Aus dem Institut für Tropenmedizin und Internationale Gesundheit
der Charité-Universitätsmedizin Berlin
und dem
Institut für Parasitologie und Tropenveterinärmedizin
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Human genetics affecting malaria susceptibility and manifestation, and *P. falciparum* genetics affecting antimalarial drug resistance

Inaugural-Dissertation
zur Erlangung des Grades eines
Doctor of Philosophy (PhD)
in Biomedical Sciences
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vorgelegt von
Welmoed van Loon
aus Wageningen, die Niederlande

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ABC: ATP-binding cassette
ACT: artemisinin-based combination therapy
AL: artemether-lumefantrine
AQ: Amodiaquine
AS: artesunate
COVID-19: Coronavirus Disease 2019
CQ: chloroquine
DARC: Duffy antigen receptor for cytokines
DHA: dihydroartemisinin
DHFR: dihydrofolate reductase
DHPS: dihydropteroate synthase
DNA: Deoxiribonuclei Acid
G6PD: glucose-6-phosphate dehydrogenase
GPI: glycosylphosphatidylinositol
Hb: Haemoglobin
HLA: Human leukocyte antigen
HRP2, -3: Histidine-rich protein 2, -3
IC50: 50% inhibitory concentration
IFN: interferon
IL: interleukin
IPTp: intermittent preventive treatment in pregnancy
IRS: indoor residual spraying
ITN: insecticide treated bed net
LAMP: loop mediated isothermal amplification
LF: lumefantrine
MBL: Mannose binding lectin
MDA: mass drug administration
MHC: Major histocompatibility complex
MiRNA: microRNA
MOI: multiplicity of infection
MQ: mefloquine NLR: NOD-like receptor
mRNA: messenger RNA
MyD88: myeloid differentiation primary response gene 88
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

PfARPS10: *P. falciparum* apicoplast ribosomal protein S10

PfATG18: *P. falciparum* autophagy-related protein 18

PfCRT: *P. falciparum* chloroquine resistance transporter

PfEMP1: *P. falciparum* erythrocyte membrane protein 1

PfFD: *P. falciparum* ferrodoxin

PfK13: *P. falciparum* Kelch-13

PfMDR1, -2: *P. falciparum* multidrug resistance protein 1, -2

PfMRP1, -2: *P. falciparum* multidrug resistance-associated protein 1, -2

Plasmodium spp: *Plasmodium species*

PPQ: piperaquine

PQ: primaquine

PRR: Pathogen-recognition receptor

PvDBP: *P. vivax* Duffy binding protein

RDT: rapid diagnostic test

RLR: RIG-I-like receptor

RNA: Ribonucleic acid

RSA: Ring-stage susceptibility assay

SEA: South-East Asia

SNP: single nucleotide polymorphism

SP: sulfadoxine-pyrimethamine

spp: *species*

TIRAP: Toll-interleukin 1 receptor domain containing adaptor protein

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TRIF: Toll-like receptor adaptor molecule 1

1. INTRODUCTION

Malaria is one of the most important diseases in human history and has evolved with humankind. The disease has already been described by Hippocrates in 400 BC, appears in Chinese writings dating 4000 years back, and DNA analysis confirmed malaria in pharaoh Tutankhamun's mummified remains from 1300 BC (Hippocrates 1849; Lalremruata et al. 2013; CDC 2022). Estimated deaths caused by malaria greatly outnumber deaths by *all* other pathogens together. The disease might even have killed half of the people that have ever lived (Whitfield 2002). Malaria parasites have shaped the human genome and *vice versa*. Malaria affected the course of civilisations throughout history. Fluctuations of agricultural expansion in Europe are closely linked with malaria burden, mighty armies have been weakened by malaria over centuries, the fall of Rome is attributed to malaria, and Alexander The Great's desire to merge East and West would have ended differently if malaria had not interfered (Carter and Mendis 2002). Today still, half of the people on earth are at risk for malaria greatly affecting societies and economies (Gallup and Sachs 2001; Sarma et al. 2019). The global community might have lost a bit of attention due to the COVID-19 pandemic, but malaria killed over half a million people in 2021, mostly children under five (WHO 2021b).

Considering the enormous burden that malaria has posed on humankind, current times mark an interesting tipping point in history: malaria related mortality has dropped dramatically in the last century. The World Health Organization sets goals including malaria eradication and elimination and the U.S. President's Malaria Initiative's vision is clear: A world free of malaria in our generation. However, today's global malaria situation is far from there.

The reduction in malaria burden over the past two decades can be attributed to improved socio-economic circumstances and hygiene globally, increased funding, political commitment, and effective interventions. Main pillars of effective interventions are vector control and case management, and the latter is greatly carried by antimalarial treatment. Alarmingly, malaria parasites have developed resistance to all antimalarial drugs available today. Tackling malaria cannot be done by one method. Malaria exists in an ecosystem and is ever evolving. Control strategies require a multi-facetted approach, which needs a deep understanding of all these facets, from the parasite's perspective to its hosts' perspective.

The malaria parasite has a complex lifecycle, an intermediate host, and a surprising capacity to evolve and adapt. It hides from our immune system, it manipulates our immune response, it develops drug resistance, and it escapes detection tools. Malaria parasites escape elimination both within the human host and on an inter-continental scale. On the other hand, humans have adapted, and are adapting, to malaria by developing genetic traits that make them more tolerant to the disease. This continuous process of co-adaptation between host and pathogen is known as the Red Queen Hypothesis, referring to a passage in Lewis Carroll's Through the Looking-Glass in which Alice and the Red Queen need to keep running to stay at the same place (Van Valen 1973). The recently suggested novel emergence of vivax malaria parasites in Africa that can infect humans previously resistant to this parasite – due to a genetic trait developed likely *because of* malaria pressure– (Golassa et al. 2020) is exemplary for the Red Queen Hypothesis.

This thesis aims to contribute to a better understanding of how both, human genetics, and malaria parasite genetics, contribute to malaria outcome. We investigated the role of two common genetic variations in humans (a microRNA variant involved in immune regulation and Duffy antigen variants known for their role in vivax malaria resistance) on malaria susceptibility and manifestation, and we examined several genetic markers of antimalarial drug resistance currently circulating in the malaria parasite population in Rwanda, East Africa.

2. LITERATURE

2.1. Malaria – the pathogen

2.1.1. *Plasmodium* life cycle

Malaria is caused by infection with the *Plasmodium* parasite. The *Plasmodium* genus is protozoan (unicellular), obligate intracellular, and has a complex lifecycle including an intermediate, invertebrate host (the *Anopheles* mosquito for most *Plasmodium* species) and a definite, vertebrate host. Over 200 *Plasmodium* species are described, of which five naturally infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. Only the latter is considered not human-exclusive and zoonotic, although there are reports of human *Plasmodium spp*. detected in great apes (Hayakawa et al. 2009; Duval et al. 2010).

The human *Plasmodium* life cycle starts with a female mosquito taking a bloodmeal and injecting sporozoites in the human blood stream. Sporozoites migrate to the liver and invade hepatocytes within minutes, where they form schizonts. After several days to weeks, these schizonts lyse and release merozoites in the blood stream. *Plasmodium vivax* and *P. ovale*, however, can form dormant stages in the liver (hypnozoites) that revive after weeks or months. Released merozoites from the liver infect erythrocytes and enter the asexual erythrocytic stage: schizonts form in erythrocytes, which lyse, and release 5-70 new merozoites in the blood stream. This cycle repeats, depending on the species, every 24 to 72 hours, allowing parasite density to increase rapidly to millions of infected erythrocytes in the blood stream. Some merozoites then differentiate into male or female gametocytes, which can be taken up by a mosquito. Male and female gametocytes further develop sexually in the mosquito, and eventually produce a zygote that differentiates into sporozoites which can be injected again in the human host (Figure 1) (Phillips et al. 2017).

Most notable species specific differences in the life cycle are: the erythrocytic cycle time causing malaria's typical recurring fever episodes (*P. knowlesi*, 24h; *P. falciparum*, *P. ovale*, *P. vivax*, 48h; *P. malariae*, 72h), the number of merozoites formed, the moment that gametocytogenesis starts (*e.g.*, *P. falciparum* completes multiple erythrocyte cycles before it differentiates into gametocytes, whereas *P. vivax* produces gametocytes continuously parallel to the erythrocyte cycles), and a dormant stage exists in hepatocytes for *P. vivax* and *P. ovale*, causing malaria relapse in some cases (Phillips et al. 2017).

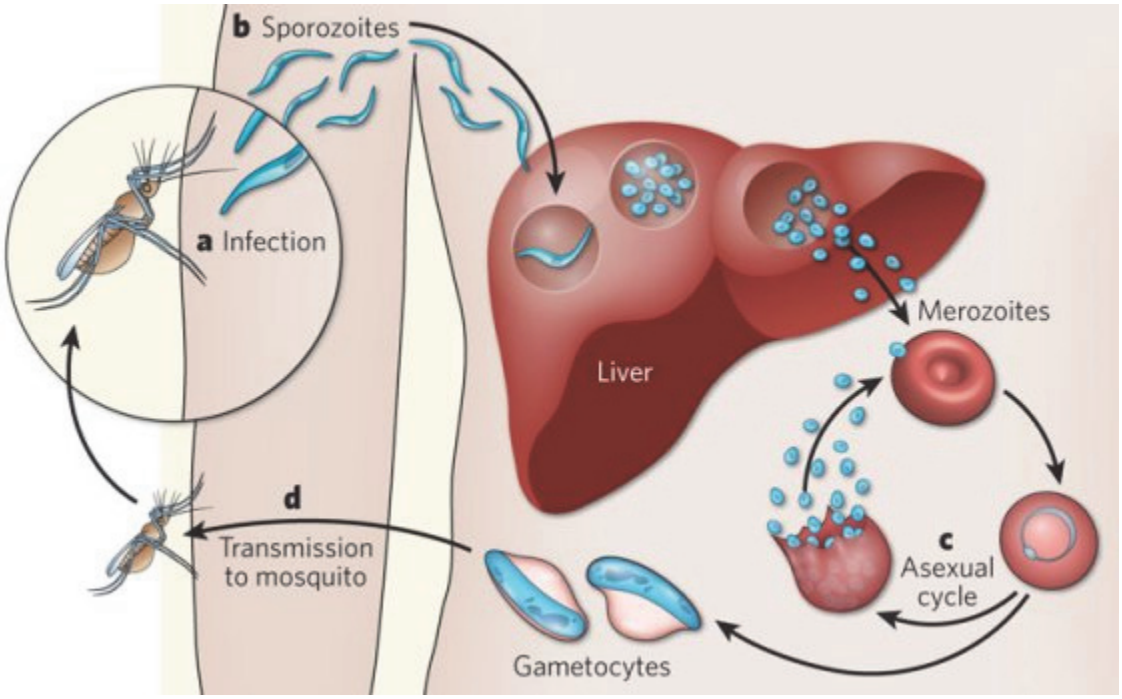

Figure 1. *Plasmodium spp.* lifecycle.

a. Infected mosquitoes inject Plasmodium sporozoites into a human host while taking a blood meal. b. Sporozoites reach the hepatocytes (liver cells) via the blood stream, where they proliferate asexually into merozoites, and invade erythrocytes (red blood cells). c. Parasites enter the asexual erythrocytic cycle, at which malaria clinically manifests with symptoms like fever and chills. This cycle repeats and parasite density increases. Female and male gametocytes are produced. d. Gametocytes are taken up by a mosquito during a blood meal, fuse to form oocysts that in turn create sporozoites. The sporozoites migrate to the mosquito's salivary glands, and the cycle of infection repeats. Adapted from Michalakis and Renaud, Nature*, 2009:462.*

During *Plasmodium*'s erythrocytic stage, haemoglobin is the parasite's primary food source, providing it with amino acids for development and growth. Up to 70% of the host's haemoglobin may be taken up and digested, which also makes room for the parasite to grow (Rosenthal and Meshnick 1996). Haemoglobin uptake happens through the parasite's food vacuole (cytostome) (Abu Bakar et al. 2010). A crucial process in this digestion is the detoxification of hematin into the compact and insoluble hemozoin crystal (Rosenthal and Meshnick 1996). This detoxification process is a drug target: chloroquine and amodiaquine interfere with hematin and avoid its crystallization in hemozoin. In addition, a by-product of haemozoin production is crucial in artemisinin activation (Foley and Tilley 1998). Hemozoin crystals have distinct chemical and visual properties and play a role in host immune sensing, and in microscopic detection of malaria.

2.1.2. *Plasmodium* and its genomic toolbox

Plasmodium has a remarkable genetic diversity, within the host, on the population level, and on an intercontinental scale. The *P. falciparum* genome was published in 2002, marking a milestone in malaria research (Gardner et al. 2002) . *Plasmodium* genomic analyses give insights in malaria epidemiology, diagnosis, treatment, vaccine development, and surveillance, but also serve as a good model to study general concepts in population genetics (Chang et al. 2012; Volkman et al. 2012; Nkhoma et al. 2013; Anderson et al. 2017).

The *Plasmodium* cell has a nucleus containing a compact, AT-rich genome of 23 MB base pairs, spread over 14 chromosomes and two other organelles containing their own set of DNAs: mitochondria and an apicoplast. During its vertebrate host stage, the parasite is exclusively haploid, *i.e.*, contains only a single set of each chromosome in the nucleus. The zygote stage in the mosquito is the only stage in which the parasite forms a diploid genome, after sexual recombination (Sinden and Hartley 1985). However, also during mitosis, *P. falciparum* recombines its antigenic *var* genes giving rise to new variants during the course of one infection (Scherf et al. 1998; Duffy et al. 2009; Bopp et al. 2013). Besides sexual and asexual recombination, *Plasmodium* integrates genomic changes by mutations, a primary example being single nucleotide polymorphisms (SNPs) and copy number variants that confer drug resistance.

Plasmodium adapts its DNA and transcription pattern *in vitro*, depending on the circumstances, *e.g.*, deletion of unnecessary genes in laboratory strains, or a switch in transcription depending on culture conditions (Biggs et al. 1989; Kafsack et al. 2014; Claessens et al. 2017; Awandare et al. 2018). Examples of genetic and epigenetic versatility in *Plasmodium* parasites in the field are the independent emergence and loss of drug resistance genes with and without drug pressure (Laufer et al. 2006), deletion of genes *histidine rich protein 2* and *-3* (*HRP2, HRP3)* assumingly due to their use in malaria detection (Poti et al. 2020), and different transcriptional signatures associated with seasonality, putatively bridging periods of low transmission (Andrade et al. 2020). In a patient with 1% parasitemia, over 85% of the parasites are estimated to have acquired a novel SNP (Bopp et al. 2013). Copy number variants are assumed to have a higher mutation rate (Preechapornkul et al. 2009).

The *var* gene family constitutes a particular group in the *Plasmodium* genome with regard to versatility. These genes encode the highly polymorphic *Plasmodium falciparum* erythrocyte

membrane protein 1 (PfEMP1), a major virulence factor due to immune evasion and intravascular sequestration (Newbold et al. 1999). *P. falciparum* contains about 60 *var* gene copies, distributed across multiple chromosomes, but only one is transcribed in any individual parasite (Chen et al. 1998; Scherf et al. 1998). The remaining genes are transcriptionally silenced, a process called allelic exclusion. However, parasites can switch the expression of a given *var* gene in a new generation and thereby evade the host's anti-PfEMP1 immunity (Scherf et al. 1998; Duffy et al. 2009; Bopp et al. 2013).

Infections in malaria endemic areas are often polyclonal, *i.e.*, patients carry multiple, genetically distinct parasite clones, referred to as multiplicity of infection (MOI). The MOI in one infection is generally estimated with three markers: merozoite surface proteins 1 and 2, and glutamine rich protein (WHO 2008). However, given *Plasmodium's* genetic diversity, much more parasite heterogeneity may be present within one host, and this diversity is likely vastly underestimated (Juliano et al. 2010; Robinson et al. 2011). Implication of this might be the under detection of alleles in minority clones, *e.g.*, mutations conferring drug resistance, or misclassification of recurrent infection after treatment.

Plasmodium's ability to adapt its genome and thereby escape treatment or detection, and possibly immunogenicity, has implications for treatment policies, surveillance, diagnosis, and vaccine development.

2.2. Malaria – the disease

2.2.1. Epidemiology

Malaria is one of most important infectious diseases worldwide, with half of the global population at risk, and more than 200 million cases and 600,000 deaths in 2020 (WHO 2021b). Over 90% of malaria related mortality and morbidity occurs in Africa, by *P. falciparum*, where it mainly affects children under five years of age and pregnant women (WHO 2021b). Enormous progress has been made in decreasing the malaria burden in the last decades: malaria deaths reduced from almost 900,000 in 2000 to about 550,000 in 2019. However, the COVID-19 pandemic has disrupted this trend (WHO 2021b).

Plasmodium vivax is geographically wider spread as compared to *P. falciparum*, as it occurs at subtropical and temperate latitudes (Sattabongkot et al. 2004) (Figure 2). It is the dominant malaria *spp* in South-East Asia (SEA) and Latin America but rather rare in Africa, which is attributed to the prevailing Duffy-negative blood group (Miller et al. 1976).

Plasmodium vivax is often referred to as clinically benign. It is generally considered to only constitute a small proportion of the global malaria problem, and was not separately reported in the World Malaria Report until 2013. However, 14 million cases were estimated in 2019, much higher than estimated by the World Malaria Report (Battle et al. 2019). *P. vivax* forms dormant stages in the liver, hypnozoites, which can relapse. These form a constant transmission reservoir if not treated correctly (Anstey et al. 2012; Baird 2013). In regions where *P. falciparum* and *P. vivax* coexist, a consistent increase in the proportion of vivax malaria has been observed (Price et al. 2020). Reliable estimates on the burdens of *P. ovale*, *P. malariae* and *P. knowlesi* are not available, but are likely responsible for less than 5% of the malaria cases globally.

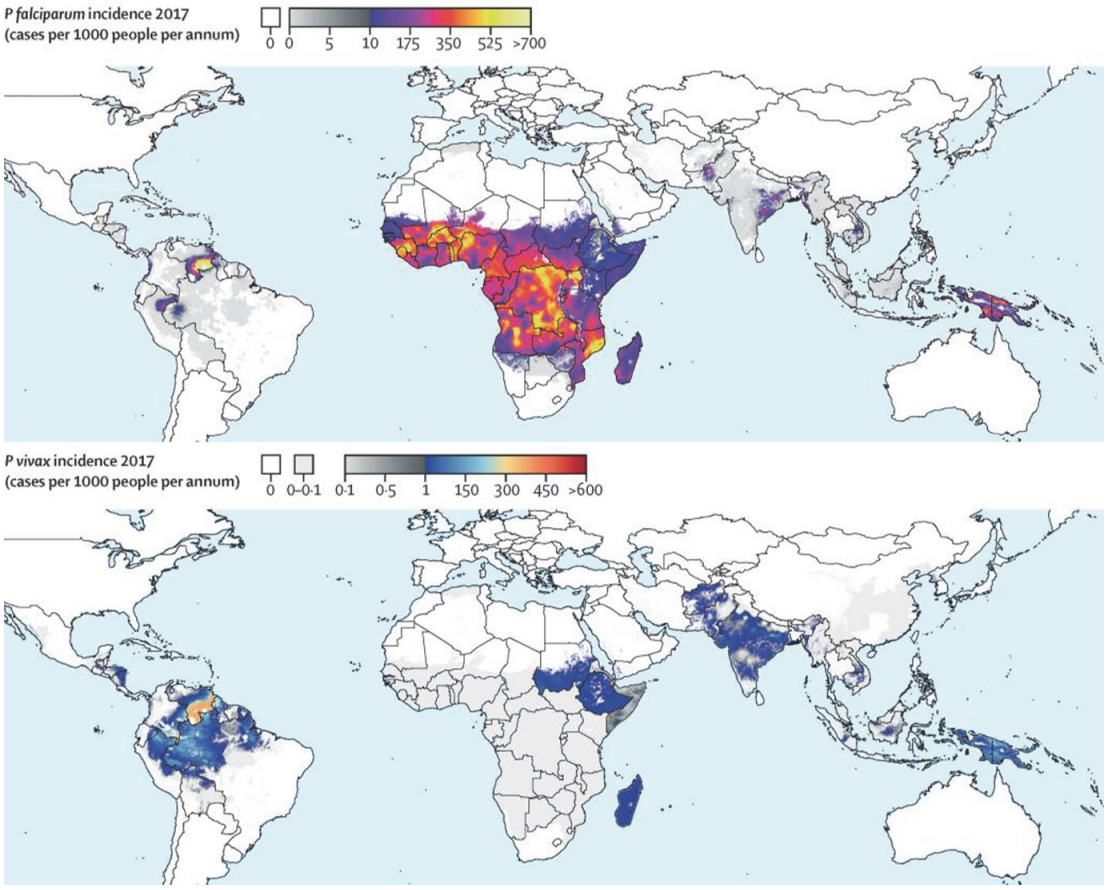

Figure 2. Estimated *P. falciparum* and *P. vivax* incidence in 2017.
Adapted from Weiss et al., Lancet*. 2019:394 and Battle et al.,* Lancet*. 2019:394.*

2.2.2. Clinical disease and pathophysiology

Malaria is a complex disease with a diverse manifestation, and its outcome depends on many factors including parasite and host genetics, previous exposure, age, nutritional status, and socio-economic factors. Without previous exposure to malaria, almost all infected

individuals become ill after infection. Malaria can be divided into two disease presentations: uncomplicated and, in case of *P. falciparum*, severe malaria.

The process from infectious mosquito bite to liver invasion and merozoite formation is asymptomatic. In early infection, during the erythrocytic cycle, a range of clinical conditions appear, such as the characteristic intermittent febrile illness, chills, headache, malaise, nausea, muscle pain, and joint aches. With increasing parasite density, pathogenic processes follow, like anaemia, metabolic acidosis, and spleno- and hepatomegaly. The development into severe malaria is a multi-system disorder and can result in permanent organ damage or death if not treated promptly. Three major manifestations of severe malaria are severe malarial anaemia, cerebral malaria (coma), and respiratory distress (WHO 2021b). Severe malarial anaemia is caused by haemolysis of parasitized erythrocytes, accelerated haemolysis of unparasitized erythrocytes, and by bone marrow dyserythropoiesis (Jakeman et al. 1999; White 2018). Cerebral malaria can result in lifelong neurological disabilities. It is caused by microvascular obstruction of sequestered infected erythrocytes in the brain, pro inflammatory cytokine cascades (a “cytokine storm”) and vascular leakage, eventually leading to brain hypoxia (Luzolo and Ngoyi 2019). Respiratory distress may be caused by metabolic acidosis or by acute lung injury. The first is due to lactic acid accumulation because of microvascular obstruction, cytokine cascades, and/or anaemia, whereas the latter is the result of inflammation driven alveolar damage (Van den Steen et al. 2013). In pregnant women, *P. falciparum* strains with specific binding affinity to the placental syncytiotrophoblast surface contributes to pregnancy-associated malaria, which can cause adverse birth outcomes and life-threatening disease in both mother and child, further discussed in another section (Rogerson et al. 2007).

P. falciparum causes infected erythrocytes to express endothelium adherence molecules allowing them to sequester in organs like brain, lungs, kidney, and placenta. The protein family causing sequestration by *P. falciparum* is encoded by many antigenically variant *var* genes, *i.e.*, the *PfEMP1* (Newbold et al. 1999). *P. vivax* lacks the *PfEMP1* ortholog, and preferentially infects reticulocytes (young erythrocytes), which prevents high parasite density (Galinski et al. 1992). However, *P. vivax* can cause also but rarely severe malaria, which is increasingly observed, but the underlying processes are largely unknown (Anstey et al. 2009; Price et al. 2009). *P. ovale*, *P. malariae*, and *P. knowlesi* infections can cause disease in non-immune hosts, but rarely severe.

Acquired immunity to malaria develops with repeated infections. Therefore, malaria is mostly life-threatening upon first exposures, which translates to high risk for children under five,

pregnant women (due to placenta-specific *P. falciparum* strains), and travellers or migrants from low or non-malaria transmission regions (Marsh and Kinyanjui 2006). Although sterile immunity does not occur, due to acquired immunity in malaria endemic regions, hosts often remain asymptomatic or present with mild disease only, associated with low parasitemia (Marsh and Kinyanjui 2006). However, different parasite strains may have differing virulence factors determining disease outcome, even in semi-immune patients. A multitude of host-factors other than acquired immunity also affects malaria manifestation and outcome, *e.g.*, genetic erythrocyte variants or nutritional status.

2.2.3. Innate and acquired immunity

The interplay between *Plasmodia* and the human host immune system is complex and subject to extensive study, *e.g.*, for vaccine development, and predicting and preventing severe outcomes. Antimalarial immunity is species and stage specific. It affects susceptibility to infection, manifestation and outcome of the disease, and treatment efficacy. From a clinical perspective, antimalarial immunity can be divided in an immunity reducing the risk of death by malaria, an immunity reducing the intensity of symptoms, and an antiparasitic immunity reducing the number of parasites. The classical categories of innate and acquired immunity, or cellular and molecular immunity overlap in these clinical immunity sections.

Innate immunity is inherently present in the host independently of previous exposure. Acquired immunity develops after repeated exposure and is seen in areas with stable malaria transmission and allows the host to prevent clinical and/or severe disease, but it does not eliminate the infection (Jeffery 1966; Reyburn et al. 2005; Tran et al. 2013; Griffin et al. 2015). Overall, malaria is an inflammatory response-driven disease, and immunity develops rather slow and incomplete (Hunt and Grau 2003; Clark et al. 2006), but immunity to death might be acquired after a single episode already (Gupta et al. 1999). Malaria parasites are sensed by host receptors at both the liver and blood stage, resulting in signalling pathway activation and cytokine production, contributing to parasite clearance and immune regulation (Langhorne et al. 2008).

Malaria directed innate immunity begins in the early pre-symptomatic phase of infection, but is also a determinant for the disease outcome (Clark and Rockett 1994; Haldar and Mohandas 2009). It limits the initial phase of parasite replication and shapes the adaptive immunity (Ockenhouse et al. 2006). Innate immunity to pathogens is largely driven by pattern recognition receptors (PRRs) sensing pathogen-associated molecular patterns (PAMPs). Genes for both PRRs (in the host) and PAMPs (in the pathogen) are highly conserved. The

Toll-like receptor (TLR) family constitute an important PRR component in antimalarial immunity. They are expressed and detect PAMPs at the cell surface or endosome membrane. Other PRRs are RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs), both present in the cytosol (Fitzgerald and Kagan 2020). For *P. falciparum*, most studied PAMPs are glycosylphosphatidylinositol (GPI) anchors, haemozoin, and immunostimulatory nucleic acid motifs. PAMP-activated TLRs induce an intracellular signalling cascade, mediated by several adapter proteins, including myeloid differentiation primary response gene 88 (MyD88), toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), and toll-like receptor adaptor molecule 1 (TRIF) (O'Neill and Bowie 2007). This cascade leads to pro-inflammatory responses that largely contribute to malaria pathogenesis (Walther et al. 2006). MyD88-dependent TLR9-mediated signalling contributes to protective antimalarial immunity (Gowda et al. 2012), and TLR2 and TLR4 may play important roles in malaria immunity and in the development of cerebral, placental, and other severe malaria pathology (Coban et al. 2007). Another class of receptors, lectin receptors, also play their part in *Plasmodium* immune sensing and pathogenesis. Pathogen recognition *via* mannose binding lectin (MBL) induces complement pathway activation, which is crucial for neutralizing invading parasites but may mediate pathogenesis (Silver et al. 2010).

An important immune regulator in the TLR machinery is the microRNA-146 family. MicroRNAs (miRNAs) form a class of small, non-coding RNAs that regulate and modulate gene expression. It is estimated that 30% of human genes are regulated by miRNAs, and they are closely involved in immune regulation in general (Mehta and Baltimore 2016). Moreover, their deregulation has been linked with malignancies and poor disease outcome. MiRNA-146a causes a negative feedback loop in TLR signalling by post transcriptionally downregulating the MyD88 pathway, thereby affecting cytokine responses (Sonkoly and Pivarcsi 2009).

Acquired immunity is initiated by antigen presenting cells, which form the main connection between the innate and acquired immunity because they generate memory (Gowda and Wu 2018). Successive *Plasmodium* infections improve this memory and the intensity of the response, mediated by T and B lymphocytes (Weiss et al. 2010; Kurup et al. 2019). Since major histocompatibility complex (MHC) class I and II molecules (crucial for inducing cellular immunity) are absent from the infected erythrocyte, it is generally assumed that the antibody-driven response has a primary role in blood-stage immunity. Indeed, Pfmep1- and merozoite specific IgG antibodies are thought to be main actors in preventing parasite replication and disease (Cohen et al. 1961; Gonzales et al. 2020).

2.2.4. Malaria in pregnancy

A particular group at risk for malaria are pregnant women. Pregnant women are more susceptible and disease. Moreover, malaria in pregnancy increases the chance of adverse birth outcomes. It can cause miscarriage, stillbirth, preterm delivery, and low birth weight (Fried and Duffy 2017) . This phenomenon has mostly been described for *P. falciparum* infection but is also seen for *P. vivax* (McGready et al. 2012). In 2020, more than 11 million pregnancies were estimated to be exposed to malaria, resulting in over 800,000 neonates with low birth weight (WHO 2020).

Despite acquired antimalarial immunity in endemic regions, first-time pregnant women are considered immune naïve to placenta-associated *P. falciparum* strains. Malaria in pregnancy is characterized by the accumulation of infected erythrocytes in the placental intervillous space, leading to macrophage infiltration, fibrinoid, and hemozoin deposits (Walter et al. 1982; Rogerson et al. 2003). This inflammatory climate at the maternal-fetal interface contributes to placental pathology and adverse outcomes for mother and child (Fried and Duffy 2017). Due to the accumulation in the placenta, parasite densities in peripheral blood can be low or even submicroscopic (Mockenhaupt et al. 2002).

P. falciparum strains associated with accumulation in the placenta form a population with a distinct affinity for binding to the glycosaminoglycan chondroitin sulfate A (CSA), expressed on the surface of the placental syncytiotrophoblasts (Fried and Duffy 1996; Rogerson et al. 2003). Binding of infected erythrocytes to CSA is mediated by the *PfEMP1* variant VAR2CSA, expressed on the erythrocyte surface (Salanti et al. 2004; Duffy et al. 2006). Immunity against pregnancy-related *P. falciparum* is acquired over successive pregnancies, in a setting of continuous exposure (Salanti et al. 2004), and is thought to be driven by antibodies directed against CSA binding, *e.g.*, by binding VAR2CSA (Fried and Duffy 1998). Primiparae form a distinct population of adult, immune naïve hosts in malaria endemic regions, as compared to multiparae. This allows for investigating the role of innate and acquired immunity during *Plasmodium* infection.

MicroRNAs (miRNAs) are key regulators of inflammatory processes (Mehta and Baltimore 2016), and play an important role in the communication between and regulation of maternal and foetal immune cells, which is essential for a successful pregnancy (Prieto and Markert 2011; Tannetta et al. 2014; Kamity et al. 2019). In addition, miRNAs may play an immunopathogenic role in *Plasmodium* infection during pregnancy (Moro et al. 2016).

2.3. Malaria shaped the human genome

Infectious diseases have posed evolutionary pressure on the human genome, creating a variety of defence mechanisms. Malaria is a major player in this concept: its long co-existence with humans and enormous effects on mortality make it the strongest known evolutionary force of the human genome (Kwiatkowski 2005). Genetic traits evolved and keep evolving in human populations to protect against *Plasmodium* infection, especially on the African continent, where *P. falciparum* poses a strong selective pressure. About 25% of the risk for malaria might be determined by human genetic factors (Mackinnon et al. 2005).

A classic example of such evolutionary pressure is the link between erythrocyte variants and resistance to malaria, initially proposed by J.B.S. Haldane in 1948, and based on the overlapping geographic regions with high frequencies of thalassemia and malaria (Haldane 1949). Since then, human genetics has been extensively studied with respect to malaria. However, disentangling the effect of genetic variation on susceptibility to and manifestation of malaria is complex and does not always clearly point into one direction. The most important known malaria resistance genes individually make up only a small proportion of the impact of host genetics on malaria, indicating that genetic malaria resistance is under multigenic control (Mackinnon et al. 2005). The immune response to some malaria antigens benefit the defence to other infectious pathogens too (Stirnadel et al. 2000). Some genetic polymorphisms may have protective effects against malaria but deleterious effects when present homozygously. This leads to the maintenance of both, or multiple variants of such polymorphisms in the host population: a principle referred to as balanced polymorphism. In addition, immune gene variants promoting strong inflammatory responses might favour the initial prevention of pathogen accumulation, but may exacerbate pathophysiology.

2.3.1. Erythrocyte variants and malaria

The Haldane malaria hypothesis was later validated for the sickle cell trait and many other erythrocyte variants, each affecting susceptibility to *Plasmodium* infection, progression to severe malaria, and/or death (Kwiatkowski 2005). Sickle cell trait, conferred by the HbS allele causing the deformed sickle haemoglobin, is the prime example of a balanced polymorphism in human populations. Whereas HbS homozygosity causes sickle cell anaemia, limiting a patient's expected life span considerably, heterozygosity confers a significantly reduced risk for *Plasmodium* infection and progression to severe malaria (estimates range from 10-90%) (Taylor et al. 2012). Other haemoglobin variants have also been linked to relative protection against malarial, *e.g.*, HbC and HbE, α- and β-thalassemia (Taylor et al. 2012). The

important role of structural haemoglobin variants on malaria is not surprising, since the parasite is very dependent on its haemoglobin environment. Further erythrocyte variants affecting malaria susceptibility and/or outcome include polymorphisms of the human glucose-6-phosphate dehydrogenase (G6PD) enzyme (Bienzle et al. 1972; Sabeti et al. 2002), the ABO blood group system, and variation in the Duffy antigen (chemokine receptor FY, DARC) (Miller et al. 1976; Hamblin and Di Rienzo 2000). A selection of these will be discussed further below.

The thalassemias constitute a broad group of haematological disorders and are the most common Mendelian diseases globally (Weatherall and Clegg 2001). They result from defective production of α- or β-globin chains in the haemoglobin molecule, caused by deletions or disruptions in one of the respective genes. The prevalences of α- and β-thalassemia have a remarkable overlap with historical malaria endemic regions (Haldane 1949). *E.g.*, α-thalassaemia occurs in up to 80% of some populations in Melanesia and South-East Asia and in up to 50% in Africa (Weatherall and Clegg 2001). Heterozygote α-thalassemia can be non-clinical or cause mild anaemia, but homozygosity for severe forms of α-thalassemia may cause severe disease or intrauterine death. Heterozygous and homozygous α+ thalassemia (*i.e.*, when only one of the two α-chain genes is disrupted) protect against severe malaria (Flint et al. 1986; Mockenhaupt et al. 2004). The mechanism of protection is not clear, but suggested are decreased parasite growth, increased antibody binding, or enhanced splenic clearance (Pattanapanyasat et al. 1999; Williams et al. 2002).

The G6PD enzyme is an essential defence against oxidative stress in the cell. Many G6PD variants exist, some conferring decreased enzyme activity, referred to as G6PD deficiency, causing haemolytic anaemia, depending on the extend of deficiency. Again, the geographical distribution of G6PD deficiency suggests evolutionary selection by malaria (Ganczakowski et al. 1995). The G6PD deficiency allele is prevalent at an estimated 8% in all malaria endemic countries and reaches more than 20% in some African populations (Howes et al. 2012). The effect of G6PD deficiency on malaria is not clear-cut. It is generally accepted that it confers some levels of protection against severe malaria, but exacerbation of severe malarial anaemia has also been demonstrated (Allison 1960; Ruwende et al. 1995; Clarke et al. 2017). The mechanisms underlying this protection is debated. Possible explanations are reduced parasite replication in G6PD-deficient erythrocytes, and early phagocytosis or infected erythrocytes (Luzzatto et al. 1969; Cappadoro et al. 1998).

The ABO blood groups also show a distinct geographical pattern indicative of selective pressure by *P. falciparum*, *i.e.*, blood group O is more frequent in malaria endemic regions with a prevalence of above 50% among African populations compared to below 50% in Caucasian populations (Cserti and Dzik 2007). The absence of A or B antigens on erythrocytes (blood group O) confers protection against severe malaria (Degarege, Gebrezgi, Ibanez, et al. 2019). This is attributed to less rosette forming in O-erythrocytes compared to A- or B-erythrocytes (Udomsangpetch et al. 1993; Rowe et al. 2007). However, the ABO blood group system has a different impact on malaria during pregnancy, and findings contrast between studies (Degarege, Gebrezgi, Beck-Sague, et al. 2019).

A remarkable example of how susceptibility to *Plasmodium* can differ between populations is the resistance to *P. vivax* infection in most of the Sub-Saharan African population. This is attributed due to the virtual absence of the Duffy antigen (Duffy antigen receptor for chemokines [DARC], Fy glycoprotein [FY], CD23) in Sub-Saharan Africa. The Duffy blood group system is characterized by a glycosylated protein expressed on the erythrocyte surface. The Duffy antigen (or receptor) has for long been considered crucial for *P. vivax* erythrocyte invasion (Miller et al. 1976; Livingstone 1984). Recent observations of *P. vivax* infected Duffy-negative patients in Africa suggest that the parasite is evolving to find new invasion pathways (Golassa et al. 2020). Two major alleles for the Duffy antigen exist, FYA and FYB, determined by a polymorphism G125A, resulting in genotypes FYA/FYB, FYB/FYB, or FYA/FYA. In addition, a silencing mutation results in Duffy blood group negativity, FYO, for one or both alleles. The FYA allele is more frequent the America's and South-East Asia, whereas the FYB allele dominates in Europe (King et al. 2011) (Figure 3). The absence of the Duffy antigen protects against *P. vivax* infection (Miller et al. 1976; Horuk et al. 1993; Howes et al. 2011), and the A or B alleles might differentially affect *Plasmodium* susceptibility and disease manifestation (King et al. 2011; Chittoria et al. 2012). Additionally, a less common Duffy antigen allele, FYX, characterized by the presence of two SNPs C265T and G298A, weakens FYB antigen expression (Höher et al. 2018). Besides the effect on parasite invasion, the Duffy antigen is multivalent receptor for chemokines thereby affecting inflammatory responses to infectious diseases (Rot 2005; Schnabel et al. 2010). The Duffy antigen also holds a key position in platelet-mediated *P. falciparum* killing (McMorran et al. 2012, 2013). Duffy antigen variants may affect these immune responses and thereby susceptibility to and manifestation of *Plasmodium spp*.

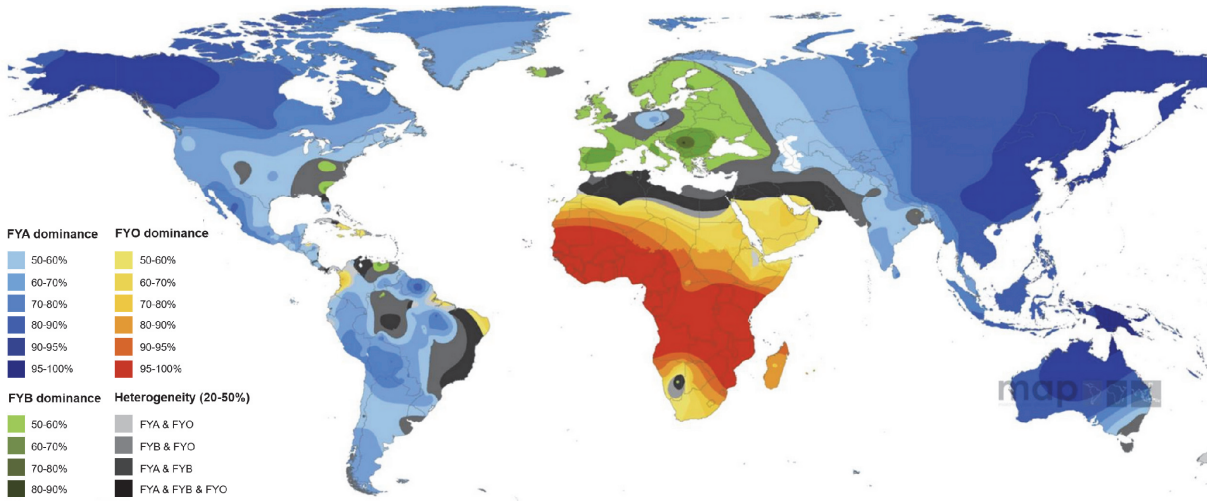

Figure 3. Global Duffy antigen allele distribution.

Areas dominated by a single Duffy allele (frequency ≥50%) are indicated by a colour scale (blue, FYA; green, FYB; orange, FYO). Areas where no single Duffy allele dominates, but two or more alleles each have frequencies ≥20%, are indicated by a grey scale. Adapted from King et al. Proc Natl Acad Sci USA*. 2011:108.*

2.3.2. Innate immune system variants and malaria

An extensive range of immune variants are reported to associate with malaria outcome, *e.g.*, variants of human leukocyte antigen (HLA) class, Interleukins, TNF and IFN, MBL, TLRs, and microRNAs (miRNAs) (Mockenhaupt et al. 2006). The miRNA-146 family is involved in the modulation of inflammatory immune responses. MiRNA-146a regulates the MyD88-dependent pathway, thereby affecting TLR signalling, which is a key pathway in the innate immune response (Taganov et al. 2006). A common miRNA-146a variant, rs2910164 G > C, shows a distinct geographic pattern, indicative of selective pressure. The C allele frequency is about 25% in European, 45% in African, and 65% in Asian populations (International HapMap Consortium no date; Belmont et al. 2003). Assumingly because of its effect on the TLR machinery, this miRNA-146a variant associates with altered susceptibility to various infectious diseases (Zhang et al. 2015; Hao et al. 2018).

2.4. Malaria control

Malaria control is the overarching term for any measures or interventions that lead to the interruption of transmission. Transmission interruption leading to zero malaria cases and the prevention of transmission reestablishment in a defined geographic area, *e.g.*, on a national level, is referred to as malaria elimination. Such requires continuous measures and

interventions in endemic regions. Reaching a malaria rate of zero cases on a global level with no remaining cases is referred to as malaria eradication.

Malaria eradication comprised one of the targets set by The United Nations' Millennium Development Goals in 2000. Largely supported by the Bill and Melinda Gates Foundation, there has been a rapid scale-up of long-lasting insecticide treated nets distribution, improved access to treatment, and more funds became available for research and development of antimalarials and vaccines. More recent and specific global targets, set by the WHO, are a 90% reduction of malaria cases and mortality by 2030 from a 2015 baseline (and a 40% reduction by 2020, which was not reached). Since 2000, enormous progress has been made in reducing malaria morbidity and mortality globally: from almost 900,000 yearly malaria deaths at the beginning of this century to about 500,000 20 years later. An estimated 1.7 billion malaria cases and 10.6 million malaria deaths have been averted globally in this period, mostly in Africa (WHO 2021b). However, malaria eradication, elimination let alone, is still not in sight in most endemic regions, global funds have plateaued since the last decade at about one-third of the WHO's estimated annual 6.8 billion USD required to reach the milestones, and the COVID-19 pandemic disrupted many malaria programmes globally (WHO 2021b).

Vector control and case management constitute the cornerstones of malaria control. Vector control includes removal or larvicidal treatment of mosquito breeding sites, indoor residual spraying (IRS) of insecticides and the distribution of insecticide treated bed nets (ITNs). Indoor residual spraying and ITNs are cost-efficient antimalarial control strategies, but heavily rely on the effectiveness of insecticides to kill *Anopheles* mosquitos. Resistance towards these insecticides is globally prevalent: in almost 70% of the reporting sites, at least one malaria vector was resistant to pyrethroids, the primary insecticide for ITNs (WHO 2021b). A novel but not yet implemented vector control technique is the generation of sterile mosquitos by genetic alteration (Macias et al. 2017).

In the absence of an effective vaccine, case management is essential to reduce or eliminate the human reservoir of malaria. Case management includes detection and treatment. Prompt and effective treatment is essential for the individual patient as it prevents development to severe malaria and death, and on a population scale as it limits further transmission. Selective pressure by antimalarial drugs has led to the emergence and spread of resistance and rendered many antimalarials less effective. Antimalarial drugs and resistance will be further discussed in other sections. Asymptomatic infection in semi-immune populations hampers early and complete case detection and therefore treatment, because these subjects

will have no or only mild symptoms and will likely not seek health care. Moreover, *P. vivax* hypnozoite carriers cannot be identified. Mass drug administration (MDA) and chemoprophylaxis overcome this. Mass drug administration for malaria is often not encouraged because it may promote emergence of drug resistance, and because it may reduce acquired immunity in populations. On the other hand, it might be the only method to eliminate the asymptomatic carrier reservoir and it significantly improves survival in vulnerable populations. Recent observations from MDA for children in Sub-Saharan Africa demonstrated that monthly administration of a combination therapy during high transmission season is extremely cost-effective: below 0.60 USD per child and a substantial reduction in malaria cases and mortality (ACCESS-SMC Partnership 2020). Intermittent preventive treatment in pregnancy (IPTp) is another example of how chemoprevention can substantially decrease malaria burden. IPTp targets pregnant women in malaria endemic regions and effectively reduces the risk of adverse birth outcomes (WHO 2021b).

Parasite-based diagnosis can be done by microscopy, by rapid diagnostic test (RDT) or by nucleic acid detection (PCR or Loop mediated isothermal amplification [LAMP]). Microscopy is the cheapest and most used method and considered the gold standard for malaria diagnosis. However, it requires trained microscopists and has a limit of detection of about 100 parasites/µL. Rapid diagnostic tests have a similar limit of detection (Britton et al. 2016). These are lateral flow devices using immunochromatography and detect malaria antigens. Nucleic acid detection in patient blood is most sensitive, it can detect a single parasite in a blood sample. However, PCR-based diagnosis is not feasible to implement at a point-of-care level because of the time, costs, and requirement of a thermocycler. Malaria specific LAMPs are a promising method because they do not require a thermocycler, only a thermostable environment around 60°C, and fewer processing steps (Selvarajah et al. 2020).

The availability of an effective malaria vaccine would be an ideal tool to speed up malaria elimination. So far, only one malaria vaccine has passed the last regulatory audits: the RTS,S/AS01 vaccine. Moreover, the RTS,S vaccine is the only human antiparasitic vaccine reaching this stage. It targets pre-erythrocytic *P. falciparum* stages. Vaccine implementation pilots started in 2016, showed good safety profiles and a vaccine efficacy of up to 36% for severe malaria (RTS,S Clinical Trials Partnership 2015; WHO 2021b). At the end of 2021, the WHO recommended the use of the RTS,S malaria vaccine in children living in regions with moderate to high malaria transmission (WHO 2021b). Another promising vaccine candidate is the R21/Matrix-M vaccine, which has successfully completed a phase II trial. It targets pre-erythrocytic *P. falciparum* stages as well and reduced clinical malaria in African children by up to 77%, which is a record malaria vaccine efficacy to date. Both vaccines

reduce clinical malaria but do not block transmission, which, in the perspective of malaria elimination, is an important requirement. Due to the COVID-19 pandemic, the development of mRNA vaccines accelerated tremendously (despite the technique being already known and available for decades (Dolgin 2021)), which is hopeful for malaria vaccine development. An mRNA-based malaria vaccine is currently in the pipeline and clinical trials are planned for 2022.

2.5. Antimalarial drugs

Antimalarial herbs and extracts have been used in traditional medicine for centuries, but the first chemically purified antimalarial, quinine, was isolated in 1820. Quinine became the first chemical compound that successfully treated an infectious disease, and it remains an important antimalarial as of today (Achan et al. 2011). In the 1940's, the cheap, safe, tolerable, and universally effective chloroquine became widely implemented, significantly decreasing the malaria burden worldwide (Cooper and Magwere 2008). However, signs of resistance were reported in the next decade, and chloroquine resistance spread globally, with dramatic effects for child mortality in Africa (Trape 2001). Other antimalarial drugs have been developed in the last century, but resistance to virtually all of these has emerged, leading to high rates of treatment failure in some regions (WHO 2021b).

A malaria patient needs rapid treatment to decrease symptoms and the risk of progression to severe disease. The symptomatic phase of the disease aligns with the erythrocytic stage of the parasite. In uncomplicated malaria, a patient requires a fast-acting drug that inhibits parasite multiplication. In severe malaria, however, death might occur within 48 hours, which is within the timespan of one generation of parasites. Thus, a drug inhibiting the progression from less harmful ring stage parasites to the virulent sequestered stages is required.

Most antimalarial drugs target asexual erythrocytic stages. However, drugs eliminating hypnozoites (*P. vivax* and *P. ovale*), gametocytes, and tissue schizonts are also on the market. Main classes are: quinoline derivatives (*e.g.*, chloroquine, amodiaquine, primaquine, piperaquine, quinine, mefloquine, lumefantrine), antifolates (*e.g.*, pyrimethamine, sulfadoxine), antibiotics (*e.g.*, doxycyclin), and artemisinin derivatives (*e.g.*, artemether, artesunate, dihydroartemisinin).

First-line antimalarials, *i.e.*, drugs of choice, currently are artemisinin combination therapies (ACTs) for uncomplicated malaria, and artesunate for severe malaria. ACT is the combination of a fast-acting artemisinin derivative that rapidly kills most parasites in blood

circulation, and a drug that clears more slowly, to kill the remaining parasites over the next days to weeks. Uncomplicated vivax malaria is treated with a combination therapy of chloroquine and primaquine; the latter is able to kill dormant liver stages (WHO 2021a).

In our studies, we identified and aimed to obtain an insight in the newly emerging artemisinin resistance in Rwanda, East Africa. To provide a complete picture of the antimalarial drug landscape globally, a brief description of the relevant drugs is provided in the next sections. A more in-depth overview is given with regard to artemisinin.

2.5.1. Quinoline derivatives

Chloroquine (CQ) belongs to the class of 4-aminoquinolines, was used to prevent and treat infection with all *Plasmodium* species up to 1990, but the emergence and wide dissemination of chloroquine resistant *P. falciparum* has limited its use to the treatment of non-falciparum malaria. Chloroquine accumulates in the parasite's digestive vacuole, where it interferes with the parasite's detoxification process of haematin into haemozoin, thereby killing the parasite (Foley and Tilley 1998).

Amodiaquine (AQ), a 4-aminoquinoline, is structurally similar to chloroquine, and shares cross-resistance with chloroquine (Basco and Le Bras 1993). However, due to its increased potency compared to chloroquine, it still reaches sufficient efficacy as treatment against chloroquine resistant *P. falciparum*. Amodiaquine is now mainly used as partner drug in the artemisinin combination therapy artesunate-amodiaquine (AS-AQ). Its mechanism of action is similar to chloroquine, involving the inhibition of haemozoin formation in the parasite's digestive vacuole (Foley and Tilley 1998).

Piperaquine (PPQ) is also a 4-aminoquinoline and structurally related to chloroquine. It shares similar pharmacological properties and is affected by cross-resistance to chloroquine. The drug is, due to resistance, only used as partner drug in ACT now, with dihydroartemisinin (DHA-PPQ). The suggested mode of action of piperaquine is similar to chloroquine (Davis et al. 2005).

Primaquine (PQ) belongs to the 8-aminoquinolines. It is the only drug available that eliminates the dormant liver stages of *P. vivax* and *P. ovale* (hypnozoites), referred to as radical cure. However, it can cause haemolytic anaemia in patients with G6PD deficiency, a prevalent genetic trait in malaria endemic regions (Carson et al. 1956). It is mainly used for

radical cure in *P. vivax* patients, as combination therapy with chloroquine (CQ-PQ), but also acts as a gametocydal drug for *P. falciparum* (WHO 2021a).

Quinine, an amino alcohol, is the oldest antimalarial used in Europe. Its use dates back to the 16th century, as an extract from cinchona tree bark, and it was purified in 1820. Resistance is present globally, although this developed remarkably slowly considering its long existence and use. Quinine is not well tolerated, and its use is largely limited to severe malaria treatment.

Mefloquine (MQ), another amino alcohol, is known for its neuropsychiatric and cardiac side effects, under the trade name Lariam. After decades of use, predominantly in chemoprophylaxis, it now has become unpopular, and it is not available in Germany anymore. Thailand used mefloquine-artesunate as an ACT for some time, which was replaced by more effective ACTs.

Lumefantrine (LF) is an amino alcohol, chemically related to quinine and has virtually never been used as monotherapy. It is used in ACT, with artemether (AL), under the tradename Coartem, which is currently the most widely used ACT globally. Lumefantrine's mechanism of action of lumefantrine is not fully understood, but it is suggestive that it forms a complex with hemin and thereby inhibits haemoglobin breakdown by the parasite (Combrinck et al. 2013).

2.5.2. Antifolates

Sulfadoxine and pyrimethamine (SP) are only used as combination, which has the distinct advantage of a single-dose therapy. It is rarely used as partner drug in ACT, with artesunate, *e.g.*, in parts of India. Because of its safety during pregnancy from 2nd trimester on and its curative and prophylactic effect, it is used as preventive treatment for malaria in pregnancy. Intermittent preventive treatment in pregnancy (IPTp) is recommended in all moderate to high malaria transmission regions in Africa (WHO 2021a). Sulfadoxine and pyrimethamine inhibit the parasitic enzymes dihydropteroate synthetase (DHPS) and dihydrofolate reductase (DHFR), respectively, thereby obstructing the parasite's folate biosynthesis pathway. Consequently, cell growth and reproduction are prevented, blocking schizont formation (Foote and Cowman 1994).

2.5.3. Artemisinin and artemisinin combination therapy

Artemisinins are the most potent, safe, and tolerable antimalarial drugs available today and their introduction might have saved millions of lives over the last decades. Artemisinin combination therapy (ACT) is the recommended first-line treatment falciparum malaria globally since 2001 (WHO 2021a).

A significant proportion of ring-stage parasites enters a state of dormancy and is not killed upon artemisinin treatment, possibly contributing to about 5-10% of treatment failure in the case of monotherapy (Ménard et al. 2005; Teuscher et al. 2010). To assure full parasite elimination, and to slow down the development of resistance, artemisinins are recommended to be co-administered with other, more slowly eliminated antimalarials, such as lumefantrine, piperaquine, amodiaquine, or mefloquine. The moslyt used ACT globally and in Africa is AL (often called co-artemether, tradename Coartem or Riamet) (Figure 4), which shows a remarkable resilience to resistance development.

Artemisinin treatment contains an artemisinin derivative (referred to as artemisinins from here on): artesunate, artemether, or dihydroartemisinin (DHA; the main and most potent metabolite of artemisinin). Artesunate administered intravenously is the WHO-recommended first-line treatment for severe falciparum malaria. Artemisinins are rapidly absorbed after oral administration and have a short elimination half-life (DHA; less than one hour). They rapidly kill both young ring-stage parasites and mature trophozoites. Parasite clearance after treatment is significantly faster compared to other antimalarials (White and Untvmity 1994).

The exact mechanism of the action of artemisinin derivatives is an active field of research, and proteomic studies suggest hundreds of artemisinin targets. A popular view is that artemisinin action is strongly linked to haemoglobin digestion by the *Plasmodium* parasite, at least partly facilitated by the highly conserved *Kelch-13* gene (Birnbaum et al. 2020). Artemisinin compounds are considered pro-drugs that require activation of their characteristic endoperoxide bridge (C-O-O-C) to be effective (Klonis et al. 2011). Main activators of artemisinins are heme and ferrous iron, which are both abundantly present in the infected erythrocyte due to haemoglobin digestion (Klonis et al. 2011). Activated artemisinins react with parasite proteins, leading to parasite damage and death, and compromise the parasite's ubiquitin-proteasome system (*i.e.*, the protein waste-disposal) (Wang et al. 2015; Bridgford et al. 2018). Ring-stage parasites are less sensitive to artemisinin pulses compared to trophozoites. The latter have a significantly higher level of haemoglobin uptake, indeed indicating the role that haemoglobin has in the activation of artemisinin. However, directly

after erythrocyte invasion, *i.e.*, in the very early ring phase, a small time-window of hypersensitivity is observed, indicating a potent artemisinin activator present in this parasite stage with low haemoglobin uptake (Klonis et al. 2013).

Figure 4. National antimalarial policy for uncomplicated falciparum malaria.

AL, artemether-lumefantrine; AS-AQ, artesunate-amodiaquine; AL-PQ, artemether-lumefantrine -primaquine; AS-PYR, artesunate-pyrimethamine; DHA-PPQ, dihydroartemisinin-piperaquine. Figure generated with data from World Malaria Report 2020, WHO 2020, created with MapChart *(https://www.mapchart.net).*

2.5.4. Antimalarial drug resistance

Antimalarials are a key component in malaria control since their introduction in the last century. Intense drug use, monotherapies in particular, puts selective pressure on the malaria parasite populations, leading to the emergence and dissemination of resistance.

Antimalarial drug resistance, in *P. falciparum* in particular, contributes significantly to the worldwide malaria burden (Marsh 1998; Murray et al. 2012).

Chloroquine resistant *P. falciparum* was reported in South-East Asia and in South America within 15 years after its introduction in the 1940's (Maberti 1960; Moore and Lanier 1961; Harinasuta et al. 1965). It arrived in East Africa in the late 1970's and swept across the continent within 15 years (Campbell et al. 1979; Fogh et al. 1979; Wernsdorfer and Payne 1991). Chloroquine resistant *P. vivax* was first reported in 1989 in Papua New Guinea and later also appeared in all *P. vivax* endemic areas (Rieckmann et al. 1989; Price et al. 2014). Today, chloroquine resistant *P. falciparum* and *P. vivax* are globally prevalent (Price et al. 2014; Ocan et al. 2019; Spotin et al. 2020). Despite pyrimethamine resistance and sulfadoxine resistance already reported in the 1950's (Hernandez et al. 1953; Maberti 1960), sulfadoxine-pyrimethamine combination therapy was introduced in the 1960's to combat chloroquine resistant malaria. Sulfadoxine-pyrimethamine resistant *P. falciparum* and *P. vivax* emerged in South-East Asia soon after (Hurwitz et al. 1981; Pinichpongse et al. 1982). Sulfadoxine-pyrimethamine resistant *P. falciparum* later arrived in East Africa where it spread continent-wide (Roper et al. 2004). Artemisinin-based monotherapies have been widely used in South-East Asia since the 1990's, and the first signs of artemisinin resistance appeared in the same region in 2007 (Noedl et al. 2008; Dondorp et al. 2009). First signs of artemisinin resistance are now, very recently, reported in East Africa (Balikagala et al. 2021; Bergmann et al. 2021; Uwimana et al. 2021; van Loon et al. 2022).

2.5.5. The Greater Mekong subregion - epicentre of antimalarial drug resistance

The emergence of chloroquine resistance, sulfadoxine-pyrimethamine resistance, and artemisinin resistance has consistently first been detected in the Greater Mekong subregion, South-East Asia. In all cases, it took roughly two decades to be detected on the African continent, always first in East Africa. Chloroquine and SP resistant *P. falciparum* strains in Africa likely originated in South-East Asia and were introduced by migrants (Wootton et al. 2002; Roper et al. 2004). In contrast, the recently detected artemisinin resistant *P. falciparum* strains in East Africa are likely of local origin, *i.e.*, artemisinin resistance emerged independently (Uwimana et al. 2020; van Loon et al. 2022).

What makes the Greater Mekong subregion a hot-spot for antimalarial resistance? Several factors might have played their part here, including political structure, socioeconomics, environmental factors, and biological factors. The region houses some remote, poor populations with limited access to health services and in some cases suspicion towards

governmental programmes (WHO 2010). Migration, *e.g.*, due to mining activities, causes a continuous influx of non-immune migrants in pockets of endemicity, under poor living and working conditions (Verdrager 1986; WHO 2010). The absence of antimalarial immunity in populations favours the emergence of drug resistance. It makes individuals more prone to develop symptoms upon *Plasmodium* infection, seek health care and receive treatment (Doolan et al. 2009), thereby increasing the drug pressure upon the parasite population. Lower levels of population immunity also increase the probability of individual parasites to survive and be transmitted (Bushman et al. 2018). Besides its effect on population immunity, low malaria transmission favours the selection of drug resistant *Plasmodium* parasites by reducing the risk of allelic outcrossing by sexual recombination in the mosquito (Anderson et al. 2000). Furthermore, the region has an abundancy of fake or poor-quality antimalarial drugs in circulation, leading to subtherapeutic drug concentrations (Dondorp et al. 2004). On the individual patient level, selection of de-novo drug-resistance in individual patients is favoured by hyperparasitaemia and low drug dosing (White et al. 2009).

2.5.6. Molecular mechanisms of drug resistance

In our studies, we focussed on antimalarial resistance in *P. falciparum* and therefore mechanisms of resistance will be discussed further with respect to *P. falciparum* only. For many antimalarials, the mechanisms of action and resistance are not fully understood. Parasite (drug) transporter proteins play an important role in antimalarial resistance, and other important proteins are the sulfadoxine and pyrimethamine target enzymes DHFR and DHPS as well as plasmepsin and Kelch-13 (K13) (Cowman et al. 1988; Ariey et al. 2014; Bopp et al. 2018). Variations in the genes encoding these proteins associate with various degrees of resistance and with treatment failure. Drug transporters and K13 will be further discussed below because of their role in general *Plasmodium* drug resistance, and in artemisinin resistance specifically.

Drug transporters

A crucial function in antimalarial drug resistance exists for the (drug) transporter proteins, of which there might be multiple in the *P. falciparum* genome (Mu et al. 2003). As seen in drug resistance in bacteria and in human cancer cells, mutations and overexpression in transporters of the ATP-binding cassette (ABC) superfamily can cause drug resistance, by facilitating the efflux of drugs from its target (Ouellette et al. 1994; Allen et al. 2000). *P. falciparum* harbours several members of this superfamily: *P. falciparum* multidrug resistance protein 1 and 2 (PfMDR1 and PfMDR2), and *P. falciparum* multidrug resistance-associated

protein 1 and 2 (PfMRP1 and PfMRP2) (Foote et al. 1989; Wilson et al. 1989; Koenderink et al. 2010). Copy number variation and single nucleotide polymorphisms of *PfMDR1* alter sensitivity *in vitro* and *in vivo* to a wide range of structurally unrelated antimalarials (Price et al. 2004; Sidhu et al. 2006; Veiga et al. 2016). Mutations in the gene encoding the *P. falciparum* chloroquine resistance transporter (PfCRT), not a member of the ABC family, are associated with chloroquine resistance (Fidock et al. 2000).

P. falciparum multidrug resistance-associated protein 1 encodes a transmembrane transporter protein, which is expressed on the food vacuole throughout the intra-erythrocytic cycle (Cowman et al. 1991). Increased *PfMDR1* copy number, common in South-East Asian but rare in African parasites, leads to increased expression, and associates with decreased susceptibility to mefloquine, lumefantrine, quinine, and artemisinin (Price et al. 2004; Sidhu et al. 2006). Five common SNPs in the *PfMDR1* gene are N86Y, Y184F, S1034C, N1042D, and D1246Y. Most research with respect to drug susceptibility has focussed on these variants, but other, rare, *PfMDR1* variants are reported too (Ahouidi et al. 2021). Increased *in vitro* tolerability of the 4-aminoquinolines chloroquine and amodiaquine is seen in case of *PfMDR1* 86Y and 1246Y (Echeverry et al. 2007; Veiga et al. 2016). On the other hand, these mutations associate with decreased sensitivity towards the amino alcohols mefloquine and lumefantrine, and towards artemisinin (Duraisingh et al. 2000; Veiga et al. 2016). It is not clear how these SNPs affect *PfMDR1*-mediated drug transport. *PfMDR1* is believed to be rather a moderator than the prime driver of chloroquine resistance (Reed et al. 2000; Babiker et al. 2001). However, the multi-valent effect the *PfMDR1* N86Y SNP has on various drugs makes it a pivotal player in multidrug resistance. Another member of the ABC family that is related to antimalarial drug resistance is the *PfMRP1* (Koenderink et al. 2010). Different SNPs occur worldwide, with varying effects on drug sensitivities, *e.g.*, I876V and K1466R.

P. falciparum chloroquine resistance transporter is an important determinant for chloroquine resistance. It is a member of the drug-metabolite transporter superfamily (Fidock et al. 2000; Martin and Kirk 2004) and located on the parasite's food vacuole, but its function remains unclear. The gene is polymorphic, but the *PfMDR1* K76T mutation occurs globally and confers chloroquine resistance (Lakshmanan et al. 2005). Similar to *PfMDR1* 86Y, *PfCRT* 76T links with decreased sensitivity to amodiaquine (Basco and Le Bras 1993), but with increased sensitivity to quinine, mefloquine and artemisinin *in vitro* (Singh Sidhu et al. 2002). The effect of this mutation on drug transport is not fully understood, although it appears to increase the export of chloroquine (Sanchez et al. 2005).

Kelch-13

Full *in vivo* artemisinin resistance does not occur so far, since it is rather a delayed parasite clearance in treated patients that is observed. Even if patients harbour parasites with such decreased artemisinin susceptibility, ACTs are curative as long as no partner drug resistance is present. However, there is consensus to refer to this decreased susceptibility as artemisinin resistance (WHO 2015).

Understanding artemisinin resistance has greatly contributed to deciphering the mechanism of action of artemisinin in parasite killing itself. Population genomics identified the key gene in artemisinin resistance in 2014: *P. falciparum Kelch-13* (*PfK13*) (Ariey et al. 2014), and genetic editing studies confirmed this (Ghorbal et al. 2014; Straimer et al. 2015). Mutations in *PfK13*, more specifically in the propeller domain (which is often referred to with *PfK13*), associate with various degrees of resistance. How these mutations affect PfK13 function and artemisinin sensitivity is remains ambiguous. The heterogeneity of *PfK13* mutations associated with similar drug sensitivity phenotypes indicates that they rather confer partial loss-of-function than a gain of function. Proposed mechanisms are: decreasing the level of activated artemisinin due to altered haemoglobin uptake (Yang et al. 2019; Birnbaum et al. 2020), or by a prolonged ring stage (Hott et al. 2015), and improved cell stress response (*e.g.*, by an enhanced ability to enter the quiescent state or by efficient degrading damaged proteins (Mok et al. 2021)). A central role is assumed for haemoglobin uptake and digestion in the parasite's food vacuole. The K13 protein is expressed in close proximity to the food vacuole, and *PfK13* mutations associate with significantly decreased expression. This leads to decreased haemoglobin uptake, which in turn results in less Fe2+-heme required for artemisinin activation (Yang et al. 2019; Birnbaum et al. 2020). In addition, *PfK13* mutations associate with increased protein folding and protein turnover, possibly facilitating rapid elimination of damaged proteins by artemisinin (Mok et al. 2021). Moreover, K13 seems to be involved in multiple metabolic pathways, including mitochondrial functions, and *PfK13* mutations may in turn affect the parasite's quiescent state during artemisinin exposure, and its ability to reinitiate growth after artemisinin-driven quiescence (Gnädig et al. 2020; Mok et al. 2021).

Most studied *PfK13* mutations are C580Y, the main artemisinin resistance allele in South-East Asia, and R539T, causing highest levels of *in vitro* resistance. Over a hundred *PfK13* mutations are reported globally. Mutations that associate with both *ex vivo/in vitro* increased artemisinin survival rates *and* with delayed parasite clearance in treated patients are categorized as validated markers of artemisinin resistance. If only the latter is observed, such

mutations are categorized as candidate markers. Ten mutations have been marked as validated markers for artemisinin resistance: F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H and C580Y (WHO 2019a). *PfK13* mutations conferring resistance cluster either at the surface of the kelch domain, possibly altering K13-substrate interaction, or in the propeller core, possibly destabilizing the protein structure (Singh et al. 2016).

2.5.7. Parasite fitness & antimalarial resistance

Observations from bacteria demonstrated that drug resistance often comes with a fitness cost (Spratt 1996). Mutations that contribute to a pathogen's drug resistance are likely to result in a loss of fitness, although other compensatory mutations may appear (Levin et al. 2000; Björkholm et al. 2001). A canonical example of ever-changing parasite genetic structures due to fitness, is the rapid return of chloroquine-sensitive *P. falciparum* strains in Malawi after the retraction of chloroquine as antimalarial treatment (Laufer et al. 2006). The fitness cost that antimalarial drug resistance might bring along in *Plasmodium* parasites is key for its success in an ecological context. Disentangling fitness in *Plasmodium* is crucial for drug discovery, designing combination therapies (Lukens et al. 2014), pathogenicity (Tukwasibwe et al. 2014), and for understanding emergence and dissemination of antimalarial resistant *Plasmodium*.

P. falciparum multidrug resistance-associated protein 1 copy number and mutations 1034C, 1042D and 1246Y impose a substantial fitness cost (25%) in in the erythrocytic cycle *in vitro* (Hayward et al. 2005; Preechapornkul et al. 2009). Field observations of increasing *PfMDR1* N86 allele prevalence after the retraction of chloroquine treatment policy or during low drug pressure in dry season suggests a fitness effect of the *PfMDR1* 86Y mutation (Ord et al. 2007; Ehrlich et al. 2020). However, this has not clearly been demonstrated *in vitro*. Of note, when accompanied by mutation *PfMDR1* 184F, a fitness cost was seen *in vitro* (Duvalsaint et al. 2021). As for the key chloroquine resistance marker *PfCRT* 76T, epidemiological studies are highly suggestive of a fitness cost (Ord et al. 2007; Ehrlich et al. 2020), but no *in vitro* data supports this. *In vivo*, *PfCRT* 76T seems to confer a loss of fitness as reflected by parasite density, but increased gametocytogenesis suggests a compensatory mechanism (Osman et al. 2007). As for *PfK13* mutations, R539T and I543T confer a significant growth defect *in vitro*, which is less for C580Y (Nair et al. 2018). This could be the reason of the *PfK13* C580Y dominance in South-East Asia, whereas *PfK13* R539T is believed to cause higher levels of artemisinin resistance (Straimer et al. 2015). Of note, *in vitro* studies demonstrate a significant difference in *PfK13* C580Y inferred fitness cost between strains with varying genetic background (Straimer et al. 2017; Stokes et al. 2021).

2.5.8. Assessing artemisinin resistance

Antimalarial drug resistance can be assessed by therapeutic efficacy trials, monitoring post-treatment selective pressure on successive infections, *ex vivo*/*in vitro* drug susceptibility tests, and by the detection of molecular markers of resistance. In standard therapeutic efficacy trials for ACT, patients are generally followed up during a 28 or 42-day period by checking blood smears or by PCR. In case of a positive result during this period, recrudescence should be distinguished from reinfection, which can be done by comparing highly polymorphic genes (*e.g.*, merozoite surface proteins 1 and 2). The partner drug efficacy in ACT efficacy trials can mask decreased efficacy of the artemisinin component. More detailed resistance data is provided by clinical efficacy trials employing artemisinin monotherapies and parasitemia assessment every 4-6 hours. The *in vivo* parasite clearance half-life that can be deducted from this approach is considered the best measure of *in vivo* artemisinin resistance. A parasite clearance half-life above 5 hours after artemisinin monotherapy is considered to reflect artemisinin resistance (WHO 2020). First cases of such delayed parasite clearance in treated patients were reported in 2007 (Noedl et al. 2008; Dondorp et al. 2009). *In vivo* therapeutic efficacy studies are costly and logistically demanding, inconvenient for the participants, and confounded by host factors such as antimalarial immunity.

Testing *Plasmodium* isolates for their artemisinin susceptibility without the interference of possible host factors is done *ex vivo*/*in vitro*. *Ex vivo* assays use parasites directly cultured from the patient, possibly including multiple clones. *In vitro* assays use culture-adapted parasites that have possibly undergone a selection process due to culture adaptation or cryopreservation. The conventional method for *ex vivo*/*in vitro* drug susceptibility testing is the assessment of the 50% inhibitory concentration (IC50). However, due to the short *in vivo* half time of the artemisinins, and its stage-specific action in the parasite, the parasite clearance half-life does not associate well with *ex vivo*/*in vitro* -obtained IC50 values. Therefore, the ring-stage survival assay (RSA) is considered the gold standard for artemisinin susceptibility testing. Parasites are tightly synchronized, and 0-3 hours post invasion rings are exposed to 700 nM dihydroartemisinin for 6 hours. The read-out is done 72 hours post drug exposure, and a survival rate above 1% is considered to reflect artemisinin resistance. The *PfK13* R539T mutation, known as the marker conferring highest levels of artemisinin resistance, has survival rates of 19-49% (Straimer et al. 2015). The *PfK13* C580Y mutation, dominant in South-East Asia, has *in vitro* survival rates of 4-24% (Straimer et al. 2015). *Ex vivo/In vitro* RSA results highly associate with *in vivo* parasite half-life (Witkowski et al. 2013).

Because of strong association, and in some cases proven causal relationship, between *PfK13* mutations and artemisinin resistance, the detection of such mutations can be used for resistance surveillance. This requires DNA extraction and purification of patient blood samples, amplification of the respective region in the *PfK13* gene, and sequencing. Known markers of resistance can be detected this way, in addition to further mutations in the same genetic region. Detection of single nucleotide polymorphisms could theoretically be made easier, *e.g.*, by avoiding DNA extraction and working on whole blood instead, or by circumventing the sequencing step by making use of specific DNA probes that will emit a signal with or without the presence of a certain mutation. Reporting of *PfK13* variations (by PCR and sequencing) since sample collection takes about three years on average (Kagoro et al. 2022).

Monitoring the selection of (suspected) markers of resistance in parasite populations can indicate the relative importance of these markers on drug susceptibility and fitness costs. It encompasses the follow up of resistance markers in parasites before treatment and after treatment in a patient cohort. This gives an insight in selection of tolerant parasites after drug pressure *in vivo*. Such studies revealed a clear opposing selective pressure on *PfMDR1* alleles by AL and AS-AQ, and AL and DHA-PPQ (Sisowath et al. 2005; Humphreys et al. 2007; Taylor et al. 2017). Similarly, *PfMDR1* genetic makeup in patient isolates differs between regions with different ACT policies in place (Taylor et al. 2017; Okell et al. 2018).

2.5.9. Status of artemisinin resistance and ACT treatment failure

By retrospective analysis, molecular markers of artemisinin resistance have been present since 2001 in South-East Asia. The first evidence of *in vivo* artemisinin resistance was observed in 2007 (Noedl et al. 2008; Dondorp et al. 2009). In 2014, the crucial gene in artemisinin resistance was identified: *PfK13*. Molecular markers that cause artemisinin resistance were pinpointed, and this list of validated and candidate markers is still growing. In the Greater Mekong subregion, ACT treatment of uncomplicated *falciparum* malaria failure now reaches levels of up to 50% (Phyo et al. 2016; van der Pluijm et al. 2019; Imwong et al. 2020). Artemisinin resistance was already present longer, which has left the partner drug unprotected and facilitated the development of treatment failures.

Genetic analyses demonstrated the independent emergence of artemisinin resistant *P. falciparum* strains across South-East Asia, notably with different *PfK13* mutations (Ashley et al. 2014; Takala-Harrison et al. 2015). In contrast to the spread of chloroquine resistant and sulfadoxine-pyrimethamine resistant *P. falciparum* resulting from only a few dominant alleles

that swept across the globe, *PfK13*-linked artemisinin resistance follows another trajectory. Across South-East Asia, *PfK13* mutations first increased in both abundancy and variation for about a decade. Then *PfK13* C580Y started to dominate in the parasite population and reaches now fixation in some regions (Figure 5). It is not clear why the C580Y allele in particular is so successful, as it does not confer stronger resistance than other alleles (Straimer et al. 2015). On the other hand, *PfK13* C580Y conferred fitness cost may be limited to local *P. falciparum* strains (Nair et al. 2018). *PfK13* C580Y *P. falciparum* isolates of local origin were found in Guyana, South America, in 2015 (Mathieu et al. 2020). However, the allele does not seem to increase in prevalence there.

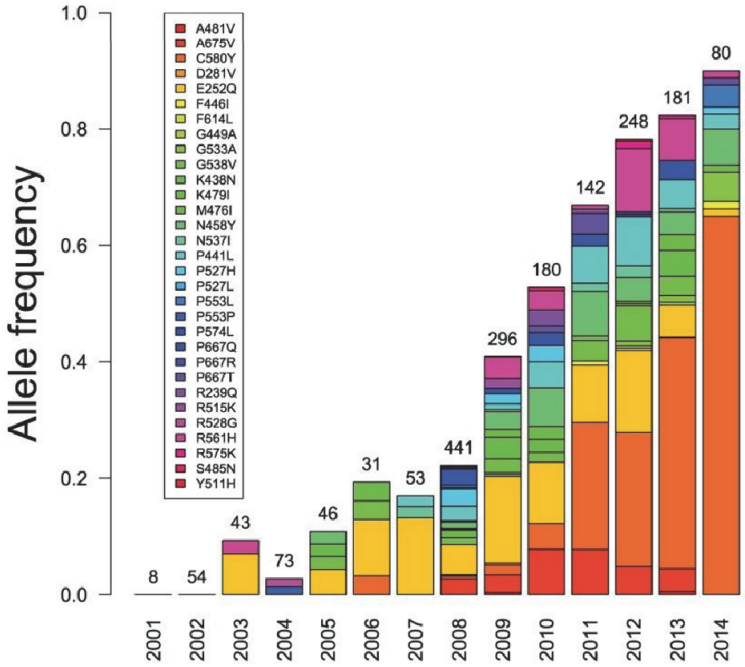

Figure 5. Frequency of different *P. falciparum Kelch-13* mutations on the Thai-Myanmar border.

Plasmodium falciparum Kelch-13 *allele frequencies from different studies combined, different alleles are indicated by colors and sample size is given on top of the bars. Adapted from Anderson et al.* Mol Biol Evol. *2017:34*

P. falciparum Kelch-13 monitoring over the last years showed heterogeneity of mutations on the African continent, but candidate or validated markers for artemisinin resistance were mostly absent (Ménard et al., 2016; Kayiba et al., 2021). Artemisinin combination therapy treatment failure has sporadically been reported in Africa (Dimbu et al. 2021; Gansané et al. 2021), but no *PfK13*-mediated artemisinin resistance could be identified until now. In Rwanda, the validated marker of artemisinin resistance *PfK13* R561H is detected at >10% in isolates from 2018/2019, and this mutation associated with delayed parasite clearance in AL treated children (Bergmann et al. 2021; Uwimana et al. 2021; Straimer et al. 2022). In

neighbouring Uganda, the candidate marker *PfK13* A675V (qualifying for validated marker by now), was also detected at >10% and associated with increased parasite half-life in artesunate treated patients and with DHA susceptibility *ex vivo*. These data are the first indications that artemisinin resistance has emerged in Africa, which might have disastrous consequences. As observed in South-East Asia, partner drug resistance may follow leading to ACT treatment failure. Predictions suggest that a 70% ACT failure rate will lead to 116,000 excess deaths annually, almost exclusively in Africa (Lubell et al. 2014).

2.6. Malaria in Mangaluru, southwest India

India contributed to 1.7% of the global malaria cases in 2020, and to approximately half of the vivax cases (WHO 2019b, 2021b). Like almost all countries in the WHO South-East Asia Region, India met the 40% reduction target in case incidence and mortality rate by 2020 compared to 2015. India's vast economic and social transformation of the last decades is paralleled by unique changes in its malaria epidemiology, such as the increase in urban malaria in some regions (NVBDCP 2016). Urban malaria in India is predominantly caused by *P. vivax* and is subject to outbreaks. It is attributed to construction sites, unplanned urbanization and migrant workers importing malaria from endemic regions of India, while settling in poor housing conditions. Since 2005, India uses ACT as first-line treatment for uncomplicated falciparum malaria (artesunate + SP + primaquine, and AL in northeastern states). Vivax malaria is treated with chloroquine + primaquine (Anvikar et al. 2016; WHO 2021b).

Sample collection for our study in India was done in Mangaluru (also Mangalore), in 2015. Mangaluru is located in the state of Karnataka, southwest India. Mangaluru is a rapidly expanding harbour and business city, with about half a million inhabitants and it experiences a large influx of work-related migrants. It is situated next to the Arabian Sea, has a hot and humid climate, with one monsoon season between May and October. Mangaluru is endemic for malaria and transmission happens year-round and peaks during the monsoon season. From 2015–2017, Mangaluru accounted for approximately half of all malaria cases in the state of Karnataka (Directorate of Health and Family Welfare Services Gov of India 2017). The city's malaria incidence decreased drastically over the last decade, bolstered by the National Vector Borne Disease Control Programme (NVBDCP) and its Urban Malaria Scheme. In 2015, 12,641 cases were recorded (Baliga et al. 2021). Most cases occur in Mangaluru's urban setting rather than rural areas, *P. vivax* dominates (70%), and most patients are young men of low-socio-economic status (*e.g.*, migrant workers) (Gai et al. 2018).

2.7. Malaria in Rwanda

Rwanda is a small, land-locked country in the East African Great Lakes region and it is one of the most densely populated countries of the continent. Malaria in Rwanda has been a major public health problem for long. Transmission occurs throughout the year, with peaks during the bi-annual rainy seasons in April-May and November-December. The dominant malaria species is *Plasmodium falciparum*, but *P. malariae*, *P. ovale*, and *P. vivax* are observed too (Bergmann et al. 2021).

In 2000, the malaria incidence was 25,000/100,000 in Rwanda and mortality was 80/100,000. Since then, malaria mortality has steadily been decreasing to a stable level of approximately 25/100,000 in 2020, whereas the 2020 incidence is back at a similar level as in 2000, with large fluctuations over the course of the past two decades (Figure 6) (WHO 2021b). The national first-line anti-malarial treatment policy changed in 2001 from chloroquine to amodiaquine and SP and to the ACT AL in 2006. In that year, a new malaria strategic plan led to large investments and scale-up of additional control measures including the distribution of long-lasting insecticide treated nets, and indoor residual spraying (Karema et al. 2020). Since 2008, a national integrated Community Case Management policy was implemented, including malaria testing and treatment of both children and adults at the community level by community health workers (Mugeni et al. 2014). Malaria incidence did indeed decrease, but dramatically peaked again in 2017 to about 72,500/100,000 (Figure 6) (WHO 2021b). Factors hypothesized to have contributed to this development include climate change, delay of ITNs supply and delivery, insecticide resistance, short-term durability of ITNs, substandard ITNs, and change in vector behaviour (Karema et al. 2020). A new strategic plan focussed on expansion of testing and treatment of adults at the community level by community health workers, expansion or IRS, and sensitization campaigns (Karema et al. 2020). Between 2017 and 2020, cases dropped by 65%, which appears not to be due to altered testing (WHO 2021b). Treatment trials conducted on children in Rwanda demonstrated good AL and DHA-PPQ treatment efficacy (>98%) after 4 weeks in 2013-2015 and 93-97% for AL in 2018 (Uwimana et al. 2019, 2021).

Sample collection for our studies in Rwanda has been done in the Huye district, located on the central plateau at 1,700 m average altitude. The region is characterized by densely populated, cultivated hills. The Huye district is at risk for malaria epidemics due to its neighbouring endemic districts, and a migration influx from the high-burden country Burundi. In 2019, 31,457 malaria cases were reported by community health workers and health facilities in the Huye district (numbers from regional malaria records Kabutare District

Hospital, Huye, Rwanda). In 2011, 2014, and in 2016, *Plasmodium spp* prevalence was 17% (PCR), 22% (PCR), and 12% (microscopy), respectively, among randomly selected children in the Huye district (Gahutu et al. 2011; Sifft et al. 2016; Nyirakanani et al. 2018).

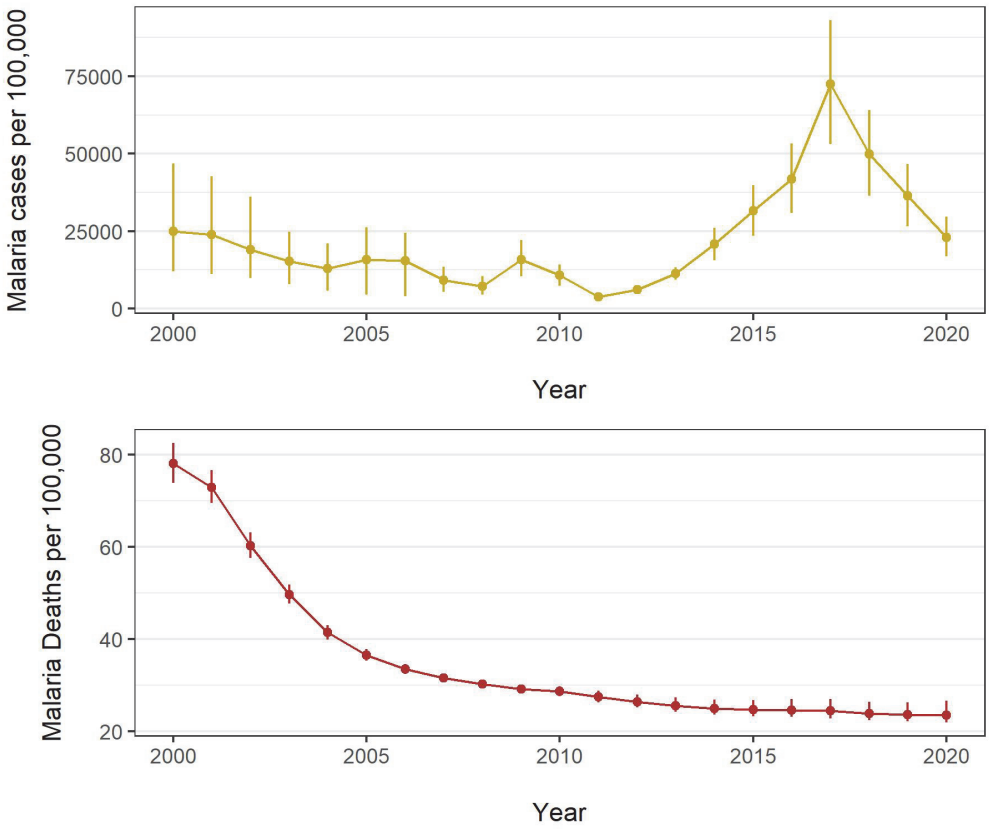

Figure 6. Malaria burden in Rwanda between 2000-2020.

Figure generated from estimated malaria incidence and mortality from World Malaria Report 2021, WHO 2021.

3. OBJECTIVES AND AIMS

3.1. Host genetic variation affecting malaria in different populations

Host genetics have a major impact on susceptibility to and manifestation of malaria. Deciphering the host genetic factors that protect against infection or disease can be used as a tool to understand *Plasmodium* biology, antimalarial immunity and pathophysiology, which in turn offers insights for vaccine development, therapeutic targets, and biomarkers. Most of this work has been done with respect to *P. falciparum*, the *Plasmodium spp.* associated with highest malaria mortality. However, *P. vivax* is a considerable public health threat too and its burden is likely underestimated. It is geographically more widespread, and severe malaria caused by *P. vivax* is reported. We investigated the role of several human genetic variants in malaria, in two vastly different settings, including a *P. vivax* endemic region.

Specific objectives:

a. To examine the association of a common variant of microRNA-146a, a major regulator of innate immune pathways, with *Plasmodium spp.* infection, parasite density, and the malaria outcomes fever, anaemia, low birth weight and preterm delivery in pregnant and delivering women in Ghana, where *P. falciparum* prevails.
b. To examine the association of the same microRNA-146a variant with *Plasmodium spp.* infection, parasite density, and the malaria outcomes fever, anaemia, thrombocytopenia, as well as increased creatinine and bilirubin concentrations in an Indian population, where *P. falciparum* and *P. vivax* co-exist.
c. To examine the association of common Duffy antigen alleles with *Plasmodium spp.* infection, parasite density, and the malaria outcomes fever, anaemia, thrombocytopenia, as well as increased creatinine and bilirubin levels in an Indian population, where *P. falciparum* and *P. vivax* co-exist.

3.2. Genetic markers of antimalarial drug resistance in Rwanda and their *in vitro* phenotype

One of the most important tools for malaria control are antimalarial drugs. However, resistance to all antimalarials in use has emerged. The artemisinins constitute the last generation of highly effective antimalarial drugs, but resistance is now prevalent in South-East Asia. Although artemisinin-based treatment remains highly effective in Africa,

artemisinin resistance leaves the partner drug in artemisinin combination therapy vulnerable for resistance development. Such has been observed in the Greater Mekong subregion, where artemisinin and partner drug resistance now cause treatment failure rates of up to 50%. A similar scenario would have devastating consequences for the African continent, which carries the main malaria burden. We investigated the prevalence of antimalarial drug resistance markers in Rwanda 2018/2019, compared them with previous data from the same region, and phenotypically characterized some suspicious *P. falciparum* isolates. The sample collection for these studies was done when data on artemisinin resistance in Africa was not available yet. Our reports on artemisinin resistant malaria in Rwanda belong to the seminal papers in this field.

d. To investigate the prevalence and trends in *PfK13* variants associated with sensitivity to artemisinin in *P. falciparum* isolates from Rwanda 2010 to 2019.
e. To characterize the *in vitro* artemisinin susceptibility of *PfK13*-mutant *P. falciparum* isolates from Rwanda.
f. To investigate the prevalence and trends in *PfMDR1* variants associated with sensitivity to various antimalarials in *P. falciparum* isolates from Rwanda 2010 to 2019.

4. ORIGINAL ARTICLES

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

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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)

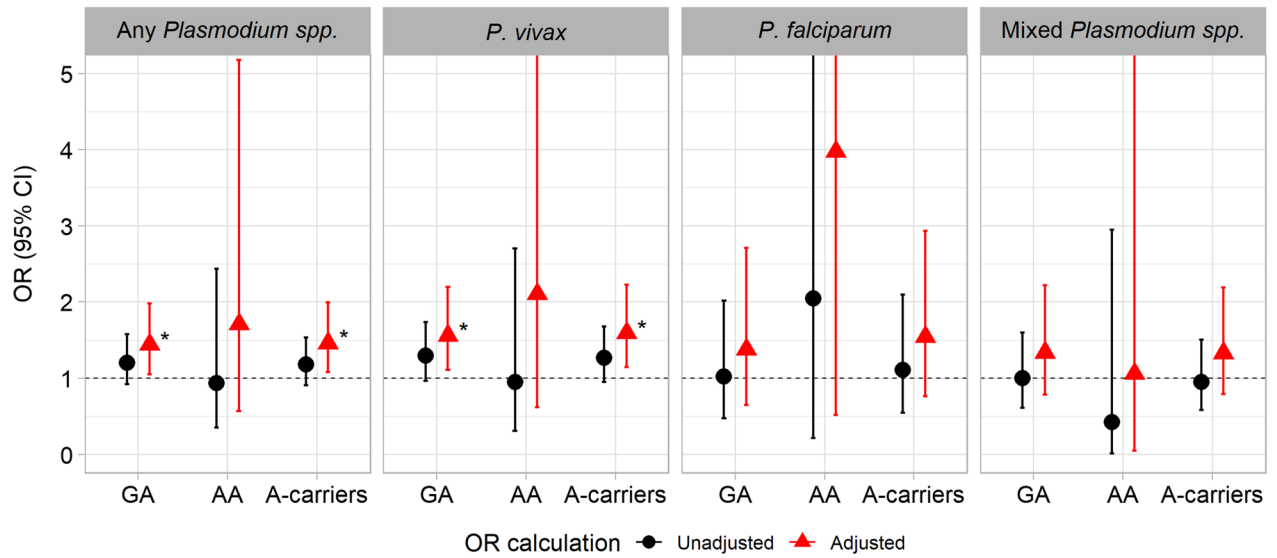

Fig. 1 *DARC* 298A carriage is associated with increased odds of malaria and vivax malaria. OR, odds ratio; 95% CI, 95% confidence interval; unadjusted, crude odds ratio; adjusted, odds ratio adjusted for age, sex, and migration status; *P < 0.05 as compared to *DARC* G298 wildtype individuals. Forest 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
Not applicable.

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

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

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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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4.3. *MiRNA-146a* polymorphism was not associated with malaria in Southern India

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MiRNA-146a Polymorphism Was Not Associated with Malaria in Southern India

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Abstract. Micro-RNAs (miRNAs) play a crucial role in immune regulation, and a common miRNA-146a polymorphism (rs2910164) increased the odds of falciparum malaria in pregnant African women. Here, we examined whether this association holds true in a different population, that is, 449 mainly male and adult malaria patients and 666 community controls in southwestern India. *Plasmodium vivax* malaria (67%) predominated over falciparum malaria (11%) and mixed species infections (22%). Overall, 59% of the study participants carried the miRNA-146a polymorphism. However, it was not associated with the odds of malaria, irrespective of parasite species. This underlines the importance of considering the complexities of clinical manifestations of malaria, genetic background, and parasite species when disentangling the role of human genetic variation, including those of miRNAs in malaria.

Understanding the immune components that predispose individuals to susceptibility to and severe manifestation of malaria is a key component of controlling and eliminating this complex disease. Micro-RNAs (miRNAs) are small, noncoding RNA molecules in a hairpin structure, and they are involved in gene regulation on a posttranslational level.[1] There is increasing interest in miRNAs because of their regulatory role at various steps in the innate and adaptive immune network, which could open up novel clinical applications. Micro-RNA-146a is an important regulator in pattern recognition receptor signaling, such as the Toll-like receptor machinery.[1,2] Micro-RNA-146a suppresses NF-kB activation by a negative regulation loop, thereby altering several inflammatory cytokines.[3,4] It also participates in hematopoietic cell regulation,[5] which might be relevant in malaria, considering its strong interference with blood cell homeostasis.

A common single-nucleotide polymorphism (SNP) in miRNA-146a, rs2910164 G>C, has been associated with modified risks for various types of cancer,[6] autoimmune disorders,[7] and infectious diseases.[8–10] The presence of the polymorphism results in reduced amounts of mature miRNA-146a in vitro.[11] Also, the SNP gives rise to a miRNA-146a molecule that is thought to target another set of genes than wild-type miRNA-146a, and, therefore, SNP heterozygosity might have a very different functional effect compared with either of the homozygous genotypes.[12]

Previously, we found that this SNP greatly increased the odds of malaria in pregnancy, particularly in primigravidae.[8] Malaria in pregnancy has a distinct pathophysiology and immunity, which differs from those of nonpregnant hosts.[13] Also, miRNAs have been shown to play a specific role at the maternal–fetal interface,[14,15] that is, the predilection site of malaria in pregnancy. Here, we examined whether miRNA-146a rs2910164 G>C also affects susceptibility to and/or manifestation of malaria in nonpregnant hosts including those with *Plasmodium vivax* infection.

The present study was conducted from June to December 2015 in Mangaluru, Karnataka, coastal southwestern India. Mangaluru is a harbor city of some 500,000 inhabitants exhibiting the peculiarity of urban malaria in the otherwise less affected state of Karnataka. Wenlock Hospital is the largest governmental health facility in Mangaluru particularly serving the socioeconomically deprived portion of the population of the Mangaluru conglomeration. Patients presenting at the outpatient department (OPD) of the Wenlock Hospital with malaria-like symptoms were referred to the malaria diagnostic unit for diagnosis using Giemsa-stained thick blood films. Patients with confirmed malaria diagnosis were consecutively recruited during the operating hours (08:00–16:00) of the OPD. Patients presenting beyond that time were not regarded. Detailed recruitment procedures, clinical and laboratory examinations, and patient characteristics have been published elsewhere.[16] Afebrile, unmatched community controls were randomly recruited across Mangaluru City Corporation in parallel, and they provided a finger-prick blood sample.[17] Informed written consent was obtained from all adult participants and from parents or legal guardians of minors. 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. In brief, a medical history was obtained, and a clinical examination was performed for all patients. Parameters assessed or calculated included weight, height, body mass index, axillary temperature, fever (> 37.5°C), hemoglobin, white blood cell and thrombocyte counts, geometric mean parasite density, parasite species (polymerase chain reaction [PCR]-confirmed), and concentrations of creatinine and bilirubin.[16] Severe malaria was defined based on the current WHO definition with some modifications,[16] and severe thrombocytopenia as < 50,000/mcL.[18] DNA was extracted (Qiamp blood mini kit, Qiagen, Hilden, Germany), and miRNA-146a rs2910164 was genotyped by melting curve analysis using commercially available primers and probes (TIB Molbiol, Berlin, Germany). Analysis was limited to patients and controls originating from Karnataka avoiding potential geographic impact on the genetic association study. Data analysis was performed using R version 3.4.3. Genotype frequencies in patients and controls were

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compared by a two-tailed Fisher's exact test, and odds ratios, 95% CIs and *P*-values were produced. Clinical parameters of patients were compared between genotypes using a two-tailed Fisher's exact test, Welch's *t*-test, Wilcoxon signed-rank test, or Kruskal–Wallis test as applicable. Adjusted odds ratios (aORs) for malaria, clinical parameters, and self-reported symptoms were derived from a logistic regression model including age and gender. In addition, all analyses were stratified by *Plasmodium* spp., that is, *P. vivax* mono-infection, *Plasmodium falciparum* mono-infection, and mixed spp. infection. A P-value < 0.05 was considered statistically significant.

The mean age (SD) of the 449 malaria patients was 33.7 (13.0) years, and it was 35.0 (15.2) years in 666 community controls. Among the patients, only 11.8% (53/449) were female, but 47.9% (319/666) among the controls ($P < 0.001$). *Plasmodium vivax* malaria was present in 67.5% (303/449) of patients, *P. falciparum* malaria in 10.5% (47/449), and 22.0% (99/449) had a mixed *P. vivax–P. falciparum* infection. Overall, 58.9% (657/1115) of the study participants were carriers of the miRNA-146a SNP (46.8% heterozygous and 12.1% homozygous). Of note, the SNP distribution did not differ between patients and controls, irrespective of stratification by parasite species (Tables 1 and 2). Geometric mean parasite density (95% CI) in individuals with a wild-type genotype, and heterozygous and homozygous polymorphism was 3,422 (2,734–4,283), 4,057 (3,310–4,971), and 3,256 (2,092–5,068), respectively ($P = 0.4$). Likewise, absolute thrombocyte counts were similar in malaria patients with three genotypes (median [range]: 110,000 [6,000–273,000], 103,000 [6,000–658,000], and 113,000 [10,000, 299,000]; $P = 0.6$), but severe thrombocytopenia was least common in wild-type patients (9.6%, 17/177) and more common in heterozygous (19.1%, 36/188) and homozygous patients (21.2%, 11/52, $P = 0.01$). In multivariate analysis, severe thrombocytopenia was increased in heterozygous (aOR [95% CI], 2.3 [1.2–4.3]; $P = 0.01$) and homozygous patients (aOR [95% CI], 2.4 [1.0–5.5]; $P = 0.04$). After stratification by parasite species, this risk increase became nonsignificant. Severe malaria was present in 3.4% (15/443) of all patients. It tended to occur more frequently in heterozygous (3.5%, 7/202) and homozygous patients (7.3%, 4/55) than those with wild-type alleles (2.2%, 4/186; $P = 0.2$). No further differences with respect to clinical parameters and self-reported symptoms were observed (data not shown).

We have previously shown that carriage of the miRNA-146a rs2910164 SNP is present in 67% of a study population of pregnant women in Ghana. It is associated with 1.6-fold increased odds of malaria and with almost 6-fold increased odds in homozygous primigravidae and primiparae.[8] In the present Indian study population, the prevalence was 59%, but the SNP was not associated with malaria. Several reasons may explain these discrepant findings. First, all pregnant African women in the previous study had *P. falciparum* infection, whereas *P. vivax* predominated in the Indian patients who largely were adult males. The observed absence of an effect of the SNP may consequently stem from the scarcity of *P. falciparum* among the Indian patients. Considering the sample size, the present study was powered to detect increased odds of falciparum malaria, at a level of almost double the value previously observed among pregnant African women.[8] Moreover, acquired immune mechanisms in the Indian adult patients may have blurred an effect of an innate immune mechanism provided by miRNA-146a variation. Malaria in pregnancy is caused by specific placenta-adhering strains of *P. falciparum*, which induce strain-specific immune mechanisms. These are absent or low in first pregnancies.[13] Potential concealment of a SNP effect is consequently less likely, particularly in first-time pregnant women who can be considered relatively immune-naive. In addition, the malaria-related effect of miRNA-146a might be pronounced in malaria in pregnancy: placental tissue exhibits a specific miRNA expression pattern, and placental miRNAs take part in gene regulation and inflammation control at the fetal–maternal interface.[14,15] Micro-RNA-146a has been hypothesized to be involved in the reaction on cell stress in the placenta.[19] In pregnancy, *P. falciparum* adheres to the placental syncytiotrophoblast, thereby evading splenic elimination.[13] This epithelium lining the intravillous space is known to produce extracellular vesicles containing miRNAs.[15] This peculiarity may partially explain the discrepant finding of the miRNA-146a SNP affecting malaria risk in pregnant women but not in predominantly adult Indian males. Furthermore, considering both, genetic diversity between Africans and Indians and the complex interaction of miRNA-146a in immune regulation,[1,3,4] it is possible that the SNP yields different effects per se in these two populations. Last, human miRNA transcripts have been shown to impair *Plasmodium* function via gene regulation[20] even though the role of the miRNA-146a SNP in this context is unknown. The scarcity of data on the actual function of this polymorphism also impedes the interpretation of the weak associations of the polymorphism with thrombocytopenia (nonsignificant following Bonferroni correction for 16 comparisons) and even more so with respect to severe malaria. Conflicting findings on thrombocyte counts in miRNA-146a knockdown and knockout mice have been reported.[5] Assuming that miRNA-146a negatively regulates inflammatory processes, a SNP-dependent alteration might result in more intense pro-inflammatory response to infection. This in turn may increase the probability of thrombocytopenia and severe malaria. However, this interpretation should be considered with care because we did not assess inflammatory mediators in the present study. Definitely, this as well as the

TABLE 1

Distribution of miRNA-146a rs2910164 in malaria patients and controls from Karnataka, India

MiRNA-146a single nucleotide polymorphism rs2910164	Total ($N = 1115$)	Controls ($N = 666$)	Patients ($N = 449$)	Odds ratio (95% CI)	*P*-value*	Adjusted odds ratio (95% CI)†	*P*-value†
Wild type	458 (41.1%)	271 (40.7%)	187 (41.6%)	1	–	1	–
Heterozygote	522 (46.8%)	316 (47.4%)	206 (45.9%)	0.9 (0.7–1.2)	0.7	0.9 (0.7–1.2)	0.5
Homozygote	135 (12.1%)	79 (11.9%)	56 (12.5%)	1.0 (0.7–1.5)	0.9	1.0 (0.7–1.5)	0.9

Micro-RNA = miRNA.

* By two-tailed Fisher's exact test.

† By logistic regression model including gender and age in years.

TABLE 2

Distribution of miRNA-146a rs2910164 in malaria patients and controls from Karnataka, India, stratified by parasite species

MiRNA-146a single nucleotide polymorphism rs2910164	*Plasmodium vivax* patients (*N* = 303)	*Plasmodium falciparum* patients (*N* = 47)	Mixed spp patients (*N* = 99)
Wild type	129 (42.6%)	21 (44.7%)	37 (37.4%)
Heterozygote	140 (46.2%)	19 (40.4%)	47 (47.5%)
Homozygote	34 (11.2%)	7 (14.9%)	15 (15.2%)

Micro-RNA = miRNA.

absence of an effect on malaria risk as seen here needs to be confirmed in larger study samples and different populations, and functional studies are needed to decipher the underlying mechanisms. Still, the present work emphasizes the role of various factors, which might influence the results of genetic association studies with respect to malaria.

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4.4. Increase in *Kelch 13* Polymorphisms in *Plasmodium falciparum*, Southern Rwanda.

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DISPATCHES

Increase in Kelch 13 Polymorphisms in *Plasmodium falciparum*, Southern Rwanda

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Artemisinin resistance in *Plasmodium falciparum* is associated with nonsynonymous mutations in the *Kelch 13* (*K13*) propeller domain. We found that 12.1% (8/66) of clinical *P. falciparum* isolates from Huye district, Rwanda, exhibited *K13* mutations, including R561H, a validated resistance marker. *K13* mutations appear to be increasing in this region.

Emerging artemisinin resistance to *Plasmodium falciparum* endangers malaria control worldwide. Currently, the resistance epicenter is the greater Mekong subregion in Southeast Asia (*1*). In sub-Saharan Africa, where illnesses and deaths from *P. falciparum* malaria are highest, such resistance may result in disastrous consequences (*2*). Early detection and close monitoring are therefore crucial.

Artemisinin resistance in *P. falciparum* is associated with *pfkelch13* polymorphisms encoding the parasite's *Kelch 13* (*K13*) propeller domain, which consequently serve as a molecular marker in surveillance (*3*). More than 200 nonsynonymous *K13* single-nucleotide polymorphisms have been reported, including 11 candidate resistance mutations (i.e., associated with delayed parasite clearance) and 9 validated mutations (i.e., also reduced in vitro sensitivity) (*4*). Compared with those from Asia, isolates from sub-Saharan Africa show pronounced heterogeneity of nonsynonymous *K13* polymorphisms, most of them rare, possibly reflecting lower drug pressure (*5*).

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Rwanda achieved substantial reductions in malaria during 2006–2011, partly due to home-based management using artemether/lumefantrine (*6*). In 2010, at our study site in Huye district, southern Rwanda, we observed a pattern in the *P. falciparum* multidrug resistance: 1 gene suggestive of intense artemether/lumefantrine drug pressure, whereas *K13* mutations were absent. However, among *P. falciparum* isolates, 2.5% in 2014 and 4.5% in 2015 harbored *K13* variants, including 2 candidate mutations (*7*,*8*). A recent report showed the presence of a validated *pfkelch13* mutation, R561H, at 2 sites in Rwanda (*9*). We conducted a cross-sectional molecular surveillance study to update records of the prevalence of *K13* variants in Huye among isolates collected in 2019.

The Study

During September–December 2019, we recruited study patients with uncomplicated malaria seeking treatment at the Sovu Health Centre and Kabutare District Hospital, Huye district, Rwanda. Huye district (population ≈390,000) is located on the central plateau of Rwanda (average altitude 1,700 m, yearly rainfall 1,200 mm, mean temperature 19°C). Malaria transmission peaks in October–November and March–May. In 2010, a total of 11.7% of children had microscopically confirmed *Plasmodium* infection (*8*).

We obtained written informed consent from all participants or from the caregivers for children; we also obtained written assent from participants 7–18 years of age. The study was approved by the Rwanda National Ethics Committee. Eligibility criteria for participants included age >1 year; a positive result on a rapid diagnostic test, SD Bioline Malaria Ag Pf/Pan (Abbott Global Point of Care, https://www.globalpointofcare.abbott); and a fever (axillary temperature ≥37.5°C) at the time they sought treatment or within 48 hours beforehand (self-reported). We collected whole blood in S-Monovette EDTA (ethylenediaminetetraacetic

acid; Sarstedt, https://www.sarstedt.com) tubes and confirmed malaria diagnosis by microscopy of Giemsa-stained thick blood smears; patients were also seen by a physician. We provided a 3-day regimen of artemether/lumefantrine for treatment, the first dose given under observation. All patients were asked to return after 3 days to evaluate residual parasitemia on Giemsa-stained thick blood smears.

Definite parasite density was counted per 200 leukocytes on Giemsa-stained thick blood smears by 2 independent microscopists, assuming a mean leukocyte count of 8,000/μL. We extracted DNA using a QIAamp DNA Blood Mini kit (QIAGEN, https://www.qiagen.com). *Plasmodium* species were typed by real-time PCRs with commercially available primers and probes for *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi* (TIB MolBiol, https://www.tib-molbiol.com) on a Roche LightCycler 480 device (https://lifescience.roche.com). *K13* was amplified (codons ≥441≤688) by using nested PCR (*3*) and sequenced by a commercial provider (Eurofins Genomics, https://www.eurofinsgenomics.com). Sequences were aligned to reference *K13* 3D7–1343700 (PlasmoDB, https://plasmodb.org) by using Geneious Prime version 2020.1 (https://www.geneious.com). We used R version 3.6.3 (https://cran.r-project.org) for statistical analysis and a binomial logistic regression model to estimate the time-trend of nonsynonymous mutations ($p<0.05$).

Of 90 patients included in the study, 74 tested positive by microscopy and PCR and 2 by PCR only. Among these patients, 51.3% (39/76) were female and 4 were pregnant; the median age was 18 years (range 2–69 years). *P. falciparum* was found in 88.2% (67/76), *P. vivax* in 7.9% (6/76), *P. ovale* in 7.9% (6/76) and *P. malariae* in 1.3% (1/76). The geometric mean parasite density, based on microscopy results, was 8,926 parasites/μL (95% CI 5,911–13,478 parasites/μL); mean temperature was 37.4°C (SD ±1.3°C). After 3 days of treatment, 61 malaria patients had a negative blood smear, 1 patient (1.6%, 1/62) had asymptomatic parasitemia (31,520 parasites/μL), and 14 patients did not return for the day 3 checkup.

None of the patients infected with *K13* variant parasites tested positive after 3 days of treatment. One pregnant patient sought treatment again. Initially, she had *K13* wild-type parasites and was given artemether/lumefantrine; her day 3 microscopy result was negative. Three weeks later, we detected *K13* R561H parasites, possibly due to reinfection, and administered quinine.

Samples from 98% (66/67) of *P. falciparum* isolates were successfully sequenced for the *K13* propeller domain. We found 5 different nonsynonymous polymorphisms in 8 isolates (Table); 3 harbored R561H, a validated resistance mutation, and the 2 candidate polymorphisms C469F and A675V (*4*). This finding suggests that the number of isolates with nonsynonymous *K13* mutations had increased significantly over the previous decade (OR 1.4, 95% CI 1.1–1.8; $p = 0.003$).

Conclusions

Of *P. falciparum* isolates from symptomatic patients in southern Rwanda, 12% exhibited nonsynonymous *K13* mutations, a significant increase (OR 1.4, 95% CI 1.1–1.8; $p = 0.003$) over the previous decade compared with their absence in 2010 and 4.5% prevalence in 2015 (*7*). Of note, the validated marker R561H alone occurred in 4.5% of the isolates collected in 2019. Recent studies report 1%–3.5% of nonsynonymous *K13* polymorphisms in parasite isolates from East Africa (*10*), whereas during 2013–2015 in Rwanda, this figure was 6.9% (*9*).

The R561H artemisinin resistance mutation is regularly observed across Asia (*10*). A recent study that reported R561H in 7.4% of isolates collected during 2013–2015 in central Rwanda and 0.7% of isolates in south-central Rwanda (*9*) suggested that this mutation emerged indigenously and independently from Asia 561H strains. We do not have data in our study to support this. None of the *K13* variant parasites showed delayed clearance in our study, which may be due to the partner drug lumefantrine still being effective, similar to observations in Southeast Asia (*11*). In addition, the absence of delayed parasite clearance despite *K13* mutations may reflect partial immunity contributing to parasite elimination (*12*).

We found other nonsynonymous polymorphisms only once among the isolates tested. C469F and A675V are considered artemisinin resistance candidate

Table. Nonsynonymous single nucleotide polymorphisms in the *Kelch 13* propeller domain of clinical *Plasmodium falciparum* isolates collected in the Huye District, Rwanda, 2010–2019*

Year	No. sequenced isolates	No. (%) isolates with nonsynonymous mutations	Amino acid changes and nucleotide changes
2010	75	0	Not applicable
2014	81	2 (2.5)	V555A, A626S
2015	66	3 (4.5)	P574L,† D648H, A675V†
2019	66	8 (12.1)	C469F,† G533A, V555A, R561H‡ (3×), A578S, A675V†

*Data during 2010–2015 derived from Tacoli et al. (*7*).
†Candidate mutations for artemisinin resistance.
‡Validated mutation for artemisinin resistance (*4*).

mutations (*4*) and have previously been seen in East Africa (*7*,*13*,*14*). G533A and V555A have also been previously reported in Africa but have not yet been evaluated for resistance (*7*,*13*). A578S is a common *K13* polymorphism across Africa but is not linked to artemisinin resistance (*1*).

Our study has clear limitations. Data from only 2 healthcare facilities, with limited catchment areas, were included. Adherence to treatment was assessed by patient self-report, and drug susceptibility testing was not performed. Future research should include ring-stage susceptibility assays to contribute to understanding the role of *K13* mutations in Africa. Separate testing for each drug in a combination for efficacy and continued surveillance for antimicrobial resistance are needed.

Our results show that *K13* mutations are present in Rwanda and that their prevalence in *P. falciparum* malaria patients in the Huye District increased from 0% in 2010 to >12% in 2019. The validated artemisinin resistance mutation R561H occurs in 4.5% of *P. falciparum* isolates being transmitted in this area. The emergence of artemisinin resistance–related mutations in Rwanda is alarming because it might indicate developing resistance against commonly used antimalarials in this region. Countermeasures need to be considered early, potentially including 3-drug antimalarial combinations (*2*).

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About the Author

Ms. Bergmann is a medical student at Charité–Universitaetsmedizin Berlin, interested in infectious disease epidemiology and tropical diseases. This manuscript forms part of her medical doctoral thesis.

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4.5. *In Vitro* Confirmation of Artemisinin Resistance in *Plasmodium falciparum* from Patient Isolates, Southern Rwanda, 2019

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DISPATCHES

In Vitro Confirmation of Artemisinin Resistance in *Plasmodium falciparum* from Patient Isolates, Southern Rwanda, 2019

Welmoed van Loon, Rafael Oliveira, Clara Bergmann, Felix Habarugira, Jules Ndoli, Augustin Sendegeya, Claude Bayingana, Frank P. Mockenhaupt

Artemisinin resistance in *Plasmodium falciparum* is conferred by mutations in the *kelch 13* (*K13*) gene. In Rwanda, *K13* mutations have increased over the past decade, including mutations associated with delayed parasite clearance. We document artemisinin resistance in *P. falciparum* patient isolates from Rwanda carrying *K13* R561H, A675V, and C469F mutations.

Artemisinin-based combination therapies (ACTs) have contributed greatly to the global decline of illness and death from malaria (*1*). However, the novel emergence of artemisinin resistance in eastern Africa has threatened the effectiveness of these breakthrough treatments (*2*–*4*). To avert potential disaster resulting from increased resistant malaria cases, the nature and extent of this resistance in Africa urgently needs to be characterized.

Artemisinin resistance is conferred by some *Plasmodium falciparum kelch 13* (*K13*) gene mutations, only a few of which are validated markers of resistance, defined by both in vitro resistance and delayed parasite clearance in treated patients. For candidate markers, only parasite clearance applies (*1*). In Rwanda, *K13* mutations have increased over the past decade. *K13* R561H, a validated marker associated with delayed parasite clearance, was recently observed in >10% of *P. falciparum*–positive samples (*2*,*3*,*5*). In neighboring Uganda, artemisinin resistance conferred by another mutation, *K13* A675V, has recently been reported (*4*). We document in vitro artemisinin resistance in 3 *P. falciparum* patient isolates from Rwanda carrying *K13* R561H, A675V, and C469F mutations.

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The Study

We recruited malaria patients in Huye District, Rwanda, during September–December 2019 and documented patient characteristics and consent, ethical clearance, and *K13* variants elsewhere (*2*). Within 6 hours of sample collection, we cryopreserved all 66 *P. falciparum* isolates in ethylenediaminetetraacetic acid by washing the red blood cell pellet, adding freezing solution (3% sorbitol, 28% glycerol, 0.65% NaCl), and freezing at −80°C. Eight of the 66 isolates carried nonsynonymous *K13* mutations (*2*). We successfully thawed and culture-adapted 4 of the isolates in which we identified *K13* mutations: R561H, the current prevalent mutation in Rwanda; A675V, found in 11% of *P. falciparum* samples in Uganda; C469F, another candidate marker; and V555A, which is of unknown significance.

We conducted a 0–3-h postinvasion ring-stage susceptibility assay (RSA) with the active metabolite dihydroartemisinin (*6*). We exposed ring stages to a 6-h pulse of 700 nmol/L dihydroartemisinin and cultured exposed and nonexposed isolates in vitro in triplicate for 72 h. We counted parasite density per ≥10,000 red blood cells on Giemsa-stained thin blood films and calculated the means of triplicates. Dividing parasite density in dihydroartemisinin-exposed cultures by the density in nonexposed cultures provided the RSA survival rate. We considered results if 72-h growth rates exceeded 1.5× rates in the nonexposed controls and had ≥3 successful independent triplicate experiments per isolate. We

also assessed 50% inhibitory concentrations (IC_{50}) (*7*). We exposed synchronized ring-stage parasites for 72 h across a range of dihydroartemisinin concentrations (0–1 μmol/L) in duplicate or triplicate and in ≥3 independent experiments. We measured growth by SYBR Green I staining (ThermoFisher, https://www.thermofisher.com) and performed photometric assessment using FilterMax F5 microplate readers (Molecular Devices, https://www.moleculardevices.com). We estimated IC_{50} using a 4-parameter fit dose-response curve. For artemisinin-susceptible parasites, 2 cultured wild-type isolates from patients in Rwanda grew too poorly for RSA and IC_{50} assays and were replaced by artemisinin-susceptible *K13* wild-type strain NF54, which is of putative African origin. We assayed isolates in parallel with NF54 and compared IC_{50} by Student *t*-test. We performed analyses using R version 3.6.3, including the drc (dose-response curve) package (https://cran.r-project.org/web/packages/drc/drc.pdf).

RSAs yielded mean ± SE survival rates of 0.2% ± 0.1% for the NF54 strain and 0.3% ± 0.1% for V555A, well below the World Health Organization–accepted 1% resistance threshold (*1*,*6*). In contrast, 3 other isolates with *K13* mutations had >1% mean survival rates: 4.7% ± 1.5% for R561H (prevalent in Rwanda), 1.4% ± 0.2% for A675V (prevalent in Uganda), and 9.0% ± 1.6% for C469F (Figure 1). Conventional susceptibility testing yielded mean IC_{50} of 4.2 ± 0.5 nmol/L for dihydroartemisinin for the NF54 strain and 3.4 ± 0.3 nmol/L for V555A. IC_{50} levels were higher in isolates with dihydroartemisinin-resistant RSA findings: 14.1 ± 4.0 nmol/L for R561H, 7.4 ± 3.3 nmol/L for A675V, and 6.9 ± 1.5 nmol/L for C469F (Figure 2).

We determined the regional origin of the 4 tested patient isolates by single-nucleotide polymorphism (SNP) barcoding. We typed 23 SNPs to group into haplotypes associated with geographic origin (*7*,*8*). The R561H isolate displayed haplotype 9 and the other isolates haplotype 22 (*8*), confirming African ancestry.

Conclusions

Artemisinin resistance is defined by RSA results and delayed parasite clearance in treated patients. In Africa, abundant *K13* variants circulate, but very few have been defined in terms of drug susceptibility (*1*). The *K13* mutation R561H, which has emerged in Rwanda (*2*,*5*), confers delayed parasite clearance (*3*). We found that a patient isolate with the R561H mutation from Rwanda was in vitro artemisinin resistant. Taken together, these results strongly suggest that R561H is a marker of resistance in Rwanda, a finding that needs to be confirmed in larger sample-size research. The same need for confirmation applies to *K13* candidate resistance markers A675V, recently characterized in Uganda (*4*), and C469F (*1*).

RSA survival rates for *K13* R561H *P. falciparum* in our study concord with levels in multiple gene-edited *P. falciparum* lines (*5*,*10*). Also in line with our findings are high RSA survival rates in A675V isolates from neighboring Uganda, where *K13* A675V was found in 11% and C469Y (but not C469F) in 2% of *P. falciparum* isolates collected during 2017–2019. Both mutations are associated with delayed parasite clearance (*4*). Isolates with increased survival rates also showed higher dihydroartemisinin IC_{50} levels. If this association is confirmed, IC_{50} assays that are much less labor-intensive could be useful for flagging isolates deserving additional testing by RSA.

The small number of isolates we evaluated was an obvious limitation of our study. Ideally, we would have compared the effects of individual mutations in wild-type isolates from Rwanda with study isolates, but the few selected performed poorly in vitro

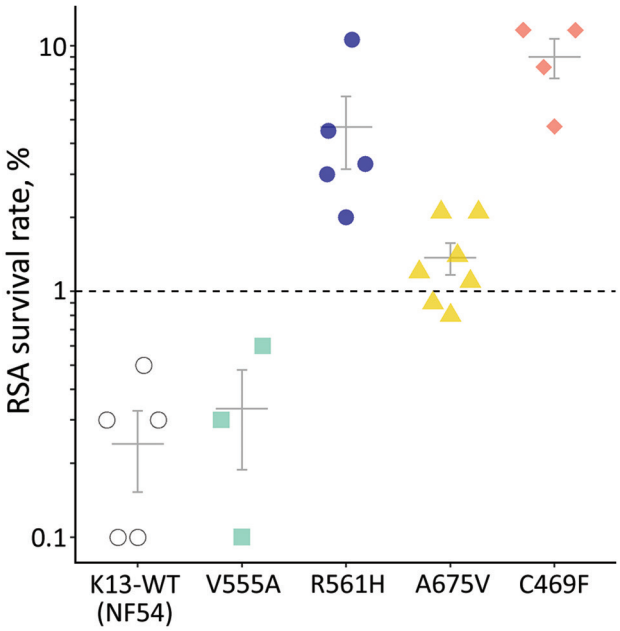

Figure 1. RSA 0–3-hour postinvasion survival rates (%) of an artemisinin-susceptible, *K13* WT *Plasmodium falciparum* strain (NF54) and 4 *P. falciparum* patient isolates from Rwanda with *K13* mutations. Each data point represents the mean of triplicate experiments. Isolate growth rates were only considered for analysis if 72-hour growth rates exceeded 1.5× rates in the nonexposed controls. Indicated error bars display the mean ± SE; dashed line indicates the 1% survival rate threshold used to define artemisinin resistance (*1*,*6*). K13, *kelch 13*; RSA, ring-stage susceptibility assay; WT, wild-type.

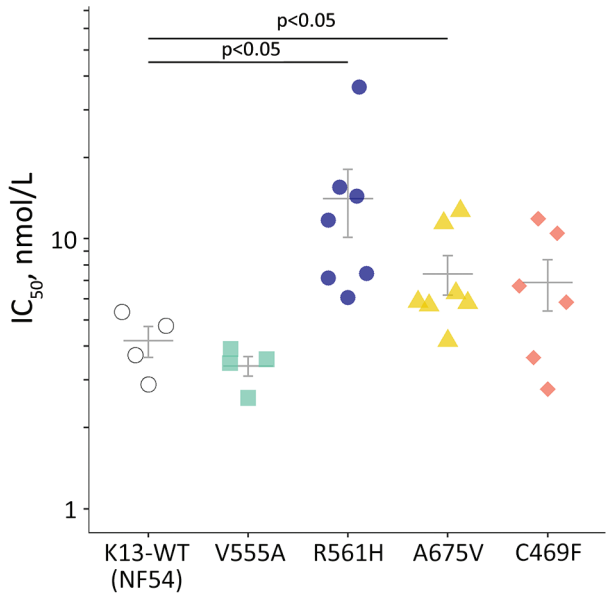

Figure 2. IC_{50} values for dihydroartemisinin for an artemisinin-susceptible, *K13* WT *Plasmodium falciparum* strain (NF54) and in 4 *P. falciparum* patient isolates from Rwanda with *K13* mutations. Indicated error bars display the mean ± SE. p values were determined by Student *t*-test. IC_{50}, 50% inhibitory concentration; K13, *kelch 13*; WT, wild-type.

and were replaced by the artemisinin-sensitive NF54 strain, enabling us to verify that the RSA was working properly. A study strength is the detailed characterization of the susceptibility and ancestry of isolates.

RSA data on suspicious *K13* isolates from Africa are scarce but essential and urgent for the situational evaluation of artemisinin resistance emerging in Africa. *K13* mutations have conferred a wide range of artemisinin susceptibility when introduced in different parasite lines (*10*). Of note, artemisinin resistance identified in Rwanda and Uganda is of indigenous origin, not imported from Asia where resistance has been prevalent for years (*1*). These 2 observations argue for the need for local characterization of artemisinin resistance in circulating parasites.

Artemisinin resistance alone does not necessarily lead to ACT treatment failure, and efficacy in Rwanda still is high (*3*). However, resistance leaves the partner drug unprotected, potentially leading to resistance developing to that component as well. Eventually, this process could result in increased ACT treatment failure, which has already been observed in southeast Asia (*11*,*12*). In Africa, this development might be delayed because of prevalent partial immunity contributing to parasite elimination and high transmission increasing the likelihood of resistance allele outcrossing. Nonetheless, in Rwanda, where artemether/lumefantrine is the first-line antimalarial drug combination, a shift in the *P. falciparum* multidrug resistance 1 (*pfmdr1*) genotype pattern over the past decade suggests an increasingly lumefantrine-tolerant phenotype (*13*,*14*), although *pfmdr1* is not a validated marker for lumefantrine resistance.

Recent research indicates that the R561H mutation is fitness neutral (*10*), implying its wider dissemination even without drug pressure. So far, a viable alternative to ACTs is not in sight. Increasing resistance, combined with the lack of effective alternative antimicrobial drugs, suggests a pessimistic scenario for sub-Saharan Africa, considering the region's high malaria burden. Large-scale monitoring, containment strategies, and early consideration of 3-drug ACTs (*15*) are required to control widespread artemisinin resistance in Africa.

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4.6. Changing Pattern of *Plasmodium falciparum Pfmdr1* Gene Polymorphisms in Southern Rwanda

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EPIDEMIOLOGY AND SURVEILLANCE

Changing Pattern of *Plasmodium falciparum pfmdr1* Gene Polymorphisms in Southern Rwanda

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ABSTRACT *Plasmodium falciparum multidrug resistance-1* gene (*pfmdr1*) polymorphisms associate with altered antimalarial susceptibility. Between 2010 and 2018/2019, we observed that the prevalence of the wild-type allele N86 and the wild-type combination NYD increased 10-fold (4% versus 40%) and more than 2-fold (18% versus 44%), respectively. Haplotypes other than NYD or NFD declined by up to >90%. Our molecular data suggest the *pfmdr1* pattern shifted toward one associated with artemether-lumefantrine resistance.

KEYWORDS multidrug resistance, *Plasmodium falciparum*, malaria, Rwanda

Treatment of *Plasmodium falciparum* malaria relies on artemisinin-based combination therapies (ACTs), comprising a fast-acting artemisinin derivative and a slowly eliminated partner drug. *P. falciparum kelch-13* (*pfkelch13*) single-nucleotide polymorphisms (SNPs) associate with decreased artemisinin susceptibility. When conferring reduced *in vitro* sensitivity and delayed parasite clearance *in vivo*, they are termed validated mutations, common in Southeast Asia (1). Recently, these were detected in East Africa (Rwanda) and associated with delayed parasite clearance (2–4). Although ACT failure remains rare in Sub-Saharan Africa (1), the emergence of non-artemisinin partner drug resistance is feared. Susceptibility to these antimalarials, including lumefantrine (LF) and amodiaquine (AQ), is influenced by the *Plasmodium falciparum multidrug resistance-1* gene (*pfmdr1*) SNPs N86Y, Y184F, and D1246Y (5–9). Individual allele combinations, or haplotypes (e.g., N86-Y184-D1246, NYD, wild-type haplotype), exhibit specific susceptibility phenotypes (9). Notably, *pfmdr1* 86Y associates with increased sensitivity to LF, mefloquine, and dihydro-artemisinin and decreased chloroquine and AQ sensitivity (5). Resistant strains spread under drug pressure but may decline without (10, 11). Rwanda has used AL as a first-line antimalarial since 2006 (12). In 2010, we reported a predominant *pfmdr1* pattern (NFD) suggestive of intense AL pressure in mostly asymptomatic preschool children in Huye, Rwanda (13). Almost a decade later, we reassessed *pfmdr1* alleles in symptomatic and largely adult patients in Huye and compared them to the 2010 findings.

In March–June 2018 and September–December 2019, we recruited 295 uncomplicated malaria patients at Sovu Health Centre and Kabutare District Hospital, Huye district, Rwanda. All reported fever in the preceding 48 h or were febrile (164/276; ≥37.5°C, axillary). The study was approved by the Rwanda National Ethics Committee, and participants or caregivers provided informed written consent. Patients were clinically examined, malaria was microscopically confirmed, and venous blood was collected into EDTA. Following DNA extraction (QIAamp DNA blood minikit; Qiagen, Germany), *Plasmodium* species was confirmed by PCR (14) in 2018 and by real-time PCR (TIB MolBiol, Germany) in 2019. Two *pfmdr1* regions (codons 61 to 236 and 1023 to 1288) were PCR amplified (15), sequenced (Eurofins Genomics,

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TABLE 1 Observed prevalence of *pfmdr1* polymorphisms and allele combinations in Huye, Rwanda, in 2010 and in 2018/2019

pfmdr1 allele or allele combination[a]	2010 (13), *n* = 104 [% (*n*)]	2018 and 2019,[b] *n* = 212 [% (*n*)]
86Y	39.4 (41)	3.8 (8)*
184F	51.9 (54)	53.8 (114)
1246Y	12.5 (13)	2.4 (5)*
N86-Y184-D1246	18.3 (19)	44.3 (94)*
N86-**184F**-D1246	38.5 (40)	50.0 (106)
86Y-Y184-D1246	23.1 (24)	1.9 (4)*
86Y-**184F**-D1246	7.7 (8)	1.4 (3)*
86Y-Y184-**1246Y**	5.8 (6)	0.5 (1)*
N86-**184F**-**1246Y**	2.3 (3)	1.9 (4)*
86Y-**184F**-**1246Y**	2.3 (3)	0
N86-Y184-**1246Y**	1.0 (1)	0

[a]Mutations are presented in boldface.
[b]*, significantly different from the respective proportion in 2010.

Germany), and aligned to reference PF3D7_0523000 (PlasmoDB; https://plasmodb.org/plasmo/app/record/gene/PF3D7_0523000) using CodonCode Aligner 4.2.5. *Plasmodium* infections can contain genetically distinct parasites and have a multiplicity of infection (MOI) of >1. To include all isolates, also those with evidence of MOI of >1 (i.e., both wild-type and mutant alleles present), we grouped alleles into combinations. In a secondary analysis, we estimated haplotype frequencies using a Bayesian model (16), which integrates unknown MOIs. The same priors were used as described previously (17). SNP and allele combination prevalence as well as haplotype frequencies in 2010 and 2018/2019 were compared using Fisher's exact test. A *P* value of <0.05 was considered significant. We used R 3.6.3 for statistical analyses.

By PCR, 90.3% (234/259) of the malaria patients in 2018/2019 had *P. falciparum* infection. Of these, 50.6% (118/233) were female, median age was 17.5 years (range, 1 to 73), and mean temperature was 37.2°C (standard deviation [SD], ±1.3°C). Good-quality sequencing reads for both *pfmdr1* regions were obtained from 90.6% (212/234) of isolates. Evidence of an MOI of >1 was present in 17.9% (38/212) of samples. The observed mutation prevalence was the following: 86Y, 3.8%; 184F, 53.8%; and 1246Y, 2.4% (Table 1). As for observed allele combinations, NFD (50%) dominated over wild-type NYD (44%). Considering haplotype frequency estimates, the reverse was seen (i.e., 39% versus 56%) (Table 2). In any case, >90% of isolates showed NFD or NYD in 2018/2019. Other nonsynonymous polymorphisms were T199S (*n* = 4), V207I (*n* = 2), T222I (*n* = 8), and Q1198K (*n* = 1), but not S1034C or N1042D.

Compared to 2010 data from the same region, the 2018/2019 *pfmdr1* allele pattern has changed: the prevalence of the 86Y mutation declined 10-fold and that of 1246Y 5-fold, whereas 184F remained basically unchanged (Table 1). Consequently, both the observed prevalence and the estimated frequency of wild-type haplotype NYD more than doubled between 2010 and 2018/2019. Allele combinations or haplotypes other

TABLE 2 Estimated haplotype frequency in Huye, Rwanda, in 2010 and in 2018/2019[a]

pfmdr1 haplotype	2010 (13) [% (95% credibility interval)]	2018 and 2019 [% (95% credibility interval)]
N86-Y184-D1246	24.0 (17.0, 32.3)	56.3 (49.6, 62.9)
N86-**184F**-D1246	38.0 (29.6, 47.0)	39.2 (32.8, 45.9)
86Y-Y184-D1246	24.3 (17.4, 32.3)	1.2 (0.4, 2.6)
86Y-**184F**-D1246	3.4 (1.2, 7.2)	0.9 (0.3, 2.2)
86Y-Y184-**1246Y**	4.6 (1.9, 8.6)	0.4 (0.0, 1.3)
N86-**184F**-**1246Y**	2.0 (0.5, 5.1)	1.1 (0.3, 2.4)
86Y-**184F**-**1246Y**	0.7 (0.0, 2.9)	
N86-Y184-**1246Y**	1.5 (0.2, 4.5)	0.5 (0.1, 1.6)

[a]Haplotype frequencies are estimated by a Bayesian model accounting for multiplicity of infection (17). Mutations are presented in boldface.

than NYD or NFD present in 2010 declined by up to >90% or disappeared, resulting in reduced genetic diversity (Tables 1 and 2).

The trends in our study accord with observations across Africa, i.e., a shift toward *pfmdr1* N86 and D1246, where AL is the major antimalarial (11). The N86 wild-type allele confers decreased LF susceptibility and increased AL failure (6, 8). So far, AL treatment failure is rare in Rwanda (1), possibly due to partial immunity and clinical artemisinin effectiveness. However, susceptibility to dihydro-artemisinin is linked to *pfmdr1* 86Y (5, 8), which almost vanished from the local parasite population. Moreover, a validated marker of artemisinin resistance, *pfkelch13* R561H, occurs in 4.5% of *P. falciparum* isolates in the same population (2). This molecular constellation, the emergence of an artemisinin resistance allele together with >95% of *pfmdr1* N86, indicates a shift toward AL-resistant genotypes in this region.

Since almost 20% of samples had evidence of an MOI of >1, we modeled haplotype frequencies, which differed from observed allele combination prevalence. This illustrates that considering one mutated allele in samples with an MOI of >1 as mutated genotypes should not be mistaken as haplotype frequency. Using MOI in analyzing temporal and/or regional allele patterns is recommended to increase comparability (16).

As limitations, we assessed *pfmdr1* alleles at two time points only, in a confined region, and lack susceptibility data. Moreover, we did not type *pfmdr1* copy number or the *P. falciparum chloroquine resistance transporter* gene, which also interfere with artemether and LF sensitivity (6, 7). We compared randomly selected, mostly asymptomatic children (13) to symptomatic, largely adult patients. Manifestation associates with *pfmdr1* SNPs (18), but not to the extent observed in our study. A strength is the comparison of molecular markers in the same district almost a decade apart.

Fifteen years after the implementation of AL as a first-line antimalarial, our study suggests the pattern in *pfmdr1* SNPs shifts toward AL resistance-associated genotypes in the Huye region. The recently demonstrated independent emergence of artemisinin-resistant *P. falciparum* strains at two sites in Rwanda underlines the importance of focal surveillance (3, 4). These developments could be the first sign of an imminent health threat to the African continent, and, in the absence of novel antimalarials, triple ACTs might be considered (19).

Data availability. Data will be made available in the WWARN repository, as a .csv file, containing Pfmdr1 genotypes and basic patient characteristics. The doi will be available when the repository is confirmed. Code for the implementation of the haplotype frequency estimation model is available at https://github.com/welmoedvl/ARTHUR_pfmdr1_haplotypefreqest.

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We have no conflicts of interest to declare.

C. Bayingana and F.P.M. designed the study. D.M., J.N., and A.S. supervised logistics. W.V.L., C. Bergmann, F.H., D.S., J.N., and A.S. were responsible for patient recruitment. W.V.L., C. Bergmann, C.T., and D.M. did the laboratory work. W.V.L. and R.O. analyzed the data. W.V.L. and F.P.M. wrote the manuscript. All authors contributed to and approved the manuscript.

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5. DISCUSSION

5.1. Host genetic polymorphisms in different malaria settings

Human genetics has co-evolved with infectious diseases, particularly in the case of malaria, thought to be the most important force that shaped the human genome (Kwiatkowski 2005). Genetic variants that affect susceptibility to and manifestation of malaria provide information about which physiological mechanisms may play a protective or hazardous role. This can further be used to explore vaccine or therapeutic targets. We investigated the role of genetic polymorphisms in malaria on the level of two different biological mechanisms, *i.e.*, in an erythrocyte receptor, and in a regulator for the innate immunity. It demonstrates the impact that common genetic variations can have on malaria, depending on the population and setting.

5.1.1. Duffy antigen variants and malaria in Mangaluru, India

We did not find an association between vivax malaria and the major Duffy alleles FYA and FYB in a case-control study in Mangaluru, southern India. However, FYB carriage (*i.e.*, hetero- or homozygous for the allele) was associated with protection from hospitalization and severe malaria in the case of *P. falciparum* infection. Carriage of another, less common, Duffy variant, DARC 298A, showed increased odds for malaria, vivax malaria in particular, and for several symptoms. Other Duffy antigen variants typed were Duffy negativity (FYO, DARC T-33C), and DARC G298A (when present with DARC 298A, defined as Duffy allele FYX). These were absent and rare, respectively.

Duffy alleles FYA & FYB

The global distribution of Duffy alleles has a strong regional signature: the FYO allele reaches frequencies of 100% in sub-Saharan Africa, the FYA allele dominates in South-East Asia, and the FYB allele dominates in Europe and the America's (Figure 3) (Howes et al. 2011). This alone already suggests that the Duffy alleles might influence malaria susceptibility or outcome. Indeed, *P. vivax* uses the Duffy antigen as its erythrocyte invasion receptor (Miller et al. 1975). Considering the burden caused by vivax malaria in Asia combined with *P. vivax* being present in Asia since the arrival of modern humans (Liu et al. 2014), it is surprising that the major vivax-resistance allele mainly occurs in Africa and not in Asia. There may be other major genetic traits in the Asian population protecting against *P.*

vivax infection. Genetic ancestry analysis suggests that FYB is the original Duffy allele (Li et al. 1997; Tournamille et al. 2004). The current abundance of FYA is suggestive for an evolutionary benefit of this allele, such as a protective effect of FYA against *P. vivax* infection.

In Brazil, FYA was indeed associated with protection against *P. vivax* infection and disease, whereas FYB was associated with increased risk (Cavasini et al. 2007; King et al. 2011; Kano et al. 2018). Regional vivax malaria incidence in India was associated with lower FYA, and with higher FYB allele frequencies (Chittoria et al. 2012). Besides the allelic effect, expression differences affect *P. vivax* invasion *in vitro* and susceptibility *in vivo* as well (Cavasini et al. 2007; King et al. 2011). Expression of the Duffy antigen is directly influenced by Duffy negativity (FYO), *i.e.*, FYO/FYO results in absence of the Duffy antigen, FYA/FYO leads to 50% less expression as compared to FYA/FYA, and the same is true for FYB (Michon et al. 2001). Moreover, 40-50% less *P. vivax* Duffy Binding Protein binds to FYA/FYA erythrocytes as compared to FYB/FYB erythrocytes *in vitro*, which is not attributable to different Duffy expression in these genotypes (King et al. 2011). There are data suggesting that FYB/FYB erythrocytes express less FY compared to FYA carrying ones (Woolley et al. 2000), although others did not observe such (King et al. 2011).

The observed FY genotype distribution among controls in our study (FYA/FYA, 43%; FYA/FYB, 44%; FYB/FYB, 13%) was similar to the one reported in a cross-sectional assessment in southern India (N = 32; 44%, 47%, 9%). In contrast with this cross-sectional study, we did not find an association between vivax malaria and FY alleles or genotypes. Our study was done in the urban setting of Mangaluru, where malaria preventive measures, and environmental and housing characteristics strongly associate with malaria (Siegert et al. 2021), possibly overshadowing the role of host genetics. Although the study size was big, only comparing vivax mono infections with controls might have led to loss of power to detect the effect of FY genotype on clinical vivax malaria. We did observe increased odds for hospitalization and severe malaria with the FYA/FYA genotype among all malaria patients. As the group of hospitalized patients almost exclusively comprised falciparum malaria patients, this may be unconnected with the Duffy receptor function for erythrocyte invasion by *P. vivax*. This might be explained by the role that the Duffy antigen plays in platelet mediated parasite killing, since Duffy antigen expression is linked to platelet factors binding to parasitized cells (McMorran et al. 2012, 2013). However, this has only been investigated with respect to Duffy negativity, and it is unclear how FYA or FYB allele differences affect this process.

Another mechanism underlying our observed effect of FYB carriage on hospitalization of malaria patients could be a less malaria-specific role of the Duffy antigen. The Duffy antigen acts a receptor for several proinflammatory cytokines secreted by immune cells (Rot 2005). In a genome-wide association study, the FYB allele was the strongest genetic determinant for high serum monocyte chemoattractant protein-1, a proinflammatory cytokine (Schnabel et al. 2010). The effect FY alleles may have on proinflammatory regulation could contribute to malaria manifestation since this is largely driven by inflammatory responses.

The Duffy alleles affect the antibody response against *P. vivax* Duffy Binding Protein (PvDBP): PvDBP-directed antibodies more effectively block PvDBP binding to erythrocytes expressing FYA compared with FYB *in vitro* (King et al. 2011; Kano et al. 2018). *P. vivax* Duffy Binding Protein is a major target for protective acquired immunity, since naturally acquired PvDBP antibodies effectively prevent malaria (Cole-Tobian et al. 2002). Consequently, PvDBP is a prime vaccine target (Grimberg et al. 2007). Further investigation of the role and mechanisms that the Duffy alleles FYA and FYB play in malaria manifestation and the effect on antimalarial immunity is required.

Duffy G298A

Duffy antigen variants DARC C265T and DARC G298A are common, and almost exclusively detected in combination with the FYB allele. When both mutations are present, this is referred to as the Duffy FYX allele, and decreased FYB expression is observed (Olsson et al. 1998; Höher et al. 2018). In line with this, a cross-sectional study on malaria patients in Brazil found that the carriage of FYX (DARC 265T and 298A) was associated with low vivax parasite density, but not with malaria *per se* (Albuquerque et al. 2010). This contrasts with our observed increased odds of malaria, and of *vivax* malaria in particular, for DARC 298A carriage in India. We did not find an association with parasite density. *In vitro* work indicates that decreased FYB expression is only decreased when both DARC 298A and DARC 265T are present (Olsson et al. 1998). In our study, DARC 265T was rare (1%), and although omitted from further analysis, this indicates that FYB expression is not the reason of our observed link between DARC G298A and malaria susceptibility. Our observations might be explained by the function the Duffy antigen has as a chemokine receptor, as described above.

5.1.2. MiRNA-146a polymorphism and malaria

We found that a common SNP in miRNA-146a (rs2910164 G > C) was associated with increased odds of falciparum malaria in pregnant women in Ghana. This was most pronounced in primigravidae, who can be considered relatively immune-naïve, indicating the protective role in innate immunity of miRNA-146a for pregnancy related malaria. No association was found between the miRNA-146a variant and malaria in predominantly adult male malaria patients in India. However, severe thrombocytopenia was increased in malaria patients who carried the SNP.

MiRNA-146a immune regulation & malaria

The miRNA-146a rs2910164 C allele frequency is about 25% in European, 45% in African, and 65% in Asian populations (International HapMap Consortium no date; Belmont et al. 2003). A polymorphism increasing malaria susceptibility is expected to be rather rare in malaria endemic regions, which conflicts with the observed geographic allele distribution. Similar discrepancies have been observed for, *e.g.*, TLR-4 variants or mannose-binding lectin deficiency (Mockenhaupt et al. 2006; Holmberg et al. 2008). Such might be explained by genetic variations causing adverse effects after the out-of-Africa-migration of humans, *e.g.*, because of increased susceptibility to other infections and sepsis (Ferwerda et al. 2007). With respect to tuberculosis, both increased and decreased susceptibility to pulmonary tuberculosis in case of miRNA-146a rs2910164 have been reported from China (Li et al. 2011; Zhang et al. 2015). These data indicate a complex role of miRNA-146a rs2910164 in the regulation of immunity to infectious diseases.

MicroRNAs are small non-coding RNA molecules that act as gene regulator, by inhibiting their expression. It has become clear that they play a key role in immune regulation and modulation (Mehta and Baltimore 2016). MicroRNA-146a causes a negative feedback loop in the TLR cascade: it is upregulated during innate pathogen recognition, downregulates the MyD88 pathway, thereby inhibiting TLR and cytokine signalling (Taganov et al. 2006; Mehta and Baltimore 2016). MicroRNA-146a has an immunomodulatory role in apicomplexan parasitic infections. *In vitro* work demonstrated that *Leishmania* infection increased levels of miRNA-146a, which, *via* the TGF-β signalling pathway, benefitted parasite killing in macrophages (Nimsarkar et al. 2020). In mouse models, elevated miRNA-146a expression associated with increased *Toxoplasma* infection. MicroRNA-146a knockout mice had decreased IFN-γ levels, protecting them during early *Toxoplasma* infection (Cannella et al.

2014). To the best of our knowledge, no work has been published that investigates the role of miRNA-146a in malaria.

Different effects of MiRNA-146a rs2910164 G > C in different populations

The rs2910164 SNP affects pre-miRNA-146a processing into mature miRNA-146a. Homozygosity results in reduced amounts of mature miRNA-146a *in vitro*, whereas heterozygosity gives rise to additional miRNA-146as: one from the leading strand and two from the passenger strand (miRNA-146a*G and miRNA-146a*C) (Jazdzewski et al. 2008, 2009). Since miRNA-146a*G and miRNA-146a*C are thought to have a different set of target genes, SNP heterozygosity may have a different functional effect compared with either of the homozygous genotypes (Jazdzewski et al. 2009).

Toll-like receptors recognize *P. falciparum* and are crucial for the initiation of an early immune response (Krishnegowda et al. 2005). This response might be influenced by the variant miRNA-146a due to altered TLR and cytokine signalling. Indeed, we did observe an association between the miRNA-146a SNP and malaria in African pregnant women. The effect was stronger for homozygote women than in heterozygous women, suggesting a cumulative effect of the variant allele. In addition, the odds for malaria were pronounced in relatively immune-naïve primigravidae carrying the SNP (van Loon et al. 2019). This indicates that the SNP mostly affects innate immunity, which is in line with the hypothesis that miRNA-146a is a major TLR response regulator. Associations with the miRNA-146a rs2910164 SNP have been reported with other infectious diseases as well: decreased susceptibility to leprosy and both increased and decreased susceptibility to tuberculosis (Zhang et al. 2015; Hao et al. 2018).

We did not find an association of the miRNA-146a rs2910164 SNP with malaria in the case-control study in India. This was also true when stratifying on *P. falciparum* infections only (van Loon et al. 2020). Several reasons may explain these discrepant findings. Firstly, since the SNP might mainly affect innate immunity, acquired immune mechanisms in the Indian adult patients (consisting of a high proportion of migrant workers possibly having regular malaria exposure), could have masked an effect. In addition, the malaria-related effect of miRNA-146a may be pregnancy-specific. Placental tissue exhibits a specific miRNA expression pattern, and placental miRNAs take part in gene regulation and inflammation control at the fetal-maternal interface (Prieto and Markert 2011; Tannetta et al. 2014; Kamity et al. 2019). MicroRNA-146a has been hypothesized to be involved in the reaction on cell stress in the placenta (Maccani et al. 2010). In pregnancy, *P. falciparum* evades splenic

elimination by adhering to the placental syncytiotrophoblast (Fried and Duffy 2017). This epithelium produces extracellular vesicles containing miRNAs (Prieto and Markert 2011). This peculiarity may partially explain the discrepant finding of the miRNA-146a SNP affecting malaria risk in pregnant women but not in predominantly adult Indian males. Furthermore, considering both, genetic diversity between Africans and Indians and the complex interaction of miRNA-146a in immune regulation, it is possible that the SNP yields different effects *per se* in these two populations. The Indian population is genetically heterogeneous characterized by a mosaic of peoples (Das 1991; Bamshad et al. 2001), and in our case-control study this might have been amplified by the urban setting of rapidly growing Mangaluru. Moreover, India's complex malaria epidemiology of *P. falciparum* and *P. vivax* (Das et al. 2012), and a study design in which the patient group vastly differed from the control group in malaria control measures and socio-economic factors, could have masked miRNA-146a effects (Siegert et al. 2021). We could not control for these differences retrospectively in the analysis because categories in these variables did not overlap.

Increased odds for severe thrombocytopenia in the Indian malaria patients might be explained by miRNA-146a-regulation of inflammatory responses. However, this interpretation should be considered with care because we did not assess inflammatory mediators in the present study. Moreover, conflicting findings on thrombocyte counts in miRNA-146a knockdown and knockout mice have been reported (Labbaye and Testa 2012).

Due to their regulatory function, stability, and distinct patterns in physiological changes, miRNAs get increasing attention because of their potential role as biomarkers or therapeutic target in disease, including malaria (Gupta and Wassmer 2021). This stresses that further understanding the regulatory role of miRNAs in malaria is required.

5.2. Antimalarial drug resistance in Rwanda

Until recently, artemisinin resistance was not observed in Africa, and ACT remains highly effective on the continent. A continuous assessment and characterization of resistant *P. falciparum* circulating on the African continent that harbours 90% of the global *P. falciparum* burden is crucial to prevent further artemisinin resistance dissemination.

We have been monitoring *PfK13* and *PfMDR1* in *P. falciparum* isolates from Huye, southern Rwanda since 2010. Rwanda's location makes it of particular interest for such surveillance because previous antimalarial resistance first arrived in East Africa before it spread over the African continent. Artemether-lumefantrine was introduced in 2006 in Rwanda, with wide

distribution and coverage (Karema et al. 2020). Data on the *PfMDR1* pattern in the Huye region from 2010 suggested substantial AL pressure on the local *P. falciparum* population (Zeile et al. 2012).

5.2.1. *PfK13* variants in Rwanda

We observed a significant increase in *PfK13* variation over the last decade in the Huye region: from the absence of nonsynonymous *PfK13* mutations in 2010, to 2.5% in 2013, 4.5% in 2015, and 12% (9% unique *PfK13* mutations) in 2019. Moreover, in 2019, we detected the validated marker for artemisinin resistance *PfK13* R561H in three (4.5%) *P. falciparum* isolates, and candidate markers *PfK13* A675V and C469F, each once (Bergmann et al. 2021). We then confirmed artemisinin resistance for these isolates *in vitro* and their local origin (van Loon et al. 2022).

Increasing PfK13 variation in Rwanda

An increase in *PfK13* polymorphisms since ACT introduction has not been reported elsewhere in Africa except at the Grande Comore Island (Huang et al. 2015). A report from Uganda even suggests a decrease in *PfK13* variation after ACT implementation (Conrad et al. 2019). This contrasts with artemisinin resistance hot-spots in South-East Asia, where *PfK13* diversity first increased for about a decade and then decreased due to the *PfK13* C580Y mutation taking the overhand (Figure 5) (Anderson et al. 2017). A possible explanation might be the time lag between artemisinin use in Africa (since the 2000's, with slow distribution) compared to South-East Asia (initially as monotherapy, since the 1980's in Cambodia (Dondorp et al. 2009)). Another factor affecting *P. falciparum* genetic diversity, and thereby *PfK13* diversity, is transmission intensity. Due to sexual outcrossing between unrelated parasite lines, parasites in a high transmission setting are more likely to lose resistance-associated mutations (Anderson et al. 2000). The polyclonal nature of *Plasmodium falciparum* infections in a high transmission setting means that remaining competitive in terms of growth rate is essential for individual parasites. Additionally, transmission affects the level of host immunity, which in turn associates with the emergence of antimalarial drug resistance (Ataide et al. 2017). This can be attributed to host immune status affecting the survival probability of individual parasites, putting more importance to parasite fitness (Bushman et al. 2018). In addition, low immunity makes malaria patients more prone to develop symptoms and get treatment, thereby increasing drug pressure in the population (Doolan et al. 2009). Rwanda has achieved a significant decline in malaria incidence in recent years of approximately 30% between 2015-2020 (WHO 2021b). This

decreased transmission intensity might have contributed to the increase in *PfK13* diversity in Rwanda.

It is remarkable that the successful *PfK13* C580Y mutation in South-East Asia, now reaching fixation in some regions (Ariey et al. 2014; Ashley et al. 2014), does not appear in Africa, apart from sporadic reports (Ndwiga et al. 2021). Only two other validated markers of artemisinin resistance were detected at >10% in South-East Asia since the emergence of artemisinin resistance: *PfK13* E252Q (not in the propeller domain), and R561H. No clear explanation has been found for the success of the *PfK13* C580Y allele compared to other *PfK13* mutations in South-East Asia, although the mutation emerged independently in multiple locations in South-East Asia (Miotto et al. 2015). *P. falciparum* isolates carrying *PfK13* C580Y are not more artemisinin resistant *in vivo* compared to isolates with other validated artemisinin resistance mutations, as measured by survival rate or treatment failure (Anderson et al. 2017). Similarly, *in vitro* artemisinin resistance is not higher in gene-edited *PfK13* C580Y mutants compared to other common artemisinin resistance markers (Stokes et al. 2021). No head-to-head comparisons are available of gametocytaemia in isolates with the C580Y mutation versus other *PfK13* mutations. Because the K13 protein is located on the digestive vacuole of the parasite, and *PfK13* mutations likely affect haemoglobin uptake, it is plausible that such mutations affect the parasite's growth (Birnbaum et al. 2020). Indeed, the *PfK13* C580Y mutation has some decreased *in vitro* fitness compared to corresponding wild type strains and compared to resistant isolates including R561H (Nair et al. 2018; Stokes et al. 2021). Importantly, the *in vitro* fitness effect depends on parental parasite strains (Stokes et al. 2021), indicating an interaction between *PfK13* C580Y and compensatory mutations to counteract its deleterious effects. Suggested genes that play a role in this genetic background are mutations in the *P. falciparum* genes for apicoplast ribosomal protein S10 (PfARPS10), ferredoxin (PfFD), multidrug resistance protein 2 (PfMDR2), and chloroquine resistance transporter (PfCRT), identified by a genome-wide association study (Miotto et al. 2015). However, gene-editing experiments combining *PfK13*, *PfFD*, and *PfMDR2* variants could not confirm this (Stokes et al. 2021).

Although more than 100 non-synonymous *PfK13* mutations have been reported in Africa, they occur at low relative frequencies (Ménard et al., 2016; Kayiba et al., 2021). It has previously been suggested that Africa harbours a large reservoir of neutral *PfK13* mutations without signs of a drug pressure selection (Amato et al. 2016). This contrasts with the recently reported high prevalence of *PfK13* R561H in Rwanda (up to 22%) and *PfK13* A675V (11%) in Uganda (Balikagala et al. 2021; Stokes et al. 2021). Genetic analyses showed that both alleles are the result of clonal expansion, and of local origin (Uwimana et al. 2020;

Balikagala et al. 2021; van Loon et al. 2022). Similar to *PfK13* C580Y, the emergence and success of these mutations in the Rwandan and Ugandan *P. falciparum* populations might be due their specific resistance or fitness features, further discussed below. Only one other *PfK13* mutation is frequently detected across Africa, although at low frequencies, *PfK13* A578S, which does not confer artemisinin resistance *in vitro* in a Dd2 strain (Ménard et al. 2016). The rarity of this mutation complicates assessing a link with delayed parasite clearance, but data from Uganda suggests such a link (Hawkes et al. 2015).

PfK13 R561H mutation emerges in Rwanda and confers artemisinin resistance

The *PfK13* R561H mutation has been circulating in South-East Asia since the 2000's (Figure 5) but was only recently found at high frequencies in Africa and in a confined region only, *i.e.*, Rwanda. *Plasmodium falciparum Kelch-13* R561H has only been reported in a few other African countries besides Rwanda, *i.e.*, in Tanzania (2/764, 0.3%, 1/422, 0.2%) and in Uganda (1/796, 0.1%), all in 2017-2019 (Bwire et al. 2020; Asua et al. 2021; Moser et al. 2021). The first *PfK13* R561H mutant samples from Rwanda date from 2012-2015 (Uwimana et al. 2020). *Plasmodium falciparum Kelch-13* R561H associates with delayed parasite clearance in patients (Uwimana et al. 2021; Straimer et al. 2022), and we demonstrated that a Rwandan patient isolate with the mutation was *in vitro* artemisinin resistant (van Loon et al. 2022). Further *in vitro* work on *PfK13* R561H so far was done with gene-edited parasite lines, confirming artemisinin resistance when the mutation is introduced in some, but not all, laboratory parasite lines (Stokes et al. 2021). The introduction of *PfK13* R561H in the West-African laboratory line 3D7, and in the South-East Asia line Dd2 resulted in similar resistance levels as compared to *PfK13* C580Y (Uwimana et al. 2020; Stokes et al. 2021). Of note, no artemisinin resistance was observed after R561H introduction in the Tanzanian lab strain F32. *Plasmodium falciparum Kelch-13* R561H was fitness neutral when introduced in 3D7, but not in the case of Dd2 (Stokes et al. 2021). These data suggest that the genetic background plays a crucial part in the level of resistance and fitness cost conferred by *PfK13* mutations, which might explain the different selection of *PfK13* mutations in Africa compared to South-East Asia. It also warrants caution for interpretation of genetic markers of resistance from different geographic regions.

Structural and functional implications of the *PfK13* R561H mutation are still unclear. Most work regarding the functionality of *PfK13* mutations has focussed on C580Y or R539T. The 561st amino acid in the *PfK13* protein is at the 3rd blade of the propeller domain, corresponding to an exposed location in its crystal structure. These properties resemble those of *PfK13* R539T, known as the marker causing highest levels of artemisinin resistance,

but contrast with *PfK13* C580Y that is located in the fourth blade in the propeller domain, in the channel that runs through the centre of the protein (Chhibber-Goel and Sharma 2019). The mutation replaces arginine with histidine, which are both positively charged amino acids and have similar properties to some extent.

Plasmodium falciparum Kelch-13 R561H clearly qualifies for a genetic marker of concern in Rwanda and might be fitness neutral. If this mutation is indeed the African counterpart of the *PfK13* C580Y mutation in South-East Asia, without intervention we can expect a quick dominance of this allele in the *P. falciparum* population. Consequently, this might leave the partner drug in ACTs unprotected and will favour the development of multi-resistant strains, eventually leading to ACT treatment failure.

Other PfK13 mutations in Rwanda

Besides R561H, we found the following other *PfK13* mutations in the Huye region, 2019: A675V, C469F, A578S, G533A, and V555A, each once. A675V and C469F are known as candidate markers of artemisinin resistance in South-East Asia. As for Africa, the *PfK13* A675V mutation was recently found at 11% in northern Uganda, neighbouring Rwanda, where it associated with prolonged parasite clearance in artesunate treated patients, and with *ex vivo* artemisinin resistance (Balikagala et al. 2021). This data actually qualifies *PfK13* A675V to be a validated marker of resistance (WHO 2021b). We confirmed *in vitro* artemisinin resistance in a Rwandan patient isolate carrying the *PfK13* A675V mutation, as well as in an isolate with C469F. An isolate with the *PfK13* V555A mutation was not artemisinin resistant *in vitro* (van Loon et al. 2022).

Other recently detected *PfK13* mutations of interest in Rwanda are P574L, Q661E, and P667S (Uwimana et al. 2021; Straimer et al. 2022). Although these mutations were rare, they represent candidate and validated markers of resistance in South-East Asia (WHO 2021b), or paralleled delayed parasite clearance in the respective Rwandan patient (Straimer et al. 2022). Further work is required to investigate their role in the Rwandan parasite population and artemisinin resistance.

5.2.2. *PfMDR1* pattern in Rwanda

Plasmodium falciparum multidrug resistance protein 1 polymorphisms and haplotypes correlate with sensitivity to artemisinin and ACT partner drugs. We observed a decrease in *PfMDR1* haplotype variation over the last decade in Huye. The dominating haplotypes are

now NFD and NYD, reflecting the decrease of *PfMDR1* mutations 86Y and 1246Y, *i.e.*, a switch back to the wild type variants at these loci. This is a problematic development because these two alleles associate with decreased lumefantrine susceptibility: the partner drug in Rwanda's first-line ACT policy.

PfMDR1 variants and drug pressure associate

In contrast with our *PfK13* observations, the trend in *PfMDR1* haplotypes that we observed in Rwanda aligns with other reports from Africa. This might be explained by the primary driver behind *PfMDR1* selection, which is possibly the withdrawal of chloroquine rather than ACT pressure. *Plasmodium falciparum multidrug resistance protein 1* initially came into focus because of its role in chloroquine resistance, but by now, polymorphisms in the *PfMDR1* gene have been associated with *in vitro* sensitivity to most antimalarials (Foote et al. 1990; Veiga et al. 2016)]. *Plasmodium falciparum multidrug resistance protein 1* is of particular interest today regarding its effect on ACT partner drug sensitivity. Increased *PfMDR1* copy number correlates with decreased sensitivity to numerous antimalarials and with treatment failure (Sidhu et al. 2006; Lim et al. 2009), but it is rare in Africa and will not be further discussed here. Common *PfMDR1* variants in Africa are N86Y, Y184F, and D1246Y (the latter is rare in West Africa), which only became widespread in Africa during the chloroquine era. In short, *PfMDR1* 86Y and 1246Y associate with decreased *in vitro* and *ex vivo* sensitivity to chloroquine and amodiaquine, and with increased sensitivity to artemisinins, lumefantrine, and mefloquine (Wurtz et al. 2014; Veiga et al. 2016; Rasmussen et al. 2017). However, sensitivity for the latter three is only modestly affected. *Plasmodium falciparum multidrug resistance protein 1* Y184F seems of little impact to drug sensitivity (Veiga et al. 2016; Rasmussen et al. 2017). Of note, *PfMDR1* N86 parasites associate with a five-fold increase in AL treatment failure (Venkatesan et al. 2014). The combination of the three common *PfMDR1* mutations make up a *PfMDR1* haplotype (*e.g.*, 86Y-184F-1246Y, or YFY), and each haplotype might differently affect antimalarial susceptibility. Consequently, the effect of individual *PfMDR1* mutations on treatment efficacy in patients is not always evident. In a pooled analysis, *PfMDR1* N86Y independently associated with ACT treatment failure in patients compared to the wild type allele (Venkatesan et al. 2014). A treatment follow-up study showed that NFD haplotype parasites survived 15-fold higher lumefantrine blood concentrations as compared to those with the YYY haplotype in patients (Malmberg et al. 2013). Spatial-temporal analyses show differential selection trends in Africa for *PfMDR1* variants after the introduction of different ACTs. A selection of N86 and D1246 is seen in regions with first-line AL policies, whereas the opposite is seen in the case of AS-AQ policies (Okell et al. 2018). Similarly, in treatment follow-up studies, *PfMDR1* variant selection is

present in treated patients. Artemether-lumefantrine selects the N86 variant, AQ selects for 86Y, DP and AQ select for the YYY haplotype (Sisowath et al., 2005; Holmgren et al., 2006; Humphreys et al., 2007; Henriques et al., 2014; Taylor et al., 2017).

Seasonal comparisons of circulating *PfMDR1* mutations suggested a fitness cost for 86Y (Ord et al. 2007). *In vitro* work indicates the same for *PfMDR1* mutations 184F and 1246Y (Hayward et al. 2005; Fröberg et al. 2013; Duvalsaint et al. 2021). However, an independent *in vitro* fitness cost for *PfMDR1* 86Y, the apparent key drug sensitivity locus in *PfMDR1*, could not be confirmed. Notably, *PfMDR1* 86Y does have an *in vitro* fitness advantage over N86, but only in combination with 184F. This leads to the hypothesis that the 184F mutation, thought of little relevance for antimalarial drug resistance, persisted under chloroquine resistance due to fitness advantages of the YF haplotype over the NY haplotype (Duvalsaint et al. 2021). It remains unclear why the 184F mutation persists with the continent-wide decline of *PfMDR1* 86Y.

The above-described differences in drug susceptibility and fitness between haplotypes demonstrate the importance of haplotype typing rather than looking into single polymorphisms in *PfMDR1*. As with chloroquine, *PfMDR1* mutations may rather contribute to increased resistance rather than being the primary cause of resistance to ACT components. Nevertheless, the changing *PfMDR1* pattern in Rwanda indicates substantial AL pressure and the now dominating haplotyopes associate with increased lumefantrine tolerance compared to previously prevalent variants (Bergmann et al. 2021; van Loon et al. 2021).

5.2.3. Molecular data for antimalarial resistance surveillance

Molecular markers of resistance against chloroquine, sulfadoxine, and pyrimethamine were only discovered after global dissemination of resistance, making these markers of little use for antimalarial resistance control. In the era of ACTs however, the role of molecular markers for surveillance, policymaking, and containment might be much bigger.

The gold standard for assessing antimalarial drug efficacy are *in vivo* therapeutic efficacy studies. These studies have ethical issues, are costly and take time. Additionally, treatment efficacy is subject to multiple host factors, *e.g.*, naturally acquired immunity and drug metabolism, possibly masking changes in drug efficacy. *In vitro*/*ex vivo* drug testing of patient or genetically altered parasite lines requires specialized laboratory facilities and staff. Moreover, not all parasite isolates will grow in culture and can therefore not be characterized.

The discovery of mutations in the *PfK13* propeller domain causing artemisinin resistance makes a targeted molecular surveillance possible (Juliano et al. 2010; Ariey et al. 2014). Particularly now, sequencing and genetic analyses are becoming cheaper and more accessible globally. Moreover, genetic markers of resistance are crucial for combination therapies surveillance, since resistance to one of the components might already settle in while clinical failure is not detectable yet due to the other, still potent, therapy component. In addition, acquired antimalarial immunity in a population might mask the presence of antimalarial resistance in the parasite population (Hastings et al. 2015).

Identification and validation of molecular for antimalarial resistance

As for *in vitro* validation of artemisinin resistance, the RSA is considered the gold standard. It accounts for the narrow time-window that *P. falciparum* has a resistant phenotype, and it somewhat mimics *in vivo* artemisinin pharmacokinetics. However, the RSA is a time-consuming assay and requires specialized malaria cell culture staff and facilities. The assay is hard to scale up and cannot be used for routine screening of artemisinin resistance. We demonstrated with a small sample set that the conventional IC50 assay is informative about the artemisinin resistant phenotype in patient *P. falciparum* isolates with *PfK13* mutations. Observed IC50 values of artemisinin resistant isolates did significantly differ from the artemisinin susceptible control strain (van Loon et al. 2022). 50% inhibitory concentration assays cannot replace RSAs but could be used to flag up isolates that deserve further attention. This is of particular interest because IC50 assays can be scaled up, and experience with IC50 drug assays is more common in laboratories compared to RSA.

To successfully apply molecular markers of resistance, the mutations to look for need to be known. To date, *PfK13* is the only gene known that independently affects artemisinin resistance. However, genome-wide association studies revealed multiple other loci that might impact artemisinin resistance in *P. falciparum*: on a *PfK13* mutant background, SNPs in *P. falciparum* genes PfARPS10, PfFD, PfMDR2, and PfCRT associated with delayed parasite clearance in artemisinin treated patients (Miotto et al. 2015). A variant in the *P. falciparum* gene for autophagy-related protein 18 (PfATG18) associated with decreased sensitivities to dihydroartemisinin, artemether and piperaquine in cultured patient isolates (Wang et al. 2016). However, such studies are not suitable to detect associations with low frequency alleles, particularly in the case of heterogeneity as seen in *PfK13*, due to low power. A causal relation of a genetic marker and drug resistance can be proven by experiments using gene-edited parasite lines, assuring identical parasites are tested with and without the marker of interest. However, the parental parasite line's genetic background likely affects the outcome

of such experiments (Stokes et al. 2021), and an induced mutation can have different effects in different parasite stains. Moreover, laboratory parasite lines have different phenotypic properties compared to real-life parasite lines (Biggs et al. 1989; Kafsack et al. 2014; Claessens et al. 2017; Awandare et al. 2018). An additional difficulty is that mutations might only cause partial resistance or have a multiplicative effect on drug resistance. Therefore, a framework that combines longitudinal data of efficacy studies, *in vitro* and *ex vivo* studies on patient isolates, and studies on gene-edited parasite lines is required to effectively screen for new molecular markers, confirm and quantify resistance, and validate molecular markers for antimalarial resistance.

Practical considerations for the molecular surveillance of antimalarial resistance

Screening for mutations in circulating parasites cannot replace *in vivo* and *in vitro* drug efficacy surveillance, but it does form an essential part of today's malaria control strategy. With established (potential) markers of resistance, combined with screening for new markers is integrated, large sample sets can be analysed, even pooled. Indeed, such studies are conducted globally, but molecular surveillance of artemisinin resistance Africa is rather linked to research projects than being structurally incorporated in public health systems.

Rolling out a dense surveillance network for molecular surveillance of artemisinin resistance in Sub-Saharan Africa will require optimization of the detection methods. In best case, detection of artemisinin resistance is done at a point-of-care level to make a well-informed decision about treatment. A similar approach exists for the detection of drug resistant tuberculosis, *i.e.*, the GenXpert (Stevens et al. 2017), although this is not really comparable with malaria because of the vast differences in incidence between these diseases.

Screening for *PfK13* variants is generally done by sequencing of PCR products. Although sample processing, storage, PCR, and sequencing is relatively cheap and fast, this process has potential to be streamlined, *e.g.*, circumventing DNA isolation and sequencing. For some known markers of resistance, LAMP assays already exist (Chahar et al. 2017; Mohon et al. 2018). The COVID-19 pandemic has demonstrated that such novel screening tests can be quickly developed and distributed. The LAMP method does not require further instruments such as a thermocycler and is versatile. It can be adapted to novel molecular markers of resistance. Moreover, the LAMP assay can be produced in any basic molecular biology laboratory: a master mix with the correct primers and micro centrifuge tubes is all what is required.

Reporting of surveillance data for markers of resistance should be sped up. Time between data collection and publication of results is about three years on average (Ehrlich et al. 2020; Kagoro et al. 2022). Considering the speed at which the *PfK13* C580Y mutation gained dominance in South-East Asia, a several years-time lag of *PfK13* variant reporting in African *P. falciparum* isolates is unacceptable. Ideally, a real-time database would be used for reporting markers of artemisinin resistance, detected by simple devices. Only when surveillance is scaled up and data is shared timely, further dissemination of artemisinin resistance in Africa can be prevented.

5.3. Conclusion and outlook

This thesis presents the work on two main malaria topics: human host genetics interfering with malaria, and parasite genetics interfering with malaria outcome. The present work is a demonstration of how genetic traits in both the host and the parasite affect malaria epidemiologically and on an individual level. We took an epidemiological approach by investigating genetic traits in host and parasite populations and put that in a molecular context either by using *in vitro* data from others (host genetics), or our own *in vitro* experiments (parasite genetics). Putting genetic factors of the host and the parasite in context will contribute to improved design and interpretation of malaria interventions.

We found that two common Duffy alleles associated with malaria susceptibility and manifestation in Southern India, a region mainly endemic for vivax malaria. The Duffy antigen is known for its crucial role in *P. vivax* susceptibility, but our data suggest additional Duffy antigen-related mechanisms that affect malaria, such as its role in platelet mediated parasite killing and proinflammatory regulation. Both mechanisms require *in vitro* confirmation regarding differences between the Duffy alleles, and epidemiological studies with special focus on severe malaria. If a common Duffy allele can be designated as a significant risk factor for severe malaria, patients should be screened for it to anticipate on, *e.g.*, increased inflammatory responses. Since the *P. vivax* Duffy Binding Protein (PvDBP) is a major vaccine target, deciphering the differential antimalarial immunity properties of the Duffy antigen alleles is crucial for successful implementation of such a vaccine. Although considered the benign, less important malaria species for long, it is increasingly recognized that *P. vivax* poses a considerable burden globally and in India specifically. It is therefore crucial to invest in a deeper understanding host genetic factors that affect *P. vivax* infection and immunity specifically.

We found that a common variant in an innate immune regulator, miRNA-146a, affects malaria susceptibility in pregnant women in Ghana, but not in India. Our results suggest a crucial role of miRNA-146a in the innate immune response to malaria, possibly pregnancy-specific. Additional epidemiology studies are required to decipher the potential pregnancy-related effect of miRNA-146a variants on malaria. Furthermore, *in vitro* work is needed to confirm the regulatory effect of antimalarial immunity by miRNA-146a, and to explore possibilities to therapeutically target poor immunity. Malaria in pregnancy is a major public health concern in Sub-Saharan Africa. If a common genetic variant can be conclusively identified as a risk factor for malaria in pregnancy, carriers of the variant should be recognized and considered a special risk group. In general, miRNAs receive increasing interest as biomarker or therapeutic target for diseases, including malaria, which even more promotes further disentangling the link between miRNA-146a and malaria.

In Rwanda, we uncovered several alarming developments in the circulating *P. falciparum* population over the past decade: a shift to *PfMDR1* haplotypes associated with increased AL tolerance, increasing diversity in the central artemisinin resistance gene *PfK13*, 4.5% prevalence of validated markers of artemisinin resistance in 2019, and *in vitro* artemisinin resistance in patient isolates from 2019. These data contribute to the recent and compelling evidence that artemisinin resistance is emerging in East Africa. As observed in South-East Asia, this facilitates the development of ACT partner drug resistance. ACT treatment failure there reaches levels of up to 50%. Such developments will have disastrous consequences on the African continent. Surveillance for markers of artemisinin resistance needs to be improved, since this will provide data essential for containment and halting further dissemination. Improved surveillance includes better methods such as molecular assays that can be employed in a decentralized manner and less labour-intensive *in vitro* assays, broader sampling techniques such as vector-based surveillance, increased sampling by developing a dense geographical surveillance network across Sub-Saharan Africa, better organization to assure systematic and continuous surveillance rather than project-based surveillance, and faster sharing of data. Surveillance data on artemisinin resistance is the cornerstone for control strategies and will provide the data for reconsideration of regional treatment policies, *e.g.*, towards triple ACTs.

A central question that remains is what constitutes the main determinants for the emergence of artemisinin resistance in Africa. The observation of independent origin of artemisinin resistant *P. falciparum* at several locations in East Africa is notable since this was not the case with chloroquine and sulfadoxine-pyrimethamine resistance dissemination in the last

century. Identifying the key factors affecting artemisinin resistance development in Africa will help predict and prevent its novel emergence and spread.

6. ZUSAMMENFASSUNG

Bedeutung von humangenetischen Polymorphismen für Risiko und Manifestation der Malaria und von parasitären Mutationen für die Therapieresistenz

Genetische Merkmale sowohl des menschlichen Wirts als auch des Malariaparasiten beeinflussen die Malaria epidemiologisch und auf individueller Ebene. In dieser Arbeit werden diese Themen miteinander kombiniert: die Genetik des Wirts, die sich auf die Malaria auswirkt, und die Genetik des Parasiten, die sich auf die Folgen der Malaria auswirkt. Indem wir unsere epidemiologischen und experimentellen Beobachtungen miteinander in Kontext stellen, wollen wir dazu beitragen, die Konzeption und Interpretation von Malariamaßnahmen zu verbessern.

Duffy-Antigen-Allele sind für ihre entscheidende Rolle in Bezug auf Anfälligkeit für Malaria vivax bekannt. Wir untersuchten, wie sie Malariaanfälligkeit und -manifestation in einer Fallkontrollstudie an 909 Malariapatienten und 909 gesunden Kontrollpersonen in Indien beeinflussten. Dabei stellten wir fest, dass das FYB-Allel vor Hospitalisierung bei Falciparum-Malaria schützt, und das DARC 298A-Allel mit einem erhöhten Malariarisiko per se (bereinigte Odds Ratio [aOR], 1,5; 95% Konfidenzintervall [CI], 1,1-2,0) und insbesondere mit einer *P. vivax*-Infektion (aOR, 1,6; 95% CI, 1,1-2,2) assoziiert ist. Da das *P. vivax* Duffy-Bindungsprotein (PvDBP) ein wichtiges Impfstoffziel ist, ist die Entschlüsselung der unterschiedlichen Eigenschaften der Duffy-Antigen-Allele in Bezug auf die Malariaimmunität von entscheidender Bedeutung.

Wir untersuchten weiterhin, wie sich eine häufige Variante in einem angeborenen Immunregulator, microRNA-146a, auf die Malariaanfälligkeit und -manifestation in zwei verschiedenen Wirtspopulationen auswirkte. Bei 509 schwangeren Frauen in Ghana erhöhte die Homozygotie für die genetische Variante die Wahrscheinlichkeit einer Infektion signifikant (aOR, 2,3; 95% CI, 1,3-4,0), insbesondere bei Primigravidae (aOR, 5,8; 95% CI, 1,6-26,0). Bei 296 entbindenden Primiparae stellten wir einen ähnlichen Effekt fest (aOR, 5,9; 95% CI, 2,1-18,0). In einer gleichartigen Fall-Kontroll-Studie in Indien wurde jedoch kein solcher Effekt festgestellt. Die erste Beobachtung deutet auf eine Rolle von microRNA-146a bei der angeborenen Immunantwort auf Malaria hin, da Primiparae in diesem endemischen Umfeld eine relativ nicht-immune Gruppe darstellen. Unsere etwas widersprüchliche zweite Beobachtung unterstreicht die Komplexität der Malariaimmunität bei unterschiedlichem genetischen Hintergrund, Parasitenart und klinischem Umfeld. Möglicherweise spielt das

microRNA-146a-Molekül eine wichtige Rolle bei schwangerschaftsbedingter Malaria und weniger bei unkomplizierter, nicht schwangerschaftsbedingter Malaria. Aufgrund des zunehmenden Interesses am Einsatz von miRNAs als Biomarker oder als therapeutisches Ziel für Krankheiten, einschließlich bei Malaria, ist ein gründliches Verständnis ihrer Rolle bei verschiedenen Malariaarten erforderlich.

Wir untersuchten darüber hinaus 288 *P. falciparum*-Isolate aus Ruanda (2010-2019) auf Marker für Malariaresistenz. Die wichtigsten Ergebnisse waren i) eine Zunahme der Diversität von PfK13 (dem zentralen Gen für Artemisinin-Resistenz) in den letzten zehn Jahren, ii) das Vorhandensein eines validierten Markers für Artemisinin-Resistenz (PfK13 R561H) bei 4,5 % im Jahr 2019, und die In-vitro-Bestätigung von Artemisinin-Resistenz bei ruandischen Patientenisolaten im Jahr 2019. Darüber hinaus beobachteten wir eine Verschiebung im Muster der PfMDR1-Haplotypen (welche die Empfindlichkeit gegenüber verschiedenen Malariamitteln beeinträchtigen), die mit einer erhöhten Artemether-Lumefantrin-Toleranz einhergeht. Diese Daten bestätigen jüngste Erkenntnisse in Bezug auf das Auftreten von Artemisinin-Resistenz in Ostafrika. Wie in Südostasien zu beobachten, erleichtert dies die Entstehung von Resistenzen gegen Artemisinin-Kombinationstherapien, die dort ein alarmierendes Ausmaß erreicht haben. Eine solche Entwicklung hätte katastrophale Folgen für den afrikanischen Kontinent. Die Überwachung von Markern der Artemisinin-Resistenz muss dringend methodisch verbessert und ausgeweitet werden, da dies einen Grundpfeiler zur Verhinderung einer weiteren Verbreitung darstellt.

7. SUMMARY

Human genetics affecting malaria susceptibility and manifestation, and *P. falciparum* genetics affecting antimalarial drug resistance

Genetic traits in both the human host and the malaria parasite affect malaria epidemiologically and on an individual level. This thesis combines these topics: host genetics interfering with malaria, and parasite genetics interfering with malaria outcome. By putting our epidemiological and experimental observations in context, we aim to contribute to improved design and interpretation of malaria interventions.

We investigated how different Duffy antigen alleles, known for their crucial role in *vivax* malaria susceptibility, affect malaria susceptibility and manifestation in a case control study among 909 malaria patients and 909 healthy community controls in India. We observed that the FYB allele associated with protection against hospitalized falciparum malaria and that the DARC 298A allele associated with increased risk of malaria *per se* (adjusted odds ratio [aOR], 1.5; 95% confidence interval [CI], 1.1–2.0) and particularly of *P. vivax* infection (aOR, 1.6; 95% CI, 1.1–2.2). Since the *P. vivax* Duffy Binding Protein (PvDBP) is a major vaccine target, deciphering the differential antimalarial immunity properties of the Duffy antigen alleles is crucial.

We studied how a common variant in an innate immune regulator, microRNA-146a, affects malaria susceptibility and manifestation in two different host populations. Among 509 pregnant women in Ghana, homozygosity for the genetic variant significantly increased the odds for infection (aOR, 2.3; 95% CI, 1.3–4.0), particularly in primigravidae (aOR, 5.8; 95% CI, 1.6–26.0). A similar effect was observed in 296 delivering primiparae (aOR, 5.9; 95% CI, 2.1–18.0). However, no such effect was seen in the case-control study in India. The first observation suggests of a role of microRNA-146a in the innate immune response to malaria, since primiparae constitute a relatively non-immune group in this endemic setting. Our somewhat conflicting, second observation underlines the complexities of malaria immunity on different genetic background, parasite species and clinical setting. Possibly, the microRNA-146a molecule has a major role in pregnancy-related malaria, and less in uncomplicated non-pregnancy related malaria. Due to the emerging interest in employing miRNAs as biomarker or therapeutic target for diseases, including malaria, a thorough understanding of their role in different malaria entities is required.

We screened for markers of antimalarial drug resistance in 288 *P. falciparum* isolates from Rwanda (2010-2019) and main findings were i) an increase in *PfK13* (the central gene in artemisinin resistance) diversity over the last decade, ii) the presence of a validated marker of artemisinin resistance (*PfK13* R561H) in 4.5% in 2019, and *in vitro* confirmation of artemisinin resistance in Rwandan patient isolates from 2019. In addition, we observed a shift in the pattern of *PfMDR1* haplotypes (known to interfere with sensitivity to various antimalarials) associated with increased artemether-lumefantrine tolerance. These data contribute to the recent and compelling evidence that artemisinin resistance is emerging in East Africa. As observed in South-East Asia, this facilitates the development of artemisinin combination therapy partner drug resistance, which has reached alarming levels there. Such developments would have disastrous consequences on the African continent. Surveillance for markers of artemisinin resistance urgently needs to be improved methodologically and expanded, since this will be a cornerstone of preventing further dissemination.

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9. LIST OF PUBLICATIONS

9.1. Peer-Reviewed Publications

1. **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;18. doi: 10.1186/s12936-019-2643-z.
2. Gai PP, **van Loon W**, Siegert K, Wedam J, Kulkarni SS, et al. Duffy antigen receptor for chemokines gene polymorphisms and malaria in Mangaluru, India. *Malar J*. 2019;18. doi: 10.1186/s12936-019-2966-9.
3. **van Loon W**, Gai PP, Kulkarni SS, Rasalkar R, Siegert K, et al. MiRNA-146a Polymorphism Was Not Associated with Malaria in Southern India. *Am J Trop Med Hyg*. 2020;102. doi: 10.4269/ajtmh.19-0845.
4. Maechler F, Gertler M, Hermes J, **van Loon W**, Schwab F, et al. Epidemiological and clinical characteristics of SARS-CoV-2 infections at a testing site in Berlin, Germany, March and April 2020—a cross-sectional study. *Clin Microbiol Infect*. 2020;26. doi: 10.1016/j.cmi.2020.08.017.
5. **van Loon W**, Gomez MP, Jobe D, Franken KLMC, Ottenhoff THM, et al. Use of resuscitation promoting factors to screen for tuberculosis infection in household-exposed children in the Gambia. *BMC Infect Dis*. 2020;20. doi: 10.1186/s12879-020-05194-1.
6. Bergmann C, **van Loon W**, Habarugira F, Tacoli C, Jäger JC, et al. Increase in *Kelch13* Polymorphisms in *Plasmodium falciparum*, Southern Rwanda. *Emerg Infect Dis*. 2021;27. doi: 10.3201/eid2701.203527.
7. Hommes F*, **van Loon W***, Thielecke M, Abramovich I, Lieber S, et al. SARS-CoV-2 infection, risk perception, behaviour, and preventive measures at schools in Berlin, Germany, during the early post-lockdown phase: A cross-sectional study. Int. *J. Environ. Res. Public Health*. 2021;18. doi: 10.3390/ijerph18052739.
8. **van Loon W**, Hommes F, Theuring S, Von der Haar A, Koerner J, et al. Renewed absence of SARS-CoV-2 infections in the day care context in Berlin, January 2021. *Clin. Infect Dis*. 2021;73. doi: 10.1093/cid/ciab199.
9. Thielecke M, Theuring S, v**an Loon W**, Hommes F, Mall MA, et al. SARS-CoV-2 infections in kindergartens and associated households at the start of the second wave in Berlin, Germany – a cross sectional study. *Eur J Public Health*. 2021.
10. **van Loon W**, Rössig H, Burock S, Hofmann J, Bernhard J, et al. Emergence of SARS-CoV-2 B.1.1.7 Lineage at Outpatient Testing Site, Berlin, Germany, January–March 2021. *Emerg Inf Dis*. 2021.

11. Theuring S, Thielecke M, **van Loon W**, Hommes F, Huelso C, et al. SARS-CoV-2 infection and transmission in school settings during the second wave in Berlin, Germany: a cross-sectional study. *Euro Surveil*. 2021;26. doi: 10.2807/1560-7917.ES.2021.26.34.2100184.
12. Gertler M, Krause E, **van Loon W**, Krug N, Kausch F, Rohardt C. Self-collected oral, nasal and saliva samples yield sensitivity comparable to professional-collected oro-nasopharyngeal swabs in SARS-CoV-2 diagnosis. *Int J Inf Dis*. 2021;110. doi: 10.1016/j.ijid.2021.07.047.
13. **van Loon W**, Theuring S, Hommes F, Mall MA, Seybold J, et al. Berlin schools in early 2021: SARS-CoV-2 infections at lockdown and with split classes. *JAMA Network Open*. 2021;4. doi: 10.1001/jamanetworkopen.2021.27168.
14. **van Loon W**, Bergmann C, Habarugira F, Tacoli C, Savelsberg D, et al. Changing pattern of *Plasmodium falciparum multi-drug resistance-1* gene polymorphisms in southern Rwanda. *Antimicrob Agents Chemother*. 2021;65. doi: 10.1128/AAC.00901-21.
15. Vinkeles Melchers NVS, Stolk WA, **van Loon W**, Pedrique B, Bakker R, et al. The burden of skin disease and eye disease due to onchocerciasis in Africa for 1990, 2020, and 2030. *PLoS Negl Trop Dis*. 2021;15. doi: 10.1371/journal.pntd.0009604.
16. Siegert K, **van Loon W**, Gai PP, Rohmann JL, Piccininni M, et al. The Effect of Socioeconomic Factors and Indoor Residual Spraying on Malaria in Mangaluru, India: A Case-Control Study. *Int J Environ Res Public Health*. 2021;18. doi: 10.3390/ijerph182211853.
17. Ruf S, Hommes F, **van Loon W**, Seybold J, Kurth T, Mall MA, Mockenhaupt FP, Theuring S. A Retrospective Outbreak Investigation of a COVID-19 Case Cluster in a Berlin Kindergarten, November 2020. *Int J Environ Res Public Health*. 2021;19. doi: 10.3390/ijerph19010036.
18. Kindzierski S[*], **van Loon W**[*], Theuring S, Hommes F, Thombansen E, Böttcher M, Matthes H, Rössig H, Weiger D, Wiesmann C, Kurth T, Kirchberger V, Seybold J, Mockenhaupt FP, Gertler M. SARS-CoV-2 infection among educational staff in Berlin, Germany, June to December 2020. *Euro Surveill*. 2022;27. doi: 10.2807/1560-7917.ES.2022.27.11.2100524.
19. **van Loon W**, Oliveira R, Bergmann C, Habarugira F, Ndoli J, Sendegeya A, Bayingana C, Mockenhaupt FP. In Vitro Confirmation of Artemisinin Resistance in *Plasmodium falciparum* from Patient Isolates, Southern Rwanda, 2019*. Emerg Infect Dis*. 2022;28. doi: 10.3201/eid2804.212269.

[*] Shared first authorship

9.2. Contributions at scientific conferences

- van Loon W, Gai PP, Hamann L, Bedu-Addo G, Mockenhaupt FP. MiRNA-146a polymorphism increases the odds of malaria in pregnancy. Conference on Tropical Medicine and Global Health (annual meeting Deutschen Gesellschaft für Tropenmedizin und Internationale Gesundheit [DTG] and Österreichischen Gesellschaft für Tropenmedizin, Parasitologie und Migrationsmedizin [ÖGTPM]), Munich, Germany, April 4-6 2019.
- van Loon W, Bergmann C, Habarugira F, Tacoli C, Savelsberg D, Oliveira R, et al. Changing pattern of *Plasmodium falciparum* multi-drug resistance-1 gene polymorphisms in southern Rwanda. 5th Kongress für Infektionskrankheiten und Tropenmedizin (KIT), virtuell, June 16-19 2021.

10. ACKNOWLEDGEMENTS

Four years ago, I enthusiastically followed my interests in epidemiology and infectious diseases by starting a malaria project in Berlin. Now, numerous experiments and one pandemic later, this turned out to be a great shot: Berlin and the research group transformed into my home, and infectious disease epidemiology became more relevant than ever. Oh. And that thesis is written! Which would obviously not have been possible without the encouragement and help of many. It builds on previous work of a dedicated scientific community, it definitely is the result of team work and involved collaborations, it required feedback and supervision, and a significant dose of moral and social support. Here we go:

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Carroll Lewis' Red Queen once said: "*Now, here, you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!*" This phrase is the analogy behind the Red Queen Hypothesis, and I consider it the glue connecting the two quite different malaria topics brought together in this thesis. I will carry this phrase with me as a drive for my work on infectious diseases, while cherishing Alice's *"Curiouser and curiouser!"*.

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12. DECLARATION OF ORIGINAL AUTHORSHIP

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.

Berlin, den 12.12.2022
Welmoed van Loon

13. EXTENDED DECLARATION OF AUTHORSHIP

Hiermit versichere ich, Welmoed van Loon, dass die folgenden Publikationen:

- **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;18. doi: 10.1186/s12936-019-2643-z.
- Gai PP, **van Loon W**, Siegert K, Wedam J, Kulkarni SS, et al. Duffy antigen receptor for chemokines gene polymorphisms and malaria in Mangaluru, India. *Malar J*. 2019;18. doi: 10.1186/s12936-019-2966-9.
- **van Loon W**, Gai PP, Kulkarni SS, Rasalkar R, Siegert K, et al. MiRNA-146a Polymorphism Was Not Associated with Malaria in Southern India. *Am J Trop Med Hyg*. 2020;102. doi: 10.4269/ajtmh.19-0845.
- Bergmann C, **van Loon W**, Habarugira F, Tacoli C, Jäger JC, et al. Increase in *Kelch13* Polymorphisms in *Plasmodium falciparum*, Southern Rwanda. *Emerg Infect Dis*. 2021;27. doi: 10.3201/eid2701.203527.
- **van Loon W**, Bergmann C, Habarugira F, Tacoli C, Savelsberg D, et al. Changing pattern of *Plasmodium falciparum multi-drug resistance-1* gene polymorphisms in southern Rwanda. *Antimicrob Agents Chemother*. 2021;65. doi: 10.1128/AAC.00901-21.
- **van Loon W**, Oliveira R, Bergmann C, Habarugira F, Ndoli J, Sendegeya A, Bayingana C, Mockenhaupt FP. In Vitro Confirmation of Artemisinin Resistance in *Plasmodium falciparum* from Patient Isolates, Southern Rwanda, 2019*. Emerg Infect Dis*. 2022;28. doi: 10.3201/eid2804.212269.

massgeblich von mir verfasst wurden.

Mögliche Übereinstimmungen mit Textpassagen aus meiner Dissertation „*Human genetics affecting malaria susceptibility and manifestation, and P. falciparum genetics affecting antimalarial drug resistance*“ stellen somit keinen Plagiatsfall dar. Dies wird bei Bedarf bestätigt durch den Betreuer der Dissertation und Co-Autoren der aufgeführten Publikationen.

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