Aus dem Institut für Tropenmedizin und Internationale Gesundheit
der Charité-Universitätsmedizin Berlin,
und dem Institut für Mikrobiologie und Tierseuchen
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Human genetic polymorphisms influencing the risk and manifestation of malaria in India and Africa

Inaugural-Dissertation
zur Erlangung des Grades eines
Ph.D. in Biomedical Sciences
an der
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vorgelegt von
Prabhanjan Gai
Biotechnologe aus Bijapur, Indien

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ACT: Artemisinin-based combination therapy

AL: Artmether/Lumefantrine

APC: Antigen presenting cell

ART: Artemisinin

ARTs: Artemisinin derivatives

CLR: C-type lectin receptor

CM: Cerebral malaria

CQ: Chloroquine

CQR: Chloroquine resistance

CSA: Chondroitin sulphate A

DARC: Duffy antigen receptor for cytokines

DC: Dendritic cell

Dhfr: dihydrofolate reductase

Dhps: dihydropteroate synthase

GC: Germinal center

GMPD: Geometric mean parasite density

ICAM-1: Intercellular adhesion molecule 1

IFN-g: Interferon-γ

IgE: Immunoglobulin E

IgG: Immunoglobulin G

IgM: Immunoglobulin G

IL: Interleukins

IRAK1: Interleukin-1 receptor-associated kinase-1

iRBC: Infected red blood cell

K13: Kelch 13

KAHRP: Knob-associated histidine-rich protein

Mal: My D88 adaptor

MBC: Memory B cell

MBL: Mannose binding lectin

MHC: Major histocompatibility complex

MiRNA: MicroRNA

NK: Natural killer cell

NLR: NOD-like receptor

P. falciparum: *Plasmodium falciparum*

P. knowlesi: *Plasmodium knowlesi*

P. malariae: *Plasmodium malariae*

P. ovale: *Plasmodium ovale*

P. vivax: *Plasmodium vivax*

PAMP: Pathogen-associated molecular pattern

PCR: Polymerase chain reaction

Pfcrt: *P. falciparum* chloroquine resistance transporter

PfEMP1: *P. falciparum* erythrocyte membrane protein 1

PfEMP2: *P. falciparum* erythrocyte membrane protein 2

Pfmdr1: *P. falciparum* multidrug resistance protein-1

Pfmrp1: *P. falciparum* multidrug resistance associated protein

Pfnhe1: *P. falciparum* sodium/hydrogen exchanger

Plasmodium spp: *Plasmodium* species

PRR: Pathogen-recognition receptor

Pvcrt-o: *P. vivax* transporter protein

PvDBP: *P. vivax* Duffy binding protein

Pvk12: *P. vivax* kelch 12

Pvmdr1: *P. vivax* multidrug resistance protein-1

RBC: Red blood cell

RDT: Rapid diagnostic test

RESA: Ring-infected erythrocyte surface antigen

SEA: Southeast Asia

SNP: Single nucleotide polymorphism

SP: Sulfadoxne/Pyrimethamine

Th1: T helper cell-1

TLR: Toll-like receptor

TNF-α: Tumor necrosis factor-α

TRAF6: TNF receptor-associated factor-6

TRAF6: Tumor necrosis factor receptor-associated factor-6

WHO: World Health Organization

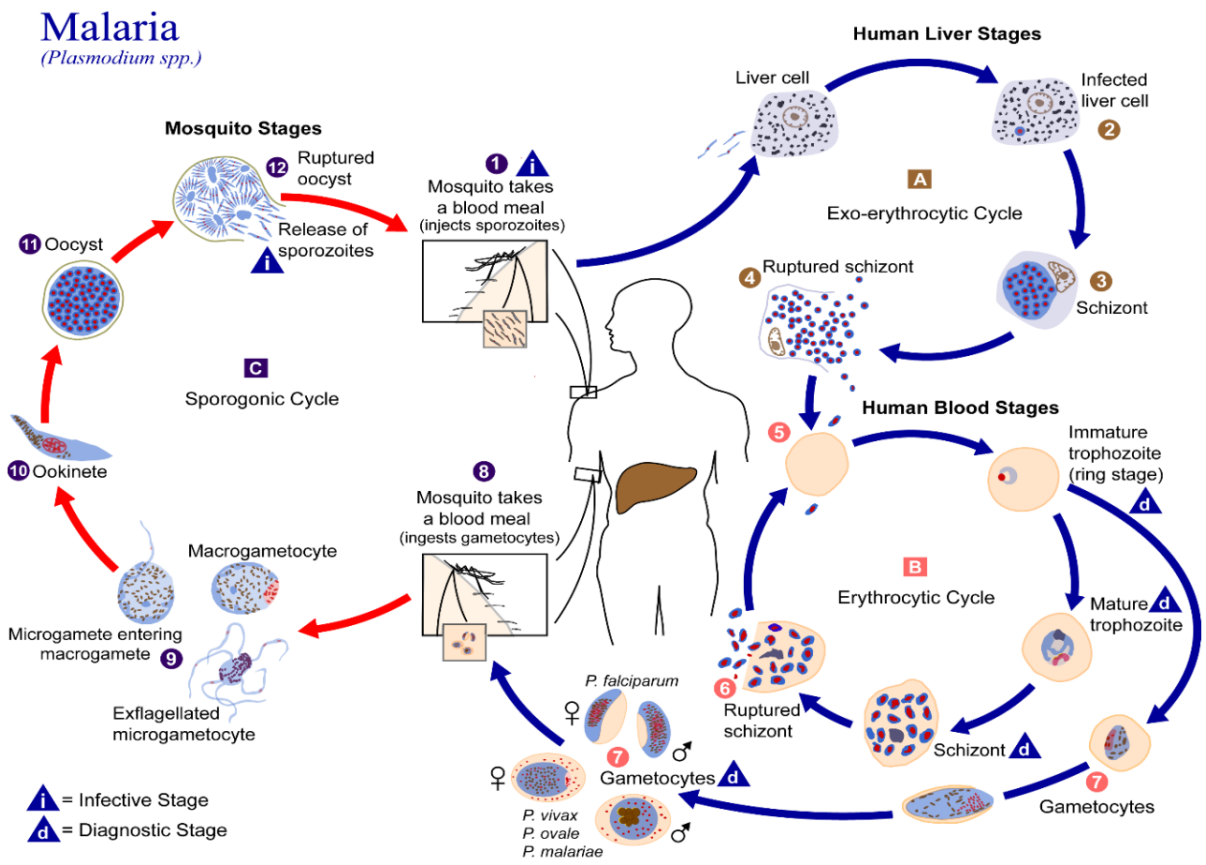
1. Introduction

1.1 Malaria

Malaria is one of the most serious and potentially fatal tropical diseases leading to approximately 400000 deaths worldwide every year (WHO, 2018). Malaria is caused by infection with parasites of the genus *Plasmodium*, which are transmitted through the bites of *Anopheles* mosquitos carrying the eukaryotic microorganisms. Out of more than 100 *Plasmodium species* identified, only five have the ability to infect and cause disease in humans, namely, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. *P. falciparum* and *P. vivax* occur in tropical and sub-tropical regions worldwide, but *P. falciparum* particularly predominates in Africa (WHO, 2018). Among the five *Plasmodium* species infecting humans, *P. falciparum* is causing potentially fatal courses of the disease including severe anaemia and organ dysfunction. *P. vivax* is the most common *Plasmodium* species infecting humans. *P. vivax* is largely considered benign but can relapse after several months or years (White, 2011). It infects individuals who are Duffy blood group positive, i.e., who present Duffy blood group antigens on the surface of their erythrocytes (Höher et al., 2018). *P. ovale* is very similar to *P. vivax* in terms of biology and morphology but can infect Duffy negative individuals. Though it is found in most tropical countries, it is relatively common in Africa. *P. ovale* is also considered to relapse after several months or years. *P malariae* is found in all malarious regions. In untreated conditions, blood stage parasites can cause chronic infection and can persist for years. *P. knowlesi* is primarily found in long and pig-tailed macaques in Southeast Asia (SEA) and was experimentally proven to infect humans in 1932 (Knowles and Gupta, 1932). Its lifecycle is rigorous with asexual replication every 24 hours.

1.2 Lifecycle of *Plasmodium* parasites

The lifecycle of *Plasmodium* parasites involves an intermediate and a definite host. Infected *Anopheles* mosquitos (intermediate host) show infectious parasite stages in their salivary gland. During their blood meal on a human host, these transmit sporozoites into the blood stream (Figure 1). Sporozoites within 10-20 minutes infect liver cells, where they undergo asexual division and form schizonts. After an incubation period of 5 days to several weeks (depending on species) schizonts rupture and release merozoites into the blood stream. In *P. vivax* and *P. ovale* infections, dormant parasite forms (hypnozoites) develop upon primary infection in liver cells, which – for so for unknown reasons – may enter into schizogony after weeks to months (White, 2011).



Adapted from CDC DPDx-Malaria | https://www.cdc.gov/dpdx/malaria/index.html

Figure 1: Life cycle of *Plasmodium* parasite in *Anopheles* mosquito and human host. (1) Mosquito releases sporozoites into the human blood stream during a blood meal (2) Sporozoites infect liver cells and (3) mature into schizonts. (4) Schizonts rupture to release merozoites. (5) Merozoites infect red blood cells. This cycle continues numerous times leading to millions of parasite-infected red blood cells.

Merozoites infect erythrocytes and further develop to form ring stage trophozoites. As before in the liver cells, the parasites undergo schizogony for 24h to 72h, after which the infected red blood cell (RBC) is lysed and new merozoites are released (Crutcher and Hoffman, 1996). This marks the completion of the erythrocytic cycle. Repeated replication results in millions of parasite-infected RBCs in the host bloodstream. Only at the level of erythrocytic asexual parasitaemia clinical manifestation occurs (Ashley et al., 2018). Within one to two weeks of asexual parasitaemia, a small proportion of the parasite biomass differentiates to form gametocytes (sexual parasite stage). The male (microgametocytes) and female (macrogametocytes) gametocytes are taken up by the *Anopheles* mosquito during a blood meal. They fuse to form a diploid zygote, which transforms into a motile ookinete that burrows in the mosquito midgut wall. Ookinetes develop into oocysts, which further mature

and replicate to release sporozoites. Sporozoites make their way to the salivary glands and are now ready to be transmitted to the human host during the mosquito's next blood meal (Aly et al., 2009).

1.3 Epidemiology of malaria

In view of the ongoing efforts on the eradication of infectious diseases in tropical and sub-tropical countries, substantial progress has been achieved in reducing the burden of malaria. However, this disease continues to be a challenging concern to the public health system laying risk of infection on millions of individuals worldwide. It is one of the most globally spread infectious diseases with 219 million estimated clinical cases and 435000 deaths in 2017 according to the World Health Organization (WHO) (WHO, 2018). More than 90% of malaria-related deaths occurred in Africa, majorly affecting children under the age of 5 years. Southeast Asia (SEA) takes the second position following Africa in terms of the estimated number of cases with 11.3 million. In Africa *P. falciparum* accounts for almost 99% of cases whereas in SEA this proportion is 63% (WHO, 2018). *P. vivax* is the most widespread malaria parasite, prevalent in Asia and South America. In 2017 about 82% of all vivax cases occurred in SEA and nearly 50% of those in India (WHO, 2018). Figures 2 and 3 display the worldwide incidence rates of *P. falciparum* and *P. vivax*, respectively, based on the data from 2017 (accessed from the malaria atlas project).

Malaria transmission is determined by several factors ranging from vectorial capacity (density, longevity and efficiency) to climate (White et al., 2014). The intensity of transmission depends largely on the vector and only a few can transmit *Plasmodium* to humans. Among more than 400 identified *Anopheles* mosquitos, around 40 vectors have been of major importance for humans and they mostly take a bite on humans during dusk and dawn (Rossati et al., 2016). Climate plays a very important role in vector survival and development. Temperature is a key factor in affecting the life cycle of a mosquito itself. Altering temperature in the water may affect the amount of the eggs hatched and could have an impact on the transition time in the development from larvae to pupae. Similarly, optimal temperatures (25°C - 30°C) are required for the development of parasites in the mosquito (Stresman, 2010). Malaria transmission can be characterized based on 4 major eco-epidemiological factors, for example in Ethiopia; a) above 2500 meter altitude, malaria free high land areas; b) 1500 – 2500, highland fringe (frequent epidemics); c) below 1500 meters, lowland areas with unstable or seasonal transmission, and d) stable malaria transmission (transmission throughout the year) (WHO, 2019). Entomological inoculation rates (infectious bites per year) vary depending on the region. For instance, they are much lower in unstable

transmission areas such as Asia and South America as compared to sub-Saharan Africa where inoculation rates can go as high as 1000 per year (Gething et al., 2011).

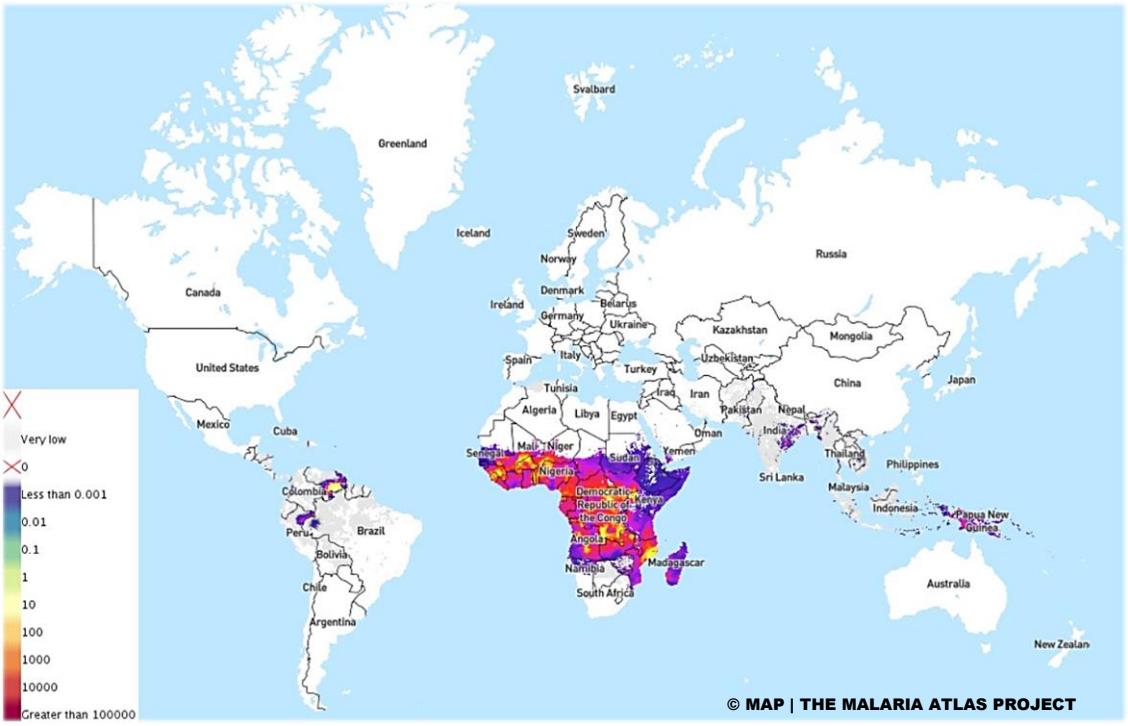


Figure 2 *Plasmodium falciparum* incidence rate globally for 2017

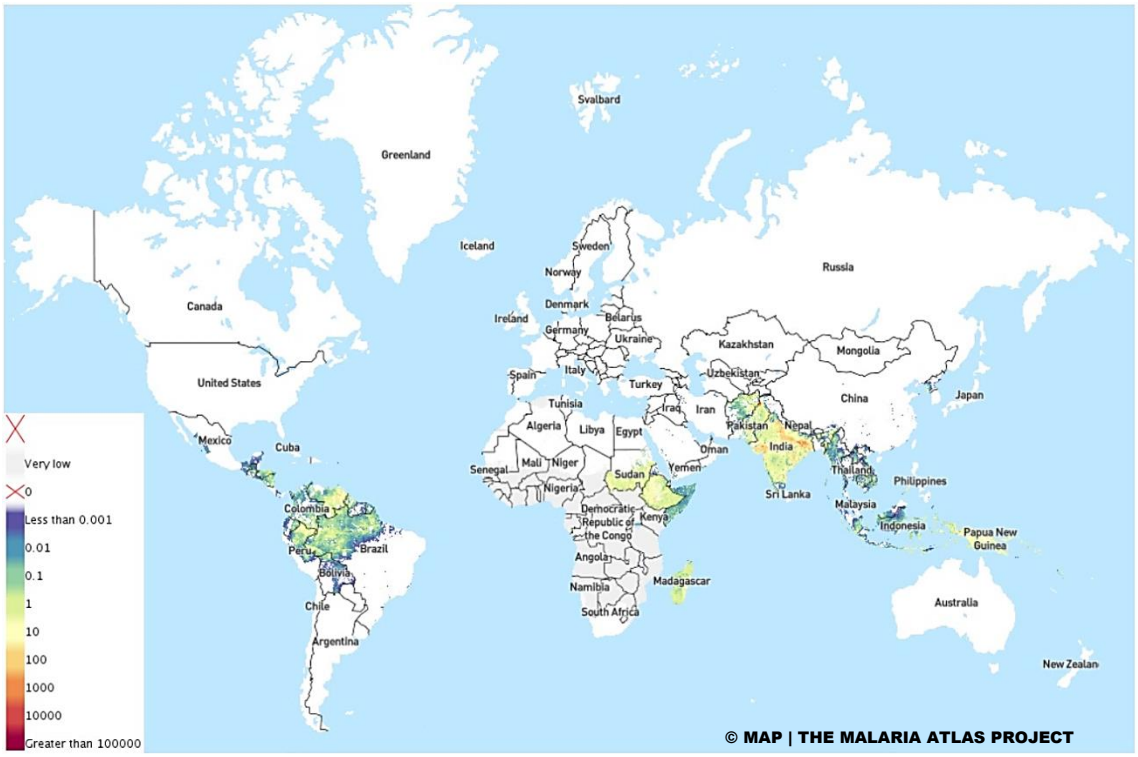


Figure 3 *Plasmodium vivax* incidence rate globally for 2017

1.4 Pathogenesis of *Plasmodium* parasite infection and its consequence

The pathogenesis of *Plasmodium* infection involves a complex interaction of the infected RBCs (iRBCs) with other cells. For instance, in *P. falciparum* infection, the parasite is able to modify the erythrocyte structurally by forming electron dense knobs of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) including knob-associated histidine-rich protein (KAHRP), PfEMP2, and ring-infected erythrocyte surface antigen (RESA) on the iRBC surface. (Sharma, 1997; Maier et al., 2009). PfEMP1 is a strain specific adhesive protein encoded by the *var* gene family, that mediates cytoadherence of infected erythrocytes, which means attachment to the surface receptors of endothelial cells, blood cells, platelets, and uninfected erythrocytes (Smith et al., 2013). iRBCs adhere to epithelial walls or to each other *via* platelet-mediated agglutination (but not exclusively) and to uninfected erythrocytes by rosetting (Pain et al., 2001; Doumbo et al., 2009). Adherence of iRBCs to the endothelial cells further leads to the sequestration of RBCs infected with mature parasites into blood vessels of vital organs including brain (cerebral malaria) and placenta. The sequestration of iRBCs may further lead to complications such as hypoxia, acidosis, inflammation and leakage (Idro et al., 2010; Sharma and Shukla, 2017). Cytoadherence and sequestration is observed only in *P. falciparum* malaria, with the possible exemption of *P. vivax* (see below).

The hall mark feature in pathogenesis of cerebral *P. falciparum* malaria is sequestration of iRBCs into the microvasculature, which leads to congestion in the venules and capillaries (MacPherson et al., 1985; Berendt et al., 1994; Ponsford et al., 2012). Moreover, sequestration of iRBCs was observed to be highest in brain vessels as compared to other organs. iRBCs isolated from Malawian paediatric cerebral malaria (CM) cases adhered to brain microvascular endothelial cells more efficiently than uncomplicated malaria related iRBCs. In contrast, the latter showed significantly increased affinity towards dermal endothelial cells (Storm et al., 2019). Sequestration and rosetting are considered to be the parasite virulence factors associated with severe malaria (Carlson et al., 1990; Pain et al., 2001).

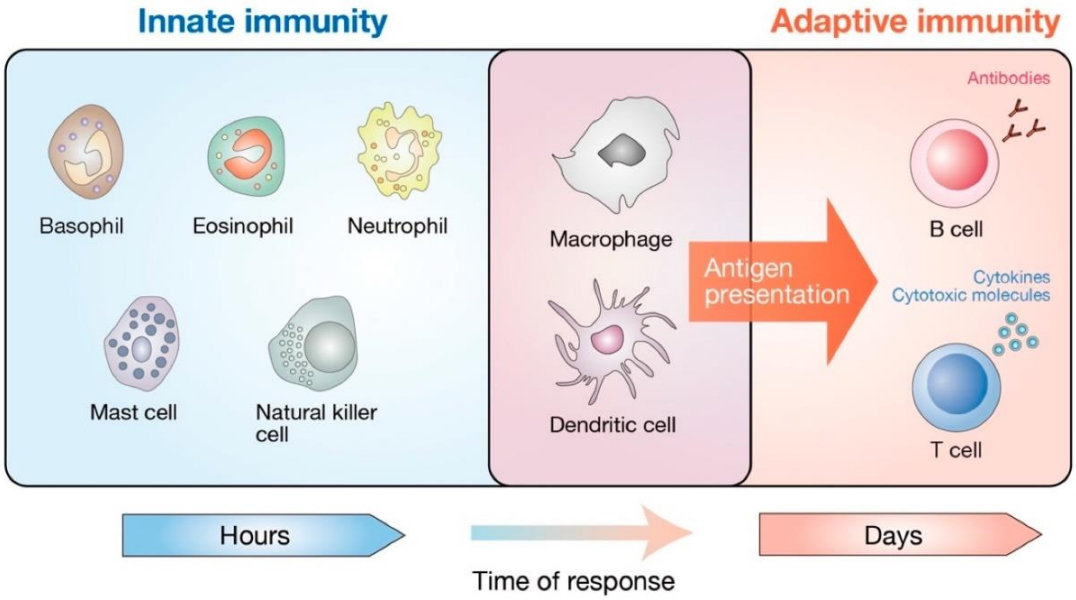
Parasite density contributes to pathophysiology; however, the density is governed by the selectivity of the parasites to RBCs. Unlike *P. falciparum*, the other malaria parasites are highly selective in invading RBCs (for example, *P. vivax* exclusively invades reticulocytes). Due to this selective feature, *P. vivax* parasitaemia does not reach high levels as seen in falciparum malaria (Simpson et al., 1999). Recent *in vitro* data suggest that *P. vivax*-infected RBCs cytoadhere to endothelial cells, *via* intercellular adhesion molecule 1 (ICAM-1) and chondroitin sulphate-A (CSA), however, at a 10 times lower frequency than *P. falciparum*-iRBCs (Carvalho et al., 2010). Whereas, in another study there was no cytoadherence to

ICAM-1 observed but to the glycosaminoglycans CSA and hyaluronic acid (Chotivanich et al., 2012).

The soluble *Plasmodium* products (malaria toxins) from the erythrocytic stages induce the production of cytokines and inflammatory mediators such as tumor necrosis factor – α (TNF-α), interferon-γ (IFN-γ), and interleukins (IL). Pro- and anti-inflammatory cytokines play a significant role in malaria parasite clearance by activation of stage specific immune responses including the activation of the phagocytic cells as an immediate response (Oyegue-Liabagui et al., 2017). For instance, the T helper cell-1 (Th1) cytokines such as TNF-α, IFN-γ and IL12 (by inhibiting parasite growth) may limit the progression of uncomplicated to severe complications (Perlmann et al., 1999; Angulo and Fresno, 2002). IL-17 and IL-22 are involved in the recruitment of neutrophils and further induce the secretion of several proinflammatory cytokines (Valeri and Raffatellu, 2016; Oyegue-Liabagui et al., 2017). However, excessive levels of inflammatory cytokines have been linked with intensified pathological complications such as severe anaemia, respiratory distress as well as cerebral malaria (Angulo and Fresno, 2002; Dunst et al., 2017). In contrast, the anti-inflammatory cytokines such as IL-4 and IL-13 play a crucial role in neutralising the outrageous Th1 cytokine response (Riley et al., 2006). Nevertheless, why only a small proportion of malaria patients develop severe disease is not entirely understood. Underlying reasons may depend on several factors such as genetic make-up, immune response, and social and geographic factors (White et al., 2014).

1.4.1 Immunity: host defence against *Plasmodium* parasites

As a consequence of *Plasmodium* infection, the human body responds by inducing complex and stage specific immunological activities. In endemic countries, malaria often does not lead to severe outcomes in infants during their first few months of life because of the passive immunity from maternal antibodies. However, from 6 months to around 5 years of age, children are highly susceptible to malaria, which may also result in severe disease, until they acquire active immunity (after several exposures) (Marsh, 1992). Immune responses are largely based on and regulated by two pillars: the innate and the adaptive immune system (Figure 4).



Adapted from Yamauchi T et al., Cells. 2019:30;8(5)

Figure 4: Essential components of the human immune system.

Innate and adaptive immunity play an integral role in defending infections. The innate immune system responds robustly to pathogen entry, first by phagocytizing and next, by activating inflammatory pathways. On other hand, adaptive immunity kick starts after a few days of infection. These responses are mediated *via* antigen presentation and are specific to the type of infection.

Innate immunity. Innate immunity is the first line of defence and induces non-specific responses. Impediments such as skin, phagocytic cells and a several antimicrobial compounds are primary factors in the innate immune system. Peripheral blood mononuclear cells (PBMCs) that oscillate between the peripheral circulation and the spleen are the first to sense and react to the infection (Ockenhouse et al., 2006). Macrophages, dendritic cells (DCs), neutrophils, monocytes and natural killer (NK) cells play important roles during the initial stages of infection. Macrophages and dendritic cells are the first to respond to the early

blood stage infection by phagocytosis of iRBCs. However, phagocytosis of iRBCs by macrophages compromises their ensuing function in inflammatory signalling to a certain extent. Whereas, DCs retain their function intact and produce cytokines and chemokines activating further immune responses (Schwarzer et al., 1992; Wu et al., 2015). Macrophages directly produce TNF-α at low levels when they come in contact with glycoproteins and glycolipids released from rupturing iRBCs. Furthermore, in the presence of other mononuclear cells, TNF-α production is significantly increased (Schofield and Hackett, 1993). In malaria mice models, IFN-γ production was associated with the regulation of initial level of parasites, and most of the early IFN-γ release was due to NK cells (De Souza et al., 1997). Moreover, increased lysis of *P. falciparum* infected erythrocytes by NK cells was observed (Orago and Facer, 1991).

Host PBMCs sense pathogen-associated molecular patterns (PAMPs) *via* evolutionarily conserved pathogen-recognition receptors (PRRs). PRRs are located at several locations including cell surface, plasma and endosomal membranes, the outer membrane of mitochondria, and cytosol. Toll-like receptors (TLRs), c-type lectin receptors (CLRs), namely, mannose and galactose binding proteins, NOD-like receptors (NLRs) and scavenger receptors, are among notable transmembrane receptors sensing *Plasmodium* parasites (Brubaker et al., 2015; Gowda and Wu, 2018). In malaria, PAMP sensing starts already in the liver. For a long time, it was assumed that the infected hepatocytes go unrecognized by the innate immune system. However, from recent studies on *Plasmodium* infected mice, one can understand that, although infected hepatocytes escape from being recognized by macrophages and dendritic cells, they are recognized by cytosolic PRRs, which further induce type I IFN responses (Liehl et al., 2014; Miller et al., 2014).

So far, 10 TLRs have been identified, which recognize a wide range of PAMPs, including those derived from *Plasmodium* parasites, bacteria and viruses. TLRs activate the downstream signalling cascade, which in turn initiates pro-inflammatory responses. These signals are mediated through adaptors such as the myeloid differentiation factor 88 (MyD88), MyD88 adaptor like protein (MAL, TIRAP), or TIR domain-containing adaptor inducing interferon-β (TRIF, TICAM-1). The pro-inflammatory response plays a central role in malaria pathogenesis (Dunst et al., 2017; Oyegue-Liabagui et al., 2017). MyD88 knockout mice infected with different *Plasmodium* strains displayed an impaired pro-inflammatory cytokine production and a reduced intensity of symptoms (Adachi et al., 2001; Franklin et al., 2007). Another study showed that MyD88-dependent TLR2- and/or TLR9-mediated signalling may play a very important role in cerebral malaria (Coban et al., 2006). Likewise, lectin receptors are also crucial in *Plasmodium* parasite recognition and disease progression. Pathogen

recognition *via* mannose binding lectin (MBL) triggers the activation of the lectin pathway (complement pathway). Activation of molecules in the complement pathway is important for neutralizing invading parasites; however, over activation of these components may mediate pathogenesis (Silver et al., 2010).

Adaptive immunity. The adaptive immune response is induced by antigen presenting cells (APCs) such as DCs. DCs are the central link between innate and adaptive immune system by presenting pathogen specific antigens, and thus generating unique memory (Banchereau et al., 2000). Subsequent infections enhance the memory and the strength of the response, and these responses are mediated by lymphocytes such as T and B cells (Wherry and Masopust, 2016). Immature DCs upon contact with antigens mature to express major histocompatibility complex molecules (MHC), co-stimulatory (CD40, CD80, CD86) and adhesion molecules on their surface. Maturation of DCs is found to be crucial in activating CD8+ and CD4+ T cells to Th cell subsets (Stevenson et al., 2011). DCs upon secreting cytokines and chemokines recruit other immune cells, which in turn modulate T and B cell responses in order to clear iRBCs (Yap et al., 2019). For instance, in individuals with partial immunity, infected hepatocytes can be cleared by parasite specific CD8+ T cells, and sporozoites invading into hepatocytes can be halted by CD4+ T cell-dependent antibody responses (Kurup et al., 2019). Increased IFNγ responses by TH1 cells in *P. falciparum* infected individuals and in rodents infected with *P. berghei* ANKA was observed (Troye-Blomberg et al., 1985; Villegas-Mendez et al., 2012). IFNγ activates macrophages, which enhances the phagocytic activity, in turn inducing the production of reactive oxygen species toxic to the *Plasmodium* parasites (Kurup et al., 2019). IL-2 derived from CD4+ TH1 cells activates NK cells and further contributes to protective immune responses (Horowitz et al., 2010). Likewise, several T cell subsets have been observed to actively modulate the adaptive immune response in malaria (Kurup et al., 2019).

On the other hand, B cells respond *via* antibody production. Upon encountering antigens, B cells differentiate into three subsets (i) short-lived plasmablasts initiating an extra-follicular antibody response, (ii) early memory germinal center (GC) independent memory B cells (MBCs) and (iii) GCs aggregating in the B-cell follicle. GCs undergo further differentiation to form GC affinity-matured MBCs and long-lived plasma cells, which protect from reinfection. Plasma cells after migrating to the bone marrow provide a continuous source of high-affinity antibodies, whereas MBCs circulate in the blood and secondary lymphoid tissue, and instantly induce effector response upon antigen encounter (Ly and Hansen, 2019). Passive transfer of immunoglobulin G (IgG) was reported to reduce parasitaemia and diminish clinical symptoms (Cohen et al., 1961; Bouharoun-Tayoun et al., 1990). IgG (opsonising

antibody) was found to play a versatile role; it may block sporozoite invasion into hepatocytes as well as merozoites entering erythrocytes, and mediate phagocytosis of iRBC expressing variant surface antigens on their surface (Hill et al., 2013). Contrarily, IgE and IgM have been negatively correlated with malaria. For instance, IgE levels were elevated in cerebral malaria as compared to uncomplicated malaria (Perlmann et al., 1994). IgM masking of protective IgG epitopes on iRBCs mediates the *P. falciparum* evasion, by protecting the parasites from phagocytosis (Barfod et al., 2011). However, the roles of IgE and IgM with respect to malaria are not well established. Nevertheless, innate and adaptive immunity are inter-connected as the cytokines produced during the innate immune response regulate the outcome of the adaptive response (Stevenson and Riley, 2004).

1.4.2 Clinical presentation: uncomplicated malaria and severe malaria

The host presents clinical symptoms shortly after the first cyclic rupture of iRBCs. The clinical outcome of malaria varies with the parasite species, age, immunity, host genetic factors, and epidemiology. Young children and pregnant women living in malaria endemic areas are at increased risk (White et al., 2014). Initial symptoms of malaria may include general weakness, fatigue, headache, muscle aches, abdominal pain and fever. Whereas in stable transmission areas, adults are more likely to have asymptomatic infections due to acquired immunity. Asymptomatic infection is defined by the presence of parasites without presenting any disease symptoms (Okell et al., 2012). Manifestation is further classified into uncomplicated and severe malaria.

Uncomplicated malaria accompanies very unspecific symptoms (fever, headache, chill, body ache, vomiting, nausea) and, therefore, clinical diagnosis gets unreliable (Bartoloni and Zammarchi, 2012). In *P. falciparum* malaria, the usual incubation period from infection to presentation of first symptoms is between 6 to 14 days. In non-endemic areas, uncomplicated malaria usually can be identified by the presence of fever (taking into account a travel history) as well as thrombocytopenia (Ashley et al., 2018). Thrombocytopenia is thought to be a common feature both in uncomplicated and severe malaria (Taylor et al., 2008; Leowattana et al., 2010). *P. vivax* generally causes an uncomplicated form of the disease. *P. vivax* preferably invades young erythrocytes (reticulocytes), thereby limiting the reproductive capacity, thus resulting in lower parasitaemia levels as compared to *P. falciparum* (Anstey et al., 2009). Uncomplicated malaria is easily treatable with several antimalarial drugs. However, in case of delayed treatments, it may lead to severe malaria in *P. falciparum* infection.

Severe malaria and related deaths are predominantly due to *P. falciparum* infection. Criteria defining severe malaria are listed by WHO, such as, impaired consciousness, acidosis, hypoglycaemia, severe malarial anaemia, renal impairment, prostration, jaundice, pulmonary oedema, abnormal bleeding, and hyperparasitaemia (WHO, 2014). However, most frequent complications include severe anaemia and cerebral malaria, (Trampuz et al., 2003). Children and pregnant women are at higher risk of severe falciparum malaria (Snow et al., 1998; Kovacs et al., 2015). The most severe complication is cerebral malaria (CM). CM is a rapidly developing encephalopathy that leads to impaired consciousness, cerebral seizures, coma, and may also lead to death. CM accounts for almost 20% of adult deaths and 15% of childhood deaths, and the majority of the deaths occur in African region (WHO, 2014; W. Wang et al., 2015). CM in adults was correlated with coma, whereas mostly causing endothelial damage and perivascular ring haemorrhages in children (Dorovini-Zis et al., 2011; Ponsford et al., 2012).

Although *P. vivax* is traditionally considered to cause less intense disease, except occasional severe anaemia, in recent years an increasing number of severe malaria cases including cerebral malaria and deaths are reported (Anstey et al., 2009; Price et al., 2009). Though *P. vivax* is endemic in Asian and Central and South American countries, severe cases and fatalities are particularly reportedly from India (Limaye et al., 2012; Jain et al., 2013; Kochar et al., 2014). Moreover, a recent review and meta-analysis of severe vivax malaria since 1900 observed that the majority of reports originate from the Indian subcontinent, and that severe thrombocytopaenia (< 50,000/μL) was a common defining symptom (Rahimi et al., 2014). Severe vivax malaria and related fatalities appear to differ with defining criteria, age, endemicity and geographical setting, and the rate of co-morbidities (Price et al., 2009; Rahimi et al., 2014).

1.4.3 Malaria during pregnancy

Pregnant women, particularly primiparae, are at increased risk of malaria, especially due to *P. falciparum* infection, which may lead to preterm delivery, low birth weight and infant mortality, (Brabin, 1983; Desai et al., 2007). Chronic infection is associated with reduced birth weight, whereas acute infection is linked to preterm delivery (Menendez et al., 2000; Tako et al., 2005). The antibodies expressed in response to iRBC adhering to the placental syncytiotrophoblast are usually absent in primigravidae, however, specific immunity develops with successive pregnancies (Mayor et al., 2011). In *P. falciparum* infection, iRBCs derived from the placenta vary in several ways as compared to the iRBCs from non-pregnant individuals. iRBCs from the placenta bind to glycosaminoglycan receptors, and chondroitin

sulphate-A (CSA) has been identified as the major receptor (Fried and Duffy, 1996). CSA is found in the placenta as a side chain of thrombomodulin (tissue anticoagulant). In pregnancy, iRBCs sequester mainly in the intervillous space of the placenta unlike in the other tissues where they adhere to the vascular walls (Muthusamy et al., 2004). Rosetting of iRBCs-RBCs and agglutination of iRBCs-iRBCs is not commonly observed (Rogerson et al., 2000). PfVAR2CSA (PfEMP1 variant) is upregulated in placental parasites. PfVAR2CSA binds to CSA and mediates sequestration (Desai et al., 2007). Along with the iRBC sequestration, immune cells are infiltrating the intervillous space (Beeson and Duffy, 2005). Parasite densities are observed to be much higher in the intervillous space than in peripheral blood, and the placenta appears blackened due to deposition of malarial pigment. The increased burden and adverse outcomes of *P. falciparum* infection in pregnancy include inflammation, membrane thickening, perivillous fibrinoid deposits and syncytial knotting resulting in an altered exchange system between the mother and fetus (Davison et al., 2000).

In comparison to *P. falciparum*, very little is known about the placental pathology in vivax malaria. Although *P. vivax* can form rosettes, cytoadhesion is uncommon (Udomsanpetch et al., 1995). However, recent evidence suggest, that *P. vivax* infection is associated with increased maternal anaemia, miscarriage, intrauterine growth restriction and low birth weight (Nosten et al., 1999; McGready et al., 2012; Bardají et al., 2017).

1.5 Host genetics: from natural selection to innate defence

Infectious diseases have exerted significant pressure on the human genome. Malaria is considered to be the strongest evolutionary force (Kwiatkowski, 2005). Conceivably, this disease is one of the oldest infectious diseases co-existing with humans causing substantial morbidity and mortality. Particularly, *P. falciparum* is assumed to be existing since approximately 100,000 years. As a result of the overwhelming aftermath of this disease, humans have evolved (and keep evolving) by developing the protective genetic traits (Kwiatkowski, 2005).

The human genetic makeup and it's role with respect to infectious diseases has been widely studied (Abel and Dessein, 1997). However, the existing understanding of evolutionary selection due to malaria is largely related to *P. falciparum* infection and very little is known for *P. vivax*. Genetic variations (mutations or single nucleotide polymorphisms) in several populations confer resistance, or increase the disease risk, and sometimes interestingly play variable roles due to selection pressure (Driss et al., 2011; Withrock et al., 2015). For

example, sickle cell disease offers protection against malaria in endemic areas and at the same time, it is associated with susceptibility to respiratory infections. Another example is Tay-Sachs disease (causes mental and physical disabilities in infants), which is highly prevalent in the regions where tuberculosis has a low prevalence (Withrock et al., 2015).

Haldane – malaria hypothesis. J.B.S Haldane was the first to hypothesize a link between genetic polymorphisms and resistance to malaria. In the Eighth International Congress of Genetics held in 1948 at Stockholm, he gave a logical explanation for the high frequency of sickle cell and thalassemia in the Mediterranean. He proposed that the 'anaemic heterozygotes' are more resistant to attacks by the *Plasmodium* parasites, and that the frequency of these disorders increases as a result of natural selection of malaria protective traits (Haldane, 1949). Within a few years' time, the Haldane malaria hypothesis was validated by A. C. Allison, wherein he observed that the global distribution of the sickle cell trait (HbAS) was in accordance with malaria endemicity and that it was associated with low parasite counts (Allison, 1954). Several studies including a meta-analysis have confirmed that in individuals with sickle trait, a parasitaemia is considerably reduced as compared to HbAA, and that HbAS also offers substantial protection from malaria progressing to severe disease and death (Olumese et al., 1997; Aidoo et al., 2002; Cauwe et al., 2007; May et al., 2007). In line with this, in a malaria endemic region of Nepal, individuals from the Tharu group have a higher frequency of thalassemia and a parallelly lower malaria incidence as compared to other ethnic groups (Modiano et al., 1991). Thus hemoglobinopathies are considered to slice the 'Gordian knot' of host-parasite interactions to confer protection against malaria (Taylor et al., 2013).

1.5.1 Role of erythrocyte polymorphisms in malaria

Erythrocyte polymorphisms involved in sickle cell anaemia, thalassemia, glucose-6-phosphate – dehydrogenase deficiency, the ABO blood group system, and not lastly Duffy blood group system play a central role in conferring protection and/or increasing the malaria risk. Notable ones with respect to the current work are discussed below.

ABO blood group system. Similar to the selective pressure on the malaria-protective sickle cell trait, *P. falciparum* malaria has been observed to influence the global distribution of ABO blood groups (Cserti and Dzik, 2007). The ABO blood group system involves three carbohydrate antigens (A-B-H). The H antigen is converted to A or B by glycosyltransferase activity in individuals expressing A, B, and AB. Whereas, a single base deletion limits such

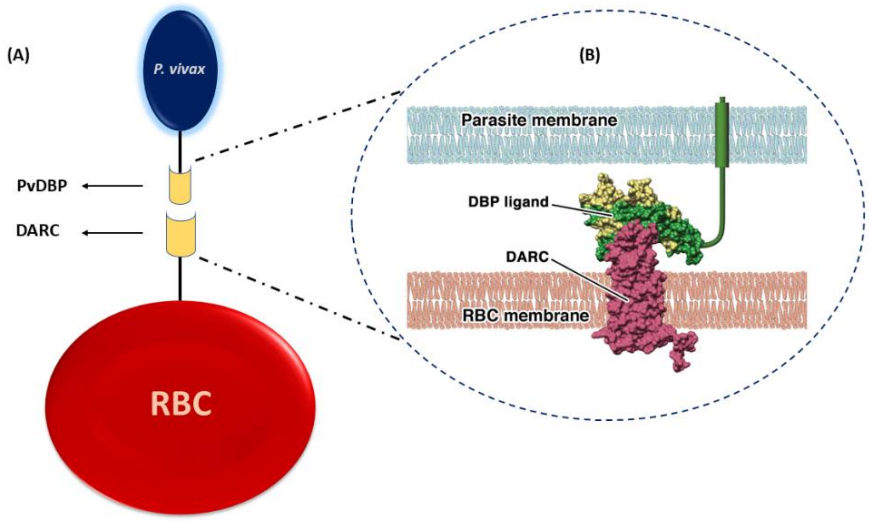
an activity in blood group O individuals (Yamamoto et al., 1990). Group A individuals have anti-B, group B individuals have anti-A, and AB individuals have neither of the antibodies in their plasma, whereas anti-A and Anti-B is present in O individuals. A and B glycosyltransferases are encoded by the *ABO* gene on chromosome 9. A and B allele products differ by four amino acid substitutions, i.e., Arg176Gly, Gly235Ser, Lue266Met and Gly268Ala. Variations at positions 266 and 268 are primary to determine A and B transferase activity (Daniels, 2005).

In malarious regions, the O blood group is reported in higher frequencies than non-O blood groups, particularly in Africa (Cserti and Dzik, 2007; Loscertales et al., 2007; Senga et al., 2007), and to exert protective effects against severe malaria across African populations (Rowe et al., 2007; Fry et al., 2008; Jallow et al., 2009; Timmann et al., 2012). In Asia, the O blood group was observed to be more common in individuals near to the equator. In Beijing, the prevalence of the group O is 29% whereas in Canton, a tropical area, group O is present at 46%. Group O was is common in Turkey and Persia. In colder regions, other blood groups, especially group A is common (Cserti and Dzik, 2007). A study from India reported that group O was common for all regions except in the North where the group B prevailed (Agrawal et al., 2014). In line with the above literature, several findings support the protective role of the group O including *in vitro* studies (Loscertales et al., 2007). For example, the RBCs belonging to group A and group B form more rosettes than group O RBCs *in vitro,* and in another study involving Malawian children, the odds of severe malaria was reduced by 66% in group O individuals (Udomsangpetch et al., 1993; Rowe et al., 1995).

In contrast to the observations mentioned above, the impact of the ABO blood group system on malaria during pregnancy is distinct and, moreover, the findings are ambiguous. For instance, blood group O was associated with an increased odds of placental malaria in primiparae but with a reduced risk in multiparae in Gambia and Malawi (Loscertales and Brabin, 2006; Senga et al., 2007). In Sudan, the blood group O and past placental infection were associated in both primi- and multiparae (Adam et al., 2007). In contrast, a Gabonese study reported a trend towards less placental malaria in women with the blood group O (Adegnika et al., 2011), and a recent study from Thailand showed that the ABO blood groups were not associated with malaria during pregnancy at all (Boel et al., 2012). In-depth, functional studies elucidating the role of the ABO antigens in malaria during pregnancy are needed.

Duffy blood group system. The Duffy blood group system (Duffy antigen receptor for chemokines, DARC, or Duffy antigens) is another influential component in *Plasmodium* infection, particularly in vivax malaria. DARC is a glycosylated erythrocyte membrane protein and the encoding gene is located on chromosome 1. A central *DARC* polymorphism, G125A, forms the basis of the Duffy blood group system, which leads to two major allele forms, i.e., glycine in FYA and aspartic acid in FYB. The resulting genotypes are FYA/FYB (phenotype; Fy (a+, b+)), FYB/FYB (Fy (a-, b+)), and FYA/FYA (Fy (a+, b-)). Another mutation, T-33C, in the GATA box silences the antigen expression (FYA^{ES} and FYB^{ES}; ES : Erythrocyte Silent) giving rise to Duffy blood group negativity (Fy (a-,b-)) (Höher et al., 2018). Duffy negativity is predominant in Africa, and it has been observed for long to confer protection against *P. vivax* and *P. knowlesi* infection (Miller et al., 1975; Horuk et al., 1993; Howes et al., 2011). In addition, C265T and G298A mutations give rise to the FYX allele, which is involved in weakening the FYB antigen expression. SNP G298A alone is not able induce this weakening effect (Höher et al., 2018).

The *P. vivax* Duffy binding protein (PvDBP) is a central ligand involved in the binding to DARC, and DARC on the reticulocyte is very essential for invasion (Figure 6). Specifically, PvDBP-RII binds to DARC by forming a heterotrimer and heterotetramer complex. DARC residues 19–30 are essential interaction sites for attachment of DBP-RII mediating the junction formation (Batchelor et al., 2014).



Adapted and modified from: Beeson JG, et al. PLoS Med. 2007;4(12):e350

Figure 5: Invasion of *P. vivax* merozoites into the reticulocyte.

(A) PvDBP mediated junction formation with DARC on the reticulocyte. (B) A model of the PvDBP binding to the DARC receptor that occurs during invasion of reticulocytes (inset of A).

DARC serves as a multi-specific receptor for a wide range of chemokines (Horuk et al., 1993; Pogo and Chaudhuri, 2000). In addition to its protective role in vivax malaria, DARC has been linked with several inflammatory and infectious diseases including increased rates of prostate cancer and asthma as well as an increased risk of HIV infection in case of Duffy blood group negativity (He et al., 2008; Horne and Woolley, 2009). Furthermore, DARC is essential for platelet-mediated killing of *P. falciparum* parasites (McMorran et al., 2012, 2013). Due to Duffy negativity and a subsequently low prevalence of vivax malaria in the African region, the resulting DARC association studies are primarily from South America and Asia.

In Brazil, FYA/FYA was reported to reduce the odds of vivax malaria, whereas the opposite was reported in another study (Albuquerque et al., 2010; King et al., 2011). In India, the FYA allele has been associated with a reduced vivax malaria incidence and the FYB allele with an increased one (Chittoria et al., 2012). Additionally, a few available studies from India did not report any association between DARC genotypes and vivax malaria (Singh et al., 1986; Kar et al., 1991; Verma and Thakur, 1993). DARC genotype associations with vivax malaria observed so far are diverse. Duffy negativity occurs only in a few tribal populations and the predominant genotype across India is FYA/FYA (Verma and Thakur, 1993; Chittoria et al., 2012). Since India contributes around 40% of *P. vivax* and 60% of *P. falciparum* cases globally, this country provides an interesting platform to examine the influence of DARC genotypes on vivax and falciparum malaria separately (WHO, 2018).

1.5.2 Polymorphisms in genes regulating immune mechanisms

TP53. TP53 is a tumour suppressor protein. Since its discovery, the role of TP53 in arresting cell cycle and apoptosis has been well studied, particularly in cancer. However, its functions outstretch from tumour suppressive activity to the regulation of metabolism, reactive oxygen level and autophagocytosis of the cell (Brady and Attardi, 2010). Recently, the role of TP53 was shown to be very crucial in modulating *Plasmodium* liver stage infection (Kaushansky et al., 2013). Mice experiments revealed that the liver-stage parasites suppress host TP53, thereby enhancing the parasite survival capacity. In contrast mice, expressing an extra copy of TP53 were able to counterbalance this effect and thus curbed the parasite proliferation (Kaushansky et al., 2013).

A single nucleotide variation at codon 72 located on the *TP53* gene leads to an amino acid substitution from proline to arginine (Pro72Arg). Arg72 allele is reported to be a more potent inducer of apoptosis and to confer various functional consequences (Dumont et al.,

2003). Additionally, this SNP is associated with an increased risk of human papilloma virus infection (HPV). Specifically, the E6 onco-protein degrades Arg72 more efficiently than the wildtype (Storey et al., 1998). Several studies have reported the association of this SNP with various cancers (Whibley et al., 2009). In addition to its role in cancer related diseases, a retrospective study from Sardinia, Italy, involving a limited number of subjects reported that Arg 72 is positively selected due to malaria and shows a malaria protective role (Gloria-Bottini et al., 2013). However, sufficiently powered case-control studies on this SNP may provide a better understanding of its association with malaria risk.

MicroRNA-146a. MicroRNAs, generally addressed as miRNAs, are a family of short length (~22 nucleotides) non-coding, evolutionarily conserved RNA molecules. MiRNAs are proved to play an imperative role in posttranslational regulation of protein expression. MiRNAs have a distinct ability of being multivalent, which means that a single miRNA is able to target multiple genes (Tanase et al., 2012). The miRNA named lin-4 was the first to be discovered in *Caenorhabdtis elegans*, which was followed by the discovery of let-7 in the same species. In the past two decades, several human miRNAs have been identified and, at present, there are approximately 2500 miRNA sequences listed in the repository (Peng and Croce, 2016). The role of miRNAs in cancer has been adequately studied so far. Their diverse function in cancer include the regulation of proliferative signalling and growth suppressors, cell differentiation, migration, and apoptosis (Tan et al., 2017). In addition, miRNAs are able to control innate and adaptive immune pathways by regulating the proteins involved (Mehta and Baltimore, 2016).

MiRNA-146a was found to interfere in TLR signalling *via* a negative feedback loop by involving two crucial components of the immune system: interleukin-1 receptor-associated kinase-1 (IRAK1) and TNF receptor-associated factor-6 (TRAF6) (Taganov et al., 2006). Moreover, a point mutation in miRNA-146a, rs2910164 G > C, was found to alter IRAK-1 and TRAP-6 levels by impairing the miRNA activity (Taganov et al., 2006). The TLR machinery and associated molecules have been shown to influence malaria risk *per se* and to play an important part in disease progression and immune tolerance (Jide et al., 2009).

SNP rs2910164 is located at position +60 upfront the first nucleotide on the passenger strand of pre-miRNA-146a (Jazdzewski et al., 2008). This SNP has been previously associated with altered risks of several cancers and autoimmune diseases, and with tuberculosis and leprosy (Li et al., 2011; Cezar-de-Mello et al., 2014; Park et al., 2016; Hao et al., 2018).

1.6 Combating malaria: a hard task in the era of growing antimalarial resistance

Nearly half of the world's population is at risk of malaria (WHO, 2018). Developing fully effective vaccines has not been successful so for. The RTS,S vaccine offers moderate protection in African children (Olotu et al., 2013), there are no highly effective vaccines available in the market covering all age groups. Hence, exercising precaution and prevention measures are of high importance. These include vector control (insecticide treated mosquito nets, indoor residual spraying, larval source management, improved housing and locality), mass drug administration, efficient diagnostics and early treatment. Accurate diagnosis of malaria is very essential to achieve an effective disease management. During the early days of infection, since malaria symptoms are very general, it is difficult to make a sound clinical diagnosis. Therefore, laboratory-based confirmation is crucial. Malaria endemic and non-endemic countries have distinct difficulties in diagnostics. In endemic countries, due to partial immunity there might be none, or very limited clinical presentation whereas, in non-endemic countries, malaria is rare and mostly imported. Microscopy is the most widely used of all diagnostic tools for routine diagnosis. However, limitations of microscopy such as wrong interpretation especially in case of mixed infections may lead to ineffective treatment outcomes (Barber et al., 2013).

The first chemically purified antimalarial, quinine, was isolated in 1820. Since then several drugs including the natural ones have been developed (Figure 5).

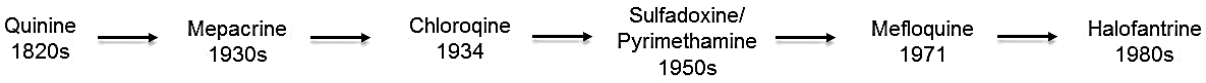


Figure 6: Notable antimalarial drugs discovered between 1820 and 1980

Quinine was isolated from the bark of the cinchona tree and it is still been regarded as one of the most efficient drugs (Achan et al., 2011). However, quinine resistant parasites were suspected for the first time in Rio de Janeiro in 1907, and later in 1910, and resistance was confirmed among German workers returning from Brazil during that time (da Silva and Benchimol, 2014). Mepacrine, which is a derivative of methylene blue, was broadly used during World War II as a prophylactic drug, but not anymore due to its strong side effects (Weina, 1998). Whereas, methylene blue and derivatives have gained renewed interest in recent years. Chloroquine (CQ) was widely used to treat all malaria forms since the 1940s.

Just after a decade of its use, the first case of resistance in *P. falciparum* was reported in 1950. Since then CQ resistance among *P. falciparum* has spread globally with few pockets of susceptibility remaining, e.g. in Central America (Payne, 1987; Wellems and Plowe, 2001). Still CQ is used for treating vivax malaria in most endemic regions.

Currently used antimalarials are divided into several classes; i.e., quinoline derivatives, antifolates, antibiotics and artemisinin derivatives (Table 1) (Cui et al., 2015). With emergence of resistance of *P. falciparum* to old drugs, artemisinin-based combination therapy (ACT) is today recommended for uncomplicated falciparum malaria (Nosten and White, 2007). The artemisinin component of the ACT rapidly eliminates parasites, whereas, the partner drug has a long-lasting effect on removal of remaining parasites and reduces the selection pressure for artemisinin resistance (Nosten and White, 2007). Among the currently administered drugs, ACTs are the most effective. Artemisinin was first isolated by Tu Youyou from the plant *Artemisia annua* (herb commonly used in Chinese medicine) in 1971. The commonly accepted theory on the mode of action of artemisinin is that haem activates this molecule, generating free radicals which have the ability to damage the proteins required for parasite survival (J. Wang et al., 2015). This drug has shown great efficacy in case of multi-drug resistant *P. falciparum*. Semi-synthetic lactol derivatives of the artemisinin molecule, namely artemether, artesunate and dihydroartemisinin display enhanced bioavailability and efficacy (O'Neill and Posner, 2004) and, therefore, they have been promising drug candidates in ACTs. However, the first evidence of resistance to artemisinin was reported in 2008 in Cambodia (Noedl et al., 2008), followed by 30 independent cases reporting resistance specifically to the dihydroartemisinin – piperaquine combination ten years later in the Southeast Asian region (Amato et al., 2018).

CQ remains the standard treatment for *P. vivax* malaria, given as CQ *plus* primaquine (Nosten and White, 2007). In areas with reportedly reduced CQ efficacy, WHO recommends ACT *plus* primaquine as an alternative treatment (WHO, 2015).

With the recent emergence of resistance to artemisinin and its derivatives (front line antimalarials), there is an immediate need for the development of new drug molecules, particularly of those with a novel mode of action. High-throughput screening techniques have allowed researchers to identify new candidates with promising antimalarial properties, some of which are under development (Tse et al., 2019).

Table 1: Classification of antimalarials and their use

Class	Drug	Use
4-Aminoquinoline	Chloroquine	Treatment of non-falciparum malaria
	Amodiaquine	Partner drug for ACT
	Piperaquine	ACT partner drug with dihydroartemisinin as ACT
8-Aminoquinoline	Primaquine	Radical cure and terminal prophylaxis of *P. vivax* and *P. ovale*; gametocytocidal drug for *P. falciparum*
	Quinine	Radical cure of P. vivax and P. ovale Treatment of *P. falciparum* and severe malaria
Arylamino alcohol	Mefloquine	Prophylaxis and partner drug for ACT for treatment of falciparum
	Lumefantrine	Combination with artemether as ACT
Sesquiterpene lactone endoperoxides	Artemether	ACT: combination with lumefantrine
	Artesunate	Treatment of severe malaria and as ACT
	Dihydroartemisinin	ACT: combination with piperaquine
Mannich base	Pyronaridine	Combination with artesunate as ACT
Antifolate	Pyrimethamine/sulfadoxine	Treatment of chloroquine-resistant parasites; Combination with artesunate as ACT
Naphthoquinone/antifolate	Atovaquone/proguanil	Combination for prophylaxis and treatment of *P. falciparum* (Malarone)
Antibiotic	Doxycycline	Chemoprophylaxis; treatment of *P. falciparum* in combination with quinine
	Clindamycin	

Adapted and modified from Cui et al, *Am. J. Trop. Med. Hyg*., 93 (Suppl 3), 2015, pp. 57–68

1.6.1 Antimalarial drug resistance: an evolving threat to global health

Parasite resistance to, and reduced efficacy of antimalarials has often been a major hurdle in fighting malaria. There are several ways to assess resistance, which include clinical trials, *ex vivo* and *in vitro* assays (*P. falciparum*) and genetic polymorphisms related to drug resistance. Genetic aberrations in the parasite genome are the central reason resulting in resistance to antimalarials, namely, mutations in or copy number variations of genes encoding drug parasite targets or in genes encoding influx/efflux pumps (White, 2004).

Resistance - *P. falciparum*. The *P. falciparum* genome encodes multiple proteins which are associated with the transport of drugs. In general, polymorphisms in transport proteins have been linked to increased efflux of drugs from the cells, which can mediate resistance (Borges-Walmsley et al., 2003). In *P. falciparum* isolates, polymorphisms in the genes multidrug resistance protein-1 (*pfmdr1*) and multidrug resistance protein-2 (*pfmdr2*), chloroquine resistance transporter (*pfcrt*), multidrug resistance associated protein (*pfmrp1)* and sodium/hydrogen exchanger (*pfnhe1*), dihydropteroate synthase (*dhps*) and dihydrofolate reductase *(dhfr)* have been associated with resistance to several antimalarials and, recently, Kelch 13 (*K13*) to delayed parasite clearance following artemisinin treatment.

Pfmdr1. Mutations in *pfmdr1* have been reported to modulate susceptibility to several antimalarial drugs (Naß and Efferth, 2019). The *pfmdr1* mutations N86Y and D1246Y have been associated with reduced sensitivity to quinine, CQ and amodiaquine, whereas, the respective wildtype alleles appear to confer resistance to lumefantrine, mefloquine and artemisinin (Cui et al., 2015). CQ resistant strains carrying *pfmdr1* N86, C1034, N1042, Y1246 displayed decreased sensitivity to quinine (Sidhu et al., 2005). The function of *pfmdr1* was questionable after observations revealed low *pfdmr-1*^{CQR} copy numbers in CQ resistant isolates (Wellems et al., 1990; Basco et al., 1995). Furthermore, a study from Thailand found no correlation between N86Y and the spread of chloroquine resistance (CQR) (Mungthin et al., 2014). These ambiguities led to the conclusion that the mutations in *pfmdr1* are not solely responsible in modulating CQ susceptibility but other mutations, i.e., in/of *pfcrt* and *pfmrp1* might be involved.

***Pfcrt*.** *Pfcrt* plays a pivotal role in CQR. So far, around 32 *pfcrt* polymorphic residues have been identified. Among them, at least 11 are linked to CQR, which include C72S, M74I, N75E, K76T, H97Q, A220S, Q271E, N326S, I356T, C350S, and R371I (Ibraheem et al., 2014). A meta-analysis showed that the risk of CQ therapeutic failure was a consequence of the presence of the K76T mutation (Picot et al., 2009). In addition to CQ, *pfcrt* haplotypes also influenced the susceptibility to amodiaquine, piperaquine and lumefantrine (Menard et al., 2006; Echeverry et al., 2007). In conclusion, *pfcrt* K76T in combination with mutations at other codons and with *pfmdr1* mutations mediates CQR by increased efflux of CQ from the digestive vacuole of the parasite (Ecker et al., 2012).

***Dhps/Dhfr*.** Dhps and dhfr are two *P. falciparum* enzymes in the folate pathway targeted by antimalarial drugs. Dhps, which is involved in catalysing the synthesis of a folate precursor is the target of the sulfur-based drugs sulfadoxine and dapsone. Dhfr reduces dihydrofolate into tetrahydrofolate and its function is disrupted by the antifolate drugs and competitive enzyme inhibitors pyrimethamine and cycloguanil (Petersen et al., 2011). Point mutations (A16V, C50R, N51I, C59R, S108N/T, V140L and I164L) in *dhfr* and in *dhps* (I431V, S436A/F, A437G, K540E, A581G and A613S/T), which accumulate in *P. falciparum* isolates, have been associated with sulfadoxine/pyrimethamine (SP) resistance (Peterson et al., 1990; Plowe et al., 1997; Sutherland et al., 2009; Chauvin et al., 2015). The triple *dhfr* mutant (N51I, C59R, and S108N) displays 225 times higher resistance to pyrimethamine *in vitro* as compared to wildtype *P. falciparum* (Nzila-Mounda et al., 1998). The quintuple mutant consisting of *dhfr* triple and *dhps* double mutations (A437G and K540E) is considered to predict clinical SP resistance (Mugittu et al., 2004). SP as a first line drug in treating falciparum malaria is no longer recommended. However, it is still used for

intermittent preventive treatment in pregnancy, and as part of ACT in India (ter Kuile et al., 2007; Anvikar et al., 2014).

***Kelch 13*.** First reports of reduced efficacy and delayed parasite clearance following treatment with artemisinin derivatives (ARTs) originated from the Cambodia-Thailand border in 2008, which happens to be the epicenter of antimalarial drug resistance (Noedl et al., 2008; Wongsrichanalai and Meshnick, 2008). ARTs are front-line antimalarial drugs currently used worldwide for the treatment of falciparum malaria. Emerging resistance to these drugs is a great threat in combating malaria. Recently, *K13* propeller variants were identified as markers of resistance to artemisinin (Ariey et al., 2014). *K13* resistance polymorphisms validated by *in vivo* and *in vitro* studies include N458Y, Y493H, R539T, I543T, C580Y, and several other candidate mutations associated with delayed parasite clearance have been reported. In addition, two non-propeller domain mutations K189T and E252Q were observed frequently in clinical studies (WHO, 2017).

Resistance - *P. vivax*. The molecular basis of *P. vivax* resistance to antimalarial drugs has not been studied as extensively as in *P. falciparum*. The orthologs of *Pfmdr1* and *pfcrt* genes, *P. vivax* multidrug resistance-1 (*pvmdr1*) and transporter protein (*pvcrt-o*) have been identified as markers of CQR in *P. vivax* isolates. Whole genome sequence analysis has revealed that *pvmdr1* harbours 24 single nucleotide polymorphisms (SNPs), whereas *pvcrt-o* only five SNPs *plus* a lysine insertion at position 10 (Orjuela-Sánchez et al., 2009). Several polymorphisms (notably, Y976F and F1076L) in *pvmdr1* have been linked with CQR. Particularly, Y976F in the *pvmdr1* gene is reported to be associated with reduced CQ sensitivity in studies from South-east Asia and with increased IC50 values for CQ *in vitro* (Suwanarusk et al., 2007, 2008; Nyunt et al., 2017). In *P. falciparum* transformed with pvcrt-o, a 2.2-fold decrease in susceptibility to CQ was observed, suggesting a possible role of pvcrt-o in *P. vivax* CQR and the K10 insertion in the *pvcrt-o* as a possible molecular marker of CQR (Sá et al., 2006). Among several mutations identified in *pvdhfr*, point mutations F57L/I, S58R, T61M, and S117T/N are considered to confer antifolate resistance in *P. vivax* (Hawkins et al., 2007). Furthermore, findings of *P. falciparum K13* polymorphisms and their association with artemisinin resistance have paved the way to investigate the orthologue *P. vivax Kelch 12* gene (pvk12). A study from Cambodia observed a *pvK12* SNP (V552I) at a low level, suggesting that the SNP is not selected by artemisinin drug pressure unlike the *K13* mutations in *P. falciparum* (Popovici et al., 2015). Nonetheless, it cannot be ruled out as the study region has not observed artemisinin resistant *P. vivax* isolates.

Note: Introduction (some parts) and discussions for the current work findings are adapted from own/co-authored publications, and their respective cross-references are provided in each section.

2. Objectives and aims

2.1 Factors influencing malaria in India

India contributes to half of the global *P. vivax* cases and at the same time, around 60% of malaria cases in this country are due to *P. falciparum*. Severe vivax malaria is uncommon, but an increasing number has been reported in recent years (Rahimi et al., 2014). Studies with thorough examination including properly set defining criteria for severe malaria are needed to understand the causal factors that lead to severity. Host genetics plays a central role in modulating the innate immune response. Associations of host polymorphism with malaria and their functional aspects with respect to *P. falciparum* infection have been studied for several decades. However, such studies are limited in vivax malaria, and particularly from India they are rare. Recently, resistance to artemisinin combination therapy has been reported from Southeast Asia, which is a huge obstacle in malaria control. Even though no such cases have been reported from India, it is of immediate need to investigate the problem proactively by screening for validated molecular markers of drug resistance.

Mangaluru, located in the Southwest of India on the banks of Arabian Sea, provides a very broad malaria scenario for addressing the afore mentioned problems. Mangaluru receives an average rainfall of 3450 mm annually with one monsoon season between May and October (ClimaTemps.com, 2017). Governmental records indicate that Mangaluru contributes to around 60% to the total malaria cases in the Karnataka state, however, with the majority of cases occurring in urban Mangaluru. Among these, 82% of infections are due to *P. vivax*. Mangaluru receives a considerable number of migrant workers due to its increasing urbanization, and it seems that there is a substantial importation of malaria from other endemic parts of India (Shivalli et al., 2016; Dayanand et al., 2017). However, the present clinical presentation of malaria, including the status of antimalarial drug resistance in Mangaluru is not well studied.

Against this background, we conducted a non-interventional malaria case-control study in Mangaluru, and particularly aimed at the following.

Specific objectives:

The primary objectives of this study were:

(i) to describe the clinical manifestation pattern of malaria and vivax malaria in particular, and

(ii) to examine the role of *DARC* gene polymorphisms in influencing susceptibility or resistance to malaria.

The secondary objective was:

(iii) to investigate the molecular markers of drug resistance in *P. falciparum* and *P. vivax* isolates.

2.2 Factors influencing malaria in Africa

In high transmission areas of Africa, namely, Rwanda and Ghana, asymptomatic infection is common. Moreover, risk and manifestation of malaria in children and in pregnant women are influenced by diverse factors including host genetics. Examining host genetic polymorphisms in such patient groups would allow a better understanding of their diverse roles.

Specific objectives:

We primarily aimed at examining the associations of

(i) *TP53* Pro72Arg SNP with *P. falciparum* infection in pregnant women and in children,

(ii) ABO genotypes in malaria during pregnancy, and

(iii) miRNA 146-a rs2910164 G > C SNP in malaria during pregnancy.

The spread of artemisinin resistant *P. falciparum* parasites poses a huge global threat. To have an overview on the spread of resistant parasites and in turn to react with immediate control measures, active screening of the validated molecular makers of resistance is needed.

(iv) Secondarily, we aimed at investigating the presence and associations of *Kelch 13* polymorphisms in *P. falciparum* isolates from Rwanda collected at different time points.

3. Original articles

3.1 Manifestation of malaria in Mangaluru, southern India

3.2 Duffy antigen receptor for chemokines gene polymorphisms and malaria in Mangaluru, India

3.3 No association of the *p53* codon 72 polymorphism with malaria in Ghanaian primiparae and Rwandan children

3.4 Reduced prevalence of placental malaria in primiparae with blood group O

3.5 MiRNA-146a polymorphism increases the odds of malaria in pregnancy

3.6 Artemisinin resistance-associated *K13* polymorphisms of *Plasmodium falciparum* in Southern Rwanda, 2010-2015

3.7 Molecular evidence for *Plasmodium falciparum* resistance to sulfadoxine-pyrimethamine but absence of *K13* mutations in Mangaluru, Southwestern India

3.8 Characterization of *Plasmodium vivax pvmdr1* polymorphisms in isolates from Mangaluru, India

Gai *et al. Malar J (2018) 17:313*
https://doi.org/10.1186/s12936-018-2462-7

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Manifestation of malaria in Mangaluru, southern India

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Abstract

Background: Severe and fatal vivax malaria is increasingly reported from India. In Mangaluru, southern India, malaria is focused in urban areas and associated with importation by migrant workers. In Wenlock Hospital, the largest governmental hospital, the clinical, parasitological and biochemical characteristics of malaria patients were assessed.

Methods: During the peak malaria season in 2015 (June to December), outpatients were interviewed and clinically assessed. Malaria was ascertained by microscopy and PCR assays, concentrations of haemoglobin, creatinine and bilirubin, as well as thrombocyte count, were determined, and severe malaria was defined according to WHO criteria.

Results: Among 909 malaria patients, the vast majority was male (93%), adult (median, 26 years) and of low socioeconomic status. Roughly half of them were migrants from beyond the local Karnataka state, mostly from northern and northeastern states. Vivax malaria (69.6%) predominated over mixed *Plasmodium vivax–Plasmodium falciparum* infection (21.3%) and falciparum malaria (9.0%). The geometric mean parasite density was 3412/µL. As compared to vivax malaria, patients with falciparum malaria had higher parasite density and more frequently showed impaired general condition, affected consciousness and splenomegaly. Also, they tended to more commonly have anaemia and increased creatinine levels, and to be hospitalized (7.3%). Mixed-species infections largely assumed an interim position. Severe malaria (3.5%) was not associated with parasite species. No fatality occurred.

Conclusion: In this study, uncomplicated cases of malaria predominated, with *P. falciparum* causing slightly more intense manifestation. Severe malaria was infrequent and fatalities absent. This contrasts with the reported pattern of manifestation in other parts of India, which requires the analysis of underlying causes.

Keywords: Malaria, India, Mangaluru, *Plasmodium vivax*, *Plasmodium falciparum*, Admission, Severe, Fatal

Background

India has achieved major reductions in the burden of malaria in the last decade. However, the country still contributes 6% of global malaria cases and accounts for approximately half of the total *Plasmodium vivax* cases worldwide [1, 2]. *Plasmodium vivax* has long been regarded a rather benign disease, irrespective of its substantial morbidity in Asia and Central and South America. However, severe vivax malaria has been increasingly reported in recent years, particularly from India [3–6]: among those hospitalized with *P. vivax* mono-infection, 11–45% developed severe malaria, including cerebral malaria and fatalities, in various settings in the country [7–9]. A recent systematic review and meta-analysis on severe vivax malaria since 1900 revealed that the majority of reports originated from India, that severe thrombocytopaenia (< 50,000/µL) was the most common defining symptom and that the overall case fatality rate was 0.3% [6]. Nevertheless, prevalence syndromes and fatality of severe vivax malaria appears to differ with defining criteria, age, endemicity and geographical setting, and rate of co-morbidities, among others [3, 6]. For instance, in one study from Western New Guinea, severe anaemia

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(haemoglobin (Hb) < 6 g/dL) was the predominant feature of severe vivax malaria [10], while respiratory distress was reported to predominate in neighbouring Papua New Guinea [11]. The role of mixed *P. vivax–P. falciparum* infections in terms of clinical manifestation and severity is not well established, not simply because of the very low sensitivity of conventional microscopy in detecting the minority species [12–14]. In mixed-species infections, both increased severity compared to *P. vivax* mono-infection but beneficial effects such as curbed peak *P. falciparum* parasite density have been observed [9, 11, 12, 15, 16].

In Mangaluru (population approximately 500,000), a harbour city in Karnataka, southern India, malaria shows particular characteristics. Located on the shores of the Arabian Sea with its hot and humid climate, Mangaluru receives an annual average of 3450 mm rainfall with one monsoon season between May and October [17]. Malaria episodes in the district reduced by two-thirds between 2005 and 2013 [18]. However, governmental records indicate a minimum of 6000 episodes of malaria per year in Mangaluru, with most cases occurring in urban rather than in rural areas. Among these, *P. vivax* dominates (82%), and migrant workers cause substantial importation of malaria from other parts of India [19, 20]. The urban nature of malaria in Mangaluru is largely attributed to the abundance of inner-city construction sites with stagnant water bodies, the migration of workers from malaria-endemic parts of India to work on these sites, and their poor housing conditions [19, 20]. *Anopheles stephensi* is the perdominant vector in this area [21].

Published data on the current clinical presentation of malaria in Mangaluru are virtually absent. The present study aimed at providing a description of the manifestation of malaria at the largest governmental health facility in Mangaluru, the 900-bed Wenlock Hospital, and to specifically assess differences between *P. vivax* and *P. falciparum* mono-infections as well as mixed-species infections.

Methods

Study site

Mangaluru (Mangalore) is a harbour city of 485,000 inhabitants (agglomeration, 624,000; 2011 national census data) located at the Arabian Sea in Karnataka, South India. Wenlock Hospital (900 beds) is the largest governmental hospital in Mangaluru offering treatment particularly for the economically deprived part of the population. In addition, several private hospitals provide health services in the absence of primary health care facilities in this urban setting. In 2014, Wenlock Hospital reported 6767 malaria cases, 80.1% being *P. vivax* mono-infections. Patients attending the outpatient department showing symptoms suspicious of malaria are directed to the hospital's malaria diagnostic unit. From June to December 2015, during the peak malaria season, malaria patients were recruited at the malaria diagnostic unit during the operating hours of the outpatient department (08:00–16:00). Patients attending at other times were not considered. Patients confirmed to have malaria were treated according to standard guidelines on an outpatient basis, i.e., chloroquine for 3 days plus primaquine for 14 days for vivax malaria; artesunate–sulfadoxine–pyrimethamine for 3 days plus single dose primaquine on the second day in case of falciparum malaria; and, artesunate–sulfadoxine–pyrimethamine for 3 days plus primaquine for 14 days in case of mixed *P. vivax–P. falciparum* infection. Admission to ward was based on the attending physician's discretion. Patients were enrolled into the study upon microscopic diagnosis of malaria, and all study participants provided informed written consent. The study protocol was reviewed and approved by the Institutional Ethics Committee of Kasturba Medical College, Mangaluru, Manipal University, and permission to conduct the study was granted by the Directorate of Health and Family Welfare Services, Government of Karnataka.

Examinations

Upon recruitment, patients were interviewed using preformed questionnaires on socio-economic parameters, education, occupation, household characteristics, and malaria-related behaviour. A medical history was taken and a clinical examination was performed in all patients applying standardized documentation forms. Weight and height were measured. Body mass index (BMI) was calculated as kg/m^2 and fever defined as axillary temperature ≥ 37.5 °C. Venous blood was collected into EDTA. Malaria parasites were counted per 200 white blood cells (WBCs) on Giemsa-stained thick blood films, and parasite species defined based on thin-film microscopy. Following DNA extraction (Qiamp blood mini kit, Qiagen, Germany), *Plasmodium* species was ascertained by nested polymerase chain reaction (PCR) assays [14]. Plasma was separated from blood by centrifugation. Routine hospital laboratory services provided results for haemoglobin (Hb) concentration (photometrically), WBCs and thrombocytes counts (Coulter principle) as well as concentrations of creatinine (Jaffé reaction), total bilirubin (DPD method), and direct bilirubin (Jendrassik–Grof method). Abnormal laboratory values were defined as: anaemia Hb < 11 g/dL (< 5 years), < 11.5 g/dL (5 to < 12 years), < 12 g/dL (12 to < 15 years, or females ≥ 15 years) and < 13 g/dL (males ≥ 15 years) [22]; leukocytosis > 10,000 WBCs/μL; thrombocytopaenia < 150,000/μL; increased creatinine > 1.4 mg/dL; increased total bilirubin > 1.2 mg/dL, and increased

direct bilirubin > 0.2 mg/dL. Severe malaria was defined based on the current WHO definition [23] with some modifications. In particular, hypotension (systolic blood pressure < 80 mmHg in adults and < 70 mmHg in children) was considered a sign of severe malaria irrespective of the absent assessment of capillary refill or impaired perfusion; acidosis and hypoglycaemia were not routinely assessed; confusion in adult patients with a Glasgow coma score > 11 was also considered indicative of severe malaria. Of note, definitions of renal impairment (plasma creatinine > 3 mg/dL, or urea > 20 mM) and jaundice (plasma bilirubin > 3 mg/dL plus parasitaemia > 100,000/μL) followed WHO criteria as did the definition of severe malarial anaemia (Hb < 5 g/dL in children, or Hb < 7 g/dL in adults plus parasitaemia > 10,000/μL; no parasite density threshold for vivax malaria).

Statistical analysis

Patients were considered for analysis if they had microscopically visible and PCR confirmed parasitaemia and, for admitted patients, if malaria diagnosis was available within 24 h. Data analysis was performed using SPSS 22 (IBM Corp., Armonk, NY, USA) and Statview 5.0 (SAS Institute Inc., Cary, NC, USA). Continuous parameters were compared between groups by Student's *t* test, analysis of variance (ANOVA), Mann–Whitney U-test, or Kruskal–Wallis test as applicable. Proportions were compared between groups by Chi square (χ^2) test or Fisher's exact test, and odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. Logistic regression was used to calculate adjusted odds ratios (aORs). Independent predictors of severe malaria were calculated by logistic regression analysis including factors showing association with severe malaria at a level of $P < 0.10$ and with backward removal of factors not associated in multivariate analysis ($P > 0.05$). A *P*-value < 0.05 was considered statistically significant.

Results

A total of 909 patients with microscopically visible parasitaemia and PCR confirmed *Plasmodium* species were analysed (Table 1). Their median age was 26 years, the vast majority was male (92.8%), and most individuals (77.8%) had migrated to Mangaluru a median period of 6 months before presentation (range 1–600 days), predominately for working (96.0%; 680/708). Roughly half of the patients were migrants originating from beyond the state of Karnataka, and among them, most (82.8%, 371/448) were from northern and northeastern Indian states (West Bengal, Jharkhand, Uttar Pradesh, Bihar, Odisha, Assam). More than half of the patients (56.1%, 510/909) were either construction workers or daily labourers, and this figure was 76.3% (283/371) among the north/northeastern migrants. Most of the patients were from a low socio-economic status (SES) background (Table 1). More than two-thirds had incomplete or no formal education. The median monthly family income was approximately €80, on which a median of four individuals (1–15) were dependent. Although electricity was available in most households, household assets were limited. Approximately 40% of the patients stated using either bed net or repellent coils for malaria prevention. Almost half of the patients (45.8%, 415/906) reported to have had malaria before, the vast majority of those within the preceding year (82.7%, 343/415). Anti-malarial treatment (chloroquine and/or primaquine) within the preceding 4 weeks was reported by 0.6% (5/908) of patients and current medication by 1.3% (12/908; most commonly antihypertensives, 4; histamine-2 blockers, 4; antidiabetics, 3).

Most of the patients had vivax malaria (69.6%, 633/909), 21.3% (194/909) harboured both *P. vivax* and *P. falciparum*, and 9.0% (82/909) of individuals had falciparum malaria. However, these proportions changed over time.

Precipitation was highest in June to August 2015 (1836 mm) and declined thereafter in September to December (543 mm). In parallel, the proportion of *P. vivax* mono-infection declined from 90.4% (104/115) in June to a low of 53.3% (97/182) in August and increased thereafter (Fig. 1). Socio-demographic factors had limited influence on parasite species. As compared to patients with *P. vivax* or mixed species infections, those with falciparum malaria tended to be older and to less commonly have formal education. The proportion of low income was highest among falciparum malaria patients and ownership of wealth indicators (e.g., TV set, motorbike) was lowest. Migration and origin did not overtly affect parasite species; nevertheless, *P. falciparum* mono-infection tended to be less common in patients originating from outside the state of Karnataka (OR, 0.75 (95% CI, 0.46–1.21), $P = 0.21$, Table 1) and this difference was statistically significant for north/northeastern migrants (5.4% (20/371) vs 11.5% (62/538); OR, 0.44 (95% CI, 0.25–0.76), $P = 0.002$). At the same time, falciparum malaria was comparatively common among construction workers/daily labourers (Table 1). In multivariate analysis, including origin and occupation (and month of recruitment), falciparum malaria was positively associated with being a construction workers/daily labourer (aOR, 2.49 (95% CI, 1.49–4.17), $P = 0.0005$) and negatively with migration from North/northeastern India (aOR, 0.33 (95% CI, 0.19–0.58), $P = 0.0001$). Malaria prevention, with the potential exception of window nets, as well as previous (Table 1) or recent (Table 2) malaria episodes did not influence parasite species.

Table 1 Characteristics of 909 malaria patients from Mangaluru, India

Parameter	All	*P. vivax*	*P. vivax/P. falciparum*	*P. falciparum*	*P*
No. (%)	909 (100)	633 (69.6)	194 (21.3)	82 (9.0)	–
Age (years; median, range)	26.0 (4.0–82)	25.0 (4–70)	27.5 (7–82)	30.5 (12–65)	0.11
Male (%, n)	92.8 (844)	93.4 (591)	92.8 (180)	89.0 (73)	0.36
Migrated to Mangaluru (%, n)	77.8 (706/907)	77.3 (488/631)	80.4 (156)	75.6 (62)	0.58
Origin (%, n)					
Mangaluru	22.1 (201)	22.6 (143)	19.6 (38)	24.4 (20)	
Karnataka	27.3 (248)	25.3 (160)	31.4 (61)	32.9 (27)	
Non-Karnataka states	49.3 (448)	50.9 (322)	46.9 (91)	42.7 (35)	
No data/unclear	1.3 (12)	1.3 (8)	2.1 (4)	0	0.31
Self-defined tribal origin (%, n)	18.3 (166)	19.0 (120)	18.0 (35)	13.4 (11)	0.47
Formal education					
None	32.8 (298)	30.2 (191)	38.1 (74)*	40.2 (33)	
Incomplete (< 10th class)	38.0 (345)	39.2 (248)	37.1 (72)	30.5 (25)	
Completed (10th class)	13.9 (126)	15.0 (95)	10.8 (21)	12.2 (10)	
Advanced [(pre-)university]	14.6 (133)	15.3 (97)	12.4 (24)	14.6 (12)	
No data/unclear	0.8 (7)	0.3 (2)	1.5 (3)	2.4 (2)	0.06
Occupation construction worker or daily labourer (%, n)	56.1 (510)	53.7 (340)	58.8 (114)	68.3 (56)*	*0.03*
Monthly family income (rupees; median, range), n = 893	6000 (0–35,000)	7000 (0–35,000)	6000 (0–30,000)	6000 (2000–20,000)*†	*0.006*
Monthly family income < median (6000 rupees)	35.2 (314/893)	33.0 (205/621)	36.3 (69/190)	48.8 (40)*	*0.02*
People living in household (number; median, range), n = 889	5 (1–70)	5 (1–70)	5 (1–40)	4 (1–25)*†	*0.02*
People per room in household (number; median, range), n = 870	4 (0.25–70)	4 (0.25–70)	4 (0.50–40)	3 (0.67–25)*†	0.09
Household characteristics (%, n)					
Electricity	94.4 (857/908)	95.3 (602/632)	92.8 (180)	91.5 (75)	0.21
Electric fan	70.0 (636/908)	70.6 (446/632)	71.6 (139)	62.2 (51)	0.26
TV set	18.9 (172/908)	21.4 (135/632)	14.9 (29)	9.8 (8)*	*0.01*
Fridge	5.2 (47/908)	5.9 (37/632)	4.6 (9)	1.2 (1)	0.19
Motorbike	3.6 (33/908)	4.9 (31/632)	1.0 (2)*	0*	*0.008*
Radio	2.8 (25/908)	3.0 (19/632)	1.5 (3)	3.7 (3)	0.48
Bicycle	1.7 (15/908)	1.7 (11/632)	1.5 (3)	1.2 (1)	0.93
Stated use of a bed net in preceding night (%, n)	39.1 (354/906)	41.0 (259/631)	35.2 (68/193)	32.9 (27)	0.17
Window nets present (%, n)	4.2 (38/906)	4.0 (25/631)	6.7 (13/193)	0†	*0.04*
IRS in preceding 6 months (%, n)	2.8 (25/906)	3.3 (21/631)	1.6 (3/193)	1.2 (1)	0.28
Stated use of repellent coils (%, n)	39.3 (356/906)	39.6 (250/631)	40.4 (78/193)	34.1 (28)	0.59
Stated use of repellent fluids (%, n)	9.7 (88/906)	10.0 (63/631)	8.3 (16/193)	11.0 (9)	0.72
Stated stagnant water bodies at home (%, n)	31.0 (281/906)	32.0 (202/631)	27.5 (53/193)	31.7 (26)	0.48
Stated malaria episode in preceding 12 months (%, n)	37.9 (343/906)	38.8 (245/631)	34.2 (66/193)	39.0 (32)	0.50
Time since last malaria episode (months; median, range), *n* = 415	5 (0.2–240)	4 (0.2–180)	6 (0.5–240)	9 (0.5–120)	0.19

* P < 0.05 as compared to vivax malaria; † P < 0.05 as compared to mixed species malaria

The geometric mean parasite density (GMPD) was 3412/μL (95% CI, 3081–3779), and it was significantly higher in *P. falciparum* and mixed-species infections than in vivax malaria (Table 3). The median duration of disease preceding presentation was 3 days (range 1–30), without differences according to parasite species (Table 2). Reported signs and symptoms did not differ with respect to parasite species even though sweating tended to be more commonly reported by vivax malaria patients as compared to patients with mixed species or falciparum malaria, and vomiting and abdominal pain was less commonly reported (Table 2). Only a few patients reported existing co-morbidities.

Upon clinical examination, patients with falciparum malaria showed increased proportions of impaired general condition, affected consciousness and splenomegaly

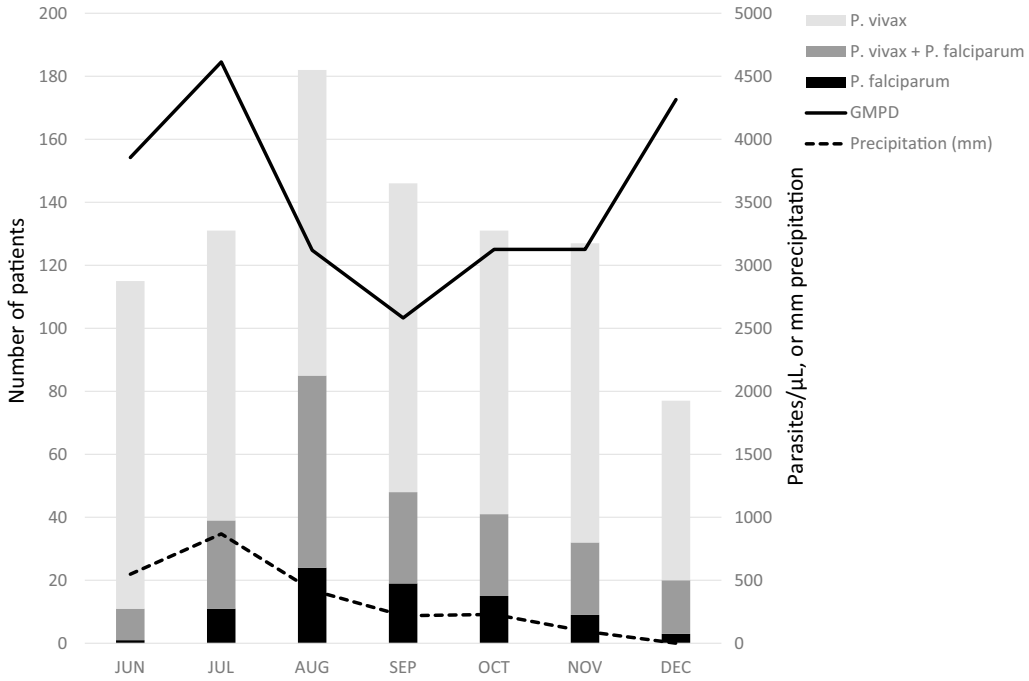

Fig. 1 Parasite species, geometric mean parasite density and precipitation according to month of presentation

(Table 3). Accordingly, 7.3% of falciparum malaria patients were admitted to ward as compared to 3.3% of *P. vivax* patients ($P=0.07$) (Table 3). Hb levels were slightly lower in *P. falciparum* as compared to *P. vivax*, and significantly reduced in mixed-species infection. Heart and respiratory rate, blood pressure, thrombocyte count as well as creatinine and bilirubin concentrations were similar between the three types of malaria. Nonetheless, hypotension was increased in mixed-species infection and increased creatinine levels ($P=0.13$) as well as severe thrombocytopaenia (< 50,000/µL; $P=0.04$) were seen twice as frequently in *P. falciparum* as compared to *P. vivax* (Table 3).

Severe malaria was rare: in 32 patients (3.5%), there was evidence of severe malaria according to the WHO definition, i.e., hypotension (15; impaired perfusion not assessed), renal impairment (5), renal impairment and respiratory distress (1), severe malarial anaemia (4), prostration (3), confusion (2), jaundice (1), and abnormal bleeding (haematemesis, 1). Impaired consciousness, convulsions, hypoglycaemia, acidosis, and pulmonary oedema were not observed. Hyperparasitaemia [> 10% infected red blood cells (RBCs)] was absent, the highest parasite density observed was 148,325/µL in a patient with mixed-species infection. Parasite species did not significantly affect the proportion of severe malaria, which occurred in 3.2% (20/633), 4.1% (8/194) and 4.9% (4/82) of cases with vivax malaria, mixed-species infection and falciparum malaria, respectively ($P=0.64$). In multivariate analysis, independent predictors of severe malaria included increasing age, female, reported diabetes mellitus, and thrombocytopaenia while increasing BMI proved to be protective (Table 4). Migration, SES, origin, previous malaria, preventive measures, and current parasite density, as well as other parameters shown in Tables 1 and 2 were not associated with severe malaria. Of the 35 (3.9%) patients who were admitted to ward, 10 were categorized as severe malaria patients. Other reasons included vomiting (5), dehydration (2), co-morbidities (2), weakness (2), suspected typhoid fever (1), jaundice (1), recent delivery (1), patient request (1), low blood pressure (1) as well as retrospectively not ascertainable causes (9). Of note, none of the patients died.

Discussion

In this study from the largest governmental hospital in urban Mangaluru, coastal southern India, almost 70% of malaria episodes were due to *P. vivax* and the vast majority of patients were managed as outpatients (96%). Severe malaria was rare (3.5%), and fatalities absent. Overall, *P. falciparum* and mixed infections showed a more pronounced manifestation than vivax malaria although respective differences were moderate. This contrasts with the severity and associated deaths of *P. vivax* infections reported from other regions of India [9–11].

Table 2 Patients' history according to malaria parasite species

Parameter	*P. vivax*	*P. vivax/P. falciparum*	*P. falciparum*	*P*
No. (%)	633 (69.6)	194 (21.3)	82 (9.0)	–
Stated history of malaria within preceding 1.5 months (%, n)	6.2 (39)	5.7 (11)	9.8 (8)	0.41
Stated duration of current episode (days; median, range)	3.0 (1–30)	3.0 (1–15)	3.0 (1–20)	0.55
Stated current signs and symptoms (%, n)				
Fever	99.7 (630/632)	99.5 (193)	100 (82)	0.79
Headache	93.5 (591/632)	95.4 (185)	96.3 (79)	0.43
Chills/shivering	88.4 (559/632)	90.7 (176)	82.9 (68)	0.18
Fatigue/weakness	86.1 (544/632)	89.7 (174)	90.2 (74)	0.29
Muscle pain	85.6 (541/632)	86.1 (167)	89.0 (73)	0.70
Sweats	75.6 (477/632)	71.5 (138)	69.5 (57)	0.31
Back pain	69.6 (440/632)	74.7 (145)	72.0 (59)	0.38
Cough	42.9 (271/632)	41.8 (81)	43.9 (36)	0.94
Nausea	41.0 (259/632)	44.0 (85/193)	41.5 (34)	0.75
Abdominal pain	30.4 (192/632)	32.0 (62)	37.8 (31)	0.39
Vomiting	27.4 (173/632)	34.5 (67)	36.6 (30)	0.06
Dyspnoea	7.1 (45/632)	3.1 (6)*	6.1 (5)	0.13
Diarrhoea	4.0 (25/632)	3.1 (6)	4.9 (4)	0.76
Stated co-morbidities (%, n)				
Diabetes mellitus	1.7 (11/632)	0.5 (1)	1.2 (1)	0.45
HIV/AIDS	0.3 (2/632)	0	0	0.65
COPD	0.6 (4/632)	0	0	0.42
Hypertension	0.8 (5/632)	3.1 (6)*	0	*0.02*
Clinical malnutrition	1.1 (7/632)	0.5 (1)	0	0.50
Chronic liver disease	0.3 (2/632)	0.5 (1)	0	0.79

* $P < 0.05$ as compared to vivax malaria; † $P < 0.05$ as compared to mixed species malaria

The present study has several specifics and limitations, which need to be considered when interpreting the findings. Wenlock Hospital is largely attended by adult male patients of comparatively low SES, more than half of whom are construction site workers or daily labourers. This pattern in turn may impact on knowledge and awareness of malaria, degree and pace of health care utilization and thus on the clinical picture [20, 24, 25]. As a result, the manifestation of malaria presented here may differ from that encountered at private and highly modern health facilities in Mangaluru, which attract a different patient population. As Wenlock Hospital caters for the vast majority of malaria patients in the city, who are predominately socio-economically underprivileged and migrant labourers [19], the present study provides a sufficiently representative picture of malaria in Mangaluru. In the present study, the definition of severe malaria largely followed the 2014 WHO criteria [23]. Some modifications, e.g., including confusion or hypotension without evidence of reduced perfusion as indicative of severe malaria may have led to an overestimation of severe malaria. On the other hand, acidosis and hypoglycaemia could not routinely be assessed, and potentially severely sick patients attending the casualty department after outpatient hours were not regarded, which may have caused a respective underestimation. Both scenarios, however, do not explain the low rates of hospitalization and severe malaria as compared to other settings in India [6, 9–11]. Although criteria for severe vivax malaria have been formulated by WHO [23], their validity has hardly been evaluated. Although more than 900 patients were analysed, the comparatively low proportion of falciparum malaria affected the statistical power of comparisons with vivax malaria. The low proportion of children (4%) among the patients examined also needs to be considered when comparing results to those of other studies with a predominantly paediatric patient population. Lastly, co-morbidities in the present study were predominantly derived from patient statements, and 1.4% of patients reported to have diabetes mellitus. Even when considering a large proportion of undiagnosed diabetes, this contrasts with the current estimate of adult diabetes prevalence of 10.4%

Table 3 Clinical and laboratory characteristics according to parasite species

Parameter	*P. vivax*	*P. vivax/P. falciparum*	*P. falciparum*	*P*
No. (%)	633 (69.6)	194 (21.3)	82 (9.0)	–
GMPD (/µL; 95% CI)	2999 (2660–3382)	4246 (3413–5283)*	5408 (3758–7780)*	*0.0005*
General condition, impaired (%, n)	4.3 (27/628)	4.7 (9/192)	16.0 (13/81)*†	*< 0.0001*
Consciousness affected (%, n)	2.2 (14/629)	3.6 (7/192)	11.1 (9/81)*†	*0.0001*
Splenomegaly (%, n)	16.6 (104/628)	20.1 (39)	29.6 (24/81)*	*0.01*
Petechia (%, n)	1.0 (6/629)	0.5 (1)	2.4 (1)	0.33
Admission to ward (%, n)	3.3 (21)	4.1 (8)	7.3 (6)	0.20
Axillary temperature (°C, mean ± SD), n = 903	37.1 ± 1.5	37.4 ± 1.5*	37.3 ± 1.7	*0.03*
Weight (kg; median, range), n = 899	54.5 (11.4–96.8)	52.8 (17.2–89.9)	52.0 (31.6–71.7)*	*0.03*
Height (cm, mean ± SD), n = 889	165.0 ± 10.4	165.0 ± 10.0	165.5 ± 8.6	0.92
BMI (kg/m^2; median, range), *n* = 887	19.8 (12.3–39.5)	19.1 (12.2–46.4)	19.2 (12.7–27.6)	*0.002*
Heart rate (/min; median, range), n = 906	99.0 (45–198)	100.0 (56–145)	104.5 (59–140)	0.16
Blood pressure, systolic (mmHg; median, range), n = 908	116.0 (71–250)	116.0 (66–217)	115.5 (78–194)	0.41
Blood pressure, diastolic (mmHg; median, range), n = 908	74.0 (27–155)	75.0 (40–161)	73 (42–109)	0.54
Hypotension (systolic BP < 80 mmHg; %, n)	1.1 (7/632)	3.6 (7)*	1.2 (1)	*0.05*
Respiratory rate (/min; median, range), n = 900	24.0 (14–44)	24.0 (15–34)	24.0 (16–44)	0.19
Thrombocytes (/µL; median, range), n = 859	110,000 (4000–326,000)	96,000 (10,600–293,000)	106,000 (6000–658,000)	0.28
Thrombocytopaenia (< 150,000/µL; %, n)	72.5 (437/603)	77.0 (141/183)	74.0 (54/73)	0.47
Severe thrombocytopaenia (< 50,000/µL; %, n)	10.9 (66/603)	14.2 (26/183)	19.2 (14/73)*	0.09
Hb (g/dL; median, range), n = 869	13.7 (5.1–20.3)	13.1 (7.0–20.5)*	13.5 (5.0–18.4)	*0.02*
Anaemia (%, n)	30.8 (188/610)	44.1 (82/186)*	41.1 (30/73)	*0.002*
Severe malarial anaemia (%, n)	0.7 (4/610)	0	0	0.43
White blood cells (/µL; median, range), n = 849	5400 (600–20,700)	5350 (700–17,400)	5500 (2900–16,400)	0.43
Creatinine (mg/dL; median, range), n = 828	0.90 (0.30–6.70)	0.90 (0.40–2.40)	0.90 (0.50–1.90)	0.73
Increased creatinine (> 1.4 mg/dL; %, n)	2.4 (14/576)	3.9 (7/179)	5.5 (4/73)	0.26
Renal impairment (creatinine > 3 mg/dL)	0.9 (5/576)	0	0	0.33
Bilirubin, total (mg/dL; median, range), n = 832	1.19 (0.20–8.10)	1.16 (0.30–8.72)	1.33 (0.37–5.85)	0.54
Increased bilirubin (> 1.2 mg/dL; %, n)	48.3 (280/580)	46.7 (84/180)	52.8 (38/72)	0.68
Bilirubin, direct (mg/dL; median, range), n = 833	0.21 (0.01–6.05)	0.22 (0.09–4.01)	0.25 (0.09–4.86)	0.41
Increased direct bilirubin (> 0.2 mg/dL; %, n)	50.0 (290/580)	51.4 (93/181)	55.6 (40/72)	0.66
Jaundice (bilirubin > 3 mg/dL and > 100,000 parasites/µL)	0	0.6 (1/181)	0	0.16

* P < 0.05 as compared to vivax malaria; † P < 0.05 as compared to mixed species malaria

in India [26]. This suggests a higher proportion of co-morbidities (potentially influencing the clinical picture) than reported in the present study.

Interestingly, falciparum malaria appeared to be a local rather than an imported disease. The prevalence of *P. falciparum* mono-infection was significantly increased in construction workers and reduced in migrants from north/northeastern India, although both factors overlapped. In the latter region, the burden of malaria is increased as is the proportion of *P. falciparum* [27]. Conceivably, migrants originating from there show a higher degree of *P. falciparum*-related semi-immunity as compared to the local Mangaluru population and consequently some degree of resistance to locally transmitted *P. falciparum* parasites. Alternative explanations include an increased relapse rates in imported *P. vivax* strains, poor compliance with the 2 weeks of primaquine treatment, or an increased rate of common infectious diseases among the economically deprived migrants giving rise to an increased *P. vivax* relapse rate [28]. In these cases, migrants would show a comparatively increased proportion of *P. vivax*.

One month after the peak of precipitation, the proportion of *P. vivax* infections was lowest whereas that of *P. falciparum* was highest. In India, both temperate and tropical types of *P. vivax* relapse (i.e., long and short latency) occur [21] but for the present study, no respective information is available. Nevertheless, it is conceivable that at the beginning of peak transmission, relapses of *P. vivax* still predominated among the attending patients

Table 4 Factors associated with severe malaria

Parameter	No.	% severe malaria	OR (95% CI)	P	aOR (95%)	P
Age (years)	909	na	1.04 (1.02–1.07)	0.0009	1.03 (1.00–1.06)	0.04
Gender						
Male	844	2.8	1		1	
Female	65	12.3	4.80 (1.89–11.84)	0.001	6.07 (2.32–15.89)	0.0002
Reported diabetes mellitus						
No	895	3.2	1		1	
Yes	13	23.1	8.96 (1.50–37.11)	0.009	13.60 (2.81–65.83)	0.001
BMI (kg/m^2)	887	na	0.88 (0.76–1.00)	0.06	0.80 (0.69–0.93)	0.005
Thrombocytopaenia						
No	227	0.9	1		1	
Yes	632	4.6	5.41 (1.35–47.11)	0.01	4.64 (1.05–20.56)	0.04
Reported COPD						
No	904	3.2	1			
Yes	4	75.0	90.52 (6.86–4755.80)	0.002	–	
Construction worker/daily labourer						
No	399	4.8	1			
Yes	510	2.5	0.52 (0.24–1.13)	0.08	–	

BMI body mass index, *na* not applicable, *OR* odds ratio, *95% CI* 95% confidence interval, *aOR* adjusted odds ratio; n = 838, $R^2 = 0.15$

and *P. falciparum* proportionally increased with increasing precipitation and thus transmission.

Reported signs and symptoms did not differ substantially with parasite species even though gastrointestinal symptoms (vomiting, abdominal pain) appeared to be slightly increased in infections comprising *P. falciparum*. Nevertheless, upon clinical examination, patients with falciparum malaria showed worse general and consciousness conditions than patients with *P. vivax* or mixed infections. This may be partly due to the increased parasite counts and reduced nutritional status observed in falciparum malaria patients. On the other hand, cytokine production, potentially affecting the general condition, is higher in *P. vivax* infection than that in *P. falciparum* infections of similar parasite numbers [29, 30]. Other features of *P. falciparum* as compared to *P. vivax* infections included the highest proportion of severe thrombocytopaenia as well as trends towards more frequently increased creatinine and bilirubin levels, more anaemia, and more common hospitalization. Altogether, these differences were modest. Mixed species infection largely assumed an interim position between the manifestations of *P. vivax* and *P. falciparum*. Notable exemptions were the highest proportions of anaemia and hypotension. Given the only minor differences in the clinical picture according to parasite species, the present study does not contribute to solving the question whether mixed-species infections do rather attenuate falciparum malaria or increase its severity [4, 11, 12, 31]. One previous hospital-based study from the southern outskirts of Mangaluru conducted more than a decade ago [32] also reported a predominance of young males among the malaria patients. Based on the quantitative buffy coat (QBC) method for diagnosis, *P. falciparum* infections (34%) were more common than in the present study but mixed infections slightly less prevalent (14%). Data are not well comparable because of a considerably larger proportion of inpatients (61%) in that study but it is noteworthy that, similar to the present study, falciparum malaria was associated with increased rates of hospitalization and of altered consciousness. In the previous study from Mangaluru, two patients (1.1%) reportedly died due to cerebral malaria (no parasite species provided).

Severe malaria due to *P. vivax* infection is increasingly reported since the year 2000, and > 40% of respective studies originate from India whereas a large number of endemic countries do not report severe vivax malaria at all [6]. The inclusion of severe thrombocytopaenia as marker of severity may partially be involved in this discrepancy. In the present study, the proportion of severe vivax malaria of 3.2% would have increased to 12.9% when considering this criterion. However, abnormal bleeding (haematemesis) was observed only in one patient with *P. vivax* infection and a thrombocyte count of 78,000/μL. Other reasons for the heterogeneity of severe vivax malaria potentially include geographical differences in the peak age of vivax malaria, endemicity, chloroquine resistance, parasite virulence, and misdiagnosis of other severe diseases [6]. Beyond doubt, *P. vivax* can cause severe and fatal disease, and the existing

evidence has thoroughly been reviewed [3, 4, 6, 33]. However, the reason for the respective geographical variation as well as the underlying pathophysiology are far from being understood.

Severe malaria in the present study occurred at similar proportions irrespective of parasite species. Reported diabetes mellitus was its strongest independent predictor followed by female gender, thrombocytopaenia, and increasing age, while increasing BMI was protective. Increased susceptibility to malaria in diabetic individuals has been reported from Africa [34] and there is strong evidence that diabetes increases the severity of a number of infectious diseases [35]. Female gender may correspond to peculiarities of health care utilization, and increasing age to (unreported) co-morbidities. Malnutrition, i.e., low BMI, is a known risk factor for malaria severity [36]. Thrombocytopaenia increased the risk of severe malaria almost fivefold. When replacing this factor with severe thrombocytopaenia in the multivariate model, the association weakened and remained significant only for vivax malaria (aOR, 3.5; 95% CI, 1.1–11.1). This might argue for severe thrombocytopaenia as an indicator of severe vivax malaria. However, the above estimates do not provide information on causation.

Conclusions

Malaria in Mangaluru, coastal southern India, affects predominantly young men of low-socio-economic status, many of whom are migrant workers from other parts of India, and rarely causes hospitalization. Severe vivax malaria does occur in this setting at a rate only slightly lower than in falciparum malaria but its prevalence contrasts with substantially higher figures reported from elsewhere in the country. Studies into pathophysiology and parasite biology are needed to disentangle the respective heterogeneity.

Authors' contributions

PPG, FPM, PG, AJ, RD and DS designed the study. PPG, KS, JW, AB, AK, SH, RD, and DS were responsible for patient recruitment, clinical and laboratory examinations. PPG, KS, SK, and RR did the PCR analyses. PPG and FPM the statistical analyses and wrote the paper with major contributions of the other authors. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The dataset generated and/or analysed in this study is not publicly available due to issues of confidentiality and ongoing analyses, but are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Institutional Ethics Committee of Kasturba Medical College, Mangaluru, Manipal University (IEC KMC MLR 05-1598), and all study patients provided informed written consent. Permission to conduct the study was granted by the Directorate of Health and Family Welfare Services, Government of Karnataka.

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Malaria Journal

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Duffy antigen receptor for chemokines gene polymorphisms and malaria in Mangaluru, India

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Abstract

Background: Duffy blood group antigens serve as receptors for *Plasmodium vivax* invasion into erythrocytes, and they are determined by polymorphisms of the Duffy antigen receptor for chemokines (DARC), also known as Fy glycoprotein (FY). Duffy negativity, i.e., absence of the antigens, protects against *P. vivax* infection and is rare among non-African populations. However, data on *DARC* polymorphisms and their impact on *Plasmodium* infection in India are scarce.

Methods: In a case–control study among 909 malaria patients and 909 healthy community controls in Mangaluru, southwestern India, *DARC* polymorphisms T-33C (rs2814778), G125A (rs12075), C265T (rs34599082), and G298A (rs13962) were genotyped. Associations of the polymorphisms with the odds of malaria, parasite species and manifestation were assessed.

Results: Among patients, vivax malaria (70%) predominated over falciparum malaria (9%) and mixed species infections (21%). *DARC* T-33C was absent and C265T was rare (1%). FYB carriage (deduced from *DARC* G125A) was not associated with the risk of malaria per se but it protected against severe falciparum malaria ($P = 0.03$), and hospitalization ($P = 0.006$) due to falciparum malaria. Vice versa, carriage of *DARC* 298A was associated with increased odds of malaria (aOR, 1.46 (1.07–1.99), $P = 0.015$) and vivax malaria (aOR, 1.60 (1.14–2.22), $P = 0.006$) and with several reported symptoms and findings of the patients.

Conclusion: This report from southern India is the first to show an independent effect of the *DARC* 298A polymorphism on the risk of malaria. Functional studies are required to understand the underlying mechanism. Moreover, FYB carriage appears to protect against severe falciparum malaria in southern India.

Keywords: Duffy, DARC, SNPs, Malaria, India, *Plasmodium vivax*, *Plasmodium falciparum*

Background

Malaria is considered a major driving force in shaping the human genome [1]. "Classical" erythrocyte variants such as the sickle-cell trait offer relative resistance against malaria and are thus subject to evolutionary selection in endemic regions. In addition, various further host genetic polymorphisms influence susceptibility to the disease and/or its manifestation [2, 3]. This includes the Duffy antigen receptor for chemokines (DARC, or Duffy antigen), which is a glycosylated erythrocyte membrane protein. The encoding *DARC* gene is located on chromosome 1. A common *DARC* polymorphism, G125A (rs12075), generates the FYA (G125) and FYB (125A) alleles. The resulting genotypes include the wildtype FYA/FYA, which correspond to the phenotype Fy (a+, b−), FYA/FYB (Fy (a+, b+)) and FYB/FYB (Fy (a−, b+)). An additional T-33C mutation silences antigen expression giving rise to Duffy blood group negativity (Fy (a−, b−)). Further single nucleotide polymorphisms (SNPs), C265T and G298A, are together responsible for weakening the

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expression of the FYB allele, whereas G298A alone is not able induce this effect [4].

In line with its function as a multi-specific receptor for a wide range of chemokines [5, 6], the absence of DARC on the erythrocyte cell surface (Duffy blood group negativity) has been associated with diverse conditions including inflammation, HIV infection, and malignancies [7, 8]. With respect to malaria, Duffy blood group negativity is predominant among Africans and renders erythrocytes resistant to invasion by *Plasmodium vivax* and *Plasmodium knowlesi* [5, 9–12]. Moreover, binding of DARC to platelet factor 4 (PF4) is essential for platelet-mediated killing of *Plasmodium falciparum* parasites [13, 14]. Associations of *DARC* genotypes with vivax malaria are reportedly conflicting. For instance in Brazil, FYA/FYA conferred reduced odds of vivax malaria [15]. However, in another Brazilian study, FYA/FYA was significantly more frequent in vivax malaria patients as compared to healthy blood donors without a history of malaria [11]. In India, the FYA allele has been associated with a reduced incidence of vivax malaria and the FYB allele with an increased one [16]. However, the few available individual studies from India did not show a link between *DARC* genotypes and vivax malaria [17–19].

In India, FYA/FYA is the predominant genotype and Duffy negativity occurs only in a few tribal populations [16, 19]. At the same time, India contributes to nearly half of the global *Plasmodium vivax* cases, and *P. vivax* and *P. falciparum* are responsible for 37% and 63% of malaria cases, respectively [20]. This provides the opportunity to study the effect of *DARC* genotypes on the risk of malaria per se, and of vivax and falciparum malaria separately. Of note, the manifestation of *Plasmodium* infection is not only caused by the infecting parasite but also by pro-inflammatory host responses, which potentially contribute to pathophysiology [21]. In this regard, the function of DARC as a receptor for diverse chemokines [5, 6] might possibly influence the clinical manifestation. Against this background, the present study aimed at describing the *DARC* genotype distribution pattern in Mangaluru and as a next step at examining the association of *DARC* genotypes with (i) malaria, (ii) malaria as caused by the various *Plasmodium* spp., and (iii) clinical presentation.

Methods

A total of 909 malaria out-patients were recruited at Wenlock Hospital, Mangaluru, Karnataka, India between June to December 2015. Wenlock Hospital (900 beds) is the largest governmental hospital in Mangaluru offering treatment particularly for the economically-deprived part of the population. In parallel, an average of 40 (26–53) healthy community controls were randomly recruited in each of the 60 census wards of Mangaluru yielding a total number of 2478 individuals. The study protocol was approved by the Institutional Ethics Committee of Kasturba Medical College, Mangalore, Manipal University, and permission to conduct the study was granted by the Directorate of Health and Family Welfare Services, Government of Karnataka. Informed written consent was obtained from all individuals enrolled in this study.

Details of the patient recruitment process as well as socio-economic, clinical and laboratory data have been reported elsewhere [22]. Briefly, socio-economic data were collected by trained interviewers from patients (cases) and controls. Venous blood was collected into EDTA from malaria patients and by finger prick blood on Whatman™ 3MM paper from controls. Malaria parasites were counted *per* 200 white blood cells (WBCs) on Giemsa-stained thick blood films, and parasite species was defined based on thin-film microscopy. Following DNA extraction (QIAamp DNA Blood Mini kit, Qiagen, Hilden, Germany), *Plasmodium* species was ascertained by semi-nested polymerase chain reaction (PCR) assays [23]. Out of 2383 *Plasmodium*-negative controls, 909 were randomly selected for this case–control study. *DARC* SNP genotyping including T-33C, G125A, C265T and G298A was achieved by melting curve analysis on the Light Cycler 480 instrument (Roche, Basel, Switzerland) using commercial primers and probes; reagent concentrations and PCR conditions are available with the manufacturer (TIB MOLBIOL, Berlin, Germany).

Data analysis was performed using *R*Studio 3.5.1 (2018) (Integrated Development for R. RStudio, Inc., Boston, USA) and SPSS 25 (IBM Corp., Armonk, USA). The distribution of *DARC* genotypes between case and controls were compared by χ^2 test or Fisher's exact test as appropriate, and odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated. Binomial logistic regression was used to calculate the adjusted odds ratios (aORs) of individuals with variant genotypes for malaria per se and for malaria separated by species with probable confounders: age, sex and migration to Mangaluru. Continuous parameters were compared using Student's *t* test, Mann–Whitney U test, or Kruskal–Wallis test as applicable. A P value < 0.05 was considered statistically significant.

Results

Essential characteristics of malaria patients and controls are displayed in Table 1. More than 90% of malaria patients were male adults. Their median age was 26 years, and more than three in four had migrated to Mangaluru a median period of 6 months before presentation (range 1–600 days). Their overall socio-economic status including educational background was low, and

Table 1 Characteristics of malaria patients and controls

Parameter	Cases	Controls	P
No.	909	909	
Male gender (%, n)	92.8 (844)	57.5 (523)	< 0.0001
Age (years; median, range)	26 (4–82)	30 (1–94)	0.0001
Migration (%, n)	77.8 (706/907)	34.4 (313/909)	< 0.0001
Socio-economic parameters			
No formal education (%, n)	33.0 (298/902)	11.1 (98/882)	< 0.0001
Occupation as construction worker or daily labourer (%)	56.1	36.3	< 0.0001
Monthly family income (rupees; median, range), cases; n = 893, controls; n = 575	6000 (0–35,000)	7000 (500–100,000)	0.06
Stated use of a bed net in preceding night (%, n)	39.1 (354,906)	54.3 (484/892)	< 0.0001
Stated use of a window net (%, n)	4.2 (38/906)	42.4 (376/890)	< 0.0001
Presence of stagnant water bodies (%, n)	31 (281/906)	3.3 (29/851)	< 0.0001
Plasmodium prevalence			
P. vivax	69.6 (633)	0	–
P. falciparum	9.0 (82)	0	–
P. vivax and *P. falciparum* mixed	21.3 (194)	0	–
Geometric mean parasite density (/µl; 95% CI)			
All patients	3412 (3081–3779)	–	–
P. vivax	2999 (2660–3382)	–	–
P. falciparum	5408 (3758–7750)	–	–
P. vivax and *P. falciparum* mixed	4246 (3413–5283)	–	–
DARC G125A genotypes (%)			
GG (FYA/FYA)	43.9	43.1	1
GA (FYA/FYB)	44.1	43.7	0.91
AA (FYB/FYB)	11.9	13.1	0.48
GA or AA (FYB carriers)	56.1	56.8	0.74
DARC G298A genotypes (%)			
GG	83.3	85.5	1
GA	15.6	13.3	0.16
AA	1.1	1.2	0.87
GA or AA	16.7	14.5	0.19

more than half of the patients were either construction workers or daily labourers [22]. In comparison, among control individuals, the proportion of males was lower, age was higher, and only a minority had migrated to Mangaluru city (each, $P < 0.0005$). Among patients, vivax malaria (70%) predominated over falciparum malaria (9%), and mixed *P. vivax–P. falciparum* infections (21%). The geometric mean parasite density (GMPD) was 3412/µl (95% CI 3081–3779).

The *DARC* SNP-33 T>C was absent in 570 random samples genotyped, and the SNP 265 C>T was rare (1% (6/564) heterozygous). These polymorphisms were thus omitted from analysis. Genotyping of the *DARC* SNPs G125A and G298A was successful in all patients and controls. *DARC* 298A occurred exclusively when also 125A was present, i.e., on an FYB background ($P < 0.0001$). In the study sample and based on *DARC* G125A, FYA/FYB (43.9%) and FYA/FYA (43.5%) were the most common Duffy genotypes (FYB/FYB, 12.5%). Of note, these genotypes did not differ between cases and controls, and thus were not associated with the odds of malaria (Table 1), irrespective of stratification by parasite species (Table 2).

In contrast, carriage of *DARC* 298A (GA or AA), i.e., genotypes involved in but not solely responsible for a weakened expression of the FYB allele [4], appeared to be more common in malaria patients (16.7%, 152/909; $P = 0.19$) and in vivax malaria patients in particular (17.7%, 112/633; $P = 0.09$) as compared to controls (14.5%, 132/909) (Table 2; Fig. 1). Adjusting for the observed differences in age, sex, and migration, carriage of *DARC* 298A was associated with increased odds of

Table 2 Genotype distribution among malaria patients and controls separated by *Plasmodium* species

SNP	Genotype	Controls, n = 909	Malaria patients		
			P. vivax, *n* = 633	*P. falciparum*, *n* = 82	*P. vivax* and *P. falciparum*, *n* = 194
DARC G125A (n, %)	GG (FYA/FYA)	392 (43.1)	280 (44.2)	36 (43.9)	83 (42.7)
	GA (FYA/FYB)	398 (43.7)	277 (43.7)	34 (41.4)	90 (46.3)
	AA (FYB/FYB)	119 (13.1)	76 (12.0)	12 (14.6)	21 (10.8)
	GA or AA (FYB carriers)	517 (56.8)	353 (55.7)	46 (56.1)	111 (57.2)
DARC G298A (n, %)	GG	777 (85.5)	521 (82.3)	69 (84.2)	167 (86.1)
	GA	121 (13.3)	105 (16.6)	11 (13.4)	26 (13.4)
	AA	11 (1.2)	7 (1.1)	2 (2.4)	1 (0.5)
	GA or AA	132 (14.5)	112 (17.7)	13 (15.8)	27 (13.9)

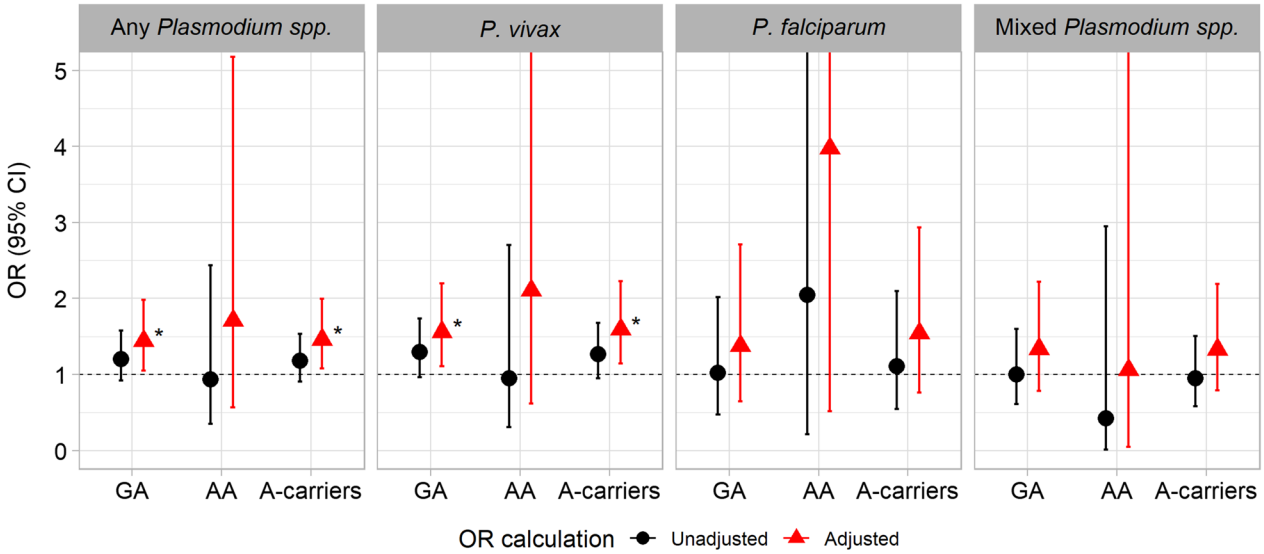

Fig. 1 *DARC* 298A carriage is associated with increased odds of malaria and vivax malaria. OR, odds ratio; 95% CI, 95% confidence interval; unadjusted, crude odds ratio; adjusted, odds ratio adjusted for age, sex, and migration status; *P < 0.05 as compared to *DARC* G298 wildtype individuals. Forest plots display the odds of malaria according to *DARC* G298A genotypes. χ^2 test or Fisher's exact test was applied to calculate unadjusted odds ratios (95% CIs). Adjusted odds ratios were derived from logistic regression models adjusting for age, sex and migration status. As compared to control individuals (14.5%, 132/909), *DARC* 298A carriage was more common in malaria patients [16.7%, 152/909; aOR, 1.46 (1.07–1.99)] and in vivax malaria patients in particular [17.7%, 112/633; aOR, 1.60 (1.14–2.22)]

malaria (aOR, 1.46 (1.07–1.99), $P = 0.015$) and of vivax malaria in particular (aOR, 1.60 (1.14–2.22), $P = 0.006$) (Fig. 1). No significant association with falciparum or mixed species malaria was observed.

In a next step of analysis among malaria patients, the proportions of hospitalization and of severe malaria were compared between *DARC* genotypes. For that, FYA/FYA and wildtype *DARC* G298, respectively, were set as reference groups. Among the patients, 3.5% (32/909) and 3.8% (35/909) of individuals were hospitalized and had severe malaria, respectively. The proportion of patients who were admitted to ward was highest in individuals with FYA/FYA (5.0%, 20/399), lower in FYA/FYB (3.5%, 14/401, $P = 0.29$) and lowest in FYB/FYB (0.9%, 1/109, $P = 0.06$). This was due to the absence of hospital admissions in patients with falciparum malaria carrying the FYB allele ($P = 0.006$, Table 3). Severe malaria due to any parasite species occurred at similar proportions in patients with the different FY genotypes, but severe falciparum malaria was absent in individuals carrying FYB ($P = 0.03$, Table 3). *DARC* 298A carriage did neither affect the proportion of hospitalized patients nor that of severe malaria. Also, it did not substantially change the associations of

Table 3 Proportion of patients with hospitalization and severe malaria according to *FY* genotypes

	FYA/FYA	FYA/FYB	FYB/FYB	FYB carriage with *DARC* 298A carriage
Hospitalization[a]				
All species	5.0% (20/399)	3.5% (14/401)	0.9% (1/109)	2.6% (4/152)
P. vivax	3.6% (10/280)	3.6% (10/277)	1.31% (1/76)	3.6% (4/112)
P. falciparum	16.7% (6/36)	0% (0/34)*	0% (0/12)	0% (0/13)
Mixed	4.8% (4/83)	4.4% (4/90)	0% (0/21)	0% (0/27)
Severe malaria[b]				
All species	3.5% (14/399)	3.7% (15/401)	2.8% (3/109)	3.3% (5/152)
P. vivax	2.9% (8/280)	3.6% (10/277)	2.6% (2/76)	3.6% (4/112)
P. falciparum	11.1% (4/36)	0% (0/34)	0% (0/12)	0% (0/13)
Mixed	2.4% (2/83)	5.6% (5/90)	4.8% (1/21)	3.7% (1/27)

[a] Of 35 patients admitted to ward, 10 were categorized as severe malaria patients. Other reasons included vomiting (5), dehydration (2), co-morbidities (2), weakness (2), suspected typhoid fever (1), jaundice (1), recent delivery (1), patient request (1), low blood pressure (1) as well as retrospectively not ascertainable causes (9)

[b] 32 patients had severe malaria according to the WHO definition, i.e., hypotension (15; impaired perfusion not assessed), renal impairment (5), renal impairment and respiratory distress (1), severe malarial anaemia (4), prostration (3), confusion (2), jaundice (1), and abnormal bleeding (haematemesis, 1). Impaired consciousness, convulsions, hypoglycaemia, acidosis, hyperparasitaemia and pulmonary oedema were not observed

* $P < 0.05$ as compared to FYA/FYA

FYB carriage with the odds of hospitalization or severe malaria (Table 3).

Lastly, signs and symptoms as well as laboratory parameters were analysed with respect to *DARC* genotypes. These did not differ significantly with the three FY genotypes. However, *DARC* 298A carriage was associated with increased proportions of patients reporting a history of muscle pain, back pain, fatigue, and at borderline, diarrhoea. Basically, the same findings were seen for vivax malaria, whereas in falciparum malaria *DARC* 298A carriage was associated with a history of sweats ($P = 0.05$) and of vomiting (Table 4). Clinically, the proportions of splenomegaly and of elevated bilirubin concentration were increased in patients with *DARC* 298A carriage as was axillary temperature ($P = 0.05$), specifically in mixed species infections ($P = 0.01$).

Discussion

The present results indicate that FYB carriage in an Indian population does not influence the risk of malaria per se, but, in case of *P. falciparum* infection, it is associated with protection from hospitalization and severe malaria. Vice versa, *DARC* 298A carriage appeared to increase the risk of malaria, and of vivax malaria in particular, and to affect the occurrence of several symptoms.

Despite its sample size the study has several limitations which need to be considered when interpreting the results: subgroups, e.g., patients with falciparum malaria, were relatively small affecting the power of analyses. Patients and controls differed in essential parameters such as age, gender, and migration status, because of which risk estimates had to be adjusted accordingly. The *DARC* polymorphisms T-33C and C265T were too rare to deduce meaningful findings. No interaction in terms of associations with malaria or signs or symptoms was seen for the FYA or FYB alleles and *DARC* G298A. Therefore, data were presented separately.

In the present study, the FYA/FYA and FYA/FYB genotypes occurred in each approximately 44%. Among more than 3000 blood donors in New Delhi, FYA/FYA and FYA/FYB were observed in 32.5% and 48.9%, respectively. Duffy blood group negativity, absent in the present study, was observed in 0.3% [24]. Of note, the proportion of Duffy blood group genotypes differs across India but findings from the South of the country closely match with the prevalence data of the present study [16]. In comparison to other ethnic groups, the predominant FYA/FYB genotype in the present study is slightly more common in Caucasians (49%) but rare in sub-Saharan Africans (1.0%) and Chinese (8.9%). This is due to a higher FYA allele frequency among Indians than in Caucasians and sub-Saharan Africans. Duffy blood group negativity is absent or very rare in all populations except for sub-Saharan Africans (68%) [24]. Carriage of *DARC* 298A was found in 15.6% of the current study participants, corresponding to an allele frequency of 0.08. Based on the 1000 Genomes Project, this matches the respective figure of 0.09 among South Asians, but it is lower than the allele frequency of 0.18 among Caucasians and higher than the value of 0.005 in Africans [4].

Duffy blood group antigens are known to play an important role in *P. vivax* malaria [5, 6, 25] and to be

Table 4 Patient history as well as signs and symptoms according to *DARC* 298A carriage and parasite species

	All malaria patients		Vivax malaria		Falciparum malaria		Mixed species malaria	
	Wildtype	*DARC* 298A carriage	Wildtype	*DARC* 298A carriage	Wildtype	*DARC* 298A carriage	Wildtype	*DARC* 298A carriage
No	757	152	521	112	69	13	167	27
Reported signs and symptoms in preceding 2 weeks								
Fever	99.6 (753/756)	100.0 (152)	99.6 (518/520)	100.0 (112)	100.0 (69)	100.0 (13)	99.4 (166)	100.0 (27)
Headache	94.0 (711/756)	94.7 (144)	93.1 (484/520)	95.5 (107)	97.1 (67)	92.3 (12)	95.8 (160)	92.6 (25)
Muscle pain	84.9 (642/756)	91.4 (139)*	84.2 (438/520)	92.0 (103)*	89.9 (62)	84.6 (11)	85.0 (142)	92.6 (25)
Back pain	69.6 (526/756)	77.6 (118)*	67.9 (353/520)	77.7 (87)*	72.5 (50)	69.2 (9)	73.7 (123)	81.5 (22)
Fatigue/weakness	86.1 (651/756)	92.8 (141)*	85.0 (442/520)	91.1 (102)	89.9 (62)	92.3 (12)	88.0 (147)	100.0 (27)
Chills/shivering	88.1 (666/756)	90.1 (137)	88.1 (458/520)	90.2 (101)	82.6 (57)	84.6 (11)	90.4 (151)	92.6 (25)
Sweats	73.5 (554/754)	77.6 (118)	76.1 (395/519)	73.2 (82)	65.2 (45)	92.3 (12)*	68.7 (114/166)	88.9 (24)*
Cough	42.2 (319/756)	45.4 (69)	41.9 (218/520)	47.3 (53)	43.5 (30)	46.2 (6)	42.5 (71)	37.0 (10)
Nausea	41.2 (311/754)	44.1 (67)	40.8 (212/519)	42.0 (47)	42.0 (29)	38.5 (5)	42.2 (70/166)	55.6 (15)
Vomiting	29.8 (225/756)	29.6 (45)	28.5 (148/520)	22.3 (25)	30.4 (21)	69.2 (9)*	33.5 (56)	40.7 (11)
Diarrhoea	3.3 (25/756)	6.6 (10)	2.9 (15/520)	8.9 (10)*	5.8 (4)	0.0 (0)	3.6 (6)	0.0 (0)
Abdominal pain	31.7 (240/756)	29.6 (45)	30.0 (156/520)	32.1 (36)	40.6 (28)	23.1 (3)	33.5 (56)	22.2 (6)
Assessed parameters								
GMPD (/µl; 95% CI)	3364 (3011–3757)	3641 (2800–4734)	2995 (2626–3415)	3036 (2253–4092)	4681 (3148–6961)	11,580 (5055–26,527)	4216 (3340–5321)	4428 (2347–8354)
Splenomegaly (%, n)	16.8 (126/751)	27.0 (41)*	14.5 (75/516)	25.9 (29)*	27.9 (19/68)	38.5 (5)	19.2 (32)	25.9 (7)
Axillary temperature (°C, mean ± SD), n = 903	37.2 ± 1.6	37.4 ± 1.5	37.1 ± 1.6	37.3 ± 1.4	37.3 ± 1.7	37.4 ± 2.0	37.3 ± 1.5	38.1 ± 1.4*
Body mass index (BMI) (kg/m^2; median, range), n = 887	19.5 (12.2–46.3)	19.3 (13.6–33.3)	19.8 (12.3–39.5)	19.3 (13.6–33.3)	19.1 (12.6–27.5)	19.7 (14.6–24.9)	19.0 (12.2–46.3)	19.3 (16.7–31.9)
Hypotension (systolic BP < 80 mm Hg; %, n)	1.6 (12/756)	2.0 (3)	1.0 (5/520)	1.8 (2)	1.4 (1)	0.0 (0)	3.6 (6)	3.7 (1)
Severe thrombocytopenia (< 50,000/µl; %, n)	12.3 (88/715)	12.5 (18/144)	10.5 (52/495)	13.0 (14/108)	21.0 (13/62)	9.1 (1/11)	14.6 (23/158)	12.0 (3/25)
Anaemia (%, n)	34.3 (248/722)	35.4 (52/147)	30.1 (151/501)	33.9 (37/109)	41.0 (25/61)	41.7 (5/12)	45.0 (72/160)	38.5 (10/26)
Increased creatinine (> 1.4 mg/dl; %, n)	3.3 (23/688)	1.4 (2/140)	2.7 (13/473)	1.0 (1/103)	4.9 (3/61)	8.3 (1/12)	4.5 (7/154)	0.0 (0/25)
Increased bilirubin (> 1.2 mg/dl; %, n)	46.8 (324/693)	56.1 (78/139)*	46.3 (221/477)	57.3 (59/103)*	53.3 (32/60)	50.0 (6/12)	45.5 (71/156)	54.2 (13/24)

* $P < 0.05$ as compared to the wildtype *DARC* G298

essential in platelet-mediated killing of *P. falciparum* [13]. However, actual findings in various populations including Indians have been ambiguous [11, 15–19]. In the present study FYA or FYB did not affect the odds of malaria, irrespective of parasite species.

This contrasts with recent report on protective effects of FYA/FYA against vivax malaria in Brazil [15]. Likewise, in one study from India, FYA was found to be associated with a reduced 5 years average incidence of vivax malaria [16]. In Brazil, no association with falciparum malaria was observed [15], in India, falciparum malaria was not analysed [16]. In another study from Brazil, FYA/FYA was associated with increased susceptibility to vivax malaria [11], and in older work from India, no impact of the Duffy blood group genotypes on vivax or falciparum malaria was observed [17–19]. The reason for these conflicting results may be related to variable proportions of *P. vivax* and *P. falciparum* among the patients included, partially low sample sizes and genetic variation among the diverse populations, including the Indian one. Of note, in the present study, falciparum malaria patients with FYA/FYA showed the highest rate of hospitalization and severe malaria, which was unexpected considering the protective effects against vivax malaria mentioned above [15, 16]. In-vitro, binding of Duffy antigens to platelet factor 4 (PF4) is crucial for platelet-mediated killing of *P. falciparum* [13, 14] even though the role of FY variation in that is unknown. One explanation for the finding of reduced odds of severe malaria in patients with FYB carriage could be that it affects binding affinity towards PF4 and thereby the capacity of platelet mediated killing. On the other hand, parasite densities and other severity markers of infection were not reduced in patients with FYB carriage. Consequently, further work is required to explain the observed association of FYB carriage with hospitalization and severe falciparum malaria.

A novel finding is that carriage of the *DARC* 298A variant increased the odds of malaria by roughly 50%. Moreover, this polymorphism was associated with increased proportions of patients reporting several signs and symptoms. This SNP has not been observed to be independently associated with malaria. One previous study from Brazil did not observe an association with malaria susceptibility when combining *DARC* C265T and G298A as a condition weakening the expression of Duffy antigens (FYX) [11]. *DARC* C265T was absent in the present study population. *DARC* 298 G>A results in an amino acid substitution in the first intracellular loop of the Duffy glycoprotein. It has been linked with reduced FYB expression only in the presence of C265T [4, 26]. On the other hand, DARC acts as a multi-specific receptor for chemokines. These include the melanoma growth stimulatory activity, interleukin-8, regulated upon activation normal T-expressed, monocyte chemotactic protein-1, neutrophil activating protein 2 and 3, epithelial neutrophil activating peptide-78, angiogenesis-related platelet factor 1, and growth-related gene alpha [5, 6]. In line with this wide-range receptor function, DARC per se has been associated with several inflammatory and infectious diseases including increased rates of prostate cancer and asthma as well as an increased risk of HIV infection in its absence [7, 27]. DARC also influences inflammation in terms of chemokine levels and leukocyte trafficking and malignancy [8]. Monocytes and neutrophils phagocytize infected red blood cells, and they are important sources of cytokines, which act as signaling molecules in activating immune responses against malaria [28]. Increased phagocytic activity via neutrophils is observed in vivax malaria [29]. A possible explanation in support of the present study findings could be the involvement of variant *DARC* 298A in altering the chemoattractant properties of the Duffy glycoprotein, leading to a modified activation of the pro-inflammatory signal cascade. Functional studies are needed to verify this hypothesis.

Conclusion

This study from southern India is the first to show an independent effect of *DARC* 298A in *Plasmodium* infection. *DARC* 298A carriage appears to be associated with increased susceptibility to malaria and to vivax malaria in particular, and to worsen several signs and symptoms. Functional studies on the role of this polymorphism are required to disentangle the underlying mechanisms. The same applies to the role of FYB genotype carriage protecting against severe falciparum malaria. Considering Duffy blood group antigens being studied as vaccine candidates against vivax malaria [30] and the present clinico-epidemiological findings, unravelling the molecular mechanisms of Duffy blood group antigens influencing malaria susceptibility and resistance is urgently needed.

Abbreviations

DARC: Duffy antigen receptor for chemokines; FY: Fy glycoprotein; *P. vivax*: *Plasmodium vivax*; *P. falciparum*: *Plasmodium falciparum*; WBCs: white blood cells; PF4: platelet factor 4; SNP: single nucleotide polymorphism; PCR: polymerase chain reaction; ORs: odds ratios; CI: confidence interval; GMPD: geometric mean parasite density; BMI: body mass index.

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Authors' contributions

PPG, FPM, PG, AJ, RD and DS designed the study. PPG, KS, JW, AB, AK, SB, RD, and DS were responsible for patient recruitment, clinical and laboratory examinations. PPG, KS, RR and SK did the PCR analyses. PPG, WVL and FPM did the statistical analyses and wrote the paper with major contributions of the other authors. All authors read and approved the final manuscript.

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Availability of data and materials
The dataset generated and/or analysed in this study is not publicly available due to issues of confidentiality and ongoing analyses, but are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The study protocol was reviewed and approved by the Institutional Ethics Committee of Kasturba Medical College, Mangalore, Manipal University (IEC KMC MLR 05-1598), and informed written consent was obtained by all study patients. Permission to conduct the study was granted by the Directorate of Health and Family Welfare Services, Government of Karnataka.

Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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Short Report: No Association of the p53 Codon 72 Polymorphism with Malaria in Ghanaian Primiparae and Rwandan Children

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Abstract. The p53 protein is a key cell-signaling mediator integrating host responses to various types of stress. A common polymorphism of the encoding *TP53* gene (codon 72, Pro > Arg, rs1042522) is associated with susceptibility to virus-related and other cancers. The p53 has also been shown to be central for successful *Plasmodium* liver stage infection. We examined whether the polymorphism is associated with *P. falciparum* infection in Ghanaian primiparae and Rwandan children. The allele frequency of *TP53* codon 72 Arg was 0.30 among 314 Ghanaian primiparae and 0.31 among 545 Rwandan children, respectively, and it was not associated with infection prevalence or parasite density. This does not exclude p53 to be of pathophysiological relevance in malaria but argues against a major respective role of the *TP53* codon 72 polymorphism.

In view of the ongoing burden of malaria, improved knowledge of pathophysiological mechanisms may provide novel targets for prevention and treatment.[1] Recently, the p53 tumor suppressor protein, a central host cell-signaling factor, has been shown to be critical for successful *Plasmodium* liver stage infection.[2] As a transcription factor, p53 responds to various stimuli to induce apoptosis or cell cycle arrest, or to integrate a variety of other host responses.[3,4] *Plasmodium* liver stage parasites suppress p53 thereby promoting survival of the infected hepatocyte. Conversely, increased levels of p53 counterbalance this suppression and reduce liver stage parasite burden.[2]

The *TP53* gene, encoding p53, shows a common single nucleotide polymorphism at codon 72 (proline to arginine, Pro > Arg, rs1042522), conferring various functional consequences including the Arg allele being more effective at inducing apoptosis.[5] This allele has been associated with altered susceptibility to several virus-related and other cancers.[4,6] Notably, indirect evidence for a malaria-protective role of the *TP53* codon 72 Arg allele is derived from a small study in Sardinia.[7]

We, therefore, examined the distribution of the *TP53* codon 72 alleles in two African populations with or without *P. falciparum* infection, i.e., placental *P. falciparum* infection in Ghanaian primiparae, and largely asymptomatic *P. falciparum* infection among Rwandan children.

Socio-demographic, clinical, and parasitological characteristics of the two cross-sectional studies and the study groups have been reported elsewhere.[8,9] In brief, 314 primiparous pregnant women were recruited in southern Ghana of whom two-thirds (by polymerase chain reaction [PCR]) had largely asymptomatic placental *P. falciparum* infection (i.e., only 6.3% of these were febrile), which nevertheless was associated with maternal anemia, low birth weight, and preterm delivery.[8] The second group involved 545 children < 5 years of age randomly selected from 24 rural villages in southern highland Rwanda of whom 16.1% were *P. falciparum* infected (by PCR; 2.9% categorized as symptomatic malaria defined as a positive blood film *plus* fever, or a history of fever within the preceding 48 hours).[9] All participants (or legal guardians) gave informed consent, and the study protocols were approved by the Committee on Human Research Publications and Ethics, School of Medical Sciences, University of Science and Technology, Kumasi, Ghana, and the National Ethics Committee, Republic of Rwanda, respectively. The DNA was extracted from stabilized blood samples (AS1 and QIAmp DNA Blood Mini Kit; Qiagen, Hilden, Germany). The Arg allele was differentiated from the Pro allele at codon 72 of *TP53* by restriction fragment length polymorphism of PCR-generated amplicons using primers and the restriction enzyme *Bst*U1 as published elsewhere.[10] Data were analyzed with Statview 5.0 (SAS Institute Inc., Cary, NC). Allele frequencies and genotypes were compared by the χ^2 test, and continuous variables by the Mann Whitney *U* test or Student's *t* test as applicable.

The distribution of *TP53* codon 72 genotypes according to *P. falciparum* infection among Ghanaian primiparous women and Rwandan children is presented in Table 1. Genotypes were in Hardy–Weinberg equilibrium among Rwandan children ($P = 0.51$) but not among Ghanaian women ($P = 0.03$). Arg allele frequencies (Ghana, 0.30; Rwanda, 0.31) and genotypes did not differ between *P. falciparum* infected and non-infected subjects. Adjusting for the age difference between infected and non-infected individuals and for further associated factors,[8,9] the lack of association between *TP53* genotypes and *P. falciparum* infection remained (data not shown). Likewise, there was no association between genotypes and peripheral blood geometric mean parasite density (/μL, 95% confidence interval [CI]), in either pregnant women (Pro/Pro, 1,096 [513–2,346]; Pro/Arg, 675 [390–1,168]; Arg/Arg, 1,449 [134–15,690]; $P = 0.52$) or in children [1,837 (978–3,449]; 968 [404–2,322]; 6,792 [615–75,062]; $P = 0.12$). In pregnant women, the *TP53* codon 72 allele had no influence on maternal anemia, birth weight, or preterm delivery, irrespective of placental malaria (data not shown), and in Rwandan children, the prevalence of symptomatic malaria did not differ significantly with genotype (Pro/Pro, 3.1% [8 of 260]; Pro/Arg, 1.8% [4 of 228]; Arg/Arg, 7.1% [4 of 57]; $P = 0.11$).

Findings from murine models indicate a critical role of p53 in susceptibility to *Plasmodium* infection in that increased levels reduce parasite liver stage burden.[2] The Arg allele of the *TP53*

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TABLE 1

Distribution of *TP53* genotypes according to *Plasmodium falciparum* infection in Ghanaian primiparae and Rwandan children

Parameter	*P. falciparum* infection*		*P*
	None	Present	
Ghanaian primiparae			
No.	107	207	
Age (years; median, range)	21.0 (16–36)	20.0 (15–33)	0.04
TP53 codon 72 genotypes			
Pro/Pro	49.5 (53)	44.9 (93)	
Pro/Arg	44.9 (48)	48.3 (100)	
Arg/Arg	5.6 (6)	6.8 (14)	0.72
TP53 codon 72 Arg allele frequency	0.28	0.31	0.46
Rwandan children			
No.	457	88	
Age (months; median, range)	31 (1–60)	35.5 (4–60)	0.02
TP53 codon 72 genotypes			
Pro/Pro	47.9 (219)	46.6 (41)	
Pro/Arg	41.6 (190)	43.2 (38)	
Arg/Arg	10.5 (48)	10.2 (9)	0.96
TP53 codon 72 Arg allele frequency	0.31	0.32	0.89

* In pregnant women and children, *P. falciparum* was detected in placental and peripheral blood samples, respectively.

codon 72 variant is pro-apoptotic, potentially protective with respect to virus-related and other cancers,[4–6] and suggested to be subject to selection because of protection against malaria.[7] In this study, we are unable to show a role of the *TP53* codon 72 allele in malaria. As a matter of fact, this study was not *a priori* designed to assess an association between *TP53* codon 72 alleles and *P. falciparum* infection. Considering the given prevalence, the sub-studies were powered (80%) to detect only substantial reductions of infection caused by the Arg allele (Ghana, OR, ≤ 0.60; Rwanda, OR, ≤ 0.50). Smaller effects may thus be discernible at considerably larger sample sizes. Nevertheless, allele frequencies in our African study populations were considerably lower than in Sardinia or other Caucasian populations,[7,11,12] and a declining Arg allele frequency toward the equator argues against selection by malaria.[11,12] Notably, respective genetic data from Africans are comparatively scarce. Genotype frequencies deviated from Hardy–Weinberg equilibrium in the Ghanaian subgroup. One reason for this might be a small sample size. Interestingly, the *TP53* codon 72 Pro/Pro genotype has been associated with reduced pregnancy rates,[13] and in fact, this genotype tended to be underrepresented among the Ghanaian primiparae. The present findings of lacking genetic association do not exclude p53 to be pathophysiologically relevant in malaria,[2] but argue against a major respective role of the *TP53* codon 72 allele. The p53 is activated by various stress signals and can induce a variety of host responses[3,4]; possibly, the biological effects conferred by this allele are not those essential in antiplasmodial defense. Alternatively, the variant may not influence *P. falciparum* infection per se, as examined in this study, but rather specific malaria entities, e.g., severe malaria, or infection risk in other populations.

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Reduced prevalence of placental malaria in primiparae with blood group O

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Abstract

Background: Blood group O protects African children against severe malaria and has reached high prevalence in malarious regions. However, its role in malaria in pregnancy is ambiguous. In 839 delivering Ghanaian women, associations of ABO blood groups with *Plasmodium falciparum* infection were examined.

Methods: *Plasmodium falciparum* infection was diagnosed in placental blood samples by microscopy and PCR assays. Present or past infection was defined as the detection of parasitaemia or haemozoin by microscopy, or a positive PCR result. Blood groups were inferred from genotyping rs8176719 (indicating the O allele) and rs8176746/rs8176747 (distinguishing the B allele from the A allele).

Results: The majority of women had blood group O (55.4%); present or past *P. falciparum* infection was seen in 62.3% of all women. Among multiparae, the blood groups had no influence on *P. falciparum* infection. In contrast, primiparae with blood group O had significantly less present or past infection than women with non-O blood groups (61.5 *vs* 76.2%, $P = 0.007$). In multivariate analysis, the odds of present or past placental *P. falciparum* infection were reduced by 45% in blood group O primiparae (aOR, 0.55 [95% CI, 0.33–0.94]).

Conclusions: The present study shows a clear protective effect of blood group O against malaria in primiparae. This accords with findings in severe malaria and *in vitro* results. The data underline the relevance of host genetic protection among primiparae, i.e. the high-risk group for malaria in pregnancy, and contribute to the understanding of high O allele frequencies in Africa.

Background

Plasmodium falciparum malaria has long been suggested to influence the global distribution of the ABO blood groups [1], similar to the selection of the malaria-protective sickle cell gene in endemic regions [2]. In fact, blood group O is particularly common in malarious regions, e.g. sub-Saharan Africa [3,4], and confers protection against potentially fatal severe malaria across African populations [5-8]. In addition, various *in vitro* observations and functional hypotheses support a protective role of blood group O (reviewed by [3,4,9]).

In contrast, findings are notably ambiguous with respect to the impact of the ABO system on malaria in pregnancy. Pregnant women – particularly primiparae – are a high risk group for *P. falciparum* infection and malaria. In areas of high transmission, malaria in pregnancy is frequently asymptomatic but consequences involve anaemia, abortion, stillbirth, low birth weight (LBW), preterm delivery (PTD), and, annually, up to 200,000 infant deaths [10]. In pregnant women, specific expression variants of the parasite's *P. falciparum* erythrocyte membrane protein-1 (PfEMP1) mediate adhesion to the placental syncytiotrophoblast (the epithelial lining of the intervillous space) and thereby placental sequestration of infected erythrocytes, deposition of haemozoin (malaria pigment), and, commonly, the local accumulation of inflammatory cells [11]. In highly endemic regions, specific immunity against these pregnancy-associated parasites is particularly low in primigravidae, and acquired only with successive pregnancies, which goes along with declining infection prevalence and clinical manifestation [10-12]. In The Gambia and Malawi, blood group O was associated with increased odds of placental malaria in primiparae but with a reduced risk in

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multiparae [13,14]. In Sudan, blood group O and past placental infection were associated in both primi- and multiparae [15]. In contrast, a Gabonese study reported a trend towards less placental malaria in blood group O women [16], and in a recent prospective study from Thailand, no association at all between ABO blood groups and malaria during pregnancy was observed [17]. Against this background of conflicting results, the present study aimed at examining the influence of blood group O on *P. falciparum* infection among pregnant women in hyper- to holo-endemic Ghana using PCR for blood group genotyping.

Methods

Eight-hundred and thirty-nine women with live singleton delivery were recruited from January 2000 through January 2001 at the Presbyterian Mission Hospital in Agogo, Ghana. The study protocol was approved by the Committee on Human Research Publications and Ethics, School of Medical Sciences, University of Science and Technology, Kumasi, and informed written consent was obtained from all women. Agogo is a community of some 30,000 inhabitants located in the forested hills of Asante Akim North District. Subsistence farming, trade and mining are the main income sources in that region, and malaria is hyper- to holo-endemic [18]. Study procedures and the characteristics of the largely asymptomatic participants have been described in detail elsewhere [12]. In brief, women were clinically examined, socio-economic data documented, and intervillous and venous blood samples were collected into EDTA. Malaria parasites in post-delivery placental and venous samples were counted microscopically on Giemsa-stained thick blood films *per* 100 high-power fields and 500 white blood cells, respectively. Placental parasite densities were expressed as parasites/100 fields and peripheral ones as parasites/μL assuming a mean white blood cell count of 8,000/μL. Leukocyte-associated haemozoin in placental samples was recorded. Following DNA extraction (QIAmp, Qiagen, Hilden, Germany), nested *P. falciparum*-specific PCR assays were performed [19]. Present or past placental *P. falciparum* infection was defined as the presence of placental parasitaemia or haemozoin by microscopy, or a positive *P. falciparum* PCR result on placental samples. Plasma concentrations of pyrimethamine, at that time recommended for malaria chemoprophylaxis in pregnancy, and of chloroquine were measured by ELISA assays [20] with limits of detection of 10 ng/mL and 5 ng/ml, respectively. Haemoglobin (Hb) was measured by a HemoCue photometer (Ångelholm, Sweden). Anaemia was defined as Hb <11 g/dL. Birth weight and gestational age were assessed within 24 hours after delivery. LBW was defined as a birth weight <2500 g. PTD was defined as a gestational age <37 weeks applying the Finnström score which correctly estimates gestational age (±3 weeks) in >95% of infants [21].

Three loci on the *ABO* gene, namely rs8176719, rs8176746, and rs8176747 were typed by melting curve analysis employing the LightCycler 480 device (Roche Diagnostics, Mannheim, Germany) and using commercially available primers and probes (TIB Molbiol, Berlin, Germany). ABO blood groups were inferred from rs8176719 (indicating the *O* allele) and from rs8176746/rs8176747 (distinguishing the *B* allele from the *A* allele) [22]. In case of ambiguous typing results ($n = 38$), PCR-generated fragments containing the polymorphisms of interest were subjected to sequencing (Eurofins, Berlin, Germany).

Continuous variables were compared between groups by *t*-test, analysis of variance, Mann–Whitney *U*-test, and Kruskal-Wallis test as applicable. Associations of blood groups with *P. falciparum* infection, anaemia, LBW, and PTD were identified by χ^2-test, and odds ratios (ORs) and 95% confidence intervals (95% CIs) calculated. Adjusted ORs (aORs) were derived from logistic regression models with stepwise backward removal of factors not associated in multivariate analysis ($P > 0.05$). For placental malaria, previously identified associated factors were *a priori* included, i.e. delivery in rainy season, age and plasma pyrimethamine concentrations [12]. A *P*-value of <0.05 was considered statistically significant.

Results

ABO genotyping was successful in 827 of 839 (98.6%) women with a live singleton delivery. The majority of women (55.4%) had blood group O. Blood groups B, A, and AB were present in 22.6, 18.0, and 4.0%, respectively. Blood groups were in Hardy-Weinberg equilibrium; genotypes are detailed in Table 1. Plasma concentrations of pyrimethamine, representing chemoprophylaxis, were observed in 36.0% (163/453) of women with blood group O, and in 33.3% (119/357) of women with non-O blood groups ($P = 0.43$; chloroquine plasma concentrations, 24.3% (110/453) *vs.* 20.2% (72/357), $P = 0.16$).

Evidence of present or past *P. falciparum* infection in placental samples (parasitaemia or haemozoin by

Table 1 ABO genotypes in 827 delivering Ghanaian women

Phenotype	Genotype	No (%)
O	O O*	458 (55.4)
B	B O	169 (20.4)
	B B	18 (2.2)
A	A O	136 (16.4)
	A A	13 (1.6)
AB	A B	33 (4.0)

*, includes genotypes OO^{24} ($n = 30$), $O^{24}O^{24}$ ($n = 1$), and $O^{2}O^{2}$ ($n = 1$).

microscopy, or a positive *P. falciparum* PCR result) was seen in 62.3% (515/827) of all women. This figure was significantly lower in women with blood group O (59.0%, 270/458) than in women with non-O blood groups (66.4%, 245/369; $P = 0.03$). Notably, this difference was pronounced among primiparae (61.5% *vs.* 76.2%, $P = 0.007$) but insignificant among multiparae (Table 2). Further analysis therefore focused on the 300 primiparous women. Among these, the prevalence of infection (regardless of diagnostic tool) appeared to be lower in women with blood group O than in any of the non-O blood groups. Based on parasite detection by microscopy, this difference was only small. Likewise, neither placental nor peripheral blood parasite densities correlated with the blood groups (e.g., placental parasite densities in blood group O *versus* non-O primiparae: 127 (95% CI, 74–216) *vs.* 102 (51–204) parasites/100 high-power fields, $P = 0.63$). In contrast, the reduced infection prevalence among blood group O primiparae was obvious taking into account PCR results, and even more so when considering placental haemozoin (Table 3). In multivariate analysis, the odds of present or past placental *P. falciparum* infection were reduced by 45% in primiparae with blood group O (Table 3), adjusting for known predictors of placental infection in this group [12], i.e. years of age (aOR, 0.92 [95% CI, 0.86–0.99]), delivery in the rainy season (aOR, 1.76 [95% CI, 1.06–2.92]), and presence of pyrimethamine in plasma (indicating compliance with chemoprophylaxis; aOR, 0.60 [95% CI, 0.35–1.00]). Breakdown by placental infection status [23] revealed that blood group O tended to protect particularly against late infections (both parasites and pigment present), while - combining microscopy and PCR results - the impact on microscopic (i.e. visible parasites) and submicroscopic infections (i.e. detectable by PCR only) was similar (Table 3).

Small numbers prevented a meaningful attribution of infection risks to individual genotypes. Nevertheless, present or past placental infection was observed in 88.9% (16/18) of primiparae with homozygous non-O genotypes, in 74.1% (80/108) of O-heterozygous women, and in 61.5% (107/174) of first-time delivering women with blood group O (c^2 trend = 8.7, $P = 0.003$). Correspondingly, the *O* allele was significantly less frequent in primiparae with present or past placental infection than in those without (0.72 *vs* 0.84, $P = 0.003$).

In multiparae, none of the above malaria-protective features of blood group O were discernible; blood group A even tended to come along with lower infection prevalence (Table 2). Particularly, already in secundiparae, present or past placental infection occurred at similar prevalence in women with blood group O and in the non-O group (66.0% (70/106) *vs* 69.2% (54/78), $P = 0.65$).

Blood group O was furthermore associated with reduced odds of maternal anaemia among all women, and among primiparae in particular (Table 4). This was due to the difference in women without placental malaria, i.e. only 10.5% (7/67) of non-malarious blood group O primiparae in this group were anaemic as compared to 33.3% (10/30) of their non-O peers ($P = 0.006$) whereas the difference was small and not significant among primiparae with evidence of present or past infection (44.9%, 48/107 *vs* 52.1%, 50/96, $P = 0.30$). Likewise, the proportions of malaria-associated LBW or preterm delivery did not differ significantly between O and non-O blood groups, neither in primiparae nor in multiparae.

Discussion

In the present study from Ghana, almost two in three women had evidence of present or past placental *P. falciparum* infection. In this highly endemic setting, blood group O was associated with protection against placental malaria among primiparae but not in multiparae, and particularly against late or chronic infections, i.e., infections characterized by the presence of pigment. This

Table 2 Prevalence of *Plasmodium falciparum* infection according to blood group and parity

	Blood group, primiparae					Blood group, multiparae				
	O	Non-O				O	Non-O			
		All	B	A	AB		All	B	A	AB
No.	174	126	72	46	8	283	237	111	102	24
P. falciparum infection, placental blood (%)										
Microscopy positive	42.5	50.0	52.8	43.5	62.5	30.0	27.0	31.5	21.6	29.2
Haemozoin positive	36.2	50.8*	52.8*	50.0	37.5	24.0	24.9	28.8	17.6	37.5
PCR positive	60.3	70.6	65.3	76.1*	87.5	55.5	58.2	60.4	54.9	62.5
Any positive finding[†]	61.5	76.2*	69.4	84.8*	87.5	57.6	61.6	64.0	57.8	66.7
P. falciparum infection, peripheral blood (%)										
Microscopy positive	24.1	29.4	33.3	26.1	12.5	17.0	11.4	14.4	8.8*	8.3
PCR positive	54.6	65.1	62.5	69.6	62.5	50.5	49.4	52.3	45.1	54.2

*Comparison to blood group O, $P < 0.05$; [†], parasitaemia or haemozoin by microscopy, or positive PCR. Parity data were missing for seven women.

Table 3 Placental *Plasmodium falciparum* infection in primiparae separated by blood group O (*n* = 174) vs non-O blood groups (*n* = 126)

Placental infection status	Blood group		OR (95% CI)	*P*	aOR* (95% CI)	*P*
	O	Non-O				
Microscopy positive (%)	42.5	50.0	0.74 (0.45-1.20)	0.20	0.80 (0.49-1.28)	0.35
Haemozoin positive (%)	36.2	50.8	0.55 (0.34-0.90)	0.01	0.61 (0.37-0.98)	0.04
PCR positive (%)	60.3	70.6	0.63 (0.38-1.06)	0.07	0.68 (0.41-1.13)	0.14
Any positive finding (%)†	61.5	76.2	0.50 (0.29-0.86)	0.007	0.55 (0.33-0.94)	0.03
Microscopic infection status (%)						
Pigment only	5.2	8.7	0.47 (0.16-1.32)	0.11	0.62 (0.23-1.67)	0.35
Pigment *plus* parasites	31.0	42.1	0.58 (0.34-1.0)	0.04	0.64 (0.38-1.09)	0.10
Parasites only	11.5	7.9	1.14 (0.46-2.85)	0.75	1.33 (0.55-3.20)	0.52
PCR-based infection status (%)						
Submicroscopic	17.8	22.2	0.56 (0.28-1.14)	0.08	0.59 (0.30-1.14)	0.12
Microscopic	42.5	50.0	0.60 (0.34-1.04)	0.05	0.64 (0.37-1.11)	0.12

Odds ratios are calculated against the respective non-infected reference group.
*Adjusted for age, rainy season, and presence of pyrimethamine in plasma (indicating intake of chemoprophylaxis).
†Parasitaemia or haemozoin by microscopy, or positive PCR.

finding accords with the clearly protective role of blood group O in severe malaria [5-8] and the moderately beneficial impact of blood group O in many studies on uncomplicated malaria (reviewed by [3,4,9]). Severe childhood malaria and malaria in pregnancy differ largely in terms of pathophysiology, immunity and clinical manifestation [10-12], but primiparae to some extent resemble young children with respect to a lack or an insufficient degree of protective immune mechanisms against *P. falciparum*. In pregnant women, *P. falciparum*-infected erythrocytes sequester in the intervillous space by adhering to ligands on the syncytiotrophoblast, followed by the local accumulation of haemozoin and inflammatory cells. Specific antibodies capable of blocking parasite adhesion prevent this placental malaria only after successive pregnancies exposed to *P. falciparum* infection [11,24,25]. This likely explains why protection against *P. falciparum* infection due to blood group O in the present study was limited to the relatively immune-naïve group of primiparae. At higher parity, the effects of adaptive immunity may override the protection afforded by blood group O.

Notably, however, two studies from The Gambia and Malawi have reported results contrasting the present finding, i.e. an increased risk of active placental malaria in blood group O primiparae and a reduced risk in multiparae [13,14]. The reason for this disagreement is unclear but may involve the classification of placental malaria by histopathological diagnosis in these previous studies and by blood film microscopy and PCR in the present. Remarkably, in both previous studies, past infections (presence of pigment only) tended to be reduced in blood group O primiparae. In the present study, much of the observed associations was based on the lowered presence of haemozoin but microscopically visible parasites also tended to be reduced in group O primiparae (Table 3). Sensitive PCR assays in the present study, considered to detect only viable parasites [26], only shifted this difference between blood groups to a higher prevalence level and, interestingly, the reduced infection rate in blood group O primiparae was observed for both microscopic and submicroscopic infections. This is in line with the lack of effect of the blood groups on parasite density observed in the present study. Part of the conflicting results with and between previous studies may result from small samples sizes [13,15,16] as well as from low prevalence of infection [15-17] in previous studies. Notably, however, in one small study from Gabon, blood group O tended to protect against placental malaria, with a larger effect in primiparae than in

Table 4 Anaemia, low birth weight, and preterm delivery separated by blood group and parity

Parameter	All		Primiparae		Multiparae	
	Blood group O	Non-O blood groups	O	Non-O blood groups	O	Non-O blood groups
Anaemia (%, n/n)	31.7 (145/458)	39.6 (146/369)*	31.6 (55/174)	47.6 (60/126)†	31.8 (90/283)	35.9 (85/237)
LBW (%, n/n)	14.6 (67/458)	17.9 (66/369)	23.0 (40/174)	30.2 (38/126)	9.5 (27/283)	11.4 (27/237)
PTD (%, n/n)	17.8 (81/454)	20.3 (74/365)	24.7 (43/174)	28.6 (36/126)	13.6 (38/279)	15.8 (37/234)

*, $P = 0.02$; †, $P = 0.005$.

multiparae [16]. In almost 1,500 women in Thailand, weekly malaria screening by peripheral blood films did not show any effect of the ABO blood groups on malaria episodes (occurring in 30% of women) [17]. However, peripheral blood film microscopy in pregnant women has a notoriously poor sensitivity [27], only 1% of placentas were malaria positive, and the majority of malaria episodes were due to *Plasmodium vivax*. Therefore, it is questionable whether these findings from Thailand can be compared with the African studies. Blood group O in the present study was also associated with reduced odds of anaemia, particularly in primiparae. Protection from malaria-related anaemia in individuals with blood group O has been observed before [28] but, remarkably, this was not the case in the present study, in which, rather, anaemia was less common among non-infected blood group O women. This finding may reflect the cumulative effect over time of reduced malaria in blood group O primiparae. Longitudinal studies will be needed to address this question.

As a limitation of the present study, protection by blood group O was statistically significant only when considering placental haemozoin or when combining results of all diagnostic means (although microscopic data showed accordant trends). Statistically significant findings were observed in the relatively small group of 300 primiparae of whom 174 had blood group O. The study was sufficiently powered to detect an influence of blood group O on present or past infection but power declined with declining diagnostic sensitivity, i.e. prevalence of infection. Limited sample sizes in subgroups may thus have interfered with the analysis of infection risks in individual genotypes and of potential modification of malaria-related outcomes, e.g., LBW. Also, data on some potentially interfering factors were not available, e.g., bed net use or transfusions. This should be kept in mind when interpreting the data.

The hypotheses on why blood group O protects from malaria, and from severe malaria in particular, include aspects of differential attractiveness to *Anopheles* vectors, shared ABO antigens with *P. falciparum*, impaired merozoite penetration as well as reduced cyto-adherence (reviewed by [3,4,9]). The A and B antigens are erythrocyte surface trisaccharides [22] and receptors for rosetting, i.e. the binding of *P. falciparum*-infected red blood cells (RBCs) to uninfected erythrocytes (an *in vitro* sign of parasites causing severe malaria) [5]. Binding of the parasite ligand, PfEMP1, to the A antigen has been shown [29]. In blood group O, the disaccharide H antigen is produced instead of A and B antigens [22], and *P. falciparum*-infected RBCs form smaller and less firm rosettes than in non-O RBCs [29]. In fact, the protective effect of blood group O against severe malaria has been shown to operate through the mechanism of reduced *P. falciparum* rosetting [5]. However, rosetting is rarely observed in placental *P. falciparum* isolates [30] and the PfEMP1 domain mediating binding to ligands on the syncytiotrophoblast differs from the binding domain involved in rosetting [29,31,32]. Nevertheless, ABO-dependent differences in cyto-adherence may still be relevant in placental malaria (reviewed by [4]): The molecules on the syncytiotrophoblast to which pregnancy-specific *P. falciparum* strains bind, i.e. chondroitin sulphate A (CSA) and hyaluronic acid [33,34], are structurally related to the A antigen which gives rise to the possibility that adhesion of infected RBCs to the syncytiotrophoblast may be influenced by ABO polymorphisms. Also, the placental proteoglycan thrombomodulin mediates part of the binding of infected RBCs [35]. Thrombomodulin and other molecules involved in cyto-adhesion, e.g., von Willebrandt factor, are affected by the ABO phenotype [36]. Recently, enhanced macrophage-mediated phagocytosis of *P. falciparum*-infected O-RBCs has been shown [37]. Considering that many women with placental malaria show a substantial inflammatory infiltrate in the intervillous space, composed mainly of monocytes and macrophages [38], ABO-dependent clearance of infected RBCs could partially explain the finding of the present study of less placental malaria in women with blood group O.

Conclusion

The present study demonstrates a protective influence of blood group O against *P. falciparum* infection in primiparae living in an area of high malaria endemicity. Considering the consequences of placental malaria in terms of infant mortality, the influence of the ABO polymorphism on malaria in pregnancy may contribute to the high frequencies of the O allele in sub-Saharan Africa.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GBA, KT, and FPM designed the study. GBA, TAE and FPM were responsible for patient recruitment, clinical and laboratory examinations. PPG and SM did the PCR analyses, TAE and KT contributed to data interpretation, and FPM did the statistical analyses. GBA and FPM wrote the paper with major contributions of the other authors. All authors read and approved the final manuscript.

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MiRNA-146a polymorphism increases the odds of malaria in pregnancy

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Abstract

Background: *Plasmodium falciparum* infection during pregnancy is a major cause of poor maternal health, adverse foetal outcome and infant mortality in sub-Saharan Africa. Genetic disposition is involved in susceptibility to malaria in pregnancy and its manifestation. MicroRNAs (miRNAs) influence gene regulation including that of innate immune responses. A miRNA-146a rs2910164 G > C single nucleotide polymorphism (SNP) has been associated with increased risks of several diseases, but no data as to malaria are available.

Methods: The association between miRNA-146a rs2910164 and *P. falciparum* infection among 509 Ghanaian women attending antenatal care (ANC) and 296 delivering Ghanaian primiparae was investigated. Malaria parasites were diagnosed by microscopy and PCR. Leukocyte-associated hemozoin in placental samples was recorded as well. Proportions were compared between groups by Fisher's exact test, and logistic regression models were used to adjust for possible confounders.

Results: By PCR, *P. falciparum* infection was detected in 63% and 67% of ANC attendees and delivering primiparae, respectively. In both groups, two in three women were either heterozygous or homozygous for miRNA-146a rs2910164. Among ANC attendees, homozygosity conferred increased odds of infection (adjusted odds ratio (aOR), 2.3; 95% CI, 1.3–4.0), which was pronounced among primigravidae (aOR, 5.8; 95% CI, 1.6–26) but only marginal in multigravidae. Likewise, homozygosity for miRNA-146a rs2910164 in primiparae increased the odds of past or present placental *P. falciparum* infection almost six-fold (aOR, 5.9; 95% CI, 2.1–18).

Conclusions: These results indicate that SNP rs2910164 G > C is associated with increased odds for *P. falciparum* infection in first-time pregnant women who are considered to lack sufficient acquired immune responses against pregnancy-specific strains of *P. falciparum*. These findings suggest that miRNA-146a is involved in protective malarial immunity, and specifically in the innate component.

Keywords: Malaria, Pregnancy, *Plasmodium falciparum*, MiRNA-146a, Polymorphism, Innate immunity

Background

Plasmodium falciparum infection during pregnancy is a major cause of poor maternal health, miscarriage, stillbirth, low birth weight (LBW), preterm delivery and infant mortality in sub-Saharan Africa. Primiparous woman exhibit an increased susceptibility to *P. falciparum* infection and consequently bear a higher risk for placental malaria (i.e., parasites and/or malaria pigment (hemozoin) discernible in placental tissue or blood), malarial anaemia and malaria-related morbidity and mortality as compared to multigravidae. The increased risk of malaria and complications is largely due to parasites exhibiting specific variants of the *P. falciparum* erythrocyte membrane protein-1, which facilitate adhesion to the syncytiotrophoblast (the surface lining the placental intervillous space), followed by the accumulation of infected erythrocytes and inflammatory cells in the placental intervillous space [1]. The acquisition of specific immune responses to syncytiotrophoblast-adhering *P. falciparum* strains increases with every consecutive pregnancy, resulting in better parasite recognition and

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reduced susceptibility and manifestation in multigravidae [2]. Moreover, due to placental sequestration, microscopy strongly underestimates actual prevalence of *P. falciparum* infection in pregnancy [3].

Host genetic variation plays an important role in susceptibility to and manifestation of malaria. The association of single nucleotide polymorphisms (SNPs) in genes encoding toll-like receptors (TLRs) and other members of the innate immune system with susceptibility to (severe) malaria in Sub-Saharan African populations [4, 5] suggests that SNPs in other immune regulators such as micro-RNAs (miRNAs) influence malaria as well. MiRNAs are a class of small, non-coding, evolutionarily conserved RNA strains of approximately 22 nucleotides, and they are involved in gene regulation by their posttranslational action at the 3′-UTR region of mRNA. They control many processes, including pathways in the innate and adaptive immune responses [6]. MiRNA-146a is involved in the innate immune response by a negative feedback loop including two key molecules downstream of the TLR machinery: interleukin-1 receptor-associated kinase (IRAK)-1 and TNF receptor-associated factor (TRAF)-6 [7]. Recent studies have shown the potential of using miRNA-146a as a biopharmaceutical agent [8, 9]. The presence of the variant C-allele in SNP rs2910164 disrupts miRNA-146 processing and leads to altered IRAK-1 and TRAP-6 expression [7]. SNP rs2910164 in the passenger strand of pre-miRNA-146a has been linked with both decreased and increased risk to various types of cancer [10], autoimmune diseases [11] and increased susceptibility of mycobacterial infections [12, 13].

In this cross-sectional study, the presence of miRNA-146a SNP rs2910164 G > C was hypothesized to affect susceptibility to *P. falciparum* infection. *Plasmodium falciparum* infection was assessed and the miRNA-146a SNP was genotyped in 805 Ghanaian pregnant women, a group at high risk of malaria.

Methods

In November and December 1998 and between January 2000 and January 2001, respectively, 530 pregnant women attending antenatal care (ANC) and 893 delivering woman were recruited at the Presbyterian Mission Hospital in Agogo, Ashanti Region, Ghana, a region holoendemic for *P. falciparum* [14]. Informed consent was obtained from all study participants (from parents or guardians of those < 18 years of age). The study protocols were reviewed and approved by the Committee on Human Research Publication and Ethics, School of Medical Sciences, University for Science and Technology, Kumasi, Ghana. Study groups, procedures and malariological indices have been described previously [15, 16]. Briefly, all women were clinically examined, socioeconomic data, gravidity or parity, fever (≥ 37.5 °C, axillary for ANC attendees and sublingual for delivering women) were documented, and samples of venous and intervillous (delivering women) blood were collected into EDTA. For the present study, 530 ANC attendees and 304 primiparae with live singleton delivery were included. The group of ANC attendees comprised of 24.9% (127/509) primigravidae, 21.2% (108/509) secundigravidae and 53.8% (274/509) multigravidae.

Plasmodium parasite density in venous and intervillous blood samples were microscopically counted on Giemsa-stained think films *per* 500 white blood cells (WBC) and *per* 100 high-power fields (HPF), respectively. The presence of leukocyte-associated hemozoin in the intervillous samples was also recorded. For ANC attendees, WBCs were counted using a Cell Counter (HC555, Clinicon, Germany) and the peripheral blood parasite density was calculated as parasites per microliter, deducing the multiplicator from the individual WBC count. Plasma and blood cells were separated by centrifugation. Genomic DNA was extracted from blood (QIAamp Blood Kit, Qiagen, Germany) and plasmodial infections and species were diagnosed by nested PCR assays [17]. "Past or present placental malaria" was defined as positivity of placental samples for *P. falciparum* infection by PCR, microscopy, and/or hemozoin detection. MiRNA-146a SNP rs2910164 genotyping was carried out by melting-curve analysis applying commercially available primers and probes (TIB Molbiol, Germany).

Haemoglobin (Hb) was measured by a HemoCue photometer (Ångelholm, Sweden), and anaemia was defined as Hb level < 11 g/dL [18]. Gestational age was assessed within 24 h of delivery by applying the morphological Finnström score and a value < 37 weeks was categorized as preterm delivery [19]; LBW was defined as < 2500 g. Pyrimethamine (PYR), then used as chemoprophylaxis, was detected by enzyme-linked immunosorbent assay based methods in urine (ANC attendees) or plasma (primiparae) [15]. Proportions of *P. falciparum* infection among women with and without the miRNA-146a SNP were compared by a two-tailed Fisher's exact test, and odds ratio (OR) and 95% confidence intervals (95% CIs) were computed. Additionally, miRNA-146a genotypes were compared with respect to the outcomes of malaria, i.e., fever, anaemia, LBW and preterm delivery. Trends, e.g., increasing infection prevalence in women with wild type alleles over heterozygosity to homozygosity for the miRNA-146a SNP, were tested by the Cochran Armitage test. Logistic regression models were used to adjust ORs of infection for known predictors, i.e., age (years), presence of PYR, and rainy or dry season (only in delivering women). All analysis was done in R version 3.4.3. A *P*-value < 0.05 was considered statistically significant.

Results

Typing of the miRNA-146a SNP was successful in 96.0% (509/530) of ANC attendees and 97.4% (296/304) of delivering women. The miRNA-146a polymorphism was present in 67.7% (heterozygous, 48.1%; homozygous, 19.6%; allele frequency, 0.44) of ANC attendees and 69.2% (48.3% and 20.9%; allele frequency, 0.45) of delivering women (Tables 1, 2, 3). In both groups, allele distribution was in Hardy–Weinberg equilibrium ($c^2 = 0.24$; $P = 0.62$, $c^2 = 0.18$; $P = 0.67$).

In peripheral blood samples of ANC attendees (mean age, 26.6 ± 6.3), malaria parasites were detected by microscopy in 32.8% (167/509), and 63.3% (322/509) were found to harbour *P. falciparum* by PCR. Infection prevalence (PCR) was higher in primigravidae (74.0%, 94/127) than in multigravidae (59.7%, 228/382; $P = 0.004$). The geometric mean parasite density (GMPD) of microscopically positive samples was 304/μL (95% CI, 259–356) for all ANC attendees, 165/μL (95% CI, 139–195) for primigravidae and 719/μL (95% CI, 536–963)

Table 1 Prevalence of *P. falciparum* infection (PCR) according to miRNA-146 genotype in pregnant women attending ANC

SNP rs2910164	Positive cases	Univariate analysis[a]		Multivariate analysis[b]	
	% (Fraction)	OR (95% CI)	*P*	aOR (95% CI)	*P*
All	63.3 (322/509)				
Wild type	55.5 (91/164)	1		1	
Heterozygote	64.9 (159/245)	1.5 (1.0–2.3)	0.063	1.4 (0.9–2.1)	0.12
Homozygote	72.0 (72/100)	2.1 (1.2–3.7)	0.0089	2.3 (1.3–4.0)	0.0053
Het. or Hom.	67.0 (231/345)	1.6 (1.1–2.4)	0.014	1.6 (1.1–2.4)	0.023
Primigravidae	74.0 (94/127)				
Wild type	60.0 (21/35)	1			
Heterozygote	75.4 (46/61)	2.0 (0.8–5.5)	0.17	1.8 (0.7–4.7)	0.20
Homozygote	87.1 (27/31)	4.4 (1.2–21.0)	0.025	5.8 (1.6–26.0)	0.012
Het. or Hom.	79.3 (73/92)	2.5 (1.0–6.4)	0.040	2.5 (1.0–6.2)	0.040
Multigravidae	59.7 (288/382)				
Wild type	54.3 (70/129)	1		1	
Heterozygote	61.4 (113/184)	1.3 (0.8–2.2)	0.24	1.3 (0.8–2.1)	0.29
Homozygote	65.2 (45/69)	1.6 (0.8–3.0)	0.17	1.8 (0.9–3.4)	0.082
Het. or Hom.	62.5 (158/253)	1.4 (0.9–2.2)	0.12	1.4 (0.9–2.2)	0.14

Allele frequencies of SNP rs2910164 G > C were 0.47 (303/644) in infected and 0.38 (142/374) in non-infected women ($P = 0.005$)

OR odds ratio, *aOR* adjusted odds ratio

[a] Fisher's exact test for independence, compared to reference (wild type)

[b] Logistic regression model, including co-predictors age, PYR in urine or plasma and number of antenatal care visits. Effect of genotype on outcome variable was compared to reference (wild type)

Table 2 *Plasmodium falciparum* (PCR) infection according to miRNA-146 genotype in placental blood of delivering primiparae

SNP rs2910164	Positive cases	Univariate analysis[a]		Multivariate analysis[b]	
	% (Fraction)	OR (95% CI)	*P*	aOR (95% CI)	*P*
Primiparae	64.9 (192/296)				
Wild type	60.4 (55/91)	1		1	
Heterozygote	60.8 (87/143)	1.0 (0.6–1.8)	1.0	1.4 (0.7–2.9)	0.38
Homozygote	80.6 (50/62)	2.7 (1.2–6.4)	0.013	5.9 (2.1–18.0)	0.0011
Het. or Hom.	66.8 (137/205)	1.3 (0.8–2.3)	0.29	2.1 (1.0–4.2)	0.038

Allele frequencies of SNP rs2910164 G > C were 0.49 (187/384) in infected (PCR) and 0.38 (80/208) in non-infected women ($P = 0.02$)

OR odds ratio, *aOR* adjusted odds ratio

[a] Fisher's exact test for independence, compared to reference (wild type)

[b] Logistic regression model, including co-predictors age, season, PYR in urine or plasma and number of antenatal care visits. Effect of genotype on outcome variable was compared to reference (wild type)

Table 3 Past or present placental malaria (PCR, microscopy, haemozoin) according to miRNA-146 genotype in delivering primiparae

SNP rs2910164	Positive cases	Univariate analysis[a]		Multivariate analysis[b]	
	% (Fraction)	OR (95% CI)	P	aOR (95% CI)	P
Primiparae	67.9 (201/296)				
Wild type	61.5 (56/91)	1		1	
Heterozygote	66.4 (95/143)	1.2 (0.7–2.2)	0.48	1.6 (0.8–3.5)	0.21
Homozygote	80.6 (50/62)	2.6 (1.2–6.1)	0.013	5.9 (2.1–19.0)	0.0013
Het. or Hom.	72.2 (145/205)	1.5 (0.9–2.6)	0.14	2.3 (1.1–4.7)	0.020

OR odds ratio, *aOR* adjusted odds ratio

[a] Fisher's exact test for independence, compared to reference (wild type)

[b] Logistic regression model, including co-predictors age, season, PYR in urine or plasma and number of antenatal care visits. Effect of genotype on outcome variable was compared to reference (wild type)

for multigravidae. 14.7% (75/509) of the ANC attendees were febrile, and 53.6% (273/509) had anaemia (mean Hb, 10.7 ± 1.4 g/dL).

Among delivering primiparae, malaria parasites were detected microscopically in 26.4% (78/296) and 45.6% (135/296) of peripheral and placental blood films, respectively. By *P. falciparum* PCR, these figures were 59.1% (175/296) and 64.9% (192/296). Past or present placental malaria, i.e. considering also deposited hemozoin, was identified in 67.9% (201/296). The GMPD of microscopically positive peripheral and placental blood samples was 709/µL (95% CI, 563–894) and 1.15/HPF (95% CI, 0.86–1.54), respectively. 4.1% (12/293) of the delivering women were febrile and 38.8% (115/296) had anaemia (mean Hb, 11.2 ± 4.1 g/dL). 25.7% (76/296) of the neonates had LBW and 26.4% (78/296) were preterm.

Carriage of the miRNA-146a SNP was associated with increased odds of *P. falciparum* infection in both ANC attendees (Table 1) and primiparae (Tables 2, 3). This finding was due to a strong respective effect among homozygous individuals, i.e., more than five-fold increased odds of infection in primigravidae and primiparae, and a lesser, non-significant one among heterozygous women. Consequently, in primigravidae and primiparae, significant trends were seen for increasing *P. falciparum* prevalence from wild type individuals over heterozygous to homozygous women (ANC attendees, Z-statistic = −2.8, $P = 0.005$; primigravidae, Z-statistic = −2.5, $P = 0.01$; primiparae, Z-statistic = −2.1, $P = 0.04$). Of note, increased *P. falciparum* prevalence was also observed among multigravid miRNA-146a SNP carriers, however, only weakly and statistically not significant.

In terms of clinical manifestation of infection, the miRNA-146a SNP did not show any significant association. In ANC attendees, fever occurred in 17.1% (28/164) of wildtype individuals and 13.6% (47/345; $P = 0.3$) of SNP carriers, and anaemia was present in 49.4% (81/164) and 55.7% (192/345; $P = 0.2$), respectively. Likewise, among primiparae, proportions did not differ between wildtype women and SNP carriers for fever (5.6%, 5/89 vs. 3.4%, 7/204; $P = 0.5$), anaemia (40.7%, 37/91 vs. 38.0, 78/205; $P = 0.7$; LBW (25.3%, 23/91 vs. 25.8%, 53/205; $P = 1.0$), and preterm delivery (26.4%, 24/91 vs. 26.3%, 54/205; $P = 1.0$). Stratification by infection status did not change this observation.

Discussion

A common miRNA-146a SNP is associated with increased odds of *P. falciparum* infection in first-time pregnant women. This suggests this regulator of inflammation and innate immune responses to be involved in susceptibility to malaria. Genetic host variation contributes to large inter-individual variation in susceptibility to and manifestation of malaria, and the high frequency of several alleles in malaria-endemic regions are considered to reflect evolutionary selection due to this disease. Examples of malaria-protective traits include haemoglobin variants, enzyme disorders, and erythrocyte membrane polymorphisms [20]; whereas polymorphisms in genes encoding innate immune factors may increase or decrease susceptibility and manifestation [21].

The present study for the first time shows an impact of a miRNA genetic variation on the risk of human malaria, even though functional investigations have previously pointed to a role of miRNAs in that disease [22–24]. As a limitation, the present cross-sectional studies were not a priori designed to show associations with genetic traits. As a matter of fact, association does not necessarily mean causality. The classification of past or present placental malaria, i.e., combining microscopy, hemozoin detection, and PCR results, was applied to yield the highest diagnostic sensitivity including recently resolved infection (hemozoin) but does not match with the otherwise known classification based on placental histopathology.

Lastly, due to the absence or late development of acquired immune mechanisms targeting the specific malaria parasites adhering to the intervillous syncytiotrophoblast [2], primigravidae and primiparae are considered relatively immune-naive. On the one hand, this facilitates the identification of the influence of genetic disposition, particularly with respect to innate immune responses. Therefore, and after having observed only weak and non-significant associations among multigravid ANC attendees, we abstained from genotyping multiparae. On the other hand, these findings need to be confirmed for other diseases entities, e.g. uncomplicated malaria or severe paediatric malaria.

Both TLR-2 and TLR-4 recognize *P. falciparum*, which initiates innate immune responses [25]. During innate recognition, miRNA-146a is up-regulated by NF-kB through a MyD88-dependent pathway. Subsequently, IRAK-1 and TRAF-6 are downregulated by miRNA-146a through posttranslational repression. MiRNA-146a thus influences TLR functionality via a negative feedback loop on the downstream mediators IRAK-1 and TRAF-6 [6, 7].

Consequently, altered TLR and cytokine signalling might influence the innate immune response to *P. falciparum* in individuals with variant miRNA-146a. The miRNA-146a rs2910164 G > C SNP, located in the passenger strand of the hairpin structured miRNA (miRNA-146a*), affects the processing of pre-miRNA-146a into mature miRNA-146a. Homozygosity for this polymorphism is associated with reduced expression of the downstream mediators, and heterozygosity with the expression of additional miRNA-146a: one from the leading strand and two from the passenger strand (miRNA-146a*G and miRNA-146a*C), which all three give rise to a mature miRNA [26, 27]. The additional mature miRNA-146a*G and miRNA-146a*C are predicted to have a distinct set of target genes, different from the mature miRNA-146a [26]. Whereas no results with respect to malaria have been published, previous studies reported associations of miRNA-146a rs2910164 G > C with increased susceptibility to pulmonary tuberculosis [12] and leprosy [13], in addition to various effects in neoplastic conditions [10].

Expanding on Haldane's malaria hypothesis, a polymorphism increasing malaria risk should be expected to be rare in endemic regions. However, in sub-Saharan Africa, miRNA-146a rs2910164 occurs in 67% (GC, 44.2%; CC, 23.0%) [28], similar to the present results, and thus more frequently than in Caucasians (41%; GC, 34.5%; CC, 6.2%) [28]. Similar discrepancies have been observed for, e.g., *TLR-4* variants or mannose-binding lectin deficiency [29, 30].

Potential explanations include alleles or genotypes, which may have become deleterious after the out-of-Africa-migration of humans, possibly because of increased susceptibility to severe bacterial infections and sepsis [31]. Alternatively, counter-selecting evolutionary forces leading to high miRNA-146a SNP frequencies in sub-Saharan Africa (which consequently would have a larger impact than malaria) are hard to imagine. With respect to tuberculosis, both increased and decreased susceptibility to pulmonary tuberculosis in case of miRNA-146a rs2910164 have been reported from China [12, 32]. Moreover, the present study showed associations with infection but not with manifestation. For a common *TLR-4* SNP in Ghana, increased susceptibility to severe malaria but a trend towards reduced mortality was found in a previous study [30]. Considering the complex roles of miRNA-146a in immunomodulation and inflammatory responses [33], more refined and prospective studies involving patients of differing ethnicities are required to disentangle the potential influences of miRNA-146a rs2910164 G > C on the various entities of malaria, i.e., from (asymptomatic) infection to (fatal) disease.

Conclusion

Homozygosity for the miRNA-146a rs2910164 SNP predisposes to *P. falciparum* infection in first-time pregnant Ghanaian women. This suggests that miRNA-146a plays an important role in the respective innate immune response but further studies are required to detail the actual pathophysiology involved. Understanding protective immunity towards malaria in pregnancy is essential to improve maternal health and for decreasing the huge share of malaria in infant mortality in sub-Saharan Africa. MiRNA-based biopharmaceuticals are an active field of research. Enhanced antimicrobial immune responses have been observed after silencing or administration of miRNAs [8, 9, 34, 35]. The findings in the present study suggest that miRNA-146a is involved in innate immunity against malaria highlighting its potency as a biopharmaceutical target.

Abbreviations

ANC: antenatal care; aOR: adjusted odds ratio; CI: confidence interval; GMPD: geometric mean parasite density; Hb: haemoglobin; HPF: high power fields; IRAK-1: interleukin-1 receptor-associated kinase; LBW: low birth weight; miRNA: micro-RNA; OR: odds ratio; PYR: pyrimethamine; SNP: single nucleotide polymorphism; TLR: toll-like receptors; TNF: tumor necrosis factor; TRAF-6: TNF receptor-associated factor-6; WBC: white blood cell.

Authors' contributions

FPM and GBA designed the study, and were responsible for patient recruitment, clinical and laboratory examinations. PPG and LH did the genotyping. WvL and FPM did the statistical analyses, and wrote the paper with major contributions of the other authors. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Written informed consent were obtained from the study participants.
The study protocol was approved by the Committee on Human Research Publication and Ethics, School of Medical Sciences, University for Science and Technology, Kumasi, Ghana.

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Artemisinin Resistance–Associated *K13* Polymorphisms of *Plasmodium falciparum* in Southern Rwanda, 2010–2015

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Abstract. Emerging artemisinin resistance is a threat to global malaria control. Mutations in the *Plasmodium falciparum* Kelch 13 (*K13*) propeller domain confer artemisinin resistance and constitute molecular markers for its detection and monitoring. We sequenced 222 *P. falciparum* isolates obtained from community children in the Huye District of southern Rwanda in 2010, 2014, and 2015 to investigate the presence of *K13* polymorphisms. No polymorphisms were observed in 2010 but they were present in 2.5% and 4.5% in 2014 and 2015, respectively. In 2015, two isolates showed candidate *K13* resistance mutations (P574L and A675V), which are common in southeast Asia and associated with delayed parasite clearance. *K13* polymorphisms in southern Rwanda are infrequent but include variants associated with artemisinin resistance. Establishing correlations with local treatment response and in vitro resistance assays are needed in addition to further monitoring *K13* polymorphisms in the study area.

Artemisinin-based combination therapy (ACT) is the mainstay of malaria treatment and control. However, emerging resistance of *Plasmodium falciparum* to artemisinin derivatives (ART) in southeast Asia may threaten the achievements of the last decade in reducing malaria morbidity and mortality. So far, ART resistance refers to delayed parasite clearance and in vitro findings, whereas actual clinical treatment failure still is rare.[1–3] Delayed parasite clearance is increasingly observed in the Greater Mekong sub-region (GMS) in southeast Asia e.g., in Cambodia,[3,4] but only occasionally in sub-Saharan Africa (SSA), for example, in three patients from Uganda.[5] ART resistance poses a serious threat to public health in SSA, potentially leading to a recurrence of the excess mortality due to drug resistance seen before the implementation of the ACTs.[6] Surveillance of ART resistance by clinical trials in SSA is therefore desirable, but its actual performance and coverage are limited by costs and logistics, above all.

Recently, Kelch 13 (*K13*) propeller variants have been identified as molecular markers of ART resistance facilitating large-scale screening and monitoring of resistance emergence and spread.[7] The *P. falciparum K13* gene encodes a Kelch protein of 727 amino acids considered to be involved in the parasite's cytoprotective and antioxidant responses.[8,9] More than 180 non-synonymous *K13* mutations have been identified so far. World Health Organization recently updated the role of *K13* polymorphisms in ART resistance[3]: validated *K13* mutations are associated with both, delayed parasite clearance as well as resistance as indicated by the in vitro ring-stage survival assay,[10] whereas candidate mutations meet only one of these requirements. Validated polymorphisms include C580Y, the most common mutant in resistant parasites,[11] in addition to Y493H, R539T, I543T, and R561H. Candidate mutations involve P574L and A675V, among others. The list of mutations associated with ART resistance is still evolving, however.[3,12]

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In SSA, available evidence points to a multitude of rare non-synonymous *K13* polymorphisms and almost absence of the validated mutations seen in southeast Asia.[3,12–14] In Rwanda, east Africa, malaria morbidity and mortality declined greatly between 2005 and 2011 following the enforcement of control activities including the adoption of artemether-lumefantrine (AL) as first-line treatment in 2005. In particular, community-level case management programs have contributed greatly to the large-scale deployment of ACTs.[15] Correspondingly, the pattern of *pfmdr1* alleles in clinical isolates from Huye District, southern Rwanda, which we examined in 2010, was suggestive of intense AL pressure on the parasite population.[16] In this study, we aimed at assessing the presence of *K13* polymorphisms in *P. falciparum* isolates collected between 2010 and 2015 in Huye District, southern Rwanda.

Plasmodium falciparum isolates were collected at three occasions between 2010 and 2015 from infected children residing in the Huye District of southern Rwanda. Huye District (population 330,000) is located on Rwanda's central plateau (average altitude, 1600–1800 m; mean temperature, 19°C; yearly rainfall, approximately 1,200 mm). Malaria transmission in the area is perennial but low; *P. falciparum* is the predominating species.[17] In 2010, children under the age of 5 years were examined to determine the prevalence of common childhood diseases.[17] In 2014 and 2015, blood samples were collected from school children alongside monitoring the effectiveness of routine deworming (manuscript in preparation). Informed consent for the participation in these studies was obtained from the children's parents or legal guardians, and the study protocols were approved by the Rwanda National Ethics Committee. Eighty-five microscopically positive samples were available for analysis from 2010, and we randomly selected the same number of *P. falciparum*-positive samples (by either microscopy or polymerase chain reaction [PCR]) from 2014 and 2015. Genomic DNA was extracted from full blood aliquots (2010, 2014) or filter paper blood spots (Whatman 3MM, Whatman, Buckinghamshire, UK; 2015) by QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). All 255 samples were confirmed to be *P. falciparum* positive by semi-nested PCR assays.[18] The *K13* propeller domain was amplified by previously published PCR assays.[7] PCR products were bidirectional sequenced (Source BioScience, Berlin,

TABLE 1

Prevalence of *K13* mutations in *Plasmodium falciparum* isolates from southern Rwanda, 2010–2015

Year	Number of sequenced samples	Non-synonymous mutations, *n* (%)	Amino acid and nucleotide changes
2010	75	0	–
2014	81	2 (2.5)	V555A (GTA→GCA)
			A626S (GCA→TCA)
2015	66	3 (4.5)	P574L (CCT→CTT)
			D648H (GAT→CAT)
			A675V (GCT→GTT)

Germany), and multiple sequence alignment was performed to detect *K13* polymorphisms using BioEdit v.7.2.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and SnapGene v.3.1 (GSL Biotech, Chicago, IL) software. The *K13* sequence of *P. falciparum* 3D7 (PF3D7_1343700) retrieved from PlasmoDB was used as reference for the alignment. Of 255 samples, 222 (87%) *K13* amplicons were successfully sequenced.

The median age of the 222 children from whom the isolates were obtained was 8 years (range, 0.3–11), and 51.8% (115/222) were girls. The geometric mean parasite density was 4,217/μL (95% confidence interval, 3,175–5,601, 199 samples); 10.4% (23/222) of samples were submicroscopic, that is, positive by PCR only. Fever (axillary temperature ≥ 37.5°C) was present in 20.8% (42/221) of the children. Intake of antimalarials, generally AL, was stated for 6.7% (5/75; preceding 2 weeks) of children in 2010, and for 42.7% (32/75, preceding month) in 2014 (no data for 2015).

Five of the 222 *P. falciparum* isolates (2.3%) revealed single nucleotide polymorphisms in the *K13* propeller domain (Table 1), all were non-synonymous. Notably, there was a nonsignificant trend toward increasing prevalence of polymorphisms, that is, none in 2010, 2.5% in 2014, and 4.5% in 2015 (χ^2 trend = 3.3; $P = 0.07$). Among the three *K13* polymorphsims detected in isolates from 2015, two, that is, P574L and A675V, are candidate mutations associated with artemisinin resistance.[3] The remaining three polymorphisms identified were novel (D648H, V555A, A626S). The presence of *K13* polymorphisms was not associated with age, sex, parasite density, fever, or pretreatment (data not shown).

We show that *K13* polymorphisms are present in southern highland Rwanda at a low frequency but include two candidate mutations previously observed in southeast Asia and associated with ART resistance. The relationship of *K13* polymorphisms with ART resistance is complex and interpretation is hampered by a multitude of naturally occurring *K13* variants and a lack of linked phenotypical data. So far, more than 20 *K13* polymorphisms associated with delayed parasite clearance have been reported with identical mutants arising independently at different locations.[3,7,12,19]

In two previous large-scale studies on African isolates, validated *K13* mutations had not been observed. In one survey across 14 African sites including 1,184 *P. falciparum* isolates collected between 2002 and 2011, 23 different mutations (15 coding ones) were observed, of which 18 were restricted to single geographical sites. Two candidate mutations were detected, namely G449D in Mali and P553L in Kenya.[13] Another study on 1,212 more recently collected African isolates (2013–2014) found 22 *K13* polymorphisms (seven non-synonymous) at allele frequencies of 1–3%.[14] A recent assessment of *K13* variants by the MalariaGEN consortium revealed 64 *K13* polymorphisms in 1,648 African samples. Of 26 non-synonymous polymorphisms, 14 were also present in isolates from southeast Asia, and seven of those are considered to confer ART resistance. The majority of African *K13* polymorphisms appeared to be of local origin, and, as compared with southeast Asian isolates, there were substantially more rare polymorphisms. The authors considered the heterogeneity of mostly rare non-synonymous polymorphisms in the African isolates as reflecting the only recent (and not universal) access to ACTs in that region and, thus, limited drug pressure and selection as compared with southeast Asia. In line with this notion, further analysis of the African isolates suggested neutral evolution of the rare *K13* polymorphisms, that is, a large reservoir of "natural" K13-propeller variants in SSA.[12]

In the present study from southern Rwanda, we detected the *K13* candidate mutations P574L and A675V. Although the A675V variant has been detected only once among more than 4,000 African *P. falciparum* isolates,[12–14] the *K13* candidate mutation P574L is reported here for the first time from Africa. Both variants are common in southeast Asia,[7,12] and both are associated with delayed parasite clearance.[11] Among the three novel polymorphisms, *K13* V555A was one of five polymorphisms detected among isolates from different areas of Rwanda in the recent KARMA (K13 Artemisinin Resistance Multicenter Rapid Assessment) project. In those isolates collected in 2012–2013, no candidate mutations were observed.[20]

The proportion of *K13* polymorphisms tended to increase over time. Although derived from a rather small single-center study, this observation may reflect the increased availability of ACTs in Rwanda during recent years.[15] Moreover, the incidence of malaria in this country has increased since 2011,[15] possibly increasing the likelihood of the random occurrence of *K13* variants. Already in 2010, we observed a *pfmdr1* allele constellation in the study area (40% *pfmdr1* N86 F184 D1246), which is indicative of intense AL drug pressure and reappearing parasitemia following treatment.[16] Against such background of a parasite population with affected susceptibility to the non-artemisinin partner drug, the potential of spreading *K13* candidate mutations is worrisome. However, and as a limitation of our study, the actual role of *K13* polymorphisms in SSA is far from being understood. It has been suggested that the link with resistance may differ geographically,[14] and that specific, non-K13 genetic factors in the local parasite population may predispose to the emergence of resistance-causing mutations.[11] Information on the respective genetic makeup of the parasite population in the study area is therefore needed in addition to results of up-to-date ACT efficacy trials and in vitro ring-stage survival assays as well as the evaluation of association between these phenotypic resistance indicators and local *K13* variants.

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Molecular Evidence for *Plasmodium falciparum* Resistance to Sulfadoxine–Pyrimethamine but Absence of *K13* Mutations in Mangaluru, Southwestern India

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Abstract. In most of India, sulfadoxine–pyrimethamine (SP) *plus* artesunate serves as first-line treatment for uncomplicated falciparum malaria. In 112 clinical *Plasmodium falciparum* isolates from Mangaluru, southwestern India, we sequenced molecular markers associated with resistance to SP, lumefantrine, and artemisinin (*pfdhfr*, *pfdhps*, *pfmdr1*, and *K13*). The *pfdhfr* double mutation 59R-108N combined with the *dhps* 437G mutation occurred in 39.3% and the *pfdhfr* double mutation *plus* the *pfdhps* double mutation 437G-540E in additional 24.1%. As for *pfmdr1*, the allele combination N86-184F-D1246 dominated (98.2%). *K13* variants were absent. No evidence for artemisinin resistance was seen. However, the antifolate resistance alleles compromise the current first-line antimalarial sulfadoxine–pyrimethamine *plus* artesunate, which may facilitate the emergence of artemisinin resistance. Artemether–lumefantrine, introduced in northeastern parts of the country, in the study area faces the predominant *pfmdr1* NFD genotype, known to impair lumefantrine efficacy. Further monitoring of resistance alleles and treatment trials on alternative artemisinin-based combination therapies are required.

Emerging artemisinin resistance of *Plasmodium falciparum* in Southeast Asia threatens global malaria control.[1] In India, the countrywide first-line antimalarial drug for uncomplicated falciparum malaria is artesunate *plus* sulfadoxine-pyrimethamine (SP) (*plus* single dose primaquine) except for the northeastern states, where artemether–lumefantrine is recommended because of intense SP resistance.[2] Sulfadoxine–pyrimethamine treatment failure rates vary greatly across India,[3] as do the frequencies of associated mutations in the parasite's *dihydrofolate reductase* (*pfdhfr*) and *dihyropteroate synthase* (*pfdhps*) genes.[4–8] Cumulative *pfdhfr* and *pfdhps* mutations render *P. falciparum* resistant to pyrimethamine and sulfadoxine, respectively.[9] Work on Asian strains suggests a predominant sequential accumulation of mutations, in that two initial mutations preferentially occur in *pfdhfr* (108N, 59R), followed by two in *pfdhps* (437G, 540E) and a third in each of *pfdhfr* and *pfdhps*.[10] Artemisinin resistance, which so far largely means delayed parasite clearance, in vitro findings, and/or associated mutations in the Kelch 13 (*K13*) propeller domain of *P. falciparum*, is spreading in mainland Southeast Asia including neighboring Myanmar,[1,11] but has not been confirmed in India so far.[12] Here, we assessed molecular markers of resistance to artesunate–SP some 2,500 km away from India's hotspot of SP resistance (and potential gateway of artemisinin resistance) in the northeastern states, namely, in the city of Mangaluru, coastal southwestern Karnataka. In addition, we assessed *P. falciparum multidrug resistance-1* (*pfmdr1*) alleles to appraise the potential of, for example, lumefantrine as an alternative to SP as partner drug.

Malaria outpatients were recruited between June and December 2015 at the malaria diagnostic unit of Wenlock Hospital, the largest governmental hospital in Mangaluru, southwestern India. All study participants provided written informed consent (of parent/guardian in case of children < 18 years of age), and the study protocol was reviewed and approved by the Institutional Ethics Committee of Kasturba Medical College, Mangaluru, Manipal University (IEC KMC MLR 05-1598). Permission to conduct the study was given by the Directorate of Health and Family Welfare Services, Government of Karnataka. Study details and clinical manifestation are presented elsewhere.[13] Patients confirmed to have falciparum malaria were treated by hospital staff according to standard guidelines on an outpatient basis, that is, artesunate–SP for 3 days *plus* single dose primaquine on the second day. Genomic DNA of patients infected with *P. falciparum* was extracted from full blood aliquots (QIAamp DNA Blood Mini Kit; Qiagen, Hilden, Germany). *Plasmodium* species was ascertained by nested polymerase chain reaction (PCR) assays.[14] In addition, following amplification, PCR products were bidirectionally sequenced (Source BioScience, Berlin, Germany), and multiple sequence alignment was performed to detect polymorphisms in *pfdhfr*, *pfdhps*, *pfmdr1*, and *K13* using BioEdit v.7.2.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and SnapGene v.3.1 (GSL Biotech, Chicago, IL) software. *Plasmodium falciparum* 3D7 (PF3D7_1343700) retrieved from PlasmoDB was used as reference for the *K13* alignments, and for *pfdhfr* and *pfdhps*, references were NCBI XM_001351443.1 and GenBank Z30654.1, respectively. We specifically analyzed the mutations *pfdhfr* N51I, C59R, S108N, I164L; *pfdhps* S436A/F, A437G, K540E, A581G, A613S/T; and *pfmdr1* N86Y, Y184F, and D1246Y. Sequencing plots did not suggest the presence of polyclonal infections.

Of the 276 patients infected with *P. falciparum*, 138 isolates (50%) were randomly selected, and of those, 112 (81.1%) were successfully typed for all alleles under study (including 53 mixed *Plasmodium vivax–P. falciparum* infections). Among the 112 patients (median age, 30.5 years; range, 10–65), 92.0% (103) were male; 79.5% (89) had migrated to Mangaluru a median of six months (range, 1–240) before presentation; and 20.5% (23) originated from Mangaluru city, 33.9% (38) from the local Karnataka state, 27.7% (31) from the northern/northeastern states, and 17.9% (20) from other regions of

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India. Their socioeconomic status was low (data not shown), and 69.6% (78) of patients were construction workers or daily laborers. The geometric mean parasite density was 9,572/μL (95% confidence interval, 7,516–12,190/μL). Each 4.5% (five) of patients was admitted to ward or had severe malaria, respectively. Intake of antimalarials (chloroquine) in the preceding 6 weeks was reported by 0.9% (two) of patients.

Only one-third of *pfdhfr* alleles were wild type, and this figure was slightly lower for *pfdhps* (Table 1). For *pfdhfr*, two-thirds of isolates exhibited the double mutation 59R-108N, whereas for *pfdhps*, the single mutation 437G dominated over the double mutation 437G-540E. Together, almost 40% of isolates showed *pfdhfr* 59R-108N *plus pfdhps* 437G in addition to one in four isolates with the *pfdhfr* double mutation *plus* the *pfdhps* double mutation. No *K13* polymorphisms were detected. Also, we observed only wild-type alleles at codon 86 of *pfmdr1* (N86), and almost exclusively so in the combination N86-184F-D1246 (NFD).

Isolates from native Mangalureans and from migrants did not differ in terms of isolates with *pfdhfr*/*pfdhps* double–single or double–double mutations (60.9% [14/23] versus 64.0% [57/89]; $P = 0.78$). Of note, this figure was not increased in migrants from the north/northeastern states (67.7%, 21/31; $P = 0.60$), but tended to do so in migrants from the local state of Karnataka (78.9%, 30/38; $P = 0.13$), and it was reduced in migrants from elsewhere in India (30.0%, 6/20; $P = 0.04$). The time since migration was not associated with carrying these SP-resistant parasites ($P = 0.74$).

We show that in coastal southwestern India, most of the *P. falciparum* isolates have mutations conferring SP resistance, whereas *K13* variants associated with artemisinin resistance are absent. Anticipating a further intensification of SP resistance as seen elsewhere in India,[3] the useful therapeutic lifetime of the current combination artesunate–SP appears limited. However, considering the fixation of *pfmdr1* N86 and the almost fixation of the NFD allele combination, artemether–lumefantrine might not be a promising candidate for replacing artesunate–SP in this area. Moreover, based on the limited dataset, SP resistance in Mangaluru seems to be a local rather than an imported problem.

TABLE 1

Prevalence of antimalarial drug resistance alleles and genotypes in Mangaluru, southern India

Gene	Allele or genotype	Prevalence (%, *n*/112)
pfdhfr	Wild type	33.9 (38)
	Double mutation (59R-108N)	66.1 (74)
pfdhps	Wild type	29.5 (33)
	Single mutation (437G)	45.5 (51)
	Double mutation (437G-540E)	25.0 (28)
pfdhfr/*pfdhps*	Wild type	26.8 (30)
	dhfr wild type + *dhps* single (437G)	6.3 (7)
	dhfr wild type + *dhps* double (437G-540E)	0.9 (1)
	dhfr double (59R-108N) + *dhps* wild type	2.7 (3)
	dhfr double (59R-108N) + *dhps* single (437G)	39.3 (44)
	dhfr double (59R-108N) + *dhps* double (437G-540E)	24.1 (27)
K13	Wild type	100 (112)
pfmdr1	Wild type	0 (0)
	86N-184F-1246Y	1.8 (2)
	86N-184F-1246D	98.2 (110)

Our data originate from a limited number of *P. falciparum* isolates and represent only a snapshot in a dynamic process of resistance development. As compared with recent molecular data from India, the observed *pfdhfr* double mutation 59R-108N (i.e., without 51I) is found rather in central India,[4,5] whereas the *pfdhfr* triple mutation (and also 164L) has become prevalent particularly in northeastern India.[6–8] Likewise, the *pfdhps* mutations 437G and 540E (and the respective *pfdhfr*/*pfdhps* combinations) are comparatively rare in central India but common in the Northeast.[4–8] Our data from southwestern India occupy a middle position in this regard: whereas *pfdhfr* 59R-108N occurs at a prevalence similar to central India,[4,5] the *pfdhps* mutations 437G and 540E are almost as common as in northeastern India.[6–8] We detected *pfdhfr* 59R-108N *plus pfdhps* 437G or *plus* 437G-540E in almost two in three isolates. In East Africa, the *pfdhfr* triple mutation, *pfdhps* 437G-540E, and their combination (quintuple mutant) strongly predict SP treatment failure.[15] At a lower level of SP resistance, for example, in Indonesia, SP treatment failure has been associated with *pfdhfr* 59R-108N *plus pfdhps* 437G, and the *pfdhfr* double–*pfdhps* double variant with high-grade resistance (RII/III).[16] Even when double *pfdhfr* mutations do not greatly intensify SP resistance as compared with the 108N core mutation alone,[9] the prevalence of *pfdhps* 437G and 540E suggests SP resistance in the study area to be pronounced but not yet highly intense. Given ongoing SP drug pressure, for example, on parasites transmitted to recently treated patients without detectable artesunate levels but fading SP concentrations, and the foreseeable, stepwise development of further *pfdhfr*/*pfdhps* mutations,[10] SP resistance is likely to intensify in the study area, eventually compromising artesunate–SP. Reassuringly, no molecular evidence for artemisinin resistance was seen in the present study. However, against the background of evidence for impaired and potentially further waning SP efficacy, *K13* mutations may emerge or spread after importation. In this regard, a limited number of *K13* mutations have recently been detected in the northeastern state of Arunachal Pradesh bordering Myanmar.[12]

To protect the artemisinin component, partner drugs should have the highest possible efficacy. India's National Drug Policy on Malaria recommends the use of artemether–lumefantrine in the northeastern states,[2] which bear intense SP resistance. The present study showed the predominance of *pfmdr1* N86 and of the NFD haplotype. In vitro, wild-type *pfmdr1* N86 reduces sensitivity to dihydroartemisinin and to the partner drugs lumefantrine or mefloquine (3- to 4-fold higher IC50s) but increases susceptibility to chloroquine, monodesethyl amodiaquine (active metabolite of amodiaquine), and, less pronounced, piperaquine.[17] In clinical trials, *pfmdr1* N86 predicts recrudescence in patients treated with artemether–lumefantrine.[18] Similarly, *pfmdr1* NFD parasites reinfecting after artemether–lumefantrine treatment have been shown to tolerate 15-fold higher artemether–lumefantrine blood concentrations than those with the opposite YYY haplotype,[19] although the central polymorphism appears to be *pfmdr1* N86.[18] Interestingly, artemether–lumefantrine and artesunate–amodiaquine select different *pfmdr1* alleles,[18] which suggests that artesunate–amodiaquine and dihydroartemisinin–piperaquine might be effective in parasites with reduced susceptibility to artemether–lumefantrine.[1,11,18]

The diversity of malaria in India, including geographically variable drug susceptibility and resistance alleles, impedes drug policy recommendations, which accurately fit for the whole of the subcontinent. India presently forms the western boundary of artemisinin-resistant malaria, and the term of the presently used first-line treatment artesunate–SP is limited. Against the background of spreading and intensifying SP resistance as seen in the present study, expanded monitoring of molecular makers and clinical trials on alternative first-line antimalarials are required.

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Characterization of *Plasmodium vivax pvmdr1* Polymorphisms in Isolates from Mangaluru, India

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Abstract. India accounts for approximately half of the global *Plasmodium vivax* cases, but information as to the presence of chloroquine (CQ) resistance is scarce. In an observational study in Mangaluru, south-western India, of 116 vivax malaria patients analyzed, 89.5% (102/114) had cleared parasitemia on days two or three of CQ treatment. Two remaining patients presented on days four and five without parasitemia. One hundred eight isolates of these 116 patients were successfully sequenced for *pvmdr1* polymorphisms. Eight non-synonymous polymorphisms but no wild-type isolate were detected. Ten *pvmdr1* haplotypes were observed with mutations T958M and F1076L occurring in all isolates, whereas the candidate CQ resistance marker Y976F was present in one isolate only. *Pvmdr1* polymorphisms were not associated with early parasite clearance. The high proportion of early parasite clearance and the virtual absence of *pvmdr1* Y976F and of sextuple *pvmdr1* mutants suggest that CQ in the study area is still sufficiently effective. However, the abundance of *pvmdr1* mutations in the local parasite population warrants monitoring.

India accounts for approximately half of the global *Plasmodium vivax* malaria cases.[1] The city of Mangaluru, located at the Arabian Sea in south-western India, shows a peculiar pattern of urban malaria with importation of plasmodia particularly from the north-eastern parts of the country.[2] Chloroquine (CQ, plus primaquine) still is the mainstay of treating vivax malaria, even though treatment failures have been reported in several Asian countries including India.[3] Chloroquine resistance has been linked to polymorphisms in the *P. vivax* multidrug resistance gene *pvmdr1*, orthologue to *Plasmodium falciparum pfmdr1*. Particularly, the substitution Y976F in *pvmdr1* gene has been associated with a reduced CQ sensitivity in few studies in Southeast Asia, especially in Thailand, Myanmar, and Indonesia.[4–6] Furthermore, *P. vivax* isolates carrying the Y976F mutation reportedly show significantly increased IC_{50} values for CQ in vitro.[7] In Madagascar, all CQ treatment failures occurred in infections with sextuple *pvmdr1* mutant parasites (S513R-G698S-M908L-T958M-Y976F-F1076L).[8] However, present knowledge on the distribution of these mutations and of the respective haplotypes remains scarce, especially in India.

A recent study from Mangaluru,[9] southern India, reported *pvmdr1* mutations including Y976F, which might reflect a trend toward emerging drug resistance. Here, we aimed at further investigating these polymorphisms to achieve a more thorough understanding of CQ resistance in the area.

Plasmodium isolates were obtained between June and December 2015 from 909 malaria outpatients attending Wenlock Hospital, the largest governmental health facility of Mangaluru. Recruitment procedures and patient characteristics have been detailed elsewhere.[10]

Six hundred thirty-three patients had *P. vivax* mono-infections and were treated with CQ for 3 days plus primaquine (0.25 mg/kg body weight) for 14 days. Patients investigated were mostly young (median age, 25 years) males (93%) with a geometric mean parasite density of 2,999 parasites/μL (95% CI, 2,660–3,382). Chloroquine intake within the 4 weeks preceding presentation was stated by < 1% of patients.[10]

Study participants were asked to return to the hospital on day 2 (48 hours) or day 3 (72 hours) of CQ treatment to evaluate parasite clearance by thick blood film microscopy. Among 633 vivax malaria patients, 114 returned for the recommended control on day 2 (81) or on day 3 (33). Two additional patients presented at days 4 and 5.

For *pvmdr1* typing, DNA was extracted from blood samples obtained from these 116 patients at initial presentation, *pvmdr1* was amplified as published elsewhere,[11] and polymerase chain reaction (PCR) products were bidirectionally sequenced (Eurofins Genomics, Berlin, Germany). Multiple sequence alignment was performed using SnapGene v. 3.1 (GSL Biotech, Chicago, IL) software and the *pvmdr1* Sal-1 strain sequence (GenBank: AY618622.1) as the reference. Data analysis was performed using SPSS v. 22 (IBM Corp., Armonk, NY).

On day 2 of CQ treatment, 87.7% (71/81) of patients presenting for a checkup had cleared parasitemia, and this figure was 93.9% (31/33) on day 3. Two further patients were free of malaria parasites when presenting on days four and five of treatment. *Pvmdr1* sequencing was successful for 108 isolates (93.1%, 108/116). Four synonymous (T529T, A970A, S1358S, and R1422R) and eight non-synonymous (S513R, T958M, Y976F, F1076L, Y1028C, L1393N, L1425R, and T1269S) point mutations were identified. All 108 *P. vivax* isolates presented the synonymous single-nucleotide polymorphism (ssSNP) T529T (A970A, 1.9% [2/108], S1358S, 8.4% [9/108], R1422R, 0.9% [1/108]) and the non-synonymous (ns) SNP T958M. Of these, 87.0% (94/108) additionally had nsSNP F1076L. The prevalence of the other nsSNPs was S513R (9.6%, 10/108), Y976F (0.9%, 1/108), Y1028C (2.8%, 3/108), L1393N (24.0%, 26/108), L1425R (0.9%, 1/108), and T1269S (3.7%, 4/108). Of note, F1076L isolates did not carry mutations T1269S and L1393N in an almost mutually exclusive manner ($P < 0.001$). Vice versa, S513R did only occur among F1076L parasites.

Ten *pvmdr1* haplotypes were recognized (Table 1), including T958M-Y976F-F1076L in one isolate (0.9%). None of

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TABLE 1

Prevalence of *pvmdr1* haplotypes and proportion of parasitemic patients on follow-up

Pvmdr1 haplotype	No.	%	Proportion of patients parasitemic on day 2 or day 3 of chloroquine treatment
S513R-T958M-Y976F-F1076L	1	0.9	1/1 (100%)
S513R-T958M-Y1028C-F1076L	3	2.8	0/3 (0%)
S513R-T958M-F1076L-L1393N	2	1.9	0/2 (0%)
S513R-T958M-F1076L-L1425R	1	0.9	0/1 (0%)
T958M-F1076L-T1269S-L1393N	1	0.9	1/1 (100%)
T958M-F1076L-T1269S	3	2.8	1/3 (33.3%)
S513R-T958M-F1076L	3	2.8	0/3 (0%)
T958M-F1076L-L1393N	9	8.3	0/9 (0%)
T958M-L1393N	14	13	1/14 (7.2%)
T958M-F1076L	71	65.7	6/71 (8.5%)

the individual polymorphisms (data not shown) or haplotypes (Table 1) were associated with day 2 or day 3 positivity.

In this study from coastal, south-western India, CQ was successful in eliminating *P. vivax* malaria in 88% and 94% of patients on days 2 and 3, respectively. In a meta-analysis of *P. vivax* CQ resistance, the earliest treatment failure occurred at a median of 14 days (range 3–28 days), and early parasite clearance correlated with treatment outcome as assessed on day 28. Of note, parasite clearance in 95% or 100% of patients by day 2 or day 3, respectively, was found to be 100% predictive of CQ sensitivity as defined by the day 28 outcome.[3] The present study was not designed as a treatment trial, but against this background, it seems justifiable to state that CQ in the study area is sufficiently effective. This is supported by the virtual absence of the candidate CQ resistance marker *pvmdr1* Y976F, the lacking association of the detected polymorphisms with follow-up positivity and the absence of sextuple *pvmdr1* mutants carrying mutation S513R and Y976F.

The high prevalence of *pvmdr1* T958M and F1076L in our study is in accordance with the genotype pattern previously reported at this location.[9,12] However, whereas the candidate marker Y976F occurred only once (0.9%) in the present study, the figure was almost 8-fold higher in a previous report.[9] The abundance of *pvmdr1* F1076L in isolates from Mangaluru has been considered an indication of emerging CQ resistance.[9,12] However, as with most previous investigations, the present data do not support a predictive role of that polymorphism. Ultimately, prolonged monitoring of treated patients is required to elucidate the role of *pvmdr1* variants in recrudescence and to enable the prompt detection of CQ resistance in south-western India.

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4. Discussion

The present work focused on the role of host polymorphisms in susceptibility to and manifestation of malaria in India and in Africa. Further aspects of investigation included the clinical picture of vivax malaria as well as the degree of antimalarial drug resistance in the study areas. The essential results of the present work involve:

In Mangaluru, India, severe malaria was rare and *P. falciparum* infection caused more intense manifestations than *P. vivax* malaria. In addition, falciparum malaria appeared to be locally acquired, rather than imported by migrant workers from endemic areas (Gai et al., 2018).

DARC FYB carriage (rs12075) in India appeared to protect against severe manifestations of *P. falciparum* infection, whereas *DARC* 298A carriage increased the odds of malaria and of *P. vivax* infection in particular (Gai et al., 2019). Among pregnant women from Ghana, blood group O reduced the odds of *P. falciparum* infection, whereas a common miRNA -146a SNP increased them (Bedu-Addo et al., 2014; van Loon et al., 2019). However, TP53 codon 72, Pro > Arg was not associated with malaria, neither in Ghanaian pregnant women nor in Rwandan children (Gai et al., 2014).

As for antimalarial drug resistance in Rwanda, molecular markers suggest the emergence of artemisinin resistance in that country (Tacoli et al., 2016). No such evidence was found in India but clinical and molecular data point to a substantial degree of SP resistance and a limited usefulness of lumefantrine as a substitute in ACT (Wedam et al., 2018). Reassuringly, no evidence of CQ-resistant *P. vivax* was observed (Tacoli et al., 2019).

4.1 Manifestation of malaria in Mangaluru, southern India

The current study provides an update on the characteristics of malaria patients as to their clinical manifestation at Wenlock Hospital, the largest governmental health facility in Mangaluru (Gai et al., 2018). Most patients had *P. vivax* malaria and uncomplicated cases prevailed largely. Patients were predominantly young males, and more than three in four had migrated to Mangaluru, commonly for working. Nearly half of the patients had a migration background from beyond the state of Karnataka, and most of those were from northern and northeastern Indian states where malaria is endemic. Most of the patients had a low socio-economic status (SES). Patients with falciparum malaria more frequently had intensified

symptoms and abnormal laboratory values than patients with *P. vivax* infection. Severe malaria was rare.

Origin, profession and parasite species

The prevalence of *P. falciparum* infection was significantly increased in construction workers; however, it was reduced in migrants from north/northeastern India. Hence, *P. falciparum* parasites in Mangaluru appear to be locally acquired, rather than imported. The north and northeastern states are known for their increased malaria burden as well as for a predominance of *P. falciparum* (Das et al., 2012). Migrants originating from these regions presumably show a higher degree of *P. falciparum*-related semi-immunity as compared to the local Mangaluru population which, in turn, may partially explain a comparatively lower proportion of falciparum malaria among the migrants. Alternatively, an increased relapse rates in imported *P. vivax* strains, poor compliance with the 2 weeks of primaquine treatment, or an increased rate of common infectious diseases among the economically deprived migrants giving rise to an increased *P. vivax* relapse rate could lead to relatively higher proportion of vivax malaria among the migrants producing the impression of a reduced *P. falciparum* infection rate (Shanks and White, 2013).

Precipitation was highest between June to August, which declined between September to December. Parallelly, *P. vivax* mono-infections declined between June to August. One month after the peak precipitation, *P. falciparum* was more common than *P. vivax*. This suggests *P. vivax* relapses predominate at the beginning of the transmission season, and subsequently, *P. falciparum* proportions increased with increasing precipitation.

Parasite species - correlation with clinical manifestation

The geometric mean parasite density (GMPD) was higher in patients with *P. falciparum* or mixed infections than in vivax malaria patients. Overall, *P. falciparum* infected patients displayed worsened general conditions and more affection of consciousness than patients with *P. vivax* or mixed infections. This could partially be related to the increased GMPD and the reduced nutritional status in falciparum malaria patients. Previous studies have observed stronger host responses including increased cytokine levels due to *P. vivax* as compared to *P. falciparum* iRBCs (Karunaweera et al., 1992; Hemmer et al., 2006). In addition, the proportion of severe thrombocytopenia was higher in falciparum than in vivax malaria. Hb levels tended to be lower and concentrations of creatinine and bilirubin to be higher. Hospitalisation was also more common among *P. falciparum* infected patients than among patients with *P. vivax* or mixed species infections.

Severe malaria was rare at only 3.5% of patients. The parasite species did not appear to affect severe malaria. In the present study, the definition of severe malaria largely followed the 2014 WHO criteria (WHO, 2014). Severe vivax malaria is increasingly reported since 2000, and more than 40% of these reports originate from India whereas several endemic countries did not report severe vivax malaria (Rahimi et al., 2014). It appears that the inclusion of severe thrombocytopaenia as a marker of severity in other studies may partially be involved in this discrepancy. If considering this criterion in the present study, the proportion of severe vivax malaria of 3.2% would have increased to 12.9%. Other reasons for the heterogeneity of severe vivax malaria may include geographical differences in the peak age of vivax malaria, endemicity, CQ resistance, parasite virulence, and misdiagnosis of other severe diseases (Rahimi et al., 2014).

Factors associated with severe malaria

Reported diabetes mellitus was found to be the strongest independent predictor of severe malaria, followed by female gender, thrombocytopenia, and increasing age whereas, increasing BMI appeared to be protective in the multivariate analysis. Diabetes was previously reported to be associated with increased susceptibility to *P. falciparum* infection in Africa, and there is strong evidence that it increases the severity of several infectious diseases (Danquah et al., 2010; van Crevel et al., 2017). Female gender may correspond to peculiarities of health care utilization and increasing age to (unreported) co-morbidities. Decreasing BMI (malnutrition) is also a known risk factor for malaria severity (Mockenhaupt et al., 2004). In the present study, thrombocytopenia independently increased the risk of severe malaria almost fivefold. In the multivariate model, upon replacing this factor with severe thrombocytopenia, the association weakened and remained significant only for vivax malaria. This might argue for severe thrombocytopenia as maker of severe vivax malaria.

4.2 The role of host genetic polymorphisms in different malaria entities

4.2.1 Duffy antigen receptors for chemokines, Mangaluru, India

The role of *DARC* gene polymorphisms with respect to malaria *per se* and particularly to *P. vivax* infections was examined (Gai et al., 2019). *DARC* polymorphisms, namely T-33C, G125A, C265T and G298A were genotyped among 909 malaria patients (cases) attending the Wenlock Hospital and 909 control individuals from Mangaluru. Carriage of *DARC* FYB (deduced from *DARC* G125A) was not associated with the risk of malaria *per se*, but it protected against severe malaria and hospitalization due to *P. falciparum* infection. In

contrast, *DARC* 298A carriage was associated with increased odds of malaria and of vivax malaria. The erythrocyte silent form (Duffy negativity, T-33C) was absent and *DARC* 265T was very rare in the present study group. Therefore, these two *DARC* polymorphisms were not considered for the analysis.

DARC FYB carriage – protection against severe falciparum malaria

Based on *DARC* G125A, FYA/FYB (44%) and FYA/FYA (44%) were the most common genotypes. These genotypes did not differ between cases and controls, irrespective of stratification by parasite species. However, *DARC* FYB carriage was absent among the hospitalised cases (P = 0.03) as well as among patients with severe falciparum malaria (P = 0.006). This contradicts to some extent with the protective effect of FYA/FYA against vivax malaria in Brazil (King et al., 2011). Moreover, in India, FYA was found to be associated with a reduced five years average incidence of vivax malaria (Chittoria et al., 2012). Considering such a protective effect of FYA/FYA against vivax malaria, it was unexpected to observe the increased proportions of hospitalization and severe malaria among FYA carriers. *In vitro*, binding of Duffy antigens to platelet factor 4 (PF4) is important for the platelet-mediated killing of *P. falciparum* (McMorran et al., 2012, 2013). A potential argument in favour of our finding of FYB carriage conferring protection against severe malaria could be that it affects the Duffy antigen - PF4 complex binding affinity, thereby altering the capacity of platelet mediated killing of *P. falciparum*. An augmented killing of that parasite could, in turn, contribute to a lessened risk of developing severe disease.

The SNP 298 G>A could potentially affect the chemoattractant property of DARC

Carriage of *DARC* 298A tended to be increased in patients and, particularly, in vivax malaria patients as compared to controls. Upon adjusting for the differences in age, gender and migration status between cases and controls, *DARC* 298A carriage was significantly associated with increased odds of malaria and of vivax malaria in particular.

To the best of our knowledge, the present study for the first time shows a significant association of the SNP *DARC* G298A with malaria. A previous study from Brazil did not observe an association with malaria susceptibility when combining *DARC* C265T and G298A as a condition for weakened expression of Duffy antigens (FYX) (Albuquerque et al., 2010).

DARC *per se* serves as a multi-specific receptor for a variety of chemokines which include the melanoma growth stimulatory activity, interleukin-8, regulated upon activation normal T-expressed, monocyte chemotactic protein-1, neutrophil activating protein 2 and 3, epithelial neutrophil activating peptide-78, angiogenesis-related platelet factor 1, and growth-related

gene alpha (Horuk et al., 1993; Pogo and Chaudhuri, 2000). Moreover, DARC has been linked with several inflammatory and infectious diseases including increased rates of prostate cancer and asthma as well as an increased risk of HIV infection in its absence (Lentsch, 2002; He et al., 2008). DARC also influences inflammation in terms of altered chemokine levels and leukocyte trafficking (Horne and Woolley, 2009). Leukocytes such as monocytes and neutrophils are largely involved in the phagocytosis of infected red blood cells. Moreover, leucocytes are also known to be an important source of cytokines, which act as signalling molecules in activating immune responses against *Plasmodium* infection (Aitken et al., 2018). A recent *in vitro* study observed an increased phagocytic activity *via* neutrophils in vivax malaria (de Leoratti et al., 2012). A possible explanation in support of observed increased risk of malaria could be that *DARC* 298A may alter the chemoattractant properties of the Duffy glycoprotein, leading to a modified activation of the pro-inflammatory signalling cascade. Nevertheless, the present study cannot provide conclusive arguments and, therefore, further functional studies are needed.

4.2.2 Lack of association of the *TP53* codon 72 polymorphism with malaria in Ghanaian primiparae and Rwandan children

A common SNP at codon 72 of the *TP53* gene (Pro > Arg, rs1042522) was not associated with malaria in the present study groups (Gai et al., 2014). This polymorphism was genotyped among two different African populations and malaria entities, i.e., placental *P. falciparum* infection in Ghanaian primiparae (n = 314), and asymptomatic *P. falciparum* infection among Rwandan children (n = 545) (Mockenhaupt et al., 2006; Gahutu et al., 2011).

The *TP53* codon 72, Arg allele frequencies (Ghana, 0.30; Rwanda, 0.31) as well as the genotypes did not differ between *P. falciparum* infected and non-infected individuals. Upon adjusting for the age difference between infected and non-infected individuals and for further associated factors (Mockenhaupt et al., 2006; Gahutu et al., 2011); the lack of association between *TP53* genotypes and *P. falciparum* infection did not change. Furthermore, the *TP53* codon 72 genotype was not associated with the peripheral blood geometric mean parasite density, neither in pregnant women nor in children. In pregnant women, the Arg allele had no influence on maternal anaemia, birth weight, or preterm delivery, irrespective of placental malaria.

TP53 codon 72 Arg variant exhibits diverse roles but may not contribute to Plasmodium defence

In mice model experiments, increased TP53 levels were associated with a reduced *Plasmodium* liver stage burden (Kaushansky et al., 2013). The *TP53* codon 72 variant Arg allele is pro-apoptotic in nature, and has potential protective roles with respect to virus-related and other cancers (Dumont et al., 2003; Whibley et al., 2009; Ricks-Santi et al., 2010). Of note, this allele is suggested to be selected in lowland Sardinia, presumably because it did provide protection against malaria (Gloria-Bottini et al., 2013). However, the present study was unable to show such a role in two malaria endemic regions of Africa. Allele frequencies in the present study groups were lower than in Sardinia as well as in other Caucasian populations (Beckman et al., 1994; Själander et al., 1996; Gloria-Bottini et al., 2013). Thus, the declining Arg allele frequency towards the equator may argue against selection by malaria (Beckman et al., 1994; Själander et al., 1996). Genotype frequencies deviated from Hardy–Weinberg equilibrium in the Ghanaian subgroup. A potential reason for this might be a small sample size. The *TP53* codon 72 wildtype genotype (Pro/Pro) has been associated with reduced pregnancy rates, and interestingly, this genotype tended to be underrepresented among the Ghanaian primiparae group (Kang et al., 2009) . Nevertheless, the present findings on the lack of genetic association do not exclude a pathophysiological relevance of TP53 *per se* in malaria but it potentially argues against a major respective role of the *TP53* codon 72 allele (Kaushansky et al., 2013). TP53 is activated by various stress signals and can induce a wide range of host responses. However, the biological effects conferred by *TP53* codon 72 allele are possibly not those which are required for defending against *Plasmodium* spp. infection (Vogelstein et al., 2000; Ricks-Santi et al., 2010). Another explanation could be that the Arg allele may not influence *P. falciparum* infection *per se*, but rather may have an effect on severe malaria or the risk of infection in other populations.

4.2.3 The impact of ABO blood groups on malaria in Ghanaian primiparae

The blood group O was associated with protection against placental malaria among primiparae and, particularly, against late or chronic infections, i.e., infections characterized by the presence of placental malaria pigment (Bedu-Addo et al., 2014). Three polymorphisms, namely rs8176719 (indicating the O allele), rs8176746/rs8176747 (distinguishing the B allele from the A allele) in the *ABO* gene were successfully genotyped among the 827 delivering women recruited at the Presbyterian Mission Hospital in Agogo, Ghana (Mockenhaupt et al., 2006). Blood group O predominated among the study participants. Primiparae with blood group O had significantly less present or past placental

malaria than women with non-O blood groups. In multivariate analysis, adjusting for the differences in age, season, and antimalarial drug intake, the odds of present or past placental *P. falciparum* infection were reduced by 45% in blood group O primiparae. Whereas in multiparae, blood group O did not show such a protective effect. In line with our findings, blood group O has been previously associated with a clear protective role in severe malaria (Rowe et al., 2007; Fry et al., 2008; Jallow et al., 2009; Timmann et al., 2012). Moreover, a moderately beneficial impact of blood group O on uncomplicated malaria was observed in several studies (Cserti and Dzik, 2007; Loscertales et al., 2007; Rowe et al., 2009).

Blood group O confers protection against P. falciparum infection in primiparae

Pathophysiology, immunity and clinical manifestation largely differ between (severe) childhood malaria and malaria in pregnancy (Rogerson et al., 2000; Mockenhaupt et al., 2006; Desai et al., 2007). Nevertheless, primiparae to some extent resemble young children in terms of an insufficient degree of protective immune mechanisms against *P. falciparum* infection. In pregnant women, *P. falciparum*-infected erythrocytes sequester into the intervillous space by adhering to ligands on the syncytiotrophoblast, which is followed by the local accumulation of haemozoin and inflammatory cells. Antibodies blocking this parasite adhesion prevent placental malaria only after several successive pregnancies exposed to *P. falciparum* infection (Fried et al., 1998; Beeson et al., 1999; Rogerson et al., 2007). This explains why protection against *P. falciparum* infection due to blood group O in the present study was limited to the relatively immune-naïve group of primiparae. However, with increasing parity, the effects of adaptive immunity may overrule the protection offered by blood group O.

Possible ways of defence against placental P. falciparum infection by blood group O

Various hypotheses have been put forward to explain the protective role of blood group O in malaria, which include differential attractiveness to *Anopheles* vectors, antigenic similarity of ABO antigens with *P. falciparum*, impaired merozoite penetration as well as reduced cyto-adherence (Cserti and Dzik, 2007; Loscertales et al., 2007; Rowe et al., 2009). Another possible explanation could be reduced rosetting observed with bloodgroup O iRBCs. The A and B antigens serve as receptors for rosetting. In blood group O individuals, the A and B antigens are replaced by the disaccharide H antigen, and *P. falciparum*-infected RBCs form smaller and weaker rosettes than in non-O RBCs (Barragan et al., 2000; Blumenfeld and Patnaik, 2004). In line with this, the protective effect of blood group O against severe malaria was demonstrated to work by reduced rosetting of *P. falciparum* iRBC (Rowe et al., 2007).

However, in placental malaria rosetting is infrequent and, moreover, the PFEMP1 binding domain differs between the rosettes and the syncytiotrophoblast (Chen et al., 2000; Rogerson et al., 2000; Fried and Duffy, 2002). Nevertheless, cytoadherence to the syncytiotrophoblast may still vary depending on the type of ABO blood group (Loscertales et al., 2007). For instance, pregnancy-specific *P. falciparum* binding ligands such as chondroitin sulphate A and hyaluronic acid (Fried and Duffy, 1996; Beeson et al., 2000) are structurally related to the A antigen and, therefore, the ABO polymorphisms may influence the adhesion of infected RBCs to the syncytiotrophoblast. Moreover, the binding of iRBCs is mediated by the placental proteoglycan thrombomodulin to some extent (Rogerson et al., 1997). Thrombomodulin and other molecules involved in cyto-adhesion to the syncytiotrophoblast (e.g., von Willebrandt factor) are affected by the ABO phenotype (Blann et al., 1996).

A recent study observed an enhanced macrophage-mediated phagocytosis of *P. falciparum*-infected O-RBCs (Wolofsky et al., 2012). Increased infiltration of immune cells including monocytes and macrophages into the intervillous space was observed in many women with placental malaria, which together with ABO mediated iRBCs clearance could potentially explain the present study finding of the blood group O related protectiveness in placental malaria (Ordi et al., 1998).

4.2.4 MiRNA-146a polymorphism – increased risk of malaria in pregnancy

The present study findings uncover the role of a common miRNA-146a (rs2910164 G > C) SNP in malaria susceptibility among pregnant women (van Loon et al., 2019). This SNP was genotyped among 530 pregnant women attending antenatal care (ANC) and among 304 primiparae with live singleton delivery recruited at the Presbyterian Mission Hospital in Agogo, Ghana (Mockenhaupt et al., 2000, 2006).

The miRNA-146a SNP carriage (GC or CC) was associated with increased odds of *P. falciparum* infection both in the ANC attendees and primiparae. This was majorly due to the strong effect among homozygous individuals. Homozygosity increased the odds of infection five-fold in primigravidae and primiparae. Moreover, in primigravidae and primiparae, significant increasing trends of *P. falciparum* prevalence from wild type individuals over heterozygous to homozygous individuals were observed. However, this SNP did not show any significant association in terms of clinical manifestation of infection.

MiRNA immunomodulatory functions and possible role of miRNA-146a SNP in malaria

MiRNAs are involved in gene regulation and subsequently regulate the factors involved in the innate and adaptive immune response pathways. TLR-2 and TLR-4 are known to recognize *P. falciparum* and initiate immune responses (Krishnegowda et al., 2005). During innate recognition of pathogens, miRNA-146a is up-regulated by NF-kB through a MyD88-dependent pathway, followed by downregulation of the key molecules in the TLR machinery such as IRAK-1 and TRAF-6 by miRNA-146a through posttranslational regulation. MiRNA-146a interferes in the TLR signalling cascade *via* a negative feedback loop on the downstream mediators IRAK-1 and TRAF-6 (Taganov et al., 2006; Mehta and Baltimore, 2016). Altered TLR mechanisms and cytokine signalling in the presence of the variant miRNA-146a allele might influence the innate immune response to *P. falciparum* infection. Homozygosity for the miRNA-146a rs2910164 G > C SNP, located in the passenger strand of the hairpin structured miRNA (miRNA-146a*), influences the processing of pre-miRNA-146a into mature miRNA-146a. The miRNA-146a rs2910164 G > C variant homozygosity has been associated with reduced expression of the downstream mediators, and heterozygosity with the expression of additional miRNA-146a: one from the leading strand and two from the passenger strand (miRNA-146a*G and miRNA-146a*C), which all together give rise to a mature miRNA (Jazdzewski et al., 2008, 2009). However, the additional mature miRNA-146a*G and miRNA-146a*C may potentially have a distinct set of target genes, unlike the mature miRNA-146a (Jazdzewski et al., 2009). Moreover, in previous studies miRNA-146a rs2910164 G > C was reported to be associated with increased susceptibility to leprosy and to various effects in neoplastic conditions (Jazdzewski et al., 2009; Hao et al., 2018). In addition, this SNP was reported to be associated with both increased and decreased susceptibility to pulmonary tuberculosis in China (Zhang et al., 2015). However, no such findings with respect to malaria have been reported so far.

Considering the complex immuno-regulatory role of miRNA-146a and its diverse effects in several infectious diseases, more studies are required to understand the functional aspects of the miRNA-146a variant with respect to malaria.

4.3 Molecular markers of antimalarial drug resistance in *P. falciparum* and *P. vivax* isolates

In view of increasing antimalarial resistance by the *Plasmodium* parasites, the current sub-studies provide an update on the molecular markers of drug resistance in *Plasmodium* isolates from highland Rwanda and costal Southwestern India.

4.3.1 *P. falciparum* – antimalarial drug resistance.

Briefly, the current study identified the presence of two candidate mutations, P574L and A675V, in the *Kelch 13* gene among *P. falciparum* isolates from Rwanda, but no mutations occurred in isolates from India (Tacoli et al., 2016; Wedam et al., 2018). In addition, this study shows that most of the *P. falciparum* isolates from India had mutations conferring sulfadoxine-pyrimethamine (SP) resistance (*dhps* & *dhfr*). Moreover, the lumefantrine resistance marker *pfmdr1* N86 wildtype was found to be fixed and the NFD (N86-184F-D1246) allele combination to approach fixation (Wedam et al., 2018).

i) *K13* mutations in *P. falciparum* isolates from Huye and Mangaluru

Currently, artemisinin-based combination therapy (ACT) is the mainstay of malaria treatment. *P. falciparum* Kelch 13 (*K13*) propeller variants have been identified as markers of artemisinin resistance (ART). The presence of *K13* polymorphisms was assessed in *P. falciparum* isolates from Huye district, Rwanda (n = 225; collected in 2010, 2014 & 2015), and from Mangaluru, Southern India (n = 112; 2015). In Rwanda, no polymorphisms were observed in isolates from 2010; however, they were present in 2.5% and 4.5% in 2014 and 2015, respectively. In contrast, no *K13* polymorphisms were present in *P. falciparum* from southern India.

K13 variants and ART resistance – state of art

In the present study from Rwanda, five non-synonymous mutations in the *K13* propeller domain (*2010*: V555A & A626S; *2015*: P574L, D648H & A675V) were observed in 2.3% of the *P. falciparum* isolates. Among the three *K13* SNPs detected in the isolates from 2015, P574L and A675V are candidate mutations associated with ART resistance (WHO, 2017). The other three polymorphisms observed were novel. The current study reported the presence of the P574L mutation for the first time in Africa, whereas the A675V variant has been detected previously only once among more than 4,000 African *P. falciparum* isolates (Taylor et al., 2015; MalariaGEN Plasmodium falciparum Community Project, 2016). Both

mutations are observed frequently in Southeast Asia (Ariey et al., 2014; MalariaGEN Plasmodium falciparum Community Project, 2016). Among the novel mutations, the *K13* V555A polymorphism was also detected in a recent study under the KARMA project (K13 Artemisinin Resistance Multicenter Rapid Assessment) in the isolates collected from different areas of Rwanda between 2010-2013 (Ménard et al., 2016). However, in that survey no candidate mutations were observed. In the present study, the proportion of *K13* polymorphisms tended to increase over time. Although derived from a rather small single-center study, our observation may reflect the increased availability of ACT in Rwanda during recent years. Moreover, malaria incidence has increased since 2011 in this country, which possibly increased the likelihood of the random occurrence of *K13* mutations (United States Agency of International Development President's Malaria Initiative Series, 2016). A study conducted by our research group in 2010 observed a *pfmdr1* allele constellation in Rwanda (40% *pfmdr1* N86 F184 D1246), which indicates intense AL drug pressure and which is known to be associated with reappearing parasitaemia following AL treatment (Zeile et al., 2012). Against such a background of affected susceptibility to the non-artemisinin partner drug, the likelihood of *K13* candidate mutations spreading is alarming.

ART resistance is spreading in mainland Southeast Asia including neighbouring Myanmar but has not yet been confirmed in India (Dondorp et al., 2009; Miotto et al., 2015). However, a limited number of *K13* mutations have recently been detected in the northeastern state of Arunachal Pradesh bordering Myanmar (MalariaGEN Plasmodium falciparum Community Project, 2016). Our current study finding reconfirms that the *K13* polymorphisms – so far – have not appeared in Southwestern India, which receives a significant number of migrant workers from the north and northeastern states of India. However, continuous monitoring of the molecular markers associated with ART resistance is needed.

ii) Molecular markers of SP and lumefantrine resistance in Mangaluru

In most parts of India, SP (*plus* single dose primaquine) is used as the first-line antimalarial drug for treating uncomplicated falciparum malaria except for the northeastern states, where artemether–lumefantrine is recommended because of intense SP resistance (Anvikar et al., 2014). This study assessed the genetic markers in *P. falciparum* associated with resistance to SP and lumefantrine.

Medium level of sulfadoxine/pyrimethamine resistance in Mangaluru

The *pfdhfr* double mutation 59R-108N was present in two-thirds of isolates, and the *pfdhps* single mutation 437G (45%) dominated over the double mutation 437G-540E (25%). Overall,

almost 40% of isolates had *pfdhfr* 59R-108N *plus pfdhps* 437G in addition to one in four isolates with the *pfdhfr* double mutation *plus* the *pfdhps* double mutation. In comparison to recent studies from India, the frequencies of the *pfdhfr* double mutation (59R-108N) and *pfdhps* 437G and 540E in the present study are similar to that of central and northeast parts of the country, respectively (Mohapatra et al., 2014; Pathak et al., 2014; Sharma et al., 2015; Patel et al., 2017). In East Africa, the *pfdhfr* triple mutation, *pfdhps* 437G-540E, and their combination (quintuple mutant) are strongly associated with SP treatment failure (Kublin et al., 2002). In Indonesia, *pfdhfr* 59R-108N *plus pfdhps* 437G were associated with low level SP treatment failure, whereas the *pfdhfr* double–*pfdhps* double variant with high-grade resistance (RII/III) (Nagesha et al., 2001). The double *pfdhfr* mutation does not greatly intensify SP resistance as compared to the single *pfdhfr* 108N mutation. However, the prevalence of *pfdhps* 437G and 540E mutations in the present study area suggests SP resistance to be pronounced but not extreme so far.

Artemether–lumefantrine may not be a good replacement for SP - artesunate

Artemether–lumefantrine is used to treat falciparum malaria in the northeastern states of India, which bear intense SP resistance (Anvikar et al., 2014). To protect the ART component, partner drugs should be highly effective. The wild-type alleles at codon 86 *pfmdr1* (N86), and N86- 184F-D1246 (NFD) combination predominated in the isolates from Mangaluru. In a recent *in vitro* study, the presence of the *pfmdr1* N86 wildtype was associated with reduced sensitivity to dihydroartemisinin and to lumefantrine, but with increased susceptibility to CQ, monodesethyl amodiaquine, and piperaquine (Veiga et al., 2016). In clinical trials, this allele predicts the recurrence of parasitaemia in patients treated with AL (Venkatesan et al., 2014). Parasites with the *pfmdr1* NFD combination, reappearing after treatment with AL, can tolerate 15-fold higher AL blood concentrations as compared to the YYY haplotype (Malmberg et al., 2013). This and the predominance of *pfmdr1* NFD argues against replacing SP-artesunate with AL. In fact, AL and artesunate–amodiaquine are observed to select different *pfmdr1* alleles, which suggests that artesunate–amodiaquine and dihydroartemisinin–piperaquine might be effective in parasites with reduced susceptibility to AL. (Venkatesan et al., 2014; Haldar et al., 2018).

4.3.2 *P. vivax* – antimalarial drug resistance

CQ, *plus* primaquine is still the first-line antimalarial for treating *P. vivax* infections in India, though there are reports of treatment failure from several Asian countries (Price et al., 2014). The *pvmdr1* gene polymorphisms associated with CQ resistance were assessed among the

P. vivax isolates from Mangaluru (Tacoli et al., 2019). Eight non-synonymous polymorphisms in the *pvmdr1* were detected, and no isolate had complete wild-type alleles. Ten *pvmdr1* haplotypes were observed with mutations T958M and F1076L occurring in all isolates, whereas Y976F, a candidate CQ resistance marker, was present in only one isolate. In a few studies from Southeast Asia, the Y976F polymorphism has been associated with reduced CQ sensitivity (Imwong et al., 2003; Suwanarusk et al., 2008; Nyunt et al., 2017). *P. vivax* isolates carrying this mutation were reported to show significantly increased IC50 values for CQ *in vitro* (Suwanarusk et al., 2007). Moreover, in Madagascar, a sextuple *pvmdr1* mutant parasite including the Y976F mutation (S513R-G698S-M908LT958M-Y976F-F1076L) was associated with CQ treatment failure (Barnadas et al., 2008).

Chloroquine appears to be sufficiently effective in Mangaluru

Among the present study participants, almost 90% had cleared parasitaemia on days 2 or 3 of CQ treatment and the rest presented on days 4 and 5 without parasitaemia. No association was observed between the individual SNPs or haplotypes and the day 2 or day 3 positivity in this study. A meta-analysis of *P. vivax* CQ resistance observed the earliest treatment failure at a median of 14 days (range 3–28 days), and early parasite clearance predicted treatment outcome as assessed on day 28 (Price et al., 2014). Although the present study was not designed as a treatment trial, our findings suggest that CQ in the study area is still effective. Moreover, the virtual absence of *pvmdr1* Y976F and the lacking association of the SNPs with day 2 or day 3 positivity support our observations. However, the high prevalence of F1076L, previously reported among the isolates from Mangaluru, has been considered as an indication of emerging CQ resistance (Joy et al., 2018; Anantabotla et al., 2019). The present findings do not support the predictive role to F1076L but alarms the need for prolonged monitoring of patients treated with CQ to identify the role of *Pvmdr1* variants and to detect CQ resistance.

4.4 Conclusion and outlook

The present thesis focuses on several aspects of malaria such as the clinical pattern of malaria in Mangaluru, India, the role of host genetic factors, and antimalarial drug resistance.

Falciparum malaria has always been under the limelight of research as it is causing fatal courses of the disease. Hence, the existing understanding is relatively more established with respect to *P. falciparum* than *P. vivax* infection. The findings from Mangaluru provide an improved understanding of clinical, parasitological and biochemical characteristics of malaria patients in this area. Our results show that uncomplicated malaria cases predominate and confirm that *P. vivax* causes less intense manifestation than *P. falciparum*. Severe malaria is rare. This contrasts with the clinical pattern reported from other parts of India. Further studies into pathophysiology and parasite biology are needed to understand the underlying causes.

The central objective of this thesis was to study how host genetic variants influence malaria, and particularly, as regards different malaria entities. Our findings provide an insight into the roles of host polymorphisms in genes related to erythrocyte structure and immune mediators. For the first time, the present work shows particular associations of two different *DARC* SNPs with malaria: (i) a protective role of FYB carriage against severe falciparum malaria and hospitalisation and, (ii) an increased risk of malaria *per se* and particularly of *P. vivax* infection due to *DARC* 298A carriage. In addition, among African individuals we show a clear protective effect of the O blood group against falciparum malaria, and *vice versa* increased odds of *P. falciparum* infection in first time pregnant women due to a common miRNA-146a SNP. A lack of association of the TP53 codon 72 Arg allele with *P. falciparum* infection was also observed. Collectively, our study findings may contribute to a better understanding of the pathophysiology of malaria. However, further functional studies involving the above-mentioned SNPs are required to unravel the exact underlying mechanisms.

The current work also identified the presence of candidate Kelch-13 mutations (P574L and A675V) in *P. falciparum* isolates from Rwanda, which are common in Southeast Asia and associated with delayed parasite clearance following of artemisinin treatment. However, such mutations were absent in the isolates from Mangaluru. Considering the ongoing SP drug pressure and the distribution pattern of *pfdhfr/pfdhps* alleles, SP resistance may likely intensify in Mangaluru and, therefore, the therapeutic lifetime of artesunate-SP, the first line antimalarial, appears to be limited. Moreover, the *pfmdr1* allele constellation suggests that artemether-lumefantrine might not be a promising candidate for replacing artesunate-SP in

the study area. Further monitoring of molecular markers and treatment trials on alternative artemisinin-based combination therapies are required. In addition, the virtual absence of the mutations in *pvmdr1* associated with CQ resistance among the *P. vivax* isolates, and almost complete clearance of parasitaemia by day 3 after treatment suggests that CQ is still sufficiently effective in Mangaluru. However the prevalence of *pvmdr1* F1076L is considered as an indication of emerging chloroquine resistance. Continuous monitoring of patients treated with CQ is required to understand the *pvmdr1* role in recrudescence, as well as to detect the CQ resistance in this region.

In view of increasing antimalarial-drug resistance, the development of promising vaccine candidates and new interventions is urgently required. Erythrocyte polymorphisms associated with host resistance in malaria have been crucial for studying host-parasite interactions. Even after several decades, novel mechanisms involved in host resistance to *Plasmodium* infection in conditions such as sickle cell trait and host-parasite receptor interactions, (e.g., ABO blood group and DARC) are still being elucidated. The key factors involved in resistance provided by erythrocyte disorders could be of potential therapeutic importance. For instance, DARC is being studied as a vaccine candidate against vivax malaria, and considering the present study findings, further understanding of the molecular mechanisms of Duffy blood group antigens influencing malaria susceptibility is needed (Singh et al., 2018). Likewise, miRNAs are actively studied as biopharmaceutical targets, and as vaccine candidates (Chakraborty et al., 2017). MiRNAs are observed to display enhanced antimicrobial function after silencing or administrating them (Drury et al., 2017). Our results suggest that miRNA-146a is involved in innate immunity against malaria highlighting its potential as a biopharmaceutical target.

5. Zusammenfassung

Einfluss humangenetischer Polymorphismen auf Risiko und Manifestation der Malaria in Indien und Afrika

Die Malaria ist eine der bedeutsamsten parasitären Krankheiten und wird durch die Infektion mit *Plasmodium*-Parasiten verursacht. Weltweit sind mehr als 3 Milliarden Menschen von einer *Plasmodium*-Infektion bedroht. Populationen in Malaria-Endemiegebieten unterliegen einer evolutionären Selektion genetischer Polymorphismen, die einen relativen Schutz gegen die Krankheit bieten. In erster Linie betreffen diese genetischen Varianten Wirtserythrozyten. Darüber hinaus beeinflussen Polymorphismen in Genen, die das Immunsystem regulieren, die Anfälligkeit sowie die Pathophysiologie der Malaria. Das diesbezügliche Wissen ist hinsichtlich der *P. falciparum*-Malaria deutlich umfassender als für die Malaria durch *P. vivax*. Unabhängig davon wird zunehmend, insbesondere aus Indien, berichtet, dass die traditionell gutartig geltende Infektion mit *P. vivax* nicht selten schwere Malaria auslöst. Die dazu verfügbaren Daten sind jedoch nicht ausreichend und uneindeutig. Ähnliches gilt für das Ausmaß und die Verbreitung von Resistenzen gegenüber den gängigen Antimalaria-Medikamenten.

In Mangaluru, Südindien, untersuchten wir zunächst das Manifestationsmuster der Malaria und insbesondere der *P. vivax*-Infektionen. Diese Studie zeigt, dass Malaria in Mangaluru größtenteils unkompliziert verläuft und vorwiegend junge Männer mit niedrigem sozioökonomischen Hintergrund betrifft, bei denen es sich hauptsächlich um Wanderarbeiter aus anderen Teilen Indiens handelt. Schwere Malaria tritt nur selten auf und geringfügig häufiger bei Infektion mit *P. falciparum* als bei Infektion mit *P. vivax.* Dies steht jedoch im Gegensatz zum berichteten, deutlich höheren Anteil schwerer *P. vivax*-Malaria in anderen Landesteilen. Zweitens untersuchten wir in einer Fall-Kontroll-Studie die Assoziation von Polymorphismen im Duffy-Antigen-Rezeptor für Chemokine (DARC) mit der Malaria. DARC ist eine zentrale Komponente bei der Invasion von *P. vivax* in die Erythrozyten. Die Ergebnisse zeigen, dass Träger der *DARC* 298A-Variante ein signifikant erhöhtes Risiko für Malaria haben, insbesondere für eine *P. vivax*-Infektion. Interessanterweise scheinen Träger der Duffy-Variante FYB gegen schwere Malaria durch *P. falciparum* geschützt zu sein.

Wir erweiterten auch das vorhandene Wissen über genetischen Assoziationen zwischen Wirtspolymorphismen und der *P. falciparum*-Infektion in Afrika bei Schwangeren und Kindern. Die Untersuchungen zeigen, dass die ABO-Blutgruppe O bei ghanaischen Erstgebärenden Schutz gegen die *P. falciparum*-Malaria verleiht, während ein häufiger Polymorphismus von miRNA-146a, rs2910164 G>C, mit einer erhöhten Wahrscheinlichkeit

einer Malaria in der Schwangerschaft assoziiert ist. Zusätzlich untersuchten wir die Assoziation einer Variante des Tumorsuppressorproteins *TP53*, von der zuvor angenommen wurde, dass sie vor Malaria schützt. Dieser Polyorphismus war jedoch nicht mit der *P. falciparum*-Malaria bei ghanaischen Primiparae oder ruandischen Kindern assoziiert.

Hinsichtlich der Malariamedikamentenresistenz untersuchten wir assoziierte molekulare Marker in *Plasmodium*-Isolaten aus Afrika und Indien. Wir beobachteten ein seltenes Auftreten von *Kelch-13*-Genvarianten in *P. falciparum*-Isolaten aus Ruanda, entdeckten jedoch zwei Mutationen (P574L und A675V), die in Südostasien mit Artemisininresistenz einhergehen. Unter den *P. falciparum*-Isolaten aus Indien waren keine *Kelch-13*-Varianten vorhanden. Sulfadoxin-Pyrimethamin (SP) *plus* Artesunat ist die derzeitige Erstlinientherapie der Malaria in Indien. Die Verteilung der Antifolatresistenz-Allele legt nahe, dass die SP-Resistenz im Untersuchungsgebiet ausgeprägt, aber noch nicht intensiv ist, wodurch die sog. therapeutische Lebensdauer von Artesunat-SP begrenzt scheint. Darüber hinaus lässt die fast vollständige Fixierung der *pfmdr1*-Allelkombination N86-184F-D1246 darauf schließen, dass Artemether-Lumefantrin, das im Nordosten Indiens eingesetzt wird, im Untersuchungsgebiet keine vielversprechende Alternative darstellt. Chloroquin (CQ) wird in erster Linie zur Behandlung von *P. vivax*-Malaria eingesetzt, und *pvmdr1*-Varianten wurden mit Resistenz in Verbindung gebracht. Das beinahe vollständige Fehlen solcher Varianten unter den *P. vivax*-Isolaten sowie die fast vollständige Parasiten-Eliminierung nach CQ-Therapie legen nahe, dass CQ im Studiengebiet immer noch wirksam ist.

Insgesamt liefert die vorliegende Dissertation neue Erkenntnisse oder erweitert das derzeitige Wissen über die Rolle von humangenetischen Polymorphismen in verschiedenen Studienumgebungen, was wiederum zu einem besseren Verständnis der Pathophysiologie der Malaria beitragen kann. Darüber hinaus verbessert diese Studie die bemerkenswert begrenzte Charakterisierung der Manifestation der Malaria in Indien. Zudem zeigen die Daten die Bedrohung der Wirksamkeit der Erstlinien-Therapeutika in Indien und Ruanda auf.

6. Summary

Malaria is one of the most relevant parasitic diseases and caused by infection with *Plasmodium* parasites. More than 3 billion people are at risk of *Plasmodium* infection worldwide. Populations in malaria endemic areas are subject to evolutionary selection of genetic polymorphisms conferring relative protection against the disease. Primarily, those genetic variants affect host erythrocytes. In addition, polymorphisms in genes related to molecules regulating the immune system also influence susceptibility to malaria as well as its pathophysiology. The available knowledge with respect to malaria-related traits is more extensive as regards falciparum malaria than vivax malaria. Moreover, *P. vivax*, traditionally thought to be benign, is increasingly reported to cause severe malaria, particularly in India. However, the available data are scarce and ambiguous. Similar limitations apply to the extent and spread of antimalarial drug resistance.

In Mangaluru, southern India, firstly, we assessed the manifestation pattern of malaria and in particular of *P. vivax* infection. This study shows that in Mangaluru malaria is mostly uncomplicated and affects predominantly young men from a low-socio-economic background, who are majorly migrant workers from other parts of India. Severe vivax malaria occurs at a rate slightly lower than in falciparum malaria but its low prevalence contrasts with considerably higher figures reported from other parts of the country. Secondly, in a case-control study, we examined the association of polymorphisms in the Duffy antigen receptor for chemokines (DARC) with malaria. DARC is a central component in *P. vivax* invasion into the red blood cells. *DARC* 298A carriage increased the odds of malaria *per se* and more prominently of *P. vivax* infection. Interestingly, FYB carriage appeared to confer protection against severe falciparum malaria.

We also expanded the existing knowledge on host genetic associations with falciparum malaria in Africa, in particularly among pregnant women and children in Africa. The O blood group was observed to confer protection against falciparum malaria in primiparous Ghanaian women, whereas a common polymorphism in miRNA 146a, rs2910164 G > C was associated with increased odds of malaria in pregnancy. In addition, we examined the association of a common *TP53* polymorphism previously suggested to be malaria protective. However, this variant was not associated with falciparum malaria among Ghanaian primiparae or Rwandan children.

As to antimalarial drug resistance, we assessed associated molecular markers in *Plasmodium* isolates from Africa and India. The present work observed an infrequent

occurrence of Kelch 13 gene variants in *P. falciparum* isolates from Rwanda but detected two mutations (P574L and A675V) associated with artemisinin resistance in Southeast Asia. Among the *P. falciparum* isolates from India, no *K13* variants were present. Sulfadoxine-pyrimethamine (SP) *plus* artesunate is the current first-line antimalarial in India. Based on the antifolate resistance allele distribution, we assume that SP resistance in the study area is pronounced but not yet intense, thereby limiting the useful therapeutic lifespan of artesunate-SP. Moreover, the near fixation of the *pfmdr1* allele combination N86-184F-D1246 suggests that artemether-lumefantrine, introduced in the northeastern parts of this country, is not a promising alternative in the study area. Chloroquine (CQ) is primarily used to treat vivax malaria and *pvmdr1* variants have been associated with CQ resistance. However, the virtual absence of such variants among the *P. vivax* isolates as well as almost complete parasite clearance within 2-3 days suggest that CQ is still effective.

Overall, the present thesis provides new insights or expands the present knowledge on the roles of host genetic variation in various study settings which, in turn, may contribute to a better understanding of malaria pathophysiology. Moreover, this study improves the remarkably limited characterisation of the manifestation of malaria in India. Most lastly, the data show the threat of the effectiveness of the first-line antimalarials in India and Rwanda.

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8. Publications and scientific contributions

8.1 Scientific articles

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Apoorv TS, Babu PP, Meese S, **Gai PP**, Bedu-Addo G, Mockenhaupt FP. Matrix metalloproteinase-9 polymorphism 1562 C>T (rs3918242) associated with protection against placental malaria. Am J Trop Med Hyg. 2015; 93:186-8.

8.2 Contributions at scientific conferences

Poster presentations

Prabhanjan Gai, Stefanie Meese, K. Thangaraj, Pramod Gai, Velavan Thirumalaisamy, Frank P. Mockenhaupt, TLR1 602S allele – Increased risk of malaria in Indian population. 9th European Congress on Tropical Medicine and International Health, Basel Switzerland, 06 – 09.09.2015.

Prabhanjan Gai, Thittayil Suresh Apoorv, Phanithi Prakash Babu, Stefanie Meese, George Bedu-Addo, and Frank P. Mockenhaupt. A common metalloproteinase-9 polymorphism (1562 C>T) protects against placental malaria. 9th European Congress on Tropical Medicine and International Health, Basel Switzerland, 06 – 09.09.2015.

Prabhanjan Gai, Frank P. Mockenhaupt, Konrad Siegert, Jakob Wedam, Archith Boloor, Suyamindra Kulkarni, Rashmi Rasalkar, Animesh Jain, Chakrapani Mahabala, Pramod Gai, Shantharam Baliga, Rajeshwari Devi, Damodara Shenoy. Manifestation of malaria in Mangalore, southern India. American Society of Tropical Medicine and Hygiene 66th Annual Meeting, Baltimore, Maryland USA, 05 – 09.11.2017.

Prabhanjan Gai, Suyamindra Kulkarni, Konrad Siegert, Jakob Wedam, Rashmi Rasalkar, Costanza Tacoli, Animesh Jain, Chakrapani Mahabala, Shantaram Baliga, Rajeshwari Devi, Damodara Shenoy, Pramod Gai, Frank P. Mockenhaupt. A TLR1 polymorphism increases the risk of vivax malaria in southern India. American Society of Tropical Medicine and Hygiene 66th Annual Meeting, Baltimore, Maryland USA, 05 – 09.11.2017.

Oral presentations

Prabhanjan Gai, George Bedu-Addo, Stefanie Meese, Teunis A Eggelte, Kumarasamy Thangraj, Frank P. Mockenhaupt. ABO blood group genotypes and risk of *P. falciparum*

malaria during pregnancy in Ghanaian women. July 2014, Paratrop, Zürich 2014, Joint Meeting – Parasitology and Tropical Medicine, Zürich, Switzerland, 16 – 19.07.2014.

Prabhanjan Gai, Konrad Siegert, Jakob Wedam, Pramod Gai, Suyamindra Kulkarni, Rashmi Rasalkar, Rajeshwari Devi, Animesh Jain, Damodar Shenoy, Frank Mockenhaupt.Malaria in Mangalore, India: a case-control study. GRK 1673 and ROKODOKO symposium “Functional molecular infection epidemiology”, RKI, Berlin, Germany, 06.04.2016

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11. Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Prabhanjan Gai

Berlin, 19.03.2020

12. Erweiterte Selbständigkeitserklärung

Hiermit versichere ich, Prabhanjan Gai, dass die folgenden Publikationen:

Gai PP, van Loon W, Siegert K, Wedam J, Kulkarni SS, Rasalkar R, et al. Duffy antigen receptor for chemokines gene polymorphisms and malaria in Mangaluru, India. Malar J. 2019;18:328.

Gai PP, Mockenhaupt FP, Siegert K, Wedam J, Boloor A, Kulkarni SS, Rasalkar R, Kumar A, Jain A, Mahabala C, Gai P, Baliga S, Devi R, Shenoy D. Manifestation of malaria in Mangaluru, southern India. Malar J. 2018;17:313.

Gai PP, Meese S, Bedu-Addo G, Gahutu JB, Mockenhaupt FP. No association of the p53 codon 72 polymorphism with malaria in Ghanaian primiparae and Rwandan children. Am J Trop Med Hyg. 2014; 90: p.1133

van Loon W, Gai PP, Hamann L, Bedu-Addo G, Mockenhaupt FP. MiRNA-146a polymorphism increases the odds of malaria in pregnancy. Malar J. 2019 Jan 14;18(1):7.

Tacoli C, Gai PP, Siegert K, Wedam J, Kulkarni SS, Rasalkar R, Boloor A, Jain A, Mahabala C, Baliga S, Shenoy D, Gai P, Devi R, Mockenhaupt FP. Characterization of *Plasmodium vivax pvmdr1* polymorphisms in isolates from Mangaluru, India. Am J Trop Med Hyg. 2016 Nov 2;95(5):1090-1093. Epub 2016 Aug 29.

Tacoli C, Gai PP, Bayingana C, Sifft K, Geus D, Ndoli J, Sendegeya A, Gahutu JB, Mockenhaupt FP. Artemisinin resistance-associated *K13* polymorphisms of *Plasmodium falciparum* in Southern Rwanda, 2010-2015. Am J Trop Med Hyg. 2016;95:1090-1093.

Bedu-Addo G, Gai PP, Meese S, Eggelte TA, Thangaraj K, Mockenhaupt FP. Reduced prevalence of placental malaria in primiparae with blood group O. Malar J. 2014;13: p.289

Wedam J, Tacoli C, Gai PP, Siegert K, Kulkarni SS, Rasalkar R, Boloor A, Jain A, Mahabala C, Baliga S, Shenoy D, Devi R, Gai P, Mockenhaupt FP. Molecular evidence for *Plasmodium falciparum* resistance to Sulfadoxine-Pyrimethamine but absence of *K13* Mutations in Mangaluru, Southwestern India. Am J Trop Med Hyg. 2018 Dec;99(6):1508-1510.

maßgeblich von mir verfasst wurden.

Mögliche Übereinstimmungen mit Textpassagen aus meiner Dissertation "Human genetic polymorphisms influencing the risk and manifestation of malaria in India and Africa" stellen somit keinen Plagiatsfall dar.

Dies wird bei Bedarf bestätigt durch den Betreuer der Dissertation und Co-Autoren der aufgeführten Publikationen.

Prabhanjan Gai

Berlin, 19.03.2020