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REVIEW

Recent advances and perspectives on tropical diseases: Malaria

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Abstract

Malaria remains a major health problem in the world. It is a neglected disease because it occurs almost exclusively in poor developing countries, which offer negligible marketable and profitable opportunities. Malaria (together with Tuberculosis), is responsible for an unprecedented global health crisis with devastating effects in developing countries. The 2011 Word Malaria Report indicated that 106 countries showed endemic malaria. Malaria control depends mainly on drug treatment, which is increasingly difficult due to the spread of drug resistant parasites and requires expensive drug combinations. Part of the inability to combat this disease is attributed to an incomplete understanding of its pathogenesis and pathophysiology. Improving the knowledge of the underlying pathogenic mechanisms of malaria transmission and of the exclusive metabolic pathways of the parasites (protozoa of the genus Plasmodium), should promote efficient treatment of disease and help the identification of novel targets for potential therapeutic interventions. Moreover, the elucidation of determinants involved in the spread of malaria will provide important information for efficient planning of strategies for targeted control.

Key words: Malaria; Anopheline mosquitoes; Plasmodium parasites

EPIDEMIOLOGY

Malaria remains one of the most deadly parasitic diseases with an estimated 216 million clinical cases and about 655 000 deaths annually. Currently 86% of deaths happen in sub-Saharan Africa, especially in children under 5 years of age[1].

Malaria burden is caused by *Plasmodium* (*P.*) parasite, transmitted through the bite of infected female anopheline mosquitoes. Four species are able to infect humans: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. Among these, *P. falciparum* is the most lethal species and, together with *P. vivax* accounts for more than 95% of malaria cases in the world[2]. More recently, cases of infections in humans due to *P. knowlesi*, a malaria parasite normally hosted by "kra" monkey, have been reported in South East Asia[3].

There is no completely satisfactory epidemiological classification of malaria. It is possible to distinguish between a high and stable malaria transmission, and a low and unstable malaria transmission strictly dependent on mosquito species (the vector of the disease), geographic and climatic factors[4]. The most common parameter used for the measurement of malaria transmission is the number of infective mosquito bite per year. Stable malaria transmission is widespread in South of the Sahara where populations are continuously challenged with malaria inoculations from childhood onwards. This continuous exposure to the infection leads to a partial immunity in adults. Unstable malaria transmission is typical of Asia and Latin America, where the populations pos-

sess no immunity because of the low rate of exposure to malaria[4].

STRATEGY OF CONTROL

In the absence of an effective vaccine, malaria control relies on a dual approach: primarily in mosquito control and secondarily in reducing the parasite reservoir in the population. World Health Organization (WHO) recommends preventive measures: (1) the use against malaria parasites of insecticide treated nets and indoor residual spraying with insecticide; and (2) the employment against malaria parasites of an intermittent preventive treatment in travellers in endemic regions, and in vulnerable populations in high transmission areas.

For the treatment of uncomplicated malaria WHO recommended from 2001 the adoption of artemisinin-based combination therapies (ACTs). These therapies are now based on the combination of an artemisinin derivative together with a blood schizonticidal drug; the most commonly used are combinations of artesunate-sulfadoxine/pyrimethamine, artesunate-amodiaquine, artesunate-mefloquine, and artemether-lumefantrine[5]. In the updated treatment guidelines, WHO strongly recommends the dihydroartemisinin-piperaquine combined therapy as the most effective ACT. In contrast, the treatment of severe malaria is normally based on the administration of parenteral antimalarial drugs, which must be immediate, appropriate and effective. Intravenous artesunate is recommended as the first choice treatment for severe malaria. Quinine constitutes a valid alternative when parenteral artesunate is not available. Following initial parenteral treatment, it is necessary to continue and complete treatment with a full course of an effective ACT.

Unfortunately, malaria parasites develop resistance to almost every known antimalarial drug including artemisinin derivatives. For this reason, new antimalarial drugs synthesized by medicinal chemists are needed, targeting metabolic pathways of the parasite and able to by-pass resistance mechanisms enacted by the parasite[6,7].

PATHOPHYSIOLOGY

The pathophysiology of malaria represents one of the most interesting aspects of this infectious disease although knowledge of the complex biochemical mechanisms involved to date remains incomplete.

In areas where malaria is highly endemic, malarious patients can experience an asymptomatic parasitemia mainly due to immunological phenomena or manifest non-specific symptoms with a gradual or a fulminant course. Generally malaria is defined as an acute febrile illness whose symptoms resemble those of common viral infections (e.g., malaise, dizziness, myalgia, nausea, vomiting, and diarrhea). This may lead to a delay in diagnosis which is not often available and reliable, particularly in rural zones. In addition to fever, typical physical signs of malaria infection include chills, headache, tachycardia, jaundice, pallor, orthostatic hypotension, hepatomegaly, and splenomegaly. Severe malaria may lead to permanent cerebral damage, acute anemia, respiratory distress and metabolic complications (especially acidosis and hypoglycemia), which may develop rapidly and progress to death within hours or days[8].

All clinical manifestations of malaria are related to the intra-erythrocytic growth phase of the parasite[9]. The so-called "primary attack" is usually atypical and may resemble any febrile illness. It occurs after a variable prepatent period, which is the time that elapses between infected mosquito bite and the appearance of symptoms. During this prepatent period, *Plasmodia* (injected in form of sporozoites) undergo schizogony in the liver (first part of asexual cycle), evolving into schizonts, then into encapsulated merozoites. Following the rupture of the hepatocytes, merozoites migrate through the bloodstream into the erythrocytes where they undergo another schizogony cycle (second part of asexual cycle)[10]. Newly formed merozoites rupture the host cell and invade new erythrocytes. It is now well known that *P. falciparum* proteases of at least two classes (serine and cysteine) play a crucial role in host cell invasion and egress processes[11], and specific inhibitors are currently the subject of extensive research into their role as possible drug candidates[12].

The lysis of erythrocytes is responsible for fever and related symptoms, while during the prepatent period some individuals may show no symptoms or only vague signs of illness such as headache, aches and pains, nausea. When the infection stabilizes the individual manifests "short term relapses" of symptoms at regular intervals of 48-72 h, depending on the *P.* species. These are intervals between two consecutive erythrocyte ruptures, corresponding to the erythrocytic phase of malaria parasites. Sporozoites of *P. vivax* and *P. ovale* are able to remain in the liver as dormant hypnozoites, capable of causing "long term relapses" after an average of 2-3 mo, sometimes even after years from the initial infection. In cases of *P. falciparum* and *P. malariae* infection, relapses from the liver do not occur. However, the blood infection may remain chronic and, if untreated, may remain chronic for years in case of *P. falciparum* and decades in case of *P. malariae*. Some of the merozoites do not undergo sporogony but develop into sexual stages microgametocytes (male) and macrogametocytes (female) instead, degenerating within 6-12 h if they are not ingested via another mosquito bite. Finally, the parasite completes its life cycle inside the mosquito midgut where a complex process of differentiation, growth and fertilization of gametocytes takes place, leading to the formation of diploid zygotes. These zygotes further differentiate into mobile ookinetes that move to the midgut surface and mature into oocysts. Thousands of sporozoites are formed within the oocyst, thereby making their way to the salivary glands. The infected mosquito is now able to transmit the infection during its next blood meal. The

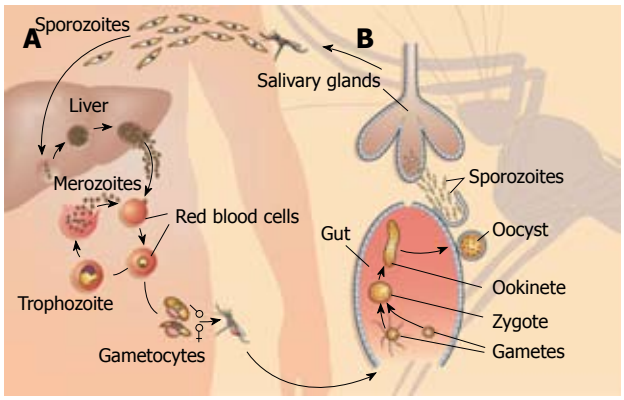

Figure 1 Plasmodium life cycle. A: In the human host, infected female *Anopheles* mosquitoes inject the sporozoite form of the parasite during a blood meal. Sporozoites reach the liver through the bloodstream, where they proliferate asexually, and then, as merozoites, invade red blood cells, evolve and eventually generate male and female gametocytes which are transmitted back to the mosquito; B: In the mosquito vector, gametocytes fuse to form zygotes and differentiate to form oocysts that duly divide to create sporozoites in the mosquito midgut. These migrate to the salivary glands, where the cycle of infection starts again.

entire *Plasmodium* life cycle is depicted in Figure 1.

Cerebral malaria is the most serious complication of malaria, and it is caused almost exclusively by *P. falciparum* as a consequence of the clogging of the cerebral microcirculation. Only a few cases of severe *P. vivax* malaria in adults have been reported to date[13]. In *P. falciparum* malaria, the infected red blood cells (RBCs) develop knobs on their surfaces, which being sticky result in increased cytoadhesion and rosette formation, particularly in cerebral vessels. The infected RBCs may also adhere to the endothelium of capillaries and venules, further blocking the blood flow. By means of this immunological strategy, the parasite remains within the vascular compartment and avoids circulating through the spleen. The increased cytoadherence and clumping of uninfected RBCs together with parasitized RBCs culminates in damage to vital organs like brain, kidneys, lungs, liver, and gastrointestinal tract, leading to the various potentially fatal complications of *P. falciparum* malaria[14]. Obstruction to the cerebral microcirculation results also in hypoxia and increased lactate production due to anaerobic glycolysis. The process of adhesion of infected erythrocytes in various tissues is called "sequestration", and is thought to be the result of interaction between parasite-encoded variant surface antigens on the outer membrane of infected erythrocytes and a range of host receptors. The process is mediated by the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) which is expressed on the surface of infected erythrocytes and anchored into the knobs, thus representing a key virulence factor for this species of human malarial parasite[15].

The rupture of RBCs by merozoites releases certain factors and toxins, which in turn induce the release of cytokines such as tumor necrosis factor (TNF) and interleukin-1 from macrophages, resulting in chills and high-grade fever. The tendency of erythrocytes to adhere to blood vessels is also thought to be related to excessively high levels of TNF. The latter is in turn a potent inducer of nitric oxide (NO) synthase type 2 and a role for NO in the pathogenesis of cerebral malaria has, therefore, been suggested[16]. The most sound hypothesis is that NO helps the body defend itself against malaria (either killing parasites or interfering with their ability to multiply), and that NO levels might even be a key factor in determining whether an individual will get a milder form of the disease or a life-threatening form[17]. Likewise, there is evidence that an excessive production of NO may contribute to the brain damage caused by strokes and to the life-threatening plunge in blood pressure, known as septic shock, that occurs in some infections.

Anstey *et al*[17] demonstrated controversial outcomes in a study conducted on a sample of Tanzanian children (with or without parasitemia). The very sickest children with cerebral malaria had the lowest levels of NO, and the highest levels were found in the children who remained healthy despite being infected with malaria organisms. The very sickest children did have high levels of TNF, and the researchers expected this to also lead to high NO levels, as previous studies had predicted. They hypothesized that the low levels of NO found in the sickest children probably occurred because the children had unusually high levels of interleukin-10, a substance made by the immune system that suppresses NO.

Overall, the immune system of the person plays a crucial role in the clinical response to infection and transmission of malaria. Humoral antibodies to sporozoites, intrahepatic parasites, merozoites, malaria toxins, parasite antigens on infected RBCs, intraerythrocytic parasites, and, within the mosquito, to parasite fertilization, have recently been identified, suggesting that cell-mediated immunity plays a role in liver and RBC invasion and parasite development. However, they may provide a form of non-sterile and age-dependent immunity termed "premunition", which has to be maintained by almost continuous exposure to the parasites, another unique feature of this infectious disease. Individuals in malariuos areas may gradually evolve from a state of immunity against clinical malaria to a state of partial immunity against the infection. Continuous exposure may be attained due to the long-term survival of parasites in the host and/or by frequent re-infections[18].

Pregnant women constitute a notable exception to this, since they are highly vulnerable to infection due to general impaired immunity, particularly during a first pregnancy. The phenomenon of the slow acquisition of immunity and the sudden reappearance of susceptibility during pregnancy are strictly interrelated. In women during pregnancy, the placenta expresses a new receptor that was previously unavailable for infected RBC adherence[19-21]. This receptor has been identified as a uniquely low-sulfated chondroitin sulfate proteoglycan (CSPG) located mainly in the intervillous space and to a lesser extent on the surface of the syncytiotrophoblast cells[22,23]. It has been demonstrated that CSPG has a minimum

motif for optimal infected RBC binding located in its polysaccharide part. This binding site is a dodecasaccharide called "sulfated glycosamino-glycan chondroitin sulfate A (CSA)" and it is composed of a chain of alternating sugars (n-galactosamine and glucuronic acid) in which sulfation of the n-galactosamine moieties occurs at carbon 4 (CSA is therefore also known as chondroitin-4-sulfate)[24-27]. Infected RBCs are tethered to the placenta surface through interactions between PfEMP1 domains and CSA, leading to inflammation and blockage of blood flow to the developing fetus. In this situation the host immune system in unable to detect and destroy infected RBCs in the spleen. Accumulation of infected RBCs in the placenta engenders monocyte/macrophage immune response, which in turn produce proinflammatory mediators[28]. This is associated with all clinical conditions of placental malaria, including, maternal anemia, low birth weight of babies, premature delivery, spontaneous abortion, stillbirth, and can lead to the death of mother and child[29-32]. Anti-adhesion antibodies from pregnant women (except primigravidas) may offer only limited and short-duration protection to the newborn[33]. In rural areas with intense malaria transmission, approximately 30% (depending on the season) of infants at three months of age are infected by *P.* species and may develop their own partially protective immunity. This natural immunity may display a spectrum of anti-parasite activity and be effective in limiting parasite infectivity and parasite replication, thus having an impact on actual parasite burden in the human host. This may also limit infectivity of gametocytes in the mosquitoes and subsequent transmission to other hosts[34].

While gradual acquisition of natural immunity is indisputable, the mechanism of stage-specific partially protective immunity in infants, adults and during pregnancy remain rather elusive[34], raising questions around the efficacy of a long-term candidate vaccine that could provide only partial immunity. Understanding the mechanisms of immune response and identification of specific immunologic determinants will be fundamental for designing and developing promising vaccine candidates, including multistage vaccines. More likely, an efficacious fight against malaria will depend upon a combination of multistage vaccines with other malaria control options, including effective chemotherapy and anti-vector programs limiting host-vector interactions.

DETERMINANTS OF MALARIA

The progression of malaria and the toll of victims in communities and countries depend on several intrinsic and extrinsic determinants which often combine in a negative way. Of the intrinsic determinants, host (human) immunity, virulence of parasite species, anopheline longevity and avidity for humans have the greatest impact on the malaria burden[35], and will be herein discussed in details. Among extrinsic determinants, climate (mainly rainfall), human activities, vector ecology and behaviour, political and economic (poverty) conditions as well as effectiveness of control and prevention efforts are the most important.

Host

The role of host as a medium for parasite development is very complex and susceptibility to infection and severity of illness of human populations exposed to malaria infection may be influenced by other intrinsic factors (e.g., age, genetic disposition). Host immunity is a crucial and poorly understood factor for survival of people infected with the malaria parasite[36]. This is particularly true with respect to the *P. falciparum* parasite which causes severe malaria, wherein many pathological processes such as hemostatic dysfunction, sequestration, systemic inflammation and neuronal damage are implicated[37,38].

Sickle cell anemia provides the best example of a change in hemoglobin structure that impairs malaria parasite growth and development. Sickle cell consists in an inherited alteration of hemoglobin in which RBCs assume an abnormal, rigid, sickle shape. Some people who have the sickle cell trait (commonly written HbAS) inherit a normal hemoglobin A (HbA) gene from one parent and an abnormal hemoglobin sickle (HbS) gene from the other. These individuals are resistant to malaria since sickle RBCs are not conducive to the parasites[39]. HbAS provides neither absolute protection nor invulnerability to malaria. Unlike people with sickle cell disease (who have mainly HbS) and people with normal hemoglobin genotype (HbAA), individuals (and particularly children) with HbAS and infected with *P. falciparum* are generally able to survive their initial acute malarial attacks. They are also referred to as "healthy carriers" because have neither symptoms and nor sickle-cell disease or sickle-cell anemia, although they carry one of the genes that cause the disease[40]. This genetic selective scenario is termed "balanced polymorphism", a concept strengthened by the observation that the heterozygous (HbAS) trait is more prevalent in regions of endemic malaria[41]. Other genetic defects related to hemoglobin structure that confer protection against malaria include hemoglobin C (HbC), hemoglobin E (HbE), α^+ thalassemia, and β-thalassemia[42]. HbS, HbC and HbE arise from a single point mutation of the gene HBB which encodes β-globin chains: Glu→Val at codon 6 for HbS, Gln→Lys for HbC and Glu→Lys for HbE[43]. Both HbC-heterozygotes and HbC-homozygotes are protected against severe malaria with the latter being to a greater extent[44-47], whereas a reduced parasite invasion by *P. falcipaum* has been observed only for HbE-heterozygotes[41,48]. In contrast, α^+ Thalassemia, arises from the disruption of only one of two identical genes encoding α-globin chains (HBA1 and HBA2, located on different chromosomes). In this particular condition, homozygous individuals are still able to produce α-globin and are only mildly anemic[49].

Erythrocyte-membrane proteins represent other key factors of malaria progress since they are extensively

involved in the parasite invasion process and host-defensive mechanisms. Ovalocytosis and GYPs-deficit (the genes encoding glycophorin A, B and C) are among significant hereditary anomalies[50,51]. The former, common in parts of Southeast Asia, consists in a deficit of an anion-exchange protein encoded by the gene *SLC4A1*, known as "band 3 protein", implicated in *P. falciparum* malaria resistance[52]. Glychoporins are sialoglycoproteins essential for host cell invasion. Their genetic deficiency makes erythrocytes resistant to invasion by *P. falciparum*[51]. Innate host resistance towards *P. vivax* is expressed by individuals who possess the Duffy blood factor on the surface of RBCs. The Duffy factor is an antigen by which the merozoites of *P. vivax* enter RBCs. Most of sub-Saharan Africans lack this antigenic factor, therefore *P. vivax* is essentially absent from most of this area. Among other innate factors associated with decreased susceptibility to severe malaria, deficiency in glucose-6-phosphate dehydrogenase is of great importance. This enzyme is responsible for preventing the formation of reactive oxygen species from heme groups, thereby slowing down parasite growth[53,54]. On the contrary, a deficit of haptoglobin (a hemoglobin-binding protein present is plasma which protects tissue from oxidative stress) is associated with an increased susceptibility to severe malaria in specific areas[55,56]. The increased cytoadherence of *P. falciparum*-infected erythrocytes in small vessels plays a critical role in the pathogenesis of severe malaria. Several endothelial cell adhesion molecules are implicated in this event, binding mainly PfEMP1. These include fatty acid translocase, intracellular adhesion molecule 1, platelet endothelial cell adhesion molecule 1[57-59]. For these host receptors for cytoadherence, a genetic polymorphism in the promoter region appears to be correlated with the frequency of severe disease[60-62]. CR1 is considered the most important surface protein that allows infected RBCs to form rosettes and its deficiency is also associated with a high level of genetic polymorphism[63].

As previously stated, interaction between malaria and the immune system is even more complex when immunological responses are directly involved. A large array of proteins responsible for antigen recognition, antibody response, inflammatory mediation, as well as other serum factors undergo genetic polymorphism in naturally exposed populations. The consequences of this immune gene polymorphism are not always predictable and may vary substantially in different malarious regions due to many others biological reasons[43]. This issue is the main hurdle to the development of an effective malaria vaccine and the main reason why the use of genotype information for improved malaria treatments and prevention remains a challenge for the future.

Parasite

Of the five *P.* species affecting humans, *P. falciparum* is the most virulent for reasons which are incompletely understood. Some genetic studies indicate that it is the most recently evolved species, whereas other studies reveal a high level of genetic variation, suggesting a large population size that has been maintained for several hundred thousand years[64].

P. vivax was the most globally widespread and most prevalent species, until the middle of the 1900s. Currently, it still has the most wide geographic distribution and constitutes the second most common cause of malaria globally with 90% of those infections occurring outside of Africa. This is partly because of its ability to complete the sporogonic cycle at a minimum lower temperature of 16 ℃, compared to 21 ℃ for *P. falciparum*, but (as mentioned above) mainly due to the Duffy polymorphism in Africans which results in a phenotype that does not allow *P. vivax* to invade RBCs. Although *P. vivax*, which can cause relapses months after an infection due to the presence of liver hypnozoites, accounts for 65% of malaria cases in Central and South America and Asia[65], it causes substantial morbidity with debilitating symptoms but it is rarely fatal. Generally, patients infected with *P. vivax* die as a consequence of splenomegaly. In relation to pregnancy, *P. vivax* (unlike *P. falciparum*) is not associated with shorter gestation or with an increased rate of miscarriage or stillbirth, is instead associated with an increased risk of low birth weight[66].

P. falciparum and *P. vivax*, which represent the two principal human malaria parasites, seem to be very different in origin and in phylogenetic relationship to other *P.* species[67]. *P. malariae* is the causative agent of quartan fever. It occurs in various tropical regions throughout the world and causes low, yet significant, morbidity and mortality levels in humans which are associated with renal complications (quartan malarial nephropathy, more common in adults than children)[68]. Patients may remain parasitemic and asymptomatic for as much as 50 years or more, even in the absence of reinfection. They usually have relapses at irregular intervals, which are actually recrudescences due to the subpatent parasitemia[69]. The reason of the long duration of *P. malariae* infection may be explained by the slow rate of erythrocytic schizogony of the parasite, and by the inefficacy of common antimalarial drugs with blood schizonticidal action in eradicating it. Another reason for such a long duration of infection arises because *P. malariae* is the species that has been infecting humans for the longest time. As a consequence, this species has adjusted to the human immune system, which is unable to completely eliminate the parasite.

P. ovale, also a relapsing species, is a rare (0.5%) cause of infection. It is found principally in sub-Saharan Africa and some islands of the western Pacific[70]. *P. ovale* infections generally follow a benign course, though rare complications may arise due to spleen rupture[69]. This species was the last of the malaria parasites of humans to be correctly described. It was considered for long time a variant form of *P. vivax* due to the fact that both were found in enlarged and stippled infected erythrocytes. In 1922, Stephens named the parasite *P. ovale* in recognition

of the oval shape of the infected erythrocytes, particularly those containing younger stages of the parasites, which may also show "spiking" or fimbriation[70]. However, ovalization and fimbriation do not occur spontaneously and cannot be detected by examination of infected blood samples: these transformations are artificially induced at the time of the smear and constitute the best diagnostic tool for species identification[69].

The fifth *P.* species affecting humans is *P. knowlesi*, an intracellular malaria parasite whose natural vertebrate host is Macaca fascicularis (the 'kra' monkey). It is now increasingly recognized as a significant cause of malaria in humans, particularly in South East Asia[71,72]. From a phylogenetic point of view, *P. knowlesi* and *P. vivax* are closely related[73], although there are important phenotypic differences between them, such as their host blood cell preference, the absence of a dormant liver stage or 'hypnozoite' in *P. knowlesi*, and length of the asexual cycle. Recently the *P. knowlesi* [H strain, Pk1(A+) clone] nuclear genome sequence has been described[74], and it provides an opportunity for comparison with the *P. vivax* genome[73] and other sequenced *P.* genomes[75-77].

Evidence is accruing that malaria parasites exhibit a wide range of inter-strain variation within species, patients, and localities, probably because they are exposed to strong selection from the human immune response and treatment with antimalarial drugs. Such a vast strain-specific diversity has a strong impact on clinical manifestations in various age groups and on malaria transmission[78,79]. However, detecting and understanding parasite maintenance in populations is complicated due to the fact that different evolutionary forces, such as copy number polymorphism and transcriptional variation, help in maintaining the genetic diversity in the parasite genome[80,81]. It is now quite clear that both *P. falciparum* and *P. vivax* use the genetic diversity they possess to fight against the antimalarial drugs and host immunity although the mechanism of maintenance of such diversity is unknown[67]. In cases of *P. falciparum* malaria, people living in endemic countries develop only partial immunity, and this immunity correlates with acquisition of strain-specific antibodies that recognize PfEMP1 proteins[33,82]. The host immune system counters the obstruction of the microcirculation by producing antibodies that interfere with the adhesion of infected RBCs and increase their detectability. The genome of the 3D7 strain of *P. falciparum* contains 59 genes for PfEMP1 proteins[76]. One gene is expressed at a time, and the parasite avoids detection by varying which PfEMP1 is produced[83,84]. This ability to switch among a large array of different adhesion ligands is almost certainly the reason why it takes children several years, and many disease episodes, to acquire substantial protective immunity to *P. falciparum* malaria[21]. Vaccination represents the most direct way to achieve host immunity prior to pathogen exposure. Unfortunately, the malarial parasite constantly changes its immune makeup in every stage of its life cycle, thereby frustrating efforts to produce an effective vaccine.

The development of *P. falciparum* resistance to the most commonly used anti-malarial drugs has been a major cause of failure of any malaria control program and of increasing malarial burden[85]. Generally, resistance depends on the chemical class of the antimalarial and its mode of action[86]. Resistance to 4-aminoquinolines, cinchona alkaloids and highly hydrophobic arylminoalcohols, arises from mutations of genes encoding vacuolar trans-membrane proteins which regulate the influx/efflux of the drug at the target[87], whereas there are no well-documented reports on development of resistance against 8-aminoquinolines. Chloroquine (CQ) resistance in *P. falciparum* is primarily attributable to single nucleotide polymorphisms in *pfcrt* (CQ resistance transporter)[88]. Mutations in *P. falciparum* multidrug resistance 1 (PfMDR1), the gene encoding the *P. falciparum* P-glycoprotein homologue-1, seem to be the main cause of resistance to mefloquine but are also implicated in CQ resistance[89,90]. Resistance to antifolates is quite common worldwide and apparently depends on a stepwise accumulation of single point mutations of genes *pfdhps* and *pfdhfr* encoding the drug targets, dihydropteroate synthase and dihydrofolate reductase, respectively[91]. Atovaquone resistance is associated with single point mutations in the cytochrome *b* gene of *P. falciparum*[92]. Although artemisinin derivatives represent the most efficacious class of antimalarial drugs, some cases of resistance have been recently detected. This may be due to mutations or amplifications of the gene encoding a PfMDR1 or mutations in the gene encoding sarco-endoplasmic reticulum calcium ATPase6[93,94]. Resistance to one chemical class of antimalarial drugs may cross-react with the others and this is the main reason for the poor efficacy of multi-target antimalarial chemotherapy. This capacity of the malaria parasite to counter the multi-target therapy arises from the fact that gene mutations usually do not act in isolation and act synergistically to encode or enhance resistance. Both mutations in different genes and sequential accumulation of mutations in a single gene may determine cross-resistance[95].

Mosquito

All human malaria is transmitted through bites by female mosquitoes of genus *Anopheles* (*An.*), but not all anophelines can be considered vectors of malaria. To become a vector, a mosquito has to be susceptible to malaria sporogony, be anthropophagic and have enough longevity to become infective to humans. Mosquito longevity is particularly important because parasite sporogony takes place inside the mosquito midgut over a time span that can vary from 8 d to 30 d, depending on ambient temperature. Usually, sporogony within the mosquito does not occur at temperatures below 16-18 ℃. The tropical regions provide ideal living and breeding conditions for the *An.* mosquitoes since temperature, rainfall and humidity are important factors for their survival and distribution. Specific breeding sites in rural areas are bodies of fresh water that are usually large, open,

sunlit and more or less permanent, e.g. swamps (near the edges if deep), weedy sides of streams and channels, rivers, ponds, tanks, wells, furrows or ditches, protected portions of lake shore, rice fields, or water seepages, which are fed from underground permanent sources. In urban and peri-urban areas the preferred breeding sites are building-constructions sites, wells, garden ponds, cisterns, overhead tanks, ground level cement tanks, water coolers[96-98]. Altitude is another limiting factor for the development of anopheline mosquitoes as, with the exception of a few species, they are generally unable to infect at above 2000 m[99].

Some *An.* species may be affected by environmental drivers and display behavioural variation (also known as behavioural resistance) within the same species. The latter may determine their occurrence, vectorial status and adaptation to a changing environment. This been recently assessed for *An. sundaicus*, an important malaria vector in coastal areas in Southeast Asian region which can has adapt to breed in a wide range of salinity conditions from fresh water to brackish water[100]. Behavioural variation has alos been observed in the *An. dirus* complex, which includes efficient malaria vectors of the Asian forested zone. Although forest seems essential for survival of this species, adaptations to orchards and wells have been recorded[101].

Information on the vectors and their precise role in malaria transmission is unclear as all the major malaria vectors are species complexes, which are morphologically indistinguishable but differ significantly in biological characteristics that are vital for malaria control. These include vectorial potential, host-preference, distribution pattern, resting behavior and response to insecticides. These sibling (or cryptic) species can be recognized only through chromosomal studies or biochemical analysis[102]. For instance, the *An. culicifacies* complex, which constitutes the main vector system in Indian with an overall rate of 60%-65% of malaria cases[103], comprises five sibling species provisionally designated as species A, B, C, D and E. Among them, species B holds special features: (1) it is the only member of *culicifacies* complex regarded as a non-vector; (2) it is found in isolation in eastern India, while in other areas of Indian sub-continent it is found in association with species C, D or E[104]; (3) it has the ability to kill malaria parasites in the midgut during early sporogony by a process of encapsulation[105]; and (4) it develops resistance towards insecticides [dichlorodiphenyltrichloroethane (DDT) and malathion] at a faster rate than species A and/or sympatric species B[106-108]. *An. anthropophagus* and *An. sinensis* constitute another example of cryptic species which are morphologically indistinguishable. They are the main vectors in central China where they mostly occur in symmetricity. Identification of their genetic diversity could facilitate studies on malaria transmission and the development of prevention strategies for malaria control[109].

Although there are 444 formally named species and 40 unnamed members of species complexes recognized as distinct morphological and/or genetic *An.* species[110], only 60 of them transmit malaria under natural conditions, and only 30 are of major importance[111]. Of these, the *An. gambiae* complex and *An. funestus* are the most efficient vectors of *P. falciparum* malaria in Sub-Saharan Africa[112]. *An. gambiae* exhibits the highest rates of sporozoite development, thus it represents one of the prime targets for genetic modification projects[113]. This species complex consists of six sibling species (*An. arabiensis*, *An. bwambae*, *An. merus*, *An. melas*, *An. quadriannulatus*, *An. gambiae* sensu stricto) with different behavioural traits[114]. Within this species complex, *An. gambiae* s. str. and *An. arabiensis* are the major vectors of human malaria in sub-Saharan Africa, with *An. merus* and *An. melas* being intermediate in importance[115]. This last two species are associated with salt-water with a localized distribution along the eastern and western coasts of Africa, respectively[116,117]. *An. bwambae* and *An. quadriannulatus* (species A, found in south-east Africa, and species B, which has been described in Ethiopia) are highly zoophilic and are never or rarely exposed to the human *P. falciparum,* thus they are not considered vectors of human malaria[115,118]. *An. bwambae* (which has only been found breeding in mineral springs) has the most restricted range, limited to the Semliki Forest of Uganda[119]. *An. gambiae* s. str. is currently in a state of diverging into different species - the Mopti (M) and Savannah (S) strains - though as of 2007, the two strains were still considered to be a single species. The *An. gambiae* s. str. genome has been sequenced by Holt *et al*[120] and revised by Sharakhova *et al*[121], though there is controversy over the choice of strain used, which is considered a hybrid of two different strains.

In the Pacific area *An. farauti* complex, *An. maculatus* and *An. flavirostris* play a predominant role in malaria transmission. These species are geographically isolated, with the latter confined mainly to the Philippines, much of Indonesia, and Sabah, Malaysia[122-124]. Actually, *An. flavirostris* is considered a subspecies of *An. minimus*, a complex that counts five sibling species and represents one of the most important vectors of human malaria in East Asian countries (Nepal, Bangladesh, north Thailand, Indonesia, south China and also in the Yaeyama Island of Japan)[125]. The *An. farauti* complex belongs to the *An. punctulatus* group, which includes at least 12 sibling species and is widespread in northern Australia (wherein it is not a malaria vector) and the islands of the south-west Pacific[126]. Four of the twelve species have been implicated as malaria vectors in Papa New Guinea[127]. *An. albimanus* is the primary coastal vector in South America, Central America and the Caribbean[128]. *An. aquasalis* is a coastal Neotropical species, considered to be the primary coastal malaria vector of *P. vivax* in Venezuela[129]. Another important malaria vector with a broad geographical distribution ranging from southern Mexico to northern Argentina is *An. darlingi*. Its degree of involvement in human malaria transmission seems to differ among localities[130]. Besides its morphological, behavioral and genetic diversity, *An. darlingi* spread

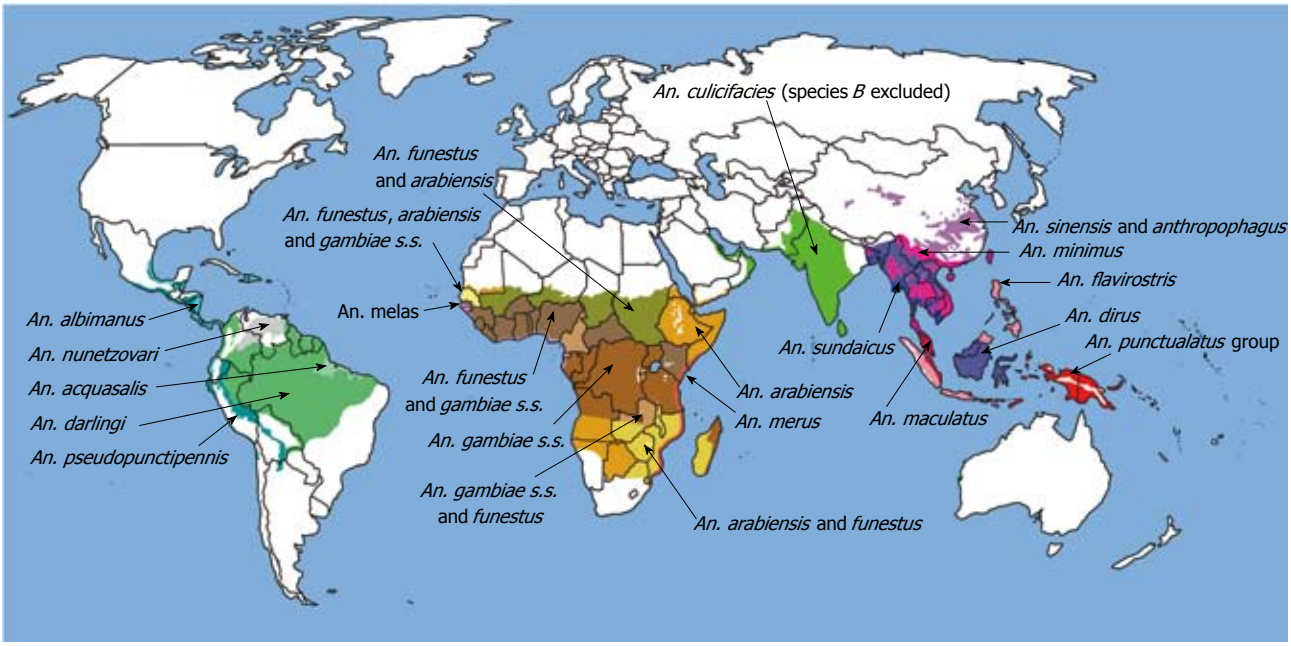

Figure 2 Map of the world showing the distribution of the most important anthropophilic *Anopheles* mosquitoes which are currently considered vectors of malaria. *An.: Anopheles; s.s.: sensu stricto*

throughout the Amazon basin (where it is considered the main vector) has been recently associated with the uncontrolled rate of deforestation[131]. *An. nuneztovari* is considered an important vector of human malaria in areas of Venezuela and Colombia, although populations of this species occupy large geographic areas (northern South America, eastern Panama, Brazil and Amazon region states)[132]. Among the dominant *Anopheles* vectors of human malaria in the Americas it is worth noting the *An. pseudupunctipennis* complex since it can survive and transmit malaria at altitudes up to 3000 m[133]. The geographic distribution of the mentioned human malaria vectors is depicted in Figure 2.

Vector control has had limited success in areas where malaria is heavily endemic, primarily due to the lack of resources for disease management. The options available for vector control are mainly, insecticides, personal protection measures, larval control, biological control and environmental management. Pyrethroids are the only insecticides that have been used for impregnation of bednets because of their very low mammalian toxicity. Trials of long-lasting insecticide-treated nets impregnated with permethrin, deltamethrin and alphacypermethrin are underway[134]. Unfortunately, there is an increasing resistance in vectors towards pyrethroids and a well established resistance towards other older insecticides used as indoor residual sprays or in public health. Mosquitoes exhibit a broad armamentarium for physiological defense. Their major mechanisms of resistance include: glutathione-S-transferase-based degradation of DDT, carboxyl esterase-dependent hydrolysis of malathion, altered acetyl cholinesterase activity to organophosphates and carbamates, cytochrome P-450 monooxygenase and *kdr* type resistance against pyrethroids[135]. Larval control may represent an alternative strategy for mosquito abatement, but it is a laborious challenge that requires practical environmental and entomological skills at all levels, and thus is only achievable in urban and peri-urban situations. Environmental control is used to prevent breeding, nesting, and feeding of vectors and, as for larval control, requires community participation and intersectoral collaboration. All these prevention measures will have a paltry impact on transmission and a limited success in decreasing the malaria burden when applied alone. Thus, newer and more advanced vector-focused approaches are needed[136].

PLASMODIUM METABOLISM AND POSSIBLE DRUG TARGETS

The malaria parasite possesses unique mechanisms for the synthesis of biomolecules and a better understanding of its metabolic pathways may lead to the development of novel therapeutic strategies.

Hemoglobin degradation pathway

The malaria parasite grows rapidly during many stages of its life cycle within the human host and exhibits a high multiplication rate. This necessitates a constant source of nutrients, both for protein biosynthesis and energy metabolism, which mainly come from ingested hemoglobin during the morphologically separate phases inside the erythrocyte (ring stage, trophozoite stage, and schizont stage)[137,138]. The parasite degrades a large amount of host cell hemoglobin by means of a variety of proteases which are thought to act in semi-ordered fashion[139], and whose importance as a drug targets is well-documented[140,141]. This massive digestive process (up to 65% of the total host hemoglobin) occurs in a specialized organelle, the

food vacuole, which requires a pH optimum in the range 4.5-5.0[142].

Two closely related aspartic proteases, termed plasmepsin (PM) I and Ⅱ, are involved in the early events of hemogloglobin degradation, promoting unfolding of globin chains and release of the heme moiety. Further digestion of globin chains is carried out by at least other two aspartic proteases, PM Ⅳ and histo-aspartic proteinase[140,143], and three cysteine proteases termed falcipains (FPs)[141,144-146]. The cysteine proteases involved in the hemoglobin catabolism are two nearly identical copies of FP-2 (FP-2 and FP-2', also known as FP-2A and FP-2B respectively)[146,147], and FP-3[144]. Both PMs and FPs are synthesized as membrane-bound proforms that are transported to the food vacuole and activated by means of overlapping, redundant mechanisms[148].

A zinc metalloprotease (named falcilysin) intervenes later in this digestive process since it is able to cleave only small polypeptides (up to 20 amino acids)[149] that are eventually shortened further by a dipeptidyl aminopeptidase 1[150]. Oligopeptides are then pumped out of the food vacuole and an amino peptidase activity within the parasite cytoplasm provides amino acids essential for parasites survival[151,152]. Despite this substantial proteolysis, malaria parasites employ only 16% of the digested hemoglobin for biosynthesis of proteins[153]. Most of newly obtained amino acids are effluxed from the infected erythrocyte to provide space for the growing parasite and maintain osmotic stability[154].

Massive degradation of hemoglobin also leads to generation of a large quantity of heme that is toxic to the parasite, promoting membrane damage due to its peroxidative properties[155]. Released hematin is detoxified to a cyclic dimer, β-hematin. The low digestive vacuolar pH promotes crystallization and polymerization of these dimers to give hemozoin pigment[156,157]. This defense mechanism from oxidative stress is most pronounced during the trophozoite stage of the parasite development[158]. More recent studies indicate that the crystallization process from β-hematin dimers to hemozoin takes place in, or closely associated with, neutral lipid nanospheres in the aqueous content of the vacuole[159]. Despite accumulating heme derived from RBC hemoglobin, malarial parasites synthesize heme *de novo* for metabolic use. The heme biosynthetic pathway of the parasite is similar to that of humans and animals, using glycine and succinyl-CoA to make δ-aminolevulinic acid (ALA), the committed precursor for heme biosynthesis. However, while the parasite makes its own ALA, it has to import ALA dehydratase and ferrochelatase from host RBC to complete the pathway[160,161].

Purine pathway

Plasmodium does not manifest the *de novo* pathway for purine nucleotide biosynthesis and depends on the salvage pathway for its supply of purine[162]. Purine uptake into the intraerythrocytic malaria parasite involves four different nucleobase/nucleoside transporters localized to the parasite plasma membrane, two with a high substrate affinity and two with a low substrate affinity[163-166]. The first comprehensive model for purine uptake by *P. falciparum* has been proposed by Quashie *et al*[167]. Their studies report on the presence of a low affinity adenosine transporter, a high affinity adenine transporter, a low affinity uptake route for adenine and a high affinity hypoxanthine/purine nucleoside transporter (PfNT1, also known as PfENT1). Recently, the role of PfNT1 in the purine salvage pathway has been called into question by other authors who assert that the high-affinity uptake components characterized by Quashie *et al*[167] are strictly related to the high-affinity intracellular metabolism (and perhaps sequestration) of purines carried out by *Plasmodium* rather than the presence of high-affinity transporters, reaffirming a low-affinity system for PfNT1[168,169]. Studies on PfNT1 knock-out parasites have also indicated, one or more additional transport pathways (of unknown affinity) for the uptake of adenine[170]. Hypoxanthine is regarded as the key metabolic precursor of all other purines. The source of hypoxanthine is believed to be the adenosine triphosphate present in the infected erythrocytes, which is catabolized to hypoxanthine *via* adenosine diphosphate, adenosine monophosphate, adenosine and inosine[171]. In *Plasmodium*, adenosine is converted to inosine by adenosine deaminase[172], which is further metabolized to hypoxanthine by purine nucleoside phosphorylase[173].

Pyrimidine and co-factors of the B-complex vitamin pathway

Unlike purine biosynthesis, the malaria parasite lacks the salvage pathway for pyrimidines and synthesizes them *de novo*[174]. To accomplish this, the parasite usually synthesizes itself the required folate co-factors (vitamin B9), starting with the condensation of pteridine with para-aminobenzoic acid to form dihydropteroic acid. The latter is then condensed with glutamic acid to dihydrofolic acid and lastly reduced to tetrahydrofolic acid, which is the active form of vitamin B_9 essential in one-carbon transfer reactions[175]. Humans only have the ability to convert folic acid into tetrahydrofolic acid and this is the basis of the selective toxicity of type- I antifolates (dihydropteroate synthase inhibitors) used occasionally as antimalarial drugs in association with type-Ⅱ antifolates (dihydrofolate reductase inhibitors)[176]. Among the six enzymes involved in pyrimidine biosynthesis, dihydroorotate dehydrogenase (the fourth one) is the most exploited as a drug target[177].

In addition to vitamin B_9, the malaria parasite is able to achieve *de novo* synthesis of other important co-factors of B complex (B_1 and B_6) which do not occur in humans and therefore represent potentially powerful drug targets[178]. Biosynthesis of thiamine pyrophosphate (the active form of vitamin B_1) takes place *via* two branches, the thiazole and pyrimidine pathways, which join to form the key intermediate thiamine monophosphate. Two different distinct pathways, generate pyridoxal 5-phosphate

(the active form of vitamin B_6). One is related to the pentose phosphate shunt and glycolysis, while the other depends on the salvage of B_6 vitamins[179]. Both vitamins B_1 and B_6 are transported primarily in erythrocytes, raising the question of why *Plasmodium* uses energy to produce its own co-factors. For vitamin B_6, the reason resides in the fact that it is tightly bound to serum albumin and hemoglobin and thus might not be available to the parasite[179]. However, it has been demonstrated that the *de novo* synthesis of vitamins B_1 and B_6 by the parasite is not sufficient for its survival during erythrocytic schizogony[180-182]. Vitamin B_1 is an essential co-factor of enzyme complexes such as pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, branched-chain 2-oxo acid dehydrogenase and transketolase, thus involving diverse and connected metabolic pathways. Vitamin B_6 acts as a carbonyl-reactive co-factor for more than 140 distinct enzymatic reactions, all involved in the conversion of amino acids *via* processes such as decarboxylation and transamination[179].

Lipid pathway

P. species, like other apicomplexan parasites, have a special type Ⅱ fatty acid biosynthetic pathway (FAS Ⅱ) whose enzymes are localized in the apicoplast[183]. This organelle is a plastid of cyanobacterial origin and it is closely associated with the mitochondrion at all stages of parasite development[184]. The apicoplast-mitochondrion association is also involved in other biosynthetic processes such as isoprenoid and ubiquinone biosynthesis[174]. *Plasmodium* FAS Ⅱ consists of a chain elongation pathway which depends on four key enzymes (in sequence: FabB/F, FabG, FabZ and FabI) and leads to the formation of fatty acids with between 10 and 14 carbons linked to an acyl carrier protein[185]. All the four key enzymes of *Plasmodium* FAS Ⅱ represent promising drug targets because of their bacterial origin[186]. Although as a rule the malaria parasite scavenges fatty acids from its host, it has been recently demonstrated that FAS Ⅱ is essential for its liver-stage development[187].

During the erythrocyte stage, the rapid growth of the parasite is fueled by other precursors such as serine, ethanolamine, and choline which represent the major building blocks for the *de novo* synthesis of structural and regulatory phospholipids. The enzymes involved in these biosynthetic pathways are either absent from humans, or markedly different from their human counterparts, and thus represent other important drug targets[186].

Glucose pathway

In the *P. falciparum* the mitochondrion is atypical. During the asexual stage, it lacks cristae, but possibly develops cristae in the gametocyte stage[188]. The role of the parasite mitochondrion has not yet been completely clarified, though the main functions of a typical mitochondrion have been assessed. It is involved in energy generation through glycolysis, electron transport *via* flavin adenine dinucleotide-linked tricarboxylic acid cycle enzymes, and intermediary metabolism[189]. Glucose is the primary source of energy but the metabolic steps involved in the conversion of glucose to lactate (approximately 85%) are essentially the same as that found in other organisms. Therefore, the glycolytic pathway of *Plasmodium* has not been investigated as a potential drug target as much as other pathways. In this context, a parasite-derived carrier protein called *P. falciparum* hexose transporter is more important as a drug target due to its superior affinity for glucose as compared to human Glucose transporter 1[190].

Other pathways under evaluation

There are several other secondary metabolic pathways of the malaria parasite that are currently under consideration as valid drug targets[191].

Antioxidant enzymes in the redox pathway, such as glutathione reductase, glutathione S-transferase, and thioredoxin reductase can be exploited as antimalarial drug targets since they differ sufficiently from the host enzymes.

Staines *et al*[192] have reported the induction by the parasite of new permeability pathways towards the membrane of infected RBCs. These pathways are mainly anion specific channels that also increase the influx of cations and small electroneutral solutes (although at a lower rate).

Plasmodial cyclin-dependent kinases are highly conserved and play a pivotal role in parasite growth and development, and are currently being exploited for the development of selective inhibitors[193].

The shikimate pathway is absent in human and all other mammals, but it is essential for parasite growth. Thus, it is being thoroughly investigated for the development of new antimalarial agents[194]. This pathway is an important metabolic route that links carbohydrate metabolism to the biosynthesis of aromatic compounds such as aminoacids and precursors of other essential cofactors (ubiquinone and folic acid).

Aquaporins are integral membrane proteins that regulate the flow of water or uncharged polar solutes. They protect the parasite from the osmotic stress encountered during its development inside the erythrocyte as well as during its passage through the kidney of the human host. Moreover, they help the parasite to acquire nutrients from the host organism and to eliminate metabolites. The structure of plasmodial aquaporins differs from from that of human aquaporins at the level of the accessible pore mouth. Therefore, aquaporins represent a valid alternative target for the development of new antimalaial agents[195].

CONCLUSION

In spite of the significant recent advances in antimicrobial chemotherapy, supportive care and vector control strategies, malaria still represents a serious threat to human heath, killing about one million people per year worldwide. Part of the inability to combat this disease is attributed to an incomplete understanding of its patho-

genesis and pathophysiology. Improving the knowledge of the underlying pathogenic mechanisms of malaria transmission and of the exclusive metabolic pathways carried out by the parasite, should promote the efficiency of the treatment of disease and help the identification of novel targets for potential therapeutic interventions. Moreover, the elucidation of determinants involved in the spread of malaria - which we have hopefully sufficiently covered within this review - will provide important information for efficient planning of strategies for targeted control.

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