The Concept of Fitness in *Leishmania*

15

Manu Vanaerschot, Franck Dumetz, Marlene Jara, Jean-Claude Dujardin, and Alicia Ponte-Sucre

Abstract

A pathogen's fitness relates to all biological processes that ensure its survival, reproduction, and transmission in specific conditions. These often include the presence of drugs, forcing pathogens to adapt and develop drug resistance in order to survive. The acquisition of a drug-resistant trait usually comes at a cost, making drug-resistant parasites less fit than their wild-type counterparts. This has important implications on the development of drug resistance and on the frequency of treatment failure cases in endemic regions. Treatment failure in patients suffering from leishmaniasis has been observed for most antileishmanials, but could not always be correlated to drug resistance of the infecting parasite. One similitude of both pentavalent antimonial and miltefosine treatment failure, however, relates to changes in parasite fitness. In the specific case of *Leishmania donovani*, for example, this may contrast with the usual fitness cost observed in natural drug-resistant organisms and highlights parasite fitness as an important

M. Vanaerschot (✉)
Department of Microbiology and Immunology, Columbia University College of Physicians and Surgeons, New York, NY, USA

F. Dumetz
Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium

M. Jara
Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru

J.-C. Dujardin
Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium

Department of Biomedical Sciences, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Antwerpen, Belgium

A. Ponte-Sucre
Laboratory of Molecular Physiology, Institute of Experimental Medicine, Luis Razetti School of Medicine, Faculty of Medicine, Universidad Central de Venezuela Caracas, Caracas, Venezuela

A. Ponte-Sucre, M. Padrón-Nieves (eds.), *Drug Resistance in Leishmania Parasites*,
https://doi.org/10.1007/978-3-319-74186-4_15

contributor to treatment failure in visceral leishmaniasis in the Indian subcontinent. In this final chapter, we will canvass the knowns and the unknowns of *Leishmania* fitness at different parasite life stages and for different *Leishmania* species and discuss its relevance for the development and spread of drug resistance and/or treatment failure in the field. We will also propose new research avenues for leishmaniasis drug development and control in the context of current elimination efforts.

15.1 Introduction

Viruses were pioneers as target for studies of the concept of fitness. In these organisms, fitness was initially defined as their ability to successfully survive, reproduce, and infect in a defined environment [1–4]. For *Leishmania*, the concept was initially related to proficiency; i.e., the complex integrated skills that allow *Leishmania* to successfully replicate and cause the disease [5]. As the life cycle of *Leishmania* oscillates between two life stages that occur in a specific host—promastigotes develop in the insect vector and amastigotes develop in mammalian hosts—*Leishmania* adapted to these environments by undergoing several developmental stages; each bears specific traits to guarantee survival, reproduction, and ultimately, transmission to a new host. The fitness of *Leishmania* is thus the amalgamation of its success in all these processes combined (reviewed in [6]). Although many of the determinants involved in these processes are becoming more and more appreciated, only few are well understood. These include determinants specific to parasite life stages such as promastigote metacyclogenesis and amastigote survival in host cells (Sect. 15.2.1) and molecular traits that contribute to the parasite's adaptive skills during its whole life cycle (Sect. 15.2.2).

Importantly, the fitness of an organism is not only dependent on that organism itself but also on the environment in which it lives (Fig. 15.1). In the case of *Leishmania*, this includes host factors such as immunity and nutritional status, whether or not the parasite can hide in certain tissues (Sect. 15.2.1.1), to even dynamic global trends that may enhance the chance for emerging infectious diseases to occur and expand swiftly [7], discussed in Sect. 15.2.3. The interaction of all these fitness determinants is complex and eventually results in the capacity of the parasite to be transmitted and to infect the next host, where it may cause disease, a process originally defined as virulence. Virulence has been used as one of the foremost markers for fitness in *Leishmania* since its expression constitutes the mechanism per excellence that permits the "survival of the best," guaranteeing successful transmission to the next host [8]. Virulence is important at both the promastigote and the amastigote stage. Its function is evident at the dynamic interface that allows integrity but at the same time guarantees communication between the organism and its host.

More recent contributor to this environment are drugs. Drugs can dramatically alter the fitness landscape for *Leishmania* parasites, selecting *Leishmania* sub-populations

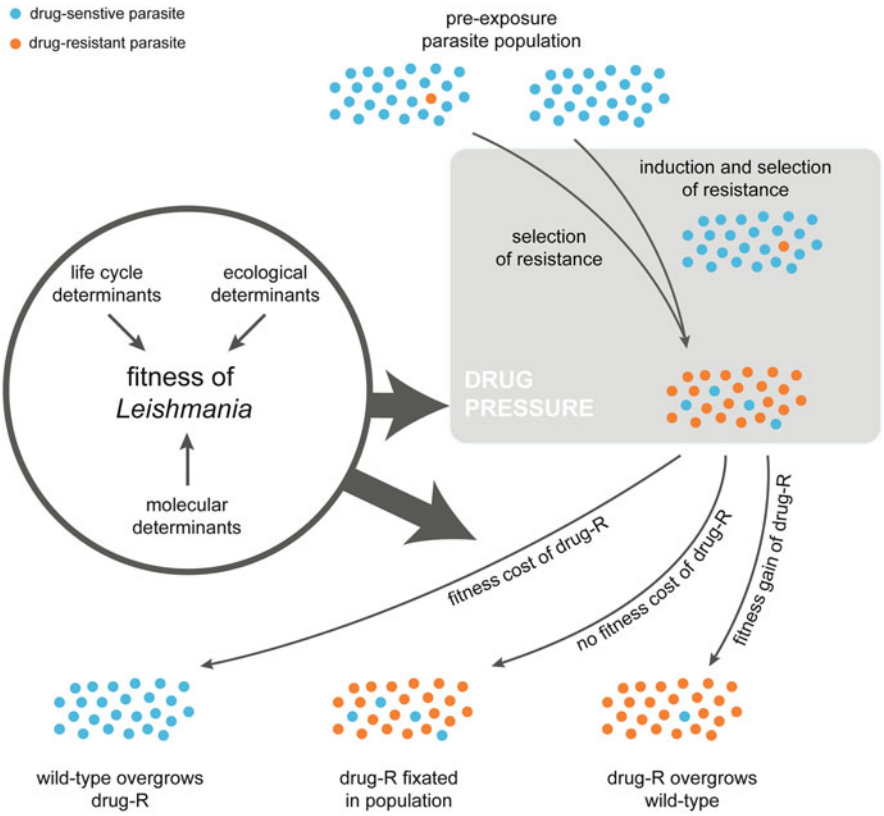

Fig. 15.1 The impact of parasite fitness on the evolution of parasite populations in the presence of drugs. Drug pressure selects for drug-resistant parasites, but sensitive parasites may potentially overcome drug treatment through mechanisms related to quiescence or hiding in niches where drug levels are low—thus without developing a classic drug-resistant phenotype. Once drug pressure is relieved again (due to, e.g., changes in treatment policies), their fitness compared to wild-type drug-sensitive parasites will decide on their future success in the population

that are able to survive drug pressure thanks to specific physiological traits—this will be discussed in Sect. 15.3 of this chapter.

15.2 The Knowns and Unknowns of *Leishmania* Fitness

15.2.1 Life Cycle Determinants

15.2.1.1 Amastigotes

Once an infected sand fly bites a mammalian host, parasites and sand fly saliva components are inoculated into the skin and invade mononuclear phagocytes in which they will develop into amastigotes. This may lead to two different outcomes:

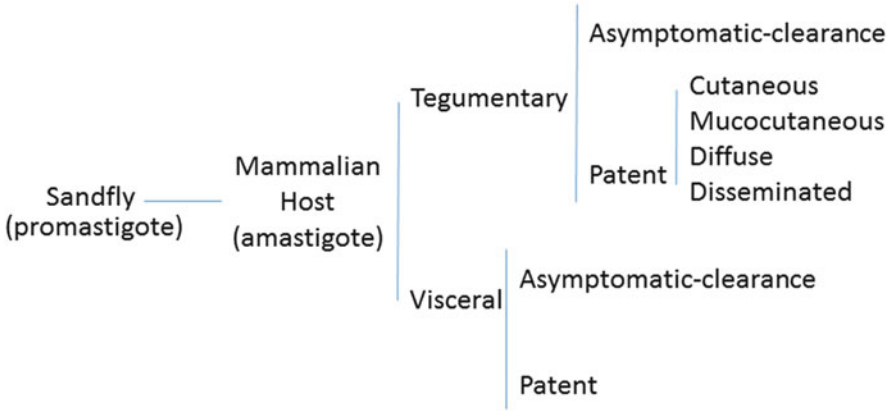

Fig. 15.2 Spectrum of clinical manifestations that may result from *Leishmania* infection in New and Old World *leishmaniasis*

either the host immune system successfully controls the infection, resulting in an asymptomatic infection, or the infection becomes patent, resulting in mild or severe disease (Fig. 15.2).

Classically, amastigotes are defined as the non-motile, parasitic forms with an ovoid or spherical body, a rod-shaped kinetoplast and a rudimentary, retracted flagellum arising from a basal body. This developmental form paradoxically lives in the immune cells that constitute the primary defense against invasion by foreign organisms, suggesting that through evolution, *Leishmania* has successfully learned to adapt to the stressful environment constituted by the intracellular milieu. *Leishmania* amastigotes are experts at exploiting host cell processes to establish infection and persist in several tissues. Although infected cells favor the immediate control of intracellular pathogens, the intracellular milieu constitutes a pathogenic protective space that drives the adaptive response of the parasite and allows it to display its florid pathogenic potential [9] and divert host mechanisms that would otherwise lead to parasite killing.

1. Immune System

   Once the primary parasite-host interaction occurs, the immune system initiates its activity with the aim to control the infection. The final end of its function could represent control of the disease, with or without sterilization, eventually leading to the asymptomatic character of the infection, or to a patent infection, either tegumentary or visceral. *Leishmania* is a versatile organism with diverse host defense evading mechanisms [9]. These host manipulation skills of the parasite are key to its survival and replication inside host cells. While being phagocytosed, *Leishmania* ensures that it is not recognized as a foreign organism by the host cell by interacting with specific surface receptors expressed by host neutrophils, dendritic cells, macrophages, and monocytes (reviewed in [10]). This is exemplified by *L. (L.) amazonensis*, which causes diffuse cutaneous

leishmaniasis (CL), a true anergic form of tegumentary leishmaniasis. As described by Zerpa et al. (Chap. 8), an initial local lesion may be the origin of the spread of parasites by lymphatic and hematic means, with the subsequent inhibition of specific cellular immunity. *L. (L.) amazonensis* expose phosphatidylserine on their surface, a signal to host immune cells to phagocytose harmless agents. This "apoptotic mimicry" of *L. (L.) amazonensis* allows it to silently enter the mononuclear phagocytes in which it multiplies, without activating the immune system, and is thought to have evolved from a few parasites with altruistic behavior for the greater good of the overall parasite population in a host—a trait that was fixated throughout the parasite's evolution [11]. Once inside the host cell, the biggest threat to the parasite is the production of reactive oxygen and nitrogen species by this host cell. However, specific molecular features of *Leishmania* will protect it from these immune effector molecules (see Sect. 15.2.2). Additionally, *Leishmania* actively inhibits the host cell from producing these toxic molecules. One intriguing example of specific molecular features triggered by *Leishmania* is the parasite-mediated activation of the host cell phosphatase SHP-1 that will inhibit host cell pathways that would normally lead to mounting an adequate anti-parasite immune response, including the production of ROS and RNS (reviewed in [6]).

The parasite also affects the immune system at a more systemic level: infected macrophages can produce high levels of activating cytokines like tumor necrosis factor α, interleukin-1, or the down-modulatory interleukin-10 and transforming growth factor-β [9]. Additionally, the parasite contributes to confuse host cells from their functions, by expressing, for example, decoy molecules on their surface or excreting molecules into the host cell that disturb cell signaling pathways [6, 12]. By affecting physiological functions of the host cell, the parasite ultimately determines its own fate and that of the host (possibly causing disease). For example, the amastigote form of the parasite can influence the phosphorylation state of host molecules, as well as the activity of mitogen-activated protein kinases [13, 14]; additionally, it can inhibit the production of superoxide and nitric oxide by infected macrophages [15], as well as macrophage activation by interferon-γ [16, 17]. Last but not least, their presence inside macrophages is effective to prevent the action of interleukin-12 [10, 18]. All these events occur upon internalization of the parasite into the parasitophorous vacuole in the newly infected host cell. However, the signaling mechanisms and pathways that are essential to prevent amastigotes disappearance and to guarantee their survival and replication inside the parasitophorous vacuole are not yet fully elucidated [10]. Since chemotherapy, especially with drugs like antimonials that need a competent immune system to exert their action mechanism, decreases the parasite load in the patient, the host immune system might be able to retake control and mount an effective response [6]. An interesting example of how determinant the immune system is on the outcome of the disease is exemplified in visceral leishmaniasis (VL) and post-Kala-azar dermal leishmaniasis (PKDL). In this case, the continuous presence of T-regulatory cells and their selective recruitment to the infected sites play a critical role in the persistence of a residual

parasite burden [19]. This continuous presence can result in visceral disease relapse after apparent cure or the development of post-Kala-azar dermal leishmaniasis [20]. On the other hand, MIL does not require a potent immune system to fully exert its action but has been reported to positively affect the immune status of VL patients [21]. Immunomodulation may thus also depend on the parasite load in the patient: a higher parasite load likely further boosts the immunomodulatory effects that are already intrinsic to any *Leishmania*.

2. Niches and Quiescence

Leishmania parasites are ancient eukaryotic organisms that have evolved into a species that has a higher diversity and adaptive capacity than its hosts. This is especially important since intracellular parasitism (rare, obligatory) associates with challenges that if not conquered mean the senescence of an organism and at the end, of a species. Thus, parasites must invade host cells successfully and be able to escape or divert intracellular mechanisms that would otherwise clear intracellular invaders. The used mechanisms include programmed cell death either by apoptosis or autophagy and machineries related to the activation of immunity like production of reactive oxygen-nitrogen intermediates and lysosomal degradation [22]. Moreover, host surveillance such as Toll-like receptors and intracellular sensor systems impose an additional challenge that intracellular parasites must overcome [22].

This means that a determinant factor that modulates the outcome of the invasion produced by *Leishmania* depends on its ability to infect alternative tissue niches within the vertebrate host, less accessible not only to the surveillance systems but also to drugs. In fact, amastigotes either remain in the original site of infection (as in the case of CL) or disseminate to other teguments (as in mucocutaneous leishmaniasis (MCL) or disseminated leishmaniasis) or to the viscera (as in VL) [20]. Interestingly, parasites are capable of invading sites other than those expected to be affected, albeit at lower levels and hereby remaining unnoticed. These places may function as hidden niches that can be (re)activated at a later moment. As such, *Leishmania* DNA has been described to be present in the bloodstream [23], in urine [24], and in apparently healthy mucosa [25] of patients suffering from cutaneous and MCL. More interestingly, as the *Leishmania* kinetoplast DNA degrades rapidly [26], this observed DNA should originate from living or recently dead parasites. In VL patients, parasites have been found in the blood [27] and skin as evidenced by the emergence of post-Kala-azar dermal leishmaniasis [28]. Interestingly, both MCL and PKDL are examples of leishmanial disease that appears many years after apparent cure. Yet the tissues and organs that are targeted are either very well perfused in the case of MCL (the mucosa) or not so perfused in the case of post-Kala-azar dermal leishmaniasis (the skin). This imposes a controversial discussion since hiding in a well-perfused tissue might result in a higher exposure to the immune system, while hiding in a less perfused organ could imply hiding from the immune system.

Host cells of *Leishmania* include macrophages, neutrophils, and dendritic cells. Upon initial infection, neutrophils are recruited to the site of sand fly bite and survival within these cells will determine the fate of the parasite. Inside

neutrophils, *Leishmania* parasites establish vacuoles that avoid lysosome fusion thus providing a protective environment for survival, if not replication. The parasite might also invade tissue cells like fibroblasts or Langerhans cells that support growth but are less able to clear parasites, perhaps due to the restricted microbicidal capacity of these host cells [22, 29]. Upon time, less neutrophils and more macrophages are infected, resulting in an active infection [22]. Amastigotes are thought to be metabolically less active than promastigotes. This is exemplified by the longer doubling time for axenic amastigotes (4 days) and amastigotes from lesions (12 days) compared to promastigotes (9 h) [30]. There is also experimental evidence showing that *Leishmania* amastigote transcription [31, 32] and translation [30, 33, 34] are significantly decreased in the amastigote stage, coinciding with lower levels of polysomes observed in axenic amastigotes [34]. Amastigotes also have a downregulated metabolism. The uptake and utilization of amino acids and glucose is diminished [35]. At the energetic level, amastigotes have lower levels of ATP than promastigotes, probably due to their attenuated oxidative phosphorylation and lower oxygen consumption [36]. Although such studies should also be performed on intracellular amastigotes, these results imply that amastigotes (or a subset of them) could be in a quiescent state, living on their reserves. This has been shown to be the case in the chronic stage of *L. (L.) major* infection in a murine model after the lesion is self-cured: persistent amastigotes could be divided into a population of amastigotes that grow at the same rate (60% of total) and another population that shows no evidence of active growth (40% of total) [37]. Interestingly, both dividing and non- or slow-dividing cells resided in the same host cells, being macrophages and dendritic cells. Quiescence among amastigotes or/and other niches of infection could be critical factors to hide from the host's immune system and eventually promote the parasite's survival.

15.2.1.2 Promastigotes

When a female sand fly bites an infected host, it will engorge *Leishmania* amastigotes and amastigote-containing cells together with the blood. These amastigotes will then transform to slender flagellated promastigotes in the abdominal midgut of the sand fly, where they need to overcome several bottlenecks in order to continue the parasite's life cycle. Alkalinization, changes in the midgut, and a decrease in the level of proteolytic activity promote the development of promastigotes in the gut of sand flies, meaning that growth and differentiation within the sand fly are linked to changes in pH, sugars, and among others, AA levels that might even modulate migration from preceding gastrointestinal portions into the cardio-esophageal valve [38, 39]. Gut epithelial cells of the sand fly will secrete a chitinous matrix that will form a peritrophic membrane encircling the blood meal and the engorged parasites, but promastigote-secreted chitinases will cause it to break down sooner than normal to allow migration of parasites to the anterior part of the sand fly [40]. To avoid excretion with the rest of the digested blood meal, promastigotes attach themselves to the microvillar lining by their flagellum (reviewed in [41]). Over the course of a few days, they will migrate to the thoracic

midgut and the stomodeal valve and will undergo a transformation from dividing non-infective promastigotes into nondividing infective metacyclic promastigotes, a process called metacyclogenesis [42]. This process is of the utmost importance for *Leishmania*, as only these metacyclic parasites will be able to successfully initiate infection of the mammalian host later on. In the anterior midgut of the sand fly, promastigotes will secrete a gel-like substance to create a plug that fills the anterior midgut and extends to the stomodeal valve into the foregut [43]. When the sand fly wants to feed, it will first have to regurgitate to overcome the obstruction by the plug, hereby expelling (metacyclic) promastigotes into the skin of the host [44] and allowing the life cycle to continue. Breaking through the peritrophic membrane, attaching to the midgut to avoid excretion and metacyclogenesis are processes that are initiated by the parasite. However, while undergoing these developmental steps, the parasite needs to continuously defend against the sand fly immune system (reactive oxidative and nitrosative stress) and compete for resources with the normal flora of the sand fly.

This complex play of various *Leishmania* promastigote differentiation stages in the metabolically different locations in the sand fly and the complexity of sand fly studies itself have hampered our understanding of the exact detail of the fitness actors at play in this part of the parasite's life cycle. However, the in vivo transmission model of *Leishmania* development that has been developed using hamsters and *Lutzomyia longipalpis* sand flies [45] opens new avenues for fitness studies, including the promastigote stages in their natural environment.

15.2.2 Molecular Determinants

Leishmania belongs to the trypanosomatid family [46], implying among others two particularities: (1) at the genome expression level, all trypanosomatids transcribe their genes in long transcripts that contain several genes, also called polycistronic expression [47, 48], and (2) at the biochemical level, they use trypanothione (two glutathione molecules linked by spermidine) as the main regulator of their intracellular reducing environment and to detoxify the cell, in contrast to other eukaryotes that only have the less powerful glutathione [49–51]. To adapt to the poor flexibility of polycistronic expression, *Leishmania* developed multiple and unique, genomic adaptations among trypanosomatids. *Leishmania* is constitutively mosaic aneuploid, meaning that a given chromosome may have a different copy number, or somy, within different cells in the total population, going from monosomy (one copy of the chromosome) to pentasomy (five copies of the chromosome) [52–54]. Evidences that mosaic aneuploidy is also present at the amastigote stage were recently described in *L. (L.) donovani* parasites isolated from hamsters [55]. This creates a vast diversity within the population, providing a high adaptive capacity of the parasite population to various kinds of stress, including drugs [56]. This adaptive capacity provided by somy variation and SNP selection was exemplified when selecting for MIL resistance in vitro: first a somy reduction of chromosome 13 carrying the *L. (L.) donovani* MIL transporter (LdMT) appeared, secondly a LdMT

deletion on one chromosome, and thirdly a nonlethal mutation on the second LdMT allele that provided good levels of resistance [57]. *Leishmania* also has other features related to genome flexibility. As such, the parasite can generate local gene copy number variations (CNV) through linear or circular extrachromosomal amplification, using direct and inverted DNA repeats [58], as well as intrachromosomal amplification (ICA) [59, 60]. This phenomenon was observed in in vitro laboratory parasites selected against many different drugs such as arsenic [61], antimonials [60, 62, 63], amphotericin-B (AMB) [64], methotrexate [65–67], and other non-antileishmanial drugs [68, 69], highlighting that this mechanism is one of the main adaptive features of *Leishmania*. Interestingly, 94% of the clinical isolates from the lowland of the Indian subcontinent assessed in a genetic diversity study showed two different ICAs, and two epidemic clones that carry these ICAs showed to have propagated successfully in India. Parasites not carrying these ICAs were also present in the Indian subcontinent but were restricted to one restricted area, the Nepalese highlands, and seemed less fit to spread throughout the Indian subcontinent [59]. This highlights once more the importance of ICAs for the parasite's adaptive capacity to survive environmental stress, be it the presence of drugs (Dumetz F. et al., unpublished data) or other selective pressures.

Such ICAs may indeed affect the metabolomic profile of the parasite: the same clinical antimonial-resistant (SSG-R) parasites from Nepal that carried an ICA at the level of argininosuccinate synthase [59], the enzyme catalyzing the transformation of citrulline in argininosuccinate, also displayed a significant increase in their argininosuccinate content as identified by metabolome studies [70]. Notably, argininosuccinate is a metabolite that is part of the urea cycle and is, among others, a basic component of the pathway that eventually leads to putrescine and trypanothione synthesis.

Trypanothione is the main active defense system of *Leishmania* against reactive oxygen and nitrogen stress (ROS/RNS). The parasite will encounter oxidative and nitrosative stress throughout its life cycle as a promastigote and an amastigote, but ROS/RNS can also be induced by drugs such as pentavalent antimonials (Sb^V), for example. *Leishmania*'s redox system consists of a cascade of enzymes with trypanothione as the main reducing agent (Fig. 15.3). When ROS and RNS are detoxified by members of this cascade (either trypanothione itself (H_2O_2 [71],

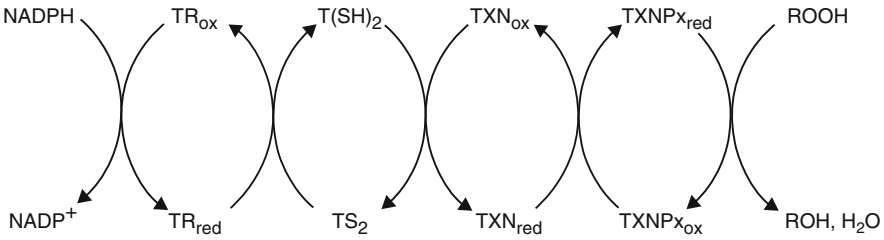

Fig. 15.3 The NADPH-dependent redox cascade with trypanothione (TSH2) as the central reductant. *TR* trypanothione reductase, *TXN* tryparedoxin, *TXNPx*, tryparedoxin peroxidase

NO. [72, 73]), tryparedoxin or tryparedoxin peroxidase (H_2O_2 [74, 75], $ONOO^-$ [74, 76], H_2O_2 + NO. [77]), the flavoenzyme trypanothione reductase (TR) will replenish the pool of reduced trypanothione (T[SH]2) from oxidized trypanothione (T[S]2) using NADPH as an electron donor (Fig. 15.3). TR is therefore thought to be a central and very important enzyme for the intracellular survival of *Leishmania* [78–80].

When promastigotes were put under pressure with Sb^{III}, which is the toxic reduced form of Sb^V that is the core component of SSG, many intermediates of the trypanothione pathway were found to be upregulated [70, 81–83], confirming earlier investigations carried out at the protein level in different *L. (L.) donovani* strains from the Indian subcontinent where an upregulation of the enzymes of the thiol pathway was observed in SSG-R *L. (L.) donovani* [84] and *L. (L.) infantum* [85] parasites.

Studies on the metabolomic profile of MIL-resistant *L. (L.) donovani* showed a large modification of the lipid composition, probably due to the mechanism of action of MIL on the membrane, but also an increase of the metabolites implicated in the thiol pathway [57, 86]. The lipid composition is also found to be changed in parasites resistant to drugs without a clear link to lipid metabolism: unsaturated phosphatidylcholine lipids and phosphatidylethanolamine were increased in SSG-R versus SSG-S parasites, suggesting an extensive change in the membrane composition of SSG-R parasites [87].

Interestingly, studies on in vitro selection of resistance against a combination of drugs identified that this requires different adaptations compared to resistance against just either one of the drugs in that combination [82]. One common factor, however, was the pivotal role of pathways regulating protection against oxidative stress and membrane composition [82]. These molecular traits of *Leishmania* are thus considered to be important molecular determinants of the parasite's adaptive capacity and therefore also its fitness.

15.2.3 Epidemiological Determinants

When talking about epidemiological determinants that might affect the fitness of *Leishmania*, we have to be aware of the fact that nowadays, and regarding the spectrum of leishmaniasis, CL and VL have undoubtedly a wider geographical distribution than before; additionally, the higher leishmaniasis incidence is a result of risk factors that can also be determinant for changes in fitness and virulence of the parasite [88]. In fact, changes in environmental conditions (i.e., temperature), human behavior (nutrition, misuse of drugs), immunogenic patient profile (co-infection with HIV), and genetic factors (parasite species) might determine the fate of the parasite-host interaction affecting directly the interplay between these two fundamental actors in the development of the disease.

Regarding climate models, it is well recognized that there will be a global average increase of air temperatures from 1 °C to 4 °C by 2100 [89]—more than ever before [90]. A consequent modification of species occurrence and distribution will occur

with up to 37% of all existent species "committed to extinction" due to climate change [91]. For parasites, an increase in organisms' virulence and transmission rates are the most commonly described responses to rising temperatures [92, 93], implying that some parasites might become more successful and increase their fitness compared to earlier times. Examples exist in the bacterial parasite *Pasteuria ramosa* [94] or in the tapeworm *Schistocephalus solidus* with increased castration rates of *Daphnia magna* or growth rates in three-spined sticklebacks at higher temperatures [95]. Besides direct effects on hosts and/or parasites, if the global warming changes parasite virulence and/or host resistance in an asynchronous way, the interactions among both organisms will also be affected [96]. Environmental changes can therefore induce adaptive peaks (different host species) to occur closer in time, easing the transfer to a new host by a proportion of the parasite population, afterward followed by the rest of the same population. These changes in the environment can also facilitate the invasion of more species that then become potential hosts suitable for ecological fitting of the parasite [90].

The importance of the immune system status for disease development can be understood by evaluating the effect of co-infection between HIV and leishmaniasis. In fact, HIV is changing the nature of the human infection, the response to treatment, and the epidemiology of leishmaniasis in different geographical areas including Africa, Europe, and Brazil. HIV patients are immunosuppressed, and treatment of VL in such patients requires a long course of treatment, resulting in an increased risk of relapse and a high chance on the development of drug resistance. Further suppression of the immune system by HIV exacerbates the situation. Both diseases drive each other at least in experimental settings [97], and patients suffering from both diseases simultaneously have higher parasite burdens and weaker or absent immune responses. This causes them to respond slowly to treatment with antimonials (SSG) [98], and their clinical improvement does not correlate with parasite clearance from splenic aspirate smears, resulting in about 60% of the patients showing relapse within 1 year, and with any antileishmanial drug used [99], with secondary resistance being common to all of them [100, 101]. As under experimental settings, the vector *Phlebotomus ariasi*, common in southern Europe, can become infected by feeding on HIV-*Leishmania* co-infected patients [97]. Questions arise about whether or not these patients can provide a human reservoir prone to modulate the epidemiology of the disease in southern Europe. This is a fundamental question since without HIV, VL patients are not infectious to this sand fly. As the courses of drug treatment should be increased concomitantly, an open question is whether this condition can lead to the emergence of primary drug resistance [102].

In the American leishmaniasis context, it is interesting to note that parasites of the *Viannia* subgenus may be infected by a specific virus (*Leishmania* RNA virus-1 or LRV1) that successfully impairs the host immune response to *Leishmania* and promotes parasite persistence [103]. In *L. (V.) braziliensis*, the presence of the RNA virus was shown to be associated to the development of mucosal disease [104] and even treatment failure [105]. The importance of viral or bacterial

endosymbiosis and how this may shape the genome and the fitness of the parasite remains to be further studied.

This takes us to the discussion that among the factors that are determinant to the outcome of an initial infection with *Leishmania*, the species constitutes one of the strongest predictors for the development of a given clinical form of disease. This is clearly exemplified in American leishmaniasis. *L. (V.) braziliensis* and *L. (L.) amazonensis* infections lead mostly to tegumentary forms of disease, while *L. (L.) infantum* has the potential to induce visceral disease. Even more, strain differences within the same species might also be associated with a given clinical form of disease [9]. As described elsewhere [20], in Peru, patients infected with *L. (V.) guyanensis* are generally more responsive to SSG than patients infected with *L. (V.) braziliensis* [106], while the opposite result was observed in Brazil [107]. In Venezuela, diffuse CL patients infected with either *L. (L.) amazonensis* or *L. (L.) mexicana* comprise a poor response to SSG [108] (Chap. 8 by Zerpa et al.). These results reveal the important role of the different epidemiological and genetic diversity of New World *Leishmania* on treatment outcome of American tegumentary leishmaniasis.

A final determinant that we will briefly discuss relates to the fact that the response to treatment in the New World differs significantly from that in the Old World, an issue that further reflects the multifactorial character of the disease. As previously mentioned, drug, host, and parasite factors contribute to the final outcome [109]. Old World leishmaniasis has a more homogeneous therapeutic outcome, except when caused by *L. (L.) aethiopica*, compared to New World leishmaniasis, where therapeutic responses are mixed and unpredictable. This implies that treatment guidelines have to be evaluated on a global basis, taking into account the vast differences between Old and New World leishmaniasis [106, 109]. This also implies a different rationale for researchers looking for determinant factors that contribute to treatment outcome, as drug resistance could be partially responsible for treatment failure, but additional factors like the epidemiological complexity of the disease due to the diversity of etiological agents and their (epi-) genetic features may dramatically complicate the panorama, especially for American tegumentary leishmaniasis treatment. However, other issues can determine the response to treatment and we will briefly refer to them herein.

Substandard product levels constitute the inevitable consequence of inadequate local regulation of pharmaceutical companies and the lack of good manufacturing practices in many countries [110]. Drugs with substandard concentrations of the active ingredient determine a poor response to treatment and can increase the risk of spread of drug-resistant (drug-R) pathogens [111]. Similarly, inadequate dosage (even higher dosage than needed) is also a positive factor that could be a selective factor for the selection of resistant parasites occurring in a patient [20, 112].

Additionally, poor hygienic measures and transmission control in clinics and hospitals in the developing world, the natural niche for leishmaniasis, lead to environmentally suboptimal disposition of the medicaments. The threat from these (and other) released medicaments is illustrated by the existence of a large reservoir of resistance genes present in the human microflora. These genes could serve as donors for the transfer of genes to human pathogens by means of horizontal gene transfer.

Little is known about the role of horizontal gene transfer in poor response to drugs in parasites like *Leishmania*. Nevertheless, we cannot exclude the relevance that this mechanism might have in this parasite [113].

15.3 Leishmaniasis Treatment Failure and Fitness

15.3.1 Fitness Cost or Not

The presence of drugs has a dramatic impact on parasite fitness and therefore also on the equilibrium that exists within the parasite populations in a region where drugs are deployed. In fact, although "fitness cost" is the most common feature observed in nature as a result of drug resistance expression, "fitness compensation" is also observed in such circumstances (reviewed in [114]).

Parasite populations under drug pressure can result in either the selection of pre-existing resistant variants that were circulating in the field or in the induction of new variants emerging under drug pressure. The level of drug pressure will play an important role in the emergence and/or spread of drug-R parasites. As mentioned earlier, both substandard drug levels and higher dosages than what is required may result in a high selective pressure for pre-existing drug-R parasites [115].

Drug pressure on a parasite population may result in parasites with a drug-R trait that may have an originally lower relative fitness compared to others in natural no-drug conditions. However, they will become more successful than drug-sensitive (drug-S) parasites in drugged conditions. This capacity to better withstand drugs may be related to genetic factors that prevent the drug from acting on its target or to factors that enable the parasite to more easily adapt to drug pressure compared to its counterparts. As discussed earlier in this chapter, these factors can be species dependent. Assessing the fitness of drug-S and drug-R parasites can therefore shed more light on the life span of a drug, as a rise in drug-R parasites leads to a more frequent appearance of treatment failure, which may eventually lead to the drug being too inefficacious to justify further use. However, the acquisition of a drug-R trait generally comes at a cost [114]. This fitness cost will make the drug-R parasite less fit compared to wild-type parasites when the drug pressure on the parasite population is low or even absent [20]. Since most *Leishmania* parasites hide in reservoirs that are generally untreated, such as asymptomatics or PKDL-patients for *L. (L.) donovani* and animal reservoirs for *L. (V.) braziliensis*, the relative fitness of drug-S and drug-R parasites in no-drug conditions will have a major impact on the speed by which drug-R parasites will spread in a parasite population.

Such a fitness assessment is hard to make and requires adequate in vitro and in vivo tools and, even more important, a set of *Leishmania* strains that are representative for the region of interest. In the context of drug resistance studies, clinical drug-R strains or strains from treatment failure patients may not always be available and therefore require substitution by strains that are made resistant in the lab. Although the resistance mechanisms generated in the lab may differ from those in the field, they do provide insights into how a drug works and what the parasite's

options are to become resistant. The fitness effects related to these resistance mechanisms, however, may play out very different when induced in an in vitro context compared to being naturally generated in a patient. This is mainly due to the lack of immune factors, different host cell niches, and other fitness determinants described earlier that are missing in a simplified in vitro context.

In the last few years, there was an appreciable upsurge of fitness studies in the context of both natural and in vitro drug resistance. Comparing a set of clinical *L. (L.) donovani* SSG-S and SSG-R strains, an increase in metacyclogenesis [116] and an increased fitness in infected mice were observed for SSG-R lines compared to SSG-S lines [117, 118]. Since SSG interacts with the immune system to reduce the parasite load in the patient, it was hypothesized that the parasite adapted to the host immune system while adapting to the drug, leading to the traits that are suggestive of a higher fitness compared to wild-type drug-S strains [6, 20]. This was further substantiated by several studies that identified specific host manipulation skills of clinical SSG-R strains that can be directly related to the increased fitness of these strains in vivo (reviewed in [20]). Interestingly, the majority of these clinical SSG-R strains isolated from SSG-treatment failure patients belong to a specific genetic group of parasites (ISC5) that has expanded significantly in the Indian subcontinent, even at times when SSG was no longer the first-line treatment [119]. This observation was confirmed by mathematical modeling studies showing that SSG-R strains must have had an increased fitness compared to SSG-S strains in order to explain their success in the field [120, 121]. Recent reports, however, indicate that the genotype related to SSG-R parasites is decreasing in prevalence since 2013 [122], possibly due to other treatment options (such as MIL) wiping out genetic diversity and reshaping the landscape of *Leishmania* genotypes circulating in the field. Initially, the higher fitness that was described for *L. (L.) donovani* SSG-R versus SSG-S strains was thought to be a unique case due to the combination of a highly adaptive parasite and a drug that interacts closely with the immune system. However, when testing clinical *L. (L.) donovani* strains from patients that failed the more recently introduced MIL treatment, an increased metacyclogenesis that translated into higher in vitro infection levels was again observed—this despite the lack of a clear in vitro miltefosine-resistant (MIL-R) phenotype in these clinical lines [123]. Phenotypes linked to an increased fitness might thus be a common trait of *L. (L.) donovani* parasites that are able to overcome drug treatment. This is further supported by studies on *L. (L.) donovani* lines that were in vitro generated to be resistant to various single and combination treatment regiments, showing a generally higher competitive fitness of resistant lines compared to their wild-type [124]. These studies identified a higher promastigote survival rate in conditions of starvation, a higher tolerance to heat shock and pH stress, and an increased survival rate in in vitro macrophages [124]. Although some of these traits seemed to be absent in some drug-R lines (combinations with amphotericin-B and the MIL-R line), there is a general trend toward a fitness increase of *L. (L.) donovani* drug-R lines, even when generated in vitro.

However, what is true for one *Leishmania* species is not necessarily true for another. In the closely related *L. (L.) infantum* species, for example, studies on

in vitro-induced MIL-R lines did not reveal similar trends [125]. These strains did not display the increased metacyclogenesis rate and even showed a lower in vitro survival rate than the wild-type control, contrasting with the *L. (L.) donovani* findings described earlier [123]. The induced *L. (L.) infantum* MIL-R line showed a similar susceptibility to nitrosative stress as the wild-type control but showed a lower capacity to induce IL-10 production in in vitro-infected macrophages [125]. While it is hard to compare fitness results between species and experimental designs due to differences in protocols, some studies have compared the effect of several drugs using the same (model) system. While the in vitro-induced MIL-R line did not show a difference or a lower in vitro infection level, the same study reports that in vitro-induced paromomycin-R lines did show a better in vitro and in vivo growth at the amastigote level and a higher tolerance for nitrosative stress, without a clear influence of metacyclogenesis as defined in their setup. Induced IL-10 levels remained unchanged in paromomycin-R vs wild-type *L. (L.) infantum* lines [125]. Also, in *L. (L.) majo* made resistant in vitro to MIL, the MIL-R strain proliferated at comparable rates as wild-type parasites and exhibited similar responses regarding programmed cell death. Interestingly, metacyclogenesis was increased in MIL-resistant *L. (L.) major*, although they proved to be less virulent both in vitro and in vivo. These results thus suggest that development of experimental resistance to MIL did not lead to an increased competitive fitness in *L. (L.) major* [126].

Assessing the fitness of drug-R or treatment failure parasites reaches an even higher level of complexity in the case of American tegumentary leishmaniasis, which comprises infection of many different *Leishmania* species. Here, treatment outcome is largely affected by the infecting (tolerant) species, although it is not clear if true parasite adaptation to the drug exists [20]. In fact, as previously mentioned, *L. (V.) guyanensis*-infected patients in Peru respond better to SSG than those infected with *L. (V.) braziliensis* [106], but the opposite occurs for Brazilian patients [107]. Venezuelan *L. (L.) amazonensis*- or *L. (L.) mexicana*-infected diffuse CL patients also often show a poor response to SSG [127–130] (Chap. 8 by Zerpa et al.). Another complicating factor for New World leishmaniasis is the existence of hybrids. The analysis of *L. (V.) braziliensis-L. (V.) peruviana* hybrids suggests that they display a growth capacity (growth rate and cell density at stationary phase) similar to that of wild-type *L. (V.) peruviana* parasites but significantly lower than that of *L. (V.) braziliensis*, thus suggesting a lower fitness of the hybrids in comparison to the *L. (V.) braziliensis* wild-type parasites [131]. How these hybrids relate to parasite fitness in the context of drug resistance and treatment failure requires more research. However, it is clear that this vast variety of *Leishmania* species and their different epidemiological and genetic context in New World *Leishmania* has a major impact on treatment outcome and makes an assessment of the fitness effects of drug resistance and tolerance in New World *Leishmania* species even more complicated than for Old World *Leishmania* species.

Another factor that affects parasite fitness and treatment outcome in New World leishmaniasis is superinfection of *L. (V.) braziliensis* by the *Leishmania* RNA virus (LRV) [132]. Taylor et al. in 1998 developed a mathematical model explaining that a

lower infectivity of superparasitized parasites might exist in contrast to the potential benefit of being infected by an organism that encodes functions as resistance to antibiotics. This is common in nature as pathogens might be infected either by plasmids, viruses, or parasites [133]. *Leishmania* superinfected with LRV has been associated with failure of SSG-treatment, most likely due to RNA factors that modulate the host's immune system, ensuring survival of *L. (V.) braziliensis* and therefore also the virus it carries. Although superinfection of *Leishmania* by a virus might induce a fitness cost in the absence of drugs, it seems to result into an advantage when the patient in which it resides is being treated.

The previously discussed quiescent-like state among amastigotes could affect their drug tolerance compared to promastigotes: if the drug depends on the action of a metabolic pathway that is downregulated in a quiescent stage or if the drug enters the cell through a transporter and this transporter is downregulated in a quiescent stage, this will result in an increased tolerance to the drug for the population with a quiescent phenotype [134]. For example, *L. (L.) amazonensis* and *L. (L.) mexicana* amastigotes have shown to be more tolerant to treatment with trivalent antimonials (Sb^{III}), which enter the cell through the aquaglyceroporin 1 transporter, compared to their respective promastigotes [135]. *L. (L.) mexicana* amastigotes are also more tolerant to exposure to pentamidine, a drug which interferes with the synthesis of DNA and the morphology of kinetoplast DNA [136, 137]. Larger studies comparing the IC_{50}s of promastigotes and amastigotes should be performed in order to extend these segregated observations. From another perspective, quiescence might explain the survival of a small population of amastigotes inside the tissue that, because of their low metabolic status, are drug tolerant or indifferent even when the majority of the population is susceptible.

Host tissue niche preference may also affect treatment outcome, as drug distribution might differ between different niches, possibly resulting in sublethal or irregular drug exposure of amastigotes and apparent clinical cure of the patient. Such niches might then serve as foci from where infection can spread again and result in PKDL or MCL [20, 138], as described earlier (Chap. 8 by Zerpa et al.). The presence of other niches of infection could explain the survival of *Leishmania* despite treatment of the host, but the fact that in most of the cases the amastigotes remain in the original lesion indicates that quiescence could be an important strategy of *Leishmania* to survive the drug pressure and the immune system.

Oversimplification of the process by which drug-R lines are selected in nature sometimes leads to the difficulty by which in vitro or in vivo experimental resistance can be attained being interpreted as an argument against fitness benefits in natural drug-R lines [125]. It is important to stress that in the field, drug-R phenotypes are selected in the context of immune systems (which are different than those of common in vivo VL models), transmission through sand flies, and additional challenges for the parasite that are not present in in vitro or in vivo selection systems in the lab. When the parasite is developing drug resistance in the field, these natural bottlenecks will also serve as positive filters for those drug-R parasites that have the best combination of traits to survive all bottlenecks. This series of bottlenecks gives

the opportunity to rare variants to become successful and may result in different traits emerging in natural drugged populations compared to lab parasite populations.

15.3.2 Drug Discoveries and Control Perspectives

The treatment of leishmaniasis has long relied on drugs based on ancient compounds with known curative but also toxic effects, such as SSG. MIL and AMB, the two most recent additions to the antileishmaniasis drug arsenal, were originally developed as antineoplastic or antifungal compounds, respectively. New compounds are in the pipeline but are not likely to evolve into an actual therapy option in the next few years to come. The search for new drugs against leishmaniasis, being a neglected tropical disease, has been hampered by the lack of public and private interest ever since the parasite was discovered. However, the lack of funding that this entailed was not the only limiting factor for drug discovery projects. The intracellular lifestyle of *Leishmania* amastigotes, the only life stage that reproduces in the host, severely complicated the development of large-scale leishmaniasis drug discovery pipelines as intracellular amastigotes could not be easily cultured in vitro. Methods to grow amastigotes extracellularly (axenic amastigotes), which are to certain extents similar to the naturally occurring intracellular amastigotes, have been developed and further optimization of these culture protocols recently allowed high-throughput screening with a high predictability of leishmanicidal intracellular activity [139]. In addition, recent efforts developed an in vitro model that allowed replication of actual intracellular amastigotes in THP-1 cells [140], providing a model that is much closer to natural infections than the axenic models, allowing *Leishmania* to grow intracellularly, invade new host cells, etc. This intracellular model may therefore also allow assessing these fitness determinants at a higher throughput. Evaluating the fitness of natural wild-type parasites and parasites resistant to experimental compounds can provide a better insight into the effect that introducing a drug in a certain geographical context may have on the local parasite population and the spread of a possible resistant phenotype. Such studies are rarely performed at early stages of leishmaniasis drug discovery but could now be encouraged by the development of such higher-throughput assays.

All monotherapies but one, AMB, have succumbed to a rise in treatment failure rates several years after their introduction. This is due to treatment failure-inducing parasites having a competitive fitness over wild-type parasites when under treatment. This not only entails that they continue to replicate in the host in the presence of the drug, but also that they are able to spread to the vector, undergo the different promastigote development stages, and eventually infect new hosts. A better knowledge on the factors important for parasite fitness in both the mammalian host and the vector might contribute to the development of innovative treatment regimens that disturb this fitness advantage of resistant parasites in a parasite population under treatment. One could consider treating patients with a combination of one or more drugs that aim to cure the patient and one other compound that has the sole purpose of allowing easy emergence of a specific resistance mechanism that induces a fitness

defect at the level of promastigote development in parasites that somehow survived exposure to the curative partner drug. This would prevent drug-R parasites to undergo full development in the vector, impeding their transmission to new hosts and preventing the spread of drug-R parasites. This is of course easier said than done, as it requires the identification of factors that are important for parasite development in both the host and the vector and subsequently the identification of a chemical compound able to induce a specific genetic change in the parasite that results in resistance in the mammalian host and impedes promastigote development in the vector. Nevertheless, innovative treatment schemes such as these exploit the parasite's ability to become drug-R but provide the benefit of prolonging the life span of the other drugs that are part of the combination treatment regimen. Designing more treatment schemes that directly affect parasite fitness in wild-type and drug-R parasites may be a way forward in rational drug design pipelines. In the *Leishmania* field, the importance of studying drug-R parasites in drug discovery projects has only recently gained more attention [141].

However, rational drug design and drug use are only two of several important aspects in leishmaniasis control. As such, the Kala-azar Elimination Program in the Indian subcontinent relied on early diagnosis, adequate treatment, and vector control. While early diagnosis and adequate treatment are pivotal to cure patients, mathematical modeling has shown that it has only little effect on eventual control of the disease at the population level, i.e., reducing infection incidence [120]. Building further upon this mathematical model, studies have estimated that 10 years of sustained suboptimal insecticidal residual spraying would be required to reach the VL elimination goal [142]. These studies highlight that transmission is a major contributor to the fitness of *Leishmania* and emphasize the importance of affecting parasite development in the vector, either by killing the vector itself or by preventing parasite development in this vector. A better understanding of the fitness factors related to promastigote development and how they can be affected may thus provide powerful new tools for leishmaniasis control.

15.4 Conclusion

Leishmania is a parasite with remarkable adaptive skills, posing major challenges for its control in endemic areas. Fitness of drug-R versus drug-S parasites plays an important role in shaping future parasite populations, and understanding the processes involved is pivotal to allow the design of new treatment strategies that defy the parasite's capacity to render new drugs useless through the development of drug resistance. It is encouraging that fitness studies are more and more performed in the context of drug resistance. However, it is often difficult to compare results between species or even between studies on the same species due to varying epidemiological and genetic contexts. Current advances in genome editing (CRISPR/Cas9) in combination with a detailed knowledge of resistance mechanisms should now allow to create genetically paired clinical isolates in the lab with and without these resistance determinants. This is well exemplified by a combinatorial genetic modeling study

that focused on a quadruple *Plasmodium* mutant resistant to chloroquine [143]. Through the creation of a battery of genetically engineered mutants, fitness studies on each of these and implementation of all data into a mathematical model, the mutational trajectory that led to this successful mutant could be reconstructed. Comparing such genetic mutants or revertants with their wild types will provide more insight into the exact fitness consequences of the phenotype, how it might have evolved and allow a more straightforward comparison of results obtained in different systems.

References

1. Bates M, Wrin T, Huang W, Petropoulos C, et al. Practical applications of viral fitness in clinical practice. Curr Opin Infect Dis. 2003;16:11–8.
2. Geretti AM. The clinical significance of viral fitness. J HIV Ther. 2005;10:6–10.
3. Quiñones-Mateu ME, Arts EJ. Virus fitness: concept, quantification, and application to HIV population dynamics. Curr Top Microbiol Immunol. 2006;299:83–140.
4. Andino R, Domingo E. Viral quasispecies. Virology. 2015;479–480:46–51.
5. Natera S, Machuca C, Padrón-Nieves M, Romero A, et al. *Leishmania* spp.: proficiency of drug-resistant parasites. Int J Antimicrob Agents. 2007;29:637–42.
6. Vanaerschot M, Decuypere S, Berg M, Roy S, et al. Drug-resistant microorganisms with a higher fitness – can medicines boost pathogens? Crit Rev. Microbiol. 2012;39:1–11.
7. Semenza JC, Rocklöv J, Penttinen P, Lindgren E. Observed and projected drivers of emerging infectious diseases in Europe. Ann N Y Acad Sci. 2016;1382:73–83.
8. Debrabant A, Nakhasi H. Programmed cell death in trypanosomatids: is it an altruistic mechanism for survival of the fittest? Kinetoplastid Biol Dis. 2003;2:7.
9. Gollob KJ, Viana AG, Dutra WO. Immunoregulation in human American leishmaniasis: balancing pathology and protection. Parasite Immunol. 2014;36:367–76.
10. Kima PE. The amastigote forms of *Leishmania* are experts at exploiting host cell processes to establish infection and persist. Int J Parasitol. 2007;37:1087–96.
11. El-Hani C, Borges VM, Wanderley JLM, Barcinski MA. Apoptosis and apoptotic mimicry in *Leishmania*: an evolutionary perspective. Front Cell Infect Microbiol. 2012;2:96.
12. Bogdan C, Röllinghoff M. The immune response to *Leishmania*: mechanisms of parasite control and evasion. Int J Parasitol. 1998;28:121–34.
13. Boggiatto PM, Jie F, Ghosh M, Gibson-Corley KN, et al. Altered dendritic cell phenotype in response to *Leishmania amazonensis* amastigote infection is mediated by MAP kinase, ERK. Am J Pathol. 2009;174:1818–26.
14. Xin L, Li K, Soong L. Down-regulation of dendritic cell signaling pathways by *Leishmania amazonensis* amastigotes. Mol Immunol. 2008;45:3371–82.
15. Van Assche T, Deschacht M, da Luz RAI, Maes L, et al. *Leishmania*–macrophage interactions: Insights into the redox biology. Free Radic Biol Med. 2011;51:337–51.
16. Matte C, Descoteaux A. *Leishmania donovani* amastigotes impair gamma interferon-induced STAT1alpha nuclear translocation by blocking the interaction between STAT1alpha and importin-alpha5. Infect Immun. 2010;78:3736–43.
17. Abu-Dayyeh I, Hassani K, Westra ER, Mottram JC, et al. Comparative study of the ability of *Leishmania mexicana* promastigotes and amastigotes to alter macrophage signaling and functions. Infect Immun. 2010;78:2438–45.
18. Ruhland A, Kima PE. Activation of PI3K/Akt signaling has a dominant negative effect on IL-12 production by macrophages infected with *Leishmania amazonensis* promastigotes. Exp Parasitol. 2009;122:28–36.

19. Rai AK, Thakur CP, Singh A, Seth T, et al. Regulatory T cells suppress T cell activation at the pathologic site of human visceral leishmaniasis. PLoS One. 2012;7:e31551.
20. Vanaerschot M, Dumetz F, Roy S, Ponte-Sucre A, et al. Treatment failure in leishmaniasis: drug-resistance or another (epi-) phenotype? Expert Rev Anti Infect Ther. 2014;12:937–46.
21. Ghosh M, Roy K, Roy S. Immunomodulatory effects of antileishmanial drugs. J Antimicrob Chemother. 2013;68:2834–8.
22. David Sibley L. Invasion and intracellular survival by protozoan parasites. Immunol Rev. 2011;240:72–91.
23. Guevara P, Rojas E, Gonzalez N, Scorza JV, et al. Presence of *Leishmania braziliensis* in blood samples from cured patients or at different stages of immunotherapy. Clin Diagn Lab Immunol. 1994;1:385–9.
24. Veland N, Espinosa D, Valencia BM, Ramos AP, et al. Polymerase chain reaction detection of *Leishmania* kDNA from the urine of Peruvian patients with cutaneous and mucocutaneous leishmaniasis. Am J Trop Med Hyg. 2011;84:556–61.
25. Figueroa RA, Lozano LE, Romero IC, Cardona MT, et al. Detection of *Leishmania* in unaffected mucosal tissues of patients with cutaneous leishmaniasis caused by *Leishmania* (*Viannia*) species. J Infect Dis. 2009;200:638–46.
26. Prina E, Roux E, Mattei D, Milon G. *Leishmania* DNA is rapidly degraded following parasite death: an analysis by microscopy and real-time PCR. Microbes Infect. 2007;9:1307–15.
27. Deborggraeve S, Boelaert M, Rijal S, De Doncker S, et al. Diagnostic accuracy of a new *Leishmania* PCR for clinical visceral leishmaniasis in Nepal and its role in diagnosis of disease. Trop Med Int Heal. 2008;13:1378–83.
28. Mukhopadhyay D, Dalton JE, Kaye PM, Chatterjee M. Post kala-azar dermal leishmaniasis: an unresolved mystery. Trends Parasitol. 2014;30:65–74.
29. Bogdan C, Donhauser N, Döring R, Röllinghoff M, et al. Fibroblasts as host cells in latent leishmaniosis. J Exp Med. 2000;191:2121–30.
30. Kloehn J, Saunders EC, O'Callaghan S, Dagley MJ, et al. Characterization of metabolically quiescent *Leishmania* parasites in murine lesions using heavy water labeling. PLOS Pathog. 2015;11:e1004683.
31. Alcolea PJ, Alonso A, Gómez MJ, Moreno I, et al. Transcriptomics throughout the life cycle of *Leishmania infantum*: High down-regulation rate in the amastigote stage. Int J Parasitol. 2010;40:1497–516.
32. Michel G, Ferrua B, Lang T, Maddugoda MP, et al. Luciferase-expressing *Leishmania infantum* allows the monitoring of amastigote population size, in vivo, ex vivo and in vitro. PLoS Negl Trop Dis. 2011;5:e1323.
33. Biyani N, Madhubala R. Quantitative proteomic profiling of the promastigotes and the intracellular amastigotes of *Leishmania donovani* isolates identifies novel proteins having a role in *Leishmania* differentiation and intracellular survival. Biochim Biophys Acta. 2012;1824:1342–50.
34. Cloutier S, Laverdière M, Chou M-N, Boilard N, et al. (2012) Translational control through eIF2alpha phosphorylation during the *Leishmania* differentiation process. PLoS One 7: e35085.
35. Saunders EC, Ng WW, Kloehn J, Chambers JM, et al. Induction of a stringent metabolic response in intracellular stages of *Leishmania mexicana* leads to increased dependence on mitochondrial metabolism. PLoS Pathog. 2014;10:e1003888.
36. Mondal S, Roy JJ, Bera T. Characterization of mitochondrial bioenergetic functions between two forms of *Leishmania donovani* – a comparative analysis. J Bioenerg Biomembr. 2014;46:395–402.
37. Mandell MA, Beverley SM. Continual renewal and replication of persistent *Leishmania major* parasites in concomitantly immune hosts. Proc Natl Acad Sci USA. 2017;114:E801–10.
38. Dillon RJ, Ivens AC, Churcher C, Holroyd N, et al. Analysis of ESTs from *Lutzomyia longipalpis* sand flies and their contribution toward understanding the insect-parasite relationship. Genomics. 2006;88:831–40.

39. Diaz E, Zacarias AK, Pérez S, Vanegas O, et al. Effect of aliphatic, monocarboxylic, dicarboxylic, heterocyclic and sulphur-containing amino acids on *Leishmania* spp. chemotaxis. Parasitology. 2015;142:1621–30.
40. Schlein Y, Jacobson RL, Shlomai J. Chitinase secreted by *Leishmania* functions in the sandfly vector. Proceedings Biol Sci. 1991;245:121–6.
41. Sacks DL, Melby PC. Animal models for the analysis of immune responses to leishmaniasis. Curr Protoc Immunol. 2001. https://doi.org/10.1002/0471142735.im1902s108.
42. da Silva R, Sacks DL. Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. Infect Immun. 1987;55:2802–6.
43. Rogers ME, Chance ML, Bates PA. The role of promastigote secretory gel in the origin and transmission of the infective stage of *Leishmania mexicana* by the sandfly *Lutzomyia longipalpis*. Parasitology. 2002;124:495–507.
44. Bates PA. Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. Int J Parasitol. 2007;37:1097–106.
45. Aslan H, Dey R, Meneses C, Castrovinci P, et al. A new model of progressive visceral leishmaniasis in hamsters by natural transmission via bites of vector sand flies. J Infect Dis. 2013;207:1328–38.
46. Barrett MP, Burchmore RJ, Stich A, Lazzari JO, et al. The trypanosomiases. Lancet. 2003;362:1469–80.
47. Sunkin SM, Kiser P, Myler PJ, Stuart K. The size difference between *Leishmania major* friedlin chromosome one homologues is localized to sub-telomeric repeats at one chromosomal end. Mol Biochem Parasitol. 2000;109:1–15.
48. Johnson PJ, Kooter JM, Borst P. Inactivation of transcription by UV irradiation of *T. brucei* provides evidence for a multicistronic transcription unit including a VSG gene. Cell. 1987;51:273–81.
49. Fairlamb AH, Blackburn P, Ulrich P, Chait BT, et al. Trypanothione: a novel bis(glutathionyl) spermidine cofactor for glutathione reductase in trypanosomatids. Science. 1985;227:1485–7.
50. Mukhopadhyay R, Dey S, Xu N, Gage D, et al. Trypanothione overproduction and resistance to antimonials and arsenicals in *Leishmania*. Proc Natl Acad Sci U S A. 1996;93:10383–7.
51. Krauth-Siegel RL, Meiering SK, Schmidt H. The Parasite-Specific Trypanothione Metabolism of *Trypanosoma* and *Leishmania*. Biol Chem. 2003;384:539–49.
52. Sterkers Y, Lachaud L, Crobu L, Bastien P, et al. FISH analysis reveals aneuploidy and continual generation of chromosomal mosaicism in *Leishmania major*. Cell Microbiol. 2011;13:274–83.
53. Sterkers Y, Lachaud L, Bourgeois N, Crobu L, et al. Novel insights into genome plasticity in Eukaryotes: mosaic aneuploidy in *Leishmania*. Mol Microbiol. 2012;86:15–23.
54. Lachaud L, Bourgeois N, Kuk N, Morelle C, et al. Constitutive mosaic aneuploidy is a unique genetic feature widespread in the *Leishmania* genus. Microbes Infect. 2013:2–7.
55. Prieto-Barja P, Pesher P, Bussotti G, Dumetz F, et al. Haplotype selection as an adaptive mechanism in the protozoan pathogen *Leishmania donovani*. Nat Ecol Evol. 2017;1:1961–9.
56. Leprohon P, Légaré D, Raymond F, Madore E, et al. Gene expression modulation is associated with gene amplification, supernumerary chromosomes and chromosome loss in antimony-resistant *Leishmania infantum*. Nucleic Acids Res. 2009;37:1387–99.
57. Shaw CD, Lonchamp J, Downing T, Imamura H, et al. In vitro selection of miltefosine resistance in promastigotes of *Leishmania donovani* from Nepal: genomic and metabolomic characterization. Mol Microbiol. 2016;99:1134–48.
58. Ubeda J-M, Raymond F, Mukherjee A, Plourde M, et al. Genome-wide stochastic adaptive DNA amplification at direct and inverted DNA repeats in the parasite *Leishmania*. PLoS Biol. 2014;12:e1001868.
59. Imamura H, Downing T, Van den Broeck F, Sanders MJ, et al. Evolutionary genomics of epidemic visceral leishmaniasis in the Indian subcontinent. Elife. 2016;5:1–39.

60. Monte-Neto R, Laffitte M-CN, Leprohon P, Reis P, et al. Intrachromosomal amplification, locus deletion and point mutation in the aquaglyceroporin AQP1 gene in antimony resistant *Leishmania* (*Viannia*) *guyanensis*. PLoS Negl Trop Dis. 2015;9:e0003476.
61. Grondin K, Papadopoulou B, Ouellette M. Homologous recombination between direct repeat sequences yields P-glycoprotein containing amplicons in arsenite resistant *Leishmania*. Nucleic Acids Res. 1993;21:1895–901.
62. Moreira DS, Monte Neto RL, Andrade JM, Santi AMM, et al. Molecular characterization of the MRPA transporter and antimony uptake in four New World *Leishmania* spp. susceptible and resistant to antimony. Int J Parasitol Drugs Drug Resist. 2013;3:143–53.
63. Brotherton M-C, Bourassa S, Leprohon P, Légaré D, et al. Proteomic and genomic analyses of antimony resistant *Leishmania infantum* mutant. PLoS One. 2013;8:e81899.
64. Singh A, Papadopoulou B, Ouellette M. Gene amplification in amphotericin B-resistant *Leishmania tarentolae*. Exp Parasitol. 2001;99:141–7.
65. Papadopoulou B, Roy G, Ouellette M. Frequent amplification of a short chain dehydrogenase gene as part of circular and linear amplicons in methotrexate resistant *Leishmania*. Nucleic Acids Res. 1993;21:4305–12.
66. Grondin K, Roy G, Ouellette M. Formation of extrachromosomal circular amplicons with direct or inverted duplications in drug-resistant *Leishmania tarentolae*. Mol Cell Biol. 1996;16:3587–95.
67. Ubeda J-M, Légaré D, Raymond F, Ouameur AA, et al. Modulation of gene expression in drug resistant *Leishmania* is associated with gene amplification, gene deletion and chromosome aneuploidy. Genome Biol. 2008;9:R115.
68. Ritt J-F, Raymond F, Leprohon P, Légaré D, et al. Gene amplification and point mutations in pyrimidine metabolic genes in 5-fluorouracil resistant *Leishmania infantum*. PLoS Negl Trop Dis. 2013;7:e2564.
69. Kumar P, Lodge R, Raymond F, Ritt J-F, et al. Gene expression modulation and the molecular mechanisms involved in Nelfinavir resistance in *Leishmania donovani* axenic amastigotes. Mol Microbiol. 2013:1–18.
70. Berg M, Vanaerschot M, Jankevics A, Cuypers B, et al. Metabolic adaptations of *Leishmania donovani* in relation to differentiation, drug resistance, and drug pressure. Mol Microbiol. 2013;90:428–42.
71. Ariyanayagam MR, Fairlamb AH. Ovothiol and trypanothione as antioxidants in trypanosomatids. Mol Biochem Parasitol. 2001;115:189–98.
72. Bocedi A, Dawood KF, Fabrini R, Federici G, et al. (2010) Trypanothione efficiently intercepts nitric oxide as a harmless iron complex in trypanosomatid parasites. FASEB J 24:1035–1042.
73. Romão PRT, Tovar J, Fonseca SG, Moraes RH, et al. Glutathione and the redox control system trypanothione/trypanothione reductase are involved in the protection of *Leishmania* spp. against nitrosothiol-induced cytotoxicity. Brazilian J Med Biol Res Rev. 2006;39:355–63.
74. Piñeyro MD, Arcari T, Robello C, Radi R, et al. Tryparedoxin peroxidases from Trypanosoma cruzi: High efficiency in the catalytic elimination of hydrogen peroxide and peroxynitrite. Arch Biochem Biophys. 2011;507:287–95.
75. Flohé L, Budde H, Bruns K, Castro H, et al. Tryparedoxin peroxidase of *Leishmania donovani*: molecular cloning, heterologous expression, specificity, and catalytic mechanism. Arch Biochem Biophys. 2002;397:324–35.
76. Alvarez MN, Peluffo G, Piacenza L, Radi R. Intraphagosomal peroxynitrite as a macrophage-derived cytotoxin against internalized Trypanosoma cruzi: consequences for oxidative killing and role of microbial peroxiredoxins in infectivity. J Biol Chem. 2011;286:6627–40.
77. Iyer JP, Kaprakkaden A, Choudhary ML, Shaha C. Crucial role of cytosolic tryparedoxin peroxidase in *Leishmania donovani* survival, drug response and virulence. Mol Microbiol. 2008;68:372–91.

78. Dumas C, Ouellette M, Tovar J, Cunningham ML, et al. Disruption of the trypanothione reductase gene of *Leishmania* decreases its ability to survive oxidative stress in macrophages. EMBO J. 1997;16:2590–8.
79. Tovar J, Wilkinson S, Mottram JC, Fairlamb AH. Evidence that trypanothione reductase is an essential enzyme in *Leishmania* by targeted replacement of the tryA gene locus. Mol Microbiol. 1998;29:653–60.
80. Tovar J, Cunningham ML, Smith AC, Croft SL, et al. Down-regulation of *Leishmania donovani* trypanothione reductase by heterologous expression of a trans-dominant mutant homologue: effect on parasite intracellular survival. Proc Natl Acad Sci USA. 1998;95:5311–6.
81. Rojo D, Canuto GAB, Castilho-Martins EA, Tavares MFM, et al. A multiplatform metabolomic approach to the basis of antimonial action and resistance in *Leishmania infantum*. PLoS One. 2015;10:1–20.
82. Berg M, Garcia-Hernandez R, Cuypers B, Vanaerschot M, et al. Experimental resistance to drug combinations in *Leishmania donovani*: Metabolic and phenotypic adaptations. Antimicrob Agents Chemother. 2015;59:2242–55.
83. Decuypere S, Rijal S, Yardley V, De Doncker S, et al. Gene expression analysis of the mechanism of natural Sb(V) resistance in *Leishmania donovani* isolates from Nepal. Antimicrob Agents Chemother. 2005;49:4616–21.
84. Decuypere S, Vanaerschot M, Brunker K, Imamura H, et al. Molecular mechanisms of drug resistance in natural *Leishmania* populations vary with genetic background. PLoS Negl Trop Dis. 2012;6:e1514.
85. Gómez Pérez V, García-Hernandez R, Corpas-López V, Tomás AM, et al. Decreased antimony uptake and overexpression of genes of thiol metabolism are associated with drug resistance in a canine isolate of *Leishmania infantum*. Int J Parasitol Drugs Drug Resist. 2016;6:133–9.
86. Canuto GAB, Castilho-Martins EA, Tavares MFM, Rivas L, et al. Multi-analytical platform metabolomic approach to study miltefosine mechanism of action and resistance in *Leishmania*. Anal Bioanal Chem. 2014;406:3459–76.
87. t'Kindt R, R a S, Jankevics A, Brunker K, et al. Metabolomics to unveil and understand phenotypic diversity between pathogen populations. PLoS Negl Trop Dis. 2010;e904:4.
88. Oryan A, Shirian S, Tabandeh M-R, Hatam G-R, et al. Genetic diversity of *Leishmania major* strains isolated from different clinical forms of cutaneous leishmaniasis in southern Iran based on minicircle kDNA. Infect Genet Evol. 2013;19:226–31.
89. CoreWriting Team, Pachauri RK. Climate Change 2014: Synthesis report contributions of working groups I, II and III to the fifth assessment report of the IPCC. 2014.
90. Brunner FS, Eizaguirre C. Can environmental change affect host/parasite-mediated speciation? Zoology. 2016;119:384–94.
91. Thomas CD, Cameron A, Green RE, Bakkenes M, et al. Extinction risk from climate change. Nature. 2004;427:145–8.
92. Kutz SJ, Hoberg EP, Polley L, Jenkins EJ. Global warming is changing the dynamics of Arctic host-parasite systems. Proc Biol Sci. 2005;272:2571–6.
93. Larsen MH, Mouritsen KN. Temperature–parasitism synergy alters intertidal soft-bottom community structure. J Exp Mar Bio Ecol. 2014;460:109–19.
94. Mitchell SE, Rogers ES, Little TJ, Read AF. Host-parasite and genotype-by-environment interactions: temperature modifies potential for selection by a sterilizing pathogen. Evolution. 2005;59:70–80.
95. Macnab V, Barber I. Some (worms) like it hot: fish parasites grow faster in warmer water, and alter host thermal preferences. Glob Chang Biol. 2012;18:1540–8.
96. Scharsack JP, Schweyen H, Schmidt AM, Dittmar J, et al. Population genetic dynamics of three-spined sticklebacks (*Gasterosteus aculeatus*) in anthropogenic altered habitats. Ecol Evol. 2012;2:1122–43.

97. Jiménez M, Alvar J, Tibayrenc M. *Leishmania infantum* is clonal in AIDS patients too: epidemiological implications. AIDS. 1997;11:569–73.
98. Rosenthal E, Marty P, Poizot-Martin I, Reynes J, et al. Visceral leishmaniasis and HIV-1 co-infection in southern France. Trans R Soc Trop Med Hyg. 1995;89:159–62.
99. Lopez-Velez R, Perez-Molina JA, Guerrero A, Baquero F, et al. Clinico epidemiologic characteristics, prognostic factors, and survival analysis of patients coinfected with human immunodeficiency virus and *Leishmania* in an area of Madrid, Spain. Am J Trop Med Hyg. 1998;58:436–43.
100. Bryceson AD, Chulay JD, Ho M, Mugambii M, et al. Visceral leishmaniasis unresponsive to antimonial drugs. I. Clinical and immunological studies. Trans R Soc Trop Med Hyg. 1985;79:700–4.
101. Davidson RN, Di Martino L, Gradoni L, Giacchino R, et al. Liposomal amphotericin B (AmBisome) in Mediterranean visceral leishmaniasis: a multi-centre trial. Q J Med. 1994;87:75–81.
102. Bryceson A. Current issues in the treatment of visceral leishmaniasis. Med Microbiol Immunol. 2001;190:81–4.
103. Ives A, Ronet C, Prevel F, Ruzzante G, Fuertes-Marraco S, et al. *Leishmania* RNA virus controls the severity of mucocutaneous leishmaniasis. Science. 2011;331(6018):775–8.
104. Cantanhêde LM, da Silva Júnior CF, Ito MM, Felipin KP, et al. Further evidence of an association between the presence of *Leishmania* RNA virus 1 and the mucosal manifestations in tegumentary leishmaniasis patients. PLoS Negl Trop Dis. 2015;9:e0004079.
105. Adaui V, Lye L-F, Akopyants NS, Zimic M, et al. Association of the endobiont double-stranded RNA virus LRV1 with treatment failure for human leishmaniasis caused by *Leishmania braziliensis* in Peru and Bolivia. J Infect Dis. 2016;213:112–21.
106. Arevalo J, Ramirez L, Adaui V, Zimic M, et al. Influence of *Leishmania* (*Viannia*) species on the response to antimonial treatment in patients with American tegumentary leishmaniasis. J Infect Dis. 2007;195:1846–51.
107. Romero GA, Guerra MV, Paes MG, Macêdo VO. Comparison of cutaneous leishmaniasis due to *Leishmania* (*Viannia*) *braziliensis* and *L.* (*V.*) *guyanensis* in Brazil: therapeutic response to meglumine antimoniate. Am J Trop Med Hyg. 2001;65:456–65.
108. Zerpa O, Convit J. Diffuse cutaneous leishmaniasis in Venezuela. Gaz méd Bahia. 2009;79:30–4.
109. Goto H, Lindoso JAL. Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. Expert Rev Anti Infect Ther. 2010;8:419–33.
110. Ponte-Sucre A, Diaz E, Padrón-Nieves M. The concept of fitness and drug resistance in *Leishmania*. In: Ponte-Sucre A, Diaz E, Padrón-Nieves M, editors. Drug Resist. *Leishmania* parasites, Consequences, molecular mechanisms and possible treatments. Vienna: Springer; 2013. p. 431–49.
111. Newton PN, Green MD, Fernández FM. Impact of poor-quality medicines in the "developing" world. Trends Pharmacol Sci. 2010;31:99–101.
112. de Mello CX, de Oliveira Schubach A, de Oliveira RVC, Conceição-Silva F, et al. Comparison of the sensitivity of imprint and scraping techniques in the diagnosis of American tegumentary leishmaniasis in a referral centre in Rio de Janeiro, Brazil. Parasitol Res. 2011;109:927–33.
113. Andersson DI, Hughes D. Antibiotic resistance and its cost: is it possible to reverse resistance? Nat Rev Microbiol. 2010;8:260–71.
114. Melnyk AH, Wong A, Kassen R. The fitness costs of antibiotic resistance mutations. Evol Appl. 2015;8:273–83.
115. Huijben S, Bell AS, Sim DG, Tomasello D, et al. Aggressive chemotherapy and the selection of drug resistant pathogens. PLoS Pathog. 2013;9:e1003578.
116. Ouakad M, Vanaerschot M, Rijal S, Sundar S, et al. Increased metacyclogenesis of antimony-resistant *Leishmania donovani* clinical lines. Parasitology. 2011;138:1392–9.
117. Vanaerschot M, Maes I, Ouakad M, Adaui V, et al. Linking in vitro and in vivo survival of clinical *Leishmania donovani* strains. PLoS One. 2010;5:e12211.

118. Vanaerschot M, de Doncker S, Rijal S, Maes L, et al. Antimonial resistance in *Leishmania donovani* is associated with increased in vivo parasite burden. PLoS One. 2011;6:e23120.
119. Imamura H, Downing T, Van den Broeck F, Sanders MJ, et al. Evolutionary genomics of epidemic visceral leishmaniasis in the Indian subcontinent. Elife. 2016;5:e12613. https://doi.org/10.7554/eLife.12613.
120. Stauch A, Sarkar RR, Picado A, Ostyn B, et al. Visceral leishmaniasis in the Indian subcontinent: modelling epidemiology and control. PLoS Negl Trop Dis. 2011;5:e1405.
121. Stauch A, Duerr HP, Dujardin JC, Vanaerschot M, et al. Treatment of visceral leishmaniasis: model-based analyses on the spread of antimony-resistant *L. donovani* in Bihar, India. PLoS Negl Trop Dis. 2012;6(12):e1973.
122. Rai K, Bhattarai NR, Vanaerschot M, Imamura H, et al. Single locus genotyping to track *Leishmania donovani* in the Indian subcontinent: Application in Nepal. PLoS Negl Trop Dis. 2017;11:e0005420.
123. Rai K, Cuypers B, Bhattarai NR, Uranw S, et al. Relapse after treatment with Miltefosine for visceral Leishmaniasis is associated with increased infectivity of the infecting *Leishmania donovani* strain. MBio. 2013;4:e00611-13.
124. García-Hernández R, Gómez-Pérez V, Castanys S, Gamarro F. Fitness of *Leishmania donovani* parasites resistant to drug combinations. PLoS Negl Trop Dis. 2015;9:e0003704.
125. Hendrickx S, Beyers J, Mondelaers A, Eberhardt E, et al. Evidence of a drug-specific impact of experimentally selected paromomycin and miltefosine resistance on parasite fitness in *Leishmania infantum*. J Antimicrob Chemother. 2016;71:1914–21.
126. Turner KG, Vacchina P, Robles-Murguia M, Wadsworth M, et al. Fitness and phenotypic characterization of Miltefosine-resistant *Leishmania major*. PLoS Negl Trop Dis. 2015;9:e0003948.
127. Padrón-Nieves M, Machuca C, Díaz E, Cotrim P, et al. Correlation between glucose uptake and membrane potential in *Leishmania* parasites isolated from DCL patients with therapeutic failure: a proof of concept. Parasitol Res. 2014;113:2121–8.
128. Ponte-Sucre A. Leishmaniasis, the biology of a parasite. In: Ponte-Sucre A, Padron Nieves M, editors. Drug Resist. *Leishmania* parasites, Consequences, molecular mechanisms and possible treatments. Vienna: Springer; 2013. p. 1–12.
129. Padron-Nieves M, Ponte-Sucre A. Marcadores de resistencia en *Leishmania*: susceptibilidad in vitro a drogas leishmanicidas vs. retencion de calceina en aislados de pacientes venezolanos con leishmaniasis cutanea difusa. Arch Venez Farmacol y Ter. 2015;32:29–33.
130. Zerpa O, Ulrich M, Blanco B, Polegre M, et al. Diffuse cutaneous leishmaniasis responds to miltefosine but then relapses. Br J Dermatol. 2007;156:1328–35.
131. Torrico MC, De Doncker S, Arevalo J, Le Ray D, et al. In vitro promastigote fitness of putative *Leishmania* (*Viannia*) *braziliensis*/*Leishmania* (Viannia) *peruviana* hybrids. Acta Trop. 1999;72:99–110.
132. Hartley M-A, Ronet C, Zangger H, Beverley SM, et al. *Leishmania* RNA virus: when the host pays the toll. Front Cell Infect Microbiol. 2012;2:99.
133. Taylor DR, Jarosz AM, Fulbright DW, Lenski RE. The acquisition of hypovirulence in host-pathogen systems with three trophic levels. Am Nat. 1998;151:343–55.
134. Maisonneuve E, Gerdes K. Molecular mechanisms underlying bacterial persisters. Cell. 2014;157:539–48.
135. Sereno D, Lemesre JL. Axenically cultured amastigote forms as an in vitro model for investigation of antileishmanial agents. Antimicrob Agents Chemother. 1997;41:972–6.
136. Callahan HL, Portal AC, Devereaux R, Grogl M. An axenic amastigote system for drug screening. Antimicrob Agents Chemother. 1997;41:818–22.
137. Kaur G, Rajput B. Comparative analysis of the omics technologies used to study antimonial, amphotericin B, and pentamidine resistance in *Leishmania*. J Parasitol Res. 2014;2014:1–11.
138. Saravia NG, Weigle K, Segura I, Giannini SH, et al. Recurrent lesions in human *Leishmania braziliensis* infection--reactivation or reinfection? Lancet (London, England). 1990;336:398–402.

139. Nühs A, De Rycker M, Manthri S, Comer E, et al. Development and validation of a novel *Leishmania donovani* screening cascade for high-throughput screening using a novel axenic assay with high predictivity of leishmanicidal intracellular activity. PLoS Negl Trop Dis. 2015;9:e0004094.
140. Tegazzini D, Díaz R, Aguilar F, Peña I, et al. A replicative in vitro assay for drug discovery against *Leishmania donovani*. Antimicrob Agents Chemother. 2016;60:3524–32.
141. Hefnawy A, Berg M, Dujardin J-C, De Muylder G. Exploiting knowledge on *Leishmania* drug resistance to support the quest for new drugs. Trends Parasitol. 2017;33:162–74.
142. Le Rutte EA, Coffeng LE, Bontje DM, Hasker EC, et al. Feasibility of eliminating visceral leishmaniasis from the Indian subcontinent: explorations with a set of deterministic age-structured transmission models. Parasit Vectors. 2016;9:24.
143. Gabryszewski SJ, Modchang C, Musset L, Chookajorn T, et al. Combinatorial genetic modeling of pfcrt -mediated drug resistance evolution in *Plasmodium falciparum*. Mol Biol Evol. 2016;33:1554–70.