Chapter 1

Introduction and Review of Literature on Redox Metabolism of *Leishmania* Parasites

Abstract

Leishmania parasites have developed different immune evasion mechanisms to survive and proliferate inside the host immune cells. One of its key defence mechanisms includes the parasite's antioxidant metabolism. All trypanosomatids, including *Leishmania,* have evolved to have unique redox metabolism to counter the oxidative stress they encounter throughout their lifecycle. These parasites are normally vulnerable to oxidative attack, and unlike higher eukaryotes, they depend on trypanothione-based redox metabolism. The absence of trypanothione and the enzymes involved in its biosynthesis, combined with the parasite's dependence on redox metabolism, establishes it as a crucial aspect of the parasite's biology and a potential therapeutic avenue. Extensive research has been conducted to understand the different components of redox biology in *Leishmania* parasites for their roles in pathogenicity. In that process, different enzymes involved in the biosynthesis of trypanothione, such as trypanothione synthetase, trypanothione reductase, and trypanothione peroxidase are studied extensively by biochemical or genetic methods. In this chapter, we provide a comprehensive review of the *Leishmania* parasite's immune evasion mechanisms, focusing on its redox metabolism, discussing key enzymes, molecular mechanisms, and potential drug targets. By integrating insights from biochemical, structural, and pharmacological studies, we highlight existing knowledge gaps and future directions in targeting redox pathways for leishmaniasis treatment.

1.1 Introduction to Leishmaniasis

Leishmaniasis is a vector-borne parasitic disease caused by more than 20 different species of *Leishmania* parasites. It is considered a neglected tropical disease (NTD), associated with conditions like poverty, malnutrition, and weak immune systems (Alvar et al., 2006; Okwor & Uzonna, 2016a). Depending on which species is involved, leishmaniasis can be categorized into three main forms: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL), varying in site of infection and severity, from self-resolving lesions to life-threatening conditions. Visceral leishmaniasis, also known as kala-azar, is the most severe, systemic form and is often fatal if left untreated, ranking it as the second-deadliest parasitic disease after malaria (World Health Organization, 2025; Pace, 2014). Post-kala-azar dermal leishmaniasis (PKDL) is a skin manifestation that often appears as a sequela of visceral leishmaniasis and serves as a reservoir for future infections. VL is caused by *Leishmania donovani* in Asia and Africa and *Leishmania infantum* in the Middle East, Central Asia, South America, and Central America (Burza et al., 2018). Globally, an estimated 50,000–90,000 new cases of visceral leishmaniasis occur each year, with only 25 to 45% being reported (World Health Organization, 2025).

The *Leishmania* parasites are transmitted through the *Phlebotomus* sandfly vector. The parasite has a complex life cycle and alternates between two distinct morphological forms during its development: flagellated promastigotes, while in the lumen of a female phlebotomine sandfly vector, and as oval-shaped intracellular amastigotes, while in the mammalian hosts. Metacyclic promastigotes are introduced into the mammalian host by the bite of an infected sandfly, where they encounter different immune cells, such as neutrophils, dendritic Cells, and macrophages, and are subsequently phagocytosed by macrophages, transforming into amastigotes. The

ability of *Leishmania* parasites to persist in a latent form within various cells, such as macrophages, and also in some cases fibroblasts, adipocytes, or adipose-tissue derived mesenchymal stem cells, elucidates the parasite's resilience, enabling survival for decades even after self-healing or treatment. The chances for *Leishmania* reactivation due to immunosuppression are a significant concern, particularly for patients in endemic regions who are undergoing transplantation or infected with HIV (Allahverdiyev et al., 2011; Conceição-Silva & Morgado, 2019). *Leishmania* parasites exhibit remarkable evolutionary adaptations, characterised by ultrastructural traits such as a singular mitochondrion, kinetoplast, glycosome, and distinctive glycocalyx plasma membrane. They have established distinctive metabolic pathways to endure adverse stress conditions within the intracellular microenvironment of the hosts (Campos et al., 2012; Comini et al., 2013a; Rodrigues et al., 2014). Understanding the biology of the *Leishmania* parasites has thus become an important area of research, driven by the parasite's complex biology and its devastating impact on public health. Moreover, limited treatment options and the emergence of drug resistance in endemic areas highlight the urgent need for targeted therapies and novel drug targets (Pinheiro & de Souza, 2022; Légaré & Ouellette, 2014; Sundar et al., 2024). By studying the underlying molecular mechanisms of infection, researchers can identify vulnerabilities in the parasite lifecycle, paving the way for new and innovative therapeutic interventions. Such knowledge is also pivotal for developing effective vaccines and diagnostic procedures aimed at controlling and eliminating leishmaniasis as a global health burden.

1.2 Pathogenesis and Establishment of *Leishmania* Infection

Leishmania infection begins when an infected female sand fly introduces metacyclic

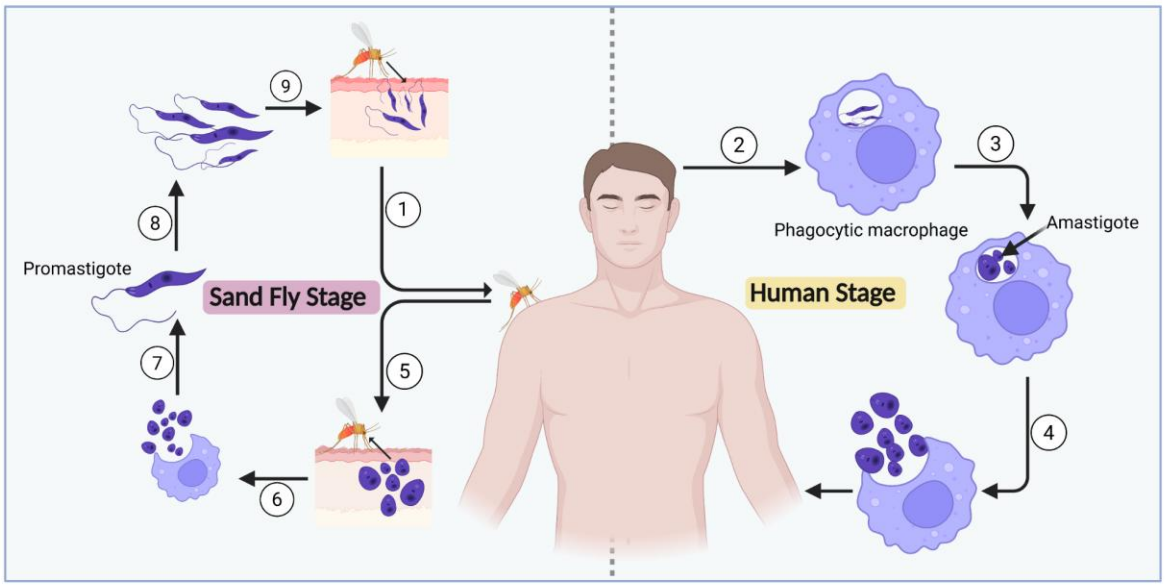

Figure 1.1: The digenic life cycle of *Leishmania* parasites. It involves the transmission of metacyclic promastigotes into the host body by the bite of a female *Phlebotomus* sandfly vector. The parasites are then phagocytosed by the macrophages and other phagocytic cells, where the parasites transform into nonmotile amastigotes. Subsequently, the amastigotes multiply within the macrophages and are released upon cell lysis, infecting new cells. When another sandfly takes a blood meal from an infected host, it ingests the macrophages containing the amastigotes, whereupon digestion, the amastigotes are released into the midgut and transform back to promastigotes.
.

promastigotes into the dermis of the mammalian host during a blood meal. This process induces tissue damage and the production of sand fly saliva, which contains chemicals that enhance parasite survival by degrading neutrophil extracellular traps (NETs) and inhibiting coagulation (Chagas et al., 2014; Giraud et al., 2019). Upon entry, *Leishmania* parasites initially interact with local macrophages and infiltrating neutrophils, which secrete antimicrobial agents and generate NETs to capture the parasites (Guimarães-Costa et al., 2014; Kennedy & DeLeo, 2009). *Leishmania* can evade these attacks via surface chemicals such as lipophosphoglycan (LPG) and enzymes like GP63, which disrupt the complement cascade and NET stability (Hallé et al., 2009; Hermoso et al., 1991). The parasites use host cell surface receptors, including mannose-fucose, Fc, and complement receptors CR1 and CR3, to enhance phagocytosis predominantly by macrophages (Akilov et al., 2007; Blackwell et al.,

1985). After internalization by the macrophages, *Leishmania* parasites form a parasitophorous vacuole (PV) that begins to acquire endocytic and lysosomal markers like Rab5, Rab7, LAMP-1, and LAMP-2, leading to the maturation and nutrient acquisition of the parasite-containing compartment (Liévin-Le Moal & Loiseau, 2016). Recent studies have shown that *Leishmania* parasites can sense the lysosomal environment (acidic pH and body temperature) within host cells, which acts as a signal for transformation into the replicative amastigote stage, thereby promoting successful intracellular development (Zilberstein, 2021). Once established, these amastigotes modulate the cellular machinery of macrophages to survive and replicate within the niche (Goto, 2025;Carneiro et al., 2021;Palomino-Cano et al., 2024a). *Leishmania* amastigotes can survive within the phagolysosome, exploiting host signalling to suppress microbicidal processes and endure. They have evolved to have various sophisticated strategies to survive and proliferate within the macrophages (Awasthi et al., 2003; Matheoud et al., 2013a). These include manipulation of the host cell signaling pathways modulating immune responses, inhibition of the phagolysosome formation, and neutralization of the oxidative attack.

1.2.1 Modulation of Immune Signalling

Leishmania interferes with macrophage activation by inactivating key signaling pathways like JAK/STAT, Ca2+-dependent protein kinase C (PKC) isoforms, and mitogen-activated protein kinases (ERK1/2). This inactivation is further facilitated by the activation of the host protein tyrosine phosphatases, such as SHP-1, which directly deactivates JAK2 and ERK1/2, resulting in reduced cytokine secretion and nitric oxide (NO) production (Barrie et al., 2024; Shio et al., 2012). *Leishmania* parasites can also induce the synthesis of certain cytokines responsible for shifting macrophage

polarization from a pro-inflammatory (M1) to an anti-inflammatory (M2) state. M1 macrophages, activated by Th1 cytokines such as INF-γ and TNF-α, are primarily-

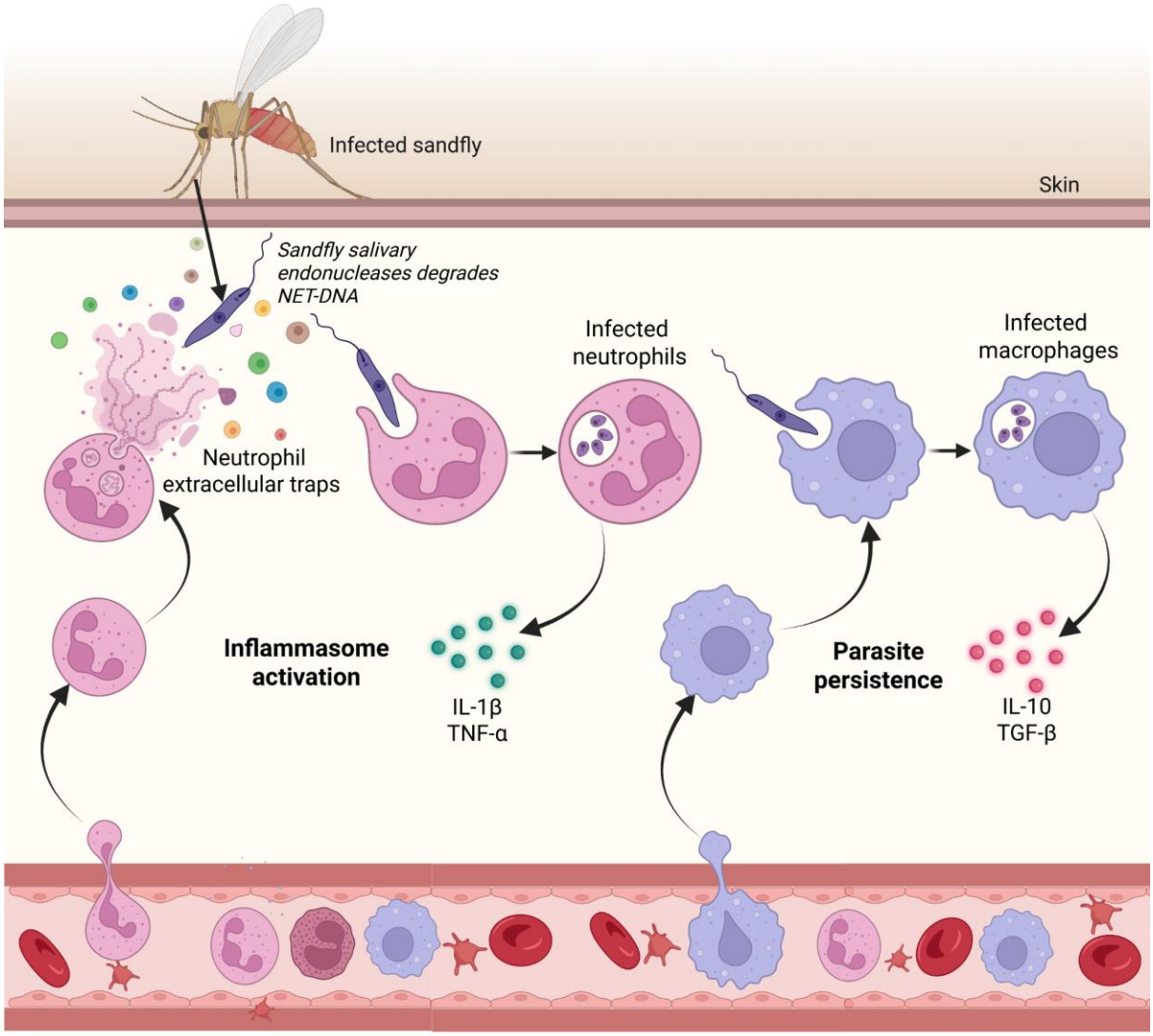

Figure 1.2: The interaction of *Leishmania* parasites with the immune cells. Upon entering the host, parasites are initially trapped by neutrophils through Neutrophil Extracellular Traps (NETs). However, endonucleases found in sandfly saliva, including Lutzomyia-derived DNase (Lundep), can partially degrade these NETs, facilitating parasite evasion. Despite this, the phagocytosis of parasites by neutrophils induces the production of pro-inflammatory cytokines, hence enhancing the host immune response. The subsequent phagocytosis by macrophages results in a shift in immune regulation, where *Leishmania* actively inhibits activation of the host immune response by producing anti-inflammatory cytokines, hence facilitating intracellular survival and persistence within macrophages.

responsible for the effective clearance of intracellular pathogens, including *Leishmania*. In contrast, M2 macrophages, induced by Th2 macrophages like IL-4 and IL-13, are associated with tissue repair and immune suppression, creating a

permissive environment for the parasite. The salivary components of the vector have an immuno-modulatory role, which enhances the production of anti-inflammatory cytokines, such as IL-10 and TGF-β. This promotes the establishment of M2 macrophages, favouring the parasite's survival within the host (Tomiotto-Pellissier et al., 2018).

1.2.2 Inhibition of Phagolysosome Maturation

Inhibiting phagolysosome biogenesis is another strategy used by *Leishmania* parasites, which is primarily achieved through lipophosphoglycan (LPG), a major surface glycoconjugate that blocks phagosomal maturation by periphagosomal accumulation of F-actin and disrupting lipid microdomains. This inhibition may lead to defective assembly of the NADPH oxidase complex and the exclusion of vesicular proton-ATPase from phagosomes, creating a non-acidic and ROS-deficient environment, a favourable environment for parasite survival (Moradin & Descoteaux, 2012;Courret et al., 2002). *Leishmania*'s surface metalloprotease, GP63, plays a critical role in the subversion of host microbicidal pathways. GP63 cleaves SNARE proteins such as VAMP8, disrupting phagolysosome biogenesis. Additionally, GP3 degrades components of the nuclear pore complex, such as Nup62, altering nucleocytoplasmic transport that hinders antigen cross-presentation, thus impairing T-cell activation (Matheoud et al., 2013b).

1.2.3 Neutralization of Reactive Oxygen Species

One of the remarkable aspects of *Leishmania* pathogenesis is the ability of amastigotes to replicate within the acidic and hydrolytic environment of the mature phagosome, adapting to oxidative and nitrosative stress via upregulation of antioxidant

systems like trypanothione and superoxide dismutase. Immune cells such as macrophages usually trigger an oxidative environment inside the cells, generating ROS as part of their immune response. *Leishmania* parasites are susceptible to oxidative stress, as these ROS and the extremely acidic surroundings damage vital macromolecules in the parasite, such as DNA, proteins, and lipids (Pal et al., 2010; Reverte et al., 2022). While most eukaryotic cells rely on a combination of enzymatic antioxidants (e.g., superoxide dismutase, catalase, glutathione reductase) and non-enzymatic antioxidants (e.g., ascorbic acid, reduced glutathione) to neutralize ROS, *Leishmania* and other trypanosomatids employ a distinctive trypanothione-based redox metabolism (Castro & Tomás, 2008a; García-Caparrós et al., 2021; Schlecker et al., 2005). This distinctive redox metabolism, centred around trypanothione, a compound of two glutathione molecules and one spermidine, is the principal antioxidant molecule. It substitutes the traditional glutathione/glutathione reductase system seen in higher eukaryotes. The trypanothione system enables *Leishmania* to maintain redox equilibrium under severe oxidative stress, facilitating its survival within macrophages and contributing to resistance against specific drugs, including antimony. The dependence of *Leishmania* on trypanothione-based redox metabolism thus represents a notable evolutionary difference, making it a potential therapeutic target for selective parasite inhibition without killing the host cell.

1.3 Redox Biology in Pathogenesis

1.3.1 Redox Homeostasis: Redox homeostasis is a crucial component of cellular metabolism in all living cells, ensuring the balance between reducing and oxidizing-

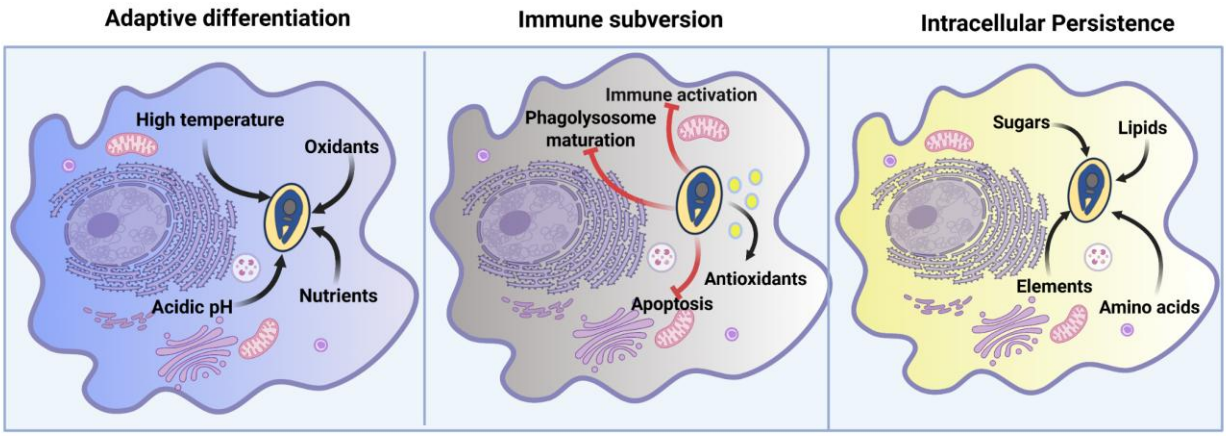

Figure 1.3: Establishment of *Leishmania* infection in the Host macrophage. Upon phagocytosis by the macrophages, in the phagolysosomal compartment, factors such as increased temperature, acidic pH, and nutrient presence induce differentiation into the amastigote form and reside within the parasitophorous vacuoles (PVs). Inside these PVs, *Leishmania* manipulates the host cellular processes by delaying phagolysosome maturation, preventing apoptosis, and minimizing immune activation via the production of anti-inflammatory cytokines, such as IL-10, thus facilitating intracellular survival and persistence. The parasite's strong antioxidant defence systems, particularly through the trypanothione-based metabolism, effectively neutralise reactive oxygen species created by the host, hence promoting long-term intracellular persistence.

reactions to maintain the physiological steady state. The maintenance of redox equilibrium relies on a precisely balanced interplay between the production of reactive species, mainly reactive oxygen species (ROS) and reactive nitrogen species (RNS), and their neutralization by antioxidants (Ushio-Fukai et al., 2021;Ursini et al., 2016). Such equilibrium is essential for various cellular processes, including energy production, signaling pathways, and detoxification of harmful metabolites. Disruption in redox balance often leads to increased intracellular concentrations of reactive oxygen species (ROS), a condition denoted as oxidative stress, causing pathogenesis such as endothelial dysfunction, neurogenerative and cardiovascular diseases, inflammation, atherosclerosis, and cancer in humans (Schieber & Chandel, 2014;Forman & Zhang, 2021). Recent discoveries have deepened our understanding of the molecular mechanisms behind this dysregulation, highlighting the crucial roles

of genomic instability, epigenetic changes, protein degradation, and metabolic reprogramming (B. Li et al., 2025).

Both external and internal factors can induce such a disbalance in the redox equilibrium or oxidative stress (Pizzino et al., 2017). Reactive oxygen species (ROS) are historically regarded as deleterious by-products, as they are key factors in various cell death pathways, including apoptosis, necrosis/necroptosis, ferroptosis, pyroptosis, and autophagic cell death (Fulda, 2016; Green & Kroemer, 2004; Ricci et al., 2004). However, recent studies indicate that ROS plays crucial roles in cellular activities such as signal transduction, cell migration, differentiation, proliferation, and immunological responses (Azad & Iyer, 2014;Manoharan et al., 2024a). For instance, the oxidation of cysteine residues in proteins is mediated by H2O2, where H2O2 oxidizes the thiolate anion (Cys-S-) to the sulfenic form (Cys-SOH), resulting in allosteric changes within the protein that modify its function (Rhee, 2006). The sulfenic form can be reduced to thiolate anions by disulfide reductases, specifically thioredoxin (Trx) and glutaredoxin (Grx), thereby restoring the protein's function to its original state. Reactive oxygen species such as superoxide anion (O2−), hydrogen peroxide (H2O2), and hydroxyl radicals (OH·) are generated as the natural byproducts of aerobic cellular metabolism, where mitochondrial respiration takes place (Magnani & Mattevi, 2019). Ionizing radiation, such as X-rays and gamma rays, UV light, and the metabolism of different drugs and xenobiotics can also generate ROS inside cells (Collin, 2019; Mailloux, 2020).

1.3.2 Antioxidant Defence System

To mitigate the potentially harmful effects of oxidants, all aerobic organisms have developed sophisticated antioxidant defense systems, comprising both enzymatic and non-enzymatic components.

1.3.2.1 Enzymatic Antioxidants: These are the endogenous enzymes that catalyze the neutralization of free radicals and convert them into less harmful substances, essentially detoxifying the cell. Key examples include Superoxide dismutase (SOD), Catalase, Glutathione peroxidase (GPx), Glutathione reductase, Ascorbate peroxidase (APX). Superoxide dismutase (SOD) catalyses the reaction between two superoxide radical anions (O_2^-) resulting in the formation of one molecular oxygen (O_2) and one hydrogen peroxide molecules (H2O2) (Diesen & Kuo, 2010). Catalase then catalyzes the reaction that converts hydrogen peroxide to oxygen and water. Glutathione peroxidases (GPx) are the most prominent family of peroxidases that use glutathione as a cofactor to transform hydrogen peroxide and other peroxides into water or corresponding alcohols (Pei et al., 2023;Le & Walter, 2007) Glutathione reductase is involved in the regeneration of glutathione, thereby responsible for maintaining the supply of reduced glutathione in the cytoplasm (Couto et al., 2016). Ascorbate peroxidase (APX) is a major antioxidant in plant cells that uses ascorbate (vitamin C) to detoxify hydrogen peroxide (Saxena et al., 2023). Other related enzymes, such as thioredoxin reductase, glutathione S-transferase, and peroxidases, are also involved in the process of reduction of oxidized thioredoxin, elimination of xenobiotics, and oxidation of various substrates (Ashraf, 2009).

1.3.2.2 Non-Enzymatic Antioxidants: These are the antioxidant molecules that can directly neutralize free radicals and reactive oxygen species (ROS) by donating electrons, stabilizing and preventing them from damaging cellular components like DNA, proteins, and lipids. Natural nonenzymatic antioxidants include glutathione, vitamin E, A, C, flavonoids, carotenoids, plant polyphenols, uric acid, allyl sulphides, theaflavin, curcumin, bilirubin, melatonin, and polyamines (Moussa et al., 2020). Vitamin A or Retinol, prevents the peroxidation of liposomes and fatty acid esters in vitro, rendering it an excellent peroxyl radical scavenger. Similar to retinols, carotenoids possess the ability to scavenge peroxyl radicals and can neutralise singlet oxygen, a highly reactive and unstable form of partly reduced oxygen (Didier et al., 2023). Vitamin E is lipophilic and concentrated in cellular membranes. operates by diminishing lipid peroxyl radicals (LOO). It operates by transferring the phenolic hydrogen atom from the chroman ring, yielding a relatively stable and unreactive resonance-stabilized tocopheroxyl radical, which cannot initiate additional lipid peroxidation. The α-tocopherol radical can be reverted to its original active form by ascorbic acid or coenzyme Q10 (Moussa et al., 2020). Vitamin C (L-ascorbic acid) is an optically active, water-soluble free radical scavenger characterised by a strongly acidic hydroxyl group (pKa = 4.2). It is an antioxidant and reducing agent that acts by donating electrons to various enzymatic and nonenzymatic reactions. for instance, it regenerates vitamin E (HO-tocopherol) from its oxidized form (O-tocopherol) back to its active state by reducing vitamin E radicals formed when vitamin E scavenges oxygen radicals. Glutathione It is the principal non-protein, thiol-containing tripeptide in all eukaryotic cells, composed of cysteine, glutamic acid, and glycine. Despite its simple structure, it plays critical roles in mitigating oxidative stress and serves as a cofactor in multiple cellular events, including cell cycle G1-to-S phase transition,

regulation of certain transcription factors, cell differentiation, programmed cell death, and resistance against pathogens. The sulfhydryl group (−SH) of cysteine participates in reduction and conjugation processes, which are typically regarded as the primary functions of GSH (Forman et al., 2009; Griffith, 1999). Due to its pivotal role in cellular defense mechanisms, glutathione is often referred to as the master antioxidant. It is abundant in the cytosol (1–11 mM), nucleus (3–15 mM), and mitochondria (5–11 mM), either interacts directly with reactive oxygen and nitrogen species (ROS and RNS, respectively) or serves as a cofactor for many antioxidant and associated enzymes such as peroxidases and transferases. Glutathione is biosynthesized in two ATP-dependent enzymatic steps. The first step is a rate-limiting step, catalyzed by gamma-glutamyl synthetase, ligating glutamate to cysteine to synthesize γ-glutamylcysteine (γ-GC). In the subsequent step, glutathione synthetase (GS) adds glycine to γ-glutamylcysteine (γ-GC), forming glutathione (GSH) (Griffith, 1999). Glutathione is present in a reduced (GSH) or oxidized (GSSG) form. Glutathione reductase (GR) continuously regenerates GSH from GSSG using NADPH as a reductant. Given its essential roles in redox equilibrium and cellular metabolism, the dysregulation of glutathione levels is associated with the pathogenesis of numerous diseases, including neurological disorders, cancer, and infectious diseases, highlighting its therapeutic promise as a target for pharmaceutical intervention (Santacroce et al., 2023).

1.4 Unique Redox Metabolism of *Leishmania* Parasites

Kinetoplastids, such as *Leishmania*, possess several unique low molecular weight thiols and redox proteins. In most eukaryotic organisms, the glutathione (GSH)/GR

and thioredoxin (Trx)/TrxR systems regulate intracellular thiol redox homeostasis; however, trypanosomatids utilise a redox metabolism centred on a low molecular mass dithiol trypanothione [bis(glutathionyl) spermidine; T(SH)2] and trypanothione reductase (TR), which maintains it in a reduced state. During infection, *Leishmania* parasites differentiate from promastigotes to intracellular amastigotes and encounter a sudden alteration in temperature, pH, and nutrients, which induces oxidative stress inside the parasite (Dolai & Adak, 2014). Moreover, inside the phagocytic cells, parasites are exposed to various reactive species that can further damage the cellular components, including DNA, lipids, and proteins (Brígido et al., 2025; Palomino-Cano et al., 2024b). The redox homeostasis in these parasites is effectively managed, enabling them to endure the oxidative burst during host infection and to adeptly adjust to the many metabolic and environmental parameters dictated by their digenetic life cycle. Besides trypanothione (T(SH)2), *Leishmania* parasites have a variety of thiol-containing low molecular weight proteins: glutathione (GSH), glutathionylspermidine (Gsp), and ovothiol (OSH), with amounts that vary according to species, life cycle, and growth phases. In contrast to human cells, *Leishmania* lacks several conventional antioxidant enzymes that neutralise reactive oxygen species (ROS) and reactive nitrogen species (RNS). These include catalase, selenium-dependent glutathione peroxidase, glutathione reductase (GR), and thioredoxin reductase (TrxR) (Krauth-Siegel et al., 2005). T(SH)2 is the most potent reducing agent of trypanosomatids in comparison to Gsp, GSH, and OvSH. This unique metabolite was identified by Fairlamb et al. in 1985 as a principal thiol compound in the African *T. brucei* due to the unusual glutathione reductase activity (Fairlamb et al., 1985a). Studying trypanothione-based redox metabolism in *Leishmania* becomes important as it

highlights a unique defence mechanism in trypanosomatids against oxidative stress and drug resistance.

1.4.1 Trypanothione-Based Redox Control

Trypanothione is pivotal in the redox metabolism of trypanosomatid parasites, preserving intracellular redox homeostasis and facilitating several metabolic and stress response processes. In contrast to glutathione in higher eukaryotes, trypanothione operates through a distinct redox cycle that involves trypanothione reductase (TryR), which utilises NADPH to restore the reduced form of trypanothione ($T[SH]_2$). Reduced $T[SH]_2$ donates electrons to tryparedoxin (TXN), a thioredoxin-like protein, which then transfers reducing equivalents to several downstream enzymes. These encompass tryparedoxin peroxidase (TXNPx), glutathione peroxidase-like enzymes, dehydroascorbate reductase, and methionine sulfoxide reductase, constituting the fundamental components of the parasite's defence against reactive oxygen and nitrogen species (ROS and RNS).

The production of T(SH)2 involves the synthesis of GSH and spermidine (Spd), followed by the conjugation of these metabolites. Similar to other organisms, GSH is synthesised in *Leishmania* by gamma-glutamylcysteine synthetase and glutathione synthetase, which are demonstrated to be indispensable for *Trypanosoma brucei* (Huynh et al., 2003; Pratt et al., 2014). While the mechanisms for supplying spermidine are not conserved across many trypanosomatids, *Leishmania* converts arginine into spermidine. African trypanosomes lack a functioning arginase and get ornithine from their mammalian host. They stimulate the host's arginase activity, initiated by a parasitic-secreted protein known as Kinesin Heavy Chain 1 (De Muylder

et al., 2013). The subsequent two processes, specifically the conversion of ornithine into spermidine, are identical in *Trypanosoma brucei* and all *Leishmania* species; however, *Trypanosoma cruzi* is auxotrophic for polyamines and acquires putrescine and spermidine from the host (Krauth-Siegel & Leroux, 2012). The enzymes of the polyamine pathway in African trypanosomes and *Leishmania* are crucial and, in certain instances, examined as potential therapeutic targets (Krauth-Siegel & Comini, 2008a).

The final phase in the production of T(SH)2 involves the conjugation of two GSH molecules with Spd. *T. cruzi, T. brucei,* and *L. major* trypanothione synthetase (TryS) facilitate both sequential reactions. *Crithidia fasciculata, T. cruzi,* and certain *Leishmania* species furthermore possess a glutathionylspermidine synthetase (GspS). Nevertheless, GspS null mutant *L. infantum* exhibits no proliferation deficit, indicating that the enzyme does not have an in vivo function (Sousa et al., 2014). TryS is crucial in *T. brucei* and *L. infantum*, as demonstrated by inverse genetics and chemical methodologies, a trait that may probably be generalised to all trypanosomatids (Comini et al., 2004;Torrie et al., 2009).Trypanothione exists in both its reduced form [$T(SH)_2$] and oxidised form [TS_2], with its reduced state being sustained by the NADPH-dependent flavoenzyme trypanothione reductase (TryR). This enzyme functions similarly to mammalian glutathione reductase but demonstrates considerable substrate specificity for positively charged glutathionyl–polyamine conjugates such as trypanothione and glutathionyl-spermidine. Thus, trypanothione reductase (TR) is the sole enzyme that links the NADPH- and thiol-based redox systems in these parasites. The trans-dominant mutant *L. donovani*, exhibiting 15% of wild-type TR activity, proliferates under standard culture conditions but has heightened susceptibility to-

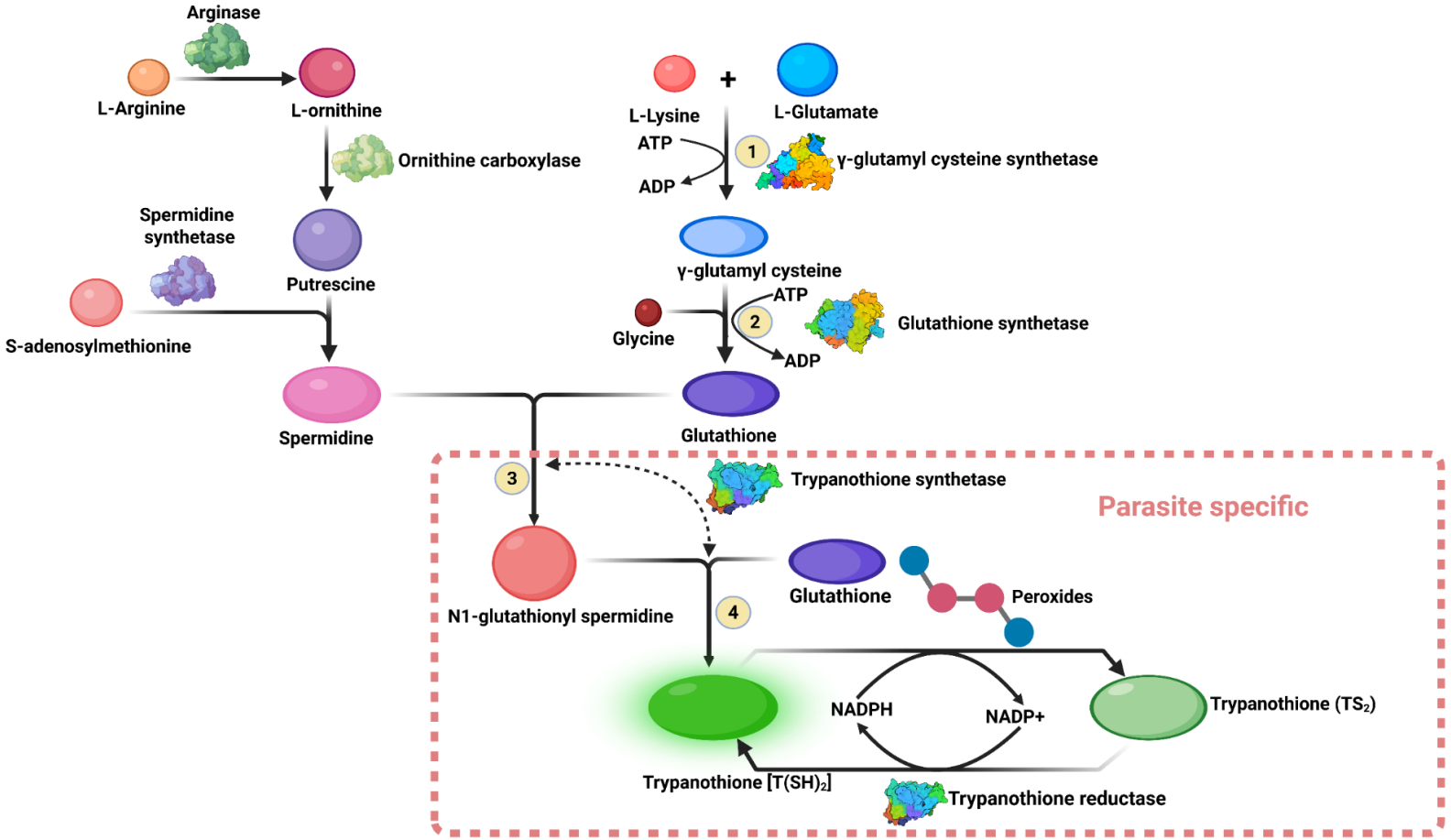

Figure 1.4: Trypanothione biosynthesis pathway in *Leishmania spp.* Trypanothione biosynthesis pathway is central to the redox metabolism and antioxidant defence mechanisms of *Leishmania* parasites. The pathway starts with the biosynthesis of glutathione (GSH) from glutamate, cysteine and glycine in two ATP-dependent enzymatic reactions catalyzed by the γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS), respectively. The polyamine, Spermidine is synthesized by ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) pathway, is conjugated with two molecules of glutathione in a reaction catalyzed by trypanothione synthetase (TryS), producing trypanothione [$T(SH)_2$]. Trypanothione is maintained in its reduced state through the NADPH-dependent reaction catalyzed by trypanothione reductase (TR). The reduced trypanothione [$T(SH)_2$] serves as a primary electron donor to various downstream redox molecules, including tryparedoxin (TXN) and tryparedoxin peroxidase (TXNPx), which neutralise reactive oxygen and nitrogen species.

oxidative stress and a reduced ability to survive within macrophages (Tovar, Cunningham, et al., 1998). Likewise, a reduction of the enzyme to below 10% of the wild-type level resulted in *T. brucei* parasites that multiply properly but exhibit sensitivity to H2O2 and are incapable of infecting mice (Krieger et al., 2000). T(SH)2 directly reduced low molecular weight metabolites, including dehydroascorbic acid and the disulfide forms of glutathione and ovothiol A (Krauth-Siegel & Comini, 2008a). It serves as the electron donor for the parasite glutaredoxins, thioredoxin, and tryparedoxin (Tpx). The Tpx specific to trypanosomatids supplies electrons for ribonucleotide reductase, methionine sulfoxide reductase, and both types of thiol peroxidases: 2-Cys peroxiredoxins (Prx) and glutathione peroxidase-type enzymes (Castro & Tomás, 2008b). These peroxidases also neutralise hydrogen peroxide, lipid hydroperoxides, and peroxynitrite, determining for fate of the host's immunological response. Moreover, trypanothione directly engages in the reduction of dehydroascorbate and glutathione disulphide, hence enhancing antioxidant defence and preserving intracellular redox balance. Several currently available anti-trypanosomatid drugs influence trypanothione metabolism (e.g., nifurtimox, melarsoprol, antimonials, and eflornithine). Considering that the enzymes implicated in the synthesis and reduction of trypanothione, together with Tpx and the cytosolic Prx and Px-type enzymes, are crucial, nearly all components of the system provide viable therapeutic targets. Therefore, the majority of drug development strategies have concentrated on the two primary enzymes, trypanothione reductase and trypanothione synthetase(Castro & Tomás, 2008b; Hiller et al., 2014).

1.4.1.1 Key Enzymes and Therapeutic Targets in Redox Biology of *Leishmania* parasites

(A) Trypanothione Synthetase: Trypanothione synthetase (TryS, EC 6.3.1.9) is a monomeric bifunctional enzyme within the acid-ammonia ligase family, capable of catalysing the formation of carbon-nitrogen bonds. The molecular weight ranges from 69 to 79 kDa and has 627 to 719 amino acid residues, organised into two catalytic domains. The enzyme was first discovered in 1990 during studies of *Crithidia fasciculata* as glutathione spermidine synthetase and subsequently cloned in *Trypanosoma cruzi* and *Trypanosoma brucei* by the Fairlamb lab in 2002 (Tetaud et al., 1998). Its crucial function in the production of trypanothione (T(SH)2) was subsequently defined, especially in *Leishmania major* in 2005(Oza et al., 2006). Structural analyses indicated that TryS consists of a C-terminal synthetase domain including an ATP-grasp fold and an N-terminal amidase domain reliant on cysteine and histidine.

The active site of the synthetase domain binds substrates, including spermidine, glutathionylspermidine, and glutathione, with essential residues such as Arg-553 and Arg-613 modulating catalytic activity (Fyfe et al., 2008). TryS mostly facilitates the conjugation of glutathione and spermidine to produce glutathionylspermidine, which is crucial for T(SH)2 synthesis, employing energy obtained from ATP hydrolysis. It also demonstrates restricted amidase activity, largely modulating T(SH)2 levels.

Structural investigations emphasise the conformational alterations required for the functional interaction between the synthetase and amidase domains, essential for cellular homeostasis under diverse pH values experienced during parasite life cycles(Ariyanayagam et al., 2003). Comparative genomic analyses highlight the parallels

and variances in TryS architecture and function among many animals, underscoring its evolutionary conservation and functional variety (Bollinger et al., 1995).

(B) Trypanothione Reductase: Trypanothione reductase (TryR) is a flavoprotein disulfide oxidoreductase found universally in all trypanosomatid species (Fairlamb et al., 1985b). It is an essential enzyme in trypanosomatid parasites, crucial for their defence against oxidative stress. TryR facilitates the conversion of trypanothione disulfide to trypanothione, which is essential for preserving the parasite's thiol-redox equilibrium (Apt, 2010; Irigoín et al., 2008). It was first isolated from *Crithidia fasciculata* and subsequently from *Trypanosoma cruzi* (Shames et al., 1986) (JOCKERS-SCHERÜBL et al., 1989). Structurally and functionally, TryR exhibits similarities to glutathione reductases (GRs), as both are homodimeric proteins of approximately 52 kDa per subunit, utilise flavin adenine dinucleotide (FAD) as a coenzyme, and employ NADPH as an electron donor. Both enzymes possess a redox-active disulfide in their active sites (Ghisla & Massey, 1989; Henderson et al., 1987; KRAUTH-SIEGEL et al., 1987).

Despite their structural similarity and about 40% sequence commonality, TryR and GR exhibit stringent substrate specificity. Glutathione reductases (GRs) and thioredoxin reductases (TrxRs) are lacking in trypanosomatids, positioning TryR as the pivotal enzyme that connects NADPH metabolism with the parasite's thiol-dependent redox system. A decrease in TryR expression undermines the parasite's resistance to oxidative stress, highlighting its critical function in cellular redox regulation (Dumas, 1997). Inhibition of TryR in *Leishmania donovani* by trivalent antimonials was reported as one of its mechanisms of action (Cunningham & Fairlamb, 1995).

Genetic validation on many kinetoplastid parasites has established TryR as essential for survival and pathogenicity, highlighting its potential as a therapeutic target (Tovar, Wilkinson, et al., 1998). TryR comprises three functional domains: a FAD-binding domain, a NADPH-binding domain, and a C-terminal interface domain. The N-terminal dinucleotide-binding motif exhibits a Rossmann fold, and the substrate-binding cleft is situated at the dimer interface, with active site residues provided by both monomers.

TryR, along with its downstream thiol products, maintains the intracellular antioxidants, critical for trypanosomatid parasites' survival, especially under the oxidative stress inside the host macrophages. The targeted inhibition studies on TryR demonstrated disruption in the redox equilibrium, resulting in increased intracellular reactive oxtgen species (ROS) levels, ultimately eliminating intracellular parasites (Turcano et al., 2018a). Crystallization of *Trypanosoma cruzi* TryR and co-crystallization with its inhibitors had identified critical residues essential for ligand binding, notably Trp21 and Met113 (Bond et al., 1999) (Khan et al., 2000; Saravanamuthu et al., 2004). Subsequent computational modelling of *Leishmania infantum* TryR has revealed some additional hydrophobic residues, including Phe396, Leu399, and Pro462, that facilitate ligand binding (Venkatesan et al., 2010). Efficient inhibitors generally exhibit elongated hydrophobic domains and a net positive charge, promoting preferential affinity for TryR compared to host GR (Faerman et al., 1996). Residues adjacent to the active site, specifically Phe396, Pro398, and Leu399, constitute a hydrophobic region, whereas comparative analysis with human GR indicates that substitutions like Met406, Tyr407, and Ala409 may be utilised in rational drug design for the selective targeting of the parasite enzyme (de Molfetta et al., 2009).

(C) γ-glutamylcysteine synthetase: γ-Glutamylcysteine synthetase (Gcs; EC 6.3.2.2) is the rate-limiting enzyme in glutathione biosynthesis, present in nearly every eukaryotic cell, from plants to animals, including humans. It facilitates the ATP-dependent conjugation of L-cysteine with L-glutamate to produce γ-glutamylcysteine. This enzyme is crucial for cellular redox equilibrium, and its genetic deletion leads to non-viability in various organisms, including fungi, mammals, Trypanosoma brucei, and *Leishmania infantum*, unless compensated by exogenous glutathione supplementation (Olin-Sandoval et al., 2012;A. Mukherjee et al., 2009a) . Increased expression levels of Gcs at both mRNA and protein levels have been linked with enhanced resistance to oxidative stress, as seen in human neuroblastoma cells.

The pharmacological inhibition of Gcs by L-buthionine-S, R-sulfoximine (BSO), a selective and irreversible inhibitor, has shown therapeutic efficacy in T. brucei infection models, suggesting the enzyme's viability as a medicinal target. Furthermore, immunisation approaches employing *Leishmania donovani* Gcs (LdGcs) in protein or DNA vaccine formulations have demonstrated protective efficacy in mouse models, thereby underscoring the enzyme's medicinal significance. Despite its crucial function in nearly all animals, Gcs demonstrates significant sequence variation and can be categorised into three separate evolutionary groupings. The initial group comprises Gcs from proteobacteria, exemplified by *Escherichia coli*; the subsequent group contains non-plant eukaryotes, such as Homo sapiens, *Saccharomyces cerevisiae*, and trypanosomatids; whereas the final group consists of α-proteobacteria and higher plants, including Pisum sativum and Glycine max. Despite generally low pairwise sequence identities among these groups (typically under 10%), structural analyses indicate a conserved core fold consisting of six antiparallel β-strands encircled by α-

helices, highlighting the evolutionary preservation of its catalytic architecture amid significant sequence variability.

(D) Glutathione synthetase: Glutathione synthetase is the enzyme (EC 6.3.2.3) that catalyzes the biosynthesis of glutathione (gamma-glutamyl-cysteinylglycine) by conjugating gamma-glutamylcysteine and glycine in an ATP-dependent reaction. GS belongs to the ATP-grasp superfamily, a group of enzymes characterized by a conserved ATP-binding domain. The enzyme is characterised as a monomer in *Streptococcus agalactiae*, a dimer in humans, or a tetramer in fission yeast. The X-ray crystallographic structures of glutathione synthetases have been documented for *Escherichia coli*, yeast, and humans. The enzyme exhibits specificity for γ-glutamyl-L-cysteine and analogous compounds, including L-γ-glutamyl-α-L-aminobutyrate, while demonstrating minimal activity with γ-glutamyl-D-amino acids. The Km values for the mammalian enzyme are: glycine (1 mM), ATP (0.05 mM), and γ-glutamylcysteine and γ-glutamyl-α-aminobutyrate, both ranging from 0.02 to 0.2 mM. A mutation in the *Glutathione synthetase gene* (*GS*) in human results in glutathione deficiency (GSD), which is a rare, autosomal recessive metabolic condition characterized by low levels of glutathione (Atwal et al., 2016). A similar functional analysis was carried out in *Saccharomyces cerevisiae*, revealing that deletion of the *GS* gene leads to glutathione (GSH) deficiency, accumulation of the intermediate γ-glutamylcysteine (γ-Glu-Cys), dependence on exogenous GSH for normal growth, and impaired growth on minimal media, while overexpression of *GS* increases enzyme activity but not GSH levels. These studies again highlighted that γ-glutamylcysteine synthetase (Gcs) is the rate-limiting step, but not GS and γ-Glu-Cys can partially compensate for GSH in oxidative stress protection but not in essential cellular function (Grant et al., 1997). Although

glutathione is not the primary antioxidant in trypanosomatids like it is in most other organisms, it serves as a crucial precursor for trypanothione synthesis. The synthesis of trypanothione is dependent on the availability of glutathione. A regulated knockout study of *Trypanosoma brucei* glutathione synthetase (*Tb*GS) showed depletion of *Tb*GS in bloodform parasites results in loss of trypanothione and cell death, rendering the glutathione synthetase one of the crucial components of the parasite's redox biology (Pratt et al., 2014). The *Leishmania donovani* glutathione synthetase shares very low sequence similarity with the human homologue (~28%). The low sequence similarity presents a favourable opportunity for targeted drug development, since it diminishes the probability of cross-reactivity with the host enzyme. Focussing on pathogen-specific proteins improves treatment accuracy and reduces any adverse effects on human cellular functions (Huggins et al., 2012;Minadakis et al., 2023).

1.4.2 Immunological aspects of redox biology

Redox homeostasis plays key roles in immunological signalling, influencing both innate and adaptive immune responses of the host. It entails the transfer of electrons and the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which function as signalling molecules. These chemicals regulate several immune cell functions, encompassing activation, differentiation, and survival. Redox signalling influences pathogen detection, cytokine synthesis, and immunological checkpoint function (Manoharan et al., 2024b; Martinvalet & Walch, 2022).

Macrophages, originating from circulating blood monocytes, are essential components of the innate immune response, acting as the primary defense against pathogens. These multifunctional immune cells surveil tissues to identify, engulf (via

phagocytosis), and eliminate invading pathogens, while also removing cellular waste(Chen et al., 2023). Macrophages, serving as antigen-presenting cells (APCs), connect the innate and adaptive immune systems by activating T cells and initiating inflammation via the release of cytokines and chemokines(Muntjewerff et al., 2020). This cascade enlists and activates supplementary immune cells, augmenting the host's defence mechanisms. The foundation of macrophage-mediated microbial eradication is in the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), especially nitric oxide (NO), which function as powerful antibacterial agents that can destroy phagocytosed infections (Morris et al., 2022). However, to maintain the oxidative stress, macrophages employ Nrf2 transcription factor to upregulate antioxidants (e.g., glutathione), counteracting ROS toxicity (Wang et al., 2019).

Macrophages possess pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs), which identify pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharides and viral RNA(Muntjewerff et al., 2020). Upon activation by interferon-gamma (IFN-γ), macrophages enhance their antimicrobial capabilities by producing enzymes, including proteases, nucleases, and lysozymes within acidified phagosomes. Additionally, they utilise antimicrobial peptides to precisely target intracellular infections via autophagy. Notwithstanding their formidable defence systems, some diseases, including *Leishmania* spp., have developed intricate techniques to circumvent or undermine these processes, transforming macrophages into reservoirs for replication instead of sites for elimination.

1.4.3 Role of Redox Metabolism in Drug Resistance of *Leishmania* parasites

The redox metabolism in *Leishmania* parasites plays a key role in developing resistance to current antileishmanial therapies. The overexpression of thiol-metabolic proteins, especially those associated with drug resistance, oxidative stress tolerance, and parasite persistence, has underscored the adaptive importance of thiol-mediated redox balance. Essential proteins such as trypanothione synthetase (TryS) and trypanothione reductase (TryR) play a critical role in the redox system and are frequently overexpressed in drug-resistant strains of *Leishmania* and *Trypanosoma*, suggesting their involvement in facilitating oxidative stress tolerance and drug resistance. Resistance to sodium antimony gluconate (SAG) and amphotericin B (AmpB) is often linked to changes in redox metabolism. Clinical isolates and laboratory-generated SbIII-resistant parasites frequently exhibit increased levels of $T(SH)_2$ in comparison to drug-sensitive strains (Haimeur et al., 2000). Increased production of P-glycoprotein-like proteins, which facilitate the efflux of metal-thiol conjugates, has been associated with resistance (Légaré & Ouellette, 2014) .

Progress in genome sequencing, RNA sequencing, and annotation of several *Leishmania* species has considerably improved our understanding of the metabolic complexities of these parasites (Berriman et al., 2005; El-Sayed et al., 2005; Ivens et al., 2005). Thiol metabolism can be enhanced by integrative omics approaches—namely transcriptomics, proteomics, and metabolomics—which together enable a systems-level comprehension of metabolic regulation and adaptation in these organisms.

Functional investigations utilising RNA interference or gene knockout methodologies have highlighted the critical importance of certain thiol metabolism enzymes for

parasite survival, as previously noted. In arsenite-resistant *L. amazonensis*, there has been an overexpression of genes encoding TryS, TryR, cytosolic tryparedoxin (cTXN), cytosolic tryparedoxin peroxidase (cTXNPx), and ornithine decarboxylase (ODC) (Hsu et al., 2008). Likewise, SAG-resistant isolates exhibit elevated levels of $T(SH)_2$ (MANDAL et al., 2007a), whilst AmpB-resistant *L. donovani* strains demonstrate enhanced expression of enzymes associated with thiol metabolism. Significantly, TryS was upregulated 2–3 fold in AmpB-resistant isolates (Equbal et al., 2014), and its expression augmented in a dose-dependent way upon H_2O_2 exposure, signifying its involvement in redox equilibrium. Nonetheless, TryS expression decreased when H_2O_2 concentrations were elevated (200 μM), indicating that the threshold for redox adaptation may have been surpassed, resulting in parasite apoptosis.

In *T. cruzi*, the overexpression of TryS improves survival under oxidative stress and develops resistance to benznidazole and nifurtimox (Mesías et al., 2019). TryR, the sole enzyme that can transfer reducing equivalents from NADPH to TS_2, is overexpressed at both the mRNA and protein levels in *L. donovani* isolates that exhibit resistance to antimonials (Koch et al., 2013). The upregulation of ODC and γ-glutamylcysteine synthetase (γ-GCS) in laboratory-generated antimonial-resistant parasites underscores the significance of thiol biosynthesis in resistance mechanisms(Wyllie et al., 2004). Field isolates have diverse expression, indicating distinct mechanisms of resistance acquisition (Decuypere et al., 2012; A. Mukherjee et al., 2009b).

In AmpB-resistant clinically isolated parasites, elevated mRNA levels of TryS and TryR highlights their role in reactive oxygen species detoxification (A. K. Mukherjee et al., 2010). The upregulation of TryR mRNA (2.8-fold) and protein (2.5-fold) in our recent investigation supports these findings (Dumas, 1997). Furthermore, the resistance

mechanisms in Amphotericin B-resistant *L. donovani* encompass modified membrane fluidity, diminished ergosterol expression, and elevated MDR1 gene expression, which collectively result in decreased drug absorption and enhanced efflux (A. K. Mukherjee et al., 2010).

Proteins such as TXN, TXNPx, and ascorbate peroxidase (APx) are also crucial to thiol-mediated redox homeostasis. $T(SH)_2$ reduces TXN and dehydroascorbate, whereas the TXN/TXNPx and Asc/APX systems regulate H_2O_2 at sub-lethal levels (Turrens, 2004). TXNPx, along with TXN, is capable of degrading peroxynitrite (Barr & Gedamu, 2003). These proteins are increased in Amphotericin B-resistant parasites and in the amastigote stage, enhancing survival (S. S. Suman et al., 2016) (Wyllie et al., 2010). The overexpression of cTXNPx imparts resistance to H_2O_2 and t-butyl hydroperoxide (Wilkinson et al., 2002) and is associated with increased infectivity and resistance to SAG in *L. donovani* (Iyer et al., 2008).

Elevated cTXNPx expression has been seen in *L. amazonensis* amastigotes, with overexpression providing a 100-fold increase in tolerance to $ONOO^-$ and facilitating lesion formation in mice (Henard et al., 2014). Likewise, the overexpression of TryR, cTXN, and cTXNPx in T. cruzi increases resistance to H_2O_2 and imparts drug resistance (González-Chávez et al., 2019). This work indicates that γ-GCS exerts greater control on $T(SH)_2$ biosynthesis compared to TryS, implying that TryS is more focused on $T(SH)_2$ utilisation. cTXN contributed 70% to peroxide reduction, followed by cTXNPx at 20% and TryR at 10%.

Additionally, rLd-cTXN was demonstrated to act as an immunomodulator by diminishing NO and ROS production from macrophages obtained from VL patients, hence fostering a Th2-skewed immune response and facilitating disease persistence

(S. K. Suman et al., 2018). In *L. major*, the overexpression of APX alleviates ROS-induced apoptosis (Dolai et al., 2008). Increased APX expression in AmpB-resistant *L. donovani* is associated with improved oxidative stress tolerance. These analyses thus indicate the overexpression of LdTryS, LdTryR, LdcTXN, LdcTXNPx, and LdAPX, thereby confirming the role of thiol pathway proteins in facilitating AmpB resistance.

Cysteine, a precursor of thiol-containing antioxidants such as glutathione and trypanothione, is essential for sustaining redox equilibrium in protozoa (Krauth-Siegel & Leroux, 2012; Nozaki et al., 2005; K. Singh et al., 2017). The cysteine synthase (CS) and serine acetyltransferase (SAT) genes in *L. donovani* are increased in amastigotes, indicating their significance for survival within macrophages (K. Singh et al., 2016). Comparable stage-specific expression of CS has been documented in *T. cruzi* (Marciano et al., 2012). The upregulation of CS in *L. donovani* correlates with drug resistance, heightened infectivity, and enhanced stress tolerance, as further substantiated in AmpB-resistant isolates (K. Singh et al., 2017).

Additional research on *L. braziliensis* and *T. rangeli* corroborates the involvement of CS in thiol-mediated resistance (Romero et al., 2014). LdCS overexpressors demonstrate increased antioxidant activity upon ROS generation, underscoring the significance of CS in adapting to oxidative stress (K. Singh et al., 2017). Prior studies have associated increased thiol levels with drug resistance (MANDAL et al., 2007b),(A. Mukherjee et al., 2009b). These findings collectively confirm that the overexpression of thiol-metabolic proteins is a primary mechanism utilised by *Leishmania* parasites to mitigate oxidative damage and treatment stress.

1.5 Current Anti-Leishmanial Drugs and Their Limitations

Due to the unavailability of vaccines, the treatment of visceral leishmaniasis solely relies on a limited number of chemotherapeutic drugs. The effectiveness of currently available drugs is not satisfactory because of limitations such as low efficacy, adverse side effects, increasing drug resistance in endemic areas, high costs, and prolonged treatment durations. Many clinical trials have already been conducted to optimize the therapeutic regimens and enhance the efficacy of the limited number of available anti-leishmanial compounds. Below are the drugs currently used for the treatment of visceral leishmaniasis:

1.5.1 Antimoniate: Antimonials have been the first-line drugs for decades against all three forms of leishmaniasis(Chowdhury et al., 2014). Currently, two pentavalent antimonial compounds are available: sodium stibogluconate (Sb) and meglumine antimoniate. The compound sodium stibogluconate acts as a prodrug, while its active form, Sb (III), kills the parasites by inhibiting the trypanothione reductase (TryR) enzyme in the trypanothione biosynthesis pathway. Additionally, a study demonstrated that the prodrug form of sodium stibogluconate, Sb(V), directly inhibits topoisomerase I activity (Krauth-Siegel & Comini, 2008b).

1.5.2 Amphotericin B: Amphotericin B (AmB) remains a cornerstone in the treatment of visceral leishmaniasis (VL), especially in regions where resistance to first-line drugs, such as sodium stibogluconate (Sbv), is prevalent (Jha et al., 1995). Clinical studies have shown high efficacy rates with AmB. The standard regimen typically involves daily infusions of 1 mg/kg over 20 days. However, this treatment protocol requires

prolonged hospitalization and careful monitoring due to potential adverse effects such as nephrotoxicity, electrolyte imbalances, and infusion-related reactions, including fever, chills, and rigors. AmB exerts its action by binding to ergosterol of the cell membrane of *Leishmania* parasites, increasing membrane permeability, leading to cell death (Ramos et al., 1996). Despite the considerable efficacy of AmB, its nephrotoxic properties have led to the development of lipid-associated formulations, called liposomal amphotericin B, which provide less renal toxicity and enhanced tolerance, thus improving treatment options for VL(Chattopadhyay & Jafurulla, 2011).

1.5.3 Liposomal Amphotericin B (L-AmB): Liposomal amphotericin B (L-AmB), also known as AmBisome®, is a lipid-based formulation developed to enhance the therapeutic index of amphotericin B. Notably, AmBisome® has demonstrated a reduced incidence of adverse effects, including nephrotoxicity and infusion-related reactions. AmBisome® has demonstrated considerable efficacy against VL in clinical trials, with single dosing between 5 and 15 mg/kg resulting in cure rates over 90%. Despite its efficacy, AmBisome® is utilised less frequently due to its restricted stability at temperatures over 25 °C and the requirement for parenteral administration. Moreover, while AmBisome® has demonstrated significant efficacy in the treatment of visceral leishmaniasis (VL), its effectiveness in cutaneous leishmaniasis (CL) has been limited, with cure rates falling below 80%. These challenges have prompted research into the development of more effective liposomal amphotericin B formulations, particularly those suitable for oral and topical administration, to enhance treatment outcomes for cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL). The development and optimisation of liposomal amphotericin B formulations in leishmaniasis treatment are prioritised to enhance drug delivery to macrophages, the

host cells for *Leishmania* parasites, while also improving patient compliance by reducing toxicity and simplifying administration protocols (Frézard et al., 2022).

1.5.4 Paromomycin: Paromomycin, an aminoglycoside antibiotic, has demonstrated efficacy as a treatment for visceral leishmaniasis (VL), particularly in regions where traditional antileishmanial therapies have diminished in effectiveness. Initially a broad-spectrum antibiotic, paromomycin was subsequently repurposed for the treatment of visceral leishmaniasis due to its ability to inhibit protein synthesis in *Leishmania* parasites by binding to ribosomal RNA. Clinical investigations demonstrated its efficacy, resulting in its approval for VL treatment in India, where it is administered intramuscularly either as a monotherapy or in combination with other agents such as miltefosine and amphotericin B. Due to its cost-effectiveness and favourable safety profile compared to pentavalent antimonials, the drug is particularly beneficial in resource-limited settings (Chunge et al., 1990). However, the emergence of potential resistance and variability in treatment response underscores the necessity for continuous monitoring and combination therapies to preserve efficacy(Croft et al., 2006).

1.5.5 Miltefosine: Miltefosine, an alkyl phospholipid compound, emerged as the first effective oral treatment for visceral leishmaniasis (VL). It was approved for VL treatment in India (2002), Germany (2004), Colombia (2005), and Bangladesh (2006) (Dorlo et al., 2012). Initially developed for breast cancer and other solid tumors (Sundar & Olliaro, 2007), miltefosine's progress as an oral cancer agent was hindered by gastrointestinal toxicity issues (Dummer et al., 1993). Demonstrating potent anti-

leishmanial activity in vitro and in animal models (Croft et al., 1987, 1996; Kuhlencord et al., 1992), oral miltefosine was clinically evaluated for VL in humans starting in 1996 (Sundar & Olliaro, 2007). Following a Phase III trial in India (2002), it achieved a remarkable 94% long-term cure rate at doses of 50–100 mg/day for 28 days (Sundar et al., 2002). Europe subsequently licensed it for HIV-VL co-infected patients in 2005 (Berman, 2005; Sindermann & Engel, 2006). Despite its oral administration advantage for domiciliary treatment, miltefosine's extended regimen and long half-life (seven days) pose challenges like poor compliance post-physical recovery and heightened risk of resistance development (Sundar et al., 2012). Concerns over teratogenicity in animals and prolonged residual presence in humans restrict its use in women of childbearing age. To mitigate these challenges and extend its clinical utility, combining short courses of miltefosine with other effective anti-leishmanial agents is proposed.

Additionally, combining Liposomal Amphotericin B with miltefosine shows promise in enhancing efficacy, preventing post-kala-azar dermal leishmaniasis (PKDL), and reducing toxicity. Short-course miltefosine regimens could offer comparable efficacy to monotherapy while improving patient compliance and reducing resistance development risks (Goswami et al., 2020).

1.5.6 Sitamaquine: Sitamaquine, a 4-methyl-6-methoxy-8-aminoquinoline compound, is another oral drug developed after miltefosine by the Walter Reed Army Institute of Research (WRAIR, USA) in collaboration with GlaxoSmithKline (UK). It demonstrated efficacy against *Leishmania donovani* promastigotes comparable to miltefosine. Clinical trials conducted in India, Kenya, and Brazil reported cure rates ranging from 27% to 87% (Dietze et al., 2001; Jha et al., 2005; Sherwood et al., 1994;

Wasunna et al., 2005). However, its further development was discontinued due to significant nephrotoxicity. While the exact mechanism of action remains unclear, it is believed to target succinate dehydrogenase, inducing oxidative stress in *Leishmania* parasites (Carvalho et al., 2011)(Loiseau et al., 2011).

1.5.7 Pentamidine: Pentamidine, an aromatic diamidine, was initially introduced in Bihar, India, as a second-line treatment for visceral leishmaniasis (VL) to address resistance to antimonial therapy. Though it exhibited cure rates comparable to first-line anti-leishmanial drugs, its efficacy was lower than amphotericin B (AmB), and its use as monotherapy was discontinued in endemic regions due to severe toxicity concerns, including cardiac complications, hypotension, diabetes mellitus, and gastrointestinal issues (V. N. Das et al., 2001). Commercially available as Pentacarinat® (Sanofi-Aventis), pentamidine is currently recommended as secondary prophylaxis in HIV-VL co-infected patients. Its mechanism of action involves the inhibition of active transport systems and interference with the mitochondrial DNA complex in *Leishmania* and other kinetoplastids (No, 2016). Despite its adverse effects, irreversible toxicity appears to be rare, and pentamidine may still have a role in cases where first-line treatments fail or are contraindicated. Additionally, in challenging cases, it could be considered as part of a combination therapy regimen, especially for cases in non-endemic regions (Piccica et al., 2021).

1.6 Antiparasitic Efficacy of Molecules Targeting Redox Metabolism

Redox-active substances, including trypanothione [$T(SH)_2$], glutathione (GSH), ovothiol, and cysteine, are proven to be essential for sustaining redox homeostasis in

trypanosomatid parasites. Several enzymes involved in biosynthesis, redox reactions, and the preservation of this equilibrium have been identified, including trypanothione synthetase (TryS), trypanothione reductase (TryR), tryparedoxin (TXN), tryparedoxin peroxidase (TXNPx), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutaredoxin (Grx), ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (AdoMetDC), and cysteine synthase (CS). These proteins are essential for parasite survival, particularly in conditions of oxidative stress and drug resistance.

These proteins have been extensively studied as potential drug targets, leading to the identification, characterisation, and development of various anti-trypanosomal compounds (Battista et al., 2020),(Comini & Flohé, 2013), (Flohé, 2012),(Huang et al., 2020),(Krauth-Siegel & Comini, 2008a),(Tomás & Castro, 2013). Extensive research have also evaluated various drug target pathways in large scales, such as folate biosynthesis, purine salvage, polyamine biosynthesis, trypanothione metabolism, cysteine biosynthesis, topoisomerases, kinases, proteases (cysteine, aspartic, serine, metalloproteases), and glycolysis with documented mechanisms of action for multiple natural and synthetic inhibitors (Nagle et al., 2014)(Nagle et al., 2014).

Conventional chemical screening utilising enzyme assays is laborious and requires substantial resources. Consequently, high-throughput screening (HTS) has been implemented. HTS resulted in the discovery of three compounds (DDD86439, DDD85811, and DDD86243) that inhibit TryS of T. brucei, exhibiting IC_{50} values of 45, 95, and 140 nM, respectively. DDD86243 interacts with the TryS catalytic site (Glu650, Asp651, Glu652, Arg553, Arg613) by hydrogen bonding (Torrie et al., 2009). Fifteen active compounds have subsequently shown anti-parasitic action against TryS in *T. cruzi, T. brucei*, and *L. infantum*. Significantly, bis-benzyl derivatives and N5-

substituted paullones (e.g., MOL2008, FS-554) showed submicromolar IC_{50} values (0.15 and 0.35 μM) against *L. infantum* TryS (Benítez et al., 2016). Multiple compound classes, including phenothiazines, polyamine-based inhibitors, nitrofurans, naphthoquinones, aminodiphenylsulfides, platinum (II) complexes, and trivalent antimonials, have demonstrated efficacy against TryR (Baiocco et al., 2009; Benson et al., 1992; Blumenstiel et al., 1999).

The crystal structure of TryR, both in isolation and in complex with substrates or inhibitors, has elucidated the selective binding of dihydroquinazolines to the active site of *T. brucei* TryR, identifying them as powerful, non-covalent inhibitors (Patterson et al., 2011). A recent work screened 30 compounds targeting *L. donovani* TryR, identifying four with selective inhibition, one of which exhibited an IC_{50} of 15.2 μM, thereby qualifying as a lead contender(Kuldeep et al., 2021). Compounds such as iso-atriplicolide, chalcones, and azoles significantly inhibit TryR and cTXNPx in vitro(Baiocco et al., 2013; Lenz et al., 2019; Ortalli et al., 2018). Chalcones specifically interact with the active sites of TryR and cTXNPx, elevate intracellular reactive oxygen species, and result in parasite mortality (Escrivani et al., 2021).

Molecular modelling and docking have elucidated critical catalytic residues and compound-binding affinities, facilitating rational drug design(da Silva et al., 2019; Ramu et al., 2017). A non-covalent LmTXNPx inhibitor was identified via enzyme-inhibitor docking, and in *L. amazonensis*, the binding of chalcone to the cTXNPx active cysteine was corroborated by CRISPR-Cas9 deletion experiments, which revealed diminished anti-leishmanial efficacy (Escrivani et al., 2021).

Inhibitors of polyamine biosynthesis enzymes (ODC, SpdS, AdoMetDC) have been identified by computational methods (Brindisi et al., 2015; Hazra et al., 2013; V. M. et

al., 2018). Structural elucidations of these enzymes and those responsible for synthesising $T(SH)_2$ have facilitated targeted pharmacological development (Ilari et al., 2017; Leroux & Krauth-Siegel, 2016). A variety of synthetic and natural compounds were evaluated, and their IC_{50} values and inhibitory mechanisms were established using high-throughput screening and enzymatic tests, although most lacked in vivo confirmation(Colotti et al., 2020;Matadamas-Martínez et al., 2019; Turcano et al., 2018b). Three non-competitive inhibitors were identified to augment metacaspase-like activity, resulting in apoptotic DNA fragmentation in *Leishmania*. These inhibitors engage with LdAPx through critical residues (Arg64, Trp67, His68, Glu96, Asp163, Phe201) via hydrogen bonding and hydrophobic interactions (Mansuri et al., 2017).

Crystallographic studies have verified that active-site residues (Trp21, Val53, Ile106, Tyr110, Met113) in *T. brucei* TryR facilitate selective binding to inhibitors, differentiating them from human glutathione reductase (GR) (Turcano et al., 2020). In *L. donovani*, the binding of inhibitors was localised to Arg235, Tyr221, Gly223, and Asn254, which are not present in human GR and TXNR, so confirming their specificity (Turcano et al., 2018b). Similarly, further specific inhibitors comprise geraniol and linalool (V. M. et al., 2018), dithioamidines aimed at ODC (Smithson et al., 2010), and pyrimidineamines for AdoMetDC (Volkov et al., 2018), along with inhibitors targeting TXN/TXNPx in *L. major* (Brindisi et al., 2015). However, further in vivo validation of these drugs using animal models is essential for verifying their efficacy (Baiocco et al., 2009; Mansuri et al., 2017; Smithson et al., 2010).

1.7 Rationale for Developing New Chemotherapeutics

The treatment of visceral leishmaniasis (VL) remains solely dependent on a limited number of chemotherapeutic agents, which are often hindered by numerous practical and clinical constraints. Nearly all of these drug candidates were not initially developed for *Leishmania* as their primary target (CHARLTON et al., 2018a), and their mechanism of action is only partially understood (van Griensven et al., 2024), frequently leading to host toxicity or poorly identified parasite-specific pathways. Due to these concerns, leishmaniasis patients often endure extended treatment regimens that are associated with significant side effects and in certain instances, treatment failure. On top of that, the increasing rate of resistance to monotherapies, for example, pentavalent antimonial and miltefosine in endemic regions, further decreases the efficacy of current therapeutics (Sundar et al., 2024). Although continuous efforts have been made by many groups of scientists all over the world, the lack of preventive vaccines, increasing cases in non-endemic areas, and the evolutionary adaptability of these parasites render new drug development an urgent need. Additionally, in contrast to diseases common in high-income nations, VL has not garnered significant investment in drug research, resulting in a stasis of treatment innovation (Lee et al., 2021; Weng et al., 2018). This reflects a significant knowledge gap in *Leishmania* biology and translates into limited treatment options for treating VL effectively. Nevertheless, the rationale for new antileishmanial drug development must address the most fundamental scientific and clinical gaps, besides overcoming the current therapeutic challenges. Recent advances in genome editing tools such as CRISPR-Cas9 and omics technologies like single-cell sequencing in trypanosomatids have enabled high-throughput genetic and transcriptomic level study in *Leishmania*, offering direct insights into gene and protein’s essentiality, functions, and their potentiality as

new drug targets (Abuchery et al., 2022; Beneke et al., 2017a). This paves the way for rational drug discovery, shifting from time and resource-consuming procedures like experimental compound screening to mechanism-based targeting of essential genes and protein complexes.

There is also an increasing recognition that drug efficacy is not solely determined by parasite susceptibility but also by host-parasite interactions (Tamir et al., 2025; Varikuti et al., 2018). For instance, the parasite's capacity to endure within macrophage phagolysosomes hinders pharmacological administration. Thus, future therapies must be designed for effective intracellular accumulation and macrophage targeting, ideally using nanoparticle carriers or prodrug strategies (Nafari et al., 2020; Palomino-Cano et al., 2024c).

1.8 Discussion

Leishmaniasis represents a significant public health concern, mainly affecting the underprivileged populations from underdeveloped and developing countries. Visceral leishmaniasis (VL) is prevalent in the Indian subcontinent, where it significantly contributes to higher mortality rates. The emergence of new VL cases in non-endemic areas, risks associated with HIV co-infection, and drug resistance collectively amplify the threat posed by leishmaniasis in this region. Current therapeutic options are associated with multiple limitations, highlighting the necessity of novel anti-leishmanial drugs. Since the development of effective therapeutics depends on a comprehensive understanding of the parasite’s vulnerabilities and strengths, in this chapter, we have extensively discussed the immune evasion and antioxidant defence mechanisms used by *Leishmania* parasites.

The redox-metabolism in *Leishmania* parasites is significantly distinct from the usual ways that higher animals fight free radicals. GSH, which is an important precursor for trypanothione ($T(SH)_2$) biosynthesis, is at the centre of this pathway. The enzyme glutathione synthetase (GS) catalyses GSH biosynthesis, hence playing an indirect yet vital role in sustaining redox balance and facilitating parasite survival in oxidative conditions. Despite extensive research on enzymes such as trypanothione synthetase (TryS) and trypanothione reductase (TryR), glutathione synthase in *Leishmania* is comparatively unexplored as a pharmacological target, despite its indispensable role in trypanothione biosynthesis.

Functional investigations in *Trypanosoma brucei* and *L. infantum* have highlighted the critical importance of the GS enzyme. Genetic knockdown tests demonstrate that GS depletion results in diminished trypanothione levels, causing oxidative imbalance and ultimately leading to parasite mortality. The low sequence similarity (~28%) of the enzyme in *L. donovani* with its human homolog presents a potential advantage for target-based drug identification, thereby reducing off-target toxicity. However, although the enzyme's essentiality is partially recognised, comprehensive genetic, structural, and functional characterisation of GS in *L. donovani* has not been studied yet. This highlights a significant knowledge gap that must be resolved to thoroughly assess its potential as an antileishmanial target.

For a genetic validation, CRISPR-Cas9-mediated gene editing can be utilised to confirm the essentiality of *L. donovani* GS throughout different life stages and stress conditions. Conditional knockdown mutations or CRISPR-induced point mutations at catalytically essential residues might elucidate the enzyme's function in parasite growth, redox resistance, and infectivity. Additionally, transcriptome profiling under oxidative stress or drug pressure may elucidate if GS is differentially regulated during

amastigote differentiation or in resistant strains. These investigations would establish the essential information required to validate GS as a therapeutic target.

The biochemical analyses, such as recombinant expression of *L. donovani* GS, along with enzyme kinetics, substrate specificity assays, and inhibition investigations, will be crucial for elucidating its catalytic mechanism and discovering small-molecule inhibitors. High-throughput screening of chemical libraries, specifically targeting ATP-grasp fold inhibitors, may uncover compounds that competitively block the GSH-synthesizing function of GS. The observed functional necessity in associated parasites, such as *Trypanosoma brucei,* along with the requirement for precise trypanothione regulation, indicates that even partial inhibition of GS could significantly impact the redox equilibrium and viability of the parasite.

Computational biology tools such as molecular docking and simulation studies provide effective ways to accelerate target validation and lead identification processes. Using the modelled 3D structure of *L. donovani* GS through modern tools like AlphaFold, we may reveal structurally conserved catalytic pockets and parasite-specific allosteric regions. In silico docking and virtual screening of these pockets may produce novel lead compounds with high binding affinity and selectivity. Moreover, comparative structural investigations of the parasite and host GS may inform the rational design of parasite-specific inhibitors. Machine learning models developed using known GS inhibitors from other organisms could also predict possible candidates for *L. donovani*, hence reducing the time of experimental screening.

The stage-specific essentiality of GS throughout the parasite's life cycle is another inadequately explored area. Considering the metabolic differences between promastigotes and amastigotes, it is crucial to determine whether GS expression or

activity fluctuates throughout differentiation or in response to host-induced stress conditions, such as acidic pH and oxidative pressure. This will be essential in developing drug compounds that effectively target the intracellular amastigote stage, the clinically significant form in humans.

In conclusion, *L. donovani* glutathione synthetase is a promising yet unexplored target in the development of novel antileishmanial treatments. A multifaceted strategy, including genetic manipulation, computer simulation, and biochemical verification, will be crucial for a thorough assessment of its druggability. Future drug development efforts should focus on addressing the current knowledge gaps, especially in GS's structural characteristics, regulatory mechanisms, and its molecular-level interactions with other pathways. These investigations may reveal a novel class of redox-targeting drugs capable of overcoming current therapy limitations and drug resistance in leishmaniasis.