1. Publishable summary

Visceral leishmaniasis (VL), one of the most-neglected infectious diseases, has an annual incidence of 500,000 cases. Early treatment is a major pillar of the current program for VL elimination on the Indian sub-continent. However, the arsenal of available drugs is very limited, and their indiscriminate use is jeopardized by drug resistance. Combination regimens for VL are under clinical development, but it will take several more years to change the drug policy. Meanwhile, the effectiveness of current drugs needs to be safeguarded in order to cure patients and ensure unremitting sustainment of VL control.

For this, the uninterrupted supply of quality drugs, the promotion of treatment compliance and, the monitoring of treatment effectiveness and of drug resistance will be pivotal. The latter demands improved knowledge and know-how, hence clinical and laboratory research are urgently needed to support the drug policy of the VL elimination program. Kaladrug-R addresses these needs: we aim to develop, evaluate and disseminate new tools for the assessment of drug resistance in *L. donovani* as well as innovative methodologies for monitoring Kala-Azar treatment effectiveness under routine conditions.

A. Activities

This aim is further articulated around 7 specific objectives that we started addressing during the first 18 months of the project.

1. Development of an innovative approach for monitoring the effectiveness of Kala-Azar drug treatments in routine conditions. Most of the laboratory work executed in this project is based on clinical activities running in reference hospitals. However, the main challenge of VL control resides outside these well-equipped centres. Furthermore, solutions for the monitoring of the effectiveness of chemotherapy should also be found outside the laboratory. Therefore, simple clinical and epidemiological tools were developed and standardized based on the case definitions and retrospective cohort monitoring methodology. The monitoring system, as proposed by the project, was discussed during the clinical meetings and approved for testing in a few pilot/sentinel sites. First reports on treatment outcomes have been produced. Though still a learning process, the cohort reporting appears to be complicated, due to the many subclasses (treatment options and case definitions. Early treatment outcomes are satisfactory with >90% initial cure rate for MIL. It is too early yet for definite outcomes. A retrospective study on health seeking behaviour and treatment effectiveness on 150 patients treated for VL in Muzaffarpur district in 2008 showed cure rates of 75% with SSG, 84% with MIL and 94% with Amphotericin B.

2. Recruitment and follow-up of cohorts of patients treated with MIL in India and Nepal, provide complete clinical documentation and obtain pre-treatment samples as well as samples from treatment failure for validation of the above mentioned assays. So far, 285 and 199 VL patients were recruited for the project in India and Nepal, respectively. They consisted in patients without any previous history of VL, but we also included patients who came to the hospital for a 'retrospective' VL relapse (having been treated before/outside the project). Only the first category of patients can be considered for estimating MIL treatment effectiveness in a correct way and it is presently too early to produce conclusions on this issue. Our clinical partners isolated a large number of clinical isolates, 166 in India and 66 in Nepal, that will be of great importance for

the project and future research on *L. donovani.*. Among them, 31 isolates were obtained from 'retrospective' VL relapse cases. So far, we do not have data on the *in vitro* MIL-susceptibility of these isolates and this will be analysed in priority in the next months. As a kind of baseline laboratory analysis, 8 Nepalese and 8 Indian clinical isolates sensitive or resistant to SSG (parasite population background expected in the region) and obtained before the onset of MIL chemotherapy in the region were submitted to the biological assays mentioned above. Our data indicated the absence of resistance to MIL in the tested baseline sample. However, the observation of a few isolates showing a lower (down to 5 times) susceptibility to MIL deserves special attention and highlights the need for a close monitoring of the situation now that MIL has been implemented. Our laboratory work on the experimental induction of MIL-resistance on *L.donovani* strains from the region, which took only 31 weeks for 2 Nepalese strains, together with the observation of few less susceptible natural strains in India indicates that it is probably only a question of time before the first MIL-resistant clinical isolates will emerge among patients.

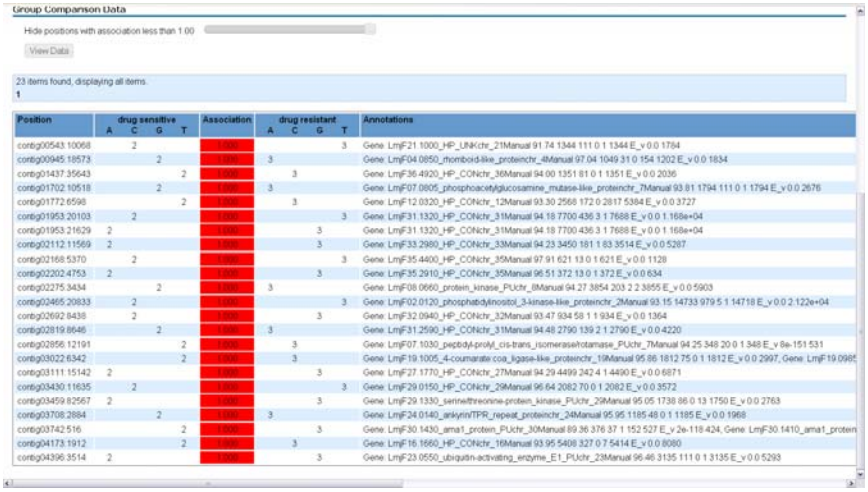

Fig.1. The 2 extreme (but bridged) poles of Kaladrug-R: clinical attention to VL patients in the Indian sub-continent and genome sequencing of clinical *L. donovani* strains revealing drug-resistant specific genetic features. Together with clinical and epidemiological tools, laboratory tools derived from this basic knowledge should contribute to a better surveillance of drug resistance in the region.

3. Development of new tools for the assessment of drug resistance in *L. donovani* parasites. Biological assays were standardised for testing the *in vitro* susceptibility to 3 drugs: (i) pentavalent antimonials (SSG) that showed decreased effectiveness in the region, (ii) miltefosine (MIL), the drug recommended for implementation in the frame of the Kala-Azar elimination program and (iii) Paromomycin (PMM), a drug which is currently in phase IV. Molecular tools for the detection of drug resistance require the identification of molecular markers. So far, this exploration was essentially done in the frame of SSG-resistance, because of the availability of clinical isolates showing naturally developed *in vitro* SSG-resistance (and thus not *in vitro* induced resistance). Studies of pre-defined targets (based on previously acquired knowledge) provided two avenues for potential exploitation. On one hand, the expression of several genes showed to be increased in SSG-resistant strains and the set of altered genes distinguished 2 populations of parasites, suggesting that parasites could follow different ways to become SSG resistant. On the other hand, we found that the fluidity of the parasite's membrane was higher in resistant parasites as compared to sensitive counterparts. This could be related to a difference in lipid composition. As an alternative way to identify molecular markers of resistance, a global approach based on full genome sequencing was followed. First, a high quality draft of the *Leishmania donovani* genome sequence was generated. A high quality draft of the *Leishmania donovani* genome sequence was generated. The high-throughput Solexa sequencing technology demonstrated its huge and unprecedented potential for diversity studies of parasites: we sequenced the whole genome of 18

clinical isolates from India and Nepal, characterised by different sensitivities to antimony and observed 6,242 high-confidence SNPs. This major breakthrough is pivotal for all experimental workpackages, especially for population genetics studies as well as for the detection of SNPs that might be interesting for the detection of resistance. Interestingly, of all the coding SNPs discovered this far, 14 have been identified where the one SNP allele is present in the entire SSG-susceptible group and a different SNP allele is the most frequent in the SSG-resistant group. Furthermore, by segregating the strains into those originating in cured (9) and non-cured (9) patients (non-responders, relapsed, dead or in follow-up), SNPs differing between the groups were detected. Interestingly, new genetic diversity elements were also encountered through full genome sequencing: each strain showed to be characterised by a unique ploidy pattern and we suspect that the increase in copy number of specific chromosomes might play a role in the phenotype polymorphism of the studied parasites. The same approach will be applied as soon as possible to the analysis of MIL-resistant isolates (experimentally induced or clinical isolates from relapsing patients). From all the work described here above, 4 tools for the assessment of drug resistance could already be assembled and one of them (MIL susceptibility in promastigotes) is already standardised and used in a clinical setting of the consortium.

4. Exploration, in experimental conditions, of the pathways leading to parasite resistance to Paromomycin. This activity was only started recently and results are very preliminary. Analysis of 8 Indian strains, most of them SSG-resistant, showed that they were not cross-resistant to PMM. Experimental induction of PMM-resistant strains is in progress and we expect that in the next months we will obtain the first resistant laboratory strains for further analysis.

5. Building models to understand the dynamics of the past spread of parasite SSG resistance as a model for resistance to MIL or future drugs. Before building a mathematical model on the emergence and spread of drug resistance, we had first to elaborate a basic transmission model for anthroponotic VL. This model can be considered as a 'première' in the epidemiological panorama of Kala-Azar. We use an SIRS type model (susceptible-infected-recovered-susceptible) described by ordinary differential equations for the dynamics of kala azar in the human and fly population. Our model considers various infection outcomes, like asymptomatic and symptomatic infections, post kala-azar dermal leishmaniasis (PKDL), and co-infection with HIV. After estimating model parameters, using data from another EC-funded project (FP6, Kalanet), the model will be used to study and predict the effects of the different intervention strategies and the risk of an emerging drug resistance.

6. Study of the impact of drug resistance on the parasite fitness.
Leishmania donovani expertly survives in two 'hostile' environments, the macrophage of the mammalian host and the sand fly. The parasite possesses a unique redox system to protect against stresses and macrophage manipulating skills to prevent outbursts of reactive oxygen and nitrogen species by the mammalian host defence mechanisms. Treating the patient, e.g. with sodium stibogluconate (SSG) that was used for over 70 years in the Indian subcontinent, imposed another more artificially imposed stress on the parasite. To evaluate how *L. donovani* copes with these stresses and to study their influence on the parasite's survival throughout the life cycle, we compared the proliferation rate, tolerance to various stresses and infection capacity of 3 Nepalese *L*. *donovani* strains in several *in vitro* and *in vivo* models. The two strains that reached a higher parasite density in the stationary phase during promastigote growth, showed a higher rate of metacyclogenesis, attained a higher *in vitro* infection level at 24 hours post infection and a marked higher and more persistent *in vivo* parasite load in mice all share a SSG-resistant phenotype. We have also data showing that primary macrophages infected with SSG-resistant parasites produce 3-4 fold more IL-10 (known disease promoting cytokine) than the sensitive ones. These observations might indicate an increased fitness of SSG-resistant *L. donovani*, which would make it one of the first organisms showing an increased fitness after acquiring resistance in stead of the usual fitness cost. This is probably due to the unique combination of a highly adaptive parasite and a drug that mainly relies on the immune system of the host. These will be verified on a larger panel of strains during the next months.

7. Getting research results into policy at regional level and disseminating the generated knowledge and the validated tools in other regions in the world endemic for leishmaniasis.
From the very beginning of the project, our aim has been to embed our activities in the frame of the running Kala-azar elimination programme (KAEP), but also to promote collaboration with other running research consortia. This determined 2 complementary strategies of dissemination. On one hand, we involved Indian and Nepalese representatives of the KAEP in our plenary coordination meetings and informed relevant stakeholders at WHO/TDR, WHO/SEARO, Ministries of health in India and Nepal about the KALADRUG project. This will be essential to disseminate major findings and tools and get an impact on the health policy at regional level. On the other hand, a close interaction is established with the 2 other FP7 consortia active in the frame of chemotherapy. We already produced together a joined review presenting the complementarity of our respective research approaches (protecting existing drugs vs developing new ones) and are collaborating for the organisation of the EC Conference on Neglected Protozoan Diseases (Paris, September 2010) that will allow to further network these consortia. Our website is operational (www.leisrisk.net/kaladrug) and regularly updated; it contains among others, general information about the project, an agenda with major dissemination milestones, an overview of our publications and presentations in congresses and all our SOPs (also available outside the consortium).

B. Expected final results and their impact

By providing knowledge and tools relevant for monitoring the effectiveness of the existing few drugs, present project will contribute to their 'protection' and establish the bases for their longer-term and more rational use. As such, it will have an impact on one of the major consequences of neglect. Our clinical and epidemiological work should contribute to (i) an early identification (in clinical settings as well as in peripheral health centres) of the persons at higher risk of treatment failure and (ii) a more rational therapeutic attitude. This would have a direct impact on treatment costs. By creating a network of institutions from Europe and the Indian sub-continent, covering a broad range of expertises (from genomics to clinical and epidemiological research), our activities will strongly support the KA elimination programme. This will be further promoted by the inclusion of 2 National managers from India and Nepal in the consortium itself. By deciphering the molecular mechanisms leading to drug resistance and providing the respective detection tools and by understanding the epidemiological dynamics of its emergence and spreading, our consortium will support control programmes relying on chemotherapy (among other methods), like the Kala-azar elimination programme. By focusing on 3 drugs (SSG, the previous first-line drug; MIL, the recently introduced one; PMM, possibly the next one), we will learn the lessons from the past, have a direct impact on the present and anticipate the future. We will here evaluate tools and strategies that will be transferred to the elimination programme, which will be facilitated by the presence of its representatives in our consortium. Through the development and evaluation of clinical tools for monitoring treatment effectiveness, we will have an impact on the therapeutic attitude of physicians. By adapting them to reference hospitals and peripheral health centres, this impact will be broad. The tools we plan to develop for the diagnosis of drug resistance are not devoted to be used at individual level only, but as well at the community level. Through a special workpackage, these laboratory tools will be simplified as much as possible. By integrating a leader institution in genome sequencing in a multi-disciplinary project with a particular emphasis on clinical and epidemiological research, present consortium will favour post-genomic translational research. Impact could be far beyond the specific objectives of present project (drug resistance, markers for population genetics, training of analysts closer to the field): e.g. comparison of the *L.donovani* genome sequence with that of other species might highlight interesting targets for new drugs or vaccines. Furthermore, the knowledge (here gathered) on how this genome can be 'modelled' under drug pressure will be an additional support to future studies.

C. Contact information project coordinator

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