Visceral leishmaniasis – malaria co-infections

van den Bogaart, E.

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The challenge of co-infections and their biological implications

The term ‘co-infection’, or alternatively ‘concomitant infection’, describes the simultaneous infection of a host by multiple pathogens. In its broadest sense, the term applies to all cases of co-existence among genetically different microorganisms, including members of the same species (e.g., those belonging to a different strain or population), for which the term of mixed infections is often preferred.

In nature, co-infections are the rule and this has been recognized since the earliest recorded times, as confirmed by the discovery of eggs from multiple helminth species in human coprolites and other human remains from prehistoric sites. Little has changed since then. Helminth co-infections continue to affect an estimated 800 million people worldwide, mainly in developing countries, while the true prevalence of co-infection is likely to exceed one sixth of the global population, with co-infections outnumbering single infections in many communities. Many of these co-infections involve globally important diseases, such as acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus (HIV), tuberculosis (TB) or malaria, which combine to devastating effect. In 2014 alone, for example, over 1 million lives were lost to HIV, one fifth of whom due to concomitant TB, the leading cause of death among people living with HIV.

As with any single infection, the epidemiological pattern of co-infections relies on a combination of ecological and biological factors, which shape transmission dynamics both at the community and the individual level. Competition for space and resources structures pathogen communities, selecting species that are better suited for survival in certain environments and promoting biodiversity, whereas host-mediated interactions drive variation in susceptibility and infectiousness within and between individual hosts. It is a truism that a host harboring any infectious agent is not the same as one that is uninfected, just as a host harboring bacteria is not the same as one harboring parasites or viruses. From the moment a host comes in contact with any infectious agent, its immune system begins to mount a characteristic array of responses that are qualitatively different for each pathogen. This diversity of immune responses is achieved through the concerted action of specialized cells and their cytokines that drive polarization of adaptive immunity into either cell- or antibody-mediated responses. Because these immune responses tend to be mutually exclusive (they antagonize each other’s actions by blocking polarized maturation of the opposite cell type or its receptor functions), any pre-existing condition able to trigger a defined cytokine milieu can potentially influence the response to a second stimulus. In other words, cytokines secreted in response to one pathogen may act synergistically, antagonistically or independently with those elicited by another pathogen, enhancing (cross-immunity), suppressing (immune-suppression) or not at all affecting the immune response to each of the two pathogens. Most of inter-microbial interactions result in immune-suppression (representative is the case of individuals developing Burkitt’s lymphoma as a result of their reduced resistance to the Epstein Barr virus following exposure to chronic malaria), but examples of negative interactions between concomitant pathogens abound in wild life as well as in experimental infections.

To name just a few, infections with Plasmodium falciparum appear to be suppressed in patients...
co-infected with measles or influenza viruses,\textsuperscript{25} while the presence of an ongoing disease, such as malaria or schistosomiasis protects the host from being re-infected with other plasmodial or schistosome species, respectively.\textsuperscript{26-28}

Not all factors affecting the course and outcome of co-infections are immunological or host-derived. Changes in the microenvironment as a result of the first infection can indirectly affect the fitness of the second infectious agent,\textsuperscript{1} while a number of pathogen-associated molecules are known to directly modulate proliferation of certain co-existent micro-organisms (e.g., the \textit{Leishmania} surface molecule lipophosphoglycan induces transcription of HIV in CD4\textsuperscript{+} T cells).\textsuperscript{19} Timing represents another critical variable in determining the outcome of co-infection, as the nature of host-pathogen interactions varies according to the stage of infection.\textsuperscript{30} As an example, the outcome of dual infections with \textit{Babesia microti} and \textit{Trypanosoma brucei brucei} in mice varies according to when the piroplasm is administered in relation to the trypanosome, with an inhibitory effect that intensifies as the time between co-infection increases.\textsuperscript{31}

Despite the widespread acceptance that different organisms co-existing in the same hosts can, and do, influence one another directly or indirectly, health workers and microbiologists seldom consider more than a single organism at a time.\textsuperscript{1} Literature describing the extent and impact of co-infections remains scanty\textsuperscript{1,8} and much of the relevant scientific evidence continues to be generated under carefully controlled laboratory conditions, in which pathogenic infections typically occur in isolation. Several reasons may account for this contrived approach to reality. The first is that the mechanisms underlying co-infection dynamics are complex to understand, requiring an integrated approach that combines ecological, biological and immunological variables.\textsuperscript{1} The second is that diagnosing a co-infection can be challenging in the field, as most infections lack pathognomonic signs\textsuperscript{32-35} and can give rise to serodiagnostic cross-reactivity.\textsuperscript{36} Clinicians usually follow a differential approach in establishing their diagnoses, making use of diagnostic procedures that hardly detect more than a single infection at a time. All this results in a systematic underestimation of co-infection burden, particularly for those conditions that do not result in an increased mortality risk.\textsuperscript{35}

Recent studies on the epidemiology of poly-parasitism, however, have provided compelling evidence that co-infections are ubiquitous and no longer represented by the one host-one pathogen paradigm, advocating for a new way of conceiving host-pathogen interactions and designing disease control programs.\textsuperscript{37}

**Visceral Leishmaniasis and Malaria, the diseases**

Visceral leishmaniasis (VL) and malaria are two severe infectious diseases caused by parasites of the genus \textit{Leishmania} and \textit{Plasmodium}, respectively. Endemic in large areas of the tropics and subtropics, the two diseases share a similar vector-borne transmission, with female insects spreading infection by feeding on the blood of vertebrate hosts. Sand flies of the genus \textit{Phlebotomus} and \textit{Lutzomya} are responsible for transmitting VL in the Old and in the New World, respectively,\textsuperscript{38} whereas \textit{Anopheles} mosquitoes act as malaria vectors throughout all endemic regions.\textsuperscript{39}

Visceral leishmaniasis, also known as kala-azar, black fever or Dumdum fever, presents as one
and the most severe of several clinical syndromes associated with human leishmaniasis; the other ones being (diffuse) cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis (MCL) and post kala-azar dermal leishmaniasis (PKDL). Cutaneous leishmaniasis is the most common form of the disease and presents with skin lesions on exposed parts of the body, which may heal spontaneously leaving disfiguring scars. In MCL, patients suffer from progressively destructive ulcerations of the mucosa, extending from the nose and mouth to the pharynx and larynx. These lesions are not self-healing and usually appear months or years after a first episode of CL. Visceral forms of leishmaniasis arise as parasites disseminate throughout the reticuloendothelial system and invade internal organs, particularly liver, spleen and bone marrow. These syndromes are typically fatal if left untreated. After treatment, VL may progress to PKDL, a dermatological sequel that is frequently observed in Sudan and more rarely in other East African countries and on the Indian subcontinent.

As many as 21 Leishmania species are pathogenic for humans, with clinical manifestations depending on complex interactions between the virulence characteristics of the infecting Leishmania species and the immune responses of its host. As a result, different species generally cause different clinical forms of the disease (e.g., members of the Leishmania tropica and Leishmania mexicana complexes usually cause CL, whereas Leishmania braziliensis is the etiological agent of most MCL cases), but a single Leishmania species can produce more than one clinical syndrome, and each syndrome can be caused by multiple species. Accordingly, several parasites causing CL can visceralize (e.g., L. tropica), but only two species routinely do so (Leishmania donovani and Leishmania infantum), and these are the causative agents of most human VL worldwide.

In contrast to the complex etiology of leishmaniasis, human malaria is caused by six species of Plasmodium only (P. falciparum, P. vivax, P. malariae, P. ovale curtisi, P. ovale wallikeri and P. knowlesi), of which P. falciparum and P. vivax are by far the most common. P. vivax is the most frequent and widely distributed cause of recurrent malaria, causing almost half the cases of malaria outside Africa. By contrast, P. falciparum, the deadliest of the six Plasmodium species, predominates across sub-Saharan Africa, where it accounts for the large majority of malaria infections and deaths. The other human malaria parasite species are less common, with P. ovale causing less than 5% of malaria cases, primarily in sub-Saharan Africa, and P. malariae being widespread but rare. Both species, along with most P. vivax infections, cause a milder and self-limiting form of malaria, known as ‘benign malaria’, that opposes to the potentially severe syndromes caused P. falciparum and P. knowlesi. The latter is a primate malaria parasite mostly found in South-East Asia that leads to severe malaria in about 2% of cases. It causes malaria in long-tailed macaques (Macaca fascicularis), but it may also infect humans, accounting for up to 70% of malaria cases in certain areas of South-East Asia.

Epidemiology of Visceral Leishmaniasis and Malaria

To date, a total of 80 countries and 1 territory on 5 continents reported endemic VL transmission (Figure 1). Of these, Bangladesh, India, Brazil, Sudan, South Sudan and Ethiopia harbor more than 90% of global VL cases. Overall, official case tolls total more than 58,000 cases of VL
per year, but this figure is believed to be a substantial underestimate of the actual disease incidence, due to poor reporting and detection of VL cases. Global estimates of VL occurrence and its underreporting levels across different endemic foci indicate that approximately 0.2 to 0.4 million people are affected by the disease each year, of whom 20,000 to 40,000 die, ranking VL as the second largest parasitic killer in the world after malaria.

Worldwide, malaria continues to take a huge toll on human health, both in terms of morbidity and mortality. Despite increasingly successful efforts to eliminate the disease, 97 countries still reported ongoing malaria transmission in 2014 (Figure 2), the majority of which (80%) occurred in just 18 countries across sub-Saharan Africa. Overall, an estimated 198 million malaria cases were reported in 2013 alone, resulting in 584,000 deaths, of which 78% were estimated to occur in children under the age of five. Whereas for *P. vivax*, three Asian countries (India, Indonesia and Pakistan) accounted for more than 80% of estimated cases, the global burden of malaria mortality and morbidity was dominated by *P. falciparum* malaria in sub-Saharan Africa: the Democratic Republic of the Congo and Nigeria together accounted for 39% of the global total of estimated malaria deaths and 34% of cases in 2013.

The epidemiological pattern of the two diseases varies upon the endemic region, reflecting differences in the parasite species, the ecology of its transmission sites and the individual susceptibility of its hosts. In the Mediterranean basin and in Latin America for example, where VL is caused by *L. infantum* (also known as *Leishmania chagasi* in South America), the
prevalence of the disease is low (≤1%) and transmission is anthropozoonotic, with domestic and wild canids acting as main reservoirs of infection.\textsuperscript{42,51} Peridomestic sandflies living in the yards of rural villages or suburbs are largely responsible for spreading the disease, although other routes of transmission, including parenteral, congenital and sexual exposures, have been occasionally reported.\textsuperscript{52,53} Children and an increasing rate of immunosuppressed individuals, such as HIV-infected patients\textsuperscript{54} and patients under immunosuppressive therapy,\textsuperscript{55,56} are mostly affected, whereas infected adults typically carry the parasite asymptomatically – between 2% and 40% of the population living in the Mediterranean region are carriers of \textit{Leishmania} parasites.\textsuperscript{57,58}

In contrast, in East Africa and on the Indian subcontinent, VL affects all age groups. Caused by \textit{L. donovani} sensu stricto, the disease is usually considered to be an anthroponosis, with PKDL cases being the putative reservoir between epidemic cycles.\textsuperscript{51} The landscape epidemiology of \textit{L. donovani} transmission differs across the Asian and African continents:\textsuperscript{42} on the Indian subcontinent, most foci are in long-established villages with sedentary populations, whereas in East Africa, migratory populations are also at high risk, including cattle herdsmen and villagers displaced by drought and warfare.\textsuperscript{42} Migration, along with lack of control measures and HIV co-infection, have been recognized as being the main factors driving the increased incidence of VL worldwide\textsuperscript{41,59,60} and the severe outbreaks that have occurred in the past, like in South Sudan, where in a context of civil war and famine, VL killed an estimated 100,000 people out of a population of 280,000 between 1984 and 1994.\textsuperscript{61} Malaria transmission is also widely heterogeneous around the world, both across and within

Figure 2. Global distribution of malaria stratified by endemicity class, 2010\textsuperscript{220} (adapted with permission).
countries, reflecting differences in the density, longevity, biting habits, and efficiency of the mosquito vector. Those that are long-lived and robust to environmental change, occur in high densities in tropical climates, breed readily, and preferentially bite humans are the most effective in transmitting the disease — e.g., the *Anopheles gambiae* complex in Africa. In most of Asia and South and Central America, where transmission is mainly low and seasonal, *P. falciparum* and *P. vivax* malaria have roughly an equal prevalence. In these areas, most people typically receive one or fewer infectious bites per year — the so-called entomological inoculation rate (EIR). By contrast, transmission intensities are much higher in much of sub-Saharan Africa and in parts of Oceania. Here, EIRs can be as high as 1,000 per year and transmission occurs all year round, resulting in a pronounced malaria morbidity and mortality among children and pregnant women, while the rest of the population has become immune. In the sub-Saharan region from Senegal to Sudan, transmission is intense but largely confined to the 3-4 month rainy season. In these areas where malaria transmission is unstable (low and erratic), full protective immunity is not acquired, and symptomatic disease can occur at all ages. In such settings, changes in environmental, economic, or social conditions — e.g., heavy rains after drought or large population movements — together with a breakdown in malaria control and prevention services (often because of armed conflicts) can result in epidemics, with substantial mortality in all age groups. Endemic *P. vivax* infections occur well beyond the tropics and subtropics with the exception of most of West and Central Africa, where the absence of the Duffy gene amongst the local populations prohibits the parasite from infecting them. Strong and complex linkages associates VL and malaria to poverty, as confirmed by the high disease prevalence among marginalized communities, in countries that are among the least developed in the world or in the poorest regions of so-called ‘middle-income’ countries (such as Bihar State in India). Poor housing conditions and environmental sanitation, along with lack of protective bed nets, increase the risk of acquiring the infection (sand flies breed in cracks of mud-plastered houses and moist soils and are attracted to crowded households), whereas malnutrition and immunosuppression increase the risk that an infection will progress to clinically manifested disease and result in severe complications.

### Biology of Visceral Leishmaniasis and Malaria

#### Parasite life cycle

Both VL and malaria are caused by dixenic protozoan parasites that cycle between an insect and a vertebrate host (Figures 3 and 4). Inhabiting the arthropod vector as free-living organisms, *Leishmania* and *Plasmodium* parasites have developed unique adaptive mechanisms that enable them to survive as obligate intra-cellular organisms in the mammalian host, where they infect professional phagocytes (such as macrophages) and hepatocytes/erythrocytes, respectively.

The cycle begins with inoculation of motile forms — *Leishmania* flagellated promastigotes and *Plasmodium* sporozoites — into the dermis of the vertebrate host. Here, in the case of *Leishmania*, the metacyclic promastigotes from the initial inoculum (or those that have been released from infected neutrophils) are quickly internalized by tissue-resident phagocytes (macrophages and Langerhans cells) and by inflammatory mono-cyte-derived dendritic cells that infiltrate from
the blood to the inoculation site, where they may facilitate parasite trafficking to the draining lymph nodes. Upon phagocytosis, metacyclic promastigotes transform into round-shaped, aflagellated amastigotes (mammalian stage) that undergo replication by binary fission, eventually overburdening the infected cell and causing its rupture. The so-released amastigotes proceed then to infect other surrounding phagocytes, perpetuating the infection to the reticulo-endothelial system via the lymphatic and blood circulations and (in case of visceral disease only) causing infiltration of the bone marrow, spleen and liver.

Conversely, the *Plasmodium* sporozoites travel to the liver within few minutes from inoculation, invading the hepatocytes where they begin to multiply. After about a week, the liver schizonts burst, releasing into the bloodstream thousands of merozoites that infect the erythrocytes and begin the asexual cycle. Inside the red blood cells, parasites undergo several developmental stages (ring form, trophozoite and schizont), marked by a progressively increasing size, alterations in cell membrane composition to facilitate importation of nutrients (inserting new parasite-derived proteins and exposing cryptic surface antigens), and accumulation of malaria pigment to dispose of the toxic heme waste product. By the end of the intra-erythrocytic lifecycle, when most of the red blood cell contents have been consumed and several nuclear divisions have taken place, the erythrocytic schizont is ready to burst and release between 6 and 30 daughter merozoites, each of which can invade erythrocytes and repeat the cycle. Illness starts 6-8 days after parasites have emerged from the liver, when total asexual
parasite numbers in the circulation reach about 50/μL of blood (roughly 100 million parasites in the blood of an adult). In *P. vivax* and *P. ovale* infections, some intra-hepatic forms remain dormant as hypnozoites for between 2 weeks and more than a year (depending on geographic origin), before awakening to cause the relapses that characterize these infections.

Few days after the peak of asexual parasitaemia, some blood-stage parasites develop into longer-lived sexual forms (gametocytes) that, upon uptake by feeding anopheline mosquitoes, initiate sporogony in the insect midgut and produce infecting sporozoites.

Similarly, sandflies become infected by ingesting *Leishmania*-harboring cells during blood meals. After entering the sandfly midgut, amastigotes undergo several morphological transformations that culminate with the development of metacyclic promastigotes, ready to infect new vertebrate hosts.

**Hemozoin**

During its intra-erythrocytic cycle, the malaria parasite feeds on host cell hemoglobin, liberating toxic free heme that is detoxified via the malaria pigment, hemozoin (HZ). Hemozoin, a yellowish, optically birefringent crystal, structurally identical to synthetic β-hematin, is composed by heme dimers that are assembled in the digestive food vacuole of the parasite, where they accumulate and persist until schizont rupture. It is estimated that at the end of the intra-erythrocytic cycle, up to 80-90% of all heme iron is localized within the parasite food vacuole, resulting in as much as 0.2-2.0 grams of HZ being produced by *P. falciparum* after each cycle, assuming 1-10% parasitaemias.

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Figure 4. Life cycle of *Plasmodium* parasites.
At the end of the asexual cycle, when the infected erythrocytes rupture, daughter merozoites are released into the blood stream along with HZ crystals, that are rapidly engulfed by the phagocytic cells in the peripheral blood and in the reticulo endothelial system. Here, HZ persists undigested, withstanding phagosome degradation and possibly outliving the cells in which it resides, as demonstrated by the persistence in the peripheral circulation of pigment-containing monocytes and pigment-containing neutrophils, and by the accumulation of HZ in various organs (including liver, spleen, and bone marrow) after extended periods of up to 270 and 196 days post-infection. Importantly, these studies noted that HZ appeared to be actively redistributed, both between organs (e.g., from liver to spleen) and inside organs (e.g., between red and white pulp in the spleen).

For a long time, HZ has been considered a metabolically inert side product of hemoglobin digestion. Recent research, however, has led to the recognition that HZ acts as a key factor in malaria-associated immunopathology, although an exact role is yet to be defined. The reported immunomodulating effects of HZ include: (1) changes in the production of pro- and anti-inflammatory cytokines, chemokines, and other effector molecules; (2) reduced phagocytic activity and oxidative burst in HZ-laden monocytes/macrophages; (3) changes in nitric oxide (NO) release, whose up- and down-regulation may contribute to the pathogenesis of severe malaria and malaria-mediated immunosuppression, respectively; (4) activation and migration of neutrophils; (5) induction of matrix metalloproteinase 9, which causes increased inflammation and extravasation of blood cells; (6) down-regulation of cyclooxygenase-2 and prostaglandin E2, thought to contribute to cerebral malaria and anemia; (7) changes in the activation status of innate immune responses, that range from Toll-like receptor-9-mediated activation of dendritic cells to impaired maturation of dendritic cells and reduced expression of cell-surface markers on monocytes; (8) impaired T-cell and B-cell responses. Some of these studies examining the effect of HZ on host immune cells have resulted in conflicting evidence, as it is the case for activation/suppression of dendritic cells, which may be partly explained in terms of dose-dependency. Specifically, smaller quantities of HZ, at the beginning of the infection, may act by activating dendritic cells, whereas larger amounts may be associated with a suppressive effect. Finally, some doubts remain as to whether these immunomodulating effects are caused by the HZ alone or by HZ-associated lipids, proteins or Plasmodium DNA.

**Immunopathogenesis of Visceral Leishmaniasis and Malaria**

As for most successful pathogens, the remarkable ability of *Leishmania* and *Plasmodium* parasites to evade host immunity and exploit its machinery is the key to their success and pervasiveness. Immunological elusion, achieved through the controlled suppression of innate and cell-mediated immunities in the case of *Leishmania* spp. or via epigenetic switch and sequestration of infected erythrocytes in the case of *Plasmodium* spp., ensures the parasite may escape host clearance and combines with pathogenic manipulation of host pathways to preserve parasite fitness. While improving the chance that the parasite may complete its life cycle, these virulence determinants come at a cost to the host, who suffers the pathological
consequences of the infection and of the immune response that ensues.\textsuperscript{117,118}

It is generally accepted that in the presence of an infection, naïve CD$^+$ T helper cells (Th)0 differentiate into either Th1 or Th2 lymphocytes, that – upon release of specific cytokine patterns – drive the immune system towards cell- or antibody-mediated responses, respectively. CD$^+$ Th1 cell-mediated responses involving type-1 cytokines (e.g., interferon-gamma (IFN-γ)) are ideally suited for clearing intracellular protozoa through activation of specific effector cells, whereas extracellular pathogens are predominantly controlled by CD$^+$ Th2 cells, through a network of type-2 cytokines (e.g., interleukin (IL)-4 and IL-5) that promote expansion of antibody-producing B cells.\textsuperscript{119} In addition, CD$^+$ Th0 cells may also differentiate into either regulatory T cells (Treg) or Th17 lymphocytes. Treg cells act by suppressing type-1 and pro-inflammatory responses through IL-10 and transforming growth factor-beta (TGF-β) signaling, whereas IL-17 and IL-6 released by Th17 lymphocytes promote inflammation (Figure 5).\textsuperscript{120}

Figure 5. Summary of the major CD4$^+$ T cell differentiation pathways and related cytokines (boxes),\textsuperscript{222} with their impact on VL and malaria diseases.\textsuperscript{222} Cross-regulating cell- and cytokine-mediated mechanisms shape the immune reaction across the four response arms and prevent from deleterious polarizations. Skewing of the initial T cell differentiation towards a T helper cell (Th)1 response is pivotal for clearing \textit{Leishmania} or early \textit{Plasmodium} infections, but it requires a T regulatory (Treg) counterbalance to mitigate their clinical course. Failure to activate Th1 cells, e.g., due to the inhibiting effect of IL-10 and its producing cells, may result in the uncontrollable proliferation of \textit{L. donovani} and \textit{P. falciparum} parasites, the latter causing severe complications under a pro-inflammatory milieu (adapted with permission).
In human VL, manifestations range from asymptomatic infection to progressive and potentially fatal visceral disease, pending on the host’s immune status. Clinically silent infections require robust cell-mediated immune responses, mounted upon a network of Th1 cytokines (Figure 5) and enforced by classically activated macrophages through a battery of microbicidal mechanisms, that include release of pro-inflammatory cytokines, activation of natural killer (NK) cells, up-regulation of inducible nitric oxide synthase (iNOS), and production of nitrogen and oxygen radical species. The observation that peripheral blood mononuclear cells (PBMCs) isolated from individuals with asymptomatic or subclinical VL infections proliferate and release IL-2, IL-12 and IFN-γ in response to *L. donovani* stimulation confirms the central role played by the Th1 immunity in parasite killing. Conversely, failure to control *L. donovani* infections has been associated with a profound unresponsiveness of PBMCs and T cells to *L. donovani* and an increased release of IL-10 by Treg cells (Figure 5). Just this cytokine, possibly in combination with IL-4, IL-13 and IgE, is believed to act as one of the main immunosuppressive factors that drives replication of *Leishmania*, as confirmed by its progressive decrease in VL patients recovering from disease and the ability of anti-IL-10 antibodies to restore antigen-mediated proliferation of PBMCs and IFN-γ release in cells isolated from VL patients. Other potentially immunosuppressive agents reported during human VL include soluble IL-2 and IL-4 receptors, IL-6, IL-13, IL-10, and TGF-β, as confirmed by their increased serum levels during symptomatic disease.

In addition to confer sterile protection against VL, cell-mediated immunity also plays a key role in controlling malaria infections, both at pre- and intra-erythrocytic stages. Early type-1 responses dominated by IFN-γ signaling have long been established as driving the pre-erythrocytic immunity against both murine and human malaria (Figure 5), as demonstrated by a wealth of *in vivo* and *ex vivo* evidence. By contrast, its protective role against blood-stage parasites has only recently been recognized, and is believed to be accomplished via macrophage activation. The observations that infected erythrocyte-specific IFN-γ responses correlate with protection against parasitaemia in animals as well as in patients, and that children with prior mild malaria display an enhanced ability to express iNOS over children with prior severe malaria converge towards an IFN-γ-led anti-parasitic effect. Consistent with this view is also the finding that tumor necrosis factor-alpha (TNF-α) and reactive oxygen intermediates induced by *Plasmodium* spp. contribute to the control and elimination of blood-stage parasites. However, whilst early activation of Th2 cells is deleterious for infection clearance and correlates with malaria severity, its involvement during the erythrocytic stage is essential for counterbalancing the Th1 cytokines and triggering antibody-mediated responses (Figure 5), important for protection against malaria. A prominent role in switching from Th1 to Th2 responses is attributed to IL-10, whose anti-inflammatory properties act by limiting the damage inflicted on normal tissues by excessive Th1 and pro-inflammatory responses, as seen in the immunopathology of severe malaria. Whilst uncontrolled release of IFN-γ, TNF-α and IL-12 caused by parasite-mediated inhibition of IL-10 and TGF-β signaling have been firmly associated with an increased risk of developing cerebral malaria, low IL-10/TNF-α ratios are considered predictive of malarial severe anemia.
Clinical presentation of Visceral Leishmaniasis and Malaria

Following an incubation period that generally lasts between 2 and 6 months, but that can be as short as a few weeks or as long as several years, VL patients present with symptoms and signs typical of a persistent systemic infection. Fever, at times intermittent and usually associated with rigor and chills, malaise, weakness, night sweats, loss of appetite and weight are common manifestations of VL, whose onset is usually insidious or subacute, with slow progression of symptoms over a period of months. In rare cases, acute febrile illness can occur with rapidly progressive symptoms. Hepato-splenomegaly resulting from parasite invasion of the reticulo-endothelial system, along with anemia caused by the persistent inflammation state, hypersplenism and/or bleeding are frequently observed, usually in combination with pancytopenia and hypergammaglobulinemia. Lymphadenopathy is frequent in East Africa, but rare outside this region, whilst melanocyte stimulation and xerosis typically occurred (today this symptom is uncommon) amongst VL patients from the Indian subcontinent, causing the characteristic skin hyper-pigmentation that has earned the disease the name of ‘kala-azar’ (black fever in Hindi). As the disease advances, the risk of co-infections (such as pneumonia, diarrhea or tuberculosis) flaring up as a result of host immunosuppression increases, leading to potential misdiagnosis and worsened prognosis. Young malnourished children are most susceptible to developing progressive infection; those who present later in the course of the disease may present with edema caused by hypoalbuminemia, hemorrhage caused by thrombocytopenia, or growth failure caused by features of chronic infection. If the disease is left untreated, death usually occurs within 2 years as a result of massive bleeding (secondary to infiltration of the hematopoietic system), severe anemia, immunosuppression, and/or secondary infections. Importantly, infection with Leishmania spp. does not always equate with clinical illness, as infected individuals, documented with a positive skin test to Leishmania antigen (Montenegro test), can remain asymptomatic for life.

The non-specific nature of VL symptoms entails its differential diagnosis encompassing a variety of other infectious and febrile systemic illnesses, including malaria. In endemic areas, indeed, malaria is often the most common cause of fever, with characteristic spikes being described every day for P. knowlesi (quotidian malaria), every 2 days for P. vivax, P. ovale and less commonly P. falciparum (tertian malaria), and every 3 days for P. malariae (quartan malaria). In practice though, this periodicity is rarely seen, particularly for P. falciparum, and is most likely to occur if the infection is left untreated and becomes synchronous. Fever presents after an incubation period of about 2 weeks (range 6 to 40 days, depending on the parasite species), and is usually associated with a set of non-specific symptoms, such as chills, sweating, malaise, headache, body aches, nausea, vomiting and orthostatic hypotension. Mild jaundice may also develop, but it is rather uncommon in children, who instead suffer from a more frequent enlargement of the liver. In individuals at risk, like non-immune persons, children 6
to 36 months of age, immunocompromised patients (including splenectomized individuals) and pregnant women, *P. falciparum* malaria and more rarely *P. vivax* malaria may progress to severe disease, sometimes very rapidly, with manifestations depending on age. Severe anemia and hypoglycaemia are more likely to occur in children, whereas acute pulmonary edema, acute kidney injury, and jaundice are more common in adults. Coma (cerebral malaria) and acidosis, on the other hand, occur in all age groups. Manifestations of complicated malaria arise as the parasitaemia exceeds 2%, and mainly result from the ability of parasitized (and non-parasitized) erythrocytes to adhere to small blood vessels; a phenomenon known as cytoadherence that cause small infarcts, capillary leakage, and organ dysfunction. These syndromes can portend a grave prognosis, with fatal outcomes in nearly all cases that fail to receive treatment, and a mortality rate of 15% to 20% amongst patients properly managed in intensive care units.

**Treatment of Visceral Leishmaniasis and Malaria**

As an effective vaccine for VL or malaria has yet to be licensed, chemotherapy remains the mainstay for treatment and prevention of these two diseases (chemoprophylaxis for malaria only). Antimicrobial drugs selected upon local drug resistance patterns, treatment guidelines, tolerability, availability, and affordability are administered as part of the standard therapeutic approach in combination with supportive care to address concomitant anemia, hemorrhagic complications, malnutrition and secondary infections.

Pentavalent antimonials, the first-line treatment in most endemic areas since the 1940s, are toxic drugs that frequently cause adverse, sometimes life-threatening effects, such as cardiac arrhythmia and acute pancreatitis. Due to their high failure rates in some areas of the Indian subcontinent, their use as first-line treatment in these regions has been dismissed in favor of conventional amphotericin B. Amphotericin B has an excellent cure rate (up to 100%), but requires slow intravenous administration, that commonly cause infusion-related reactions – e.g., fever, chills, thrombophlebitis – and occasionally serious toxicity – e.g., hypokalemia, nephrotoxicity, myocarditis, and even death. Liposomal amphotericin B (AmBisome) – a relatively new drug that combines high efficacy with low toxicity – is currently the preferential treatment in high-income countries. Until recently, its use in developing countries was precluded by its high market price, but since it became available at a preferential pricing, the situation has partially improved. Currently, liposomal amphotericin B has been included as first-line VL treatment in East Africa in the 2010 revised World Health Organization (WHO) recommendations. Miltefosine, which was initially developed as an anti-cancer drug, is the first effective oral drug for VL. Registered for use in India since March 2002, the drug showed a final cure rate of 94-97% in India and of 85% in Bangladesh at the dose of 2.5 mg/kg/day for 28 days, while the results of clinical trials conducted in East Africa and Brazil are yet to be published (with the exception of one study conducted in north Ethiopia). Its main limitations include high cost, gastrointestinal adverse effects, occasional hepatic and nephrotoxicity, and teratogenicity (hence its preclusion to pregnant women). In addition, its long half-life also makes it vulnerable to rapid development of drug resistance, as confirmed by the high failure rates that are already
Paromomycin (formerly known as aminosidine) is a broad-spectrum aminoglycosidic antibiotic with good anti-leishmanial activity. Registered in India in 2006, the drug showed excellent efficacy and safety in a Phase III clinical trial conducted in India, and an efficacy of 84.3% in East Africa. Major advantages of this drug include its low cost and its good tolerability, while the need for a prolonged parenteral administration (three weeks) and for serum transaminase monitoring limits its desirability. Sitamaquine (WR-6026) is an oral 8-aminoquinoline drug at developmental stage that has completed phase II trials in India, Kenya and Brazil, where it showed cure rates ranging from 27% to 87%, and several cases of serious renal adverse events. Combination therapy is the suggested way forward to increase treatment efficacy, prevent the development of drug resistance, reduce treatment duration and perhaps decrease treatment costs. The association of sodium stibogluconate and paromomycin was found to be safe and effective in early trials conducted in India and East Africa, while drug combinations including liposomal amphotericin B and miltefosine have been studied in India and are currently under evaluation in Bangladesh and East Africa.

Artemisinin-based combination therapies (ACTs) are the cornerstone for the treatment of malaria nowadays. Recommended in 2001 by the WHO as the first-line treatment of uncomplicated P. falciparum malaria (severe P. falciparum malaria is treated initially with intravenously administered artesunate, followed by a suitable oral drug (combination)), these treatments have substantially contributed to the reduction of global malaria morbidity and mortality since their use became widespread approximately 10 years ago. In the S currently recommended ACTs, two active anti-malarial drugs with different mechanisms of action (one being an artemisinin derivative, such as artemether or artesunate) combine to improve treatment efficacy and minimize the spread of drug resistance, despite the first cases of reduced susceptibility have already been reported in South-East Asia. Artemisinin is a natural product that is isolated from Artemisia annua (Asteraceae), a medicinal plant that has been used for over a thousand years in China. Its semi-synthetic derivatives artesunate and artemether are the two most widely used oral artemisinins, available either alone or in combination. Amodiaquine, mefloquine, chloroquine and their historic parent compound quinine are an important group of 4-aminoquinoline drugs that act as blood schizonticidals against all Plasmodium species pathogenic to man, and as gametocytocidals against P. vivax and P. malariae in the case of mefloquine and quinine. Quinine is a quinidine alkaloid contained in the bark of the cinchona tree (Cinchona calisaya and Cinchona succirubra). Its use as an anti-malarial agent dates back as early as the 1600s, when malaria patients were cured with infusions of bark obtained from plants growing in the Peruvian Amazon. Nearly 400 years later, quinine remains an important drug for treating severe P. falciparum malaria (in the absence of i.v. artesunate), despite its toxicity particularly when used for extended periods of time. Sulfadoxine and pyrimethamine are two schizonticidals that act by inhibiting the tetrahydrofolate synthesis pathway of the malaria parasite. Their use in fixed-dose associations (Fansidar) produces synergistic effects sufficient to cure sensitive malaria strains. The radical cure of P. vivax and P. ovale requires primaquine, an 8-aminoquinoline that targets all stages of the Plasmodium life cycle (including hypnozoites), preventing late relapses. Primaquine
metabolites, however, cause severe hemolytic anaemia and methemoglobinemia in patients who are genetically deficient in glucose-6-phosphate dehydrogenase (G6PD),\cit{184} imposing a pre-screening requirement for G6PD deficiency that limits its use. Tafenoquine, a novel 8-aminoquinoline with tissue-schizonticidal activity, currently still remains in clinical trials.\cit{183}

**The search for new drugs**

Current therapeutic options for VL rely on a handful of drugs that suffer from major limitations, such as severe toxicity, high costs, difficult route of administration and increasing inefficacy due to emergence and spread of resistance. New, safer and more effective oral treatments are urgently needed to improve clinical resolution of the disease and to reduce its transmission rates across endemic areas. Unfortunately, the lack of a profitable market and effective mechanisms related to public health policy, financing, and drug discovery and development expertise and capacity has largely inhibited the pharmaceutical industry from investing in the development of new drugs for VL and other neglected tropical diseases.\cit{185} – during 1975-2004, only 21 (1.3%) out of 1556 approved drugs were specifically developed to address neglected tropical diseases.\cit{186} Recent changes in the drug discovery model for these diseases (product development and private-public partnerships) have dramatically improved their drug Research & Development landscape.\cit{185,187} Nevertheless, the current pipeline for anti-leishmanial drugs remains substantially weak, with few new chemical entities expected to enter the market in the coming years.\cit{188}

One of the main difficulties that hinders the systematic search for new anti-leishmanial compounds is the complexity of performing adequate phenotypic screening.\cit{188} The *Leishmania* life cycle is composed of two stages, the free living promastigote stage found in the vector, and the obligate intracellular amastigote stage that lives in the vertebrate host. Whilst promastigotes can be easily adapted for *in vitro* culturing and testing due to their axenic requirements, *in vitro* maintenance of the clinically relevant stage of disease necessitates a suitable host cell model that inevitably interferes with the standard high-throughput parasite viability assays. As a result, screening of potential anti-leishmanial compounds has relied for decades upon the use of promastigotes, and only more recently of axenic amastigotes, for which simple and efficient *in vitro* assays have been made available.\cit{189-195} However, lack of consistency between insect and mammalian stage drug susceptibilities has been frequently reported, emphasizing the need of testing against the intracellular amastigote stage already at primary hit-discovery screens.\cit{196,197} Assessment of anti-leishmanial activity against the vertebrate host stage traditionally relies on phenotypic assays, with major problems related to data quality and poor performance.\cit{198,199} With the advent of new technologies, however, it has now become possible to increase the throughput of these very labor-intensive assays. Two main methods are in use for the detection of intracellular parasites: plate-reader-based methods that rely on reporter constructs\cit{197-199} and automated high-content microscopy-based counting. Both platforms have radically improved the screening capacity for leishmaniasis,\cit{202-205} but bear the disadvantages of using transgenic *Leishmania* spp. in the case of reporter-gene assays or requiring a complex technology in the case of automated microscopy. This precludes their broad implementation and routine application to clinical isolates, compelling
the development of a simpler and more widely applicable assay.

Phenotypic screens are also key to the discovery of new anti-malarial drugs, as confirmed by the observation that only a minority of compounds currently in clinical testing had molecularly defined targets from the outset.\textsuperscript{206} The majority, on the contrary, are derived from phenotypic screening, with their pharmacological targets being identified only at a later stage. This renaissance of phenotype-based screening in the drug discovery against malaria has followed the recent advances in assay automation, image capture and analysis technology, which altogether have noticeably reduced the costs of assaying a compound against primary human erythrocytes infected with \textit{P. falciparum}. As a result, a total of ~6 million compounds have been screened to date, of which, excitingly, more than 25,000 have shown half-maximal (IC\textsubscript{50}) activity at approximately 1 $\mu$M or lower against \textit{P. falciparum}.\textsuperscript{207} This, along with the new therapeutic agents currently in development, highlights how dramatically improved is the malaria drug pipeline over the past 5 years. It should be noted, however, that most of the medicines under development act as schizonticidals. In an era where the malaria research agenda is set towards eradication rather than control, different approaches are required, including the use of drugs that block transmission and kill the dormant liver stages of \textit{P. vivax} and \textit{P. ovale}.\textsuperscript{206} In neither case, unfortunately, the biology can be perfectly reproduced in high-throughput screening formats, as confirmed by the absence of models for the early-stage gametocytes and the primate dormant forms.\textsuperscript{206} For these reasons, it is important that the investment in the primary biology continues so that screening against all phases of gametogenesis and primate hypnozoites, or even human cells infected with \textit{P. vivax}, can eventually be performed. New classes of drugs for such stages of malaria parasites would truly be transformative and accelerate the decline of malaria – a change that is urgently needed over the next 20 years.\textsuperscript{206}

\textbf{Co-infections with Visceral Leishmaniasis and Malaria}

Although concomitant infections by multiple pathogens are ordinary events in nature, many of their fundamental patterns remain undescribed, including the mechanisms that govern the type and magnitude of co-infections and their burden on human health.\textsuperscript{8} Interest in unraveling these critical issues has raised in recent years, but the focus has remained disproportionately confined to a small group of infections, at the expense of the global killers that thrive amongst the world’s poorest.\textsuperscript{8} In this respect, co-infections with VL and malaria are no exception. Despite the anything-but-negligible co-infection rates that have been sporadically reported across various African and Asian countries,\textsuperscript{207-211} the literature is silent on this subject, and there is virtually no information on how often these co-infections occur and with which consequences for the patient. Given the high burden that VL and malaria impose worldwide, this knowledge gap appears somehow paradoxical, particularly in light of the fact that all conditions apply for the two infections to co-occur and cross-interact in the same host (Table 1).

The first of these conditions requires VL and malaria to share part of their geographical and ecological distribution. Malaria is widespread across much of the tropics and subtropics and so is VL to a lesser extent, resulting in an extensive overlap throughout most \textit{L. donovani} foci and
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a part of *L. infantum* ones (Figure 6). Namely, people living in East Africa (Sudan, South Sudan, Ethiopia, Uganda, Kenya, Somalia and Eritrea) are at high risk of co-acquiring VL and malaria, as confirmed by the co-infection prevalences found amongst VL patients (20.8% and 6.4% in two studies conducted in east Uganda and 10.7% in Sudan), while a moderate to low risk exists for people inhabiting certain districts of Brazil and the Indian subcontinent (co-infection rates of 1.2% and 5.9% were found amongst Bangladeshi VL patients and Indian patients with fever and splenomegaly, respectively). The risk of co-acquiring VL and malaria is particularly high for children, who have not yet developed immunity to the diseases, and live in endemic communities where infected adults act as reservoirs of infection. Nomadic populations, and men who work in agricultural or pastoral settings are also at increased risk (particularly those who sleep outside or under acacia-balanite trees), due to the increased time spent outdoors and in proximity of vector breeding sites.

The current knowledge on the immuno-patho-genetic mechanisms of VL and malaria supports the view that the two parasites may interact with each other in the co-infected host, either directly

<table>
<thead>
<tr>
<th>Table 1. Selected features of VL and malaria forming the rationale for the co-occurrence and cross-interaction of these two infections.</th>
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<tbody>
<tr>
<td><strong>Visceral Leishmaniasis</strong></td>
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<tr>
<td>Endemic in East Africa, in the Indian subcontinent and in Latin America.</td>
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<tr>
<td>Transmitted by sand flies that breed and shelter in moist soils (cracks of plastered houses, termite hills, etc.).</td>
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<td>Transmission throughout the year, but higher during or shortly after the rainy season.</td>
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<tr>
<td>Incubation period averages 2 to 6 months.</td>
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<td>Children and young adults at higher risk.</td>
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<td>Poor housing, crowded households, HIV and malnutrition are main risk factors.</td>
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<tr>
<td>Parasites infect phagocytes of the spleen, liver, and bone marrow.</td>
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<tr>
<td>Suppression of cell-mediated immune responses by IL-10 predisposes to development of clinical disease.</td>
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Figure 6. Overlap in the geographical distribution of VL- and malaria-endemic areas, as achieved by graphically overlapping Figure 1 and Figure 2.

This map is intended as a visual aid only and not as a definitive source of information about disease endemicity.
or through the immune system. In humans, *Leishmania* and *Plasmodium* parasites have different host cell tropisms (phagocytes in the case of *Leishmania*, hepatocytes and erythrocytes in the case of *Plasmodium*), but the two infections share a number of microenvironments that either support the parasite life cycle or participate in the pathophysiology of disease. For example, the liver which hosts the pre-erythrocytic stage of the malaria cycle is scattered with HZ-laden macrophages (Kupffer cells) during acute malaria and with inflammatory granulomas surrounding infected Kupffer cells during VL. Again, deposits of HZ are found in the spleen, which in turn undergoes extensive microarchitectural remodeling following infections with visceralizing *Leishmania* spp. These alterations at cellular and tissue level, arisen in response to one infection, bear the potential to impact the course and pathophysiology of the other infection, and this holds true to a greater extent for any systemic effect or response (immunological and non) mounted by the host. For example, an ongoing pro-inflammatory and type-1 response initiated in response to a leishmanial infection could potentially improve clearance of *Plasmodium* liver forms, whereas the severe anemia induced by VL could act as a deterrent to blood-stage malaria, in line with previous evidence suggesting that anemia may offer protection against malaria infection.

Conflicting data have emerged from the very few studies that have examined the course of malaria and leishmaniasis co-infections so far. In 1954, the first experimental study ever conducted on this subject demonstrated that multiplication of *Plasmodium berghei* parasites in golden hamsters was inhibited by the concomitant infection with *L. infantum*, but not vice versa. Thirty years later, Coleman *et al.* observed that mice co-infected with *L. mexicana amazonensis* and *Plasmodium yoelii* suffered from an exacerbated course of leishmaniasis, which was subsequently attributed to the *Plasmodium chabaudi* chabaudi-triggered release of splenic IL-4 in mice co-infected with *L. infantum*.

Whatever the nature of these interactions taking place during *Leishmania-Plasmodium* co-infections, it is clear that their outcome depends upon multiple factors, amongst which the immunocompetency of the host, the EIR, and the stage and order with which the two infections superimpose. Consequently, an arrange of outcomes, rather than a single one, appears to be a more likely scenario, which may have partially contributed to making these co-infections poorly apparent and, as such, prone to neglect.

**Thesis outline**

Despite cases of co-infection with VL and malaria are frequently encountered across co-endemic areas and the two infections have been shown to cross-interact in co-infected animal models, little is known on the extent and course of these co-infections in humans, and even less is known on the nature of the interactions that may take place in the co-infected host.

It has been the aim of the research described in this thesis to provide more insights into the clinical epidemiology and bio-immunology of these co-infections. Designed on the initial, circumstantially-underpinned hypothesis that in co-endemic areas, malaria frequently superimposes in VL patients and exacerbates host pathology as a result of immunological interactions, this dissertation combines clinical and field evidence gathered from patients hospitalized in East Africa with *in vitro* data on the mutual effects of *L. donovani*
and *P. falciparum* or its waste product HZ on and through the innate immune system, respectively.

In relation to clinical epidemiology, the research aimed at exploring the prevalence, features and risk factors for VL-malaria co-infections amongst VL patients hospitalized in two different bioclimatic regions of East Africa, marked by high VL endemicity and different malaria transmission patterns. Accordingly, **chapter 2** describes a case-control study performed on a dataset gathered by Médecins sans Frontières (MSF) at Amudat Hospital, north-east Uganda, whereas the results of a multi-center retrospective survey conducted in east Sudan are presented in **chapter 3**.

With regard to the immunological interactions taking place during these co-infections, an initial exploratory survey was conducted on naturally infected patients from Gedarif State, Sudan. This study, reported in **chapter 4**, compared the cytokine profiles of co-infected patients over the ones of VL and malaria mono-infected patients and healthy endemic controls. In **chapter 5**, the effect of *in vitro* concomitant exposure of *L. donovani* and *P. falciparum*-infected erythrocytes on the phenotype and function of human dendritic cells is presented.

One of the common obstacles when dealing with complex models such as those involving two or more pathogens, is the unsuitability or under-performance of existing methodologies, as it was our case when trying to measure by microscopy the growth of *Leishmania* intracellular amastigotes in the presence of other phagocytic meals. The problem was subsequently circumvented by developing a set of two new *Leishmania* viability assays. The first consists of a quantitative reverse-transcriptase PCR that simultaneously monitors viability of the *Leishmania* parasite and its host cells [**chapter 6**], while the second is a simple enzymatic assay that enables assessment of parasite viability at high throughput [**chapter 7**]. This latter assay was next applied to the study of HZ-mediated effects on the ability of human and murine macrophages to sustain *Leishmania* invasion and replication [**chapter 8**].
References


