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Role of ATP2B4 and human malaria : looking for functional genetic variants associated with malaria

Samia Nisar

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UNIVERSITÉ D'AIX-MARSEILLE

Theories and Approaches of Genomic Complexity

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Sujet de la thèse

Role of *ATP2B4* and human malaria : looking for functional genetic variants associated with malaria

présentée par

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Le 11 September 2020

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Co-Directeur de thèse

Directeur de thèse

DEDICATED TO MY LOVING PARENTS

For their endless love, support and encouragement

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Samia Nisar

Abstract

Malaria causes approximately one million fatalities per year, mostly among Africa. The severity of the disease is influenced by complex interactions between many factors including host genetics. My thesis work concerns the identification and the functional characterization of the genetic variants involved in resistance/susceptibility to severe malaria (SM) by combining bioinformatics and genetic approaches. Several GWAS of SM have identified various loci. Nevertheless, only a limited number of loci and genetic variants have been replicated in independent populations. The first part of my project was to systematically identify the regulatory effect of all the SNPs in linkage disequilibrium (LD) with the tagSNPs associated with SM in several populations. By using different bioinformatics tools, we prioritized the potential causal variants and interestingly found 5 best candidates (rs11240734, rs1541252, rs1541253, rs1541254, and rs1541255) located close to each other on chromosome 1 in *ATP2B4* region. Our bioinformatic results predicted that they have a regulatory effect, whereas the tagSNPs are unlikely functional. *ATP2B4* is the main calcium pump of erythrocytes (the host cells of the pathogenic stage of malaria parasites) and alteration of its expression may affect the intraerythrocytic calcium concentration and thus the structure and development of intraerythrocytic stages of the parasite. By genotyping these SNPs in a Senegalese population, we confirmed the association of tagSNP (rs10900585) and the 5 candidates (major allele as the risk allele) with SM. Moreover, a meta-analysis confirmed the association of this tagSNP with SM in several populations. Furthermore, we have genotyped *ATP2B4* variants (rs10751450, rs10751451, rs10752452) that have shown to be in an enhancer and to alter *ATP2B4* expression and assessed their association with SM. Interestingly, we evidenced a close LD between these SNPs and rs11240734, rs1541252, rs1541253, rs1541254, and rs1541255, and detected a significant association with SM in the Senegalese population. Moreover, haplotype analysis revealed that the individuals homozygous for the major haplotype were found to have a higher risk of developing SM. In the next part, we investigated the functional impact of the candidate variants on gene regulation by using different functional approaches. Luciferase assay results indicated a significant promoter activity for both risk (major alleles) and the non-risk haplotype (minor alleles) with non-risk haplotype inducing higher transcriptional activity. Furthermore, the region showed a significant enhancer effect on the *ATP2B4* promoter. However, the major haplotype (risk) exhibited significantly higher enhancer impact than minor haplotype (non-risk) which confirmed that the regulatory region exhibits allele-dependent enhancer activity in

K562 and suggested that our regulatory region is an epromoter (promoter with enhancer function). Using CRISPR-Cas9, we performed the knockout of the region containing the SNPs and found a decrease in expression of the gene compared to WT for total expression as well as for two long transcripts, which confirmed that our regulatory region with rs11240734, rs1541252, rs1541253, rs1541254, and rs1541255 has an enhancer function. Additionally, the impact of deletion on intracellular calcium concentration was measured using flow cytometry and the results indicated a significant increase in intracellular calcium in deleted clones. In conclusion, we identified an enhancer that controls *ATP2B4* expression and is perturbed by several genetic variants associated with severe malaria. This also fosters the development of therapeutic strategies based on the modulation of *ATP2B4* expression and calcium levels.

Résumé

Le paludisme cause environ un million de décès par an, principalement en Afrique. La gravité de la maladie est influencée par de nombreux facteurs incluant les facteurs génétiques de l'hôte. Mon travail de thèse porte sur l'identification et la caractérisation fonctionnelle de variants génétiques impliqués dans la résistance/susceptibilité aux formes graves (FGs) de paludisme en combinant des études bioinformatiques et génétiques. Plusieurs études d'association sur l'ensemble du génome ont permis d'identifier des loci associés aux FGs. Néanmoins, seul un nombre limité de loci et de variants ont été répliqués dans des populations indépendantes. La première partie de mon projet a été d'évaluer systématiquement l'effet régulateur potentiel des SNPs en déséquilibre de liaison (DL) avec les tagSNPs associés aux FGs. En utilisant des outils bioinformatiques, nous avons priorisé ces variants et avons identifié que les 5 meilleurs candidats (rs11240734, rs1541252, rs1541253, rs1541254 et rs1541255) sont situés à proximité les uns des autres sur le chromosome 1 dans la région du gène *ATP2B4*. Nos résultats ont ainsi permis de prédire que ces variants auraient un effet régulateur, alors que les tagSNPs seraient peu fonctionnels. La protéine *ATP2B4* est la principale pompe calcium des érythrocytes (les cellules hôtes du stade pathogène des parasites) et l'altération de son expression pourrait donc affecter la concentration en calcium intra-érythrocytaire et le développement des stades intra-érythrocytaires du parasite. Nous avons confirmé l'association du tagSNP (rs10900585) avec les FGs au Sénégal et la méta-analyse confirme cette association dans plusieurs populations. Nous avons montré que les 5 candidats (allèle majeur comme allèle à risque) sont également associés aux FGs au Sénégal. Pour les variants rs10751450, rs10751451 et rs10752452 précédemment décrits comme fonctionnels et altérant l'expression d'*ATP2B4*, nous avons obtenu une association avec les FGs dans notre population d'étude. Un fort DL a été observé entre ces 3 SNPs et les 5 SNPs candidats et l'analyse des haplotypes a révélé que les individus homozygotes pour l'haplotype composé des allèles majeurs présentaient un risque plus élevé de développer une FG. Dans la partie suivante, nous avons étudié l'effet fonctionnel de ces variants sur la régulation du gène *ATP2B4* en utilisant différentes approches fonctionnelles. Les expériences de gène rapporteur ont permis de montrer une activité promoteur et amplificateur pour la région contenant les 5 SNPs candidats. L'haplotype à risque correspondant aux allèles majeurs a un effet promoteur moins élevé que l'haplotype correspondant aux allèles mineurs. En revanche l'activité amplificateur est plus élevée pour l'haplotype à risque avec les allèles majeurs. Ces résultats suggèrent que cette région ayant un effet promoteur aurait aussi une fonction amplificateur

(epromoter) et que l'effet régulateur est allèle dépendant. Dans les cellules K562 dans lesquelles nous avons enlevé la région contenant les SNPs avec la technologie CRISPR-Cas9, l'expression du gène *ATP2B4* est moins élevée que dans les cellules sauvages. Ce résultat est significatif à la fois pour l'expression globale de tous les transcrits mais aussi spécifiquement pour les deux longs transcrits confirmant ainsi la fonction promoteur et amplificateur de cette région. Nous avons également montré par cytométrie en flux que cette délétion a pour effet une augmentation significative du calcium intracellulaire. En conclusion, nous avons identifié une région avec un effet promoteur et amplificateur dont l'effet sur l'expression d'*ATP2B4* varie en fonction des allèles des SNPs associés aux FGs. Ainsi nos résultats permettent de proposer de nouvelles stratégies thérapeutiques basées sur la modulation de l'expression d'*ATP2B4* et du calcium intracellulaire.

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List of Abbreviations

SNP	Single nucleotide polymorphism
PMCA4	Plasma membrane calcium ATPase 4
CSP	Circumsporozoite protein
TRAP	Thrombospondin-related adhesive protein
iRBC	Infected red blood cell
RDTs	Rapid diagnostic tests
PfHR2	Plasmodium falciparum histidine-rich protein 2
pLDH	Plasmodium spp. Lactose dehydrogenase
TNF	Tumor necrosis factor
IL	Interleukin
PfEMP1	Plasmodium falciparum erythrocyte membrane protein1
ICAM-1	Intracellular adhesion molecule 1
VCAM 1	Vascular adhesion molecule 1
CD36	cluster determinant 36
MAC-1	Macrophage 1 antigen
LFA-1	Lymphocyte function-associated antigen 1
pRBC	Parasitized red blood cell
EPO	Erythropoietin
IFN	Interferon
NOS	Nitric oxide synthase
MSP	Merozoite surface protein
AMA-1	Apical membrane antigen 1
EBA	Erythrocyte binding antigen
LSA	Liver stage antigen
DC	Dendritic cells
MHC	Major histocompatibility complex

SSP	Sporozoite surface protein
ESP	Exported surface protein
CMI	Cellular mediated inhibition
CTLs	Cytotoxic T lymphocytes
Ig	Immunoglobulin
ADCI	Antibody-directed cellular inhibition
MSP	Merozoite surface protein
GPI	Glycosylphosphatidylinositol
PAMP	Pathogen associated molecular pattern
TLR	Toll-like receptors
PGE2	Prostaglandin E2
EPCR	Endothelial protein C receptor
CSA	Chondroitin sulfate A
LFA-1	Lymphocyte function associated antigen 1
BBB	Blood brain barrier
CM	Cerebral malaria
TDT	Transmission disequilibrium test
G6PD	Glucose-6-phosphate dehydrogenase
CRISPR	Clustered regularly interspaced short palindromic repeats

Introduction

A. INTRODUCTION TO MALARIA

1. Malaria epidemiology

1.1. Brief history

The term malaria is derived from the Italian word ‘mal’ ‘aria’ which means ‘bad air’ from an early association of the disease with marshy areas. Towards the end of the 19th century, Charles Louis Alphonse Laveran, a French army doctor, identified malaria parasites in fresh blood in 1880. He must have had remarkable eyesight as this was 10 years before Dimitri Romanowski developed the methylene blue staining method. It was not until 17 years later in 1897 that Ronald Ross demonstrated that these parasites were transmitted by mosquitoes providing insight on the lifecycle of these parasites then known as *Plasmodium* (Bruce-Chwatt 1981; Capanna 2006). Modern techniques in molecular biology and genetic anthropology have revealed that *P. falciparum* diverged from *P. reichenowi* some five to ten million years ago, parallel to the divergence of Homo from chimpanzees (Sallares, Bouwman et al. 2004). The most recent common ancestor of *P. falciparum* existed in tropical Africa some 100,000 years ago (Conway, Fanello et al. 2000). In contrast to *P. falciparum*, the species *P. ovale*, *P. vivax* and *P. malariae* transferred laterally from primates to humans (Sallares, Bouwman et al. 2004). Malaria represents one of the strongest selective pressures on the history of the human genome (Hirayasu, Ohashi et al. 2012) and is responsible for several clinical forms (Kwiatkowski 2005).

1.2. Present-day burden

One hundred and forty years after Laveran’s discovery, malaria remains a global burden with statistics according to the WHO report 2019 estimating that it affects approximately 228 million individuals. Its greatest impact is exerted in sub-Saharan Africa with most cases reported in children less than five years of age and pregnant women (Beier 1998).

At present, the various scientific discoveries have enabled the better understanding of the disease, the parasite, its vector, and the host, Thanks to the means organized in the fight against malaria at the scientific research and measures undertaken by the various global institutions (in particular, the accessibility of mosquito nets, insecticide, the rapid increase of diagnostic tests and greater availability of antimalarial drugs), the number of malaria cases recorded worldwide between 2000 and 2016 decreased by 18% and mortality rate being associated has decreased by 48%. Ninety-one countries and territories are today considered endemic for malaria against 108 in 2000 (Figure 1) (Organization 2016).

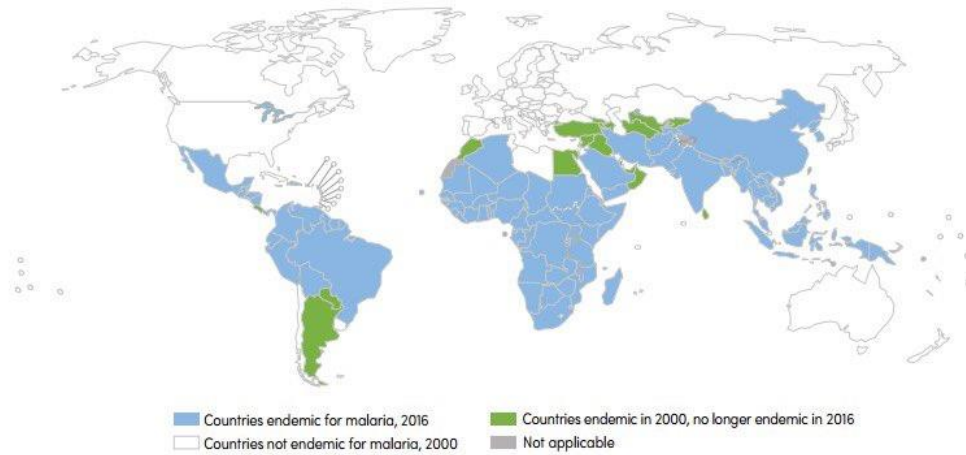


Figure 1. Malaria endemic countries in 2000 and 2016. Based on (WHO, 2017).

In 2018, an estimated 228 million cases of malaria occurred worldwide, compared to 231 million cases in 2017 and 251 million cases in 2010. Regardless of this decline, malaria still remains a huge public health burden. Around the world, Africa is the most impacted with 93% of malaria-related cases and deaths, followed by South-East Asia from the southeast (3.4%), the eastern Mediterranean region (2.1%) and Central America and south (<1%) (Figure2). The African population lacks access to the resources to prevent and treat the disease (Organization 2019).

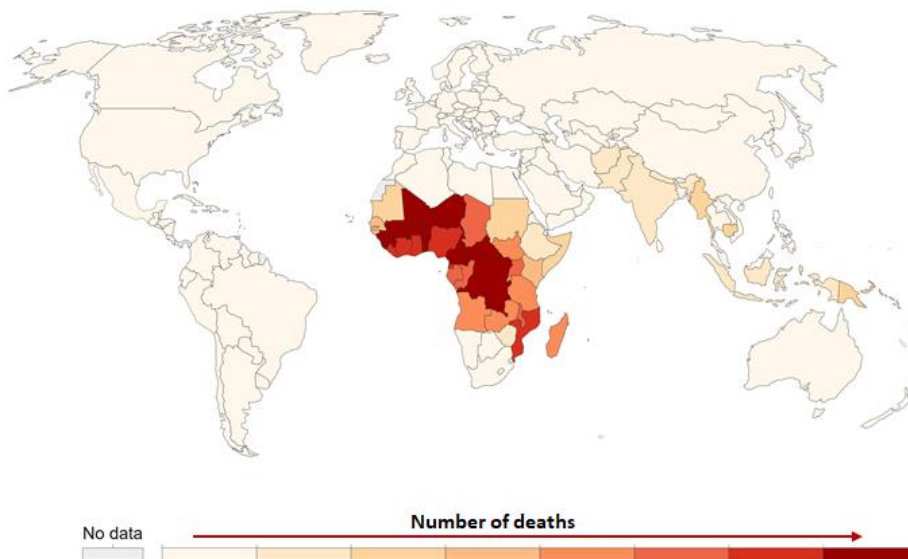


Figure 2. Death rate from malaria in 2018 (WHO, 2019), depicting Africa with most number of deaths.

2. THE PROTAGONISTS

Malaria is an infectious disease caused by protozoan parasites of the genus *Plasmodium* that are transmitted by the bite of a female mosquito infected with the genus *Anopheles* during its blood meal. The severity of the disease depends on multiple factors related to different protagonists: the host, the vector and the parasite.

2.1. The Pathogenic Agent

The *Plasmodium* is unicellular eukaryote belonging to the phylum *Apicomplexa*, so named because of the apical complex allowing them to invade the host cells. There are more than 150 species of *Plasmodium*, among them, the five are responsible for the disease in humans includes, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi* (Singh, Sung et al. 2004).

Plasmodium falciparum is the most dangerous of the five species. It's of great significance because of the highly pathogenic nature causing the most severe form of malaria with the highest number of reported cases predominantly in Africa (Beier 1998) with a mortality peak of about 1 million deaths in 2003 (Gething, Casey et al. 2016). However, *P. falciparum* is not only restricted to Africa but it's found all over the world (Figure 3).

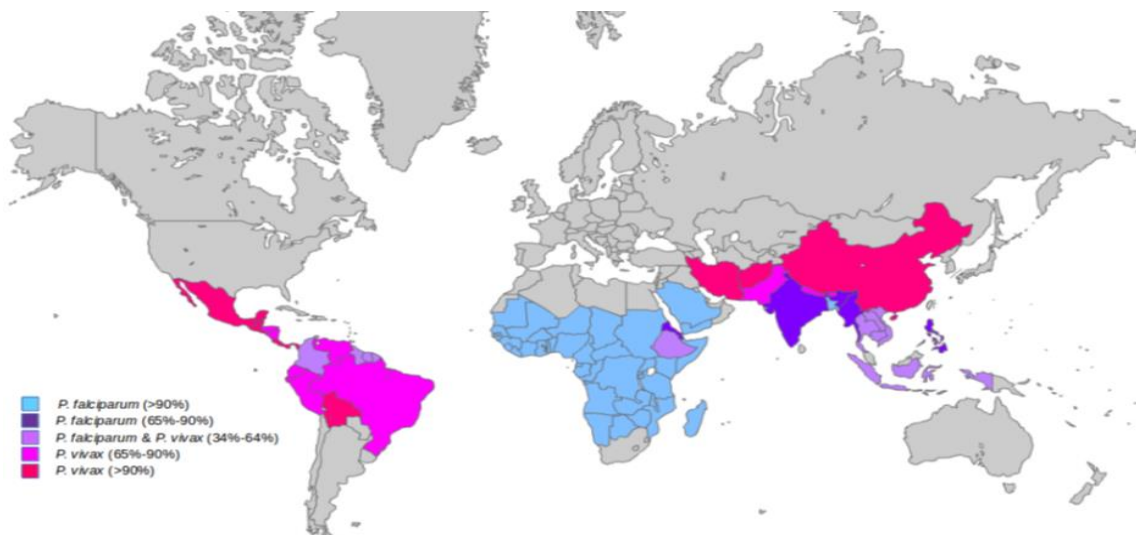


Figure 3. Geographical distribution of *P. falciparum* and *P. vivax* in 2016. Based on (WHO, 2016).

Plasmodium vivax has a more extensive geographical distribution than *P. falciparum*, namely in Asia, South America and Oceania (Figure 3). This distribution can be explained by the fact that *P. vivax* can grow in the vector at lower temperatures; it can survive at higher altitudes

and in cooler climates and finally it can remain dormant in the liver as hypnozoites for years. Although cases of *P. vivax* malaria are in Africa, the risk of infection with this species is quite low because of the absence of Duffy protein (a gene producing a protein necessary for *P. vivax* to invade Erythrocytes) in many African populations. It causes periodic fevers recurring every 48 hours (Mercereau-Puijalon and Menard 2010; Howes, Reiner Jr et al. 2015).

Plasmodium ovale occurs mainly in intertropical Africa in the center and west, as well as in Asia (Figure 4) where they cause mild fever with late relapses (about 5 years) (Autino, Noris et al. 2012; Sutherland 2016; Rutledge, Böhme et al. 2017; Zaw and Lin 2017).

Plasmodium malariae is found in sub-Saharan Africa, in much of South-East Asia, Indonesia, many islands in the western Pacific and the basin regions Amazonian South America (Figure 4) (Sutherland 2016; Rutledge, Böhme et al. 2017). *P. malariae* is responsible for quartan fevers (every 72 hours) with late relapses (up to more than 20 years) but not from persistent parasites in the liver stage (hypnozoites) as in *P. vivax* and *P. ovale* but in the blood stage (Collins and Jeffery 2007).

The species *Plasmodium knowlesi* is zoonotic being transmitted from macaque monkeys to humans by the vector *Anopheles leucosphyrus* (Coatney 1971; Cox-Singh, Davis et al. 2008). This strain causes cyclic periodic fevers every 24 hours and is mostly present in Southeast Asia. *P. knowlesi* parasitaemia is associated with severe or even fatal forms of malaria (Cox-Singh and Singh 2008; Millar and Cox-Singh 2015; Shearer, Huang et al. 2016).

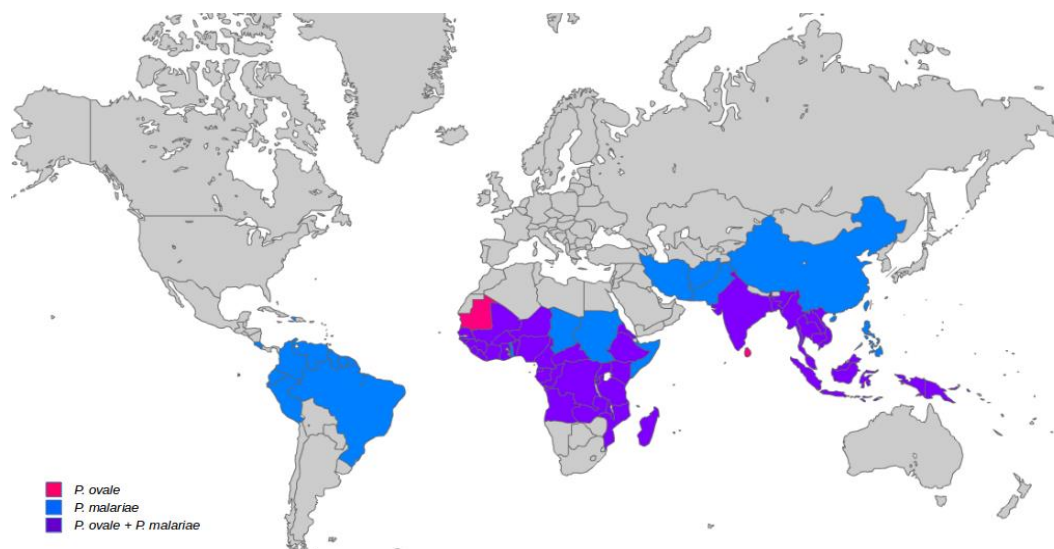


Figure 4 Geographical distribution of *P. ovale* and *P. malariae* in 2016, showing the occurrence of Plasmodium species and co-infection across the world. Based on (Rutledge, Böhme et al. 2017).

2.2.The Vector

There are about 500 different species of Anopheles mosquitoes including 70 that can transmit malaria to humans. Of these, only 41 are considered vectors of major importance (DVS: Dominant Vector Species), that are, responsible for the majority of human infections (Autino, Noris et al. 2012). *Anopheles gambiae*, the most prevalent vector in Africa, is responsible for most of the infections. The characteristics which make them more dangerous are:

- A longer lifespan (allowing the parasite to finish its development).
- Greater resilience to climate change.
- Anthropophilic character (that is, their tendency to perform their blood meal preferentially on the human rather than on the animal).
- Endophilic nature (that is, their tendency to eat and rest inside the houses).

Only female Anopheles mosquitoes are hematophagous (feeds on blood) and transmit the disease (Organization 2013).

Besides, Africa has temperate and stable weather conditions enabling the *Anopheles* host to thrive and hence transmit the parasites to the human host (Singh, Sung et al. 2004).

2.3.Lifecycle of malaria parasite

Plasmodium life cycle is complex and takes place successively in two hosts: (i) Man or intermediate host (asexual or schizogonic) and (ii) the anopheles or definitive host (sexual phase) (Figure 5)

The cycle in the human host, is also divided into two phases of asexual multiplication:

- the hepatic or exoerythrocytic phase, which corresponds to the incubation phase (asymptomatic phase).
- The blood or erythrocyte phase, responsible for clinical manifestation of the disease.

2.3.1. Asexual Phase in Human host

The life cycle of *Plasmodium* begins with a bite of an infected female *Anopheles* mosquito to the human host during a blood meal (Figure 5). It can be divided into various stages, starting with sporozoite entry into the blood stream. Infective sporozoites from the salivary gland of Anopheles mosquito are injected and deposited into the skin human host and are released slowly into the circulation (Doolan, Dobaño et al. 2009; Pasvol 2010; Nkhoma, Nair et al. 2012).

Once in the human blood stream, *P.falciparum* sporozoites reach the liver, penetrates the hepatocytes and this initiates the asexual reproduction (schizogony), an asymptomatic phase. Invasion is mediated by specific binding of the parasite circumsporozoite protein (CSP) and thrombospondin-related adhesive protein (TRAP) to heparan sulphate proteoglycans on the hepatocytes. Each sporozoite gives rise to thousands of merozoites inside the hepatocyte and each merozoite can invade erythrocyte upon release from liver initiating a blood stage infection (Jones and Good 2006; Greenwood, Fidock et al. 2008). In *P. vivax* and *P. ovale*, some of the parasites can remain in the liver for years as hypnozoites and may cause recurrent episodes of malarial disease. (www.malariasite.com).

To invade the RBC, the parasite must engage binding receptors and undergo apical orientation, junction formation and signaling. The parasite invades a vacuole derived from the RBC's plasma membrane and enters the vacuole by making a junction. Inside the erythrocyte, the merozoites develop to a 'ring' stage and then to a trophozoite which replicates to form a multinucleated schizont containing an average of 10 merozoites. When the schizont matures, the erythrocyte ruptures and releases merozoites that infect new erythrocytes and so repeat the cycle, taking approximately 48 hours. These repeated cycles lead to a rapid exponential growth of infected erythrocytes and during this blood stage, clinical symptoms of malaria appears.

2.3.2. Sexual phase in Anopheles mosquito

At some point in the life cycle, a proportion of merozoites follow a different developmental path, and rather than maturing into schizont, some of the trophozoites develop into the sexual stage of the parasite, forming either a female or male gametocyte. The stimuli triggering gametocytogenesis are not fully understood but likely result from various forms of stress, including pressure exerted by host immune system.

Gametocytes are the infective stage of the parasite and are ingested by female Anopheles mosquito into its midgut, where the female gametocyte matures to a macrogamete. Meanwhile, male gametocyte undergoes replication and exoflagellation to produce microgametocytes. These gametes fuse to form a diploid zygote. The motile zygote (ookinete) migrates through, and penetrates on the outer surface of the gut wall (as an oocyst). Asexual division within this oocyst produces numerous haploid sporozoites which migrate to the mosquito's salivary gland ready to be transmitted to the human host in a blood meal (Carter 1988; Eichner, Diebner et al. 2001).

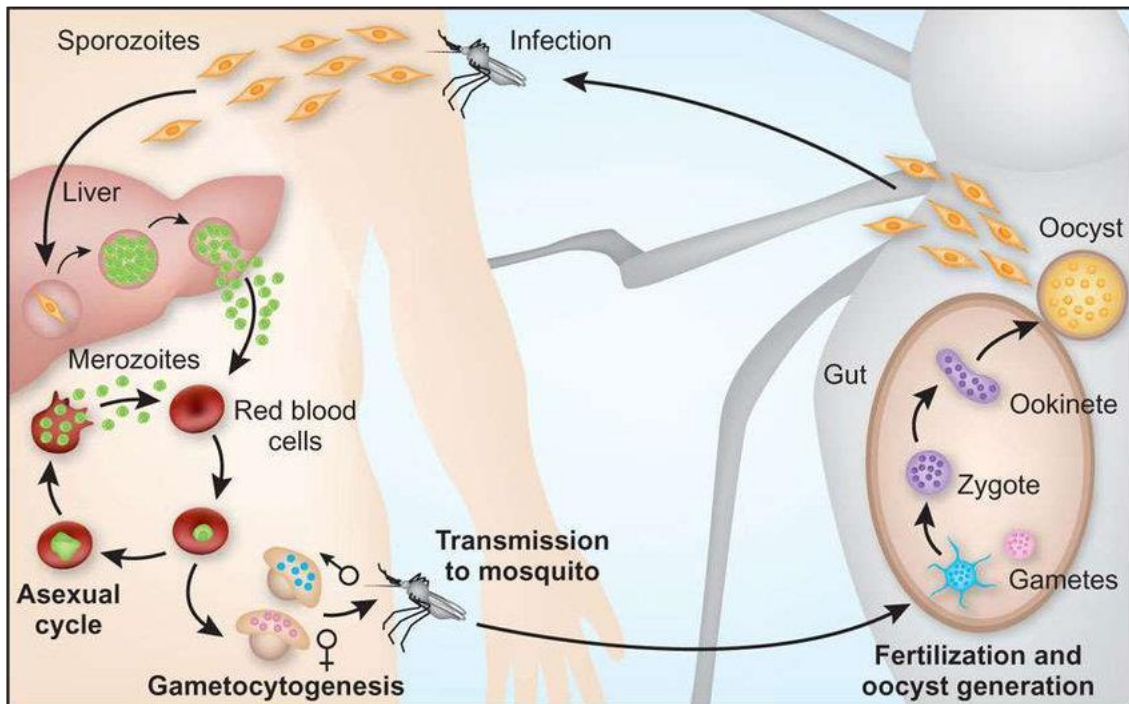


Figure 5. Life cycle of malaria parasite in human and mosquito.

Parasites in sporozoites form are inoculated into the host's subcutaneous tissue during the bite of the female Anopheles. They enter the vascular system and migrate to the liver where they infect the hepatocytes. Within these hepatocytes, they divide and upon rupture and release thousands of matured parasites as merozoites into the bloodstream, which invade the red blood cells and multiply there, destroying the red blood cell and releasing new merozoites. Some of them invade new erythrocytes; others are reprogrammed to undergo gametocytogenesis. Once mature, they enter the peripheral circulation to be ingested by a mosquito during his blood meal. Ingested gametocytes in the mosquito mature in the form of a microgamete male and female macrogamete. The fusion of these gametes forms a zygote, which develops into a mobile ookinete migrating through the epithelium of the intestinal mucosa and encysts to become an oocyst. The rupture of this oocyst releases mobile haploid sporozoites which move towards the salivary glands. The sporozoites may be injected with a new blood meal. Adapted from (Pasvol 2010).

B. PATHOPHYSIOLOGY OF MALARIA

1. Clinical forms

Once infected with *Plasmodium* infection, the response of the host can be extremely variable ranging from asymptomatic infection, to uncomplicated/mild or even severe attacks, which can lead to the death of the individual. The severity of these clinical manifestations is determined by complex interactions between the host, the parasite and environmental factors (Figure 7). The outcome of an infection and progression into pathology depends on the specific and dynamic combination of host and parasite properties. Clinical disease also changes with age, immunity and transmission rates. Uncomplicated malaria occurs in semi-immune individuals while severe malaria affects non-immune subjects, with the group most at risk in endemic areas being children under five years of age (Rogier, Commenges et al. 1996). However, depending on the *Plasmodium* species, the clinical forms of the disease are not necessarily the same.

Our project will focus only on *P. falciparum* species, which is the most hazardous and is responsible for most of the death. *P. falciparum* spreads promptly in the blood and creates blood clots in small vessels, resulting in serious anemia, cerebral malaria and eventually death.

Malaria disease can be categorized as:

1.1. Asymptomatic malaria

Asymptomatic malaria can be caused by all *Plasmodium* species. Asymptomatic forms of infection occur without symptoms and they represent a vast hidden reservoir of active infection which allows the persistence of the parasite and its possible spread to others human hosts. It is difficult to understand why some people develop clinical manifestations during malaria infection while others remain asymptomatic (Day and Marsh 1991; Trape, Rogier et al. 1994; Daubersies, Sallenave-Sales et al. 1996), The exposure, the general state of health and the physiological condition of the host, especially variations in immunity, the parasitic factors involved in the virulence of the infection or the environment can influence the progression of malaria infection towards an asymptomatic form. Asymptomatic malaria infections are common in humans living in endemic regions, including in low- transmission as in Peru, Brazil or Colombia (Alves, Durlacher et al. 2002; Roshanravan, Kari et al. 2003; Alves, Gil et al. 2005; Fugikaha, Fornazari et al. 2007; Cucunubá, Guerra et al. 2008; Imwong, Nguyen et al. 2015).

Indeed, in these regions, the asymptomatic forms have a prevalence four to five times higher than symptomatic infections. However, little is known about the silent or long-term consequences of asymptomatic health infections. A study conducted in the Sumba region (Indonesia), has shown that compared to unaffected individuals, people with asymptomatic infections have higher levels of inflammation markers, while low platelet count and hemoglobin and thus have suggested that asymptomatic malaria infections have subtle health consequences, especially in children, and should be regarded as potentially harmful (De Mast, Brouwers et al. 2015).

1.2.Uncomplicated malaria:

Uncomplicated or mild malaria is characterized by the presence of asexual form of *Plasmodium* in the blood associated with different symptoms, without any complications. The presence of *Plasmodium* is observed directly by microscope or detected by malaria Rapid Diagnostic Tests (RDTs). RDTs offer the potential to provide accurate diagnostic to all at-risk populations. Malaria RDTs rely on the detection of parasite specific antigens (proteins) circulating in the bloodstream. The most common antigens for RDTs are *P.falciparum* histidine-rich protein 2 (*PfHR2*) and *Plasmodium spp.* Lactose dehydrogenase (pLDH).

An infected individual develops mild malaria when his parasitemia (number of iRBCs per μL of blood) passes a certain threshold, pyrogenic threshold. If the parasitemia remains below the pyrogenic threshold, it falls in asymptomatic infection. A study has shown that the individual's risk of fever was multiplied by almost 44 fold when the parasitemia passed this threshold depending on age (Rogier, Commenges et al. 1996).

The main clinical symptoms of mild malaria are a combination of fever, chills and sweat, headache, vomiting, watery diarrhea, anemia, and swelling of the spleen (splenomegaly). In addition to these symptoms, children can experience convulsions, coughing and rapid shallow breathing. Such symptoms are caused by the rupture of iRBCs and by the release of pyrogens like $\text{TNF-}\alpha$, $\text{IL-1}\beta$ and IL-6 by macrophages and provoke mild malaria.

1.3.Severe malaria

Severe malaria is usually caused by infection with *Plasmodium falciparum*, though less frequently can also be caused by *Plasmodium vivax* or *Plasmodium knowlesi*. Severe malaria is a complex disorder that affects several tissue and organs. The criteria for severe malaria in both endemic and non-endemic settings as defined by the World Health Organization are characterized by:

- Deep Coma (cerebral malaria) (Blantyre score <3 for children and Glasgow score <9 for adults).
- Impaired consciousness
- extreme weakness
- Multiple convulsions
- Pulmonary edema
- Acute renal failure
- Severe anemia (Hb <5g/dL)
- Hypoglycemia (blood glucose level <40mg/dl)
- Acidosis (pH <7.35 plasma bicarbonate <15 mmol/L)
- Parasitemia > 2,500,000 parasites / μ L blood

In Africa, most of the deaths due to severe malaria occur among Children and the outcomes are in the form of following three major clinical manifestations that may occur separately or in combination: severe anemia, cerebral malaria and respiratory distress (Marsh, Forster et al. 1995; Organization 2000).

1.3.1. Cerebral malaria

Cerebral malaria is the most severe neurological complication of infection due to *P.falciparum* and is defined by the WHO as a severe malaria attack characterized by a deep coma (inability to locate a painful stimulus, Blantyre score <3 for children and Glasgow score <11 for adults).

Two factors are associated with cerebral malaria:

a. The Reduced deformability of iRBCs - Deformability is an essential feature of red blood cells (RBCs) that enables them to travel through even the smallest capillaries of the human body. An infected RBC undergoes changes as the parasite grows within it. In *P. falciparum* infections, iRBCs have been observed to become rigid and spherical losing their elasticity. Mechanically, the iRBC gradually loses its deformability, having difficulty in passing through the microvasculature and becomes more easily adhered to the vascular walls and to other cells (Paulitschke and Nash 1993). Although this mechanism enhances the splenic clearance of iRBCs, on the other hand, it leads to the blockage in the microvasculature, causing blockage of the blood's pathway which stops blood flow, leading to a shortage of oxygen and nutrients to those areas of the brain. The occlusion of the small vessels occurs throughout the brain which manifests as cerebral malaria (Miller, Baruch et al. 2002).

b. In *P. falciparum* malaria, mature forms of the parasite are not usually observed by microscopy as they adhere to the endothelium of microvasculature, a phenomenon known as cytoadherence (Berendt, Ferguson et al. 1994). In addition, uninfected RBCs and platelets have been observed to stick to iRBCs also known as rosetting which together with cytoadherence are believed to be involved in the development of severe malaria (Wahlgren, Barragan et al. 1998). Major organs such as the brain, lungs, liver and the placenta undergo obstruction due to the sequestration of non-infected and infected RBCs affecting their function (Miller, Good et al. 1994).

Pathological examinations of individuals who have acceded to severe malaria reveal this sequestration in major organs suggesting the association of severe malaria and cytoadherence (Fried and Duffy 1996; Newbold, Warn et al. 1997). In the brain, this manifests as cerebral malaria while in pregnant mothers as placental malaria. Cytoadherence and rosetting have been associated with the presence of the surface molecules called *Plasmodium falciparum* erythrocyte membrane protein 1 (*PfEMP 1*) which binds to receptors expressed on the surface of endothelial cells of the brain including ICAM-1 (intracellular adhesion molecule 1), VCAM-1 (vascular adhesion molecule 1), CD36, thrombospondin and E-selectin that renders the iRBCs 'sticky' causing them to form rosettes with non-infected RBCs and also, enabling them to sequester to capillaries or tissues (Pasternak and Dzikowski 2009).

The sequestration alters blood flow in the brain and causes hypoxia (Figure 6). A pro-coagulant state caused by the presence of the parasite also seems to play an important role in the pathophysiology of cerebral malaria. Studies have shown that sequestration can lead to activation of endothelial cells which initiate the coagulation cascade which itself is accompanied by thrombocytopenia (Francischetti, Seydel et al. 2008). This altered hemostasis, combined with obstruction by parasitized erythrocytes, could be the cause of hemorrhages observed in patients who died following brain damage caused by malaria (Gay, Zougbedé et al. 2012).

Blood platelets also play an interesting role in the pathology of severe malaria by several mechanisms including their ability to regulate the production of surface receptors like ICAM-1 and pro-inflammatory cytokines like interleukin 6 (IL-6) by endothelial cells via the production of IL-1 (Hawrylowicz, Howells et al. 1991). Platelets also contribute to the modulation of endothelial cell metabolism by regulating the expression of prostaglandin. Their adhesion to the endothelium, a process that takes place at the start of a proinflammatory response, is also an aspect by which activated platelets promote the sequestration of leukocytes and erythrocytes. Indeed, the platelets express on their surface fibrinogen and the

ICAM-2 and P-selectin receptors which can interact respectively with the MAC-1 receptor (Macrophage 1 antigen) and the LFA-1 (Lymphocyte Function-associated Antigen 1) and PSGL-1 ligands (P-selectin glycoprotein ligand 1). Likewise, infected erythrocytes can form an infected endothelium-platelet-erythrocyte complex by the interaction between the parasite antigen *PfEMP-1* of the parasite expressed on the surface of the parasitized cells and the CD36 receptor expressed on the surface of activated platelets (Schofield and Grau 2005).

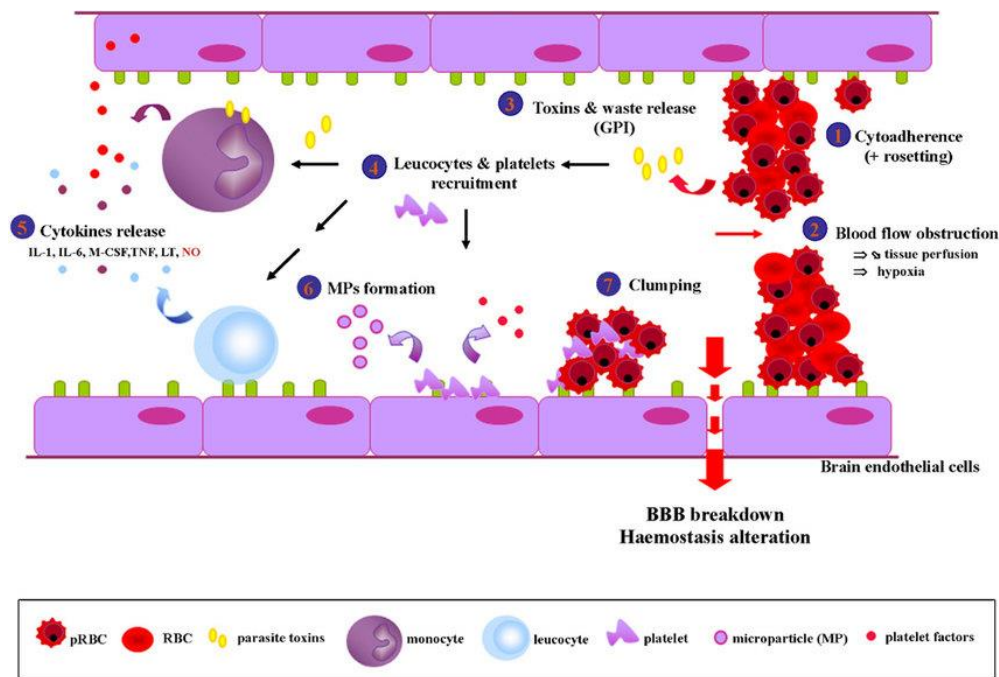


Figure 6. Brain endothelium during cerebral malaria pathogenesis.

Different mechanisms that can contribute to the pathogenesis of cerebral malaria. This figure shows a schematic representation of the cerebral endothelium and events that may play a role in the pathogenesis of cerebral malaria. The mature forms of parasitized red blood cells (pRBCs) adhere to the endothelial cells of the brain micro-vessels through interactions between their membrane proteins, called *PfEMP-1*, and the cell adhesion molecules expressed on the surface of endothelial cells (1). This cytoadherence, combined with other phenomena such as rosetting (aggregation of several red blood cells RBCs and pRBCs together), clumping (aggregation of platelets and RBCs) (7), the decrease in deformability of RBCs, cause a vascular obstruction which leads to a defect in tissue perfusion, hypoxia (2), and creates a toxic local environment, to which is added the reduction in the elimination of parasitic waste. The second consequence of this parasitic sequestration in micro-vessels is the release of toxins and parasitic factors (3) which can lead to the recruitment of host cells, such as leukocytes and platelets (4). These cells produce mediators (5, 6), including pro-inflammatory cytokines which can have a deleterious local or systemic effect and are responsible for the activation of endothelial cells. This cellular activation (also caused by RBCs) can lead to the alteration of homeostasis, to apoptosis of endothelial cells, and to the rupture of the blood-brain barrier (caused in particular by an increase in inter-endothelial permeability); rupture probably followed then by other secondary neuropathological events, at the level of the cerebral parenchyma, which could lead to a cerebral edema or a coma (Gay, Zougbedé et al. 2012).

The mortality rate in adults and children due to cerebral malaria is about 20% and most deaths happen within 24hrs of admission, before antimalarial drugs may have had time to work. While in adults, cerebral malaria is a multiorgan disease. After a few days of illness patients normally present with fever, discomfort, body and joint pain, headache and eventually coma. The immune mechanisms involved in cerebral malaria have been described in another section (Idro, Jenkins et al. 2005).

1.3.2. Severe malarial anemia

Severe malaria anemia is defined by low hemoglobin level below 5g / dl (Organization 2000) and is characterized by lysis of both healthy and infected red blood cells (Jakeman, Saul et al. 1999), and an abnormal erythrocyte production (dyserythropoiesis) (Thawani, Tam et al. 2014). The elimination of infected red blood cells takes place via two processes. (i) The rupture of schizonts at the end of the erythrocyte cycle (Phillips & Pasvol, 1992) and (ii) The splenic clearance during which infected red blood cells are either phagocytosed by macrophages (Buffet, Safeukui et al. 2011).

During severe malaria anemia, the healthy/uninfected red blood cells are also lysed and eliminated. Studies suggest the establishment of an apoptotic mechanism that could also be responsible for the destruction of healthy red blood cells (Jakeman, Saul et al. 1999; Totino, Daniel-Ribeiro et al. 2016).

In addition, the decrease in erythrocyte production again involves several mechanisms. Under physiological conditions, erythropoiesis is the process for the renewal of red blood cells. This process takes place in the bone marrow and involves the proliferation and maturation of erythrocyte under the action of a cytokine erythropoietin (EPO) primarily produced by the kidneys. During malaria infection, this process is bothered by, among other things, a decrease in the synthesis of EPO (mainly in adults) and inhibition of the response of erythrocyte progenitors to EPO due to an imbalance in the pro and anti-inflammatory cytokine balance.

Many studies have shown that an excessive release of IFN- γ and TNF- α , associated with NO (nitric oxide), contribute to dyserythropoiesis and erythrophagocytosis (Menendez, Fleming et al. 2000; Perkins, Were et al. 2011).

1.3.3. Respiratory distress

Acute respiratory distress syndrome (ARDS) is one of the major complications associated with malaria. This syndrome is characterized by the inability of lungs to function properly causing an increased respiratory rate of the patient and possibly dyspnea (shortness of breath) that can cause death. ARDS is primarily caused by the damage to the lining of the capillaries by a subsequent infection which consequently makes the walls more permeable, resulting in accumulation of fluid and, thus, an inability of the lung to ensure the exchange of oxygen and carbon dioxide (Taylor, Hanson et al. 2012). Like cerebral malaria, the mechanisms involved in the development of ARDS are the increased inflammatory response following schizont rupture, endothelial cell activation and the sequestration of infected red blood cells, leading to obstruction of micro-vessels and pulmonary hypoxia. Disruption of the integrity of the alveoli-capillary barrier causes the passage of plasma liquid into the alveoli and eventually leading to pulmonary edema (Van den Steen, Deroost et al. 2013).

2. Factors determining the severity of malaria infection

The severity of the *Plasmodium* infection is determined by complex interactions between factors related to the host and the parasite, but also environmental and socio-economic factors (precise in Figure 7) which results in diverse clinical outcomes ranging from asymptomatic infections to severe forms of disease as described above.

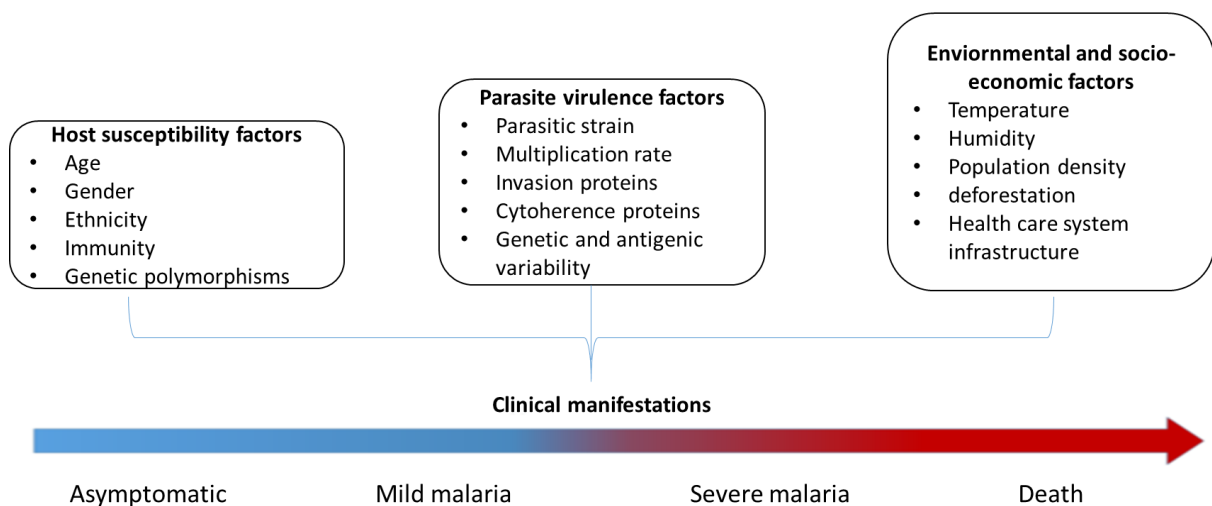


Figure 7. Factors determining the severity of human malaria.

Susceptibility to *Plasmodium* infection results from a complex combination of factors from of the host, the parasite and the environment. The degree of susceptibility can lead to various clinical manifestations, ranging from asymptomatic infections to severe attacks and death.

2.1.Parasite factors

The capability of the parasites to multiply, to adhere to the tissues of the host and to produce toxic substances or genetic and antigenic variation to induce immune damaging responses to host is considered to be the virulence factors. A study conducted in Thailand showed in vitro that *P. falciparum* isolates from people with severe malaria had multiplication rates three times higher than those from patients with uncomplicated malaria (Chotivanich, Udomsangpetch et al. 2000). Different studies have put evidences of polymorphic differences in surface antigen levels involved in the erythrocytes invasion, namely the family of merozoite surface proteins (MSP), Apical membrane antigen 1 (AMA1), erythrocyte binding antigen 175 (EBA-175). These polymorphisms would allow the parasite to escape the immune response (Boutlis, Tjitra et al. 2003; Nankabirwa, Wandera et al. 2013; Maketa, Mavoko et al. 2015). Along with invasion proteins, there are cytoadherence proteins which plays a role in the sequestration of the parasite and are responsible for the pathogenesis (Miller, Ackerman et al. 2013). All *Plasmodium* species invade and replicate within their hosts' erythrocytes, but the virulence of *P. falciparum* is linked to expression of the protein PfEMP1 on the surface of infected erythrocytes. PfEMP1 expressed by *Plasmodium* can bind to different host receptors, permitting them to sequester and obstruct small blood vessels and leading to damage in deep tissues of vital organs, including the lungs, kidneys, and placenta. Moreover, sequestration within the brain can cause life-threatening pathologies in a syndrome known as cerebral malaria (Wassmer, Taylor et al. 2015). Cytoadherence and sequestration evolved to enable *P. falciparum* to avoid passage through the spleen; thus, this process successfully allows the parasites to escape filtration and mechanical clearance from circulation. Furthermore, the specific pathogenicity of *P.falciparum* is credited to its ability to modify the surface of human infected red blood cells (RBCs). These modifications impart cytoadhesive properties to the red blood cells. To evade the arm of the host's adaptive immunity, the parasites have evolved unique mechanisms of tightly controlled alternating expression of adhesive surface protein variants (Duraisingh and Horn 2016) called the process of antigenic variation.

2.2.Environmental and socio-economic factors

Climate and environmental changes are driving the expansion of many vector species and the intensification of transmission of pathogens (Patz, Olson et al. 2008). For example, in several tropical regions, deforestation has resulted in an increased rate of human bites by previously

zoophilic vectors and the appearance of new malaria epidemics (Vittor, Gilman et al. 2006; Cox-Singh, Davis et al. 2008). Mosquitoes survive better under conditions of high humidity. They also become more active when humidity rises. This is why they are more active and prefer feeding during the night – the relative humidity of the environment is higher at night. Also, historic temperature increases have also been implicated in the spread of malaria in new habitats and new regions (Patz and Olson 2006).

Environmental changes linked to economic development can also cause an increased transmission of malaria. Indeed, following the elimination of malaria in the Demerara estuary in Guyana (by spraying DDT), the human population has increased rapidly, land use activities have shifted from livestock to rice cultivation more profitable, and mechanization on the roads and in the fields replaced the horse, the mule, and the oxen. These changes have caused such imbalance between man and cattle that *Anopheles aquasalis*, a very abundant species all along the coast, but which was so far "fixed" by the livestock, shifted its focus from cattle to humans (Giglioli 1963). Poverty is another economic factor that could be considered as an important factor for intense malaria transmissions. Most of the families in deprived areas do not have access to prevention methods such as mosquito nets or insecticides, medical care and antimalarial treatments (Sachs and Malaney 2002). Different studies conducted in Sudan and in Zimbabwe have shown that the time spent to visit a health care center and associated transportation costs can influence the decision to seek early treatment against malaria and therefore results in delay diagnosis and treatment (Malik, Hanafi et al. 2006; Mutsigiri-Murewanhema, Mafaune et al. 2017). However, to overcome the problem of long distances between communities and health facilities, which can lead to delays in diagnosis and treatment, Zimbabwe introduced a treatment community burden of malaria by village health workers in 2012.

The other consequence of poverty determining the outcome of malaria infection is malnutrition that constitutes a significant risk of severe malaria and is associated with infant mortality (Mutsigiri-Murewanhema, Mafaune et al. 2017). Different studies suggest that improving nutritional status helps to reduce the number of malaria-related deaths because it decreases the severity of episodes of malaria (Caulfield, Richard et al. 2004). Improper nutrition affects the functionality of all body systems. So children being underweight are more vulnerable to malaria due to impaired immune systems (Scrimshaw and SanGiovanni 1997).

2.3.Host factors

Apart from parasite and environmental factor, various host factors also contribute to the disease severity, such as age, gender, ethnicity, immunity and genetic factors (Figure 7).

Studies in areas of high and moderate transmission have shown that relative frequencies of severe anaemia and cerebral malaria (the two main presentations of severe malaria in African children) vary with age (Angyo, Pam et al. 1996; Greenwood 1997; Organization 2000). It is known that in a holoendemic area, the age group 1 – 5 years is the most vulnerable to severe and complicated malaria since their immunity to malaria is still very poor. Children under 5 years of age are at the highest risk for the disease with mortality rate of 10-20%. (Jelliffe and Stanfield 1978; Hendrickse 1991; Achidi, Salimonu et al. 1996; Streatfield, Khan et al. 2014; Seydel, Kampondeni et al. 2015).

Considering the gender, evidence suggests that given equal exposure, adult men and women are equally vulnerable to malaria infection, except for pregnant women who are at greater risk of severe malaria in most endemic areas (Reuben 1993). Although malaria affects both men and women, gender roles and gender dynamics give rise to different vulnerabilities, such as exposure patterns. For example, traditional gender roles may involve men working in the fields at dusk or women gathering water early in the morning, exposing them to peak mosquito-biting times (Reuben 1993; Vlassoff and Manderson 1998; Cotter, Sturrock et al. 2013). It is well established that *P.falciparum* is an evolutionary driving force that has shaped the human genome (Kwiatkowski 2005). Strikingly, this selective pressure led to the emergence of a particular population in Africa that is relatively protected against malaria. The Fulani from Sudan and West Africa constitute a particularly interesting ethnic group because of their lower susceptibility to malaria as compared to other sympatric populations such as the Dogon in Mali (Dolo, Modiano et al. 2005), the Mossi and Rimaibé in Burkina Faso (Modiano, Petrarca et al. 1995; Modiano, Petrarca et al. 1996) and the other ethnic groups of Darawesh village in Sudan (Creasey, Giha et al. 2004).

Host immune and genetic factors will be described in details as follows.

C. HOST IMMUNE FACTORS

In areas with low transmission or in regions of seasonal malaria infections, people generally develop severe malaria during an infection, such as in Sri Lanka (Alles, Mendis et al. 1998). Numerous studies have shown that due to subsequent regular and repeated exposure (Marsh and Kinyanjui 2006; Langhorne, Ndungu et al. 2008), individuals living in highly endemic areas where malaria prevails all year round, develop protective immunity but not sterilizing (that is, it does not eliminate the parasite from the individual) called "premunition". This premunition is labile (it disappears between 1 and 2 years if the subject leaves the endemic area) and its development is progressive (Griffin, Hollingsworth et al. 2015), with the successive emergence of clinical immunity (or "anti-disease") offering protection against simple forms and complications by the disease, followed by anti-parasitic immunity allowing low parasitemia by controlling parasite multiplication. Children of 6 months to 5 years of age have the lowest levels of protective immunity and are most likely to develop high parasitemia with risks of complications and death (Marsh and Kinyanjui 2006). Infants (<6 months) benefit from some protection, probably due to antibodies acquired from their mother, but also due to the presence of fetal hemoglobin within erythrocytes (Amaratunga, Lopera-Mesa et al. 2011), responsible for an altered representation of *PfEMP1* on the surface of infected red blood cells and therefore impaired cytoadherence, as well as constituents of breast milk rich in antibodies (Hawking 1954; Kassim, Ako-Anai et al. 2000; Kicska, Ting et al. 2003).

1. Mechanism of protective immunity

Immunity against malaria is complex and stage specific. Sterile immunity against the liver stage can be induced in animals and humans with irradiated and genetically attenuated sporozoites. Irradiated sporozoites-induced protective immunity has been shown to involve CD4⁺ producing IFN- γ , and CD8⁺ T cells directed against liver stage parasites, and in vivo depletion of these effectors has led to blood infection upon a live sporozoite challenge (Schofield, Villaquiran et al. 1987; Romero, Maryanski et al. 1989; Scheller, Green et al. 1997). When a mosquito bites a person and injects sporozoites, antibodies to the sporozoites could protect both through opsonization leading to the clearance of sporozoites before reaching the hepatocyte (Schofield, Villaquiran et al. 1987; Nussenzweig and Nussenzweig 1989) and by blocking invasion of hepatocytes by the parasite (Fidock, Pasquetto et al. 1997; Silvie, Franetich et al. 2004). Circumsporozoite protein (CSP) is the most abundant protein on the sporozoite and participates in binding to liver cells. Together with liver stage antigen-1

(LSA-1), LSA-3, sporozoite surface protein (SSP2) and exported surface protein (ESP), CSP constitutes target of neutralizing antibodies (John, Zickafoose et al. 2003).

The parasite developing in the host hepatocyte is the major target of protective immunity directed against the pre-erythrocytic stage. CD4⁺ and CD8⁺ T cells can indeed recognize parasite-derived peptides presented by MHC class II and I molecules respectively, on the surface of infected hepatocyte. Furthermore, it has been shown that the sporozoites are drained from the skin inoculation site to lymph node where they can prime T cell response specific for the parasite infected hepatocyte through antigen presentation by dendritic cells (DCs) (Chakravarty, Cockburn et al. 2007). In BALB/c mice, activated CD8⁺ T cells produce IFN- γ which proceeds and induce the production of IL-12. IL-12 in turn induces IFN- γ production in Natural killer (NK) cells in a positive feedback loop. The IFN- γ then activates nitric oxide (NO), subsequently eliminating the infected hepatocytes or the intra-hepatic schizont via toxicity, or cellular mediated inhibition (CMI) (Good and Doolan 1999). Distinct mechanisms can be induced in mice with different genetic backgrounds. In most cases, however there is an absolute requirement of CD8⁺ T cells and IFN- γ , and the strikingly short time to re-infection in adult subjects suggests that immunity against the pre-erythrocytic stage is not particularly effective, and early studies with direct blood stage challenge indicate that immune adult remains protected even if this stage of the life cycle is bypassed (Marsh and Kinyanjui 2006) (Figure 8)

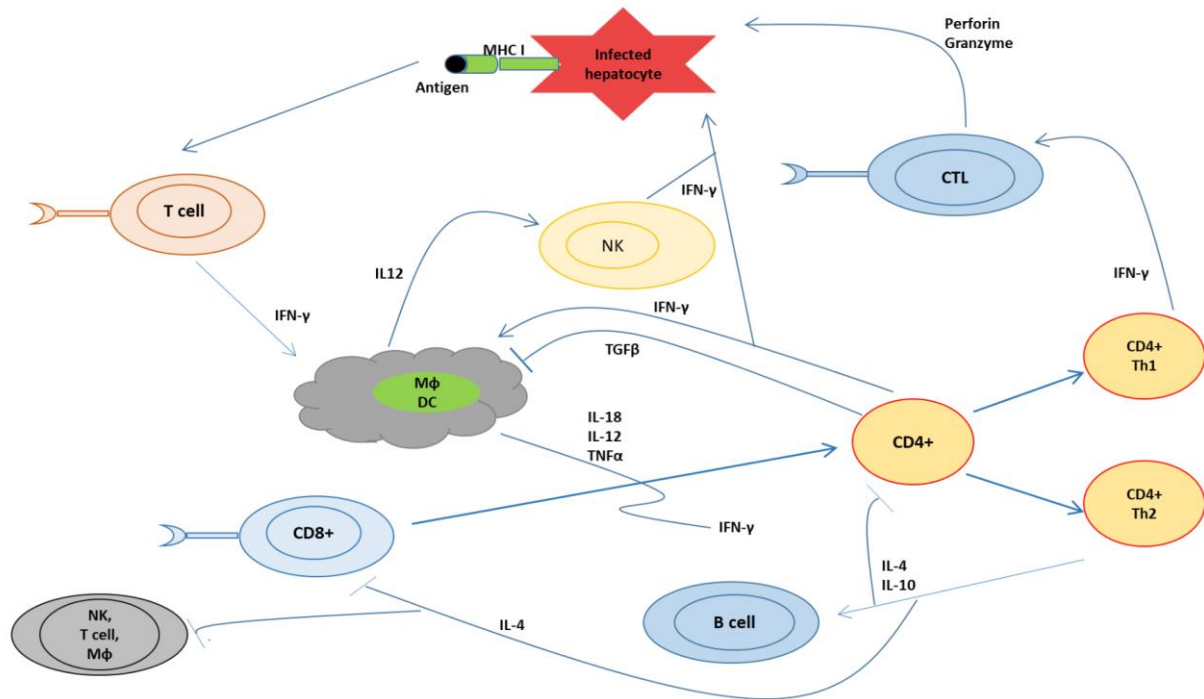


Figure 8. Mechanisms of exo-erythrocytic protective immunity.

Infected hepatocytes present MHC class I sporozoite peptide complexes on their surface leads to the recognition by cytotoxic T-lymphocytes (CTLs) to kill the infected cell. Antigen presenting cells activate CD4+ T cells, and produce IL12 and IL18 that stimulate the production of IFN- γ by NK and CD4+ T cells, which is able to trigger a cascade of immune reactions and lead to the death of intracellular parasite within the hepatocyte. The cytokines are major inducers of Th1 and Th2 subset development. Th1 develops from Naïve CD4+ T cells which are important for abolition of parasite. IFN- γ is the promising cytokine of Th1 cells and promotes the microbicidal activity of macrophage and production of cytokines (TNF, IL-12, and IL-18). The development of Th1 response can be antagonized directly by IL-4 and TGF- β and indirectly by IL-10 that can inhibit the production of proinflammatory cytokines. B-cell proliferation is induced by IL-10 that is crucial for the development of malarial antibodies. Adapted from (Malaguarnera and Musumeci 2002).

Furthermore, Asexual blood stage of *P.falciparum* is thought to protect against malaria and both antibody-dependent and cell mediated mechanisms contribute in providing immunity against the erythrocytic stage.

T cells are essential for the acquisition and maintenance of protective immunity against asexual blood stage of malaria. The regulatory and effector functions of CD4+ cells are evident in both humans and mice. In mice, evidence is based on adoptive transfer of protection by such cells and an increased susceptibility to CD4+ T cells depleted mouse (Langhorne, Simon-Haarhaus et al. 1990). The existence of functionally distinct CD4+ T cells in humans is indicated by results of in vitro experiments with PBMCs from naturally

P.falciparum exposed individuals (Troye-Blomberg, Perlmann et al. 1999). It is suggested that CD4+ T cells of the Th1 type activate macrophages and other cells to produce TNF, NO and other mediators through the release of inflammatory cytokines, while CD4+ cells of Th2 type functions in humoral immunity as helper cells for B cells (Abbas, Murphy et al. 1996).

Antibodies are generally accepted markers of infection and are also effectors of protection against the blood stage of malaria. Antibodies play an important role in protection against malaria and this was first demonstrated by the passive transfer of antibodies from immune adults to children with acute *P.falciparum* infection were successfully used to treat patients in Thailand (Sabchareon, Burnouf et al. 1991). Bouharoun-Tayoun *et al.* observed significant differences in the distribution of immunoglobulin G (IgG) in protected and non-protected individuals. It was found that cytophilic subclasses (IgG1 and IgG3) were prevalent in protected individuals while, IgG2 and IgG4 were abundant in non-protected individuals (Bouharoun-Tayoun and Druilhe 1992). In Burkina Faso, it was observed that the IgG2 antibodies can participate in protection because certain individuals have a frequent mutated allele of Fc γ RIIa that can bind IgG2, along with IgG3 and IgG1 (Aucan, Traoré et al. 2000). There are several studies which demonstrate the protective effect of IgG3 against *P.falciparum* malaria, although there are some contradictory results too. Generally, the most prevalent isotype of antibody responses associated with protection is IgG3, which is a short lived subclass compared to other IgG subclasses. Serum antibodies to merozoite surface protein (MSP) 2 and MSP3, which have been associated to protection are almost exclusively of the IgG3 class (Oeuvray, Bouharoun-Tayoun et al. 1994; Taylor, Allen et al. 1998; Roussilhon, Oeuvray et al. 2007). High levels of the cytophilic IgG3 subclass have been associated with reduced parasitemia, and protection against mild and severe malaria (Roussilhon, Oeuvray et al. 2007; Giha, Nasr et al. 2010). Some candidate genes have been associated with IgG1 or IgG3 subclass levels. These include *HBB*, *IL4*, *TNF*, and *FCGR2A*, which have also been associated with both malaria resistance and IgG levels (Kwiatkowski 2005; Carpenter, Rooth et al. 2009; Tangteerawatana, Perlmann et al. 2009). This suggests that those genes control the production of cytophilic IgG subclasses. More generally, genes that have been associated with malaria resistance may be associated with the level of protective IgG subclasses (Afridi, Atkinson et al. 2012).

The acquired humoral protection against malaria might involve immune phagocytosis, antibody-dependent cellular inhibition (ADCI), or related mechanisms. The most efficient *in vivo* model for the antibody mediated parasite control in malaria endemic areas requires the

participation of monocytes through the Fc receptors after binding to their parasite target and has been called as antibody-dependent cellular inhibition (ADCI) (Khusmith and Druilhe 1983; Lunel and Druilhe 1989). This hypothesis was strengthened by the results of a comparative in-vivo/in-vitro model where the antibodies which proved to be protective in-vivo by passive transfer of IgG in humans, had no effect in-vitro on the growth of *P.falciparum* asexual blood stages unless they cooperated with blood monocytes. Conversely, antibodies that were not protective in-vivo were unable to exert in-vitro an antimalarial effect by cooperation with monocytes (Bouharoun-Tayoun, Attanath et al. 1990). It is thought that at the time of schizont rupture, there occurs a contact between some merozoites surface components and cytophilic antibodies which are bound to monocytes via their Fc fragment helps to trigger the release of soluble mediators which diffuse in the culture medium and blocks the division of surrounding intraerythrocytic parasites. TNF is involved in this mechanism, although it is yet to establish if TNF requires help from another unidentified component to access for intraerythrocytic parasite. It is a known fact that SNPs present in the promoter region of TNF tend to affect the levels of malaria antibodies (Migot-Nabias, Mombo et al. 2000) and so can also affect the Antibody dependent cellular immunity (ADCI) defense mechanism (Figure 9).

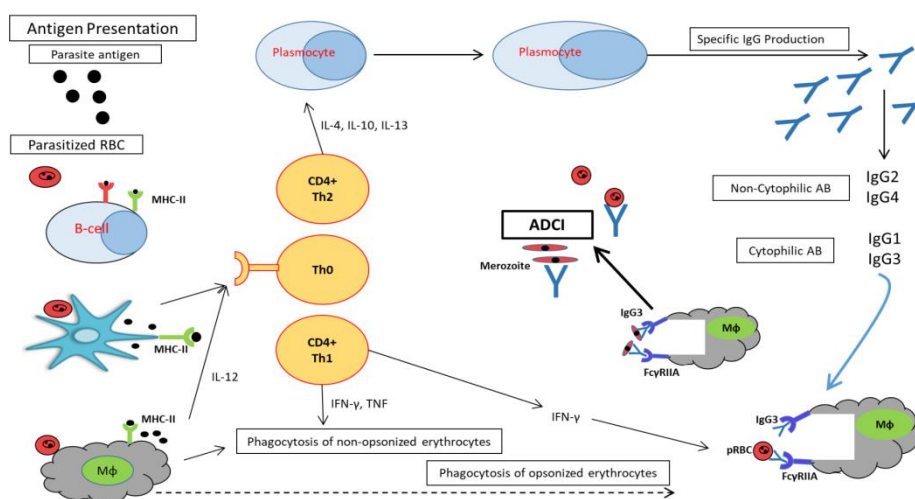


Figure 9. Mechanism of Antibody dependent cellular immunity (ADCI).

The malarial antigens or parasitized RBCs (pRBCs) are recognized by antigen presenting cell (APC), which leads to the production of IFN- γ by T cells and the activation of monocytes. The process of immune phagocytosis is increased, and antibody-dependent cellular inhibition is initiated by the binding of merozoites via cytophilic antibody to Fc γ IIA receptor on monocytes with a release of chemokines, which blocks the division of intraerythrocytic parasite. Noticeably, IgG2 can be cytophilic for individuals carrying a frequent mutated Fc γ RIIA receptor. Adapted from (Stevenson and Riley 2004).

Although, the production of TNF- α may be essential for protection against *P.falciparum* malaria (Taverne, Bate et al. 1990) but, high levels of TNF- α have also been shown to be associated with severe complications (Shaffer, Grau et al. 1991). Studies in African children have shown that the raised levels of the inflammatory cytokines TNF- α , interleukin (IL)-1 β and IL-6 are associated with cerebral malaria (Kwiatkowski, Sambou et al. 1990).

2. Induction of fever

Lysis of infected erythrocytes releases not only merozoites into the bloodstream but also parasitic exoantigens like hemozoin and glycosylphosphatidylinositol (GPI). These exoantigens are pathogen associated molecular patterns (PAMP) which are recognized by Toll-like receptors (TLR), expressed by cells of the innate immune system (macrophages and dendritic cells), the activation of which stimulates production of cytokines and pro-inflammatory pyrogenic mediators.

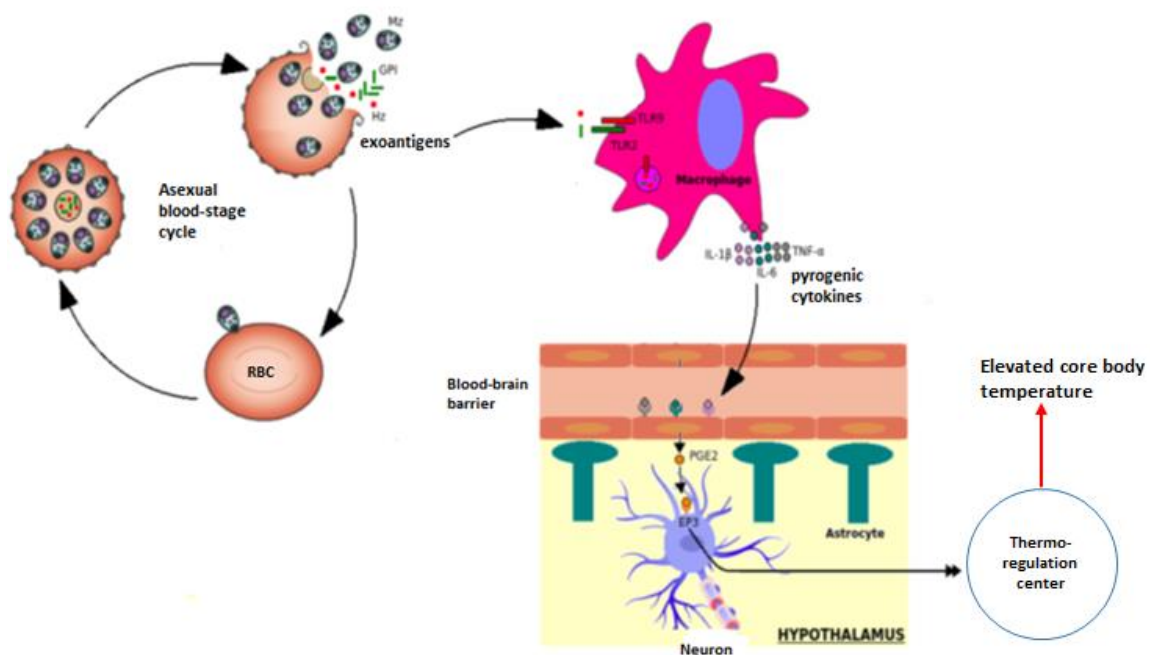


Figure 10. Mechanisms of induction of malaria fever.

During the blood stage of malaria infection, the lysis of infected erythrocytes releases parasitic exo-antigens (GPI, hemozoin, DNA) which are recognized by Toll-like receptors (TLR) on the surface of phagocytes and other innate immune cells. These cells then produce pro-inflammatory pyrogenic mediators and cytokines (TNF- α , IL-1 β , and IL-6) which act on hypothalamic endothelial cells and induce the production of PGE2. PGE2 then binds to the EP-3 receptors of hypothalamic nerve cells, causing the body temperature to rise. Abbreviations: Hz, hemozoin; GPI, glycosylphosphatidylinositol; Mz, Merozoite; TLR, Toll-like receptor. Adapted from (Oakley, Gerald et al. 2011).

The recognition of the parasitic GPI by the TLR2 receptor and that of the covered plasmodial DNA hemozoin by TLR9 results in the production of pyro genes molecules like TNF- α , IL-

1 β , and IL-6 by macrophages. These pyrogenic cytokines cross the bloodstream to reach the thermoregulatory regions of the hypothalamus where they induce prostaglandin E2 (PGE2) production by the endothelial cells and perivascular associated with the blood-brain barrier. The PGE2 then attaches to the nerve cell receptor EP-3 in hypothalamus leading to an increase in temperature (Figure10) (Kwiatkowski 2005; Oakley, Gerald et al. 2011).

3. Immunopathogenesis of cerebral malaria

Three major, interdependent mechanisms contribute to the pathogenesis of cerebral malaria (Storm and Craig 2014; Wassmer, Taylor et al. 2015):

- The obstruction of the cerebral micro-vessels following the sequestration of infected erythrocytes.
- The induction of an exacerbated pro-inflammatory response and the activation of endothelial cells.
- The activation of cytolytic CD8+ T cells and the alteration of the blood-brain barrier.

Vascular occlusion leading to cerebral malaria is based on the ability of infected erythrocytes to be sequestered in the cerebral micro-vessels. This sequestration can be broken down into several phenomena including, cytoadherence, rosette formation (interaction of infected red blood cells and non-infected) and the formation of platelet aggregates (interaction between infected red blood cells on the one hand and with the endothelium on the other, by platelets) (Grau and Craig 2012).

During infection with *P. falciparum*, the red blood cell is remodeled by the parasite causing the formation of small membrane protuberances called knobs. These have been identified as the site of contact between the endothelial cells and the malaria proteins expressed on surface of infected red blood cells, including *PfEMP1* (*P. falciparum* erythrocyte membrane protein 1) (Kilejian 1979; Rowe, Claessens et al. 2009; Smith, Rowe et al. 2013). Cytoadherence of infected red blood cells involves the binding of this parasite protein to adhesion molecules expressed on the surface of endothelial cells of different tissues, such as *ICAM-1* and *EPCR* in brain, *CSA* in placenta and *CD36* in macrophages (Figure 11).

The variable cytoadhesive surface antigens are collectively known as *Plasmodium falciparum* erythrocyte membrane protein 1 (*PfEMP1*). In the genome of *P. falciparum*, *PfEMP1* is encoded by about sixty var genes, which are expressed in a mutually exclusive manner to avoid the simultaneous recognition by the host's immune system (i.e: only one var gene is expressed at a time). Over time an antibody-mediated response targeting the expressed form

of *PfEMP1* develops and can greatly reduce the parasites population in blood circulation, often to such low parasitemia that infected cells cannot be detected by microscopic examination of stained blood smears (Miller, Baruch et al. 2002).

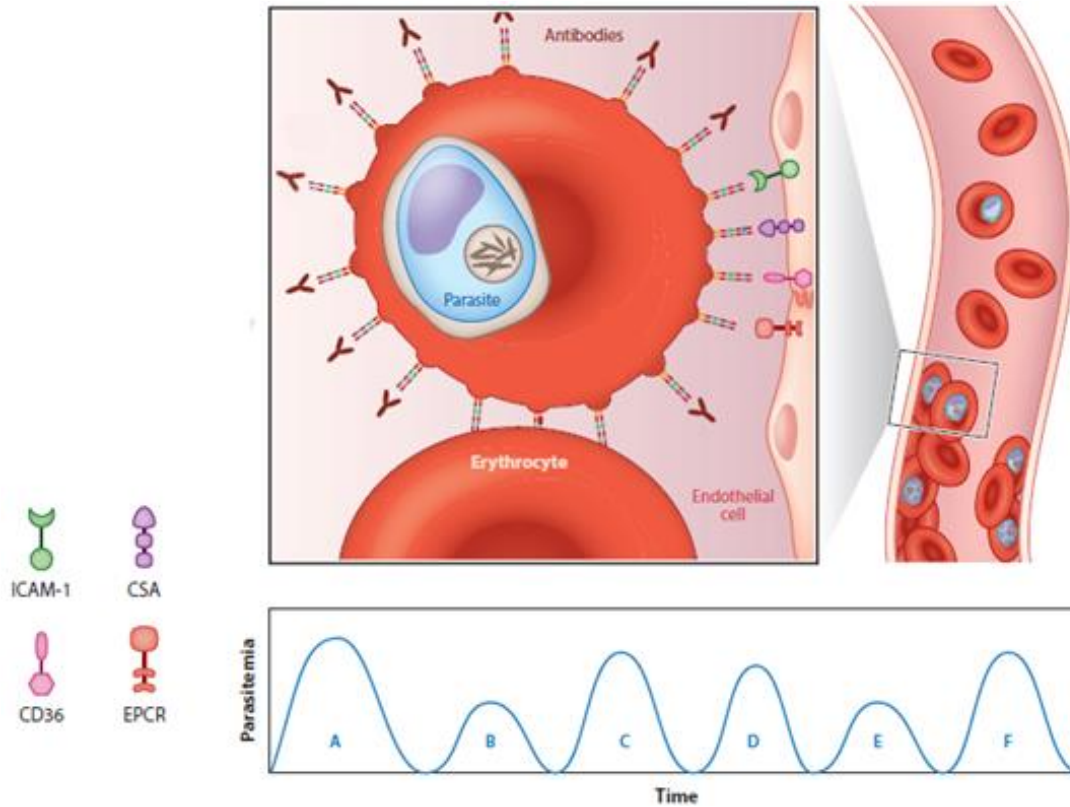


Figure 11. Cytoadhesion and antigenic variation by *Plasmodium falciparum* parasites.

An infected red blood cell presenting PfEMP1 antigen on its surface that binds to the endothelial cells of different tissues, including ICAM-1 (intercellular adhesion molecule 1) and EPCR (endothelial protein C receptor) in brain., CSA (chondroitin sulfate A) in placenta, CD36 (cluster determinant 36) in macrophages. Infected cells can also adhere to uninfected red blood cells. Adherence to the vascular endothelium results in sequestration away from the circulation and obstruction of blood vessels. Antibodies recognize the specific forms of PfEMP1, disturbing adhesion and leading to the destruction of the parasite population. Populations of parasites that have switched to expressing alternative forms of PfEMP1 can then expand, resulting in sequential waves of parasitemia over time. Adapted from (Deitsch and Dzikowski 2017).

However, small numbers of parasites within the infecting population can switch expression to a *var* gene that encodes a *PfEMPI* variant that has not yet been exposed to the immune system. This alteration allows the parasites to once again undergo clonal expansion until they trigger another antibody-mediated response. As a consequence, this switching in *var* gene expression is supposed to allow the parasites to evade immunity and maintain chronic infections characterized by oscillating waves of parasitemia (Miller, Good et al. 1994). The parasites strongly maintain the mutually exclusive expression of *var* gene to avoid the premature exposure of its antigenic repertoire, while the remainder of the gene family remains transcriptionally silent (Scherf, Hernandez-Rivas et al. 1998; Dzikowski, Frank et al. 2006; Voss, Healer et al. 2006).

The other mechanism behind cerebral malaria is the induction of an exacerbated pro-inflammatory response and endothelial cell activation. In fact, these cytokines, and more particularly TNF- α and IL-1 β , activate the endothelial cells (Dunst, Kamena et al. 2017) which:

- Increase the expression of adhesion molecules such as *ICAM1*, *VCAM* and *E-selectin* on their surface, exacerbating the phenomenon of sequestration of infected red blood cells, platelets and leucocytes.
- Secrete in turn pro-inflammatory cytokines and chemokines which also induces local inflammation causing local inflammation and endothelial activation could be exacerbated.

Finally, another important immunological actor seems to intervene in the pathogenesis of cerebral malaria is the CD8 + T lymphocyte (Figure 12)

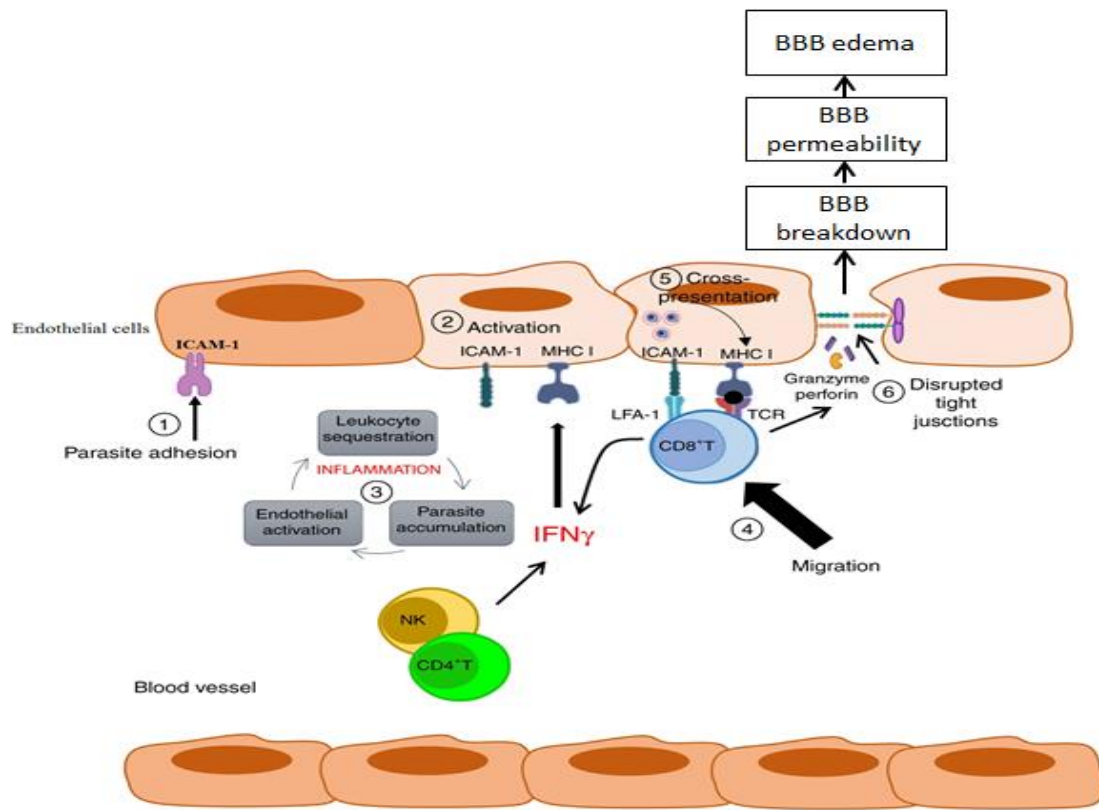


Figure 12. Immunopathogenesis of CM.

In response to the blood stage infection, various cytokines such as TNF and IFN- γ are produced by the immune cells. These cytokines stimulate the up-regulation of the adhesion molecules in the brain endothelial cells, (1) resulting in the sequestration and binding of the infected RBCs to *ICAM-1* receptor expressed on brain endothelial cells, initiating and amplifying the local immune response. (2) Interaction between iRBC and endothelial cells induces the brain endothelial cells to secrete cytokines, and adhesion receptors (CD36, *ICAM-1*), as well as major histocompatibility complex (MHC) class I. (3) These cytokines recruit leukocytes ($CD4^+$ T cells, NK, neutrophils, monocytes) and platelets, that together with iRBC accumulate in the microvessel and the activated endothelial cells. Secretion of IFN- γ by $CD4^+$ T and NK cells induce the activation and proliferation of T cells and increases the expression of adhesion molecules and MHC-I in the endothelial cells. (4) Parasite specific- $CD8^+$ T cells that have been primed in the spleen, migrate into the brain and bind to endothelial *ICAM-1* via LFA-1. (5) IFN- γ -stimulation enhances the antigen processing capability of endothelial cells, to present the malaria antigen loaded on MHC-I to specific- $CD8^+$ T cells. (6) Upon antigen recognition, $CD8^+$ T cells produce IFN- γ and secrete granzyme and perforin that will disrupt the tight junctions, leads to obstruction and increased permeability (edema). Adapted from (Howland, Claser et al. 2015).

Cells activated endothelial cells, in addition to an over expression of ICAM-1 allowing retain the CD8 + T lymphocyte via an interaction with lymphocyte function associated antigen 1 (LFA-1) at its surface, are able to present parasite antigens to the same lymphocyte via the MHC Class I (Howland, Poh et al. 2013; Howland, Poh et al. 2015). This direct interaction between brain endothelial cells and CD8+ T would result in their activation and perforin release (Shaw, Stewart-Hutchinson et al. 2015). Indeed, many experimental studies of cerebral malaria in mice have identified the pathogenic role of these cells, offering different mechanisms related to perforin release, including cytolytic or apoptotic processes and the rupture of the blood-brain barrier followed by other secondary neuropathological events, at the level of the cerebral parenchyma, which could lead to a cerebral edema or a coma (Hunt, Golenser et al. 2006; Haque, Best et al. 2011). The role of T cells in causing brain vasculature injury associated with cerebral malaria is well established in mice, but in the case of humans, a recent study has shown that T cells target blood vessels in brains of children with cerebral malaria. This finding build a bridge between mouse and human cerebral malaria studies by implicating the role of T cells in the development of disease pathology. The results of this study demonstrated an increased accumulation of CTLs along the walls of the brain blood vessels in cerebral malaria tissue samples. In addition, the CTLs were shown to contain and release the effector molecules which damage cells, suggesting that CTLs play a critical role in CM by damaging the BBB. This suggest that the mouse model for CM may accurately reflect the human disease pathology (Rénia, Grau et al. 2020).

D. HUMAN GENETICS AND MALARIA

1. Candidate gene approach

The candidate gene approach directly tests the effects of mutations located in specific genes for their association with the disease. This approach consists of selecting genes whose function can be related to pathophysiology of malaria. Thus, in the case of malaria, those are essentially the genes coding for proteins involved in cytoadherence, in the host's immune response against parasitic infection or in the physiology of red blood cells. The polymorphisms studied are mostly the variations that impact the gene expression and the activity of their products. Once these criteria have been established, two possibilities can be considered: a case-control association study or a family based association study.

Case-control association approach is most represented in the study of the implication of candidate genes in the pathology of malaria. Genetic association tests rule in principle on a possible difference in the frequency of a specific mutation between a case population and control population group. If the mutation is found to be significantly more frequent in patients compared to the healthy individuals, this means that (1) either the mutation is causal and directly involved in the pathology of malaria, (2) either it is in linkage disequilibrium with another polymorphism which is directly involved in the disease, (3) that this difference is just the consequence of a genetic background. Moreover, this latter case represents population stratification that is a difference in allelic frequency in a subgroup of one of the concerned populations. A major characteristic of case-control studies is that data are collected retrospectively and as a result may give rise to bias. This is a particular problem associated with case-control studies and therefore needs to be carefully considered during the design and conduct of the study. This is an important parameter which makes case-control studies difficult to design because it is necessary to find a perfectly control population. Moreover, the two groups must be matched on certain parameters, particularly age and gender.

Family-based association studies, also called Transmission Disequilibrium Tests (TDTs) were developed to combine the test of linkage and association of candidate genes using affected individuals and their parents. It effectively compares allele frequencies in affected individuals with those predicted from their parental genotypes. Compared to case-control studies, TDT is unaffected by population stratification. The advantage is that it does not require the selection of control group and thus avoids selection bias. Spielman *et al.* first developed the TDT for quantitative traits to study the families with at least one affected offspring and two parents, where one of the parents should be heterozygous for the allele associated with the disease (Figure 13) (Spielman, McGinnis et al. 1993). The basic principle of the TDT is to compare the number of times an allele of interest, in a studied polymorphism, is transmitted from heterozygous parents to affected offspring with the number of times some other alleles are transmitted from these same heterozygous parents to affected offspring. It measures the over-transmission of an allele from heterozygous parents to affected offsprings. If this test, which corresponds to Mac Nemar χ^2 test, is significant, the association thus detected can then be interpreted in two ways: either the polymorphism associated is the causal allele responsible for the disease, or the polymorphism associated is in linkage disequilibrium with the causal allele. The TDT test has been extended for the quantitative traits and families with several affected and unaffected children (Horvath, Xu et al. 2001). Despite of the advantages, very

few studies have been performed using this test as it is much more difficult to recruit a cohort comprising large number of nuclear families with at least one of the parents heterozygous for the studied polymorphism.

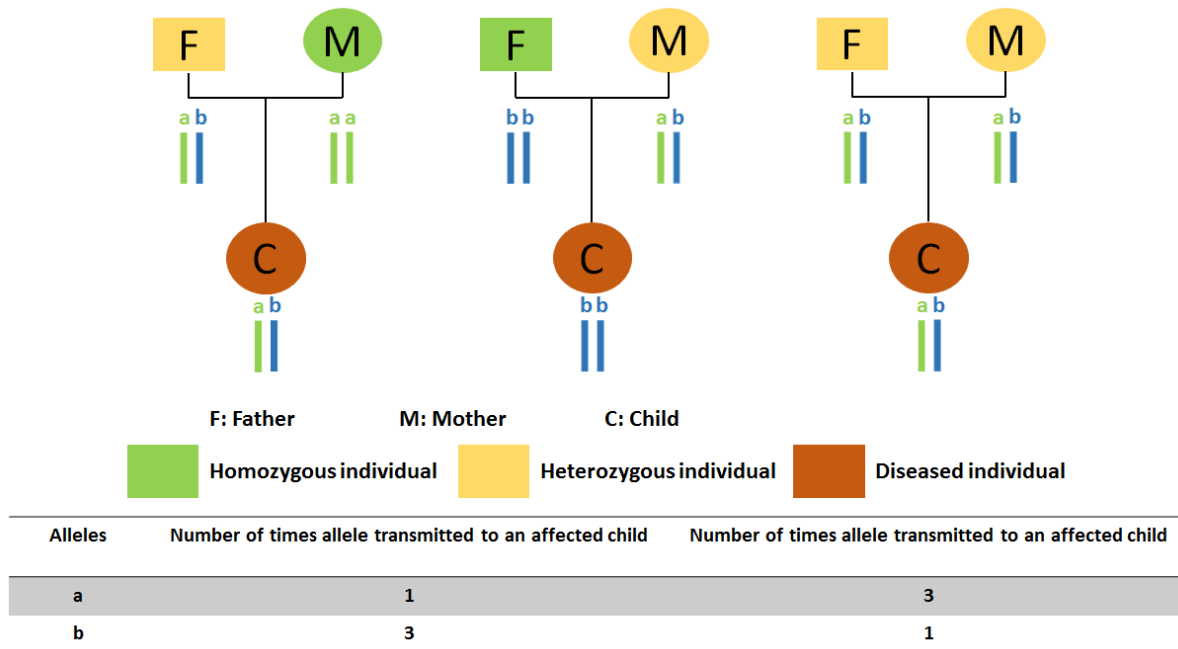


Figure 13. Illustration of principle of TDT.

TDT evaluates the preferential transmission of a particular marker allele from heterozygous parents to affected children. This test compares the frequency of the allele transmitted by the parents to the affected children with the frequency of the non-transmitted allele by Chi square test. This analysis needs three family members (mother, father and child) and one of the parents should be heterozygous for the studied polymorphism. In case of bi-allelic polymorphism, such as SNP, represented by alleles a and b, the principle of TDT helps to establish the table for allelic transmission. The application Mac Nemar Chi square test in this data provides a statistical evidence of association between the studied polymorphism and the disease. If the transmission of allele b is significantly higher than 50%, it means that allele a is associated with susceptibility. If the transmission of allele b is significantly lower than 50%, it means that allele a is associated with resistance. The significant result suggests that the polymorphism is either directly associated in the development of disease or is in linkage disequilibrium with the causal mutation.

The rationale of candidate gene approach states that a major component of quantitative genetic variation of phenotype under investigation is caused by functional mutation of putative gene. Candidate genes are generally the genes with known biological function directly or indirectly regulating the developmental processes of the investigated traits, which could be confirmed by evaluating the effects of the causative gene variants in an association analysis (Tabor, Risch et al. 2002). It does not require large families with both affected and

unaffected members, but can be performed with unrelated cases and controls or with small families (e.g: a proband and parents). Furthermore, this approach is better suited for detecting genes underlying common and more complex diseases where the risk associated with any given candidate gene is relatively small (Collins, Guyer et al. 1997).

The first critical step in conducting a candidate gene study is the choice of a suitable candidate gene that may plausibly play a relevant role in the process of disease under investigation and the suitable polymorphism that might influence the trait of interest and further, the role of this gene is tested in a sample of randomly chosen subjects with the disease (cases) and without disease (controls) (Malhotra and Goldman 1999).

However, candidate gene approaches have been criticized owing to low replication of results and analysis of a relatively few number of genes. The practicability of this approach is largely limited by its reliance on existing knowledge about the known or presumed biology of the phenotype under investigation, and unfortunately the detailed molecular anatomy of most biological traits remains unknown (Tabor, Risch et al. 2002).

1.1. RBC polymorphisms

Some genetic mutations resulting in protection against malaria are highly prevalent in regions where malaria is endemic. Haldane (1949) first suggested a “balanced polymorphism” where the homozygote’s hematological disadvantage is balanced by the resistance to malaria displayed by the heterozygote (HALDANE 1949). Among the known alterations conferring resistance against malaria includes various blood group polymorphisms such as hemoglobinopathies (thalassaemias, HbS, HbC and HbE), erythrocyte polymorphism and newly identified gain-of-function *PIEZO1* mutation as shown in Table 1.

1.1.1. Hemoglobin disorders

Hemoglobin is made up of four globin chains which, consists of two alpha and two beta chain ($\alpha_2 \beta_2$). Genes in the α -globin and β -globin regulate globin chain production. The hemoglobinopathies are the most known single-gene disorder and most common are the α and β -thalassaemias, where there is deficient synthesis of globin proteins, and three structural variants of the β -globin chain, hemoglobin S (HbS), hemoglobin C (HbC) and hemoglobin E (HbE). Evolutionary pressure from *P.falciparum* malaria on human populations has selected

various erythrocyte polymorphisms that protect against severe complications and death from the disease.

HbS (rs334) results from mutation of sickle hemoglobin (HbS), a glutamate-to-valine substitution in the sixth position of the β -globin chain. Molecular-genetic evidence suggests that this mutation has been selected independently at least five times in Africa, Arabia, and India. Although the homozygous SS (sickle cell disease) state is often fatal to young children, high frequencies (in some regions 20%) of the HbS gene are maintained by the protection that the benign heterozygous AS (sickle trait) state can confer to children against life-threatening *P.falciparum* malaria. The individuals carrying sickle cell trait are found to be protected from severe malaria although contradictory results were obtained in some other studies due to the low frequency of the mutation in the studied populations. (Hill, Allsopp et al. 1991; Williams, Mwangi et al. 2005). A few studies have demonstrated a reduced risk for uncomplicated malaria and anemia in children with sickle trait (Allen, Bennett et al. 1992; Williams, Mwangi et al. 2005; Williams, Mwangi et al. 2005; Kreuels, Kreuzberg et al. 2010) and it was demonstrated that it may lead to a lower frequency of stunting in children with HbAS genotype (Kreuels, Ehrhardt et al. 2009). HbAS children generally have lower parasite densities than AA children during episodes of asymptomatic infection and symptomatic malaria (Williams, Mwangi et al. 2005; Williams, Mwangi et al. 2005; May, Evans et al. 2007).

There are some hypothesis to answer the question that how HbS can provide protection against severe malaria. It has been assumed that it is partly due to reduction of hemoglobin solubility under low oxygen tension (Pasvol, Weatherall et al. 1978) and increased sickling of parasitized cells with an enhanced elimination by the reticuloendothelial system (Luzzatto, Nwachuku-Jarrett et al. 1970). It has been suggested that HbAS causes a reduced cytoadherence of parasitized red blood cells (Cholera, Brittain et al. 2008) and it enhances immune response against *P.falciparum* infected erythrocytes (Verra, Simapore et al. 2007). It has been shown in one study that a degradation product of iRBCs containing HbS is able to inhibit actin polymerization in vitro and may confer protection from severe malaria (Cyrklaff, Sanchez et al. 2011).

Gene name & symbol	Protein & function	Localization	Phenotype	References
<i>PIEZO1</i>	Piezo1, mechanosensitive ion channel protein	16q24.3	↓ SM	(Zarychanski, Schulz et al. 2012; Ma, Cahalan et al. 2018; Nguetse, Purington et al. 2020)
Hemoglobin S (HBB)	HbS, component of hemoglobin	11p15.5	↓ UM and SM	(Allison 1954; Agarwal, Guindo et al. 2000; Ayi, Turrini et al. 2004; Cholera, Brittain et al. 2008)
Hemoglobin C (HBB)	HbC, component of hemoglobin	11p15.5	↓ UM and SM	Agarwal, Guindo et al. 2000; (Friedman, Roth et al. 1979; Modiano, Luoni et al. 2001; Fairhurst, Fujioka et al. 2003; Fairhurst, Baruch et al. 2005)
Hemoglobin E (HbE)	HbE, component of hemoglobin	11p15.5	↓ SM	(Hutagalung, Wilairatana et al. 1999) (Chotivanich, Udomsangpetch et al. 2002)
α -thalassemia (HbA)	α -thalassemia, component of hemoglobin	16p13.3	↓ SM	(Allen, O'donnell et al. 1997; May, Evans et al. 2007)
β -thalassemia (HBB)	β -thalassemia, component of hemoglobin	11p15.5	↓ SM	(Pasvol, Wainscoat et al. 1982; Luzzi, Merry et al. 1991)

Glucose-6-phosphate dehydrogenase (G6DP)	G6PDA, pentose phosphate pathway enzyme	Xq28	↓ UM and SM	(Allison and Clyde 1961; Bienzle, Lucas et al. 1972; Cappadoro, Giribaldi et al. 1998; Guindo, Fairhurst et al. 2007)
Pyruvate kinase (PKLR)	PKR, glycolytic enzyme	1q21.3	↓ Parasitemia	(Ayi, Min-Oo et al. 2008)
Blood groups (ABO)	Blood group antigen, Glycoproteins on RBCs	9q34.13	↓ SM	(Fischer and Boone 1998; Lell, May et al. 1999; Barragan, Kreamsner et al. 2000; Rowe, Handel et al. 2007; Paré, Chasman et al. 2008)
Duffy antigen (DARC)	Duffy antigen, chemokine receptor	1q23.1	↓ SM	(Tournamille, Colin et al. 1995)
Ovalocytosis (SLC4A1)	Protein band 3, anion transporter of RBCs	17q21.31	↓ SM	(Cortés, Benet et al. 2004; Cortés, Mellombo et al. 2005)

Table 1. Genetic polymorphism of erythrocytes system implicated in resistance to malaria.

↓ Decrease, UM: uncomplicated malaria, SM: severe malaria.

Thalassemias are a group of inherited hematological disorders affecting the synthesis of hemoglobin and causes reduction or abrogation of globin chains. Both α and β chains can be affected thus, determining two major varieties of disorders: α - and β - thalassemia. For α -thalassemia, the common molecular defects are deletions involving either one, two, three or four alleles at the α locus, deletions of both HbA genes cause α^0 -thalassemia (a lethal condition leading to foetal hydrops syndrome) or α^+ -thalassemia is one of the pair of genes is deleted. Similarly, β -thalassemia can also be completely (β^0 causing severe anemia) or partially (β^+ resulting in a mild or asymptomatic condition). The α - and β - thalassemia occurs throughout sub-Saharan Africa and the Mediterranean region, the Middle East, the Indian-Subcontinent and South-East Asia.

α^+ -thalassemia protects against severe malaria in both heterozygous and homozygous state without affecting parasitemia (Allen, O'donnell et al. 1997), this was supported by epidemiological evidence in two different African populations (Williams, Wambua et al. 2005; May, Evans et al. 2007). The molecular mechanism of protection is unclear as there is no evidence of reduced rate of invasion or growth of the parasite by this trait, however, these altered erythrocytes have been observed to bind more malaria immunoglobulin than normal erythrocytes suggesting an immune mediated mechanism of protection (Luzzi, Merry et al. 1991; Williams, Weatherall et al. 2002). A study in Kenya has shown negative epistatic interaction between the two most common factors conferring protection against malaria, HbS and α -thalassemia, despite the individual protective effect: Children who are HbAS and either heterozygous or homozygous for α -thalassemia showed an incidence of severe malaria compared to group of normal hemoglobin (Williams, Mwangi et al. 2005).

β^+ - thalassemia is also shown to protect against severe malaria by an alteration of cytoadherence and rosetting of *P.falciparum* infected erythrocytes (Udomsangpetch, Sueblinvong et al. 1993). There is also an evidence of enhanced phagocytosis in β^+ - thalassemic infected erythrocytes (Ayi, Turrini et al. 2004).

1.1.2. Erythrocyte Enzymatic disorders

Glucose-6-phosphate dehydrogenase (G6PD) is a housekeeping enzyme which is a key component in the phosphate pathway, employed by erythrocytes to handle oxidative damage. G6PD is

encoded by a 16.2 kb gene found on X chromosome. Approximately 160 genetic variants causing clinical deficiency of G6PD have been characterized but only few of them have been observed at high frequencies in malaria endemic areas. The A-variant 202A/376G G6PD is considered to be the most common G6PD allele in Africa with two non-synonymous SNPs at position 202 and 376 of the coding sequence. There have been contrasting results with association of G6PD deficiency with protection against malaria. Studies have indicated that male hemizygotes and female homozygotes were protected from malaria although a few authors have suggested that it is the female heterozygotes who receive protection, while a case-control study in Gambia and Kenya showed female heterozygotes and G6PD-deficient male hemizygotes were protected from severe malaria (Ruwende, Khoo et al. 1995). Another study in Mali reported that hemizygous males and not heterozygous females were protected against severe disease (Guindo, Fairhurst et al. 2007). *In vitro* experiments have also provided conflicting results: parasite growth was not inhibited in hemizygous G6PD deficient erythrocytes in short-term cultures without oxidative stress according to some studies (Friedman 1979), while opposite was observed in other (Friedman 1979; Roth, Raventos-Suarez et al. 1983; Usanga and Luzzatto 1985); nevertheless, the inhibition occurred consistently in oxidant stress condition (Martin 1994). The G6PD deficient erythrocytes are suggested to be more susceptible to oxidative stress and undergo phagocytosis at an earlier stage than normal infected ones (Cappadoro, Giribaldi et al. 1998).

1.1.3. Erythrocyte membrane polymorphism

ABO blood group: The A and B antigens are trisaccharide structures attached to a variety of glycolipids and glycoproteins on the erythrocyte surface. Group O individuals lack the terminal glycosyltransferases necessary to produce the A or B antigens and carry the disaccharide H antigen. The A and B trisaccharides are thought to act as receptors for rosetting on uninfected erythrocytes (Barragan, Kremsner et al. 2000). Blood group O is known to protect against malaria as rosettes formed by infected O blood group RBCs are small and are easily disrupted compared to blood group A, B or AB (Rowe, Handel et al. 2007). Besides, there is evidence suggesting blood group A is associated with higher incidence of severe malaria (Fischer and Boone 1998; Lell, May et al. 1999). Moreover, a meta-analysis also supported the protective role of blood groups in severe malaria where Blood group O has shown confers protection to severe disease.

The meta-analysis restates the observation of protection conferred by blood group O. (Panda, Panda et al. 2011).

1.1.4. *PIEZO1*

A recent study has identified a novel gain-of-function mutation (*E756del*) in the mechanically activated channel *PIEZO1*, present in one-third of the African population and is associated with malaria resistance. This mutation in *PIEZO1* gene, which encodes a protein that detects pressure, can dehydrate red blood cells. In a mouse model, this mutation made it more difficult for the *Plasmodium* to infect red blood cells. It's a deletion of three DNA bases. (Ma, Cahalan et al. 2018). A similar variant was already known to produce a rare disease called hereditary xerocytosis, which dehydrates red blood cells, causing anemia, an enlarged spleen and other symptoms (Zarychanski, Schulz et al. 2012; Albuissou, Murthy et al. 2013; Bae, Gnanasambandam et al. 2013).

A very recent case-control study conducted in Gabon to test the association of *PIEZO1* *E756del* and malaria severity. It has been shown that malaria parasites grow poorly in human red blood cells from people with the *E756del* mutation as it produces misshapen, partially dehydrated RBCs, making it more difficult for malaria parasites to exist in RBCs which reduces the diseases's virulence and provides resistance from malaria. The red blood cells, although deformed, don't take the characteristic crescent shape found in sickle cell disease. Moreover, this allele has been demonstrated *in vitro* to prevent cerebral malaria infection. People have two copies of the *PIEZO1* gene, one inherited from each parent. One gene with the *E756del* mutation appears to provide malaria resistance. The variant was found to be strongly associated with protection against SM in heterozygous individuals. The surface expression of the *P. falciparum* virulence protein PfEMP-1 has shown to be significantly reduced in infected cells heterozygous for *PIEZO1**E756del*, a phenomenon that has been observed with other protective polymorphisms, such as hemoglobin C and suggest that the mechanism of protection may be related to impaired export of *P. falciparum* virulence proteins (Nguetse, Purington et al. 2020).

1.2.Cytoadherence molecules

The pathogenesis of severe malaria is attributed to cytoadherence or sequestration of *P.falciparum*-infected red blood cells in small blood vessels. On the host side, different adhesion molecules like ICAM-1, CD36 and CR1 serves as binding receptors for PfEMP1 and several genetic studies have been performed to determine whether some polymorphisms of these genes may be involved in the susceptibility/resistance to severe malaria (Table 2).

Gene name & symbol	Protein & function	Localization	Phenotype	Allele	References
Intercellular adhesion molecule 1 (<i>ICAM1</i>)	CD54, receptor of iRBCs on endothelium	19p13	↑ SM, CM	M29	(Fernandez-Reyes, Craig et al. 1997)
Thrombospondin receptor (<i>CD36</i>)	CD36, receptor of infected erythrocytes	7q11	↓ SM, CM	CD36 deficiency	(Pain, Urban et al. 2001)
Platelet endothelial cell adhesion molecule (<i>PECAMI</i>)	CD31, receptor of iRBCs on endothelium	17q23	↑ SM, CM	V125	(Casals-Pascual, Allen et al. 2001; Kikuchi, Looareesuwan et al. 2001)
Complement receptor 1 (<i>CR1</i>)	CR1, immune-regulatory protein	1q32	↓ SM, malarial anemia	Several	(Rowe, Moulds et al. 1997; Waitumbi, Opollo et al. 2000; Cockburn, Mackinnon et al. 2004; Panda, Panda et al. 2012)

Table 2. Adhesion molecules involved in resistance to malaria.

↑ increase, ↓ Decrease, SM: severe malaria, CM: cerebral malaria.

ICAM-1 (intercellular adhesion molecule-1), encoded by *ICAM1*, serves as an endothelial receptor for iRBCs. A polymorphism (M29) in the N-terminus domain, present at higher frequencies in African population was associated to susceptibility to CM due to higher affinity for the infected RBCs (Fernandez-Reyes, Craig et al. 1997). However, this association was not confirmed in Gambia (Bellamy, Kwiatkowski et al. 1998), which might be due to high polymorphisms of its parasite ligand PfEMP1, thus differing between the two areas (Mazier, Ntcheu et al. 2000).

Another antigen CD36, encoded by *CD36*, is a receptor for different molecules like thrombospondin (TSP) and is expressed by platelets and dendritic cells as well as by endothelium. A missense mutation at exon 10 of CD36 (T188G) determines a null phenotype where this glycoprotein is abrogated. This deficiency was associated with a reduction of parasite sequestration and malaria protection against severe malaria (Pain, Ferguson et al. 2001).

1.3.Immune genes

Various immunogenic variants tend to confer resistance or susceptibility to malaria (**table 3**).

Human leukocyte agent (HLA) system is the name of the major histocompatibility complex (MHC) in humans and is a group of genes found on chromosome 6 which encodes cell-surface antigen-presenting proteins and many other genes. HLA class I molecules (HLA-A, HLA-B, HLA-C) present peptides from inside the cell, and HLAs corresponding to MHC class II (HLA-DP, HLA-DQ, HLA-DR) present antigens from outside the cell to T-lymphocytes. Population genetic studies of the Sardinian population suggested an interaction between HLA polymorphisms and susceptibility to malaria (Contu, Carcassi et al. 1998). The frequency of HLA alleles differed greatly between populations living at different altitudes of the island which suggests a reflection of malaria transmission intensity and selective pressure exerted by malaria (Piazza, Mayr et al. 1985). HLA genes are highly polymorphic and are thought to evolve through natural selections by infectious agents. It was confirmed in a large case control study conducted in Gambia, where the HLA class I allele HLA-B53 and of the HLA class II haplotype were associated with protection from severe malaria. These two variants are also found in higher frequencies in African populations as compared to other population groups suggesting their protective effect against malaria (Hill, Allsopp et al. 1991). Further antigen screening of the pre-

erythrocytic stage of *P.falciparum* showed that HLA-B53 binds to liver surface antigen 1 (LSA-1) which tend to protect against malaria (Hill, Elvin et al. 1992).

Tumor necrosis factor-alpha (TNF- α) is a Th1 cytokine and is involved in multiple inflammatory and immune responses and plays an important role in the pathogenesis of many infectious diseases including malaria. *TNF* gene is highly inducible and the polymorphisms in the promoter region could therefore effect *TNF* production. Polymorphisms in *TNF* promoter have been associated with severe malaria. A large case-control study in Gambia revealed a high risk of death due to cerebral malaria (CM) associated with *TNF-308A* allele, a variant of the *TNF* promoter region known to affect the *TNF* production (McGuire, Hill et al. 1994). A study in Sri Lanka found that those who carried *TNF-308A* allele had an increased risk of severe infectious diseases in general (Wattavidanage, Carter et al. 1999), whereas a study in Kenya showed an increase in infant mortality and malaria morbidity (Aidoo, Terlouw et al. 2002). Other TNF promoter allele, *TNF-376A* has been associated with susceptibility to CM (Knight, Udalova et al. 1999), whereas the *TNF-238A* allele has been associated to severe malarial anemia and severe malaria in Gambia (McGuire, Knight et al. 1999). Another case-control study in Southwest Nigeria conducted to investigate the association between *TNF-308* and *TNF-238* and the clinical outcome of malaria in a Nigerian population, which showed an association of *TNF-238* in the clinical outcome of malaria, while no association was found between *TNF-308* and malaria outcome (Olaniyan, Amodu et al. 2016). A longitudinal study in Burkina Faso suggests that several *TNF*-promoter SNPs are involved in the regulation of parasite density and susceptibility to mild malaria (Flori, Delahaye et al. 2005). Knight *et al* indicated in his study that the polymorphism *TNF-376* affected the expression of *TNF* gene *in vitro*, altered the binding affinity of a ubiquitous transcription factor *OCT-1* and is associated with increased susceptibility to CM in Gambian children (Knight, Udalova et al. 1999).

Interferon- γ encoded by *IFNG*, is a key immunological mediator that is believed to have both protective and pathological role in malaria (Stevenson and Riley 2004). Various studies conducted in naturally exposed individuals have shown that malaria induced *IFN- γ* production is associated with lower infection rates and resistance to co-infection as well as to reduced risk of clinical cases (Deloron, Chougnet et al. 1991; Luty, Lell et al. 1999; Doodoo, Omer et al. 2002). Associations between *IFNG* SNPs (*IFNG-183T*) and (*IFNG-874*) and susceptibility to severe

malaria have been reported in a family-based association study conducted in Mali (Cabantous, Poudiougou et al. 2005) and a large case-control study of Gambian children (Koch, Rockett et al. 2005). A study of *IFNGR1*, which encodes the ligand binding α chain of the IFN- γ receptor, found that, in Gambian ethnic group Mandinka, heterozygotes for *IFNGR1*-56T/C polymorphism were protected against CM and fatal outcome, while the people carrying the *IFNGR1*-470 in/del polymorphism, which has significant effect on gene expression (Koch, Kwiatkowski et al. 2006), were protected against severe malaria in general (Koch, Awomoyi et al. 2002).

Gene name & symbol	Protein & function	Localization	Phenotype	Allele	References
Human leukocyte antigen (<i>HLA</i>)	HLA, antigen presentation and recognition	6p21	Acquired immunity	<i>HLA-B53</i>	(Hill, Elvin et al. 1992): Bennett et al., 1993: Gilbert et al., 1998: Young et al., 2005
Tumor necrosis factor (<i>TNF-α</i>)	TNF, pro-inflammatory cytokine	6p21	↓ SM: malarial anemia, cerebral malaria	<i>TNF-238A</i> , <i>TNF-308A</i>	(McGuire, Hill et al. 1994; McGuire, Knight et al. 1999)
Interferon gamma (<i>IFNG</i>)	IFN- γ , immune-regulatory cytokine	12q66	↓ SM: control of parasite infection	Several SNPs	(Deloron, Chougnnet et al. 1991; Bach, Aguet et al. 1997; Luty, Lell et al. 1999; Plebanski and Hill 2000; Dodoo, Omer et al. 2002; Koch, Awomoyi et al. 2002; Cabantous, Poudiougou et al. 2005; Koch, Rockett et al. 2005; Koch, Kwiatkowski et al. 2006)
Interleukin 1 (<i>IL1/IL1B</i>)	IL1, pro-inflammatory cytokine	2q12.21	↑ SM: malarial anemia, cerebral malaria	Several SNPs	(Walley, Aucan et al. 2004)
Interleukin 10 (<i>IL10</i>)	IL10, IgG class switching	1q31	↓ SM	haplotype	(Kurtzhals, Adabayeri et al. 1998; Wilson, Rockett et al. 2005; Ouma, Davenport et al. 2008)

Interleukin 12 (<i>IL12B</i>)	IL12, Th1 cytokine	5q31	↓ SM	Several SNPs	(Stevenson, Su et al. 2001; Morahan, Boutlis et al. 2002; Marquet, Doumbo et al. 2008; Shimokawa, Nishiyama et al. 2009; Phawong, Ouma et al. 2010)
Fc receptor (<i>FCGR2A</i>)	<i>FCGR2A</i> , IgG receptor	1q23	↑ SM, ↓ parasitemia	H131, R131	(Shi, Nahlen et al. 2001; Cooke, Aucan et al. 2003)
<i>NOS</i> (<i>NOS2A</i>)	<i>NOS2</i> , production of nitric oxide	17q11	contradictory	Several SNPs	(Burgner, Xu et al. 1998; Kun and Mordmuller 1998; Kun, Mordmüller et al. 2001)
Natural cytotoxicity receptor (<i>NCR3</i>)	<i>NKp30</i> , activated NK cell receptor	6p21	↑ UM	NCR3-412C	(Delahaye, Barbier et al. 2007; Mavoungou, Held et al. 2007)
Toll-like receptor 4 (<i>TLR4</i>)	TLR4, pathogens recognition	9q32	↑ SM	299G, T3991	(Mockenhaupt, Cramer et al. 2006; Mockenhaupt, Hamann et al. 2006)
Toll-like receptor 9 (<i>TLR9</i>)	TLR9, pathogens recognition	3p21.3	↑ SM, parasitemia	T-1237C, T-1486C	(Leoratti, Farias et al. 2008; Sam-Agudu, Greene et al. 2010; Esposito, Molteni et al. 2012; Sawian, Lourembam et al. 2013; Kar, Panigrahi et al. 2015)

Table 3. Immunogenic variants involved in resistance to malaria.

↑ increase, ↓ decrease, SM: severe malaria, UM: uncomplicated malaria.

Fcγ Receptors, belong to immunoglobulin superfamily and have a role in inducing phagocytosis, pro-inflammatory cytokine production, antigen presentation and antibody responses. *FcγRIIA* is IgG receptor capable of binding immunoglobulin subtypes IgG1-3 and it is able to bind C-reactive protein (CRP) with high affinity. However, both recognition of IgG and CRP by *FcγRIIA* are influenced by polymorphism within the gene. A single nucleotide change (G-A) within the *FCGR2A* alters a histidine (H) to an arginine (R) residue at position 131 and changes its function in vitro. The presence of H131 allele is essential for efficient binding of igG2 subtypes while R/R131 genotype has preferential affinity for IgG3 and IgG1 subtypes (Warmerdam, Van de Winkel et al. 1990). A pioneer birth cohort study in this regard was conducted in infants in Western Kenya. The results showed that infants with the R/R131 genotype were protected against high-density *P.falciparum* infection as compared to children with H/R131 genotype (Shi, Nahlen et al. 2001). It is thought that R/R131 genotype is being associated with susceptibility while, *FcγRIIa*-H/H131 genotype is associated with protection, although the results have been contradictory. Follow-up studies in Thailand and Gambia showed that homozygous for the H131 genotype were susceptible to CM (Omi, Ohashi et al. 2002; Cooke, Aucan et al. 2003).

Interleukin-10 is a Th2 cytokine and is involved in differential control of IgE and IgG4 levels. The functional importance of *IL-10* mediated upregulation of IgG4 in malaria infection is unclear. *IL-10* is a major anti-inflammatory cytokine, and a low *IL-10/TNF* ratio has been associated with malaria anemia (Kurtzhals, Adabayeri et al. 1998). In a case-control study conducted in Gambia, *IL-10* haplotype (HAP3) was associated to protection against severe malaria (Wilson, Rockett et al. 2005) while, haplotypes of *IL-10* promoter variants were associated with protection against severe malaria and increased *IL-10* production in Kenya (Ouma, Davenport et al. 2008). This indicates that *IL10* variants are related to susceptibility and resistance to malaria and thus influence the outcome of malaria.

Interleukin-12 subunits are encoded by *IL12A* and *IL12B* respectively. *IL12* has a protective role in malaria through its ability to promote *IFN-γ* and development of Th1 cells (Stevenson, Su et al. 2001). *IL12B* polymorphism in the promoter regions has been shown to affect *IL12B* gene expression (Shimokawa, Nishiyama et al. 2009). Homozygosity for the *IL12B* promoter variant (3'UTR-2) was associated with increased mortality in Tanzania but this finding was not

replicated in Kenyan individuals with severe malaria (Morahan, Boutlis et al. 2002). A study conducted in Mali indicates that heterozygosity in *IL12B* *pro* variant is associated with cerebral malaria (Marquet, Doumbo et al. 2008). Functional analysis suggested that the heterozygous genotype at risk is associated with reduced expression of gene producing *IL12B* and a low level of *IL12* in cerebral malaria (Marquet, Doumbo et al. 2008). Altogether this finding suggested that *IL-12* may be a protective factor against cerebral malaria.

Natural cytotoxicity receptor 3 (*NCR3*) gene encodes a protein *NKp30* which is an activated NK cell receptor involved in the lysis of tumor cells. A single nucleotide polymorphism in *NCR3* promoter region (*NCR3-412 G/C*) has been associated with mild malaria susceptibility (Delahaye, Barbier et al. 2007). The role of NK receptors in malaria was further elaborated in a study which showed that *NKp30* and *NKp46* tend to interact with duffy binding like domain (DBL) of *P.falciparum* present on the surface of iRBCs and this interaction was found to be direct, specific and functional, leading to perforin production and release of granzymes B thus killing of iRBCs (Mavoungou, Held et al. 2007). Moreover, *NCR3* has been shown to be associated with mild malaria in Congo which revealed that *NCR3-412C* (rs2736191) carriers had more frequent mild malaria attacks (Baaklini, Afridi et al. 2017).

Nitric oxide synthase 2 (*NOS2*) gene encodes for the inducible NO synthase (iNOS). Several studies have explored the genetic variants that are functionally associated with an increased NO bioavailability in the affected individuals and these variants are found to be linked to a decrease in mild malaria and risk of severe malaria. A study in Gabon showed that *NOS2-954C* allele has been associated with elevated levels of NO synthase and also showed protection from severe malaria and resistance to reinfection (Kun and Mordmüller 1998; Kun, Mordmüller et al. 2001). A cisgenic variant of *NOS2* (rs6505496) found to increase the NO bioavailability and confer increased susceptibility to asymptomatic infection but protect from cerebral malaria (de Jesus Trovoada, Martins et al. 2014). In Gambian children, a *NOS2* microsatellite polymorphism has been associated to severe malaria (Burgner, Xu et al. 1998). The *NOS2-1173T* allele was associated with protection against severe malaria in Tanzania supporting the role of this effector molecule in malaria (Hobbs, Udhayakumar et al. 2002).

Toll-like receptors (TLRs) are critical mediators of the inflammatory response to malarial infection. The genetic polymorphisms affecting the the function of TLRs might be partially responsible for the inter-individual variation in the disease manifestation. Of the ten TLRs in humans, the most notable are TLR4 and TLR9, whose activation and changes in expression are thought to potentially affect the clinical outcome of malaria (Corr and O'Neill 2009). While TLR4 may interact with extra-cellular ligands (such as *Pf*GPI; *Plasmodium falciparum* Glycosylphosphatidylinositol) as well as intracellular ligands as it is located both on the cell surface and on endosomes, TLR9 interacts only with intracellular ligands owing to its endosomal localization (Krishnegowda, Hajjar et al. 2005).. A study suggested that the non-synonymous SNP (D299G) in TLR4 influenced TLR4 signaling and was thus functionally important (Long, O'Connor et al. 2014). This polymorphism was found to be associated with an increased risk of severe malaria and low birth weight in the Ghanian children (Mockenhaupt, Cramer et al. 2006). On the other hand, the inhibition of TLR9 activation by its antagonist or deficiency in mice confers protection against cerebral malaria(Coban, Ishii et al. 2007; Franklin, Ishizaka et al. 2011), rather, the two most common promoter variants of *TLR9*, T-1237C (rs5743836) and T-1486C(rs187084), have a cis-regulatory effect on *TLR9* expression (Campino, Forton et al. 2009) with various consequences for disease outcome or altered cytokine levels in malaria(Sam-Agudu, Greene et al. 2010). The two TLR9 SNPs are associated with higher levels of IFN γ in children with cerebral malaria, implying a causality to the association (Sam-Agudu, Greene et al. 2010). The SNP T-1486C was found to be associated with high parasitemia and in Brazil as well as as low birth weight in Ghanian children (Leoratti, Farias et al. 2008) suggesting the role of these polymorphisms in the pathogenesis of malaria.

2. Genome scan

Genome scan refer to genetic methods of systematically looking for chromosomal intervals that are inherited as a specific trait. While candidate gene approaches identify only a limited number of genes by making a prior hypothesis on the potential role of certain genes according to their function, genome scans allow the identification of susceptibility/resistance genes without making hypothesis on gene function.

Both genetic linkage and association analyses aim at identifying the molecular genetic basis of diseases or traits. The primary difference between these two approaches is that linkage analysis looks at the relation between the transmission of a locus and the disease/trait within families,

whereas association analysis focuses on the relation between a specific allele and the disease/trait within a population of unrelated subjects. In the past decades, many genetic variants underlying diseases or traits have been identified and different types of variants can be detected by these two methods.

2.1. Genetic linkage

Genetic linkage analysis is used to identify regions of the genome that contain genes that predispose to disease. In malaria, several genetic linkage analyses have been performed to look for the locus involved in parasitemia and mild disease which are as under:

A number of studies (Table 4) have pointed out that the genetic control of parasitemia is complex, involving several genetic factors rather than a single major gene effect (Garcia, Cot et al. 1998; Rihet, Traoré et al. 1998). In an effort to detect genes implicated in control of parasitemia, a non-parametric linkage analysis was performed on a small Cameroonian population revealed that 5q31-33 region was related to *P.falciparum* parasitemia (Garcia, Marquet et al. 1998), along with 4 other chromosomal regions containing immune genes: 6p21 (*HLA*-tumor necrosis factor), 2q13-q21 (interleukin 1a and b genes); 14q11 (locus encoding the α chain of the T-cell antigen receptor), 7q35 (gene cluster encoding the β subunit of the T-cell receptor), but no linkage was found with these 4 markers (Garcia, Marquet et al. 1998).

In order to determine whether the region 5q31-33 was also linked to *falciparum* parasitemia in other African population with different genetic background, a second linkage analysis was evaluated in an urban population of Burkina Faso (153 sibs from 34 families) and the results of this study showed a strong linkage between parasitemia and the 5q31-33 locus (Rihet, Traoré et al. 1998). These results were again verified by an additional linkage analysis in a rural population of Burkina Faso with malaria transmission rates much higher than the urban population (Flori, Kumulungui et al. 2003) and a genome-wide linkage study conducted in the population of Dielmo, Senegal and highlighted a significant link between the region 5q31 and the mean parasite density (Sakuntabhai, Ndiaye et al. 2008). These results have also been reproduced in a murine model of *Plasmodium chabaudi* malaria infection, where a quantitative locus of malaria resistance has been mapped in homologous regions of 5q31-q33 in humans (Hernandez-Valladares, Rihet et al. 2004).

Study population	Locus	Sample size	Strategy	References
Cameroon	5q31-33	26 sibling pairs, 9 families	Linkage analysis on 5 candidate regions	(Garcia, Marquet et al. 1998)
Burkina Faso (urban)	5q31-33	153 sibling pairs, 34 families	Linkage analysis on 5q31-33	(Rihet, Traoré et al. 1998)
Burkina Faso (rural)	5q31-33	178 sibling pairs, 44 pedigrees	Linkage analysis on 5q31-33	(Flori, Kumulungui et al. 2003)
Burkina Faso (urban)	IL12B (5q31-33)	153 sibling pairs, 34 families	Linkage analysis	(Barbier, Atkinson et al. 2008)
Senegal	5q31	878 individuals	Genome-wide linkage analysis	(Sakuntabhai, Ndiaye et al. 2008)
Senegal	5q31-33 (<i>ARHGAP26</i>)	626 individuals	Family-based association study	(Milet, Nuel et al. 2010)
Burkina Faso	<i>IRF1</i> (5q31-q33)	190 individuals	Association study	(Mangano, Luoni et al. 2008)
Burkina Faso	17p12	118 families	Linkage analysis on 17p12 and 6p21.3	(Brisebarre, Kumulungui et al. 2014)

Table 4: Linkage or association studies of loci with *P.falciparum* parasitemia.

Study population	Locus	Sample size	Strategy	References
Burkina Faso	6p21-23 (<i>TNF</i> , <i>NCR3</i>)	34 families	Linkage analysis on MHC region and association study	(Flori, Sawadogo et al. 2003; Flori, Delahaye et al. 2005; Delahaye, Barbier et al. 2007; Baaklini, Afridi et al. 2017)
Burkina Faso	6p21.3	63 families	Genome-wide linkage analysis	(Brisebarre, Kumulungui et al. 2014)
Ghana	10p15	68 families	Genome-wide linkage analysis	(Timmann, Evans et al. 2007)
Senegal	6p25.1, 12q22	626 individuals	Genome-wide linkage analysis	(Milet, Nuel et al. 2010)
Ghana	5q31-33 (<i>IL3</i>)	1015 individuals	Association study	(Meyer, Calixto Fernandes et al. 2011)
Brazil	IL10	141 individuals		(Domingues, Kanunfre et al. 2016)

Table 5: Linkage or association of loci with Mild malaria.

Several polymorphisms of the candidate genes from this region have been investigated, particularly, *IL12B* gene polymorphisms (*rs2288831*, *rs3213099*, *rs3181221*, *rs3213105*, *rs11574790*), were found to be linked but not associated with Parasitemia in Burkina Faso population (Barbier, Atkinson et al. 2008) and *IRF1* gene polymorphism which was associated with protection against *P.falciparum* infection (Mangano, Luoni et al. 2008).

On the other hand, no linkage has been observed between the region 5q31-q33 and the parasite density in a Senegalese population, but a significant association was found between the intensity of the plasmodial infection and a polymorphism (*rs830532*) of the *ARHGAP26* (Rho GTPase activating protein 26) gene in the 5q31-q33 region in a family based association study (Milet, Nuel et al. 2010). This gene encodes a protein involved in the transduction pathway of the integrin signal, which may promote the adhesion of iRBCs to the endothelium of micro vessels and thus contributing to disease (Davis, Lee et al. 2013). Although no linkage was observed with 5q31-33 region in this Senegalese population, but there are some evidences of linkage between parasitemia and 20p11-q11 region (Milet, Nuel et al. 2010).

In another study conducted on the families from rural and urban districts from Burkina Faso, a significant linkage was observed between parasitemia and the chromosome regions 6p21.3 and 17p12. The region 6p21.3 contained 5 genes (*TNF*, *LTA*, *LTB*, *LST1* and *NCR3*) while the region 17p12 contained 11 genes (*MAP2K4*, *MYOCD*, *ELAC2*, *ARHGAP44*, *COX10*, *DNAH9*, *HS3ST3B1*, *HS3ST3A1*, *CDRT15*, *ZNF18*, and *SHISA6*) (Brisebarre, Kumulungui et al. 2014).

Mild/uncomplicated malaria attacks are diagnosed on the basis of *P.falciparum* parasitemia, fever and clinical symptoms including vomiting, headache, pain and diarrhea. The chromosomal region 6p21-23 of major histocompatibility complex (MHC) containing the tumor necrosis factor alpha (*TNF α*) gene was the first identified locus to be linked to mild malaria in Gambian children (Jepson, Sisay-Joof et al. 1997). Another study in Burkina Faso (34 families) also confirmed this linkage between MHC region and mild malaria susceptibility (Flori, Sawadogo et al. 2003) (Table 5). Five genes were found under the linkage peak including *TNF*, *NCR3*, *LTA*, *LTB*, and *LST1*.

More research revealed that *TNF* polymorphisms (*TNF-1031*, *TNF-308*, *TNF851* and *TNF1304*) and *NCR3* (*NCR3-412*) were associated with uncomplicated episodes of malaria (Flori, Delahaye et al. 2005; Delahaye, Barbier et al. 2007; Baaklini, Afridi et al. 2017). Genome-wide linkage analysis that detected a linkage between 6p21.3 locus and parasitemia, showed that this same

locus is significantly linked to mild malaria in Burkina Faso. The region contained the five genes (*TNF*, *LTA*, *LTB*, *LST1* and *NCR3*), thus supporting the studies that have already shown the *TNF* and *NCR3* association with mild malaria (Flori, Delahaye et al. 2005; Delahaye, Barbier et al. 2007; Nguyen, Baaklini et al. 2017), supporting their involvement in malaria pathogenesis. Genetic variation within the promoter likely alter the binding affinity of the transcription factors to the promoter region, thus influencing gene expression and malaria outcome.

Another genome-wide linkage analysis on 68 families recruited from rural areas of Ghana (West Africa) detected a significant link between susceptibility to uncomplicated malaria and the 10p15 chromosomal region (Timmann, Evans et al. 2007). This region contains several functional candidate genes, including *IL15RA* (interleukin-15 receptor alpha chain), *IL2RA* (alpha chain of interleukin 2 receptor) and *GATA3* (GATA-binding protein 3).

Evidence for linkage between susceptibility to mild malaria has also obtained in a Senegalese population on 6p25.1 and 12q22 regions (Milet, Nuel et al. 2010). The 12q22 chromosomal region contains three potential candidate genes, *PLXNC1*, *UBE2N* and *SOCS2*, all of which are involved in the immune response. The 6p25.1 region includes the *LYRM4* gene which plays an important role in multiple cellular processes, including macrophage activity.

2.2.Genome-wide association studies (GWAS)

GWAS is an experimental design used to detect associations between genetic variants and traits in samples from populations. The path from GWAS to biology is not straightforward because an association between a genetic variant at a genomic locus and a trait is not directly informative with respect to the target gene or the mechanism whereby the variant is associated with phenotypic differences.

The advancement of high-throughput technologies in the recent years has extended the analysis of genetic determinants of the complex diseases. Unlike candidate gene approach that required the prior hypothesis about a gene, it's now possible to perform GWAS in order to identify the susceptibility/resistance genes without any necessity of prior hypothesis with the use of these technologies. These approaches make use of high density array with thousand to millions of SNPs; however, require large number of subjects to acquire statistical power. Moreover, it requires the analysis of two independent cohorts: a discovery cohort to identify interesting chromosomal regions and a replication cohort to confirm the association results.

Genome-wide association studies seek to identify the vast majority of common loci variants in the human genome that affect disease susceptibility by comparing allele frequencies in individuals with the disease and healthy controls. Since the completion of the Human Genome Project (Consortium 2001), genetic epidemiology has entered an era of single nucleotide polymorphisms. With the reduction in the cost and commercial availability of SNP genotyping, many researchers have taken advantage of these technologies, which has led to the publication of hundreds of GWAS seeking to find the genetic causes of many diseases and other traits. For example, the Wellcome Trust Case-Control Consortium (Consortium 2007), conducted association studies of seven common diseases (including bipolar disorder, coronary artery disease, Crohn's disease, hypertension, rheumatoid arthritis, Type I and II diabetes) with a total of 14,000 cases and 3000 shared controls whose results were then replicated successfully. Since then, many important GWAS datasets have been generated in African populations, notable among them being studies on malaria conducted by the Malaria Genomic Epidemiology Network (MalariaGEN) (Jallow, Teo et al. 2009; Band, Le et al. 2013). MalariaGEN is a multi-centre initiative that aims to gain a better understanding of the genetic epidemiology of malaria throughout the malaria-endemic world.

Some landmark genome wide association studies (GWAS) have been conducted in African, European, Asian European and Asian populations (Table 6). Application of GWAS to populations in Africa could provide insights into pathways controlling resistance to malaria as well as genetic origins of related diseases.

The first GWAS on SM by genotyping 500,000 SNPs in a discovery cohort of 2,500 children from The Gambia, and replication in an additional 3,400 children was conducted by Jallow *et al* (Jallow, Teo et al. 2009). Strikingly, neither any new gene nor any of the well-known erythrocyte variants that have been selected by malaria, other than *HbS* were identified in this study. Several reasons could be assumed for these results including: considerable phenotypic heterogeneity, with overlapping clinical entities (82 of children with severe malaria had cerebral malaria, 30% had severe anemia and 11% had respiratory distress), population stratification (as reported by authors), differences in haplotype structure between the ethnic groups, or the lack of fine mapping of the SNPs (single nucleotide polymorphisms) dataset within these regions.

A large GWAS conducted by *Timmann et al* among children in Ghana to analyze a total of 5,010,634 SNPs in 1325 cases of severe malaria (severe anemia or cerebral malaria) and 828

controls, associated two previously unknown resistance loci with severe malaria (Timmann, Thye et al. 2012). One of the loci was identified on chromosome 1q32 within the *ATP2B4* gene, which encodes for *PMCA4*, the main calcium pump of erythrocytes. Also, the alteration of its expression may disrupt the homeostasis of intraerythrocytic calcium concentration and thus, affect the development and structure of intraerythrocytic stages of parasite (Stauffer, Guerini et al. 1995) and the second loci was identified on chromosome 16q22.2 within *MARVELD3* gene, which encodes a tight-junction protein that is expressed on endothelial cells and could play a role in microvascular damage caused by the adhesion of pRBCs to endothelium (Steed, Rodrigues et al. 2009). Moreover, the results of this study also confirmed earlier reports on the protective effects of sickle cell and blood group O. A replication analysis was performed for 1320 additional cases and 2222 controls from the same population. All these results, with the exception of those for the *MARVELD3* gene, were replicated in Gambia ($P < 0.05$) (Timmann, Thye et al. 2012).

In an attempt to assess the contribution of previously associated genes, a large multicenter case-control study of severe malaria conducted on 11,890 cases and 17,441 controls from 12 sites in Africa, Asia and Oceania. To overcome the heterogeneity of different studied populations, a Bayesian statistical framework was used. A total of 55 SNPs were evaluated in 27 genetic regions. Strong evidence of association ($P < 10^{-4}$) was obtained for five of the genes studied (*ABO*, *ATP2B4*, *CD40L*, *G6PD* and *HBB*), and no interaction was detected between these genes (Network, Rockett et al. 2014). The associations of *HbS* (rs334) and *ABO* (rs8176719) with resistance to severe malaria were confirmed in this study. However, both protective and susceptible polymorphisms have been identified for *ATP2B4* gene, and the association between the *CD40LG* variant and protection detected for the whole study population was not consistently found when the various sites were considered separately. This suggests that genetic studies of severe malaria are more complex than previously thought and that it is difficult to reproduce the previous results.

Study population	Locus	Sample size and phenotype	Reference
Gambia	<i>HbB (HbS)</i>	Discovery: 1060 SM, 1500 Controls Replication: 1087 SM, 2376 Controls	(Jallow, Teo et al. 2009)
Ghana	<i>ATP2B4, MARVELD3, ABO, HBB</i>	Discovery: 1325 SM, 828 Controls Replication: 1320 SM, 2222 Controls	(Timmann, Thye et al. 2012)
12 locations in Africa, Asia, Oceania	<i>HBB, ABO, ATP2B4, G6PD, CD40L</i>	11,890 SM, 17,441 Controls	(Network, Rockett et al. 2014)
Burkina Faso, Cameroon, Gambia, Ghana, Malawi, Mali, Tanzania	<i>FREM3, GYPE, GYPB, GYPA, ATP2B4, HBB, ABO</i>	Discovery: 5633 SM, 5919 Controls Replication: 13,946 SM/Controls	(Network 2015)
Tanzania	<i>HBB, IL-23R, IL12RB2</i>	914 subjects (446 cases, 465 controls)	(Ravenhall, Campino et al. 2018)
12 locations, 10 in Sub-Saharan Africa and others in Vietnam and Papua New Guinea	<i>EPHA7, FREM3, ATP2B4, ABO, HBB</i>	16115 SM and 20229 controls	(Network 2019)

Table 6: Genome wide association studies for severe malaria.

Another multicentre GWAS comprising 5633 children with severe malaria and 5919 controls from The Gambia, Kenya and Malawi was conducted by genotyping 2.5 million SNPs and imputing 10 million SNPs, with statistical approaches used to account for heterogeneity between populations (Band, Le et al. 2013). This study identified a new locus close to *FREM3* gene and a cluster of three glycoprotein genes (*GYPE*, *GYPB* and *GYPA*) and confirmed association with *HBB*, *ABO* and *ATP2B4* genes (Network 2015). But again, the associated polymorphisms of severe forms of malaria found in previous studies have not all been found, showing that the realization of such studies in the African populations remains very difficult.

Candidate SNPs were then evaluated in the replication cohort, which included 13,946 subjects from Burkina Faso, Cameroon, Gambia, Ghana, Malawi, Mali and Tanzania (Network 2015). An

analysis of the total of 25,498 samples revealed a strong association with a particular SNP (rs184895969) located close to the *FREM3/GYPE* locus, with a minor allele providing about 40% protection and is common in Kenyan population. Glycophorins are abundant in the erythrocyte membrane and are essential for the free flow of these cells in the bloodstream. Here, the SNPs for which strong evidence of association with severe malaria was obtained were located within or close to genes encoding key erythrocyte proteins. Interestingly, the *FREM3/GYPE* locus has been identified as a putative region of ancient balancing selection, as it is part of a haplotype common to human and chimpanzees. This study highlights the scientific interest of large multisite genetic analyses, but also shows that performing such studies in African populations remains highly challenging.

Another GWAS of severe malaria performed in Tanzanian population using haplotype-based selection methods to a case-control dataset for 914 participants (446 cases and 465 controls) and 15.2 million SNPs. This study has not only confirmed the expected association with the sickle cell trait but also has revealed multiple novel loci under association or selection with new target genes in immunological pathways. Positive association has been identified within two interleukin receptors (*IL-23R* and *IL-12RB2*) and Kelch-like protein *KLHL3* as well as near significant effects of Major Histocompatibility Complex (MHC) haplotypes (Ravenhall, Campino et al. 2018).

A very recent GWAS of severe malaria conducted in 12 study locations including 10 in Sub-Saharan Africa and other in Vietnam and Papua New Guinea in over 17,000 individuals. This study confirmed the association of *FREM3*, *ATP2B4*, *ABO*, *HBB* with malaria and also identified a novel variant on chromosome 6 located close to *EPHA7* locus associated with malaria (Network 2019).

It is thought that the regulatory genetic variants alter severe malaria resistance. Although, there have been several GWAS, but a limited number of loci and genetic variants have been identified and replicated in GWAS including *ATP2B4* (rs10900585, rs4951377) on chromosome 1, *FREM/GYP* (rs186873296) on chromosome 4, *EPHA7* (rs62418762) on chromosome 6, *ABO* (rs8176719) on chromosome 9 and *HBB* (rs334) on chromosome 11.

OBJECTIVES AND JUSTIFICATION OF PROJECT

The overall objective of my work is the identification and functional characterization of the genetic variants that are associated and involved in resistance or susceptibility to malaria in the Senegalese population by combining different bioinformatics and functional approaches.

With my colleagues, we started our work from the GWAS data of severe malaria in several populations and looked for the loci with significant association signals. We found that only a limited number of these loci were replicated in independent populations. The aim was to identify the potential causative genetic variants located in these loci and then demonstrate their functional activity. At first, we used different bioinformatics approaches to look for the SNPs in linkage disequilibrium with the tagSNPs associated with severe malaria in several population. These bioinformatics tools allowed us to identify 5 putative regulatory candidate SNPs located very close to each other on chromosome 1 with *ATP2B4* gene. For the association studies, first we confirmed the association of the tagSNP (rs10900585) with severe malaria in our Senegalese population and then performed meta-analysis to validate the results. Further, the association of the 5 candidate variants was assessed with severe malaria in Senegal.

We further wanted to understand the functional role of the 5 candidate variants with malaria by using different functional approaches. We focused particularly on the combined effect of 5 polymorphisms. The goal was to check the impact of the region on gene expression and for this we performed luciferase reporter assay. The next step was to validate the the impact of SNPs on its target and for this we used the technique of Crispr-Cas9 to delete the regulatory region where SNPs are located and checked the regulation of the target gene by qPCR. The last step was to check the expression of PMCA4 protein in the deleted clones as well as the impact of deletion on the intracellular calcium concentration by flow cytometry.

The project allowed us to identify a regulatory region that acts as an enhancer, controls the *ATP2B4* expression and perturbed by the genetic variants that are associated with severe malaria.

To summarize, this project aimed;

- To perform bioinformatics analysis to identify the potential candidate variants from the various severe malaria loci based on GWAS data.
- To evaluate the association of the tagSNP and the regulatory variants with severe malaria in Senegalese population.
- To evaluate the functional impact of the putative candidate variants on the gene regulation using functional assays.
- To check the expression of PMCA4 protein in the deleted clones.
- To check the effect of the variants on intracellular calcium concentration.

Results

Article: Association of *ATP2B4* variants with severe malaria in Senegal and their impact on *ATP2B4* regulation

Over the past decades, genome-wide association studies (GWAS) have revolutionized the field of complex disease genetics. It involves the testing of genetic markers across the genomes of many individuals to identify the genotype-phenotype associations and thus providing numerous compelling associations for the human complex traits and diseases. Despite the clear successes in identifying the novel disease susceptibility genes, it implicates the entire genome in disease predisposition and most of the association signals reflect the variants and genes with no direct biological relevance to the disease. It does not necessarily pinpoint the causal variant and the underlying mechanisms leading to disease outcome. Therefore, bioinformatics and functional studies are required to reveal the causal variants and related mechanisms.

Several GWAS of severe malaria have identified various loci (with significant association signals) with only a limited number of loci and genetic variants replicated in independent populations (Jallow, Teo et al. 2009; Timmann, Thye et al. 2012; Band, Le et al. 2013; Network 2015; Ravenhall, Campino et al. 2018; Band, Le et al. 2019). We were therefore interested in identifying potential causal variants located in these loci and to determine their functional role. Therefore, we systematically identified the regulatory effect of all the SNPs in linkage disequilibrium with the tagSNPs (rs10900585, rs4951377, rs186873296, rs62418762 and rs8176719) associated with severe malaria in several populations and further investigated the best candidates for their functional impact on gene regulation. We identified 125 SNPs in close linkage disequilibrium with the tagSNPs. To prioritize the potential variant, we perform bioinformatics analysis using IW-scoring and ReMap tools. Additionally, the localization of SNPs in the DNase 1 hypersensitivity region and histone marks peaks (H3K4me1, H3K4me3, H3K27ac) was evaluated. Interestingly, we found 5 best candidate variants (rs11240734, rs1541252, rs1541253, rs1541254, rs1541255) located close to each other on chromosome 1 in *ATP2B4* region based on the bioinformatics analysis. In addition, these 5 SNPs were in the top 10 ranks in IW-scoring and with a high number of ReMap ChIP-seq peaks. In contrast, the tagSNPs (rs10900585, rs4951377) had a very low IW-score and were neither localized in ReMap peaks nor the histone marks peaks, which suggested that they have no functional role. Convincingly, our bioinformatics approach allowed us to identify 5 new potential candidates

which may be responsible for the malaria susceptibility. Among the loci that have been replicated, *ATP2B4* on chromosome 1 has been identified as a severe malaria resistance locus. It has been suggested that regulatory genetic variants alter resistance to severe malaria and has been reported that variants localized in *ATP2B4* enhancer influence its expression (Lessard, Gatof et al. 2017). *ATP2B4* encodes for *PMCA4* (Plasma membrane calcium ATPase4), which is a major calcium pump of the red blood cells and the alteration of its expression may affect the intraerythrocytic calcium concentration and thus the structure and development of intraerythrocytic stages of the malaria parasite.

We accessed the association of tagSNP and the 5 selected candidates in a Senegalese population. Recruitment of the samples was carried out from 2012 to 2015 in two Senegalese sites: at Principal Hospital of Dakar and regional hospital of Tambacounda. The study group comprised of severe malaria patients and control group. There were 90 cerebral malaria and 27 non-cerebral malaria cases. Non-cerebral malaria cases were defined as severe malaria without any neurological symptoms; however, they presented isolated symptoms of severe malaria such as severe anemia, respiratory distress, hypoglycemia, or hypoxia. Subsequently, 196 samples including 27 SM and 90 CM samples were collected. 79 healthy individuals were recruited as the control group for the study (Table 4).

	Cerebral malaria (N=90)	Non-cerebral malaria (N=27)	Controls (N=79)
Gender			
Female	28	10	41
Male	57	17	38
Age			
Median	22	8	33
25 th and 75 th percentile	(12-41)	(3-33)	23-51

Table 7. Gender and age information of the Senegalese study population.

We found an association of the variants with severe malaria with major allele as the risk allele. Moreover, we also performed a meta-analysis and confirmed the association of tagSNP (rs10900585) as it has been associated with severe malaria in several populations. A recent study

has shown *ATP2B4* variants (rs10751450, rs10751451, rs10752452) to be located in an enhancer and altering *ATP2B4* expression but they were not genotyped (Lessard, Gatof et al. 2017). As these SNPs were found to be in close LD with our candidates, so we also genotyped them and accessed a significant association with severe malaria. Moreover, we conducted haplotype analyses for the most frequent haplotypes in our population (major alleles haplotype and minor alleles haplotype) and found that the individuals homozygous for the major haplotype have a higher risk of developing SM.

Luciferase reporter assay allowed us to access the functional role of these variants. The region showed a significant promoter activity for both risk (major alleles) and the non-risk haplotype (minor alleles). However, the non-risk haplotype induced higher transcriptional activity than risk haplotype, suggesting a differential allelic promoter activity. Moreover, by observing the individual impact of each SNP and showing that each SNP has a specific impact on the expression and it was revealed that the 5 SNPs may be functional variants and it could be probably their combined effect involved in susceptibility to the SM as already revealed by haplotypes.

Furthermore, the region showed a significant enhancer effect on *ATP2B4* promoter. However, the major alleles haplotype (risk) exhibited significantly higher enhancer impact than minor alleles haplotype (non-risk). The results confirmed that the regulatory region exhibits allele-dependent enhancer activity in K562 and suggested that our regulatory region is an epromoter (promoter with enhancer function).

CRISPR-Cas9 mediated genome editing allowed us to validate the function of epromoter by performing a knockout of the regions containing 5 SNPs (rs11240734, rs1541252, rs1541253, rs1541254, rs1541255) and 8 SNPs (rs10751450, rs10751451, rs10751452, rs11240734, rs1541252, rs1541253, rs1541254, rs1541255). Expression was observed for total *ATP2B4* as well as for two long transcripts (*ATP2B4-203* and *ATP2B4-204*) encoding for PMCA4a and PMCA4b. Decrease in gene expression was observed with both the deletions for total *ATP2B4* compared to WT. For the expression analysis of two long transcripts of *ATP2B4*, there was also a significant decrease in the deleted clones compared to WT cells. These results validated the epromoter function of our regulator region. However, no significant difference in expression of

ATP2B4 was observed for the two deletions which suggest that the regulatory region with our selected 5 variants is enough to regulate *ATP2B4* expression.

Additionally, by measuring the intracellular calcium concentration in the deleted and WT cells, the deleted clone (with 5 variants) showed an increased intracellular calcium concentration which represents less *ATP2B4* expression leading to a decrease in calcium efflux.

In conclusion, we identified an enhancer that controls the expression of *ATP2B4*, and that is perturbed by several genetic variants associated with severe malaria. This suggests that we identified causal variants within a locus identified through GWAS of severe malaria. This also fosters the development of therapeutic strategies based on the modulation of *ATP2B4* expression and calcium levels.

**Identification of *ATP2B4* regulatory element containing functional genetic variants
associated with severe malaria**

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Abstract

Background. Genome-wide association studies (GWAS) for severe malaria have identified 30 genetic variants that are mostly located in non-coding regions, with only few associations replicated in independent populations. In this study, we aimed at identifying potential causal genetic variants located in these loci and demonstrate their functional activity.

Results. We systematically investigated the regulatory effect of the SNPs in linkage disequilibrium with the tagSNPs associated with severe malaria in several populations. Annotating and prioritizing genetic variants led to the identification of a regulatory region containing 5 *ATP2B4* SNPs in linkage disequilibrium with the tagSNP rs10900585. We confirmed the association of rs10900585 and also found significant associations of severe malaria with our candidate SNPs (rs11240734, rs1541252, rs1541253, rs1541254, and rs1541255) in a Senegalese population. Then, we showed that this region had both promoter and enhancer activities and that both individual SNPs and the combination of SNPs had regulatory effects using luciferase reporter assays. In addition, CRISPR/Cas9-mediated deletion of this region decreased *ATP2B4* transcript and protein levels and increased Ca²⁺ intracellular concentration in K562 cell line.

Conclusions. Taken together, our data show that severe malaria associated genetic variants alters the activity of a promoter with enhancer function. We showed that this regulatory element controls the expression of *ATP2B4* that encodes a plasma membrane calcium-transporting ATPase 4 (PMCA4), which is the major calcium pump on red blood cells. Altering the activity of this regulatory element affects the risk of severe malaria probably through calcium concentration effect on parasitaemia.

Background

Malaria caused by *Plasmodium falciparum* parasites is a major cause of morbidity and mortality in many developing countries predominantly in endemic areas of Sub-saharan Africa. The disease outcome is variable and may be subjective to a combination of various factors including host genetic factors, parasite virulence as well as environmental factors (Miller, Baruch et al. 2002; Mackinnon, Mwangi et al. 2005). *P. falciparum* infection causes various clinical phenotypes from asymptomatic parasitemia and uncomplicated malaria to severe malaria (including severe anemia, cerebral malaria and respiratory distress) (Weatherall and Clegg 2002).

There is a growing body of evidence that human genetic factors influence the outcome of infection since the discovery of the protective effect of the sickle cell mutant (rs334). Heritability studies reported that genetic factors explain from 20% to 25% of the variations in malaria phenotypes (Rihet, Traoré et al. 1998; Mackinnon, Mwangi et al. 2005; Damena, Denis et al. 2019). Genetic linkage analyses conducted in Africa provided evidence of linkage of uncomplicated malaria with chromosome 6p21 (Jepson, Sisay-Joof et al. 1997; Flori, Delahaye et al. 2005; Brisebarre, Kumulungui et al. 2014), whereas parasitemia was genetically linked to chromosome 5q31-q33 (Garcia, Marquet et al. 1998; Rihet, Traoré et al. 1998; Flori, Kumulungui et al. 2003; Sakuntabhai, Ndiaye et al. 2008; Milet, Nuel et al. 2010; Brisebarre, Kumulungui et al. 2014). Moreover, several GWAS of severe malaria have identified several malaria resistance loci (Jallow, Teo et al. 2009; Timmann, Thye et al. 2012; Band, Le et al. 2013; Network 2015; Ravenhall, Campino et al. 2018; Network 2019). However, a limited number of loci and genetic variants were identified and replicated in GWAS: *ATP2B4* on chromosome 1, *FREM3-GYP A/B* on chromosome 4, *EPHA7* on chromosome 6, *ABO* on chromosome 9, and *HBB* on chromosome 11. rs334 that is a nonsense polymorphism has been shown to be the main causal SNP within the

HBB locus (Pule, Chimusa et al. 2017), and a copy number variant within *GYP A/B* locus was shown to confer protection against malaria in East Africa (Leffler, Band et al. 2017). The specific variant named DUP4 encodes hybrid glycophorin proteins that may alter the invasion of red blood cells by the parasite (Sim, Chitnis et al. 1994; Mayer, Cofie et al. 2009). Besides, it is thought that regulatory genetic variants alter severe malaria resistance. In this way, Lessard et al (2017) evidenced that an enhancer containing rs10751450, rs10751451, and rs10751452 influence the expression of *ATP2B4*, which encodes for PMCA4 (Plasma membrane calcium transporting ATPase4), the major Calcium pump of red blood cells (Lessard, Gatof et al. 2017). Identifying causal variants from GWAS results remains challenging, although some important results have been obtained. In particular, it is thought that unknown non-coding variants may alter the expression of genes associated with malaria phenotypes.

Here we looked for regulatory SNPs in linkage disequilibrium with tagSNPs, for which association with severe malaria has been replicated in independent populations. We prioritized and annotated the SNPs, and further investigated the best candidates for their functional activity. We provide evidence that several SNPs alter the activity of an enhancer and the expression of *ATP2B4*, modulate intracellular calcium concentration, and are associated with severe malaria in a Senegalese population.

Results:

Prioritization and annotation of putative regulatory SNPs in linkage disequilibrium with tagSNPs associated with severe malaria

We focused our analysis on the SNPs, for which significant GWAS signals have been replicated: rs4951377 and rs10900585 at the *ATP2B4* locus, rs186873296 at the *FREM3-GYP A/B* locus, rs62418762 at the *EPHA7* locus, and rs8176719 at the *ABO* locus. We excluded the coding variation rs334, which captured the GWAS signal at the *HBB* locus. We identified 126 SNPs in linkage disequilibrium with the malaria-associated SNPs, on the basis of a r^2 higher than 0.6. We excluded rs181620317 that is a missense variant within the *FREM3* coding sequence, and further prioritized the SNPs for their potential regulatory effect using the IW-scoring (<https://www.snp-nexus.org/IW-Scoring/>) (Wang, Dayem Ullah et al. 2018). Moreover, we investigated the ability of sequences containing the SNPs to bind transcription factors using the Remap tool (Chèneby, Gheorghe et al. 2018), which integrates public ChIP-seq data sets. In addition, we checked whether the 125 SNPs have been annotated as eQTLs (<https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php>) or identified as regulatory SNPs using the massive parallel reporter assay named Sure-seq (van Arensbergen, FitzPatrick et al. 2017). Supplementary Table 1 shows the rank of the SNPs based on the IW-score, the number of peaks of ChIP-seq for each SNP, and the annotation of the SNPs related to gene expression levels. Table 1 shows the best SNPs on the same basis.

As shown in Figure 1, the number of peaks identified by ChIP-seq negatively correlated with the rank of the corresponding SNPs ($\rho=-0.652$, $P=2.17 \cdot 10^{-11}$). The highest numbers of peaks were observed for rs1541252, rs1541253, rs1541254 and rs1541255 ($n>90$), which were ranked second, 10th, 6th and 7th based on the IW-score, respectively (Table 1). The best SNP according to the IW-scoring method was rs11240734, for which 54 peaks of ChIP-seq were registered in the Remap catalog. Strikingly, those 5 SNPs are close to each other on chromosome 1 in *ATP2B4* region. These SNPs are located within a DNase I hypersensitivity region, and peaks of

H3K4me1, H3K4me3, and H3K27ac marks in K562 cell line, as shown in Figure 2. Similar epigenomic marks have been found at these positions in erythroblasts (Supplementary Figure 1). rs11240734 and rs1541252 have been annotated as eQTLs, according to Haploreg, whereas rs1541255 was found to have a regulatory effect, according to the Sure-seq method (van Arensbergen, FitzPatrick et al. 2017).

Interestingly, the TaqSNPs rs4951377 and rs10900585 that are in linkage disequilibrium with rs11240734, rs1541252, rs1541253, rs1541254, and rs1541255 had a low IW-score, and were ranked at positions 51 and 60, respectively (Supplementary Table 1). In addition, there were neither peaks of ChIP-seq in the Remap catalog nor peaks of H3K4me1, H3K4me3, and H3K27ac marks in K562 cell line for both SNPs (Figure 2), suggesting that they have no functional role. In contrast, rs10751450 that was ranked third based on the IW-score is located within a DNase I hypersensitivity region, and peaks of H3K4me1 and H3K27ac marks in the K562 cell line (Figure 2), and has been shown to be located within a regulatory region (Lessard, Gatof et al. 2017). Similar epigenomic profiles were observed in erythroblasts (Supplementary Figure 1). In addition, 46 peaks of ChIP-seq were evidenced in the region containing rs10751450 on the basis of the Remap catalog. The two additional SNPs rs10751451 and rs10751452 that are close to the rs10751450 (Lessard, Gatof et al. 2017) were ranked here 12th and 13th based on the IW-score, whereas rs10751451 was annotated as an eQTL according to Haploreg (Table 1). Figure 2 shows the position of the region containing rs10751450, rs10751451, and rs10751452 and that containing rs11240734, rs1541252, rs1541253, rs1541254, and rs1541255. Overall, SNP prioritization and annotation allowed us to identify 5 new SNPs with potential regulatory effect, which may drive the observed association of their tagSNPs with malaria susceptibility.

Association between the *ATP2B4* tagSNP rs10900585 and severe malaria

We first evaluated whether the tagSNP was associated with malaria in our study population living in Senegal. One hundred and ninety-six individuals from the Senegalese cohort, including 117 subjects with severe malaria and 79 controls, were successfully genotyped by sequencing (Table 1). The frequencies of observed genotypes conformed to the Hardy-Weinberg Equilibrium ($P=0.52$) (Supplementary Table 2) and the allelic frequencies for rs10900585-T and G were found to be 0.59 and 0.41, respectively. We detected a significant association ($P= 0.029$; OR= 1.94) of *ATP2B4* rs10900585 with SM (Table 2) without the effect of any covariate. The TT genotype that was more frequent among cases (48.7%) than controls (32.9%) and was identified as the risk genotype. Nevertheless, there was no significant association at the 5% significance level when taking into account age as a covariate ($P= 0.055$) (Supplementary Table 3).

We added 13 independent populations to our study population living in Senegal for performing a meta-analysis. All the studies included a control group and a case group with severe malaria patients (Table 3). There were no deviations from Hardy Weinberg equilibrium ($P>0.05$). The meta-analysis included 12794 cases and 19898 controls. There was a significant heterogeneity among the studies ($I^2=66\%$; $\tau^2=0.0174$; $P=0.0003$). Thus, random effect model was used for calculating combined Odd Ratio and 95% confidence interval (CI). There was an association of rs10900585 with SM under the genetic dominant model (OR=1.12; 95% confidence interval= 1.02-1.23). Figure 3 shows the corresponding forest plot.

Association of rs11240734, rs1541252, rs1541253, rs1541254, and rs1541255 with severe malaria in Senegal population.

We further investigated the genetic association of the 5 SNPs newly identified as potential regulatory polymorphisms by SNP prioritization and annotation analysis. These 5 SNPs were in a 600 bp DNA fragment that we sequenced to determine the genotypes of 79 controls and 117 severe malaria subjects. None of polymorphisms deviated from the Hardy-Weinberg equilibrium with a significance level of 0.05 (Supplementary Table 2). For the statistical analysis, we compared subjects carrying the homozygous genotype for major alleles with those carrying either the heterozygous genotype or the homozygous genotype for minor allele. The analysis of individual markers revealed significant associations with disease for all these polymorphisms (P value= 0.002), with an estimated OR of 2.52 (1.39-4.58) (Table 2), indicating a stronger association compared to the tagSNP. Among the severe malaria individuals, 53,8% carried the rs11240734TT, the rs1541252CC, the rs1541253CC, the rs1541254GG and the rs1541255AA genotypes whereas only 31,6% of the control individuals carried these genotypes. In addition, we found a strong linkage disequilibrium between all the 5 SNPs as well as with the tagSNP with a r^2 varying from 0.72 to 1 (Figure 4a). In particular, complete linkage disequilibrium was observed between the polymorphisms rs11240734, rs1541252 and rs1541253. The association of the 5 SNPs with severe malaria remained significant (P=0.006) after taking into account age as a covariate (Supplementary Table 3).

Association of rs10751450, rs10751451, and rs10751452 with severe malaria in Senegal population.

Although suggested as regulatory polymorphisms (Lessard, Gatof et al. 2017), the SNPs rs10751450, rs10751451 and rs10751452 have never been genotyped and tested for their

association with malaria. We then tested whether these 3 SNPs were associated with severe malaria. The frequencies of observed genotypes conformed to the Hardy-Weinberg Equilibrium (Supplementary Table 2). The analysis indicated a significant association between malaria disease and these SNPs, rs10751450 (P=0.016; OR 2.07), rs10751451 (P=0.002; OR 2.49) and rs10751452 (P=0.006; OR 2.27) (Table 2). This association remained significant when taking into account age as a covariate (Supplementary Table 3). Noticeably, in our study population, these SNPs were found to be in strong linkage disequilibrium between them as well as with our 5 new candidate SNPs (Figure 4b).

Haplotype analysis of the *ATP2B4* SNPs

We therefore conducted a haplotype analysis, combining rs11240734, rs1541252, rs1541253, rs1541254 and rs1541255. In total, we observed 5 haplotypes showing various frequencies, 0.65 for haplotype (1) with the major alleles (rs11210734T-rs1541252C-rs1541253C-rs1541254G-rs1541255A), 0.33 for haplotype (2) with the minor alleles (rs11210734C-rs1541252T-rs1541253T-rs1541254C-rs1541255G), 0.015 for the haplotype (3) (rs11210734T-rs1541252C-rs1541253C-rs1541254C-rs1541255A), 0.002 for haplotype (4) (rs11210734C-rs1541252T-rs1541253T-rs1541254C-rs1541255) and 0.002 for haplotype (5) (rs11210734C-rs1541252T-rs1541253T-rs1541254G-rs1541255A). We first investigated the relationship between severe malaria and the two most frequent haplotypes (1 and 2). The haplotype (1) was found to be associated with an increased risk of developing SM (P=0.005) using chi-square test. Among severe malaria subjects, 55.8 % were homozygous for the susceptibility haplotype (1) and 34.5 % were heterozygous whereas only 31.6 % of the control subjects carried this haplotype at

homozygote state. Statistical analysis indicated that individuals homozygous for haplotype (1) with the major alleles have a higher risk of developing a severe form ($P=0.002$, $OR=2.52$) compared to heterozygous and homozygous haplotypes for minor alleles. This association remained significant when including age as a covariate in the statistical model ($P=0.006$) (Table 4). We further performed the haplotype analysis by including rs10751450, rs10751451, rs10751452, rs11240734, rs1541252, rs1541253, rs1541254 and rs1541255. Again the two most frequent haplotypes were analyzed. The haplotype (CCTTCCGA) was found to be associated with an increased risk of developing SM ($P=0.011$) using chi-square test. Among the SM individuals, 47.8% were homozygous and 27.4% were heterozygous for the susceptible haplotype (CCTTCCGA) while only 26.6 % of the control subjects carried this haplotype in homozygous state. By accessing the relationship with SM, haplotype (CCTTCCGA) with major alleles at homozygous state was found to be significantly associated with an increased risk of SM ($P=0.003$, $OR=2.67$). The association remained significant by taking age as a covariate ($P=0.005$) (Table 4).

Lower promoter activity and higher enhancer activity are associated with the risk haplotype of severe malaria

To evaluate the potential effect of these variants in regulating *ATP2B4* expression, we cloned the region comprising the 5 SNPs into a luciferase reporter construct and we measured relative transcriptional activity in the K562 cell line. We evaluated both the risk haplotype, combining the major alleles, and non-risk haplotype, combining the minor alleles. We also used site-directed mutagenesis to modify each SNP and estimate their individual impact (Figure 5a). Interestingly,

this regulatory region showed a strong promoter activity compared to the SV40 promoter for both the risk haplotype with major alleles ($P < 1.10^{-4}$, 10.9 fold increase) and the non-risk haplotype with minor allele ($P < 1.10^{-4}$, 17.7 fold increase). Moreover, the risk haplotype was shown to have reduced transcriptional activity than the non-risk haplotype ($P < 1.10^{-4}$). Hence, our results revealed differential allelic promoter activity in K562 cell line showing that the non-risk haplotype exhibited 1.6 fold increased transcriptional activity compared to the risk haplotype. In addition, we observed that each SNP has a specific impact on expression and their combination reflects the combined effect observed. These results indicate that these 5 SNPs may be functional variants and that we must consider that it is their combined effect, which is probably involved in the susceptibility to severe forms as suggested also by the haplotypes naturally present in individuals living in endemic areas.

To functionally confirm that this genomic region may have enhancer function, we performed luciferase promoter assays by cloning it downstream the luciferase reporter gene derived by a promoter region of *ATP2B4* of 780 bp (Figure 5b). We first validated the promoter activity of this 780 bp region compared to SV40 promoter ($P < 1.10^{-4}$, 3.8 fold increase). We further confirmed the enhancer activity of the 600 bp region ($P < 0.01$, 1.3 fold increase for the risk haplotype as compared to *ATP2B4* promoter). Moreover, we showed that the region containing the major alleles exhibited an enhancer activity that was significantly higher than that of the region containing the minor alleles ($P = 0.01$). Also, there was a 1.2 fold increase in activity of the major allele haplotype relative to the minor allele haplotype. These data confirm that this regulatory region exhibits allele-dependent enhancer activity in K562 cell line. Altogether, our results suggested that this regulatory region is a promoter with enhancer function, also named Epromoter (Dao and Spicuglia 2018). Furthermore, they suggest that genetic variants within this regulatory

region orient the function of the region towards a promoter activity or towards an enhancer activity.

Genome editing confirmed the regulatory activity of the region containing the SNPs

To analyze the function of the Epromoter element, we deleted the DNA region containing the malaria-associated SNPs (rs11240734, rs1541252, rs1541253, rs1541254 and rs1541255) in the K562 cell line, using the CRISPR/Cas9 mediated genome editing (Figure 6a). We used 2 independent guide RNAs resulting in a 506-bp deletion. To evaluate the effect of this deletion on *ATP2B4* expression, we isolated individual clones to control the genomic sequence and selected 3 deleted clones with the expected sequence, with no additional edits. Expression analysis for total *ATP2B4* showed a 3.3 fold decrease in gene expression in deleted clones as compared to WT cells ($P < 1.10^{-4}$) (Figure 6b). We also estimated the expression of the two long transcripts (*ATP2B4-203* and *ATP2B4-204*) of *ATP2B4* (encoding for PMCA4a and PMCA4b) and we showed a 2.8 fold decrease in deleted clones compared to WT cells ($P < 1.10^{-4}$) (Figure 6b). These results support the hypothesis that this region has enhancer function.

We then introduced a pair of guide RNAs resulting in a 1262-bp deletion to remove the 8 SNPs (rs10751450, rs10751451, rs10751452, rs11240734, rs1541252, rs1541253, rs1541254 and rs1541255) (Figure 6a). We selected 3 clones after verifying the genomic sequences. We have observed that clones with this large 1262-bp deletion had a similar reduction in expression compared to the small 506-bp deletion both for total expression and for long transcripts of *ATP2B4*. No significant difference in expression of *ATP2B4* was observed between these two deletions suggesting that the minimal region of 506 bp including (rs11240734, rs1541252,

rs1541253, rs1541254 and rs1541255) is enough to regulate the expression of *ATP2B4* (Figure 6c).

K562 deleted clones exhibited higher intracellular calcium concentration

We measured intracellular calcium concentration by cytometry in K562 WT cells and 2 clones deleted for the region containing rs11240734, rs1541252, rs1541253, rs1541254 and rs1541255. Figure 7 shows the results for one experiment with clone Δ ATP2B4_2 and one experiment with clone Δ ATP2B4_3. They indicated an increase of calcium concentration in deleted clones. Six and two experiments were performed with Δ ATP2B4_2 and Δ ATP2B4_3 clones, respectively. Comparing mean of the MFI yielded significant differences when including only Δ ATP2B4_2 clone results ($P= 0.016$) or including both Δ ATP2B4_2 and Δ ATP2B4_3 results ($P= 0.002$). In the same way, meta-analyses taking into account MFI, SD, and sample size provided evidence of an increase of calcium concentration when including only the results obtained with Δ ATP2B4_2 clone ($P<0.001$) or including both Δ ATP2B4_2 and Δ ATP2B4_3 results ($P<0.001$). Overall, these results indicate that deleted clones expressed *ATP2B4* at a low level, leading to a decrease of calcium efflux and an intracellular accumulation of calcium.

PMCA4 protein is not expressed in K562 deleted clones

The expression of the protein PMCA4 has been assessed by cytometry in K562 WT cells and in the deleted clones Δ ATP2B4_2 and Δ ATP2B4_3. Three independent experiments have been performed for each clone and a representative FACS plot is shown in Figure 8. The results

showed that the PMCA4 protein is not detected in the deleted clones, whereas this protein is expressed in K562 WT cells; MFI difference between WT and deleted clones is significant ($p=0.002$). Meta-analyses that took into account MFI, SD, and sample size further provided evidence of a significant decrease of protein level in deleted clones ($P<0.0001$). Indeed, the protein was undetectable in the deleted clones.

Discussion

Several GWAS have identified and replicated tagSNPs associated with severe malaria. Most of the causal variants remain, however, unknown. Here we identified 5 *ATP2B4* regulatory SNPs associated with severe malaria in a Senegalese population and in linkage disequilibrium with the tagSNPs identified by GWAS (rs4951377 and rs10900585). We showed that these 5 SNPs are located in a promoter region that displays enhancer activity (Epromoter) described recently in our laboratory (Dao, Galindo-Albarrán et al. 2017).

From the tagSNPs replicated at least once in an independent population, we identified 125 non-coding SNPs in linkage disequilibrium with the tagSNPs associated with severe malaria. We prioritized the candidate regulatory SNPs using bioinformatic tools. Strikingly, the top 10 ranked SNPs by the IW-scoring method were on chromosome 1 within *ATP2B4*. When combining the IW-scoring method with the annotation of ChIP-seq peaks at each SNP, rs1541252, rs1541253, rs1541254, and rs1541255 appeared to be the most promising candidates. In addition, rs11240734 that was ranked first based on the IW-score and fifth based on the number of ChIP-seq peaks was close to rs1541252, rs1541253, rs1541254, and rs1541255, suggesting that these SNPs may be located within a regulatory element. This hypothesis is also supported by the co-location of the SNPs with epigenomic marks associated with promoter or enhancer activity, such

as H3K4me1, H3K4me3, and H4K27ac. In the same way, rs11240734, rs1541252, rs1541253, and rs1541254 were reported as eQTLs in blood cells (Westra, Peters et al. 2013), whereas rs1541252 and rs1541253 have been shown to be associated with PMCA4 levels (Zámbó, Várady et al. 2017). Moreover, rs1541255 was shown to be a regulatory variant on the basis of a reporter assay (van Arensbergen, Pagie et al. 2019). Noticeably, the tagSNPs rs4951377 and rs10900585 previously associated with severe malaria were ranked 51th and 60th using the IW score, respectively. In addition, they were located within regions showing neither H3K4me1, H3K4me3, H3K27ac marks nor evidence of binding transcription factors, suggesting that they are probably not the causal SNPs. Nevertheless, rs4951377 was an eQTL in blood cells (Westra, Peters et al. 2013), illustrating that an eQTL is not necessarily a regulatory variant. It is very likely that the association signal of rs4951377 with gene expression is explained by the effect of functional SNPs in linkage disequilibrium with rs4951377.

In addition, our bioinformatics analysis confirmed that rs10751450, rs10751451, rs10751452 ranked 3th, 12th and 13th, respectively, could be regulatory SNPs as previously suggested (Lessard, Gatof et al. 2017). Interestingly, these SNPs have shown to be located within an enhancer altering *ATP2B4* that codes for PMCA4 (Plasma membrane calcium transporting ATPase4) (Lessard, Gatof et al. 2017). This enhancer region is roughly 700 bp away from the region containing rs11240734, rs1541252, rs1541253, rs1541254, and rs1541255, which is potentially a regulatory region according to our results.

We genotyped rs10900585, rs11240734, rs1541252, rs1541253, rs1541254, rs1541255, rs10751450, rs10751451, and rs10751452 in a Senegalese population and assessed their association with severe malaria. Our result with the tagSNP rs10900585 was consistent with the previous finding revealing the same risk genotype (Timmann, Thye et al. 2012), whereas our

meta-analysis integrating our Senegalese population validated the association of the tagSNP with severe malaria. In addition, we provided evidence of an association of rs11240734, rs1541252, rs1541253, rs1541254, rs1541255, rs10751450, rs10751451, and rs10751452 with severe malaria in Senegal. Interestingly, rs11240734, rs1541252, rs1541253, rs1541254, rs1541255, rs10751450, and rs10751451 that were imputed in the most recent GWAS of severe malaria (Network 2019) showed a high Bayes Factor value ($BF > 1.8 \times 10^7$) that was consistent with an association; the SNPs were, however, not genotyped in this study. In the same way, rs1541255 was associated with severe malaria in Kenya (Ndila, Uyoga et al. 2018), whereas rs11240734, rs1541252, rs1541253, rs1541254, rs10751450, rs10751451, and rs10751452 were not genotyped in this population.

Furthermore, when analyzing the combination of genetic variants rs11240734, rs1541252, rs1541253, rs1541254, and rs1541255 located within the regulatory region, we found a strong haplotype association with severe malaria and we observed that the majority of the Senegalese individuals exhibited either the haplotype combination of all the major alleles or the haplotype combination of all the minor alleles whereas only few individuals carried other haplotypes. Furthermore, we tested the regulatory activity of this region containing the SNPs using the luciferase reporter assay. We showed that this region had a promoter and an enhancer activity and that these activities were modulated by the SNPs and their combination. Interestingly, we observed higher enhancer activity with the haplotype combining the major alleles whereas this haplotype showed lower promoter activity compared to the haplotype with the minor alleles. This result supports a hypothetical model of regulation proposed recently by (Gao, Xia et al. 2018; Hua, Ahmed et al. 2018; Ying, Li et al. 2018), whereby genetic variants can impose a regulatory switch between the enhancer and the promoter activity. Our results further suggest that there was

an effect of the combination of several SNP alleles on gene expression, thus reflecting the interaction between these SNPs. Although, little is known about haplotype's influence on gene expression, Ying et al (Ying, Li et al. 2018) recently proposed a method to detect eQTL haplotypes and suggested that they are enriched in regulatory regions such as promoters or enhancers. Our finding is also supported by a study providing evidence at numerous loci that “multiple enhancer variants” cooperatively contribute to altered expression of their gene targets and that target transcript levels tend to be modest (Corradin, Saiakhova et al. 2014). This raised the question of the natural combination of alleles in African populations under selective pressure and supports our finding that the combination of these 5 SNPs was responsible for susceptibility to severe malaria and that there is not a single causal variant.

CRISPR/cas9 genome editing allowed us to assess the impact of this regulatory region containing rs11240734, rs1541252, rs1541253, rs1541254, and rs1541255. Also, we showed that the deletion of this region decreased both the global *ATP2B4* expression and specifically the expression of the long transcripts in the K562 cell line, supporting its regulatory activity and suggesting that it is a promoter with enhancer function (Epromoter). Consistently, decreasing expression of the transcripts in the clones with a deletion of this regulatory region results in the absence of the PMCA4 protein in these clones.

In addition, CRISPR/Cas9-mediated deletion of this region increased intra-cellular calcium concentration. After deleting a region containing rs10751450, rs10751451, and rs10751452, Lessard et al showed a reduction in *ATP2B4* expression and a cytoplasmic calcium accumulation (Lessard, Gatof et al. 2017). Hence, we included these SNPs in our study and we obtained similar association results when adding rs10751450, rs10751451, and rs10751452 in the haplotype analysis but also similar decrease in *ATP2B4* expression in the two different clones with either 5

or 8 SNPs deletion. This finding supports that rs11240734, rs1541252, rs1541253, rs1541254, and rs1541255 affect the *ATP2B4* gene expression as much as rs10751450, rs10751451, and rs10751452 and contribute to development of severe malaria. Here, we have demonstrated the potential impact of these variants in regulating *ATP2B4* expression and intracellular calcium levels. We hypothesize that changes in calcium homeostasis may affect the growth of parasite in red blood cells and may also affect clumping of infected red blood cells and their sequestration to the brain micro vessels, thus contributing to severe malaria susceptibility. In addition, our results support the hypothesis that a calcium-activated potassium channel may be activated resulting in potassium efflux, dehydration, red blood cell volume loss, increased mean corpuscular hemoglobin concentration, and reduced parasitemia in malaria patients (Lessard, Gatof et al. 2017). Indeed rs1541252 and rs1541255 were found to be associated with mean corpuscular hemoglobin concentration in African-American children and parasitemia in malaria patients, respectively (Li, Glessner et al. 2013; Ndila, Uyoga et al. 2018).

Noticeably, it is not excluded that the modulation of intracellular Ca^{2+} concentration alters the physiology of other cell types. For example, the regulation of intracellular Ca^{2+} signaling is a major determinant of CD8+ T cell responsiveness which may have an important role in the determining of severe malaria. Moreover, the PMCA4 protein is expressed both in the red blood cell and in the brain with PMCA4b and PMCA4a the most abundant isoforms in red blood cells and in brain, respectively (Caride, Filoteo et al. 2007). Because these isoforms are tightly and specifically regulated in tissues and cells, we can therefore propose that dysregulation of calcium homeostasis in the brain could also be directly involved in cerebral malaria susceptibility as for different brain disorders such as Alzheimer's disease and Parkinson's disease. PMCA4 which is the major isoform present on endothelial cells may also play crucial role in severe malaria

through its role as negative regulator of vascular endothelial growth factor (VEGF)-activated angiogenesis. In cerebral malaria, the binding of parasitized erythrocytes to the cerebral endothelium and the consequent angiogenic dysregulation play a key role in pathogenesis (Furuta, Kimura et al. 2010). VEGF, a regulator of endothelial inflammation and integrity, is involved in modulating tissue pathology in response to *P. berghei* infection. Indeed, a potent inhibitor to VEGF signaling pathway aggravated dramatically the course of *P. berghei* infection (Canavese and Crisanti 2015).

Conclusions

We identified a new regulatory region that controls the expression of both *ATP2B4* mRNA and PMCA4 protein and that affects intracellular calcium levels. We also showed that the activity of this regulatory region was perturbed by 5 genetic variants associated with severe malaria. This suggests that we identified causal variants within a locus identified through GWAS of severe malaria. This also fosters the development of therapeutic strategies based on the modulation of *ATP2B4* expression and calcium levels. It should be stressed, however, that the effect size of *ATP2B4* was modest in our study population. This is consistent with previous heritability studies (Ndila, Uyoga et al. 2018; Network 2019; Damena and Chimusa 2020). More generally, genetic variants in *ATP2B4*, *FREM3-GYP A/B*, *EPHA7*, *ABO*, and *HBB*, which have been identified by several GWAS studies, explain 11% of the genetic contribution to variation in severe malaria susceptibility (Network 2019). In this way, a polygenic effect has been proposed by Damena et al (2019), suggesting that many genetic variants with a small effect size remain to be discovered. Interestingly, the heritability was found to be concentrated on chromosomes 5, 11 and 20

(Damena and Chimusa 2020). These findings are reminiscent of the genetic linkage of parasitemia to chromosome 5q31-q33 detected in several independent populations (Garcia, Marquet et al. 1998; Rihet, Traoré et al. 1998; Flori, Kumulungui et al. 2003; Sakuntabhai, Ndiaye et al. 2008; Milet, Nuel et al. 2010; Brisebarre, Kumulungui et al. 2014). Interestingly, some GWAS signals that have not been replicated were found on chromosomes 5 and 11. Also, further studies focusing on chromosomes 5, 11 and 20 may lead to the identification of new severe malaria susceptibility loci.

Materials and methods

Study Subjects, blood samples and Phenotypes

Malaria patients were identified in two major Senegalese sites, the principal hospital of Dakar and the regional hospital of Tambacounda, between 2012 to 2015. Malaria patients have been described (Thiam, Baaklini et al. 2018). The study groups comprised of severe malaria patients and control individuals. There were 90 cerebral malaria cases and 27 severe noncerebral cases, as described (Thiam, Baaklini et al. 2018). The control samples (n=79) were obtained from healthy volunteers living in Dakar. Written informed consent was obtained for each patient or their accompanying family members. The study was approved by the institutional research ethics committee of the Université Cheikh Anta Diop. Study form mentioning age, gender, ethnicity, and other clinical data was filled for each subject.

Venous blood samples were collected in Vacutainer ACD tubes (Becton Dickinson, Rutherford, USA). Biological data including parasite density, hematology, and other characteristics were recorded on the day of admission. For all patients, the presence of *Plasmodium falciparum* was

determined by at least two trained biologists by microscope examination of thin and thick smears using an oil immersion lens at 100x magnification, prior to anti-malarial treatment. If no asexual parasite was observed, the slide was considered negative. On the contrary, if the asexual parasites were observed then the slide was considered as positive and the number of parasitized erythrocytes were counted per μl of blood.

Bioinformatic prioritization and functional annotation of genetic variants

A systematic literature search was performed using the PubMed database to identify all the GWAS on severe malaria. Significant GWAS signals replicated at least in an independent population were identified and the corresponding tagSNPs (rs4951377, rs10900585, rs186873296, rs62418762 and rs8176719) were selected for further analysis. Haploreg v4.1, a tool including linkage disequilibrium (LD) information from the 1000 Genome project was used to identify the SNPs in linkage disequilibrium with these tagSNPs. This analysis on African population and LD threshold $r^2 > 0.6$ resulted in the selection of 125 SNPs. To prioritize the SNPs with putative functional significance, we performed bioinformatic analysis using IW-Scoring annotation tool (<https://www.snp-nexus.org/IW-Scoring/>) (Wang, Dayem Ullah et al. 2018) and Remap 2018 based on DNA-binding ChIP-seq experiments (Chèneby, Gheorghe et al. 2018). We also checked whether these SNPs have been annotated as eQTLs (<https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php>) or identified as regulatory SNPs using the massive parallel reporter assay named Sure-seq (van Arensbergen, FitzPatrick et al. 2017). Additional analysis has been performed to evaluate whether the candidate SNPs are

located within a DNase I hypersensitivity region, peaks of H3K4me1, H3K4me3, and H3K27ac marks in K562 cell line and visualized them in UCSC genome browser.

DNA extraction, DNA amplification and genotyping

Genomic DNA was extracted from 200µl of peripheral whole blood samples using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and was further amplified by Illustra GenomiPhi V2 DNA Amplification kit (GE healthcare Life Sciences, Velizy-Villacoublay, France) according to the manufacturer's instructions.

We genotyped the polymorphisms in the Senegalese population, which consisted of 117 individuals with severe malaria and 79 healthy controls. In total, we genotyped 9 SNPs of *ATP2B4* including rs11240734, rs1541252, rs1541253, rs1541254 and rs1541255, the TaqSNP rs10900585 and three additional SNPs (rs10751450, rs10751451 and rs10751452) previously described as regulatory variants (Lessard, Gatof et al. 2017). Genotyping was performed using either the Sanger sequencing or the TaqMan SNP genotyping assays (Applied Biosystems, ThermoFisher). All the primer pairs for sequencing were designed using Primer 3 software. PCR amplification of DNA fragments containing rs11240734, rs1541252, rs1541253, rs1541254 and rs1541255 was performed with forward (5'-TCAGGCCTAGCTATCAGTTCAG-3') and reverse (5'-CGAGTAGCCGTCCGAAGTC-3') primers, respectively. For the tagSNP rs10900585, the forward and the reverse primers were (5'-GGGATGAGGAGGCTTACAGG-3') and (5'-GAGGTTGAGGTGAGCGGATC-3'), respectively.

PCR amplification was carried out in 50µl reaction volume with 12.5 ng of genomic DNA, GoTaq G2 2X ready to use Master Mix (Promega, cat#M7433) and 10µM of each primer. For rs10900585, thermocycling started with a single denaturation step for 2 minutes at 95°C,

following 35 cycles of denaturation for 40 seconds at 95°C, annealing for 30 seconds at 65°C and extension at 72°C for 1 minute. One final extension step was added for 10 minutes at 72°C. While for rs1541252 thermo cycling started with a single denaturation step for 2 minutes at 95°C, following 35 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 60°C and extension at 72°C for 40 seconds. Final extension for 5 minutes at 72°C. The PCR products were submitted to electrophoresis on 2% agarose gel to verify the product size and then purified with PCR Purification kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Five µl of purified PCR products were sequenced using Sanger method (GATC biotech, Germany or Eurofins genomics, Germany).

For rs10751450, rs10751451 and rs10751452, we used TaqMan genotyping assays C_31796478_10, C_31796479_10 and C_31796480_10, respectively. PCR amplification was performed with the QuantStudio 6 Flex Real-Time PCR Systems (Applied Biosystem, ThermoFisher Scientific) in a final volume of 5 µl containing 2.1 µl of master Mix (Applied Biosystem, ThermoFisher Scientific), 0.06 µl of TaqMan probe and 1.84 µl H₂O and 1 µl of DNA (10-15ng/µl).

Luciferase Gene Reporter Assay and site-directed mutagenesis:

Promoter activity.

A 601bp DNA fragment upstream the *ATP2B4* translation start site (chromosome 1: 203682549-203683049 according to the hg38 assembly) was cloned into the MluI-XhoI sites of pGL3-basic vector (Promega, Madison, WI, USA, cat# E1751), which contained the firefly luciferase coding sequence (GeneCust Custom Services, Luxembourg). We obtained two different pGL3 constructs

containing either minor or major alleles of the 5 SNPs (rs11240734, rs1541252, rs1541253, rs1541254 and rs1541255). From the minor allele construct, we generated additional constructs by replacing the minor allele with the major allele of each SNP individually. The site-directed mutagenesis was performed using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA, USA, cat#E0554S) and primers designed by NEBaseChanger tool provided by the supplier (detail sequences provided in (Supplementary Table 4). K562 cells (ATTC CLL-243) were grown in Gibco RPMI 1640 medium (Thermo Fisher Scientific Waltham, MA, USA) supplemented with 10% of FBS (fetal bovine serum). K562 transfection was performed with Neon™ Transfection system (Invitrogen) according to the manufacturer's instructions. For each out of 9 tests performed, 10⁶ cells were co-transfected with 1µg of control vectors 1) negative control vector (empty pGL3-basic vector (cat# E1751)) or 2) positive control vector (pGL3-promoter vector (cat# E1761)) or with 1µg of construct to be tested (pGL3-basic vector containing either the minor alleles CTTTCG, or the major alleles TCCGA or one of these combinations TTTCG, CCTCG, CTCCG, CTTGG and CTTCA)) and 200 ng of pRL-SV40 (a plasmid encoding renilla luciferase from Promega (cat# E2231)), which was used as a transfection efficiency control. Transfected cells were maintained at 37°C in 5% CO₂ for 24 hours. Values of Firefly and renilla luciferase were obtained by analyzing 20µl of cell lysate according to the standard instructions provided in the Dual Luciferase kit (Promega cat# E1910) in a TriStar LB 941 Multimode Microplate Reader (Berthold technologies, Thermo Fisher Scientific, Waltham, MA, USA). Firefly luciferase activity of each sample was normalized to renilla luciferase and expressed as the fold change of the empty vector control.

Enhancer activity.

A 780 bp fragment of *ATP2B4* promoter (chromosome1: 203626081-203626860 (hg38 assembly)) was cloned into pGL3-Enhancer vector (Promega, cat#E1771, Promega) between MluI- Xho I sites and the 601 bp fragment of *ATP2B4* enhancer (chromosome 1: 203682549-203683049 according to the hg38 assembly) containing respective minor and major alleles of the 5 SNPs was cloned into BamHI-SalI sites. Luciferase assay were performed in K562 cells. We transfected 10^6 cells by NeonTM Transfection system (Invitrogen, ThermoFisher Scientific) with 1 µg of control vectors (empty pGL3-basic vector or positive control pGL3 promoter vector) or with 1µg of construct to be tested and 200 ng of renilla luciferase plasmid (pRL-SV40, Promega, cat#E2231) as an internal control. Twenty-four hours after transfection luciferase, the activity was analyzed.

CRISPR-Cas9 genome editing in K562 cells

Using computational algorithms with prioritization for on-target efficiency and reduced off-target effects (available online CRISPR design tool provided by IDT), we identified guide RNAs to target *ATP2B4* specific region. Chemically synthesized oligoribonucleotides were manufactured by IDT: the crRNAs (35 mer with specific part to DNA target sequence) and the universal tracrRNAs (67 mer). A two-part system where synthetic crRNA (5 µl of 100 µM) and tracrRNA (5 µl of 100 µM) were annealed to form an active gRNA complex. Cas9 RNP complexes were assembled in vitro by incubation of 3.4 µl Cas9 protein (62 mM) with 4.8 µl active gRNA complex (cr:tracrRNA) and 1.8 µl of PBS. To generate the genomic deletion two different RNP complexes were simultaneously electroporated with Neon transfection system into $5 \cdot 10^5$ K562 cells. The small deletion of 506 bp (containing the 5 SNPs) was obtained by using 2 µl gRNA1 and 2 µl of gRNA2 (details of sequences in (Supplementary Table 5). The large deletion of 1262 bp (containing the 8 SNPs) was performed by using gRNA2 and gRNA3 (Supplementary Table

5). Those bulk cultures transfected with tandem gRNA were plated clonally in 96-well plates at limiting dilution less than 0.5 to avoid mixed clones. After approximately 14 days of clonal expansion, amplification of DNA was performed directly from the clones by using the 2X Phire Tissue Direct PCR Master Mix containing Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific), with 20 μ l of dilution buffer and 0.5 μ l of DNA Release additive, which allowed to improve the release of DNA from the cells. Clones were screened for deletion by PCR using primers F1 and R1 for the small deletion detection that would produce a specific short fragment of 335 bp in the presence of deletion and a long fragment of 841 bp in the absence of deletion (Supplementary Table 5). Primers F2 and R2 were used for large deletion detection that would produce amplicons of 355 bp in presence of deletion and 1617 bp in the absence of deletion (Supplementary Table 5). PCR was performed using 10 μ l of 2x Master Mix and 10 μ M of each primer, using the following cycling conditions: single denaturation step for 5 minutes at 98°C, following by 35 cycles of denaturation for 5 seconds at 98°C, annealing for 5 seconds at 60°C and extension at 72°C for 30 seconds. A final extension for 1 minute at 72°C is added. After electrophoresis on 1% agarose gel, we selected homozygotes deleted clones showing only amplicons of 335 bp or 355 bp for the small and the large deletions, respectively. Selected clones were sequenced using the Sanger method and only the clones with the appropriate sequence with no additional edits were kept for gene expression analysis.

Reverse transcription-quantitative PCR

Total RNA was extracted for each selected clone and wild-type K562 cells using the RNeasy mini kit (Qiagen, Hilden, Germany). One μ g of RNA per clone was converted into cDNA using

Superscript VILO Master Mix (Invitrogen, ThermoFisher Scientific). Three independent RNA/cDNA preparations were performed for each clone. Real-time quantitative PCR (RT-qPCR) was subsequently performed using SYBR select Master Mix (ThermoFisher Scientific) on a QuantStudio 6 Flex instrument. Primers were designed using Primer 3 software to span exon 1 of *ATP2B4* (F3 and R3) to specifically quantify the long transcripts (ENST00000357681, ENST00000367218) or to span exon 19 and exon 20 (F4 and R4) to quantify all the *ATP2B4* transcripts (ENST00000357681, ENST00000367218, ENST00000341360, ENST00000458092 and ENST00000356729) (Supplementary Table 6). PCR efficiency was validated by performing serial dilution analysis. Gene expression was normalized to that of actin and the relative expression was calculated by the Δ CT method. The data reported correspond to the mean of triplicates from three independent experiments per clone and expressed as fold change relative to wild-type cells. Three independently generated clones were analyzed for gene expression quantification of *ATP2B4*.

Calcium measurement by flow cytometry in K562 clones and wild-type cells

The intracellular quantity of calcium was measured in K562 cells and Δ ATP2B4_2 and Δ ATP2B4_3 clones, by flow cytometry, using the Fluo-4, AM kit (Molecular Probes/ThermoFisher Scientific), following manufacturer's instructions. Briefly, samples of 10^6 cells were harvested for each cell type. The cells were pelleted and resuspended in 1 ml of culture medium without serum. One ml of either Fluo-4-AM (labeled cells) or kit buffer (control cells) was added to the cells, prior to 1 hour incubation at 37°C. The cells were then directly observed on an LSRFortessa X-20 cytometer (BD Biosciences). Cells were gated on forward/side-light

scatter and the fluorescence intensity of 20,000 cells was measured at the FITC excitation/emission wavelength, for each sample.

Detection of PMCA4 by flow cytometry in K562 clones and wild-type cells

The presence of PMCA4 in K562 cells and clones Δ ATP2B4_2 and Δ ATP2B4_3, was detected by flow cytometry, using the specific antibody JA9 (ABCAM, ab2783). The labeling was realized according to a slight modification of the manufacturer's protocol. For each cell type, 2×10^6 cells were harvested, pelleted and fixed in 2 ml of 80% methanol (5 min). After centrifugation, the cells were incubated in 2 ml of 1xPBS / 1% BSA, during 30 min at room temperature, in order to block non-specific interactions. Half of each cell type was then incubated with JA9 antibody ($2 \mu\text{g}/1 \times 10^6$ cells) for 30 min at room temperature. The other half was used for isotypic control (ab170190, $2 \mu\text{g}/1 \times 10^6$ cells). After centrifugation, the cells were incubated with the secondary antibody (goat anti-mouse IgG1 APC, ab 130786) at 1/200 dilution in 1xPBS / 1% BSA, for 30 min at room temperature. Finally, cells were washed twice in 1xPBS and observed on a LSR Fortessa X-20 cytometer (BD Biosciences). Cells were gated on forward/side-light scatter and the fluorescence intensity of 20 000 cells was measured at the APC excitation/emission wavelength, for each sample.

Statistical analysis

A Chi2 test was used to determine whether the genotype distribution in healthy Senegalese subjects conformed to Hardy-Weinberg equilibrium. None of the polymorphisms deviated from

Hardy-Weinberg equilibrium in tests with a threshold for significance of $P=0.05$ carried out in Haploview software. Chi² test and logistic regression analysis were carried out with SPSS (statistical software version 20) to assess the association between the SNPs and SM in unrelated Senegalese subjects. Differences were considered to be significant if the P-value obtained in a two-tailed test was < 0.05 . The odds ratio (OR), 95% confidence intervals (CIs) and the influence of covariates on the phenotype were evaluated by logistic regression analysis. Meta-analysis of genetic association was performed using MetaGenyo (Martorell-Marugan, Toro-Dominguez et al. 2017). Statistical differences between luciferase constructs or between clones were performed using Student's t-tests. Mixed models were also used to take into account the triplicates performed for each independent experiment. P values <0.05 were considered statistically significant. Plot generation was performed using GraphPad Prism. Statistical analysis to evaluate difference in the calcium concentration between deleted and wild-type clones was based on the non-parametric Wilcoxon test using SPSS, and on a meta-analysis approach using Open Meta-Analyst software (Wallace, Dahabreh et al. 2012).

Supplementary information

Additional file 1. Fig. S1. Epigenomic marks in erythroblast. **Table S1.** Number of ChIP-seq peaks and IW-scoring rank for each SNP. **Table S2.** Results of the Hardy-Weinberg Equilibrium for *ATP2B4* variants. **Table S3.** Association of *ATP2B4* SNPs with Severe Malaria in the Senegalese population.

Table S4. Primers sequences for Site-directed mutagenesis of candidate variants for luciferase assay. **Table S5.** Guide RNA and primers sequences for *ATP2B4* knockout via CRISPR-Cas9

genome editing and screening. **Table S6.** Primer sequences (F3/R3) specific for quantification of long transcripts and (F4/R4) for the quantification of all transcripts of *ATP2B4*.

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Ethics approval and consent to participate

The Comité d’Ethique de la Recherche de l’Université Cheikh Anta Diop de Dakar has approved the protocols. Each participant or their parents or legal guardians for any minors prior to inclusion has received written or verbal information in their native language, and has given a written consent.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Contributions

SM and PR designed and supervised the project. SN genotyped most of the genetic variants, whereas MT, AT and FG participated in genotyping. AT extracted the DNA samples in collaboration with RND, and performed whole genome amplification. OK, AT and BM were involved in the recruitment, the follow-up of Senegalese individuals and the collection and interpretation of biological data including parasite density, hematology, and other characteristics from different areas (Dakar and Tambacounda), under the supervision of AD. MT provided guidance for CRISPR/Cas9 technology and gene reporter experiments. SN performed qPCR, CRISPR/Cas9 and gene reporter experiments. LB has participated in gene reporter experiments. BP performed cytometry experiments for intracellular calcium levels and membrane protein levels. SN, FR, and PR performed bioinformatic analyses. SN, PR, and SM performed statistical analyses. SS provided useful comments on biological interpretation, whereas SN, SM and PR interpreted the results and wrote the paper. SM and PR supervised the whole research and

provided guidance. All authors have received the manuscript and approved the final version before submitting the article.

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Figure legends

Figure 1: Plot showing the integrated results for the prioritization of best candidate SNPs based on IW scoring analysis on X-axis and no. of ChIP-seq peaks on Y-axis.

Figure 2: Visual representation of the position of 5 *ATP2B4* candidate variants (rs11240734, rs1541252, rs1541253, rs1541254 and rs1541255; encircled red) on chromosome 1, located within a DNaseI hypersensitivity region and peaks of H3K4me3, H3K4me1 and H3K27ac histone marks. The 2 tagSNPs (rs10900585 and rs4951377; encircled green) are located neither in peaks of ChIP-seq in the ReMap catalogue nor in other epigenomic marks. The three additional regulatory variants (rs10751450, rs10751451 and rs10751452; encircled blue) previously identified as functional SNPs are shown.

Figure 3: Forest plot showing meta-analysis for association between rs10900585 and SM.

Figure 4: Linkage disequilibrium (LD) (r^2) plot for the *ATP2B4* variants in Senegalese population. a) LD between the candidate 5 SNPs and the tagSNP rs10900585 b) LD between the 8 candidate SNPs and the tagSNP rs10900585 displaying r^2 from 0.72-1.

Figure 5: Luciferase reporter assay accessing the promoter and enhancer activity of *ATP2B4* variants in K562 cells.

a) Promoter activity of the DNA region containing *ATP2B4* Major and Minor haplotypes as well as the individual polymorphism. b) Enhancer activity of *ATP2B4* SNPs with Major and Minor haplotypes. SV40 Promoter and Basic vector were used as positive and negative controls respectively. Data presented as the mean \pm standard deviation of three independent experiments.

**** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Figure 6: CRISPR-Cas9 mediated genome editing.

a) Principle strategy for the generation of DNA knockouts. gRNAs (gRNA1, gRNA2 and gRNA3) were designed flanking the genomic target to delete the two targeted DNA segments comprising either the 5 (506bp) or the 8 (1262bp) candidate SNPs, creating double-strand breaks (DSBs) at 3 bp upstream of the PAM. The resulting DSB is repaired by the NHEJ pathway. The genomic deletion is detected by PCR using primers (F1, R1) and (F2, R2) providing the amplicons of 335bp and 355bp, respectively. b) qPCR analysis of gene expression in wild type K562 cells and the deleted *ATP2B4* clones (for the 5 candidate SNPs) to quantify either all the transcripts or only the two long transcripts of *ATP2B4*. c) qPCR analysis of gene expression in wild type K562 cells and the deleted *ATP2B4* clones (for the 8 SNPs) to quantify either all the transcripts or only the two long transcripts of *ATP2B4*. Error bars show s.d. (n=3 independent RNA/cDNA preparations): ****P<0.0001.

Figure 7: Visualization of calcium concentration of K562 WT (pink) and deleted cells (blue). An increased intracellular calcium concentration displayed in clone Δ ATP2B4_2 a) and, for clone Δ ATP2B4_3 b), respectively.

Figure 8: PMCA4 protein expression using flow cytometry analysis. Representative FACs plot showing isotype (blue) and JA9 (pink) peaks visualized by a APC conjugated secondary antibody in K562 WT cells a), Δ ATP2B4_2 deleted clone b) and Δ ATP2B4_3 deleted clone c). These were visualized using FlowJo software.

Figure S1. Epigenomic marks in erythroblast. Visual representation of the position of 5 *ATP2B4* candidate variants (rs11240734, rs1541252, rs1541253, rs1541254 and rs1541255; encircled red) and 3 previously identified regulatory variants (rs10751450, rs10751451 and rs10751452; encircled blue) on chromosome 1, located within a DNaseI hypersensitivity region and peaks of H3K4me3, H3K4me1 and H3K27ac histone marks in erythroblast.

Table 1: Numbers of ChIP seq peaks and IW scoring rank for the promising candidates.

SNP rsID	Genomic coordinate (hg38)	IW-scoring rank	Number of ChIP-seq peaks	eQTL hits
rs11240734	203682695	1	54	3 hits
rs1541252	203682798	2	90	3 hits
rs10751450	203681816	3	46	
rs2228445	203698280	4	18	3 hits
rs202111522	203682602	5	43	
rs1541254	203683011	6	104	
rs1541255	203683012	7	103	
rs10594838	203685540	8	2	
rs10625220	203686622	9	15	
rs1541253	203682911	10	149	
rs8176719	133257721	11	7	14 hits
rs10751451	203681849	12	49	3 hits
rs10751452	203681901	13	50	

Table 2: Association of *ATP2B4* SNPs with Severe Malaria in the Senegalese population-based study including 117 severe malaria cases and 79 healthy controls. P-values and OR were calculated according to a logistic regression test without any covariate.

SNP	Position ^a	Minor Allele	MAF ^b	Risk Genotype	Controls %	Severe Malaria %	OR	95% CI	P
rs10900585 (T>G)	203684896	G	0.41	TT	32.9	48.7	1.94	1.07-3.50	0.029
rs11240734 (T>C)	203682696	C	0.40	TT	31.6	53.8	2.52	1.39-4.58	0.002
rs1541252 (C>T)	203682799	T	0.40	CC	31.6	53.8	2.52	1.39-4.58	0.002
rs1541253 (C>T)	203682912	T	0.40	CC	31.6	53.8	2.52	1.39-4.58	0.002
rs1541254 (G>C)	203683012	C	0.43	GG	31.6	53.8	2.52	1.39-4.58	0.002
rs1541255 (A>G)	203683013	G	0.40	AA	31.6	53.8	2.52	1.39-4.58	0.002
rs10751450 (C>T)	203681817	T	0.41	CC	32.9	50.4	2.07	1.15-3.75	0.016
rs10751451 (C>T)	203681850	T	0.40	CC	34.2	56.4	2.49	1.38-4.50	0.002
rs10751452 (T>C)	203681902	C	0.39	TT	35.4	55.6	2.27	1.26-4.09	0.006

^a Data represent the position on chromosome 1 according to human hg38 coordinates.

^b MAF (Minor allele frequency) was estimated from Senegalese healthy controls.

OR, odds ratio; CI, confidence interval.

Table 3: Number of cases and control individuals for each study population used for meta-analysis

Reference	PMID	Year	Population	No. of cases	No. of controls	Genotype (cases)			Genotype (control)			HW P value
						TT	TG	GG	TT	TG	GG	
Clarke <i>et al.</i>	25261933	2014	Mali	427	322	228	164	35	156	141	25	0.99
Clarke <i>et al.</i>	25261933	2014	Burkina Faso	843	720	388	377	78	365	292	63	0.99
Clarke <i>et al.</i>	25261933	2014	Ghana(Navrongo)	614	165	334	246	34	86	74	5	0.27
Clarke <i>et al.</i>	25261933	2014	Ghana(Kumasi)	761	1231	294	365	102	405	586	240	0.99
Clarke <i>et al.</i>	25261933	2014	Nigeria	76	34	25	39	12	15	15	4	0.99
Clarke <i>et al.</i>	25261933	2014	Kenya	1624	3762	688	786	150	1644	1689	429	0.99
Clarke <i>et al.</i>	25261933	2014	Tanzania	425	451	178	199	48	182	211	58	0.99
Clarke <i>et al.</i>	25261933	2014	Malawi	1374	2562	654	595	125	1159	1105	298	0.99
Clarke <i>et al.</i>	25261933	2014	Vietnam	779	2456	746	33	0	2367	89	0	0.99
Ndila <i>et al.</i>	30033078	2018	Kenya	2200	3762	989	973	238	1639	1688	435	0.99
Timmann <i>et al.</i>	22895189	2012	Ghana (GWA)	1325	828	510	624	191	269	406	153	0.99
Timmann <i>et al.</i>	22895189	2012	Ghana (replica)	1320	2222	507	622	191	697	1095	430	0.99
Timmann <i>et al.</i>	22895189	2012	Gambia	909	1304	408	402	99	517	608	179	0.99
Nisar <i>et al.</i>		2020	Senegal	117	79	57	44	16	26	41	12	0.99

Table 4: Haplotype association analysis (two most frequent haplotypes) for the 5 and 8 SNPs in Senegalese population including severe malaria cases and healthy controls. P-values and OR were calculated according to a logistic regression.

Major haplotype	Minor haplotype	Risk haplotype	P value	SMvsCtrl	
				OR	95% CI
TCCGA	CTTCG	TCCGA/TCCGA	0.002 ^a	2.52 ^a	1.39-4.58 ^a
			0.006 ^b	2.38 ^b	1.29-4.40 ^b
CCTTCCGA	TTCCTTCG	CCTTCCGA/CCTTCCGA	0.003 ^a	2.67 ^a	1.41-5.04 ^a
			0.005 ^b	2.53 ^b	1.32-4.86 ^b

^a The statistical model comparing individuals homozygous for major allele haplotype with individual heterozygous or homozygous for minor haplotype without any covariate.

^b The statistical model by taking into account age as a covariate.

OR, odds ratio; CI, confidence interval.

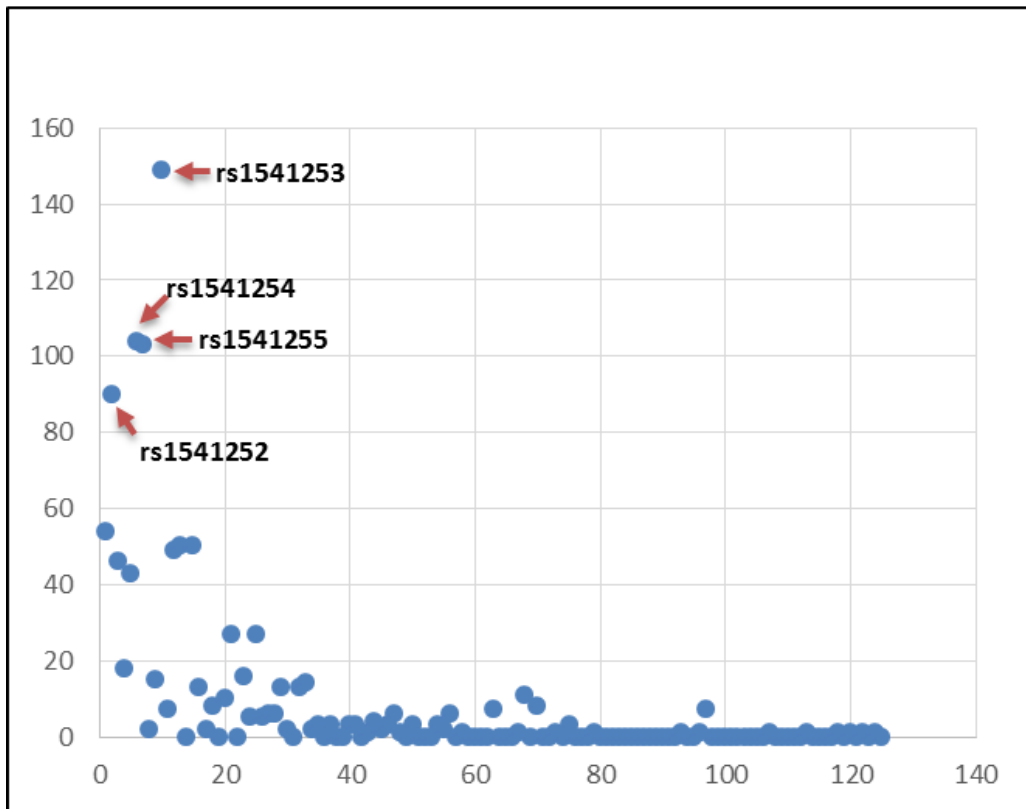


Figure 1

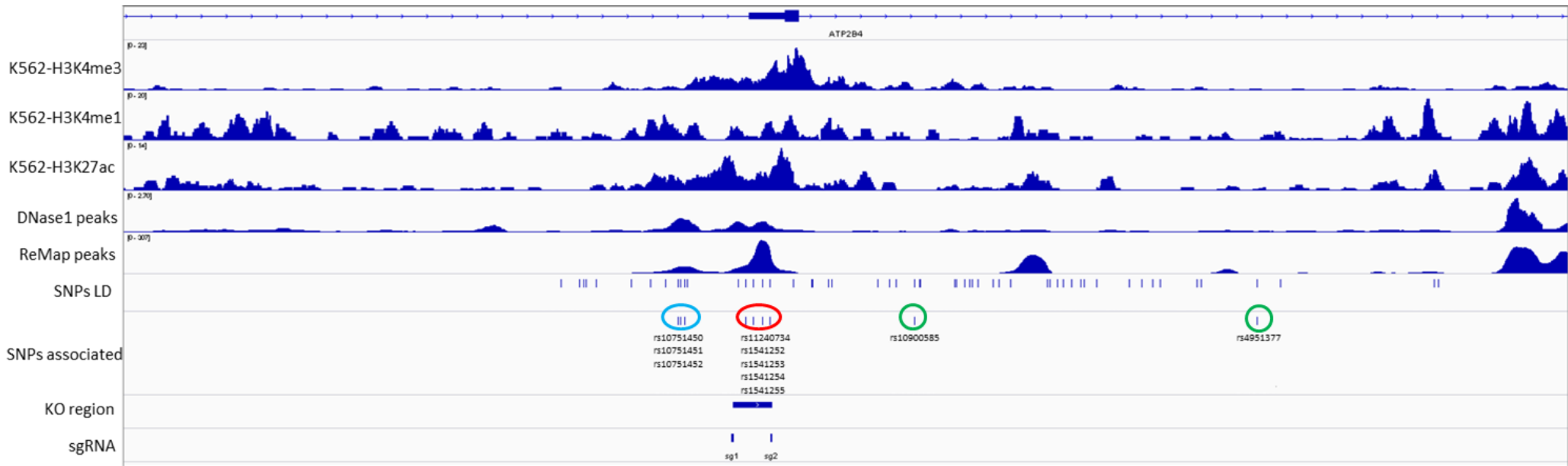


Figure 2:

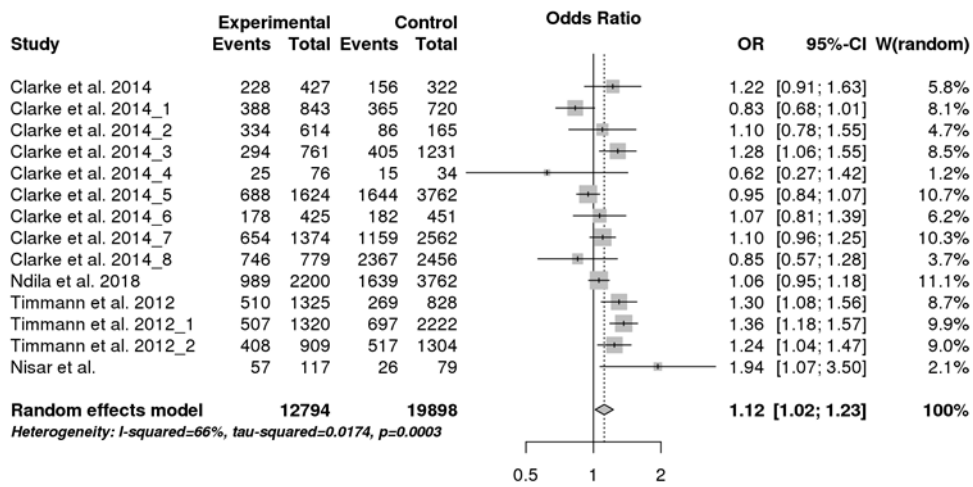


Figure 3

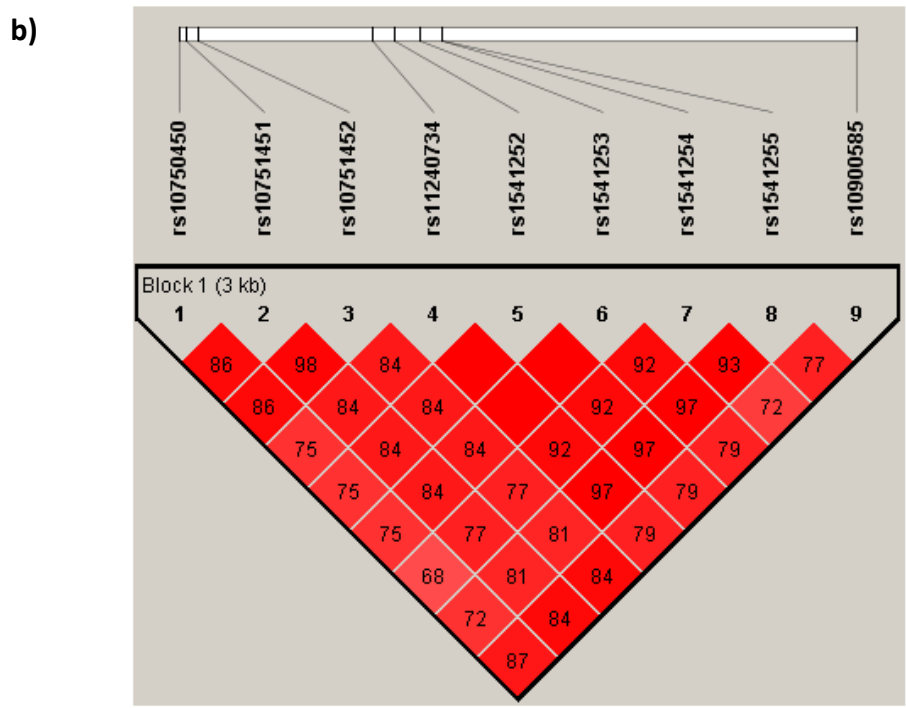
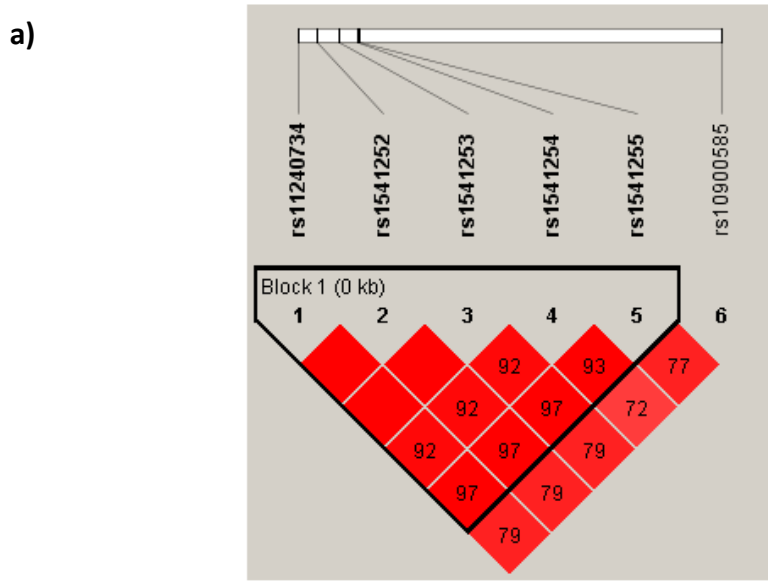


Figure 4

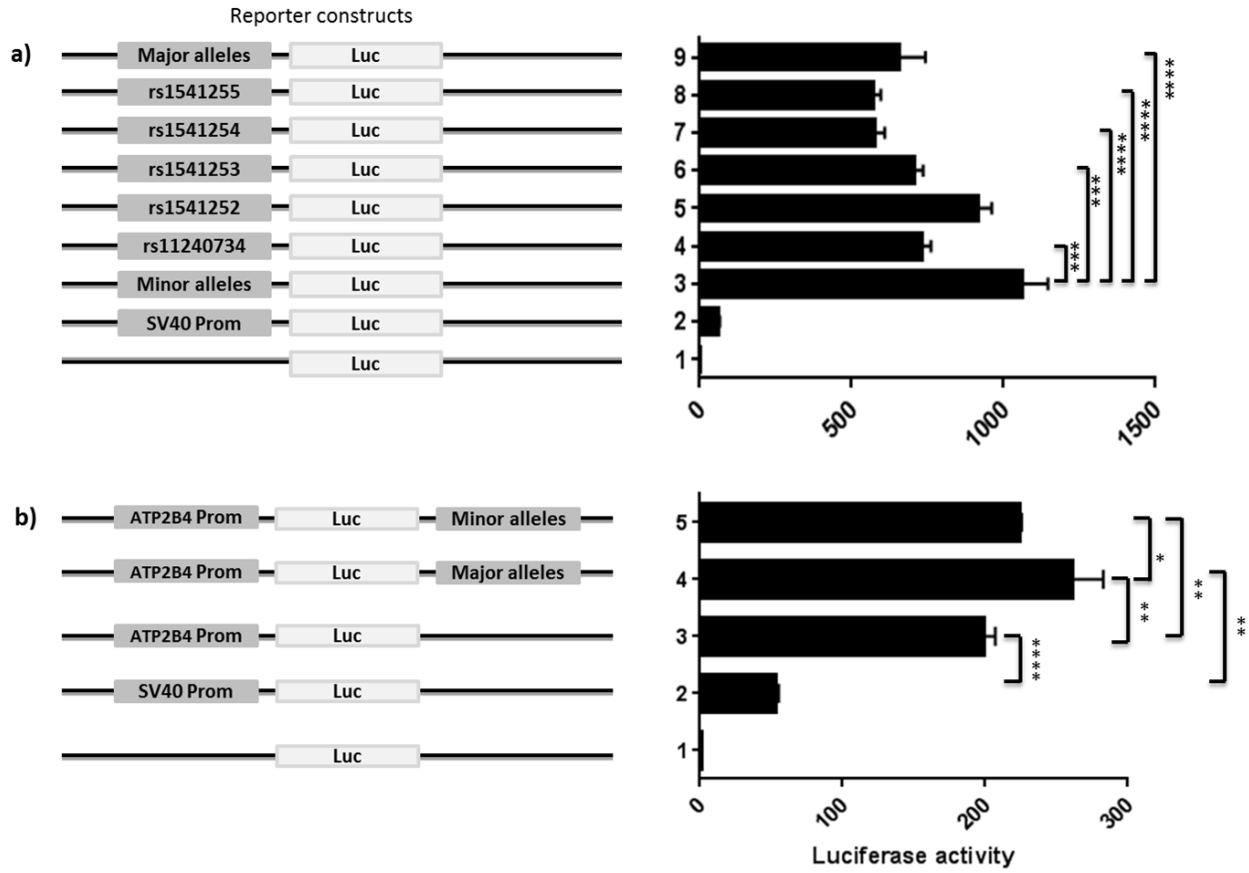


Figure 5

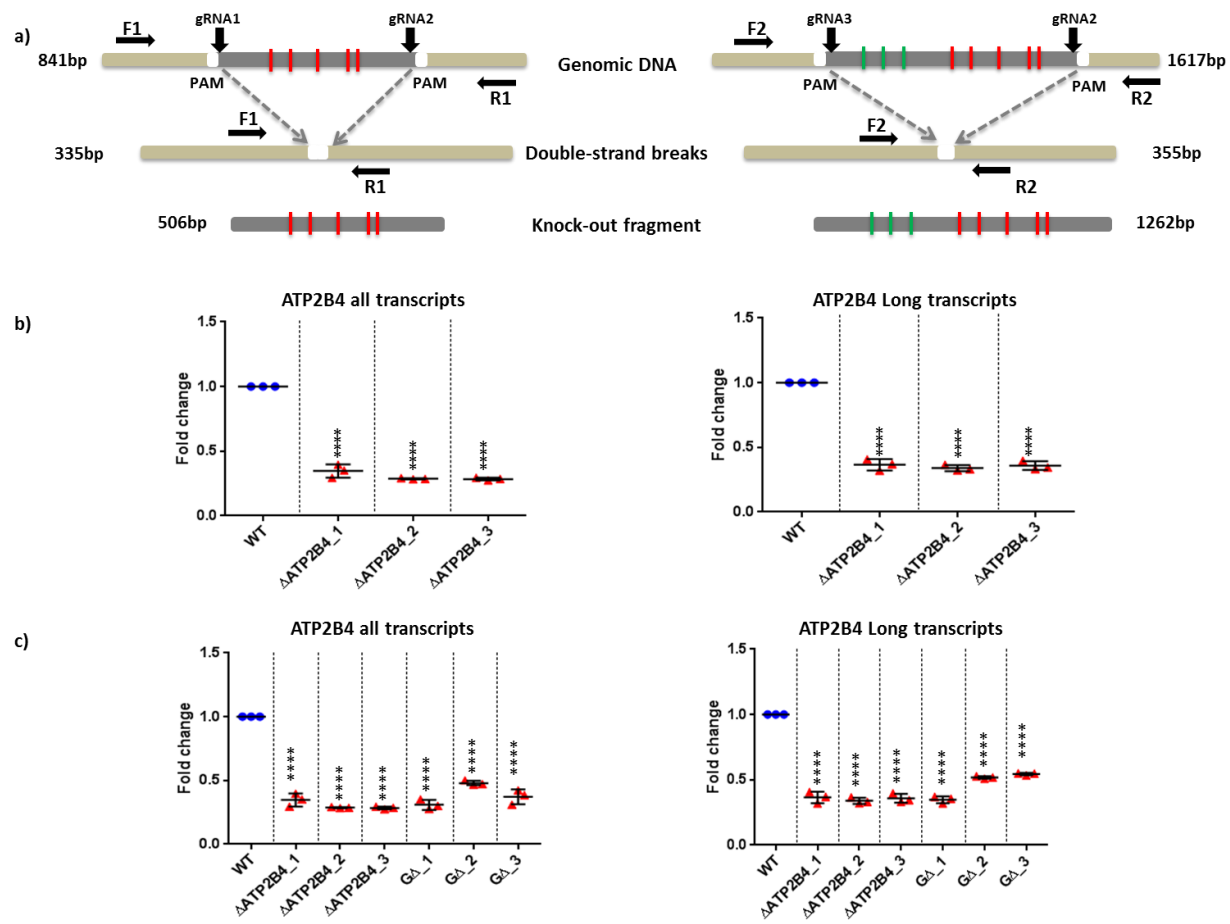


Figure 6

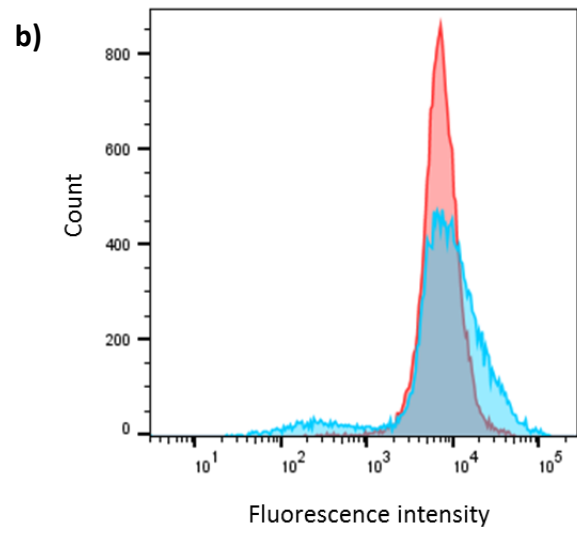
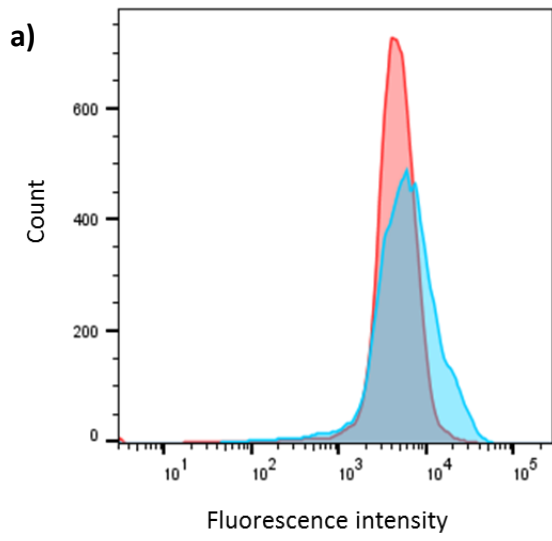


Figure 7

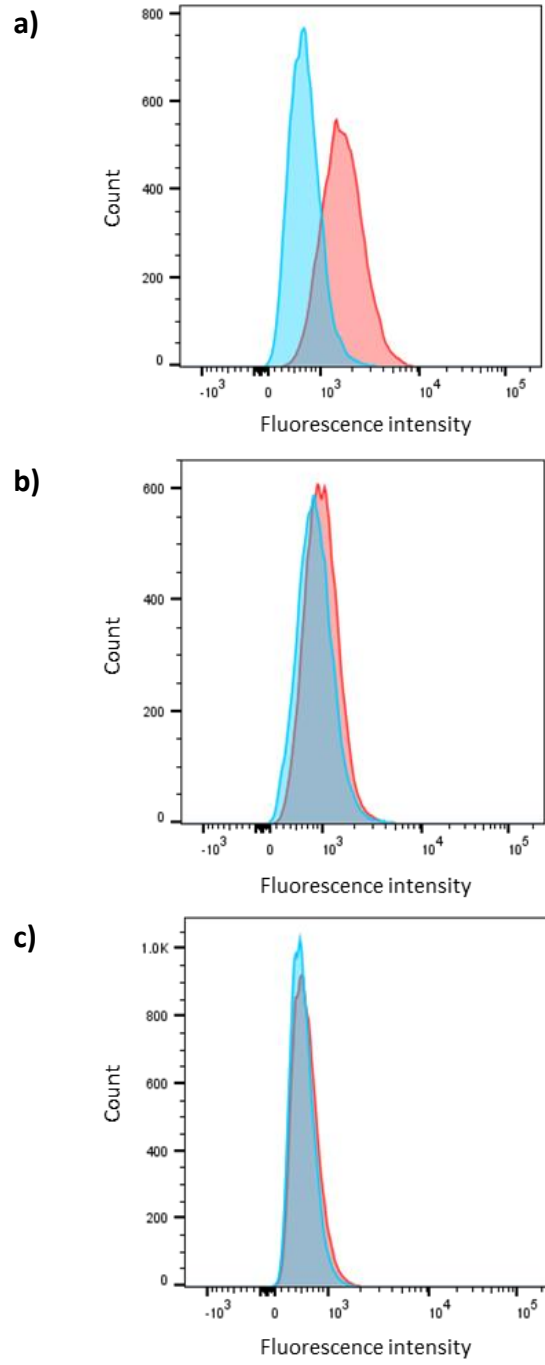
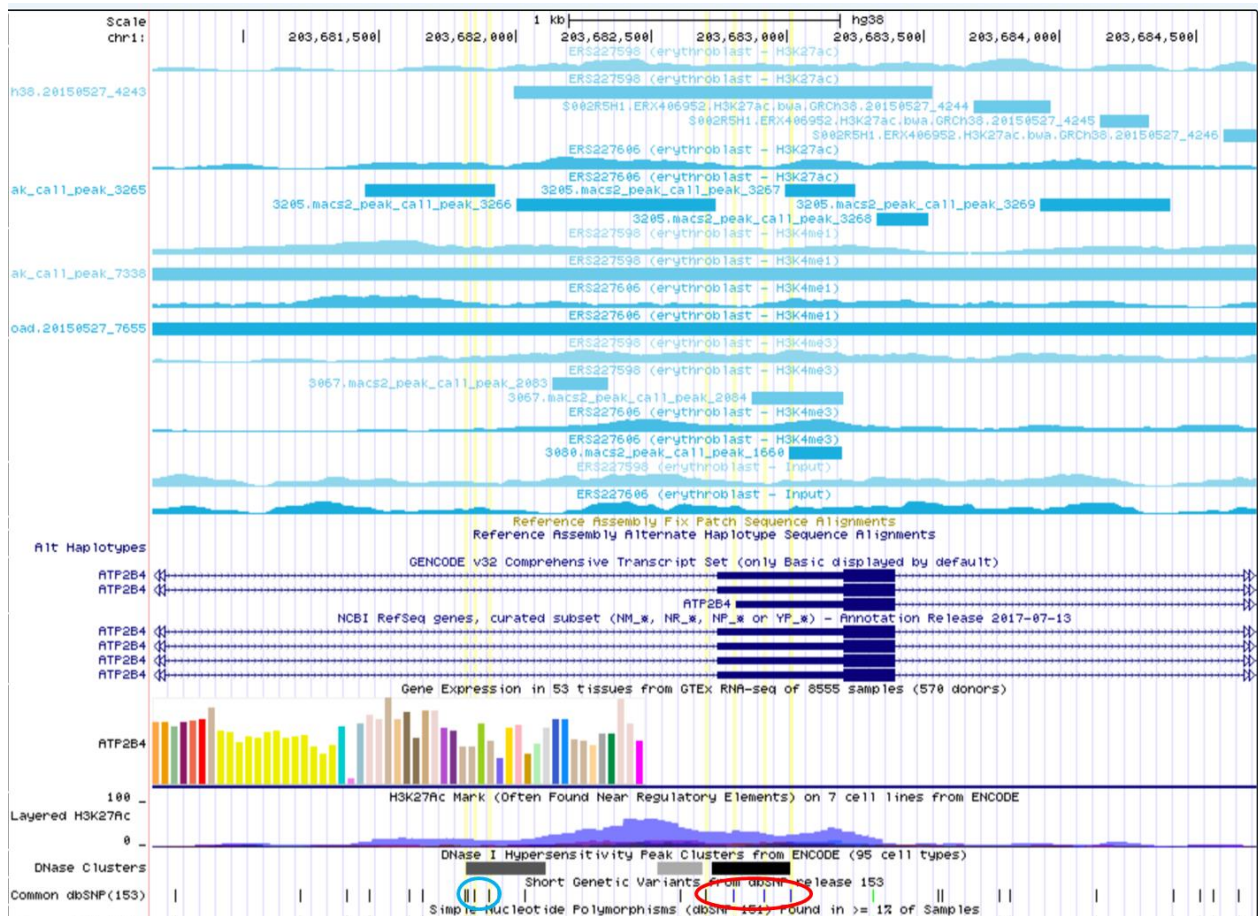


Figure 8



Supplementary figure 1

Supplementary Table 1: Number of ChIP-seq peaks and IW-scoring rank for each SNP

rsID	Genomic coordinates	IW-score	IW scoring Rank	Number of ChIP-seq peaks	eQTL hits
rs11240734	chr1: 203682695	3,4634	1	54	3 hits
rs1541252	chr1: 203682798	3,1663	2	90	3 hits
rs10751450	chr1: 203681816	3,1011	3	46	
rs2228445	chr1: 203698280	2,7194	4	18	3 hits
rs202111522	chr1: 203682602	2,4339	5	43	
rs1541254	chr1: 203683011	2,5458	6	104	
rs1541255	chr1: 203683012	1,8505	7	103	
rs10594838	chr1: 203685540	1,7405	8	2	
rs10625220	chr1: 203686622	-0,041	9	15	
rs1541253	chr1: 203682911	1,1953	10	149	
rs8176719	chr9: 133257721	2,482	11	7	14 hits
rs10751451	chr1: 203681849	1,0658	12	49	3 hits
rs10751452	chr1: 203681901	0,7364	13	50	
rs1999063	chr6: 92558036	-0,1021	14	0	
rs200499024	chr1: 203681934	0,0246	15	50	
rs11240733	chr1: 203681463	-0,2697	16	13	
rs6594007	chr1: 203685643	-0,3419	17	2	3 hits
rs184527160	chr4: 143782986	-0,3847	18	8	
rs4951369	chr1: 203683554	-0,4386	19	0	
rs192832154	chr4: 143712081	-0,3659	20	10	
rs10736845	chr1: 203681657	-0,4748	21	27	3 hits
rs4951070	chr1: 203683569	-0,5606	22	0	3 hits
rs1419114	chr1: 203683315	-1,218	23	16	3 hits
rs115426616	chr4: 143829454	-0,7402	24	5	
rs10751449	chr1: 203681655	-0,852	25	27	
rs7551442	chr1: 203685992	-1,098	26	5	3 hits
rs11240731	chr1: 203681207	-0,9579	27	6	
rs145677206	chr4: 143714066	-1,1407	28	6	
rs7551560	chr1: 203686141	-1,1388	29	13	3 hits
rs184063934	chr4: 143712635	-1,2999	30	2	

rs76471583	chr4: 143682248	-1,3356	31	0	
rs4951381	chr1: 203691709	-1,462	32	13	3 hits
rs4951074	chr1: 203691652	-1,39	33	14	3 hits
rs7539122	chr1: 203685914	-1,4746	34	2	3 hits
rs183974736	chr4: 143820702	-1,5281	35	3	
rs141787228	chr6: 92561006	-1,5312	36	0	
rs4951081	chr1: 203702748	-1,7027	37	3	2 hits
rs146104413	chr4: 143727260	-1,6695	38	0	
rs7546390	chr1: 203680294	-1,8079	39	0	
rs6692632	chr1: 203685417	-1,7525	40	3	
rs143128154	chr1: 203687259	-1,2236	41	3	
rs181423417	chr4: 143757217	-1,9533	42	0	
rs201385421	chr1: 203680619	-2,1083	43	1	
rs186873296	chr4: 143781320	-2,1521	44	4	
rs6594006	chr1: 203685609	-2,1009	45	2	3 hits
rs6658130	chr1: 203685439	-2,2154	46	3	3 hits
rs182386242	chr4: 143781807	-2,2226	47	6	
rs10900588	chr1: 203687685	-2,1508	48	1	3 hits
rs142731296	chr4: 143721082	-2,3307	49	0	
rs148995556	chr4: 143834360	-2,3027	50	3	
rs10900585	chr1: 203684895	-2,392	51	0	
rs184820425	chr4: 143703359	-2,3098	52	0	
rs189887464	chr4: 143814352	-2,343	53	0	
rs189149534	chr4: 143735452	-2,368	54	3	
rs190618313	chr4: 143683055	-2,3782	55	2	
rs2365859	chr1: 203688760	-2,3568	56	6	
rs149914432	chr4: 143745524	-2,4268	57	0	
rs6697384	chr1: 203708436	-2,4504	58	1	
rs12035565	chr1: 203680749	-2,3356	59	0	
rs4951377	chr1: 203689342	-2,4195	60	0	3 hits
rs184497846	chr4: 143826117	-2,4907	61	0	
rs4951378	chr1: 203689653	-2,5181	62	0	3 hits
rs4951375	chr1: 203686656	-2,689	63	7	3 hits

rs4707740	chr6: 92475691	-2,5897	64	0	
rs151041851	chr4: 143709357	-2,6522	65	0	
rs188245886	chr4: 143685416	-2,6877	66	0	
rs10900589	chr1: 203687845	-2,7977	67	1	3 hits
rs2183001	chr6: 92559278	-2,755	68	11	
rs183414953	chr4: 143687477	-2,7972	69	0	
rs147037418	chr4: 143783020	-2,8153	70	8	
rs4707739	chr6: 92475162	-2,8479	71	0	
rs4951370	chr1: 203683776	-2,8554	72	0	
rs184627523	chr4: 143813545	-2,9012	73	1	
rs151007681	chr4: 143693392	-2,9308	74	0	
rs2365860	chr1: 203687302	-2,9416	75	3	3 hits
rs6594008	chr1: 203685722	-2,8381	76	0	3 hits
rs6594011	chr1: 203686826	-3,0051	77	0	
rs142537619	chr4: 143740251	-3,0594	78	0	
rs7546599	chr1: 203680539	-3,2011	79	1	
rs186896080	chr4: 143707920	-3,0982	80	0	
rs4951371	chr1: 203683817	-3,1091	81	0	
rs144794297	chr4: 143681764	-3,107	82	0	
rs10793762	chr1: 203684969	-3,0229	83	0	
rs7547793	chr1: 203684415	-3,1917	84	0	
rs4347266	chr1: 203687049	-3,0943	85	0	
rs187764080	chr4: 143694376	-3,1214	86	0	
rs186094021	chr4: 143784432	-3,1403	87	0	
rs148311540	chr4: 143831995	-3,1777	88	0	
rs146553388	chr4: 143678651	-3,2106	89	0	
rs184114500	chr4: 143716592	-3,2087	90	0	
rs12122293	chr1: 203687988	-3,2423	91	0	
rs192804806	chr4: 143814874	-3,271	92	0	
rs187377484	chr4: 143840985	-3,2947	93	1	
rs6696548	chr1: 203686938	-3,3281	94	0	
rs10900586	chr1: 203684956	-3,3152	95	0	
rs182977569	chr4: 143707465	-3,3377	96	1	

rs2365858	chr1: 203688821	-3,3986	97	7	3 hits
rs3851298	chr1: 203695918	-3,3045	98	0	
rs147565832	chr4: 143764457	-3,3684	99	0	
rs116706045	chr4: 143828597	-3,349	100	0	
rs7555703	chr1: 203684561	-3,325	101	0	
rs6594010	chr1: 203686753	-3,3861	102	0	
rs145945511	chr4: 143785346	-3,3956	103	0	
rs143493147	chr4: 143823879	-3,3901	104	0	
rs141987247	chr4: 143687265	-3,4094	105	0	
rs138428794	chr4: 143732433	-3,4284	106	0	
rs7514742	chr1: 203680587	-3,5388	107	1	
rs149060195	chr4: 143732407	-3,5054	108	0	
rs9345242	chr6: 92517194	-3,519	109	0	
rs6692627	chr1: 203688091	-3,5304	110	0	
rs72926662	chr6: 92598571	-3,5808	111	0	
rs184908374	chr4: 143744599	-3,5872	112	0	
rs186790584	chr4: 143758986	-3,6109	113	1	
rs183166203	chr4: 143734504	-3,6343	114	0	
rs62418762	chr6: 92508979	-3,6525	115	0	
rs189723550	chr4: 143764152	-3,6609	116	0	
rs192563008	chr4: 143824275	-3,6974	117	0	
rs10944597	chr6: 92494331	-3,6797	118	1	
rs77397300	chr4: 143752045	-3,7663	119	0	
rs185357240	chr4: 143779806	-3,7691	120	1	
rs187621111	chr4: 143687488	-3,8762	121	0	
rs9353916	chr6: 92480915	-3,8775	122	1	
rs7535676	chr1: 203684657	-3,9418	123	0	
rs184895969	chr4: 143777374	-3,9361	124	1	
rs147881070	chr4: 143747987	-4,0485	125	0	

Supplementary table 2: Results of the Hardy-Weinberg Equilibrium for *ATP2B4* variants.

SNP	H-W (P-value)
rs10900585	0.52
rs11240734	0.27
rs1541252	0.27
rs1541253	0.27
rs1541254	0.77
rs1541255	0.27
rs10751450	0.76
rs10751451	0.53
rs10751152	0.76

Supplementary table 3: Association of ATP2B4 SNPs with Severe Malaria in the Senegalese population. P-values and OR was calculated according to logistic regression test taking age as covariate.

SNP	Position ^a	Minor Allele	MAF ^b	Risk Genotype	Controls %	Severe Malaria %	OR	95% CI	P
rs10900585 (T>G)	203684896	G	0.41	TT	32.9	48.7	1.82	0.99-3.34	0.055
rs11240734 (T>C)	203682696	C	0.40	TT	31.6	53.8	2.38	1.29-4.40	0.006
rs1541252 (C>T)	203682799	T	0.40	CC	31.6	53.8	2.38	1.29-4.40	0.006
rs1541253 (C>T)	203682912	T	0.40	CC	31.6	53.8	2.38	1.29-4.40	0.006
rs1541254 (G>C)	203683012	C	0.43	GG	31.6	53.8	2.38	1.29-4.40	0.006
rs1541255 (A>G)	203683013	G	0.40	AA	31.6	53.8	2.38	1.29-4.40	0.006
rs10751450 (C>T)	203681817	T	0.41	CC	32.9	50.4	1.98	1.06-3.67	0.030
rs10751451 (C>T)	203681850	T	0.40	CC	34.2	56.4	2.23	1.21-4.12	0.011
rs10751452 (T>C)	203681902	C	0.39	TT	35.4	55.6	2.05	1.11-3.78	0.021

^a Data represent the position on chromosome 1 according to human hg38 coordinates

^b MAF (Minor allele frequency) was estimated from Senegalese healthy controls.

OR, odds ratio; CI, confidence interval.

Supplementary table 4: Primers sequences for Site-directed mutagenesis of candidate variants for luciferase assay.

SNP	Forward Primer	Reverse Primer
rs11240734 (T/C)	ATACTTATGGTTGCTCTAATGGTTTCTTTTG	CCAGCATCTGCAAGGGCA
rs1541252 (C/T)	TCAGGCCTAGCTATCAGTTCAG	CGAGTAGCCGTCCGAAGTC
rs1541253 (C/T)	CCTGGACAACCACTATCATCAC	GTAGGTAGGTTCTAGCTTAG
rs1541254 (G/C)	AGACTTCATAGGGAAGAAAGGATCTAGAC	GGCAGCGAGAGGAGGAAG
rs1541255 (A/G)	GACTTCATACAGAAGAAAGGATCTAGACTTCG	TGGCAGCGAGAGGAGGAA

Supplementary table 5: Guide RNA and primers sequences for ATP2B4 knockout via CRISPR-Cas9 genome editing and screening.

Guide RNA	Sequence 5'-3'	Coordinates (hg19)
gRNA1	TCCTCTACATTGGAGTTTAC	chr1:203651648-203651667
gRNA2	TAGACTTCGGACGGCTACTC	chr1:203652154-203652173
gRNA3	AGACTCTGGTGCTCACTCAC	chr1:203650890-203650909
F1	GGCCACCCTTCAGATCACTT	chr1:203651422-203651441
R1	GCCTCCCTGTCTCAACTTCT	chr1:203652243-203652262
F2	CTGAAACTGAGAAGGGGCCT	chr1:203650729-203650748
R2	TGGGTTCGTCATTTGCCTG	chr1:203652326-203652345

Supplementary table 6: Primer sequences (F3/R3) specific for quantification of long transcripts and (F4/R4) for the quantification of all transcripts of ATP2B4.

Guide RNA	Sequence 5'-3'	Coordinates (hg19)
ATP2B4_del_F3	CAAGAGTCTGGCCCGAGTTA	chr1:203595946-203595965
ATP2B4_del_R3	TCTGCTGTTGAGATCGTCCA	chr1:203596006-203596025
ATP2B4_del_F4	TTGGAGAACTTCTGTGGGGC	chr1:203693094-203693113
ATP2B4_del_R4	AGGAGATCACCAAGGATGCC	chr1:203696590-203696609

Complementary result 1: Association between *ATP2B4* tagSNP and 5 candidate variants with mild malaria in Senegal

During this thesis, we also accessed the association of tagSNP (rs10900585) and the *ATP2B4* variants (rs11240734, rs1541252, rs1541253, rs1541254 and rs1541255) with mild malaria in Senegalese cohort. From 2012 to 2015, samples were taken at Principal Hospital of Dakar and regional hospital of Tambacounda in Senegal comprised of 75 mild malaria (MM) patients and 79 healthy controls.

The genotyping was performed using Sanger sequencing. The fragment of the *ATP2B4* gene containing the polymorphisms was amplified by PCR with 5 μ L of whole genomic DNA, 25 μ L of Master Mix GoTaq G2 Hot Start (Promega Corporation, Madison, WI USA) according to the manufacturer's instructions and 3 μ L of mixture of primers sense F: 5' TCAGGCCTAGCTATCAGTTCAG 3' and antisense R: 5' CGAGTAGCCGTC CGAAGTC 3' of 10 μ M each. The amplification was carried out in Mastercycler (Eppendorf, Hamburg, Germany) following 35 cycles with a T_m of 60 ° C. After amplification, the PCR product was checked on 1.5% agarose gel. The amplicon was then further purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Sequencing was performed by GATC (Constance, Germany) from 5 μ L of PCR product (20-80 ng / μ L) and 5 μ L of F primer at 5 μ M. The chromatograms were read with Chromas 2.6.5 software (South Brisbane, Australia).

For all the SNPs including tagSNP (rs10900585) and 5 potential candidate variants (rs11240734, rs1541252, rs1541253, rs1541254 and rs1541255), the observed genotype frequencies in the control group conformed to the Hardy – Weinberg equilibrium ($P > 0.5$ for all SNPs). In order to access the association of the variants with MM, logistic regression analysis was performed. We compared the subjects carrying the homozygous genotype for major alleles with those carrying either the heterozygous genotype or the homozygous genotype for minor allele. A significant association was detected when comparing control group with MM ($P < 0.0001$) which revealed that the major allele of all the SNPs was found to be associated with an increased risk of developing MM without the effect of any covariates (Table 5). The percentage of individuals having MM phenotype, who are carrying the major alleles (rs10900585-TT, rs11240734-TT,

rs1541252-CC, rs1541253-CC, rs1541254-GG and rs1541255-AA), was found to be 2 times higher than control group for all the SNPs (62.7% compared to 32.9% in control group for rs10900585, 62.7% compared to 31.6% in control group for rs11240734, rs1541252, rs1541253 and 64.0% compared to 31.6% for rs1541254, rs1541255, respectively) as presented in the contingency tables (Tables 6-11), indicating the number and percentage of each genotype in mild malaria and control groups.

Table 8. Association of *ATP2B4* variants with mild malaria in Senegalese population including 75 MM cases and 79 healthy controls. P-value and OR was calculated according to logistic regression test without any covariate.

SNP	Minor Allele	Risk Genotype	Controls %	Mild Malaria %	OR	95% CI	P
rs10900585 (T>G)	G	TT	32.9	62.7	3.42	1.76-6.64	<0.0001
rs11240734 (T>C)	C	TT	31.6	62.7	3.63	1.86-7.06	<0.0001
rs1541252 (C>T)	T	CC	31.6	62.7	3.63	1.86-7.06	<0.0001
rs1541253 (C>T)	T	CC	31.6	62.7	3.63	1.86-7.06	<0.0001
rs1541254 (G>C)	C	GG	31.6	64.0	3.84	1.97-7.49	<0.0001
rs1541255 (A>G)	G	AA	31.6	64.0	3.84	1.97-7.49	<0.0001
rs10751450 (C>T)	T	CC	32.9	64.0	3.62	1.86-7.05	<0.0001
rs10751451 (C>T)	T	CC	34.2	64.0	3.42	1.76-6.64	<0.0001
rs10751452 (T>C)	C	TT	35.4	64.0	3.23	1.67-6.26	<0.0001

Table 9. Contingency table for rs10900585

Genotype/Phenotype	TT	TG	GG	Total
Ctrl	26 (32.9%)	41 (51.9%)	12 (15.2%)	79 (100%)
MM	47 (62.7%)	24 (32.0%)	4 (5.3%)	75 (100%)
Total	73 (47.4%)	65 (42.2%)	16 (10.4%)	154 (100%)

Table 10. Contingency table for rs11240734

Genotype/Phenotype	TT	CT	CC	Total
Ctrl	25 (31.6%)	44 (55.7%)	10 (12.7%)	79 (100%)
MM	47 (62.7%)	23 (30.7%)	5 (6.7%)	75 (100%)
Total	72 (46.8%)	67 (43.5%)	15 (9.7%)	154 (100%)

Table 11. Contingency table for rs1541252

Genotype/Phenotype	CC	CT	TT	Total
Ctrl	25 (31.6%)	44 (55.7%)	10 (12.7%)	79 (100%)
MM	47 (62.7%)	23 (30.7%)	5 (6.7%)	75 (100%)
Total	72 (46.8%)	67 (43.5%)	15 (9.7%)	154 (100%)

Table 12. Contingency table for rs1541253

Genotype/Phenotype	CC	CT	TT	Total
Ctrl	25 (31.6%)	44 (55.7%)	10 (12.7%)	79 (100%)
MM	47 (62.7%)	23 (30.7%)	5 (6.7%)	75 (100%)
Total	72 (46.8%)	67 (43.5%)	15 (9.7%)	154 (100%)

Table 13. Contingency table for rs1541254

Genotype/Phenotype	GG	CG	CC	Total
Ctrl	25 (31.6%)	40 (50.6%)	14 (17.7%)	79 (100%)
MM	48 (64.0%)	21 (28.0%)	6 (8.0%)	75 (100%)
Total	73 (47.4%)	61 (39.6%)	20 (13.0%)	154 (100%)

Table 14. Contingency table for rs1541255

Genotype/Phenotype	AA	AG	GG	Total
Ctrl	25 (31.6%)	44 (55.7%)	10 (12.7%)	79 (100%)
MM	48 (64.0%)	22 (29.3%)	5 (6.7%)	75 (100%)
Total	73 (47.4%)	66 (43.0%)	15 (9.7%)	271 (100%)

Complementary result 2: Association of rs10751450, rs10751451 and rs10751452 with mild malaria in Senegal

As the 3 SNPs that are in strong LD with the tagSNP and 5 candidate SNPs was found to be associated with SM, we also assessed their association with MM in our Senegalese cohort. rs10751450, rs10751451 and rs10751452 were genotyped by Taqman assays (C_31796478_10, C_31796479_10 and C_31796480_10) respectively. Each reaction well was added with 1.84µl of H₂O, 2.1µl of 2X Taqman master mix, 0.06µl of Taqman probes and 1µl of DNA (10 – 15ng/µl). Amplification was performed with polymerase activation at 95°C for 10 minutes, followed by 40 cycles of denaturation (95°C, 15s) and annealing/extension (60°C, 1min). After amplification, allelic identification was performed by the integrated software of the Quantstudio 6 Flex System (Real – time PCR Software v1.3).

From the observed genotypes, contingency tables were constructed (Tables 12-14). The frequencies of observed genotype in control group conformed to the Hardy – Weinberg equilibrium ($P > 0.4$ in 3 SNPs). For the statistical analysis, we compared subjects carrying the homozygous genotype for major alleles with those carrying either the heterozygous genotype or the homozygous genotype for minor allele. The results revealed a significant association of all the SNPs with the disease ($P < 0.0001$), where the major allele homozygotes showed a higher risk of developing MM compared to healthy controls (Table 5).

Table 15. Contingency table for rs10751450

Genotype/Phenotype	CC	CT	TT	Total
Ctrl	26 (32.9%)	41 (51.9%)	12 (15.2%)	79 (100%)
MM	48 (64.0%)	23 (30.7%)	4 (5.3%)	75 (100%)
Total	74 (48.1%)	64 (41.6%)	16 (10.4%)	154 (100%)

Table 16. Contingency table for rs10751451

Genotype/Phenotype	CC	CT	TT	Total
Ctrl	27 (34.2%)	41 (51.9%)	11 (13.9%)	79 (100%)
MM	48 (64.0%)	23 (30.7%)	4 (5.3%)	75 (100%)
Total	75 (48.7%)	64 (41.6%)	15 (9.7%)	154 (100%)

Table 17. Contingency table for rs10751452

Genotype/Phenotype	TT	CT	CC	Total
Ctrl	28 (35.4%)	40 (50.6%)	11 (13.9%)	79 (100%)
MM	48 (64.0%)	23 (30.7%)	4 (5.3%)	75 (100%)
Total	76 (49.4%)	63 (40.9%)	15 (9.7%)	154 (100%)

Together, these results indicated that the risk genotype for the MM is the similar as for the SM in our Senegalese population.

Complementary result 3: Effect of the region on the regulation of nearby genes

Our putative regulatory region with enhancer function may regulate the expression of various genes even when being distant from their targets. Using bioinformatics search, we found a physical interaction specifically in T cell between *ATP2B4* and *LAXI* gene which shows that there could be an impact of our region on the regulation of *LAXI* (Figure 14). *LAXI* is a membrane protein implicated in the stimulation of T lymphocytes and its expression in K562 cell line is almost negligible. However, it was found to be expressed well in Jurkat cell line which is an immortalized line of human T lymphocyte cells. It also participates in the activation of T lymphocytes, has a crucial part in parasite control during infection (Togni, Lindquist et al. 2004).

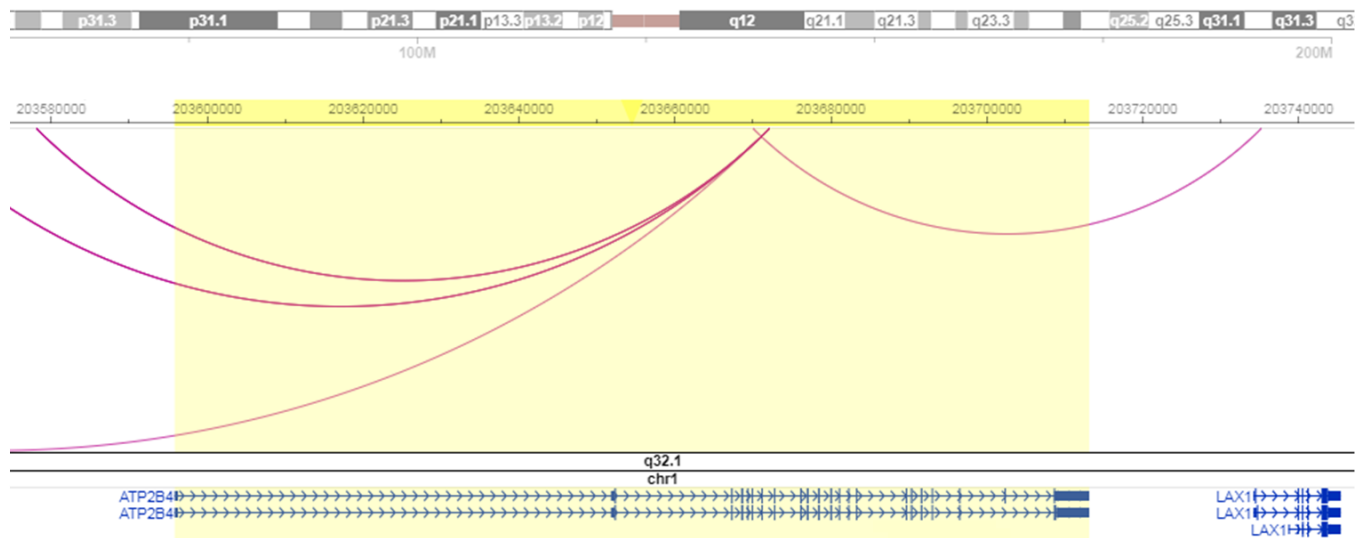


Figure 14. Interaction between *ATP2B4* and *LAXI*.

Visualization of the interaction (highlighted in yellow) between *ATP2B4* and *LAXI* in WashU Epigenome Browser.

As the bioinformatics study revealed the interaction between the two genes, so we further performed the functional studies to check the impact of our regulatory region on the expression of *LAXI* gene by luciferase reporter assay and CRISPR-Cas9 mediated genome editing in Jurkat cell line under stimulation with PMA/ionomycin. The cells were grown in Gibco RPMI 1640 medium (Thermo Fisher Scientific Waltham, MA, USA) supplemented with 10% of FBS.

To perform Luciferase assay, 3 different constructs were used. To check the promoter activity, an 810bp fragment of *LAXI* promoter (chromosome 1: 203764726-203765535 (hg38 assembly)) was

cloned into the MluI-XhoI sites of the pGL3-basic vector (Promega, Madison, WI, USA, cat# E1751), which contained the firefly luciferase coding sequence (GeneCust Custom Services, Luxembourg). To check the impact of our regulatory region on *LAXI*, the 810bp fragment of *LAXI* promoter and the major and minor alleles constructs (601bp) were cloned into pGL3-Promoter vector (Promega) between MluI- Xho I sites and the 601 bp fragment of *ATP2B4* containing respective minor and major alleles of the 5 SNPs was cloned into BamHI-SalI sites (Figure 15). Empty pGL3-Basic vector and pGL3-promoter vectors were used as positive and negative controls (Figure 16).

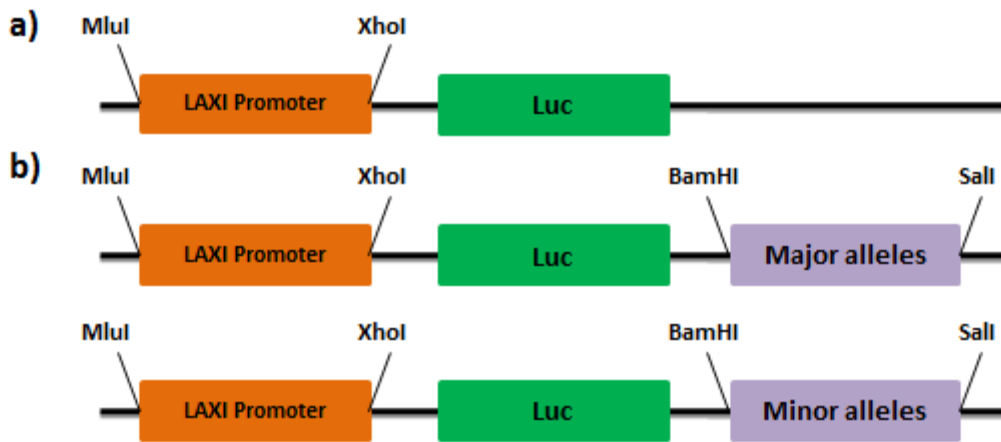


Figure 15. Different constructs used for the Luciferase reporter assay.

a) Construct to test the Promoter activity of *LAXI* (810bp fragment). b) Construct to check the impact of region (601bp) with major and minor allele on *LAXI* promoter.

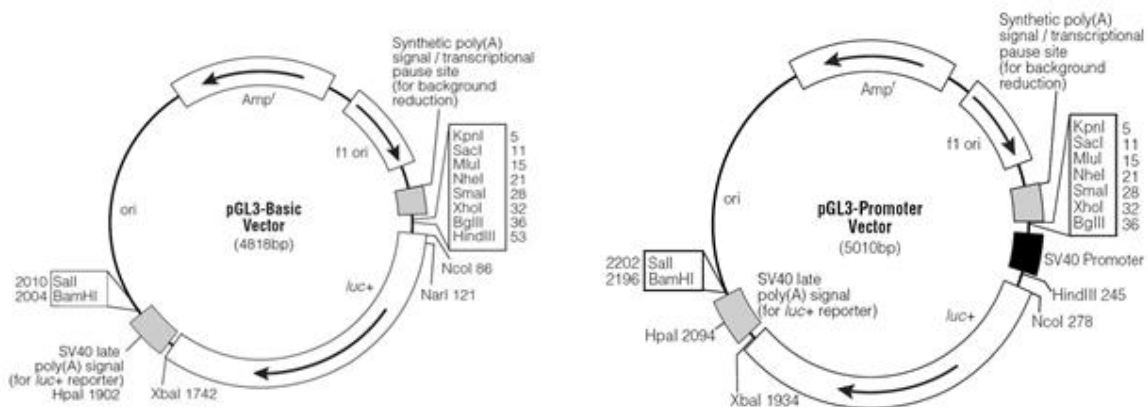


Figure 16. PGL3-Basic and pGL3-Promoter vectors to serve as negative and positive controls

For each of the 9 tests, 10^6 cells were co-transfected with 1 μg of the target region (810bp) and 1 μg each of negative control (pGL3-basic vector) and positive control (pGL3-promoter vector). 200ng of Renilla was used as transfection efficiency control. Transfection was performed with NeonTM Transfection system (Invitrogen) following the manufacturer's guidelines. Electroporation conditions were kept at 1200v-40ms-1 pulse. Transfected cells were cultured for 24 hrs and then stimulated for 6 h and 24 h with 20ng/ml PMA and 2.5 μM Ionomycin. The luciferase activity was then measured using Dual-Luciferase Reporter Assay kit (Promega) on a Tristar LB 941 Multimode Microplate Reader (Berthold technologies, Thermo Fisher Scientific, Waltham, MA, USA). For each sample, firefly luciferase values were normalized to Renilla luciferase values and the results are represented as fold change of the basic vector control.

Luciferase assay results revealed the promoter activity of the 810 bp region of *LAXI* compared to SV40 promoter ($P < 0.001$) after 24 h of stimulation with PMA/Ionomycin. Interestingly, our putative regulatory region showed a silencer activity for the *LAXI* gene compared to *LAXI* promoter ($P < 0.0001$) irrespective of the alleles after both 6 h and 24 h of stimulation (Figure 17). This result confirms that apart from a promoter, our region has an impact on the regulation of the neighboring genes. But unlike *ATP2B4*, the regulatory region exhibit allele-independent silencer activity on *LAXI* in Jurkat cells.

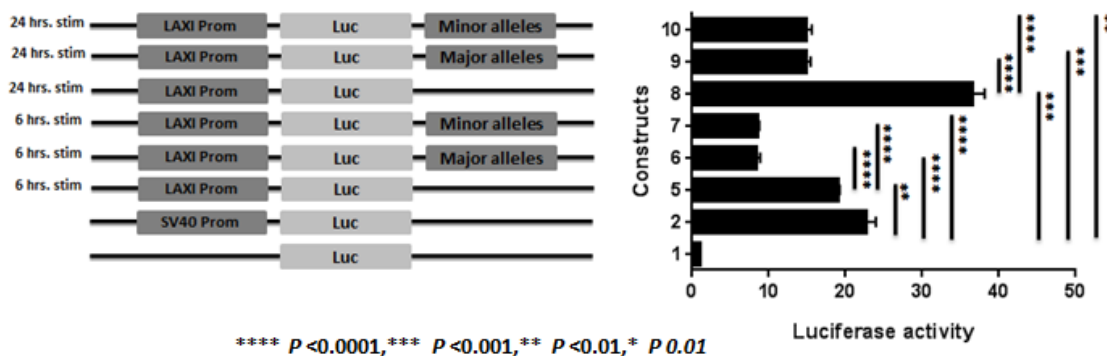


Figure 17. Luciferase reporter assay results for *ATP2B4* variants in Jurkat cells.

SV40 Promoter and Basic vectors were used as positive and negative controls respectively. The *ATP2B4* major and minor alleles haplotypes showing the silencer activity for *LAXI* under stimulation conditions. Data presented as the mean \pm standard deviation of three independent experiments **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

To further validate this silencer activity, we performed CRISPR-Cas9 deletion of our region with 5 SNPs and another with 8 SNPs in Jurkat cells. Independent guide RNAs were used to obtain 506bp and 1262bp deletions. In order to generate the genomic deletions, the respective RNP complexes for the two deletions were electroporated using Neon transfection system (Thermo Fisher Scientific). After 24-48h of transfection, the transfected cells were plated in 96-well plates at limiting dilution (0.5 cells per 100 μ l per well) for the clonal expansion. Individual cell clones were screened for homologous allele deletion after 10-14 days by direct PCR using Phire Tissue Direct PCR Master Mix (Thermo Fisher Scientific) according to manufacturer's protocol. Allowing the detection of knockout or wild-type alleles, specific for the two deletions, forward and the reverse primers were designed producing 335 and 355 bp amplicons in the presence of deletion while 841bp and 1617bp in the absence of deletion. The amplicons were observed on 1% agarose gel and the clones were considered to have undergone homologous allele deletion if they had no wild-type band and at least one deletion band of expected size for the small and large deletions. Selected clones were sequenced using the Sanger method and only the clones with the appropriate sequence with no additional edits were kept for gene expression analysis.

Total RNA from Jurkat from WT and deleted clones in non-stimulated or PMA/Ionomycin treated conditions was extracted using RNeasy mini kit (Qiagen). One microgram of RNA was reverse transcribed into cDNA using Superscript VILO Master Mix (Thermo Fisher Scientific). The Real-time PCR was performed using Power SYBR Master Mix (Thermo Fisher Scientific) on a QuantStudio 6 Flex (life technology) instrument. To quantify the *LAXI* transcript, forward primer 5'-AGAAATTCTGAGAGCCCGGAG-3' and reverse primer 5'-GATACCCACCGCGTACTCTG-3' were used. Expression of the gene was normalized to that of HPRT. Relative expression was calculated by the Δ CT method, and the data reported correspond to the mean of triplicates from three independent experiments per clone and expressed as fold change relative to wild-type cells.

To analyze *LAXI*, we observed an effect of the stimulation, and there was an increased production of *LAXI* under stimulation (Figure 18).

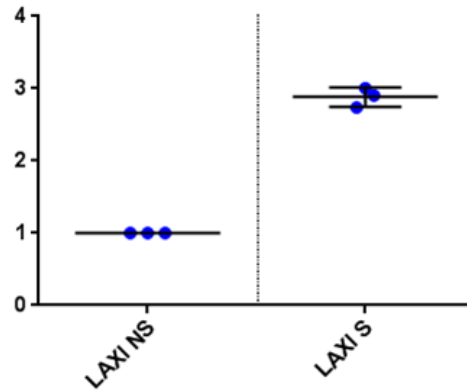


Figure 18. qPCR analysis of *LAXI* gene expression in stimulated (S) and non-stimulated (NS) WT Jurkat cells showing an increased expression of *LAXI* under stimulation.

The results of deletions confirmed the silencer activity of the region on *LAXI*. Upon the deletion of the fragment there was an increased expression of *LAXI* in the stimulated clones with both small and large deletions compared to WT stimulated ($P < 0.01$) (Figure 19).

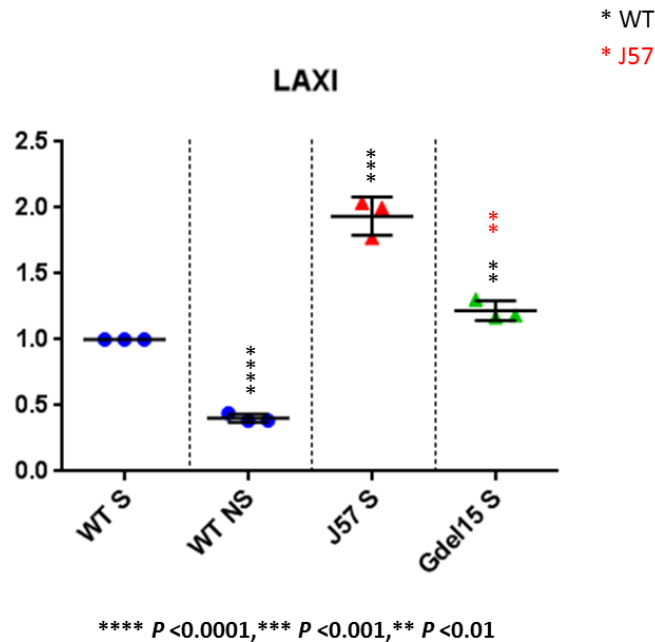


Figure 19. CRISPR-Cas9 mediated genome editing in Jurkat cells.

qPCR analysis of gene expression in stimulated (S) and non-stimulated (NS) wild type Jurkat cells and the stimulated (S) deleted clones for small deletion (J57) and large deletion (Gdel15), respectively. Error bars show s.d. (n=3 independent RNA/cDNA preparations).

However, the silencer activity with the regulatory region (containing 5 SNPs) was much stronger than the large region (containing 8 SNPs). The statistical difference between the two regions was significant ($P < 0.01$) which shows that there is an impact of the regions on the expression of *LAXI* and small region 506bp with 5 SNPs (rs11240734, rs1541252, rs1541253, rs1541254 and rs1541255) has a higher impact on the regulation of *LAXI* than the large region. These results indicate that the 5 SNPs in our regulatory region may be functional variants that have more important role in the *LAXI* regulation.

Together, all these results reveal the importance of our regulatory region with the 5 functional variants in the expression of genes. It acts as an epromoter for *ATP2B4* on one side and also it acts as a silencer for the *LAXI* gene.

Discussion

1. Association of *ATP2B4* locus with severe malaria in Senegal

Genome-wide association studies have identified several malaria associated loci and their corresponding variants but only a limited number of identified loci and genetic variants have been replicated in independent populations. Most of the causal variants remain, however, unknown (Jallow, Teo et al. 2009; Timmann, Thye et al. 2012; Band, Le et al. 2013; Network 2015; Ravenhall, Campino et al. 2018; Band, Le et al. 2019). The loci that have been replicated and their corresponding variants includes *ATP2B4* (rs10900585, rs4951377) on chromosome 1, *FREM3-GYP A/B* (rs186873296) on chromosome 4, *EPHA7* (rs62418762) on chromosome 6, *ABO* (rs8176719) on chromosome 9, and *HBB* (rs334) on chromosome 11. We successfully genotyped the *ATP2B4* tagSNP rs10900585 and confirmed its association with severe malaria in Senegalese population with major allele T as the risk allele. Polymorphism rs10900585 with T as risk allele for developing severe malaria compared to different control groups are shown in distinct populations with a very significant P-value and a significant OR in locations in Africa, Asia and Oceania such as Kenya, Mali, Burkina Faso, Ghana, Nigeria, Tanzania, Malawi, and Vietnam (Bedu-Addo, Meese et al. 2013; Network, Rockett et al. 2014; Ndila, Uyoga et al. 2018). *ATP2B4* encodes for *PMCA4* (Plasma membrane calcium ATPase4), which is a major calcium pump of the red blood cells and therefore defined as fine tuner of cytosolic Ca^{2+} concentrations in RBCs (Stauffer, Guerini et al. 1995; Brini and Carafoli 2009). Being located in the second intron of *ATP2B4*, the SNP rs10900585 has no apparent regulatory function, but might affect mRNA splicing and hence influence protein expression and activity (Faustino and Cooper 2003). Since rs10900585 has been associated with severe malaria in several populations with heterogeneous genetic backgrounds living in areas of variable transmission, we therefore, performed a meta-analysis that confirmed the association of the tagSNP rs10900585 and suggests that the SNP is significantly associated with an increased risk of developing SM. It tends to confirm the involvement of *ATP2B4* in susceptibility to SM.

2. Bioinformatics analysis for the identification of potential causal non-coding variants and their association with severe malaria

Despite the clear success of GWAS in identifying the novel disease susceptibility genes, it implicates the entire genome in disease predisposition and leads to the identification of risk haplotype which have some biological relevance to the disease. It does not necessarily pinpoint

the causal variant and the underlying mechanisms leading to disease outcome. Therefore, bioinformatics and functional studies are required to reveal the causal variants and related mechanisms. Although rs10900585 is reported as having an association with severe malaria, it doesn't necessarily represent the causal variant. As an association with a disease can be detected whether the SNP is indirectly associated with the disease or in strong linkage disequilibrium with the causal variant, which is directly associated with the disease (Balding 2006).

From the tagSNP replicated at least once in independent population, we identified the SNPs in linkage disequilibrium with the tagSNPs associated with severe malaria. After identifying the SNPs in LD, prioritized the candidate regulatory variants focusing on i) the ranking of the SNPs for their regulatory activity using IW-scoring method, ii) the ability of the sequences containing the SNPs to bind transcription factors ChIP-seq peaks. The results revealed the top 10 SNPs on chromosome 1 within *ATP2B4* gene, whereas the 11th SNP was in the *ABO* gene on chromosome 9. The three best SNPs were rs11240734, rs1541252, and rs10751450. Then, we combined the results of above analysis at each SNP which revealed rs1541252, rs1541253, rs1541254, and rs1541255 as the most promising candidates with highest numbers of ChIP-seq peaks. In addition, rs11240734 that was ranked first based on the IW-score and fifth based on the number of ChIP-seq peaks was close to rs1541252, rs1541253, rs1541254, and rs1541255, suggesting that these SNPs may be located within a regulatory element. This suggestion was further validated by the co-location of the SNPs within epigenomic marks associated with promoter or enhancer activity, such as H3K4me1, H3K4me3, and H4K27ac. rs11240734 and rs1541252 have been annotated as eQTLs, according to Haploreg, whereas rs1541255 was found to have a regulatory effect, according to a massively parallel reporter assay (van Arensbergen, FitzPatrick et al. 2017). Prominently, the tagSNPs, rs4951377 and rs10900585 that were found to be associated with severe malaria were ranked 51th and 60th and were neither located within regions showing neither H3K4me1, H3K4me3, H3K27ac marks nor evidence of binding transcription factors, suggesting that they are probably not the causal SNPs. Nevertheless, rs4951377 was an eQTL in blood cells (Westra, Peters et al. 2013), illustrating that an eQTL is not necessarily a regulatory variant. It is very likely that the association signal of rs4951377 with gene expression is explained by the effect of functional SNPs in linkage disequilibrium with rs4951377.

We genotyped the 5 promising newly identified candidate variants of *ATP2B4* (rs11240734, rs1541252, rs1541253, rs1541254, rs1541255) located very close to each other and evidenced much stronger association with SM in Senegalese cohort compared to the tagSNP. Moreover, rs11240734, rs1541252, rs1541253, and rs1541254 were reported as eQTLs in blood cells (Westra, Peters et al. 2013), while rs1541252 and rs1541253 have been shown to be associated with *PMCA4* protein levels (Zámbó, Várady et al. 2017). When checked for the LD, the 5 SNPs were found to be in close LD with each other and with the tagSNP and we found that the majority of Senegalese individuals exhibited either the haplotype combination of all the major alleles of the SNPs or the haplotype combination of all the minor alleles while only a few individuals carried the other haplotypes. So, we assessed the association of the two most frequent (major and minor) haplotypes and found a strong haplotype association with severe malaria. The major allele haplotype was found to be significantly associated with an increased risk of developing SM. This further validated the association of *ATP2B4* locus with the disease.

In addition, the third best candidate based on IW scoring was found to be rs10751450. Interestingly, rs10751450 has been shown to be located within an enhancer altering *ATP2B4* expression (Lessard, Gatof et al. 2017), and was associated with mean corpuscular hemoglobin concentration (Aste, Elding et al. 2016). It has been indicated through Mendelian randomization correlation that the association of the SNP with SM depends on its effect on mean corpuscular hemoglobin concentration (Band, Le et al. 2019). Moreover, close to rs10751450, the two other SNPs, rs10751451 and rs10751452 that were ranked 12th and 13th are also located within the same enhancer. This enhancer region is roughly 700bp away from the region containing rs11240734, rs1541252, rs1541253, rs1541254 and rs1541255, which is potentially a regulatory region based on our results. So, our bioinformatics analysis confirmed that rs10751450, rs10751451 and rs10751452 could be the regulatory SNPs as previously suggested (Lessard, Gatof et al. 2017). Although these three SNPs have been imputed (Network 2019), but to the best of our knowledge, these have not been genotyped in African populations. Therefore, we genotyped these SNPs and assessed their association with severe malaria in a Senegalese population. The SNPs were found to be in close LD with the tagSNP, with the 5 identified candidate SNPs as well as among themselves. The haplotype analysis again showed the major allele haplotype as the most frequent and also significantly associated with the SM. Our results confirm the potential role of rs10751450, rs10751451 and rs10751452, and at the same time that

of the enhancer perturbed by this genetic variation. More particularly, rs10751451 is an eQTL in blood cell (Westra, Peters et al. 2013) and may disrupt GATA1 motif (Lessard, Gatof et al. 2017).

Our results indicated that the bioinformatics tools should help to identify the putative regulatory variants that can be validated further via functional assays. It allowed us to identify 5 new SNPs with potential effect and which may be responsible for the malaria susceptibility.

3. Functional analysis of the putative regulatory variants and their impact on gene regulation

We decided to test the best candidates by experimental approaches (Luciferase reporter assay and CRISPR-Cas9 deletion of the region). This allowed us to show the effects of the variants in K562 cells. K562 are multipotent malignant hematopoietic cells which can differentiate into recognizable progenitors of erythrocytes, granulocytic and monocytic series. We observed that the region containing the SNPs regulates the expression of *ATP2B4* gene that encodes for Plasma membrane calcium ATPase 4 and is the main pump for the homeostasis of Ca^{2+} in the erythrocytes. We showed that this region had a promoter and an enhancer activity and that these activities were modulated by the SNPs. Luciferase reporter assay results suggested that there was an effect of the combination of alleles on gene expression, thus reflecting the interaction between these SNPs. Interestingly, the risk haplotype (with major alleles) showed a lower promoter activity but a significant higher enhancer activity in K562 cell line which suggested that the region is an epromoter (a promoter with an enhancer function), and it also showed that the region exhibits an allele-dependent enhancer activity. Andersson *et al.* hypothesized that there could be an inverse relation between the promoter and enhancer function of a region, the stronger the promoter activity, the weaker will be the enhancer activity and vice versa (Andersson, Sandelin et al. 2015).

Although little is known about the influence of the haplotype on gene expression, a recently proposed method to detect eQTL haplotypes suggested that they are enriched in regulatory regions such as promoters or enhancers (Ying, Li et al. 2018). Our finding is also supported by a study providing evidence at numerous loci that “multiple enhancer variants” cooperatively

contribute to altered expression of their gene targets and that target transcript levels tend to be modest (Corradin, Saiakhova et al. 2014). This raised the question of the natural combination of the alleles in African populations under selective pressure and strengthen our finding that the combination of 5 SNPs was responsible for the susceptibility to severe malaria and not a single causal variant.

To further validate the impact of our potential regulatory region as epromoter, we performed CRISPR-Cas 9 mediated deletion of the region containing rs11240734, rs1541252, rs1541253, rs1541254 and rs1541255 in K562 cells, which resulted in decreased expression of *ATP2B4* compared to non-deleted WT cells. We showed that the deletion of this region decreased both the global *ATP2B4* expression and specifically the expression of the two long transcripts of *ATP2B4* in K562 cell line, supporting its regulatory activity and validating that our regulatory region is an epromoter.

Besides, the enhancer that we identified likely alters the expression of nearby genes. So we also checked the effect of the region on the regulation of nearby genes. Using bioinformatics search, we found a physical interaction between *ATP2B4* and *LAXI* gene. It is noteworthy that rs11240734, rs1541252, rs1541253, and rs1541254 have been associated with the expression of *LAXI* (Westra, Peters et al. 2013), which is a membrane protein implicated in the stimulation of T lymphocytes and its expression in K562 cell line is almost negligible. However, it was found to be expressed well in Jurkat cell line which is an immortalized line of human T lymphocyte cells. Interestingly, our luciferase reporter assay clearly revealed a silencer effect of our regulatory region containing the SNPs on *LAXI* (Figure 20). Moreover, CRISPR-Cas9 deletion validated this silencer activity. Upon deletion of the region, an increased expression of *LAXI* was observed.

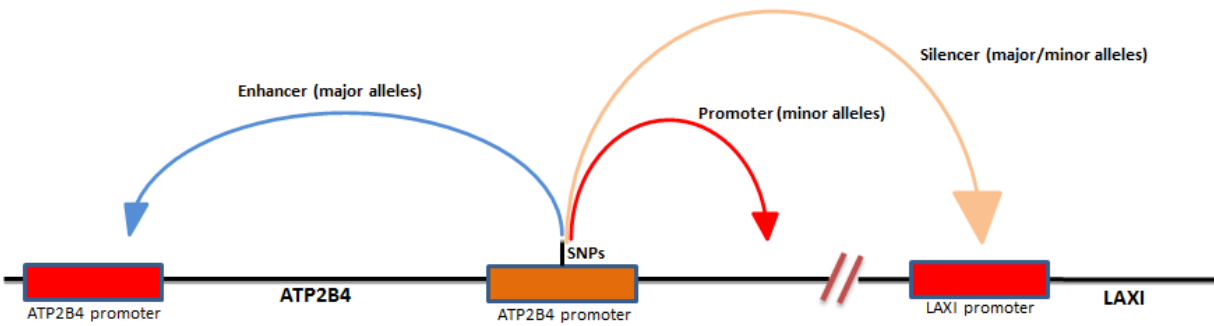


Figure 20 Effect of variants on *ATP2B4* and *LAXI*.

The candidate variants found to have an allele specific expression of *ATP2B4*. They showed a promoter activity with minor alleles while, an enhancer effect with the major alleles. On the other hand, the variants showed a silencer effect on *LAXI* gene, irrespective of the alleles.

4. Functional hypothesis for the impact of *ATP2B4* variants in red blood cells

Mean corpuscular hemoglobin concentration (MCHC) was found to be significantly lower in patients with malaria. This may be due to the parasite's primary target being RBCs, resulting in an accelerated removal of both parasitized and non-parasitized RBCs by the destruction of RBCs (Kitua, Smith et al. 1997; Price, Simpson et al. 2001). Lessard *et al* have proposed that a calcium activated potassium channel may be activated resulting in potassium efflux, dehydration, red blood cell volume loss, increased mean corpuscular hemoglobin concentration, and reduced parasitemia in malaria patients (Lessard, Gatof et al. 2017).

Our results revealed the association of major alleles of the *ATP2B4* variants with the severe malaria susceptibility and that could have an impact on the red blood cells. We demonstrated the potential impact of these variants in regulating *ATP2B4* expression and the intracellular calcium levels. We hypothesize that changes in calcium homeostasis may affect the growth of parasite in red blood cells and may also affect clumping of the infected red blood cells and their sequestration to the brain microvessels and, thus contributing to severe malaria susceptibility. Moreover, our results support the hypothesis that a calcium activated potassium channel may be activated resulting in potassium efflux, dehydration, RBC volume loss, increased MCHC and reduced parasitemia in patients (Lessard, Gatof et al. 2017). Indeed, polymorphisms rs1541252

and rs1541255 were found to be associated with mean corpuscular hemoglobin concentration in African-American children and parasitemia in malaria patients, respectively (Li, Glessner et al. 2013; Ndila, Uyoga et al. 2018). So as our results indicated that the major alleles were found to have an enhancer effect on the two long transcripts of *ATP2B4*. These long transcripts correspond to PMCA4a and PMCA4b and are the most studied transcripts based on the available data. PMCA4a is expressed in brain and PMCA4b is more expressed in the red blood cells (Caride, Filoteo et al. 2007). Therefore, a plausible explanation could account for how the variants could impact the red blood cells. As the *ATP2B4* variants are located in an enhancer so it could be hypothesized that these variants increased the expression of the gene, which resulted in an increased efflux of Ca^{2+} from the red blood cell leading to reduced mean corpuscular hemoglobin concentration and, red blood cell dehydration and thus increased parasite multiplication and malaria susceptibility (Figure 21).

A decrease of Ca^{2+} concentrations in the parasitophorous vacuole, in which the parasite resides and develops, was shown to affect parasite maturation (Gazarini, Thomas et al. 2003). Altered expression, activity or Ca^{2+} affinity of *PMCA4* might change the intracellular Ca^{2+} homeostasis and therefore lead to an impaired intraerythrocytic growth and development of *P. falciparum* (Timmann, Thye et al. 2012). But, since calcium signaling plays an important role in the activation of many different cell types, *ATP2B4* variants might affect malaria pathogenesis by an alternative mechanism. Modified cytosolic Ca^{2+} homeostasis might also lead to an altered activation of platelets and endothelial cells influencing platelet-mediated parasite killing and endothelial sequestration being a key factor of malaria pathogenesis (McMorran, Marshall et al. 2009; Bridges, Bunn et al. 2010).

Recent studies have identified an allele of *PIEZO1*, E756del that results in a gain-of-function mutation, also resulting in an increased intracellular Ca^{2+} concentration, a dehydration of the red blood cells and low parasitemia in vitro. Moreover, in a mouse model, this mutation made it more difficult for the *Plasmodium* to infect red blood cells. Also, it has been shown that malaria parasites grow poorly in human red blood cells from people with the *E576del* mutation as it produces misshapen, partially dehydrated RBCs, making it more difficult for malaria parasites to exist in RBCs which reduces the disease virulence and provides resistance from malaria (Ma,

Cahalan et al. 2018; Nguetse, Purington et al. 2020). Similar mechanisms may be altered by *ATP2B4* variants.

Considering the RBC hypothesis, we could expect that the parasitemia is associated with *ATP2B4* variants in vivo. Thus, as a future perspective, it will be interesting to assess the association between the *ATP2B4* variants with parasitemia in an African population and also the effect of variants in vitro on parasite growth.

Another indication we could expect from this hypothesis is that the risk alleles for the severe malaria should also be the risk ones for developing mild malaria. To assess this, we checked the association of the variants with mild malaria in our Senegalese cohort and found a significant result which indicated that the major alleles were also associated with the risk of developing mild malaria.

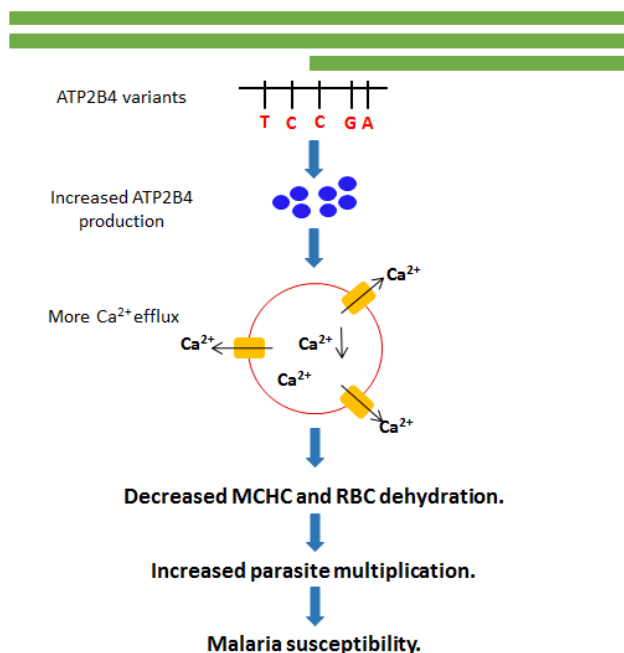


Figure 21. Impact of *ATP2B4* variants on red blood cell.

Cells with the major alleles of variants having an enhancer effect on the long transcripts of *ATP2B4*, results in an increased expression of the gene and probably the PMCA4a and PMCA4b proteins. The RBC with increased *ATP2B4* experience more Ca²⁺ efflux from the intracellular space, resulting in reduced MCHC and, dehydration and malaria susceptibility. Green bars represent the two long and short transcripts of *ATP2B4*.

5. Functional hypothesis for the impact of *ATP2B4* variants in immune cells

Noticeably, it is not excluded that the modulation of intracellular Ca^{2+} concentration alters the physiology of other cell types. For example, the regulation of intracellular Ca^{2+} signaling is a major determinant of CD8+ T cell responsiveness which may have an important role in severe malaria. A possible hypothesis for the effect of *ATP2B4* variants in immune cells could be that the *ATP2B4* variants result an increase in the gene expression and so does the *PMCA4* on the cell surface leading an increased Ca^{2+} efflux from the intracellular space. Ca^{2+} is important in both clumping of infected red blood cells and in their sequestration to the brain micro vessels, both of which contribute to severe malaria susceptibility through the occlusion of the blood vessels. Moreover, the PMCA4 protein is expressed both in the red blood cell and the brain with PMCA4a and PMCA4b as the most abundant isoforms in brain and red blood cells, respectively (Caride, Filoteo et al. 2007).

Ca^{2+} signaling is essential for many cellular processes including regulation of gene transcription and cell activation (Feske 2007). Moreover, modified activation of platelets, including intraerythrocytic parasite killing and their involvement in iRBC sequestration, as well as endothelial cells, affecting iRBC cytoadherence, were proposed as alternative mechanisms by which altered Ca^{2+} homeostasis might affect malaria pathology (McMorran, Marshall et al. 2009; Bridges, Bunn et al. 2010; Timmann, Thye et al. 2012). Since Ca^{2+} signaling plays a crucial role in the differentiation and activation of several effector mechanisms of various immune cells, modified Ca^{2+} homeostasis might also alter immune responses in *P. falciparum* infections and thereby malaria manifestation (Feske 2007). In this way, a recent study using mouse model has demonstrated the gain-of-function *PIEZO1* expression in T cells that contributes to protection against cerebral malaria. The mutation causes an increase in calcium level of T cells which helps in activation and thus protects from cerebral malaria (Ma, Cahalan et al. 2018).

Studies have shown that pRBCs adhesion to ECs induces cell apoptosis via over expression of cytoadherent molecules (Pino, Vouldoukis et al. 2003). *In vivo* studies in mice model of CM have implicated cytotoxic effector CD8+T lymphocytes in development of murine CM pathogenesis by direct cytotoxicity against ECs (Claser, Malleret et al. 2011). Thus, apoptosis and cytotoxicity may lead to disruption of BBB and endothelial damage. Internalization of plasmodial antigens by

endothelial cells leads to the presentation of the parasite's peptides on the endothelium. These plasmodial peptides are then presented to CD8+T lymphocytes via MHC class I (Jambou, Combes et al. 2010). In mice, CD8+T lymphocytes seem to play a role in the evolution of cerebral complications due to malaria through their ability to infiltrate the brain and contribute to the permeability of the blood-brain barrier (Nitcheu, Bonduelle et al. 2003) (Figure 22).

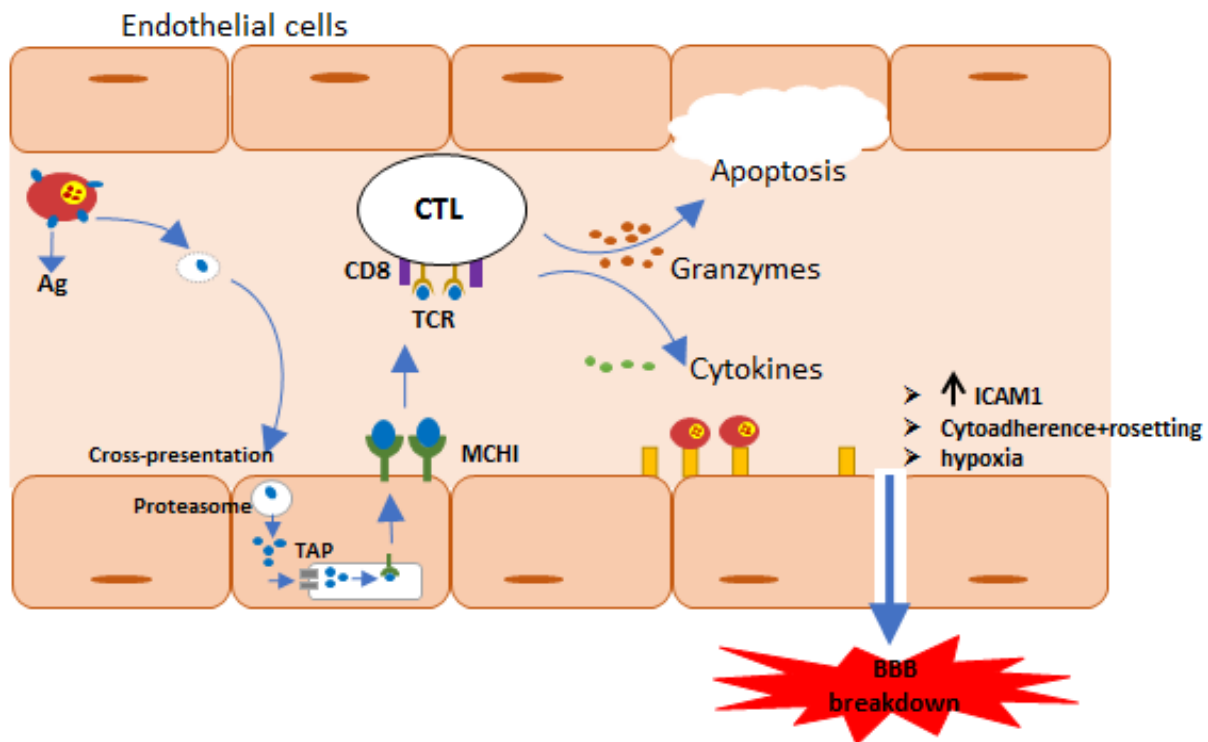


Figure 22. Endothelial cells damage by CD8+ T cells.

The parasite antigens are internalized into the endothelial cells and are further degraded by the proteasome, and the resulting peptides are transported to MHC I in the ER by TAP (transporters associated with antigen processing). Within the ER, the MHC I and peptides form a stable complex that is then transported to the cell surface of endothelial cells. Upon recognition via TCR and CD8 receptors, Cytotoxic T lymphocytes (a) leads to the release of granzymes that results in apoptosis of endothelial cells, and (b) release of cytokines that leads to an increase expression of *ICAM1*, cytoadherence, rosetting, hypoxia and ultimately Blood Brain Barrier (BBB) breakdown, resulting in disease susceptibility (CM). Adapted from (Rénia, Grau et al. 2020).

However, in vitro, there was still a direct contact between activated CD8⁺T lymphocytes and endothelial cells of the brain (Lou, Dayer et al. 1996). The results of these studies suggest a prominent role of activated CD8⁺T cells in the mouse model of malaria, which is probably also the case in humans. The role of T cells in causing brain vasculature injury associated with cerebral malaria is well established in mice, but in the case of humans, a recent study has shown that T cells target blood vessels in brains of children with cerebral malaria. This finding build a bridge between mouse and human cerebral malaria studies by implicating the role of T cells in the development of disease pathology. The results of this study demonstrated an increased accumulation of CTLs along the walls of the brain blood vessels in cerebral malaria tissue samples. In addition, the CTLs were shown to contain and release the effector molecules which damage cells, suggesting that CTLs play a critical role in CM by damaging the BBB. This suggest that the mouse model for CM may accurately reflect the human disease pathology (Rénia, Grau et al. 2020). Moreover, CD8⁺T cells are the major producers of granzyme which causes endothelial cell death by apoptosis. It has been shown that the proportion of granzymes CD8⁺ T cells allowed the discrimination between uncomplicated and severe malaria, suggesting their role in the pathogenesis of malaria complications (Kaminski, Riehn et al. 2019).

In the blood stage of *P. falciparum*, CD8⁺ T cells are thought to play a minor role as the activation of CD8⁺ T cells depends on the expression of antigens via MHC I and red blood cells lack MHC I expression. In murine malaria models, however, it could be demonstrated that parasite-specific CD8⁺ T cells are induced during blood-stage malaria, probably through cross presentation of parasite antigens (Lundie, de Koning-Ward et al. 2008) or even endothelial cells (Turner, Lavstsen et al. 2013) and contribute to the development of experimental cerebral malaria (Lundie, de Koning-Ward et al. 2008; Swanson, Hart et al. 2016). There is compelling in vivo and in vitro evidence that the endothelium has the capability of acquiring and crosspresenting *P.falciparum* antigens during the infection which is an essential step for the antigen specific TCR ligation and cytolysis of endothelial cells. This has been proposed as the main mechanism behind BBB breakdown (Howland, Claser et al. 2015).

Moreover, our putative regulatory region may also regulate the expression of various genes even when being distant from their targets. It is noteworthy that rs11240734, rs1541252, rs1541253, and rs1541254 have been associated with the expression of *LAX1* (Westra, Peters et al. 2013).

LAXI is a gene expressed in the Jurkat cells, and it was found to be the target of this our regulatory region. The gene's protein takes part in the inactivation of MAP kinases, and the adaptive immune response. *LAXI* also participated in the activation of T lymphocytes and has a crucial part in parasite control during infection (Togni, Lindquist et al. 2004). Accordingly, we also evaluated the effect of the region on *LAXI* and found a significant allele-independent silencer activity which shows that our regulatory region has a role in the regulation of *LAXI*. *LAXI* functions as a negative regulator in lymphocyte signaling. It inhibits TCR signaling when it captures Lck (lymphocyte specific protein tyrosine kinase) (Zhu, Granillo et al. 2005).

T cell receptor (TCR) signaling is initiated by engagement of TCRs along with coreceptors CD4 and CD8 with MHC-peptide complexes on the surface of antigen presenting cells (APCs) (Brownlie and Zamoyska 2013). During TCR signaling, binding of the TCR activates Lck. Activated Lck then phosphorylates ITAM (immunoreceptor tyrosine-based activation motif) and another tyrosine kinase ZAP70. Phosphorylated ZAP70 then recruits and phosphorylates LAT (linker for activation of T cell). Together these events set off a complex and tightly regulated signaling cascade leading to proliferation (Brownlie and Zamoyska 2013; Li and Rudensky 2016) (Figure 23).

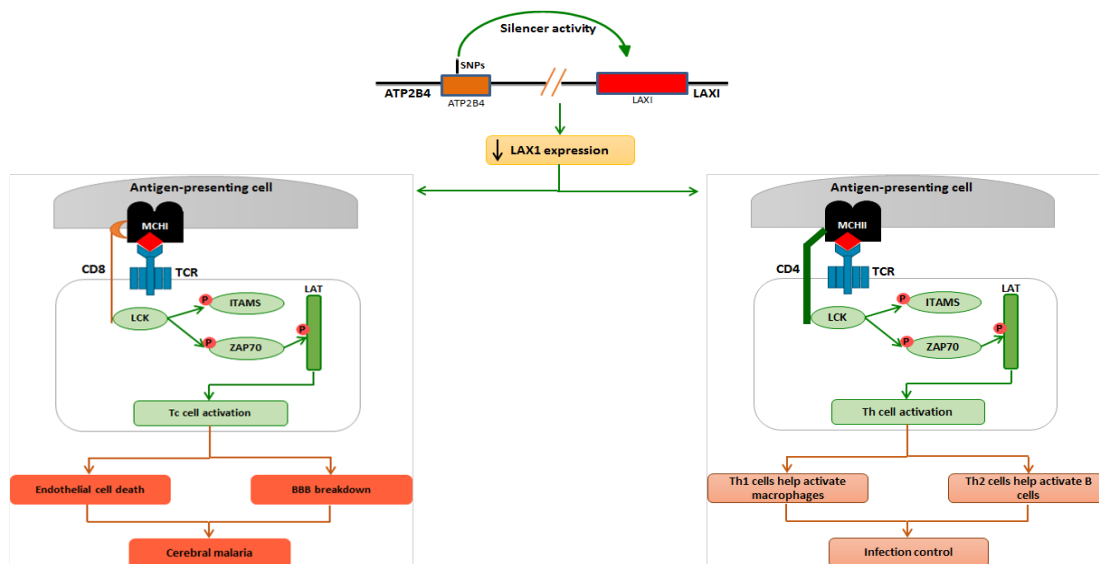


Figure 23. Interaction of *ATP2B4* variants with *LAXI* and their impact on *LAXI* regulation.

The region seems to have a silencer activity on *LAXI*, thus, decreases the *LAXI* expression and the protein. *LAXI* inhibits TCR signaling and functions as negative regulator in lymphocyte signaling. The decrease in the *LAXI* enhances the TCR signaling leading to the activation of T cells.

In conclusion, we identified a new regulatory region that controls the the expression of *ATP2B4*, and that is perturbed by several genetic polymorphisms associated with severe malaria in a Senegalese population. These variants have a role in the control of intracellular calcium concentration. This suggests that we have identified causal variants within a locus that have been identified by GWAS of severe malaria. This could also be beneficial in the development of therapeutic approaches based on the modulation of *ATP2B4* expression and calcium levels.

Moreover, for the future perspectives, several hypotheses can be useful for further exploring the role of *ATP2B4* and malaria. It has been shown in vitro that there is an effect of *PIEZO1* mutation on the calcium level in red blood cell and also it has been shown to be associated with cerebral malaria. Also, it could be imagined that there are several mutations in *ATP2B4* and *PIEZO1* that jointly influence the calcium concentration in RBC. It is expected that combined alleles of two genes jointly alter calcium levels, parasite growth in vitro and in vivo, and the risk of clinical malaria. Besides, the enhancer containing our regulatory SNPs may alter *LAX1* expression, TCR signaling, T cell activation, and the risk of severe malaria. Further studies are required to evaluate these hypotheses.

More broadly, our bioinformatics and genetic study could also help in the identification of causal variants involved in the pathophysiology of several other diseases. Knowing that many projects are based on Whole genome sequencing for individuals that will give the association of all the SNPs with the phenotype, but the problem of how to identify the causal variants will remain in place. So our approach will be helpful in the identification and prioritization of the causal SNPs and then further validate them functionally using experimental methods to explore the mechanism.

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