Ahyun Hong

Estudo da regulação dos transportadores de ferro e heme na interface *Leishmania*-hospedeiro: efeito da deficiência em ferro na virulência de *Leishmania (L.) amazonensis*.

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Orientadora: Maria Fernanda Laranjeira da Silva

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Dedication

A dedication to my mom, who has been my academic inspiration from day one, instilling in me in the belief that *"learning is the easiest thing in the world, so never stop learning and strive for your highest"*. This is for you, Mom.

나의 영원한 롤모델, "*세상에서 배우는 것 만큼 쉬운 것이 없으니 항상 공부하고 최고를 향해 노력해야 한다*" 는 믿음을 실천하고 끈임없이 보여주는 우리 엄마에게 바칩니다.

Epigraph

“When they go low, we go high”

Michelle Obama

Acknowledgment

Before all else, I wouldn't and couldn't have reached this milestone without the unwavering support of my family. Across 20,000 kilometers and a 12-hour time difference, you were the wind beneath my wings, always believing in me even when I questioned myself. Thank you for always being my greatest cheerleaders.

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1. Introduction

1.2. History of leishmaniases and genus *Leishmania*

The protozoan parasite belonging to the *Leishmania* genus, causative agent of zoonotic vector-borne diseases collectively known as leishmaniases, owes its nomenclature to the collaborative efforts of Scottish pathologist Lieutenant General Sir William Boog Leishman and Irish doctor Charles Donovan. This nomenclature originated from their pivotal discovery of the parasite responsible for visceral leishmaniasis during the early 20th century[1].

Sir William Boog Leishman, who served with the British Army in eastern India, initially identified the organism in smears extracted from the spleen of a soldier who succumbed to an unidentified disease in Dumdum, India. Subsequently, Charles Donovan corroborated Leishman's findings by examining smears from patients in southern India. Remarkably, up until this juncture, the causative organism had been suggested to be trypanosomes. It was only after British medical doctor Sir Ronald Ross contested this classification, asserting that the organism was a distinct parasite, that it was officially named *Leishmania donovani*. However, it's noteworthy that descriptions of leishmaniases appear in various ancient histories, predating the formal identification of the *Leishmania* parasite[2, 3].

In the 7th century BCE, within the library of Assyrian King Ashurbanipal, a recorded description mirroring the clinical manifestations of cutaneous leishmaniases was discovered on a clay tablet[4]. Subsequently, a paleoparasitological study in 2006, conducted by a group from Munich, Germany, analyzed 91 ancient Egyptian mummies dating from 2050 to 1560 BCE and 70 Christian Nubian mummies from 550 to 1500

AD, revealing a 120 bp fragment of a conserved region of leishmanial mitochondrial DNA in four Egyptian and nine Nubian mummies. Further characterization through direct DNA sequencing unveiled that these mummies were infected with *L. donovani*. Additionally, the Eber Papyrus from 1500 BCE mentioned cutaneous leishmaniases as a "Nile Pimple."[2] Even in South America, evidence of *Leishmania*-infected macrophages was detected in a Peruvian mummy from 800 BCE, suggesting the presence of leishmaniases prior to European colonization[5].

Leishmania, a parasite found in tropical and subtropical regions worldwide, has a debated origin for its different species. The place of origin of the *Leishmania* genus is believed to have occurred in the Mesozoic era (252-266 MYA) before the breakup of the supercontinent Pangaea[2, 6]. Presently, there are twenty-two known species found in human hosts, classified under the subgenera *L. (Leishmania)*, *L. (mundinia)*, and *L. (Viannia)*[7, 8].

1.3. The public health aspect of leishmaniases

1.3.1. Clinical manifestations

Leishmaniases stand among the neglected tropical diseases identified by the World Health Organization (WHO) and are estimated to rank ninth in terms of disease burden among infectious diseases globally[9]. These diseases manifest in a diverse range of clinical presentations, spanning from self-healing cutaneous lesions to mucosal lesions and potentially life-threatening visceral forms. While most instances of cutaneous leishmaniasis (CL) result in self-healing skin lesions, they often leave permanent scars. The enduring nature of these scars can give rise to self- and social

stigmas, impacting the affected individual's quality of life and leading to psychosocial burdens[10]. In certain cases, CL can progress into more severe forms, including mucocutaneous (MCL), diffuse (DCL), or disseminated (DL) cutaneous leishmaniases. Visceral leishmaniasis (VL), commonly known as kala-azar, represents the most severe manifestation of leishmaniases. Clinical manifestations of VL encompass non-tender splenomegaly, with or without hepatomegaly, and individuals with underlying health conditions may develop post-kala-azar dermal leishmaniasis (PKDL)[10].

1.3.2. One Health and leishmaniases

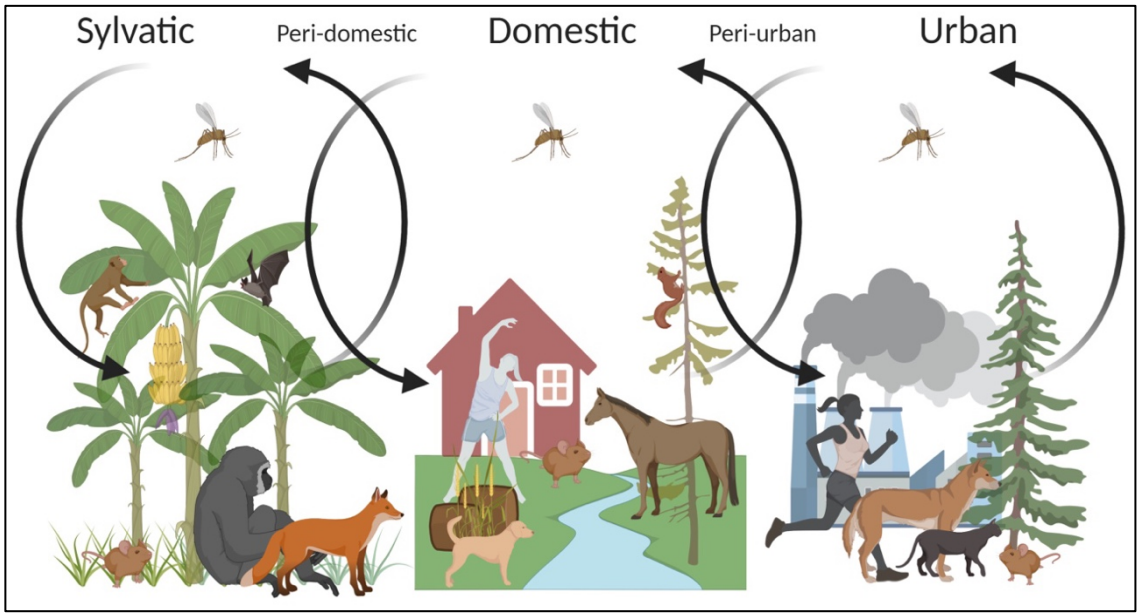

Figure 1: The transmission cycles of zoonotic leishmaniases.

Sylvatic leishmaniases can spill over into humans living in proximity to forest foci of transmission, mainly due to deforestation or other factors affecting the ecological balance. As indicated by arrows, sand fly vectors, originally breeding in natural forest environments, adapt to peri-domestic and domestic settings, eventually infiltrating densely populated urban areas. *Note*: From "One Health Approach to Leishmaniases: Understanding the Disease Dynamics through Diagnostic Tools", by A. Hong, R.A Zampieri, J.J. Shaw, L. M. Floeter-Winter, M.F. Laranjeira-Silva, 2020, *Pathogens*, 9 (10). DOI: 10.3390/pathogens9100809.

The transmission dynamics of *Leishmania* are intricately woven within the interactions of human and animal hosts, parasites, and the sandfly vector, creating a complex scenario. These dynamics are further complicated by environmental changes and socioeconomic factors, such as inadequate housing, unsanitary conditions, malnutrition, and migration. Human activities can reshape the composition and behavior of sand fly vectors, and zoonotic leishmaniases showcase a wide diversity of mammalian reservoirs globally. Sylvatic transmission is influenced by the presence of wildlife in and around human settlements, with various animals, including rodents, foxes, dogs, cats, primates, hyraxes, and bats, serving as reservoir hosts for different *Leishmania* species. Urbanization and deforestation can create new breeding habitats for vectors, fostering spillover events across ecosystem boundaries. Recognizing the interdependence among humans, animals, and the environment is crucial, especially as over 60% of human infectious diseases are zoonotic, emphasizing the intricate connections within this dynamic system (Figure 1) [Reviewed in [10]].

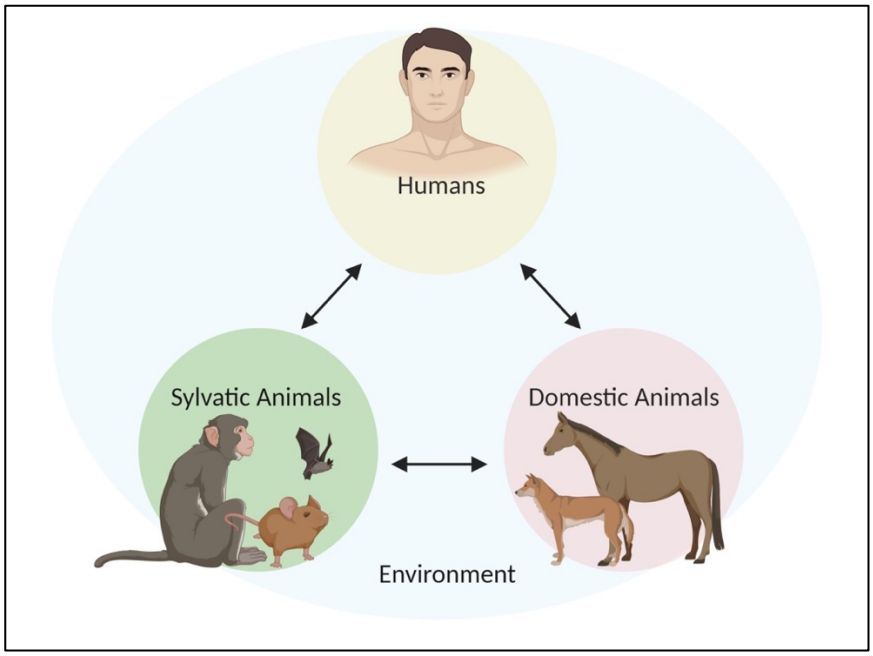

Figure 2: The One Health diagram illustrating the interactions between humans, sylvatic, and domestic animals within the shared environment.

Note: From "One Health Approach to Leishmaniases: Understanding the Disease Dynamics through Diagnostic Tools", by A. Hong, R.A Zampieri, J.J. Shaw, L. M. Floeter-Winter, M.F. Laranjeira-Silva, 2020, *Pathogens,* 9 (10). DOI: 10.3390/pathogens9100809.

The One Health approach is a global strategy that advocates for collaborative efforts across multiple sectors and disciplines, encompassing human, animal, and environmental health, acknowledging their intricate connections (Figure 2). The emergence and resurgence of infectious diseases are influenced by diverse anthropogenic elements, often unintentionally contributing to the phenomenon. These include environmental factors like climate change and deforestation, population movements such as migration and increased international travel, socioeconomic and political-driven factors like poverty, lack of political will, and conflict, as well as genetic factors, including host adaptation and susceptibility to infection. Given the intricate transmission cycle of leishmaniases, the adoption of the One Health approach

is imperative, not only for a deeper understanding of the disease itself but also for effective control strategies [Reviewed in [10]].

1.3.3. Diagnosis of leishmaniases

Diagnosing leishmaniases typically involves assessing clinical symptoms in patients and conducting one or more laboratory tests [Reviewed in[10]]. A traditional approach is parasitological diagnosis, which involves examining tissue aspirates or biopsies from suspected individuals with VL or CL using optical microscopy or culturing samples for further analyses[11]. Polymerase Chain Reaction (PCR)-based methods have become a key diagnostic tool, rapidly confirming diagnoses and aiding in disease control and surveillance by identifying parasite species and their geographical distribution[12, 13]. Quantitative PCR (qPCR) is commonly used in clinical laboratories to detect parasites in skin lesions and blood samples, offering a quantitative approach for diagnosis and treatment monitoring[14, 15]. High-resolution melting (HRM) analysis, a variation of qPCR, is emerging as a highly sensitive method for distinguishing *Leishmania* species[16, 17]. Immunological tests like the Montenegro Skin Test (MST), or also widely known as the Leishmanin Skin Test (LST) remain in use, especially in remote areas[11, 18], but their reliability is limited, particularly for detecting relapses, due to the persistence of specific antibodies after recovery and lack of species specificity[11].

1.3.4. Treatment for leishmaniases

Current treatment options for leishmaniases encompass a range of therapeutic strategies designed to combat infections caused by *Leishmania*. Pentavalent

antimonials remain a cornerstone of CL treatment, alongside the emergence of novel oral and topical alternatives in recent years[19]. Miltefosine, the sole oral medication currently approved for the treatment of leishmaniasis, targets both promastigote and amastigote form of the parasites, proving effective against both VL and CL[20, 21]. Eukaryotic protein kinases have been identified as potential targets for rational drug design against leishmaniasis, offering new avenues for therapeutic interventions[22]. The development of effective vaccines is a critical focus, with ongoing efforts to enhance cell-mediated immunity and CD8+ T cell responses to improve treatment outcomes and control disease progression[23]. The search for novel therapeutic agents and vaccines continues to address the challenges posed by leishmaniases, emphasizing the importance of improving our understanding of *Leishmania* essential metabolic pathways for development of new targeted drugs and innovative approaches in combating these complex diseases[24-27].

1.4. The life cycle of *Leishmania*

Leishmania parasites undergo a complex life cycle involving two distinct developmental forms—amastigotes and promastigotes—each adapted to different hosts. In the vertebrate host, *Leishmania* takes on the amastigote form, characterized by oval-shaped cells within parasitophorous vacuoles (PV) of macrophages or phagolysosomes. Following a blood meal from an infected vertebrate host, the invertebrate vector ingests macrophages containing amastigotes, which transform into non-infective procyclic promastigotes in the sandfly's digestive tube. These promastigotes, with a long, slender shape and a flagellum at the anterior pole, are crucial for the parasite's life cycle and adaptation to different hosts[28].

The sandfly's saliva is integral to *Leishmania* transmission, facilitating attachment to the sandfly midgut and supporting the parasite's developmental stages. *Leishmania* replication and infectivity are enhanced through sequential blood meals, leading to increased parasite loads and lesion frequency in the mammal host[29]. Additionally, *Leishmania* exosomes play a protective role, contributing to the parasite's survival and transmission within the sandfly vector[30]. Upon reaching a specific density, procyclic promastigotes differentiate into infective metacyclic promastigotes, capable of invading the sandfly's esophagus and proventriculus. During a subsequent blood meal, regurgitation ensures the inoculation of infective forms into a new vertebrate host, where they undergo differentiation into amastigotes, completing the life cycle and ensuring parasite propagation[31, 32].

1.4.1. Immune response and life cycle of *Leishmania*

Macrophages and various immune cells play pivotal roles in both innate and adaptive immunity during the life cycle of *Leishmania*. Macrophages, key participants in the innate immune response, serve as one of the main host cells for *Leishmania* parasites. Upon infection, *Leishmania* manipulates macrophage functions to establish a conducive environment for its survival and replication, influencing the parasite's life cycle and pathogenesis[33]. Additionally, other immune cells such as dendritic cells, neutrophils, and natural killer cells contribute to the innate immune response against *Leishmania* infection[34-36].

In terms of adaptive immunity, macrophages and dendritic cells serve as antigen-presenting cells, initiating the activation of T lymphocytes and shaping the adaptive immune response against *Leishmania*[35, 36]. The crucial crosstalk between

macrophages and T cells orchestrates an effective immune response to control *Leishmania* infection[35]. Furthermore, the modulation of macrophage polarization, transitioning from M1 to M2 phenotypes, can impact the immune response against *Leishmania*, underscoring the significance of macrophage plasticity in shaping the immune response[37].

1.5. Iron and heme metabolism in *Leishmania*

1.5.1. Iron availability and life cycle of *Leishmania*

Numerous studies have highlighted the significant impact of nutrient availability within PV on parasite replication and disease virulence, as evidenced by the limited availability of essential micronutrients like iron and heme within PV[38-40]. Iron, a crucial element for various biological processes, serves as a cofactor for several enzymes essential to *Leishmania*, contributing to its oxidation-reduction potential and facilitating electron transfer reactions. Of particular importance among these enzymes are iron-dependent superoxide dismutases (FeSODs), which play a crucial role in protecting the parasite against free radicals[41]. Both the host and the parasite rely on iron for essential biological functions, and the host's ability to restrict iron access to parasites stands as a key defense mechanism in infection control[42]. Notably, the natural resistance-associated macrophage protein 1 (Nramp1) transporter functions to remove iron and other divalent cations from late endocytic compartments of macrophages, impacting host susceptibility to intracellular pathogens such as *Leishmania*, *Salmonella*, and *Mycobacterium*[43-46]. That being said, for *Leishmania* to replicate within PV, the parasites face the dual challenge of acquiring iron and competing with the host's Nramp-

1, underscoring the intricate interplay between the parasite and the host's defense mechanisms in the context of nutrient availability.

Besides, iron availability within the sandfly vector can influence the developmental changes of *Leishmania* parasites from procyclic promastigotes to metacyclic promastigotes. *Leishmania* relies heavily on iron for intracellular replication, and alterations in iron availability appear to trigger developmental changes in the parasite[47]. The generation of reactive oxygen species (ROS) mediated by iron has been implicated in *Leishmania* differentiation, emphasizing the pivotal role of iron in the parasites' developmental processes[47]. The modulation of iron acquisition and utilization pathways in *Leishmania* can impact differentiation processes, influencing the parasites' infectivity and survival within both the vector and mammalian hosts (Figure 3)[40]. Furthermore, *Leishmania*-mediated suppression of iron export in macrophages promotes parasite replication, underscoring the importance of iron availability in the host-parasite interaction. The interaction between *Leishmania* and macrophages, influenced by iron availability, can impact the parasites' ability to establish infection and replicate within the host[48].

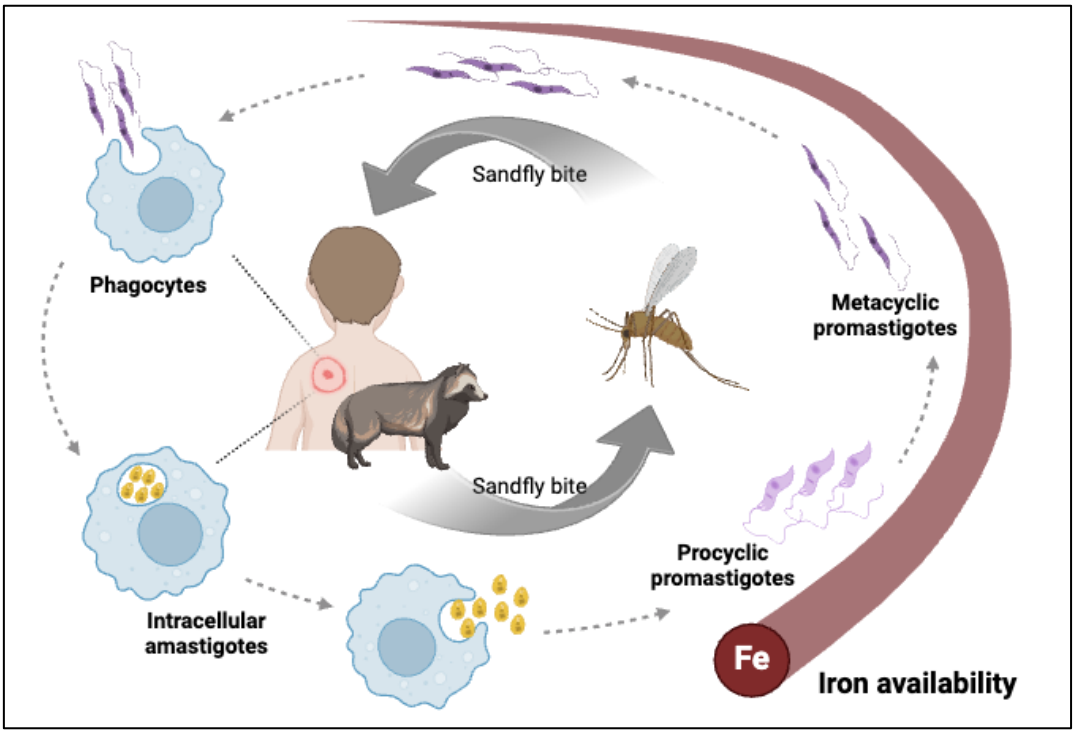

Figure 3: Life cycle of *Leishmania* and iron availability within the sandfly vector.

Upon a blood meal, the midgut of a sandfly becomes rich in iron due to hemoglobin breakdown. During this phase, ingested amastigotes transform into procyclic promastigotes, initiating their replication. After a few days, the parasites cease replication and enter a "sugar meal phase," characterized by limited iron availability within the invertebrate host. In the subsequent blood meal, metacyclic promastigotes are transmitted to the mammalian host by sandflies. Inside the host's macrophages, these promastigotes transform into amastigotes within the parasitophorous vacuoles, completing the life cycle. The low iron availability within the parasitophorous vacuoles in macrophages triggers the upregulation of proteins involved in iron acquisition. [Figure modified from [47]].

1.5.2. Iron and heme transporters in *Leishmania*

In recent years, a series of proteins crucial for iron transport and metabolism in *Leishmania* were identified and characterized, as illustrated in Figure 4. The initial discovery involved *Leishmania* Iron Transport 1 (LIT1), characterized as a member of the ZIP family and identified as an iron transporter expressed on the plasma membrane of intracellular amastigotes[49]. Following this, *Leishmania* Ferric Reductase 1 (LFR1) was identified as a key player in reducing the insoluble form of iron (Fe^{3+}) to its soluble

counterpart (Fe^{2+}), enabling its translocation across membranes[50]. Subsequent investigations led to the identification of *Leishmania* Iron Regulator 1 (LIR1) during the analysis of transcriptomic data from iron-depleted parasites. *LIR1*, a member of the major facilitator superfamily (MFS), emerged as a membrane transporter responsible for regulating intracellular iron levels by facilitating the export of this essential transition metal[51]. The comprehensive understanding of these iron-related proteins provides valuable insights into the intricate mechanisms governing iron homeostasis in *Leishmania*.

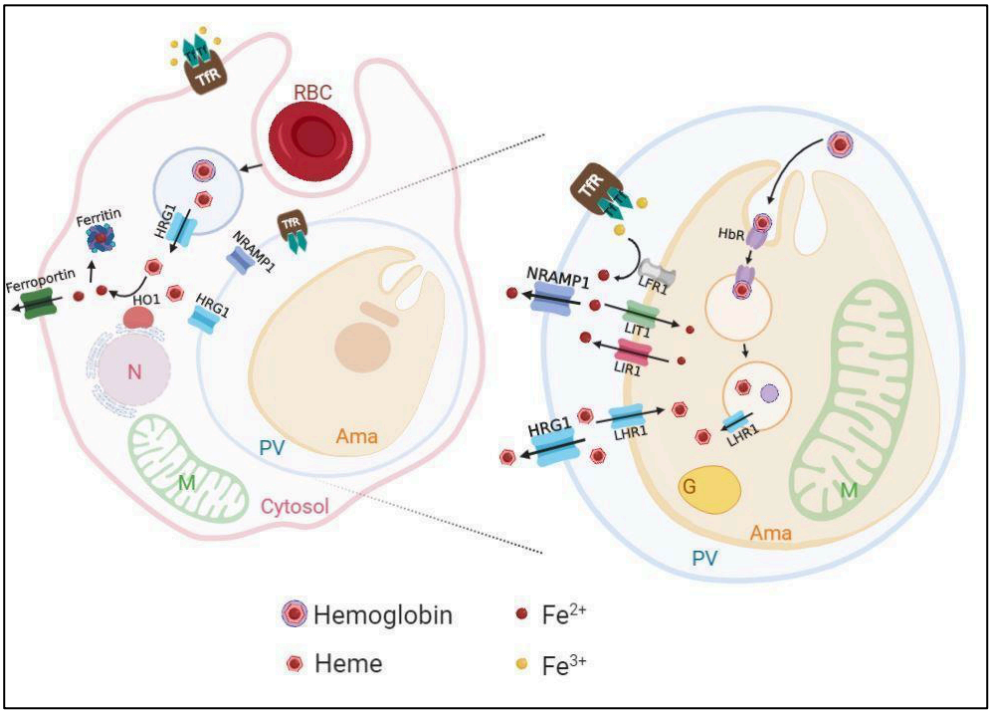

Figure 4: *Leishmania* iron and heme traffic pathways within host macrophages.

When hemoglobin is degraded through erythrophagocytosis, heme is translocated to the cytosol by HRG1 and broken down by HO1 (HMOX1). The released iron is stored in ferritin or exported by ferroportin. *Leishmania* PVs resemble phagolysosomes that contain HRG1, which is then likely to compete with the parasites for heme-iron. NRAMP1 is postulated to transport iron from the phagolysosome although the source of this iron is unknown. Transferrin (Tf) bound iron is internalized by the transferrin receptor (TfR) via endocytosis which fuses with the phagosome. Fe^{+3} is released by the low pH into the phagosomal lumen. *Leishmania* utilizes a ferric reductase (LFR1) to reduce Fe^{+3} to Fe^{+2}, and a ferrous iron transporter (LIT1) transports Fe^{+2} into the cytosol. Cellular iron levels in the parasite are maintained by the iron exporter LIR1. *Leishmania*

must also acquire heme, via heme transporter LHR1 or hemoglobin, via endocytosis of the hemoglobin receptor (HbR). **Abbreviations*: Ama, amastigote; G, glycosome; HbR, hemoglobin receptor; HRG1, heme-responsive gene 1; HO1; heme oxygenase 1; LFR1, *Leishmania* ferric reductase 1; LIT1, *Leishmania* iron transporter 1; LIR1, *Leishmania* iron regulator 1; LHR1, *Leishmania* heme response 1; M, mitochondria; N, nucleus; NRAMP1, natural resistance-associated macrophage protein 1; PV, parasitophorous vacuole; RBC, red blood cell; Tf, transferrin; TfR, transferrin receptor. *Note*. From "Iron and Heme Metabolism at the Leishmania-Host Interface", by M.F. Laranjeira-Silva, I. Hamza, J.M. Pérez-Victoria, 2020, *Trends Parasitol, 36* (3), p. 279-289. DOI: 10.1016/j.pt.2019.12.010.

Heme stands as another indispensable nutrient, acting as a cofactor for proteins involved in crucial cellular and physiological functions such as oxygen storage and transport, signal transduction, and oxidative metabolism[52-54]. The intricate process of heme biosynthesis involves eight highly conserved reactions leading to the incorporation of Fe^{2+} into protoporphyrin IX (PPIX), with several enzymes participating in this pathway (Figure 5) [51, 55-57]. Despite the absence of a complete heme biosynthetic pathway in *Leishmania*, the presence of hemoproteins within *L. amazonensis* hints at the existence of a mechanism for heme uptake from the environment.

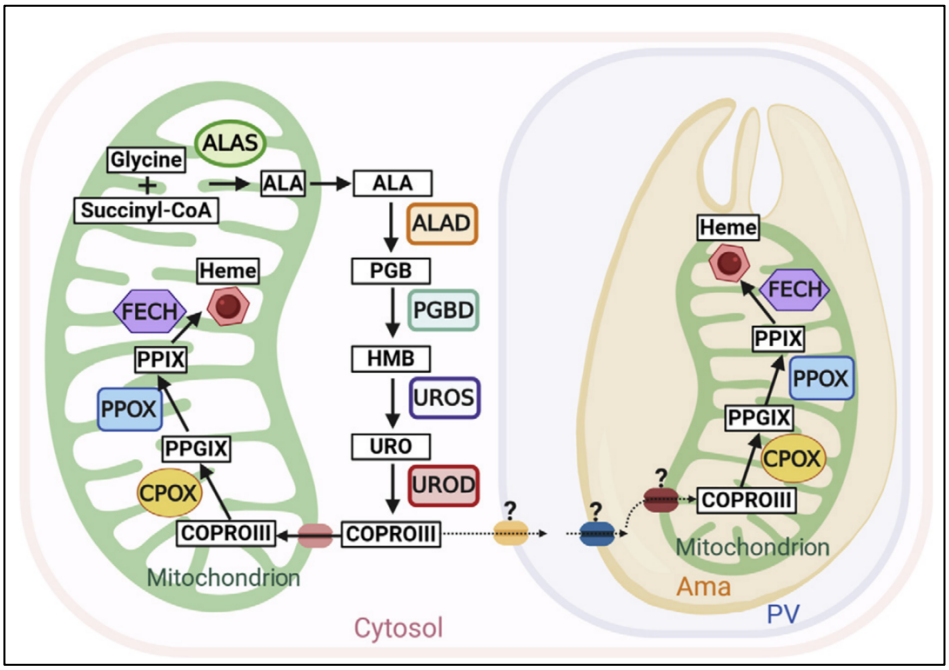

Figure 5: Mammalian and *Leishmania* heme biosynthesis pathways.

The synthesis of heme in the mammalian mitochondrion initiates in the mitochondrial matrix with the production of 5-aminolevulinic acid (ALA), catalyzed by 5-aminolevulinate coproprohyrinogen III (COPROIII). COPROIII is then transported back to the mitochondrial matrix, where the final three enzymatic reactions take place. In *Leishmania*, mitochondria exclusively possess the last three enzymes of the heme biosynthetic pathway, which were acquired through lateral gene transfer (LGT) from γ-proteobacteria. These enzymes include coproporphyrinogen oxidase (CPOX), protoporphyrinogen oxidase (PPOX), and ferrochelatase (FECH). Enzymatic substrates and products are shown in black/white boxes.***Abbreviations*: ALA, 5-aminolevulinic acid; ALAS, 5-aminolevulinate synthase; Ama, amastigote; COPROIII, coproporphyrinogen III; CPOX, coproporphyrinogen oxidase; FECH, ferrochelatase; HMB, hydroxymethylbilane; PGB, porphobilinogen; PGBD, porphobilinogen synthase; PPGIX, protoporphyrinogen IX; PPIX, protoporphyrin IX; PPOX, protoporphyrinogen oxidase; PV, parasitophorous vacuole; URO, uroporphyrinogen III; UROD, uroporphyrinogen decarboxylase; UROS, uroporphyrinogen III synthase. *Note*. From "Iron and Heme Metabolism at the Leishmania-Host Interface", by M.F. Laranjeira-Silva, I. Hamza, J.M. Pérez-Victoria, 2020, *Trends Parasitol, 36* (3), p. 279-289. DOI: 10.1016/j.pt.2019.12.010.

In mammals, Heme Responsive Gene 1 (HRG1) is responsible for heme transport, residing in the plasma membrane, lysosomal membrane, and endosome membrane. In macrophages, HRG1 plays a crucial role in maintaining iron homeostasis and transporting heme from the phagolysosome to the cytoplasm during

erythrophagocytosis[58]. Following this, *Leishmania* Heme Response 1 (LHR1) was identified due to its similarity to *C. elegans* HRG1. LHR1 was found to facilitate heme transport across the plasma membrane and regulate intracellular heme levels in *L. amazonensis*. The LHR1 protein, with four transmembrane domains, is situated at both the plasma membrane and endocytic compartment membrane of the parasite[58-60]. Although LHR1 and HRG1 share common functionalities, their sequence identity is notably weak. Specifically, LHR1 transmembrane residues Y18, Y80, and Y129, which are absent in the HRG family, play a crucial role in heme uptake, influencing the development of cutaneous lesions[59, 60].

The loss of an allele (single knockout, SKO), two or more alleles (double knockout, DKO or KO) of *LIT1*, *LFR1*, *LIR1*, or *LHR1* results in severe defects in the differentiation and/or multiplication of these parasites in the host. Specifically, the knockout of *LIT1* disrupts the intracellular growth of amastigotes within macrophages and hampers cutaneous lesion development during *in vivo* mice infection[49]. *LFR1* knockout interferes with differentiation into metacyclic promastigotes and amastigotes, and compromises parasite viability after intracellular transport facilitated by *LIT1*[50]. Regarding *LIR1*, its knockout results in an increased sensitivity to iron toxicity and significantly impaired infectivity[51]. Additionally, a single knockout of *LHR1* appears less effective in uptaking heme, leading to an inability to replicate intracellularly after infecting macrophages and exhibiting defects in cutaneous lesion development[58-60].

1.6. Iron deficiency anemia and leishmaniases in Brazil

The coexistence of iron deficiency, a prevalent global nutritional concern, and the high prevalence of leishmaniases in specific regions of Brazil, notably the

northeastern part, raises questions about potential impact of one of these health issue on the other. Iron deficiency is a widespread problem, especially among vulnerable populations such as children under 5 and women in Brazil, as indicated by the National Demography and Health Survey (PNDS)[61].

The overlapping occurrence of anemia and leishmaniases in the same geographic regions highlights the need for comprehensive investigations into potential interactions or influences between the two conditions. The northeastern region, known for its higher prevalence of anemia and significant leishmaniases burden, presents a unique context for such research. Despite the evident coexistence, there is a surprising lack of studies exploring how pre-existing anemia might impact the progression or severity of leishmaniases in affected individuals. On the flip side, a recent study exposed the consequences of localized *L. major* infection on systemic iron homeostasis and unveiled the potential role of oral iron supplementation in restoring balance[62]. As we unravel the interaction between iron deficiency and leishmaniases, ongoing and future research efforts could further investigate the complex dynamics of these two health issues. This exploration may uncover potential correlations, shed light on the mechanisms involved, and contribute to more targeted public health interventions. Exploring the relationship between nutritional status and infectious diseases is crucial for developing holistic strategies to address health challenges in specific populations.

The primary objective of this work was to explore the mechanisms of iron and heme metabolism regulation at the *Leishmania*-host interface. The core hypothesis was that *Leishmania* growth and virulence are intricately linked to the host's iron and heme levels. The project specifically examined the cross-regulation between iron and heme transport mediated by *LIT1* and *LHR1* in the parasite. To assess the importance of host

heme and iron status in *L. amazonensis* infection progression, control or iron-deficient (anemic) mice were infected with *L. amazonensis*.

The overarching goal was to enhance our understanding of *Leishmania*-host interactions, focusing on iron and heme metabolism pathways at both cellular and systemic levels. The results obtained from these studies contribute to elucidating the genetic and molecular basis of how essential nutrients and cofactors are acquired by pathogens to overcome nutritional barriers imposed by the host. Given the potential similarities in these pathways among different pathogenic species, the outcomes may pave the way for the development of novel antiparasitic agents.

2. Conclusion

This project represents a thorough exploration of the intricate regulatory mechanisms governing iron and heme transporters at the *Leishmania*-host interface. With a primary focus on unraveling the cross-regulation between the iron transporter, LIT1, and the heme transporter, LHR1, our investigation has encountered unexpected challenges during the validation of reported "*lit1-/lit1-*" *L. amazonensis* clones. Surprising findings, such as comparable *LIT1* expression in both WT and mutant clones, prompted a critical reevaluation of our strategies, leading to a necessary shift to CRISPR/Cas9 genome editing approach. The genomic plasticity challenges of *Leishmania*, particularly the recovery of open reading frame (ORF) copies during subculturing, underscore the intricacies of achieving complete knockouts. These observations prompt inquiries into the potential indispensable role of *LIT1* in *L. amazonensis*, deviating from prior findings. This underscores the need for deeper exploration into the intricate interplay between *Leishmania* genome dynamics and gene function.

Our phenotypic characterization of LIT1 and LHR1 overexpressors in *LHR1* SKO and *LIT1* PKO parasite cell lines unveiled captivating insights into the intricate interplay between iron and heme availability, gene expression, and *Leishmania* pathogenesis. While normal culture conditions exhibited compromised replication in mutants, overexpression of *LIT1* or *LHR1* restored promastigote growth profiles to WT levels. In heme-depleted media, mutants showed altered replication rates, with *LIT1* PKO promastigotes potentially indicating a reduction in intracellular iron content. In contrast, *LIT1* PKO demonstrated a failure to replicate within macrophages, as did *LIT1* and *LHR1* overexpressors in *LIT1* PKO parasite cell lines. This observation, together with previous discoveries from the literature[49], raises the possibility that *LIT1* could be

indispensable for the intracellular replication of amastigotes within the mammalian host. Therefore, it is essential to repeat the experiment to obtain more conclusive outcomes. Our subsequent *in vivo* studies further supported these findings, revealing delayed lesion development in *LIT1* PKO infections, underscoring the critical role of *LIT1* in lesion progression. Once again, the overexpression of *LIT1* and *LHR1* did not restore infectivity under the tested condition, prompting inquiries into *LIT1*'s specific role in intracellular replication and cutaneous lesion development, as well as the potential significance of host iron and heme reserves in the context of cutaneous lesion development. This complex scenario highlights the need for further investigations to decipher the specific contributions of *LIT1* and *LHR1* in shaping the intricate dynamics of host-parasite interactions and underscores the intricate mechanisms underlying *Leishmania* infection and pathogenesis.

Our investigation into the impact of iron deficiency anemia on cutaneous leishmaniasis lesion progression provides compelling preliminary insights, though challenges hinder conclusive evidence. Inducing iron deficiency anemia in mice and infecting them with WT *L. amazonensis* revealed an apparent trend of delayed lesion development, suggesting a potential influence of the host's heme and iron status on infection progression. However, due to experimental challenges and an insufficient sample size, our parasite load analysis remains inconclusive, necessitating an expanded sample size for robust statistical analysis in upcoming experiments. Remarkably, the observed trend indicates a delayed lesion development in iron-deficient mice infected with the WT strain. Ongoing experiments within our laboratory aim to overcome these challenges and provide a more comprehensive understanding of the intricate dynamics of cutaneous leishmaniasis progression under conditions of iron deficiency anemia.

The broader significance of this research goes beyond the cross-regulation between the two transporters, reaching into the complex pathways of host's iron and heme metabolism at both cellular and systemic levels. The knowledge gleaned from this study holds promise for identifying novel chemotherapeutic targets for leishmaniases. Therefore, this project lays the groundwork for ongoing exploration in this field.

3. Abstract

Leishmaniasis, a disease caused by protozoan parasites of the genus *Leishmania*, affects millions of people around the world. Nutrient availability within parasitophorous vacuoles profoundly influences parasite replication and virulence during infection. *Leishmania* faces the challenge of acquiring essential nutrients, particularly iron and heme, from the host, as it lacks iron storage proteins and heme biosynthesis capacity. The acquisition is vital for survival, despite the cytotoxic potential of iron and heme. This project explored deep into the intricate regulatory mechanisms governing iron and heme transporters at the *Leishmania*-host interface. Challenges encountered during the validation of *LIT1* mutant prompted a shift to CRISPR/Cas9 genome editing, stressing the genomic plasticity challenges of *Leishmania*. Phenotypic characterization of LIT1 and LHR1 overexpressors unraveled insights into the interplay between iron, heme, and *Leishmania* pathogenesis. While knockout mutants exhibited compromised replication in normal conditions, overexpression restored growth in heme-depleted media. Notably, *LIT1* proved essential for intracellular replication, supported by *in vivo* infection showing delayed lesion development in *LIT1* PKO mutants. The broader significance of the study goes beyond advancing our comprehension of host-parasite interactions in leishmaniases, highlighting the crucial influence of the host's iron and heme status on disease progression. Additionally, the identification of potential chemotherapeutic targets not only offers new directions for ongoing exploration in this field but also holds promise for the development of innovative strategies to combat leishmaniases and enhance treatment outcomes.

4. Resumo

Leishmaniose, uma doença causada por parasitas protozoários do gênero *Leishmania*, afeta milhões de pessoas do mundo. A disponibilidade de nutrientes dentro dos vacúolos parasitóforos influencia profundamente a replicação e virulência do parasita durante a infecção. *Leishmania* enfrenta o desafio de adquirir nutrientes essenciais, especialmente ferro e heme, do hospedeiro, já que carece de proteínas de armazenamento de ferro e capacidade de biossíntese de heme. A aquisição desses nutrientes é vital para a sobrevivência, apesar do potencial citotóxico do ferro e do heme. Este projeto explorou os mecanismos regulatórios que regem os transportadores de ferro e heme na interface hospedeiro-*Leishmania*. Desafios encontrados durante a validação do mutante *LIT1* promoveram uma mudança para a edição de genoma CRISPR/Cas9, ressaltando os desafios de plasticidade genômica da *Leishmania*. A caracterização fenotípica de superexpressores de LIT1 e LHR1 expandiu nosso conhecimento sobre a relação entre ferro, heme e a patogênese da *Leishmania*. Enquanto os mutantes nocaute de *LIT1* exibiram replicação comprometida, a superexpressão restaurou o crescimento em meio deficiente em heme. Notavelmente, *LIT1* se mostrou essencial para a replicação intracelular, o que foi confirmado na infecção *in vivo* com o desenvolvimento de lesões significativamente menores com os nocautes *LIT1*. A significância mais ampla do estudo vai além do avanço da nossa compreensão das interações hospedeiro-parasita nas leishmanioses, destacando a influência crucial do status de ferro e heme do hospedeiro na progressão da doença. Além disso, a identificação de alvos terapêuticos promete novas estratégias inovadoras para combater as leishmanioses e melhoras os resultados do tratamento.

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