EXPERT REVIEWS

Treatment failure in leishmaniasis: drug-resistance or another (epi-) phenotype?

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Two major leishmaniasis treatments have shown a significant decrease in effectiveness in the last few decades, mostly in the Indian subcontinent but also in other endemic areas. Drug resistance of *Leishmania* correlated only partially to treatment failure (TF) of pentavalent antimonials, and has so far proved not to be important for the increased miltefosine relapse rates observed in the Indian subcontinent. While other patient- or drug-related factors could also have played a role, recent studies identified several parasite features such as infectivity and host manipulation skills that might contribute to TF. This perspective aims to discuss how different parasitic features other than drug resistance can contribute to TF of leishmaniasis and how this may vary between different epidemiological contexts.

Leishmania is a kinetoplastid parasite that can cause a spectrum of different clinical forms of leishmaniasis, ranging from self-resolving cutaneous leishmaniasis (CL), disfiguring mucocutaneous leishmaniasis (MCL), to fatal visceral leishmaniasis (VL). The species involved vary greatly between geographical regions: from the Indian subcontinent (ISC) where the viscerotropic species *Leishmania donovani* is predominantly present to Latin America where several *Leishmania* species causing VL, CL and MCL can coexist [1]. Noteworthy, in many cases infections remain asymptomatic [2].

The parasites are transmitted between vertebrate hosts by sand flies in which they develop as an extracellular flagellated form (the promastigote). After the bite of the sand fly, highly infective metacyclic promastigotes will successfully colonize host cells of the reticuloendothelial system and transform into the obligate intracellular amastigote form. Control of leishmaniasis relies mainly on vector control, early diagnosis and adequate treatment. Especially the latter is a tough nut to crack, since *Leishmania* are masters in adaptation.

Pentavalent antimonials (SSG) were long the only antileishmanial agent available but showed a progressively decreasing efficacy. This was especially true in the ISC (more particularly Bihar), where dosages were increased in several rounds until the maximum acceptable toxicity was reached. But even after these measures, treatment failure (TF) rates exceeded 60% in high endemic areas at the end of the 1990s (reviewed in [3]). Around the same time, the highly efficacious amphotericin B (AMB) was officially introduced, and its safer liposomal formulation continues to yield high cure rates up to now [4]. In the Kala-Azar Elimination Program – a joint effort between India, Nepal and Bangladesh to reach VL elimination by 2015 – the oral drug miltefosine (MIL) was chosen as the first-line treatment as it is easier to administer and induces less severe side effects [5]. However, MIL is teratogenic and should therefore not be prescribed to women of childbearing age who cannot guarantee contraception during and at least 2 months after treatment [6]. In addition, recent reports showed a decreased efficacy of MIL, with relapse rates of up to 20% [7,8].

In Africa, the duration of VL treatment has been significantly reduced without a loss of efficacy from 30 days of SSG monotherapy to 17 days of SSG when administered in combination with paromomycine [9]. However, there are worrying reports on an increasing unresponsiveness to AMB in Sudan [10]. The high HIV prevalence in Africa should most definitively be taken into account when studying treatment outcome, since drugs may have a

Table 1. Parasitic factors other than direct drug resistance that may contribute to treatment failure in leishmaniasis.

	Mechanism	Example
Species-specific drug tolerance	Inherent genetic and metabolic diversity	American tegumentary leishmaniasis
Increased infectivity	Increased metacyclogenesis at promastigote level	SSG-failure *Leishmania donovani* MIL-relapse *L. donovani*
Specific host manipulation skills		
– Extra immune suppression – Reduced exposure to drug	IL-10 and Tregs induction Induced overexpression of host cell MRP1 to export the drug	SSG-failure *L. donovani* SSG-failure *L. donovani*
Increased tolerance to effector molecules of immune system	Resistance to reactive oxygen and/or nitrogen species	SSG-R *L. braziliensis*
Quiescence/niche preference	Hypothesis	Not described but might play a role in PKDL, MIL relapse and asymptomatics
Infection with *Leishmania* RNA virus	Hypothesis	Not described, but might play a role in the development of MCL

MCL: Mucocutaneous leishmaniasis; MIL: Miltefosine; MRP1: Multidrug resistance protein 1; PKDL: Post-kala-azar dermal leishmaniasis; SSG: Pentavalent antimonials.

differential effectiveness in HIV-infected versus non-HIV-infected cases: MIL and SSG have a similar effectiveness in non-HIV-infected men, but MIL was found to be less effective in HIV-coinfected cases compared with SSG [11]. Importantly, the prolonged VL treatment of HIV-coinfected cases may contribute to the selection of drug-resistant parasites. European patients have the luxury to be treated in optimal settings (essentially with SSG and liposomal AMB [12]); therefore, TF is mainly limited to HIV-coinfected patients. In Latin America, the treatment of CL and MCL mainly relies on SSG, also MIL has been used for some types of localized CL. The efficacy of SSG in this subcontinent is however unpredictable, and TF rates span from 7% in Bolivia up to 39% in Colombia. In Old World CL, SSG is still the drug of choice for CL with a TF rate of around 13% [9].

A decrease in effectiveness is part of any drug lifecycle in the arms race opposing humans to pathogens. A thorough understanding of this phenomenon is crucial to avoid current and upcoming antileishmanial drugs to succumb to the same fate as SSG and MIL. While TF definitively has a complex origin, in the present review we focus on the contribution of the parasite and more specifically, we show that drug resistance (DR) should not be considered as the only parasitological contributor (TABLE 1).

Leishmaniasis TF: the paradox of parasite DR

TF in leishmaniasis (and other infectious diseases) is known to have a multifactorial origin, involving features related essentially to the host (such as immunity, genetics, nutritional status), the drug (quality, pharmacokinetics, etc.) and the parasite (DR, coinfection with other pathogens, etc.). Parasite resistance to a drug is thus only one of many possible contributors of TF, and the two concepts are not at all synonyms, even if they are often confounded in literature. TF is a clinical phenotype expressed by the patient in whom clinical symptoms do not improve at the end of a complete treatment (nonresponse) or reappear

after initial cure (relapse); in some reports, the term 'clinical resistance' is used for TF, increasing the risk of ambiguity. In contrast, DR is a feature of parasite strains that express a significantly lower susceptibility to a given drug than sensitive strains. DR most often emerges when pathogens are exposed to nonlethal concentrations of drugs due to inadequate treatment (underdosing, low treatment compliance) or environmental contamination of the drug and is a result of *Leishmania*'s exceptional adaptive skills (reviewed in [13,14]). DR is thus an adaptive trait that emerges and spreads within the parasite population after exposure to the drug and must therefore be distinguished from drug tolerance that is an innate feature due to intrinsic metabolic properties of some species or life stages (e.g., *Leishmania braziliensis* is *in vitro* more tolerant to MIL than *L. donovani* [15], and *Leishmania* promastigotes are naturally tolerant to SSG, in contrast to amastigotes [16]). DR develops in a given patient, but the DR phenotype is measured experimentally (essentially *in vitro*) with isolated parasites. This entails several potential biases due to the selection associated with the isolation and cultivation processes or the properties of the chosen susceptibility assays themselves.

SSG is the only drug so far for which both TF and DR have been detected in immunocompetent patients, allowing the analysis of the connection between both conditions (parasite mechanisms of SSG-R are reviewed in [17]). DR strains of *L. donovani* [18] and *L. braziliensis* [19], as defined *in vitro* with an intracellular amastigote susceptibility test, were indeed isolated from patients with TF (nonresponse at the end of complete treatment). However, the same proportion of DR strains could also be isolated at the onset of treatment in VL and CL patients showing clinical cure, even after twelve months of follow-up [18,19]. This indicates that the current susceptibility tests are poor predictors of treatment outcome. We hypothesize that certain parasitic phenotypes (further called epiphenotypes)

that matter in the patient are not expressed by the parasite or not perceived by the tests. These epiphenotypes could theoretically be directly related to DR (like a pump that would not be expressed in a given *in vitro* system). However, we focus our review on other parasite adaptations that may contribute to TF in the patient even in the absence of classical DR such as virulence or quiescence. Epiphenotypes are not always detected using standard *in vitro* drug susceptibility assays and their detection may require an adaptation of current *in vitro* susceptibility tests, with cytokines to better mimic an immunological environment for example, or the application of other assays to measure virulence or other epiphenotypes.

Recent findings on a second drug, MIL, highlight that DR is not the only feature that must be examined when addressing TF from the parasite perspective. In Nepal, TF (relapse after 6–12 months) is indeed increasingly reported (up to 20%), but none of the strains isolated from the MIL-failing patients showed to be resistant *in vitro* [7]. This is surprising since MIL-relapse could be relatively easily induced in *in vitro* models (reviewed in [20]). While other factors could have played a role in TF, like a lower exposure to the drug [21], we examined additional features of the parasites and described a higher infectivity of strains isolated from TF patients [22]. The higher infectivity of MIL-relapsed strains can thus be considered as an epiphenotype that contributes to TF. This might only be the tip of the iceberg and raises fundamental questions. What are all the adaptations encountered in DR strains? Which are the ones linked to the mode of action of the drugs, and which are 'accompanying measures'? And, most provocatively, what is the weight of 'true' DR (thus direct adaptation to the drug) in patient treatment outcome? While trying to answer these questions, we deliberately focused our attention on *L. donovani* and VL in the ISC, currently the best documented paradigm on DR. In a later section, we will complement our review with information obtained on American tegumentary leishmaniasis (ATL), in another epidemiological context.

Parasite traits other than DR that may favor TF in VL patients

Host manipulation

Intrinsically, *Leishmania* disposes of several host defense evading mechanisms that eventually contribute to its own survival and subsequent transmission success. This manipulation occurs at various levels, from fooling host cells by expressing decoy molecules on their surface to actively secreting molecules into the host cell to affect its signaling pathways (reviewed in [23]). Patients with VL are known to suffer from IL-10-mediated immune suppression [24], and Tregs have proven to play a major role in this [25]. Treatment reduces the parasite load in the patient and thereby allows the host immune system to retake control and mount an effective response. This is especially the case for drugs that require interaction with a competent immune system to fully exert their action mechanism. SSG is such a drug: it directly kills the parasite through Sb^{III} (the reduced form of the active component Sb^V in SSG), but

also interacts with host cells to activate an efficient leishmanicidal immune response (reviewed in [17]).

Interestingly, SSG-R *L. donovani* exhibits additional host manipulation skills due to a higher concentration of terminal glycoconjugates (*N*-acetylgalactosamine residues) on their surface compared with SSG-S strains [26]. This induces a surge of IL-10 that precludes an effective immune response (inhibiting production of reactive oxygen and nitrogen species by host cells) and increases the expression of the host's transporters MRP1 and MDR1 – which export the drug from the host cell [17–19]. This glycan thus prevents host cells to clear their intracellular parasites even in the presence of therapeutic concentrations of the drug. The continuous presence of Tregs and their selective recruitment to the infected sites also play a critical role in the persistence of residual parasite burden [25], which can result in VL relapse or the development of post-kala-azar dermal leishmaniasis (PKDL), another form of TF. The presence of residual IL-10 and TGF-β in some SSG-relapsed cases together with the elevation of these cytokines in PKDL has already been indicated [27]. Ganguly *et al.* demonstrated an increase of Tregs within tissue from the lesions of PKDL patients [28]. Saha *et al.* also demonstrated that the production of IL-10 and TGF-β and the expansion of Tregs play important roles in the exacerbation of Indian PKDL. Interestingly, infection with SSG-R *L. donovani* has shown to induce Tregs that mediate their suppressive activity not only through IL-10, as is the case for wild-type SSG-S parasites, but also by TGF-β production [29]. The persistence of these cells in combination with the SSG-R-specific immunomodulatory skills is likely the basis for parasite persistence and subsequent SSG-TF. Importantly, AMB has the ability to decline both IL-10 and TGF-β levels in patients, which may partly explain why AMB is still effective in patients who failed SSG treatment [27].

While MIL does not require a potent immune system to fully exert its action, it is known to positively affect the immune status of VL patients [30]. As yet, specific immunomodulatory skills of strains from MIL-relapsed patients have not been identified. However, immunomodulation can 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*, wild-type or not. The observed increased infectivity of *L. donovani* strains isolated from SSG- and MIL-failed patients (Preadaptations of parasites in the sand fly section) may thereby also contribute to more immunomodulation and increased parasite survival.

To recapitulate, treatment of leishmaniasis may apparently result in the development of new parasitic mechanisms that enable a more efficient manipulation of host cells and the host immune system by *Leishmania*, promoting its persistence.

Parasite niches & quiescent stages

Another important factor to consider in TF is whether parasites might be able to infect alternative tissue niches within the vertebrate host that are less accessible to drugs. In the case of treatment with antimonials, it is well known that the drug

concentration differs greatly between organs, with the liver being one of the organs with a higher Sb^V concentration [31]. Depending on the *Leishmania* species, amastigotes are thought to remain in the original site of infection (CL) or disseminate to other teguments (MCL) or viscera (VL). However, sites other than those expected to be diseased have also been found to be infected with parasites, though at a low level, so that they generally remain unnoticed. In CL/MCL, for example, *Leishmania* DNA has also been reported in the bloodstream [32,33], urine [34] and apparently healthy mucosa [35]. This observed DNA should originate from living amastigotes or from recently dead parasites because nuclear and kinetoplast *Leishmania* DNA degrades rapidly [36]. In VL patients, parasites have been found in the blood [37] and skin as evidenced by the emergence of PKDL [38]. Especially, the latter is a less perfused organ where drug distribution might not be optimal, possibly resulting in sublethal drug exposure of amastigotes. This is exemplified by observations made in Rhesus monkeys where the skin showed a relative lower, but rather constant concentration of antimonials several months after standard dose treatment [39]. After apparent clinical cure of the patient, such niches might serve as foci from where infection can spread again and eventually cause TF. Noteworthy, these underexposed niches may not only be foci of parasite survival but also might contribute to the development of DR. The clinical importance of these niches is well exemplified by the emergence of PKDL in individuals who previously received SSG for treatment of VL. Interestingly, since MIL and AMB are introduced in the ISC, PKDL prevalence has dropped, further highlighting the link of PKDL development and specific drug treatment [40].

Besides different tissues, *Leishmania* also infects different types of cells: amastigotes have been found in a variety of host cells such as macrophages, neutrophils and dendritic cells. The metabolic state of amastigotes may differ between these different types of host cells, as exemplified by fibroblasts, where amastigotes appear to be in a nonreplicative state [41]. Such a quiescent-like state might be less susceptible to drugs due to their decreased metabolism like the persisters in mycobacteria [42,43] or the *Plasmodium* hypnozoite that can be in a dormant stage. Amastigotes, in general, are far less active than promastigotes, and this is already apparent right after promastigote to amastigote differentiation: this differentiation step is accompanied by a cell size reduction of 20–30 μm^3 to 5–8 μm^3, meaning a factor of 3.7. Interestingly, this reduction in cell size is not accompanied with a similar reduction of protein content as this decreases only 2.3-fold [44,45]. Therefore, this results in amastigotes having an increased protein concentration compared with the promastigote and the host cell itself. There is also experimental evidence showing that *Leishmania* amastigote transcription [46,47] and translation [48,49] are significantly decreased in the amastigote stage, coinciding with lower levels of polysomes observed in axenic amastigotes [49]. Although such studies should also be performed on intracellular amastigotes, also taking free ribosomes into account, these results imply that amastigotes (or a subset of them) could be in a quiescent state,

living on their reserves. The persister-like cells in *Mycobacteria* that show a significantly reduced growth rate and metabolism have a reduced susceptibility to antibiotics. Interestingly, these persisters can revert back to dividing cells when drug pressure is alleviated, allowing continuous persistence of infection despite several rounds of drug treatment, which contributes to TF without the necessity of DR [42,43]. Similar mechanisms may thus be at play in *Leishmania* infection as well: if *Leishmania*'s capacity to infect nontraditional niches or cell types in which they have an altered metabolic state is somehow encoded in the parasite genome, treatment of the host might cause a selection for this specific parasite trait and cause a progressive increase in TF rates over time.

Preadaptations of parasites in the sand fly

In previous sections, we mainly highlighted parasite traits at the intracellular amastigote level that may affect treatment outcome in the patient. However, recent studies have shown that molecular adaptations related to TF are already present in the sand fly stage, the promastigotes. As such, both SSG-resistant *L. donovani* and *L. donovani* isolated from MIL–TF patients (some of which were SSG-sensitive) have shown an increased rate of metacyclogenesis *in vitro* [22,50]. Metacyclogenesis is the process whereby noninfectious procyclic promastigotes differentiate into infectious promastigotes – an increased metacyclogenesis therefore translates into an increased infectivity to host cells as shown by *in vitro* and *in vivo* experiments [50–52]. It is remarkable that (genetic) preadaptations in the sand fly may lead to TF in the infected host. It is therefore fundamental to note that even a promastigote trait may lead to differential behavior in the host either at the individual amastigote level or at the amastigote population level. For example, the observed increased metacyclogenesis equals to an increased infectivity and results in a higher parasite load which in its turn may lead to a more efficient immunomodulation of the host, precluding the immune system to aid drugs in trying to eliminate the parasite (see also Host manipulation section). This increased infectivity also relates directly to the increased parasite load and increased severity of disease observed in dogs and in patients where SSG treatment failed [53–57].

However, a higher infectivity may also result in parasites infecting host cells located in specific niches that are less accessible to drugs (such as the skin) and from where parasites may re-emerge after treatment ('Parasite niches & quiescent stages' section).

Contrast & extrapolation to other epidemiological contexts

The epidemiological context of leishmaniasis can significantly add to the complexity of assessing which parasite features relate to TF in leishmaniasis. In this mindset, we chose to contrast the above perspective on TF in VL in the ISC with that of TF in American tegumentary leishmaniasis (ATL). This not only allows us to assess the applicability of the findings described above to other species, but also to identify additional pathogen-

related features besides classical DR that may play a role in treatment outcome. This is particularly relevant when considering the major differences at epidemiological level: the zoonotic context of ATL and thus the lower drug pressure on the parasite population compared with anthroponotic VL of the ISC.

A first feature appearing clearly in TF of ATL is the weight of the *Leishmania* species itself. In Peru, for example, patients infected with *Leishmania* (*Viannia*) *guyanensis* are generally more responsive to SSG treatment than patients infected with *L.* (*V.*) *braziliensis* [58], while the opposite result was observed in Brazil [59]. In Venezuela, diffuse cutaneous leishmaniasis patients infected with either *L.* (*L.*) *amazonensis* or *L.* (*L.*) *mexicana* comprise a poor response to SSG [60]. The different intrinsic tolerance or susceptibility of various species to drugs is probably playing a major role in determining ATL treatment outcome. For example, the median effective dose (ED_{50}) of MIL in *L. braziliensis* clinical isolates before the implementation of MIL in Latin America was higher than in *Leishmania lainsoni* (>30 and 1.89–3.37 μg/ml, respectively) and *L. donovani* from ISC (0.09–5.7 μg/ml) – an observation explained by the intrinsic lower expression of the LdMT–LdRos3 complex (the transporter of MIL) in *L. braziliensis* [15].

Second, it is not clear if true DR (thus resulting from parasite adaptation to the drug) exists in ATL-causing species. In an exhaustive report, we found that most of the isolates of *L. braziliensis* showed *in vitro* ED_{50} values above 60 μg/ml SSG and we hardly encountered SSG-sensitive parasites [19]. This contrasts with another report on *Leishmania panamensis* where 16 out of 19 isolates were SSG-sensitive before treatment [61], a difference which could – again – be explained by a species-specific effect. In the zoonotic context of ATL, secondary DR could be acquired as shown in Colombia by the isolation of SSG-sensitive and SSG-resistant parasites before and after TF, respectively, in the same patient [19,61]. However, this explanation is unlikely in the *L. braziliensis* study in which most of the isolates were already SSG-R before the onset of treatment and had probably never been in contact with SSG due to the large untreated animal reservoir [19]. Anyway, the ED_{50} of SSG in *L. braziliensis*, reflecting either tolerance or true DR, was a poor predictor of TF as we found parasites with low susceptibility in cured as well as in failing patients [19]. This obviously raises the same question as in *L. donovani* about the existence of epiphenotypes that would be responsible for TF.

This brings us to the third point of interest: also in ATL, the specific contribution of parasite physiology to TF must be better understood [62,63]. In natural SSG-S and SSG-R *L. braziliensis* isolates, some have reported that: cross-resistance to SSG and nitric oxide is observed [64,65]; mice infected with SSG-R or NO-resistant *L.* (*V.*) *braziliensis* [64], NO-R *L* (*L.*) *amazonensis* [66] or *L.* (*V.*) *braziliensis* [66] presented larger lesions that healed more slowly and contained higher parasite loads; IL-4 production was strongly increased in SSG-R-infected animals as well as arginase I expression, contributing to a Th2 immune response favoring *Leishmania* survival [64]. This indicates that despite the different epidemiological and

genetic context of New World *Leishmania*, similar phenomena as in those described for *L. donovani* in the ISC may occur.

A fourth path deserves particular attention in the context of ATL: parasites of the *Viannia* subgenus may be infected by a specific virus (*Leishmania* RNA virus-1), shown to allow subversion of the host immune response to *Leishmania* and promote parasite persistence [67]. This was demonstrated in experimental models, and research is currently in progress to document the prevalence of *Leishmania* RNA viruses in clinical isolates and assess a possible relationship with metastasis, MCL and treatment outcome. All along evolution, viral or bacterial endosymbionts have shaped the biology and the genome of protists, and these should thus definitively be taken into consideration to allow a holistic perception of the links between the parasite and TF.

Implications for fundamental research

The previous sections highlight that the parasite contribution to TF should not necessarily be related to DR *sensu stricto*, but might also be due to many other (epi-)phenotypes. This challenges the traditional view of parasitologists on what type of studies are needed to identify parasite factors that may contribute to TF.

This is especially true for studies on *in vitro*/*in vivo* induction of DR/TF. Classically, these studies are performed on so-called 'lab strains' that were isolated several decades ago, are well adapted to culture conditions and often come from a geographical area remote from the area of interest. Using recently isolated strains from the endemic region itself would dramatically increase the relevance and applicability of these studies to the real-life situation. Also the experimental setup is crucial for this aspect: in nature, only intracellular amastigotes are exposed to the drug. Studies on promastigotes are thus only relevant to study promastigote-specific phenotypes, such as metacyclogenesis, which may relate to treatment outcome in the host. Ideally, such promastigote phenotypes should be studied in the vector itself, and not only *in vitro* as is the case up till now. Although axenic promastigotes or axenic amastigotes may help identifying specific targets of a drug, such a setup lacks the host cell, a component that has a crucial impact on parasite survival in the presence (and absence) of drugs. Considering recent studies showing that SSG-R parasites manipulate their host cell to reduce exposure to SSG [26], the host cell proves to be an essential player in the parasite–drug relationship. This should be interpreted in the broadest sense, not only at the level of drug action, but also at the level of how parasitic mechanisms of adaption to the drug may affect their survival in the host. Working on *in vitro* intracellular amastigotes is thus recommended, but the outcome of these tests is dependent on many variables [68]. For example, the type of host cell used may not only affect the *in vitro* susceptibility of the parasite to drugs [69], but may also impact whether or not specific niche preferences or even quiescent-like amastigotes are detected (Parasite niches & quiescent stages section). However, these assays remain *in vitro* models and are far less complex than the true

interaction between the drug, the parasite and the host. Understandably, *in vivo* models perform better in terms of relevance since they allow parasites to fully exert their host manipulation skills (if any), but the choice of which animal model to apply is not straightforward. Mice are often used as they come in many (genetically manipulated) forms, but their susceptibility to *Leishmania* infection can differ between different mice strains and the infecting *Leishmania* species [68,70]. Hamsters or cotton rats are a better model for VL since they better mimic the progressive liver, spleen and bone marrow pathology as seen in human VL [70,71]. Even though hamster and cotton rat models also have limitations, such as a higher cost and the lack of inbred strains for knockout immunological or drug–parasite–host interaction studies, we assume that these models are the most relevant to field conditions. Differentiating between 'true' DR-related phenotypes and other phenotypes that may affect treatment outcome cannot be achieved by applying only one of the experimental models above. Combining various standardized *in vitro* and *in vivo* assays is fundamental to be able to dissect which factors are at play at different TF foci and to identify molecular markers that can be used to monitor the spread of those parasites that form a threat for treatment effectiveness.

Studying TF-related parasite phenotypes is one thing, but avoiding their emergence is another. Adequate treatment is crucial to avoid the emergence of parasites that may overcome host treatment. The recent findings of children being underexposed to MIL, and the consequently higher TF rate in this age group specifically raises the concern that pharmacokinetic studies during clinical development were inadequate [72]. The mechanisms by which *Leishmania* might evade a new drug and the speed by which they develop should also be assessed before officially releasing new drugs. Moreover, new concepts of targeting this intracellular parasite would be welcomed. Such concepts might, for example, include combination regimens of drugs that target the parasite directly and drugs that re-enforce the host, or drugs that would counteract the development of resistance or relapse to the first drug. Naturally, this requires extensive insights into how the parasite interacts with its hosts to avoid its clearance. For example, *in vitro* and *in vivo* studies have shown that an increased cholesterol intake provides protection against *L. donovani* infection and facilitates its intracellular killing [73,74]. One might thus hypothesize that nutritional aid or cholesterol-increasing food additives during and after treatment might reduce the chance on relapse. Multidisciplinary research will be crucial to identify such new ways to treat leishmaniasis patients and at the same time guarantee long-term effectiveness of the therapy provided.

Implications for public health
Due to a different epidemiological context, TF in New world leishmaniasis is generally more complex and unpredictable as compared with Old World leishmaniasis. This implies that treatment guidelines have to be re-evaluated on a global basis considering the huge differences between Old and New World

leishmaniasis [75]. Most DR parasites are, for now, still susceptible to other drugs such as AMB, and also drugs for other (non-infectious) diseases, such as imipramine, show promising results to eliminate wild-type and DR *Leishmania* [76]. Since the latter are already US FDA approved, such drugs may bring new hope for a swift release of new antileishmanial drugs into the field after the necessary clinical trials. However, treating patients infected with parasites with one of the phenotypes discussed above (increased infectivity, dormancy, etc.) may be more challenging since it requires the release of progressively more effective drugs, highlighting the need for new treatment strategies like those discussed earlier (Implications for fundamental research section). In addition, it is commonly thought that even after clinical cure is achieved (either at the end of treatment or at the end of the follow-up period), parasites will still remain in the host. There is thus an important distinction to be made between clinical cure, which is based on symptoms, and parasitological cure, which is likely not achievable. This is exemplified by parasite re-emergence in cured VL patients when they become immunosuppressed or when PKDL develops. Successfully cured patients could thus be considered to enter a state similar to asymptomatic patients.

These asymptomatics currently pose major questions to fundamental and public health researchers. Why do some remain asymptomatic and some develop disease? What is their spreading potential, are there enough parasites available to infect sand flies? The role of the parasite in why these individuals remain asymptomatic is not at all understood. Until now, there are no sufficient sensitive molecular methods available to study these low amounts of parasites at different '-omic' levels (genome, transcriptome) in an untargeted approach. The metabolic status of parasites carried by asymptomatic individuals thus remains a major question. Are they quiescent-like cells – as the persister Mycobacteria – that can be reactivated after the first exposure? Are they more sensitive to the immune response? And what is their drug susceptibility? Many of the epiphenotypes that may contribute to TF can thus also be of interest for more public health-related issues such as asymptomatics. The importance of detailed clinical information about the patient should not be underestimated, since the immune status and other host-related factors surely affect the host–parasite relationship, and therefore also the kinetics of infection, disease progression and treatment outcome.

Conclusion
TF in leishmaniasis is a complex phenomenon that encompasses many host-related factors, parasite-related factors and factors that lie on the interface between the host and the parasite. Recent studies highlighted that DR is not the only parasitic phenotype that may contribute to TF in leishmaniasis patients. The increased infectivity and host manipulation skills of SSG-R *L. donovani* are a striking example of epiphenotypes that can be related to TF. However, there is as yet no answer to the 'chicken or the egg' question: did SSG-resistant strains evolve from more infectious or host manipulative parasites, or vice

versa? Or is this a result of coevolution? Given that an increased virulence among *L. donovani* strains is a mutual trait in SSG and MIL–TF, one might hypothesize that virulence might be the main factor that contributes to TF and that the SSG-resistance trait is perhaps of secondary importance. Recent reports even suggest that it might have initially emerged because of environmental contamination with arsenic [77].

MIL-TF being related to an increased infectivity of the parasite and not to DR emphasizes the need to study parasites in their original clinical context and not standardly in the context of *in vitro* determined phenotypes – such as DR – which may not always be relevant to the *in vivo* situation. It is also a reminder to broaden our perspective when deciding which tests to apply on isolates from TF patients. However, differences in epidemiological context between geographical regions imply a different rationale for researchers looking for parasitic factors that contribute to TF: in ATL, for example, the diversity of etiological agents and their (epi-)genetic features may dramatically complicate the panorama of factors that may contribute to TF compared with VL.

Leishmania proves to be a genus of versatile organisms with exceptional adaptive skills that enable it to escape from most of the current treatment regimens. Better insights into how *Leishmania* and other pathogens exactly contribute to TF in the patient – and the evolutionary mechanisms that are responsible for it – are crucial to revise the current strategies by which drugs are designed and applied. Continuous monitoring in the field will be crucial to detect new mechanisms by which pathogens can escape treatment of the host and monitor the spread of those pathogen strains which may pose a public health risk in the affected regions.

Expert commentary & five-year view

TF is one of the biggest threats to life as we know it. The world population level continues to increase and changes how people interact with their environment, often favoring the transmission of various pathogens. Drugs, mostly antibiotics, are massively applied on both humans and animals and are challenged by the development of drug-resistant pathogens.

These (multi-)drug-resistant pathogens led to a significant increase of disability-adjusted life years and public health costs. Even more alarming is that the rate by which pathogens adapt to treatment of the host is faster than the rate by which new drugs emerge from drug discovery pipelines. However, DR is not the only adaptation that the pathogen may undergo in response to treatment of the host, and this is often overlooked. TF in leishmaniasis teaches us that pathogens may also persist after treatment through the adaption of their infectivity and/or host manipulation skills. These so-called epiphenotypes are different from DR and do not only pose a problem for the current drugs that lose effectiveness, but also for future drugs since they may imply a fitness advantage compared with wild-type parasites [78] – putting even more pressure on current drug development strategies to identify more effective drugs.

To avoid similar phenomena in other leishmaniasis endemic regions or even other pathogens, it is crucial to obtain insights in how these epiphenotypes evolved in a pathogen population under drug pressure. Funding, however, is nowadays still too much focused on designing new drugs through classical discovery pipelines. What is needed are radically new treatment strategies that avoid such epiphenotypes to emerge in pathogen populations, and hereby ensure long-term effective treatment for infectious diseases.

Key issues

- Drug resistance (DR) is commonly considered as the main pathogen-related phenotype that contributes to treatment failure (TF) in the patient.
- In *Leishmania donovani*, the infectivity of the parasite correlates better with TF in the patient than DR.
- Recent studies indicate that host manipulation skills of the parasite may also contribute to TF.
- Parasite adaptations favoring TF may already be present in the sand fly stage.
- Other parasitic factors unrelated to DR may play a role in TF and should be further explored: localization in preserved niches, quiescence, presence of virus in the parasites.
- Pathogen phenotypes other than DR should also be standardly assessed in TF studies.
- A better insight into how these epiphenotypes evolved under drug pressure is crucial to revise current strategies by which drugs are designed and applied in the field.

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