(Post-) Genomic approaches to tackle drug resistance in *Leishmania*

MAYA BERG[1]†, AN MANNAERT[1]†, MANU VANAERSCHOT[1]†, GERT VAN DER AUWERA[1]† *and* JEAN-CLAUDE DUJARDIN[1,2]*

[1] *Molecular Parasitology Unit, Department of Biomedical Sciences, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium*
[2] *Department of Biomedical Sciences, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium*

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SUMMARY

Leishmaniasis, like other neglected diseases is characterized by a small arsenal of drugs for its control. To safeguard the efficacy of current drugs and guide the development of new ones it is thus of utmost importance to acquire a deep understanding of the phenomenon of drug resistance and its link with treatment outcome. We discuss here how (post-) genomic approaches may contribute to this purpose. We highlight the need for a clear definition of the phenotypes under consideration: innate and acquired resistance versus treatment failure. We provide a recent update of our knowledge on the *Leishmania* genome structure and dynamics, and compare the contribution of targeted and untargeted methods for the understanding of drug resistance and show their limits. We also present the main assays allowing the experimental validation of the genes putatively involved in drug resistance. The importance of analysing information downstream of the genome is stressed and further illustrated by recent metabolomics findings. Finally, the attention is called onto the challenges for implementing the acquired knowledge to the benefit of the patients and the population at risk.

Key words: *Leishmania*, drug resistance, antimonials, miltefosine, genomics, metabolomics.

INTRODUCTION

Leishmania (Kinetoplastida, Trypanosomatidae) are protozoan parasites responsible for leishmaniasis, a disease characterized by two major clinical manifestations: (1) cutaneous leishmaniasis (CL), which can lead to disfiguring lesions and (2) visceral leishmaniasis (VL), a systemic disease which is fatal in the absence of treatment. According to a recent estimate, there are approximately 0·2 to 0·4 million new VL cases and 0·7 to 1·2 million CL cases every year (Alvar *et al*. 2012). The disease is endemic worldwide in tropical and sub-tropical regions, including in Southern Europe (Dujardin *et al*. 2008). It is noteworthy that infection with *Leishmania* does not necessarily lead to disease and, depending on the regions and the species, asymptomatic *Leishmania* carriers are more abundant than leishmaniasis patients (Ostyn *et al*. 2011). Co-infection with HIV is an increasing feature and may lead to a clinical activation of the parasites that is extremely difficult to manage.

* Corresponding author. Molecular Parasitology Unit, Department of Biomedical Sciences, Institute of Tropical Medicine, Nationalestraat, 155; B-2000 Antwerpen, Belgium. Tel: 32.3.2476355. Fax: +32.3.2476359. E-mail: jcdujardin@itg.be
† Authors contributed equally to this manuscript

The parasite is transmitted by sandflies to a broad range of mammals, including humans, in which they develop within macrophages and other cells of the reticulo-endothelial system. During their life cycle (Fig. 1) the parasites are thus exposed to very different environments in which they are present in different life stages. In the sandfly (and this can be mimicked by *in vitro* culture), the parasite is first a procyclic promastigote, a replicating flagellated form, while later on it stops dividing and undergoes a series of molecular modifications that will 'programme' it for its future intracellular life (metacyclic promastigote). After transmission to a mammalian host, the parasite is phagocytosed by macrophages and it is able to survive and replicate within the phagolysosome as a small, non-flagellated parasite, the amastigote. These morphological transformations of the parasites are characterized by significant changes in gene and protein expression (Alcolea *et al*. 2010; Tsigankov *et al*. 2012), hence extreme care is required in the biological stage of the parasite when comparing different strains at molecular level.

In nature, different scenarios of this life cycle can be encountered, from zoonotic cycles in the Amazonian jungle, in which humans are accidental hosts, to anthroponotic cycles where humans are thought to be the only host. This, together with the multiplicity of biotopes in which the parasite can be found, has led to a significant diversification of

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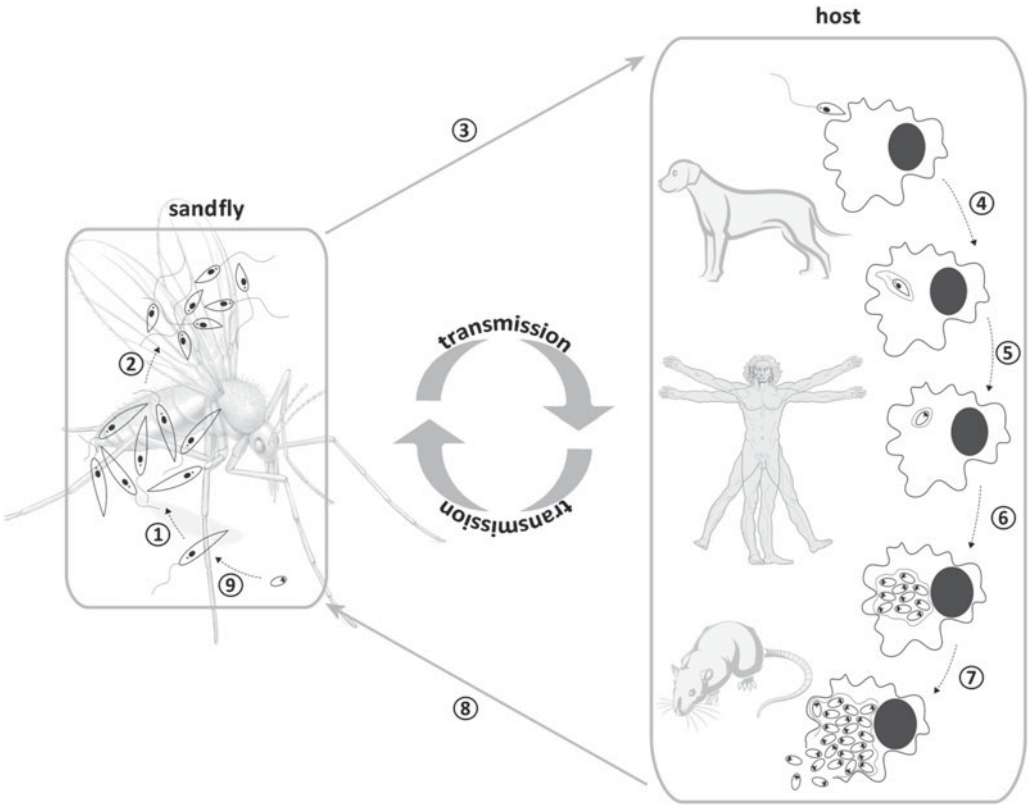

Fig. 1. Life cycle of *Leishmania*. In the vector, the sandfly, *Leishmania* grows as extracellular promastigotes, which will multiply (1) and undergo a transformation into metacyclic (infectious) forms (2). When an infected sandfly takes a blood meal, promastigotes will be injected in the host, which can be humans, dogs, rodents, etc., depending on the infecting *Leishmania* species (3). Metacyclic promastigotes will successfully infect host cells (4) and transform into intracellular amastigotes (5). These amastigotes will multiply (6) and eventually infect other neighbouring host cells (7). When another sandfly takes a blood meal, it will also imbibe amastigotes and/or amastigote containing host cells (8) which will transform into promastigotes in the sandfly gut (9) and enable a continuation of *Leishmania*'s life cycle.

Leishmania parasites, highlighted among others by the clinical polymorphism. Consequently, the taxonomy of the genus *Leishmania* is rather complex and is still a subject of controversy (see Van der Auwera *et al*. 2011). *Leishmania* is considered to reproduce essentially in a clonal way, but automictic and allomictic recombination may occur (Rougeron *et al*. 2009). In the absence of obligate sexuality, the species definition in *Leishmania* is thus ambiguous and the current taxonomy is based on a phylogenetic definition of species and describes 14 medically relevant species.

Leishmaniasis, like other infectious diseases, is the result of an evolutionary arms race between pathogens and their hosts, which after millennia has often led to a mutual non-destruction pact resulting in peaceful cohabitation as long as a biological equilibrium is maintained. However, medical interventions and other ecological interferences may lead to new episodes in the competition between pathogens and their hosts, through the development of drug resistance. Recent developments in 'omics technologies provide us a new perspective on this phenomenon. In the present review, we aim first to describe and clearly define the concept of drug resistance, then to review the advances brought by (post-)genomic approaches for its understanding, and finally to discuss how to translate these findings into tools for improving patient health.

CHEMOTHERAPEUTIC ARSENAL: THE NEED TO SAFEGUARD THE FEW AVAILABLE DRUGS

In the absence of an effective vaccine (Singh and Sundar, 2012), the control of leishmaniasis is essentially dependent on chemotherapy and vector control (Singh *et al*. 2012). However, the arsenal of drugs currently available is limited and drug efficacy is sooner or later jeopardized by drug resistance. For more than half a century *antimonials* (SSG) successfully constituted the first-line treatment of leishmaniasis. However, it showed severe side effects including pancreatitis, cardiac and renal toxicity (Sundar and Rai, 2002). This, together with the high treatment failure rate (up to 65%) and the emergence of resistance in the State of Bihar, India, made the drug obsolete in the Indian subcontinent

(Sundar *et al*. 2000). However, it is still used in first line in Africa and Latin America. *Miltefosine* (MIL), an alkylphosphocholine originally developed as an anticancer drug, has been used since 2005 in first line for the oral treatment of VL in the Indian subcontinent (Sundar *et al*. 2008, Government of India Guidelines), but has shown variable efficacy in other clinical forms (Minodier and Parola, 2007). Its major limitation is teratogenicity, so that pregnancy is a contra-indication for treatment, ruling out women of childbearing age who constitute an estimated 25% of all VL patients (Olliaro *et al*. 2005). The long half-life (150 h) of the drug and its misuse may facilitate the emergence of drug resistance. Alarming rates of increased MIL-treatment failure have already been reported in India (Sundar *et al*. 2012), Bangladesh (Rahman *et al*. 2011) and Nepal (Rijal *et al*. 2013), but this could not (yet) be related with clear-cut MIL resistance of the infecting parasite. The polyene macrolide antibiotic amphotericin B (AmB) has proven to be highly effective for the treatment of VL caused by SSG-resistant *Leishmania donovani* (SSG-R). It is an unpleasant drug because of its toxic side effects and the need for hospitalization of the patient during the 4 week course of treatment. However, expensive lipid-associated formulations of this drug have been made (Ambisome®) and have a reduced toxicity and an extended plasma half-life, therefore possibly requiring only a single infusion (Matlashewski *et al*. 2011). Treatment failure and relapse during amphotericin B treatment occur rarely, except among HIV-infected patients (mainly due to relapse and reinfection) (Lachaud *et al*. 2009). The anti-leishmanial activity of the aminoglycoside paromomycin (PMM) was already known in the 1960s (Kellina, 1961; Neal, 1968), and although PMM shows an efficacy of 95% and is relatively cheap, it is still under phase IV clinical trials – a reflection of the limited funding available. PMM is unlikely to ever become a monotherapy for VL, due to the risk of resistance emergence (Hendrickx *et al*. 2012) and the existence of other treatment regimens available (MIL, single dose liposomal AmB and combination schemes). Combination therapy may reduce both the treatment duration and the chance on the emergence of drug-resistant parasites while still guaranteeing an excellent efficacy, as shown by the first clinical trial of different combination regimens against VL in the Indian subcontinent (Sundar *et al*. 2011).

Although combination therapy is supposed to slow down the emergence of resistance, it may still be challenged by double (or cross) resistance as shown by recent experimental work (García-Hernández *et al*. 2012). Since leishmaniasis, like other neglected tropical diseases, affects the poorest people in the poorest regions of the world, the interest of the pharmaceutical industry in developing new drugs has been somewhat limited. Patients' need-driven initiatives were launched (see for instance www.dndi.org) to counter this neglect. However, it will take several more years before the rare new lead compounds, if any, reach patients, and experience from the past suggests that resistance to these new drugs will emerge sooner or later. To safeguard the efficacy of current drugs and guide the development of new ones, it is thus of utmost importance to acquire a deep understanding of the phenomenon of drug resistance and its link with treatment outcome.

TREATMENT FAILURE, INNATE AND ACQUIRED DRUG-RESISTANCE: THE NEED FOR CLEAR DEFINITIONS

Understanding drug resistance requires first of all clear conceptual definitions, as there is often confusion of terms in the literature. On one hand, an important distinction is needed between treatment failure and drug resistance. Treatment failure is the clinical phenotype: it occurs when a patient does not respond to a drug, or when the treatment was not effective in the long run, resulting in non-response (persistence of symptoms at the end of therapy) or relapse (re-occurrence of the disease in an earlier apparently successfully treated patient). In general, treatment failure can occur due to several treatment-related (e.g. compliance, dosage, quality), parasite-related (e.g. intrinsic or acquired drug resistance) or host-related (e.g. pharmacogenetics, immune response) factors (Croft *et al*. 2006). In contrast, drug resistance is a pathogen's phenotype resulting in its capacity to survive a given drug concentration, as measured with an *in vitro* susceptibility assay (see below). On the other hand, a clear distinction is also required between intrinsic and acquired resistance, (1) the former resulting from a biochemical feature of the pathogen, independent and prior to contact with the drug that allows it to tolerate that drug, while (2) acquired resistance is an adaptation selected through contact with the drug. Given the biochemical variation between the constitutive species, it is not surprising that different species may react differently to various anti-leishmanial therapeutic compounds (Croft *et al*. 2006). Most of the data in this regard are gathered from clinical observations, but *in vitro* susceptibility assays have confirmed the occurrence of significant intrinsic species-specific differences. For instance, using parasites collected before the implementation of MIL, Yardley *et al*. (2005) showed a significantly higher tolerance of *Leishmania braziliensis* to MIL than *L. donovani*. This also shows that any study on drug resistance in *Leishmania* must take into account species diversity and that results obtained with one species or particular strain cannot *a priori* be extrapolated to another one.

The current standard tool to evaluate whether an organism is resistant to a drug is exposing it to the

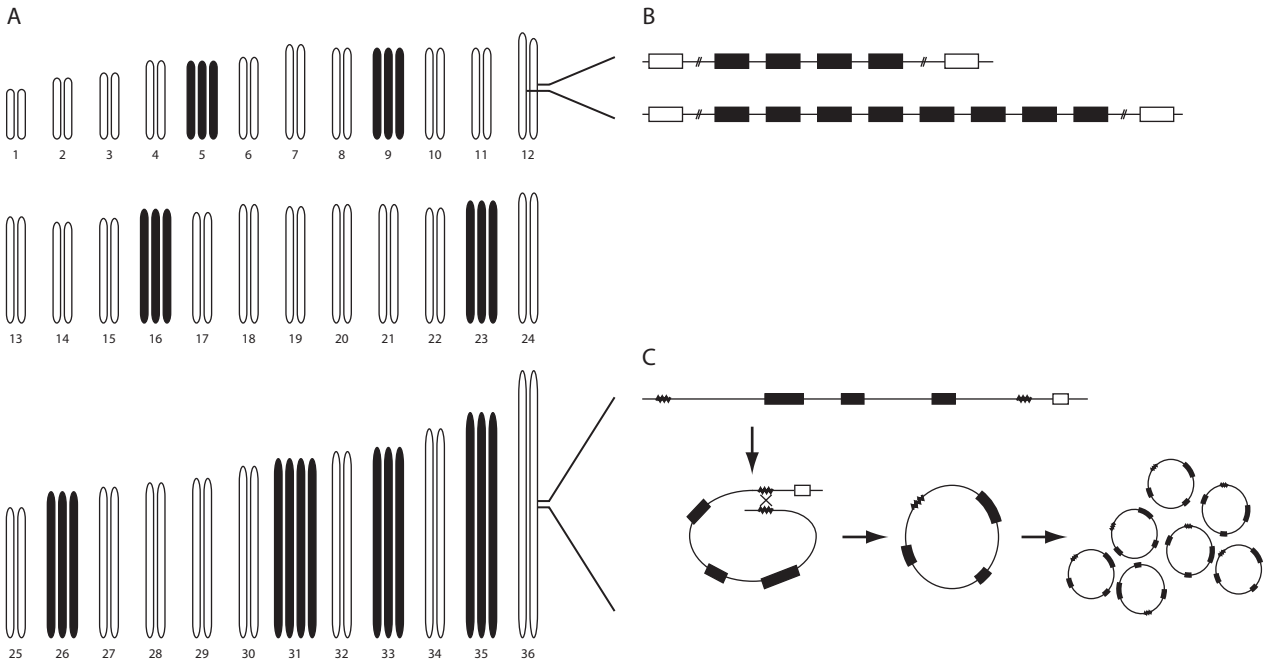

Fig. 2. Structural diversity of the *Leishmania* genome. (A) Different *Leishmania* species show aneuploidy. This example represents the karyotype of the *L. donovani* reference strain, one of many circulating aneuploid karyotypes. Most chromosomes are disomic (white), but trisomy and tetrasomy (black) are common. (B) The number of gene copies can vary between species, between strains of the same species and even between homologous chromosomes of one strain, causing chromosome size polymorphism. (C) Extra-chromosomal amplification of genes on circular episomes can occur through homologous recombination between direct repeats (zigzag lines). Episomes can be replicated, and copy numbers can differ between strains or species. Genes are represented by boxes, amplified genes (B and C) are shown in black.

drug under controlled conditions, and comparing its response with other known susceptible organisms. In the case of SSG, this involves a laborious *in vitro* test where intracellular amastigotes are exposed to SSG (Croft *et al*. 2002). The necessity to test the intracellular form of the parasite, and thus using a rather complex *in vitro* system with different cell types, is due to the intrinsic insensitivity of the extracellular promastigote stage of *Leishmania* to SSG (Vermeersch *et al*. 2009). In contrast, for MIL, studies have shown that *in vitro* amastigote and promastigote susceptibilities are correlated, indicating that screening of large sets of strains for MIL resistance might also be done at the easier-to-assess promastigote level (Kulshrestha *et al*. 2013). Besides being time-consuming and far from standardized, *in vitro* assays (even those running on intracellular amastigotes) also constitute a reductionist representation of the natural conditions to which these organisms are naturally exposed in treated patients. In case of acquired resistance to SSG, this is especially highlighted by the low predictive value of the current *in vitro* SSG susceptibility test for treatment outcome of the infected patient: in Nepal, strains that were defined *in vitro* to be SSG-R were found both in patients that were cured with SSG as in patients where SSG treatment had failed (Rijal *et al*. 2007). In view of the strong dependence of the mode of action of SSG on host factors, this is most likely due to the lack of immunogenic factors in the current gold standard SSG susceptibility test. Such scenarios might also apply for other (new) drugs. In this context, there are many expectations from molecular tools to complement and improve the *in vitro* tests. This requires elucidation of the molecular mechanisms leading to resistance, a task that must take into consideration the limits of the currently defined phenotypes: a marker of (*in vitro* defined) drug resistance could be *a priori* different from a marker of treatment failure.

GENOMIC FEATURES OF *LEISHMANIA*

Before addressing the genomic approaches used for understanding drug resistance in *Leishmania*, an overview of the major features of its genome is required. The genomes of several sequenced *Leishmania* species (*Leishmania major, L. infantum, L. braziliensis, L. donovani, L. mexicana* and *L. tarentolae*) are relatively well conserved, with a high degree of synteny (conservation of gene order) and few species-specific genes, in contrast to their phenotypic diversity (Ivens *et al*. 2005; Peacock *et al*. 2007; Downing *et al*. 2011; Rogers *et al*. 2011; Raymond *et al*. 2012). Genetic variation is usually measured as sequence divergence, but in the case of *Leishmania*, structural polymorphisms such as gene and chromosome copy number differences are a major source of genetic variation (Fig. 2). Inter- and intraspecific chromosome size polymorphisms,

as already observed in early karyotyping experiments, are the result of expansions or contractions of gene family arrays or other tandem repeats (Pagès *et al*. 1989; Inga *et al*. 1998). In addition to tandem duplications or deletions of genes, *Leishmania* is also able to produce extra-chromosomal, linear or circular amplicons (episomes) of chromosome fragments containing one or more genes by homologous recombination (Ouellette *et al*. 1991; Grondin *et al*. 1993; Leprohon *et al*. 2009). The number of chromosomes has long been the subject of debate, as it was unclear whether *Leishmania* is diploid or aneuploid, but recent genome and karyotype studies of several *Leishmania* species, strains and clones have found compelling evidence for aneuploidy (Downing *et al*. 2011; Rogers *et al*. 2011; Sterkers *et al*. 2011). The presence of different chromosome copy numbers between species, between strains and even between cells of the same population confirmed that aneuploidy is a constitutive feature of this parasite (reviewed by Mannaert *et al*. 2012 and by Sterkers *et al*. 2012). This structural variation can be regarded as a gene dosage adjustment system in the absence of transcriptional regulation. Trypanosomatid genes are organized in unidirectional, polycistronic transcription units, which are post-transcriptionally spliced and polyadenylated, and regulation of gene expression likely occurs by mRNA degradation, translational control or protein degradation (Clayton and Shapira, 2007). The ability to adjust the copy number of genes or entire chromosomes may provide a selective advantage, enabling the parasite to adapt to changing environments, e.g. in case of drug pressure (Mannaert *et al*. 2012). Both sequence and structural polymorphisms could contribute to an altered tolerance of the parasite to drugs (Leprohon *et al*. 2009; Billal *et al*. 2011). Therefore, it is highly important to identify all genetic changes, single nucleotide polymorphisms (SNPs), copy number variations (CNVs) and ploidy changes that can be linked to the phenotype of interest of the parasite. These changes can be found by comparing drug-resistant with drug-sensitive *Leishmania* strains, by targeting specific genes of which the involvement in drug resistance is known or suspected, or by using a whole genome approach to identify all possible changes.

TARGETED APPROACHES FOR UNDERSTANDING DRUG RESISTANCE

When specific genetic targets are suspected to be involved in drug resistance, one can focus on that target using a variety of tests of which the majority are based, at least in part, on the polymerase chain reaction (PCR). As such, the genetic sequence of that target, its expression at RNA or protein level and/or its activity through reporter assays might be evaluated. Searching for single nucleotide polymorphisms (SNPs) in the DNA sequence of specific enzymes, transporters or other biologically active proteins that are specifically targeted by a drug or used for its import or export, has proven to be a successful approach for *in vitro* induced miltefosine-resistant (MIL-R) strains. Mutations in *LdMT*, an inwards translocator of MIL, or its beta subunit *LdRos3* were found to be responsible for *in vitro*-induced MIL resistance (reviewed by Pérez-Victoria *et al*. 2006). In a single natural *L. infantum* isolate from an Algerian HIV-co-infected VL patient in France, a mutation in the same *LdMT* was also found (Cojean *et al*. 2012). However, such variation was not observed in dozens of clinical *L. donovani* strains isolated from Indian and Nepalese patients that relapsed after MIL treatment (Bhandari *et al*. 2012), highlighting that natural drug resistance can be more complex than *in vitro* induced resistance. Similarly, the gene expression of *LdMT* and *LdRos3* was comparable between pre-treatment, post-treatment and relapse isolates of Nepalese and Indian patients treated with MIL (Bhandari *et al*. 2012) despite earlier reports that an *in vitro*-induced lower expression of these genes can contribute to MIL resistance (Sánchez-Cañete *et al*. 2009). Analysing the genetic sequence of specific enzymes and transporters thought to be related to the transport and action mechanism of SSG (Fig. 3) in clinical SSG-S and SSG-R isolates proved equally unsuccessful as for studies on MIL resistance, showing no genetic variation in the selected targets that could be related to the SSG-R phenotype (Mandal *et al*. 2010; Decuypere *et al*. 2012). Assessing the expression of these genes with real-time quantitative PCR (Q-PCR) proved more informative with, for instance, different candidate marker genes involved in thiol metabolism showing a possible link with SSG resistance. Interestingly, the eventual genes of interest differed between *Leishmania* species (Decuypere *et al*. 2005, 2008, 2012; Carter *et al*. 2006; Adaui *et al*. 2011*a*, *b*; Mukhopadhyay *et al*. 2011; Kumar *et al*. 2012). In addition, variability was also found between studies on strains of the same *Leishmania* species of the same geographical region. These apparent inconsistencies can at least partly be explained by SSG resistance having a multifactorial origin (Downing *et al*. 2011) and by findings showing that the parasite's genetic background is an important contributor to determine which SSG resistance mechanism applies (Decuypere *et al*. 2012).

Importantly, the mode of action of anti-leishmanial drugs may imply direct and indirect mechanisms, the latter through the host cell (Fig. 3). Hence, the parasite can also express its drug resistance through manipulation of the host. This is especially true for SSG-R *L. donovani*, which have shown to modulate the host to (1) reduce the presence of the drug in the host cell and (2) minimize SSG's immuno-modulating action (reviewed by Vanaerschot *et al*. 2012). Various targeted reporter

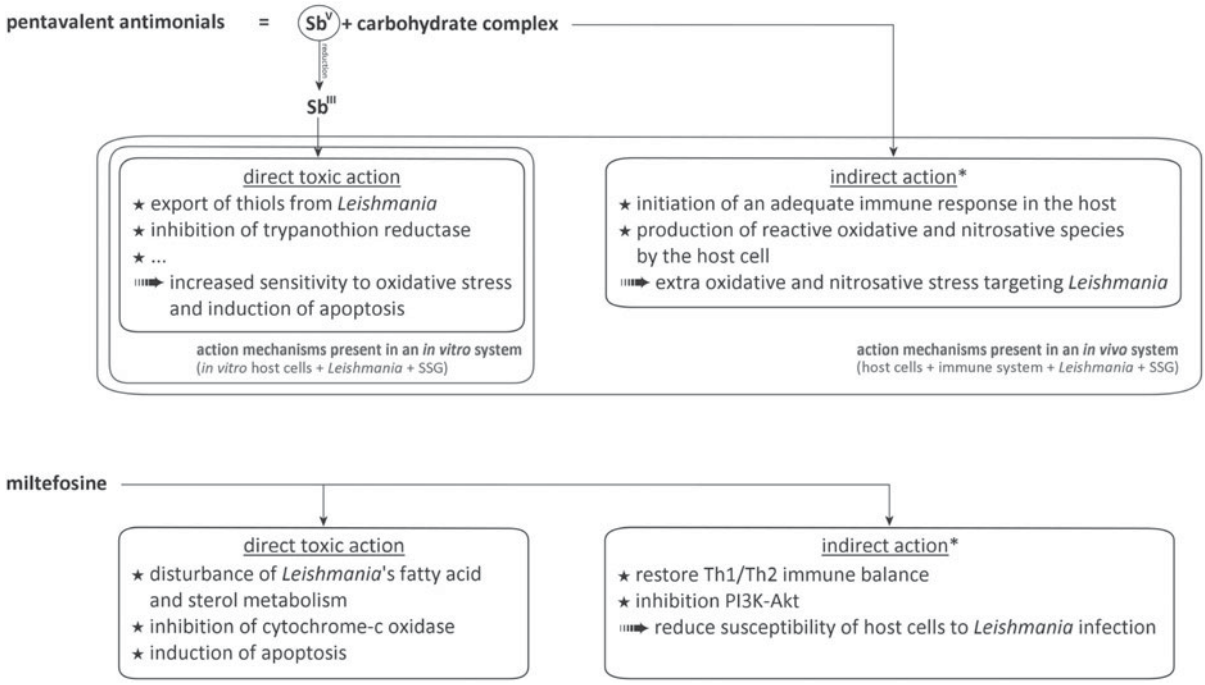

Fig. 3. The mode of action of two major drugs against leishmaniasis. * The indirect action of pentavalent antimonials has been shown to be indispensable for the drug's *in vivo* action, while the importance of MIL's indirect action mechanism is still under debate. The action mechanisms of pentavalent antimonials and MIL are more thoroughly reviewed in Vanaerschot *et al*. (2012) and Dorlo *et al*. (2012), respectively.

assays have demonstrated that SSG-R *L. donovani* induces IL-10 in its host cells, and that this is responsible for a higher gene expression of the host cell's multidrug resistance-associated protein 1 (MRP1) and permeability glycoprotein (P-gp), which can export SSG out of the host cell (Mukherjee *et al*. 2013). Also the gene expression of a key enzyme in glutathione synthesis was downregulated in SSG-R *L. donovani* infected host cells, causing a lower activation of SSG into its direct toxic form in the host cell (Carter *et al*. 2006; Haldar *et al*. 2010). Targeting the expression of cytokines can also significantly contribute to a better understanding of drug resistance and treatment failure for drugs where the host factors are, or are suspected to be, important for the mode of action (Thakur *et al*. 2003; Narayan *et al*. 2009; Costa *et al*. 2011).

UNTARGETED GENOMIC APPROACHES FOR UNDERSTANDING DRUG RESISTANCE

Since drug resistance in *Leishmania* can be multifactorial (Ashutosh *et al*. 2007; Chawla *et al*. 2011), without *a priori* knowledge an untargeted approach would be most suitable to detect the genetic changes leading to resistance. In the pre-genomics era, drug resistance studies were mainly limited to a handful of genes with suspected involvement in resistance, but the advent of genomics and next-generation sequencing technologies has lifted this research field to a new level. Before genome sequencing became well established, functional cloning has proven valuable to detect genes involved in drug resistance (reviewed by Clos and Choudhury, 2006). This method was used to identify the MIL transporter *LdMT* as responsible gene in MIL resistance by functional rescue: MIL-R parasites were transfected with cosmids from genomic DNA from a susceptible *L. infantum* line, and only the parasites transfected with the 'sensitive' *LdMT* regained sensitivity to MIL (Pérez-Victoria *et al*. 2003). A similar approach was used to identify another gene, P299, as a MIL marker that also provides protection against SSG (Choudhury *et al*. 2008).

Since drug resistance often appears to be mediated by gene amplification (Beverley, 1991), it is desirable to use a method that can detect copy number variations in the entire genome, such as full genome microarrays, comparative genome hybridization arrays and whole genome sequencing (WGS). WGS has the advantage of being able to detect mutations, copy number variations and ploidy changes simultaneously. A study of 17 clinical *L. donovani* lines with differential *in vitro* SSG susceptibility identified SNP and structural markers that were associated with SSG resistance, including 34 SNPs only present in four resistant lines, positive selection in genes associated with drug resistance, differential gene copy numbers and an episome containing an acid phosphatase and the MAPK1 gene (Downing *et al*. 2011). In a full genome microarray-based gene expression profiling study, the same MAPK1 gene

was also found overexpressed in SSG-R clinical *L. donovani* strains, together with histones H1, H2A and H4 (Singh *et al*. 2010). Another study found no ploidy or copy number changes related to resistance in the genome of multiple independent *L. major* lines with experimentally induced MIL resistance, but identified mutations in the *LdMT* gene and in a putative pyridoxal kinase gene (PK) in all resistant mutants (Coelho *et al*. 2012). Full genome microarrays are mostly used for gene expression profiling of different stages in the life cycle of *Leishmania*, but they are also useful for drug resistance studies to detect genes that are differentially expressed in drug-resistant and sensitive strains. Since regulation of gene expression is mainly post-transcriptional, changes in mRNA are often, though not always, the result of gene amplifications, deletions, or ploidy changes. Underlying genomic changes can be confirmed by Southern blot or comparative genome hybridization. Modulated expression of several chromosomes, gene overexpression and downregulation in experimental methotrexate-resistant *L. major* and *L. infantum* lines (MTX-R) and in experimental SSG-R *L. infantum* lines were the result of aneuploidy, circular gene amplification (dihydrofolate reductase-thymidylate synthase, MTX-R; ABC protein MRPA, SSG-R), linear gene amplification (pteridine reductase 1, MTX-R), and gene deletion (folate transporter FT1, MTX-R), respectively (Ubeda *et al*. 2008; Leprohon *et al*. 2009). In SSG-R mutants of *L. amazonensis*, higher expression levels of the MRPA locus were due to the presence of a linear amplicon or by aneuploidy of the whole chromosome (do Monte-Neto *et al*. 2011). Other overexpressed genes were related to thiol metabolism, but some did not have a direct link with drug resistance (nucleobase transporter NT3). In many cases, the differentially expressed or mutated genes are unknown or their involvement in drug resistance is unclear. Even if a link with drug resistance is more obvious, their contribution should be validated experimentally.

POST-GENOMIC APPROACHES TO COMPLEMENT GENOMIC DATA

Exploiting genome information to unravel pathogen biology has become the prime objective of numerous post-genomic studies. The metabolome has a privileged position in a systems biology context: it is situated at the other end of the modern 'dogma' of molecular biology, with information flowing from the genome, via the transcriptome and the proteome to the metabolome (Breitling *et al*. 2012). The metabolome is considered as the closest representation of the phenotype and represents the collection of metabolites, which are the final products of gene expression. Metabolite levels are not easily predicted by protein or transcript levels due to the highly non-local control structure of the metabolic network (e.g. enzyme inhibitors) and thus require separate analysis (Scheltema *et al*. 2010). Moreover, genome, transcriptome and proteome studies have limitations to functionally annotate identified sequences, whereas the metabolome consists of fewer components that are key actors of universal cellular processes underlying the relevance of this 'omics platform (Scheltema *et al*. 2010). In trypanosomatids such as *Trypanosoma* and *Leishmania*, metabolomics are of particular interest because the gene expression is regulated at the post-transcriptional level (De Gaudenzi *et al*. 2011; Requena, 2011) and hence genome and transcriptome studies might have certain limitations when studying the rapid effects of drug treatment or the mechanisms of drug resistance (Berg *et al*. 2012). For the moment, there is no consensus concerning the relative importance of changes in mRNA levels during parasite development (Lahav *et al*. 2011): some microarray analysis studies indicated the occurrence of substantial changes during promastigote to amastigote differentiation (>10%, Almeida *et al*. 2004; Saxena *et al*. 2007), whereas other studies suggested that transcription is constitutive throughout the parasite life and that there is a surprisingly low level of differentially expressed stage-specific genes (0·2–5% of total genes) (Akopyants *et al*. 2004; Cohen-Freue *et al*. 2007; Haile and Papadopoulou, 2007; Leifso *et al*. 2007). Within this limited set of genes, a dramatic downregulation of gene expression from stationary phase promastigotes to amastigotes was observed, highlighting the hypothesis of a pre-adaptation at metacyclic promastigote stage for intracellular survival as amastigote (Alcolea *et al*. 2010). Quantitative proteomics analyses showed a weak correlation to gene expression (20–30% correlation between changes in mRNA and protein levels, Lahav *et al*. 2011; Kramer, 2012) and have so far – similar to transcriptomics studies – mainly focused on the identification of stage-specific changes rather than on the investigation of resistance mechanisms, although useful contributions have been made to study SSG resistance (El Fadili *et al*. 2009; Singh *et al*. 2007; Walker *et al*. 2012). Compared to transcriptomics, proteomics studies revealed a higher level of differentially expressed stage-specific proteins for different *Leishmania* species (5–12%, *L. donovani* by Bente *et al*. 2003; *L. mexicana* by Nugent *et al*. 2004; *L. infantum* by Acestor *et al*. 2002), suggesting that *Leishmania* protein expression levels are likely regulated at the posttranscriptional or translational level (Cohen-Freue *et al*. 2007; Lahav *et al*. 2011). The metabolome will be affected to an even greater degree, due to the exponential effect of changes on the proteomic level (changes of a single enzyme will affect multiple metabolites) and due to the high connectivity in the metabolic network. A metabolomics study by Silva and colleagues

showed that there is a distinct variation in the metabolome during development in culture of *L. donovani* promastigotes: 26% of the total number of putatively identified metabolites was statistically different (Silva *et al*. 2011).

Although the technological developments are very recent and still being optimized, current liquid chromatography mass spectrometry LC-MS (Hilic-Orbitrap) platforms enable the metabolic profiling of multiple strains, thereby paving the way for large-scale integrative 'omics studies (e.g. diversity studies) (reviewed by Berg *et al*. 2012). In metabolomics studies, powerful biochemical, statistical, computational and bioinformatics tools are combined to generate a unique and much-needed dimension to better understand the parasite biology (Lakshmanan *et al*. 2011). For example, hypothesizing that gene dosage plays a major role in rapid evolutionary adaptation and phenotypic diversity of the parasite, metabolomics is currently used to exploit the impact of structural genome variation on the metabolic profile. Fig. 4 illustrates the complementarity of metabolomics and genomics. A recent metabolomics study (Berg, unpublished results) on three strains with a different SSG-susceptibility clearly reveals dramatic metabolic differences (1) between the different strains, (2) between different promastigote growth stages within the same strain, and (3) when a resistant strain is maintained under drug pressure. Parallel WGS of these strains showed that SNP analysis only allows distinction between strains. No distinction can be made at SNP level within a given strain between life stages or under drug pressure, while genome structure does show some differences. The functional impact of these structural changes at genome level is yet unclear, but we recommend the following: (1) genome structure should definitively be integrated into genome studies and (2) a particular attention should be paid to the choice of the life stage, not only for metabolomics studies, but also for genomics.

Current metabolomics studies have focused on metabolic changes related to SSG resistance in stationary phase promastigotes of *L. donovani* and have revealed modifications in complete pathways of the SSG-R strains. For instance, changes in the glycerophospholipids might be responsible for the increased fluidity of the plasma membrane of SSG-R parasites (Mukhopadhyay *et al*. 2011), possibly affecting the transport of antimonials inside the parasitic cell (t'Kindt *et al*. 2010). SSG-R parasites also upregulate both the transsulfuration pathway (biosynthesis of cysteine) and the urea cycle (synthesis of putrescine), possibly leading to higher levels of glutathione and trypanothione and an enhanced protection against oxidative stress (Canuto *et al*. 2012; Berg, unpublished results). Last but not least, increased levels of amino acids and purine nucleosides in SSG-R strains might serve as a survival kit during the initial stage of infection in nutrient-poor host cells.

For a true systems biology approach of genomics and metabolomics, some issues still need to be tackled. The interpretation of the identified genetic diversity elements in relation to specific phenotypic diversity remains challenging. A certain fraction of the genomic variation remains silent at the metabolic level, further complicating the integration of both global molecular profiles. Also, metabolic signatures reveal little about the genetic regulatory networks causing the metabolomic phenotype variation of the pathogen population. With quantitative trait loci mapping (QTL mapping), hotspots with system-wide effects can be highlighted (Fu *et al*. 2009), allowing to detect specific metabolite levels that serve as phenotypic indicators of the existence of underlying genetic contributors (Huang *et al*. 2009). Moreover, metabolic traits of the organism under study can be related to genetic polymorphisms in genome-wide association studies (GWAS), hereby throwing new light on the functions of certain genes (Homuth *et al*. 2012) and differentiating 'driver' mutations from biologically neutral 'passenger' changes.

EXPERIMENTAL VALIDATION OF GENES PUTATIVELY INVOLVED IN DRUG RESISTANCE

Functional analysis of differential genes in drug-resistant strains is essential to discriminate drivers from passengers and to understand their role in the resistance mechanism(s). Several genetic manipulation techniques, such as targeted gene replacement (knockout) or transfection with wild type or mutated genes (overexpression), are available for *Leishmania*. Unfortunately, gene knockdown by RNA interference is limited to *L. braziliensis* and other *Viannia* species, since no RNAi machinery is found in species of the *Leishmania* subgenus (Robinson and Beverley, 2003; Peacock *et al*. 2007). Gene knockout can be complicated by the ability of *Leishmania* to amplify genes or whole chromosomes, resulting in failure to create homozygous knockouts of essential genes, but this can be overcome by episomal gene complementation and conditional knockout (Murta *et al*. 2009; Morales *et al*. 2010). Due to differences in the environment, the mechanisms responsible for drug resistance in natural strains in the host are not necessarily the same as in experimental strains under laboratory conditions. Therefore, validation of identified genetic markers in naturally drug-resistant strains can be complicated. Even if genetic changes can be attributed to acquired drug resistance in experimental strains, translation to the field, where changing environments and host immunity add to the complex conditions, is not always straightforward.

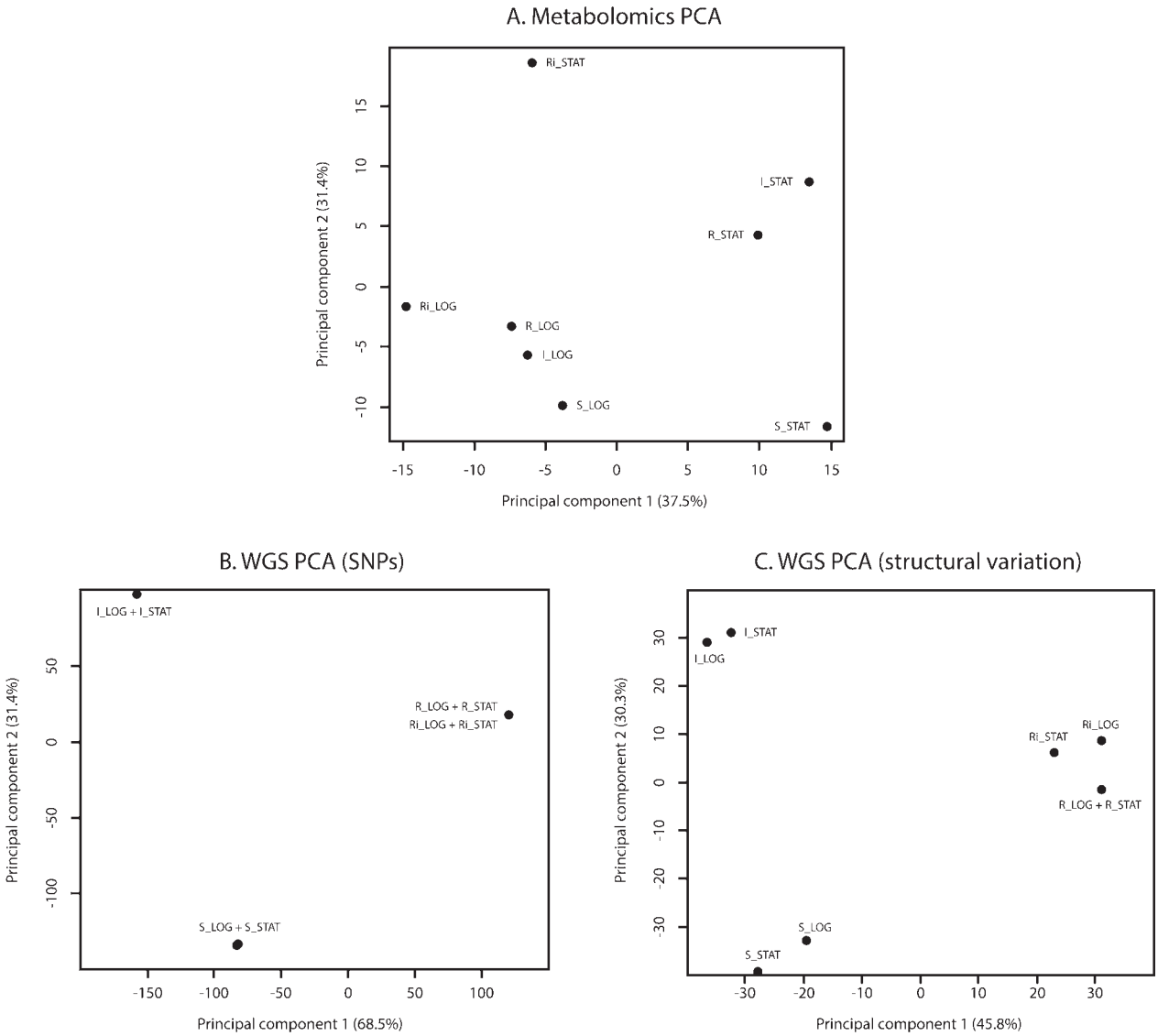

Fig. 4. Principal component analyses (PCA) based on a parallel metabolomics and genomics study of 3 *L. donovani* strains at two different growth stages of the promastigote life form or under Sb^{III} drug pressure. S: strain sensitive against both trivalent and pentavalent antimonials Sb^{III}-S/Sb^{V}-S; I: strain sensitive against trivalent antimonials but resistant against pentavalent antimonials Sb^{III}-S/Sb^{V}-R; R: strain resistant against both trivalent and pentavalent antimonials Sb^{III}-R/Sb^{V}-R; Ri = resistant strain (R) grown under Sb^{III} drug pressure for two weeks; LOG = logarithmic promastigote growth stage; STAT = stationary promastigote growth stage. (panel A) PCA plot of metabolomics results based on quantitative information of 326 putatively identified metabolites shows that separation is based on variability caused by (1) promastigote growth related differences (LOG strains cluster together and are clearly separated from STAT strains by PC1) and by (2) strains and drug pressure (S, I, R, Ri, separated by both PC1 and PC2). (panel B) PCA based on the SNPs determined with WGS shows a separation of the three strains. The two different growth stages of each strain (LOG, STAT) and the effect of Sb^{III} drug pressure during two weeks cannot be distinguished based on SNPs only, the dots overlap for each strain. (panel C) PCA based on structural variation (local copy number variation + ploidy changes) determined with WGS shows a separation by strain and drug pressure, but small differences between the life stages of each individual strain (for S, I and Ri) can be distinguished as well.

TRANSLATION TO THE FIELD/IMPROVING HEALTH PRACTICES

It is evident that generation of all the above-mentioned knowledge is only a first step towards improving the management of parasite resistance in practice. The next big challenge lies in implementing the acquired knowledge to the benefit of the patients and the populations at risk, outside the context of well-defined and well-funded clinical studies (summarized in Fig. 5). Here we need to make a clear distinction between poor and limited resource settings on the one hand, and highly equipped laboratories on the other hand.

In well-equipped clinical laboratories, which are typically found outside the impoverished endemic areas, one could revert to high-end applications for scoring resistance-related markers, both of a genetic

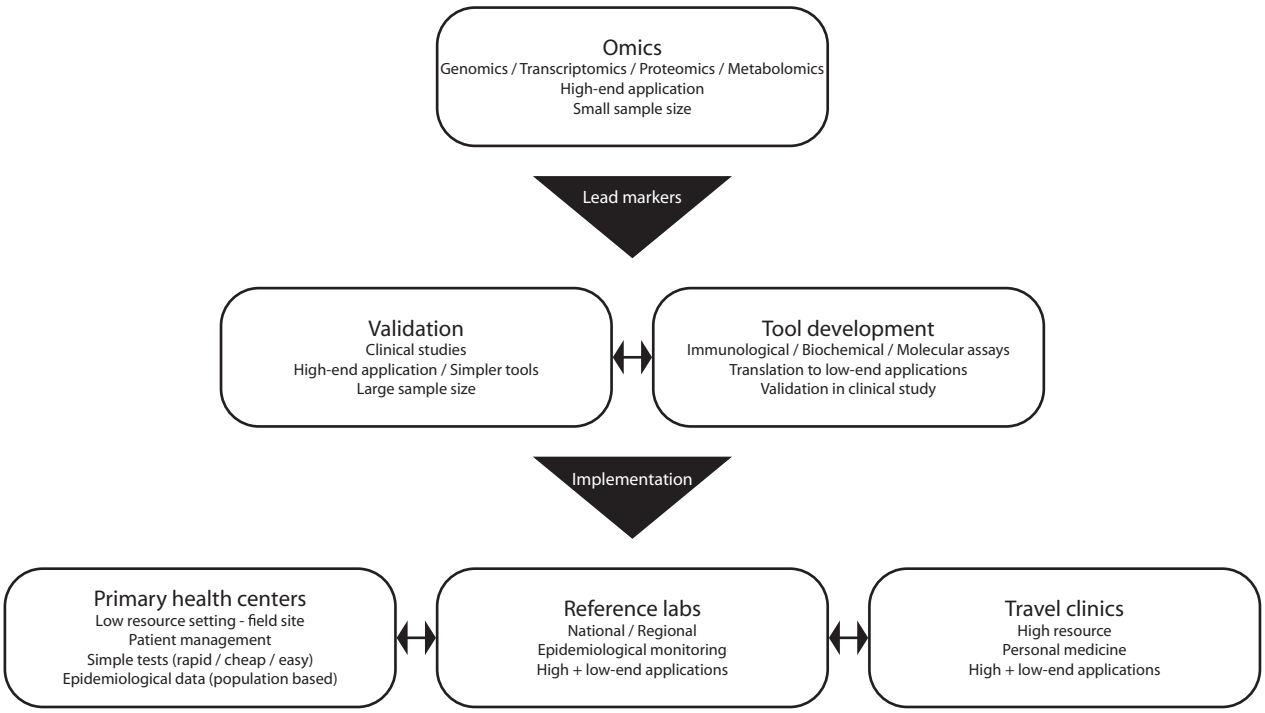

Fig. 5. Schematic overview of the steps involved in translating 'omics knowledge into disease management. Identified potential markers (top) need validation in large-scale clinical trials. In parallel, simpler assays can be developed that can be validated and used in the clinical studies. Finally, all available tools need to be implemented at different levels (bottom).

or a biochemical nature. Even full-genome sequencing directly on clinical samples and automated extraction of relevant genomic information may in time be feasible. Alternatively, biochemical or genetic PCR-based assays are even easier to implement and would not require any investments beyond currently available assays routinely used in such laboratories. Nevertheless, centres dealing with few cases on an annual basis would probably outsource the resistance profile determination to avoid the heavy investment in validation studies, as pharmaceutical companies have little to gain by producing quality-controlled and ready-made kits for a disease that is of marginal importance in the developed world. Outsourcing of resistance profiling is also common practice for other infectious agents, such as HIV.

A bigger problem is represented by limited-resource laboratories, which often have no access to even simple techniques such as PCR. Unless one could translate the acquired knowledge into a cheap and simple prognostic test that could score for parasite resistance in a given patient, treating clinicians would probably have to rely on available epidemiological data. Such epidemiological data are however typically scarce, and keeping them up-to-date requires intensive monitoring programmes that continuously scout for emerging and innate resistance in circulating *Leishmania* strains in different regions and for different drugs. Such screening must rely on a representative sampling, both in human and – if relevant – animal hosts, and could make use of the aforementioned high-end applications, provided policy makers would be willing to invest in well-equipped regional or national laboratory infrastructure.

Whichever the context, it is clear that translating knowledge on *Leishmania* resistance and treatment failure into useful tools is not a trivial exercise, and involves stakeholders at every level: scientists, health-policy makers, governments, private partners and non-profit organizations. We make strong advocacy for involving these parties from the very early steps of development on, to ensure that our knowledge on parasite resistance does not accumulate into merely another scientific publication, but is used for the benefit of the fight against leishmaniasis.

CONCLUSION

In this most recent episode of the arms race with human hosts, *Leishmania* have demonstrated unique and complex adaptive skills. Genome plasticity offers the parasite multiple solutions to the stresses induced by drugs. This represents a challenge for any new drug implemented in the field and justifies a close monitoring of drug efficacy. Similarly, the capacity to induce drug resistance should be integrated at early stage in research and development pipelines, and be used to guide drug development. Untargeted genomic approaches offer an unprecedented support to understand and monitor drug resistance, but should be complemented by downstream 'omics approaches, which are closer to the phenotype itself. The next

challenges reside in (1) the integration between the different 'omics layers, paving the way towards systems biology of drug resistance and (2) the translation of the findings for a direct benefit of the patients and the population at risk. Next-generation sequencing platforms are currently under continuous innovation, simplifying operational steps, allowing genome-wide studies on single cells and making the technology less expensive. Whole genomics and transcriptomics are thus becoming more and more accessible, which will undoubtedly contribute to a better understanding of biological phenomena such as drug resistance.

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