DISSERTATION

# Resistenz-Entwicklung von *Plasmodium falciparum* gegen Artemether-Lumefantrin in Süd-Ruanda

# Trends in Artemether-Lumefantrine resistance of *Plasmodium falciparum* in Southern Rwanda

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

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Datum der Promotion: 30.06.2024

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## List of abbreviations

| | |
|---|---|
| ACD | acid citrate dextrose |
| ACT | artemisinin combination therapy |
| AL | artemether-lumefantrine |
| ART | artemisinin (including artemisinin derivatives) |
| AS-AQ | artesunate-amodiaquine |
| CHUB/UTHB | centre hospitalier universitaire de Butare/ University Teaching Hospital of Butare |
| CM | complete culture medium |
| CQ | chloroquine |
| DBS | dried blood spots |
| DHA | dihydroartemisinin |
| DHFR | dihydrofolate reductase |
| DHPS | dihydropteroate synthase |
| DMSO | dimethyl sulfoxide |
| DNA | desoxyribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid |
| eIF2 | eukaryotic translation initiation factor |
| GMS | Greater Mekong Subregion |
| $H_2O_2$ | hydrogene peroxide |
| IC50 | half maximal inhibitory concentration |
| IRS | indoor residual spraying |
| IIH | Institute of International Health, Berlin, Germany |
| K13 | *Plasmodium falciparum* Kelch-like protein 13 |
| l | liter |
| LMF | lumefantrine |
| MFQ | mefloquine |
| mg | milligram |
| MOI | multiplicity of infection |
| NaCl | Sodium chloride |
| nmol | nanomole |
| ng | nanogram |

| | |
|---|---|
| ns | nonsynonimous |
| NFD haplotype | *pfmdr1* N86-Y184F-D1246 haplotype |
| NYD haplotype | *pfmdr1* wildtype, N86-Y184-D1246 |
| PCR | polymerase chain reaction |
| *P. falciparum* | *Plasmodium falciparum* |
| *pfcrt* | *Plasmodium falciparum* chloroquine resistance transporter gene |
| PfCRT | *Plasmodium falciparum* chloroquine resistance transporter |
| *Pfcytb* | *Plasmodium falciparum* cytochrome b complex gene |
| *pfdhfr* | *Plasmodium falciparum* dihydrofolate reductase gene |
| *pfdhps* | *Plasmodium falciparum* dihydropteroate synthase gene |
| *pfk13* | *Plasmodium falciparum* Kelch 13 gene |
| *pfmdr1* | *Plasmodium falciparum* multidrug resistance 1 |
| *pfpm2/3* | *Plasmodium falciparum* plasmepsin 2/3 gene |
| *P. malariae* | *Plasmodium malariae* |
| *P. ovale* | *Plasmodium ovale* |
| *P. vivax* | *Plasmodium vivax* |
| RBC | red blood cell |
| RDT | rapid diagnostic test |
| RT PCR | real-time PCR |
| RSA | ring-stage survival assay |
| SD | standard deviation |
| SNP | single nucleotide polymorphism |
| *Taq* polymerase | *Thermus aquaticus* DNA polymerase |
| TES | therapeutic efficacy studies |
| OR | Odds ratio |
| WBC | white blood cell |
| WHO | World Health Organization |
| xg | relative centrifugal force |
| °C | degree Celsius |
| µl | microliter |

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## Abstract

Since the implementation of artemisinin combination therapy, malaria morbidity, and mortality has drastically declined globally. However, artemisinin partial resistance has emerged in Southeast Asia, impeding effective malaria control. In Africa, where most malaria cases and deaths occur, dissemination of such resistance will have disastrous consequences. Genetic markers in the malaria parasite *Plasmodium falciparum* are an essential tool to assess the status of resistance. Mutations in the *Kelch-13* (*pfk13*) gene are associated with artemisinin partial resistance. Certain allele combinations in the *multi-drug resistance-1* (*pfmdr1*) gene associate with altered dihydro-artemisinin (DHA) and lumefantrine susceptibility. *In vitro* ring-stage survival assays uncover prolonged parasite clearance after DHA treatment.

We assessed the prevalence of *pfk13* mutations in 2019 and *pfmdr1* in 2018/2019 in southern Rwanda, where artemether-lumefantrine (AL) is the first-line treatment for uncomplicated malaria since 2006. Isolates with relevant *pfk13* mutations were subjected to ring-stage survival assays (RSA) and IC50 measurements.

We recruited 205 patients in 2018 and 90 patients in 2019 with positive rapid tests and symptoms of uncomplicated malaria in two health centers in the Huye District. Among the PCR-confirmed cases, 90.3% (167/183 [2018] and 67/76 [2019]) had a *P. falciparum* infection. In 2019, 12.1% of the *P. falciparum* isolates harbored non-synonymous *pfk13* mutations. Mutations R561H (3/66; 4.5%, validated resistance marker), C469F, A675V (candidate markers), and G533A, V555A and A578S were present. Compared to data from 2010, 2014, and 2015 from the same region, the prevalence of non-synonymous *pfk13* mutations increased significantly (OR 1.4, 95% CI 1.1, 1.8; P = 0.003). RSAs showed elevated mean survival rates above the WHO resistance threshold of 1%: for R561H (4.7% ± 1.5%), A675V (1.4% ± 0.2%), and C469F (9.0% ± 1.6%). Correspondingly, IC50 values were 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. In 2018/2019, *pfmdr1* mutations N86Y, Y184F, and D1246F were present in 3.8%, 53.8%, and 2.4%, respectively. Compared to data from 2010, N86Y occurred ten and D1246F five times less frequently, whereas the Y184F prevalence remained similar. NYD and NFD allele combinations were dominant, and genetic diversity had declined since 2010.

In conclusion, *pfk13* mutations conferring resistance to artemisinin combined with *pfmdr1* allele combinations that associate with decreased lumefantrine susceptibility are present in Rwanda. While no treatment failure has been observed, AL is in danger of becoming less effective. The introduction of triple artemisinin combination therapies might be a first step to preserving the efficacy of these antimalarials.

## Zusammenfassung

Seit der Einführung der Artemisinin-Kombinationstherapie sind Morbidität und Mortalität der Malaria weltweit stark zurückgegangen. In Südostasien erschwert jedoch eine partielle Artemisinin-Resistenz eine wirksame Malariakontrolle. In Afrika, wo die meisten Erkrankungen und Todesfälle auftreten, wird die Verbreitung einer solchen Resistenz katastrophale Folgen haben. Genetische Marker beim Malariaparasiten *Plasmodium falciparum* sind ein wichtiges Instrument zur Einschätzung der Resistenz-Situation. Mutationen im *Kelch-13* (*pfk13*)-Gen gehen mit einer partiellen Artemisinin-Resistenz einher. Bestimmte Allel-Kombinationen im *Multi-Drug-Resistance-1* (*pfmdr1*)-Gen sind mit einer veränderten Wirksamkeit von Dihydro-Artemisinin (DHA) und Lumefantrin assoziiert. *In vitro* Ringstage Survival Assays (RSA) weisen eine verzögerte Parasitenbeseitigung nach DHA-Behandlung nach.

Wir untersuchten die Prävalenz von *pfk13* Mutationen im Jahr 2019 und von *pfmdr1* in 2018/2019 in Süd-Ruanda, wo Artemether-Lumefantrin (AL) seit 2006 die Erstlinientherapie für unkomplizierte Malaria ist. Isolate mit relevanten *pfk13* Mutationen wurden einem RSA und IC50-Messungen unterzogen.

In zwei Gesundheitszentren im Distrikt Huye rekrutierten wir 205 Patienten im Jahr 2018 und 90 Patienten im Jahr 2019 mit positivem Schnelltest und Symptomen unkomplizierter Malaria. Bei 90,3 % (167/183 [2018] und 67/76 [2019]) der PCR bestätigten Fällen stellten wir eine *P. falciparum*-Infektion fest. 2019 wiesen 12,1 % der *P. falciparum*-Isolate nichtsynonyme *pfk13* Mutationen auf. Der validierte Resistenzmarker R561H (3/66, 4,5 %), die Kandidatmarker C469F und A675V, und G533A, V555A und A578S wurden nachgewiesen. Im Vergleich zu den Jahren 2010, 2014 und 2015 aus derselben Region stieg die Prävalenz nichtsynonymer *pfk13* Mutationen signifikant an (OR 1,4, 95 % CI 1,1, 1,8; P = 0,003). Die RSA zeigten erhöhte mittlere Überlebensraten oberhalb der WHO-Resistenzschwelle von 1 %: 4,7 % ± 1,5 % für R561H, 1,4 % ± 0,2 % für A675V und 9,0 % ± 1,6 % für C469F. Dementsprechend lagen die IC50-Werte bei 14,1 ± 4,0 nmol/l für R561H, 7,4 ± 3,3 nmol/l für A675V und 6,9 ± 1,5 nmol/l für C469F. In den Jahren 2018/2019 waren die *Pfmdr1*-Mutationen N86Y in 3,8 %, Y184F in 53,8 % und D1246F in 2,4 % der Isolate vorhanden. Verglichen zu Daten aus 2010 ging die Prävalenz von N86Y um das Zehnfache und von D1246F um das Fünffache zurück, während die Y184F-Prävalenz ähnlich blieb. Die

Allelkombinationen NYD/NFD waren dominant, und die genetische Vielfalt von *pfmdr1* nahm seit 2010 ab.

Zusammenfassend zeigt diese Studie in Ruanda das Aufkommen von *pfk13* Mutationen, die Resistenz gegen Artemisinin verleihen und von *pfmdr1* Allelkombinationen, die mit einer verminderten Lumefantrin-Empfindlichkeit einhergehen. Obwohl bisher kein Therapieversagen beobachtet wurde, ist die Wirksamkeit von AL gefährdet. Die Einführung von Dreifach-Artemisinin-Kombinationstherapien könnte ein erster Schritt zur Erhaltung der Wirksamkeit sein.

# 1 Introduction

Worldwide, at least 241 million people had malaria in 2020 and approximately 627,000 died, mostly (77%) children under the age of 5 years. Malaria is endemic in many tropical and subtropical countries (Figure 1), but approximately 95% of infections happen in Sub-Saharan Africa. From 2000 to 2019, the global incidence rate of cases per 1000 inhabitants at risk decreased from 81 to 56 (WHO, 2021) - partly due to intense investments in vector control and worldwide distribution of artemisinin combination therapies (Bhatt et al., 2015; Weiss et al., 2019). But in 2020 in comparison to 2019, the case incidence rate increased again to 59, 14 million more cases occurred, and 69 0000 more deaths were reported to WHO. Approximately 68% of these deaths were connected to the COVID-19 pandemic, which disrupted malaria prevention, diagnostics, and treatment (WHO, 2021).

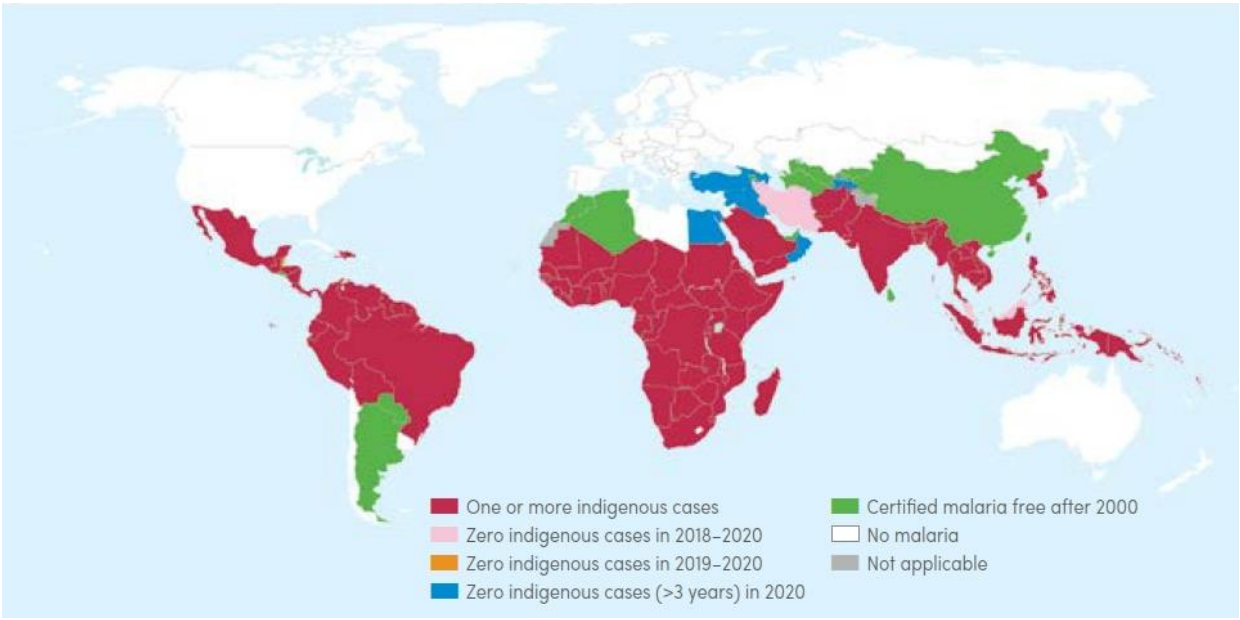


**Figure 1**: Malaria endemic countries in 2020. Source: WHO, 2021

In addition to fewer control activities implemented or maintained during the pandemic, resistance to artemisinin combination therapies in Southeast Asia threatens the progress achieved in malaria control in recent years. Emerging resistance in Africa would disastrously affect malaria case incidence and mortality. Surveillance is needed to understand the current dimension and to develop adequate response strategies.

## 1.1 The disease

Malaria is a vector-borne parasitic disease caused by female *Anopheles* mosquitoes transmitting *Plasmodium* parasites. Six different species of the protozoan genus *Plasmodium* infect humans: *P. falciparum*, *P. vivax, P. malariae, P. knowlesi,* and *P. ovale,* recently subdivided into the two distinct species *P. ovale wallikeri* and *P. ovale curtisi* (Sutherland et al., 2010). Most infections are caused by *P. falciparum* and *P. vivax* but they differ in geographic distribution: in Africa *P. falciparum* is most common and in Southeast Asia, *P. vivax* infections and coinfections occur more commonly (WHO, 2021). Disease severity and mortality vary between the different species: *P. falciparum* is the deadliest, and of the other species, only *P. vivax* and *P. knowlesi* can lead to severe malaria (WHO, 2014).

Symptoms develop during asexual reproduction when the parasites cyclically infect the red blood cells of the human host and grow exponentially, leading to increasing parasitemia, hemolysis, cytokine release, and blood clotting. In uncomplicated malaria, patients report myalgia, fatigue, abdominal symptoms, and fever. In areas with high transmission and partial immunity, people may be asymptomatic. Clinical findings include anemia, mild jaundice, and hepatosplenomegaly. (White et al., 2014).

Severe malaria is defined by complications including organ failures or metabolic abnormalities and patients are at a much higher risk of death. In a trial investigating case fatality in Africa, 8.5% of the infected children died due to severe *falciparum* malaria, even though they received appropriate treatment (Dondorp et al., 2010). Patients may suffer from organ dysfunctions such as acute respiratory distress, kidney failure, and cerebral malaria. Laboratory findings include hyperparasitemia, hemolysis, renal abnormalities, severe anemia, and hypoglycemia. In *P. falciparum* malaria, these complications are caused by hyperparasitemia and sequestration of parasitized erythrocytes in postcapillary venules, which can lead to obstruction of small vessels and thus impair organ perfusion (Dondorp et al., 2008; WHO, 2014).

During pregnancy, the sequestration of parasites in the placenta endangers both the mother and the unborn child. Additional to impaired placental function, peripheral blood smears might not detect circulating parasites, which can delay diagnosis and treatment (Mockenhaupt et al., 2002). Placental malaria can lead to growth retardation of the fetus, low birth weight, stillbirth, and maternal death.

Recurrent or persistent malaria can last for months or years and is caused by either dormant stage (hypnozoite) revival in *P. vivax* and *P. ovale* malaria (Krotoski, 1985), recrudescence after ineffective treatment, or reinfection. Children especially are at risk of negative effects, such as anemia, malnutrition, and impaired cognitive development (Tapajós et al., 2019).

## 1.2 The parasite and its life cycle

The life cycle of the human-pathogenic *Plasmodium* species includes two hosts: *Anopheles* mosquitoes, in which the sexual replication takes place, and humans, in whom the parasites multiply asexually by schizogony (Figure 2). Through mosquito bites, sporozoites invade the human bloodstream and travel to the liver. In this exo-erythrocytic cycle, sporozoites infect hepatocytes, multiply by asexual division, and form schizonts. *P. vivax* and *P. ovale* can enter a dormant stage in hepatocytes, called hypnozoites, that may survive for months to years before reactivating again (Krotoski, 1985). After an asymptomatic incubation period of one to several weeks, liver schizonts release thousands of merozoites, which then infect red blood cells (RBCs), starting the erythrocytic cycle. Following RBC invasion, the parasites undergo erythrocytic schizogony forming trophozoites.

To grow, trophozoites feed on host cell hemoglobin, which is transported into the parasite's digestive vacuole – a lysosome-like structure. Hemoglobin is broken up into globin and free heme, which is toxic to the parasites, and transformed into less-toxic crystalline hemozoin, also called malaria pigment (Oliveira et al., 2002). Many antimalarial drugs and resistance mechanisms affect this feeding process. For example, *P. falciparum* K13 plays a role in hemoglobin endocytosis and intraerythrocytic growth of young ring-stage trophozoites (Birnbaum et al., 2020).

Trophozoites mature into schizonts. When RBCs rupture, schizonts release newly formed merozoites into the bloodstream, which infect further RBCs. This asexual blood stage is the symptomatic phase of a *Plasmodium* infection and leads to intermittent fever episodes through the cyclic release of merozoites and reactive cytokines. Sexual gametocytes form within 10 days after asexual parasitemia. When a mosquito takes up gametocytes during a blood meal, they can reproduce sexually in the midgut, and eventually sporozoites develop and travel to the mosquito's salivary glands. With the bite of an infected mosquito, the cycle begins again.

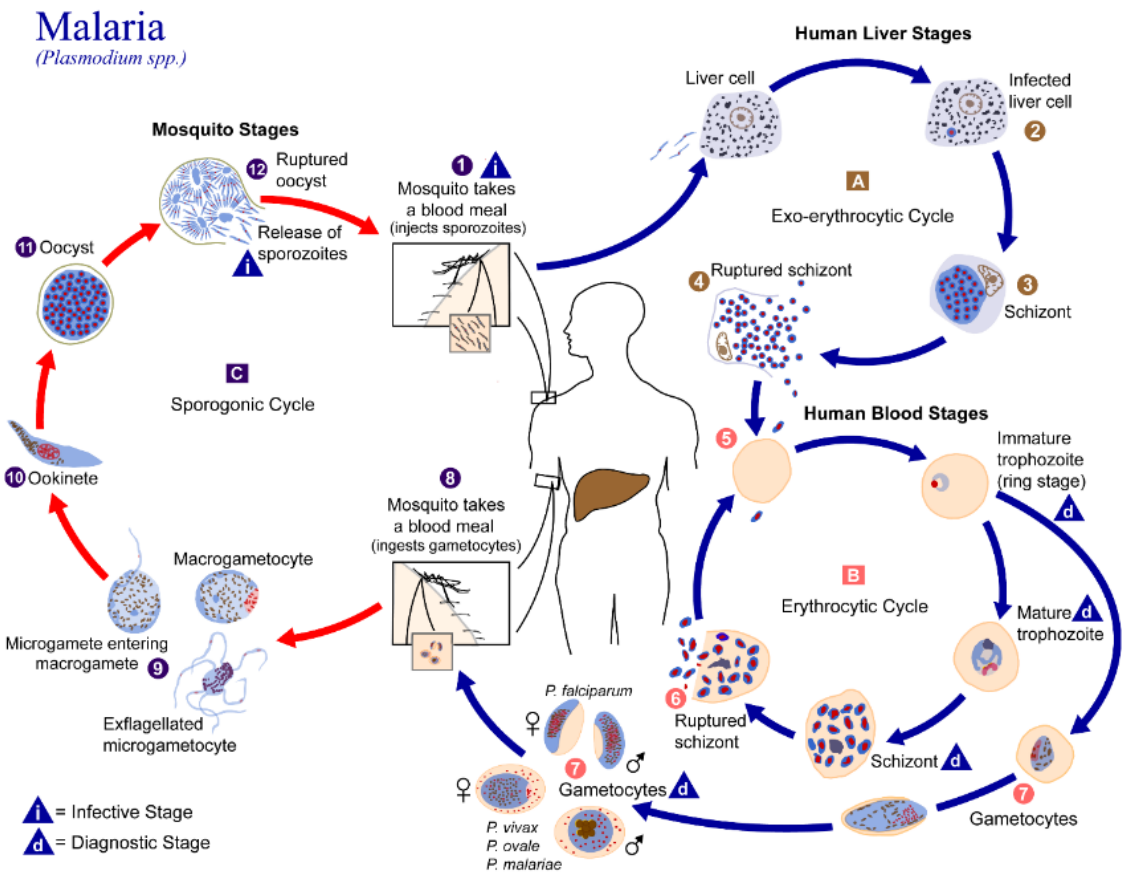


**Figure 2**: Life cycle of *Plasmodium* species (CDC, 2002)

## 1.3 Malaria management and control measures

Controlling malaria requires vector control measures, correct diagnosis, and effective antimalarial drugs. For disease prevention and vector control in endemic areas, the WHO recommends the use of insecticide-treated bed nets and the spraying of indoor surfaces with long-lasting insecticides. Breeding sites and larvae habitats outside are other targets to reduce mosquito populations. Experiments with genetically modified *Anopheles* mosquitoes, which are unable to spread malaria, are ongoing (WHO, 2022). Once a person is infected, health workers diagnose malaria mainly by microscopy of blood smears or by rapid diagnostic tests. Thick blood smears are used for diagnosis, while experienced microscopists can differentiate *Plasmodium* species on thin blood smears. Rapid diagnostic tests (RDTs) allow for a safe and quick diagnosis and are used widely at the community level for screening and if microscopy is not available. Most RDTs are based on color change if malaria antigens such as *Plasmodium* lactate dehydrogenase

(pLDH), aldolase, and *P. falciparum* specific histidine-rich protein-are present in the drop of blood applied to the test strip (White et al., 2014). Different drugs exert an antimalarial activity as presented in the next chapter. Besides treatment, some of them are used for prophylaxis for travelers from non-endemic regions and intermittent preventive therapy, currently recommended especially for pregnant women. (WHO, 2022).

## 1.4 Antimalarial treatment

A broad range of antimalarial drugs is available, with diverse modes of action (Table 1). Most affect parasites in the blood stages, while only primaquine is active against hypnozoites. The main drug target sites include the digestive vacuole, the mitochondrion, and the apicoplast. Many drugs influence the hemoglobin degradation process in the digestive vacuole, increasing toxic heme levels and disrupting hemozoin formation. Currently, due to resistance development against most known antimalarials, high efficacy, and few side effects, WHO recommends a combination therapy with an artemisinin (ART) derivative as the first-line treatment for uncomplicated *P. falciparum* malaria in most countries. First-line treatment for severe malaria is intravenous artesunate (WHO, 2022).

**Table 1**: Classes of antimalarials, examples of drug agents and modes of action.

| Class of drugs | Example | Mode of action |
|---|---|---|
| 4-aminoquinolines | Chloroquine<br>Amodiaquine<br>Piperaquine | Accumulation of toxic-free heme and inhibition of hemozoin formation (Combrinck et al., 2013) |
| 8-aminoquinolines | Primaquine | CYP-mediated toxic $H_2O_2$ accumulation (Camarda et al., 2019) |
| Aryl-amino alcohols | Quinine<br>Mefloquine<br>Lumefantrine<br>Halofantrine | Prevention of heme detoxification in the digestive vacuole (Combrinck et al., 2013)<br>Possibly another target in the parasite cytosol (Veiga et al., 2016) |
| Antifolates | Pyrimethamine<br>Trimethoprim<br>Proguanil<br>Sulfonamides | Inhibition of proteins in the folate pathway (DHFR & DHPS) needed for DNA synthesis (Foote and Cowman, 1994) |
| Naphthoquinones | Atovaquone | Inhibition of cytochrome bc1 complex in the plasmodial mitochondrion (Fry and Pudney, 1992) |
| Antibiotics | Doxycycline,<br>Clindamycin | Inhibition of apicoplast (relict plastid) protein synthesis (Dahl et al., 2007) |
| Artemisinins | Artesunate,<br>Artemether,<br>Dihydroartemisinin | Heme-mediated protein degradation (Wang et al., 2015) and proteasome dysfunction (Bridgford et al., 2018) |

### 1.4.1 Artemisinin-based therapy

The plant *Artemisia annua* was historically used in China as an herbal remedy against fever. Researchers found that the extracted component artemisinin has antimalarial properties. For her contributions to this discovery, the Nobel prize committee awarded Professor Youyou Tu the Nobel Prize in Physiology or Medicine in 2015 (Tu, 2011; “nobelprize.org,” 2023).

Artemisinin derivatives (ARTs) act through the active metabolite dihydroartemisinin (DHA). In the red blood cells, *Plasmodium* parasites feed on host hemoglobin. The reduced heme iron ($Fe^{2+}$ heme) accumulating during hemoglobin degradation activates

ARTs (Wang et al., 2015). When activated, ARTs damage many essential parasitic proteins, e.g., for hemoglobin degradation and antioxidant defense (Wang et al., 2015; Ismail et al., 2016), and inhibit the proteasome, which would normally degrade the damaged proteins (Bridgford et al., 2018).

Artemisinins exert a parasite stage-specific activity and are especially effective against very young ring-stage parasites (2-4h post-invasion) and trophozoite-stage parasites (Klonis et al., 2013). Gametocytes are killed to a certain degree, with mature gametocytes being the least susceptible (Adjalley et al., 2011).

Compared to other antimalarials, artemisinins clear parasites very quickly in the first days of infection, but the very short plasmatic elimination half-life can lead to the recrudescence of parasites after ending monotherapy. To limit recrudescence and resistance formation, artemisinin combination therapies (ACTs) with longer-acting partner drugs are recommended. First-line combinations include artemether-lumefantrine (AL), artesunate-amodiaquine (AS-AQ), artesunate-mefloquine, artesunate-pyronaridine, and dihydroartemisinin-piperaquine (WHO, 2022).

Artemisinins are generally well tolerated, and serious drug-related adverse events occur rarely (Sinclair et al., 2009; Dondorp et al., 2010). Hyperparasitemic patients can develop delayed hemolytic anemia after artemisinin therapy, probably caused by artemisinin-induced splenic pitting, i.e., the spleen extracting parasites from RBCs and releasing them back into the blood stream. This can lead to high levels of once-infected RBCs with a shortened life span (Jauréguiberry et al., 2014).

AL is currently the first-line treatment of uncomplicated malaria in Rwanda. The dose is chosen adapted to body weight, and tablets are taken twice daily for three days to cover two asexual parasite life cycles. AL should be ingested with fatty foods or liquids to ensure absorption of the highly lipophilic lumefantrine. It is not recommended for pregnant women in the first trimester (WHO, 2022).

## 1.5 Antimalarial resistance

*Plasmodium* parasites can develop resistance to antimalarial drugs, in the worst case leading to treatment failure. Drug resistance does occasionally occur in *P. vivax,* but this discussion is outside the scope of this thesis. Instead, *P. falciparum*'s ability to develop resistance will be discussed in more detail.

In the late 1950s, when chloroquine was the first-line antimalarial globally, chloroquine-resistant *P. falciparum* isolates emerged in Southeast Asia, spread to East Africa in the 1970s and across the African continent (Campbell et al., 1979; Mita et al., 2009). In some countries, chloroquine resistance is said to have increased malaria mortality in children from two-fold to as much as six-fold (Trape, 2001). Other antimalarials and combination therapies were implemented, but resistance emerged quickly, e.g., against sulfadoxine-pyrimethamine (Mita et al., 2009).

At the beginning of the 21st century, concerted efforts including the scale-up of vector control and worldwide access to artemisinin-based drugs reduced malaria cases and deaths globally in the following 15 years (Bhatt et al., 2015; Weiss et al., 2019). But during this time, evidence of decreasing susceptibility to ACTs emerged; prolonged parasite clearance following ART therapy with a half-maximal inhibitory concentration (IC50) value up to 4 times higher than in cured patients was first shown in 2007 in western Cambodia (Noedl et al., 2008; Dondorp et al., 2009). Delayed parasite clearance following therapy with artesunate, or artemisinin-based combination therapy was linked to increased survival of ring-stage parasites, termed artemisinin partial resistance (WHO, 2020).

Artemisinin partial resistance has since spread and emerged indigenously in the Greater Mekong Subregion (GMS). In Thailand and western Cambodia, five-fold increased rates of treatment failure rates following therapy with artesunate-mefloquine or dihydroartemisinin-piperaquine were observed (Ashley et al., 2014). Van der Pluijm and colleagues have confirmed increasingly prevalent multi-drug resistance in the GMS following dihydroartemisinin-piperaquine treatment resulting in treatment failure (van der Pluijm et al., 2019).

The impact of this emerging resistance could be catastrophic. Using a mathematical model with an ACT failure rate of 30% across the malaria-endemic world, excess mortality of 116,000 deaths per year was projected due to artemisinin resistance (Lubell et al., 2014). *P. falciparum* has developed resistance to almost all known antimalarials to the extent that treatment efficacy is decreasing dramatically in the GMS alongside the emergence of multi-drug resistant strains (van der Pluijm et al., 2019). Fast detection and continuous monitoring of drug resistance are crucial to enable fast treatment and policy changes.

### 1.5.1 Detection and assessment of artemisinin resistance

Artemisinin partial resistance can be assessed by different means. Therapeutic efficacy studies are the gold standard to assess clinical resistance and are used to adapt national treatment policies. Malaria patients are followed prospectively with directly observed treatment, quality measurements of antimalarial drugs, and a follow-up with regular parasite counts over 28 to 42 days depending on the medication used. If treatment failure exceeds 10 percent of tested patients after 28 to 42 days, treatment policy changes are required (WHO, 2020). Persistent parasitemia on day three of treatment (day 3 parasitemia) is a surrogate parameter for a quick and simple assessment of effective ART treatment (Stepniewska et al., 2010). Laboratory testing for drug resistance includes determining the IC50, defined as the drug concentration needed to inhibit parasite growth by 50%. Since artemisinin resistance affects especially ring-stage parasites, IC50 measurements might be slow to detect it. To test specifically for ring-stage parasite resistance, a ring-stage survival assay was developed to assess *ex vivo* and *in vitro* parasite survival in synchronized parasite cultures after DHA pulse treatment (Witkowski, 2013).

### 1.5.2 Molecular markers for antimalarial resistance

Besides labor-intensive experiments, molecular markers constitute a way of resistance surveillance. Certain genes or parts of genes encode proteins, mostly transporters, which influence drug susceptibility (Figure 3). When parasites develop coding mutations in these genes, they might lead to drug resistance, constituting a molecular marker. In *P. falciparum* malaria, relevant genes include *pfk13*, *pfmdr1*, *pfcrt, pfpm2*, *pfdhfr*, *pfdhps*, and *pfcytb* (WHO, 2020).

Single point mutations in *pfk13* are associated with artemisinin resistance. Mutations and amplification in the *Plasmodium falciparum* multi-drug resistance transporter 1 (*pfmdr1*) gene affect the susceptibility to artemisinins and combination partner drugs (Veiga et al., 2016). Both were studied further and will be discussed in more detail in the next subsections. Chloroquine-resistance transporter (*pfcrt*) gene mutations confer resistance to chloroquine, but also to amodiaquine, and piperaquine. Dihydrofolate reductase (PfDHFR) and dihydropteroate synthase (PfDHPS) are enzymes in the folate pathway targeted by pyrimethamine, proguanil, and sulfadoxine, respectively, and gene mutations can render these antifolates ineffective. Plasmepsins are proteins involved in

hemoglobin degradation and *plasmepsin 2/3* (*pfpm2/3*) gene copy number affects piperaquine effectiveness. Atovaquone selects for *cytb* mutations since it inhibits the cytochrome b complex needed for electron transport in the plasmodial mitochondrion (WHO, 2020; Wicht et al., 2020).

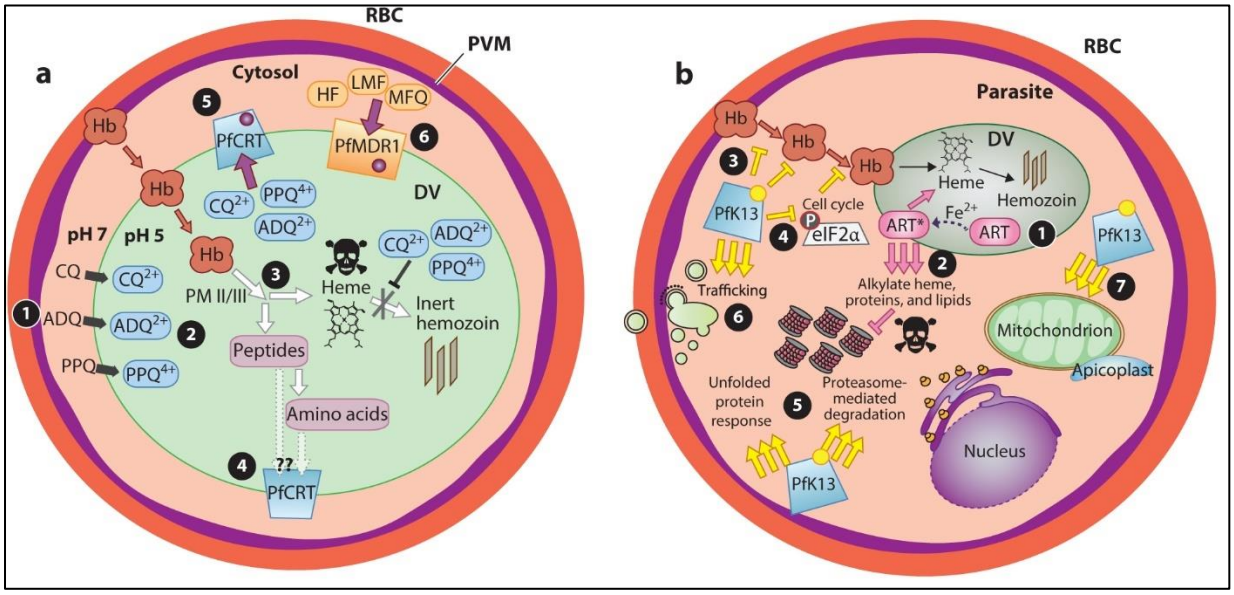


**Figure 3**: Modes of action of selected antimalarial drugs and respective resistance mechanisms. During the erythrocytic cycle, *Plasmodium* parasites grow in the red blood cell (RBC) encompassed by the parasitophorous vacuole membrane (PVM). Heme is degraded in the digestive vacuole (DV) into non-toxic hemozoin, a process inhibited by chloroquine (CQ), amodiaquine (ADQ), piperaquine (PPQ) and artemisinins (ART), and necessary to activate ART. When activated, ART damage various parasitic biomolecules and inhibit proteasome-mediated degradation. K13 is involved in hemoglobin endocytosis. Mutations in *pfk13* elicit decreased protein activity and thus limit ART activation. Other mechanisms in *pfk13* mutations might also mediate ART resistance, e.g., eIF2-mediated cellular stress response. The membrane proteins PfMDR1 and PfCRT influence drug susceptibility. Mutations in *pfcrt* lead to the efflux of antimalarials from the DV, away from the target site. *Pfmdr1* mutations presumably lead to decreased transport of halofantrine (HF), lumefantrine (LMF), and mefloquine (MFQ) into the DV, so that they can exert their antimalarial activity in the cytosol. Adapted from Wicht et al., 2020.

### 1.5.3 *pfk13*: a molecular marker for artemisinin resistance

In 2014, a molecular marker for artemisinin resistance was identified. Non-synonymous single nucleotide polymorphisms (nsSNPs) at certain loci in the gene for Kelch-like protein 13 (*pfk13)* were associated with artemisinin resistance *in vitro* (Ariey et al., 2014) and with

delayed parasite clearance following DHA exposure in clinical isolates in Southeast Asia (Ashley et al., 2014).

Birnbaum and colleagues found that the protein pfk13 is involved in hemoglobin endocytosis, especially in young ring-stage parasites. *Pfk13* mutations appear to cause reduced levels or less activity of the protein, leading to lower levels of hemoglobin in the parasite. As discussed before, artemisinin must be activated by heme produced during hemoglobin digestion. Thus, in *pfk13* mutant parasites, ART are not activated as effectively, and ring-stage parasites might survive treatment (Birnbaum et al., 2020).

Over 200 nsSNPs in *pfk13* have been reported, but for most of them, their significance for artemisinin resistance is still under investigation. To date, 10 polymorphisms have been confirmed to cause delayed clearance in patients, as well as *ex vivo* or *in vitro* resistance, and are thus referred to as validated mutations (**Table 2**). 13 further polymorphisms were associated with delayed clearance in patients, but not (yet) with *ex vivo* or *in vitro* resistance, called candidate mutations (WWARN Group, 2019; WHO, 2020).

**Table 2**: Candidate and validated resistance mutations in the *pfk13* gene

| **Candidate** | | **Validated** | |
|---|---|---|---|
| P441L | P667T* | F446I | P574L |
| G449A | G538V | N458Y | I543T |
| C469F/Y | V568G | M476I | P553L |
| A481V | R515K | Y493H | R561H |
| P527H | F673I | R539T | C580Y |
| N537I/D | A675V | | |
| R622I | | | |

Source: WHO, 2020

Since its discovery, the C580Y SNP emerged as the most important molecular marker for artemisinin resistance in the GMS causing high rates of treatment failures following different ACT treatments (Phyo et al., 2016; van der Pluijm et al., 2019). In Africa, various other *pfk13* mutations are present with partly unclear significance. First signs of ART resistance are currently emerging as presented in the discussion chapter, and R561H is the relevant marker spreading in Rwanda (Uwimana et al., 2020) (Figure 4).

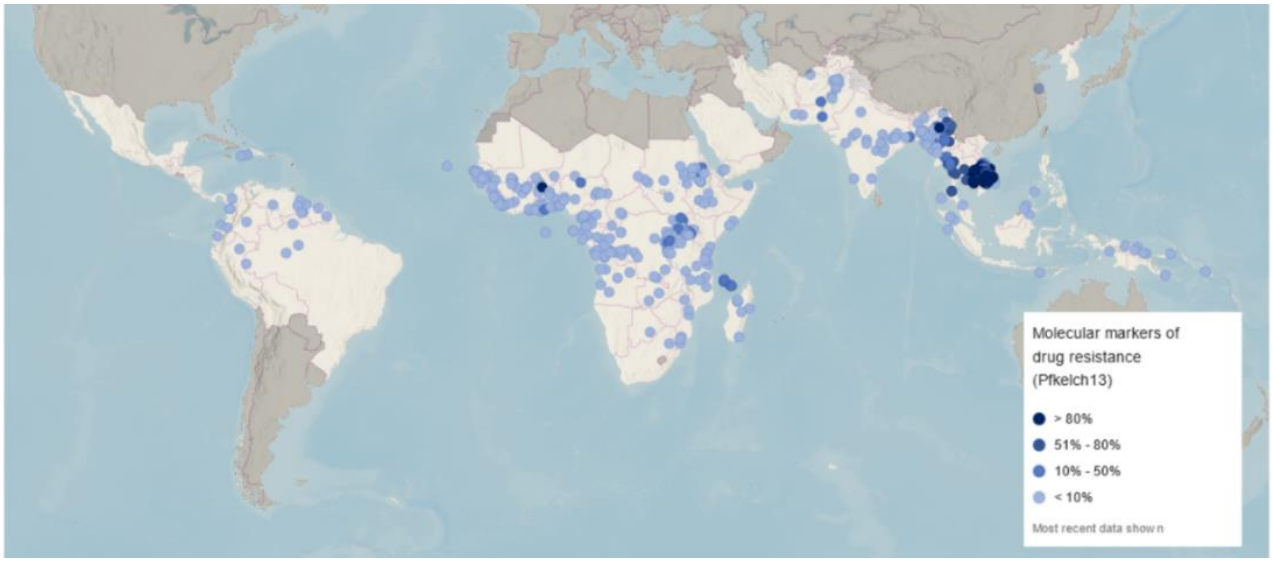


**Figure 4**: Proportion of *Plasmodium falciparum* isolates carrying *Plasmodium falciparum* K13 (*pfk13)* markers for artemisinin resistance in malaria-endemic countries. In Southeast Asia, molecular markers are common, while they occur rarely in Africa. (WHO Global Malaria Programme, 2022)

### 1.5.4 *Pfmdr1*: a molecular marker for multi-drug resistance

The *P. falciparum multidrug resistance transporter (pfmdr1)* gene encodes a P-glycoprotein, which acts as an influx-mediating transporter at the membrane of the digestive vacuole. *Pfmdr1* mutations can lead to a decreased uptake of antimalarials into the digestive vacuole, leading to higher drug concentrations in the parasite cytosol (Veiga et al., 2016). Depending on the target site of the drug, mutations in these transporters have different effects on parasite susceptibility. The most relevant *pfmdr1* mutations include amino acid changes at positions 86, 184, and 1246 (Malmberg et al., 2013; Okell et al., 2018). In a single parasite, multiple SNPs can be present and as allele combinations, they can be grouped into haplotypes. *Pfmdr1* mutations affect the efficacy of many artemisinin partner drugs and are useful to assess artemether-lumefantrine susceptibility. Interestingly, the SNP N86Y increases susceptibility to lumefantrine, and DHA (Veiga et al., 2016). Wildtype parasites (*pfmdr1* N86-Y184-D1246, NYD haplotype) exhibit higher IC50 values when subjected to AL and a higher risk for recrudescence after therapy with AL (Venkatesan et al., 2014). Also, the NFD haplotype (N86-Y184F-D1246) showed reduced AL sensitivity compared to YYY (N86Y-Y184-D1246Y) (Malmberg et al., 2013; Okell et al., 2018). Amodiaquine, mefloquine, and chloroquine susceptibility are influenced by *pfmdr1* SNPs, partly on the background of *pfcrt* mutant parasites

(Venkatesan et al., 2014; Veiga et al., 2016). Lumefantrine is active in the digestive vacuole (Combrinck et al., 2013), but it might also target a structure in the cytosol, which would explain its heightened efficacy when ejected from the digestive vacuole (Veiga et al., 2016).

Besides mutations, higher gene copy numbers of *pfmdr1* render AL treatment failure more likely (Venkatesan et al., 2014) and induce resistance to mefloquine and higher IC50 values for DHA, artesunate, quinine, and halofantrine *in vitro* (Price et al., 2004). They are common in Southeast Asia but occur rarely in Sub-Saharan Africa (Venkatesan et al., 2014).

## 1.6 Rwanda

Rwanda is a small country in East Africa, sharing borders with Burundi, the Democratic Republic of Congo, Uganda, and Tanzania. Malaria is transmitted throughout the year, with peaks during two rainy seasons from October to November and from March to May. The study area was the Huye district in the south of Rwanda. It has a population of approximately 390,000 people and is located at an average altitude of 1700 m on the central plateau of Rwanda.

Rwanda has an obligatory health insurance system. Access to health services is provided by community health workers in the villages, in community centers, and at primary health posts. In case of more severe illness, health centers refer patients to district hospitals, and these to university or referral hospitals. Due to strong efforts in both vector control and treatment, malaria cases have declined in recent years. In 2017, 5,940,533 malaria cases were confirmed and 376 people died, while in 2020 a total of 2,043,392 malaria cases and 149 deaths were reported to WHO (Figure 5) (WHO, 2021). In the Huye district, malaria prevalence data are available from a few years ago. In 2010, 11.7% of PCR-positive malaria cases were found among 749 mostly asymptomatic children (Gahutu et al., 2011). In 2014, 22.4% of 1089 school children between 6-10 years old were malaria positive by PCR (Sifft et al., 2016).

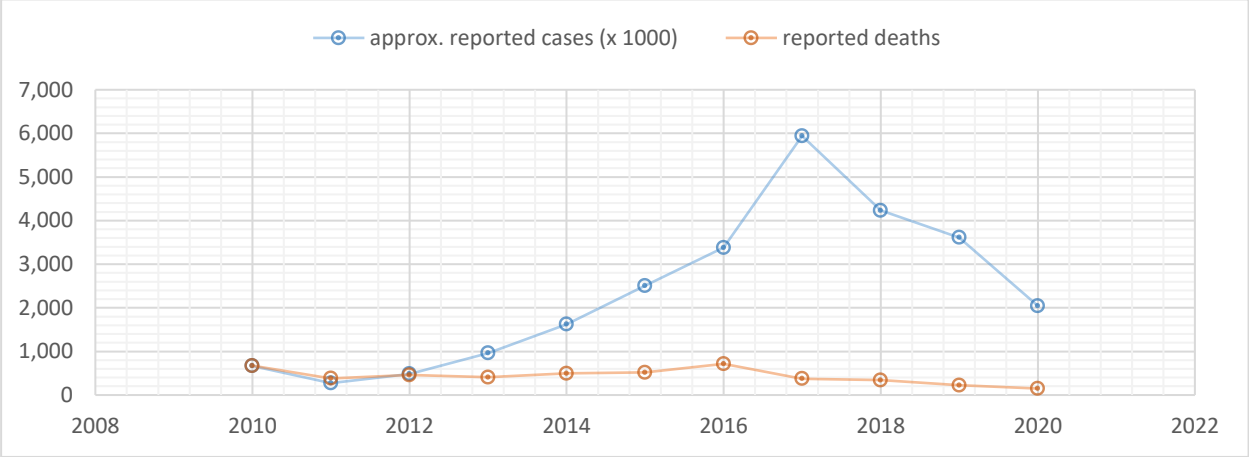


**Figure 5**: Malaria cases and deaths in Rwanda. Data based on the WHO world malaria report 2021 (WHO, 2021)

Since 2006, the first line treatment for uncomplicated malaria is AL, mainly given on a community-based level by community health workers who confirm the diagnosis with rapid diagnostic tests and distribute medication (Karema et al., 2020). Malaria treatment is provided by the government for those with low income. The community treatment system allows patients to be diagnosed and treated quickly. Indoor residual spraying with insecticides in households is performed regularly (twice yearly since 2009). 4.8 million insecticide-treated bed nets were delivered in 2020 (WHO, 2021).

Due to the easy access and rapid treatment distribution in the communities, drug pressure is very high on the local parasite population. Previous antimalarial resistance in Africa has often first been shown in East Africa before spreading across the continent (Mita et al., 2009). If ART resistance spreads, Rwanda could be among the first countries in Africa to see this emergence.

Previous studies by our research group have found signs suggesting a high artemether-lumefantrine drug pressure on the local parasites and their slow adaptation. In 2011 isolates from 104 children in the Huye District were characterized and showed a high prevalence of *pfmdr1* Y184F (>50%) and the NFD haplotype (38.5%) (Zeile et al., 2011). *Pfk13* non-synonymous SNPs including a candidate mutation for resistance, P574L, were later found from 2010 to 2015 (Tacoli et al., 2016).

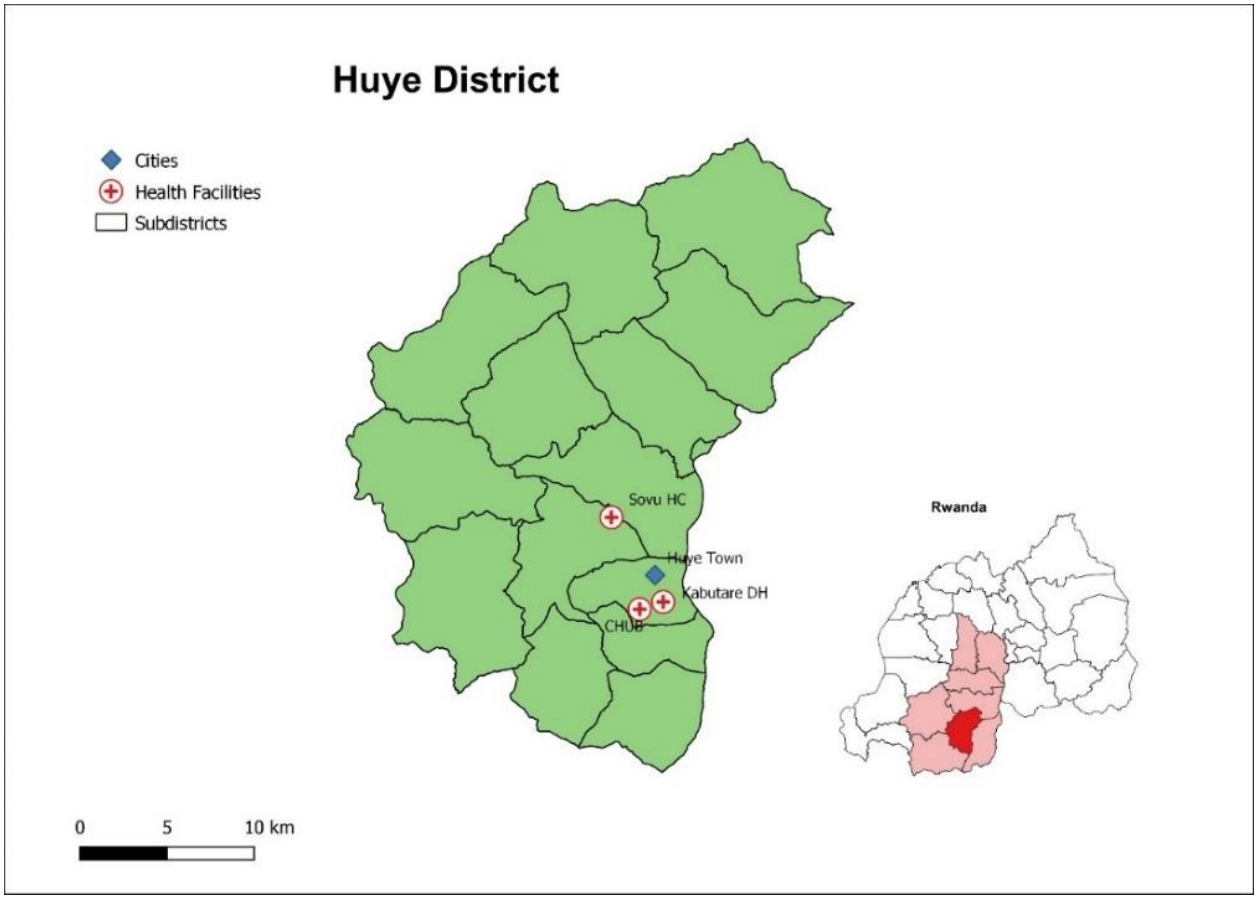


**Figure 6**: Study area with health facilities, where patient recruitment or sample processing took place. Source: Clara Bergmann

## 1.7 Study aim

Antimalarial resistance has historically emerged in Southeast Asia and spread to Sub-Saharan Africa after decades of use of certain drugs, and artemisinin resistance has already been confirmed in Southeast Asia. High drug pressure and previously reported sporadic candidate *pfk13* mutations in the study area necessitate continued surveillance in Rwanda. With this observational study, we aimed at assessing the prevalence of molecular markers of artemisinin resistance and their resistance characteristics after 15 years of AL use in Huye, southern Rwanda. Resistance characteristics such as day 3 positivity, *in vitro* survival, and IC50 values were investigated. To gain an understanding of the trend of emerging mutations in recent years the results were compared to previous research in the study area.

Objective 1: To genotypically characterize *Plasmodium falciparum* isolates from patients with uncomplicated malaria for *pfk13* and *pfmdr1*.

Objective 2: To test whether patients present with persistent parasitemia on day three of ACT treatment.

Objective 3: To establish parasite cultures and to test whether isolates with relevant mutations show prolonged survival rates in *in vitro* RSAs and elevated IC50 values.

# 2 Materials and Methods

## 2.1 Personal contribution

The research project in 2019 was a collaboration between CHUB and IIH and built on the preparation and activities of the 2018 research team. Together with Welmoed van Loon, I was responsible for implementing and coordinating the study in Rwanda from September to December 2019. We prepared materials and protocols in Berlin and took care of the material transport to Rwanda and sample shipment to Berlin. We trained the study team and managed the finances and materials in Rwanda. I did the patient data entry. Following sample shipment to Berlin, I did DNA extraction, *Plasmodium* species real-time PCR, and *pfk13* and *pfmdr1* typing in collaboration with Welmoed van Loon. I prepared the first manuscript of the paper on *pfk13* data and revised the other manuscripts on *pfmdr1* data and the *in vitro* resistance experiments.

## 2.2 Patient enrolment and management

The study sites were Sovu Health Center and Kabutare district hospital in Huye, Southern Province of Rwanda. The Rwanda National Ethics committee gave ethical approval. Patient recruitment took place from March to June 2018 and from September to December 2019.

Before conducting the study, the team was trained in the necessary procedures, and Standard Operating Procedures were made available to everyone involved. Patients presenting with non-severe malaria diagnosed by rapid diagnostic tests were asked to participate in the study and to give their informed consent. Consent forms were provided in Kinyarwanda, the local language, and a study nurse informed each patient about the aims and procedures of the study. Inclusion criteria were age above one-year, positive rapid diagnostic test, and fever at present or within the last 48 hours. Study participation was voluntary, and patients could withdraw their agreement at any time. All members of the research teams treated the obtained information as confidential.

**Figure 7**: Sovu Health Center, where malaria patients were recruited. Photo: Clara Bergmann

A laboratory technician confirmed the diagnosis by microscopy of a Giemsa-stained thick blood smear. A study nurse measured and documented axillary temperature and height, weight, and mid-upper arm circumference and took a blood sample. A maximum of 8 ml venous blood was collected into one ethylene diamine tetra-acetic acid (EDTA) and one acid citrate dextrose (ACD) tube. For backup, the collected whole blood was used to obtain dried blood spots (DBS) on filter paper. Hemoglobin was measured using a HemoCue photometer.

A study doctor saw each patient for an interview and clinical examination to check for signs of severity and document additional symptoms. Malaria treatment was provided. Non-pregnant patients received AL (Coartem), the current first-line treatment in Rwanda (Ministry of Health Rwanda, 2013), and pregnant patients in the first trimester received quinine. The first dose was given under observation. In case of vomiting within 30 minutes, a second dose was given to ensure adequate absorption. A community worker interviewed each patient and noted demographic and socio-economic factors, as well as malaria history and preventive measures. Patients were asked to return to the health center to check for persisting parasitemia on thick blood smears after three days of treatment.

## 2.3 Laboratory procedures

**Table 3**: List of solutions

| Solutions | Chemicals |
|---|---|
| freezing solution | 3.0% sorbitol<br>28.0% glycerol<br>0.65% Sodium chloride (NaCl) in distilled water |
| thawing solution | 12% and 1.6% NaCl in distilled water |
| complete culture medium | 10.43 g/l RPMI-1649 medium powder<br>2 g/l D-glucose<br>292 mg/l L-glutamine<br>5 g/l Sodium bicarbonate ($NaHCO_{3)}$<br>6 g/l HEPES<br>50 mg/l Gentamicin<br>27 mg/l Hypoxanthine powder<br>5 g/l Albumax II in distilled water |
| washing medium | 500 ml RPMI 1640<br>50 ng/ml gentamicin |

### 2.3.1 Blood smear preparation, staining, and microscopy

Thick and thin blood films were prepared by adding one drop of whole blood to a microscopy slide. The blood was then spread by another slide to either thinly fan out at the end to produce a thin smear or spread in a circle for a thick smear. Blood smears were air-dried, and thin smears were additionally fixed on the glass slide with methanol for 30 seconds. Slides were stained with 5-10% Giemsa staining for 20 minutes. Staining was rinsed with fresh water and the film was dried. Blood films were observed under the microscope with immersion oil.

Thick smears were used for diagnosis. Thin smears were used to determine parasite species and parasitemia by experienced microscopists at CHUB. Specific parasitic morphology of the different *Plasmodium* species allows for differentiation on a blood smear. Parasitemia was counted by two trained microscopists per 200 White Blood Cells (WBC), assuming a mean leukocyte count of 8.000 WBCs per microliter of blood.

### 2.3.2 Serum collection and cryopreservation of isolates

The full blood samples were taken to the laboratory of the University Teaching Hospital of Butare (CHUB), Rwanda, for further processing. Whole blood aliquots from ACD tubes were centrifuged, and the serum and buffy coat were taken off and stored separately at -20°C. The red blood cell pellet was then washed three times with washing medium, diluted in an equal volume of freezing solution (added dropwise to prevent lysis of the RBCs), and frozen at -80°C. At the end of the collection periods, frozen samples were shipped on dry ice to the Institute of International Health (IIH), Berlin, Germany.

### 2.3.3 DNA extraction and *Plasmodium* species assessment

At IIH, DNA was extracted from 200 uL whole blood aliquots and dried blood spots using the QIAGEN DNA blood mini kit and following the company's instructions. Previous students at IIH typed samples from 2018 for the *Plasmodium* species *P. falciparum, P. vivax, P. ovale, P. malariae,* with a nested PCR assay (Snounou et al., 1993), while we typed samples from 2019 with a real-time multiplex PCR assay, commercially available from TIB Molbiol, Berlin, Germany.

### 2.3.4 Nested polymerase chain reaction for *pfk13* and *pfmdr1* amplification

A nested PCR was used for multiple experiments for this thesis, allowing for the exact amplification of *Plasmodium falciparum* genes with commercially available *Taq* polymerase and primers. With a thermocycling machine, the DNA strands are first split in the denaturation step, primers adhere to DNA strands (called annealing) and the *Taq* polymerase elongates the DNA. The temperature and run time of the program was only slightly adjusted for the different experiments according to respective protocols.

A nested PCR was used to amplify the *pfk13* gene of *Plasmodium falciparum* samples from codons 441 to 688 (Ariey et al., 2014). The *pfmdr1* gene was amplified in two regions by nested PCR assays from codons 61 to 236 and codons 1023 to 1288 following an established protocol (Li et al., 2014). Successful amplification of the PCR products for *pfk13* and *pfmdr1* was assessed on 1% and 1.3% agarose gel electrophoresis, respectively and samples were sent for Sanger sequencing (Eurofins Genomics, Ebersberg, Germany).

### 2.3.5 Thawing of cryopreserved isolates

Cryopreserved isolates were carefully thawed to avoid RBC lysis for further culturing. First, the volume of the frozen sample was estimated. A fifth of the estimated volume of 12% NaCl thawing solution was added and gently mixed for three minutes. The suspension was transferred to a 50ml conal tube and 10x the RBC volume of 1.6% NaCl thawing solution and complete culture medium (CM) was added dropwise. The sample was centrifuged, and the supernatant was discarded. It was then washed two times in 5ml CM, before resuspending in 3ml CM and adding uninfected O+ RBCs.

### 2.3.6 Percoll density gradient centrifugation and Ring-Stage Survival Assay

Isolates with relevant *pfk13* mutations were thawed and cultured for *in vitro* characterization with a 0-3-hour post-invasion RSA following an established protocol (Witkowski, 2013). Once the parasites grew steadily for 2 weeks, they were considered culture adapted. RSAs require initial synchronization of the parasite cultures, which was done by double 5% sorbitol treatment followed by heparine addition (15 U/ml of sodium heparin in 3ml CM) and Percoll density gradient centrifugation to synchronize parasites to the late schizont stage. For Percoll density gradient centrifugation, the heparinized culture was layered carefully on the 70% Percoll solution and centrifuged for 15 minutes at 1100xg. The upper medium layer was aspirated and discarded, then the schizont layer was transferred to a new tube and washed 3 times with CM. The pellet was cultured with unparasitized erythrocytes for 3 hours. Percoll synchronization was evaluated on smears and deemed successful if ring-parasitemia was above 0.5%. Sorbitol (5%) was used to clean away the remaining schizonts.

For RSA, the parasitemia was set to 1% by adding noninfected erythrocytes, and hematocrit was modified to 2% by adding CM. Experiments were done in triplicate on 24-well plates. For 6 hours a pulse treatment of 700 nmol/l DHA or dimethyl sulfoxide (DMSO) for controls was added to parasite cultures. Afterward, to remove DHA, cultures were washed three times with CM. Exposed and nonexposed parasite isolates were cultured for three days.

Parasite survival rates were determined per ≥10,000 RBCs on thin blood films stained with 10% Giemsa staining. The mean parasite survival rates of the triplicates were calculated. RSA survival rates were determined by dividing the parasite density of DHA-treated parasites by the density of non-treated parasite cultures. Growth rates after 72

hours above 1.5 x in nontreated isolates and more than 3 independent triple experiments per isolate provided grounds for evaluation of the results.

### 2.3.7 IC50 determination for different DHA concentrations

IC50 determination was done on synchronized ring-stage parasites twice or three times and in more than three independent runs. The parasites were treated with a range of DHA concentrations (0 – 1 µmol/l). Growth assessment was done using a whole-cell SYBR Green I-based fluorescence assay (Machado et al., 2016). The intercalating dye adheres to double-stranded DNA, which is absent in erythrocytes, allowing for parasite growth quantification. 100µl of SYBR Green I staining was added to 96 well plates, incubated for one hour, and washed and fluorescence was measured with a FilterMax F5 micro-plate reader. The IC50 was then estimated based on a 4-parameter fit dose-response curve.

### 2.3.8 SNP Barcoding for origin determination

In 2014 Preston and colleagues proposed SNP barcoding as an effective tool to assess the origin of *Plasmodium* parasites (Preston et al., 2014). They identified 23 SNP sites at highly conserved areas with no recombination in extra-nuclear DNA of the apicoplast (relict plastid) and mitochondrion of *Plasmodium*. Taking these 23 SNPs, 34 distinct haplotypes were identified and traced to different origins (East Africa, West Africa, Southeast Asia, Oceania, and South America). Most haplotypes were unique to a region, whereas some were seen in multiple regions. The overall predictive accuracy was 92.1%. 23 SNP locations were amplified by PCR using pre-defined primers and protocols. The SNPs are covered by 10 PCR amplicons, referred to as *mt* and *apico1-9*. Primer sets for these amplicons were used, and the PCRs were run for 35 cycles. The SNPs were sequenced and used to match the isolates with the described haplotypes (Hu et al., 2021).

## 2.4 Data analysis and visualization

**Table 4**: Software and data resources used for data analysis and visualization

| | **Software and data resources** |
|---|---|
| *pfk13* reference alignment | Geneious Prime version 2020.1 |
| *pfmdr1* reference alignment | CodonCode Aligner version 4.2.5 |
| statistical analysis | R version 3.6.3 |
| data entry | Microsoft Excel Version 2013 |
| map creation | QGIS Version 3.16.11 – Hannover |
| Geographical data for maps | Openstreetmap, www.openstreetmap.org |
| country border data for maps | GADM 3.6, https://gadm.org/download_country.html |
| source for reference genes | PlasmoDB, plasmodb.org |
| *pfk13* reference | Pfk13 PF3D7_1343700 |
| *pfmdr1* reference | Pfmdr1 PF3D7_0523000 |

Collected data were entered into Microsoft Excel Version 2013. R version 3.6.3 was used to statistically analyze the data. In all statistical tests, a p-value < 0.05 was considered statistically significant.

### 2.4.1 Evaluation of sequencing results

To determine the prevalence of *pfk13* mutations for 2019 isolates, sequencing data were aligned to the lab strain 3D7 reference. The chromatogram was first preprocessed to obtain high-quality reads and exclude inaccuracies. Then the locations of SNPs different from the reference were identified.

### 2.4.2 Determination of *pfk13* prevalence development

To evaluate whether *pfk13* mutations are increasing in Huye or occur at similar low prevalence rates, published data from the study area obtained in 2010, 2014, and 2015 were used (Tacoli et al., 2016) and related to the 2019 *pfk13* prevalence results. The trend over time in the study area was calculated with a binomial logistic regression model.

### 2.4.3 *Pfmdr1* analysis

To assess the most common *pfmdr1* genotypic profiles of the 2018 and 2019 isolates, the samples were Sanger sequenced for *pfmdr1* mutations. Reference alignment was done with the same prepositions as for *pfk13*.

*Plasmodium* infections in one person can be caused by multiple strains simultaneously present in the bloodstream, also called a multiplicity of infection (MOI). Taylor and coworkers argue that for proper comparison of resistance distribution at different study sites or time points, not marker prevalence (i.e., the proportion of blood samples carrying a specific mutation) but marker frequency (i.e., the proportion of parasite clones carrying said mutation) should be determined (Taylor et al., 2014). They developed a Bayesian statistical method to take varying levels of MOIs into account, which we used to calculate haplotype frequency. The 2018/2019 SNP and haplotype prevalence data were then compared to *pfmdr1* typing data from Huye from 2010 by Fisher's exact test (Zeile et al., 2011).

### 2.4.4 RSA survival rate calculation

For RSA survival rates the dose-response curve package of R version 3.6.3 was used. IC50 values of the NF54 lab strain and clinical isolates were compared by Student *t*-test.

### 2.4.5 Map creation

The maps for this thesis (see Figure 6 and Figure 9), were created by Clara Bergmann with QGIS Version 3.16.11 – Hannover under the GNU General Public License using data freely available from OpenStreetMap and country spatial data from GADM 3.6.

# 3 Results

## 3.1 Descriptive statistics

From March to June 2018 and September to December 2019, 295 patients were included in our study based on a positive rapid diagnostic test for malaria and a history of fever or fever at present. Of these, 259 were found malaria positive by PCR. 234 of 259 samples (90.3%) tested positive for *Plasmodium falciparum* by PCR, 13 samples were positive for *P. ovale* (5.0%), 24 samples were positive for *P. vivax* (9.3%) and *P. malariae* was found in 30 samples (11.6%). Of the patients with *P. falciparum* malaria, 50.6% were female (118/233) and 8 of these women were pregnant. The median age was 17.5 years ranging from 1 to 73 years of age. 37.2°C (standard deviation [SD] ±1.3°C) was the mean temperature of all patients.

**Table 5**: Patient characteristics and behavior in 2018 and 2019

| **Parameter** | **2018** | **2019** |
|---|---|---|
| Number (N°) of included patients | 205 | 90 |
| Malaria positive by PCR | 183 | 76 |
| Mean age | 17 years (range: 1.5 – 80) | 18 years (range: 2-69) |
| N° female patients (proportion) | 108 (59.7%) | 39 (51.3%) |
| N° of pregnant patients | 3 | 5 |
| Mean axillary temperature | 37.2°C | 37.4°C |
| Mean hemoglobin | 12.7 g/dl (range: 6.9 -19.1) | 13.5 g/dl (range: 8.0-18.4) |
| Reported bed net usage last night | No data available | 60 (66.7%) |
| Antimalarial intake <2 weeks prior | 8 (3.9%) | 7 (7.8%) |

Source: Clara Bergmann

After three days of receiving their treatment, 222 patients tested negative on blood smear for malaria. 71 patients did not come back. Two returning patients had a positive blood smear for malaria. In 2018, one patient presented with low day 3 parasitemia of 6/200 WBCs. The *pfk13* sequencing data for this patient were not available. In 2019, one patient presented after three treatment days with persisting parasitemia of 31,520 parasites/µL without any symptoms. Sequencing data revealed that this patient did not harbor a *pfk13* variant parasite, and of patients with a variant parasite, none tested positive after receiving treatment for three days.

One pregnant patient presented twice during the 2019 study period and was initially given AL because she was in her second trimester. The first sample showed no mutation and after receiving her treatment, she tested negative on a blood smear after three days. Interestingly, on her second visit three weeks later, *pfK13* R561H parasites were detected in the sample, and she was treated with quinine.

## 3.2 *Pfk13* typing results

Of the isolates from 2019, 66 out of 67 *P. falciparum* samples (98%) were successfully amplified and sequenced for the *pfk13* gene. After alignment, we found 5 different non-synonymous single nucleotide polymorphisms (SNPs) in 8 different isolates including 3 isolates with the polymorphism R561H. Also, C469F, V555A, G533A, A578S, and A675V were found in one isolate respectively. To evaluate the development in recent years, we compared data on reported *pfk13* SNP prevalence from previous studies from our research group in the same area to our current study (see Table 6). Tacoli and coworkers investigated blood samples obtained from school children with malaria from the Huye District in 2010, 2014, and 2015 and analyzed them for *pfk13* mutations. (Tacoli et al., 2016). When correlating the prevalence of *pfk13* mutations with the 2019 data), the increase of polymorphisms in the number of isolates over the past 9 years was significant (OR 1.4, 95% CI 1.1–1.8; p = 0.003).

**Table 6**: Trend in *Plasmodium falciparum K13* nonsynonymous single nucleotide polymorphisms (nsSNPs) over the course of 9 years, in Huye, southern Rwanda.

| | **2010** | **2014** | **2015** | **2019** |
|---|---|---|---|---|
| Number of sequenced isolates (% of nsSNPs) | 75 (0) | 81 (2.5%) | 66 (4.5%) | 66 (12.1%) |
| nsSNPs found | None | V555A<br>A626S | **P574L**<br>**A675V**<br>D648 | **R561H*** (3x)<br>**C469F**<br>**A675V**<br>V555A<br>G533A<br>A578S |

Bold: candidate markers for artemisinin partial resistance. asterisk*: validated resistance mutation. Table adapted from Bergmann et al., 2021, and Tacoli et al., 2016.

## 3.3 *Pfmdr1* typing results

The analysis of *pfmdr1* was done on both samples from 2018 and 2019 with a total of 212 out of 234 isolates (90.6%) successfully sequenced (van Loon et al., 2021). The SNP 86Y was found in 3.8% of the samples. Mutations at 184F were prevalent in more than half of the samples (52.8%), while only very few isolates exhibited 1246Y (2.3%). Meanwhile, we found the allele combination NFD more often (50%) in the samples than NYD (44%). Compared to samples collected in 2010 (Zeile et al., 2011), the proportion of NYD increased significantly from almost 20% to 44%, while the prevalence of other haplotypes significantly decreased in that period (see Table 7).

Of the 212 samples sequenced, 38 (17.9%) had an MOI >1 in at least one of the investigated loci. When we considered this in secondary analysis, the haplotype frequency was also high at 39% for NFD and 56% for NYD (for more details, see table 2 in van Loon et al., 2021).

**Table 7**: Observed prevalence of *pfmdr1* polymorphisms and allele combinations in Huye, Rwanda, in 2010 and 2018/2019.

| ***Pfmdr1* allele or allele combination** | **2010(12) N= 104 (%[n])** | **2018 & 2019 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 |

Mutations are marked in bold. *asterisk marks proportions which were significantly different in 2018/2019 to 2010. Source: van Loon et al., 2021

### 3.4 RSA, IC50 results, and SNP barcoding

The isolates with possibly relevant *Pfk13* mutations were culture-adapted for RSA. Unfortunately, not all clinical isolates with mutations could be successfully adapted to the culturing environment. Thawing and culturing were successful for four isolates with *Pfk13* mutations A675V, C469F, and V555A, and R561H. Two *Pfk13* wild-type isolates from Rwanda did not grow enough in culture to use as controls for the RSA. Thus, we used the artemisinin-susceptible lab strain NF54, which originates presumably in Africa. RSA survival rates were below one percent for NF54 (0.2 % ± 0.1 %) and V555A (0.3% ± 0.1 %). Survival rates above one percent for seen for R561H (4.7 % ± 1.5%), A675V (1.4 % ± 0.2 %), and C469F (9.0% ± 1.6%) (see Figure 8). Corresponding to these RSA results, mean IC50 values for treatment with DHA were lower for artemisinin-susceptible NF54, resulting in 4.2 ± 0.5 nmol/l and V555A (3.4 ± 0.3 nmol/l). C469F isolates had a mean IC50 to DHA of 6.9 ± 1.5 nmol/l, A675V of 7.4 ± 3.3 nmol/l, and the experiments with R561H yielded an IC50 of 14.1 ± 4.0 nmol/l for R561H (see Figure 8).

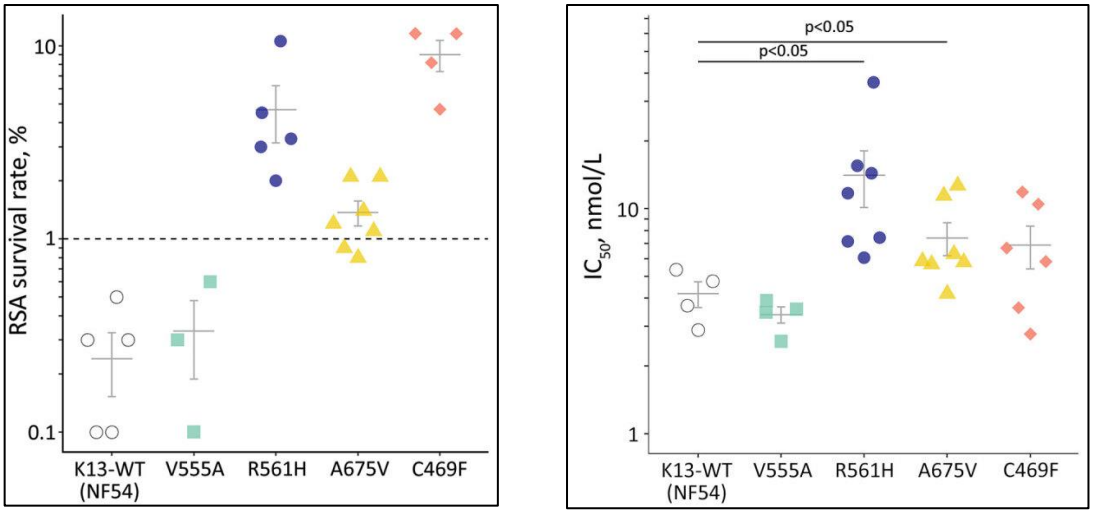


**Figure 8**: RSA survival rates (%) and IC50 values of *pfk13* wildtype NF54 strain and 4 clinical isolates with *pfk13* mutations. One data point depicts the mean of three experiments. Source: van Loon et al., 2022

SNP barcoding was done to determine the origin of the isolates. Haplotype 9 was expressed by the isolate carrying R561H and is associated with either West or East African origin. All other isolates displayed haplotype 22, which demonstrates East African origin (Preston et al., 2014).

# 4 Discussion

## 4.1 Short Summary

In Rwanda, artemether-lumefantrine (AL) is the artemisinin combination therapy (ACT) used for uncomplicated malaria since 2006. Home-based management with community health workers diagnosing malaria with rapid tests and antimalarial medication readily available lead to high drug pressure on the parasites. Previous results showed candidate *pfk13* markers present in Rwanda and *pfmdr1* mutations suggestive of high AL drug pressure (Zeile et al., 2011; Tacoli et al., 2016). We aimed at updating our knowledge on the current prevalence of molecular resistance markers in Huye, southern Rwanda by characterizing the genotypic *pfk13* and *pfmdr1* profile of *Plasmodium* parasites from patients with uncomplicated malaria and assessing the *in vitro* resistance phenotype. Due to a long-standing collaboration with the university hospital in Huye in Rwanda, we can compare data from patients recruited in 2018 and 2019 to data from patients recruited in 2010 and 2015 from the same study area and see the temporal effects.

In 2019, we genotyped *Plasmodium falciparum* samples from 90 patients and detected *pfk13* single nucleotide polymorphisms (SNPs) in 12.1% of successfully sequenced samples (8/66). Importantly, we found the mutation R561H in 4.5% of the isolates but no association with persistent parasitemia on day 3 blood smears. Compared to previous results from Huye, the prevalence of nsSNPs in the *pfk13* gene has significantly increased over the past 10 years ($p = 0.003$). While in 2010 no *pfk13* mutations were observed, in 2015 first candidate markers were detected (Tacoli et al., 2016; Bergmann et al., 2021). Further *in vitro* characterization of our isolates revealed elevated RSA mean survival rates for R561H, C469F, and A675V. SNP barcoding determined their African origin (van Loon et al., 2022).

In 2010, Zeile and colleagues assessed genetic resistance markers in 104 *P. falciparum* positive isolates from children in the Huye district and typed them for antimalarial resistance markers, including *pfdhfr*, *pfdhps, pfcrt,* and *pfmdr1*. For *pfmdr1*, the samples predominantly exhibited the haplotype NFD (almost 40%) and NYD (almost 20%) (Zeile et al., 2011). Our samples show a drastic and statistically significant increase with almost 45% exhibiting NYD and 50% of NFD.

## 4.2 *Pfk13* mutations in Rwanda and their significance

The proportion of *pfk13* mutations is increasing in Huye in southern Rwanda. Until recently, ART-resistance-associated *pfk13* mutation prevalence in local parasite populations was regarded as widespread and fixed in Southeast Asia and low to nonexistent in Sub-Saharan Africa (Amato et al., 2016; Ménard et al., 2016; Ocan et al., 2019). In Rwanda, the current prevalence is much higher. In line with our study results, another study in Rwanda showed a prevalence of 6.9% *pfk13* polymorphisms in samples collected from 2013 – 2015 (Uwimana et al., 2020) while more recently 13 – 14% of isolates already carried R561H (Stokes et al., 2021; Uwimana et al., 2021).

### 4.2.1 De novo emergence or import of artemisinin resistance?

Historically, mutations conferring resistance to pyrimethamine and chloroquine have developed in Asia and then spread to Africa. Multiple genome-wide analyses have shown that ART resistance mutations currently seen on the African continent seem to have emerged indigenously (Amato et al., 2016; Uwimana et al., 2020). In accordance, SNP barcoding showed the African origin of our isolates (van Loon et al., 2022).

### 4.2.2 *Pfk13* R561H: detection in Rwanda

The mutation R561H is a validated resistance marker in Southeast Asia. The WWARN consortium showed its association with delayed parasite clearance (WWARN Group, 2019). In Africa, its significance remained to be elucidated. It has been detected in three African countries: the Democratic Republic of Congo (Ménard et al., 2016), in Tanzania (Bwire et al., 2020; Moser et al., 2021), and Rwanda. Studies show a hotspot in Rwanda with its prevalence ranging from 0.7% to as high as 21.9% in different locations (Uwimana et al., 2020, 2021; Bergmann et al., 2021; Stokes et al., 2021; Straimer et al., 2021). For an overview, see Figure 9 and Table 8.

In Huye, we did not observe an association of *pfk13* R561H with day 3 parasitemia, but in other Rwandan locations, evidence for clinical resistance has been reported. In 2018, a therapeutic efficacy study in Rwandan children with *P. falciparum* malaria treated with AL showed a prevalence of *pfk13* R561H in 13% of 218 pretreatment samples and day 3 parasitemia in more than 10% of the isolates at two of the three Rwandan sites. In Masaka, day 3 parasitemia was significantly associated with the presence of R561H mutations. Still, drug efficacy measured on day 28 was high ranging from 93.8 to 97.2%

corrected by PCR (Uwimana et al., 2021). In another trial, additional *pfk13* characterization showed 21.9% (16/73) of samples from the Rwandan capital city Kigali carrying *pfk13* R561H, and they were associated with prolonged parasite clearance half-lives of more than 5 hours (6 of 9 patients) and day 3 parasitemia (5 of 9 patients). AL treatment efficacy after 28 days remained high at 94.4% (Straimer et al., 2021).

*In vitro* data remain scarce for clinical isolates from Africa. In gene-edited lab strains of putative African origin and expressing R561H, RSA levels have been elevated to a mean of 6.6% similar to our isolates, while the most important Southeast Asian SNP C580Y expressed in the African lab strain 3D7 averaged a 4.8% survival rate. In contrast with C580Y, R561H did not show a fitness cost in 3D7 (Stokes et al., 2021).

In summary, R561H seems to be the most important mutation spreading in Rwanda. The data available suggest that it has the properties to confer *in vitro* ART resistance at an equal level as the most important mutation C580Y in Southeast Asia, fulfilling the WHO criteria for a validated resistance marker also on the African genetic background.

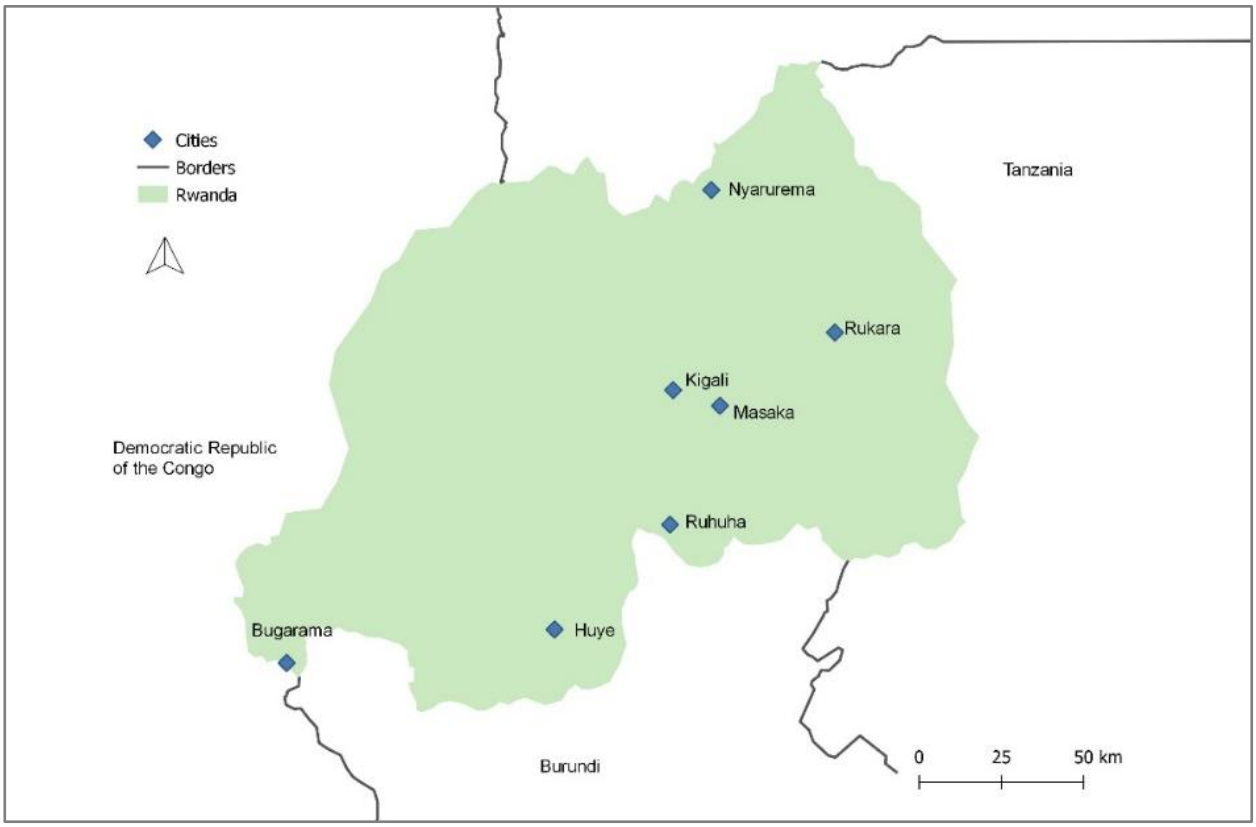


**Figure 9**: Sites in Rwanda, where research on antimalarial treatment efficacy and resistance markers was carried out. Source: Clara Bergmann

**Table 8**: Detected *Plasmodium falciparum* K13 (*pfk13)* mutations associated with artemisinin resistance in different sites (see Figure 9) in Rwanda from 2012 to 2019 and data on clinical treatment outcome.

| Study site | Study years | R561H | Candidate SNPS | Clinical outcome | Reference |
|---|---|---|---|---|---|
| **Nyarurema (n=73)** | 2012-2015 | 0 | 469F (1) | Data combined from 4 locations (Kibirizi + Bugarama): D3+: 2/413 isolates | (Uwimana et al., 2020) |
| **Rukara (n=134)** | 2012 - 2015 | 1 | none | 15/334 recrudescent infections | (Uwimana et al., 2020) |
| **Masaka/ Ruhuha (n=257)** | 2013-2015 | 19 (7.4%) | 469Y (1), P574L (1) | PCR corrected efficacy**: 95.2% (AL treatment) | (Uwimana et al., 2020) |
| **Huye (n=66)** | 2015 | 0 | A675V (1), P574L (1) | No data available | (Tacoli et al., 2016) |
| **Masaka (n=51)** | 2018 | 10 (20%) | P441L (1), 449A (1) | PCR corrected efficacy*: 97.0% D3+: 8/51 (16%) associated w/ *pfk13* mutations | (Uwimana et al., 2021) |
| **Rukara (n=82)** | 2018 | 8 (22%) | P441L (3), 449A (3), 469F (3) , P574L (2) | PCR corrected efficacy*: 93.8% D3+: 12/82 (15%) | (Uwimana et al., 2021) |
| **Bugarama (n=85)** | 2018 | 0 | P441L (1), 449A (1), 469Y (1), 469F (1) | PCR corrected efficacy*. 97.2% | (Uwimana et al., 2021) |
| **Huye (n=66)** | 2019 | 3 (4.5%) | 469F (1), A675V (1) | 1 D3+ (no *pfk13* mutation) | (Bergmann et al., 2021) |
| **Kigali (n=73)** | 2018-2019 | 16 (21.9%) | 469F (3), P574L (1) | Slow parasite clearance >5h (in 6/9 *pfk13* mutant), D3+:5/9 PCR corrected efficacy*: 94.4% | (Straimer et al., 2021) |

D3+ = parasitemia on day 3 of treatment. PCR-corrected efficacies were assessed on day 28 (*) or 42(**) of treatment.

### 4.2.3 Other *pfk13* mutations

In neighboring Uganda, recently published studies have also shown East African artemisinin resistance associated with *pfk13* candidate markers C469Y and A675V. In

240 malaria patients treated with intravenous artesunate monotherapy recruited from 2015 to 2019, *pfk13* mutations A675V (11.5% in 2019) and C469Y (4.2% in 2019) were detected and exhibited *in vivo* delayed parasite clearance (> 5 hours) and elevated RSA survival for A675V (Balikagala et al., 2021). In 2021, in 99 malaria patients treated with artemether-lumefantrine, the isolates from northern Uganda showed decreased susceptibility (elevated IC50) to DHA and lumefantrine. The RSA results confirmed increased parasite survival for isolates with the C469Y mutation. Interestingly, analysis of growth inhibition assays on previously collected isolates from 2015-2021 showed an association between C469Y and decreased lumefantrine susceptibility (Tumwebaze et al., 2022),

A675V was also detected in Rwanda at a low prevalence and expressed elevated *in vitro* RSA survival rates. At the same locus but with a different polymorphism, the candidate marker C469F showed elevated *in vitro* RSA results for our isolate.

#### 4.2.4 *Pfmdr1* assessment

The artemisinin partner drugs are meant to clear all remaining parasites from the bloodstream and preserve an effective treatment. Reduced *pfmdr1* genetic diversity and NFD/NYD haplotype predominance in our isolates are indicative of a possible selection for parasites with reduced AL susceptibility and increased risk for recrudescence after AL treatment (Venkatesan et al., 2014; van Loon et al., 2021).

The high proportion of NFD/NYD in our isolates adds to evidence from other locations in Rwanda with also NFD (43%) and NYD (48%) haplotypes being predominant. In those samples, they were not associated with recrudescence of infection or selected for in post-treatment isolates yet (Uwimana et al., 2021).

### 4.3 Limitations and strengths

Limitations of our study include a rather small sample size and a confined area of patient recruitment with only two health facilities included. Treatment compliance was not assessed and drug concentrations in the bloodstream of our patients were not measured. Treatment efficacy was only assessed on thick blood smear after 3 days of treatment following recommendations (Stepniewska et al., 2010) and not all patients returned. *Pfk13* typing results were not available for the 2018 isolates. Trend assessment is biased since we compare our mostly adult population to children, and malaria prevalence has

changed over the years. RSA results of isolates harboring *pfk13* nsSNPs were only compared to a lab strain and not to clinical isolates. Additionally, not all relevant isolates could be successfully cultured.

The strengths of our study include that the data were collected at the same facilities at different time points enabling us to highlight trends in the prevalence of genetic markers. Our isolates were characterized in-depth by both typing genetic markers as well as generating *in vitro* data, the latter being very rare for African strains.

## 4.4 Implications for further research

### 4.4.1 What data are missing?

The interplay of transmission intensity, immunity, and spread of resistant strains is not well understood, but immunity has a relevant impact on parasite clearance. A study in Mali has shown that antibodies present in the bloodstream led to shortened parasite half-life when treated with i.v. artesunate (Lopera-Mesa et al., 2013). In Southeast Asia in a multinational cohort, plasma antibody levels, and parasite clearance are inversely correlated, with partially immune patients being able to clear *pfk13* mutant parasites faster than wild-type parasites (Ataide et al., 2017). It will be interesting to see a comparable study in Africa, where transmission intensity and immunity levels differ.

Also, partner drug resistance needs to be studied further. As an example, only *pfmdr1* has been identified so far as a possible marker of lumefantrine resistance. Further study into the genetic background and possible other resistance markers should be carried out. Background markers influencing artemisinin resistance in Southeast Asia include for example *pfmdr2*, *arps*, ferredoxin, and *pfcrt* (Miotto et al., 2015). In Uganda, *pfcoronin* and *falcipain2a* mutations were associated with elevated RSA survival (Tumwebaze et al., 2022)

### 4.4.2 What are possible next steps?

Resistant *P. falciparum* with *pfk13* and *pfmdr1* mutations are becoming more widespread in Rwanda endangering ACT efficacy. Thus, the current treatment with AL is in danger of emerging treatment failure. WHO recommends policy changes if treatment failure occurs in more than 10% of patients for 28 to 42 days, post-treatment (WHO, 2020). According to the most recent data from a therapeutic efficacy study, this is not yet the case in

Rwanda (Uwimana et al., 2021). Nonetheless, continued efforts are required, and possible strategies need to be developed to evade the spread of clinical resistance. Strategies could include new combinations of ACTs, for example, artesunate-amodiaquine. A review has shown inverse selective pressure on *pfmdr1* variant parasites when using artesunate-amodiaquine and AL. Multiple first-line therapies or regularly changing drugs could be a way of reducing continuous selective pressure on *Plasmodium* parasites (Okell et al., 2018). Alternatively, adding a third component to current ACTs might be a way to reduce resistance formation (White, 2019). In a recent study, the efficacy and safety of triple ACT therapies including DHA-piperaquine + mefloquine and AL + amodiaquine were tested in the GMS and the Democratic Republic of Congo. The combinations were well-tolerated and highly effective (van der Pluijm et al., 2020). New antimalarials with distinct modes of action should be deployed when available.

One major problem is the time lag between sample collection and published data on resistance. The implementation of a comprehensive molecular surveillance system could bring us closer to real-time monitoring (Nsanzabana, 2021). This should include ART resistance markers but also monitoring of partner drug resistance. Even so, molecular data cannot replace results from therapeutic efficacy studies, which are needed before introducing policy changes.

Developing a response strategy to emerging ART resistance while continuing vector control measures and resistance surveillance will be a big challenge for the public health sector in endemic countries in Africa, especially during the ongoing COVID-19 pandemic.

# 5 Conclusion

The present research aimed to update the knowledge on molecular resistance marker prevalence in Huye, southern Rwanda. Based on molecular data from isolates collected in 2019, we showed the prevalence of *pfk13* mutations conferring artemisinin resistance. Additionally, we observed a predominance of *pfmdr1* wildtype parasites possibly conferring decreased lumefantrine susceptibility. This adds on to evidence of emerging *pfk13* mutations and *pfmdr1* haplotypes in other locations in Rwanda (Uwimana et al., 2020, 2021).

We are also able to show that the parasite population has changed from 2010 to 2018/2019 and seems to have adapted to the drug pressure. Treatment remains efficacious in sight of the limited available data of our day 3 parasitemia results and one therapeutic efficacy study in the country (Uwimana et al., 2021). Nonetheless, the *in vitro* experiments show that the mutations in Rwandan isolates do confer resistance. These data are extremely worrisome.

Besides continued surveillance and resistance characterization, an adequate response strategy needs to be developed to enable quick deployment of countermeasures when treatment failure increases or spreads. As a first step, the introduction of triple artemisinin combination therapies could slow this development. Alternatively, other artemisinin combination therapies could be introduced to reduce the selection of resistant parasites.

Emerging resistance to artemisinin combination therapy endangers the recent successes of malaria control in endemic regions. A combination of different measures and global collaboration also in pandemic times is urgently needed to respond to emerging ART resistance in Africa.

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# Eidesstattliche Versicherung

„Ich, Clara Bergmann, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema:

Resistenz-Entwicklung von *Plasmodium falciparum* gegen Artemether-Lumefantrin in Süd-Ruanda
Trends in Artemether-Lumefantrine resistance of *Plasmodium falciparum* in Southern Rwanda

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Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

# Anteilserklärung an den erfolgten Publikationen

Clara Bergmann hatte folgenden Anteil an den erfolgten Publikationen:

Publikation 1: Bergmann, C., van Loon, W., Habarugira, F., Tacoli, C., Jäger, J.C., Savelsberg, D., Nshimiyimana, F., Rwamugema, E., Mbarushimana, D., Ndoli, J., Sendegeya, A., Bayingana, C., Mockenhaupt, F.P., 2021. Increase in kelch 13 polymorphisms in plasmodium falciparum, southern Rwanda. Emerging Infectious Diseases 27, 294–296.

Beitrag im Einzelnen: In Ruanda war ich zusammen mit Welmoed van Loon Ansprechpartnerin und Koordinatorin der Studie. Wir waren zuständig für die Planung, die Durchführung, das Training des Teams, Rekrutierung der Patienten und Logistik der Materialien und Proben. Ich habe die Datenerfassung in Excel durchgeführt. In Deutschland habe ich in Kollaboration mit Welmoed van Loon im Labor an der Extraktion der DNA, der Durchführung der PCR und der Gelelektrophorese gearbeitet und ca. die Hälfte der Proben verarbeitet. Bei der Datenauswertung habe ich mitgeholfen. Ich habe die Literaturrecherche durchgeführt sowie den ersten Entwurf des Manuskripts verfasst. Dabei habe ich den Entwurf für die Tabelle verfasst.

Publikation 2: van Loon, W., Bergmann, C., Habarugira, F., Tacoli, C., Savelsberg, D., Oliveira, R., Mbarushimana, D., Ndoli, J., Sendegeya, A., Bayingana, C., Mockenhaupt, F.P., 2021. Changing pattern of Plasmodium falciparum multi-drug resistance-1 gene polymorphisms in southern Rwanda. Antimicrobial Agents and Chemotherapy 65 (9): e0090121.

Beitrag im Einzelnen: Zusätzlich zu den Proben aus Publikation 1 wurden weitere im Jahr 2018 gewonnene Proben untersucht. Bei einem Teil dieser Proben und der Proben aus Publikation 1 habe ich die Extraktion der Plasmodien-DNA aus den Blutproben, Durchführung der PCR und Gel-elektrophorese zur *pfmdr1* Charakterisierung durchgeführt. Das Manuskript habe ich überarbeitet.

Publikation 3: van Loon, W., Oliveira, R., Bergmann, C., Habarugira, F., Ndoli, J., Sendegeya, A., Bayingana, C., Mockenhaupt, F.P., 2022. In Vitro Confirmation of Artemisinin Resistance in Plasmodium falciparum from Patient Isolates Southern Rwanda, 2019. Emerging Infectious Diseases 28(4), 852-855.

Beitrag im Einzelnen: Die Proben aus Publikation 1 wurden hier weiter charakterisiert, an deren Gewinnung und Verarbeitung ich beteiligt war (s. Publikation 1). Zudem habe ich das Manuskript überarbeitet.

_____

Unterschrift, Datum und Stempel des erstbetreuenden Hochschullehrers

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Unterschrift der Doktorandin

## Article 1

Bergmann, C., van Loon, W., Habarugira, F., Tacoli, C., Jäger, J.C., Savelsberg, D., Nshimiyimana, F., Rwamugema, E., Mbarushimana, D., Ndoli, J., Sendegeya, A., Bayingana, C., Mockenhaupt, F.P., 2021. Increase in kelch 13 polymorphisms in plasmodium falciparum, southern Rwanda. *Emerging Infectious Diseases* 27, 294–296. DOI: https://doi.org/10.3201/eid2701.203527

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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DOI: https://doi.org/10.3201/eid2701.203527

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 (ethylenediamine-

tetraacetic 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. Separately testing 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*).

**Acknowledgments**

We are grateful to the staff of Sovu Health Centre and Kabutare District Hospital for their collaboration and help for over 10 years.

This study was financially supported by grant GRK2046 from the German Research Foundation (DFG), which also supported W.L.C. Bergmann was supported by DFG grant GRK2290. The funding bodies had no role in designing the study, collecting, analyzing, or interpreting data, or writing the manuscript.

## 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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## Article 2

van Loon, W., Bergmann, C., Habarugira, F., Tacoli, C, Savelsberg, D., Oliveira, R., Mbarushimana, D., Ndoli, J., Sendegeya, A., Bayingana, C., Mockenhaupt, F.P., 2021. Changing pattern of *Plasmodium falciparum multi-drug resistance-1* gene polymorphisms in southern Rwanda. *Antimicrobial Agents and Chemotherapy*. 65(9)
DOI: https://doi.org/10.1128/AAC.00901-21

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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,

**Citation** van Loon W, Bergmann C, Habarugira F, Tacoli C, Savelsberg D, Oliveira R, Mbarushimana D, Ndoli J, Sendegeya A, Bayingana C, Mockenhaupt FP. 2021. Changing pattern of *Plasmodium falciparum pfmdr1* gene polymorphisms in southern Rwanda. Antimicrob Agents Chemother 65:e00901-21. https://doi.org/10.1128/AAC.00901-21.



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**Received** 30 April 2021
**Returned for modification** 19 May 2021
**Accepted** 28 June 2021
**Accepted manuscript posted online** 6 July 2021
**Published** 17 August 2021

**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.

## ACKNOWLEDGMENTS

We are grateful to the staff of Sovu Health Centre and Kabutare District Hospital for their collaboration and help.

This study was financially supported by grant GRK2046 from the German Research Foundation (DFG) to W.V.L. and C.T. C. Bergmann was supported by DFG grant GRK2290. The funding bodies had no role in designing the study, collecting, analyzing, or interpreting data, or writing the manuscript.

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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## Article 3

van Loon, W., Oliveira, R., Bergmann, C., Habarugira, F., Ndoli, J., Sendegeya, A., Bayingana, C., Mockenhaupt, F. P., 2022. In Vitro Confirmation of Artemisinin Resistance in Plasmodium falciparum from Patient Isolates, Southern Rwanda, 2019. *Emerging infectious diseases*, 28(4), 852–855.
DOI: https://doi.org/10.3201/eid2804.212269

DISPATCHES

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

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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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DOI: https://doi.org/10.3201/eid2804.212269

## 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

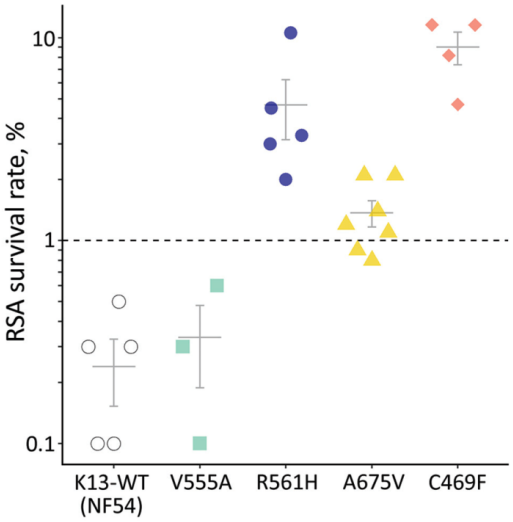


**Figure 1.** RSA 0–3-hour postinvasion survival rates (%) of an artemisinin-susceptible, *K13* WT *Plasmodium falciparum* strain (NF54) and 4 *P. falciparum* patient isolates from Rwanda with *K13* mutations. Each data point represents the mean of triplicate experiments. Isolate growth rates were only 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.

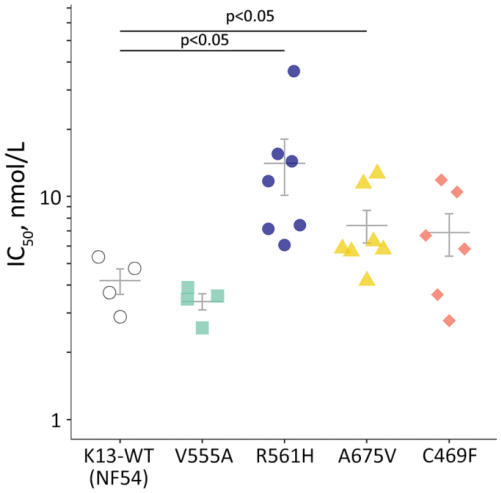


**Figure 2.** $IC_{50}$ values for dihydroartemisinin for an artemisinin-susceptible, *K13* WT *Plasmodium falciparum* strain (NF54) and in 4 *P. falciparum* patient isolates from Rwanda with *K13* mutations. Indicated error bars display the mean ± SE. p values were determined by Student *t*-test. $IC_{50}$,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

## Acknowledgments

We are grateful to the staff of Sovu Health Centre and Kabutare District Hospital for their collaboration and help. We thank Megan Peedell and Katja Puestow for their support in the laboratory.

The German Research Foundation financially supported this study through grants to W.v.L. (GRK2046) and R.O. and C.B. (GRK2290). The funding bodies had no role in designing the study, collecting, analyzing, or interpreting data, or writing the manuscript.

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## Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

## Complete Publications List

1. van Loon, W., Oliveira, R., **Bergmann, C**., Habarugira, F., Ndoli, J., Sendegeya, A., Bayingana, C., Mockenhaupt, F. P., 2022. In Vitro Confirmation of Artemisinin Resistance in Plasmodium falciparum from Patient Isolates, Southern Rwanda, 2019. *Emerging infectious diseases*, 28(4), 852–855. Impact Factor 16.126

2. van Loon, W., **Bergmann, C.**, Habarugira, F., Tacoli, C, Savelsberg, D., Oliveira, R., Mbarushimana, D., Ndoli, J., Sendegeya, A., Bayingana, C., Mockenhaupt, F.P., 2021. Changing pattern of *Plasmodium falciparum multi-drug resistance-1* gene polymorphisms in southern Rwanda. *Antimicrobial Agents and Chemotherapy*. 65(9) Impact Factor 5.938

3. **Bergmann, C**., van Loon, W., Habarugira, F., Tacoli, C., Jäger, J.C., Savelsberg, D., Nshimiyimana, F., Rwamugema, E., Mbarushimana, D., Ndoli, J., Sendegeya, A., Bayingana, C., Mockenhaupt, F.P., 2021. Increase in kelch 13 polymorphisms in plasmodium falciparum, southern Rwanda. *Emerging Infectious Diseases* 27, 294–296. Impact Factor 16.126

4. Friesen, J., **Bergmann, C.**, Neuber, R., Fuhrmann, J., Wenzel, T., Durst, A., Müller, M., Ignatius, R., 2019. Detection of Enterobius vermicularis in greater Berlin, 2007-2017: seasonality and increased frequency of detection. *European Journal of Clinical Microbiology and Infectious Diseases*.38(4):719-723. Impact Factor 2.837

5. Hommes, F., van Loon, W., Thielecke, M., Abramovich, I., Lieber, S., Hammerich, R., Gehrke-Beck, S., Linzbach, E., Schuster, A., von dem Busche, K., Theuring, S., Gertler, M., Martinez, G. E., Richter, J., **Bergmann, C**., Bölke, A., Böhringer, F., Mall, M. A., Rosen, A., Krannich, A., Keller, J., Bethke, N., Kurzmann, M., Kurth, T., Kirchberger, V., Seybold, J., Mockenhaupt, F.P. and BECOSS Study Group, 2021. 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. *International journal of environmental research and public health*, *18*(5), 2739. Impact Factor 4.614

## Acknowledgements

I want to thank my supervisor Prof. Frank Mockenhaupt for his invaluable supervision, guidance, and continuous support throughout this project in Rwanda and Berlin. I am gratefully indebted to his very valuable comments while writing the manuscript and this thesis.

I am grateful to IRTG2290 and the DFG for the financial support for taking part in this program, and for the opportunity to attend seminars and the conference in South Africa. Thank you to my fellow Ph.D. candidates and doctoral students in the program for including me in the group and making South Africa a very special memory.

A heartfelt thank you goes to the collaborating staff in Rwanda at Sovu Health Center, Kabutare district hospital, and CHUB for their invaluable contributions to the project.
I want to thank the wonderful team at IIH Berlin for the support in the lab, insights into tropical medicine, and the great friendly atmosphere.

I want to express my profound gratitude to Welmoed van Loon for both her valuable critical input, her infectious curiosity, and her friendship. Without her input and contribution, I could not have written this thesis successfully. Our teamwork both in Rwanda and in Berlin shaped my understanding of working in science.

Finally, I am endlessly grateful to my family and friends for their encouragement and support throughout my studies and the process of researching and writing this thesis. Thank you to Doug and Julie for their feedback. Thank you to my mother Ulrike Baum-Bergmann for her unconditional support, to my twin sister Lena for always being there, and to Steffen for his great cooking and emotional support in the final phase of writing. To Philipp and Clara for their feedback and the great time spent together.

Only a few people can be named here, but so many more helped me on the way.
Thank you all.