Faculty of Pharmacy, Biomedical and Veterinary Sciences

Department of Biomedical Sciences

Role of gene dosage in the acquisition of antimony resistance in *Leishmania donovani*: experimental evidence

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Cover picture: THP1 infected cells with *Leishmania major* and stained with DAPI (blue), anti-alpha tubulin (green) and anti-HASPB (red). The picture is a gift of Dr. Helen Price from the Keele University.

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A ma sœur, *to my syster,*

Que cette petite contribution soit une source d'inspiration,

may this small contribution inspire you

A ma mère et ma grand-mère, *to my mother and my grand-mother,*

Mes deux plus grandes fans,

my two biggest fans

A mon père et mon grand-père, *to my father and my grand-father,*

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"*If two points are destined to touch, the universe will always find a way to make the connection- even when all hope seems to be lost. Certain ties cannot be broken. They define who we are and who we become. Across space, across time, among paths we cannot predict- nature will always find a way*." Everyone Has a Story, Savi Sharma

This quote from one of my favourite book is exactly the way I can picture my PhD time working at the ITM. Similar to everything else in life, it is all about meeting certain people at the right time. This little contribution is not only about science, it is also about people.

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Summary:

Leishmania donovani is the etiological agent of visceral leishmaniasis (VL) in the Indian sub-continent (ISC). *Leishmania* are Protozoa alternating between two life forms: the extracellular promastigotes in the insect vector and the intracellular amastigotes in the mammalian host. Antimony, in its pentavalent form, was the first line drug in the ISC from 1923 to 2005 when it was abandoned because of treatment failure and drug resistance. I was involved in the first large scale phylogenomic study of *L. donovani* in the ISC. This study -undertaken with *in vitro* cultivated promastigotes- provided two main findings that underlie this PhD. Firstly, two genetically very different populations were encountered in the ISC: (i) the main population, endemic in the Gangetic plains and called the Core Group (CG), and (ii) another population found in the Nepalese high-lands and called ISC1. Secondly, the isolates showed a high level of aneuploidy and 100% of the CG isolates showed a unique ancestral character, the intra-chromosomal amplification (ICA) of the H- and M-loci. However, the link between these genomic features and drug resistance was not clear in this study.

The main hypothesis of this PhD was that gene dosage would play a major role in the development of antimonial resistance in the ISC. My overall goal was to analyse in experimental conditions the emergence of antimonial resistance upon *in vitro* selection. The specific aims were to study (i) if the same genomic adaptations would be encountered as in natural populations, (ii) in which order they would appear and (iii) to validate the importance of these adaptations for antimonial resistance. In the first part of the PhD, I characterized the stability of the parasite genome during the life cycle. For the first time, the genome and transcriptome of *Leishmania* strains were compared *in vitro*, in the sand fly *Phlebotomus argentipes*, and in golden Syrian hamsters. While the genome sequence itself was stable, a drastic and strain- specific modulation of parasite's aneuploidy was observed inside the mammalian host. This was highly correlated with transcription levels, supporting the adaptive function of gene dosage. This observation showed the limits of phylogenomic studies based on isolated promastigotes: as such, many adaptations linked to drug resistance and present in the host could have been lost/diluted in culture. In the second part of this PhD, I analysed the molecular adaptations developed by ISC1 and CG strains during *in vitro* selection of resistance to trivalent antimonials (Sb^{III}). The ISC1 strain required more time to become resistant than the CG ones and also needed many more molecular adaptations, as demonstrated by untargeted genomics and metabolomics. This led us to the hypothesis that CG strains were pre-adapted to Sb^{III} resistance, which we demonstrated by exposing different strains directly to a maximal concentration of Sb^{III}: in contrast to ISC1 strains which all

died, CG ones all survived this major stress. The amplification of the H-locus, in particular the MRPA gene, responsible for Sb^{III} sequestration, was shown by over-expression to be a major driver of Sb^{III} resistance. While this gene was naturally amplified in CG strains, it was amplified in the selected Sb^{III}-resistant ISC1 strain by the generation of additional copies of a whole arm of chromosome 23, bearing the MRPA gene. Gene dosage is thus a very important mechanism for the acquisition of drug resistance and MRPA amplification is the first one emerging in natural and experimental conditions.

This work paves the way for further basic studies on the adaptive skills of *Leishmania* and it highlights the need for surveillance of parasites in endemic regions, in particular in the context of running control programmes. Furthermore, the genotypic and phenotypic adaptations present in the field should be taken into consideration in R&D for new anti-*Leishmania* drugs.

Samenvatting:

Leishmania donovani veroorzaakt viscerale leishmaniase (VL) in het Indische subcontinent (ISC). *Leishmania* zijn Protozoa die alterneren tussen twee levensvormen, namelijk de extracellulaire promastigoten in de insect vector en de intracellulaire amastigoten in de zoogdier gastheer. Vanaf 1923 was pentavalent antimonium het eerste geneesmiddel in het ISC, maar door therapiefalen en geneesmiddelenresistentie werd het afgeschreven in 2005. Ik was betrokken bij de eerste grote fylogenomische studie van *L. donovani* in het ISC. Deze studie – op basis van *in vitro* gekweekte promastigoten – resulteerde in twee nieuwe inzichten die de aanzet vormden voor dit doctoraat. Ten eerste vonden we twee genetisch verschillende populaties binnen het ISC: (i) de kerngroep, een populatie endemisch in de Indo-Gangesvlakte, en (ii) ISC1, een populatie die circuleert in de Nepalese berggebieden. Ten tweede vertoonden de isolaten sterke aneuploïdie en alle kerngroepisolaten vertoonden een uniek ancestraal kenmerk, namelijk een intrachromosomale vermeerdering van het H- en M-locus. Het verband tussen deze genomische kenmerken en geneesmiddelenresistentie was echter niet duidelijk op basis van de fylogenomische studie. De belangrijkste hypothese van dit doctoraat was dat gendosering een belangrijke rol speelt in de ontwikkeling van antimoonresistentie in het ISC. Mijn doel was om onder experimentele condities het ontstaan van antimoonresistentie na *in vitro* selectie te bestuderen. De specifieke doeleinden zijn om (i) de natuurlijke genomische adaptaties experimenteel te bevestigen, (ii) de volgorde waarin deze adaptaties verschijnen te bepalen, en (iii) het belang van deze adaptaties voor antimoonresistentie te valideren. In het eerste deel van het doctoraat heb ik de stabiliteit van het parasietgenoom doorheen de levenscyclus gekarakteriseerd. Voor de allereerste keer is het genoom en transcriptoom van *Leishmania*-stammen *in vitro*, in de zandvlieg *Phlebotomus argentipes* en in de goudhamster vergeleken. Hoewel de genoomsequentie stabiel bleef, werd er een drastische en stamspecifieke verandering van aneuploïdie binnen de zoogdier gastheer geobserveerd. Dit was sterk gecorreleerd met de transcriptiewaarden, wat de adaptieve functie van gendosering ondersteunt. Deze observatie toont de tekortkoming aan van fylogenomische studies die gebaseerd zijn op promastigoten: veel adaptaties die aanwezig zijn in de gastheer en gekoppeld zijn aan geneesmiddelenresistentie kunnen verloren gaan na kweken van de parasieten in cultuur. In het tweede deel van dit doctoraat bestudeerde ik de moleculaire adaptaties van ISC1 en kerngroep stammen tijdens een *in vitro* selectie experiment met trivalente antimoon (Sb^{III}) resistentie. De ISC1 stam had meer tijd nodig om resistent te worden dan de kerngroepstammen, en ondervond ook meer moleculaire adaptaties zoals aangetoond met behulp van niet-gerichte genomica en metabolomica. Dit leidde tot de hypothese dat

kerngroepstammen vooraf aangepast waren aan Sb^{III}-resistentie, wat we bevestigden door verschillende stammen direct bloot te stellen aan de maximale Sb^{III} concentratie: in tegenstelling tot de ISC1 stammen die allemaal stierven, overleefden alle kerngroepstammen deze stressvolle behandeling. Overexpressie analyse toonde aan dat de vermeerdering van het H-locus – voornamelijk het MRPA gen dat verantwoordelijk is voor Sb^{III} opslag – een belangrijke motor is van Sb^{III} resistentie. Terwijl het MRPA gen van nature vermeerderd was in de kerngroepstammen, was het ook vermeerderd in de geselecteerde Sb^{III} resistente ISC1 stam doordat een volledige arm van chromosoom 23 met het MRPA gen in meerdere kopieën voorkwam. Gendosering is dus een heel belangrijk mechanisme voor de verwerving van geneesmiddelenresistentie en MRPA vermeerdering is het eerste mechanisme dat verschijnt in natuurlijke en experimentele condities. Dit werk laat toekomstige fundamentele studies toe over de adaptieve capaciteiten van *Leishmania*, en het benadrukt het belang van toezicht op parasieten in endemische gebieden, vooral binnen de context van lopende controleprogramma's. Tenslotte, de genotypische en fenotypische adaptaties in de natuurlijke context moeten in rekening gebracht worden in R&D voor nieuwe anti-*Leishmania* geneesmiddelen.

Abbreviations

A-D

ABC: ATP binding cassette

ACD: Active case detection

AmB: Amphotericine B

AN: Anthroponotic

AQP1: Aquaglyceroporine 1

As: Arsenic

ASS: Argininosuccinate synthase

AZN: Anthropozoonotic

BRCA2: BReast CAncer 2

Cd: Cadmium

CG: Core group

CL: Cutaneous leishmaniasis

CNV: Copy number variation

DAT: Direct agglutination test

DCL: Diffuse cutaneous leishmaniasis

DDT: Dichlorodiphenyltrichloroethane

DHFR: Dihydrofolate reductase

E-H

ECA: Extrachromosomal amplification

GSH: Glutathione

HSP70: Heat shock protein 70

HTBF: Terbinafine resistance locus protein

I-L

ICA: Intrachromosomal amplification

IFNα: Interferon α

IRS: Indoor residual spraying

ISC: Indian sub-continent

ITN: Insecticide treated nets

ITS1: Internal transcribed spacer 1

IV: Intravenous

KAEP: Kala-azar Elimination Programme

KAtex: Kala-azar latex agglutination test

kDNA: kinetoplastic DNA

LAMP: Loop mediated isothermal amplification assay

LMWA: Low molecular weight antigen

LPG: lipophosphoglycan

M-P

MAPK: Mitogen activated protein kinase

MCL: Muco-cutaneous leishmaniasis

MIL: Miltefosine

MLEE: Multilocus enzyme electrophoresis

LxMT: Miltefosine transporter

MTX: Methotrexate

MPK1: Mitogen-activated protein kinase 1

MRE11: Meiotic Recombination 11

MRPA: Multi resistance protein associated

MTX: Methotrexate

$NADP^+$: Nicotinamide adenine dinucleotide phosphate

NTD: Neglected Tropical Disease

PCD: Passive case detection

PI3K: Phosphoinositide 3-kinase

PKC: Protein kinase C

PKDL: Post Kala azar dermal leishmaniasis

PM: Peritrophic matrix

PMM: Paromomycin

PSG: Promastigote secreting gel

PTPase: Protein tyrosine phosphatase

PTU: Polycistronic transcription unit

PV: Parasitophorous vacuole

Q-T

R&D: Research and Development

RAG: Repeat alignment groups

rK39: recombinant kinesin 39

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

SAcP: Secreted Acid Phosphatase

SAG: Sodium Antimony Gluconate

Sb: Antimony

SHP1: Src homology PTPase 1

SIDER: Short Interspersed Degenerate Retroposons

SL: Splice leader

SNP: Single Nucleotide Polymorphism

SOD: Super oxide dismutase

SSG: Sodium Stibogluconate

STAT1: Signal transducer and activator of transcription 1

sv: stomodeal valve

TDR1: Thiol dependant reductase 1

TGF: Transforming growth factor

TR: Trypanothione reductase

TS: Trypanothione synthase

TSS: Transcription starting site

U-Z

VL: Visceral leishmaniasis

WGS: Whole genome sequencing

WHA: World Health Assembly

WHO: World Health Organisation

Chapter 1:

Leishmania and Leishmaniasis around the world and in the Indian sub-continent

1.1 Generalities about Leishmania and leishmaniasis

1.1.1 History of the discovery in India

Leishmania, and more particularly *Leishmania donovani*, has a strong connexion with the Indian Sub-Continent (ISC), since it was first described by Lieutenant General Sir William Boog Leishman and then confirmed by Captain Charles Donovan both engaged in the British army in India in the 1900's [1]. In 1903, Sir Leishman described *"… a species of Trypanosoma as the cause of one of the indefinite varieties of fever occurring in that country (India), in which the presence of malaria parasites in the blood is not determined…for want of the better name, I may speak of it as Dum-Dum fever…"* [2]. A couple of months later, Captain Donovan was describing a similar phenomenon in Indian patients and on the same type of histological preparation. At the time, Ronald Ross started to investigate the spreading of malaria and kala-azar. Although he lacked the time to investigate the last point he established the symptomology as: "phase 1, acute fever with enlargement of the spleen and liver, phase 2, acute enlargement of the spleen and liver, with a low fever, phase 3, cachexia, with no fever" [3]. The disease was then called Dum-Dum fever, or kala-azar, black fever in Hindi, or later on visceral leishmaniasis (VL). The parasite responsible for this particular clinical manifestation was then named after its discoverers: *Leishmania donovani*.

1.1.2 Taxonomy and life cycle

Leishmania is a genus of early branching Eukaryotes from the *Euglenozoan* clade belonging to the class of *Kinetoplastea* and to the *Trypanosomatids* family [4,5]. All *Leishmania* species are parasitic life forms. Based on multilocus enzyme electrophoresis (MLEE), the *Leishmania* genus was described as being composed of about 20 species, most of them clinically relevant [6]. The classification of the different *Leishmania* species can be superposed upon their geographic repartition: (1) the Old World species mainly composed of *L. donovani*, *L. infantum*, *L. major* and *L. tropica* and (2) the New World species with *L. braziliensis*, *L. peruviana*, *L. panamensis* and *L. guyanensis* (Table 1) [7–10]. Most of the species of the *Leishmania* genus are anthropozoonotic meaning that they infect humans and wilds mammals act as a reservoir and participate in the transmission [11]. Only *L. donovani* is traditionally described as strictly anthroponotic. Even though some evidence of cattle [12] and dogs [13] positive for *L. donovani* in Nepal and Bangladesh have been found, their role as reservoirs has not been demonstrated yet.

Table 1: Main *Leishmania* species of clinical interest with their geographic area and their respective sand fly vector. Lu. *Lutzomyia*, P. *Phlebotomus*, VL, Visceral Leishmaniasis, CL, Cutaneous Leishmaniasis, MCL, Mucocutaneous Leishmaniasis, DCL, Diffuse Cutaneous Leishmaniasis, AN, Anthroponotic, AZN, Anthropozoonotic

Sub-genera	Species	Area	Vector	Clinical manifestation	Transmission mode	Ref
L. (Leishmania)	*L. donovani*	India, Bangladesh, Nepal, Ethiopia, Soudan, North Soudan, Kenya, Sri Lanka*	*P. argentipes*, *P. orientalis*, *P. martini*	VL, PKDL, CL*	AN	[7–9]
	L. infantum syn. chagasi	France, Spain, Portugal, Italy, Malta, Cyprus, Algeria, Tunisia, Iraq, Asia, South America	*P. ariasi*, *P. perniciosus*, *Lu. longipalpis*, *Lu. evansi*, *Lu. olmeca*	VL	AZN	[7,8]
	L. major	North Africa, central and west Asia	*P. papatasi*, *P. duboscqi*, *P. salehi*	CL	AZN	[7]
	L. arabica	Saudi Arabia	*Ph. papatasi*	CL	AZN	[10]
	L. tropica	Central and west Asia and Western India	*P. sergenti*	CL	AZN	[7]
	L. aethiopica	Ethiopia and Kenya	*P. longipes*, *P. pedifer*	CL	AZN	[7]
	L. mexicana	Belize, Colombia, Costa Rica, Ecuador, El Salvador, Guatemala, Honduras, Mexico, USA	*Lu. olmeca*, *Lu. columbiana*, *Lu. ayacuchenisis*, *Lu. longipalpis*, *Lu. ylephiletor*, *Lu. cruciata*	CL, DCL	AZN	[7]
	L. amazonensis	Brasil, Bolivia, Colombia, French Guyana	*Lu. longipalpis*	CL, DCL	AZN	[7]
L. (Viannia)	*L. braziliensis*	Argentina, Belize, Bolivia, Brazil, Colombia, Costa Rica, Ecuador, French Guyana, Guatemala, Honduras, Nicaragua, Panama, Peru, Venezuela	*Lu. longipalpis*, *Lu. whitmani*, *Lu. umbralitis*, *Lu. ylephiletor*, *Lu. ovallesi*, *Lu. trapidoi*, *Lu. wellcomei*, *Lu. intermedia*, *Lu. correrai*	CL, MCL	AZN	[7]
	L. peruviana	Peru	*Lu. peruensis*, *Lu. verrucarum*	CL	AZN	[7]
	L. guyanensis	Brazil, Colombia, French Guyana, Guyana, Venezuela	*Lu. umbralitis*, *Lu. anduzei*, *Lu. ovallesi*	CL	AZN	[7]
	L. panamensis	Colombia, Costa Rica, Honduras, Nicaragua, Panama	*Lu. trapidoi*, *Lu. hartmanni*, *Lu. panamensis*, *Lu. gomezi*	CL	AZN	[7]
	L. naiffi	Brasil, French Guyana	*Lu. ayrozai*, *Lu. squamiventris*	CL	AZN	[7]
	L. lainsoni	Brazil	*Lu. ubiquitalis*	CL	AZN	[7]

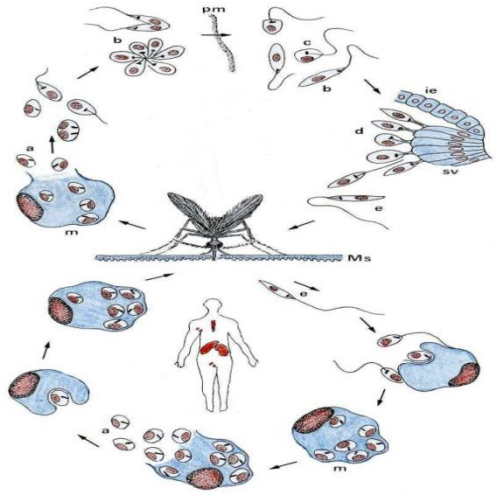


Figure 1: *Leishmania donovani* life cycle (modified from http://www.parasitologie.univ-montp1.fr/english_vers/en_leish2.htm).

During its life cycle (Figure 1), *Leishmania* alternates between two different hosts: (1) a blood-feeding phlebotomine vector (over 90 different species of phlebotomine sand flies are vectors) where the parasite develops as a promastigote, an extracellular flagellated pin-head cell and (2) a mammalian host where the parasite establishes itself as amastigote, a shortly flagellated ovoid intracellular but extra-cytoplasmic cell, inside professional phagocytic cells (m) such as macrophages and dendritic cells [14]. After a female sand fly takes a blood meal on an infected mammal, the parasites first develop in the abdominal midgut, inside the peritrophic matrix (PM), a non-cellular structure composed of chitin surrounding the blood meal. Inside the PM amastigotes are released from their host cell and differentiate into procyclic promastigotes, their highly proliferative form (a). The differentiation process is composed of several different morphological changes. Inside the PM highly replicative forms with long cell body and long flagellum called nectomonads are present (b and c). They secrete chitinases in order to rupture the PM and are finally released inside the lumen of the midgut and adhere to the microvilli by their flagellum [15,16]. At that moment, they shorten to become leptomonad forms and two different populations of promastigotes will differentiate. The first one is composed of non-proliferative forms called metacyclic promastigotes (e) after undergoing the process of metacyclogenesis. Metacyclics are the only promastigotes able to infect a mammal [17]. The second population is called haptomonads (d). They adhere to and cluster on the chitin lining of the stomodeal valve (sv) and start secreting a gel composed of filamentous proteophosphoglycan, the promastigote secreting gel (PSG). The combined action of the PSG secretion and the destruction of the valve facilitates the reflux of parasites when the fly takes another blood meal [18,19]. At that moment, metacyclics enter in the body of the mammalian host to be first taken up by neutrophils, as they are the first cell type to arrive at the biting site. One of the proposed mechanisms for *Leishmania* parasites getting inside macrophages is the "Trojan horse". *Leishmania* is present inside neutrophils as promastigotes. They further elicit neutrophils apoptosis to boost the inflammation and to recruit more macrophages [20]. It is at that moment that

macrophages, amongst other professional phagocytic cells, phagocyte *Leishmania* promastigotes, forming a phagosome (m). The phagosome will then fuse with lysosomes in order to degrade the parasite. It is the combination of the increase in temperature (26°C to 37°C) and the decrease of pH (7 inside the midgut of the phlebotomine host versus 5.5 inside the lysosome of the macrophage) that is the signal for promastigotes to differentiate into amastigotes [21]. Once the amastigotes are established, they persist inside the host cell in a vesicle formed by the fusion of the phagosome and the lysosomes; this vesicle is then called the parasitophorous vacuole (PV) [22]. The parasite will replicate inside the PV until the host cell bursts and releases its content, including the amastigotes that will be taken up by other macrophages or dendritic cells to perpetuate the colonisation inside the target organs.

1.2 Leishmaniasis: geographical distribution, economic impact, pathology and diagnosis

1.2.1 Epidemiology and pathology

Leishmaniasis is a vector-borne complex of diseases with a global incidence estimated to be 1.5 to 2 million cases per year and up to 350 million people at risk in 98 endemic countries [23]. *Leishmania* is therefore the 2nd largest parasite killer after *Plasmodium*. In May 2013, the 66th World Health Assembly adopted resolution WHA66.12 calling on the World Health Organisation (WHO) member states to intensify their fight against Neglected Tropical Diseases (NTDs), including Leishmaniasis [24]. They urged the member states to implement the recommendations from 2012 based on the WHO document called "*Accelerating work to overcome the global impact of neglected tropical diseases, a roadmap for implementation*" [25]. Leishmaniasis is part of the 17 neglected tropical diseases affecting one billion people worldwide (from http://www.who.int/neglected_diseases/diseases/en/ accessed on the 11/07/2017). Like all the other NTDs, there is a strong link between leishmaniasis and poverty [26]. Indeed, the poorest are the most exposed to the disease for multiple reasons such as housing conditions: cracks in the walls facilitate the rest of the sand flies or improper doors facilitating entry of the sand fly [27,28]. In Latin America, poor sanitation and irregular waste collection was associated with an increased risk of being infected by the parasite as it gives a favorable ground for sand fly breeding [27]. Although insecticide impregnated bed nets have been shown to be effective in the control of leishmaniasis, the high cost of such protective accessories often prevents poor people in endemic areas from having effective protection [27].

Leishmania is present nearly everywhere in the inter-tropical area. The clinical manifestations of leishmaniasis are multiple and show four different forms (see table 1):

- Cutaneous leishmaniasis (CL) is non-lethal and relatively the least physically damaging form of the disease but still socially impacting [29,30]. The pathology presents a dermatological panel: an ulcer or a nodular lesion at or near the biting site of the sand fly. CL is the most common clinical presentation of leishmaniasis, it is caused by many different species in the Old World and in the New World, e.g. *L. major*, *L. tropica*, *L. braziliensis*, *L. amazonensis*, etc… (Table 1). According to the WHO, the global incidence of CL is around 700.000 to 1.3 million annual new cases. Ten countries (Afghanistan, Colombia, Brazil, Algeria, Peru, Costa Rica, Iran, Syria, Ethiopia, and Sudan) account for 75% of all the CL cases (Table 1, Figure 2A and 3A) [23,31].
- Muco-cutaneous leishmaniasis (MCL) is non-lethal but the most physically impacting clinical manifestation, causing not only physical damages but also a high socio-economic burden [32]. MCL causes destruction of the facial mucosa: nasal septum, oral cavity, pharynges and larynges [33]. The species causing most cases of MCL is *L. braziliensis*. The reasons why *L. braziliensis* causes CL and sometimes MCL are not clear even if the presence of a *Leishmania* RNA virus seems to be one of the factors for MCL [34,35]. Few cases are reported every year and 90% of those cases come from Bolivia, Brazil and Peru [31] (Table 1).
- Visceral leishmaniasis (VL) is the only fatal form of leishmaniasis *per se*. The disease starts with acute fever and mild hepatosplenomegaly. The progression of the disease will lead to regular peaks of fever, anaemia, and cachexia and an increased swelling of the spleen and of the liver. It is lethal in 95% of the cases if untreated. Two species are responsible for 90% of the VL cases worldwide: *L. donovani* and *L. infantum (*syn. *chagasi)*, which are endemic in Ethiopia, India, Somalia, South Sudan, Sudan and Brazil (Table 1, Figure 2B and 3B) [23,31]. It is noteworthy that VL is also endemic around the Mediterranean Basin, including South Europe.
- Post Kala-azar dermal leishmaniasis (PKDL) is a complication of *L. donovani*-VL. However clinical signs are extremely different from VL and usually appear months or years after the clinical cure of VL. The disease is characterised by an aggressive and slow progression of the parasite in the skin that appears as macular, maculo-papular or nodular lesions [36].

Interestingly, a large population of infected people in endemic regions remains asymptomatic. It has been shown in India and Nepal that the prevalence of asymptomatic infection was 8 times higher than VL prevalence, only 1 out of 50 seropositive patients would develop VL within 18 months after detection [37]. The role played by asymptomatic carriers in the transmission is thought to be extremely important but yet is poorly understood.

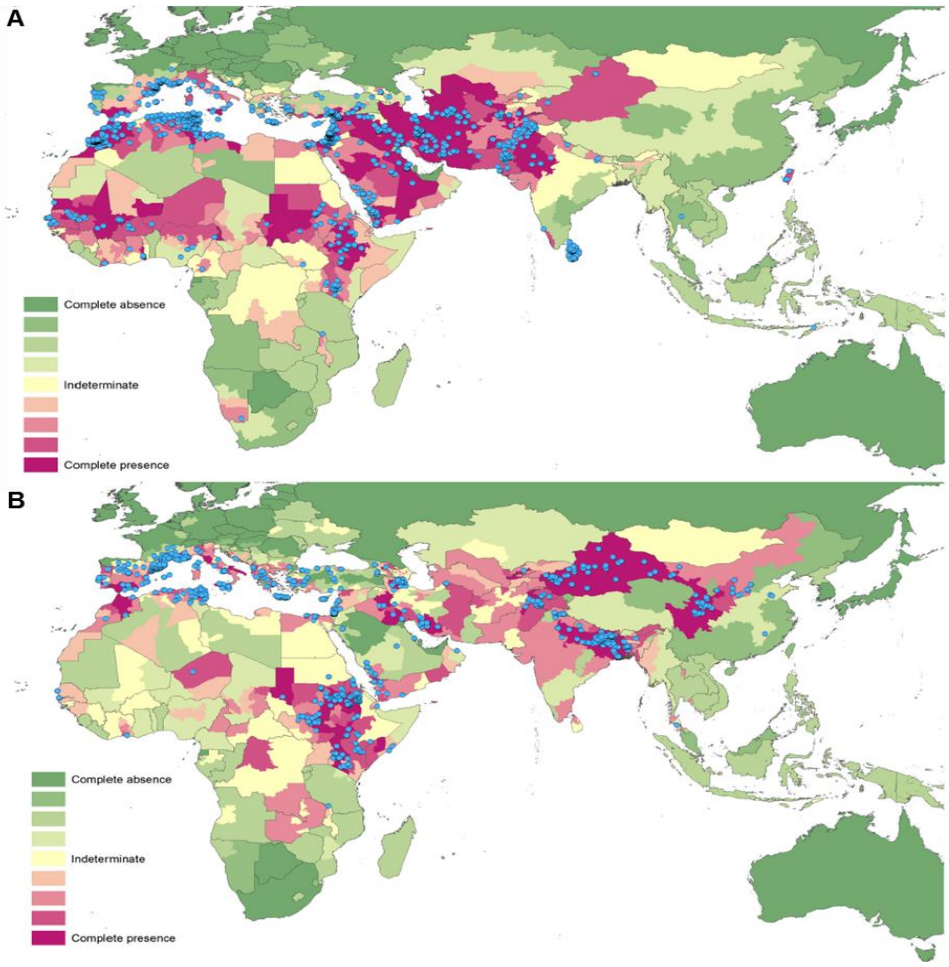


Figure 2: Spatial repartition of (A) cutaneous leishmaniasis and (B) viceral leishmaniasis in the Old World. The colors represent the absence or the presence of the disease. Blue dots indicate precisely infection pockets or the barycentre of a polygon made by small infection pockets (from Pigott, DM *et al.* Elife 2014 [38]).

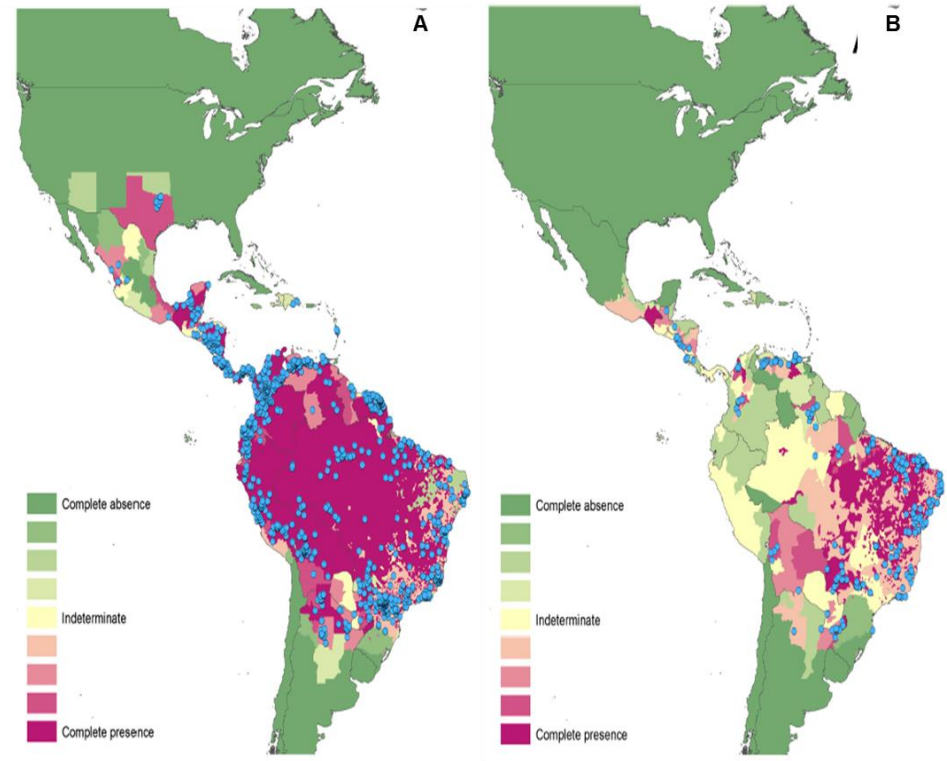


Figure 3: Spatial repartition of (A) cutaneous leishmaniasis and (B) visceral leishmaniasis in the New World. The colors represent the absence or the presence of the disease. Blue dots indicate precisely infection pockets or the barycentre of a polygon made by small infection pockets (from Pigott, DM *et al.* Elife 2014 [38]).

1.2.2 Transmission

We can distinguish 3 different types of transmission patterns (Table 1).

- Zoonotic transmission: in that case wild animals are considered as a reservoir and humans are accidental hosts. The paradigmatic species is *L. braziliensis* in the Amazonian forest where rodents and sloths are infected but do not show any symptoms, while humans develop severe lesions.
- Anthropo-zoonotic transmission: here human and animals are both reservoir for the parasite. The examples of *L. mexicana* and *L. infantum* are the most striking. The former causes CL in humans and in rodents and the latter causes VL in humans and dogs.
- Anthroponotic transmission: humans are the main reservoir during the life cycle. *L. donovani* and *L. tropica* are the two paradigmatic species.

A few cases of human to human transmission have been reported through exchange of needles by drug users [39,40] or blood transfusion [41].

1.2.3 Diagnosis

The diagnosis of leishmaniasis is ideally based on clinical manifestations coupled with epidemiological features and laboratory analyses. Laboratory diagnostics of leishmaniasis can be done indirectly, looking for host antibodies, or directly, looking for the parasite itself (antigens or DNA). The following list of approaches is particularly focusing on VL diagnostics, even if some described diagnostic tools are used for the other forms of the pathology.

- Indirect tests are named as such because they do not detect the parasite or a sub-product of the parasite but the presence of antibodies in the mammalian host serum, plasma or whole blood. There is a large diversity of techniques with a large spectrum of sensitivity and specificity. Noteworthy, serology is generally used for VL diagnostics given its Th2 profile. The most sensitive one is the Direct Agglutination Test or DAT. The DAT is composed of *L. donovani* trypsinised, coloured and lyophilised promastigotes that agglutinates within 18h if put in contact with positive host serum [42]. According to a meta-analysis including 30 studies, the sensitivity and specificity of DAT were estimated at 94.8 and 97.1%, respectively, while its performance was not influenced by the region or the parasite's species [43]. Another point in favour of DAT is its cheap price. On the other hand, even if easy to use it requires some lab equipment and the help of a skilled technician for the reading of the results. In order to tackle these issues a dipstick-based recombinant test called rK39 has been developed. K39 is a conserved antigen of 39 amino acids from the kinesin 39 protein present in the *L. donovani* complex, fixed on a strip in its recombinant form. With this strategy one can detect antibodies directly from a very small drop of blood, making it non-invasive. The technique has shown a good sensitivity (87-99%) and specificity (87-97%) in the ISC when tested on symptomatic VL-patients [44–46]. However, while the DAT is extremely sensitive in all endemic regions for every species of *Leishmania*, rK39 has a sensitivity of only 67% in sera samples from Sudan. The high sensitivity of the rK39 test in India is due to the fact that K39 elicits a higher anti-rK39 antibody response in the ISC than in East Africa [44]. This can be explained by the genetic variation in the kinesin 39 gene between parasites from East Africa and from the ISC. The price of rK39 (1USD/strip), its sensitivity and its non-

invasiveness makes it the first line diagnostic test in India. There is one biological issue when working with indirect tests, i.e. the immunocapacity of the patient. They can show false positive results due to antibody persistence years after successful treatment or false negative due to a weak immune response of a patient.

- There are 4 main direct diagnostic test types: microscopy, parasite culture, PCR/PCR-associated techniques and antigen detection. Microscopy remains the gold standard for VL diagnostics. It consists of the direct visualisation of amastigotes fixed and Giemsa stained from a clinical sample such as lymph node, bone marrow or spleen aspirates. This technique has a sensitivity inferior to 50% but a specificity higher than 95% [47,48] and is very simple to put in place in all conditions since it requires only a microscope and the technical skills to detect the amastigotes. Another way of detecting the parasite is to try to cultivate it *in vitro* on a biphasic medium composed of blood agar and a liquid phase. This technique is extremely reliable however the cultivation success rate in confirmed VL cases is lower than 50% [49]. PCR based parasite detection relies on DNA extraction from the patient sample and subsequent specific detection of *Leishmania* DNA. Many different diagnostic PCR tests have been developed over the years targeting different sequences (HSP70 [50], kDNA minicircle [51,52], ITS1 [53] and mini-exon [54]) with very variable sensitivity and efficiency [55]. However, all the described techniques require a fully equipped molecular biology laboratory which can be complicated in the field. Several attempts have been made to develop Loop-mediated isothermal amplification assays (LAMP) to technically simplify the direct detection and making it easily accessible in non-urbanised areas [56,57]. The last direct detection method is antigenic based. The only one for *Leishmania*, KAtex (Kala Azar latex agglutination test), detects a low molecular weight antigen of carbohydrate nature by agglutination with monoclonal antibodies coated on latex beads. It can detect *Leishmania* antigens from a urine sample with a relatively high sensitivity (75%) and a nearly perfect specificity (~100%) [58,59].

1.3 Drugs, their mode of action and clinical resistance

Nowadays, few *Leishmania* specific drugs are approved for human use and a few compounds are in the R&D pipeline. Four classes of drugs are available and drug resistance for some of them is already in the field. This section details the drugs, their mode of action (if known) and whether resistance exists and in what proportion.

1.3.1 Antimonials

Antimonials were the first drugs discovered with an anti-*Leishmania* activity. In fact, antimony was first used in its trivalent form, Sb^{III} as tartar emetic (a double salt of tartaric acid with potassium and trivalent antimony) against CL in 1912 by Vianna [60] and its efficiency against VL was confirmed in 1915 in South America [61] and 1916 in India [62,63]. However, Sb^{III} demonstrated a high toxicity and a poor stability in tropical conditions [64]. It was replaced by its pentavalent form Sb^{V}, as urea stibamine, in 1923 and successfully tested on VL patients in Calcutta by Dr. Brahmachari until 1937 [65]. At that time, pentavalent antimonial sodium stibogluconate (SSG) or sodium antimony gluconate (SAG, Pentostam™ or solustibosan), a chelate of Sb^{V} with gluconate was discovered and successfully administrated in China. Ten years later, the French discovered meglumine antimoniate (Glucantime™). In the late 40's, the Sb^{V} treatment success rate was about 90% in India and kept the same level of efficiency until the late 70's [63]. Since then, Sb^{V} efficiency has been constantly decreasing reaching 36% in a study performed in Bihar in 1997 [66]. The mode of action of SSG and SAG is the same. First, they are taken up by the macrophage and reduced to Sb^{III} by the macrophage thiol detoxification pathway then Sb^{III} enters inside the parasitophorous vacuole and kills the parasite [67]. Alternative hypotheses on the direct action of SSG on the parasite without macrophage reduction have been put forward [68]. However, Sb^{V} has itself an immunomodulatory action on the macrophage. Studies of the effect of Sb^{V} on uninfected hematopoietic cell lines show selective inhibition of PTPases (Protein Tyrosine Phosphatase), Src homology PTPase1 (SHP-1), SHP-2, and PTP1B suggesting a potential role in signal transduction in the *Leishmania*-killing activity [69]. When applying Sb^{V} on different cancer cell lines a synergistic activity of SSG and alpha interferon (IFNα) has been observed that abrogates IFNα resistance by activating STAT1 (Signal transducer and activator of transcription 1) [70]. STAT1 is a very important mediator of the IFN response that triggers a Th2 response leading to cytotoxicity. Sb^{V} also has the capacity to trigger the activation of several kinases implicated in signal transduction such as: phosphoinositide 3-kinase (PI3K), playing a major role in cell survival, protein kinase C (PKC), which is implicated in many signal transduction pathways, and some mitogen-activated protein kinases (MAPK) involved in cell multiplication regulation. This activation coupled with the microbicide activity of the macrophage is enough to kill the parasite [71]. As said previously, *Leishmania* downregulates the phagocyte response. A study demonstrated that Sb^{V} could induce a protective effect against the *Leishmania*-induced downregulation of ROS (reactive oxygen species). This was explained by a direct interaction between the drug and the phagocyte initiating the production of ROS by enhancing the activity of the NADPH [72]. Talking about the effect of Sb^{V} exposure on

the host cell is very important since Sb^{V} has no effect on promastigotes. However, inside the parasite, Sb^{III} targets the redox metabolism of the cell by (i) mediating the thiol-efflux and (ii) inhibiting the key redox enzyme, trypanothione reductase (TR). First, Sb^{III} is combined with thiols like glutathione (GSH) and trypanothione ($T(SH)_2$) and the Sb^{III}-thiol complex is sequestrated before being exported outside of the cell. Inhibition of TR probably occurs by the covalent bond between Sb^{III} and the reduced cysteine residues in TR's active site [73]. The continuous oxidative metabolism of the parasite leads to formation of GSSG and TS_2, which under normal conditions would be reduced by TR to maintain the redox potential. However, in *L. donovani* the presence of Sb^{III} impairs TR activity leading to continuous accumulation of dithiols [74]. Those two actions combined lead to an increase of the redox potential that ends by apoptosis-like cell death of the amastigote.

1.3.2 Amphotericin B

Amphotericin B or AmB was first used as an antifungal and is often use as a second line drug for leishmaniasis. In the particular case of India, AmB was moved to a first line drug in Sb^{V} refractory regions in 2000 upon recommendation by the Indian National Expert Committee [75]. The AmB mechanism of action is well known. AmB binds to the ergosterol of the fungi cell wall and creates pores in the cell wall leading to a rapid leakage of monovalent ions (K^+, Na^+, H^+ and Cl^-) [76,77] and small organic molecules [78]. The effect of AmB on *Leishmania* promastigotes demonstrates a similar mode of action and cell death by osmotic shock due to net salt influx across the pores formed by AmB molecules has been described [79,80]. The chemical properties of AmB make it poorly soluble in water and thus it was first combined with deoxycholate which increased the solubility and allowed intravenous (IV) administration [81]. However, AmB deoxycholate treatment showed several side effects such as fever, thrombophlebitis and occasionally hypokalaemia. To reduce the side effects and increase the half-life of the compound, liposomal AmB (Ambisome® from Gilead) was developed allowing a higher concentration of the drug, thereby reducing the time for hospitalisation. Liposomal AmB greatly reduced side effects but needed to be preserved at 25°C maximum [82]. This particular technical issue associated with the mode of administration requiring several days of hospitalisation. In regions where hospitals can be hundreds of kilometres from the patient, amphotericin B is not the drug of predilection in such endemic areas. Noteworthy, AmB has a success rate of ~100% including on SSG-R parasites [83]. However, several studies have assessed the efficiency and the feasibility of a single-dose liposomal AmB injection of 20 $mg.kg^{-1}$ [84,85] and the WHO recommended in 2010 this therapeutic option in the ISC [86]. The main drawback of liposomal AmB remains the

price (Table 2). It is up to now the most expensive anti-*Leishmania* drug available making it hardly affordable in some endemic countries.

1.3.3 Miltefosine

Miltefosine (MIL) or hexadecylphosphocholine is the first oral drug developed against *Leishmania* but was originally formulated as an antineoplastic agent in cutaneous metastases of breast cancer [87,88]. Coincidently, its activity against *L. donovani* was demonstrated at the same time [89]. Miltefosine was, up to 10 years ago, a second line drug against *Leishmania* in the ISC but the emergence and spreading of antimony resistance in the region led the international and regional authorities to move it to a first line drug. The mode of action of miltefosine is not clearly known but two main mechanisms have been described: induction of apoptosis-like cell death [90] and disturbance of the lipid-dependent cell signalling pathways [90]. The regimen to cure VL using miltefosine is 100 $mg.kg^{-1}$ of body weight/day for 28 days in adults weighing more than 50 kg, 50 mg/kg/day in adults weighing less than 50 kg, and 2.5 mg/kg/day in children (maximum dose, 100 mg/day). The main drawback of miltefosine is its teratogenicity [91]. In the case of suspected pregnancy or pregnancy miltefosine should not be administrated.

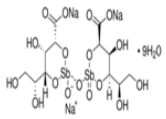
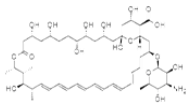
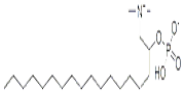
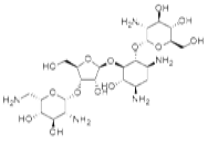
In the ISC, miltefosine treatment failure was first documented in the absence of parasitological resistance [92,93]. However, recent studies have reported for the first time field isolates from patients treated with miltefosine being resistant to the drug *in vitro*, two *L. donovani* strains were isolated from immunocompetent patients in India [94] and one *L. infantum* strain isolated from a HIV+ patient in Spain [95]. Interestingly, after sequencing the genome of the two Indian isolates and of the Spanish one, the two studies described nucleotide insertions in the Miltefosine Transporter (LxMT) as already described in the *in vitro* selection of resistant strains [96,97].

1.3.4 Paromomycin

Paromomycin (PMM), initially named aminosidine, is an antibiotic against most Gram- and many Gram+ bacteria, discovered from filtrates of *Streptomyces krestomuceticus* in the 1950s. Interestingly it was described during the 1960s to have anti-protozoan properties including *Leishmania* [98,99]. During the 1990s, the WHO/TDR started to develop a parenteral formulation leading to the conduct of several clinical trials on parenteral and topical PMM [100,101]. Topical presentation of 15% PMM combined with 12% of methyl benzethonium or 10% paraffin showed good activity on CL lesions from Old World and New World *Leishmania* species [102,103]. In 2006, a parenteral version of PMM was

licenced based on the results of a clinical trial performed in India [101]. The treatment regimen against VL is daily injection at 15 $mg.kg^{-1}$ for 21 days with a cure rate of 93% [104,105]. The mechanism of action of paromomycin is not totally deciphered yet but it is known that PMM inhibits RNA synthesis and decreases membrane fluidity and permeability by increasing the amount of polar lipids in the membrane [106]. It was also proposed that paromomycin would interfere with mitochondrial activity acting on metabolite levels upstream of the respiratory chain, leading to a progressive decrease of the energy supply [107]. Up to now no PMM field resistance was reported; this may be due to the fact that paromomycin has not been extensively used as a monotherapy. Mostly PMM is now used in combination therapy with other anti-*Leishmania* drugs such as miltefosine or SSG.

Table 2: Description of the most commonly used drugs against visceral leishmaniasis.
* for full treatment of a patient weighing 30kg (WHO (2010) Costs of medicines in current use for the treatment of leishmaniasis. 1-2 p.) [123]

	Sodium Stibogluconate	Liposomal Amphotericin B	Miltefosine	Paromomycin
Chemical structure				
Administration	i.v. infusion	i.v. infusion	Oral	i.m.
Regimen	20 mg/kg/day for 28 days	2 mg/kg/day for 5 days or 1 single injection of 7.5 mg/kg	100 (bodyweight >25kg) mg/day for 28 days	15mg/kg/day for 21 days
Toxicity	High toxicity, possible cardiac arrhythmia, nephrotoxicity and hepatotoxicity	Limited nephrotoxicity and mild procedure side effects	Teratogenicity, mild gastro-intestinal toxicity, nephrotoxicity and hepatotoxicity	Nephrotoxicity, hepatotoxicity both extremely rare
Treatment failure	>60% in the ISC	10%	6%	<5%
Cost of the drug (USD)*	21	675 (2-4d) or 900 (1d)	150	15
Advantages	Cheap	Highest therapeutic index of all the VL drugs, short	Oral route is a plus on the field, no need of hospitalisation	Cheapest drug available, no need for prolonged hospitalisation since the injection can be given as ambulatory care
Disadvantages	Prolonged cure with painful injection, requires high quality control, highly toxic and high parasite resistance in the ISC	Expensive, requires excellent preservation (<25°C) and requires i.v. infusion	Low compliance, relatively expensive, possible teratogenicity makes it forbidden for pregnant women, resistance (?)	Low efficency in monotherapy in East Africa, potential for resistance (?) and prolonged treatment favours non termination

1.3.5 Combination therapy

As seen previously, all the available drugs against *Leishmania* target different biological mechanisms inside the parasites but also all have different side effects. Combination therapy was implemented for two reasons. Firstly, to facilitate a lower dose of both drugs and/or for a shorter period to limit the side effects and long term hospitalisation. The second principle of the combination therapy is to limit the possibility for the parasite to become resistant against either one or both drugs. It has been shown that raising resistance *in vitro* against 2 different drugs is extremely difficult especially if the two drugs target two different metabolic pathways [108,109]. This would increase the life of both drugs. Ideally the cocktail should be made of a fast acting compound to quickly reduce the parasite burden and a slow-acting one to ensure long term elimination of the parasite [110]. Several clinical trials have been carried out in the Indian subcontinent where antimonials are not a therapeutic option anymore. The first combination tested in the ISC was 20 $mg.kg^{-1}$ of SSG plus 18 $mg.kg^{-1}$ of PMM for 21 consecutive days with more than 90% efficacy. Regarding efficacy there is no advantage of using SSG+PMM together since PMM efficacy alone is about 92% and SSG is now less than 50% in monotherapy [111]. Other drug combinations were tested such as liposomal AmB and miltefosine, liposomal AmB and PMM and miltefosine and PMM with more than a 97% success rate for all of them (see table 3) [112]. As expected, side effects linked to the treatment were also drastically reduced compared to the control group treated with only AmB. Based on the results of the previous study, feasibility, safety and efficacy of combination treatment were assessed in Bangladesh with globally a better success than the control group treated with Ambisome and with a comparable efficacy to the Indian trial (see Table 3). Here again very few side effects were reported and no relapse or PKDL was observed at 6 months post-treatment [113]. As before, the use of AmB is linked to good storage conditions, notably a temperature lower than 25°C, and it needs an intra-venous infusion that requires a nurse and a medical facility. Those 2 conditions could be complicated to meet in remote areas. The combination MIL/PMM is more promising in areas where access to medical care is more difficult. Indeed miltefosine is an oral drug and paromomycin requires an intra-venous injection. However, the teratogenicity of miltefosine excludes the use of that therapy for pregnant women. That particular treatment is also the most cost-effective followed by liposomal AmB and PMM [114].

Table 3: Dosage and efficacy of various combination treatment regimens tested in Bihar and Bangladesh.

Combination	Dosage	Cure rate	
		India (Bihar) [112]	Bangladesh [113]
L-AmB + MIL	Single injection of 5 mg.kg^{-1} L-AmB + 7 days 50-100 mg MIL	97.5%	94.4%
L-AmB + PMM	Single injection of 5 mg.kg^{-1} L-AmB + 10 days 11 mg.kg^{-1} intramuscular PMM	97.5%	99.4%
MIL + PMM	50-100 mg.day^{-1} MIL + 11 mg.kg^{-1} per day PMM for 10 days	98.7%	97.9%

1.4 Control of visceral leishmaniasis in the Indian sub-continent: the Kala-Azar Elimination Programme (KAEP)

Three countries, India, Bangladesh and Nepal, decided to coordinate their efforts to control kala-azar. Based on this idea, in December 2004 the Regional Technical Advisory Group on kala-azar elimination prepared a document paving the way for the KAEP [115], which officially started when the ministers of health of the 3 countries signed it in May 2005. The up-front goal of the KAEP is "to contribute to improving the health status of vulnerable groups and at risk population living in kala-azar endemic areas of Bangladesh, India and Nepal by the elimination of kala-azar so that is no longer a public health problem" by reducing the incidence of the disease to less than 1/10,000 by 2015 [116]. Noteworthy, this is an elimination program and not an eradication one (the latter meaning no case at all). When antimony resistance grew in the region, the complete VL control strategy had to be redesigned to counter this new challenge and currently, the KAEP stands on 5 major principles [115–117]:

(i) Early diagnosis of symptomatic patients and complete treatment. This primarily requires a strict and uniform clinical definition between the different countries: record a fever for more than two weeks and no response to antibiotics and/or antimalarials. The second requirement concerns laboratory diagnostics. For this, DAT and rK39-dipsticks were selected as the first screening methods and positive cases confirmed by microscope examination of spleen or bone marrow aspirates. The third requirement is to use an effective drug to treat the confirmed VL cases. The WHO first recommended the use of MIL to reach the elimination goals but the teratogenicity of the drug made it less attractive in the field. In addition, an increase of MIL treatment failure

was observed [92] and recently, the first two clinical MIL-R isolates were reported [94]. As said previously (Table 2), a single dose of liposomal AmB showed a high success rate and was then preferred to MIL. For the success of this diagnosis/treatment strategy, time is an extremely important factor. The smaller the amount of time there is between diagnosis and treatment, the lower the chance the patients escapes the treatment for structural reasons. In respect to this last point the best option is the combination of rK39 diagnostic test and single liposomal AmB treatment, as the whole operation can be done in one day.

(ii) Preventing new infections by vector surveillance and management. This point is often addressed by indoor residual spraying (IRS) of insecticides (DDT or pyrethroids) or by avoiding contact with the vector using insecticide treated nets (ITN). To be efficient, IRS has to encompass the careful spraying of houses with paying particular care to the roof and animal shelters and special attention to cracks and crevices in which the sand flies seek shelter. Community involvement is very important in any vector management strategy. In practice, the community can guide spraying teams to find the vector's breeding sites. As for other vectors, *P. argentipes* developed resistance against DDT, especially in the Indian province of Bihar where DDT was extensively used for decades. DDT resistant *P. argentipes* can also be observed in the neighbouring regions of Nepal. However, in the rest of Nepal, where pyrethroids are used, the vector shows a high susceptibility to DDT [118,119]. Counterintuitively, ITN does not seem to be very effective to reduce the vector population. Only few studies have been conducted to assess this point and they often contradict each other regarding transmission [120]. However, all the observations suggest that the majority of the transmission occurs outside the house. This specifically highlights the large gap in knowledge on sand fly behaviour and that not all insect vectors can be tackled the same way. Thus, further basic studies on vector biology and behaviour are needed.

(iii) Disease surveillance. To be efficient, disease surveillance has to be done using two approaches: passive case detection (PCD) and active case detection (ACD). PCD is based on spontaneous presentation of VL patients to the health centres. ACD is the opposite principle, meaning that health professionals are (pro-)actively visiting villages, and it depends on the governments approach to fighting infectious diseases (possible ACD strategies are reviewed and discussed in Singh et al in 2011 [121]). ACD have the advantage to reduce the time between onset of symptoms and treatment by about 50% compared to PCD [122], strengthening point number 1 on early diagnosis and chemotherapy.

(iv) Social mobilization. The first purpose is to raise public awareness on VL symptoms and transmission patterns; it is important to promote early diagnosis (point

1) and prevention (point 2) by improving IRS and reducing human-vector control. This 4th principle is a very important aspect of the KAEP since knowledge will be passed through to the next generations. The second aspect is to build partnerships between state, national and international agencies/organisations like the WHO, OneWorld Health, DNDi and pharmaceutical companies. As stated previously, leishmaniasis is a disease mostly affecting the poorest who are already populations at risk for many other physiological disorders that are risk factors for developing many infectious diseases. That particular fraction of the population has other, sometimes more important, needs and the KAEP has also a priority for building partnerships with other initiatives such as programmes aiming to improve nutritional status or to eliminate poverty.

(v) Clinical and operational research. None of the previous points can be reached without fully efficient long term treatment, effective diagnosis and monitoring tools, clear clinical guidelines and more knowledge about the parasite spreading, the drug resistance emergence and the vector behaviours.

Elimination's deadline was first set up for 2015. During an intermediate meeting this deadline was postponed to 2017. At the time of the final call to judge the efficacy of the program, several indicators are showing a drastic decrease in the number of VL cases on the ISC. However, the scientific community agrees that this effort has to be maintained, since elimination was not reached. In addition, the epidemiology of VL in the ISC is known to be cyclic with epidemics every 15 years: further attention is thus required to ensure that the currently low endemicity is really a consequence of the KAEP and not part of a natural cycle.

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Chapter 2:

Leishmania' s surviving abilities and adaptive skills

The *Leishmania* life cycle itself illustrates the capacity of the parasite to resist stress and to adapt to different environments. Alternating from the midgut of the host vector to the intracellular phagolysosomal compartment of the host macrophage requires different biological mechanisms to survive. Also, while residing inside a key cell of the immune system is a way of subverting it, it also requires specific adaptations. Finally, human activities, mainly the use of drugs, is an additional stress put on the parasite. This chapter gives an overview of the different strategies that the parasites developed to circumvent those different stresses and the variety of unique biological tools it could use to succeed.

2.1 Leishmania donovani survival

The complexity of the life cycle and the diversity of hosts highlights the variety of stresses that *Leishmania* has to face when switching from one host to the other. These include (i) oxidative stress due to heme digestion after a blood meal of the insect vector [1], midgut proteases [2], and the vector immune response [3], (ii) upon transmission in a mammalian host, complement lysis in the blood [4] and exposure to reactive oxygen and nitrogen species (ROS and RNS) in the phagolysosome of the host macrophage [5]. The different adaptive mechanisms selected by *Leishmania* result from thousands of years of co-evolution between the parasite and its two different hosts. The survival capacity of *Leishmania* relies on host manipulation to prevent its anti-leishmanial action and on a unique redox system protecting against the host ROS and RNS production.

2.1.1 Survival against the mammalian host immune response

Being an intracellular pathogen living inside a macrophage exposes *Leishmania* to further stresses but also allows a very fine manipulation of the immune response. The mammalian immune system deals with intracellular organism through the adequate Th1 response also called cellular response. The main trigger of the Th1-response is the interferon gamma (IFN-γ) that stimulates the host cell to kill the intracellular invader via the production of superoxide radical ($O_2^{\cdot-}$) and nitric oxide radical ($NO^{\cdot}$) [6]. In the phagolysosome both radicals react with hydrogen (H^+) to form other reactive oxygen and reactive nitrogen species (ROS and RNS) such as hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$) or peroxynitrite ($ONOO^-$) [7]. These different immune effectors will then interact with several pathogen targets such as thiols, metal cores, protein tyrosine residues, nucleic acids and lipids, leading to damage of the DNA, oxidative degradation of lipids, protein inactivation and eventually death of the intracellular invader [6]. Infecting ROS- or RNS-deficient mice with *L. donovani* showed that ROS-deficient mice were not able to control the parasite load at an early stage of infection (until week 2 p.i.) while RNS-deficient mice were unable to control the infection at a later stage [8].

2.1.2 *Leishmania*: an expert in subverting the mammalian host immune response

As seen previously, there is a need for the parasite to trick the immune system from the very beginning of the infection. Therefore, the parasite enters the cell in a "silent" way through specific receptors so as not to alarm the antimicrobial defences [9–13]. At first, *Leishmania* metacyclics are opsonised by complement proteins targeting their phagocytosis via Complement receptor 1 (CR1) or CR3 [14]. Then the differentiation process from metacyclic promastigote to amastigote takes place. The full differentiation process takes 120h [15], during which the parasite delays the maturation and the acidification of the phagolysosome by releasing the promastigote specific surface glycolipid, LPG (lipophosphoglycan) [16–18]. *Leishmania* manipulates the host immune system to redirect it to a Th2 response, which is not the adequate response for intracellular organisms. This relates directly to the intracellular survival abilities of *Leishmania*. Indeed, to elicit a Th2 response, foreign antigens have to be presented via the class II major histocompatibility complex (MHC class II) after degradation of the phagocytosed organism. *Leishmania* surviving perfectly inside the macrophage limits class II presentation of the antigen [19]. This directly affects the activation of the T-cells which is further increased by the ability of the *Leishmania* to downregulate the expression of the co-stimulatory factor CD80 (or ligand B7-1) at the surface of the macrophage [20,21]. In the case of non-co-stimulation by CD80, the signal sends the T-cell into anergy. *Leishmania*'s elongation factor-1α (EF1α) and fructose-1,6-biphosphate aldolase were also shown to be exported outside of the PV to bind to the Scr homology 2 domain tyrosine phosphatase 1 or SHP-1. The deactivation of SHP-1 leads to the inactivation of the macrophage [22]. Many more mechanisms are involved in the active subversion of the immune system by *Leishmania* to finally achieve the reorientation of the immune response from Th1, by decreasing the expression of Th1 profile cytokines such as Interleukin 12 (IL-12) and increasing the expression of Th2 promoting cytokines such as IL-10 and TGF-β (transforming growth factor beta) , which are ineffective to clear *Leishmania* infection, [23–25]. More recently, the concept of inactive manipulation via the quiescent state of a part of the amastigote population emerged [26]. They have a lower RNA synthesis rate, lower protein turnover [26] thus leading to a longer generation time compare to promastigotes: 12 days for amastigotes against 9h for promastigotes [27,28]. Reducing the total metabolism in such a way and reducing the generation time is a good way to escape the immune system but also allows a higher tolerance to drugs since they will be taken up less and metabolized by the parasite.

2.1.3 Trypanothione: a unique *Trypanosomatids* feature for *Leishmania*' s survival

Leishmania as the other members of the *Trypanosomatids* family [29], use trypanothione (2 glutathione molecules linked by spermidine) as the main regulator of their intracellular reducing environment, in contrast to other eukaryotes that use glutathione [30–32].

2.1.3.1 Role of Trypanothione inside the intracellular compartment

Trypanothione is the main active defense system of *Leishmania* against ROS and RNS. The parasite encounters oxidative and nitrosative stress throughout its life cycle both as promastigote and amastigote, but ROS/RNS can also be induced by drugs such as pentavalent antimonials, for example. *Leishmania*' s redox system consists of a cascade of enzymes coupled with trypanothione as the main reducing agent (Fig. 4). When ROS and RNS are detoxified by members of this cascade either by trypanothione itself (H_2O_2 [33], $NO^{\cdot}$[34,35]), tryparedoxin or tryparedoxin peroxidase (H_2O_2 [36,37], $ONOO^-$ [36,38], H_2O_2 + $NO^{\cdot}$ [39]), 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. 1). TR is therefore thought to be an important central enzyme for the intracellular survival of *Leishmania* [40–42].

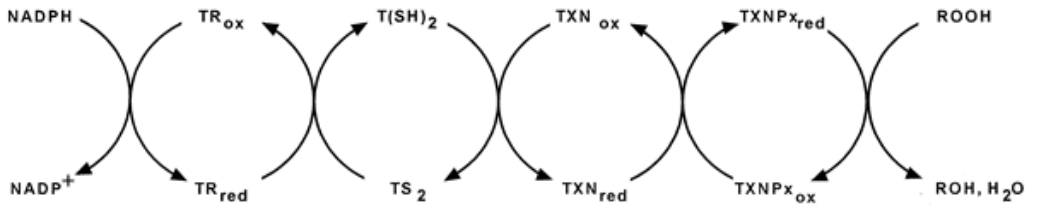

Figure 1: The NADPH dependent redox cascade with trypanothione (TSH_2) as the central reductant. TR: trypanothione reductase, TXN: tryparedoxin, TXNPx: tryparedoxin peroxidase [43].

Trypanothione is not the only thiol present in *Leishmania,* glutathione (GSH), cysteine and ovothiol are also participating in ROS detoxification, albeit in a lesser extent [32,44].

As mentioned before, superoxide or $O_2^{\cdot-}$, is a major player in the immune response against intracellular pathogens. A class of enzymes, superoxide dismutase (SOD) are able to transform $O_2^{\cdot-}$ into H_2O_2 that can be then detoxified by other factors such as the cascade described in Fig. 1. *Leishmania* encodes several iron-containing SODs that proved their importance in the defence against $O_2^{\cdot-}$ [45], in intracellular survival [46] and in virulence [46].

2.1.3.2 Role of Trypanothione against antimonials

The trypanothione pathway has been shown to be the target of antimonials. Sb^{V} can be reduced by eukaryotic cells through two different mechanisms: the spontaneous non-enzymatic reduction (by glutathione inside the macrophage and mainly by trypanothione inside the parasite) and the enzymatic reduction.

- Spontaneous, non-enzymatic reduction: Glutathione (GSH), as part of the thiol agents of every eukaryotic cell (*Leishmania* or macrophage), has strong reducing properties and is known *in vitro* to reduce Sb^{V} into Sb^{III} in specific conditions: acidic pH (4.7 < pH < 5.2) and a temperature of 37°C. In those condition GSH reduces Sb^{V} with a stoichiometry of 5 to 1 ($SbO_3^- + 5GSH + H^+ \leftrightarrow Sb(GS)_3 + GSSG + 3H_2O$) [47]. On the parasite side, cysteine and trypanothione ($T[SH]_2$) are likely to reduce Sb^{V} into Sb^{III} since they work in physiological conditions, and are more efficient compared to glutathione. Cysteine is able to catalyse the reducing reaction due to its charged amino-group and trypanothione is a dithiol and thus has a higher reducing power than monothiols like GSH. As consequence, the amount of $T[SH]_2$ needed to operate the reaction is reduced compared to the use of GSH; only 2 $T[SH]_2$ are required to the reduction of Sb^{V} ($SbO_3^- + 2\ T[SH]_2H^+ + H^+ \leftrightarrow SbT[S]_2 + T[S]_2H^+ + 3H_2O$). This reaction as well as the reaction involving cysteine can occur at pH 5 or 7 but showed optimal kinetics at the most acidic pH, potentially explained by the need for protons in the reducing reaction [48,49]. GSH, $T[SH]_2$ and cysteine dependant reactions shows optimal kinetics under an acidic pH. This particular condition is reached inside the lysosomes, endosomes and phagolysosomes of the macrophage. As with every anti-*Leishmania* drug, Sb^{V} has to transit via the phagolysosome where the pool of resident thiols present there can generate Sb^{III} [47,48] before reaching the residing amastigote where it has its toxic activity.
- Enzymatic reduction: *Leishmania* encodes for many enzymes that allow Sb^{V} reduction in Sb^{III} in optimal conditions. It is the case for the thiol dependant reductase 1 (TDR1) using GSH as reducing agent allowing a much faster reduction of Sb^{V} than the spontaneous reaction previously described. More specifically, TDR1 is 10 times more expressed in amastigotes than in promastigotes. This can be one of the factors to explain the 50 to 600-fold difference in susceptibility to Sb^{V} between the two life forms [50].

Once the process of Sb^{V} reduction in the cytoplasm is finished, antimony, in the form of $SbT[S]_2$, has to be externalized from the cell to complete the process of detoxification. This is mainly ensured by ATP binding cassette (ABC) transporters, the primary member of that family being MRPA, previously called P-gpA (P-glycoprotein A), a vesicular

transmembrane transporter (described below). Other ABC transporters have been described as implicated in the efflux of antimonials like the half transporter ABCI4 shown to increase tolerance to some heavy metals including Sb^{III}, As^{III} and Cd^{II} [51]. In naturally resistant field isolates of *L. infantum* and *L. donovani* from Crete, overexpression of Pgp170, an ABC transporter, in SSG-resistant isolates showed a higher extrusion rate than in sensitive isolates expressing Pgp170 at a lower level [52]. An overexpression study of another ABC transporter, LABCG2, in *L. major* proved its efficiency to extrude Sb^{III} via exocytosis through the flagellar pocket [53,54].

2.2 *Gene expression in Trypanosomatids and genomic adaptions to drug pressure*

2.2.1 Gene expression in *Trypanosomatids*: an atypical eukaryote

The genome size of *Leishmania donovani* is 33MB and it includes the nuclear genome organised into 36 chromosomes and the kinetoplastic DNA (kDNA) organised into mini- and maxi- circles located in the kinetoplast [55]. Gene transcription in *Trypanosomatids* occurs in polycistronic transcription units (PTUs), i.e. long transcripts that contain several genes. Coding regions (arrow boxes in Figure 2) mostly do not contain introns and there is no transcription regulatory elements in the genome (Figure 2) [56,57]

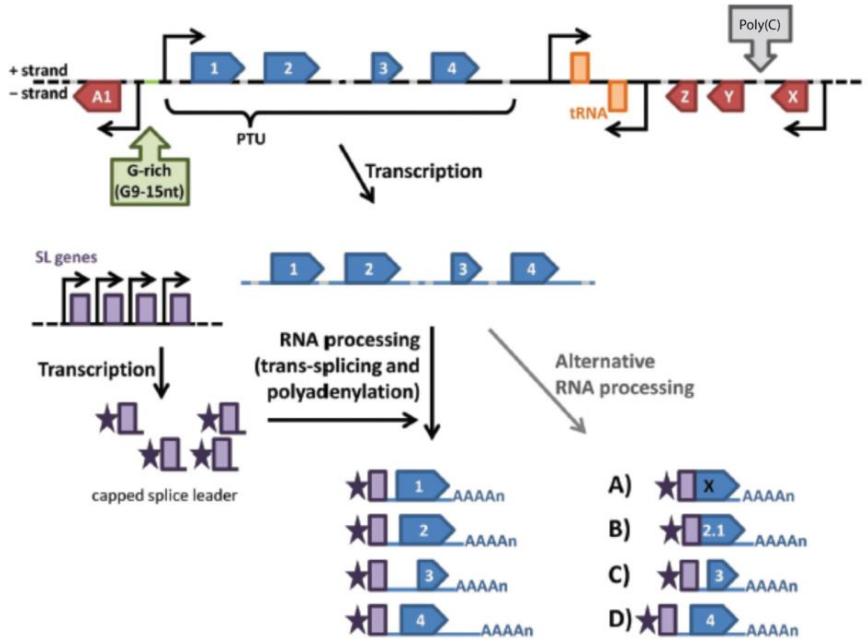

Figure 2: Polycystronic expression in Trypanosomatids (modified from Teixeira, SM *et al.* [58]).

PTUs are separated from each other by convergent or divergent strand-switch regions marked by G-rich genomic regions hallmarks of the presence of a base-J (specific epigenetic modification unique for *Trypanosomatids*) [59,60] and the extensive presence of tRNA-coding unit clusters [61]. PTUs are transcribed in pre-mRNAs by the RNA Polymerase II from the transcription start sites (TSS, indicated by black arrows in fig.2). Pre-mRNAs are maturated into mRNAs by individualisation at the level of the Poly(C) regions (accumulation of cytosine) in the intergenic regions [62]. Those Poly(C) regions guide the insertion at the 5′ end of the splice-leader sequence (SL), a 39 nucleotide-species specific sequence coded in the mini-exon locus of every *Trypanosomatid*. A poly(A) tail is added at the 3′ end of each mRNA [63]. Alternative processing, either alternative splicing in 5′ or alternative polyadenylation, can occur by the inclusion or exclusion of regulatory elements altering gene expression [64]. Gene expression in *Leishmania* is mostly controlled at the post-transcriptional level by the interaction of cis-regulating elements, called SIDER2 for Short Interspersed DEgenerate Retroposons, with RNA-binding proteins acting on the mRNA stability and the translation rate [65,66].

2.2.2 Stress response and genomic adaptations

2.2.2.1 Constitutive and mosaic aneuploidy

Leishmania exhibits multiple genomic features unique amongst eukaryotes and Trypanosomatids. It is one of the rare organisms, and to date the only reported member of the Trypanosomatid family, to show well tolerated, even beneficial, constitutive aneuploidy.

Aneuploidy, which results in the loss or gain of one or more chromosomes copies, is not to be confounded with another phenomenon called polyploidy, when the entire set of chromosome is multiplied [67]. Polyploidy has being shown to be a driver for evolution, to be relatively common in plants and to occur occasionally in invertebrates, fishes and amphibians [68,69]. Polyploidy in Trypanosomatids was recently described in a species infecting cattle, *Trypanosoma congolense*. A full triploid genome was observed in one field isolate while *T. congolense* is generally described as being disomic [70]. Aneuploidy in Metazoa is often lethal during embryogenesis or leads to death shortly after birth, like for example trisomy 18 or Edwards syndrome in humans [71]. It also produces severe abnormalities, monosomy X with Turner syndrome being one of the most known example in humans [72]. After ontogenesis and embryogenesis aneuploidy in human is generally linked with tumor formation (reviewed in [73]). However, recent research showed that it is not rare to find aneuploid cells in the liver without link to any carcinogenic mechanism: this mechanism would allow adaptation to xenobiotic or nutritional injury [74].

The situation might be different in unicellular micro-organisms, pathogenic or not, where aneuploidy could be a response to stress [75]. Fluconazole-resistant *Candida albicans* clinical isolates have been shown to carry three copies of chromosome 4, the chromosome bearing a gene associated with fluconazole resistance [76]. Fluconazole stress also triggers aneuploidy in the haploid fungus, *Cryptococcus neoformans*, leading to a duplication of chromosome 1 carrying two genes associated with fluconazole resistance [77]. All the previously cited organisms are either haploid or diploid and they generate aneuploidy when facing a stress, usually of one chromosome at the time. The particularity of *Leishmania* is its constitutional aneuploidy in *in vitro* culture, inside the vector and inside the mammalian host [55, 78, 82]. Remarkably, chromosome 31 was shown to be tetrasomic in every species investigated so far [78]. Due to the lack of regulation at the initiation start, this mechanism was hypothesized to operate the up-regulation of some necessary genes. In addition to the aneuploidy within a single cell, the population is mosaic meaning that a given chromosome may have a different somy in different cells from a population, going from monosomy (1 copy of the chromosome)

to pentasomy (5 copies of the chromosome) [79–81]. Evidence that mosaic aneuploidy is also present at the amastigote stage was recently described in *L. donovani* parasites isolated from hamsters [82]. This creates a vast diversity within the population, providing a high adaptive capacity of the parasite population to various kinds of stress, including drugs [83,84].

2.2.2.2 Local copy number variation

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 extra-chromosomal amplification, using direct and inverted DNA repeats (Figure 3 and [85]), as well as intra-chromosomal amplification (ICA) or chromosomal deletion [86,87]. This strategy allows the parasite to increase/decrease the expression level of one gene or of a set of genes. It was described that *Leishmania* genome contains many repeated sequences split into 507 identified repeat alignment groups (RAG) in the *L. major* genome. RAGs are constituted of direct or indirect repeated sequences sharing a high level of similarities on the same chromosome. They either belong to ancestral retrotransposon associated with SIDER1 and SIDER2 sequences or just unique repeated element [85]. What triggers the amplification of a specific part of the genome is not known but the molecular mechanisms of intra- or extrachromosomal amplifications have been revealed.

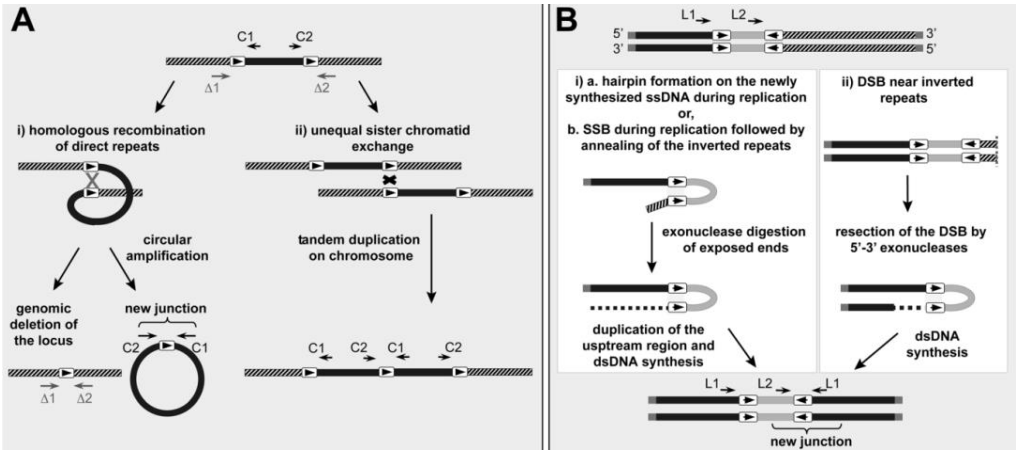

Figure 3: Mechanism of formation of local CNV from A) direct repeats and B) indirect repeats (from Ubeda, J.M. *et al.* [85]). When the local CNV is formed from 2 direct repeats it can generate different types of local CNV by i) homologous recombination of direct repeats forming an extrachromosomal circular amplicon with a loss of the genomic locus or ii) by unequal sister chromatid exchange leading to tandem duplication on the chromosome or intra-chromosomal amplification (ICA). B) When the local CNV is generated from indirect repeats it forms an extrachromosomal linear amplicon.

In 2001, a *L. major* homolog of *E. coli recA*, a gene playing a central role in homologous recombination and DNA repair, was identified. This recombinase, called LmRAD51, was demonstrated to have the same properties as *recA*: LmRAD51 binds DNA and repairs DNA damage [88]. Later on, LiRAD51 was shown to interact with another protein Breast Cancer 2, or LiBRCA2, in a complex to repair DNA. Knock-out of LiBCRA2 by gene replacement led to a decrease in DNA recognition by the LiRAD51/LiBRCA2 complex and homologous recombination efficiency; in addition, in *BRCA2* null mutants, RAD51 was no longer localized in the nucleus [89]. LiRAD51 was identified as playing a major role in homologous recombination and thus in the formation of circular amplicons [85]. In 2014, the protein responsible for the formation of the linear extra-chromosomal amplifications, Meiotic Recombination 11 or LiMRE11 was characterized [90].

2.2.2.3 Genomic adaptions in the context of drug resistance

i. In vitro selections

Gene amplifications (in different forms) were observed in experimentally selected drug resistant parasites. This concerned many different drugs such as arsenic, antimonials, Amphotericin B, methotrexate (MTX), a dihydrofolate reductase (DHFR) inhibitor, and other non-anti-*Leishmania* drugs, hereby highlighting that this mechanism is one of the main adaptive features of *Leishmania* (Table 4). As shown in Table 1, selection experiments have been extensively carried out but only two were found on *L. donovani*, one study performed on axenic amastigotes using Nelfinavir, an anti-HIV drug [91], and one on promastigotes using miltefosine as the selective drug [84].

Table 4: Non-exhaustive overview of the different drug selections performed in *Leishmania* where genome adaptations were assessed.

Species	Life stage	Type of adaptation	Ref
AmB			
L. tarentolae	AmB	2 ECAs from 2 different chromosomes	[92]
As^{III}			
L. tarentolae	Promastigotes	Circular ECA of the H- locus	[93]
L. tarentolae	Promastigotes	H- locus ECA (by direct repeats)	[94]
Nelfinavir			
L. donovani	Axenic amastigotes	Locus deletion in chr10 Modulation of aneuploidy	[91]
Mil			
L. donovani	Promastigotes	Reduction of somy of chr13 carrying LdMT Positive selection of a lethal SNP in LdMT	[84]
L. infantum	Intracellular amastigotes	Indel in LiMT Modulation of the karyotype	[95]

MTX			
L. tarentolae	Promastigotes	H- locus ECA (by long inverted repeats)	[94]
L. tarentolae	Promastigotes	Different amplifications of the H- locus containing *ptr1*	[96]
L. tarentolae	Promastigotes	Circular ECA of the H- locus	[97]
L. major	Promastigotes	Amplification of the H- and R- locus	[98]
L. major	Promastigotes	Circular ECA of the R- locus (DHFR-TS) Linear ECA of the H- locus (PTR1) Gene deletion (folate transporter on chr10)	[99]
L. tropica	Promastigotes	Co-existence of linear and circular ECAs of the H- locus	[100]
Sb^{III}			
L. guyanensis ***L. amazonenesis*** ***L. braziliensis*** ***L. infantum***	Promastigotes	Genomic amplification of MRPA (mechanism not characterised)	[101]
L. guyanensis	Promastigotes	Subtelomeric deletion of chr31 (AQP1) G133D in AQP1 reducing Sb^{III} influx Amplification of MRPA via partial chromosomal amplification	[87]
L. infantum	Promastigotes	ECA of the H- locus Modulation of aneuploidy	[83]
L. major	Promastigotes	Telomeric deletion of chr31 including AQP1 ICA of chr34 including APX and G6PDH	[102]
L. infantum	Axenic amastigotes	Increased copy number of MRPA (mechanism not described)	[103]
Terbinafine or primaquine or choloroquine			
L. major	Promastigotes	ECA of the H- locus	[104]
5-fluorouracil			
L. infantum	Promastigotes	DHFR amplification Locus deletion in chr10 Point mutations in 3 genes	[105]

Looking only at antimony resistance selection studies, we can actually notice that two major genomic adaptations are generally distinguished (i) H- locus/MRPA amplification and (ii) AQP1 inactivation. MTX is also showing either amplification of the H- and/or R-locus, the only AmB selection showed the selection of two ECA from different chromosomes and Mil showed systematically the appearance of a SNP or indel in the miltefosine transporter. Other drugs showed different genomic adaptions.

ii. In the case of *L. donovani* in the ISC

As outlined in chapter 1, antimonials have been extensively used for about 90 years in the fight against leishmaniasis. It is thus one of the most popular choices when it comes to investigating drug resistance mechanisms in *Leishmania*. Many studies have reported amplifications (ICA or ECA) or deletions of parts of the genome of different *Leishmania* species. This paragraph will compare findings on the recently circulating population of *L. donovani* in the ISC with results of *in vitro* antimony resistance selection.

From all the previous work done on *in vitro* selected parasites, performed on various *Leishmania* species, we know that all the *Leishmania* species from the Old World can generate amplicons. In contrast, amplicons were so far not reported in Neotropical species from the *Viannia* sub-genus. It has been hypothesized that the species from the *Leishmania Vianna* sub-genus cannot make linear or circular amplicons because of the presence of the RNAi machinery which is absent in all the *Leishmania Leishmania* species [106]. In 2016, a WGS study on 204 clinical isolates from VL confirmed patients was published. Interestingly, 94% of the clinical isolates from the lowland of the ISC gathered in one monophyletic group, called the Core group (CG), while the remaining parasites clustered in a phylogenetically distant group, called ISC1 which is endemic in the Nepalese highlands. Interestingly, all parasites of the CG showed two different ICAs, while these were absent in ISC1 [86]. In the same study the emergence of the CG and the two ICAs characteristic of it was dated around 1850 (Figure 4A and 4B). These ICAs corresponded to the amplification of the H- and M- locus. Even if the presence of those two ICAs is a characteristic of the CG not all the genetic groups that compose it carry the same copy number of the H- and the M- locus (Fig 4D and 4E). Interestingly ISC5 has on average the highest copy number of the whole CG for both of the ICAs.

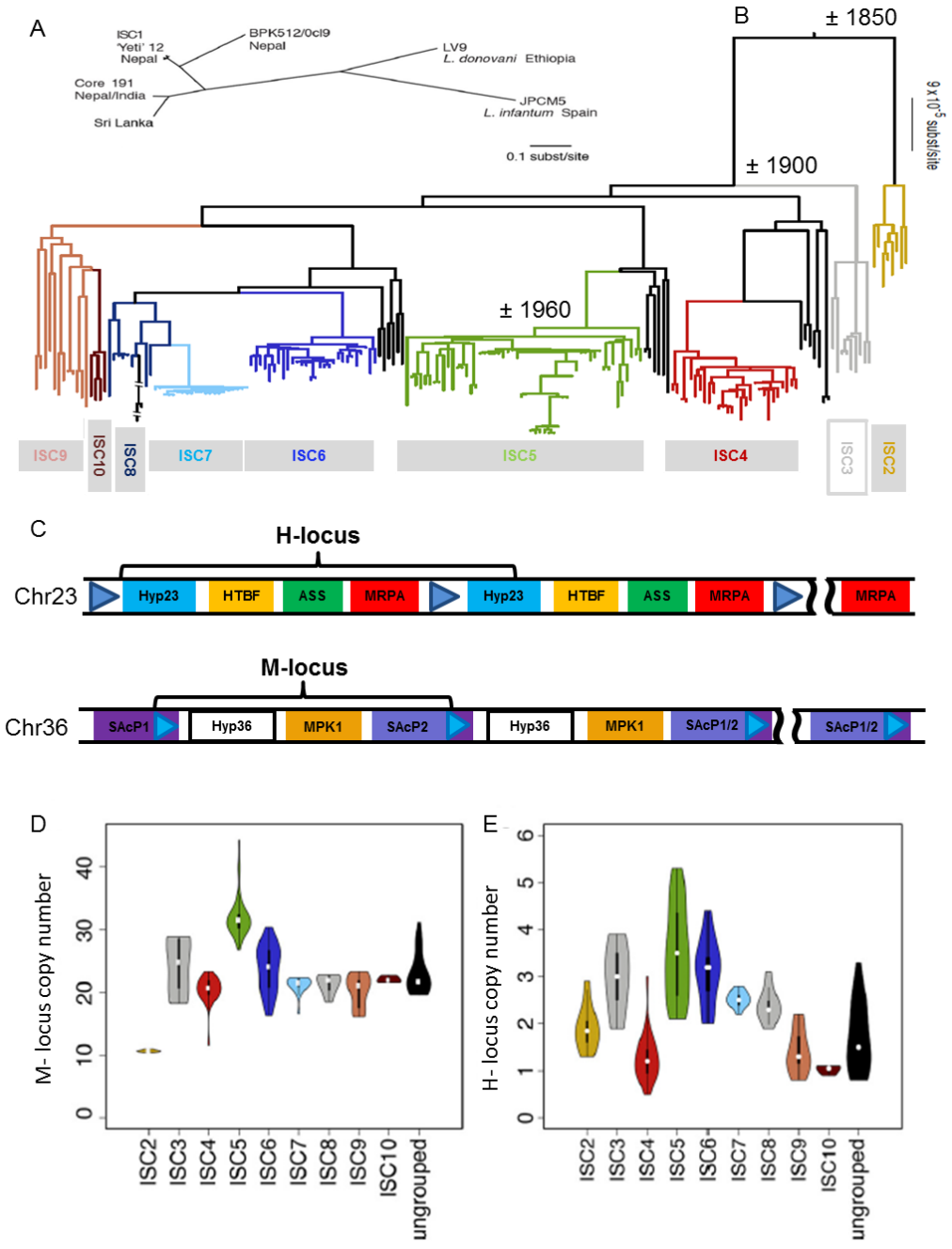

Figure 4: Genetic structure of the *L. donovani* population in the ISC and two of their distinctive genomic characteristics. (A) Phylogenetic overview of the *L. donovani* complex including strains from the ISC and, strains from East Africa as well as *L. infantum* from Spain. (B) Maximum likelihood tree over all the *L. donovani* strains from

the Core group. Years near the different nodes represent the estimated year were the divergence occurred (modified from Imamura *et al*. [86]). (C) Graphical representation of the two ICAs of the H- and M- locus. The blue triangles represent the direct repeats used to amplify each locus. (D and E). Violin plot showing the copy number of the H- and M- locus respectively. ISC1 is not represented but all the isolates of this group have 1 copy of each locus.

Structure of the H- locus of *L. donovani* in the ISC

The H-locus is present on chromosome 23. It was probably formed by the recombination of 2 direct repeats as previously described by Leprohon *et al.* in 2009 [83]. It is composed of four CDS:

(i) Hypothetical protein (LdBPK_230007600) renamed here hyp23. It is a gene of 558 bp coding for a 185 amino acid protein (21.6 kDa) of unknown function.

(ii) HTBF or H- locus terbinafine resistance locus protein (LdBPK_230007700) a gene of 570 bp coding for a protein of 189 amino acids for a molecular mass of 21 kDa. It is the analog of the *Saccharomyces pombe* YIP1 protein essential for vesicle trafficking [107]. Terbinafine is a sterol synthesis inhibitor targeting squalene epoxidase mainly used against dermatophyte fungi [107]. HTBF was described as mediating terbinafine extrusion in *L. major* [108].

(iii) MRPA or multi resistance protein associated, formally known as P-gpA (LdBPK_230007800), is a gene of 4710 bp leading to the expression of the protein of 1347 amino acids for a molecular mass of 149.6 kDa. MRPA is an ABC transporter family member and has been extensively described as playing a major role in metal resistance such as arsenicals and antimonials [103,109–111]. MRPA does not transport free As^{III} or Sb^{III} outside of the cell but a metal-thiol conjugate like the bacterial arsAB ATPase that also extrude metals outside of the cell. MRPA was described as being a pump present at the vesicular membrane where the Sb-thiol complex is sequestrated. Confocal microscopy images have shown the presence of the MRPA at the plasma membrane indicating fusion between the vesicles and the cell membrane leading to the extrusion of the metal-thiol complex in the extracellular compartment [109,112].

(iv) ASS or argininosuccinate synthase (LdBPK_230007900) is a gene of 1257 bp making a protein of 418 amino acids for a molecular mass of 46.3 kDa. ASS is normally an enzyme part of the urea cycle in the other eukaryotes that condenses citrulline and aspartate into argininosuccinate. The urea cycle is part of the arginine pathway and leads to glutathione synthesis. As stated previously, the final step of the arginine metabolism in *Leishmania* is the synthesis of trypanothione. However, in *Trypanosomatids* the urea cycle is not complete and one enzyme is missing, argininosuccinate lyase [113]. Comparative genomics in *Trypanosomatids* and in *Leishmania* has shown that ASS seemed to be a lateral gene transfer from bacteria [114,115]. In *Leishmania*, ASS might play a role in infectivity and survival in the mammalian host. Firstly, proteomic analyses have shown that ASS was more expressed in antimony resistant axenic amastigotes than in promastigotes [116]. Interestingly, argininosuccinate was found to be upregulated in the stationary phase SSG-R promastigote from the ISC [117]. ASS was shown to be upregulated in *Leishmania* in response to oxidative stress and arginine starvation. A drastic decrease of the parasite's viability was observed *in vivo* when ASS was down-regulated by gene replacement [118, 119].

- Structure of the M- locus in *L. donovani* of the ISC

The M- locus is present on chromosome 36. It is composed of two CDS, one hypothetical protein (LdBPK_360076700) renamed hyp36 and the mitogen-activated protein kinase 1 or MPK1 (LdBPK_360076800). The M- locus is probably the result of the recombination between two secreted acid phosphatases, SAcP1 and SAcP2, that are 99% identical [120].

(i) Hypothetical protein (LdBPK_360076700) here renamed as hyp36 is a gene of 558bp coding for a protein of unknown function of 469 amino acids and 50.7 kDa. However, we know from a gene replacement study investigating that particular genomic region that this gene is not essential to either promastigote or amastigote survival [121].

(ii) MPK1 (LdBPK_360076800) or mitogen-activated protein kinase 1 is a gene of 1077 bp coding for a 358 amino acids protein of 41.1 kDa. MAP kinases are implicated in environment sensing and in signal transduction in other eukaryotes [122]. Fifteen MAP kinases have been

identified in the *Leishmania* genome [123]. Amongst these 15 MPKs, MPK1 was described in *L. mexicana* to be essential for amastigote proliferation inside the host macrophage [121]. Overexpression of MPK1 in one ISC *L. donovani* strain showed a decrease of tolerance to Sb^{III} and Sb^{V} [124]. The same year the same group described the negative correlation between MPK1 expression and P-gp pump expression. Noteworthy, P-gp location was cytoplasmic and not at the intracellular vacuolar membrane [125]. Recently MPK1 was described as interacting with HSP70 and HSP90 in the formation for the foldosome which is very important to check for good conformation of the proteins [126]. The foldosome was shown to play a crucial role in *Leishmania* differentiation and amastigote viability [127].

The emergence of the ISC5 group, a very particular genetic group composed of a majority of SSG resistant isolates or isolates linked to SSG treatment failure, was estimated to occur around 1960. It is worthy to note that the genetic difference between the different ISC group is very small. The major genetic marker of the ISC5 group is an insertion of 2 nucleotides within the gene coding for aquaglyceropin-1 or AQP1 (LdBPK_310005100) generating a frame shift that shorten the protein, making it non-functional. AQP1 is a glycerol, water, urea, dihydroxyacetone, methylglyoxal and polyols transporter located at the posterior end of the promastigotes of *L. donovani* [128]. As for bacteria, humans and yeast, aquaglyceroporins are influx pumps and in *Leishmania* are responsible for the intake of Sb^{III} and As^{III} [129–132]. This was particularly investigated in *L. tarentolae* [133,134]. The deletion [87,102], the fatal mutation [87] or the post-transcriptional manipulation of AQP1 [133] are well known antimony resistance mechanisms after *in vitro* selections. However, it is the first time that a study reports this many different molecular adaptions in so many clinical isolates.

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Rationale, hypothesis, objectives and methodologies

In the Indian Sub-Continent (ISC), antimonials were used since the beginning of the 20th century, first as a trivalent form, later as a less toxic pentavalent form (SSG). At the end of the century, antimonials were abandoned in the ISC because of the high rate of SSG-resistance (SSG-R). Antimonials were replaced by Miltefosine, the first oral drug to treat VL, but after 10 years of use, the efficacy of this drug also started to decline. Now, VL is treated with Ambisome alone, one of the last effective drugs, and combination therapy is under evaluation. There are no other drugs available and it will take years before new ones (if any) become accessible to the patients.

It is important to understand why and how efficient compounds fail. It can allow for better protection of these drugs in the places where they are still used: for instance, SSG is still implemented in Latin America and East Africa and lessons/knowledge/markers associated with the SSG-R of the ISC could be useful to monitor the emergence and spreading of SSG-R in these other regions. Furthermore, this knowledge can be helpful for developing new drugs that will not target the same pathways as a drug for which the parasite is already resistant to. Finally, understanding drug resistance could allow for the rescuing of old drugs with complementary molecules that would overcome a parasites drug resistance mechanisms. This was recently illustrated by the complementation of SSG with imipramine to counterbalance parasite host manipulation.

My work was dedicated to improving the understanding of SSG-resistance. This topic has been investigated in several previous studies in a fragmented way and in different species. The originality of this work is that it is embedded in a long-term and holistic study on SSG-resistance in the ISC, starting from a clinical study in Nepal in 2002, continuing with the molecular characterisation of clinical resistance by targeted and untargeted methods and now entering into an experimental phase. Such integrated work on drug resistance in *Leishmania* is unprecedented.

At the beginning of my PhD, I actively contributed to a phylogenomic study aiming to understand the dynamics of emergence and spread of SSG-resistance in the ISC (Imamura et al., 2016). This study was based on data from whole genome sequencing of 204 isolates, at the promastigote stage. Among others, we described the occurrence of two very different *L. donovani* populations in the ISC, the main one (called the Core Group or CG) endemic in low lands and containing 9 different genetic groups (ISC2 to ISC10) and a second one in the high lands and named ISC1. This latter population is new and started to spread in Nepal. A striking difference between CG and ISC1 is the presence of two intra-chromosomal amplifications (ICAs) in all strains of the CG: a first ICA on chromosome 23, corresponding to the H-locus previously linked to antimony resistance *in vitro*, and a second ICA, the newly described M-locus on chromosome 36.

An additional adaptation was encountered in all isolates of the genetic group ISC5, a 2-nt indel introducing a stop codon in the AQP1 gene, encoding a transporter of Sb^{III}. The phylogenomic study allowed for dating of the emergence of these 2 molecular adaptations: (i) the H- and M-loci would have emerged first, around 1850 and (ii) the AQP1 indel is estimated to have appeared around 1960 (Imamura *et al.*, 2016). Besides these specific gene amplifications, a high level of aneuploidy was encountered in ISC, but the somy of specific chromosomes could not be related to SSG-resistance, hereby contrasting with reports on experimentally selected resistance.

In the present work, the **general aim** was to analyse in experimental conditions the emergence of SSG resistance upon *in vitro* selection of ISC parasites, in order to know (i) if the same genomic adaptations would be encountered as in natural populations, (ii) in which order they will appear and (iii) to validate their role in SSG-resistance in the specific context of *L. donovani* in the ISC.

The first hypothesis underlying this work was that gene dosage, through somy variation or local copy number variation (CNV), is the main driving mechanism to adapt to the environment or to drug exposure.

A second hypothesis emerged during the progress of the thesis, i.e. the biological pre-adaptation of CG strains to develop antimony resistance.

The **first objective** was to characterise the stability of the genome throughout the life cycle, including in the different environments encountered by the parasites (Chapter 3). This allowed for refinement of the bio-informatics methods used for gene dosage analysis and it provided a conceptual context for the next studies to be done on drug pressure. In this study we performed WGS and transcriptomics on promastigotes coming from different environments such as *in vitro* conditions as well as sand fly derived-promastigotes from the natural vector of *L donovani* in the ISC, *P. argentipes*. The genome and transcriptome of amastigotes extracted from infected hamsters were studied as well. Genome structure evolution of amastigotes was further studied by sequencing the genome of amastigotes at different time points. This was performed on 3 different strains with different antimony susceptibility profiles. This study showed that the karyotype of *L. donovani* strains varied rapidly during the promastigotes to amastigotes differentiation.

A **second objective** was to generate lines resistant to Sb^{V} and to Sb^{III} using strains representative of the genomic diversity of *L. donovani* in the ISC, to understand the impact of the genomic background on the development of resistance. We did not succeed

to select resistance to Sb^{V} (Chapter 5), but we obtained parasites resistant to Sb^{III}. Therefore, the second part of the thesis focused on the characterisation of the time to Sb^{III} resistance (time needed to reach resistance) and the type of resistance (molecular mechanisms associated to Sb^{III} resistance). This was studied by a combination of untargeted genomic and metabolomic approaches (Chapter 4). This study showed among others that ISC1 strain required more time and molecular adaptations to reach Sb^{III} resistance, hereby demonstrating the importance of the genetic background in the development of resistance. Our results supported the hypothesised role of gene dosage in the development of SSG-resistance and highlighted the role of the H-locus. We experimentally verified that within the H-locus, the main driver of resistance was MRPA. Altogether these findings supported the hypothesis of pre-adaptation of CG strains to the development of SSG-resistance. This was further verified by a 'flash' selection in which we demonstrated that parental CG could survive the highest concentration of Sb^{III} and develop normally, while ISC1 strains could not. These experimental results shed a new light on those obtained in the phylogenomic study. Lessons and the implications of this work were discussed in the final discussion.

Chapter 3:

Aneuploidy and gene expression in **Leishmania** *across the different hosts*

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Modulation of aneuploidy in *Leishmania donovani* during adaptation to different *in vitro* and *in vivo* environments and its impact on gene expression.

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3.1 Abstract

Aneuploidy is usually deleterious in multicellular organisms, but appears to be tolerated and potentially beneficial in unicellular organisms, including pathogens. *Leishmania*, a major protozoan parasite, is emerging as a new model for aneuploidy: *in vitro* cultivated strains are highly aneuploid, with inter-strain diversity and intra-strain mosaicism. The alternation of two life stages in different environments (extra-cellular promastigotes and intra-cellular amastigotes) offers a unique opportunity to study the impact of environment on aneuploidy and gene expression. We sequenced the whole genome and transcriptome of *Leishmania donovani* strains, throughout their adaptation to *in vivo* conditions mimicking natural vertebrate and invertebrate host environments. Nucleotide sequences were almost unchanged within a strain, in contrast to highly variable aneuploidy. Although high in promastigotes, *in vitro,* aneuploidy dropped significantly in hamster amastigotes, in a progressive and strain-specific manner, accompanied by the emergence of new polysomies. After a passage through a sand fly, smaller yet consistent karyotype changes were detected. Changes in chromosome copy number were correlated with the corresponding transcript levels, but additional aneuploidy-independent regulation of gene expression was observed. This impacted stage-specific gene expression, downregulation of the entire chromosome 31, and upregulation of gene arrays on chromosome 5 and 8. Aneuploidy changes in *Leishmania* are probably adaptive and exploited to modulate dosage and expression of specific genes; they are well tolerated, but additional mechanisms may exist to regulate transcript levels of other genes located on aneuploid chromosomes. Our model should allow studies of the impact of aneuploidy on molecular adaptations and cellular fitness.

3.2 Importance

Aneuploidy is usually detrimental in multicellular organisms, but in several micro-organisms it can be tolerated and even be beneficial. *Leishmania* – a protozoan parasite killing more than 30,000 persons each year – is emerging as a new model for aneuploidy studies as unexpectedly high levels of aneuploidy are found in clinical isolates. *Leishmania* lacks classical regulation of transcription at initiation through promoters, so aneuploidy could represent a major adaptive strategy of this parasite to modulate gene dosage in response to stressful environments. For the first time, we document the dynamics of aneuploidy throughout the life cycle of the parasite, *in vitro* and *in vivo*. We show its adaptive impact on transcription and its interaction with regulation. Besides offering a new model for aneuploidy studies, we show that further genomic studies should be done directly in clinical samples without parasite isolation and that adequate methods should be developed for this.

3.3 Introduction

Aneuploidy is usually deleterious in multicellular organisms (e.g. trisomy 21 or cancers in humans), but tolerated and potentially beneficial in some unicellular organisms [1,2]. The best-studied models are fungi, especially *Saccharomyces* and *Candida*, in which aneuploidy can be encountered in natural isolates as well as in laboratory strains [1,3–5]. The phenomenon may concern more than one chromosome [6] and it was reported to promote adaptation to stress [6,7]. More specifically, in pathogenic fungi it seems to contribute to virulence [8] and appears as an intermediate step during the development of drug resistance [9].

Leishmania (Protozoa, Kinetoplastida, Trypanosomatidae) are parasites of high medical and veterinary importance and are emerging as a new model for aneuploidy. Several reports describe aneuploidy in *Leishmania*, in contrast to other members of that family, like *Trypanosoma brucei* or *T. congolense* [10,11]. Although baseline somy is 2N, high levels of aneuploidy are found in all *Leishmania* species studied to date. Whole genome sequencing of 204 *L. donovani* strains from the Indian subcontinent recently revealed tremendous inter-strain diversity of karyotypes, with up to 22 polysomic (somy greater than 2) chromosomes out of 36 in single strains [12]. Only chromosome 31 was tetrasomic in all 204 strains as well as in other species, while only chromosome 25 remained disomic in all strains [11,12]. Furthermore, this variation also exists as mosaicism within strains: in the population of cells constituting a strain, fluorescence *in situ* hybridization (FISH) shows that individual cells have different somies for a given chromosome [13]. Lastly, aneuploidy has been shown to play a role in the development of drug-resistance, as in pathogenic fungi [14,15].

The high inter-strain and intra-strain diversity of karyotype in *Leishmania* may play a major role in the biology and evolution of the parasite. On one hand, the fluctuations in somy allow the parasites to 'manage' heterozygosity and as such aneuploidy could participate in a novel parasexual model of reproduction [16]. On the other hand, aneuploidy has been proposed to be one of the strategies used by *Leishmania* for modulating gene expression [14,17]. In the absence of transcription regulation at initiation through promoters [18], increased chromosome or gene copy number represents a solution for the parasite to increase transcript levels [14].

In natural transmission of *Leishmania*, extra-cellular flagellated promastigotes proliferate in the midgut of sand flies, from where they are introduced into vertebrate hosts during the next blood meal. These promastigotes are taken up by phagocytic cells in the vertebrate, where they transform into amastigotes; these intra-cellular forms then

transform back to promastigotes when taken up by a new sand fly. Considering the postulated major impact of aneuploidy on gene dosage and its potential functional implications, we investigate here (i) how *L. donovani* genome diversity and, in particular, aneuploidy evolves during the parasite's life cycle and its adaptation to the different environments and (ii) what is the impact of the observed changes on the transcriptome. We followed the whole genome variation of different strains, using experimental infections of Syrian golden hamster and sand flies to investigate *in vivo* amastigote and promastigote stages respectively and comparing them with *in vitro* maintained parasites. We document an unexpected modulation of aneuploidy during the life cycle and dissect transcriptional impact related to adaptation and regulation.

3.4 Materials and Methods

3.4.1 Parasites

Three *L. donovani* lines were used in this study: the SSG-sensitive and reference strain for *L. donovani* in the ISC (ISC6 genetic group) [12,19], MHOM/NP/03/BPK282/0 (the derived clone 4 was used for the *L. donovani* reference genome, the latter also referred to as BPK282A1 [19]); the SSG-resistant strain (35) MHOM/NP/03/BPK275/0 (ISC5 genetic group (12)); and the *in vivo* maintained in hamster strain MHOM/SD/62/1S-CL2D originally obtained from Henry Murray (Weill Cornell Medical College, New York, USA) [21,22].

3.4.2 Experimental models

The main experiment of this study was performed using the uncloned *L. donovani* line MHOM/NP/03/BPK282/0. The genome sequence and karyotype of the clinical line and the derived clone 4 promastigotes were found to be identical. Genome changes of the BPK282/0 line were followed in a series of *in vitro* and *in vivo* experiments using golden Syrian hamsters and *Phlebotomus argentipes* to mimic the natural vertebrate and invertebrate hosts of *L. donovani* in ISC, respectively. All life stages, environments, sample names and analyses undertaken are outlined in the experimental flowchart in Fig. 1A. In the case of BPK275/0 and 1S, the experimental set-up was simpler (detailed in Figs. 1B and 1C); for 1S originating from Sudan, *P. orientalis* was used as it is the natural sand fly vector of *L. donovani* in East Africa.

Promastigotes were maintained in culture in M199 (Gibco) supplemented with 20% (v/v) heat-inactivated fetal calf serum (FCS), 4 mM $NaHCO_3$, 100 μM adenine, 7.6 mM hemin, pH7.6 at 26°C for 7 days. For DNA/RNA extraction, promastigotes were harvested in logarithmic phase (day 4) by pelleting for 10min at 1700g at room temperature. Hamsters

were infected and amastigotes were purified as described elsewhere [23]. Hamster P1 and hamster P2 were maintained for 3 months each, while hamster P3 and P4 were maintained until clinical signs of illness in order to recover enough amastigotes to extract DNA and RNA. Parasites were re-suspended at $2x10^8$ amastigotes per ml in PBS and pelleted for DNA/RNA extraction. Hamster care and experimental procedures were performed under approval of the Animal Ethic Committee of the Institute of Tropical Medicine Antwerp (approval BM2013-8) and were compliant with the national and international laws for the protection and welfare of animals. The generation of sand fly-derived promastigotes was performed at the secure sand fly insectary at the Department of Parasitology in Prague as previously described by Sadlova *et al.* [24] with the following modifications: *P. argentipes* (origin India, old laboratory colony) or *P. orientalis* (origin Ethiopia 2010) were used for feeding on the blood containing promastigotes or hamster-derived amastigotes. After 5 days, mature infection was reached and 15 *P. argentipes* and 10 *P. orientalis* whole midguts were individually transferred into M199 medium with 10% FCS and fluorocytosin for five days. Three re-isolates were then pelleted and cryopreserved for DNA/RNA extraction, which was performed using the AllPrep DNA/RNA mini kit (Qiagen) following manufacturer instructions. In the case of Ld1S, DNA extractions were performed before cryopreservation with DNeasy blood and tissue kit from Qiagen.

3.4.3 Genome and transcriptome sequencing

PacBio sequencing was used to generate a new *LdBPK282* reference genome ftp://ftp.sanger.ac.uk/pub/project/pathogens/Leishmania/donovani/LdBPKPAC2016beta/ (see Supplementary Methods S1, end of this chapter, and Appendix A1 to 3 for details) and the European Nucleotide Archive accession number PacBio read data is ERP022358; DNA from all other samples was sequenced on Illumina platforms. Briefly, DNA sequencing libraries were prepared following different methods: (i) using Nextera XT DNA library prep kit (Illumina Inc.) with 1 ng of input genomic DNA according to manufacturer instructions and quantified by qPCR using KAPA Library Quantification Kits optimized for Roche® LightCycler 480 (Kapa Biosystems) on a LightCycler 480 (Roche). This was followed by paired-end sequencing (2 X 100bp) on a HiSeq 1500 platform producing an average depth of 51X for diploid chromosomes and the European Nucleotide Archive accession number of these Nextera reads is ERP022358; (ii) using TruSeq DNA library preparation by shearing genomic DNA into 400–600-base pair fragments (Covaris Adaptive Focused Acoustics technology), followed by generating 125bp paired-end reads on the HiSeq 2000 v4 according to the manufacturer's standard sequencing protocol [25]. Raw sequence data was deposited in the European Nucleotide

Archive with the accession number ERP017317; (iii) for all the Ld1S samples, the libraries were prepared using KAPA Hyper Prep Kit (Kapa Biosystems) with an input of 0.3-0.6 μg of sheared genomic DNA (Covaris E201) according to manufacturer instructions. The final libraries were quantified with the Library Quantification Kit (Kapa Biosystems). Each library was sequenced in paired-end mode, 2x101bp, in a fraction of one sequencing lane of an HiSeq2000 flow cell v3 (Illumina Inc.) according to standard Illumina operation procedures (see Supplementary Methods for a table specifying which library method was used for which sample).

RNASeq libraries were prepared using the Illumina TruSeq Stranded mRNA Sample Prep kit according to the manufacturer's standard protocol apart from the PCR amplification, which was performed using KAPA Hifi Polymerase. 75 base pair paired-end reads were generated on the HiSeq 2000 v4 according to the manufacturer's standard sequencing protocol. Raw sequence data was deposited in the European Nucleotide Archive with the accession number ERP017437.

3.4.4 Genome data analysis

The methods for mapping the reads, SNP and indel calling and local copy number assessment are described in detail in Supplementary Methods S1 (end of this chapter).

3.5 Somy estimation

For somy estimation we used 3 different methods based on the depth variability and total read depth of the sample. Somy values were first estimated using the median depth across each chromosome [12,19], allowing for the evaluation of sequencing quality depth statistics (average and standard deviation). While this method works optimally for samples with an average depth greater than 20, those whose average is below this threshold were analyzed using a bin-based method. This method splits each chromosome up into 2500 bp bins and a chromosome-wide median read depth is calculated from these bins. These chromosome-wide binned read depths dr_i are normalized against the median depth of neighboring chromosomes dm (see below) to get the chromosome somy value S_i, using the formula $S_i=2*dr_i/dm$, where i is the chromosomes number (1 … 36). This method has been shown to be more suitable for depths around 4x coverage and is able to handle larger depth variation than the simple median method [12,19]. For the aM(P/sf) P1 hamster sample, whose mean depth was less than 0.8, its somy was estimated based on the number of reads per 1000 bp and median absolute values of its somy values could not be computed.

Previous studies have shown some correlation between chromosome depth and length for some sequencing runs [11,19]. We thus consider this to be caused by sequencing artifacts and applied a bias correction to the estimate of the median depth *dm*. Instead of calculating one *dm* based on all chromosomes, this value was calculated based on the median values of neighboring chromosomes, whose lengths are similar since chromosomes are numbered according to increasing size. Thus, somy values S_i were calculated using a *dm* based on the median read depth of 15 chromosomes: the 7 neighboring chromosomes on each side and the chromosome itself. When a chromosome does not have 7 neighboring chromosomes on one side, mirror imaged values were used, so that some depth values were used twice.

The range of monosomy, disomy, trisomy, tetrasomy and pentasomy was defined, as previously described [11,12,19], to be the full cell normalized chromosome depth or somy of S, $S<1.5$, $1.5\leq S< 2.5$, $2.5\leq S< 3.5$, $3.5\leq S< 4.5$, $4.5\leq S<5.5$, respectively. Neighboring median correction was performed for DNA somy values but not for RNA read depth (see *RNA-S*, below) where a depth bias associated with chromosome length was not observed. To characterize statistical variation of *S*-value based on median, we used median absolute deviation, abbreviated as MAD in the text.

Comparison of *S*-values between samples was calculated using the Mann–Whitney U test in SciPy with median depth values for 2500bp bins in two samples as input. We used two criteria for detecting significant differences, respectively biological and statistical: (i) *S*-values should differ by more than 0.5 with a shift from one somy distribution range to another, for instance from 1.5-2.5 to 2.5-3.5, together with (ii) a p value $\leq 10^{-5}$. Heatmaps were created using the heatmap3 package [26] in R (R Development Core Team 2015). In addition to the observed copy number variation and sequence quality, we inspected all the depths along chromosomes using scripts partially based on a python 2D plotting library Matplotlib [27]. We remind that sequencing libraries were generated from DNA extracted from a population of cells, so that somy values calculated from sequencing data are an average across the potentially variable somy of these cells. For this reason, somy may be non-integer values, representing the mean value of a mixed population.

3.5.1.1 Transcriptome data analysis

The amount of transcripts was quantified by assessing read depth as described in (12, 19). For each chromosome, the average depth of transcripts was used to compute a RNA-based relative somy value, here called *RNA-S*. The correlation between DNA and RNA depth, namely between *S* and *RNA-S*, was calculated and visualized with SciPy [28].

Differentially expressed genes were identified using DEseq 1.18.0 [29]. Gene depth information (12; 19) was converted to a count of reads per gene and then DEseq default parameters were used for the calculations except in estimate dispersions, where method='pooled' and fitType ='local'. Library depth normalization was already performed in the somy calculation steps, therefore, for each sample, we set a normalization factor to 1 during DEseq calculations. We used a fold change cut off ≥ 2, and a Benjamini-Hochberg adjusted *p* value ≤ 0.05 to define differentially expressed genes. The percentage of DE genes per chromosome is defined as (the number of DE genes per chromosome)/(number of total genes per chromosome)*100.

Gene ontology information was extracted from the gene information file of *L. major* Friedlin (http://tritrypdb.org/common/downloads/release8.1/LmajorFriedlin/txt/TriTrypDB-8.1_LmajorFriedlinGene.txt), which contains comprehensible genome information for LmjF including ortholog, annotations and GO term information. The ortholog information in the file was used to transfer the annotations from LmjF to LdBPKv2.

To identify genes whose transcriptional level changed more than expected based on changes in chromosome somy, we selected chromosomes in which there were only two distinct DNA somy states in samples from a same life stage (here, promastigotes): ProM (I) 20, ProM (P) Sandfly 3, ProM (A) R3 and ProM (A) R10. After determining two groups with different somy, we calculated ratios of all possible pairwise combinations of the two groups and obtained an average value: the denominators were normalized RNA CDS depth values of the group with a smaller somy value and the numerators were normalized RNA CDS depth values of the group with a higher somy value. The ratios for each CDS were in chromosome position order. Normalized RNA CDS read counts were deposited to GEO with its accession number GSE97453.

3.6 Results

3.6.1 *In vitro* and *in vivo* dynamics of *L. donovani* genome

We tracked genome diversity by WGS (sample details in supplementary Table S1A) at different levels (SNPs, indels, CNVs and somy) during *in vitro* and *in vivo* maintenance of our reference BPK282/0 line. Briefly, the experiment started with *in vitro* cultured promastigotes, and we compared i) promastigotes re-isolated from sand flies infected by cultured promastigotes, ii) amastigotes during short-term and iii) long-term adaptation to hamster, iv) promastigotes re-isolated from sand flies infected by hamster-derived

amastigotes, v) during adaptation to *in vitro* culture of amastigote-derived promastigotes (see flowchart in Fig. 1A).

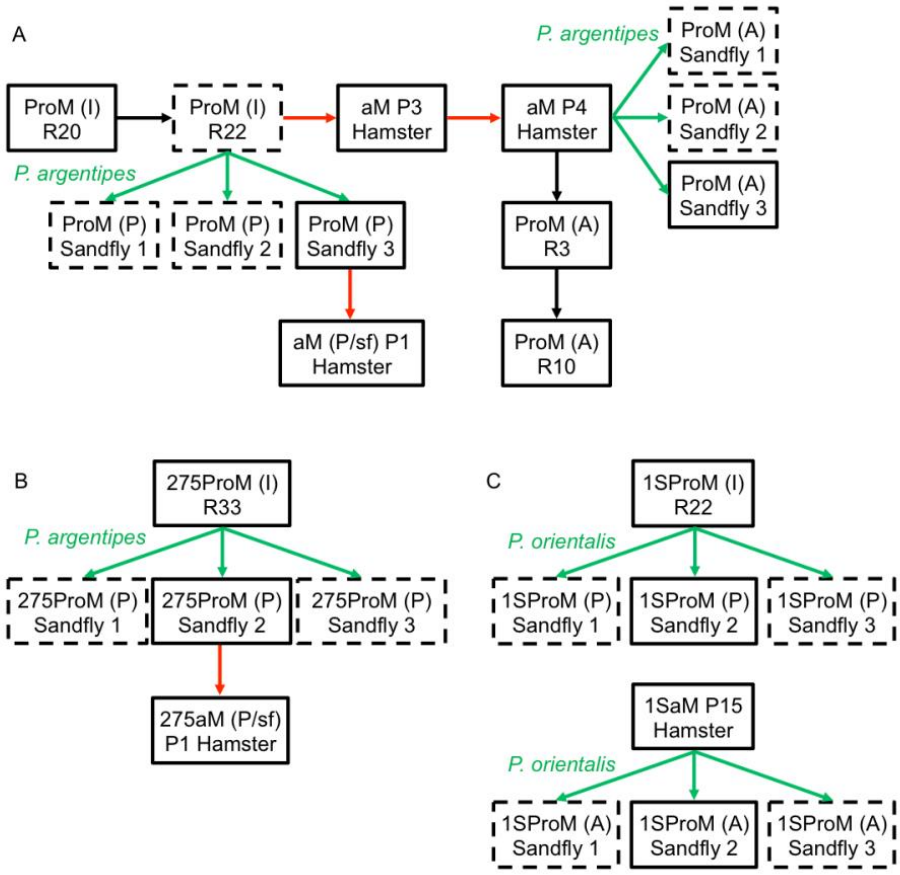

Figure 1. Flowchart showing the experimental history of the 3 *L. donovani* strains. Historic for **(A)** BPK282/0, **(B)** for BPK275/0 and **(C)** for 1S-2D. black arrows indicate *in vitro* passage (R, number of sub-inoculations since isolation from the patient or the animal); red arrows indicate a passage through hamster (P, number of passages in the animal); green arrows indicate a transmission through the natural vector. Samples that were submitted to DNA and RNA sequencing are mentioned in full line boxes while sample for which only DNA sequencing was performed are indicated in dashed boxes. ProM, promastigotes, aM, amastigotes while the letters under brackets indicated the life stage and the environmental conditions from where the parasites are coming from, I, *in vitro* promastigotes; A, amastigotes from hamster; P, promastigotes from *in vitro* culture; P/sf, sand fly originated promastigotes.

When the nucleotide sequences of all samples were compared, we detected no local CNVs (Table S2A) or indels and only 5 heterozygous SNPs in the nuclear genome, which changed frequencies during the experiment (Table S2B). Among these, 4 heterozygous SNPs on chromosomes 24, 32 and 25 were not present in cultured promastigotes, but were detected only in the adapted amastigotes (aM P3 and aM P4, see Fig. 1) and were maintained in the subsequent *in vitro*-grown and sand fly derived promastigotes. We also observed the disappearance of a heterozygous SNP located in chromosome 35 in sand fly-derived promastigotes, where promastigotes were used as inoculum (Table S2B). No new SNPs were detected in the kDNA maxi-circle.

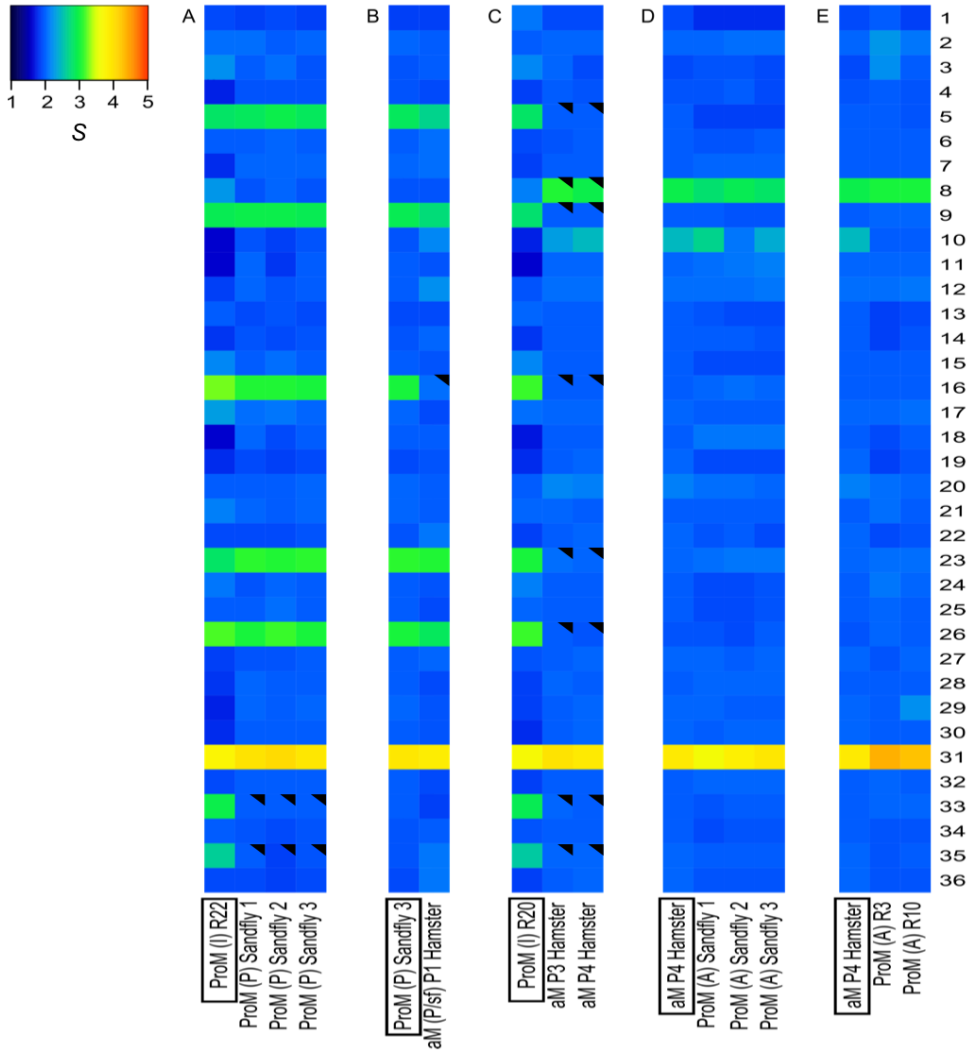

Figure 2. Dynamics of aneuploidy of *L. donovani* BPK282/0 during adaptation to different *in vitro* and *in vivo* environments : **A)** promastigotes re-isolated from sand flies; **B)** amastigotes, during short-term adaptation; **C)** amastigotes during long-term adaptation; **D)** promastigotes re-isolated from sand flies infected by hamster-derived amastigotes; **E)** during adaptation to *in vitro* culture of amastigote-derived promastigotes. Median normalized read depth of chromosomes found within each cell population for each of the 36 chromosomes (y-axis) and each sample (x-axis). The colour key shows the normalized chromosome read-depth (*S*) and the distribution frequency. The *S*-range for monosomy is $S < 1.5$ (dark blue), disomy: $1.5 \leq S < 2.5$ (light blue), trisomy: $2.5 \leq S < 3.5$ (green), tetrasomy: $3.5 \leq S < 4.5$ (orange) and pentasomy: $4.5 \leq S < 5.5$ (red). A filled black triangle on the upper right corner indicates a significant change of S (≥ 0.5, with a shift from one S-range to another and p-value $\leq 10^{-5}$), in comparison to the *S*-value of the sample framed in black box.

In contrast with the overall high conservation of the nucleotide sequence of the parasite genome, we observed drastic changes in karyotype in the different life stages and/or environments. For each chromosome, (i) median somy value *S* within each sample and its corresponding median absolute deviation (MAD) across reads of that chromosome and (ii) statistical significance of *S*-differences between samples were calculated as described in Materials and Methods (Data Set S1A1); (iii) when referring to integer somy (di-/tri-/tetra-somy), we followed the rules described previously (11,12,19). When we inoculated *in vitro* adapted promastigotes (with aneuploidy affecting 8/36 chromosomes) into a sand fly, and re-isolated parasites at the late stages of infection (5 days in the fly followed by 5 days propagation in culture), we observed that the *S*-value of chromosomes 33 and 35 significantly decreased from 2.95 ± 0.62 to 2.00 ± 0.14 (average of 3 sand fly samples) and from 2.64 ± 0.59 to 1.92 ± 0.11, respectively, while the rest of the karyotype remained unchanged (Fig. 2A, Table S3 and Data Set S1A1). Importantly, we observed identical karyotypes in promastigotes isolated from 3 independent flies, suggesting that these aneuploidy changes were not random (Data Set S1A1 and A2). Next, we inoculated the promastigotes isolated from one sand fly into hamsters, and after 2 months of infection we analyzed the karyotype of the purified amastigotes (aM (P/sf)P1). We found that overall aneuploidy pattern of these amastigotes was similar to that of the inoculum, except for chromosome 16 for which *S* dropped from 3.03 ± 0.17 to 2.08 where MAD could not be measured aM (P/sf) due to insufficient depth coverage. Aneuploidy changes were more pronounced between *in vitro*-adapted promastigotes and the derived amastigotes after 3 and 4 consecutive passages between hamsters, meaning respectively 12 and 19 months after first inoculation of promastigotes. In

promastigotes, the *S*-value of 7 chromosomes (Table S3) ranged between 2.60 ± 0.45 and 3.16 ± 0.51 and decreased significantly in amastigotes, ranging between 1.98 ± 0.07 and 2.06 ± 0.07. *S* value of chromosome 31 remained in the range of tetrasomic chromosomes ($3.5 \leq S < 4.5$) and did not change significantly among samples. In contrast, the *S*-value of two chromosomes increased from promastigotes to amastigotes: (i) chromosomes 8, from 2.15 ± 0.4 to 3.07 ± 0.12 in P3 and 2.95 ± 0.16 in P4 (both differences significant) and (ii) chromosome 10, from 1.74 ± 0.37 to 2.30 ± 0.10 in P3 (not significant) and 2.47 ± 0.14 in P4 (marginally significant) (Fig. 2C, Data Set S1A1).

Subsequently, *in vivo*-adapted amastigotes (aM P4) were used (i) to infect sand flies, to assess the stability of aneuploidy patterns during transmission (Fig. 2D) and (ii) after isolation, to follow karyotype during the short-term adaptation of amastigote-derived promastigotes to *in vitro* culture after 3 and 10 passages, respectively (Fig. 2E). We did not observe any significant karyotype changes during these experiments: we noted a slight decrease in *S*-value of chr10 during early *in vitro* cultivation from 2.47 ± 0.14 to 1.99 ± 0.12 (Fig. 2E, marginally significant).

3.6.2 Karyotype dynamics is strain-specific

Given the observed flexibility in aneuploidy patterns of BPK282/0, we further explored whether this was a specific feature of this particular line or if it could be also found in other *L. donovani* lines. To address this question, we analyzed (i) another uncloned *L. donovani* clinical line from the Indian Sub-Continent (ISC) BPK275/0 (SSG-resistant), and (ii) a cloned laboratory strain, Ld1S, isolated in Sudan in 1962 and propagated for this study either *in vitro* for approximately 200 generations or *in vivo* for at least 15 passages in hamsters (Fig. 1B, C and Data Set S1A1).

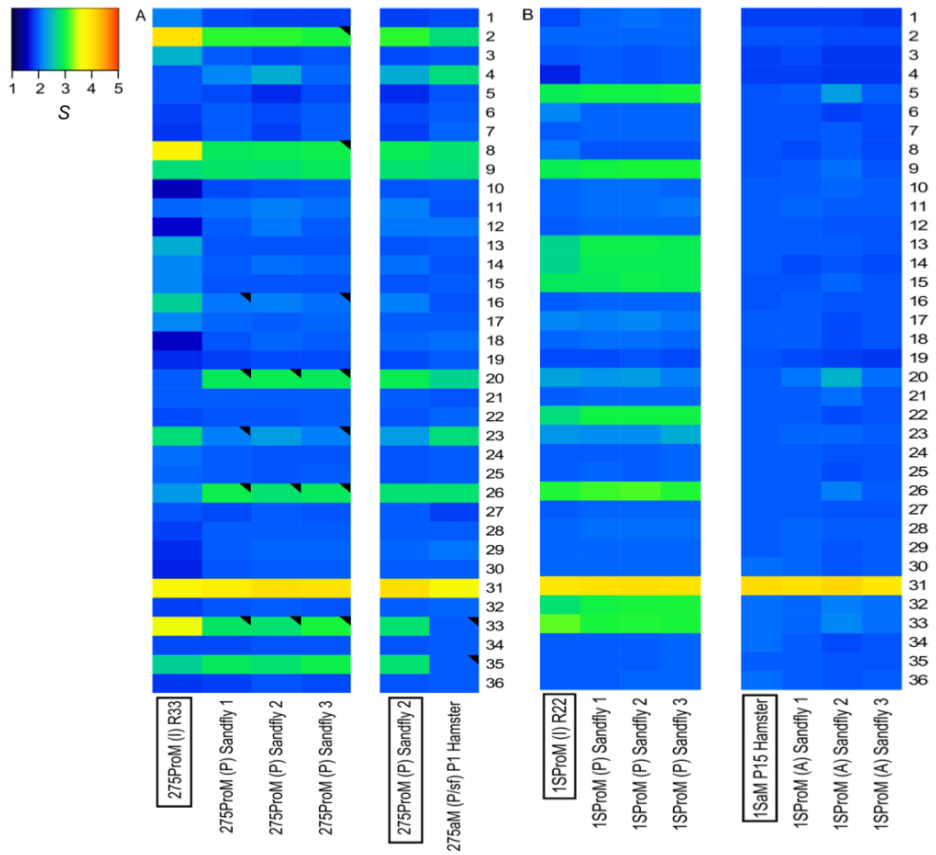

Figure 3. Dynamics of aneuploidy of *L. donovani* BPK275/0 (A) and Ld1S (B) during adaptation to different *in vivo* environments. See caption of Fig2 for details. See Data Set S1 A1 and A2 for all the pair wise comparisons.

After development into sand flies of *in vitro* adapted promastigotes of BPK275/0 (275ProM (I) R33), we observed that *S*-values of 7 chromosomes consistently changed (Table S3). For example, in sand fly 1, (i) *S*-values of chromosomes 2, 8 and 33 decreased significantly from 4.00 ± 0.90 to 3.10 ± 0.18, from 3.80 ± 1.05 to 2.89 ± 0.22 and from 3.57 ± 0.92 to 2.82 ± 0.12, respectively; (ii) *S*-values of chromosomes 16 and 23 decreased significantly from 2.64 ± 0.56 to 2.10 ± 0.10 and from 2.72 ± 0.50 to 2.17 ± 0.13, respectively; while (iii) *S*-values of chromosomes 20 and 26 significantly increased from 2.00 ± 0.44 to 2.88 ± 0.16 and from 2.26 ± 0.45 to 2.94 ± 0.14, respectively (Fig. 3A, Data Set S1A1 and Table S3). Aneuploidy patterns in sand fly 3 was identical, while in sand fly 2, two out of the 7 varying chromosomes (16 and 23) also decreased, but difference did not reach the threshold of significance (Fig. 3A, Data Set S1A1 and Table S3). Next, we inoculated promastigotes isolated from one fly to hamster, and analyzed the karyotype in the

amastigotes isolated 2 months after infection. *S*-values changed as follows: (i) for chromosomes 33 and 35, significant decrease from 2.73 ± 0.23 to 1.97 ± 0.17 and from 2.76 ± 0.19 to 2.00 ± 0.16, respectively; (ii) for chromosomes 4 and 23, increase (marginally significant) from 2.29 ± 0.23 to 2.76 ± 0.25 and from 2.34 ± 0.17 to 2.77 ± 0.21, respectively (Fig 3A, Data Set S1A1, Table S3). In contrast, we did not observe major changes in aneuploidy patterns for the Ld1S strain after passage through sand fly, whether *in vitro*-grown promastigotes or hamster-adapted amastigotes were used as source of infection (Fig. 3B, Table S3, Data Set S1A).

3.6.3 Changes in chromosome copy number correlate with overall transcriptome levels

It has been proposed that *Leishmania* uses aneuploidy to increase mRNA levels and we have previously shown a correlation between chromosome copy number and transcript levels in one promastigote sample of BPK282/0cl4 [19]. Thus, we hypothesized that the observed variation in aneuploidy is potentially adaptive and has a functional impact on the parasite's biology. Therefore, we would expect that increase or decrease in chromosome copy number would be mirrored by a proportional change in transcript levels derived from this chromosome. To address this hypothesis, we performed RNAseq analysis (detailed in supplementary Table S1B) to obtain transcriptome profiles of selected representative samples of BPK282/0 and BPK275/0 for which we previously assessed aneuploidy pattern (see details on Fig. 1). For each chromosome, we computed the median transcript level (further called *RNA-S*, see Materials and Methods) and compared this with DNA-based *S*-values. From this, we could observe that for all chromosomes except chromosome 31, changes in *S*-value were generally mirrored by alterations of *RNA-S* in the same direction, as illustrated in Fig. 4, Fig. S1A and Data Set S1 B. Within each sample, correlation between *S* and *RNA-S*, excluding chromosome 31, was significant and the coefficient of determination r^2 ranged from 0.2 to 0.7 with $P < 0.01$, except for ProM (A) R3 (Table S4). Overall, when all samples were combined, we found a significant correlation, (i) with $r^2 = 0.395$ and *p*-value = $1.05*10^{-43}$ without chromosome 31 and (ii) $r^2 = 0.341$ and *p*-value = $1.34*10^{-37}$ when chromosome 31 was included.

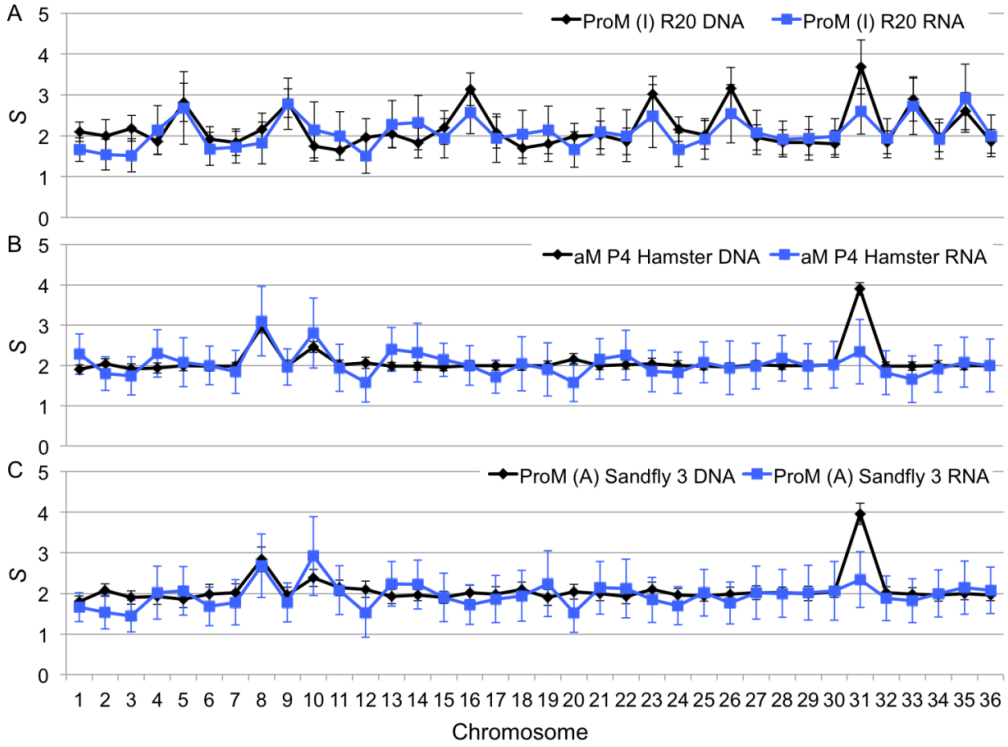

Figure 4. Impact of variable aneuploidy of *L. donovani* BPK282 on transcriptome during adaptation to different environments. Three life stages/environments are considered, in which aneuploidy is different: **(A)** *in vitro* promastigotes (R20), **(B)** amastigotes adapted to hamster (P4) and **(C)** sand fly-derived promastigotes. The black and blue lines correspond to S-values and RNA-S, respectively. Each error bar corresponds to a median absolute deviation. Details on the correlation between S and RNA-S can be found in Table S4 and the values are in Data set S1 B.

Table S4: Correlation between genome and transcriptome. The values of Pearson correlation coefficient r, determinant r × r (r2), and its statistical significance between S and RNA-S are shown. Chromosome 31 was tetrasomic in all samples and was excluded from this analysis because its transcription level was systematically close to the disomic level.

		Length	r	r*r	P-v
275ProM (I) R33	275ProM (I) R33 RNA	35	0.6166	0.3802	8.00E-05
275ProM (P) Sandfly 2	275ProM (P) Sandfly 2 RNA	35	0.6384	0.4076	3.68E-05
275aM (P/sf) P1	275aM (P/sf) P1 RNA	35	0.818	0.6691	1.98E-09
ProM (I) R20	ProM (I) R20 RNA	35	0.668	0.4462	1.16E-05
aM P3 Hamster	aM P3 Hamster RNA	35	0.6694	0.448	1.10E-05
aM P4 Hamster	aM P4 Hamster RNA	35	0.6508	0.4235	2.31E-05
ProM (A) R3	ProM (A) R3 RNA	35	0.2569	0.066	1.36E-01
ProM (A) R10	ProM (A) R10 RNA	35	0.447	0.1998	7.10E-03
ProM (P) Sandfly 3	ProM (P) Sandfly 3 RNA	35	0.6954	0.4836	3.54E-06
aM (P/sf) P1	aM (P/sf) P1 RNA	35	0.7292	0.5317	6.77E-07
ProM (A) Sandfly 3	ProM (A) Sandfly 3 RNA	35	0.5197	0.2701	1.38E-03

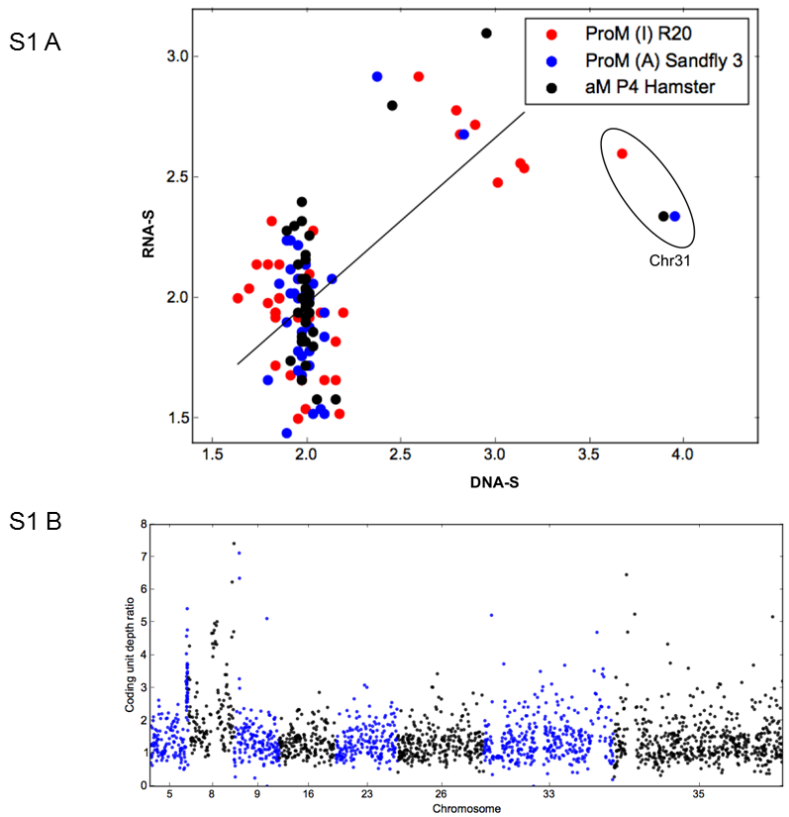

Fig S1 (A) Correlation between S and RNA-S during adaptation to different life stages/environments. The correlation coefficient between S and RNA-S in promastigotes (in vitro and from sand fly) and amastigotes was r = 0.65 (P = 7.7 × 10−12). Chromosome 31 somy values were excluded for the correlation calculation but were included in the scatter plot to illustrate their behaviour. **(B) Manhattan plot for individual gene expression in promastigotes showing variable aneuploidy.** Samples considered here are ProM (I) R20, ProM (P) sand fly 3, Prom (A) R3, ProM (A) R10. These samples represent the same life stage, but they differ in the somies of chromosomes 5, 8, 9, 16, 23, 26, 33, and 35. RNA depth ratios between trisomic and disomic groups in these chromosomes are shown: the x axis represents each coding unit along chromosomes, and the y axis the average RNA depth ratios. Colours of dots were alternated for each chromosome.

Although overall chromosomal transcription levels correlated with the somy of the respective chromosomes, this does not exclude that the transcription of some specific genes might not follow this overall trend. A potential mismatch between chromosomal and local gene transcription level was thus checked for samples of a same life-stage, in order to minimize the effect of regulation occurring between life stages (see below). We

chose 4 promastigote samples of BPK282/0 [ProM (I) 20, ProM (P) Sandfly 3, ProM (A) R3, ProM (A) R10] and focused on chromosomes showing significant changes in *S*-values, i.e. chromosomes 5, 8, 9, 16, 23, 26, 33 and 35. For these chromosomes (Fig. S1B), we compared average transcript depth ratios for each RNA coding unit between the samples with overall trisomic ($2.5 \leq S$-value < 3.5) and disomic chromosomes ($1.5 \leq S$-value < 2.5) and found 13 to 18% of 'outlier' genes in these 8 chromosomes (Table S5 and Data Set S1 C). Interestingly, most of these outliers (80%) were over-expressed. Chromosomes 5 and 8 called further attention (Fig.5). Specifically, we found 40 transcripts at the end of chromosome 5, from LdBPK_050017400 to LdBPK_050021300, which were more highly expressed than other genes in strains with trisomic chromosome 5. Remarkably, the 40 transcripts belong to one transcription unit (starting after a strand-switch region) and 38 of them encode for snoRNAs: this entire polycistronic unit thus seemed to be regulated independently from the rest of the chromosome. Chromosome 8 in turn, showed an array of amastin genes (from LdBPK_080011900 to 13000) whose expression is 4-fold higher in samples with trisomic chromosome 8 than in samples with disomic one (Fig. 5, Data Set S1 C); the array was a small fraction of a large transcription unit and interestingly, another amastin array (from LdBPK_080013400 to 13800)situated in the same transcription unit was not over-expressed.

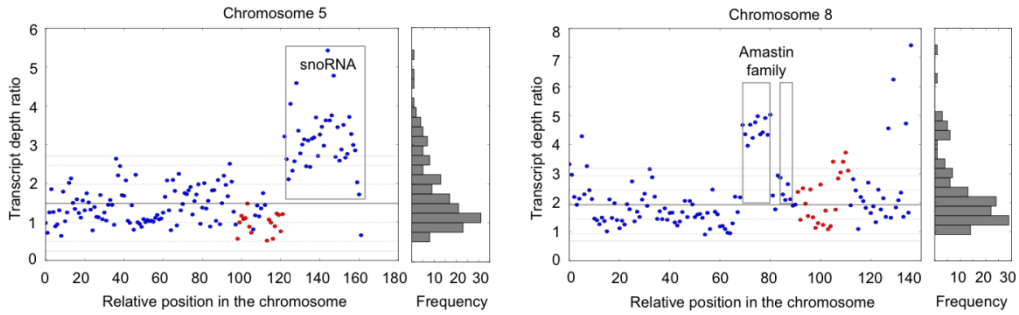

Figure 5. Individual gene expression in promastigotes showing variable aneuploidy. Samples considered here are: ProM (I) 20, ProM (P) Sandfly 3, Prom (A) R3 and ProM (A) R10. Transcript depth ratios between samples with trisomic and disomic chromosomes 5 and 8 are shown: each single dot represents a transcript, blue ones are on the (+) strand and red ones on the (-) strand. The x-axis represents each coding unit along the chromosome and the y-axis the average transcript depth ratios and their corresponding histogram shows their distribution. The snoRNA cluster on chromosome 5 and the amastin family are marked with the rectangular box. The thick dark grey horizontal lines indicate the median depth and the faint grey lines represent 1 MAD

(median absolute deviation), 2 MAD, and 2.5 MAD away from the median depth, respectively.

Table S5: Proportions of outlier transcripts compared to the S value of their chromosomes for chr 5, 8, 9, 16, 23, 26, 33, and 35. Description of the number of transcripts on the chromosomes concerned and the number of transcripts not following the general expression (up or down) of the chromosomes according to their S values.

Chromosome	All coding units*	Outliers	% of outliers	Up	Down
Ld05	162	27	17%	27	0
Ld08	137	23	17%	22	1
Ld09	168	26	15%	18	8
Ld16	177	31	18%	22	9
Ld23	200	25	13%	22	3
Ld26	283	38	13%	29	9
Ld33	378	56	15%	46	10
Ld35	546	98	18%	73	25

*=All coding units whose depth ratio is defined.

3.6.4 Individual gene expression in different life stages characterized by a shared aneuploidy pattern

It is known that the expression of some *Leishmania* genes is differently regulated from one life stage to the other, over a short period during the life cycle [30]. To integrate this, we compared the transcriptome of two consecutive amastigote samples with two *in vitro*-grown derived-promastigotes (aM P3 & aM P4 vs ProM(A) R3 & ProM(A) R10). These two amastigote samples originated from two consecutive passages in hamsters, and the promastigotes are derived from the last amastigote passage, thus we considered each stage-specific pair of samples as biological replicates. Importantly, all these 4 samples had identical aneuploidy patterns (Fig2C and E) allowing the identification of variation in the transcriptome independent of gene dosage. With a cut-off for differential expression (DE with 2-fold change and Benjamini-Hochberg corrected p value < 0.05), we found that 589 genes (6.8%) were upregulated in promastigotes (in comparison with amastigotes (Data Set S1 D1), while only 261 genes (3.0%) were expressed at higher levels in amastigotes (Data Set S1 D2); with the remaining 7831 genes (90.2 %) being constitutively expressed. When looking closely at the function of the DE genes in the two life stages, we identified that, in promastigotes, several genes belonging to categories

related to carbon, lipid and fatty acid metabolism, translation, protein modification, membrane transport, DNA replication and nucleosome assembly, and the function of the flagellum (Table S6). We could not find genes belonging to these categories in the upregulated genes in amastigotes. Instead, we found 25 genes coding for amastins and amastin-like proteins to be upregulated in amastigotes, and only 5 in promastigotes. We also analyzed the relative distribution of the overexpressed genes over the different chromosomes. We found the highest percentage of upregulated genes in amastigotes on chromosomes 8 and 10 while chromosomes 17 and 19 contained the highest percentage of genes upregulated in promastigotes (Fig. 6, Data Set S1 D1 and D2).

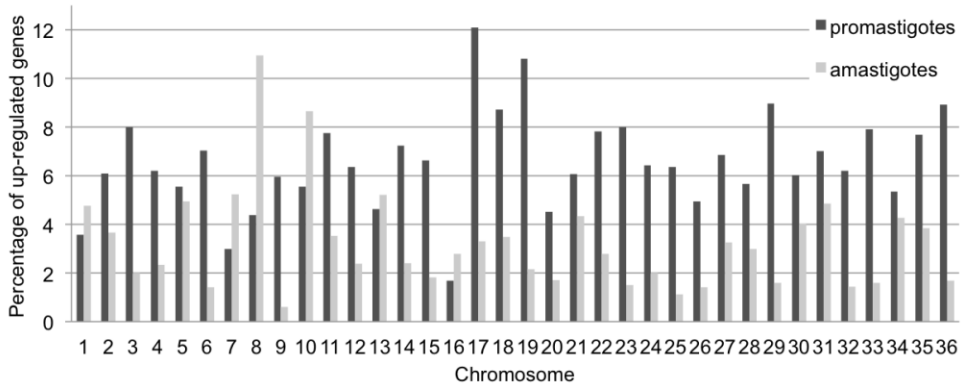

Figure 6. Aneuploidy-independent changes of transcriptome during parasite differentiation. The samples considered here have the same aneuploidy pattern and correspond to (i) adapted amastigotes of hamsters (aM P3 & P4 taken together) and (ii) promastigotes freshly differentiated from these (ProM(A) R3 and R10 taken together). The histogram shows the percentage of up-regulated coding units with ≥ 2 fold changes and Benjamini-Hochberg corrected p value ≤ 0.05 in each chromosome based on the DEseq analysis.

Table S6: GO terms based on biological process for differentially upregulated genes in amastigotes or promastigotes. Genes were considered upregulated if their ratio was ≥2-fold and P value was ≤0.05 (DEseq analysis). Amastin, which is not associated with any GO term, was added to the table manually.

	Up-regulated in promastigotes	Up-regulated in amastigotes
GO annotation with p-value <0.05	**372/589 (63.15%)**	**91/261 (34.9%)**
Acid phosphatase	4 (0.68%)	0
ATP binding	3 (0.51%)	0
ATP metabolism	17 (2.88%)	0
Heat-shock protein binding	0	2 (0.76%)
Heme binding	5 (0.85%)	0
Lipid	11 (1.87%)	1 (0.38%)
- Ergosterol + Phospholipid	4 (0.68%)	0
- Fatty acid metabolism	3 (0.51%)	0
- Lipid metabolism	3 (0.51%)	1 (0.38%)
L-methionine salvage	4 (0.68%)	0
Metabolic process	16 (2.72%)	0
Microtubule	9 (1.53%)	0
Nucleic acid	17 (2.89%)	19 (7.28%)
- DNA binding	3 (0.51%)	0
- DNA repair	0	1 (0.38%)
- DNA replication	3 (0.51%)	1 (0.38%)
- RNA binding	11 (1.87%)	13 (5%)
- RNA processing	0	4 (1.53%)
Nucleosome assembly	15 (2.55%)	1 (0.38%)
Oxidation-Reduction	7 (1.19%)	2 (0.76%)
Protein	80 (13.6%)	9 (3.45%)
- Protein folding	27 (4.6%)	1 (0.38%)
- Protein binding	11 (1.88%)	5 (1.91%)
- Post translational modification	10 (1.7%)	3 (1.15%)
- Proteasome	10 (1.7%)	0
- Proteolysis	22 (3.74%)	0
Response to ROS	9 (1.53%)	0
Sugar	14 (2.38%)	0
- Carbohydrate metabolism	10 (1.7%)	0
- Glucose metabolism	3 (0.51%)	0
- Gluconeogenesis	1 (0.17%)	0
Transcription	0	3 (1.15%)
Translation	17 (2.89%)	5 (1.91%)
Transport	9 (1.53%)	4 (1.53%)

3.7 Discussion

Primarily, this study shows that passage and adaptation of both *in vitro* and *in vivo* clinical lines of *L. donovani* results in little or no variation in SNP, indel and CNV, while large changes in aneuploidy occur over short periods of time, such as one passage through a sand fly (12.5 generations; 2.5 generations/day, over a period of 5 days [31]) or a mammalian host (approximately 5 generations, assuming a generation time of 12 days [32] over 2 months). Whether this is due to preferential growth of subpopulations with different genomic variants or *de novo* generation of genomic changes remains to be investigated further. Although our experimental setup included mostly uncloned isolates, and thus potentially subpopulation of different karyotypes, it should be noted that even in clonal populations, individual parasites were reported to differ in their karyotype based on WGS-based somy measurements in fact representing the population average [13]. Mosaicism likely explains the non-integer values that we observed for *S*-values of individual samples or for differences between samples. This mosaic aneuploidy is supposed to provide strong adaptive advantage for the whole population rather than for the single cell [16], with beneficial aneuploidies being selected in response to environmental changes.

Regardless of the mechanisms underlying this genome plasticity, it is likely that such changes in aneuploidy are adaptive, as similar changes have been observed in response to drug pressure or change in environment in *Leishmania* during *in vitro* experimental evolution studies and also in other eukaryotic pathogens [15,33,34]. Several indicators of adaptive benefits were observed here. Aneuploidy patterns were mirrored across replicates in the sand fly and also in the transcriptome changes (except for chromosome 31), indicating both a shared adaptation and a potential function of the genomic variation in adjusting gene dosage. Such changes were also found to be progressive. Amastigote adaptation to hamsters resulted in somy decrease at P1 (chromosome 16) which continued at P3 and P4 (chromosomes 5, 9, 23 and 26). Conversely, increases in somy were observed for chromosomes 8 and 10, upon hamster passaging (Fig. 4). These two chromosomes also had increased regulation of individual genes in amastigotes compared to promastigotes (Fig. 6). Chromosome 8 contains several copies of δ-amastins [35], genes only found in Trypanosomatids that possess an intracellular life-stage, in which they are highly expressed [36]. RNAi knockdown experiments have also shown that δ-amastin expression in *L. braziliensis* is needed for survival and proliferation of intracellular parasites in *in vitro* macrophage infections and in *in vivo* infection of mice [36]. Similarly, chromosome 10 contains the *gp63* gene array, a major virulence factor in promastigotes [37], and protection factor in intracellular amastigotes [38]. Thus, the

increased somy of chromosome 8 and 10 in hamster-adapted amastigotes may provide adaptive advantages due to increased copy of δ-amastins and *gp63* genes, although further experiments are required to confirm this.

Noteworthy, strain-specific differences were observed here. Like BPK282/0, BPK275/0 also showed a plastic karyotype, but increases in somy were detected in the sand fly (chromosomes 20, 26) and at early stage of adaptation to hamster (chromosomes 4 and 23). In contrast, the karyotype of Ld1S was very stable in the sand fly, and all chromosomes (except 31) were disomic in amastigotes isolated from P15 hamster. This could be explained by the genetic distance of the latter strain, originating from East Africa, although this karyotype stability could be a consequence of its historical *in vivo* laboratory passaging. This strain specificity is in line with our previous phylogenomic study in natural populations of *L. donovani* (promastigotes), where parasites belonging to the same phylogenetic clade showed a higher karyotypic similarity than those from different clades [12]. This suggests that the optimal karyotype depends on the genetic background of a particular strain [39], a feature that warrants further investigation in this and other *Leishmania* species.

Although this study addressed the gene-dosage adaptive role of aneuploidy, this strategy may also be beneficial in other areas such increasing diversity and heterozygosity, especially given the minimal sexual recombination described in this organism [13]. We demonstrated this effect in natural populations of *L. donovani*, where we found that epidemic disomic clones emerging from aneuploid ancestors showed a significantly lower degree of heterozygosity [12]. Although this effect could not be studied here due to the scarcity of heterozygous sites in BPK282/0, this phenomenon is being investigated in the Ld1S strain (Prieto *et al*. 2017, submitted).

Although several life stage comparison transcriptome studies have been undertaken previously on the short-term differentiation [40,41], the potential impact of changes in aneuploidy patterns on transcription profiles has not been studied. Our study clearly shows that life-stage/environment-induced changes in aneuploidy have a great impact on the transcriptome, but at the same time they do not remove the effect of stage-specific regulation of transcriptome, which is required during the transition from one stage to the other. While chromosome copy number is mirrored by transcript levels in this study, these should be also reflected at the protein and metabolite levels to have a phenotypic effect. Although genes are constantly transcribed in a poly-cistronic manner by this process, it is likely that only a few specific genes required for adaptation to a given environment are the main drivers, with the expression level of other genes regulated via post-transcriptional mechanisms (33). In our dataset, we observed that although overall

correlation between the quantity of DNA and RNA exists, not all individual transcripts found on variable chromosomes change to the same extent: this was most obvious in the case of snoRNAs in chromosome 5 and one amastin array in chromosome 8. Chromosome 31 constituted a major exception to the DNA/RNA correlation, as it behaved transcriptionally as a disomic chromosome, despite being tetrasomic. Tracking heterozygous sites and comparing alternative allele-frequency in both DNA and RNA should allow answering if the four chromosomes are transcriptionally active: this was not possible with BPK282/0 because of the lack of heterozygosity in that strain.

Leishmania amastigotes are thought to exist in a semi-quiescent state with reduced growth, metabolism and energy-consumption [32,42]. This hypothesis is supported here with several genes being downregulated in amastigotes compared to promastigotes, primarily those involved in metabolism, nucleosome assembly, and protein assembly and processing (Table S6). Reduced somy was also associated with the amastigote stage in hamsters, where disomy was more common than in *in vitro*-grown promastigotes (Fig. 2, [12]). Thus, during *in vitro* growth, when nutrient availability is not limiting, high levels of aneuploidy are allowed, while during the adaptation to semi-quiescent state, minimal levels of somy would be required.

Overall, this study highlights the effect of life stage and *in vitro* maintenance on the chromosomal copy number of a given strain. This would have a potential impact on molecular epidemiological studies performed on *in vitro* cultivated promastigotes, as done previously (11, 12). While this effect may be small, some genomic features could be diluted/altered during *in vitro* maintenance, even if this was rather brief (about 20 sub-inoculations after isolation from the patient). We showed that chromosome copy number is the most sensitive to environmental changes, which possibly explains the lack of correlation between aneuploidy and drug resistance of natural isolates (12), while this is a common feature during experimental selection [14,15]. This highlights the importance of using clinical samples and minimal laboratory passaging when linking genomic adaptations to drug or immune pressure in future studies.

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3.9 Supplementary Methods:

3.9.1 PacBio sequencing and assembly of L. donovani BPK282A1 reference genome

The reference strain BPK282A1 previously sequenced in 2011 [12] (known as LdBPKv1) was re-sequenced using SMRT WGS (P5C3 chemistry) from Pacific Bioscience RSII. Reads were assembled with the PacBio *de novo* assembly program RS_HGAP Assembly.2.1.0. This produced 616,963 post-filtered reads, a polymerase read N50 value of 11,717 bp, polymerase read length of 8,488 bp, polymerase read quality of 0.836. These reads were assembled and refined into 130 contigs with median 131-fold coverage using default parameters. The sufficient coverage over 100 made it possible for RS_HGAP to perform base error corrections. These contigs were assembled by a succession of methods summarized in Appendix 1; for chromosome building, they were aligned against the *L. major* genome release-8.1 [13].

For ABACAS assembly, the key parameters used were p=nucmer, minimum percent identity 15, minimum contig coverage 15. These relaxed parameters are suitable for very long PacBio contigs. Contigs that were not mapped along were found to be duplicates of the main assembly and un-assembled contigs were not used for further analysis.

PBjelly PBSuite_14.6.24 was applied for gap correction of the contigs using the PacBio reads. This was followed by two rounds of base correction using Quiver. Eight iterations of iCORN v0.97 using Illumina reads from BPK282A1 fixed 70 base errors, 711 insertions, 81 deletions and identified no heterozygous sites. This step was crucial to fix insertions and deletion errors for which Illumina reads were more suitable. Gene annotation was performed using the command line version of Companion v1.0.1, which carried out both *de novo* gene prediction and gene annotation transfer from *L. major* Friedlin [14]. The final product of these processes was named LdBPKv2. Annotations of kDNA maxi-circles, which corresponds to Ld37, and ribosomal DNA in chromosome 27, on chromosome 2, were performed by RATT [15], since the initial Companion annotation did not annotate these regions properly.

We used the results of REAPR [16] and Companion to evaluate the relative accuracy of genome assembly among LdBPKv1, LdBPKv2 (*L. donovani*), LinJPCM5 (*L. infantum*) and LmjF reference. It was not optimal to evaluate the quality of PacBio assembly whose N50 is over 11,000 bp with REAPR by using Illumina reads with short insert size. Nevertheless, REAPR provided objective evaluation of the relative accuracy of the Leishmania genomes and the summary of the results of REAPR and Companion were given in Appendix 2. Examples of improvements are mentioned in Appendix 3: (A) Extension of the chromosome sequence up to the telomeres; (B) Increased number of annotated genes, here 18 additional genes in chromosome 27, that were previously missed in LdBPKv1; (C) mapping of tandem arrays, here the mini-exon locus; (D) complete kDNA maxi-circle. Appendix 4 provides the conversion table from LdBPKv1 to LdBPKv2 and appendix 5 the ortholog information for selected multi copy genes.

Gaps present in LdBPKv2 were mostly introduced during ABACAS assembly and comparisons to other reference indicated that most of large gaps were over estimated. At the time of writing, the improvement of various kinetoplastids genomes is underway, and therefore we did not pursue the further improvement of the genome since it would not affect our results.

3.9.2 Read mapping SNP and small insertions/deletions detection

Reads were mapped to the reference *L. donovani* genome LdBPKv2 using Smalt v7.4 (http://www.sanger.ac.uk/science/tools/smalt-0). Smalt options for exhaustive searching for optimal alignments and random mapping of multiple hit reads were used. Picard v1.85 (http://broadinstitute.github.io/picard/) were used for merging and sorting bam files and marking duplicated reads. This procedure was similar to one described in the previous analysis (12).

SNPs and small insertions and deletions (indels) were called using population-based Unified Genotyper method in Genome Analysis Toolkit v3.4 (GATK: https://software.broadinstitute.org/gatk/). SNP clusters (more than 3 SNPs within 10 bases of each other) were noted but we maintained these SNPs for analysis. Low quality SNPs were filtered using GATK Variant Filtration with QD < 2.0 || MQ < 40 || FS > 60.0 || ReadPosRankSum < -8.0. To avoid false negatives, the SNP quality cut off was set to 300. Afterwards all candidate SNPs were visually inspected in the Integrative Genomic Viewer (IGV_2_3_47) [17] and SAMtools to avoid false positives. SnpEff v4.1 [18] was used to classify all SNPs and indels based on their functional impact such as frame shift, non-synonymous, synonymous change and intergenic mutation. SNPs and indels were compiled in a population genetic variation variant calling format (vcf) file. From this vcf file alternative allele and depth information was extracted for further analysis. To ensure the accuracy of variant calling, we also used the base variation data published in previous reports [12,19,20]. Variants common to all strains were excluded from analysis since our focus was to identify genetic variants different among the samples and these variants are generally associated with technical errors.

3.9.3 Local copy number variation detection

Normalized read-depth per haploid genome (haploid depth d) was defined as a raw depth (d_r), divided by the median depth of its corresponding chromosome (d_{ch}), and then d is defined as d_r/d_{ch} (see 11 and 12). Here depth of each position was measured instead of binned depth used elsewhere in the paper.

3.10 Supplementary material legends

Table S1. Sequencing details from (A) DNAseq and (B) RNAseq. (A) Displays sample name and sequence ID associated with the library preparation method and the use we had of each sample in the study. Depth details for each sample are also mentioned. (B)

Details of each sample used for the transcriptomic analysis including sample and sequence name and their respective mapping efficiency and sequencing depth statistics.

Table S2. Local copy number variation of major amplicons during adaptation of BPK282/0 to different environments. Three loci (H-locus, M-locus and chr6 'Yeti' amplicon) known to vary in copy number in natural populations (12) were analysed. For each amplicon and the different genes present in the respective amplicons, we consider the average copy number per haploid genome. The chr6 'Yeti' amplicon was observed so far only in ISC001 isolates of L. donovani (Highland parasites from Nepal, genetically very different from main population of the lowlands (12)) and never in the lowland parasites: in current study, the locus remained clearly single-copy in all life stages studied. The depth values are based on median haploid depths that are not affected by somy variations (see Supplementary Method 1). In the table, the normalised standard deviations (Norm std) and raw median depth (DEPTH) were given for assessing the quality and valiability of depth of each sample. Library preparation N means Illumina Nextera and this was shown since the depth variation tended to be different from those of other library preparations.

Table S3. SNP frequency variation during adaptation of BPK282/0 to different environments. The table shows the number of reads for DNA seq and RNA seq. The ratio x/y means the reference base count x and the alternative allele count y. Only the SNPs varying in frequency over the course of the experiments, whose accuracy was also verified in IGV, are shown. BPK282 is the reference strain from which we created the reference and Leishmania donovani in the Indian subcontinent is known to have very few heterozygous SNPs (12).

Chapter 4:

In vitro *selection of* Sb^{III} *resistant* **L. donovani** *field isolates from the ISC*

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Molecular pre-adaptation to antimony resistance in *Leishmania donovani* of the Indian sub-continent

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4.1 Abstract:

Antimonials (Sb) were used for decades for chemotherapy of visceral leishmaniasis (VL). Now abandoned in the Indian sub-continent (ISC) because of *Leishmania donovani* resistance, this drug offers a unique model for understanding drug resistance's dynamics. In a previous phylogenomic study, we found two distinct populations of *L. donovani*, the core group (CG) in the Gangetic plains and ISC1 in the Nepalese highlands. Sb-resistance was only encountered within the CG, and a series of potential markers were identified. Here, we analysed the development of resistance to trivalent antimonials (Sb^{III}) upon experimental selection, in ISC1 and CG strains. We observed that (i) baseline Sb^{III}-susceptibility of parasites was higher in ISC1 than in the CG; (ii) time to Sb^{III}-resistance was higher for ISC1 parasites than for CG strains; (iii) untargeted genomic and metabolomic analyses revealed molecular changes along the selection process: these were more numerous in ISC1 than in CG. Altogether these observations led to the hypothesis that CG parasites are pre-adapted to Sb^{III}-resistance. This hypothesis was experimentally confirmed by showing that only wild type CG strains could survive a direct exposure to the maximal concentration of Sb^{III}. The main driver of this pre-adaptation was shown to be MRPA, a gene involved in Sb^{III} sequestration, and amplified in an intra-chromosomal amplicon in all CG strains characterised so far. This amplicon emerged around 1850 in the CG, well before the implementation of antimonials for VL chemotherapy and we discuss here several hypotheses of selective pressure that could have accompanied its emergence.

4.2 Importance:

The "antibiotic resistance crisis" is a major challenge for scientists and medical professionals. This steady rise in drug resistant pathogens also extends to parasitic diseases with antimony being the first anti-*Leishmania* drug that fell in the Indian sub-continent (ISC). Leishmaniasis is a major but neglected infectious disease with limited therapeutic options. Therefore, understanding how parasites became resistant to antimonials is of imperious need. In this study, we experimentally characterised the dynamics of this resistance acquisition and showed for the first time that some *Leishmania* populations of the ISC were pre-adapted to antimony resistance likely driven by environmental factors or by drugs used in the 19th century.

4.3 Introduction

Leishmaniasis is one of the 17 neglected tropical diseases (NTDs) recognised by WHO and is responsible every year for the death of about 30 000 persons in 98 endemic countries where 350 million people are at risk of contracting the disease [1]. In the Indian sub-continent (ISC), leishmaniasis is present in its more lethal form, visceral leishmaniasis (VL). Its etiological agent, *Leishmania donovani* is a dimorphic parasite transmitted by a phlebotomine vector [1,2]. Since 1923, pentavalent antimonials (Sb^{V}) have been used to treat VL in the ISC and in other regions of the world. Sb^{V} is an immune-modulator active on the macrophage [3] and its reduced form, Sb^{III}, is directly toxic for the parasite [4,5]. However, antimonials have lost their efficacy in the ISC towards the end of the 20th century, among others because of drug resistance (DR) [6]. In the ISC, antimonials were then replaced by Miltefosine (MIL), but the efficacy of this drug is also declining [7]: first cases of MIL-resistance were recently reported [8]. A few drugs remain available (Amphotericin B and Paromomycin) and in the expectation of new compounds, it is essential to protect the current arsenal, among others by using new formulations or combination therapy. Moreover, it is important to understand in depth the mechanisms and dynamics of DR.

A recent phylogenomic study of 204 *L. donovani* isolates shed a new light on the emergence and spreading of antimonial-resistant (Sb-R) parasites in the ISC [9]. First, the study described a natural history frame for the VL epidemics: the main population of *L. donovani* emerged about 150 years ago in North-East India and after a succession of bottlenecks and expansions, it generated a series of subpopulations, currently defined as ISC2 to ISC10 and gathered within a main Core Group (CG). In addition, another genetic group (ISC1) which diverged much earlier from the CG, was discovered in the highlands of Nepal. Second, the study highlighted a series of features relevant in the context of Sb-resistance: (i) all 191 isolates (Sb-R or -sensitive) from the CG harboured two intra-chromosomal amplifications of sets of adjacent genes (ICAs), called the M- and H-loci; these genes were not amplified in isolates from the ISC1 subpopulation. The H-locus was reported elsewhere to be a major driver of Sb^{III}-resistance, because of the presence of one gene, MRPA, encoding a member of the multidrug-resistance protein family that sequesters Sb-thiol complexes into intracellular vesicles [10–12]. (ii) Within the CG, a particular genetic group, ISC5, is concentrating most of the strains isolated from antimonial treatment failure patients. All 52 strains of that group harbour a 2-nt insertion in the AQP1 gene, creating a non-functional variant of this transporter known to be involved in the uptake of Sb^{III} [13,14]. (iii) Other genomic adaptations to Sb^{III}, involved in

detoxification mechanisms and increased levels of thiols reported in experimental resistance [15], were not encountered (yet) in the natural population under study.

Considering the anthroponotic nature of VL in the ISC and the high transmission documented in the Gangetic plains, we may assume that the CG represents the parasite population that has been under strong antimonial pressure over decades. The situation might be different in the highlands, where ISC1 was documented. In this region, VL cases were sporadic and *L. donovani* transmission was only reported recently [16]; hence we may hypothesise that antimonial pressure was historically lower in these regions than in the lowlands. In this context, the present study aimed at answering two main questions. First, does the dynamics of drug resistance emergence differ depending on the genetic background, known to be different between ISC1 and CG strains? Second, do we observe similar molecular adaptations when selecting antimonial resistance in the laboratory compared to natural selection in the field, and is the succession of events comparable?

These questions were answered, as a first step, in the context of Sb^{III} exposure. We first measured the intrinsic Sb^{III} susceptibility of promastigotes from 10 *L. donovani* strains representing the genetic diversity in the ISC. We then identified three reference strains among these (one from ISC1 and two from CG) and experimentally selected Sb^{III} resistance at the promastigote stage. We analysed two parameters: (i) the time-to-resistance, i.e. the number of weeks required upon stepwise selection to achieve resistance and (ii) the type of resistance, i.e. the nature and dynamics of molecular adaptations developed by resistant parasites. This was done by untargeted genomic and metabolomic analyses and targeted transcript level analyses. Validation of main drivers of resistance was done by over-expression of candidate genes.

4.4 Results

4.4.1 Intrinsic promastigote Sb^{III} susceptibility of *L. donovani* strains from ISC1 is higher than the core group

We first assessed the intrinsic Sb^{III} susceptibility of promastigotes representative of the genomic diversity encountered in Nepal [9]. For this we chose three cloned strains of ISC1 group and seven strains of the CG (from ISC3, 4, 5, 6 and 9 groups) (Table S1). The *in vitro* susceptibility assay revealed a similar EC50 to potassium antimony tartrate (PAT) among CG promastigotes, with a mean value of 177.05 +/-36.06 $\mu g \cdot ml^{-1}$; this contrasted with the different SSG susceptibility profiles observed for intracellular amastigotes of these strains as shown in previous studies [9]. In contrast, promastigotes from ISC1 were much more susceptible to PAT than promastigotes from the CG, with a mean value of

44.2 +/-7.4 µg.ml^{-1} (Fig.1). The difference observed between CG and ISC1 strains was statistically significant (t-test, *p*-value = 6.7·10^{-11}).

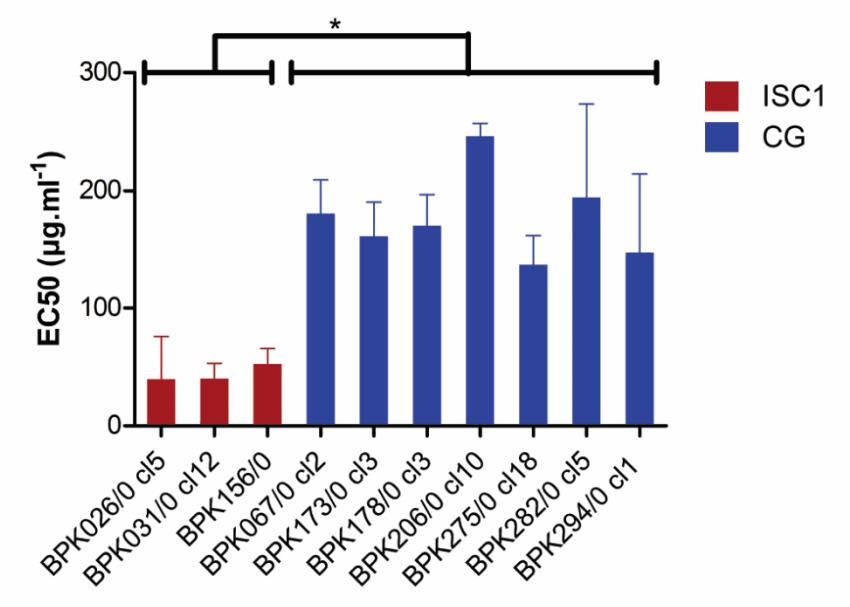

Figure 1: SbIII susceptibility of promastigotes from ISC1 and core group (CG) strains. The three ISC1 strains (red) and the seven strains of CG (blue) are representative of the overall genetic diversity previously described in [9]. Sb^{III} susceptibility for each strain was determined by three independent experiments using potassium antimony tartrate as Sb^{III} source. * p<0,05, unpaired t-test.

Table S1: *L. donovani* strains used. SSG activity indexes were calculated as previously described in [Rijal, S, Yardley, V., *et al.* Microbes and Infection 1-7, 2007], an activity index of 1 corresponds to an EC50 ranging from 7 to 18 µg Sb^{V}·ml^{-1}, while an activity index of 6 corresponds to an EC50 higher than 60 µg Sb^{V}·ml^{-1}. Isolates with an activity index of 1 or 2 are considered sensitive while those showing an activity index of 3 or more are considered resistant.

ISC group	Strain	Treatment outcome	SSG activity index	Used for	H-locus	M-Locus
					Full depth	Full depth
ISC1	MHOM/NP/02/BPK026/0 cl5	SSG definite cure	1	Susceptibility, selection and flash selection	1.59	0.72
	MHOM/NP/02/BPK156/0	SSG definite cure	-	Susceptibility, flash selection	0.97	0.78
	MHOM/NP/02/BPK031/0 cl12	SSG definite cure	1	Susceptibility, flash selection	1.61	0.80
ISC3	MHOM/NP/02/BPK067/0 cl2	-	1	Susceptibility, flash selection	4.30	9.27
ISC4	MHOM/NP/03/BPK206/0 cl10	SSG definite cure	1	Susceptibility, flash selection	2.84	9.10
ISC5	MHOM/NP/03/BPK275/0 cl18	SSG non response	>6	Susceptibility, selection and flash selection	3.85	14.36
	MHOM/NP/02/BPK173/0 cl3	SSG non response	>6	Susceptibility, flash selection	4.50	15.23
ISC6	MHOM/NP/03/BPK282/0 cl4	SSG definite cure	1	Susceptibility, selection and flash selection	4.24	12.78
	MHOM/NP/02/BPK178/0 cl3	SSG definite cure	2	Susceptibility, flash selection	3.96	12.21
ISC9	MHOM/NP/03/BPK294/0 cl1	SSG definite cure	1	Susceptibility, flash selection	4.41	10.53
				Average ISC1	1.39	0.77
				std	0.30	0.03
				Average CG	4.01	11.93
				std	0.52	2.22

4.4.2 Stepwise selection of Sb^{III} resistance in promastigotes

We aimed to compare the dynamics of Sb^{III}-resistance selection in three *L. donovani* strains differing in (i) intrinsic promastigote Sb^{III} susceptibility (BPK026: EC50 = 14.21 $\mu g \cdot ml^{-1}$, ISC1; BPK282: EC50 = 146.9 $\mu g \cdot ml^{-1}$, CG/ISC6 and BPK275: EC50 = 110.4 $\mu g \cdot ml^{-1}$, CG/ISC5), (ii) intracellular amastigote Sb^{V} susceptibility (sensitive, BPK026/282 vs resistant, BPK275) and (iii) genomic background (Table S1). The selection process was performed in quadruplicates (each replicate called line A, B, C or D), starting with 87.5 $\mu g \cdot ml^{-1}$ of PAT for both CG strains, a concentration close to their EC50. For BPK026 the selection process was initiated with 10.93 $\mu g.ml^{-1}$ of PAT, as its EC50 was much lower. The two CG strains showed similar selection dynamics with the four replicates of each strain successfully reaching the highest PAT concentration (700 $\mu g \cdot ml^{-1}$) within four rounds of selection (Fig. 2A). These lines will be further called BPK282 Sb^{III}-R and BPK275 Sb^{III}-R. The selection dynamics for BPK026 were rather different: one replicate (line D)

did not survive the first selection round, line A was cleared during the third round of selection (87.5 μg·ml^{-1}) and line C did not survive the sixth round (350 μg·ml^{-1}). Thus, one replicate only (line B) was successfully selected to survive the highest PAT concentration (further called BPK026 Sb^{III}-R) (Fig. 2B). Time-to-resistance was defined as the time needed for each line to display a wild-type growth curve in presence of 700 μg·ml^{-1} of PAT, following the stepwise selection process. Accordingly, time-to-resistance was estimated to 20 weeks for the two CG strains whereas 35 weeks were required for BPK026 line B to reach the same level (Fig. 2C). In order to evaluate the stability of the resistance phenotype, all the Sb^{III} resistant lines as well as their parental strains were maintained for 20 weeks without PAT. The withdrawal of the drug pressure did not impact their growth rate nor their Sb^{III} susceptibility (EC50>700 μg·ml^{-1}) (data not shown).

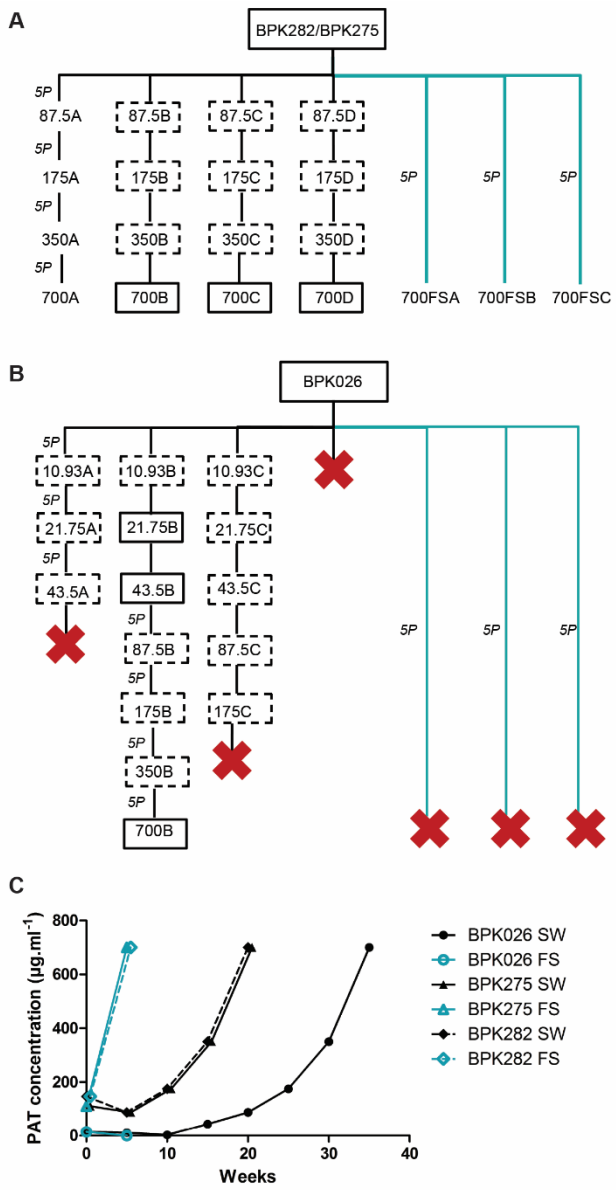

Figure 2: Dynamics of Sb^{III} resistance selection. (A, B) Flowchart of Sb^{III} resistance selection for CG strains BPK282 and BPK275 **(A)** and ISC1 BPK026 **(B)**. Boxes indicate the Sb^{III} increasing concentrations (10.93 to 700 µg·ml^{-1}), black lines are for the step-wise selection (SW) and blue lines for the flash selection (FS) of the different replicates (populations A to D). Red crosses show replicates that did not survive the selection pressure. Full black boxes: samples analysed at genomic and metabolomics levels; dashed-line boxes: samples analysed at genomic level only; no box: samples not analysed. **(C)** Time to resistance.

To fully characterise the antimony resistance profile of the selected lines, we assessed the Sb^{V} susceptibility of intracellular amastigotes by applying SSG pressure on infected macrophages. Analysis of parental lines confirmed previous results: (i) intracellular amastigotes of both BPK026 and BPK282 clearly showed a dose-response curve typical of Sb^{V}-sensitive strains with EC50s of 10.34 and 6.70 $\mu g \cdot ml^{-1}$ respectively (Fig. S1); (ii) in contrast, for BPK275, similar infection indices were observed from 0 to 60 $\mu g \cdot ml^{-1}$, characteristic of Sb^{V}-resistant amastigotes. After Sb^{III} resistance selection of promastigotes, BPK275 Sb^{III}-R amastigotes remained resistant to Sb^{V}; BPK026 Sb^{III}-R and BPK282 Sb^{III}-R both showed more than 6-fold increased EC50 to Sb^{V} (respectively 67.38 and 42.07 $\mu g \cdot ml^{-1}$) (Fig. S1).

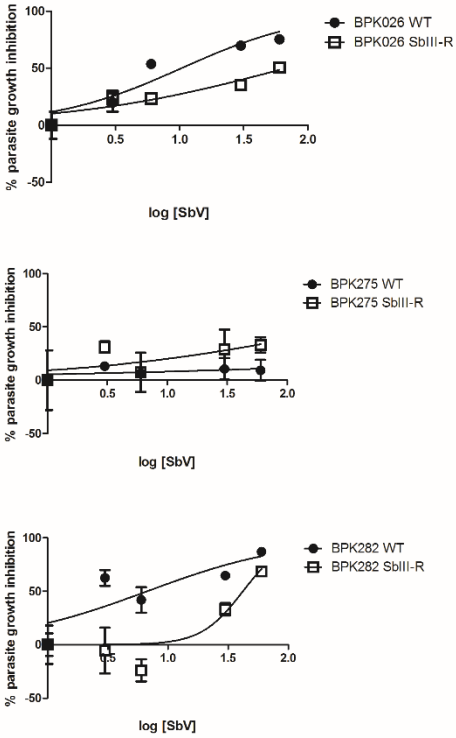

Figure S1: Dose response curve of Sb^{V} against WT and Sb^{III}-R intracellular amastigotes of BPK026, BPK275 and BPK282. Data are means of four replicates.

4.4.3 Untargeted genomic analysis during stepwise Sb^{III} resistance selection

For all parasite lines, samples were collected at each selection round for whole genome sequence analysis of nuclear DNA (see Fig. 2A and B for details). We first looked at the DNA sequence itself to detect single nucleotide polymorphisms (SNPs) and small nucleotide insertions or deletions (INDELs). The average coverage of all the analysed samples was 13.5x ±3.9 ranging from 7.4 to 24.9x and >98% of the genome was covered. All along the selection process, the two CG strains did not show any de novo SNP or

INDEL in the whole nuclear genome; among 303 heterozygous sites in the CG strains, no significant changes in allele frequency were detected. In BPK026 Sb^{III}-R line B, we did not observe any new INDEL during the selection process, but in two different genes, a heterozygous SNP appeared at round four, under a PAT pressure of 87.5 $\mu g \cdot ml^{-1}$. These two de novo SNPs respectively introduced a stop codon at position 1699 of LdBPK_210017400 (formerly LdBPK_211220.1) coding for an intraflagellar transport protein and a non-synonymous mutation H1104T in LdBPK_120011000 (formerly LdBPK_120530) coding for a fusaric acid resistance protein-like. In subsequent selection rounds, the frequency of the mutated alleles progressively increased together with the PAT concentration (Fig. 3A), from 0.067 to 0.667 (LdBPK_210017400,) and from 0.5 to 1 (LdBPK_120011000). Among the 11,189 heterozygous sites of BPK026, no change in allele frequency was observed during the selection process.

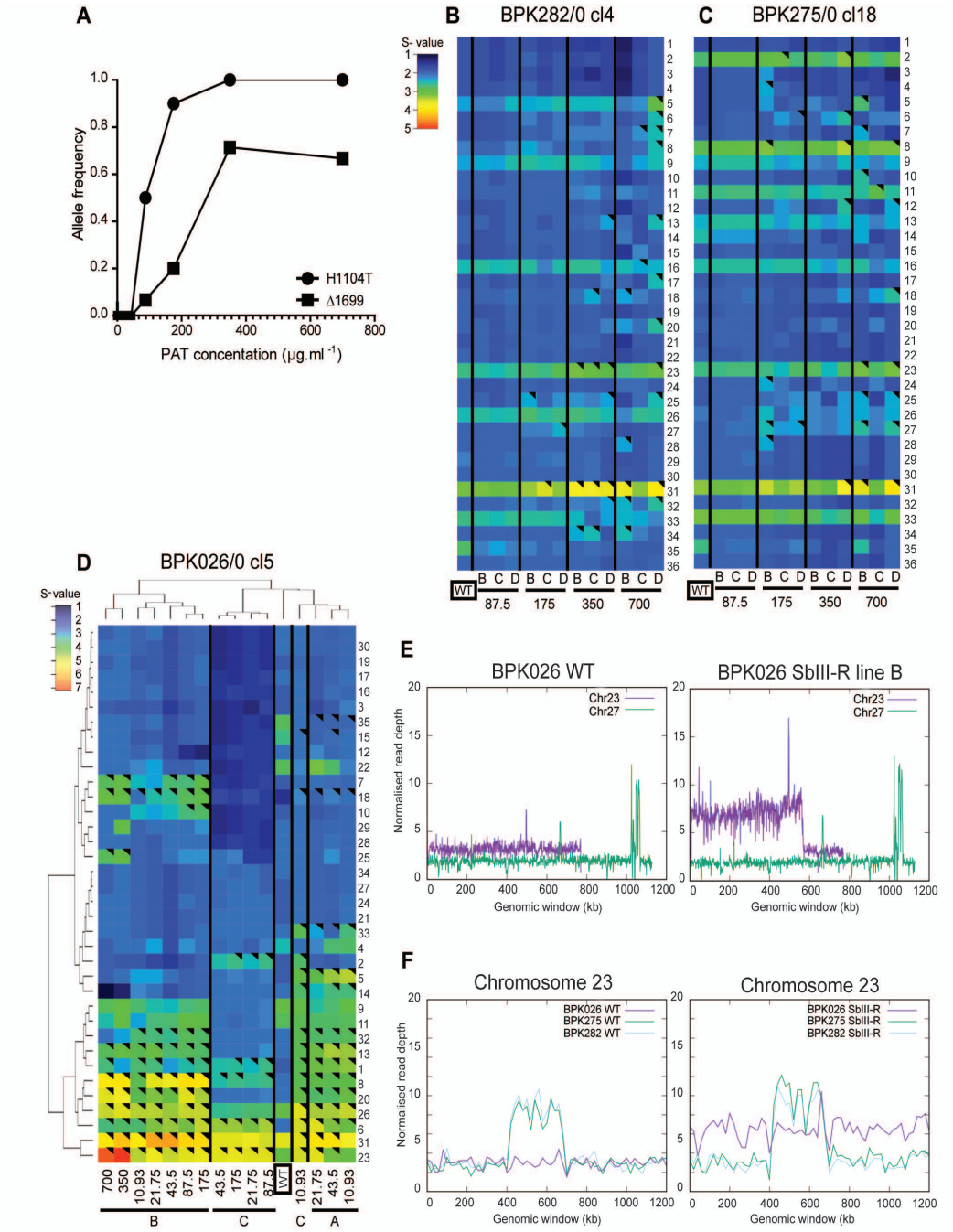

Figure 3: Genomic analysis of the selected Sb^{III} resistant strains. (A) Evolution of allele frequency over the Sb^{III} resistance selection process of BPK026 for a fusaric acid resistance protein-like (LdBPK_120011000 - H1104T) and the intraflagellar transport protein (LdBPK_210017400 – Δ1699). **(B-C)** Heat map representing the karyotype dynamics across the Sb^{III} resistance selection of BPK282 and BPK275. **(D)** High hierarchical

representation of BPK026 karyotype evolution. Heatmaps show median normalized read depths of chromosomes found within each cell population for each of the 36 chromosomes (y-axis) and each sample (x-axis), replicate populations called A, B, C or D are shown for each selecting Sb^{III} concentration (10.93 to 700 $\mu g \cdot ml^{-1}$), the samples "WT" framed in a black box represent the parental Sb^{III} sensitive population for each strain. The color key for panels B and C shows the normalised chromosome read depth (S). S ranges are as follows: monosomy, S<1.5 (dark blue); disomy, 1.5≤S<2.5 (light blue); trisomy, 2.5 ≤ S < 3.5 (green); tetrasomy, 3.5 ≤ S < 4.5 (orange); pentasomy, 4.5 ≤ S < 5.5 (red). In panel D, the range is the following: monosomy, S<1.5 (dark blue); disomy, 1.5≤S<2.5 (blue); trisomy, 2.5 ≤ S < 3.5 (light blue); tetrasomy, 3.5 ≤ S < 4.5 (green); pentasomy, 4.5 ≤ S < 5.5 (yellow) hexasomic, 5.5 ≤ S < 6.5 (orange), heptasomic 6.5 ≤ S < 7.5 (red). A black triangle in an upper right corner indicates a significant change of S- value (>0.5, with a shift from one S range to another and a *P* value of ≤ 10^{-5}) in comparison to the S value of the starting population (sample '0' framed in a black box). **(E)** The left panel shows the normalized read depth of chromosome 23 (trisomic) compared to the normalized read depth of chromosome 27 (disomic) in the parental BPK026 (BPK026 WT) and the right panel shows the normalized read depth for these two chromosomes in BPK026 Sb^{III}-R. **(F)** Normalized read depth of the fragment of chromosome 23 harboring the H- locus. The left panel displays the parental strains (BPK026 WT, BPK275 WT and BPK282 WT) while the right panel shows the Sb^{III}-R line B for the three isolates.

In a second stage, we looked at genome structure changes, more specifically somy and local copy number variations (CNVs), both known to occur when *Leishmania* is under selective pressure. The karyotype variability contrasted sharply with the conservation of the nucleotidic sequence itself reported above. However, this variability was much higher in BPK026 than in the two CG strains, with 153 significant somy changes among all lines of BPK026 vs 31 and 28 significant changes in BPK282 and BPK275, respectively (Fig. 3B, C and D). Interestingly, at each round of selection, karyotypes of distinct replicate lines were rather similar for the CG strains, while different 'scenarios' of aneuploidy were followed by the three replicate lines of BPK026. Five chromosomes (8, 18, 23, 25 and 31) showed an S-value increase in one or more lines of each strain, the most extensive ones being observed for chromosomes 23 and 31 (Fig. 3 and Data Set 1).
Interestingly, the S-value increase of chromosome 23 in BPK026 Sb^{III}-R was due to the amplification of a large segment of this chromosome (from position 10,048 to 567,768, Fig. 3E): at 700 $\mu g.ml^{-1}$, this fragment showed a copy number of 7.1 ± 1.3, while the small segment (from 569,158 to 768,383) showed a copy number similar to the WT (3.0 ± 0.4). This large sub-chromosomal amplification resulted in the specific amplification of 140

ORFs of which 52.85% are putatively annotated and 47.15% correspond to hypothetical proteins. Interestingly, among these genes, 6 (3,8%) were coding for proteins involved in redox pathways, while none of these were found in the small fragment. In addition, several genes of the large fragment have been previously reported to be involved in (i) drug resistance: LdBPK_230007200 & 7300, previously LdBPK_230230.1, pentamidine-resistance protein; LdBPK_230007700 & 7800, previously LdBPK_230280 and 290, respectively terbinafine resistance locus and MRPA within the H-locus, LdBPK_230009800, previously LdBPK_230500, trypanothione synthetase and (ii) virulence: LdBPK_230018500 & 18600, hydrophilic acylated surface proteins a and b, previously not assembled, and LdBPK_230020400, previously LdBPK_231430, membrane-bound acid phosphatase 2. All other protein categories analysed by gene ontology were represented in comparable proportions in both fragments (Data set 2 A and B).

Local CNVs like H- and M- loci ICAs, that are constitutive of the CG [9], did not vary in copy number in the CG lines, they also did not appear as ICA or ECAs in BPK026 Sb^{III}-R (Fig. 3F). Altogether, the untargeted genomic analysis revealed that along the selection process, BPK026 developed more molecular adaptations than the two CG strains, including amplification of genes known to be involved in drug resistance and virulence.

4.4.4 Targeted transcript level analysis of Sb^{III}-resistant selected lines and validation of main drivers

Given the general amplification of the H- and M-loci in the CG strains [9,17] as well as the observed changes in chromosome 23 during the Sb^{III} selection process, we performed quantitative RT-PCR assays to compare the expression levels of genes present in the H- and M-loci in the parental and Sb^{III}-resistant lines here generated. With respect to M-locus, we observed a higher expression of the two tested genes (Hyp36 and MPK1) in parental (WT) CG lines vs BPK026, that reflected the intra-chromosomal amplification (ICA) of the M-locus; however, Sb^{III} resistance in both BPK026 and the CG lines was not accompanied by a further increase of the expression of these genes (Fig. 4A). The H-locus was the most interesting target; as for the M-locus, levels of MRPA, HTBF, Hyp23 and ASS transcripts were slightly higher in WT-CG lines than in WT-BPK026 (1.5 to 3.0 fold increase) which also reflected the ICA of the H-locus in CG lines. After selection of Sb^{III} resistance, there were no significant changes of the expression of these genes within the CG lines, but in BPK026, the level of the four genes increased 1.5 to 3.5 fold, reaching the expression levels of the CG lines (Fig. 4A). This fitted with the increased copy number observed for the large fragment of chromosome 23 (harbouring among others the H-locus) mentioned above.

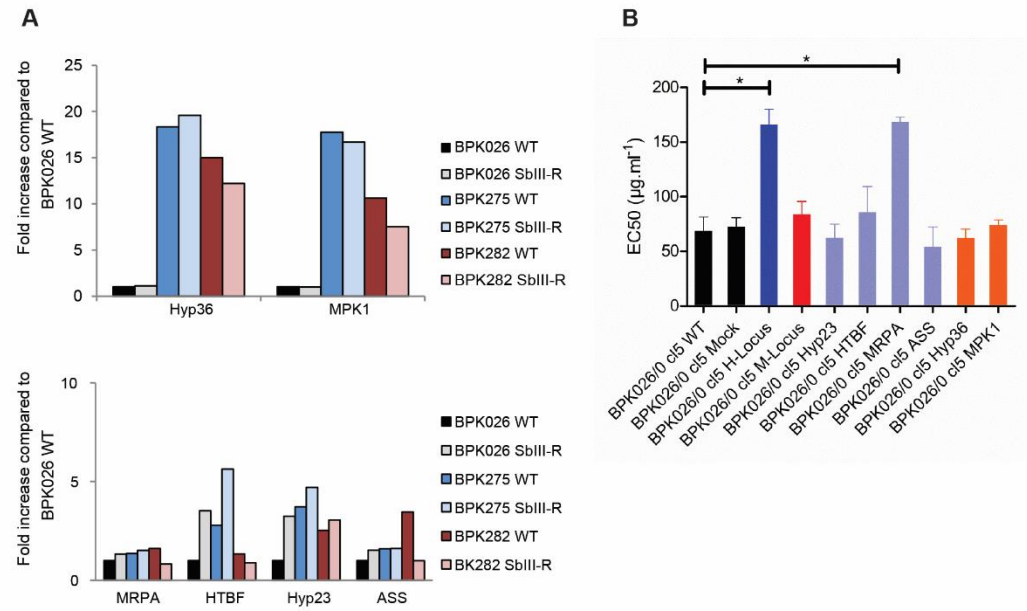

Figure 4: Importance of H- and M-loci for Sb^{III} resistance. (A) Expression levels of genes of the H- and M-loci in parental lines (BPK026 WT; BPK282 WT; BPK275 WT) and one respective Sb^{III}-resistant population (BPK026 Sb^{III}-R pop B; BPK282 Sb^{III}-R pop B; BPK275 Sb^{III}-R pop B) were measured by qRT-PCR. Data were first normalised to the stable SAT and LdBPK_240021200 genes then rescaled to the levels of BPK026 WT. Data are mean of three technical replicates. **(B)** Sb^{III} susceptibility of BPK026 WT and BPK026 WT transfected with the H- or M-loci or with the individual genes of these loci. Data are means of three independent experiments, * $p < 0{,}05$ unpaired *t*-test.

In order to confirm the importance of the H-locus and its amplification for Sb^{III} resistance (through ICA or partial increase of chromosome 23's genomic material), the full H-locus was over-expressed in BPK026 promastigotes using a cosmid carrying that region; similar over-expression of the M-locus was done as control. This experiment revealed that over-expression of the H-locus was associated with an increase of Sb^{III} tolerance of about 3-fold (p-value $6.8 \cdot 10^{-3}$), in contrast to over-expression of the M-locus that did not impact drug susceptibility. We further dissected the importance of the different genes constituting both H- and M-loci, by over-expressing them separately in BPK026. For one gene only, MRPA, over-expression was associated with an increase in Sb^{III} tolerance (3-fold, p-value $8.7 \cdot 10^{-3}$) (Fig. 4B).

4.4.5 Untargeted metabolomics analysis of Sb^{III}-resistant selected lines

An untargeted metabolomics analysis was performed on the 3 parental lines, their respective fully resistant lines (700 $\mu g \cdot ml^{-1}$) and several intermediate resistant lines (21.75 and 43.5 $\mu g \cdot ml^{-1}$) of BPK026 line B. In total, we detected 300 metabolites (Data set 3A). Principal Component Analysis (PCA) was carried out as a first exploratory analysis of this metabolomics dataset (Fig. 5A). The two first principal components (PC1 and 2) explained 33.02% and 16.03% of the total variation and clearly separated two experimental sets: (i) BPK026-parental and resistant line and (ii) BPK275-parental and resistant lines B,C and D together with BPK282-parental and resistant lines B,C and D. For BPK026, the two intermediate resistant populations clustered at an intermediate position between the parental strain and the fully resistant line. PCA results were confirmed by analysis of individual metabolites. The number of metabolites significantly differing between parental and the fully resistant lines ranged as following: 52 (BPK026), 24 (BPK282) and 17 (BPK275) highlighting the higher amount of changes occurring in BPK026 Sb^{III}-R strain (Fig. 5B, Table 1). A comparison of all the resistant lines showed the metabolic similarity of the CG strains BPK282 and BPK275 (Fig. 5A and B, Data set 3A, C). This general trend was confirmed when analysing specific classes of metabolites; amongst them two classes represented significant common changes during all the comparisons we performed: glycerophospholipids (GPL) and amino acids.

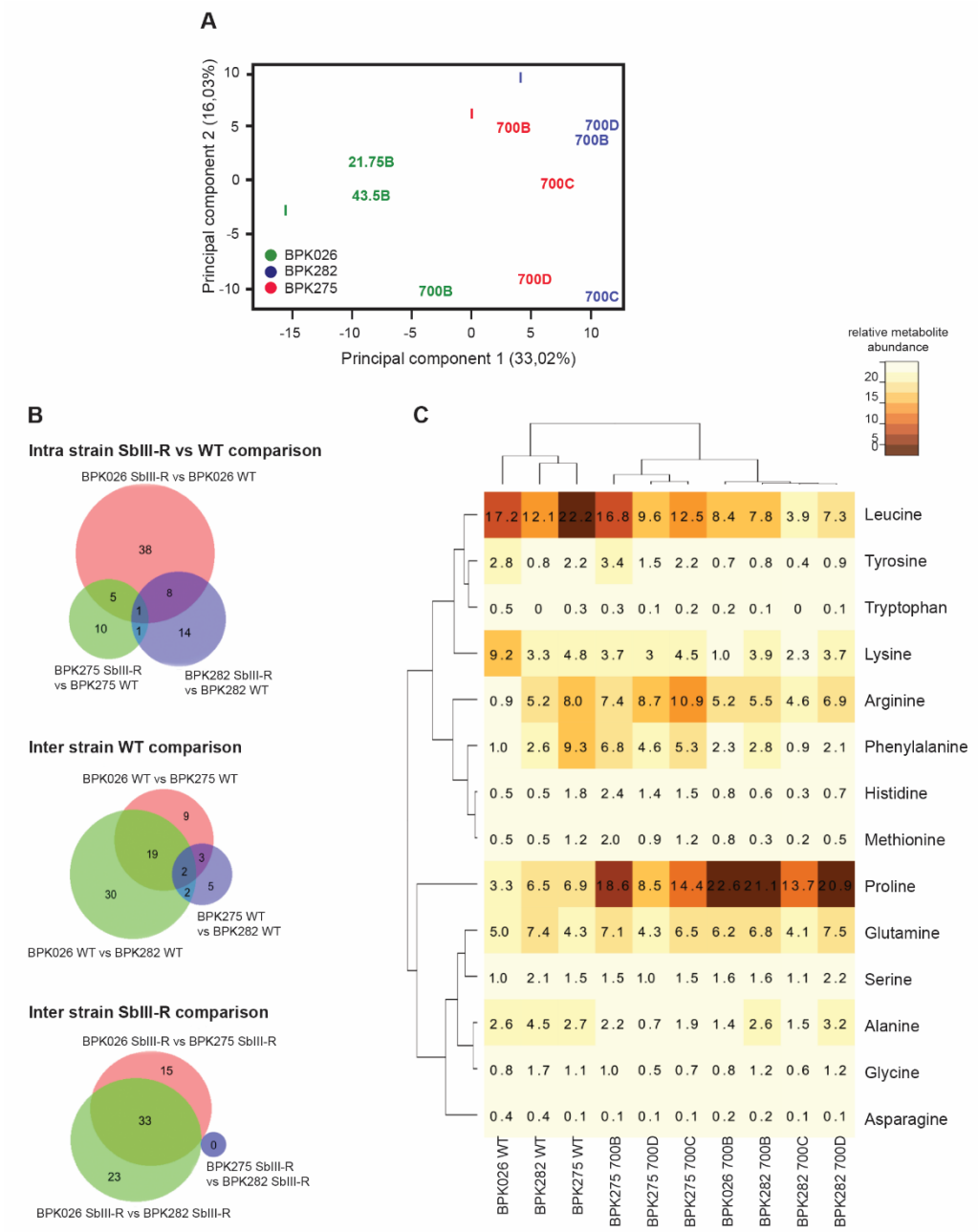

Figure 5: **Metabolomics analysis of the selected Sb^{III} resistant strains. (A)** principal component analysis based on 300 metabolites and including the initial parental strains (I) BPK026 -green, BPK275 -red and BPK282 –blue, the final populations resistant to 700

μg.ml^{-1} of Sb^{III} (BPK026 700B; BPK275 700B, C and D; and BPK282 700B, C and D) as well as BPK026 intermediates (21.75B and 43.5B respectively resistant to 21.75 and 43.5 μg·ml^{-1} of Sb^{III}). **(B)** Venn diagrams showing metabolites significantly differing between strains. **(C)** High hierarchical representation of amino acids abundance in the different strains (the parental strains BPK026 WT, BPK282 WT, BPK275 WT and the selected populations resistant to 700 μg·ml^{-1} of Sb^{III} (BPK275 700B, C and D; BPK026 700B; BPK282 700B, C and D).

Table 1: Differential metabolite abundance between Sb^{III}-R and parental lines of each of the three strains here considered. Only the biologically (2 fold changes) and statistically significant ($p < 0.05$) differences are indicated.

Classes of metabolites	BPK026 Sb^{III}-R/BPK026 WT	BPK282 Sb^{III}-R/BPK282 WT	BPK275 Sb^{III}-R/BPK275 WT
Increased			
Amino-acids, peptides and amino-acid conjugates	**9**: arginine, proline, isoleucine, glutamylamino-butanoate, N2-succinyl-ornithine, glutamylcysteine, prolyl-proline, phenylalanine, glutamylalanine	**1**: Proline	**2** : Acetyl-lysine, prolylhydroxyproline
GPLs	**7**: 1 saturated or lowly unsaturated, 6 highly unsaturated	**3**: 1 saturated or lowly unsaturated, 2 highly unsaturated	-
Thiols	**1**: glutathione	-	-
Nucleobases and nucleosides	-	**1**: xanthine	-

Others	**23**: Flumipropyn; magnoshinin; clobenpropit; PC(20:4(5Z,8Z,11Z,14Z)/18:4(6Z,9Z,12Z,15Z)); 2'-Hydroxyfurano[2",3":4',3']chalcone; coenzyme M 7-mercaptoheptanoylthreonine-phosphate heterodisulfide; PC(22:5(4Z,7Z,10Z,13Z,16Z)/22:5(4Z,7Z,10Z,13Z,16Z)); 5-Hydroxy-6,7,3',4',5'-pentamethoxyflavanone 5-O-rhamnoside; N4-Phosphoagmatine; (S)-AMPA; 5-L-Glutamylglycine; Uplandicine; 1-Pyrroline; Piperideine; 4-Aminopyridine; Histamine; y-Glu-Thr; carnitine; dihydro-4,4-dimethyl-2,3-Furandione; hexitol; 3 4 5-trihydroxy-hexanoicacid, rhamnose, deoxygalactopyranose, fucose 1 5-Anhydrosorbitol; methylglutaric acid, adipic acid, solerol, 2 2-dimethylsuccinic acid, aceto-hydroxybutanoicacid; xylitol, arabitol; 4-Guanidinobutanal	**2**: 5-Hydroxy-6,7,3',4',5'-pentamethoxyflavanone 5-O-rhamnoside; 1-Pyrroline	**2**: 7-oxo-8-amino-nonanoicacid 8-amino-7-oxo-nonanoicacid; 2-ethyl-2-Hydroxybutyric acid, 2-hydroxy-3-methylpentanoic acid, hydroxy(iso)caproic acid, X-hydroxy-hexanoic acid
Total	**40**	**7**	**4**
Decreased			
Amino-acids, peptides and amino-acid conjugates	**2**: Proline-betaine; trimethyl-lysine	**4**: glutamylalanine; succinyldiaminopimelate, N2-Succinyl-L-arginine; X-amino-pentanoic acid, valine	**3**: isoleucine, glutamylcysteine, argininosuccinate
GPLs	**5**: 4 saturated or lowly unsaturated, 1 highly unsaturated	-	**1**: phosphorylethanolamine
Thiols	-	**1**: trypanothione-disulfide	-

Nucleobases and nucleosides	-	-	**1**: IMP
Others	**5**: Creatine; cyclohexane-1,3-dione; Spisulosine, 1-deoxy-sphinganine; 2-keto-3-methyl-valerate, 2K-4CH3-pentoate, X-oxo-X-methyl-pentanoicacid, 2-ketoisocaproate, X-oxo-hexanoicacid; S-adenosylmethionine	**12**: Allantoin; 2-ethyl-2-Hydroxybutyric acid, 2-hydroxy-3-methylpentanoic acid, hydroxy(iso)caproic acid, X-hydroxy-hexanoic acid; N-Acetylglutamine; y-Asp-Asp-Pro; 5-L-Glutamylglycine; clobenpropit; guvacine; (S)-AMPA; cholinephosphate; fagomine; dehydroalanine; SP(34:0)	**8**: Flumipropyn; glucolepidiin; 5-Hydroxy-6,7,3',4',5'-pentamethoxyflavanone 5-O-rhamnoside; y-Lys-Val; PC(22:5(4Z,7Z,10Z,13Z,16Z)/22:5(4Z,7Z,10Z,13Z,16Z)); PC(22:5(4Z,7Z,10Z,13Z,16Z)/20:2(11Z,14Z)); 1-eicosanoyl-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-sn-glycero-3-phosphocholine; coenzyme M 7-mercaptoheptanoylthreonine-phosphate heterodisulfide
Total	**12**	**17**	**13**

GPL composition evolved differently over the selection process of the different strains: we observed 13 changes between BPK026 WT and BPK026 Sb^{III}-R, while only four changes were detected in BPK282 Sb^{III}-R compared to BPK282-WT and lipid composition was unchanged between all BPK275 lines. When GPL composition of BPK026 WT was compared to the two WT strains of CG taken together, we observed lower amounts of 10 GPL in the former (three unsaturated/lowly unsaturated and seven highly unsaturated) and three were more abundant (lowly unsaturated). When doing the same comparison between resistant lines of the three strains, no difference was observed (Table 2). This indicated that upon Sb^{III} resistance selection of BPK026, the level of 13 GPL evolved towards levels encountered in CG lines.

Table 2: Changes in glycerophospholipid abundance.

[a] BPK026 Sb^{III}-R population B

[b] BPK282 and BPK275 Sb^{III}-R are the average values of the three resistant replicates (populations B, C and D)

[c] CG Sb^{III}-R is the average of BPK282 and BPK275 Sb^{III}-R replicates.

	BPK026 Sb^{III}-R[a] vs BPK026 WT		**BPK282 Sb^{III}-R[b] vs BPK282 WT**		**BPK275 Sb^{III}-R[b] vs BPK275 WT**		**BPK026 WT vs BPK282 WT**		**BPK026 WT vs BPK275 WT**		**BPK026 Sb^{III}-R vs CG Sb^{III}-R[c]**	
	Up (Log2>1)	Down (Log2 <1)	Up (Log2 >1)	Down (Log2 <1)	Up (Log2>1)	Down (Log2 <1)	Up (Log2 >1)	Down (Log2 <1)	Up (Log2 >1)	Down (Log2 <1)	Up (Log2 >1)	Down (Log2 <1)
Saturated and low unsatured GPL (≤3)	0	1	1	0	0	0	0	3	0	0	0	0
High unsaturated GPLs (>3)	8	4	3	0	0	0	0	4	3	3	0	0

Amino acids were also significantly altered in the current experimental set. Again, more amino acids changed during Sb^{III}-resistance selection of BPK026 (4) than in BPK282 (2) and BPK275 (2). These four amino acids deserved a particular attention due to their variation in abundance as well as their biological importance. Two amino-acids varied in all resistant lines vs their WT parental strain: (i) proline abundance increased 6.76 fold in BPK026 Sb^{III}-R, 2.8 fold in average in BPK282 Sb^{III}-R lines and 2 fold in average in BPK275 Sb^{III}-R lines; (ii) leucine abundance decreased 2 fold in BPK026 Sb^{III}-R, 1.9 fold in BPK282 Sb^{III}-R lines and 1.7 fold in BPK275 lines. Arginine was less

WT compared to the CG strains (5.7 fold decrease compared to BPK282 WT and 8.8 fold decrease compared to BPK275 WT); interestingly, the levels of arginine did not change significantly during the Sb^{III} selection of CG lines, but it increased 5.7 fold in BPK026 Sb^{III}-R vs the WT parental strain (Fig. 5C, Data set 3A), thus reaching the levels encountered in CG lines. Finally, lysine, was more abundant in BPK026 WT than in CG WT strains and dropped drastically (9.2 fold) in BPK026 Sb^{III}-R, while levels did not change in CG resistant lines.

4.4.6 Adaptation of *L. donovani* strains to a high concentration of Sb^{III}

The previous results suggested that promastigotes of CG strains are better prepared than BPK026 to Sb^{III} exposure. To test this pre-adaptation hypothesis in extreme conditions, we assessed the possibility for the parasites to survive the highest concentration of Sb^{III} without the stepwise selection previously undertaken. The 10 strains described above and representing ISC1 (BPK026, BPK156 and BPK031) and CG (BPK067, BPK206, BPK275, BPK173, BPK282, BPK178, BPK294) (Fig. 1 and Table S1) were used for a 'flash selection', meaning direct exposure of a wild-type strain to 700 $\mu g \cdot ml^{-1}$ of PAT during five weeks, passaging the parasites every seven days and performing a growth curve for five weeks. Interestingly we observed from the first day a drastic reduction of parasite numbers for all strains and already a complete loss of BPK031 (ISC1). The two other ISC1 strains suffered of a drastic decrease but living parasites could still be observed after seven days in at least one replicate. After five weeks, no living parasites were detected in any of the three ISC1 strains. In sharp contrast, all seven CG strains recovered their original growth rate after four passages only (Fig. 2A, 2C and Fig. 6).

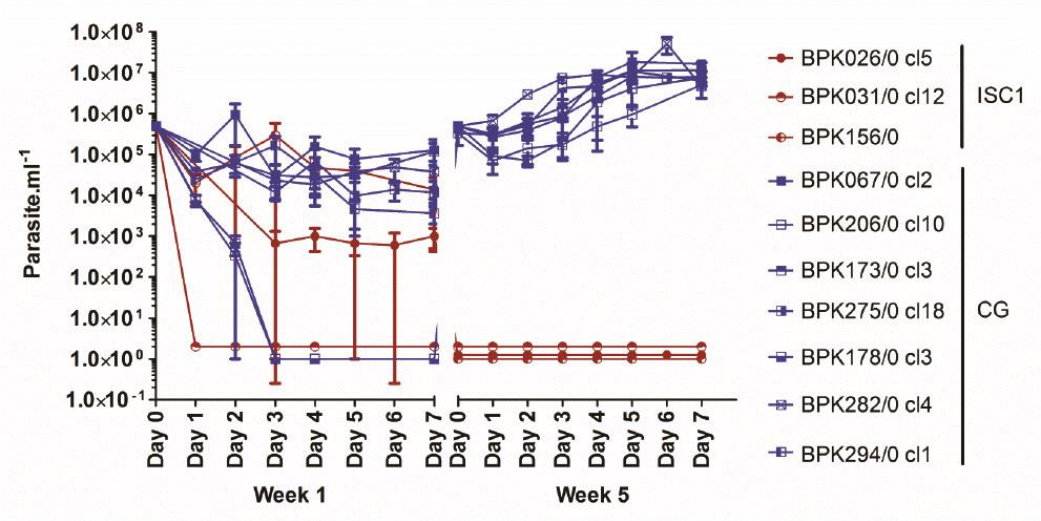

Figure 6: Flash selection with 700 µg.ml^{-1} of PAT on ISC1 and CG strains. Growth curves over five weeks of three ISC1 strains (red) and seven CG strains (blue) in presence of 700 µg·ml^{-1} of PAT . Each point represents the average value of promastigote counts in three independent flasks.

4.5 Discussion

The objective of our study was to analyse experimentally the dynamics of Sb^{III} resistance emergence in ISC strains from different genetic backgrounds, to characterise molecular adaptations developed by resistant lines and to compare them to the adaptations encountered in a clinical context. Our study was performed *in vitro*, with promastigotes and the reduced form of antimony, Sb^{III}. As such, it does not a priori take into account resistance mechanisms related to the immune-modulatory effect of Sb^{V} neither the reduction of Sb^{V} to Sb^{III}, but mechanisms related to transport and detoxification of Sb^{III}. This study therefore constitutes a first step in the analysis of *Leishmania* adaptations to antimonials but should be complemented in the future by similar work with amastigotes and Sb^{V}.

We showed here that (i) Sb^{III} susceptibility of promastigotes was higher in ISC1 than in the CG (Fig. 1 and Table S1); (ii) following a classical drug resistance selection scheme, time to Sb^{III} resistance (Fig. 2C) was higher for ISC1 parasites (35 weeks) than for CG strains (20 weeks); (iii) untargeted genomic and metabolomic analyses revealed that molecular changes associated with the acquisition of Sb^{III} resistance were more numerous in the ISC1 strain compared to the CG's; (iv) during selection, genomic changes were

very similar in the replicates of each CG strain, while very different scenarios were observed among replicates of ISC1, one of them only leading to full resistance. Altogether these observations led to the hypothesis that CG promastigotes are pre-adapted to Sb^{III} resistance. This hypothesis was experimentally tested by a "flash selection", i.e. a direct exposure of WT parasites to the maximal concentration of Sb^{III} (Fig. 6 and Table S1). The fact that none of the ISC1 strains survived the flash selection while all the CG strains could recover a growth comparable to the WT after five weeks confirmed the pre-adaption hypothesis.

Interestingly, based on our phylogenomic study, we estimated the emergence of the Indo-Nepalese populations of the CG around 1900 [9]. This corresponds more or less to the first use of Sb^{III} in clinical practice (1919), before being replaced by Sb^{V} in 1923, because of toxicity [9]. In the anthroponotic context of *L. donovani* transmission in the ISC, it is possible that these four years of application of Sb^{III} provided the selective pressure to the Indo-Nepalese ancestors of the CG. However, there are two alternative explanations. On one hand, the wide-spread contamination of drinking water by arsenic, shown to occur in the ISC [18], could have provided a strong selective pressure. Arsenic and antimonials share the same sequestration mechanism via MRPA over-expression [12]. Furthermore it was demonstrated that *L. donovani* could develop resistance to Pentostam after exposure to arsenic [19]. On the other hand, considering (i) the importance of the H-locus amplification in the pre-adaptation here reported, (ii) the occurrence of this ICA throughout the whole CG (India, Nepal and Bangladesh) and (iii) the estimated emergence of the whole CG around 1850, we may conclude that this ICA was already present in 1850, at the onset of the first reported epidemics of kala-azar. Noteworthy, *Leishmania* was only discovered half a century later, and for decades, this epidemic disease of the ISC was considered as "quinine-resistant malaria" [25]. In other words, *L. donovani* was for decades under pressure of other drugs, such as quinine and this could also have played a role in the pre-adaptation.

The untargeted genomic approach here used revealed the importance of genome structure variation for parasite adaptation to drug pressure. We did not identify any SNP/indel (BPK282 and BPK275) or very few (BPK026), in contrast to significant chromosome amplifications, total or partial, and occurring rather early during the selection process (Fig. 3B, C and D). This fits perfectly with the findings of Shaw *et al.* [20], who showed that during experimental selection of Miltefosine resistance, aneuploidy was the first adaptation of the parasite, before the appearance and fixation of SNPs. Similar results were encountered in the context of Sb^{III} resistance in *L. major*, *L. guyanensis* and *L. amazonensis* [11,14,21]. Karyotype modulation has been described as a

powerful strategy of gene dosage regulation [22] playing a role in environmental stress and in drug resistance [29].

Relatively to the two CG strains, aneuploidy changes were more abundant in BPK026 Sb^{III}-R, as expected for a less/non pre-adapted strain. Among affected chromosomes, two deserved a particular attention, as they were also showing somy changes in one or more CG lines, chromosomes 23 and 31. In BPK026, somy change of chromosome 23 involved one fragment only, containing among others the H-locus, genes involved in drug resistance and virulence (Data set 2A). The same fragment amplification was previously observed during *in vitro* selection of Sb^{III} resistant *Leishmania Viannia guyanensis*, which is known to be unable to generate ICAs or ECAs [11]. Interestingly, the position of the chromosomal breakage corresponded to a strand-switch region and to the localisation of the centromere of chromosome 23 as described recently for *L. major* [26]. In BPK282 and BPK275, somy changes of chromosome 23 were less pronounced, and pre-adaptation likely stems from the already present amplification of the H-locus, under the form of ICA. We experimentally confirmed the link between the amplification of the H-locus and a higher tolerance to Sb^{III} by over-expression and demonstrated that among the genes present in the H-locus, MRPA was the driver (Fig. 4B). The case of chromosome 31 is also interesting, as it contains the gene encoding AQP1, known to be the transporter responsible for the uptake of Sb^{III} [13] and inactive in all parasites of ISC5 because of a 2-nt indel [9]. Such indel was not observed in BPK282 and BPK026 during the Sb^{III} resistance selection, and we did not find any other down-regulation mechanism, at genomic (like the sub-telomeric deletion of AQP1 as reported elsewhere [23]) or transcriptomic levels (qPCR, not shown). Further work is required to understand the meaning of the somy increase of chromosome 31 [29]. Bringing together our experimental and phylogenomic data (presence of H-ICA in all strains of CG), we believe that MRPA is likely the earlier driver of Sb^{III} resistance. Later on, other genomic adaptations likely appear, such as AQP1 inactivation which is present only in ISC5 and did not emerge (yet) under our experimental conditions.

Untargeted metabolomics analyses provided results complementary to the genomic study. They clearly supported the pre-adaptation hypothesis of CG strains and showed that during the Sb^{III} selection process, the metabolism of BPK026 evolved towards levels of CG WT strains. This was best illustrated by GPLs and amino-acids. GPLs play an important role in membrane fluidity as shown earlier [30,31] and thus in drug resistance since it can act on the absorption of the drug. Amino acids were also modulated, notably proline and arginine (Fig. 5C). Proline levels actually increased in all lines following Sb^{III}-resistance selection. This is not unexpected since proline has been shown to enhance cell

survival during environmental stresses, in particular, proline is required for volume recovery during osmotic stress responses [24]. Increased levels of proline have been observed in other Sb^{III}-resistant strains selected *in vitro*, and addition of proline to the culture medium led to increased parasite tolerance to Sb^{III}, probably because of its protective role against drug-induced oxidative stress [31]. Arginine levels, on the other hand, remained unchanged in the Sb^{III}-R CG strains but were increased to CG's level in Sb^{III}-R ISC1, perfectly fitting with the pre-adaptation hypothesis. Arginine is part of the urea cycle, leads to the synthesis of ornithine, polyamines and ultimately the anti-oxidant trypanothione. Increased levels of thiols and of other metabolites linked to thiol production have been associated to a better protection against Sb-induced oxidative stress and were demonstrated in other studies on SSG-R parasites [15, 27, 30]. Lysine levels also supported the pre-adaptation hypothesis, remaining unchanged in WT or Sb^{III}-R CG strains but decreasing to CG levels in Sb^{III}-R ISC1. Further studies are however required to understand the implication of lysine in parasite susceptibility/resistance to Sb^{III}.

In summary, we reported for the first time the pre-adaptation of a whole *Leishmania* population to drug-resistance. The pre-adaptation to Sb^{III} here shown does not mean that all the *L. donovani* parasites of the CG would cause clinical resistance to SSG and many of them did indeed show susceptibility to Sb^{V} ([9] and BPK282 WT in this paper). However, under the adequate pressure, they could rapidly become resistant, which was not the case of ISC1 parasites. We believe that MRPA amplification (thus increased sequestration of Sb^{III}) is a first and important line of defence of the parasites, providing them a grade of tolerance to Sb^{III}. Additional adaptive layers - occurring later on during the evolution of the CG- were likely required for a higher resistance to Sb^{III} (e.g. AQP1 inactivation, leading to decreased uptake of the drug) or Sb^{V} (e.g. the presence of unique glycans driving the subversion of macrophages) and ultimately for full clinical resistance. Our findings highlight the importance of genetic diversity in the development of resistance and the need for a close monitoring of parasites populations before and after implementation of a new drug. Similarly, research and development of new drugs should take into account this concept of pre-adaptation and include a challenge of new compounds to parasites with different pre-adaptation features.

4.6 Material and Methods

4.6.1 Strains and culture conditions

Ten strains representing the diversity of *L. donovani* in the ISC were used in the present study (Table S1 and [9]). Promastigotes were maintained in HOMEM (Gibco) supplemented with 20% (v/v) heat-inactivated foetal calf serum (FCS) at 26°C. Growth curves of the strains were performed by daily manual counting for seven days using Uriglass counting chambers (A. Menarini Diagnostics).

4.6.2 Antimony susceptibility tests

Sb^{III} susceptibility tests were performed using potassium antimony tartrate (PAT) (Sigma Aldrich) as source of Sb^{III}. The EC50 were determined on logarithmic stage promastigotes after a 24h exposure to PAT, with a resazurin assay previously described [28]. Briefly, promastigotes were exposed to a semi-logarithmic concentration range of PAT from 2 to 700 $\mu g \cdot ml^{-1}$. EC50 were calculated with GraphPad Prism using a sigmoidal dose-response model with variable slope.

For the Sb^{V} susceptibility assay, primary peritoneal macrophages from Swiss OF1-mice (Charles River) were infected with day 7 stationary phase promastigotes as previously described [28]. After 24h of infection, infected macrophages were washed and fresh medium containing SSG (Calbiochem) at a concentration range of Sb^{V} between 3 to 60 $\mu g \cdot ml^{-1}$ was added for five days before final wash and Giemsa staining. The number of infected macrophages and the amount of amastigotes per infected macrophage were determined by manual counting. These numbers were used to establish the infection index (% infected macrophages * amastigotes/infected macrophage). Percentage of parasite growth inhibition was calculated as [1-(InfIndx/InfInd0)]*100 where InfInd0 and InfIndx are infection indices of untreated infected cells and cells treated with different SSG concentrations, respectively. EC50 were calculated with GraphPad Prism using a sigmoidal dose-response model with variable slope.

4.6.3 Sb^{III}-resistance selection

For each strain, selection of resistant parasites was initiated in quadruplicates in 200 µl on a 96-well plate. Briefly, in each well, 105 logarithmic phase promastigotes were incubated for seven days with PAT at concentrations ranging from 0.68 $\mu g \cdot ml^{-1}$ to 700 $\mu g \cdot ml^{-1}$ following a two-fold increase. This first step allowed the estimation of the highest concentration tolerated by the parasites at a given time point. Parasites were then transferred into 5 ml culture medium with the selected concentration of PAT and

maintained for five weeks before starting a new selection round. Selection rounds were performed successively with 10.3 $\mu g \cdot ml^{-1}$; 21.75 $\mu g \cdot ml^{-1}$; 43.5 $\mu g \cdot ml^{-1}$; 87.5 $\mu g \cdot ml^{-1}$; 175 $\mu g \cdot ml^{-1}$; 350 $\mu g \cdot ml^{-1}$ and 700 $\mu g \cdot ml^{-1}$ of Sb^{III} for BPK026 and 87.5 $\mu g \cdot ml^{-1}$; 175 $\mu g \cdot ml^{-1}$; 350 $\mu g \cdot ml^{-1}$ and 700 $\mu g \cdot ml^{-1}$ of Sb^{III} for BPK282 and BPK275. A rapid procedure for Sb^{III}-resistance selection (called Sb^{III} flash selection) was also attempted. Wild-type parasites from different genetic groups described in [9] were cultured in parallel in triplicates with and without 700 $\mu g \cdot ml^{-1}$ of PAT (Table S1). Counting was performed every day for 5 weeks as above. Parasites were subcultured at 5.10^5 parasites$\cdot ml^{-1}$ at day 7 when possible. When parasite concentration was too low, cells were pelleted and resuspended in fresh medium at a lower PAT concentration.

4.6.4 DNA preparation for NGS and data analysis

Parasites were pelleted after five weeks of culture with a given concentration of PAT. DNA isolation was done using QIAamp DNA Blood Mini Kit (Qiagen) and DNA concentration was assessed with the Qubit® DNA broad range DNA quantification kit (Thermo Fisher). Sequencing libraries were prepared with the TruSeq DNA library prep kit (Illumina) according to the manufacturer's instructions. The libraries were quantified by qPCR using the KAPA Library Quantification Kit optimised for Roche® LightCycler 480 (KAPA biosystems). The libraries were 2x100 bp paired-end sequenced with the Illumina Hiseq 2000 platform by the Beijing Genomics Institute (BGI) according to standard protocols. Ploidy, single nucleotide polymorphisms (SNPs) and local copy number variations (CNVs) were determined as described in Imamura *et al.* in 2016 [9] using the BPK282v2 PacBio reference genome accessible via ftp://ftp.sanger.ac.uk/pub/project/pathogens/Leishmania/donovani/LdBPKPAC2016beta/. Sequences are available at the European Nucleotide Archive under the following access number PRJEB22849. Gene identity (id.) in the manuscript is given with LdBPK v2 as annotated elsewhere [18]. However, LdBPK v1 gene id., as used at GeneDB (http://www.genedb.org/Homepage) is also mentioned to facilitate comparisons with other studies. Biological and statistical significance of chromosome copy number (S-value) comparison between samples was done according to two criteria as previously described [29]. Briefly, the somy of a given chromosome in a population of cells is known to be a continuous variable with a multimodal distribution of S-values because of aneuploidy mosaicism. Under this model, the S-value of homologous chromosomes is thus considered to be significantly different if (i) S- values differ from more than 0.5 with a somy distribution shift from one mode to another and (ii) p-value is $\leq 10^{-5}$.

4.6.5 Metabolites extraction, LC/MS protocol and data analysis

For each analysed sample metabolites were extracted from four replicates of logarithmic phase promastigotes (three days of culture) using a 1:3:1 (v:v:v) chloroform/methanol/water solvent as previously described [30]. Samples were analysed with an Orbitrap Exactive (Thermo Fisher Scientific) mass spectrometer coupled to a 2.1 mm ZIC-HILIC column (Sequant) at Glasgow Polyomics (University of Glasgow, Glasgow, Scotland) as previously described [31]. All samples were analysed in randomised order and in the same analytical batch. Several quality control samples including solvents blanks, pooled samples, and authentic standard mixes were also measured in the same run to verify LC-MS stability and allow the identification of contaminants [32]. Data analysis was performed in R using the packages XCMS [33] and mzMatch.R [34]. The workflow was described in detail [30]. R was also used to generate heatmaps and PCA plots based on all metabolites in the dataset with measurements across all samples. For each metabolite, we computed log2 fold changes (Log2FC), i.e. the log 2 ratio of average metabolite abundance between two conditions, for comparisons at the level of individual metabolites. The associated p-value was obtained by performing a Student t-test for each comparison subsequently corrected with the Benjamini-Hochberg algorithm to limit the False Discovery Rate (FDR) to 5%. Differences in the amount of metabolites were considered as significant if Log2FC was > 1 or <-1 and the corrected p-value < 0.05.

4.6.6 Single gene over-expressers

Each gene of the H- and M-loci was over-expressed in BPK026/0 cl5 after cloning in pLEXSY-hyg2 (Jena Bioscience) (Table S2). All genes except MRPA were PCR-amplified from BPK282 DNA using Phusion® high-fidelity polymerase (NEB) with overhang primers (Table S2) for subcloning purposes in pGEMT (Promega) before final cloning in pLEXSY-hyg2. MRPA was amplified using iProof™ High-Fidelity DNA Polymerase (Bio-Rad) with GC-buffer plus 10% DMSO and directly cloned in the pLEXSY-hyg2 using In-Fusion® HD Cloning Kit (Clontech). Every PCR-amplified sequence was sequenced at the VIB Genetic Service Facility (VIB Genetic Service Facility – Antwerp, Belgium). Promastigotes of BPK026/0 cl5 were then transfected using GenePulser Xcell (Bio-Rad) as described previously [35]. Transfected parasites were selected with 50 μg·ml-1 of hygromycin (Jena Bioscience) then maintained with 100 $\mu g \cdot ml^{-1}$.

4.6.7 Cosmid isolation and transfection

A cosmid bank of *L. donovani* MHOM/NP/03/BPK190 (ISC4) had already been prepared in the laboratory of Dr. Joachim Clos in Hamburg [36,37]. Cosmids carrying copies of the

H- locus (pcosTL-H-Locus) and the M- locus (pcosTL-M-Locus) were isolated by colony lifting. Briefly, the cosmid bank was plated on LB agar Ampicillin plates and a replica of each plate was taken on a Nylon membrane (Amersham Biosciences). Probes specific for LdBPK_360076800 (MPK1) and LdBPK_230007800 (MRPA) (Table S2) were DIG-labelled by PCR using DIG-dUTP (Roche), and hybridization was performed overnight at 65°C. The membrane was developed using anti-DIG antibody (Roche) associated with alkaline phosphatase and NBT as revealing agent. Positive signal colonies were isolated from the plate and colony PCR was used to confirm the presence of the sequence of interest. The two BPK026/0 lines carrying cosmids were obtained by nucleofection of 10 μg of pcosTL H-Locus or pcosTL M-Locus DNA using Basic Parasite Nucleofector® Kit 1 (Lonza) and Nucleofector® II (Amaxa Bioscience) according to manufacturer instructions. Parasites were selected at 50 $\mu g.ml^{-1}$ and maintained in 100 $\mu g.ml^{-1}$ of G418 (Sigma).

4.6.8 RNA extraction and real-time qPCR analysis

A total amount of 108 logarithmic promastigotes were pelleted for RNA extraction using RNAqueous-Micro Total RNA Isolation Kit (Ambion). RNA quantification prior to cDNA synthesis was done using Qubit and the Qubit RNA BR assay (Life Technologies). Synthesis of cDNA was performed using Transcriptor Reverse Transcriptase (Roche) according to manufacturer instructions. qPCRs were run on LightCycler480 (Roche) with SensiMix™ SYBR® No-ROX Kit (Bioline) as SYBRgreen source, primers used are displayed in supplementary material (Table S2). Expressions levels were assessed with qBase+ (Biogazelle) using for normalisation two genes previously shown to be very stable, SAT (LdBPK_340035000) [38] and LdBPK_240021200 [29]. The expression of both normalisation targets was confirmed by qBase+ as stable during the course of the experiment. Data were then rescaled to BPK026 WT. A fold increase of 1,5 compared to BPK026 WT was considered as significant.

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4.9 Supplementary material legends

Data set 1**: Quantitative changes in S- value observed in Fig. 3B, C and D.** (Available on http://www.itg.be/files/docs/Dataset1-Genomics.xlsx)

Data set 2**: Gene ontology analysis for chromosome 23.** (Available on http://www.itg.be/files/docs/Dataset2-CNVsGOChr23.xlsx)

Data set 3**: Metabolomics data -tab A** metabolite abundances and Log2 fold changes; **tab B** differential metabolite abundance comparing parental strains; **tab C** differential metabolite abundance comparing Sb^{III}-R lines. (Available on http://www.itg.be/files/docs/Dataset3-Metabolomics.xlsx)

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Chapter 5:

Selection of SSG-resistance on L. donovani amastigotes: in vitro, with intracellular parasites and in vivo, using a hamster model

As developed in chapter 2, antimony resistance has been mostly studied on promastigotes using the reduced form of the drug, Sb^{III}. The initial PhD proposal aimed at comparing the promastigote Sb^{III} selection presented in chapter 4 to a Sb^{V} resistance selection of amastigotes (i) *in vitro*, using infected macrophages and (ii) *in vivo,* using Golden Syrian hamsters as mammalian hosts. This should allow us to compare molecular adaptations of promastigotes (*a priori* in the context of transport -in and out- of Sb^{III} and detoxification) and amastigotes (*a priori* in the context of the immuno-modulatory effect of Sb^{V}, transport of Sb^{V} and reduction of Sb^{V} to Sb^{III}). The present chapter will describe the resistance selection on amastigotes and will try to explain why they were not successful.

5.1 In vitro selection of SSG resistant L. donovani using infected macrophages

The procedure used is based on the work published by Hendrickx *et al.* in 2012 for the selection of paromomycin resistant *L. donovani* [1].

5.1.1 Methodology

Briefly, primary mouse peritoneal macrophages (PPM) were isolated from OF1 mice and plated into two 96-well plates, one plate serving for the selection procedure and one plate for Giemsa staining to control infection levels. PPM were infected with BPK282/0 cl4 and BPK026/0 cl5 stationary phase promastigotes in full RPMI supplemented with 10% heat-inactivated FBS (HIFBS), 1% penicillin/streptomycin and 0.5% gentamycin. After 24h incubation, the remaining extracellular promastigotes were washed away with warm RPMI and infected macrophages were exposed to different doses of SSG (Calbiochem) starting from 0.68 to 60 $\mu g.ml^{-1}$ diluted into full RPMI (Figure 1). A positive control using 0.3 μM of AmB (Sigma) was included to observe total clearance of parasites [2]. After 5 days of drug exposure, the macrophages were washed 3 times with warm RPMI. In the first set of plates, infected macrophages were covered with HOMEM + 20% HIFBS and placed at 26°C until promastigote appearance. In the second set of plates, infected macrophages were fixed with methanol, stained with Giemsa and infection levels were monitored by manual counting. The principle sustaining this approach is that in infected macrophages treated with the highest concentration of drug, no amastigotes should remain and thus no promastigotes should grow after transfer of the treated macrophages to HOMEM. This effect should gradually diminish following the decreasing SSG gradient (Figure 2, upper panel). The highest concentration at which the 4 replicates would provide growing promastigotes would then be selected to go into the next round of selection. Based on this principle we performed the experiment twice with BPK282/0 cl4 and BPK026/0 cl5.

	1	2	3	4	5	6	7	8	9	10	11	12	[SSG] (μg.ml^{-1})
A									A	A	A	A	60
B													45
C													30
D													22,5
E									A	A	A	A	15
F													11,25
G													7,5
H													0

Figure 1: Plate plan for SSG selection on intracellular amastigotes. The blue squares represent the wells with BPK282/0 cl4 infected macrophages and the red ones the BPK026/0 cl5 infected macrophages. Empty coloured squares indicate SSG-treated infected macrophages (concentrations indicated on the right side) and squares with A indicate infected macrophages treated with 0.3 μM of AmB.

5.1.2 Results and discussion

As described in chapter 4 the EC_{50} of intracellular amastigotes vs SSG is 10.70 μg.mL^{-1} and 6.7 μg.mL^{-1} for the ISC1 strain BPK026/0 cl5 and the CG strain BPK282/0 cl4, respectively. We therefore expect that at the end of the selection process (60 μg.mL^{-1}) both strains would have an increase of EC_{50} of respectively 6 and 10 fold. The results observed in the 2 experiments made with each strain diverged drastically from those expected. Four days after the transfer of treated macrophages in HOMEM, we could observe promastigotes in all wells containing infected macrophages exposed to SSG (even at the highest concentrations). In contrast, no promastigotes were recovered from the wells exposed to AmB (Figure 2, lower panel, A9-12 and E9-12). When looking at the Giemsa-stained plates we could observe many extracellular parasites that were not taken up by the macrophages. Despite their abnormal morphology, some of them had apparently been able to survive and replicate once put under good conditions for promastigote replication. As already mentioned, SSG is not active on promastigotes so, unlike AmB, if extracellular promastigotes remain in the wells after the infection process, they are not killed by the drug. While enquiring in other laboratories undertaking this type of experiments, we realised that the presence of extracellular parasites was common. To counter this problem, we are now using horse serum in the macrophage incubation

medium in order to block their replication [3]. We did not have time to repeat the intracellular amastigotes selection under those new conditions.

	1	2	3	4	5	6	7	8	9	10	11	12	[SSG] (μg.ml^{-1})
A	-	-	-	-	-	-	-	-	A-	A-	A-	A-	60
B	-	-	-	-	-	-	-	-					45
C	-	-	-	-	-	-	-	-					30
D	-	-	-	-	+	-	-	-					22,5
E	+	-	-	-	+	+	-	-	A-	A-	A-	A-	15
F	+	+	+	+	+	+	+	+					11,25
G	+	+	+	+	+	+	+	+					7,5
H	+	+	+	+	+	+	+	+					0

	1	2	3	4	5	6	7	8	9	10	11	12	[SSG] (μg.ml^{-1})
A	+	+	+	+	+	+	+	+	A-	A-	A-	A-	60
B	+	+	+	+	+	+	+	+					45
C	+	+	+	+	+	+	+	+					30
D	+	+	+	+	+	+	+	+					22,5
E	+	+	+	+	+	+	+	+	A-	A-	A-	A-	15
F	+	+	+	+	+	+	+	+					11,25
G	+	+	+	+	+	+	+	+					7,5
H	+	+	+	+	+	+	+	+					0

Figure 2: Theoretical (upper panel) and observed (lower panel) results of the SSG-resistance selection on intracellular amastigotes. Blues squares represent wells with macrophages infected with BPK282/0 cl4 and red squares wells with macrophages infected with BPK026/0 cl5. In both cases, all those wells were treated with different concentration of SSG as indicated in the last column. Squares with an A are the controlled wells were infected macrophages by the two different strains were treated with 0.3 μM

of AmB. A + sign means that promastigotes were supposed to be observed or were observed after the end of the drug exposure and a week in culture under promastigote growth conditions. A – sign means that no promastigotes were supposed to be observed or were not observed after the end of the drug exposure and a week in culture under promastigote growth conditions.

5.2 In vivo SSG resistance selection on L. donovani

5.2.1 Methodology

After consulting Prof. Simon Croft from LSTMH in London, the following protocol for *in vivo* selection was applied. 10^7 stationary phase promastigotes of BPK282/0 cl4 and BPK026/0 cl5 were injected by intra-cardiac injection into 8 golden Syrian hamsters. Since the metabolisms of hamsters and humans largely differ we had to adapt the human SSG regimen (20 mg/kg/day for 5 days) to hamsters based on the following formula taking into account the body surface area (BSA) [4]:

$$HED = Animal\ dose\ x \frac{animal\ Km}{human\ Km} \quad \Leftrightarrow \quad Animal\ dose = HED\ x\ \frac{human\ Km}{animal\ km}$$

where HED represents the Human Equivalent Dose in $mg.kg^{-1}$ and K_m is a constant depending on each animal species based on the BSA. SSG was solubilised in water at a stock concentration of 20 $mg.mL^{-1}$. The average weight of a hamster is 50 g, therefore we estimated the maximal dose of SSG to administrate per day at 148 mg. After three weeks of infection, three infected animals were treated with the maximum dose of SSG (148 mg), 3 others were treated with 1.48 mg and 2 control infected animals were treated with PBS. All injections of drug were intra-peritoneal once a day for 5 days. Hamsters were euthanized 48h after accomplishment of the full treatment course, their liver and spleen were extracted, grinded into 1 ml of HOMEM and 100 µl was transferred in 5 ml HOMEM. A higher dilution was performed by diluting 100x the previous culture. Cultures were incubated at 26°C and promastigote growth was monitored after 2 and 5 days.

5.2.2 Results and discussion

The experiment was repeated twice with BPK282/0 cl4 and with BPK026/0 cl5 and the same results were obtained. After 2 days in culture all cultures coming from SSG treated animals were positive for promastigote growth. The culture coming from PBS-treated animal was also positive. At day 5 they all reached the same visual density as the PBS-treated samples. These results suggested that the treatment did not work. The fact that

the same results were obtained for the two SSG-S strains pointed toward a problem of protocol.

To understand this unexpected result, we contacted an expert in pharmacology of anti-leishmanial drugs, Prof. Thomas Dorlo from the Netherlands Cancer Institute. During our exchanges, we concluded that the results were likely linked to a problem of pharmacodynamics. Actually, SSG is normally administered in humans (i) by intra-venous infusion, meaning a long injection time and (ii) directly into the bloodstream. When injected intra-venous, all formulations of Sb^{V} are quickly absorbed by the blood and plasma resulting in a Sb^{V} peak in blood of about 12-15 $mg.L^{-1}$ at about 2 hours post treatment. There is no ethical and easy way to perform IV injection on hamsters and thus the use of the intra-peritoneal (IP) route was the only possibility. A second intra-cardiac injection, and all the subsequent ones, would not be accepted by the animal ethical committee as it is an extremely invasive procedure. We even doubt that the animal would survive a repetitive intra-cardiac injection procedure for 5 consecutive days. Considering that IP injections do not deliver the drug into the bloodstream and the SSG half-life is only 0.85 hours in initial phase on human subjects, we concluded that SSG was administered in a non-adequate way to be fully efficient. From the results of the parasite culture we even doubt that the drug reached the parasite. In a recent study using miltefosine and paromomycin, drugs respectively administrable *per os* and by IP injection, a successful *in vivo* selection was performed on golden Syrian hamster [5] showing that such types of experiment are feasible for other drugs if the adequate administration route is followed. We did not find any report of successful *in vivo* SSG–resistance selection of *Leishmania*, using the hamster model or any other animal model.

5.3 References

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Chapter 6:

General discussion

6.1 Importance of studies on drug resistance

Leishmaniasis is endemic worldwide in tropical and sub-tropical regions, but the problem of treatment failure and drug resistance has always been more critical in the Indian Sub-continent (ISC), likely resulting from a combination of factors, biotic and abiotic. The anthroponotic nature of the transmission with no proven animal reservoir plays a central role in this story, as the parasites are more exposed to drug pressure than in zoonotic forms where they spend most of their time in un-treated animals. The strong and old drug pressure is specific of the ISC: *L. donovani* is also present in East Africa and there, VL is treated with SSG since the 70's, which likely explains why SSG is still efficient in that region. In contrast, in the ISC, SSG is being used since the early 20th century and the parasites have had time to adapt. The poor health status and the poverty of the population living in ISC constitute an aggravating factor, with their correlates of co-infections, malnutrition, decreased immunity and access to drugs. Also, the quality of care is the most compromised in VL-endemic regions of ISC like Bihar.

The fate of antimonials during the 20th century perfectly illustrates these concepts and constitutes a paradigm for studies on drug resistance. Understanding the different mechanisms and the different environmental parameters underlying the emergence and spreading of drug resistance in *Leishmania* is a priority for multiple reasons. Firstly, learned lessons may allow for better strategies and tools for surveillance of the efficacy of upcoming drugs: this is urgent, as the efficacy of the drug that followed SSG, miltefosine, seems already compromised only 10 years after its implementation and the first miltefosine-resistant strains are emerging [1]. Secondly, the accumulated knowledge may guide the development of new drugs that should be equally effective against drug-sensitive and -resistant parasites present in the circulating population [2]. Thirdly, understanding resistance may allow for rescuing old drugs by adding a complementary compound to inactivate/counter the resistance mechanism (e.g. imipramine and sitamaquine in SSG-R and MIL-R *L. donovani* [3,4]). Fourthly, from a basic point of view, drug resistance is a unique model to study the adaptive skills of pathogens and their real-time evolution, which can be done both in a natural context and in an experimental one.

6.2 Drug resistance in the ISC: from phylogenomic to experimental studies

In 2016, we paved the road to the first holistic study on SSG resistance in the ISC, with a phylogenomic study of 204 confirmed VL clinical isolates from India, Nepal and Bangladesh, including SSG-S and SSG-R samples [5]. This study revealed several

findings that are supported by the experimental work presented here. First, it highlighted a unique phylo-geographic context and the presence of 2 genetically distinct populations of parasites. The main one is endemic in the lowlands all across the Gangetic plain: this relatively young population was called the Core group (CG) and emerged around 1850 in the North-East of the ISC. The second one, the ISC1 group, is located in the Nepalese highlands and diverged much earlier from the CG, being positioned on phylogenetic trees at an intermediate position between CG and East-African *L. donovani*. In contrast to the high genetic homogeneity existing within the CG, parasites from ISC1 are quite different: more than 50,000 SNPs differentiate them from the CG, there are many local CNVs, but most importantly, ISC1 parasites lack two intra-chromosomal amplifications (ICAs): the H- and the M-locus. Remarkably, both ICAs were present in 100% of the CG isolates, implying that they constituted ancestral characters that were present at the onset of that population in 1850. The presence of these ICAs all over the recent history of the CG also suggests a strong selective advantage: episomes, the other form of gene amplification encountered in *Leishmania*, are commonly observed under stress, but they generally disappear when the selective pressure is removed. Amplification of the H-locus had already been reported upon experimental selection of resistance [6], but its presence in the whole CG suggested that it was a cornerstone of the history of that group. In the same phylogenomic study, an ICA of the M-locus was described and reported to show the same phylogenomic features as the H-locus (being present in 100% of the CG isolates). Hence, this second locus also formed a basis for the experimental work presented here. Within the CG, another striking finding of the phylogenomic study was the definition of a particular group, ISC5, which emerged after 1960 and cumulated with the two ICAs a two nucleotides indel in the AQP1 gene, making the corresponding transporter of Sb^{III} non-functional. Inactivation of AQP1 is one of the molecular adaptations ascribed to antimonial resistance during *in vitro* selections [7]. All 52 isolates of ISC5 showed this 2-nt indel and it is in this group that we encountered most parasites isolated from SSG treatment failure and showing SSG-resistance *in vitro*. Last but not least, the phylogenomic study revealed an unprecedented level of aneuploidy. This remarkable propriety of *Leishmania* to tolerate and take advantage of chromosome copy number variation, or aneuploidy, constitutes a very important strategy for resisting different stresses [8]. However, it could not be linked to antimony resistance in the phylogenomic study.

The present PhD thesis aimed to further explore, experimentally, some of the hypotheses generated by this phylogenomic work, in particular that gene dosage is the main driver that triggers the antimony resistance either through somy variation of whole

chromosomes or at a local level, through copy number variation (CNV). More specifically, I aimed to analyse in experimental conditions the emergence of SSG resistance upon *in vitro* selection, in order to know (i) if the same genomic adaptations would be encountered as in natural populations, (ii) in which order they appear and (iii) to validate the role of these adaptations in SSG-resistance in the specific context of *L. donovani* in the ISC.

6.3 Is the genome stable in Leishmania?

The first experimental chapter focused on aneuploidy. While several authors had reported a link between chromosome copy number and experimentally selected drug resistance, it was curious that this link did not appear at all in clinical isolates. Therefore, the question of the impact of the environment on the genome structure of the parasite was raised in Chapter 3. In that study, we followed the stability of the genome of a few *L. donovani* reference strains throughout their life cycle, *in vitro* and *in vivo* (hamster and sand fly). We demonstrated an important karyotype modulation between life stages, more importantly in the amastigotes within the hamster. While *in vitro* propagated promastigotes were very aneuploid (up to 8 chromosomes showing a somy > 2), in the mammalian host, most of the polysomic chromosomes evolved progressively toward disomy, but new strain-specific polysomies also appeared. Thus, we can assume that the karyotype of the parasites in the patient at the moment of isolation was different from the one observed from *in vitro* grown promastigotes. The whole phylogenomic study described above was performed on cultivated promastigotes, and it is likely that chromosomal changes related to SSG-resistance could have been diluted/lost during the karyotype reshuffling accompanying *in vitro* isolation and maintenance. Hence, we are now exploring different methods to investigate the genome of the parasite directly from the clinical sample. Several approaches were imagined such as whole genome amplification of the whole sample coupled with deep sequencing; parasite DNA enrichment by elimination of the host methylated DNA; and genome capture of the parasite. The last option was the most satisfactory so far and the direct *in situ* sequencing of the collection of clinical samples is now currently being performed in the laboratory in order to re-visit the possible link between SSG-resistance and chromosome copy number in natural conditions.

Aneuploidy should also be studied at the single-cell level. Indeed, *Leishmania* is well known to be “mosaic aneuploid” meaning that different cells can harbour different polysomies [9]. This particularity gives an enormous repertoire of possibility to *Leishmania* to generate genomic diversity at two different levels, gene copy number and

SNP allele frequency, representing a major adaptive advantage. It was demonstrated in collaboration with the team of Dr. Gérald Späth at the Pasteur institute, that the parasite populations coming from different organs of the same infected hamster were showing different haplotypes, possibly associated with fitness gains in the respective environments [10]. This is opening a fascinating domain of investigation which requires technological advances among others to study the genome at the single-cell level (see future prospects).

Chapter 3 also provided a mine of useful information about the link between aneuploidy and gene expression. As said in chapter 2, *Leishmania* gene expression is not regulated at the initiation stage, but is constitutive and polycistronic. Gene dosage might thus be an adaptive strategy to modulate transcript levels. We performed RNAseq analyses of cultured promastigotes and hamster-derived amastigotes and compared the level of the expression at chromosome level in different somy variants. As expected we observed a general modulation of the transcript level following the somy of the associated chromosome. However, 10.2% of the transcripts were not following the somy trend and showed stage specific variation (6.8% up-regulated only in promastigotes and 3.0% up-regulated only in amastigotes). This shows that variation of transcript abundance in *Leishmania* is the result of (i) adaptive changes (somy) and (ii) post-transcriptional regulation. Among other post-transcriptional regulatory mechanisms, it has been shown that *Leishmania* regulate the RNA abundance of transcripts harbouring a SIDER2 signal [11]. Regarding the two ICAs of interest in the present work, we found that the local amplification of the respective genes was indeed accompanied by higher transcript levels (in comparison to the adjacent genes in the chromosome that were not amplified). However, during the life cycle, we did not observe -in contrast to the aneuploidy- any change in the copy number of ICAs in either of the corresponding transcript levels.

6.4 Selecting SSG-resistance: different compounds, different life stages

During the second part of the PhD thesis, my focus was to experimentally validate the adaptations seen in the phylogenomic study as well as the dynamics of their appearance in different parasite genetic backgrounds. As highlighted in the introduction, studying antimony resistance can be done with two compounds (Sb^{V} or Sb^{III}) and with two life stages (promastigotes or amastigotes). The clinically most relevant approach would be to select resistance against Sb^{V} on *in vivo* amastigotes. This was attempted at early stages of this PhD. We infected golden Syrian hamsters with our *L. donovani* reference strain and treated them with SSG through intra-peritoneal injection, aiming to progressively increase the dose to ultimately select resistance. We abandoned this approach for several

reasons. On one hand, infection cycles in hamsters were taking 3 weeks plus few weeks of propagation time *in vitro* but adapting the three strains to hamster took about 3 years and Sb^V-resistant parasites would never have been available for molecular studies before the end of the PhD. On the other hand, there was a pharmacokinetic issue with IP injections that did not deliver the drug into the blood stream. In addition, the SSG half-life is only 0.85 hours at initial phase, hence we concluded that SSG was administered to the hamster in a non-adequate way to be fully efficient. Actually, SSG is normally administered to humans by intra-venous infusion, meaning a long injection time and directly into the bloodstream. When injected intravenously, all formulations of Sb^V are quickly absorbed by the blood and plasma resulting in an Sb^V peak in blood of about 12-15 $mg.L^{-1}$ at about 2 hours post treatment. There is no ethical and easy way to perform such kind of procedure on a laboratory animal. So far, nobody has succeeded in selecting *in vivo* resistance to antimonials, in contrast to paromomycin and miltefosine, which can be administered IP and *per os*, respectively [12].

As a parallel strategy, we also attempted to select resistance to Sb^V *in vitro*, in intracellular amastigotes. Following this approach, we were again confronted by difficulties because of the presence of extra-cellular promastigotes after *in vitro* infection of macrophages. This is not a problem when selecting resistance to drugs which are active against both parasite life stages, like paromomycin or miltefosine [13–15], but it is a major issue in the case of Sb^V which is not active on promastigotes. It took us months to solve the problem of extracellular parasites, among others through the implementation of horse-serum to kill the extra-cellular stages [16]. Here again, we had to abandon this approach because of time limitations. Noteworthy, *in vitro* selection of resistance to Miltefosine in intracellular amastigotes also failed in *L. donovani*, while curiously it was successful in *L. infantum* [17]. Last but not least, it is likely that resistance selection on amastigotes take much more time than selection of resistance promastigotes, because of their respective generation time, respectively 12 days and 9 hours [18]. However, it was shown that amastigote replication might not be essential to select miltefosine resistant parasites [15].

6.5 Preadaptation of promastigotes to Sb^{III}

We thus decided to focus the experimental work on promastigotes, in the context of resistance to Sb^{III}. As such, we were *a priori* excluding resistance mechanisms related to (i) immunomodulation and subversion of the macrophages by Sb^V, (ii) transport of Sb^V through the macrophage membrane and (iii) reduction of Sb^V to Sb^{III} by the macrophage. On the contrary, mechanisms related to the uptake/efflux of Sb^{III} by the parasite as well as detoxification were *a priori* accessible. The originality of our selection experiment was

its phylogenomic context and the fact that we undertook the selection on different genetic backgrounds of *L. donovani*. The main findings of the study were the following. Firstly, all the strains of the CG tested showed a higher tolerance to Sb^{III} than the one measured in ISC1 strains, and this was independent of their Sb^{V} susceptibility phenotype (measured previously on intracellular amastigotes). Secondly, this higher tolerance was clearly driven by the amplification of the H-locus and more specifically by the MRPA gene. Thirdly, it took about twice as long for ISC1 strains to develop full resistance to Sb^{III}, when comparing to CG ones (35 vs 20 weeks). Fitting with a longer time-to-resistance, ISC1 strains also needed much more molecular adaptations to become fully resistant to Sb^{III}, than CG strains. Metabolomics clearly showed that ISC1 strains developed several adaptations to reach levels already present in ISC1 strains (see for example the amino-acids and glycerophospholipids). Altogether, this suggested a certain degree of pre-adaptation in the CG, likely provided by the ancestral amplification of the H-locus under an ICA form. To our surprise, the ISC1 strain did not develop an ICA or an episomal amplification of the H-locus upon selection of Sb^{III} resistance, but an amplification of a whole arm of chromosome 23, containing the H-locus, and also other genes including some involved in virulence. In the meantime, we observed in other contexts that *Leishmania* could use different genomic strategies for a gain or loss of function of a given gene, a concept known as the 'many-roads-to-drug resistance' [2]. During the selection experiment, we did not find any alterations of AQP1 (neither at genome or expression level), as observed in the ISC5 group in the phylogenomic study. Under exposure to Sb^{III}, the first line of parasite adaptation is thus to amplify in one way or another MRPA, thereby favouring drug sequestration. This is now observed experimentally and it fits with the fact that H-locus amplification represents the first adaptation appearing in the CG around 1850.

As written earlier, this pre-adaptation does not mean that the strains are fully resistant to Sb^{III}, neither to Sb^{V}. It is clear that only a fraction of CG strains were indeed resistant to Sb^{V} in clinical conditions. This PhD thesis only shows that CG parasites are somehow more tolerant to Sb^{III}, but that under conditions of higher stress they may quickly become resistant to Sb^{III}. Second lines of adaptation not reported in the present experimental work are likely required for developing full resistance to Sb^{V} and cause treatment failure. Further work on *in vivo* amastigotes should bring more light on these mechanisms. What is the selective pressure that caused the pre-adaptation of the CG strains in natural context remains to be demonstrated. We discussed three hypotheses in this work.

(i) Assuming 1850 as the origin of the CG, we need to consider selective pressures available at that time. Neither antimonials or *Leishmania* were discovered in the middle of the 19th century: the strange disease causing a darkening of the skin (kala-azar) and killing ten thousands of people in epidemics was considered as malaria and treated with quinine, hence it was sometimes reported as 'quinine-resistant' malaria [19]. We may thus assume that VL was treated during more than half a century (from 1850 until 1916 when Sb^{III} was first introduced) with quinine sulphate. Looking at the evolution of quinine resistance in *Plasmodium falciparum*, a local CNV was found to modulate quinine tolerance. The amplification of the part of the genome coding for pfmdr1, an ABC transporter responsible for the sequestration of quinine in non-acidic vacuole before extrusion from the parasite, was found in several different field isolates across the world [20]. The similarity with *Leishmania* exposed to antimony is striking as we know that MRPA is an ABC transporter that has the same mode of action than pfmdr1. To support this theory of quinine exposure linked to amplification of ABC transporter like MRPA, it has been shown in *L. major* that selecting for primaquine-resistant parasites was leading to amplification of the H- locus [21].

(ii) Assuming 1900 as origin of the Indo-Nepalese sub-population within the CG (strictly speaking, the pre-adaptation was only tested in CG parasites of that region, as we did not test the susceptibility to Sb^{III} in CG isolates from Bangladesh that diverged in 1850), this would fit with the first implementation of Sb^{III} in the region in 1916 and its replacement by Sb^{V} in 1923 because of toxicity issues. Accordingly, the Indo-Nepalese parasites were directly exposed to Sb^{III} for 7 years, which in an anthroponotic transmission context could provide a strong selective pressure.

(iii) Last but not least, the Arsenic hypothesis should also be considered. India and Nepal are two countries where, due to the combination of biogeochemical and hydrological factors, the natural level of arsenic in drinking water is exceeding the WHO guidelines value of 10 $\mu g.L^{-1}$ [22]. This contamination was further amplified in the 70's with the gold mining activities and the little rehabilitation of the closed mining sites [23]. The contaminated water is not only used for drinking, exposing all the living creature to high doses of arsenic, but it is also used for culture irrigation and high amounts of arsenic were detected in rice and vegetables [24], likely increasing the exposure to arsenic for the mammals and of the sand flies. As said in chapter 2, arsenic and antimony have very similar chemical properties and selecting arsenic resistant *L. tarentolae* led to amplification of the H- locus, following the driving force of MRPA metal-thiol extrusion properties [25–27]. To support this theory, it was shown that arsenic resistant *L. donovani*

selected *in vivo* by adding arsenic in the mice drinking water, were cross-resistant to SSG [28]. Finally retrospective epidemiological data showed a striking overlap between reported cases of SSG-treatment failure in India and areas with more than 10 $\mu g.L^{-1}$ of arsenic in groundwater [29].

6.6 Implications of present work and recommendations

In the current context of the few anti-*Leishmania* drugs available, management of drug resistance is of the utmost importance. Therefore, clinical and public health measures are required, including: (i) the completion of treatment, which can be achieved by directly observed therapy, (ii) the assessment of the good quality of the drug by pharmaco-vigilance networks, or (iii) simpler schemes to treat the patients to make them more compliant to the full regimen. Such measures should of course be integrated in general control strategies, together with vector control, vaccination (when available) or improvement of the general health status. More specifically, some findings made in the present PhD and in complementary studies to which I contributed have a clinical and public health impact and as such they can lead to specific recommendations.

Surveillance of drug resistance is a major need in the context of infectious diseases and it should be closely coupled with clinical monitoring of treatment efficacy, as this can contribute to a change drug policy in a region, obviously if alternative drugs are available. Present work focused on resistance to SSG, a drug which is not used anymore in the ISC, but still used in other regions, among others in East Africa. In this context, there is *a priori* no need for a surveillance of SSG-resistance in the ISC, but it might be interesting to follow-up the parasite populations circulating in the region and check if SSG-resistance is indeed disappearing now that the corresponding drug pressure is removed. In theory, it should be the case, but previous work showed that fitness of SSG-R parasites remained high even in the absence of the drug [30]. This could have an impact on the efficacy of following drugs, like miltefosine [31] or if SSG would be re-considered in the future, for instance in combination therapy. We identified molecular markers of SSG-resistance and these could be indeed used for this post-SSG-removal surveillance. Noteworthy, the 'many-roads-to drug resistance' concept here illustrated by the several mechanisms leading to MRPA amplification, indicates that a single PCR assay is not enough. On the contrary, untargeted methods like whole genome sequencing should be preferred, as they can identify different types of molecular adaptations. The same approach could be used for molecular surveillance of SSG-resistance in East-Africa where SSG is still used: most likely the same molecular modifications should be associated with resistance, but considering the genomic distance between East African

and ISC *L. donovani*, a validation should first be undertaken. The same principles are obviously applicable for molecular surveillance of resistance to other drugs: from the moment a drug is launched in clinical practice, resistance should be closely monitored among parasites.

In terms of molecular adaptations linked to SSG resistance, the present work also showed a nice parallel between the findings of phylogenomic work on clinically resistant isolates and experimental studies on selected resistant strains. A similar observation was made in the context of miltefosine, where the main transporter of the drug LdMT was found to be mutated in experimental resistance and also in the few clinically resistant isolates [1]. Accordingly, laboratory studies on drug resistance should be further encouraged as they have a good predictive value of what will occur in clinical conditions. Obviously in the case of SSG, these studies should be upgraded, for instance by improvements of the protocols for Sb^V resistance selection in amastigotes. As mentioned above, untargeted molecular methods should be preferred for characterisation of resistant strains and for further monitoring. However, considering the results described in chapter 3, it is obvious that molecular surveillance should be done directly on clinical samples and not on isolates, where biases can be brought in by isolations and *in vitro* maintenance. New sequencing methods based on *Leishmania* genome capture and amplification should therefore be preferred.

The present work contributed to highlighting the genetic and phenotypic differences between ISC1 and the CG. There is thus in the ISC, a new variant of *L. donovani* (ISC1), that originated from the Nepalese highlands, a region where *L. donovani* transmission was previously not reported. Recent findings suggest that it expanded in Nepal [32] and its fate in the other parts of the ISC is unknown. Another similar case was described in Sri Lanka, where variants intermediate between CG and ISC1 parasites were detected in VL but also in CL cases [33]. We know that ISC1 parasites are more susceptible to SSG, but we don't have information on those of Sri Lanka. Similarly, we don't know how these new variants will react to new drugs like miltefosine or AmB. In the context of the kala-azar elimination programme it is thus very important to follow the emergence and spread of these new variants as these could jeopardise the control activities and also reshape the epidemiological and clinical panorama of *L. donovani* infections in the future. Here again, molecular tools are available and they should be applied directly on clinical samples.

The pre-adaptation hypothesis, the occurrence of SSG-S and SSG-R parasites in the ISC as well as the presence of new variants of *L. donovani* (ISC1 or Sri-Lanka ones) highlight the occurrence of extensive phenotypic diversity in the ISC. This dimension should be integrated into drug discovery R&D pipelines. On one hand, recently isolated parasites representing the major phenotypes encountered in the ISC should be included in screening phases. In a sister PhD of the unit, the inclusion of SSG-S and SSG-R strains in secondary screening of a library of compounds revealed that about half of the molecules that were active against an old East African reference strain were inactive against fresh clinical isolates of the ISC. On the other hand, the same clinical isolates should be used for experimental resistance selection against the most promising compounds. Determination of the time-to-resistance and characterisation of the type of resistance could be compared to those observed in the case of antimonials or miltefosine and they should contribute to identifying the mode of action of new compounds or develop companion diagnostics for monitoring of resistance during clinical trials.

In conclusion, the present work highlights the importance of the legacy of a *Leishmania* population, resulting from the parasite's interaction with drugs, host and environment and the unique adaptive skills of these parasites. These cannot be neglected in any public health intervention and should also be taken into consideration in the development of new tools for surveillance and control.

6.7 Prospects for further work

Several new research lines could stem from the present PhD. Some more applied approaches were already described in the previous section. Here I briefly list some of the basic research lines that could be further pursued.

The functional role of the second ICA (corresponding to amplification of the M-locus) described in the phylogenomic study should be assessed. The present study did not find evidence of an involvement in resistance to Sb^{III}. A central gene in this ICA is MPK1: studies on the role of MPK1 in *L. mexicana* have described MPK1 as essential for the intracellular survival of the parasite [34]. In another study, experimental overexpression of MPK1 in *L. donovani* led to a decrease of Sb^{III} tolerance in the parasite by modulating the activity of the P-gp glycoprotein, which was identified as not being MRPA [35,36]. This contrast with our work should be elucidated. Finally, more recently, MPK1 was described as a partner of HSP70 and HSP90 in the foldosome, an important protein complex responsible for the proper folding of proteins exposed to stresses [37]. Due to

the high number of copies of that gene in parasites of the CG, this gene should be further analysed in the context of resistance to stress.

The different hypotheses for the selective pressure at the origin of the Sb^{III} pre-adaptation of the CG should be further tested. Even if it is known that exposure to As can select cross-resistance to antimonials [38], it is important to test how a strain of ISC1 would react to an As-selective pressure. Similarly, the 'malaria' hypothesis could be tested by exposing ISC1 parasites to quinine or quinine derivatives.

The experimental selection of antimony resistance should be pursued, among others, by completing Sb^{V}-resistance selection in intracellular amastigotes and *in vivo*. As mentioned earlier we recently found a way to counter the presence of the extracellular promastigotes issue while performing macrophages infection, through the addition of horse serum. Applying the already published strategy of successive passages inside the macrophages under drug pressure with promastigote expansion between the selection cycles should be further attempted. Similarly, a satisfactory SSG-resistance selection model *in vivo* is still needed. For this, two alternative scenario can be drafted: (i) improving the treatment administration on the hamster model by trying intra-muscular injection (ii) using a mouse model where injecting intra-venous is possible on the tail-vein. Even if the mouse model is not optimal for VL, when compared to the hamster, it would probably simplify the procedure and mimic the treatment administration performed in the clinic.

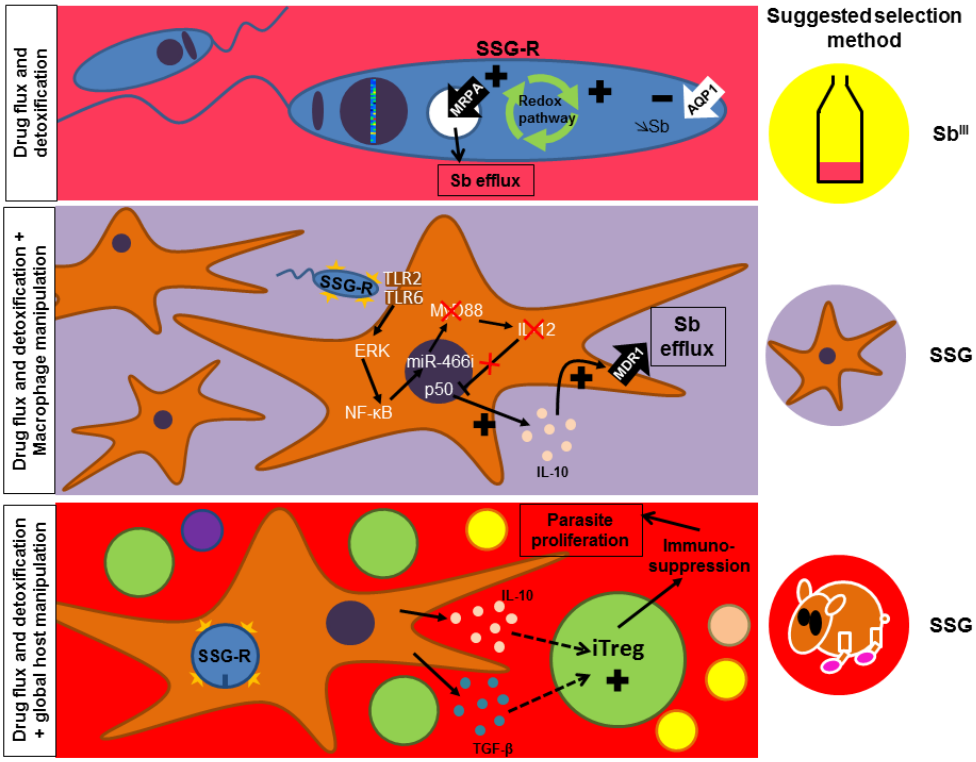

Figure 1: General summary of the adaptations developed by SSG-R field isolates from the ISC (literature and this study). The panels are organised according to the experimental model in which these adaptations were described; + and – stand for increased or decreased function. The upper panel displays the adaptations observed *in vitro* using Sb^{III} on promastigotes: resistance to Sb^{III} is brought about by increasing sequestration and efflux (MRPA), an increase of detoxification and a decrease of Sb^{III} uptake (AQP1). The middle panel concerns the manipulation of macrophages by SSG-R parasites (*in vitro*) and represents an adaptation to Sb^{V}. Parasite surface glycans (yellow spikes) interact with TLR2/6, causing a cascade of signalling events leading to inactivation of MyD88, downregulation of IL-12 and upregulation of IL-10 [39]. Consecutive to this IL-10 over-production the macrophage upregulates the efflux pump MDR1 that is responsible for Sb^{V} efflux directly from the host macrophage [40]. The lower panel shows the general immuno-suppressive activity of SSG-R parasites observed *in vitro* on key immune effectors such as iTregs [41]. On the right are indicated the resistance selection methods that are recommended to study the respective adaptations, *in vitro*/promastigotes/Sb^{III}, *in vitro*/intracellular amastigotes/SSG and *in vivo*/intracellular amastigotes/SSG.

One of the hypothesis described above is the constant arsenic pressure on the human host. As said previously, arsenic is present in the groundwater and it is thus generally present in the environment. Therefore, not only are humans are in close permanent contact with arsenic, but the sand fly population is as well. Male and female sand flies are feeding from natural sources of sugar such as plant nectar, while only females require blood, for oviposition [42]. Thus, sand flies ingest the nectar of plants containing arsenic. In other words, arsenic exposure in the sand fly could be a hidden factor leading to the pre-adaptation to antimony resistance in *L. donovani* on the ISC. This hypothesis could be tested by feeding sand flies on sugar containing arsenic and exposing parasites to increasing concentrations.

Knowing that aneuploidy can be very different *in vivo* and *in vitro*, it would be highly relevant to revisit the possible role of chromosome copy number change in natural populations of the parasite. As mentioned earlier, this requires methods allowing for sequencing the parasite directly from clinical samples. Such methods are in development in the unit, but confronted with challenges related to the sensitivity or to bio-informatics processing of the data. A next and even more challenging step should be to undergo single-cell genomics *in vivo*. Technological developments are required, including whole genome amplification with protocols respecting ploidy or single-cell sorting technologies.

This work emphasized the essential role played by constitutional aneuploidy in *Leishmania*. However the origin of this intriguing phenomenon are currently not understood. In other eukaryotes, aneuploidy is generally linked to a problem of chromatid segregation during cell division. We demonstrated that this mechanism was one of the way the parasite is using to adapt to its environment and to drug pressure. In Prieto-Barja *et al* [10], we demonstrated the rapid aneuploidy turnover of parasites stemming from a clonal population when exposed to different environments: this highlights the quick regeneration of the mosaic cell population. Systematic selection of a particular karyotype, and of a particular haplotype, linked to a specific environment suggests that this mechanism is not random. It is currently unknown if the modulation of aneuploidy in parasite populations is due (i) to pre-existing aneuploidy in a few cells (mosaicism), which is later on selected because of increased fitness, or (ii) to de novo aneuploidisation emerging in response to a given signal. Targeting the mechanisms controlling or driving this aneuploidy could be a strategy for new anti-*Leishmania* drugs.

Last but not least, the complex relationship discovered here between genome dosage and transcriptome should be completed by studies at other 'omics levels. Systems Biology studies including proteomics and metabolomics should help verifying if copy number variation of a given chromosome does indeed result in dosage of all proteins coded on this chromosome and the corresponding metabolites. Such studies could be helpful in identifying the main drivers of genome variation.

6.8 Conclusion

We believe that this work contributes to improving our understanding of *Leishmania* biology. Firstly, even if chromosome amplification was known for a long time to be involved in resistance to stress, it is the first time that the karyotype plasticity was assessed during the life cycle *in vitro* and *in vivo* and shown to vary between life stages. We also showed that aneuploidy had a major impact on transcriptome expression, and that this effect was intertwined with aneuploidy-independent regulation of gene expression. This huge potential for plasticity provides an adaptive advantage for a parasite that lacks regulation of transcription at initiation and for which gene amplification is the only way to increase the expression of a given product. Our findings emphasise the need to develop *in situ* sequencing methods to assess the karyotype of the parasite inside the host. Secondly, we described here the first case of pre-adaption to drug resistance in *Leishmania*, identified its main driver, the amplification of the gene encoding MRPA, and showed that the time-to-resistance and the type-of-resistance was very different in non-pre-adapted strains like those of ISC1.

6.9 References

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Annexe 1:

Collaborative work

"Treatment failure in leishmaniasis: drug-resistance or another (epi-) phenotype?" Expert Rev Anti Infect Ther. 12: 937–46, 2014.

Vanaerschot M, **Dumetz F**, Roy S, Ponte-Sucre A, Arevalo J, Dujardin J-C.

Abstract:

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.

"*In vivo* characterization of two additional *Leishmania donovani* strains using the murine and hamster model." Parasite Immunology, 38, 2016.

Kauffmann F, **Dumetz F**, Hendrickx S, Muraille E, Dujardin J-C, Maes L, Magez S and De Trez C

Abstract:

Leishmania donovani is a protozoan parasite causing the neglected tropical disease visceral leishmaniasis. One difficulty to study the immunopathology upon *L. donovani* infection is the limited adaptability of the strains to experimental mammalian hosts. Our knowledge about *L. donovani* infections relies on a restricted number of East African strains (LV9, 1S). Isolated from patients in the 1960s, these strains were described extensively in mice and Syrian hamsters and have consequently become 'reference' laboratory strains. *L. donovani* strains from the Indian continent display distinct clinical features compared to East African strains. Some reports describing the in vivo immunopathology of strains from the Indian continent exist. This study comprises a comprehensive immunopathological characterization upon infection with two additional strains, the Ethiopian *L. donovani* L82 strain and the Nepalese *L. donovani* BPK282 strain in both Syrian hamsters and C57BL/6 mice. Parameters that include parasitaemia levels, weight loss, hepatosplenomegaly and alterations in cellular composition of the spleen and liver, showed that the L82 strain generated an overall more virulent infection compared to the BPK282 strain. Altogether, both *L. donovani* strains are suitable and interesting for subsequent in vivo investigation of visceral leishmaniasis in the Syrian hamster and the C57BL/6 mouse model.

This work was carried out by Dr. Florence Kauffman from the Vrije Universiteit Brussel (VUB) in the Laboratory of Cellular and Molecular Immunology. I was responsible for the characterisation of the different strains that were used for this work. I performed the molecular typing by PRC-RFLP to assess the diversity of the strains at the beginning and at the end. I also participated to the interpretation of the results and to the review of the final manuscript.

"Evolutionary genomics of epidemic visceral leishmaniasis in the Indian subcontinent." eLife, 5, 2016

Imamura H, Downing T, Van den Broeck F, Sanders MJ, Rijal S, Sundar S, Mannaert A, Vanaerschot M, Berg M, De Muylder G, **Dumetz F**, Cuypers B, Maes I, Domagalska M, Decuypere S, Rai K, Uranw S, Raj Bhattarai N, Khanal B, Kumar Prajapati V, Sharma S, Stark O, Schönian G, De Koning HP, Settimo L, Vanhollebeke B, Syamal Roy, Ostyn B, Boelaert M, Maes L, Berriman M, Dujardin J-C and Cotton JA

Abstract:

Leishmania donovani causes visceral leishmaniasis (VL), the second most deadly vector-borne parasitic disease. A recent epidemic in the Indian subcontinent (ISC) caused up to 80% of global VL and over 30,000 deaths per year. Resistance against antimonial drugs has probably been a contributing factor in the persistence of this epidemic. Here we use whole genome sequences from 204 clinical isolates to track the evolution and epidemiology of *L. donovani* from the ISC. We identify independent radiations that have emerged since a bottleneck coincident with 1960s DDT spraying campaigns. A genetically distinct population frequently resistant to antimonials has a two base-pair insertion in the aquaglyceroporin gene LdAQP1 that prevents the transport of trivalent antimonials. We find evidence of genetic exchange between ISC populations, and show that the mutation in LdAQP1 has spread by recombination. Our results reveal the complexity of *L. donovani* evolution in the ISC in response to drug treatment.

This publication is one of the output publications of the FP7 consortium *KalaDrug*. In that publication I was implicated in the characterisation of the two intra-chromosomal amplifications of chromosome 23, the H- locus, and of chromosome 36, the M- locus. In order to do this I had to use sequencing results and classical molecular biology techniques. I was also involved in the review of the final manuscript. This work is the foundation of everything that is developed in this thesis.

"Multiplexed Spliced-Leader Sequencing: A high-throughput, selective method for RNA-seq in Trypanosomatids" Scientific Reports 7: 3725, 2017.

Cuypers B, Domagalska MA, Meysman P, de Muylder G, Vanaerschot M, Imamura H, **Dumetz F**, Verdonckt TW, Myler PJ, Ramasamy G, Laukens K, and Dujardin JC

Abstract:

High throughput sequencing techniques are poorly adapted for in vivo studies of parasites, which require prior in vitro culturing and purification. *Trypanosomatids*, a group of *kinetoplastid* protozoans, possess a distinctive feature in their transcriptional mechanism whereby a specific Spliced Leader (SL) sequence is added to the 5'end of each mRNA by trans-splicing. This allows to discriminate *Trypanosomatid* RNA from mammalian RNA and forms the basis of our new multiplexed protocol for high-throughput, selective RNA-sequencing called SL-seq. We provided a proof-of-concept of SL-seq in *Leishmania donovani*, the main causative agent of visceral leishmaniasis in humans, and successfully applied the method to sequence *Leishmania* mRNA directly from infected macrophages and from highly diluted mixes with human RNA. mRNA profiles obtained with SL-seq corresponded largely to those obtained from conventional poly-A tail purification methods, indicating both enumerate the same mRNA pool. However, SL-seq offers additional advantages, including lower sequencing depth requirements, fast and simple library prep and high resolution splice site detection. SL-seq is therefore ideal for fast and massive parallel sequencing of parasite transcriptomes directly from host tissues. Since SLs are also present in Nematodes, Cnidaria and primitive chordates, this method could also have high potential for transcriptomics studies in other organisms.

This publication was leaded by another PhD student of the lab, Bart Cuypers. For this work I was involved in the data analysis and in the critical revision of the final manuscript.

"Asexual maintenance of genetic diversity in the protozoan pathogen *Leishmania donovani*." Nature Ecology & Evolution, 2017 Dec;1(12):1961-1969.

Prieto-Barja P, Pescher P, Bussotti G, **Dumetz F**, Imamura H, Kedra D, Domagalska MA, Chaumeau V, Himmelbauer H, Pages M, Sterkers Y, Dujardin JC, Notredame C and Spaeth GF

Abstract:

The parasite *Leishmania donovani* causes a fatal disease termed visceral leishmaniasis. The process through which the parasite adapts to environmental change remains largely unknown. Here we show that aneuploidy is integral for parasite adaptation and that karyotypic fluctuations allow for selection of beneficial haplotypes, which impact transcriptomic output and correlate with phenotypic variations in proliferation and infectivity. To avoid loss of diversity following karyotype and haplotype selection, *L. donovani* utilizes two mechanisms: polyclonal selection of beneficial haplotypes to create coexisting subpopulations that preserve the original diversity, and generation of new diversity as aneuploidy-prone chromosomes tolerate higher mutation rates. Our results reveal high aneuploidy turnover and haplotype selection as a unique evolutionary adaptation mechanism that *L. donovani* uses to preserve genetic diversity under strong selection. This unexplored process may function in other human diseases, including fungal infection and cancer, and stimulate innovative treatment options.

This publication is the sister paper of my work presented in chapter 3. It represents the part of the study on allele frequency variation due to karyotype variability induced by long term *in vivo* maintenance of *L. donovani*. This question could not be addressed in our work due the low number of heterozygous sites in the CG strains. In this work I participated to the *in vivo* work with the clinical isolates and to the critical revision of the manuscript.

Chapter 17: The concept of fitness in *Leishmania* in the 2nd edition of Drug Resistance in *Leishmania* Parasites, *in press,* Springer, May 2018.

Vanaerschot M, **Dumetz F**, Jara M, Dujardin JC and Ponte-Sucre A

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 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.

Curriculum Vitae

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EDUCATION

Oct 2012 – to present	**Ph.D. "Role of gene dosage in the acquisition of antimony resistance in *L. donovani*: experimental evidence"** Molecular Parasitology Unit, Institute of Tropical Medicine, Antwerp, Belgium Supervisors: Prof. Dr. Jean Claude Dujardin (primary supervisor), Dr Géraldine De Muylder University of Antwerp, Belgium
2010 – 2012	**M.Sc. in Molecular and Cellular Biology specialty Medical Parasitology and Mycology "Methodology for the study of *Leishmania major* interaction between MAP kinase 7 and Metacaspase".** Molecular Parasitology and Signaling, Institut Pasteur, Paris, France. Supervisors: Dr. Gerald Spaeth (primary supervisor), Dr Mariko Dacher Université Pierre et Marie Curie (Paris VI), France
2007 – 2010	**B. Sc. Biology specialty Biotechnology** Thesis: "Establishment of cellular culture for *Cryptosporidium parvum*." Supervisors: Dr. Anne Dumoulin, Dr Jerome Follet Université Libre des Sciences et Technologies, Lille, France

WORK EXPERIENCE

2010-2011	**INSERM U1014**, Hôpital Paul Brousse, Villejuif, France First year M. Sc. internship "Investigating the role of activating ubiquitination of NF-κB pathway in antiviral innate immunity."
2009-2010	**Laboratoire de Biotechnologie et Gestion des Agents pathogènes en agriculture**, Institut Supérieur d'Agriculture, Lille, France "*Cryptosporidium* detection in micro-shell (*Ostracoda*)"

PUBLICATIONS

Cuypers B, Berg M, Imamura H, **Dumetz F**, De Muylder G, Domagalska MA, Rijal S, Bhattarai NR, Maes I, Sanders M, Cotton J, Meysman P, Laukens K, Dujardin JC "Integrated genomic and metabolomic profiling of ISC1, an emerging *L. donovani* population in the Indian sub-continent." *Submitted*

Dumetz F, Cuypers B, Imamura H, Zander D, D'Haenens E, Maes I, Domagalska MA, Clos J, Dujardin JC, De Muylder G. "Molecular pre-adaptation to antimony resistance in *Leishmania donovani* of the Indian sub-continent" *Accepted with minor comments in mSphere*

Prieto-Barja P, Pescher P, Bussotti G, **Dumetz F**, Imamura H, Kedra D, Domagalska MA, Chaumeau V, Himmelbauer H, Pages M, Sterkers Y, Dujardin JC, Notredame C and Spaeth GF "Asexual maintenance of genetic diversity in the protozoan pathogen *Leishmania donovani*." (2017) Nature Ecology & Evolution 1(12):1961-1969.

Cuypers B, Domagalska MA, Meysman P, de Muylder G, Vanaerschot M, Imamura H, **Dumetz F**, Verdonckt TW, Myler PJ, Ramasamy G, Laukens K, and Dujardin JC (2017) "Multiplexed Spliced-Leader Sequencing: A high-throughput, selective method for RNA-seq in Trypanosomatids". Scientific Reports 7: 3725.

Dumetz F, Imamura H, Sanders M, Seblova V, Myskova J, Pescher P, Cuypers B, De Muylder G, Späth GF, Bussotti G, Vermeesch JR, Berriman M, Cotton JA, Volf P, Dujardin JC, Domagalska MA (2017) "Modulation of Aneuploidy in *Leishmania donovani* during Adaptation to Different In Vitro and In Vivo Environments and Its Impact on Gene Expression". MBio 8(3): e00599-17.

Kauffmann F, **Dumetz F**, Hendrickx S, Muraille E, Dujardin J-C, Maes L, Magez S and De Trez C (2016) "In vivo characterization of two additional *Leishmania donovani* strains using the murine and hamster model.", Parasite Immunology, 38.

Imamura H, Downing T, Van den Broeck F, Sanders MJ, Rijal S, Sundar S, Mannaert A, Vanaerschot M, Berg M, De Muylder G, **Dumetz F**, Cuypers B, Maes I, Domagalska M, Decuypere S, Rai K, Uranw S, Raj Bhattarai N, Khanal B, Kumar Prajapati V, Sharma S, Stark O, Schönian G, De Koning HP, Settimo L, Vanhollebeke B, Syamal Roy, Ostyn B, Boelaert M, Maes L, Berriman M, Dujardin J-C and Cotton JA (2016) "Evolutionary genomics of epidemic visceral leishmaniasis in the Indian subcontinent." eLife, 5.

Vanaerschot M, **Dumetz F**, Roy S, Ponte-Sucre A, Arevalo J, Dujardin J-C (2014) "Treatment failure in leishmaniasis: drug-resistance or another (epi-) phenotype?" Expert Review of Anti-Infective Therapy, 12(8).

Vanaerschot M, **Dumetz F**, Jara M, Dujardin, J-C Ponte-Sucre A. Chapter 17: The concept of fitness in *Leishmania* in the 2nd edition of Drug Resistance in *Leishmania* Parasites, Springer *(to be published in May 2018)*

FUNDING & AWARDS

- **Research Fellowship (2014 and 2015)**

Funding Body: Les Amis de l'Institut Pasteur de Bruxelles
Title: Role of DNA methylation on transcription regulation and effect on drug resistance in *Leishmania donovani* (MetLeish).

- **Short term fellowship (1 month – January 2015)**

Funding Bodies: European Molecular Biology Organization - EMBO (id: ASTF 616-2014) and FWO (Flemish Research Foundation)
Title: Isolation, modification and characterisation of a *Leishmania donovani* field-isolate episome.
Location and local supervisor: Bernard Nocht Institute for Tropical Medicine, Hamburg, Germany. Supervised by Prof. Joachim Clos.

- **Zoetis Travel Award (May 2017)**

Competitive travel award to go to WorldLeish6 in Toledo, Spain, in May 2017

CONFERENCES AND INVITED TALKS

Dumetz F, Imamura H, Seblova-Hrobarikova V, Myskova J, Pescher P, Cuypers B, Vanaerschot M, De Muylder G, Mannaert A, Vermeesch J, Spaeth GF, Volf P,

Dujardin JC, Domagalska MA. Oral communication: "Genome and transcriptome dynamics during the life cycle of *Leishmania*." IVth Trypanosomatid Parasites meeting, Institut Pasteur, Paris, France. (May 2015).

Dumetz F. Invited Seminar: "Genome and transcriptome dynamics during the life cycle of *Leishmania*. (May 2016) Faculty of Biology, Pontifica Universidad Catolica del Ecuador, Quito, Ecuador.

Dumetz F, Imamura H, Cuypers B, Domagalska M, Rijal S, Dujardin JC, De Muylder G. Oral communication: "*L. donovani* from the Indian Subcontinent is pre-adapted for a rapid development of antimonial resistance driven by aneuploidy." (October 2016) Second International ParaFrap Conference, Ile des Embiez, France.

Dumetz F, Imamura H, Sanders M, Seblova-Hrobarikova V, Myskova J, Sadlova J, Pescher P, Vanaerschot M, Cuypers B, De Muylder G, Bussotti G, Späth GF, Vermeesch J, Cotton JA, Volf P, Dujardin JC, Domagalska MA. Oral communication: "Aneuploidy variation during the life cycle of *Leishmania donovani*." (May 2017). Annual meeting of the Belgian Society of Parasitology and Protistology, Brussels, Belgium

Dumetz F, Cuypers B, Imamura H, Domagalska MA, D'Haenens E, Maes I, Rijal S, Sundar S, Roy S, Dujardin JC, De Muylder G. Oral communication: "Pre-adaptation to antimonials is general in *Leishmania donovani* in the Indian subcontinent." (May 2017). Sixth World Conference on Leishmaniasis, Toledo, Spain

Dumetz F. Invited lecture: "*Leishmania*, from the field to the lab", University of Ohio, Athens, Ohio, USA (October 2017)

Dumetz F, Cuypers B, Imamura H, Domagalska MA, De Muylder G, Dujardin JC. Oral communication: "Main populations of *L. donovani* in the Indian sub-continent differ in their pre-adaptation to antimony resistance." Vth Trypanosomatid Parasites meeting, Institut Pasteur, Paris, France. (December 2017).

MENTORING OF MSc STUDENTS

Jan-June 2015	Matilda Svensson, MSc thesis "Argininosuccinate Synthase role in antimony resistance in *Leishmania donovani*"
Oct 2013-June 2014	Natania Peelman, MSc thesis "Functional Analysis of the MRPA episome in *Leishmania donovani*"