalloproteinases) and parasite proteinases (cysteine proteinases, metalloproteinases and serine proteinases) affect the dynamics of the *Leishmania* infection [64–65]. These findings and *in vitro* advances hint at a previously unrecognized potential mechanism of *Leishmania* pathogenesis mediated by *L*EVs in the complex study of dynamic molecular mechanisms of *Leishmania* life cycle suggesting significant roles in the pathogenesis of different parasite species for future applications of *L*EVs and therapeutic use of hosts *in vivo* [66–67–68]. A thorough understanding of these parasites and its activity in different contexts of host and disease development reinforce the importance of characterizing specific *L*EVs pathways.

 doi:10.20944/preprints202105.0754.v1

3. *Leishmania* extracellular vesicles (*L*EVs)

In this review article, the term "*Leishmania* extracellular vesicles" (*L*EVs) is used to refer to these naturally released spheroidal nanoparticles of polydispersed suspensions surrounded by a lipid layer of membrane [69–70] [Figure 6]. Extracellular vesicles are heterogeneous, membrane-limited structures playing important roles in numerous biological processes, both pathophysiological and physiological, and specific intercellular crosstalk between cells, transferring information through cargo and modulating the immune system of the host [71–72–73–74–75]. Similar, to other trypanosomatids, Tritryps (the major human pathogens *Trypanosoma* (*Schizotrypanum*) *cruzi*, *T. (Trypanozoon) brucei* and *L.* (*L.*) *major*) may produce extracellular vesicles, thus including exosomes/ exosome-like vesicles and secrete proteins transported in vesicles by selective and efficient pathway from the endoplasmic reticulum (RE) to the Golgi apparatus and FP [23–43–70]. *In vitro*, temperature shift has noticeable effects on increase of proteins secretion via exosome-like vesicles on the surface of the *Leishmania* parasites cultured during their replication and development into metacyclic promastigotes [11–75–76]. Emerging technologies have been adapted for the studies of extracellular vesicles from cells and for the study of parasites *in vitro*, in the way to growth the knowledge about *Leishmania*-host-cell interactions and how enabling broad scale analysis the cargo of their production of extracellular molecules [77–78–79–80–81]. Several approaches can help in better understanding the role of parasite extracellular vesicles [74–78] [Figure 7]. *L*EVs are exported by classical or non-classical molecular mechanisms and are involved in the transfer of biologically active molecules, including lipids, metabolites, miRNAs, nucleic acids and proteins [79–82-83]. Extracellular vesicles effector cargo can be delivered into the host target cells, stimulating both pro- and anti-inflammatory immune responses [32–41–84–85–86]. These vesicles have been implicated in phenotypic cellular changes and in intracellular communication, such as antigen deliver and transport of macromolecular messages of proteins and acid nucleic with regulatory potential [87–88–89–90–91]. In recent years, research efforts have been made by the scientific community in developing experimental approaches to study the parasitic extracellular vesicles in response to the recent expansion of interest in the knowledge-based role of *L*EVs in the pathogenesis of leishmaniasis [92–93–94–95–96]. However, underlying reports of described functions attributed to *L*EVs include the export of proteins, the relation with environmental changes and the modulation of cytokine responses [41–77–97–98–99]. Although its role in infectivity and development of parasitic infection still poorly understood, there are irrefutable evidences that *L*EVs have important functions, needing more studies to clarify research gaps [32–100–101–102–103]. Available data on *L*EVs strongly suggest that these vesicles may represent the future of biomarkers in medicine or be the vectors of information able to modify the range of genes expressed within recipient cells [55–58–104–105–106]. Considering the *Leishmania* morphological forms, we expect that further studies shed light on the molecular factors involved in *Leishmania* pathogenesis, exploring the effect of *L*EVs on the recipient host immune response [32–74–88–96–97]. Advances in mass spectrometry (MS) in parallel are contributing for complex proteins identification and the accurate analysis of the secretome segregating by living cells (microscopic unicellular and multicellular organisms) into the extracellular space including adhesion molecules, chemokines, cytokines, enzymes and factors, being successfully applied for the discovery of protein biomarkers in extracellular vesicles [32–65–108–109–110]. Thereby, after 2007, there are a growing number of several experimental studies *in vitro* and *in silico* over the last decade regarding the role of *L*EVs, as well as studies on extracellular vesicles from others pathogenic unicellular eukaryotes, including microaerophilic extracellular protozoan parasites, in their biogenesis pathway and infectious strategy [32–74–92–102–111]. These studies have been focused on the following points: how mechanisms the parasites generate extracellular vesicles transferring a wide range of molecules; how they use the extracellular vesicles as vehicles for cell signaling and host immune system manipulation and how they elicit the pro-inflammatory response and cause disease, playing a key role in macrophage modulation [92–113–114-115–116]. Exosomes from procyclic and stationary phase (metacyclic-like) *L.* (*L.*) *infantum chagasi* display discrete protein profiles with approximately 50 known virulence factors detected, including molecules for immunomodulation and evasion (GP63, EF1α, Oligopeptidase), increased pathogenicity (Casein kinase, KMP-11, Cysteine Peptidase and BiP)

and parasite protection (Peroxidoxin) [36]. It is known that GP63 is significantly down regulated and changes its location in the parasite as promastigotes transform to the amastigote form within infected macrophages [93–58]. Considering this change in the localization of GP63 within the parasite, it is not known whether later stage macrophage infections, that harbor amastigotes forms, would continue to release GP63 in exosomes [35–41–93-94–99]. Therefore, it remains unknown whether parasite molecules that are synthesized in amastigote forms within macrophages in long-term infections are released in exosomes [36–41–53-54]. The great majority of *L*EVs studies using different species have shown that a low percentage (5-9%) of exosomal proteins have signal peptide found, striking resemblance to higher eukaryotes and their ancient universal origins, suggesting that a great part of proteins belonging to the secretome are non-conventionally secreted without signal peptide [32–75–77–88–60–117]. In addition, studies reveal differences in the intramembranous particles (integral proteins) distribution, where particle aggregation on specialized regions of the *Leishmania* membrane reflect their composition, function, density, also according to the parasitic evolutionary form: promastigote membrane richer in whole proteins than the amastigote membrane [35–40–42–53–118]. The FP is an invagination of the cell membrane at the base of the flagellum, and the flagellum membrane have a smaller amount of intramembranous particles when compared to the parasite body [42–45]. However, in the region of the membrane around the FP is possible to find specialized domains with a high concentration of these proteins, forming zones of flagellar adhesion [42–45–119]. The overall outcomes from the *in vitro* study will contribute to evaluate the immune modulation of host cells by *L*EVs [32–36–79–97–99]. The fully understanding of the cargo profile of *L*EVs promoting macrophage and lymphocyte activation, or by contrary, inducing cell deactivation will allow a better appreciation at the interactions established between the parasites and the host cell [32–41–54–66–92]. It knowledge can be a significant contribution for development of efficient prophylactic and therapeutic approaches, as *L*EVs are the most biomimetic nanovectors for a variety of molecules, including proteins, nucleic acids and chemicals in unexplored parasite molecular mechanisms [73–74–78–120]. Take into account the high biological similarity between *Leishmania* and other pathogens within the family Trypanosomatidae [73–74–78]. In addition, some insights of *L*EVs biology might have relevance to parallel studies applied for other prot ozoan parasites such as *Toxoplasma* and *Plasmodium* [73–74–78]. While *L*EVs were found to play an active role in the mammalian host, little is understood about their effects on the sand fly, or how they might affect the insect infection by the parasite [32–41–73–74–78]. For this reason, the *L*EVs isolation from different developmental stages has suggesting possible alterations they undergo into the parasites life cycle [32–41–73–74–78]. Exosomal proteins within both parasite phases can be the focus to structure original studies by which to examine potential target for intercellular modulation [32–41–73–74–78]. These findings regarding the composition of the *L*EVs proteome raise many questions regarding their function and provide compelling evidence that *L*EVs play an active molecular role in the parasite's development within the vertebrate and invertebrate hosts in their specificities pathways [32–41–73–74–78]. Currently, the knowledge of *L*EVs biogenesis, subtypes, cargo and molecular mechanisms of shuttling remains incomplete being placed on broad fields of study including the standardization and the experimental model validation in the comparison of results to broaden expertise in exosomal production of *Leishmania*.

4. Guidelines in terms of producing exosomes from parasite cells grown in culture media (serum-exofree media)

Molecular and morphological evidence indicates that microbial secreted- extracellular vesicles transport a plethora of lipids, metabolites, nucleic acids and proteins cargoes, they play a prominent role in modulating immunity during their biogenesis to capture cell-specific proteins and that host-derived extracellular vesicles control distinct infection levels [32–71–91–106]. Despite this, extracellular vesicles mechanisms of such selective packaging remain unknown [32–71–72]. Methodological advances in differential extracellular vesicles isolation have allowed the extracellular vesicles subtypes characterization, connecting to origins, sizes, constituents and their complex and diverse/ specific functions [32–81–91–118]. According to ‘‘Minimal Information for Studies of

Extracellular Vesicles'' (MISEV2018), extracellular vesicles is a heterogenous group of vesicles which can be defined as apoptotic bodies, exosomes and microvesicles [72–80–81]. The International Society for Extracellular Vesicles (ISEV) implemented a gold standard for extracellular vesicles isolation and analysis [72–80–81]. However, Medical Parasitology studies still has no consensus on ultrasensitive detection of extracellular vesicles-subtypes specific biomarkers, originating in endosomes, exosomes, and derivatives of plasma membrane or ectosomes, defaulting to attribute their specific biogenesis pathways [83–106]. In this regard, towards criteria for padronizing research can be considered in future studies [Table 2] [72–80–81–83]. These criteria represent an initial guidelines in terms of producing exosomes from parasite cells grown in culture media (serum-free media) [80–81–83]. On a complementary basis, such criteria may be applied in order to follow up the next steps: 1. a. optimization of the cell culture conditions and the harvest conditions; b. cell culture and exosome production and culture adherent cells to confluence (e.g. RPMI 1640 media w/ 10% ultrapure FBS that provides the highest level of exosome depletion, 1 mM sodium-pyruvat in cell culture bottles 225 cm2 in 37°C w/ 5% CO2); c. removal of medium form confluent cells with addition of 50 mL of fresh medium; d. after 3 days the cell-conditioned medium is removed and two centrifugation steps are performed on the pre conditioned medium prior to pre-enrichment (300 x g for 10 min at 2-8°C and 2000 x g for 30 min 2-8°C) [80]. 2. a. test for exosome release; b. depending on the cell type and their exosome production efficiency it is possible to pull out exosomes from the solution directly without any pre-enrichment step and perform (e.g. flow analysis); c. this is very useful in order to check the status of the cells exosome release efficiency and find the correct time for exosome harvest; d. Dynabeads™ CD9, Dynabeads™ CD63 or Dynabeads™ CD81 for exosomes do contain exosomal markers, host cell first and then *L*EVs (e.g. flow analysis [80]. 3. a. confirmation of the vesicle nature using multiple methods - NA and protein detection, Electron Microscopy (EM), specific markers found in non-exosome vesicles such as markers found in intracellular compartments (e.g. ER, Golgi or others) [80–81–83–118]. 4. a. key questions asked by the extracellular research community; b. current recommendations: estimation of size distribution using e.g. nano tracking analsysis (NTA); c. combine NTA with Transmission Electron Microscopy (TEM); d. verify the presence of miRNA/mRNA; d. Western blotting (WB) targeting lipid bound targets such as tetraspanins (3 different), cytosolic proteins such as TSG101, annexins, rabs etc. absence or under-representation of ER (e.g. hsp90B1, calnexin), Golgi (GM130), mitoch (cytC) or nucleus (histones); e. flow cytometry may easily be performed to support the WB data (membrane anchored targets such as tetraspanins) using Dynabeads™ as solid support which can be introduced and detected in the flow instrument [80]. 5. a. consider the question "can the exosome harvest be used directly or do I need to pre-enrich?"; b. the flow signal you will get might vary from very low/ nothing or up to signals that are actually sufficient for further detailed analysis - depending on this signal the researcher may decide to continue to do direct isolation with the Dynabeads™ as they are compatible with many different downstream [80]. These steps can contribute to ascribe specific functions to *L*EVs, or to subtypes of *L*EVs, and report specific information beyond description of function in a crude, potentially contaminated and heterogeneous isolates [80–81–83].

5. Discussion

Extracellular vesicles are naturally secreted by all cells, including *Leishmania* species, and can be found in all body fluids and are involved in a variety of mechanisms such as the obsolete molecules eradication, dissemination of oncogenes from cancer cells and cell-to-cell communications including the spread of pathogens [74–120–122]. The importance of extracellular vesicles have sparked great interest among the research community owing to their prominent potential in diagnostics and therapeutic approaches, including those determining the interaction outcome between cells [71–72–123–124]. The overall research has mainly focused on characterization of *L*EVs in different clinical forms especially in visceral strains and in *L.* (*L.*) *mexicana* proteomics under the same conditions, providing valuable information on how polymorphisms in the *L*EVs protein could affect biological

cell-to-cell interactions between parasites and host-parasite or the leishmanicidal activity [32–36–99–125–126]. Despite this, comparative analysis of reproducible isolation and characterization of *L*EVs from procyclic and metacyclic-like cultured *in vitro* of a wider panel of *Leishmania* species stills scarce [74–120–122]. Therefore, *L*EVs are effective macrophage immunomodulators of early host inflammatory responses, but the exact mechanisms behind this are yet to be characterized [36–78–94–126–127]. The presence of *L*EVs released from *L.* **(***L.***)** *donovani* modify IFNγ-induced pro-inflammatory or anti-inflammatory cytokine-production by human monocytes cultured [66–85–97–128]. B-1 cells may produce a high quantity of IL-10 contributing for parasite persistence and maintenance of memory cells; *in vivo* analysis of *L*EVs released from *L. (L.) amazonensis* and *L.* **(***L.***)** *infantum chagasi* incite pro-inflammatory activity increasing parasite burden by B-1 cells [87–97–98]. In this way, *L*EVs released from promastigote forms modulate murine B-1 cells' responses against *Leishmania in vivo* and *in vitro* but remain poorly characterized [97]. *In vitro* studies reported *L*EVs released from *L.* **(***L.***)** *infantum chagasi* and *Leishmania (Viannia) braziliensis* induced IL-10, but they were not able to induce significant levels of pro-inflammatory cytokines in the same way that *L*EVs released from *L. (L.) amazonensis* can elicit IL-6, NO and TNF-α production [35–87–97–98–99]. Mouse model reports has shown the display of CD4+ lymphocytes inoculated with *L*EVs presented exacerbated pathology expressing immunosuppressive cytokines IL-4 and IL-10 [71–75–78–97–129]. IL-17A promotes progression of cutaneous leishmaniasis during the course of *L.* **(***L.***)** *major* infection in murine models (i.e. neutrophil recruitment) and susceptibility during experimental visceral leishmaniasis caused by *L.* **(***L.***)** *donovani* [41–66–85–97–129]. While in macrophages infected with *L. (L.) amazonensis* EV-mechanisms, induce pro-inflammatory cytokines IL-12, IL-1β and TNF-α production by others macrophages to control *Leishmania* infection by Th1 immune response [87–97–98]. In addition, egestion experiments of *L*EVs from sand fly infectious inoculum reported IL-1β induction via inflammasome by neutrophils is important for *L.* **(***L.***)** *donovani* visceralization remaining an unexplored field in its entirety [77–82–130]. Proteomics has increased the knowledge about virulence factors differentially expressed in the different parasite stages and its functional activity induced by *L*EVs released from *L.* **(***L.***)** *infantum chagasi*, though comparative proteomics of *L*EVs production during the parasite cycle *in vivo* are still necessary [35–36–77–79–99]. Promastigotes from *L.* **(***L.***)** *infantum chagasi* and *L.* **(***L.***)** *major* infected sand fly midgut secrete *L*EVs sand fly-egested via different routes containing different amounts of virulence factor GP63 [75]. Virulence factors hypothesized may then become a part of the specific *L*EVs molecular signatures during the parasite evolution affecting the host immune system, far no significant differences between strains in the number of proteins from the secretome [77–117]. *L*EVs contains both proteins and nucleic acids with no ability to replicate, expressing a higher number of virulence factors and some differentially protein expression in evolutive stage to increase virulence induced by *L*EVs from drug-resistant parasites [77–97]. The drug-resistant *L*EVs are secreted by parasite and from a nucleosome with the human histones in the host chromatin during the disease evolution [77]. Drug-resistance mechanisms can induce changes in the morphology, size, and distribution of *L*EVs, remaining far from being completely elucidated, and some may be non-specific adaptations that provide a general fitness gain allowing the parasite to survive in stressful conditions [77]. Comparative studies of *L*EVs released from *L.* **(***L.***)** *donovani*, *L.* **(***L.***)** *major*, *L.* **(***L.***)** *infantum* chagasi strains had shown resistance to antimony, miltefosine and amphotericin B, contributing for future analyses with larger sets of strains and replicates and their variability and robustness of potential biomarkers of *Leishmania* [77–99]. Future

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 May 2021 doi:10.20944/preprints202105.0754.v1

studies may explore all of these aspects and shed light on how *L*EVs, especially those released by drug-resistant parasites, contribute to the survival of *Leishmania* during its life cycle, offering potential developments for diagnosis, follow-up of treatment responses, monitoring disease progression, determining the prognosis and devising new vaccine targets [77]. In this context, comparative studies of leishmanicidal activity repurposing existing drugs for new uses can be test approached as it was proposed by an experimental study of buparvaquone BPQ, a drug used for veterinary treatment of theileriosis, showed promising activity against *Leishmania* [67]. The development, optimization and evaluation of the physical and chemical characteristics of nanostructured lipid carriers (NLCs) for BPQ encapsulation and solubility evaluation were compared *in vitro* reducing by half promastigotes of *L. (L.) amazonensis*, *L. (L.) brasiliensis* and *L. (L.) infantum* samples [67]. Another very important point for comparative studies of *L*EVs is the poorly elucidated *L. (V.) guyanensis* parasites-LRV1 endovirus mutualistic relationship by extracellular vesicles, representing a potential way for the proteomics to better understanding how adaptation mechanisms work on co-evolution *Leishmania*-virus affecting the parasite pathogenesis [71]. An experiment conducted for comparative evaluation of the interactions of isolated *L*EVS released by promastigotes and amastigotes of cutaneous and visceral *Leishmania* species with mouse macrophages was proposed in the original research findings of Gabriel (2017) [92]. *L*EVs evidenced the capacity to modulate the activity of the macrophage for the M2 stage, which favors the parasite survival [71]. *L*EVS from (*L. (L.) infantum chagasi*, *L. (L.) amazonensis*, *L. (Viannia) shawi* and *L. (V.) guyanensis* significantly increased the urea production, with the exception of *L*EVS of *L. (L.) infantum* amastigotes [92]. Conversely, *L*EVS caused significant nitric oxide (NO) inhibition [92]. These results indicate that the *L*EVs of species cutaneous and visceral lesions of *Leishmania* and both morphological forms of the parasite drive macrophage for an anti-inflammatory phenotype [92]. *L*EVs play an important role in the infectivity and modulate the host's immune response by the promastigotes and amastigotes forms of *Leishmania* [35–71–77]. In this way, the parasites are distinguished following their morphology, environmental and nutritional requirements and by their stimulus *in vitro*, metabolism, motility, growth rate and multiplication in the host or in medium cultures and, more recently, by the molecular expression of specific *L*EVs [35–71–77]. Furthermore, parasite morphology associated biological functions linked with *L*EVs modulate host immune responses are an important open field to investigate virulence factors and the mechanisms for survival and their broad immunological roles inside the cells in vertebrate hosts, attempting to the development of parallel studies with invertebrate hosts [35–71–77]. This may be the starting point for the development of new preventive and therapeutic strategies with more efficient pharmacological targets against *Leishmania* which continues difficult to prevent, and treat and, whose current available drugs are toxic and not completely effective [35–71–77]. Therefore, more studies on *L*EVs are needed, as one of the fundamental points for the control of endemic parasitic diseases is the understanding of mechanisms involving the pathogen host interaction [35–71–77].

5. Conclusions

In conclusion, this review brings about evidence suggesting that *Leishmania*, similarly to other eukaryotes, use extracellular vesicles to play an important role in the infectivity and in the modulation of the host's immune response, with a particular focus on the establishment of infection in humans and other animals. However, the knowledge in this field still has research gaps to be filled to understand the mechanisms and conditions of the functional biological aspects for the release of

*L*EVs involving the parasite-host interactions [35–77]. At the same time, nano-drug delivery system based formulations are the innovative approach towards to improve anti-leishmanial therapeutic [126]. In addition, according pathways analysis, similar approaches could be used to identify the targets of *L*EVs *in vivo*. Therefore, the future molecular Parasitology research in this field is very important for the control of Leishmaniases worldwide.

Author Contributions: All authors provided critical feedback and helped shape the research, analysis and manuscript.

Ethical Statement for Animal Studies: Ethics Committee on Animal Use of the Federal Rural University of Amazônia (CEUA-UFRA, authorization No. 034/2014).

Acknowledgments: Áurea Gabriel gratefully acknowledge the Laboratory of Professor Gabriela Santos-Gomes IHMT-UNL-Portugal for her permanent support, scientific guidance and thesis supervision; and the financial support of Portuguese Foundation for Science and Technology (FCT), through the research project GHTM–UID/Multi/04413/2013 and Portugal-Brazil research project PTDC/SAU-PAR/28459/2017 EXOTRYPANO IHMT-NOVA/ FMV-ULisboa/ UFRN. This research was supported by the Brazilian Coordenação de Aperfeiçoamento de Pessoal no Ensino Superior (CAPES): Doctoral scholarship (Á. G.). All the authors of the manuscript thank and acknowledge their respective laboratories, institutes and universities.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Atlas interactivo de leishmaniasis en las Américas: aspectos clínicos y diagnósticos diferenciales. Washington, D.C.: Organización Panamericana de la Salud; 2020. Licencia: CC BY-NC-SA 3.0 IGO. Available online: https://infectologia.org.br/wp-content/uploads/2020/09/atlas-interactivo-de-leishmaniasis-en-las-americas.pdf. (accessed on 10 February 2021).
2. WHO. Leishmaniasis. Available online: https://www.who.int/health-topics/leishmaniasis#tab=tab_1. (accessed on 10 February 2021).
3. Ashford, R.W. The leishmaniases as emerging and reemerging zoonoses. Int J Parasitol. 2000 Nov; 30(12-13):1269-81. doi: 10.1016/s0020-7519(00)00136-3. PMID: 11113254.
4. Gabriel, Á.M. *et al.* "Cutaneous Leishmaniasis: The Complexity of Host's Effective Immune Response against a Polymorphic Parasitic Disease." *Journal of immunology research* vol. 2019 2603730. 1 Dec. 2019, doi:10.1155/2019/2603730
5. Guégan J.F. Review of the Biogeography of Host-Parasite Interactions by Serge Morand and Boris R Krasnov. *Parasit Vectors*. 2011;4:34. Published 2011 Mar 14. doi:10.1186/1756-3305-4-34
6. Conceição-Silva F, Morgado FN. *Leishmania* Spp-Host Interaction: There Is Always an Onset, but Is There an End? Front Cell Infect Microbiol. 2019 Sep 19;9:330. doi: 10.3389/fcimb.2019.00330. PMID: 31608245; PMCID: PMC6761226.
7. Mabbott NA. The Influence of Parasite Infections on Host Immunity to Co-infection With Other Pathogens. Front Immunol. 2018 Nov 8;9:2579. doi: 10.3389/fimmu.2018.02579. PMID: 30467504; PMCID: PMC6237250.
8. The *PLOS Neglected Tropical Diseases* Staff (2016) Correction: A Historical Overview of the Classification, Evolution, and Dispersion of *Leishmania* Parasites and Sandflies. PLoS Negl Trop Dis 10(6): e0004770. https://doi.org/10.1371/journal.pntd.0004770
9. Wamai RG, Kahn J, McGloin J, Ziaggi G. Visceral leishmaniasis: a global overview. J Glob Health Sci. 2020 Jun;2(1):e3. https://doi.org/10.35500/jghs.2020.2.e3
10. Kevric I, Cappel MA, Keeling JH. New World and Old World *Leishmania* Infections: A Practical Review. Dermatol Clin. 2015 Jul;33(3):579-93. doi: 10.1016/j.det.2015.03.018. PMID: 26143433.
11. Frederick L. Schuster, James J. Sullivan Cultivation of Clinically Significant Hemoflagellates Clinical Microbiology Reviews Jul 2002, 15 (3) 374-389; DOI: 10.1128/CMR.15.3.374-389.200
12. Okwor, I., & Uzonna, J. (2016). Social and Economic Burden of Human Leishmaniasis. *The American journal of tropical medicine and hygiene*, *94*(3), 489–493. https://doi.org/10.4269/ajtmh.15-0408
13. Santiago E. Hernández-Bojorge, Gerardo G. Blass-Alfaro, Marissa A. Rickloff, Manuel J. Gómez-Guerrero, Ricardo Izurieta, Epidemiology of cutaneous and mucocutaneous leishmaniasis in Nicaragua, Parasite Epidemiology and Control, Volume 11, 2020, e00192, https://doi.org/10.1016/j.parepi.2020.e00192.

14. Carvalho, S., Vieira, T. M., Moura, A., & Andrade, M. C. (2020). Should an intersection between visceral leishmaniasis endemicity and the COVID-19 pandemic be considered? *Medical hypotheses*, *144*, 110289. https://doi.org/10.1016/j.mehy.2020.110289
15. Melaku Taye Amogne, Dawit Kebede Huluka, Hiruy Araya Tesfay et al. Visceral leishmaniasis in an Ethiopian patient with COVID-19: a case report, 12 November 2020, PREPRINT (Version 1) available at Research Square [https://doi.org/10.21203/rs.3.rs-105371/v1]
16. Rutte, Epke A Le Coffeng Luc E, Muñoz, Johanna Vlas, Sake J de Modelling the impact of COVID-19-related programme interruptions on visceral leishmaniasis in India, *Transactions of The Royal Society of Tropical Medicine and Hygiene*, Volume 115, Issue 3, March 2021, Pages 229–235, https://doi.org/10.1093/trstmh/trab012
17. Galvão EL, Assis TSM, Pedras MJ, Cota GF, Simões TC, Rabello A. Economic impact of localized cutaneous leishmaniasis on adult patients of a referral service in Belo Horizonte, Minas Gerais State, Brazil. Cad Saúde Publica. 2020;36(7): e00136419. doi: 10.1590/0102-311x00136419. Epub 2020 Jul 17. PMID: 32696829.
18. Maia-Elkhoury ANS, Sierra Romero GA, O. B. Valadas SY, L. Sousa-Gomes M, Lauletta Lindoso JA, Cupolillo E, et al. (2019) Premature deaths by visceral leishmaniasis in Brazil investigated through a cohort study: A challenging opportunity? PLoS Negl Trop Dis 13(12): e0007841. https://doi.org/10.1371/journal.pntd.000784113.
19. Musa, A. M. Khalil, E. A. G. Younis, B. M. Elfaki, M. E. E. Elamin, M. Y. Adam, A. O. A. Mohamed H. A. A., Dafalla, M. M. M. Abuzaid, A. A. El-Hassan, A. M."Treatment-Based Strategy for the Management of Post-Kala-Azar Dermal Leishmaniasis Patients in the Sudan", Journal of Tropical Medicine, vol. 2013, Article ID 708391, 5 pages, 2013. https://doi.org/10.1155/2013/708391
20. Aline Araujo Zuma, Sara Teixeira de Macedo-Silva, Anushree Achari, Jayaraman Vinayagam, Pinaki Bhattacharjee, Sourav Chatterjee, Vivek Kumar Gupta, Amanda Cristina de Sousa Leite, Lucas Souza de Castro, Parasuraman Jaisankar, Wanderley de Souza, Furan derivatives impair proliferation and affect ultrastructural organization of *Trypanosoma cruzi* and *Leishmania amazonensis*, Experimental Parasitology, 2021, 108100, ISSN 0014-4894, https://doi.org/10.1016/j.exppara.2021.108100.
21. Gontijo Bernardo, Carvalho Maria de Lourdes Ribeiro de. Leishmaniose tegumentar americana. Rev. Soc. Bras. Med. Trop. [Internet]. 2003 Jan [cited 2021 Mar 18]; 36(1): 71-80. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0037-86822003000100011&lng=en. http://dx.doi.org/10.1590/S0037-86822003000100011.
22. Purse BV, Masante D, Golding N, Pigott D, Day JC, Ibañez-Bernal S, et al. (2017) How will climate change pathways and mitigation options alter incidence of vector-borne diseases? A framework for leishmaniasis in South and Meso-America. PLoS ONE 12(10): e0183583. https://doi.org/10.1371/journal.pone.0183583
23. Lage, D.P., Ribeiro, P.A.F., Dias, D.S. *et al.* A candidate vaccine for human visceral leishmaniasis based on a specific T cell epitope-containing chimeric protein protects mice against *Leishmania infantum* infection. *npj Vaccines* 5, 75 (2020). https://doi.org/10.1038/s41541-020-00224-0
24. Klatt, S., Simpson, L., Maslov, D. A., & Konthur, Z. (2019). *Leishmania tarentolae*: Taxonomic classification and its application as a promising biotechnological expression host. *PLoS neglected tropical diseases*, *13*(7), e0007424. https://doi.org/10.1371/journal.pntd.0007424
25. Burki F, Roger AJ, Brown MW, Simpson AGB. The New Tree of Eukaryotes. Trends Ecol Evol. 2020 Jan; 35(1): 43-55. doi: 10.1016/j.tree.2019.08.008. Epub 2019 Oct 9. PMID: 31606140.
26. Bowden, S. E. & Drake, J. M. (2013) Ecology of multi-host pathogens of animals. *Nature Education Knowledge* 4(8): 5
27. Artun O, Kavur H. Investigation of the spatial distribution of sandfly species and cutaneous leishmaniasis risk factors by using geographical information system technologies in Karaisali district of Adana province, Turkey. J Vector Borne Dis 2017; 54: 233-9 doi: 10.4103/0972-9062.217614
28. Iliopoulou, P., Tsatsaris, A., Katsios, I., Panagiotopoulou, A., Romaliades, S., Papadopoulos, B., & Tselentis, Y. (2018). Risk Mapping of Visceral Leishmaniasis: A Spatial Regression Model for Attica Region, Greece. *Tropical medicine and infectious disease*, *3*(3), 83. https://doi.org/10.3390/tropicalmed3030083
29. S. Khademvatan, S. Salmanzadeh, M. Foroutan-Rad, S. Bigdeli, F. Hedayati-Rad, J. Saki, E. Heydari-Gorji, Spatial distribution and epidemiological features of cutaneous leishmaniasis in southwest of Iran, Alexandria Journal of Medicine, Volume 53, Issue 1, 2017, Pages 93-98, ISSN 2090-5068, https://doi.org/10.1016/j.ajme.2016.03.001.

30. Miranda LdFC, Pacheco RdS, Pimentel MIF, Salgueiro MdM, da Silva AF, de Mello CX, et al. (2019) Geospatial analysis of tegumentary leishmaniasis in Rio de Janeiro state, Brazil from 2000 to 2015: Species typing and flow of travelers and migrants with leishmaniasis. PLoS Negl Trop Dis 13(11): e0007748. https://doi.org/10.1371/journal.pntd.0007748
31. Ostad, M., Shirian, S., Pishro, F., Abbasi, T., Ai, A., & Azimi, F. (2016). Control of Cutaneous Leishmaniasis Using Geographic Information Systems from 2010 to 2014 in Khuzestan Province, Iran. *PloS one*, *11*(7), e0159546. https://doi.org/10.1371/journal.pone.0159546
32. Dong G, Filho AL and Olivier M (2019) Modulation of Host-Pathogen Communication by Extracellular Vesicles (EVs) of the Protozoan Parasite *Leishmania*. *Front. Cell. Infect. Microbiol.* 9:100. doi: 10.3389/fcimb.2019.00100
33. Bifeld E., Chrobak, M., Zander, D., Schleicher, U., Schönian, G. & Clos, J., 2015 Geographical sequence variation in the *Leishmania major* virulence factor P46. Infection, Genetics and Evolution. 30: 195–205.
34. Schwabl, P., Boité, M.C., Bussotti, G. *et al.* Colonization and genetic diversification processes of *Leishmania infantum* in the Americas. *Commun Biol* **4,** 139 (2021). https://doi.org/10.1038/s42003-021-01658-5
35. Elmahallawy, E.K.; Alkhaldi, A.A.M. Insights into *Leishmania* Molecules and Their Potential Contribution to the Virulence of the Parasite. *Vet. Sci.* 2021, *8*, 33. https://doi.org/10.3390/vetsci8020033
36. Forrest, D. M., Batista, M., Marchini, F. K., Tempone, A. J., Traub-Csekö, Y. M. Proteomic analysis of exosomes derived from procyclic and metacyclic-like cultured *Leishmania infantum chagasi*, Journal of Proteomics, Volume 227, 2020, 103902, ISSN 1874-3919, https://doi.org/10.1016/j.jprot.2020.103902.
37. Maharana, B. R., Tewari, A. K., & Singh, V. (2015). An overview on kinetoplastid paraflagellar rod. Journal of parasitic diseases: official organ of the Indian Society for Parasitology, *39* (4), 589–595. https://doi.org/10.1007/s12639-014-0422-x
38. Harshima Wijesinghe, Nayana Gunathilaka, Saveen Semege, Nishantha Pathirana, Nuwani Manamperi, Chandu de Silva, Deepika Fernando, "Histopathology of Cutaneous Leishmaniasis Caused by Leishmania donovani in Sri Lanka", BioMed Research International, vol. 2020, Article ID 4926819, 8 pages, 2020. https://doi.org/10.1155/2020/4926819
39. Nogueira PM, de Menezes-Neto A, Borges VM, Descoteaux A, Torrecilhas AC, Xander P, Revach OY, Regev-Rudzki N, Soares RP. Immunomodulatory Properties of *Leishmania* Extracellular Vesicles During Host-Parasite Interaction: Differential Activation of TLRs and NF-κB Translocation by Dermotropic and Viscerotropic Species. Front Cell Infect Microbiol. 2020 Jul 29; 10: 380. doi: 10.3389/fcimb.2020.00380. PMID: 32850481; PMCID: PMC7403210.
40. Giraud, E., Martin, O., Yakob, L. *et al.* Quantifying *Leishmania* Metacyclic Promastigotes from Individual Sandfly Bites Reveals the Efficiency of Vector Transmission. *Commun Biol* **2,** 84 (2019). https://doi.org/10.1038/s42003-019-0323-8
41. Lambertz, U., Silverman, J. M., Nandan, D., McMaster, W. R., Clos, J., Foster, L. J. & Reiner, N. E. 2012. Secreted virulence factors and immune evasion in visceral leishmaniasis Journal of Leukocyte Biology 91: 887-899.
42. Sunter J, Gull K. Shape, form, function and *Leishmania* pathogenicity: from textbook descriptions to biological understanding [published correction appears in Open Biol. 2018 Aug; 8(8):]. *Open Biol*. 2017; 7(9):170165. doi:10.1098/rsob.170165
43. Silva, C.G., SILVA, R.M.M., Diniz, V.A., and Côrte-Real, S. Ultraestrutura de Parasitos do Gênero *Leishmania*. In: Conceição-Silva, F., and Alves, C. R., comps. *Leishmanioses do continente americano* [online]. Rio de Janeiro: Editora Fiocruz, 2014, pp. 53-67. ISBN 978-85-7541-568-9. https://doi.org/10.7476/9788575415689.0004
44. Halliday C, Yanase R, Catta-Preta CMC, Moreira-Leite F, Myskova J, Pruzinova K, et al. (2020) Role for the flagellum attachment zone in *Leishmania* anterior cell tip morphogenesis. PLoS Pathog 16(10): e1008494. https://doi.org/10.1371/journal.ppat.1008494
45. Ana Paula Rocha Gadelha, Narcisa Leal Cunha-e-Silva, Wanderley de Souza, Assembly of the *Leishmania amazonensis* flagellum during cell differentiation, Journal of Structural Biology, Volume 184, Issue 2, 2013, Pages 280-292, ISSN 1047-8477, https://doi.org/10.1016/j.jsb.2013.09.006.
46. Beneke T, Demay F, Hookway E, Ashman N, Jeffery H, Smith J, et al. (2019) Genetic dissection of a *Leishmania* flagellar proteome demonstrates requirement for directional motility in sand fly infections. PLoS Pathog 15(6): e1007828. https://doi.org/10.1371/journal.ppat.1007828

47. Takahashi EA, Masoud L, Mukbel R, Guitian J, Stevens KB (2020) Modelling habitat suitability in Jordan for the cutaneous leishmaniasis vector (*Phlebotomus papatasi)* using multicriteria decision analysis. PLoS Negl Trop Dis 14(11): e0008852. https://doi.org/10.1371/journal.pntd.0008852
48. Cincurá, C., de Lima, C., Machado, P., Oliveira-Filho, J., Glesby, M. J., Lessa, M. M., & Carvalho, E. M. (2017). Mucosal leishmaniasis: A Retrospective Study of 327 Cases from an Endemic Area of *Leishmania* (*Viannia*) *braziliensis*. *The American Journal of Tropical Medicine and Hygiene*, *97*(3), 761–766. https://doi.org/10.4269/ajtmh.16-0349
49. Paulo G. Hofstatter, Giulia M. Ribeiro, Alfredo L. Porfírio-Sousa, Daniel J. G. Lahr The Sexual Ancestor of all Eukaryotes: A Defense of the "Meiosis Toolkit": A Rigorous Survey Supports the Obligate Link between Meiosis Machinery and Sexual Recombination (2020) BioEssays, ISSN: 1521-1878, Vol: 42, Issue: 9, Page: e2000037 https://doi.org/10.1002/bies.202000037
50. Shaik, J.S; Dobson, D.E; Sacks, D.L; Beverley, S.M *Leishmania* Sexual Reproductive Strategies as Resolved through Computational Methods Designed for Aneuploid Genomes. Genes 2021, 12, 167. https://doi.org/10.3390/ genes12020167
51. Young J, Kima PE. The *Leishmania* Parasitophorous Vacuole Membrane at the Parasite-Host Interface. Yale J Biol Med. 2019 Sep 20; 92 (3):511-521. PMID: 31543712; PMCID: PMC6747952.
52. Rybak, K., & Robatzek, S. (2019). Functions of Extracellular Vesicles in Immunity and Virulence. *Plant physiology*, *179*(4), 1236–1247. https://doi.org/10.1104/pp.18.01557
53. Soulat D and Bogdan C (2017) Function of Macrophage and Parasite Phosphatases in Leishmaniasis. *Front. Immunol.* 8:1838. doi: 10.3389/fimmu.2017.01838
54. Silverman, J. M. & Reiner, N. E., 2012. *Leishmania* exosomes deliver preemptive strikes to create an environment permissive for early infection. Frontiers in Cellular and Infection Microbiology 1: 1-8.
55. de Freitas E Silva, R., & von Stebut, E. (2021). Unraveling the Role of Immune Checkpoints in Leishmaniasis. *Frontiers in immunology*, *12*, 620144. https://doi.org/10.3389/fimmu.2021.620144
56. Christensen SM, Belew AT, El-Sayed NM, Tafuri WL, Silveira FT, Mosser DM (2019) Host and parasite responses in human diffuse cutaneous leishmaniasis caused by *L. amazonensis*. PLoS Negl Trop Dis 13(3): e0007152. https://doi.org/10.1371/journal.pntd.0007152
57. Diniz, João Luiz Cioglia Pereira, Costa, Manoel Otávio da Rocha, & Gonçalves, Denise Utsch. (2011). Mucocutaneous Leishmaniasis: clinical markers in presumptive diagnosis. *Brazilian Journal of Otorhinolaryngology*, *77*(3), 380-384. https://doi.org/10.1590/S1808-86942011000300018
58. Bennis, I., De Brouwere, V., Belrhiti, Z., Sahibi, H., & Boelaert, M. (2018). Psychosocial burden of localised cutaneous Leishmaniasis: a scoping review. *BMC public health*, *18*(1), 358. https://doi.org/10.1186/s12889-018-5260-9
59. Hong, A.; Zampieri, R.A.; Shaw, J.J.; Floeter-Winter, L.M.; Laranjeira-Silva, M.F. One Health Approach to Leishmaniases: Understanding the Disease Dynamics through Diagnostic Tools. *Pathogens* **2020**, *9*, 809. https://doi.org/10.3390/pathogens9100809
60. Ricardo Gonçalves, Stephen M. Christensen, David M. Mosser, Humoral immunity in leishmaniasis – Prevention or promotion of parasite growth?, Cytokine: X, Volume 2, Issue 4, 2020, 100046, ISSN 2590-1532, https://doi.org/10.1016/j.cytox.2020.100046.
61. Bilgic-Temel, A., Murrell, D. F., & Uzun, S. (2019). Cutaneous leishmaniasis: A neglected disfiguring disease for women. *International journal of women's dermatology*, *5*(3), 158–165. https://doi.org/10.1016/j.ijwd.2019.01.002
62. Atayde VD, Hassani K, da Silva Lira Filho A, Borges AR, Adhikari A, Martel C, Olivier M. *Leishmania* exosomes and other virulence factors: Impact on innate immune response and macrophage functions. Cell Immunol. 2016 Nov; 309: 7-18. doi: 10.1016/j.cellimm.2016.07.013. Epub 2016 Jul 28. PMID: 27499212. Copy
63. Arraché Gonçalves, G., Eifler-Lima, V.L. & von Poser, G.L. Revisiting nature: a review of iridoids as a potential antileishmanial class. Phytochem Rev (2021). https://doi.org/10.1007/s11101-021-09750-8
64. Carolina Piña-Vázquez, Magda Reyes-López, Guillermo Ortíz-Estrada, Mireya de la Garza, Jesús Serrano-Luna, "Host-Parasite Interaction: Parasite-Derived and -Induced Proteases That Degrade Human Extracellular Matrix", Journal of Parasitology Research, vol. 2012, Article ID 748206, 24 pages, 2012. https://doi.org/10.1155/2012/748206
65. Mercado-Camargo J, Cervantes-Ceballos L, Vivas-Reyes R, Pedretti A, Serrano-García ML, Gómez-Estrada H. Homology Modeling of Leishmanolysin (GP63) from *Leishmania panamensis* and Molecular Docking of

Flavonoids. ACS Omega. 2020 Jun 10; 5 (24):14741-14749. doi: 10.1021/acsomega.0c01584. PMID: 32596611; PMCID: PMC7315592.

66. Silverman, J. M., Clos, J., de'Oliveira, C. C., Shirvani, O., Fang, Y., Wang, C., Foster L. J. & Reiner, N. E. 2010. An exosome-based secretion pathway is responsible for protein export from *Leishmania* and communication with macrophages. Journal of Cell Science 123: 842-852.
67. Monteiro, L., Loebenberg, R., Cotrim, P., Fotaki, N., & Bou-Cracha, N. (2015). A potential nanotechnology based drug delivery for the treatment of Leishmaniases. Abstract from AAPS Annual Meeting, 2015, Orlando, USA United States.
68. Dong, G., Wagner, V., Minguez-Menendez, A., Fernandez-Prada, C., & Olivier, M. (2021). Extracellular vesicles and leishmaniasis: Current knowledge and promising avenues for future development. *Molecular Immunology*, *135*, 73-83. https://doi.org/10.1016/j.molimm.2021.04.003.
69. Gabriel Á M.; Coelho da Rocha L., Passero, F., Alexandre-Pires, G., Santos-Gomes, G. Morfometria aplicada à micromorfologia para a caracterização de subpopulações heterogéneas de vesículas extracelulares de *Leishmania* Proceedings of the X Jornadas Cientificas do Instituto de Higiene e Medicina Tropical, IHMT – Lisbon, Portugal, December 2019; IHMT-UNL: Lisbon, Portugal, 2019. Available online: http://www.ihmt.unl.pt/wp-content/uploads/2019/12/Livro-de-Resumos.pdf (accessed on 10 February 2021).
70. Izadora Volpato Rossi, Maria Alice Ferreira Nunes, Sandra Vargas-Otalora, Thalita Camelo da Silva Ferreira, Mauro Cortez, Marcel Ivan Ramirez, Extracellular Vesicles during TriTryps infection: Complexity and future challenges, Molecular Immunology, Volume 132, 2021, Pages 172-183, ISSN 0161-5890, https://doi.org/10.1016/j.molimm.2021.01.008.
71. Martin Olivier, Dario S Zamboni, *Leishmania Viannia guyanensis*, LRV1 virus and extracellular vesicles: a dangerous trio influencing the faith of immune response during muco-cutaneous leishmaniasis, Current Opinion in Immunology, Volume 66, 2020, Pages 108-113, ISSN 0952-7915, https://doi.org/10.1016/j.coi.2020.08.004.
72. Susmita Sahoo, Marta Adamiak, Prabhu Mathiyalagan, Franziska Kenneweg, Sabine Kafert-Kasting, Thomas Thum Therapeutic and Diagnostic Translation of Extracellular Vesicles in Cardiovascular Diseases Roadmap to the Clinic. Circulation. 2021; 143: 1426–1449. DOI: 10.1161/CIRCULATIONAHA.120.049254
73. Regev-Rudzki, N., Wilson, D. W., Carvalho, T. G., Sisquella, X., Coleman, B. M., Rug, M., Bursac, D., Angrisano, F., Gee, M., Hill, A. F., Baum, J. & Cowman, A. F. 2013. Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles. Cell 153: 1-14.
74. Szempruch AJ, Dennison L, Kieft R, Harrington JM, Hajduk SL. Sending a message: extracellular vesicles of pathogenic protozoan parasites. Nat Rev Microbiol. 2016 Nov; 14 (11): 669-675. doi: 10.1038/nrmicro.2016.110. Epub 2016 Sep 12. PMID: 27615028.
75. Atayde VD, Aslan H, Townsend S, Hassani K, Kamhawi S, Olivier M. Exosome Secretion by the Parasitic Protozoan *Leishmania* within the Sand Fly Midgut. Cell Rep. 2015 Nov 3;13(5):957-67. doi: 10.1016/j.celrep.2015.09.058. Epub 2015 Oct 22. PMID: 26565909; PMCID: PMC4644496.
76. Hassani, K., Antoniak, E., Jardim, A. & Olivier, M. 2011. Temperature-induced protein secretion by *Leishmania mexicana* modulates macrophage signalling and function. Plos Neglected Tropical Diseases 7: 5.
77. Douanne N, Dong G, Douanne M, Olivier M, Fernandez-Prada C (2020) Unravelling the proteomic signature of extracellular vesicles released by drug-resistant *Leishmania infantum* parasites. PLoS Negl Trop Dis 14(7): e0008439. https://doi.org/10.1371/journal.pntd.0008439
78. Torrecilhas AC, Soares RP, Schenkman S, Ferna´ndez-Prada C and Olivier M (2020) Extracellular Vesicles in Trypanosomatids: Host Cell Communication. Front. Cell. Infect. Microbiol. 10:602502. doi: 10.3389/fcimb.2020.602502
79. Pérez-Cabezas, B., Santarém, N., Cecílio, P., Silva, C., Silvestre, R., A M Catita, J., & Cordeiro da Silva, A. (2018). More than just exosomes: distinct *Leishmania infantum* extracellular products potentiate the establishment of infection. *Journal of extracellular vesicles*, *8*(1), 1541708. https://doi.org/10.1080/20013078.2018.1541708
80. Gabriel Á M.; Pedersen K. W., Santos-Gomes, G. Standardization of an extracellular vesicles research checklist in Medical Parasitology. Proceedings of the XI Jornadas Cientificas do Instituto de Higiene e Medicina Tropical, IHMT – Lisbon, Portugal, 10 December 2020; IHMT-UNL: Lisbon, Portugal, 2020.

Available online: https://www.ihmt.unl.pt/livro-de-resumos-das-xi-jornadas/ (accessed on 10 February 2021).

81. Théry C. *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines *Journal of Extracellular Vesicles* 2018, Vol. 7, 1535750 DOI: 10.1080/20013078.2018.1535750
82. Lambertz, U; Oviedo Ovando M.E. ; Unrau, P.J; Myler, P.J.; Reiner, N.E.Small RNAs derived from tRNAs and rRNAs are highly enriched in exosomes from both old and new world *Leishmania* providing evidence for conserved exosomal RNA Packaging. 2015 BMC Genomics 16,151
83. Pedersen KW, Kierulf B, Neurauter A. Specific and Generic Isolation of Extracellular Vesicles with Magnetic Beads. Methods Mol Biol. 2017; 1660: 65-87. doi: 10.1007/978-1-4939-7253-1_7. PMID: 28828649.
84. Hui WW, Emerson LE, Clapp B, Sheppe AE, Sharma J, del Castillo J, et al. (2021) Antigen-encapsulating host extracellular vesicles derived from *Salmonella*-infected cells stimulate pathogen-specific Th1-type responses in vivo. PLoS Pathog 17(5): e1009465. https://doi.org/10.1371/journal.ppat.1009465
85. Chawla, B., Jhingran, A., Panigrahi, A., Stuart, K. D. & Madhubala, R. 2011 Paromomycin affects translation and vesicle-mediated trafficking as revealed by proteomics of paromomycin–susceptible and resistant *Leishmania donovani.* Plos One 6: 10.
86. Valéria de Lima Kaminski, Joel Henrique Ellwanger, José Artur Bogo Chies, Extracellular vesicles in host-pathogen interactions and immune regulation — exosomes as emerging actors in the immunological theater of pregnancy, Heliyon, Volume 5, Issue 8, 2019, e02355, ISSN 2405-8440, https://doi.org/10.1016/j.heliyon.2019.e02355
87. Cronemberger-Andrade, A., Aragão-França, L., Araujo C. F., Rocha, V. J., Borges-Silva, M. C., Figueiras, C. P., Oliveira, P. R., Freitas, L. A. R., Veras, P. S. T. & Pontes-de-Carvalho, L. 2014. Extracellular vesicles from *Leishmania*-infected macrophages confer an anti-infection cytokine-production profile to naïve macrophages. Plos Neglected Tropical Diseases 8: 11.
88. Marcilla, A., Martin-Jaular, L., Trelis, M., Menezes-Neto, A., Osuna, A., Bernal, D., Fernandez-Becerra, C., Almeida, I. C. & del Portillo, H. A. 2014. Extracellular vesicles in parasitic diseases. Journal of Extracellular Vesicles 3: 1-15.
89. Yáñez-Mó, M., Siljander, P. R., Andreu, Z., Zavec, A. B., Borràs, F. E., Buzas, E. I., Buzas, K., Casal, E., Cappello, F., Carvalho, J., Colás, E., Cordeiro-da Silva, A., Fais, S., Falcon-Perez, J. M., Ghobrial, I. M., Giebel, B., Gimona, M., Graner, M., Gursel, I., Gursel, M., … De Wever, O. (2015). Biological properties of extracellular vesicles and their physiological functions. *Journal of extracellular vesicles*, 4, 27066. https://doi.org/10.3402/jev.v4.27066
90. O'Brien, K., Breyne, K., Ughetto, S. *et al.* RNA delivery by extracellular vesicles in mammalian cells and its applications. *Nat Rev Mol Cell Biol* **21,** 585–606 (2020). https://doi.org/10.1038/s41580-020-0251-y
91. Sukhvinder Gill, Ryan Catchpole, Patrick Forterre, Extracellular membrane vesicles in the three domains of life and beyond, *FEMS Microbiology Reviews*, Volume 43, Issue 3, May 2019, Pages 273–303, https://doi.org/10.1093/femsre/fuy042
92. Gabriel Á M.; Coelho da Rocha L., Passero, F., Santos-Gomes, G. Interações experimentais entre macrófagos de roedores e nanovesículas de *Leishmania* Proceedings of the VIII Jornadas Cientificas do Instituto de Higiene e Medicina Tropical, IHMT – Lisbon, Portugal, December 2017; IHMT-UNL: Lisbon, Portugal, 2017. Available online: http://www.ihmt.unl.pt/wp-content/uploads/2017/12/Livro-de-Resumos_8Jornadas_Cientificas.pdf (accessed on 10 February 2021).
93. Anna Gioseffi, Tim Hamerly, Kha Van, Naixin Zhang, Rhoel R Dinglasan, Phillip A Yates, Peter E Kima *Leishmania* infection alters extracellular vesicles Life Science Alliance Oct 2020, 3 (12) e202000742; DOI: 10.26508/lsa.202000742
94. Soto-Serna LE, Diupotex M, Zamora-Chimal J, Ruiz-Remigio A, Delgado-Domínguez J, Cervantes-Sarabia RB, Méndez-Bernal A, Escalona-Montaño AR, Aguirre-García MM, Becker I. *Leishmania mexicana*: Novel Insights of Immune Modulation through Amastigote Exosomes. J Immunol Res. 2020 Dec 2;2020:8894549. doi: 10.1155/2020/8894549. PMID: 33344659; PMCID: PMC7728480.
95. Reis NFdC, Dupin TV, Costa CR, Toledo MdS, de Oliveira VC, Popi AF, Torrecilhas AC and Xander P (2020) *Leishmania amazonensis* Promastigotes or Extracellular Vesicles Modulate B-1 Cell Activation and Differentiation. *Front. Cell. Infect. Microbiol.* 10:573813. doi: 10.3389/fcimb.2020.573813
96. Schorey, J. S., Cheng, Y., Singh, P. P. & Smith, V. L. 2015. Exosomes and other extracellular vesicles in host–pathogen interactions. EMBO reports 16: 24-43.

97. Barbosa, F., Dupin, T. V., Toledo, M., Reis, N., Ribeiro, K., Cronemberger-Andrade, A., Rugani, J. N., De Lorenzo, B., Novaes E Brito, R. R., Soares, R. P., Torrecilhas, A. C., & Xander, P. (2018). Extracellular Vesicles Released by *Leishmania* (*Leishmania*) *amazonensis* Promote Disease Progression and Induce the Production of Different Cytokines in Macrophages and B-1 Cells. *Frontiers in microbiology*, *9*, 3056. https://doi.org/10.3389/fmicb.2018.03056

98. Toledo MDS, Cronemberger-Andrade A, Barbosa FMC, Reis NFC, Dupin TV, Soares RP, Torrecilhas AC, Xander P. Effects of extracellular vesicles released by peritoneal B-1 cells on experimental *Leishmania* (*Leishmania*) *amazonensis* infection. J Leukoc Biol. 2020 Dec; 108(6):1803-1814. doi: 10.1002/JLB.3MA0220-464RR. Epub 2020 Apr 30. PMID: 32356366.

99. Marshall, S., Kelly, P.H., Singh, B.K. *et al.* Extracellular release of virulence factor major surface protease *via* exosomes in *Leishmania infantum* promastigotes. *Parasites Vectors* **11,** 355 (2018). https://doi.org/10.1186/s13071-018-2937-y

100. Bruna Sabatke, Bruno Gavinho, Veronica Coceres, Natalia de Miguel, Marcel I. Ramirez, Unveiling the role of EVs in anaerobic parasitic protozoa, Molecular Immunology, Volume 133, 2021, Pages 34-43, ISSN 0161-5890, https://doi.org/10.1016/j.molimm.2021.02.007.

101. Khosravi M, Mirsamadi ES, Mirjalali H, Zali MR. Isolation and Functions of Extracellular Vesicles Derived from Parasites: The Promise of a New Era in Immunotherapy, Vaccination, and Diagnosis. *Int J Nanomedicine*. 2020; 15: 2957-2969 https://doi.org/10.2147/IJN.S250993

102. Yesica Romina Nievas, Ayelen Lizarraga, Nehuen Salas, Verónica Mabel Cóceres, Natalia de Miguel Extracellular vesicles released by anaerobic protozoan parasites: Current situation Cellular Microbiology Volume 22, Issue 11 e13257 28 August 2020 https://doi.org/10.1111/cmi.13257

103. Olajide JS, Cai J. Perils and Promises of Pathogenic Protozoan Extracellular Vesicles. Front Cell Infect Microbiol. 2020 Aug 14; 10: 371. doi: 10.3389/fcimb.2020.00371. PMID: 32923407; PMCID: PMC7456935.

104. Théry, C., Clayton, A., Amigorena, S. & Raposo, G. 2006. Current protocols in cell biology Ch. 3 Exosomes from cell culture supernatants and biological fluids (Eds Bonifacino,, J. S., Dasso, M., Harford, J. B., Lippincott-Schwartz, J. & Yamada, K. M.) vol. 30: 22-29.

105. Deolindo, P., Evans-Osses, I. & Ramirez, M. I. 2013. Microvesicles and exosomes as vehicles between protozoan and host cell communication. Biochemical Society Transactions 41: 252-257

106. Zhang, Y., Liu, Y., Liu, H., & Tang, W. H. (2019). Exosomes: biogenesis, biologic function and clinical potential. *Cell & bioscience*, *9*, 19. https://doi.org/10.1186/s13578-019-0282-2

107. de Jesus JB, Mesquita-Rodrigues C, Cuervo P. Proteomics advances in the study of *Leishmania* parasites and leishmaniasis. Subcell Biochem. 2014; 74: 323-49. doi: 10.1007/978-94-007-7305-9_14. PMID: 24264252.

108. Rodríguez-Vega A, Losada-Barragán M, Berbert LR, Mesquita-Rodrigues C, Bombaça ACS, Menna-Barreto R, Aquino P, Carvalho PC, Padrón G, de Jesus JB, Cuervo P. Quantitative analysis of proteins secreted by *Leishmania* (Viannia) *braziliensis* strains associated to distinct clinical manifestations of American Tegumentary Leishmaniasis. J Proteomics. 2021 Feb 10; 232: 104077. doi: 10.1016/j.jprot.2020.104077. Epub 2020 Dec 10. PMID: 33309930.

109. Noor Z, Ahn SB, Baker MS, Ranganathan S, Mohamedali A. Mass spectrometry-based protein identification in proteomics-a review. Brief Bioinform. 2021 Mar 22; 22 (2):1620-1638. doi: 10.1093/bib/bbz163. PMID: 32047889.

110. Soumen Mukherjee, Shanta Dutta, Ashish Swarup Verma, Malay Kumar Saha, Chapter 34 - Nanoparticle synthesis harnessing benign green routes, Editor(s): Ashish S. Verma, Anchal Singh, Animal Biotechnology (Second Edition), Academic Press, 2020, Pages 689-708, ISBN 9780128117101, https://doi.org/10.1016/B978-0-12-811710-1.00038-0.

111. Sharma M, Morgado P, Zhang H, Ehrenkaufer G, Manna D, Singh U. Characterization of Extracellular Vesicles from Entamoeba histolytica Identifies Roles in Intercellular Communication That Regulates Parasite Growth and Development. Infect Immun. 2020 Sep 18;88(10):e00349-20. doi: 10.1128/IAI.00349-20. PMID: 32719158; PMCID: PMC7504958.

112. Diaz-Garrido, N.; Cordero, C.; Olivo-Martinez, Y.; Badia, J.; Baldomà, L. Cell-to-Cell Communication by Host-Released Extracellular Vesicles in the Gut: Implications in Health and Disease. Int. J. Mol. Sci. 2021, 22, 2213. https://doi.org/10.3390/ijms22042213

113. Montaner, S., Galiano, A., Trelis, M., Martin-Jaular, L., Del Portillo, H. A., Bernal, D. & Marcilla, A. 2014. The role of extracellular vesicles in modulating the host immune response during parasitic infections. Frontiers in Immunology 5 (433): 1-8.

114. Hellard E., Fouchet D., Vavre F., Pontier D. Parasite-Parasite Interactions in the Wild: How To Detect Them? (2015) *Trends in Parasitology*, 31 (12), pp. 640-652. https://doi.org/10.1016/j.pt.2015.07.005
115. Li X, Zhou Z. [Progress on the mechanism of extracellular vesicles involves in pathogen infection: Review]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2020 May; 36(5):467-473. Chinese. PMID: 32696760.
116. Babatunde, K. A., Yesodha Subramanian, B., Ahouidi, A. D., Martinez Murillo, P., Walch, M., & Mantel, P. Y. (2020). Role of Extracellular Vesicles in Cellular Cross Talk in Malaria. *Frontiers in immunology*, *11*, 22. https://doi.org/10.3389/fimmu.2020.00022
117. Rosa Milagros Corrales, Denis Sereno, Françoise Mathieu-Daudé, Deciphering the *Leishmania* exoproteome: what we know and what we can learn, *FEMS Immunology & Medical Microbiology*, Volume 58, Issue 1, February 2010, Pages 27–38, https://doi.org/10.1111/j.1574-695X.2009.00608.x
118. Gabriel, Á. M., Rafael-Fernandes, M., Passero, F., Alexandre Pires, G., Santos-Gomes, G. Inserts on comparative scanning electron microscope study of *Leishmania* exosomes. Proceedings of the VI WORLDLEISH – Toledo, Spain, 16-20 May 2017; Instituto de Salud Carlos III and *Plos* collections, 2017.
119. Wheeler, R. J., Sunter, J. D., & Gull, K. (2016). Flagellar pocket restructuring through the *Leishmania* life cycle involves a discrete flagellum attachment zone. *Journal of cell science*, *129*(4), 854–867. https://doi.org/10.1242/jcs.183152
120. Jain, S.; Santana, W.; Dolabella, S.S.; Santos, A.L.S.; Souto, E.B.; Severino, P. Are Nanobiosensors an Improved Solution for Diagnosis of *Leishmania*? *Pharmaceutics* **2021**, *13*, 491. https://doi.org/10.3390/pharmaceutics13040491
121. Li, MY., Liu, LZ. & Dong, M. Progress on pivotal role and application of exosome in lung cancer carcinogenesis, diagnosis, therapy and prognosis. *Mol Cancer* **20,** 22 (2021). https://doi.org/10.1186/s12943-021-01312-y
122. Théry, C. 2011. Exosomes: secreted vesicles and intercellular communications F1000. Biology Reports vol. 3:15 pp. 1-8.
123. Weber, B., Franz, N., Marzi, I. *et al.* Extracellular vesicles as mediators and markers of acute organ injury: current concepts. *Eur J Trauma Emerg Surg* (2021). https://doi.org/10.1007/s00068-021-01607-1
124. Carroll-Portillo, A., Surviladze, Z., Cambi A., Lidke, D. S. & Wilson, B. S. 2012. Mast cell synapses and exosomes: membrane contacts for information exchange. Frontiers in Immunology 3: 46.
125. Olivier, M., Gregory, D. J., & Forget, G. (2005). Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. *Clinical microbiology reviews*, *18*(2), 293–305. https://doi.org/10.1128/CMR.18.2.293-305.2005
126. Jamshaid, H., Din, F.u. & Khan, G.M. Nanotechnology based solutions for anti-leishmanial impediments: a detailed insight. J Nanobiotechnol **19,** 106 (2021). https://doi.org/10.1186/s12951-021-00853-0
127. Lässer, C., Eldh, M. & Lötvall, J. 2012. Isolation and characterization of RNA-containing exosomes. Journal of Visualized Experiments 59: 1-6.
128. Valverde JG, Paun A, Inbar E, Romano A, Lewis M, Ghosh K, Sacks D. Increased Transmissibility of *Leishmania donovani* From the Mammalian Host to Vector Sand Flies After Multiple Exposures to Sand Fly Bites. J Infect Dis. 2017 Apr 15; 215 (8): 1285-1293. doi: 10.1093/infdis/jix115. PMID: 28329329; PMCID: PMC5853764.
129. Terrazas, C., Varikuti, S., Kimble, J., Moretti, E., Boyaka, P. N., & Satoskar, A. R. (2016). IL-17A promotes susceptibility during experimental visceral leishmaniasis caused by *Leishmania donovani*. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 30(3), 1135–1143. https://doi.org/10.1096/fj.15-277202
130. Passelli K, Billion O and Tacchini-Cottier F (2021) The Impact of Neutrophil Recruitment to the Skin on the Pathology Induced by *Leishmania* Infection. *Front. Immunol.* 12:649348. doi: 10.3389/fimmu.2021.649348

Figures

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 May 2021 doi:10.20944/preprints202105.0754.v1

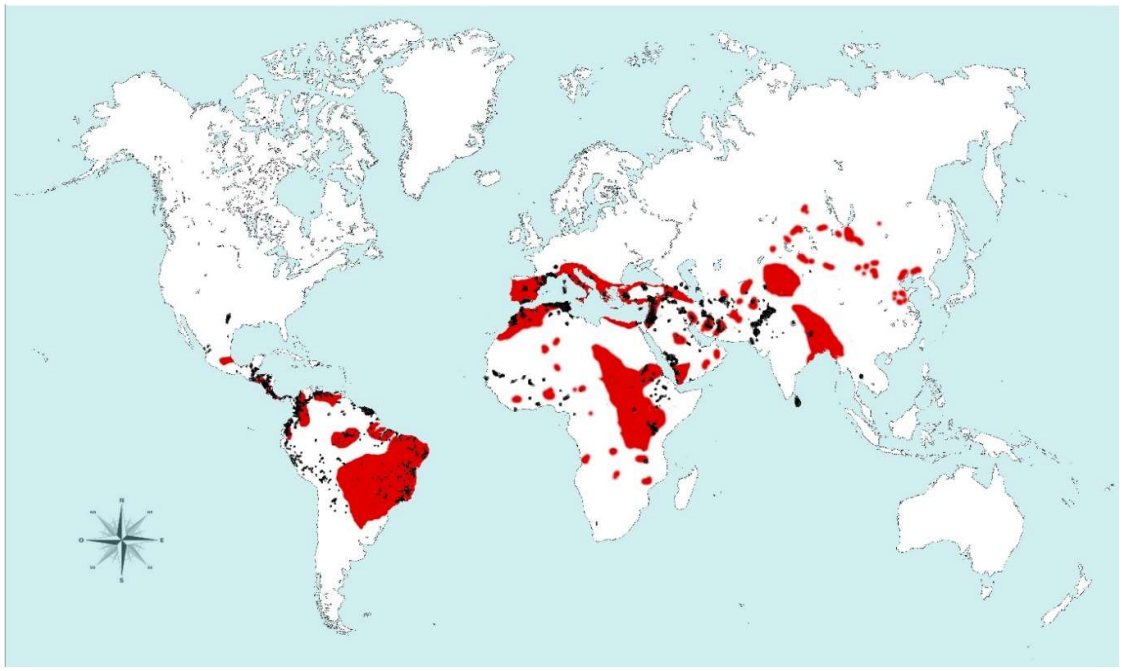
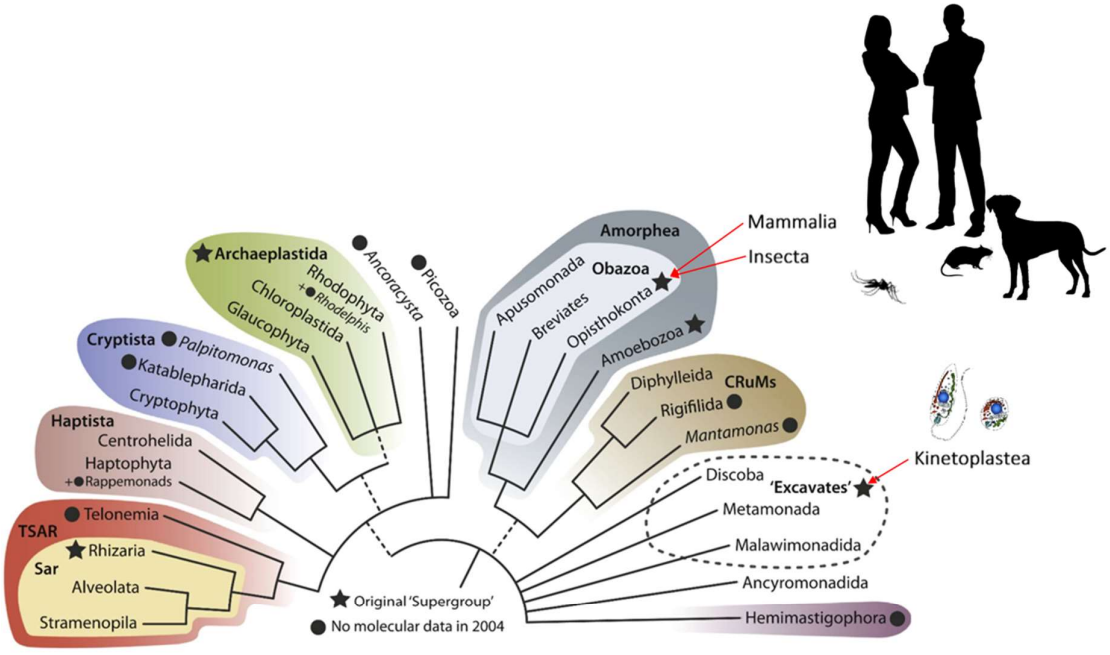


Figure 1. Global map of the occurrence of cutaneous leishmaniasis (highlighted black dots) and visceral leishmaniasis (red areas). Adapted from WHO [2].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 May 2021 doi:10.20944/preprints202105.0754.v1



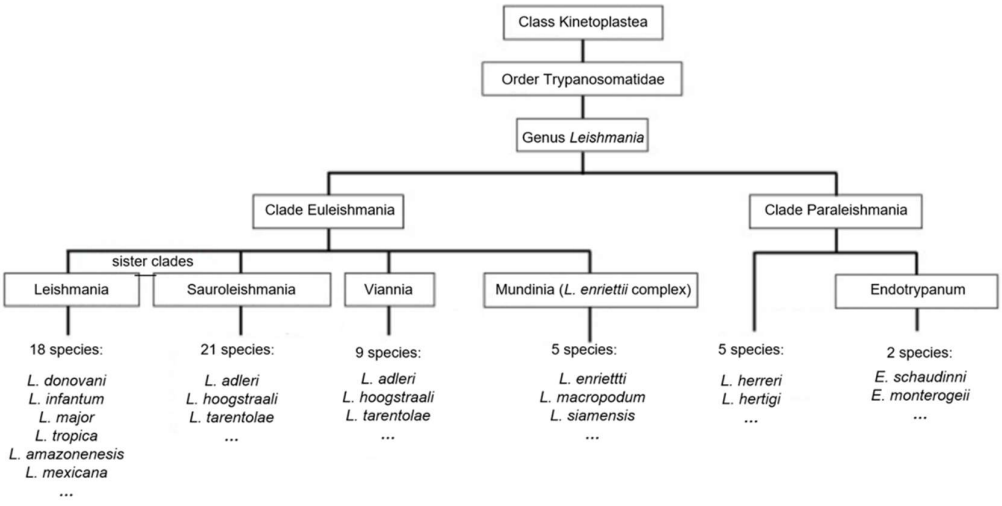


Figure 2. Genus *Leishmania*: Tree of Eukaryotes and Taxonomy [24–25].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 May 2021 doi:10.20944/preprints202105.0754.v1

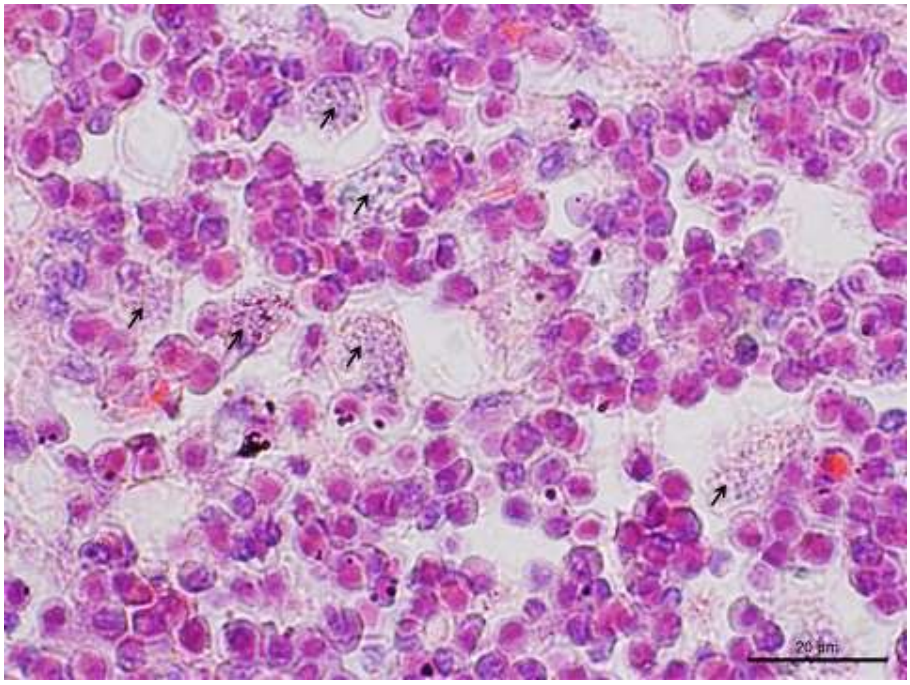


Figure 3. Canine leishmaniasis (CanL) caused by zoonotic *L. infantum*. Submandibular lymph node photomicrography. Medullary cords presenting numerous forms amastigotes (arrows). Morphology assessments by hematoxylin and eosin (H&E) stain. Scale-bar 20 µm

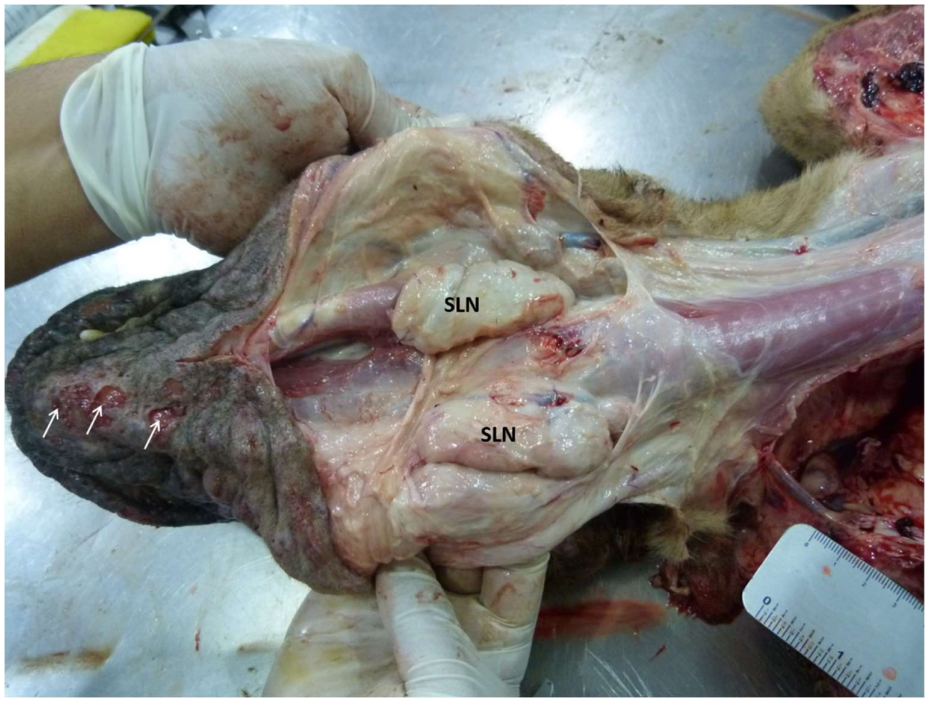


Figure 4. Canine leishmaniasis (CanL) in endemic area of Amazonia-Brazil. Dermatitis in facial area with ulcerations (arrows). Submandibular lymph nodes (SLN) increased in size (lymphodenomegaly).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 May 2021 doi:10.20944/preprints202105.0754.v1

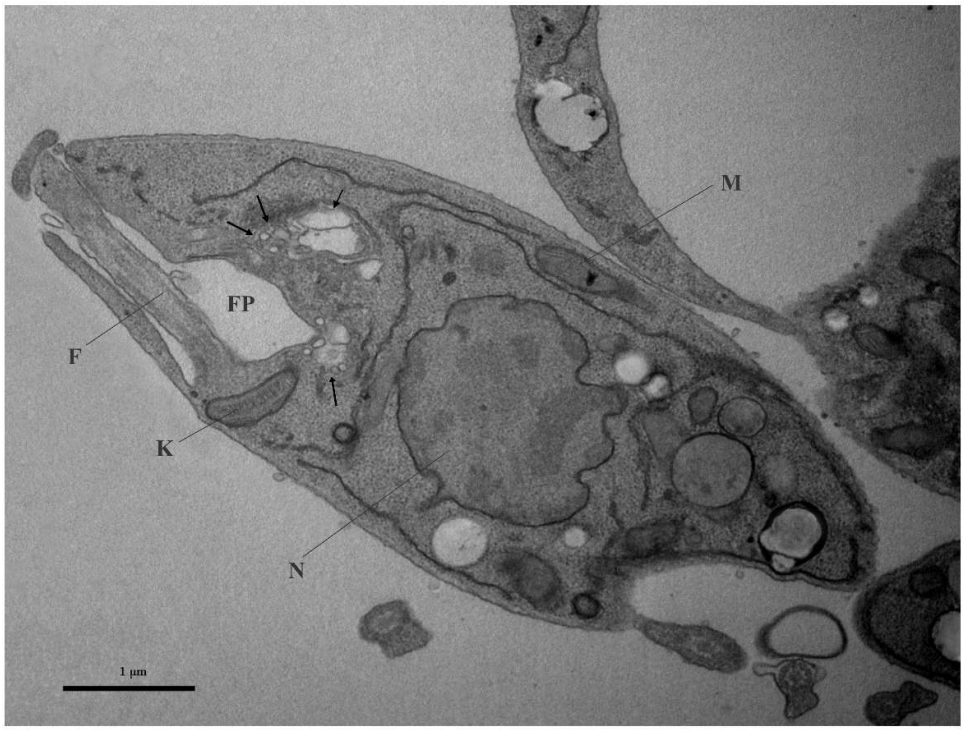


Figure 5. Tubular structures and vesicles (arrows) participating in the endocytosis and exocytosis process can be seen in the flagellar pocket (FP) of *L.* (*L.*) *amazonensis* promastigotes (MHOM/BR/2009/M26361 strain). F = flagellum; K = kinetoplast; M = mithocondria; N = nucleus. Scale-bar 1 µm

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 May 2021 doi:10.20944/preprints202105.0754.v1

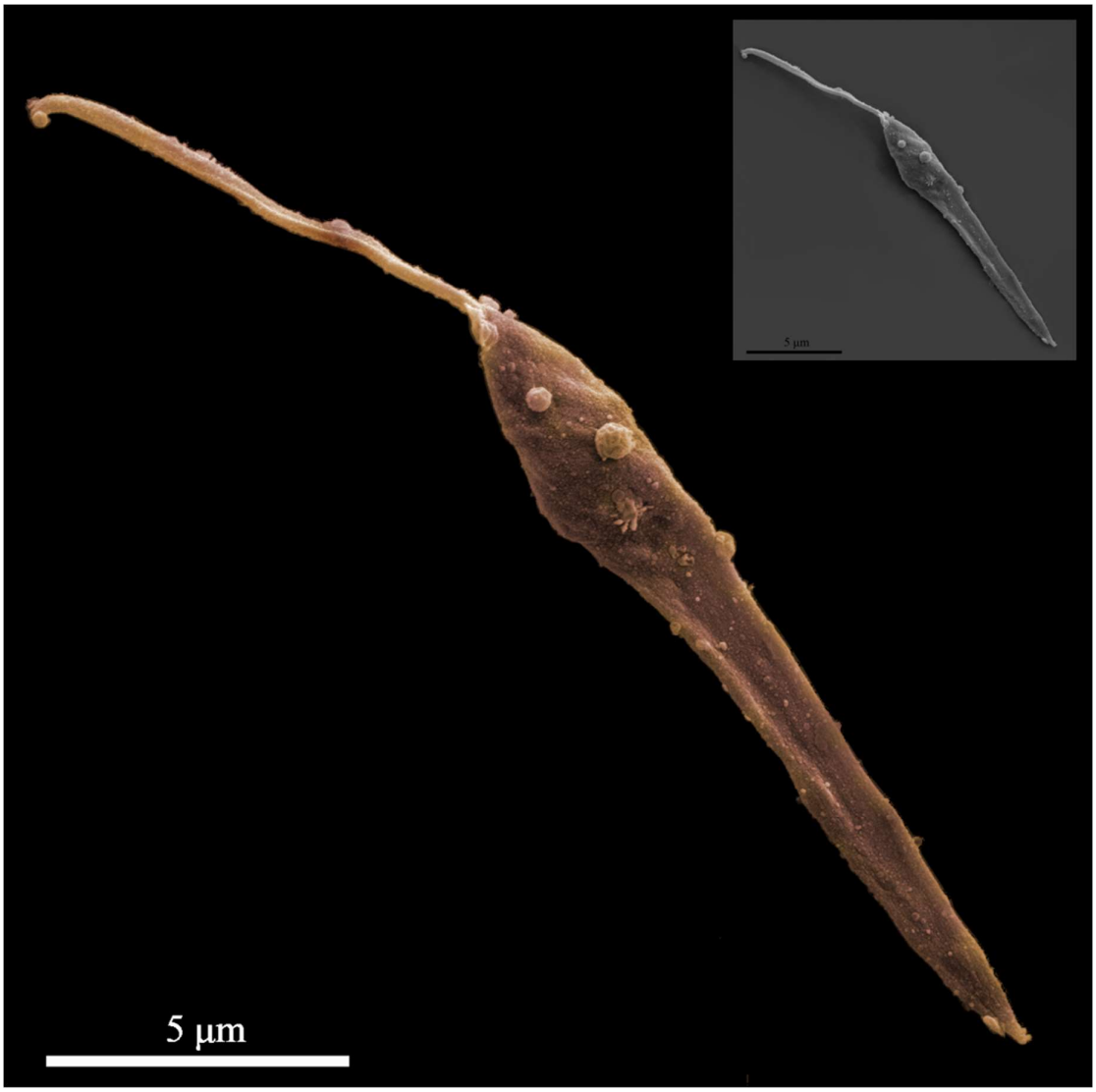


Figure 6. Heterogeneous group of membrane-bound extracellular vesicles secreted by *Leishmania*. Representative SEM photomicrograph showing surface membrane vesicular forms (light points, rounded and amorphous vesicles) of *L.* (*L.*) *amazonensis* promastigotes (MHOM/BR/2009/M26361 strain). Scale-bar 5 µm.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 May 2021 doi:10.20944/preprints202105.0754.v1

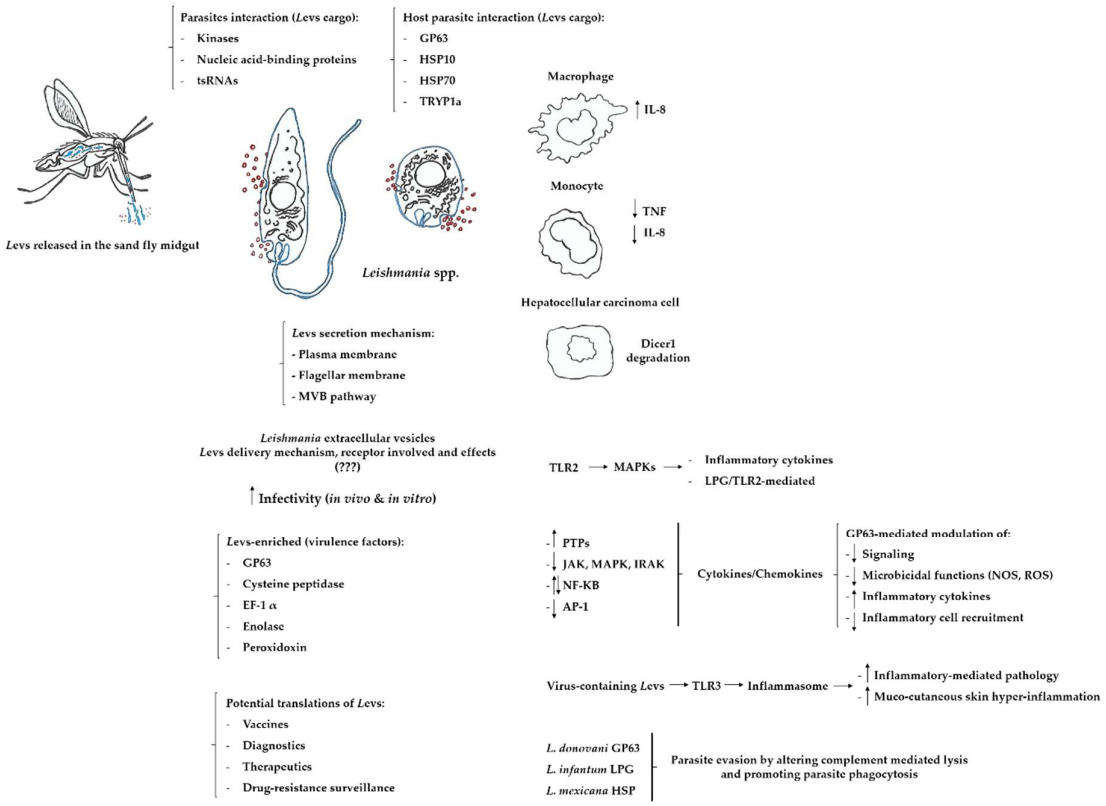


Figure 7. *Leishmania* extracellular vesicles *L*EVs cargo: parasites and host cells interactions [68–74–78].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 May 2021 doi:10.20944/preprints202105.0754.v1

Tables

Table1: Old and New World species of *Leishmania* causing visceral and cutaneous leishmaniasis: geographic distribution, vectors, hosts and reservoirs [4–9–10].

Species	Geographic distribution	Vectors	Transmission cycle	Hosts	Reservoirs
Complex ***L. (L.) donovani***	Old World	*Sergentomyia* species	Predominant anthroponotic	Rodents, Humans	Humans
L. (L.) infantum	New and Old World	*Phlebotomine* Sand Fly Species	Zoonotic	Dogs, humans	Dogs
L. (L.) aethiopica	Ethiopia	*Ph. sergenti, Ph. longipes, Ph. pedifer*	Zoonotic	Rodents, wild canids, dogs	Hyraxes
L. (L.) major	Asia & Africa	*Phlebotomus papatasi, Ph. duboscqi, Ph. salehi*	Zoonotic	Small rodents, dogs, humans	Small mammals and birds
L. (L.) tropica	Asia, Africa and Mediterranean	*Ph. sergenti*	Predominant anthroponotic	Rodents, wild canids, dogs	Humans, hyraxes
L. (L.) amazonensis	South America	*Lu. flaviscutellata, Lu. reducta, Lu. olmeca olmeca, Lu. nuneztovari*	Zoonotic	Terrestrial forest rodents, marsupials, wild canids, humans	Rodents, edentates, marsupials, wild canids
L. (L.) garnhami	Venezuelan Andes	*Lu. youngi*	Zoonotic	The opossum *Didelphis marsupialis* and humans	Marsupials
L. (L.) mexicana	USA (Texas), Central and South America	*Lutzomyia olmeca olmeca, Lu. diabolica, Lu. anthophora, Lu. columbiana, Lu. ayacuchenisis, Lu. ylephiletor, Lu. cruciata, Lu. longipalpis*	Zoonotic	Rodents: *Ototylomys phyllotis, Nyctomys sumichrasti, Heteromys desmarestianus, Sigmodon hispidus, Neotoma albigula, Proechimys* sp., *Oryzomys* sp., *Nectomy* sp, *Neacomys* sp,	Rodents, edentates, marsupials

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 May 2021 doi:10.20944/preprints202105.0754.v1

				Dasyprocta sp; *marsupials: Marmosa* sp, *Metachirus* sp, *Didelphis* sp., *Philander* sp; wild canids: *Cerdocyon thous*; humans	
L. (L.) pifanoi	Apparently limited to Venezuela	*Lu. flaviscutellata, Lu. olmeca bicolor*	Zoonotic	Humans. wild rodents, *Rattus rattus*	Rodents
L. (L.) venezuelensis	Venezuela, in the States of Lara and Yaracuy	*Lu. olmeca bicolor, Lu. rangeliana*	Zoonotic	Cats and humans	Rodents: *Sigmodon hispidus, Rattus rattus*
L. (L.) shawi	Brazilian Amazon Region	*Lu. whitmani complex*	Zoonotic	Monkeys: *Cebus apella, Chiropotes satanas*; edentates: *Choloepus didactylus, Bradypus tridactylus*; procyonids: *Nasua nasua*; humans	Monkeys, edentates, procyonids
L. (V.) braziliensis	Central & South America	*Lu. intermedia, Lu. whitmani, Lu. wellcomei, Lu. migonei, Lu. neivae, Lu. davisi, Lu. ovallesi, Lu. carrerai carrerai, Lu. spinicrassa, Lu. trapidoi, Lu. gomezi, Lu. ylephiletor, Lu. Umbralitis, flaviscutellata, Lu. olmeca*	Zoonotic	Rodents: *Oryzomys concolor, O. capito, O. nigripes, Akodon arviculoides, Proechimys* sp., *Sigmodon hispidus, Bolomys lasiurus, Rhipidomys leucodactylus, Rattus rattus*; Marsupials:	Humans. Terrestrial rodents and some marsupials

 doi:10.20944/preprints202105.0754.v1

				Didelphis marsupialis; dogs, cats and horses	
L. (V.) colombiensis	Colombia, Panama, Venezuela, forests of Brazil and Peruvian lowlands, others Latin American countries	*Lu. hartmanni, Lu. gomezi, Lu. panamensis*	Zoonotic	Sloth *Choloepus hoffmanni* and humans	Edentates
L. (V.) guyanensis	South America	*Lu. umbratilis, Lu. anduzei, Lu. Ovallesi, Lu. whitmani*	Zoonotic	Rodents, edentates: *Choloepus didactylus, Tamandua tetradactyla*; marsupials, humans	Rodents, edentates, marsupials
L. (V.) lainsoni	Forested areas of Brazil, Peru and Bolivia	*Lu. ubiquitalis, Lu. velascoi*	Zoonotic	Rodents *Agouti paca* and humans	Rodents
L. (V.) lindenbergi	Degraded forest in Belém, Pará, Brazil	*Currently unknown Lu. antunesi is highly suspected*	Zoonotic	Humans	It is suspected that the wild animal reservoirs are probably terrestrial
L. (V.) naiffi	States of Pará and Amazonas (Brazil), French Guyana	*Lu. ayrozai, Lu. paraensis, Lu. squamiventris*	Zoonotic	Nine- banded armadillo *Dasypus novemcinctus*, humans	Edentates
L. (V.) panamensis	Central America	*Lu. trapidoi , Lu. ylephiletor, Lu. gomezi, Lu. panamensis, Lu. hartmanni*	Zoonotic	Rodents: *Heteromys* sp.; edentates: *Choloepus hoffmanni*,	Rodents, edentates, marsupials, procyonids, monkeys

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 May 2021 doi:10.20944/preprints202105.0754.v1

				Bradypus infuscatus, *B. griseus*; marsupials, procyonids: *Bassaricyon gabbi*, *Nasua nasua*, *Potos flavus*, *monkeys*: *Aotus trivirgatus*, *Saguinus geoffroyi*; hunting dogs, humans	
L. (V.) peruviana	South America	*Lu. peruensis*, *Lu. verrucarum*	Zoonotic	Humans and dogs. Rodents: *Phyllotis andinum*; Marsupials: *Didelphis marsupialis*	Rodents, marsupials

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 May 2021 doi:10.20944/preprints202105.0754.v1

Table 2: EV research checklist for *Leishmania* extracellular vesicles (*L*EVs) [80–81].

Parameters	Mandatory	Mandatory if applicable	Encouraged
Nomenclature	Generic term extracellular vesicle (EV): With demonstration of extracellular (no intact parasites) and vesicular nature per these characterization and function guidelines Generic term extracellular particle (EP): No intact parasites but MISEV guidelines not satisfied	***	Generic term extracellular vesicle (EV) + specification (size, density, other) Specific term for subcellular origin: Ectosome, microparticle, microvesicle (from plasma membrane), exosome (from endosomes), with demonstration of the subcellular origin Other specific term: with definition of specific criteria
Collection and pre-processing Culture Conditioned medium **General cell characterization Medium used before and during collection (additives, serum, other)**	Nature and size of culture vessels, and volume of medium during conditioning Number of parasites/ml, or /surface area and % of live/ dead cells at time of collection (or at time of seeding with estimation at time of collection) Frequency and interval of CM harvest	Exact protocol for depletion of EVs/EPs from additives in collection medium Specific culture conditions (treatment, % O2, …) before and during collection	 ***
Collection and pre-processing Storage and recovery	Storage and recovery (e.g., thawing) of culture medium, before EV isolation (storage temperature, vessel, time; method of thawing or other sample preparation) Storage and recovery of EVs after isolation (temperature, vessel, time, additive(s)…)	***	***
EV separation and concentration **Experimental details of the method**	***	Centrifugation: reference number of tube(s), rotor(s), adjusted k factor(s) of each centrifugation step (= time+ speed+ rotor, volume/density of centrifugation conditions), temperature, brake settings Density gradient: nature of matrix, method of generating gradient, reference (and size) of tubes, bottom- up (sample at bottom, high density) or top-bottom (sample on top, low density), centrifugation speed and time (with brake specified), method and volume of fraction recovery Chromatography: matrix (nature, pore size,…), loaded sample volume, fraction volume, number Precipitation: references, ratio vol/vol or weight/vol fluid, time/temperature of incubation, time/speed/temperature of centrifugation Filtration: reference of filter type (=nature of membrane, pore size…), time and speed of centrifugation, volume before/after (in case of concentration) Antibody-based : reference of antibodies, mass Ab/ amount of EVs, nature of Ab carrier (bead, surface) and amount of Ab/carrier surface Other: all necessary details to allow replication Additional step(s) to concentrate Additional step(s) to wash matrix and/or sample	***
EV separation and concentration **Specify category of the chosen EV separation/concentration method**	***	***	High recovery, low specificity = mixed EVs and non- EV components Intermediate recovery, intermediate specificity = mixed EVs with limited non-EV components Low recovery, high specificity = subtype(s) of EVs with as little non-EV as possible High recovery, high specificity = subtype(s) of EVs with as little non-EV as possible
EV characterization **Quantification**	Volume of fluid, and/or cell number, and/or tissue mass used to isolate EVs Global quantification by at least 2 methods: protein amount, particle number, lipid amount, expressed per volume of initial fluid or number of producing cells/ mass of tissue Ratio of the 2 quantification figures	***	***
EV characterization **Global characterization**	Transmembrane or GPI-anchored protein localized in cells/parasites at plasma membrane or endosomes Cytosolic protein with membrane-binding or - association capacity Assessment of presence/absence of expected contaminants	Presence of proteins associated with compartments other than plasma membrane or endosomes Presence of soluble secreted proteins and their likely transmembrane ligands	Topology of the relevant functional components
EV characterization **Single EV characterization**	Images of single EVs by wide-field and close-up: e.g. electron microscopy, scanning probe microscopy, superresolution fluorescence microscopy Non-image-based method analysing large numbers of single EVs: NTA (Nanoparticle tracking analysis), Tunable resistive pulse sensing (TRPS), Fluorescence Correlation Spectroscopy(FCS), high-resolution flow cytometry, multi-angle light-scattering, Raman spectroscopy, etc.	***	***
Functional studies	Dose-response assessment Negative control = nonconditioned medium, biofluid/tissue from control donors, as applicable Quantitative comparison of functional activity of total fluid, vs EV-depleted fluid, vs EVs (after high recovery/low specificity separation) Quantitative comparison of functional activity of EVs vs other EPs/fractions after low recovery/high specificity separation	***	Quantitative comparison of activity of EV subtypes (if subtype-specific function claimed) Extent of functional activity in the absence of contact between EV donor and EV recipient
Reporting	Submission of data (proteomic, sequencing, other) to relevant public, curated databases or open-access repositories	Temper EV-specific claims when MISEV requirements cannot be entirely satisfied	Submission of methodologic details to EV-TRACK (evtrack.org) with EV-TRACK number provided (strongly encouraged) Data submission to EV-specific databases (e.g., EVpedia, Vesiclepedia, exRNA atlas)