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Antimalarial resistance in Mozambique: Characterization of molecular markers and assessment of *Plasmodium falciparum* susceptibility to Artemisinin based Combination Therapy

Clemente da Silva

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Antimalarial resistance in Mozambique: Characterization of molecular markers and assessment of *Plasmodium falciparum* susceptibility to Artemisinin based Combination Therapy

Autor: Clemente da Silva

Orientadora: Doutora Fátima Nogueira

Coorientadora: Doutora Sonia Enosse

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Dedication

To my beloved wife Nilza da Silva and son Teomário Clemente da Silva

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God gives us life and then lets us choose our own path. I have chosen to follow the path He has written, and I am so grateful for it! Of course, with my limitations and difficulties, but that is just part of the journey. I thank God for the gift of life, for guiding me with His wisdom, for placing the right people in my path at the right time. Everything that has happened to me along the way has been incredible, and I would not change a thing! I am so excited to see what the future holds, and I know that God is with me in every step of the way.

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RESUMO

Introdução: A malária continua a ser um dos mais graves problemas de saúde pública na África Subsaariana e Moçambique é o quarto maior contribuinte mundial, com 4,7% dos casos da doença e 3,6% do total de mortes devido à malária. O seu controlo assenta no combate ao vetor e no tratamento dos casos confirmados com medicamentos antimaláricos. A vigilância molecular da malária é um instrumento importante para monitorizar a propagação da resistência aos medicamentos antimaláricos. O principal objetivo deste estudo foi estudar a resistência aos ACTs através da caraterização de marcadores moleculares e da avaliação da suscetibilidade ex vivo do *Plasmodium falciparum* aos medicamentos antimaláricos administrados em Moçambique.

Métodos: Este projecto de tese consistiu em estudos transversais ao longo do pais incluindo ensaios de susceptibilidade do *P. falciparum* aos farmacos e a genotipagem. Os ensaios de suscetibilidade foram realizados em 43 amostras positivas para malária não complicada na província de Maputo entre maio e julho de 2022. Adicionalmente, o perfil dos polimorfismos de nucleotídeo único (SNPs) *pfk13* e *pfmdr1,* bem como a variação do número de cópias (CNV) *pfpm2* e *pfmdr1* de isolados fenotipicamente suscetíveis aos antimaláricos foi avaliado por sequenciação de Sanger e reação em cadeia da polimarase quantitativa em tempo real (qPCR), respetivamente. Paralelamente, com o objetivo de caraterizar marcadores moleculares de resistência aos antimaláricos, foi realizado um estudo transversal que recrutou ~600 participantes com infeção por malária detectada por Testes de Diagnóstico Rápido (TDR), de três locais de estudo diferentes (Niassa, Manica e Maputo) entre abril e agosto de 2021. O software SIFT (Sorting Intolerant from Tolerant) foi utilizado para prever se uma substituição de aminoácidos afecta a função da proteína Kelch 13.

Resultados: Quanto aos ensaios de susceptibilidade, as taxas de sobrevivência revelaram a ausência de parasitas sobreviventes quando expostos a 700 nM de diidroartemisinina (DHA), 200 nM de piperaquina (PPQ) e amodiaquina (AQ). As taxas foram inferior a 1%, 10% e 45%, que são os limiares para r*ing stage susceptibility assay* (RSA), *piperaquine susceptibility assay* (PSA) e *amodiaquine susceptibility assay* (AQSA), respetivamente.

Quanto à genotipagem, não foi detectada qualquer mutação validada no gene de resistência à artemisinina, *pfk13,* nos locais do estudo. No entanto, foram detectadas mutações não sinónimas com uma prevalência de 10,2%, 6% e 5% em Niassa, Manica e Maputo, respetivamente. 56,3% das mutações não-sinónimas registadas deveu-se à substituição na primeira base do codão, 25% na segunda base e 18,8% na terceira base. Além disso, 50% das mutações não-sinónimas apresentaram um *SIFTscore* inferior ao limiar, 0,05, pelo que se previu que fossem deletérias. Após a ddPCR detectou-se CNVs de *pfpm2* e *pfmdr1* em 5,7% (13/229) das amostras de alta qualidade.

Conclusão: Os parasitas em circulação nos locais estudados continuam a apresentar uma elevada sensibilidade aos antimaláricos, sem mutações associadas à resistência à artemisinina e ao seu parceiro. A monitorização contínua da suscetibilidade do parasita aos ACT e a vigilância molecular devem ser intensificadas para reduzir as hipóteses de resistência à artemisinina num futuro próximo.

Palavras-chave: *Plasmodium falciparum*, suscetibilidade *ex vivo*, resistência antimalárica, vigilância molecular da malária, Moçambique

ABSTRACT

Introduction: Malaria remains one of the most serious public health problem in sub-Saharan Africa and Mozambique is the world's fourth largest contributor, with 4.7% of disease cases and 3.6% of total deaths due to malaria. Its control relies on the fight against the vector and treatment of confirmed cases with antimalarial drugs. Malaria Molecular surveillance is an important tool for monitoring the spread of antimalarial drug resistance. The main goal of this PhD thesis was to study *Plasmodium falciparum* resistance to ACTs by characterizing molecular markers and assessing its *ex vivo* susceptibility to antimalarial drugs administered in Mozambique.

Methods: *Ex vivo P. falciparum* susceptibility assay was performed in 43 non-complicated positive malaria samples from Maputo province between May and July 2022. Additionally, the profile of *pfk13* and *pfmdr1* single-nucleotide polymorphisms (SNPs), as well as *pfpm2 and pfmdr1* copy number variation (CNV) from isolates phenotypically susceptible to antimalarials was assessed by Sanger sequencing and quantitative polimarase chain reaction (qPCR) respectively. Additionally, a cross-sectional study was conducted with the objective of characterising antimalarial resistance molecular markers. The study recruited approximately 600 participants with malaria infection, detected by rapid diagnostic tests (RDT), from three different study sites (Niassa, Manica and Maputo) between April and August 2021. To achieve the second objective, a novel, medium-throughput, quadruplex droplet digital PCR (ddPCR) assay was developed to simultaneously quantify the copy number of *pfpmp3*, *pfpmp2*, and *pfmdr1* loci in the clinical samples. Sorting Intolerant from Tolerant software was employed to predict the impact of amino acid substitutions on Kelch 13 protein function.

Results: Survival rates revealed the absence of surviving parasites when exposed to 700 nM of dihydroartemisinin (DHA), 200 nM of piperaquine (PPQ) and amodiaquine (AQ). The survival rate was less than 1%, 10% and 45%, which are the thresholds for RSA, PSA and AQSA, respectively. As for molecular characterization of resistance markers, no *pfkelch13*-mediated artemisinin resistance gene mutation was detected in our study settings. However, non-synonymous mutations were detected at prevalence of 10.2%, 6% and 5% in Niassa, Manica and Maputo, respectively. Most (56.3%) of the reported non-synonymous mutations were due to substitutions at the first base of the codon, 25% at the

second base and 18.8% at the third base. Additionally, 50% of non-synonymous mutations showed a SIFTscore below cut off value of 0.05, therefore, they were predicted to be deleterious. Following ddPCR and the application of quality control standards, we detected CNVs in 13 of 229 high-quality samples (prevalence of 5.7%). Overall, our study showed a low level of resistance CNVs present in the parasite population across all three-collection sites, including various combinations of *pfmdr1*, *pfpmp2*, and *pfpmp3* CNVs.

Conclusion: These results suggested that *P. falciparum* isolates from studied settings are still showing high sensitivity to antimalarial, with no mutations associated to artemisinin and its partner drug resistance. Continued monitoring of parasite susceptibility to ACTs and molecular surveillance should be intensified to reduce chances of artemisinin resistance in the near future.

Key words: *P. falciparum, ex vivo* susceptibility*,* antimalarial resistance, molecular surveillance, Mozambique

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ACT	Artemisinin-based combination therapy
AQ	Amodiaquine
AQSA	Amodiaquine Survival Assays
ddPCR	droplet digital Polimarase Chain Reaction
DHA	Dihydroartemisinin
DHS	Demographic and Health Survey
ELISA	Enzyme-Linked Immunosorbent Assay
IMASIDA	*Inquérito de Indicadores de Imunização, Malária e HIV/SIDA*
IRS	Indoor residual spraying
LLIN	Long-lasting insecticide-treated net
MISAU	*Ministério da Saúde*/Ministery of Health
PNCM	National Malaria Control Programme
PPQ	Piperaquine
PSA	Piperaquine Survival Assays
PVM	Parasitophorous vacuolar membrane
PY	Pyronaridine
qPCR	Quantitative Polimarase Chain Reaction
RDT	Rapid Diagnostic Tests
RSA	Ring-Stage Survival Assays
SIFT	Sorting Intolerant from Tolerant
SNP	Single Nucleotide Polymorphism
TABS	Targeted amplicon based next generation sequencing
WHO	World Health Organization

1. INTRODUCTIO

1.1. Malaria

Malaria remains one of the most serious public health problems in sub-Saharan Africa (Sato, 2021). The WHO considers malaria to be endemic in 85 countries worldwide (**Figure 1**). Between 2000 and 2023, 15 out of 25 countries that were malaria endemic in 2000 achieved 3 consecutive years of zero indigenous malaria cases were certified malaria free (WHO, 2023d).

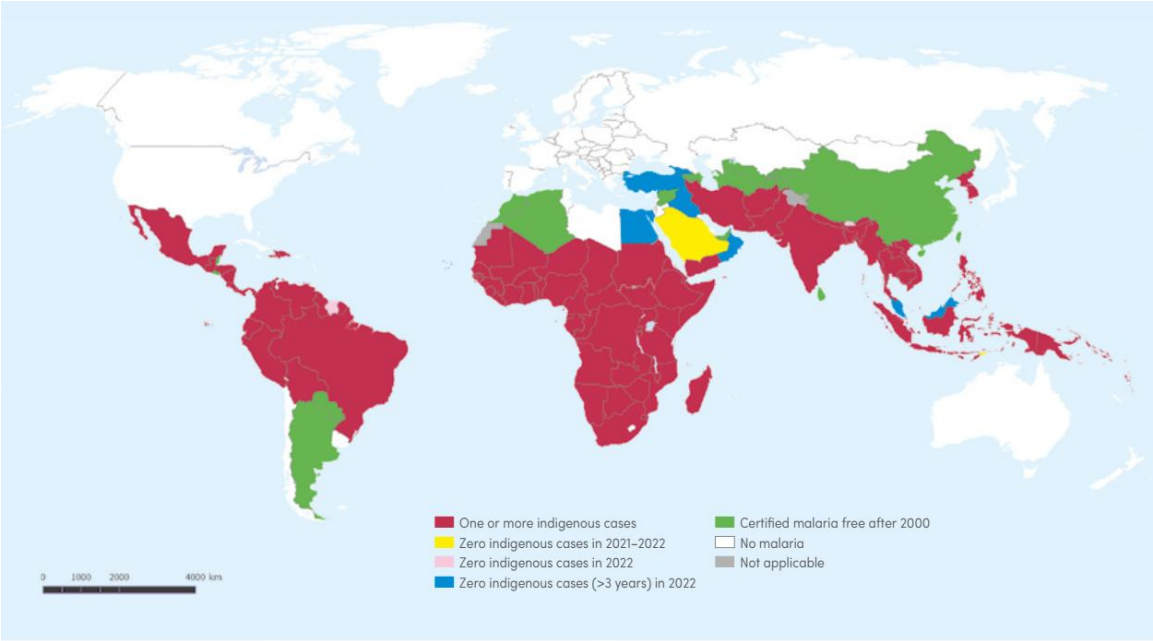

Figure 1: Countries with indigenous cases in 2022. Source (WHO, 2023d)

The latest global malaria report estimates that in 2022 there were 249 million cases and 608,000 deaths. There was an increase of 5 million cases compared with 2021. Four African countries including Mozambique, account for 48% of the global contribution in malaria cases. Mozambique is the fourth largest contributor representing 4.7% of reported cases and fifth in mortality with 3.6% of total malaria deaths (**Figure 2 and 3)** (WHO, 2022, 2023d).

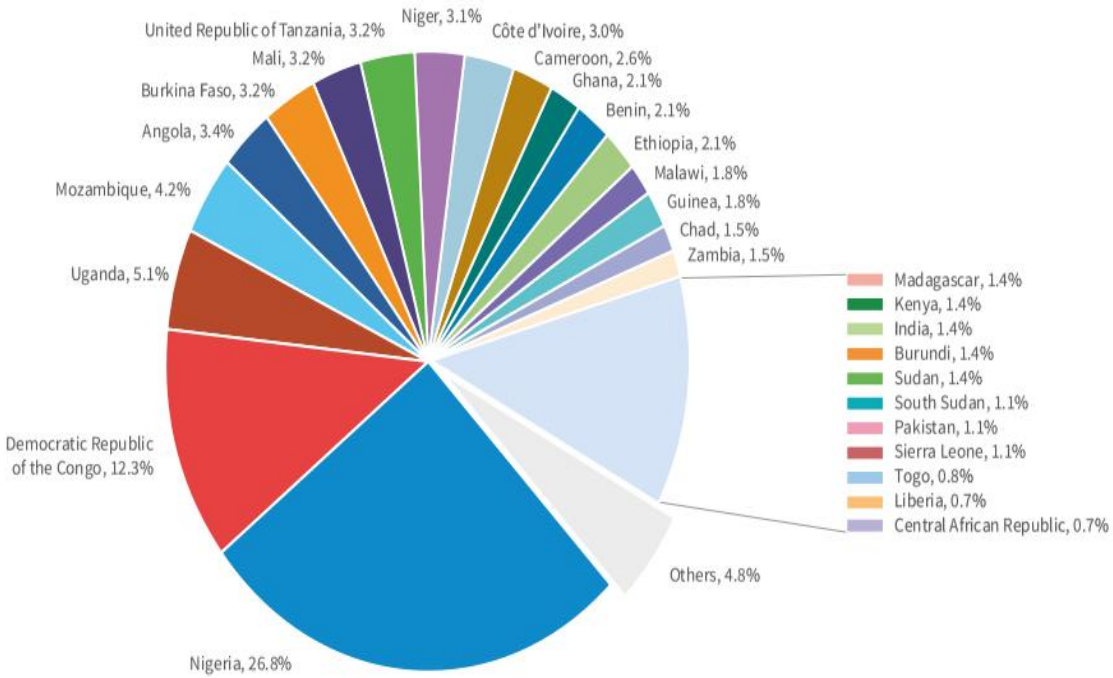

Figure 2: Estimated malaria cases in the WHO Region in 2022. Source (WHO, 2023d)

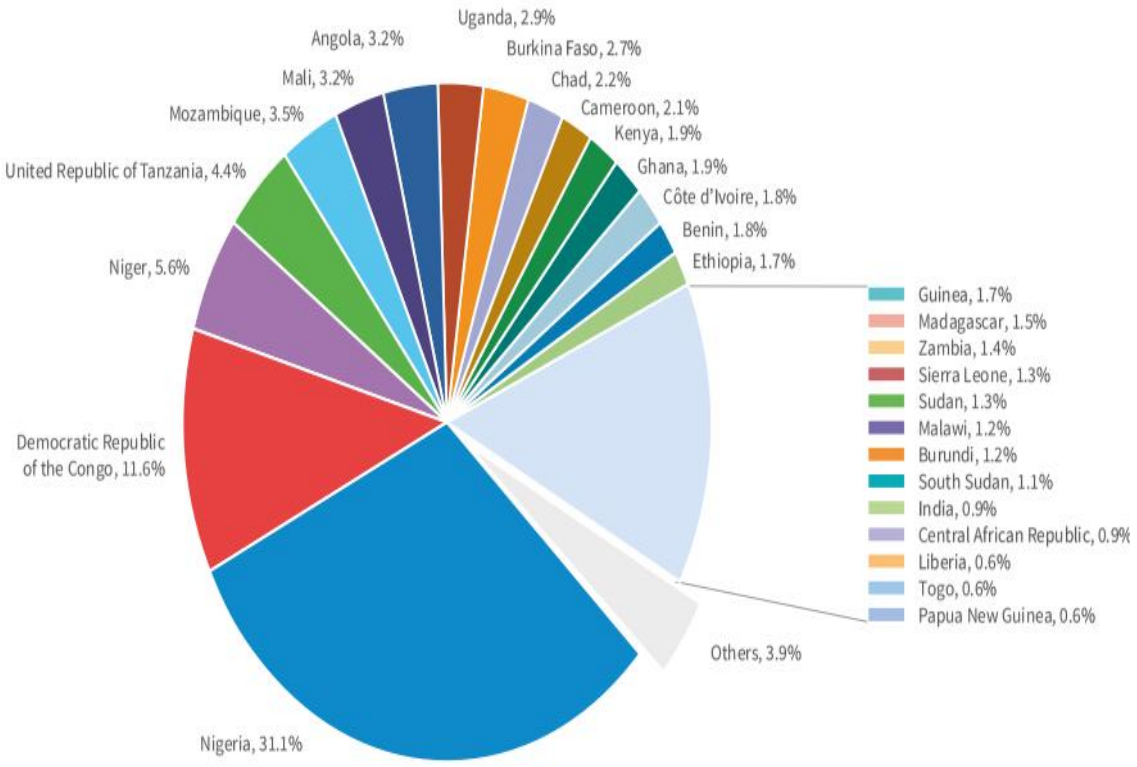

Figure 3: Estimated malaria deaths in the WHO Region in 2022. Source (WHO, 2023d)

1.2. Malaria in Mozambique

Mozambique is located on the east coast of Africa. The country is divided into three topographical regions by the Zambezi and Save Rivers, which together make up 11 provinces. The northern region, comprising the provinces of Niassa, Cabo Delgado and Nampula, is located north of the Zambezi River. It includes the narrow coastline that runs between the hills and the coast, as well as the mountains of Niassa, Namuli and the Macondes plateau. The central region, which includes the provinces of Zambezia, Tete, Sofala and Manica, is located between the Zambezi River and the Save River. The southern region, which includes the provinces of Gaza, Inhambane, Maputo and Maputo City, is situated to the south of the Save River and extends from the mountainous relief inland (with the Mashonaland platforms and the Lebombo Mountains) to the lowlands on the coast(INE, 2019).

The climate is tropical humid, with two distinct seasons: a hot, rainy season from November to March and a dry season from April to October (Arroz, 2016; INE, 2019).

However, it should be noted that climatic conditions depend on altitude. It is a fact that rainfall is heavy along the coast and decreases to the north and south. Annual rainfall varies from 500 to 900 mm depending on the region, with an average of 590 mm. It is also a fact that cyclones are common during the wet seasons (INE, 2019).

Malaria is endemic throughout the country, ranging from hyper-endemic zones along the coast, meso-endemic zones in the flatlands of the interior and some hypo-endemic zones in the highlands of the interior. Several factors contribute to this endemicity, from climatic and environmental conditions such as favourable temperatures and rainfall patterns, as well as favourable breeding sites for the vector. Most of the country has year-round transmission, with peaks during the rainy season, from December to April (INE, 2019).

According to the National Survey on Malaria Indicators that took place in 2018, there has been progress in malaria prevention between 2015 and 2018, where the distribution of mosquito nets treated with long-lasting insecticide increased from 37.9% to 51%, and the percentage of pregnant women covered in terms of medical follow-up and counselling increased from 22.4% to 40.6% (INE, 2019). In general, the prevalence of malaria at national level has not changed significantly, having fallen by one unit from 2015 (40%), 2018 (39%) (INE, 2019).

The latest Demographic and Health Survey (DHS) 2022-2023, reported that the prevalence of malaria at national level is 32%, a reduction of 7% compared to the average observed over the last 10 years (**figure 4).**

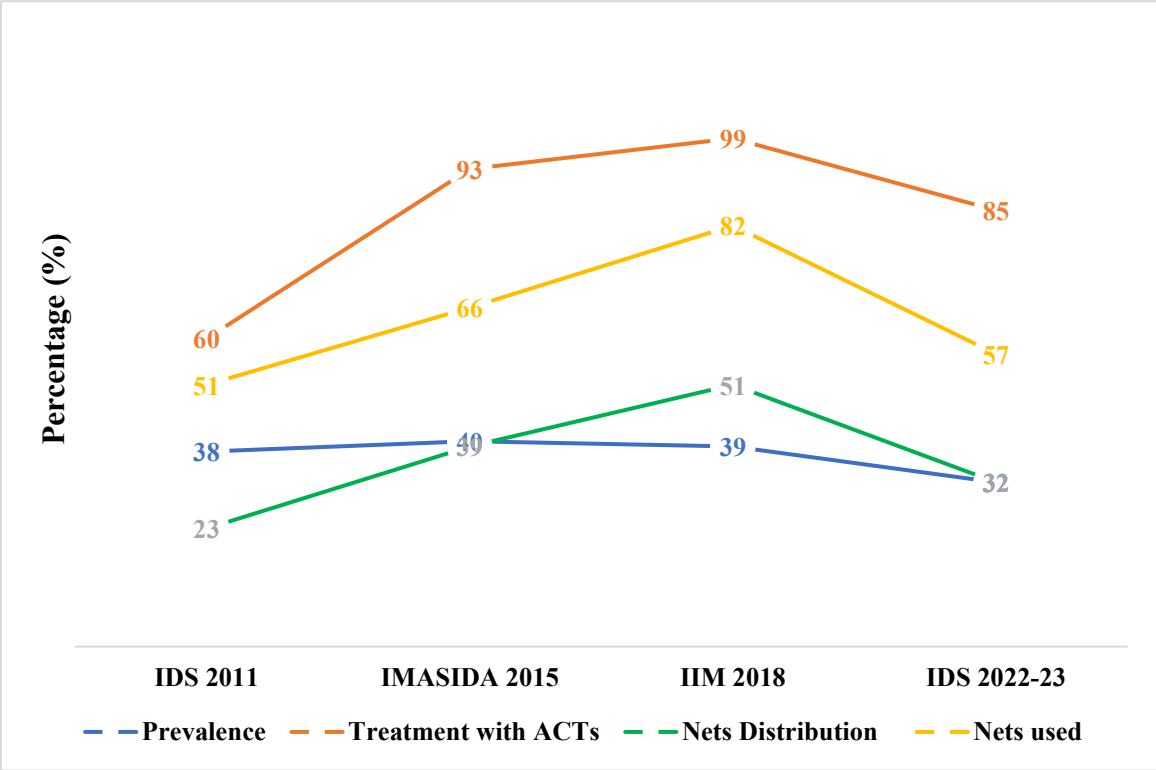

Figure 4: Malaria prevalence, ACTs and mosquito nets distribution across the country between 2011-2022. *IDS*- Demographic and Health Survey; *IMASIDA*- Malaria and HIV AIDS indicators survey; *IIM*- Malaria indicators survey.

The latest survey showed dramatic changes in the prevalence profile, where comparing to the last survey (INE, 2019) malaria prevalence has decreased in all provinces but Sofala and Nampula (INE, 2023). **Figure 5** depicts the prevalence of malaria in children under 5 years at provincial level (INE, 2023). Ironically, this reduction in prevalence comes at a time when the national coverage of mosquito net distribution, the use of mosquito nets and the treatment with ACTs of malaria cases have decreased (**figure 4**) (INE, 2023).

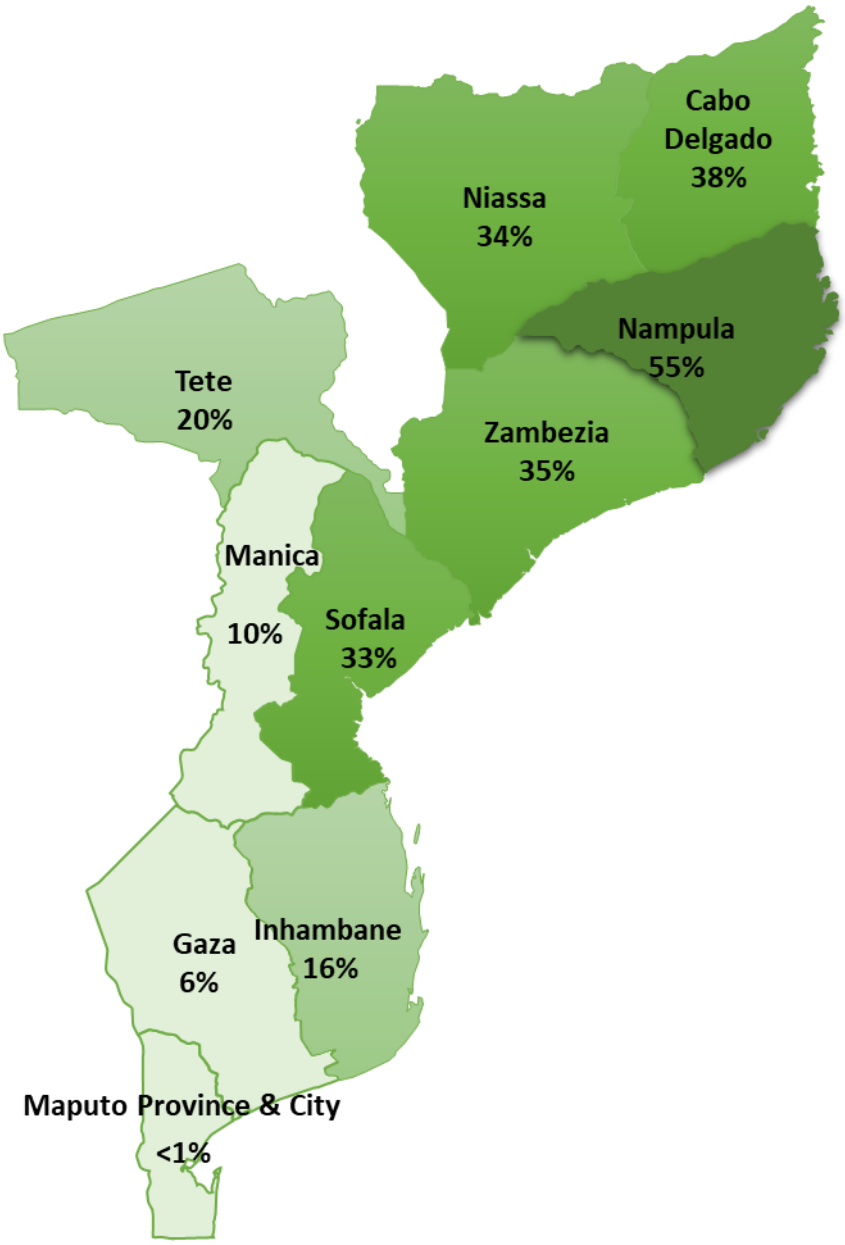

Figure 5: Prevalence of malaria in children under 5 years of age at provincial level, 2022/3. Maputo province and Maputo city represented the low prevalence settings, with approximately one percent prevalence each. Moderate prevalence settings are Gaza, Inhambane, Manica and Tete. The remaining provinces are higher in prevalence.

1.3. Etiological agent and vector

1.3.1. Malaria parasite systematic classification

The malaria parasite has the following taxonomic classification:

Kingdom: Protista

Phylum: Apicomplexa

Class: Haematozoa

Order: Haemosporida

Family: Plasmodiidae

Genus: *Plasmodium*

Species: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*

Malaria is caused by protozoan parasites from the genus *Plasmodium*. These parasites are transmitted to humans by the bite of an infected female Anopheles mosquito (Adedeji et al., 2020; Jansson et al., 2021). There are only five species of Plasmodium that infect humans and cause malaria worldwide, namely *P. falciparum, P. vivax, P. ovale* (divided into *P. ovale curtisi* and *P. ovale wallikeri*), *P. malariae* and *P. knowlesi*. The first four are specific to humans, while *P. knowlesi* is naturally maintained in monkeys and causes zoonotic malaria on a large scale in Southeast Asia (Sato, 2021). *P. falciparum* is the species that causes the majority of mortality and morbidity. It is responsible for more than 90 per cent of all malaria infections, while infections by *P. malariae and P. ovale* are observed in 9% and 1% respectively. The main malaria vectors in Mozambique belong to the *Anopheles funestus* and *Gambiae* groups (INE, 2019; MISAU, 2013).

1.4. *P. falciparum* Life cycle

The life cycle of *P. falciparum* is as follows: the parasite takes place in two hosts. The definitive host is an invertebrate (mosquito), where sexual reproduction takes place. The

intermediate host is a vertebrate (human), where asexual reproduction takes place (**Figure 6**) (Gujjari et al., 2022; White et al., 2014).

A *P. falciparum* infection begins with the injection of its infective forms, called sporozoites, into the human dermis during a female Anopheles mosquito blood meal. The sporozoites enter the vessels and migrate to the liver, where they leave the sinusoids through the endothelial cells and enter the hepatocyte. They actively invade the cell by crossing it until they find a suitable hepatocyte. They then form a parasitophorous vacuolar membrane (PVM) and undergo schizogony (Maier et al., 2019; Silvie et al., 2008; Sturm et al., 2006). Schizogony starts when a *Plasmodium* merozoite invades a red blood cell. Once inside, the merozoite first transforms into a ring-stage parasite, and then into a trophozoite. During this process, the parasite reshapes the host cell substantially and consumes its cytoplasm. This is followed by the schizont stage, during which the number of nuclei increases. Schizogony concludes with cellularisation and the release of the daughter cells. This process occurs 34–38 hours post invasion (hpi). A proportion of the asexually reproducing merozoites are reprogrammed to undergo gametocytogenesis.The gametocytes sequester and develop in the bone marrow. Once mature, they enter the peripheral circulation and are ingested by a mosquito, where they emerge as extracellular male and female gametes in the midgut (Chappell et al., 2020; Gujjari et al., 2022; Mok et al., 2015). The micro and macrogamete fuse to form a zygote, which transforms into an oocyst within 24 hours. This oocyst then migrates through the epithelium of the mosquito's midgut and encysts, undergoing asexual sporogenic replication (Ararat-Sarria et al., 2020; Chappell et al., 2020; Maier et al., 2019). The motile sporozoites are released into the haemocele by the rupture of the oocyst and pass into the salivary glands where they can be injected into the next human host (Maier et al., 2019).

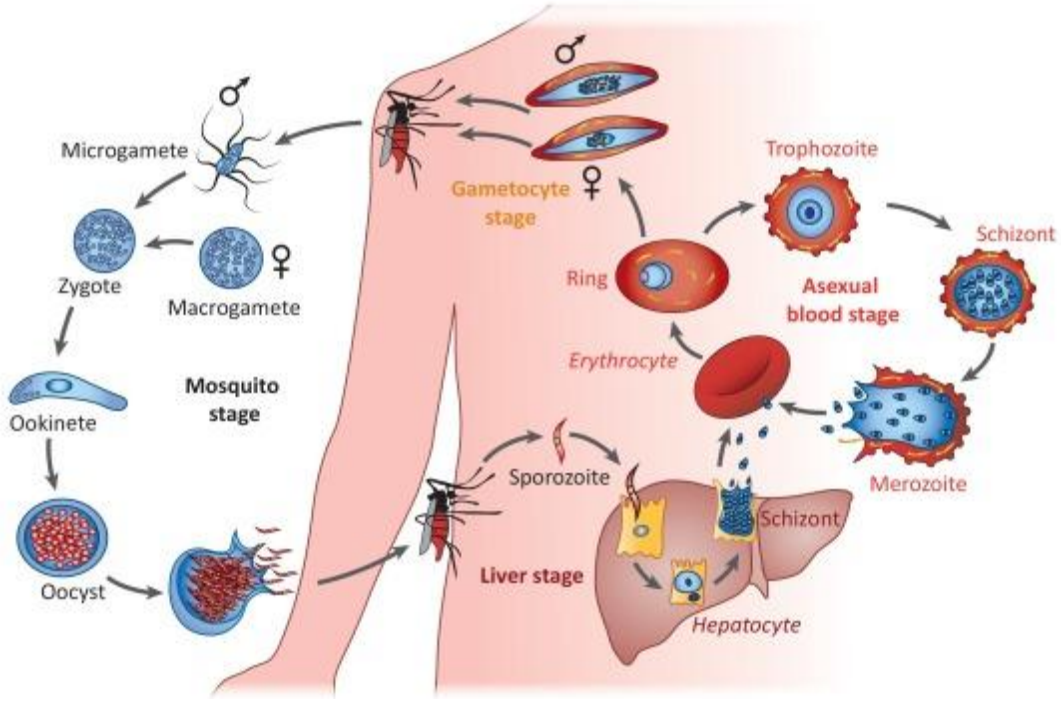

Figure 6: *P. falciparum* life cycle. Adapted from: (Maier et al., 2019)

1.5. Prevention

1.5.1. Vector Control

Vector control is a highly effective way of reducing malaria transmission and is a vital component of malaria control and elimination strategies (WHO, 2023b). The main vector control strategy implemented by Mozambique's National Malaria Control Programme (PNCM), which is in line with WHO recommendations, is universal coverage with long-lasting insecticide-treated nets (LLINs), which is complemented by indoor residual spraying (IRS), in several provinces. Historical and recent attempts to eliminate malaria in low transmission zones in southern Mozambique have been unsuccessful (Sharp et al., 2007). Residual malaria transmission persists in these areas, despite increased coverage with primary interventions: LLINs and IRS. (Galatas et al., 2020; Montoya et al., 2022).

1.5.2. Vaccine

Malaria vaccines have been developed using a number of different strategies. These include pre-erythrocytic vaccines, antibody-based subunit vaccines, vectored vaccines, whole sporozoite vaccines, genetically attenuated parasites, sporozoite subunit vaccines, erythrocytic vaccines, sexual phase vaccines, transmission blocking vaccines, and synthetic peptides and conjugate vaccines (Mills et al., 2021; Takashima et al., 2021). Two malaria vaccines are recommended for use in children living in moderate to high malaria transmission areas. Both can reduce uncomplicated and severe malaria by approximately 40% and 30% respectively (WHO, 2023c).

a) RTS,S vaccine

The first one, RTS,S, is a recombinant subunit vaccine expressed in yeast that uses the hepatitis B surface antigen to transport epitopes derived from the circumsporozoite (CSP) protein of *P. falciparum* (Ballou, 2009; Garçon et al., 2003). RTS,S induced a significant reduction in malaria in children living in areas of moderate to high malaria transmission 3 countries, namely Ghana, Kenya and Malawi (WHO, 2022; World Health Organization, 2021). The immune response induced by RTS,S does not interfere with the infectivity of *Plasmodium* gametocytes for mosquitoes. Even after vaccination, most children will carry parasites that will infect mosquitoes, so transmission in the population will remain unchanged (Zavala, 2022).

b) R21/Matrix-M

Two years later, the WHO approved a second malaria vaccine (R21/Matrix-M) for use in malaria endemic countries, including Mozambique. R21/Matrix-M is a pre-erythrocytic stage vaccine and targets the circumsporozoite protein (CSP) of *P. falciparum* (WHO, 2023a). This vaccine is made of the R21 molecule, which is a virus-like particle. It is characterised by the fusion of the central repeats of Asn-Ala-Asn-Pro (NANP) and the C-terminal sequence of the CSP protein with the hepatitis B surface antigen (HBsAg) (Collins et al., 2021; Datoo et al., 2024). This vaccine was recently introduced into the vaccination plan for children under 2 years of age in Mozambique in August 2024 (UNICEF Mozambique, 2024).

1.5.3. Preventive chemotherapy

Given the lack of an effective vaccine, parasite prevention and control hinges on the use of chemotherapy (WHO, 2023b). There are multiple chemoprevention strategies, including intermittent preventive treatment of malaria in pregnancy (IPTp), perennial malaria chemoprevention (PMC), intermittent preventive treatment of malaria in school-age children (IPTsc), seasonal malaria chemoprevention (SMC) and mass administration of antimalarials (WHO, 2023b).

a) Intermittent preventive treatment of malaria in pregnancy (IPTp)

IPTp is a strategy used in areas where malaria is endemic. The target group is pregnant women, who should receive the antimalarial sulphadoxine-pyrimethamine (SP), known as fansidar, from the second trimester of pregnancy at intervals of at least one month, so that they receive at least 3 doses, to reduce the disease burden in pregnancy and the adverse outcomes of pregnancy and childbirth. (MISAU, 2018; WHO, 2023b). IPTp is highly cost-effective, widely accepted, viable and justified by results obtained over time (Bardajı et al., 2012).

b) Perennial malaria chemoprevention (PMC)

PMC is a strategy that replaces intermittent preventive treatment of malaria in children (IPTi) used in areas of moderate to high malaria transmission in children belonging to age groups with a high risk of contracting severe malaria, aged between 12 and 24 months. The aim of this approach is to reduce the burden of disease. According to the WHO, this approach is recommended in regions where the prevalence of malaria is above 10% or where the annual incidence of parasites exceeds 250 per 1000 (WHO, 2023b) . In Mozambique, the PMC was incorporated in the NMCP's 2023-2030 strategic plan.

c) Seasonal malaria chemoprevention (SMC)

The eligibility for seasonal malaria chemoprevention (SMC) is clearly defined by the seasonality of malaria transmission and the age groups at risk of severe malaria. The use of monthly cycles of SP plus amodiaquine (AQ) for 3 consecutive days has been proven effective in African children under 5 years of age (WHO, 2023b). In Mozambique, this

approach was rigorously assessed as part of a research project coordinated by the Malaria Consortium (MC) during the peak malaria transmission season (December to March) in Nampula province. The results were clear: children in districts where SP+AQ was distributed were 86% less likely to develop clinical malaria than those in districts without SMC (www.malariaconsortium.org/blog/advancing-the-conversation-on-smc-at-astmh-2022). From 2023 onwards, this activity is implemented on a programmatic basis, as part of the 2023-2030 strategic plan.

d) Intermittent preventive treatment of malaria in school-age children (IPTsc)

IPTsc is a chemopreventive strategy that administers the same drug as that used for IPTp. The target group is children aged 5-15 who live in malaria-endemic areas with moderate to high perennial or seasonal transmission. (WHO, 2023b).

e) Mass drug administration of antimalarials (MDA)

MDA is the use of specific antimalarial drugs in areas of moderate to high transmission of *P. falciparum*, in emergencies or during periods of health service breakdown. It is an effective short-term strategy for reducing the burden of disease and can also reduce transmission in areas of low to moderate transmission (Cirera et al., 2020; WHO, 2023b). Mozambique has also joined these campaigns. Until now, only MDA with dihydroartemisinin piperaquine had been implemented in Magude, Maputo province (Cirera et al., 2020; Gupta et al., 2018), the district of Manjacaze (Gaza) and in four districts of the Cabo Delgado province, namely Ibo, Ancuabe, Mecufe and Palma. Preliminary analyses showed a reduction in malaria cases over a period of two to three months.

1.6. Malaria cases Management

1.6.1. Diagnosis

Malaria is diagnosed at the laboratory level in Mozambique using either optical microscopy to analyse a blood sample or a Rapid Diagnostic Test (RDT) based on antibodies that detect histidine-rich proteins (HRP2) (MISAU, 2017). Given the country's limited resources and endemic disease status, the use of RDTs for diagnosis is crucial. This test is not as cost-effective or technician- or electricity-intensive as other diagnostic techniques (Hasselback

et al., 2014). The use of RDTs is a WHO recommendation for diagnosing all patients who visit health and community facilities (MISAU, 2017) due to the advantages aforementioned.

1.6.2. Antimalarials for uncomplicated *P. falciparum* malaria treatment

In October 2021, the WHO recommended the use of an RTS vaccine against malaria to protect children over the age of 5 months. However, the vaccine has low efficacy and its distribution has not yet covered many countries (WHO, 2022, 2023b; World Health Organization, 2021). Malaria control remains firmly rooted in vector control and the administration of antimalarial drugs (INE, 2019; WHO, 2022).

The treatment for children and adults with uncomplicated malaria caused by *P. falciparum* is one of the following artemisinin-based combination therapies (ACTs) (WHO, 2023b). These combination were recommended by WHO in 2001 (Mutabingwa, 2005):

- Artemether-lumefantrine
- Artesunate-amodiaquine
- Artesunate-mefloquine
- Dihydroartemisinin-piperaquine
- Artesunate + sulphadoxine-pyrimethamine
- Artesunate-pyronaridine

ACTs combine an artemisinin derivative (dihydroartemisinin, artemether and artesunate) (D. Wang et al., 2018; Yu et al., 2021) with a partner drug in which the role of the artemisinin derivative is to reduce the number of parasites during the first 3 days of treatment, while the role of the partner drug is to eliminate the remaining parasites (WHO, 2018). ACTs have been used in Mozambique to treat uncomplicated malaria caused by *P. falciparum* since 2009. AL is the treatment of choice and AS+AQ is the alternative in case of contraindication (MISAU, 2017).

Artemisinin derivatives

Artemisinin derivative are phytoconstituent isolated in 1972 from the Chinese medicinal plant *Artemisia annua* L, containing a sesquiterpene lactone structure with an internal peroxide bridge (Adebayo et al., 2020; Belete, 2020; Ruwizhi et al., 2022; Vennerstrom et al., 2004). These derivatives were synthesised between 1972 and 1975 and were launched onto the world market in the 80s (Faurant, 2011). Their active compound is **dihydroartemisinin (DHA**) (D. Wang et al., 2018; Yu et al., 2021). DHA eliminates malaria parasites by damaging their membranes, creating a disruption in their mitochondrial function, causing oxidative stress through the excessive production of reactive oxide species (ROS) (Yu et al., 2021; Zhang et al., 2010). Artemisinin derivatives, which aim to induce rapid elimination of the parasite and clinical improvement of the patient, have a relatively short half-life of ~2.5h (Stover et al., 2012; WHO, 2018) and therefore not recommended to be used as monotherapy (Stover et al., 2012).

Artemether acts on plasmodial transport proteins, interfering with mitochondrial electron transport and the production of free radicals, ensuring a reduction in antioxidants and glutathione in the blood (Belete, 2020; Golenser et al., 2006; Stover et al., 2012). This drug, like other artimisinin derivatives, has a relatively short half-life, so it is not recommended as monotherapy. Although it induces rapid elimination of the parasite and clinical improvement of the patient (Stover et al., 2012).

Artesunate's mode of action is not completely known. It is thought to act throughout the phases of the asexual intra-erythrocytic schizogonic cycle and act on young gametocytes. Mechanism of action involves the generation of reactive oxygen species and free radicals, disruption of hemoglobin digestion, interference with the ubiquitin-proteasome pathway, and targeting multiple stages of the malaria parasite's lifecycle. When used as monotherapy, artesunate is associated with a high recrudescence rate (Belete, 2020; Ruwizhi et al., 2022; Sodiomon, Bienvenu Sirima & Adama, 2007)

1.6.2.1. Partner drugs

Lumefantrine

This drug has a relatively long half-life, although its action is slow. It helps to eliminate the remaining parasites circulating in the blood and consequently reduces the rate of recrudescence. While the exact mechanism of action is not yet well defined, it is clear that lumenfantrine blocks the parasite's detoxification pathway by inhibiting the formation of β-hematin. (Stover et al., 2012). AL efficacy depends on accurate dosage, treatment adherence, and food intake (Lefèvre et al., 2013).

Amodiaquine

Amodiaquine is a quinoline derivative with modifications to its side chain and belongs to the class of 4-aminoquinolines (Adebayo et al., 2020). Amodiaquine has a longer half-life than artesunate, appoximately 5h. After oral administration of amodiaquine, it is rapidly absorbed, taking about 5 hours to do so, and is then transformed into its active compound, desethylamodiaquine, which remains in the blood for more than 6 days (Krishna & White, 1996). The mechanism of action of this drug is blood schizonticidal. For this reason, the ASAQ combination is recommended for the treatment of uncomplicated malaria, as it is more effective than monotherapy with amodiaquine in children, improving the cure rate and reducing gametocythaemia (Adebayo et al., 2020; Adjuik et al., 2002; Ruwizhi et al., 2022).

Mefloquine

Mefloquine was first developed by the US Army in the 1970s to treat malaria in military personnel shortly after the Vietnam War and subsequently commercialised worldwide (Martins et al., 2021). This drug was continuously used by the military until 2013, due to possible side effects (Schneiderman et al., 2018).

Mefloquine has a half-life of approximately 2 weeks and a rapid elimination rate from gametocytes and the asexual phase of the blood (Martins et al., 2021). Although the mechanism of action of mefloquine is still unknown, studies associate this drug targets the *P. falciparum* 80S ribosome to inhibit protein synthesis (Wong *et al.* 2017).

Sulphadoxine-Pyrimethamine (SP)

Sulphadoxine is an antifolate with a structure similar to that of p-aminobenzoic acid. Sulphadoxine blocks the production of dihydrofolic acid, which is essential for nucleic acid biosynthesis, by inhibiting the key enzyme dihydropteroate synthase (Nkosi-Gondwe et al., 2023). In combination with pyrimethamine (P), a diaminopyrimidine, which belongs to the antifolate class and is used for the treatment of uncomplicated malaria. SP (Fansidar®) acts on the asexual erythrocytic forms of malaria parasites (trophozoites and schizonts) by blocking two enzymes involved in folinic acid biosynthesis (Haldar, K., Bhattacharjee, S. and Safeukui, 2018).

Despite the functions of this combination, the WHO does not recommend its use for treatment due to parasite resistance, but rather for chemoprevention (WHO, 2023b). In Mozambique, this drug is used in three chemoprevention approaches: intermittent preventive treatment of malaria in pregnancy (IPTp), chemoprevention of perennial malaria (PMC) and chemoprevention of seasonal malaria (SMC).

Pyronaridine (PY)

PY is a derivative of 10-phenyl aminobenzo and belongs to the 4-aminoquinoline family and was synthesised in the 1970s after the parasite developed resistance to chloroquine (Auparakkitanon et al., 2006). Pyronaridine (PY) is another partner drug used in combination with artesunate for the treatment of uncomplicated malaria caused by *P. falciparum e P. vivax* (Poravuth et al., 2011; Rueangweerayut et al., 2012).

The mechanism of action of this drug remains to be unravelled, but available data shows that it is active against all asexual stages of *P. falciparum* (Adebayo et al., 2020; Looareesuwan et al., 1999). Its mechanism of action involves inhibiting the formation of β-hematin, through the formation of a drug-hematin complex, inhibition of glutathione (GSH)-dependent degradation of haematin and increased haematin-induced lysis of red blood cells (Auparakkitanon et al., 2006; Croft et al., 2012).

In Mozambique, the ASPY combination is not yet in use, but there are already discussions about introducing this formulation to reduce the pressure that AL is suffering.

Piperaquine (PPQ)

Piperaquine is a bisquinoline that has a half-life of 20-30 days, which gives it a terminal elimination function (Chan et al., 2018). It has been developed in the 1960s by Shangai Pharmaceutical Industry Research (China) and Rhone Poulec (France) and widely used in China for over 20 years (Biagini et al., 2005). Its mecanisms of action is based on its accumulation in high concentrations in the parasite's digestive vacuole, inhibiting the conversion of toxic haem to non-toxic haemozoin crystals during parasite haemoglobin digestion leading to death of the parasite (K. et al., 2017; Ross et al., 2018).

In Mozambique, the combination of DHA and PPQ was used as a chemopreventive prophylaxis in line with WHO recommendations in emergencies to reduce the transmission chain and the disease burden (WHO, 2023b). This drug has also been administered in areas of near elimination, such as the Magude district in Maputo province (Gupta et al., 2018).

1.6.3. Resistance to antimalarials

Antimalarial drug resistance is defined as the ability of the parasite to survive and/or multiply despite the administration and absorption of a drug at doses equal to or greater than those usually recommended, but within the individual's tolerance limits (World Health Organization, 2018). This phenomenum is diferent from Therapeutic failure, which is defined as the inability to clear malaria parasitaemia or prevent recrudescence in an individual after administration of an antimalarial (Fatunla et al., 2023).

Over time, the WHO has changed its therapeutic approach with regard to the drugs used to treat malaria due to the emergence of resistance to successively introduced antimalarials (Plowe et al., 2007; Wongsrichanalai et al., 2002; World Health Organization, 2018).

Historically, cases of resistance has their origin in Southeast Asia (Dondorp et al., 2009; World Health Organization, 2018) and then spread to the east coast of Africa and spread to the rest of the continent (Wongsrichanalai et al., 2002). This phenomenon of resistance has led the WHO, since 2006, to recommend the treatment of uncomplicated *P. falciparum* malaria cases with combined therapies based on artemisinin derivatives (ACTs) in all countries endemic for the disease, including Mozambique (World Health Organization, 2017).

P. falciparum artemisinin partial resistance which refers to a delay in the clearance of parasites from the bloodstream following treatment with an ACT merged in Cambodia in 2009 (Dondorp et al., 2009; Noedl et al., 2010), and consequently spread to the Upper Mekong Region (GMS) in Southeast Asia (SEA). Resistance to all antimalarial drugs has now been described (World Health Organization, 2018; Xu et al., 2018). With the emergence and spread of resistance to ACTs in SEA and the recent detection of cases of resistance in Africa (World Health Organization, 2018; Xu et al., 2018) there is a pressing need to monitor the spread of resistant parasites.

1.6.3.1. Surveillance of antimalarial resistance

Parasite resistance to antimalarials is the biggest constraint in controlling malaria and reducing mortality from *P. falciparum* (World Health Organization, 2018). This occurs through the selection of mutant genes in the parasite (Noedl et al., 2010; Woodrow & Krishna, 2006).

WHO's current recommendation is to use therapeutic efficacy studies (TES) to monitor efficacy and detect treatment failures in patients. As an additional option, they advise monitoring molecular markers and conducting in vitro/ex vivo assessments of parasite susceptibility assays (Nsanzabana, 2021; Nsanzabana et al., 2018; World Health Organization, 2018). TES are extremely challenging to implement due to the significant costs involved in preparing the logistics and the inability to develop this test in areas of low malaria transmission, due to the scarcity of patients. The analysis results are influenced by other factors, including the participant's immunity (Nsanzabana et al., 2018).

The most effective laboratory methods for monitoring parasite sensitivity to antimalarials are: i) characterisation of molecular resistance markers (A. R. Taylor et al., 2017) and ii) *ex vivo*/*in vitro* susceptibility assays of parasites against the antimalarials in use (Nsanzabana et al., 2018). These methods provide powerful information on the parasite susceptibility and are easly be applied in all transmission settings. The main drawback of this method has to do with the need of well-equipped laboratory infrastructure and highly trained staff requirement (Nsanzabana et al., 2018).

1.6.3.1.1. Surveillance of molecular resistance markers to antimalarials

Molecular markers of resistance to antimalarials, which are theoretically the earliest way of detecting emerging resistance, are based on genetic alterations that make the parasite resistant to the drugs normally used in the treatment and prophylaxis of malaria (Fidock, 2000; Nsanzabana, 2021; Nsanzabana et al., 2018; Reed et al., 2000; Wongsrichanalai et al., 2002).

a) *P. falciparum* resistance to Chloroquine (CQ)

Chloroquine (CQ) monotherapy was the elected approach in order to eradicate malaria in the 20th century (Amambua-Ngwa et al., 2023). Suddenly this approach had to be changed due to the emergence of parasite resistance to the same drug in the late 1950s. It was first observed in Southeast Asia (SEA), and subsequently reached and spread across Africa (Amambua-Ngwa et al., 2023; Wellems & Plowe, 2001). The main cause of *P. falciparum's* resistance to CQ is the significantly lower accumulation of CQ in the digestive vacuole when compared to susceptible parasites, caused by increased efflux, so that it does not reach sufficiently high levels to inhibit heme polymerisation in resistant parasites (Krogstad et al., 1987; Sanchez et al., 2003).

CQ is no longer used for the treatment of *P. falciparum* malaria but is still widely used to treat uncomplicated malaria caused by *P. vivax* in GMS (Rovira-Vallbona et al., 2023). *P. falciparum* resistance to chloroquine is associated to mutation in *P. falciparum* chloroquine resistance transporter (*pfcrt*) codon K76T (the substitution of lysine (K) by threonine (T) at position 76) in *pfcrt gene* along with other mutations (C72S, M74I, N75E, A220S, Q271E, N326S, I356T and R371I) (WHO, 2019) (**table 2**), forming the resistant haplotype CVI(E/D)T (Fidock, 2000). These mutations when associated with mutations in the *pfmdr1* (*P. falciparum multidrug resistance 1 transporter*; PF3D7_0523000) gene can induce a reduction in the susceptibility of *P. falciparum* to other drugs such as Piperaquine, Mefloquine or lumefantrine (Veiga et al., 2016). A recent study has shown that the evolution of *P. falciparum* resistance to chloroquine is mediated by the putative amino acid transporter gene *pf*aat1 (Amambua-Ngwa et al., 2023).

In Asia and Africa specifically in regions where AQ is not widely used, the prevalence of *the pfcrt* 76**T**, is less than 3% (Cheng et al., 2021; Kale et al., 2024).

Table 1: Molecular markers of *P. falciparum* resistance to antimalarial drugs. *P-Plasmodium*; *pfK13-P. falciparum* Kelch 13; *pfmdr1- P. falciparum multidrug resistance 1 protein*; *pfpm2/3-P. falciparum* plasmepsin 2/3; *pfcrt: P. falciparum* chloroquine resistance transporter; *pfdhfr-P. falciparum* dihydrofolate reductase; *pfdhps- P. falciparum* dihydropteroate synthase; Adapted from (WHO, 2018, 2019).

Antimalarial	Molecular Markers	
	Genes	Mutations
Artemisinin and its derivatives	*pfk13* (Validated SNP)	F446L, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L, C580Y
	pfk13 (Candidate or associated SNP)	P441L, G449A, C469F, A481V, P527H, N537I, G538V, V568G, P574L, F673I, and A675V
Chloroquine	*pfcrt*	K76T + (C72S, M74I, N75E, A220S, Q271E, N326S, I356T and R371I)
	pfmdr1 (in combination with *pfcrt* mutations only)	N86Y, Y184F, S1034C, N1042D and D1246Y
Lumefantrine	*pfmdr1* (Not validated yet)	N86Y
Piperaquine	*pfpm2/3*	Multiple copies of *Pfpm2/3*
Amodiaquine	*pfmdr1* (Not validated yet)	N86Y
Pyrimethamine	*pfdhfr*	N51I, C59R, S108N and I164L
Sulfadoxine	*pfdhps*	S436A/F, A437G, K540E, A581Gand A613T/S
Mefloquine	*pfmdr1*	Multiples copies of *pfmdr1*
Pyronaridine	Not validated yet	

b) *P. falciparum* Resistance to Sulfadoxine–pyrimethamine (SP)

P. falciparum resistance to sulfadoxine–pyrimethamine is mediated by a combination of single-nucleotide polymorphisms (SNPs) at specific codons, 16, 51, 59, 108, and 164 of the *P. falciparum* dihydrofolate-reductase (*pfdhfr*) gene, and in codons 437, 540, 581, and 613 of the *P. falciparum* dihydropteroate-synthetase (*pfdhps*) gene. These encodes two enzymes involved in the folate biosynthesis pathway (Desai et al., 2016; Hastings et al., 2002; Oguike et al., 2016; Okell et al., 2017; Sirawaraporn et al., 1997; P. Wang et al., 1997).

Results from surveillance of these SNPs, have revealed several important genotypes associated with SP failure. The quintuple mutant haplotype (IRN ISGEAA) which is a combination of *pfdhfr* mutations N51I, C59R, and S108N and *pfdhps* mutations A437G and K540E has been associated with SP treatment failure and reduced the prophylactic period (Desai et al., 2016; Okell et al., 2017).

The prevalence of quintuple mutante in SE Asia and Africa is around 65% (Sugaram et al., 2020) and 75% (Flegg et al., 2022) respectively.

c) *P. falciparum r*esistance to artemisinin derivatives

In vivo and *ex vivo* assays accompanied by genomic analyses have associated partial resistance of *P. falciparum* to artemisinin and its derivatives with mutations in the Kelch 13 protein. The *pfkelch13* gene (PF3D7_1343700) is located on chromosome 13 and encodes a Kelch-like protein that responds to oxidative stress (Amato et al., 2017).

The presence of certain SNPs in the gene *pfk13* (World Health Organization, 2018) correlated with increased parasitaemia clearance time after treatment with ACTs. They are considered molecular markers of resistance for monitoring resistance to these combinations (Ariey et al., 2014; World Health Organization, 2018). Mutations in this gene allows the parasite to decrease the haemoglobin-derived heme, consenquently reducing the activation of artemisinin, which allows the parasite to survive in the initial stage of infection (Xie et al., 2020).

To date, more than 150 non-synonymous mutations have been reported in this gene (Amato et al., 2017). Some of the mutations are validated Single Nucleotide Polymorphisms (SNPs), used for the surveillance of delayed clearence of malaria parasites (F446L, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L, C580Y). Others are called candidates since they are still under evaluation namely, P441L, G449A, C469F, A481V, P527H, N537I, G538V, V568G, P574L, F673I, and A675V (WHO, 2019) (**table 2)**.

The most predominant validated SNP is C580Y, it was detected almost all continents (Jun Li et al., 2019; Ndwiga et al., 2021a; She et al., 2020) . Recently, *P. falciparum* parasites carrying the validated SNPs F446I, M476I, P553L, R561H, P574L, C580Y and A675V

have emerged and expanded or been identified in Africa (Ndwiga et al., 2021a), in Angola, Ghana (Matrevi et al., 2022), Mali, Rwanda (Bergmann et al., 2021; Straimer et al., 2022; Uwimana et al., 2020, 2021). In Mozambique, available studies on the prevalence of *pfK13* SNPs, refer to parasite samples collected prior to 2018 and none of them reported a validated mutation (Chidimatembue et al., 2021; Escobar et al., 2015; Gupta et al., 2018; Preston et al., 2014). Therefore, the present study was designed to update the current profile of SNPs in *pfk13* associated with artemisinin resistance in Mozambique.

In Mozambique, several studies have been published reporting the prevalence of SNPs in *pfK13* and *pfmdr1* genes (Chidimatembue et al., 2021; Escobar et al., 2015; Gupta et al., 2018; Raman et al., 2011; Silva et al., 2023; Thomsen et al., 2013). However, most of them are restricted to the south of the country. Regarding the regions where malaria is most prevalent, we found no recent publications describing the situation of aforementioned SNPs. The article written in the context of this PhD (da Silva et al., 2023) updated the profile of SNPs in *pfK13* to artimisinin derivatives.

The validated *pfk13* SNPs associated with *P. falciparum* partial resistance to artemisin and its derivates has reached highest prevalence in South East Asia (WHO, 2023d). In Africa it varied between 0.05% to 40% (Aninagyei et al., 2020; Bakari et al., 2024; Bergmann et al., 2021; Bwire et al., 2020; D. et al., 2023; Ishengoma et al., 2024; Kirby et al., 2023; Ndwiga et al., 2021b; Uwimana et al., 2020; van Loon et al., 2024).

d) *P. falciparum* resistance to artemisinin partner drugs

Piperaquine (PPQ)

The parasite resistant forms, are able to efflux piperaquine from the digestive vacuole, thus preventing this drug from binding to the heme and inhibiting its detoxification (Wicht et al., 2020). Piperaquine-resistant *P. falciparum* parasites showed an in increase in CNVs of *pfpm2/3 genes* (Benoit Witkowski et al., 2017a) and SNPs in the *pfcrt* (T93S, H97Y, F145I, I218F, M343L, C350R and G353V), a transporter resident in the membrane of the digestive vacuole (WHO, 2019). Studies showed prevalence of 23%, 54.3% and >30% in Cambodia (Lim et al., 2009), Vietnam (Phuc et al., 2017) and other African countries (Leroy et al., 2019) respectively.

Amodiaquine (AQ)

Similarly, to chloroquine, the amodiaquine resistant parasite has reduced capacity to retaining amodiaquine in the digestive vacuole (Ginsburg et al., 1998; Krogstad et al., 1987; Sanchez et al., 2003). The WHO restricted its clinical use in monotherapy in the 1990s due to hepatotoxicity and agranulocytosis, effects that also contraindicate its use as a prophylactic drug. However, recent recommendations states that this antimalarial can still be used in context of chemoprevention (section **1.3**) (Adebayo et al., 2020; Adjuik et al., 2002; Ruwizhi et al., 2022). Previous studies showed that the amodiaquine selects for *pfmdr1* polymorphism (SNP) in codon N86Y (Ginsburg et al., 1998; Holmgren et al., 2007). In Africa the prevalence of this SNP is 2.1% in Zambia (Mulenga et al., 2021). Slightly higher prevalences, 5.9% and 19.58% were observed in Ivore Cost (Konaté-Touré et al., 2024) and Burkina Faso (Somé et al., 2016). One of the highest prevalence (72%) of this mutation was observed in Iran (Ursing et al., 2006).

Mefloquine (MQ) and Lumefantrine (L)

Multiples copies of *pfmdr1* gene has been linked to resistance to Mefloquine (MQ) (WHO, 2018, 2019). Recent study from Mozambique showed a 3.9% prevalence of *pfmdr1* CNVs, molecular markers of *P. falciparum* resistance to MQ (Brown et al., 2024), which is far below 19% and 21% reported in Cambodia (Lim et al., 2009), and African respectively (Leroy et al., 2019).

As for *pfmdr1* polimorphism, the haplotypes N86, 184F and D1246 are selected by treatment with artemether+lumefantrine (Mbaye et al., 2016; Nguetse et al., 2017; Otienoburu et al., 2019). The prevalence of this haplotype was above 90% in Cambodia (Boonyalai et al., 2021), 20% in Vietnam (Rovira-Vallbona et al., 2023) between 20%-50% in Afica (Chidimatembue et al., 2021; Gupta et al., 2018; Ishengoma et al., 2019; Jian Li et al., 2014; Serrano et al., 2021; Silva et al., 2023). Studies reported that lumefantrine selects for *pfmdr1* polymorphism (SNP) in codon 86Y as well (WHO, 2018, 2019).

e) Prevalence of *P. falciparum* mutations associated with antimalarial resistance in Mozambique

The molecular marker associated with cloroquine is becoming scarce in Mozambique. Recent studies have definitively shown a prevalence of less than 1% of the pfcrt gene in codon 76T (Chidimatembue et al., 2021; Silva et al., 2023). This has to do with the fact that it was withdrawn from malaria treatment policies in Mozambique (MISAU, 2017).

As is the case worldwide, in Mozambique the prevalence of the quintuple mutant is above 72%. It increases as we move from north to south, reaching 95%. This is why SP is no longer used for treatment but for chemoprevention in pregnant women and infants (Silva et al., 2023). An additional mutation within *pfdhp*s at A581G defines the sextuple mutant (IRN ISGEGA), which has been associated with inefficacy of IPTp-SP, resulting in low infant birth weight and increased placental inflammation (Gutman et al., 2015; Harrington et al., 2009; Minja et al., 2013). The sextuple mutant has not yet been detected in Mozambique, which is why IPTp-SP and PMC continue to be applied. The WHO has stated that even if the sextuple mutant is detected, there is no reason to withdraw IPTp-SP, given the additional benefits this drug confers on women and children (Matambisso et al., 2024).

As for the SNPs and CNVs of interest for the context of this PhD, It is important to note that Mozambique is still one of the countries that has not reported a pfk13 validated mutation yet. This is despite the fact that several studies have been conducted in the country, including those by (Chidimatembue et al., 2021; da Silva et al., 2023; Escobar et al., 2015; K. et al., 2017; Serrano et al., 2021; Silva et al., 2023). However, over time, various synonymous and non-synonymous mutations have been discovered. The latter may in turn be deleterious and serve as a source for new candidate mutations or mutations associated with the resistance of P. falciparum to artemisinin derivatives. Da Silva and colleagues definitively identified 16 non-synonymous SNPs in samples collected in three different settings in 2021. The same author also reported another 32 non-synonymous mutations from samples collected in 2015 and 2018 in six provinces (da Silva et al., 2023; Silva et al., 2023) (**table 2**). The mutation A578S was reported in both studies, and its common in Africa (Ajogbasile et al., 2022; Igbasi et al., 2019; Ouattara et al., 2015b). In

2018 and 2015, Gupta and Escobar detected 1 non-synonim mutation each, F656I and V494I respectively.

Table2: List of *Pfk13* non-synonimous mutations reported in Mozambique.

Study Sites	Sampling Year	*Pfk13* Non-synonimous mutatios	Reference
Maputo, Manica, Niassa	2021	G449R, V454E, E455Q, D464H, D464Y, W470R, E509D, Q654H, V494L, C532W, I543S, A578S, A578P, V581A, Q661H, L663I	da Silva et al., 2023
Maputo, Gaza, Sofala, Tete, Zambezia, Cabo Delgado	2015 & 2018	K372E, H384R, V386I, V386L, R404K, G436S, F442L, V454I, K480R, F483S, F483L, S485G, V494I, V520F, N537D, P553T, A578S, E596G, R597G, L598S, N599S, N599D, E605G, K607E, L631F, D641N, F656I, R659G, Q661R, F662S	da Silva et al., 2023
Gaza, Sofala, Tete, Cabo Delgado	2015	F656I	Gupta et al., 2018
Maputo	2010	V494I	Escobar et al., 2015

As for *pfmdr1* polymorphism, SNP in codon N86Y is rare, with a prevalence of more than zero only identified in the study by Gupta et al. in 2015 (Gupta et al., 2018). The prevalence of this haplotype N86, 184F and D1246 selected by treatment with artemether+lumefantrine was between 20%-50% (Chidimatembue et al., 2021; Gupta et al., 2018; Serrano et al., 2021; Silva et al., 2023). Recent study from Mozambique showed a 3.9% prevalence of *pfmdr1* CNVs, molecular markers of *P. falciparum* resistance to MQ (Brown et al., 2024). Same study, reported 2.2% prevalence of *pfpm2* and 2.2% of *pfpm3* CNVs, associated with *P. falciparum* resistance to piperaquine.

To avoid redundancy, more detailed information on the prevalence of *P. falciparum* mutations associated with antimalarial resistance in Mozambique and globally can be found in Chapter 1 of the thesi`s results section.

1.6.4. Malaria molecular surveillance methods for the detection of genetic Mutations associated with anti-malarial resistance

To date, several molecular methods have been developed for the assessment of this known resistance to the most used anti-malarial drugs. These resistances are usually linked to SNPs or CNVs (Nsanzabana et al., 2018). Therefore, the majority of the methods are designed to target these types of mutations.

In general, all the methods have the same starting point, which is to take blood samples from the participants where, in areas with difficult access or few resources, dried blood sample (DBS) filter papers are used for better preservation (Mayor et al., 2023). Parasite DNA can then be extracted using commercial kits and/or the Chelex method, so that the genetic material is ready for genotyping (Nsanzabana et al., 2018).

a) Real time polymerase chain reaction (qPCR)

Real time PCR (qPCR) is the molecular technic developed with the aim to amplify the targeted nucleic acid to a detectable level in order to identify and quantify fragments of interest through the quantitative relationship between fluorescent signals (He et al., 2021; Nsanzabana et al., 2018). qPCR is relatively quantitative and require the use of standard curves and extrapolation based on patterns to calculate the target concentration of the nucleic acid in the original sample (Artika et al., 2022). Of all its advantages, the fact that gel electrophoresis is not required for confirmation of the PCR product stands out (Gautam & Kumar, 2020; Josko, 2010) . Reduced risk of contamination and the process can be monitored in real time on a computer screen and requires a small amount of template DNA (Gautam & Kumar, 2020).

To detect changes in gene copy number, fluorescent agents are used in the PCR mixture in order to detect the generated copies as it accumulates in real time during the PCR cycles. The fluorescence intensity that is generated is extrapolated to the total amount of amplicon (He et al., 2021; Nsanzabana et al., 2018). The initial concentration of the template is determined by the detection of amplicons during the early exponential phase of the PCR (Smith & Osborn, 2009).

In the context of antimalarial resistance, this assay is used to detect and measure 18S rRNA from *P. falciparum* parasites in infected red blood cells (Ballard et al., 2019; Grabias et al., 2019; Murphy et al., 2012), determine copy number variations (CNVs) of *pfmdr*1 and *plasmepsin 2 or 3* gene (*pfpm2,* PF3D7_1408000 and *pfpm3,* PF3D7_1408100) (Boonyalai et al., 2021; Gupta et al., 2018; Humphreys et al., 2007; Lim et al., 2009; Ngalah et al., 2015; Sidhu et al., 2006; Benoit Witkowski et al., 2017a).

b) droplet digital PCR (ddPCR)

The main characteristic of ddPCR is absolute quantification (Hou et al., 2023). Conventional PCR methods are only semi-quantitative and require agarose gel electrophoresis to detect the amplified product. Digital PCR does not need any referencing or extrapolation, as after the amplification reactions are complete, it precisely and discretely measures the number of initial targeted molecules (Hindson et al., 2011). Advantages of ddPCR over other forms of PCR and sanger sequencing are its higher sensitivity, precision, and accuracy (Srisutham et al., 2021; S. C. Taylor et al., 2017; Z. Wang et al., 2019). In the context of antimalarial resistance, this method has had so many important applications such as the quantification of parasite density, measurement of copy number variations (CNVs) of *pfmdr*1 and as well as the *plasmepsin 2 or 3* gene (Brown et al., 2024; Srisutham et al., 2021)

c) Sanger sequencing

This was the first DNA sequencing method that was introduced in the 70s. This made the human genome-sequencing project possible until the first decade of the 21st century (Eren et al., 2022). This method is based on the selective introduction of polymerization terminating fluorescently labelled dideoxynucleotides (ddNTPs) (Nsanzabana et al., 2018; Sanger et al., 1977). This technique has the following advantages, little dependence on computer tools and can sequence much longer fragments than next generation sequencing (NGS), as well as its accuracy to detect SNPs and small insertions/deletions (indels) (Verma et al., 2017). However, its limitations are related with not being able to detect CNVs or structural rearrangements. It cannot target diferent fragments of interest in the same run and requires a higher concentration of DNA template than NGS (Frost, Amy and van Campen, 2024).

This technique has been widely used in the identification of the SNPs associated with *P. falciparum* resistance to antimalarials (Chidimatembue et al., 2021; da Silva et al., 2023; Maiga et al., 2024; Serrano et al., 2021).

d) Next Generation Sequencing

Next-generation sequencing (NGS) is a technology for the molecular surveillance of changes in the DNA sequence of any organism. In some cases, these changes can be associated with disease or resistance to drugs phenotypes (Talundzic et al., 2018).

The advent of NGS, also known as massively parallel sequencing or deep sequencing (Koboldt et al., 2010), has brought about significant advancements in this field compared to conventional methods. One such improvement is the ability to generate larger amounts of sequencing results (few hundreds of gigabytes) while requiring a significantly smaller amount of DNA template (Larson et al., 2023; Talundzic et al., 2016, 2018).

Furthermore, its increased accuracy, for instance, in identification less frequently observed alleles and allowd sequencing multiple sample in the same run, what turns this approach less less expensive and faster approach for molecular epidemiology (Talundzic et al., 2016, 2018; Tang et al., 2017). The main limitations of NGS are the need for sophisticated softwares for results interpretation and data analyses, well-trained personel, well-equipped infrastructure including computers and data storage system (Cloud or local server) (Abbasi & Alexandrov, 2021; Alkan et al., 2011; Ashton et al., 2015; Benítez-Páez & Sanz, 2017).

There are three types of NGS, namely whole genome sequencing (WGS), whole exome sequencing (WES) and Targeted amplicon-based sequencing (TABS). WGS consists of sequencing the entire genome, providing the most comprehensive coverage, highlighting its suitability for novel gene discovery and research applications (Nakagawa et al., 2015; Tang et al., 2017). WES, consist in sequencing exons, the protein coding regions (Petersen et al., 2017). In the context of this thesis, we were focused on targeted amplicon based sequencing (see method in the chapter 3).

e) Targeted amplicon based next generation sequencing

Due to the high costs of WGS (24800 USD) (Schwarze et al., 2018) and the low nonspecificity when compared to TABS (300 USD) (Bewicke-Copley et al., 2019), recent studies in the molecular surveillance of pathogens have focused on TABS, since it can sequence and identify small genetic variations in very specific regions and a relatively short

time (Abbasi & Alexandrov, 2021; Deurenberg et al., 2017; Sanschagrin & Yergeau, 2014). It is based on identifying specific regions of interest in the genome for the characterisation and detection of genotypes of interest with relatively less demand of bioinformatics tools (Abbasi & Alexandrov, 2021; Bewicke-Copley et al., 2019).

This method uses PCR to produce amplicons and barcoding samples in order to be mixed into pools allowing multiple samples to be sequenced on a single sequencing run (Bybee et al., 2011). It is usefull for the detection of rare mutations in complex samples, in sequencing of the bacterial 16S rRNA gene across multiple species, a widely used method for phylogeny and taxonomy studies (Kozich et al., 2013).

Recently in Mozambique, molecular surveillance by TABS has been instumental, particularly in surveillance of antimalarial drug resistance, genetic diversity of the malaria parasite to support stratification of malaria transmission, tracking imported malaria cases and conducting surveillance during antenatal consultations (Brokhattingen et al., 2024; Matambisso et al., 2024; Mayor et al., 2022, 2023; Silva et al., 2023).

1.6.5. *Ex vivo* susceptibility of *P. falciparum* to antimalarials

In *vitro* (Bacon et al., 2007; L. et al., 2003)*/ex vivo* assays are the straight forward way to determine parasite resistance. In *vitro/ex vivo P. falciparum* susceptibility assay to antimalarial are based on observation of parasite development in blood cells through thick films, isotopic assays, quantification of parasite proteins and the use of DNA dye intercalators (Maji, 2018; Nsanzabana et al., 2018) using laboratory created cultured parasites (in vitro) or fresh blood samples collected from patients (*ex vivo*) (Nsanzabana, 2021).

WHO microtest (parasite maturation; based on morphology) (Rieckmann et al., 1978). It is a tool that provides information on the quantitative drug response of *P. falciparum*, regardless of the patient's immune status. This assay was developed to assess parasite susceptibility to SP in the 1980s and was later used in tests against other drugs. It is based on counting parasite growth by microscopy after a minimum of 48 hours' exposure to the drugs (Nguyen-Dinh et al., 1985).

Isotopic test

In culture, the only cells that synthesise genetic material during their growth are those of the plasmodium and not the blood cells. *Plasmodium spp.* use exogenous purines. Radiolabelled hypoxanthine (3H) is a DNA precursor that is easily incorporated into *Plasmodium* nucleic acids, which is why it is preferentially used as a radioisotope in these assays (Maji, 2018; Nsanzabana et al., 2018). The principle of the assay is based on measuring the growth of the parasite by adding a radioactive dye to the culture, which is incorporated into the parasite's DNA. The incorporation of (3H) hypoxanthine is directly proportional to the number of erythrocytes infected with *P. falciparum* in the in vitro drug susceptibility assays (Maji, 2018; Nsanzabana et al., 2018).

Enzyme-linked immunosorbent assay (ELISA) (protein quantification e.g. histidine-rich protein – HRPII and lactate dehydrogenase- LDH) (Makler & Hinrichs, 1993; Noedl et al., 2002) is an immunological assay widely used, based on the interaction between the targeted protein the primary antibody against the antigen of interest (Hayrapetyan et al., 2023). Therefore, the survival rate of the parasites is measured by tracking the concentration of proteins produced by the cultured parasites after at least 48h exposition to drugs (Makler & Hinrichs, 1993; Noedl et al., 2002).

Flow cytometry is the method used for the determination of parasite **(**labelled with fluorophores**)** viability. It rapidly analyzes cells as they passed lasers. Each cell is screened for visible light scatter and fluorescence parameters (McKinnon, 2018). It is used for antimalarial resistance surveillance to determine the survival rate of the parasites after 48h of exposition to Drugs by counting infected red blood cells labelled with fluorophore by *Plasmodium* that have grown in the culture medium (Pattanapanyasat et al., 1997).

Use of DNA intercalators, this method is based on the use of fluorescent agents, such as SYBER Green I, DAPI, which, when bound to the parasite's DNA, emit light that is detected by flow cytometerand the parasite's DNA is quantified (Wirjanata et al., 2015).

Ring stage survival assay is the reference assay to assess *in vitro/ex vivo P. falciparum* artemisinin partial resistance detected in the ring stage (Amaratunga et al., 2014;

Witkowski et al., 2013). Most of the tests used in susceptibility assays detect mutations that occur in more than one parasite stage, which reduces their sensitivity and specificity in detecting *P. falciparum* resistance to artimisinin derivatives. The Ring stage survival assay is the only method aimed at detecting alterations that occur exclusively in the ring stage. It can assess the survival rates by counting the proportion of viable parasites after exposition to drug, through microscopy or flow cytometry (Amaratunga et al., 2014).

The advantages and disadvantages of afore described laboratoy methods used to assess *in vitro or ex vivo* susceptibility of *P. falciparum* to antimalarials are portrayed in **table 3.**

In Mozambique, although there are some publications describing the therapeutic efficacy of ACTs (Abacassamo et al., 2004; Nhama et al., 2014, 2021; Salvador et al., 2017), this data is more than four years old and does not reflect current situation. The manuscript submitted to malaria journal in the context of this PhD reports for the first time *P. falciparum* ex vivo ring stage (RSA), piperaquine (PSA) and Amodiaquine (AQSA) susceptibility assays to three antimalarials in use in Mozambique DHA, PPQ and AQ respectively. The great advantages of these assays is that it can provide useful profile of parasite susceptibility to antimalarial drugs. However, the performance depend on the availability of well-equipped laboratories to validate new molecular markers of antimalarial drug resistance (Bacon et al., 2007), which is not ease to get in majority of malaria endemic coutries like Mozambique.

Table 3: Advantages and disadvantages of laboratoy methods used to assess in vitro or ex vivo susceptibility of *P. falciparum* to antimalarials. Adapted from (Nsanzabana et al., 2018)

Type of assays	Advantages	Disadvantages
WHO microtest	Do not require heavy equipment	Labour intensive Requires quality assured microscopy Difficult to standardize Expensive
Isotopic Test	Automatic reading	Use of radioactive reagents Require heavy equipment Expensive
ELISA	Relatively low cost	High inter-variability between laboratories and users
Flow Cytometry	Highly sensitive method	Require heavy equipment Expensive
SYBR green	Low cost Procedures involves few steps	Underestimation of parasitaemia Require heavy equipment Interaction between drugs and the dye
RSA, PSA & AQSA	Do not require heavy equipment (except when using flow cytometry)	Labour intensive (microscopy) Requires quality assured microscopy Difficult to standardize (microscopy) Expensive

2. GOALS OF THIS THESIS

This research protocol was conceived as part of PhD in Biomedical Sciences in the second quarter of 2019. The reason for choosing the topic, ***Antimalarial resistance in Mozambique: Characterization of molecular markers and assessment of P. falciparum susceptibity to ACT***, with the malaria situation in Mozambique as follows:

Malaria is among the top three causes of hospitalisation and death among pregnant women and children in Mozambique, hence the importance of this disease in the country's public health. Control of this disease is mainly based on vector surveillance and the use of antimalarials to kill *P. falciparum*. Recently, a vaccine was introduced for children under two years of age. Antimalarials approach have been changing for various reasons, including the emergence of parasite resistance to the drugs. This thesis was focused on in vitro monitoring of parasite resistance to antimalarials. In Mozambique, several studies have been published on the prevalence of molecular markers of resistance to ACTs, in particular *pfmdr1* and *pfK13*. There are some publications describing the therapeutic efficacy of ACTs. However, the data is more than four years old and does not reflect current status.

The WHO recommends monitoring the molecular markers of malaria and the susceptibility of *P. falciparum* every 2 years. Hence our proposal to update the phenotypic and genotypic profile of the malaria parasite with the published articles and the third one to be submitted to the malaria journal in the context of this PhD.

2.1. Main Goal

The main goal of this thesis was to study resistance to ACTs by characterising molecular markers and assessing the *ex vivo* susceptibility of *P. falciparum* to antimalarial drugs administered in Mozambique.

This thesis follows the research paper style. Two specific objectives were proposed to achieve the main objective, resulting in three papers described below.

2.2. Specific goals

2.2.1. Chapter 2: Characterise molecular markers of *P. falciparum* resistance to ACTs in Maputo, Sofala, Manica and Niassa provinces.

a) **Estimate the prevalence of mutations in genes associated with *P. falciparum* resistance to ACTs, namely *pfmdr1*, *pfK13*.**

b) **To analyse the genetic diversity of the parasites by Next Generation Sequencing (NGS).**

The activities conducted in the context of this chapter resulted in two published manuscriprt. Where, in the first one we estimated the prevalence of mutations in *pfK13* gene associated with resistance to ACTs. Moreover, the second paper was related to the estimation of the prevalence of mutations, copy number variation of *pfmdr1*, *pfpm2/3* associated with resistance to amodiquine and piperaquine. We included **chapter 4** to present the result of the epidemiology of malaria and the results of the targeted amplicon-based next generation sequencing of eight genotyped samples from Maputo.

2.2.2. Chapter 3: To determine *ex vivo* susceptibility of *P. falciparum* to antimalarial drugs in use in Maputo, Mozambique.

c) **Determine the *ex vivo* of *P. falciparum* susceptibility to artemisinin derivatives by RSA.**

d) **Estimate the IC50 of *P. falciparum* to the component drugs of ACTs not derived from artemisinin.**

e) **Carry out *in vitro/ex vivo* susceptibility assays on new drugs to discover new genes associated with *P. falciparum* resistance.**

The activities conducted in the context of this chapter resulted in one manuscriprt in preparation. Where we specifically assessed the *ex vivo* susceptibility of *P. falciparum* isolates from Maputo to dihydroartemisinin (DHA), piperaquine (PPQ), and amodiaquine

(AQ). Furthermore, using the isolates that passed the phenotypic assays, SNPs in *pfk13* and *pfmdr1* and CNVs of *plasmepsin* (*pfpmp2*) and *pfmdr1* genes were characterised.

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4. RESULTS

4.1. Chapter 1: Paper I: Mapping Antimalarial Drug Resistance in Mozambique: A Systematic Review of *Plasmodium falciparum* Genetic Markers Post-ACT Implementation

Reference:

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In this review, where I am co-author, I played an important role in the formal analysis and investigation.

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Review

Mapping Antimalarial Drug Resistance in Mozambique: A Systematic Review of *Plasmodium falciparum* Genetic Markers Post-ACT Implementation

Celso Raul Silambo Chaves [1], Clemente da Silva [1], Acácio Salamandane [2] and Fatima Nogueira [1,*]

[1] Global Health and Tropical Medicine GHTM, Associate Laboratory in Translation and Innovation Towards Global Health, LA-REAL, Instituto de Higiene e Medicina Tropical-IHMT, Universidade NOVA de Lisboa UNL, Lisbon, Rua da Junqueira, 100 1349-008, Lisboa, Portugal; a21002189@ihmt.unl.pt (C.R.S.C.); a21000955@ihmt.unl.pt (C.d.S.)
[2] Faculdade de Ciências de Saúde, Universidade Lúrio, Campus Universitário de Marrere, 4250 Nampula, Mozambique; salamandane@gmail.com
* Correspondence: fnogueira@ihmt.unl.pt

Abstract: Malaria continues to be a significant public health burden in many tropical and subtropical regions. Mozambique ranks among the top countries affected by malaria, where it is a leading cause of morbidity and mortality, accounting for 29% of all hospital deaths in the general population and 42% of deaths amongst children under five. This review presents a comparative analysis of data on five critical genes associated with antimalarial drug resistance**:** *pfmdr1*, *pfcrt*, *pfk13*, *pfdhfr*, and *pfdhps*, along with the copy number variation (CNV) in genes *pfmdr1* and *pfpm2/3*. These are genes associated with parasite response to antimalarials currently used to treat uncomplicated *P. falciparum* malaria in Mozambique. The review synthesizes data collected from published studies conducted in Mozambique after the introduction of artemisinin-based combination therapies (ACTs) (2006) up to June 2024, highlighting the presence or absence of mutations in these genes across Mozambique. We aimed at mapping the prevalence and distribution of these molecular markers across the country in order to contribute to the development of targeted interventions to sustain the efficacy of malaria treatments in Mozambique. Four databases were used to access the articles: PubMed, Science Direct, Scopus, and Google scholar. The search strategy identified 132 studies addressing malaria and antimalarial resistance. Of these, 112 were excluded for various reasons, leaving 20 studies to be included in this review. Children and pregnant women represent the majority of target groups in studies on all types of antimalarials. Most studies (87.5%) were conducted in the provinces of Maputo and Gaza. The primary alleles reported were *pfcrt* CVMNK, and in the most recent data, its wild-type form was found in the majority of patients. A low prevalence of mutations in the *pfk13* gene was identified reflecting the effectiveness of ACTs. In *pfk13*, only mutation A578S was reported in Niassa and Tete. CNVs were observed in studies carried out in the south of Mozambique, with a frequency of 1.1–5.1% for *pfmdr1* and a frequency of 1.1–3.4% for *pfpm2*. This review indicates that molecular markers linked to malaria resistance show considerable variation across provinces in Mozambique, with most up-to-date data accessible for Maputo and Gaza. In contrast, provinces such as Zambezia and Inhambane have limited data on several genes, while Nampula lacks data on all drug resistance markers.

Keywords: *Plasmodium falciparum*; malaria drug resistance; molecular markers; artemisinin-based combination therapy; Mozambique

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1. Introduction

Malaria remains one of the most pressing public health challenges in many tropical and subtropical countries, impacting millions of lives across the region [1–3].

Mozambique faces a substantial malaria challenge, being one of the countries with the highest number of cases and ranking fourth globally in terms of the malaria burden [4]. To combat malaria effectively, Mozambique must address both the vector and the administration of antimalarial drugs [5].

The ongoing fight against malaria has been complicated by the emergence and spread of *Plasmodium falciparum* resistance to antimalarial drugs, which compromises the efficacy of treatment regimens and poses a significant threat to malaria control efforts [6–8]. Understanding the distribution of molecular markers of antimalarial resistance is essential for monitoring and managing drug resistance, revising treatment guidelines, and informing the development of new antimalarial drugs.

In Mozambique, as in other parts of sub-Saharan Africa, *P. falciparum* is the predominant malaria parasite. The region has seen various waves of drug resistance, particularly to chloroquine (CQ), sulfadoxine-pyrimethamine (SP), and, more recently, to artemisinin-based combination therapies (ACTs) [1]. Molecular markers have been instrumental in tracking and understanding these resistance patterns [1,9].

Chloroquine was the first-line treatment for uncomplicated malaria in Mozambique for almost 50 years until 2004, when sulfadoxine-pyrimethamine (SP) and amodiaquine (AQ) were introduced due the emergence and spread of resistance to chloroquine [10]. The primary molecular marker associated with chloroquine resistance is the *P. falciparum* drug resistance transporter (*pfcrt*) gene, particularly the single-nucleotide polymorphism (SNP) K76T [11]. Several studies have reported high frequencies of this mutation in Mozambique and neighboring countries such as Tanzania and Malawi, reflecting the extensive spread of CQ resistance (CQ-R) in the region [12–14]. The haplotype defined by specific mutations at amino acid positions 72–76 of *pfcrt*, CVIET, has been associated with CQ-R (in Africa), while the haplotype CVMNK is associated with CQ susceptibility (CQ-S) [15,16]. Following the decline in CQ efficacy, artesunate plus SP was introduced in Maputo Province between 2004 and 2006 as the mainstay for malaria treatment [10]. However, during this pilot study, molecular markers associated with SP resistance (SNPs in the genes dihydropteroate synthase, *pfdhps,* and dihydrofolate reductase, *pfdhfr*) increased dramatically [10,17,18]. SP resistance is associated with the SNPs A16V, N51I, C59R, S108N, and I164L in the *pfdhfr* gene, which confer resistance to pyrimethamine, and I431V, S436A/F, A437G, K540E, A581G, and A613S/T in the *pfdhps*, which confer resistance to sulfadoxine [17,19]. Parasites with multiple SNPs in both *pfdhfr* and *pfdhps* were categorized as follows: a quadruple mutant (*pfdhfr* 51I + 59R + 108N and *pfdhps* 437G [IRNG]) was classified as "partially resistant"; a quintuple mutant (*pfdhfr* 51I + 59R + 108N and *pfdhps* 437G + 540E [IRNGE]) as "fully resistant"; and a sextuple mutant (*pfdhfr* 51I + 59R + 108N and *pfdhps* 437G + 540E + 581G or 613S/T [IRNGEG or IRNGES/T]) as "super resistant" [20].

These findings led to a change in the national malaria treatment policy in 2008 to the use of ACTs. In Mozambique, the recommended treatment for uncomplicated *P. falciparum* malaria consists of: artemether-lumefantrine (AMT-LUM) (first line of treatment since 2006 [21], artesunate-sulfadoxine and pyrimethamine (AS-SP), artesunate-amodiaquine (AS-AQ), artesunate-mefloquine (AS-MEF), dihydroartemisinin-piperaquine (DHA-PPQ), and artesunate-pyronaridine AS-PY [10,22]. In ACTs, artemisinin derivatives (short half-life; <6 h) are combined with long-acting antimalarial drugs like AQ, MEF, PPQ, LUM, and pyronaridine (PY) to treat uncomplicated malaria [22–24]. Regarding ACT partner drugs, the primary genes associated with resistance are *pfcrt*, *pfmdr1*, *pfpm2/3,* and the above-mentioned *pfdhfr* and *pfdhps* [25]. Multiple copies (or copy number variations, CNV) of the *P. falciparum* multidrug resistance 1—*pfmdr1* gene are established markers for resistance to MEF (MEF-R) [26,27]. Additionally, SNPs in *pfmdr1* have been linked to altered parasite tolerance or susceptibility to several antimalarial drugs, including quinine (QN), AQ, CQ, MEF, and lumefantrine (LUM) [28]. The key *pfmdr1* SNPs associated with drug resistance include N86Y, Y184F, S1034C, and N1024D [29–34]. The N86Y mutation is related to increased CQ-R and increased sensitivity to MEF [35]. Parasites carrying

pfmdr1 haplotype 86Y Y184 show increased susceptibility to LUM and MEF [36]. The role of the *pfmdr1* N86, 184F, and 1246D alleles, as well as *pfmdr1* CNV, in *P. falciparum's* response to AMT-LUM remains debated [37].

In recent years, resistance to ACTs has been reported in Southeast Asia in 2008 [25,38,39]. Recently, SNPs associated with resistance to artemisinins in Africa [40–42] were identified. Resistance to artemisinin derivatives is characterized by delayed parasite clearance times and is linked to SNPs in the Kelch13 protein coded by the gene *pfk13*. In particular, F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, and C580Y are currently considered validated molecular markers of drug resistance by WHO [38,39,41]. This study's objective is to provide a comprehensive analysis of prevalence and distribution of the molecular markers of antimalarial resistance in Mozambique. By mapping the prevalence and distribution of these markers, this research aims to contribute to supporting the development of targeted interventions to maintain the effectiveness of malaria treatments in Mozambique.

2. Methods

2.1. Selection of Relevant Literature

This study was conducted according to the recommendations of the Preferred Reporting Items for Systematic Reviews (PRISMA) [43,44]. Briefly, the search terms and criteria for the inclusion or exclusion were previously defined to be searched across various databases. After conducting the article search, the selection of the studies based on the inclusion criteria were assessed by two independent researchers. In cases of disagreement, a third researcher was consulted to solve the dispute. Following the selection of articles for inclusion in the study, a thorough analysis was conducted to extract the most important findings and conclusions. Subsequently, these data were organized and presented in tables or figures. The databases searched were Scopus, PubMed, and Web of Science, in addition to isolated searches for relevant articles found on Google Scholar. A total of 132 articles published from 2007 to July 2024 complied with the inclusion criteria in the title, keywords, or summary. Aligning with the national rollout of ACTs in 2006 [21], and to capture the progress made in molecular monitoring of antimalarial resistance, a 17-year study period was chosen.

2.2. Eligibility Criteria of Studies Include in the Review

The inclusion criteria were all original articles addressing molecular marker of antimalarial drug resistance published in indexed journals (PubMed, Science Direct, Scopus, and Google Scholar) using the keywords: "*pfpm2/3* OR *pfmdr1* OR *pfk13* OR *pfdhps* OR *pfdhfr* OR '*pfcrt*' OR 'copy number variation', AND 'Mozambique".

2.3. Screening and Data Extraction

The articles selected for the study were exported to Microsoft Excel to remove duplicates. The selection of articles was carried out by reading the titles and abstracts and then the full text. The studies were systematized by authorship, year, sociodemographic data, sample size, allele or gene, amino acid, haplotype, type of mutation, CNV, respective prevalence, antimalarial drug, and main conclusions. The quality assessments of the studies were performed using a tool for assessing risk of bias in randomized studies (Cochrane ROB2) and a tool for assessing risk of bias in non-randomized studies (ROBINS-I).

3. Results and Discussion

3.1. Basic Characteristics of Included Studies

The search strategy identified 132 studies, from which 43 duplicates were removed. After screening titles and abstracts, 56 studies were excluded. Of the remaining 33 studies, 13 were excluded after full-text review, leaving 20 studies for inclusion in this review (Figure 1).

Children under 5 years of age and pregnant women comprise most targeted groups for all types of antimalarials, followed by children and adolescents up to 15 years. Few studies have focused on adult patients. The genes *pfdhfr* and *pfdhps*, associated with SP resistance, were identified in studies focusing on patients of all ages and sexes [45,46]. Regarding the gene *pfk13*, associated with resistance to artemisinin derivatives, the study by Da Silva [47] included both children and adults of both sexes, while the study by Escobar [48] was focused on adult patients of both sexes.

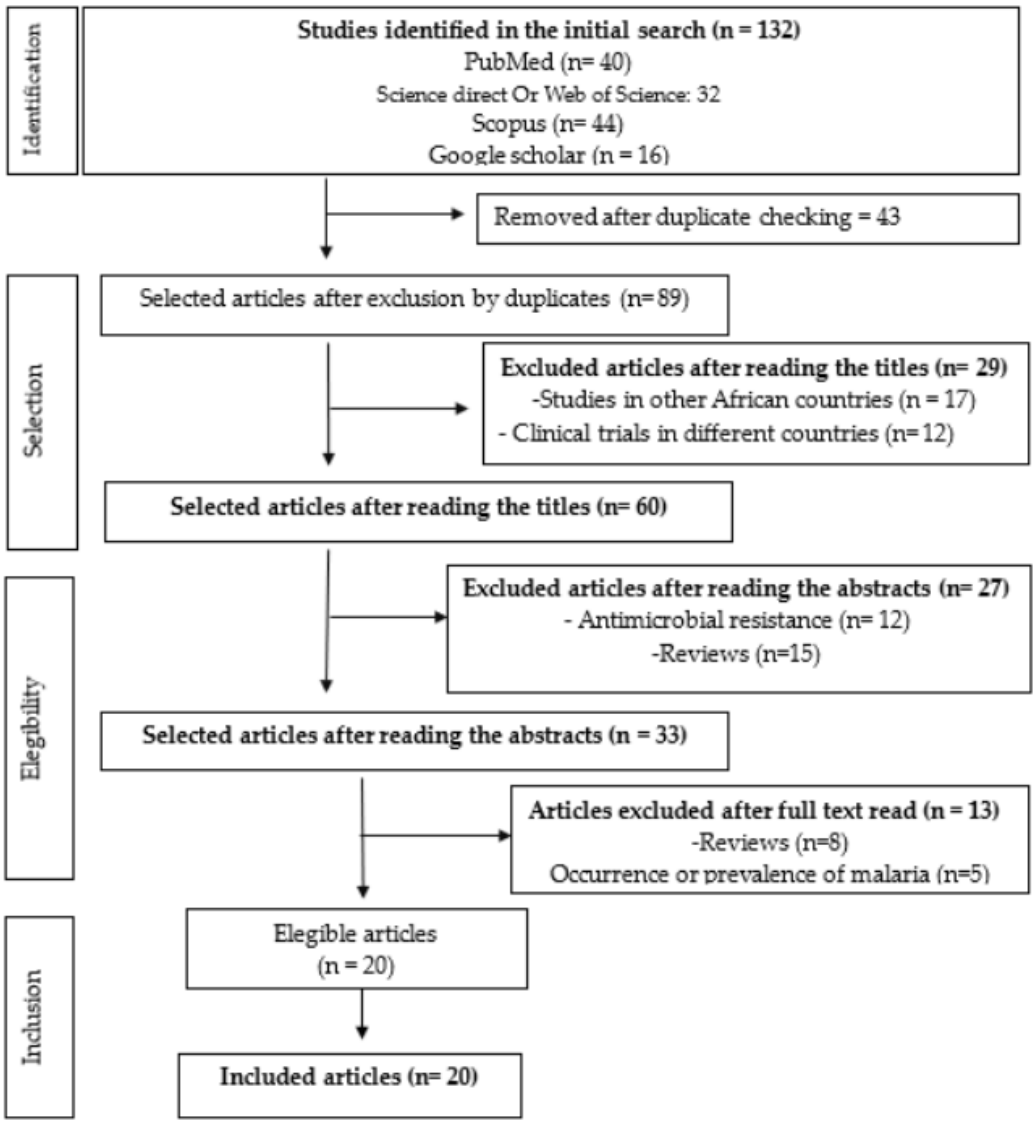

Figure 1. PRISMA diagram of the systematic review. Steps followed by this systematic review according to the PRISMA ("Preferred Reporting Items for Systematic Reviews and Meta-Analyses") guidelines.

The information about the 20 studies included in this review is summarized in Table 1 and Figure 2 and detailed in the Supplementary Materials (Tables S1- S5). Most studies (17/20; 85%) were conducted in southern Mozambique, specifically in the provinces of Maputo and Gaza (Table 1). It is important to emphasize that 40% (8/20) of the total articles included in this study addressed three genes (*pfcrt*, *pfdhfr*, and *pfdhps*, *pfmdr1*, *pfk13* and CNVs *pfpm2*/*pfpm3*/*pfmdr1*) in different provinces [49–55] (detailed in Supplementary Materials).

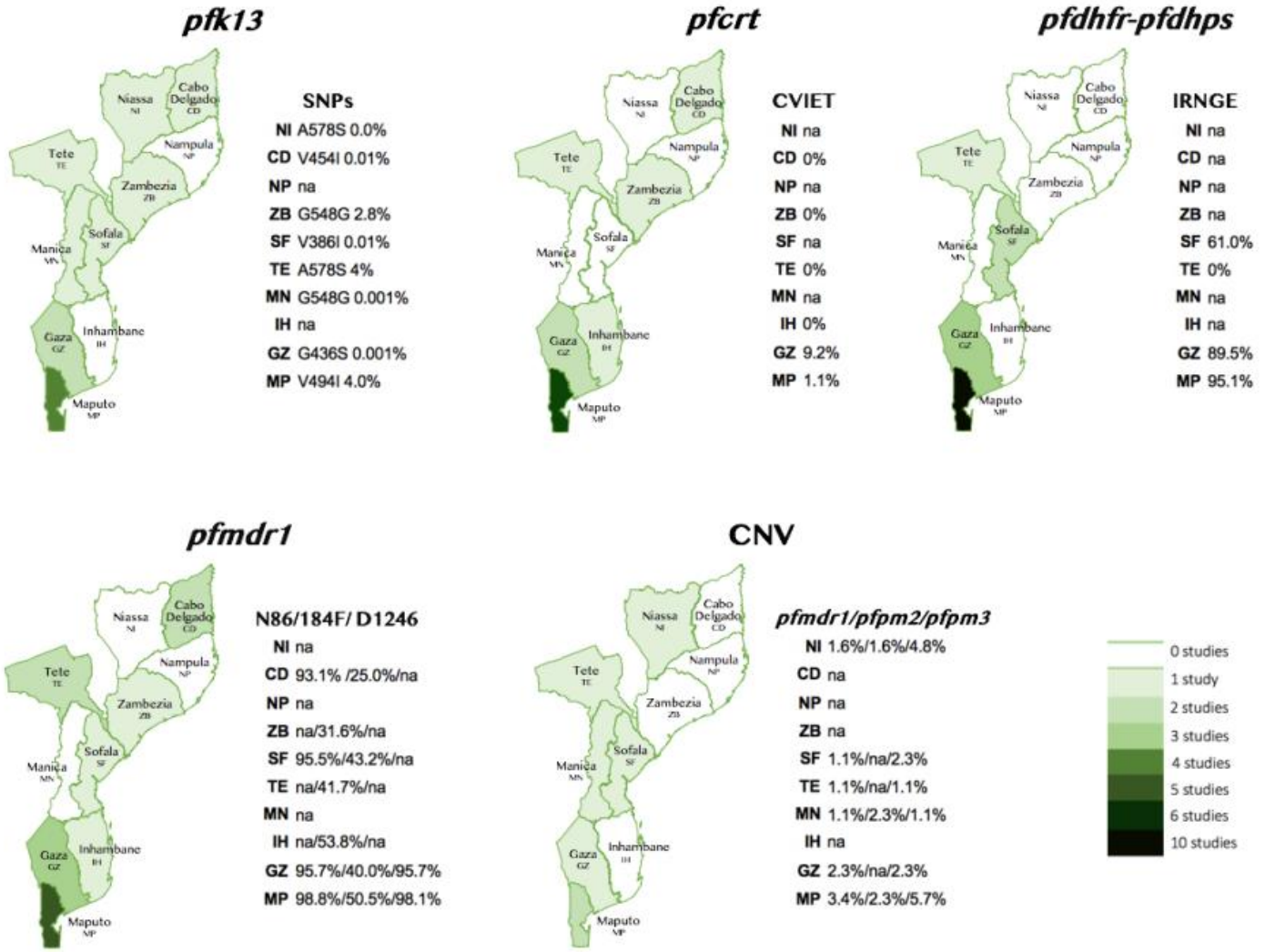

Figure 2. Geographical distribution of studies and the most recent data available for each molecular marker. Colored rectangles represent the number of studies identified for each molecular marker; CNV, copy number variation; SNPs, single nucleotide polymorphisms; CVIET, amino acid positions 72-76 of *pfcrt*; IRNGE, amino acid positions 51I + 59R + 108N of *pfdhfr* and *pfdhps* 437G + 540E of *pfdhps*; na, not available; NI, Niassa; CD, Cabo Delgado; NP, Nampula; ZB, Zambesia; SF, Sofala; TE, Tete; MN, Manica; IH, Inhambane; GZ, Gaza; MP, Maputo.

Table 1. Summary of the studies included in the review. SNP, single-nucleotide polymorphism; CNV, copy number variation.

Mutation	Gene	Province	Nº of Studies (2008–2024)	References	Year of Sample Collection *
SNP	*pfcrt*	Maputo	6	[10,50–57]	2015 - 2019 [51]
		Gaza	2		2015 [53]
		Inhambane	1		2018 [54]
		Zambezia			
		Tete			
		Cabo Delgado			
	pfdhfr, pfdhps	Maputo	10	[10,45,46,50,51,53,55,57–62]	2015 - 2019 [51]
		Gaza	3		2014 - 2015 [50]
		Tete	1		2015 [53]
		Sofala	2		
		Cabo Delgado	1		2015 [53]
	pfmdr1	Maputo	5	[10,50–55,57,63]	2015 - 2019 [51]

		Gaza	**3**		2014 - 2015 [50]
		Inhambane	**1**		2018 [54]
		Zambezia			
		Tete	**2**		
		Sofala	**1**		
		Cabo Delgado	**2**		
	pfk13	**Maputo**	**4**	[47,48,51,52,54]	2021 [47]
		Gaza	**1**		
		Zambezia			
		Tete			
		Sofala			
		Manica			
		Cabo Delgado			
		Niassa			
CNV	***pfpm2/ pfpm3/ pfmdr1***	**Maputo**	**2**	[52,53,64]	2021 [64]
		Gaza	**1**		2015 [53]
		Tete			
		Sofala			
		Manica			2021 [64]
		Niassa			

* indicates the year the samples were collected, providing the most recent data on the prevalence of the corresponding molecular marker.

A total of nine studies (45%) monitored the *pfcrt* gene (associated with CQ-R) (Table 1): six in Maputo; two in Gaza; and one for Tete, Zambezia, Cabo Delgado, and Inhambane. *pfdhr* and *pfdhps* genes, associated with SP resistance, were found in 13 studies (65%); 10 of these studies were conducted in Maputo (Table 1). Of these 13 studies, 9 involved children, 4 pregnant women, and 1 adults (Table S2).

A total of nine studies were found addressing the *pfmdr1* gene (Table 1). Five of these focused on Maputo and two on Gaza, with five involving children and four pregnant women (Table S3). Five studies were found examining the *pfk13* gene (Table 1), including two involving adults and pregnant women and three involving children, mainly in Maputo (Table S4). Finally, three studies investigating CNV were identified; all included pregnant women and children, with two in Maputo and one in Niassa, Manica, Sofala, Tete, and Gaza, respectively. This overview highlights a concentration of studies in the Maputo and Gaza provinces (in the south of the country; Figure 2) and a predominance of research involving children and pregnant women (detailed in Supplementary Materials).

Malaria remains a significant public health concern in Mozambique, with *Plasmodium falciparum* being the predominant species responsible for the disease [65]. Understanding the genetic variants associated with drug resistance is crucial for developing effective treatment strategies and transmission control of the disease. This review reveals substantial regional variability in genetic mutations associated with malaria drug resistance in Mozambique (Figure 2). While the data are more robust for Maputo and Gaza, significant gaps remain for other provinces, underscoring the need for further research to monitor genetic variations over time. For instance, research on *pfcrt* gene polymorphisms, which encode CQ-R, primarily focused on two southern provinces. The prevalence of *pfcrt* gene was detected as between 40 and 84% of patients [10,56,61]. In the past, these data were important for changing the malaria treatment, which at the time was based on chloroquine, in line with what was happening in all malaria-endemic countries [66]. In Gaza and Maputo, a moderate to high prevalence of the 76T *pfcrt* SNP (CQ-R) was found during the first years after the introduction of ACTs [10,55]. However, more recent studies conducted in the same provinces after the discontinuation of chloroquine (samples collected 2017–2019) have identified a high prevalence of CQ-S *P. falciparum* genotypes [52–54]. This shift suggests a reduced selective pressure from CQ. Similar trends have been observed in

other sub-Saharan African countries, including Kenya, Malawi, Sierra Leone, Ghana, Angola, and Ivory Coast, where CQ-S *P. falciparum* genotypes have re-emerged [67–70].

3.2. Antimalarial Resistance Associated Polymorphisms

3.2.1. *pfcrt*

The majority of the studies (87.5%) addressing the *pfcrt* gene were conducted in the provinces of Maputo and Gaza (Tables 1 and S1), with only one study addressing multiple provinces, namely Inhambane, Zambezia, Tete, and Cabo Delgado (see Table S1). The most recent evaluation of *pfcrt* CVIET haplotype was from 2024 and revealed prevalences of 1.1% in Maputo; 9.2% in Gaza; and 0% in Inhambane, Zambezia, Tete and Cabo Delgado (see Figure 2 and Table S1). The most prevalent haplotype was CVMNK (CR-susceptible), found in 92.2% of patients sampled in Cabo Delgado, Tete, Zambezia, and Inhambane in 2021 [53]. CVIET (CR-resistant) was reported in 7.8% of patients sampled in Inhambane, Zambezia, Tete, and Cabo Delgado [56], and 76T was found in 84% of adult patients of both genders in Maputo province in 2024 [71], 48.8% in children aged 2 to 3 months, and 46.4% in pregnant women [62]. The gene *pfcrt* confers resistance to a wide range of quinoline and quinoline-like antimalarial drugs in *P. falciparum*, with local drug histories driving its evolution and, thus, the drug transport specificities. For example, the change in prescription practice from CQ to PPQ in Southeast Asia has resulted in *pfcrt* variants that carry additional SNPs (H97Y, F145I, M343L, or G353V), leading to PPQ resistance [77]. There is a notable gap in the current understanding of specific *pfcrt* SNPs in Mozambique, particularly the ones associated to PPQ-R in Southeast Asia. Evaluating these markers in Mozambique could provide essential information for updating malaria treatment guidelines and managing potential PPQ-R as drug policies shift in the country.

3.2.2. *pfdhr* and *pfdhps*

The prevalence of the SP-resistance haplotype IRNGE was high in Maputo (95.1%) and Gaza (89.5%) (Figure 2). The main studied mutations occurred at amino acid positions 51, 59, 108, 164, 437, 540, and 58, either individually or in combination within the *pfdhfr* or *pfdhps* genes, resulting in multidrug resistance haplotypes (see Table S2). Taking into account the most recent results, in the Maputo province, the most frequent haplotype was IRNGE, with 95.1% prevalence in samples collected in 2015–2019 [51] and 94.2% in samples collected in 2016–2019 (Table S2) [62]. In Gaza, the sextuple IRNGEG haplotype was observed in 8% of the samples and the quintuple IRNGE in 55% [72]. Studies from the central (Tete and Sofala) [53] and southern (Gaza and Maputo) [73] regions reported higher prevalence of SNPs in the *pfdhfr* or *pfdhps* in various combinations than the northern (Cabo Delgado) provinces [53] (Table S2).

In Mozambique, SP is used for intermittent preventive treatment in pregnancy (IPTp) and has been linked to the accumulation of SP-resistant mutations in *pfdhfr* and *pfdhps* [10,50,61]. This may facilitate the selection of resistant parasites due to the repeated exposure to SP. Nevertheless, despite widespread SP resistance, studies indicate that administering three or more doses of SP to pregnant women may still confer a protective benefit against *P. falciparum* [62]. The geographical distribution of *pfdhfr* and *pfdhps* SNPs studies in Mozambique reveals uneven coverage across provinces, with a significant focus on the southern region, particularly Maputo (10; Table S2), with a limited number of studies conducted in other provinces (three in Gaza [10,50,52], , two in Sofala [53,60] and one in Cabo Delgado and Tete [53]; Table S2). The remaining five provinces do not have published information (Figure 2). In Maputo, high prevalence rates of mutations associated with SP-R were reported, such as 51I (36.6–88%) in *pfdhfr* and 59R (52.4–91%), 108N (50.4–99.2%), 540E (7.9–94.9), and 437G (42–96.2%) in *pfdhps*. The quintuple mutant IRNGE was reported in multiple studies with high prevalence, namely, 94.2% in samples from 2016 to 2019 [62] and 95.1% in samples from 2015 to 2019 [51] (Table S2). This underscores

substantial SP resistance in Mozambique and follows the trend of other African countries, such as Ghana and Nigeria [74,75].

3.2.3. *pfmdr1*

SNPs in *pfmdr1* were studied in all provinces except Niassa, Manica and Nampula (Figure 2 and Table S3). The latest reported prevalences of SNPs were N86, found in 93.1% in Cabo Delegado, 95.7% Gaza, 95.5% in Sofala, and 98.8% in Maputo, respectively, and 184F, reported in 41.7% in Tete, 43.2% in Sofala, 50.5% in Maputo, and 53.58% in Inhambane, respectively (Figure 2).

The *pfmdr1* encodes a protein involved in drug transport within the parasite and plays a key role in susceptibility to the key antimalarial ACTs. Although mutations in *pfmdr1* are not directly responsible for resistance to artemisinins, they influence the effectiveness of partner drugs, such as LUM or AQ, and the haplotype NFD has been associated with higher susceptibility to these partner drugs [29–34]. After Mozambique transitioned from chloroquine to ACTs for malaria control, the prevalence of *pfmdr1* mutations changed, with the NFD haplotype (amino acids 86/184/1246) variant becoming more common [52,54]. The current data reveal a significant geographical gap in the country regarding studies on the *pfmdr1* gene. Most research has been concentrated in Maputo (5) [51,52,55,57,63] and Gaza (3) [10,53,76]. There are limited data from other provinces, like Cabo Delgado and Tete (2) [53,54] or Zambezia, Inhambane (1) [5], and Sofala (1) [53]. This regional imbalance of studies leaves large parts of Mozambique underrepresented, especially in the northern and central provinces. For instance, no studies have been recorded in Nampula for *pfmdr1* (or any other molecular marker), and in Sofala, the only study available is based on samples collected nearly a decade ago (2015) [53]. The most recent studies have identified an appreciable prevalence of mutations in *pfmdr1,* namely, the SNPs N86 (98.8%) and 184F (75.4%) in Maputo (samples collected in 2015–2019) [51] and the haplotype NFD in Inhambane 74.4%, Cabo Delgado 66.7%, Tete 11.0%, or Zambezia 50.0% (samples collected in 2018) [54]. Similar trends have been observed in several other African countries [76–79].

3.2.4. *pfk13*

Polymorphisms of *pfk13* associated with multidrug resistance in *P. falciparum* were investigated in five studies (Table S4). Most studies (75%) were conducted in eight provinces, except Inhambane and Nampula. Only one study examined multiple provinces (Figure 2 and Table S4). A low frequency of *pfk13* was observed in all provinces where studies were conducted (Figure 2). Maputo and Tete, with 4% each, were the provinces with the highest prevalence of *pfk13* SNPs (Figure 2). Notable, findings included the synonymous mutation at codon 469 (TGC to TGT) in one sample and at codon 548 (GGC to GGT) in three samples from Zambezia province (Mopeia city [54]). Two studies were identified for the SNPs: 494I [52] and 578S [52], both with 4% prevalence and both in samples from Maputo province [48] (Table S4). Neither of these two SNPs is currently considered a validated molecular marker of drug resistance by WHO [38,39,41].

The prevalence of *pfk13* SNPs varies by region; in 2019, it was 45.4% in Southeast Asia compared to a much lower prevalence of 7.6% in Africa [66]. In Mozambique, 8/10 provinces have evaluated the presence of *pfk13* SNPs, and none of the validated or candidate mutations have been identified so far. Similar findings have been reported in other African countries like Gabon [80], Senegal [81], Kenya, and Ethiopia [82], where low frequencies of *pfk13* SNPs have been observed. However, A578S was detected in samples from Niassa and Tete provinces [54,83], as well as in Uganda and Gabon [84]. The identification of independent emergence of *pfk13* SNPs (with partial resistance to ACTs) in the African region, especially in Rwanda and Uganda [85–88], highlights the importance of surveillance efforts to obtain genotypic data and map the extent of *pfk13* SNPs throughout the

WHO African Region [89]. The recent detection of SNPs M476I, P553L, R561H, P574L, and C580Y in Africa serves as an early warning signal [40–42,90].

3.2.5. Copy Number Variations in *pfmdr1* and *pfpm2/3*

Figure 2 summarizes the latest prevalence rates and primary study provinces, and Table S5 displays detailed data collected from various populations (children, adults, pregnant women) between 2015 and 2023. Only three studies were found investigating the prevalence of copy number variations (CNVs) in *pfmdr1* and *pfpm2* (Table 1). Two studies, Brown et al., 2024 [64] and Gupta et al., 2018 [53], covered multiple provinces, while the third study (Gupta et al., 2020 [52]) focused solely on Maputo province (Table S5). *Pfmdr1* CNV prevalence rates were as follows: 4.8% in the north (Niassa); between 1.1%, 2.3% in Tete, Manica, and Sofala (center); and 5.7% in the south (Maputo; Figure 2). Regarding plasmepsins (*pfpm2* and *pfpm3*) CNVs, prevalence rates were higher in the southern provinces of Gaza and Maputo (3.4% for *pfpm2* and 2.3% for *pfpm3*) compared to the northern and central provinces (Niassa, Tete, Manica, and Sofala), where the prevalence ranged from 1.1% to 1.6% for *pfpm2* and 1.6% to 2.3% for *pfpm3* (specifically 1.6% for *pfpm3* in Niassa and 2.3% in Manica).

There are only three studies assessing CNVs of *pfmdr1*, *pfpm2*, and *pfpm3* in Mozambique; one assessing all three [64]; and two assessing *pfmdr1* and *pfpm2* [52,53]. These revealed prevalence rates ranging from 1.1 to 5.7% for *pfmdr1*, 1.1 to 3.4% for *pfpm2*, and 1.6 to 2.3% for *pfpm3* [10,22,57]. Studies from Mozambique revealed a much lower prevalence of *pfmdr1* CNV than other African countries, namely Kenya (6.2%), Ghana (18%), Tanzania (10.2%), West Ethiopia (8.4%), and North of Ethiopia (54.14%) [76,91–94]. Observations from Mozambique, on the other hand, are in line with studies from other African countries like Nigeria or Democratic Republic of Congo, where increased CNV was not observed for *pfmdr1* [95,96]. Regarding *pfpm2* prevalence, the two studies recorded in Mozambique also reported much lower prevalences than others from Africa (7.7% in Tanzania [91] and 67.9% in Guinea Equatorial [97], but comparable to, e.g., Liberia or Uganda, increased copies of *pfpm2* were not observed [98,99]).

Copy number variation (CNV) has also been found to play a significant role in the development of antimalarial drug resistance. One copy of *pfmdr1* is associated with slower clearance of parasites after PPQ treatment as compared to more copies of *pfmdr1* [107], while having two copies of *pfpm2* is associated with slower clearance [101,102], after PPQ treatment. This inverse selection pressure argues in favor of keeping these molecular markers under constant surveillance.

4. Conclusions

Although malaria is endemic throughout the country, the central and northern regions of Mozambique have the highest incidences, especially the provinces of Zambezia, Nampula, and Cabo Delegado, which are most affected by the disease [103]. This situation may be associated with the fact that these are coastal provinces, with climatic conditions and socio-economic factors favorable for the proliferation of the malaria vector. However, most studies on monitoring molecular markers of resistance to antimalarials are concentrated in the southern region of the country.

Maputo province has had the highest number of and more up-to-date studies conducted (17), followed by Gaza (4) and Tete, Sofala, and Cabo Delgado (3). Other provinces such as Manica and Niassa (2), Zambezia, and Inhambane (1) have limited studies, while no studies have been reported from Nampula. This review highlights the concentration of research efforts primarily in Maputo, reflecting a potential need for further investigation to gather more recent data on these genetic markers in the underrepresented provinces.

To address the disparities in research distribution and the underrepresentation of northern and central provinces in Mozambique, future studies should prioritize

comprehensive investigations into molecular markers of antimalarial resistance in regions with high malaria incidence, such as Zambezia, Nampula, and Cabo Delgado. These provinces are not only heavily affected by the disease, but also exhibit unique climatic and socio-economic conditions that may influence resistance patterns.

Expanding research into these areas will provide critical insights into the regional dynamics of resistance, enabling more targeted and effective malaria control strategies. Additionally, establishing collaborative research networks and strengthening local laboratory capacities in underrepresented provinces could ensure a more equitable distribution of scientific efforts. This approach will contribute to the development of a robust national framework for monitoring and combating antimalarial resistance, ultimately improving public health outcomes across the country.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1.

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Abbreviations

ART: artemisinin; LUM: lumefantrine; CQ: chloroquine; QN: quinine; PPQ: piperaquine; DHA: dihydroartemisinin; AQ: amodiaquine; MEF: mefloquine; PY: pyronaridine; SP: sulfadoxine-pyrimethamine; As: artesunate; AMT: artemether. SNP, single-nucleotide polymorphisms; CNV, copy number variation; ACT, artemisinin-based combination therapy.

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Supplementary materials:

The supplementary materials can be found at www.mdpi.com/xxx/s1.

4.2. Chapter 2: Characterisation molecular markers associated with *P. falciparum* resistance to ACTs in Maputo, Sofala, Manica and Niassa provinces.

4.2.1. Paper II: Anti-malarial resistance in Mozambique: Absence of *Plasmodium falciparum* Kelch 13 (K13) propeller domain polymorphisms associated with resistance to artemisinins

Reference:

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In this manuscript, I had the following roles:

- Participated in the study design
- Conducted the field activities (sample collection)
- Conducted laboratory activities and analisys of results
- Writing of the first draft of the manuscript

Errata Chapter 2: Paper II

Methods, page 3, first paragraph

The Pfk13 fragment, containing the main polymorphisms associated with resistance to artemisinin, was amplified by nested PCR as described by Escobar and co-workers [29], with slight adjustments. Briefly, specific primers were developed for this purpose (forward— 5′-CTA TAC CCA TAC CAA AAG ATT TAA GTG-3′, reverse—5′-GCT TGG CCC ATC TTT ATT AGT TCC C-3′), obtaining a fragment of 902 bp (from codon 4152 to codon 71523).

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Anti-malarial resistance in Mozambique: Absence of *Plasmodium falciparum* Kelch 13 (K13) propeller domain polymorphisms associated with resistance to artemisinins

Clemente da Silva[1*], Daniela Matias[1], Brigite Dias[1], Beatriz Cancio[1], Miguel Silva[1], Ruben Viegas[1], Nordino Chivale[2], Sonia Luis[3], Crizolgo Salvador[2], Denise Duarte[1], Paulo Arnaldo[2], Sonia Enosse[4] and Fatima Nogueira[1]

Abstract

Background Malaria remains one of the most serious public health problems in sub-Saharan Africa and Mozambique is the world's fourth largest contributor, with 4.7% of disease cases and 3.6% of total deaths due to malaria. Its control relies on the fight against the vector and treatment of confirmed cases with anti-malarial drugs. Molecular surveillance is an important tool for monitoring the spread of anti-malarial drug resistance.

Methods A cross-sectional study recruited 450 participants with malaria infection detected by Rapid Diagnostic Tests, from three different study sites (Niassa, Manica and Maputo) between April and August 2021. Correspondent blood samples were collected on filter paper (Whatman® FTA® cards), parasite DNA extracted and *pfk13* gene sequenced using Sanger method. SIFT software (Sorting Intolerant From Tolerant) was used, predict whether an amino acid substitution affects protein function.

Results No *pfkelch13*-mediated artemisinin resistance gene mutation was detected in this study settings. However, non-synonymous mutations were detected at prevalence of 10.2%, 6% and 5% in Niassa, Manica and Maputo, respectively. Most (56.3%) of the reported non-synonymous mutations were due to substitution at the first base of the codon, 25% at the second base and 18.8% at the third base. Additionally, 50% of non-synonymous mutations showed a SIFTscore bellow cut off value of 0.05, therefore, they were predicted to be deleterious.

Conclusion These results do not show an emergence of artemisinin resistance cases in Mozambique. However, the increased number of novel non-synonymous mutations highlights the relevance of increasing the number of studies focused on the molecular surveillance of artemisinin resistance markers, for its early detection.

*Correspondence:
Clemente da Silva
a21000955@ihmt.unl.pt
[1] Global Health and Tropical Medicine (GHTM), Department of Medical Parasitology, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira Nº100, 1349-008 Lisbon, Portugal
[2] Instituto Nacional de Saúde (INS), Av. Eduardo Mondlane Nº 1008, Caixa Postal 264, Maputo, Mozambique
[3] Hospital Provincial de Matola, 2CPV+55, Matola, Mozambique
[4] Malaria Consortium, Av. Lucas Elias Kumato, Nº 118. Bairro da Sommershield, Maputo, Mozambique

Background

Malaria remains one of the most serious public health problem in sub-Saharan Africa [1] and Mozambique is the world's fourth largest contributor, with 4.7% of disease cases and 3.6% of total deaths due to malaria [2]. In 2021, the World Health Organization (WHO) recommended the use of a malaria vaccine to protect children, however, the vaccine has low efficacy and its distribution is not yet widespread in Mozambique [3]. Therefore, malaria control relies on the fight against the vector and the administration of anti-malarial drugs [4].

Plasmodium falciparum, the most virulent of the five species that infect humans, has developed resistance to the successively introduced anti-malarial drugs, including to the currently recommended artemisinin-based combination therapy (ACT) introduced in Mozambique in 2009 [5, 6]. Artemisinin and its derivatives play an important role in killing *P. falciparum* by inhibiting the activity of phosphatidylinositol-3-kinase (PfPI3K) [7, 8] and like any other drug, it may have side effects, but they are poorly expressed [7, 9, 10]. Historically, cases of anti-malarial resistance have their starting point in Southeast Asia [6, 11], move through east Africa's coast and spread to the rest of the continent [12–16]. In Mozambique, ACT using artemether–lumefantrine (AL) or artesunate–amodiaquine (AS–AQ) is currently the first-line treatment for uncomplicated malaria [1, 17–21].

Mutations in the *pfk13* gene have been correlated with delayed parasite clearance (tolerance) after administration of ACT [7, 21]. Some of the mutations are validated single nucleotide polymorphisms (SNPs), used as molecular markers for the surveillance of artemisinin-resistant malaria parasites (F446L, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L, C580Y) (WHO, 2019). Recently, *P. falciparum* parasites carrying the validated SNPs F446I, M476I, P553L, R561H, P574L, C580Y and A675V have emerged and expanded or been identified in Africa [22], in Angola, Ghana [23], Mali, Rwanda [24–27]. In Mozambique, available studies on the prevalence of *pfK13* SNPs, refer to parasite samples collected prior to 2018 [28–31]. Thus, the present study was designed to update the current profile of SNPs in *P. falciparum pfk13* associated with artemisinin resistance in 3 provinces (Niassa, Manica and Maputo) of Mozambique.

Methods

Ethical considerations

The study protocol obtained ethical clearance by the National Bioethics Committee for Health of Mozambique (CNBS—IRB00002657) (Ref: 131/CNBS/2021) dated: March 2021.

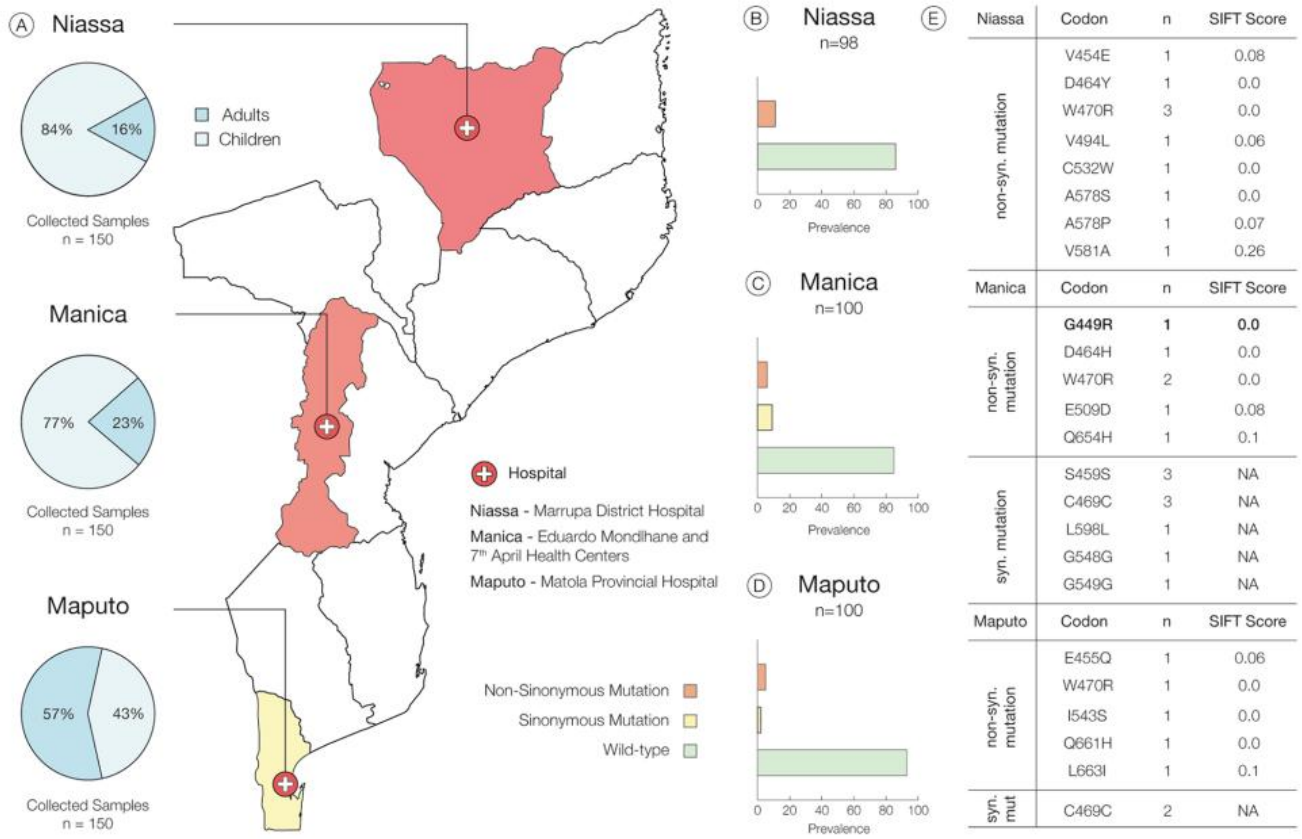
Study settings and sample collection

Malaria is endemic throughout Mozambique, ranging from hyper-endemic areas along the coastline, meso-endemic areas in the interior lowlands and some hypo-endemic areas in the interior highlands. Several factors contribute to this endemicity, ranging from climatic and environmental conditions, such as favourable temperatures and rainfall, as well as favourable breeding sites for the vector. Most of the country has year-round throughout the year, with peaks during the rainy season from December to April [4]. Patient recruitment took place in 3 different epidemiological settings, in northern (Hospital distrital de Marrupa in Niassa), central (*Centro de Saúde Eduardo Mondlhane* and *Centro de Saúde 7 de abril* in Manica) and southern (*Hospital provincial da Matola* in Maputo) area of Mozambique, between April and August of 2021. One hundred and fifty samples were collected in each study site (Fig. 1A). The choice of Niassa, Manica and Maputo provinces not only is aligned with the study scientific goals but also with the objectives of *Instituto Nacional de Saúde*, and National Malaria Control Programme Mozambique, contributing to the systematic mapping of malaria cases at the national level.

A total of 450 participants of all ages with malaria positive Rapid Diagnostic Test (RDT) were recruited and provided 100 µL of blood samples on filter papers (Whatman® FTA® cards), after written informed consent. All dried blood spot samples were then stored under − 20 °C until they were used for genotyping.

Characterization of *Pfk13* gene polymorphisms

Parasite genomic DNA from dried blood spots was extracted using the Chelex method [32], and DNA was stored at − 20 °C. Real-time PCR was used for *P. falciparum* confirmation. PCR reactions targeting the 18S rRNA gene were conducted as described in Rosanas-Urgell et al. 2010, with modifications. Briefly, forward primer 5′-TATTGCTTTTGAGAGGTTTTGTTACTTTG-3′ and reverse primer ACCTCTGACATCTGAATACGA ATGC and the probe FAM-ACGGGTAGTCATGAT TGAGTT-MGB-BHQ were used. PCR reaction mixture consisted of 7.5 µL of 2X (NZYTECH, Portugal), 600 nM of each primer and 200 nM of FAM™-labeled probe (IDT Integrated DNA Technologies, USA), 1 µL of genomic DNA and water up to 15 µL. PCR conditions: 50 °C for 2 min and 95 °C for 10 min; these were followed by 40 cycles at 94 °C for 30 s and a final cycle at 60 °C for 1 min. Triplicate samples were assayed in the Bio-Rad 500 Real Time PCR System™ (Applied Biosystems, USA). All reactions were performed with positive controls (DNA from 3D7 strain of *P. falciparum* culture).



Niassa	Codon	n	SIFT Score
non-syn. mutation	V454E	1	0.08
	D464Y	1	0.0
	W470R	3	0.0
	V494L	1	0.06
	C532W	1	0.0
	A578S	1	0.0
	A578P	1	0.07
	V581A	1	0.26
Manica	**Codon**	**n**	**SIFT Score**
non-syn. mutation	**G449R**	**1**	**0.0**
	D464H	1	0.0
	W470R	2	0.0
	E509D	1	0.08
	Q654H	1	0.1
syn. mutation	S459S	3	NA
	C469C	3	NA
	L598L	1	NA
	G548G	1	NA
	G549G	1	NA
Maputo	**Codon**	**n**	**SIFT Score**
non-syn. mutation	E455Q	1	0.06
	W470R	1	0.0
	I543S	1	0.0
	Q661H	1	0.0
	L663I	1	0.1
syn. mut	C469C	2	NA

Fig. 1 *Plasmodium falciparum* k13 polymorphisms profile in Mozambique. **A** Study sites and sample size. n-sample size in each province. Pie chart depict the percentage of children (Beau blue) and adults (Steel blue) enrolled in the study. **B–D** Prevalence of *pfk13* mutations in Niassa, Manica, and Maputo, respectively. Non-synonymous mutation (orange), synonymous mutation (yellow) and Wild type (green). **E** Mutations in 3 provinces and the respective codons and SIFT scores. Syn- Synonymous; Non.Syn- Non-synonymous. The candidate marker codon reported as G449A instead of G449R observed in our analyses (in bold). Figure was created using Illustrator version 26.3

The *Pfk13* fragment, containing the main polymorphisms associated with resistance to artemisinin, was amplified by nested PCR as described by Escobar and co-workers [29], with slight adjustements. Briefly, specific primers were developed for this purpose (forward—5′-CTATACCCATACCAAAAGATTTAAGTG-3′, reverse—5′-GCTTGGCCCATCTTTATTAGTTCCC-3′), obtaining a fragment of 902 bp (from codon 412 to codon 723). PCR conditions [33]: 94 °C 3 min; [94 °C 30 s, 57 °C 30 s, 72 °C 30 s] 10×; [94 °C 30 s, 55 °C 30 s, 72 °C 30 s] 30×; 72 °C 3 min. PCR products were analysed by electrophoresis on a 2% agarose gel stained with GreenSafe Premium (Nzytech, Portugal) to confirm amplification of targeted fragments. All positive PCR products were then purified using SureClean Plus (Bioline) and shipped to Eurofins Genomics (GATC services, Germany), to proceed with Sanger sequencing. Successfully sequenced samples were aligned to the PF3D7_1343700 gene using Multalign software (http://multalin.toulouse.inra.fr/; free online) and/or BioEdit version 7.2 for mutation detection. Bar charts with prevalence of artemisinin resistance markers were generated using GraPhpad Prism 8.01 software. In order to predict the potential impact of non-synonymous SNPs on protein function, we used SIFT software (Sorting Intolerant From Tolerant, free online, https://sift.bii.a-star.edu.sg/index.html). SIFT software, takes into account the position at which the variation takes place and the type of amino acid change, then, chooses related proteins and obtains an alignment of these proteins with the query [34]. Finally, it calculates the probability that this particular amino acid change will is tolerated [34]. If the calculated value is less than a cut-off of 0.05, the substitution is predicted to be deleterious, and the opposite is considered not deleterious [34–36].

Results

Study participants

From the 450 participants included in the study, one third were from each of the three study provinces (Fig. 1). Most of the participants were males, representing 57, 53% (259/450). The age ranged from 6 months to 74 years,

and the overall majority (%) were children between 1 to 12 years old, and the average was 15 years old.

Artemisinin resistance *pfk13* polymorphism profile

From selected samples, 66, 2% (298/450) were confirmed for *P. falciparum* by real time PCR, and DNA was successfully sequenced for *pfk13*, where 98, 100 and 100 were from Niassa, Manica and Maputo, respectively (Table 1). No validated SNP for artemisinin derivate resistance was detected in our samples. However, in Niassa, 10.2% (9/98) of samples harbored nine different non-synonymous mutations (Fig. 1B, Table 1). In Manica and Maputo, five different non-synonymous mutations were detected in 6 (6/100) and 5 (5/100) samples, respectively (Fig. 1C, D). 56.3% (9/16) of the non-synonymous mutations reported in this study were by substitution at the 1st base of the codon, 25% (4/16) at the second base and 18.8% (3/16) at the third base. The non-synonymous mutation, W470R, was detected in all provinces and its prevalence decreases from north to south with 3%, 2% and 1% in Niassa, Manica and Maputo, respectively. Synonymous mutations were only detected in samples from Manica and Maputo (Fig. 1E).

Table 1 Single nucleotide polymorphisms identified in pfk13 gene in samples collected in Niassa, Manica and Maputo, Mozambique duiring 2021

Codon	Type of mutation	Reference» mutant	Study site	n/N
G449R	NS	ggt» **c**gt	Manica	1/98
V454E	NS	gta» g**a**a	Niassa	1/100
E455Q	NS	gaa» **c**aa	Maputo	1/100
S459S	S	tcg» tc**c**	Manica	3/98
D464Y	NS	gat» **c**at	Manica	1/98
D464H	NS	gat» **c**at	Niassa	1/100
C469C*	S	tgc» tg**t**	Manica	3/98
			Maputo	2/100
W470R	NS	tgg» **a**gg	Manica	2/98
			Niassa	3/100
			Maputo	1/100
V494L	NS	gtt» **c**tt	Niassa	1/100
E509D	NS	aga» a**a**a	Manica	1/98
C532W	NS	tgt» tg**g**	Niassa	1/100
I543S	NS	att» a**g**t	Maputo	1/100
G548G	S	ggg» gg**t**	Manica	1/98
G549G	S	tct» t**t**t	Manica	1/98
A578S	NS	gct» **t**ct	Niassa	1/100
A578P	NS	gct» **c**ct	Niassa	1/100
V581A	NS	gtt» g**c**t	Niassa	1/100
L598L	S	tta» **c**ta	Manica	1/98
Q654H	NS	caa» ca**c**	Manica	1/98
Q661H*	NS	caa» ca**c**	Maputo	1/100
L663I	NS	caa» ca**c**	Maputo	1/100

n: number of samples containing mutant allele; N: number of samples sequenced at locus; NS: non-synonymous SNP; S: synonymous SNP; * has previously been reported

Putative functional impact of amino acid substitutions caused by the non-synonymous SNPs

With a cut-off score of 0.05, we predicted that V454E, V494L, A578P, V581A, E509D, E455Q and L663I mutations would be tolerated or have a non-deleterious effect on protein function, with equal score or higher than 0.05 as shown in the Fig. 1E. For the remaining SNPs, were predicted to affect protein function [34–36] with a score of 0.00 (Fig. 1E).

Discussion

In Mozambique, the first-line for uncomplicated malaria treatment is AL and ASAQ the second-line and an alternative in case of contraindications [17]. Surveillance of validated and/or resistance-associated SNPs of the aforementioned artemisinin-based combinations is a powerful weapon to control the spread of parasite resistance through early detection of mutations [37–39]. Moreover, these mutations are being associated with the reduction in *pfkelch13* function, a protein required for parasite-mediated endocytosis of host haemoglobin in the newly invaded intra-erythrocytic ring stages [11, 39].

No validated mutations [6] for artemisinin resistance was observed from this study samples. Furthermore, 16 non-synonymous and 5 synonymous mutations were detected (Table 1). However, out of these 21 point mutations, four have been previously reported in Africa, namely C469C [40, 41], A578S [41–43], Q661H [41, 44], C532W [45] and one mentioned as candidate marker; at codon 449. Although this study observed a G449R instead of G449A as reported by the WHO [46]. The remaining 16-point mutations are reported here for the first time. In Mozambique, two other non-synonym mutations have been detected before, V494I in two samples from Maputo [29] and F656I in one sample (location not specified) [30]. In Africa, six non-synonymous SNPs validated as markers of artemisinin resistance, have been reported M476I, P553L, R561H, P574L, C580Y and A675V [22, 24, 26, 27, 45, 47]. In Rwanda, another SNPs were also detected P667S, Q661E [27], V55A, C532W and G533A [45]. Patients infected with Q661H or P667S had a Parasite Clearance Half-life > 5 h [27]. Q661H has not yet been validated as a marker yet, but the fact that it has been identified in more than one region requires closer monitoring. Although these results illustrate an emergence of resistance cases in Africa, the non-synonymous mutations reported in this study is not enough to

raise an alarm in Africa as well as in Mozambique. Furthermore, the latest therapeutic efficacy study in Mozambique confirmed the susceptibility of *P. falciparum* to the drugs currently in use [19], however, continuous surveillance of ACT resistance markers is urgently needed.

The functionality of the protein was predicted. Thus, the obtained SIFT scores (Fig. 1) for V454E, V494L, A578P, V581A, E509D, E455Q and L663I indicate that these mutations are tolerated or have non-deleterious effect on protein function, because they are equal or above the cut off value of 0.05 [34–36]. For the remaining non-synonymous mutations, SIFT scores predicted them to be deleterious, with a score of 0.00, bellow cut off value of 0.05 [34–36]. SIFT issued a warning stating that there is low confidence in this prediction, since these substitutions may have been predicted to be deleterious just because the sequences used were not diverse enough [34].

These results showed that we do not have an emergency of artemisinin resistance cases, even though novel non-synonymous mutations were observed, highlighting the relevance of increasing the number of studies focused on the molecular surveillance of resistance markers to ACT.

Conclusions

- No *pfkelch13*-mediated artemisinin resistance validated gene mutation was found from this study samples. Non-synonymous mutations were detected in all three provinces, with a prevalence of 11.22%, 6% and 5% in Niassa, Manica and Maputo, respectively.
- These results are limited to the selected provinces and districts. Hence the need to replicate these experiences and always with the aim of better informing PNCM decision-making and ultimately the genomics of malaria in Mozambique to become a programmatic activity. Further research is currently being done and will provide more information on the resistance profile to *Pfkelch13* [48].

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Author contributions
CIS, BD, BC, DM, MS, RV and DD, FN: Conducted laboratory activities; CIS, BD, BC, DM, MS, and RV: Wrote the first draft of the manuscript; CIS, NC, SL, CrS: Conducted the field activities (sample collection); FN, CIS, PA and SE: Designed the study protocol and reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The data and materials analysed in this study are available from the corresponding author.

Declarations

Competing interests
The authors declare no conflict of interest.

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4.2.2. Paper III. Antimalarial resistance risk in Mozambique detected by a novel quadruplex droplet digital PCR assay

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In this manuscript, where I share the first co-authorship with Noah and Caroline, I had the following roles:

- Participated in study design,
- Collecting samples from health facilities
- DNA extraction and analysis of multiplex ddPCR results
- Writing initial draft of the paper

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Antimalarial resistance risk in Mozambique detected by a novel quadruplex droplet digital PCR assay

Noah Brown,[1] Clemente da Silva,[2] Caroline Webb,[1] Daniela Matias,[2] Brigite Dias,[2] Beatriz Cancio,[2] Miguel Silva,[2] Ruben Viegas,[2] Crizolgo Salvador,[3] Nordino Chivale,[3] Sonia Luis,[4] Paulo Arnaldo,[3] Julia Zulawinska,[1] Christopher C. Moore,[5] Fatima Nogueira,[2] Jennifer L. Guler[1,5]

AUTHOR AFFILIATIONS See affiliation list on p. 9.

ABSTRACT While the *Plasmodium falciparum* malaria parasite continues to cause severe disease globally, Mozambique is disproportionally represented in malaria case totals. Acquisition of copy number variations (CNVs) in the parasite genome contributes to antimalarial drug resistance through overexpression of drug targets. Of interest, piperaquine resistance is associated with plasmepsin 2 and 3 CNVs (*pfpmp2* and *pfpmp3,* respectively), while CNVs in the multidrug efflux pump, multidrug resistance-1 (*pfmdr1*), increase resistance to amodiaquine and lumefantrine. These antimalarials are partner drugs in artemisinin combination therapies (ACTs) and therefore, CNV detection with accurate and efficient tools is necessary to track ACT resistance risk. Here, we evaluated ~300 clinically derived samples collected from three sites in Mozambique for resistance-associated CNVs. We developed a novel, medium-throughput, quadruplex droplet digital PCR (ddPCR) assay to simultaneously quantify the copy number of *pfpmp3, pfpmp2*, and *pfmdr1* loci in these clinical samples. By using DNA from laboratory parasite lines, we show that this nanodroplet-based method is capable of detecting picogram levels of parasite DNA, which facilitates its application for low yield and human host-contaminated clinical surveillance samples. Following ddPCR and the application of quality control standards, we detected CNVs in 13 of 229 high-quality samples (prevalence of 5.7%). Overall, our study revealed a low number of resistance CNVs present in the parasite population across all three collection sites, including various combinations of *pfmdr1*, *pfpmp2*, and *pfpmp3* CNVs. The potential for future ACT resistance across Mozambique emphasizes the need for continued molecular surveillance across the region.

KEYWORDS malaria, drug resistance, copy number variation, droplet digital PCR, plasmepsin, multidrug resistance

Despite decades of research, the *Plasmodium* malaria parasite continues to cause severe disease in humans (1). The global burden of malaria cases reached 247 million and 619,000 deaths in 2021, and the African country of Mozambique accounted for 4.7% of these cases (2). *P. falciparum* is the world's most deadly malarial parasite and causes infections across this region (3). Research on drug resistance is particularly important since there is no widely employed vaccine and antimalarial drugs are a major tool for control of malaria. Due to Mozambique's sizable contribution to annual malaria cases, it is crucial to investigate the potential rise of antimalarial resistance in this area.

Artemisinin combination therapies (ACTs) are the most common treatment for *P. falciparum* malaria infections (4). Globally, ACTs are administered with several different partner drugs; in Mozambique, the first-line treatment for uncomplicated malaria is ACT with artemether–lumefantrine (AL), dihydroartemisinin–piperaquine (DHAP), or

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Address correspondence to Fatima Nogueira, FNogueira@ihmt.unl.pt, or Jennifer L. Guler, jlg5fw@virginia.edu.

Noah Brown, Clemente da Silva, and Caroline Webb contributed equally to this article. Author order was determined alphabetically.

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artesunate–amodiaquine (AS–AQ) (5). Due to reports of low ACT efficacy in Africa (3, 6–9), we aim to determine the risk of AL, AS-AQ, and DHAP resistance emergence in Mozambique.

Mutations in the *pfkelch13* gene are the primary markers of artemisinin partial resistance and are actively being monitored globally. However, the high efficacy of ACTs is in large part due to the inclusion of partner drugs (10, 11). Unfortunately, resistance to the majority of ACT partner drugs has been reported in Africa and Asia (7, 11, 12). Parasite genotypes associated with this resistance predominantly involve copy number variations (CNVs), which increase the transcription of genes encoding for proteins that confer resistance. Key resistance-associated CNVs include *pfmdr*1 (for lumefantrine and amodiaquine) as well as *pfpmp2* and *pfpmp3* genes (for piperaquine) encoding a multidrug efflux pump and digestive proteases, respectively (12–17). It is important to actively monitor these CNVs, particularly in countries like Mozambique where resistance may only just be emerging (18).

In this study, we assessed the presence of resistance-associated CNVs in clinical samples from Mozambique using a novel "quadruplex" droplet digital PCR (ddPCR) assay. This nanodroplet-based method involves end-point PCR and fluorescent probes to amplify and quantify gene copies of interest (19). DdPCR presents an attractive tool for the rapid detection of resistance genotypes in malaria-endemic areas for several reasons. When compared with Sanger sequencing, ddPCR is able to detect minor variants with high accuracy, particularly at lower DNA concentrations (20, 21). When compared to standard quantitative PCR, ddPCR can reduce average costs per sample by 72% by requiring fewer runs to evaluate more loci (22). Furthermore, ddPCR requires less hands-on time per sample due to a lower requirement for replicates and serial dilutions, thus decreasing the chance of technical errors (23). Finally, ddPCR requires sub-nanogram levels of DNA that are easily attainable from minimally invasive collection methods like dried blood spots. While instrument access may limit use in some areas, droplet-based PCR assays are becoming more popular for molecular surveillance (22, 24).

To further extend the benefits of ddPCR, we developed a quadruplex assay, where the three CNV loci (*pfmdr1, pfpmp2,* and *pfpmp3*) and a control gene (*pfhsp70*) are evaluated simultaneously. This approach improves assay efficiency and technical consistency over duplex assays (21). Following optimization, the ddPCR assay proved accurate and sensitive and identified a low but appreciable level of resistance-conferring CNVs across three Mozambique provinces. This study supports the continued need for molecular surveillance across this region to improve the detection of ACT resistance before it becomes more widespread.

RESULTS

The optimized quadruplex ddPCR assay is accurate, specific, and sensitive

The development of a two-color "quadruplex" assay requires optimization of primer annealing temperature, dilutions, and cycling number (see *Materials and Methods* and Fig. S1 to S3). Copy number determination is based on comparing positive-droplet populations (Fig. 1), which represent the relative proportions of DNA targets in the sample. In our studies, *P. falciparum hsp70* is used as a single copy "reference gene" to determine the copy number of the other "target genes" (i.e., target-CN/reference-CN ≠1 indicates a change in copy number status). With appropriate parasite DNA input (>0.05 ng), the resulting quadruplex assay has 16 distinguishable orthogonal droplet clusters (Fig. 1).

When comparing duplex versus quadruplex assays, we observed no significant difference in copy numbers of *pfmdr1*, *pfpmp2*, and *pfpmp3* using a laboratory parasite line with a known gene copy number (Fig. S4). Additionally, we confirmed that the assays are *P. falciparum*-specific (Fig. S5) and lack interference by human DNA (Fig. S6), which represents the majority of DNA in clinical surveillance samples.

Assessments that used DNA from laboratory parasite lines also displayed the accuracy and sensitivity of the novel quadruplex assays. As expected, we observed single copies

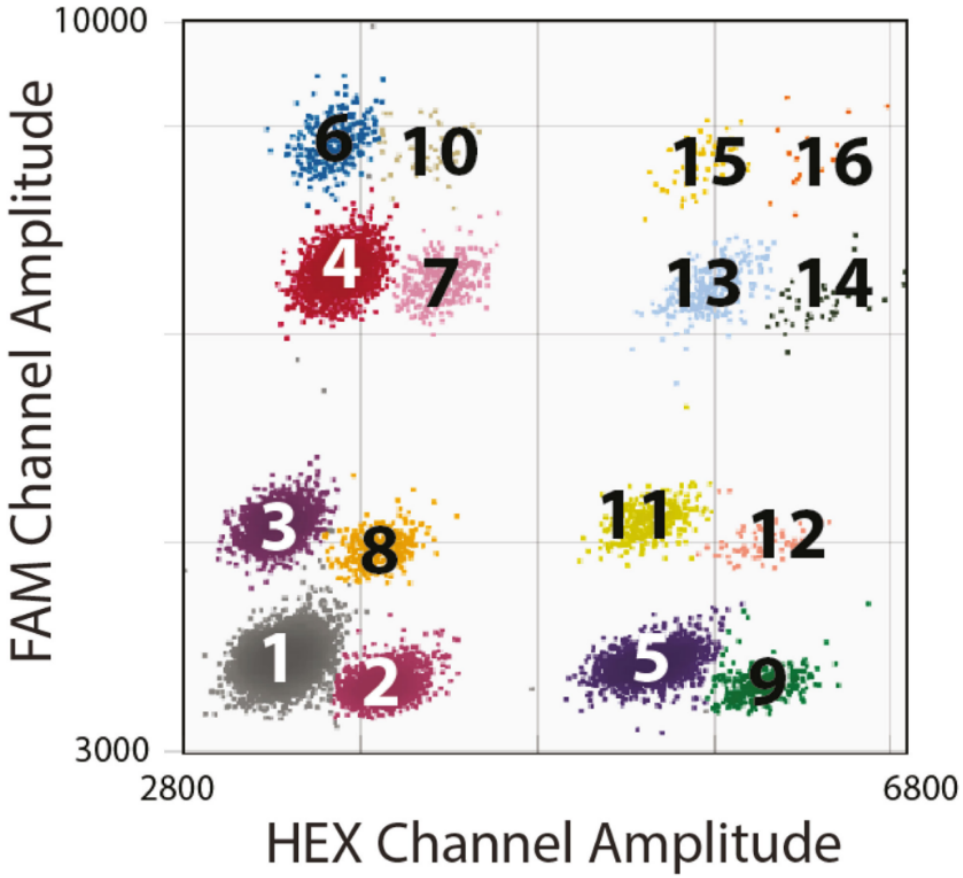

FIG 1 Two-dimensional plot of the quadruplex ddPCR assay. Sixteen clusters of droplets (maximum possible) represent (1): droplets containing no target DNA (negative population), (2): droplets containing at least one copy of *pfhsp70,* (3): droplets containing at least one copy of *pfmdr1,* (4): droplets containing at least one copy of *pfpmp3,* (5): droplets containing at least one copy of *pfpmp2,* (6): droplets with both *pfpmp3* and *pfmdr1,* (7): droplets with both *pfpmp3* and *pfhsp70,* (8): droplets with both *pfmdr1* and *pfhsp70,* (9): droplets with both *pfhsp70* and *pfpmp2,* (10): droplets with *pfmdr1*, *pfhsp70*, and *pfpmp3,* (11): droplets with *pfpmp2* and *pfmdr1,* (12): droplets with *pfhsp70*, *pfmdr1*, and *pfpmp2,* (13): droplets with *pfpmp2* and *pfpmp3,* (14): droplets with *pfhsp70*, *pfpmp2*, and *pfpmp3,* (15): droplets with *pfmdr1*, *pfpmp2*, and *pfpmp3,* (16): and droplets with *pfmdr1*, *pfpmp2*, *pfpmp3*, and *pfhsp70*.

for all ddPCR gene targets for the *NF54* parasite line (Fig. 2A), a three-copy *pfmdr1* CNV for the *Dd2* line (25, 26) (Fig. 2B), and a four-copy *pfpmp2* CNV for the PM2GT clone F4 due to episomal expression (27) (Fig. 2C). When we evaluated the sensitivity of the quadruplex assay across a 5-log range (Fig. S7 and S8A, R_2 of 0.9766), we detected positive droplets down to 0.08 pg of total parasite DNA (0.00008 ng, Fig. S7A).

To explore the impact of genotype mixtures on CNV detection using the novel quadruplex assay, we evaluated mixtures of *P. falciparum* laboratory parasite lines with different genotypes. We were able to detect copy number changes in assays where the CNV+ sample is diluted at various proportions with a CNV- sample (Fig. S9). Based on these data, we can reliably detect CNVs when they are the major genotype (>75%). We also determined that a copy number of >1.5 represents a duplication event in at least 75% of the parasite genomes within the sample. Based on confidence intervals (CIs) from individual experiments, we may be able to detect CNVs at lower levels (i.e., ~50% of the population, Fig. S9A). Therefore, in high-quality samples (low 95% CI), copy numbers between 1.2 and 1.5 were noted as potentially CNV+; this threshold is in line with previous studies that used 1.2 as a cutoff for CNV detection (22).

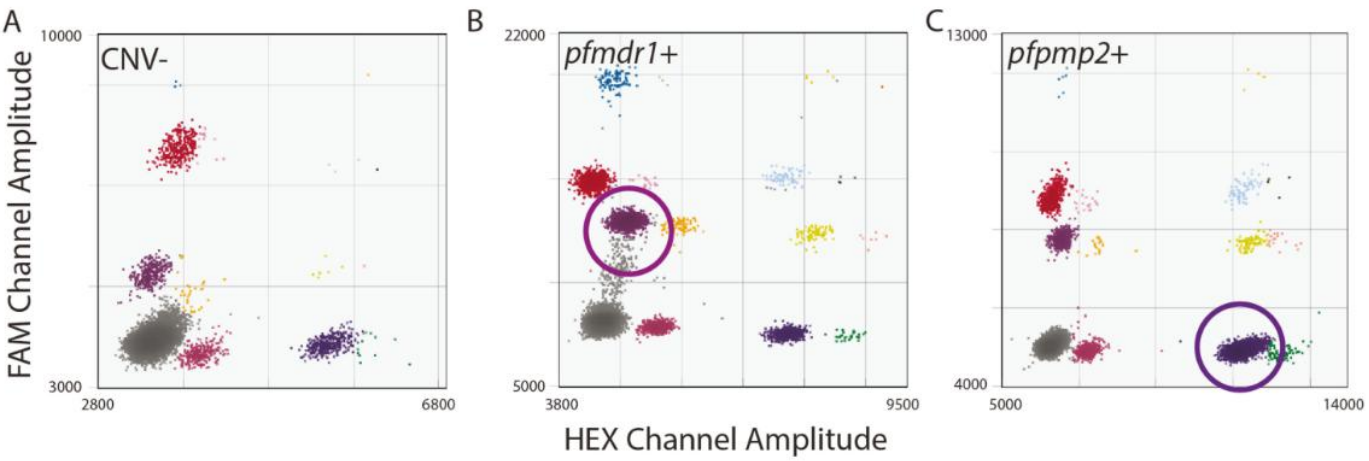

FIG 2 Parasite lines with known CNVs show the accuracy of the quadruplex ddPCR assay. All samples were run at 60 cycles. Clusters of droplets are colored as in Fig. 1. (A) Sample: laboratory-cultured *P. falciparum NF54* DNA with no known CNVs in loci of interest (0.08 ng DNA, 17,739 total droplets, CN: 0.94/1.01/1.09; *pfpmp3, pfpmp2,* and *mdr1,* respectively). Note: this sample is also part of the dilution series represented in Fig S7. (B) Sample: laboratory-cultured *P. falciparum Dd2* DNA with three copies of *pfmdr1* (0.02 ng DNA, 18,400 total droplets, CN: 2.94; *mdr1*). (C) Sample: laboratory-cultured *P. falciparum* PM2GT clone F4 DNA with four copies of *pfpmp2* (0.02 ng DNA, 9,690 total droplets, CN: 4.43; *pfpmp2*).

Assessment of clinical samples reveals a low level of CNVs across the Mozambique parasite population

Of the 297 clinical *P. falciparum* samples collected from Mozambique (Fig. 3A), we were able to assess 296 samples (99%, Table S1). The majority of samples (229/279, 77%) passed quality screening (λ = 0.005–1.1) due to low 95% CI (Fig. 3B; Table S2; Fig. S8B). When separated by province, we considered 87% of Manica samples, 82% of Maputo

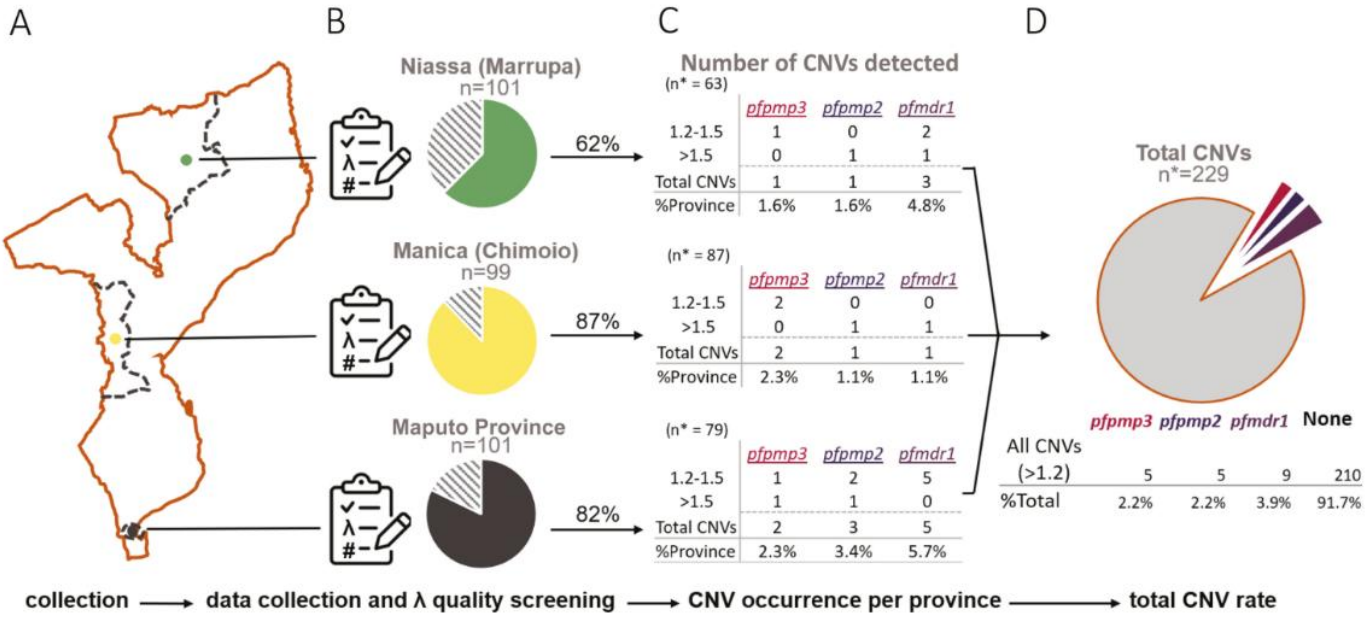

FIG 3 Summary of sample processing and results by province. (A) Depiction of Mozambique sites where samples were collected. Provinces are depicted in dashed lines. The city of collection within the province is depicted with a colored dot. (B) Sample quality control preformed from ddPCR data. Names of the province and city of collection (in parentheses) above pie charts representing the pass rate of quality control using λ (see Materials and Methods); the dashed portion represents failed quality control. *n* = total number of samples collected. (C) Results of ddPCR CNV analysis per-target in each province. n* = total number of samples passing quality screening in each province; 1.2–1.5 represents "potential CNVs"; >1.5 represents "called CNVs"; total CNVs are the sum of the CNVs > 1.2; %Province = Total CNVs/n*. (D) The cumulative total of all CNVs (>1.2) observed from all provinces. n* = combined total samples that passed quality screening.

samples, and 62% of Niassa samples as high-quality samples (Fig. 3B). Clinical samples with measurable DNA levels fell within the ddPCR positive droplet range of our standards (Fig. S8A), confirming the presence of *P. falciparum* DNA in these samples. During these studies, we detected high-quality clinical samples with as few as 80 positive droplets per target (with a mean of ~16,000 total droplets), which equates to ~0.02 ng of parasite DNA input based on our standard curve (Table S2; Fig. S8A).

Together, we measured a mean copy number for high-quality samples across all loci of 0.95 copies (mean of 0.92, 0.95, and 0.99 for *pfpmp3, pfpmp2,* and *pfmdr1,* respectively; Fig. S10A; Table S2), indicating that the assay did not exhibit locus-specific detection bias. We identified four different high-quality clinical samples that harbored definitive CNVs (>1.5 copies) with six total CNVs (Fig. 3C; Fig. S10A); the samples were spread across the three provinces (one from Maputo, two from Niassa, and one from Manica). Three of the CNVs included the *pfpmp2* gene, two included *pfmdr1*, and one included *pfpmp3*. When we manually inspected ddPCR plots, all of these samples showed high-quality droplet formation and locus detection (Fig. S10B through E). By including samples with copy numbers between 1.2 and 1.5, we identified nine additional high-quality clinical samples across all three provinces that were potentially positive for CNVs in at least one of the three loci (nine for *pfmdr1*, five for *pfpmp3*, and five for *pfpmp2*). Some samples were positive for two CNVs; one sample showed both *pfmdr1* and *pfpmp2* CNVs (Fig. S10C) and four samples with both *pfpmp2* and *pfpmp3* CNVs (Fig. S10D, Table S2).

A portion of samples were rerun to assess the reproducibility of copy number estimates using the quadruplex assay (17 CNV- samples and 13 CNV+ samples). All CNV- samples yielded reproducible calls (Table S1). Despite an additional 18 months of DNA storage, the majority of high-quality samples with potential CNVs (>1.2 copies) also yielded reproducible calls (83% for *pfmdr1*, 67% of *pfpmp3*, and 83% for *pfpmp2*, Table S1).

DISCUSSION

We identified optimal ddPCR conditions through extensive experimentation including evaluations of cycle number, dilution range, quality control metrics, and potential effects from contaminating DNA from the human host or non-*falciparum* species (Fig. S1 to S7). We established that our novel assay is accurate (Fig. 2; Fig. S4 and S9), specific (Fig. S5 and S6), and sensitive (Fig. S7). We employed metrics to determine sample quality and identified a copy number range for CNV detection in clinical samples. Using this refined approach, we identified a low level of CNVs in clinical parasite populations in Mozambique.

The clinical samples evaluated in our study were prepared from dried blood spots collected after screening for *P. falciparum*. Despite the high sensitivity and accuracy of the ddPCR assay, some samples proved unquantifiable due to low DNA content or quality (Fig. 3B; Table S1). These assay failures were likely due to the age of the purified DNA; in the current study, there was a minimum of 8 months between DNA extraction and ddPCR analysis (maximum of 28 months), which in addition to degradation from freeze–thaw and shipping temperatures (see *Materials and Methods*) could contribute to declining sample quality. Indeed, when samples were repeated ~20 months following the initial runs, we had a failure rate of >50% (Table S1); most of these samples failed due to low positive droplet numbers indicating DNA degradation as the cause.

The *P. falciparum* CNV frequency that we observed in this study was in the range found in previous studies from Mozambique (7, 18). Although we only detected potential CNVs in a total of 13 samples (Table S2), this is appreciative in a small sampling of clinical infections (13/229, 5.7% total; *pfpmp3*: 2.2%; *pfpmp2*: 2.2%; and *pfmdr1*: 3.9%, Fig. 3). For comparison, one study estimated 1.1% (4/350) and 1.4% (5/350) *pfpmp2* and *pfmdr1* CNV prevalence, respectively (18). Another Mozambican study identified multiple copies of *pfpmp2* and *pfmdr1* in 11.3% (8/71) and 16.7% (2/12) of samples, respectively (7).

The incidence of *pfmdr1* CNVs (copy number of >1.2) in our study was approximately twice as common as the *pfpmp2 or pfpmp3* loci (Fig. 3; Fig. S10A). Additionally, of the

nine *pfmdr1* CNV+ samples, three samples also harbored *pfpmp2* or *pfpmp3* CNVs (Table S2). Concomitant CNVs may reflect an increase in parasite adaptation to DHAP, which has been directly linked with all three CNVs in genome-wide association studies (8, 16). Interestingly, previous studies from Mozambique did not detect *pfpmp3* CNVs in clinical samples. Although at a very low rate, we detected samples with CNVs (copy number of >1.2) that encompassed both *pfpmp2* and *pfpmp3* genes (3/229, Fig. S10D; Table S2). The report of *pfpmp2/3* co-amplification is consistent with studies from Cambodia, where the CNV region spanned the two neighboring genes (28).

Our data are consistent with the observation that the piperaquine partner drug has not yet been widely used to treat uncomplicated malaria in Mozambique. However, its use during complicated malaria and MDA may select resistant parasite populations. As far as we are aware, this drug has been used for MDA in three Mozambique provinces; DHAP MDA was applied between 2015 and 2017 in the Magude district (29, 30), which is approximately 100 km from the current study health facility (*Hospital provincial da Matola*) in the Maputo province, and in 2021 and 2023 in Cabo Delgado, which is outside of the region of testing in this study. Although a recent study that used the same clinical samples as our study did not detect validated mutations in *pfkelch13* (31), the use of MDA in the region and our identification of a low rate of *pfmdr1* and *pfpmp2/3* CNVs necessitate continued molecular surveillance to better understand genotype dynamics across this region. Fortunately, the prevalence of resistance CNVs in Mozambique remains far below that reported in other countries such as Cambodia (23%) (17), Vietnam (54.3%) (32), and other African countries (average of 21.1% for *pfmdr1* and >30% for *pfpmp2*) (7).

Systematic monitoring of resistance markers from malaria-infected patients using sensitive and accurate assays can assist in the prevention of treatment failure due to resistance (4, 33–35). Our novel quadruplex ddPCR assay is specific for *P. falciparum* loci (Fig. S5), and not suitable for molecular surveillance of *P. ovale* infections, which cause malaria infections in this region of Africa (36, 37). The assay is capable of detecting *P. falciparum* CNVs that are not yet prominent in a parasite population but may be emerging over time (potential CNVs in high-quality samples, >1.2 copies). Therefore this tool, and others capable of detecting minor parasite genotypes, are an important component of malaria resistance surveillance programs; malaria-endemic regions with emerging resistance CNVs can increase malaria prevention efforts to protect from future widespread antimalarial resistance.

MATERIALS AND METHODS

Laboratory & control parasites, culturing, and genomic DNA extraction

Laboratory parasite DNA from *P. falciparum* NF54 and Dd2 (MRA1000 and 156, respectively) were used to design and validate assay efficacy before clinical sample use. Parasite culture was carried out as previously (38). Briefly, *P. falciparum* parasites were grown *in vitro* at 37°C at 3% hematocrit (serotype A-positive human erythrocytes) in RPMI 1640 medium (Invitrogen, Waltham, MA, USA) containing 28 mM NaHCO3 and 25 mM HEPES and supplemented with AlbuMAX (TM) II Lipid-Rich BSA (Invitrogen, 11021029) in sterile, sealed flasks, flushed with 5% O_2, 5% CO_2, and 90% N_2 (39, 40). Cultures were maintained with media changes three times each week and sub-cultured as necessary to keep parasitemia below 3%.

DNA from laboratory parasite lines was isolated as previously (41). Parasites were lysed with 0.15% saponin (Sigma Life Science) and washed with 1 x PBS to remove RBC membranes. Then, cells were treated with 200 ug/mL proteinase K (Thermo Fisher Scientific) and 0.1% L-loril sarkosil (Teknova Inc, Hollister, CA, USA) at 37° overnight. DNA was extracted using phenol/chloroform/isoamyl alcohol (25:24:1), pH 7.8–8.1 (Invitrogen), and MaXtract high-density gel tubes (Qiagen) twice, and twice more with only chloroform (Sigma-Aldrich) using standard methods. A final precipitation step with 100% ethanol removed the remaining salts and phenol. The resulting DNA was quantified

using the Qubit double-stranded DNA High-Sensitivity kit as per the manufacturer's recommendations (Invitrogen).

DNA from a laboratory parasite line harboring ~4 copies of *pfpmp2* maintained on the chromosome and episomes was provided by the Michael Klemba (PM2GT clone F4) (27). Clinical samples obtained for this study came from the University of Virginia Medical Center from patients with clinical malaria, as determined by rapid diagnostic tests and peripheral blood smears (see ethical approval above). Blood was obtained from these patients within 24 hours of phlebotomy and used to extract DNA from malaria parasites using the method described above.

Area of study, clinical isolates, and genomic DNA preparation

Mozambique clinical samples used in this study were obtained from patients admitted in health facilities from three settings, namely, Northern (Hospital distrital de Marrupa in Niassa), Central (Centro de Saúde Eduardo Mondlhane and Centro de Saúde 7 de abril in Manica), and Southern (Hospital provincial da Matola in Maputo) areas of Mozambique, between April and August of 2021, as previously described (31). The choice of Niassa, Manica, and Maputo provinces not only aligns with our scientific goals but also with the objectives of Instituto Nacional de Saúde and National Malaria Control Programme Mozambique, contributing to the systematic mapping of malaria cases at the national level.

A total of 450 participants of all ages with malaria-positive rapid diagnostic test (RDT) were recruited and provided 100 µL of blood samples on filter paper (Whatman FTA cards), after written informed consent. All dried blood spot samples were then stored under −20°C until they were used for genotyping.

Parasite genomic DNA from dried blood spots was extracted using the Chelex method (42), and DNA was stored at −20°C. Real-time PCR for *P. falciparum* confirmation, targeting the 18S rRNA gene, was conducted as described in da Silva et al. (31). *P. falciparum* positive DNA samples (n = 297) from three provinces (Maputo n = 97, Niassa n = 100, and Manica n = 100) were assessed at the University of Virginia (shipped at room temperature, stored at −20°C). Clinical isolate DNA concentration ranged from 0.1 to 2.0ng/µL with an average concentration of ~0.4 ng/µL. Samples were coded at collection, and location details were blinded during experimentation.

Droplet digital PCR quadruplex assays and CNV analysis

To achieve optimal primer/probe concentrations, the two-color quadruplex assay was developed as two individual duplex assays on either fluorescence channel and then merged into a single quadruplex assay with small adjustments (Table 1: primer and probe concentrations). Because the ddPCR droplet-analyzer used in this study possesses two fluorescence channels (FAM and HEX), quadruplexing requires an intentional decrease in the fluorescence amplitude of one of the assays on each channel to distinguish the populations. The amplitude of these droplet clusters can be affected by several factors including the age of the primers and probes, purity of the input DNA, and interexperimental variables like UV exposure and droplet generation variance (43). Generally, when shifts in amplitude occur from interexperimental factors, they affect all clusters equally, allowing for accurate assignment of droplet clusters.

Primer and probe sequences (Table 2) were BLASTed against Pf3D7 and human genomes to confirm the absence of potential secondary targets and assessed for potential dimer formation using the IDT OligoAnalyzer tool. The specificity for *Plasmodium* genes was also confirmed by the lack of amplification of human genomic DNA (Fig. S6). Optimal assay temperature was assessed by performing assays in duplex over a thermal gradient of annealing/extension temperatures and determining the condition with the highest average amplitude Fig. S1).

Purified bulk DNA (~10 ng/µL) from laboratory parasite lines was digested with BamHI (New England BioLabs) for 12 hours at 37°C and then diluted to 0.004 ng/µL for input into ddPCR assays (total of 0.02 ng). Extracted DNA (5 µL) from clinical samples

was directly digested with BamHI and diluted at a ratio of 1:2-1:10 into the ddPCR assay (Table S1; Fig. S2). Some samples were run undiluted to account for low parasite DNA amount. The BamHI restriction enzyme was chosen specifically due to a predicted restriction site located between the tandem *pfpmp2* and *pfpmp3* genes, while generating similar lengths for all target-containing DNA fragments including *pfhsp70 and pfmdr1* (10–60 kb).

Fresh primer/probe master mixes were prepared for each assay at 10x final concentrations (Tables 1 and 2). Each 23 µL ddPCR reaction contained 5 µL of restriction-digested DNA (diluted as mentioned above), 1:10 dilution of each 10 x primer/probe mix (four sets), and 1:2 dilution of ddPCR Supermix for probes (Bio-Rad Laboratories). Reactions underwent droplet generation using a QX200 Droplet Generator with droplet generation oil for probes (Bio-Rad Laboratories) and subsequent PCR using a C1000 Thermal Cycler (Bio-Rad Laboratories). Thermal cycling conditions are as follows: 10 min at 95°C initial denaturation step, 1 min at 95°C second denaturation step, and 2 min at 58°C annealing and extension step (ramp rate of 1°C per second), the second denaturation step and the annealing/extension step repeated 60 times, and then 10 min at 98°C to halt the reaction. This longer cycling program (60 cycles versus the standard 40 cycles) increased ddPCR assay success for lower-quality clinical samples (Fig. S3). Samples were kept at 4°C until use for analysis (no longer than 24 hours).

After amplification, probe fluorescence was read using a QX200 Droplet Reader (Bio-Rad Laboratories) and analyzed using QuantaSoft Software version 1.7.4 (Bio-Rad Laboratories). Populations were manually defined as instructed by the manufacturer using QuantaSoft software. Samples were only considered valid if a minimum of 10,000 total droplets were observed. Poisson confidence intervals provided by the software were used to assess sample quality.

Samples were screened for quality using the lambda (λ) value in accordance with the Minimum Information for Publication of Quantitative Digital PCR Experiments (22, 45, 46), where λ = ln(#accepted droplets/#negative droplets). Samples passed quality screening if λ was between 0.005 and 1.1. This threshold was chosen as it represented high-quality clinical samples, where the difference between the maximal 95% CI value and the determined copy number was less than 30% of the copy number value ([(MaxCI – CopyNumber) / CopyNumber] <30%, Fig. S11, blue box). Furthermore, during this calculation, if the accuracy (defined as: %100 * |1 – CopyNumber|) deviates from the expected value of "1", it is either a product of insufficient quantification or potential CNV signal (Fig. S11, green box).

CNVs were identified with QuantaSoft Software. Each droplet was automatically designated as positive or negative for template DNA through the detection of the fluorescence signal after amplification (Fig. 1). The relative abundance of a single-copy reference (*pfhsp-70*, PF3D7_0818900) compared to a target amplicon was used to detect CNVs (*pfmdr1*, PF3D7_0523000; *pfpmp2*, PF3D7_1408000; *pfpmp3*, PF3D7_1408100, e.g., a target/reference abundance of 2:1 indicates a duplication of the target). Samples that passed quality screening (see above) and possessed a ratio ≥1.5 target/reference were considered positive for CNVs. Samples with copy numbers between 1.2 and 1.5 were manually inspected and considered potentially positive if samples were high quality and droplet populations were well-resolved.

TABLE 1 Optimized quadruplex assay reaction primer and probe concentrations[a]

Target	Probe concentration (nm)	Fluorophore	Primer concentration (nm)
pfpmp2[a]	125	HEX	2,500
pfhsp70[b]	125	HEX	800
pfpmp3[a]	50	FAM	400
pfmdr1	125	FAM	600

[a]Bold targets indicate assays with higher fluorescence amplitudes on ddPCR plots.
[b]Used as a single-copy reference gene.

TABLE 2 Primer and probe sequences for the quadruplex ddPCR reaction

Target	Forward primer	Reverse primer	Probe
pfpmp2[a]	5′-ATGGTGATGCAGAAGTTGGA-3′	5′-AACATCCTGCAGTTGTACATTTAAC-3′	5'-/5HEX/-CAGGATCTGCTAATTTATGGGTCCCA/3IABkFQ/-3'
pfhsp70[b]	5′-TGCTGTCATTACCGTTCCAG-3′	5′-AGCAGCTGCAGTAGGTTCATT-3′	5'-/5HEX/AGATGCTGGTACAATTGCAGGA/IABkFQ/-3'
pfpmp3[a]	5′-CCACTTGTGGTAACACGAAATTA-3′	5′-TGGTTCAAGGTATTGTTTAGGTTC-3′	5'-/56FAM/CCAACACTCGAATATCGTTCACCAA/IABkFQ/-3'
pfmdr1[b]	5′-TGCCCACAGAATTGCATCTA-3′	5′-TCGTGTGTTCCATGTGACTG-3′	5'-/56FAM/ACCCTGATCGAAATGGAACCT/IABkFQ/-3'

[a]These primers and probes were previously reported in (28).
[b]These primers and probes were previously described in (44).

High-resolution melting for evaluating *Plasmodium* species

High-resolution melt (HRM) assays were used to validate the *Plasmodium* species of samples used to test the specificity of the quadruplex assay (47). Plasmid controls containing partial species-specific 18S rRNA gene sequences were obtained through BEI Resources (Manassas, VA, USA) (*P. falciparum* (MRA-177, lot: 70042650), *P. vivax* (MRA-178, lot: 70041752), *P. malariae* (MRA-179, lot: 70051095), and *P. ovale wallikeri* (MRA-180, lot: 70043212). Each 25 µL reaction contained 2 x HRM PCR Master Mix (Type-it HRM PCR Kit, Qiagen), 700 nM of primers targeting the multicopy 18S rRNA gene: 5′-GTT CCT CTA AGA AGC TTT-3′ and 5′-TAA CGA ACG AGA TCT TAA-3′ (48) (IDT, USA), ~10 ng of the template DNA, and RNase-free water. The PCR was performed with the following cycling conditions: 95°C for 5 min, 45 cycles of 95°C for 10 s, 55°C for 20 s, followed by an HRM ramp from 65°C to 95°C, with an increase of 0.1°C every 2 s on the Rotor-Gene Q real-time PCR instrument with a 72-well rotor (Qiagen). Rotor-Gene Q software (version 2.3.5, build 1; Qiagen) was used to plot the change in fluorescence versus temperature (dF/T), which allowed us to compare the HRM peaks of the clinical samples and plasmid controls. Multiple peaks are diagnostic of genomic DNA samples (from clinical or laboratory sources) from specific *Plasmodium* species (47). These peaks are due to amplification of distinct 18S rRNA gene copies present in the genomes (# of total 18 s rRNA genes in genome/# of 18 s rRNA genes with primer homology: *P. falciparum* 5/5; *P. vivax* 3/2; and *P. ovale wallikeri* 2/2).

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AUTHOR AFFILIATIONS

[1]Department of Biology, University of Virginia, Charlottesville, Virginia, USA
[2]Global Health and Tropical Medicine, GHTM, Associate Laboratory in Translation and Innovation Towards Global Health, LA-REAL, Instituto de Higiene e Medicina Tropical, IHMT, Universidade NOVA de Lisboa, UNL, Lisbon, Portugal
[3]Instituto Nacional de Saúde, Maputo (INS), Maputo, Mozambique
[4]Hospital Provincial de Matola, Matola, Mozambique

[5]Division of Infectious Disease and International Health, University of Virginia, Charlottesville, Virginia, USA

AUTHOR ORCIDs

Clemente da Silva http://orcid.org/0000-0003-2083-2579
Fatima Nogueira http://orcid.org/0000-0003-0313-0778
Jennifer L. Guler http://orcid.org/0000-0001-6301-4563

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AUTHOR CONTRIBUTIONS

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ETHICS APPROVAL

The study protocol obtained ethical clearance from the National Bioethics Committee for Health of Mozambique (CNBS—IRB00002657, Ref: 131/CNBS/2021, dated: March 2021). The University of Virginia Institutional Review Board for Health Sciences Research provided ethical approval for samples used as species controls in this study from patients admitted into the University of Virginia hospital (IRB-HSR protocol #21081). All samples were handled in accordance with approved protocols and in agreement with the ethical standards of the Declaration of Helsinki. A waiver for informed consent was provided through the University of Virginia health facilities because our study design met the following criteria: (i) the research involved minimal risk to subjects, (ii) the waiver does not adversely affect the rights and welfare of subjects, and (iii) the research could not practicably be carried out without the waiver.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental figures (AAC00346-24-s0001.docx). Figures S1 to S11.
Table S1 (AAC00346-24-s0002.xlsx). Clinical sample ddPCR assessment including dilutions and repeats.
Table S2 (AAC00346-24-s0003.xlsx). Clinical sample CNV calls and quality metrics.

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SUPPLEMENTAL FIGURES

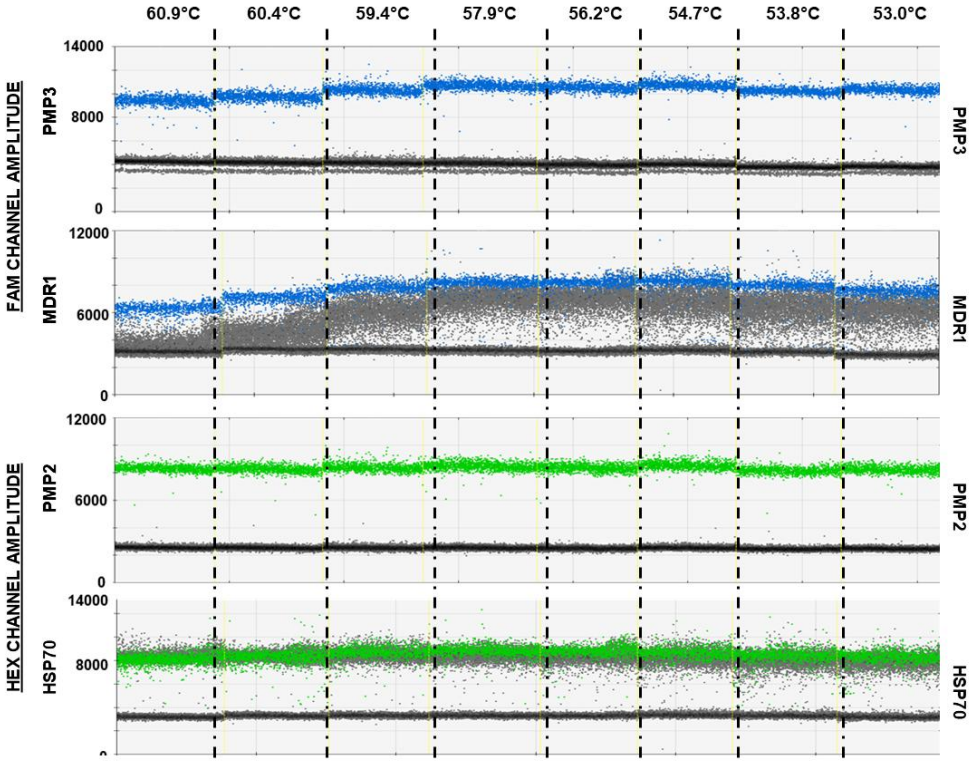

Figure S1: Thermal gradient of primer annealing temperature. Sample: laboratory cultured *3D7 P. falciparum* DNA, 0.025ng total. Cycling condition: 60 cycles across a thermal gradient, in duplex (*pfpmp2*-HEX with *pfpmp3*-FAM and *pfhsp70*-HEX *with pfmdr1*-FAM). Droplets without assignment (grey) are double positives for that assay.

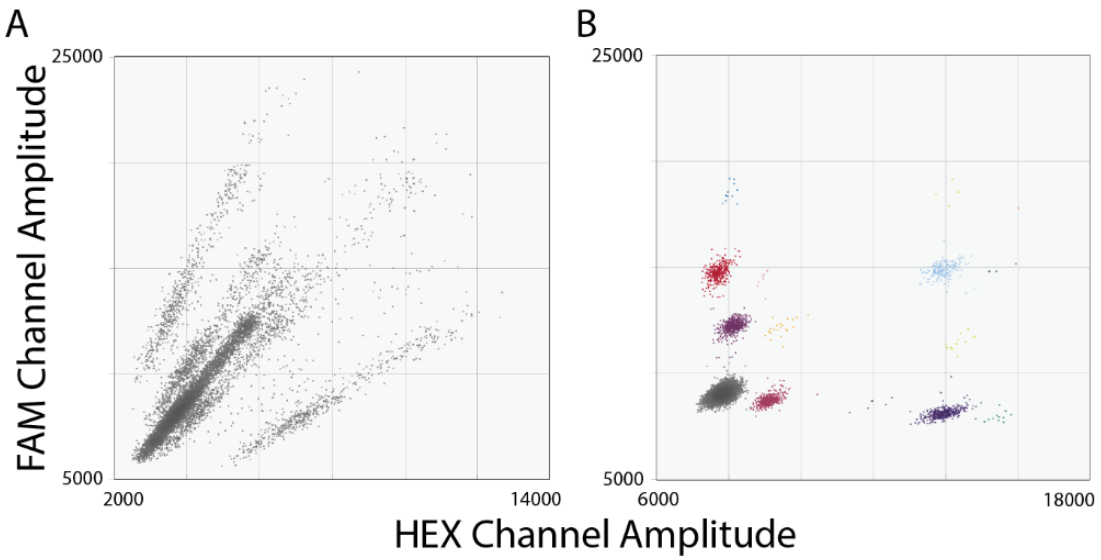

Figure S2. Clinical sample dilution improves quadruplex ddPCR amplification. Sample: 002A from Maputo, 5µl total. Cycling condition: 60 cycles. **A.** Neat condition (no dilution, 11160 total droplets). **B.** Diluted 1:10 before droplet partitioning and amplification (17358 total droplets). Clusters of droplets are colored as in Figure 1.

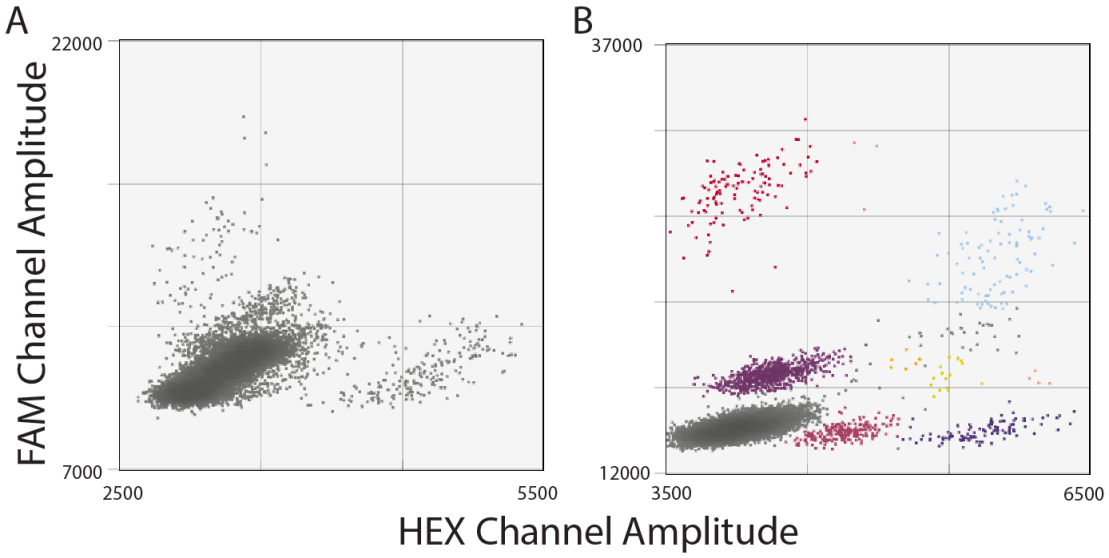

Figure S3: Longer cycling program improves quadruplex ddPCR amplification. Sample: laboratory cultured *Dd2 P. falciparum* DNA, 0.02ng total. **A.** 40 cycle PCR condition (15631 total droplets). **B.** 60 cycle PCR condition (12751 total droplets). Clusters of droplets are colored as in Figure 1.

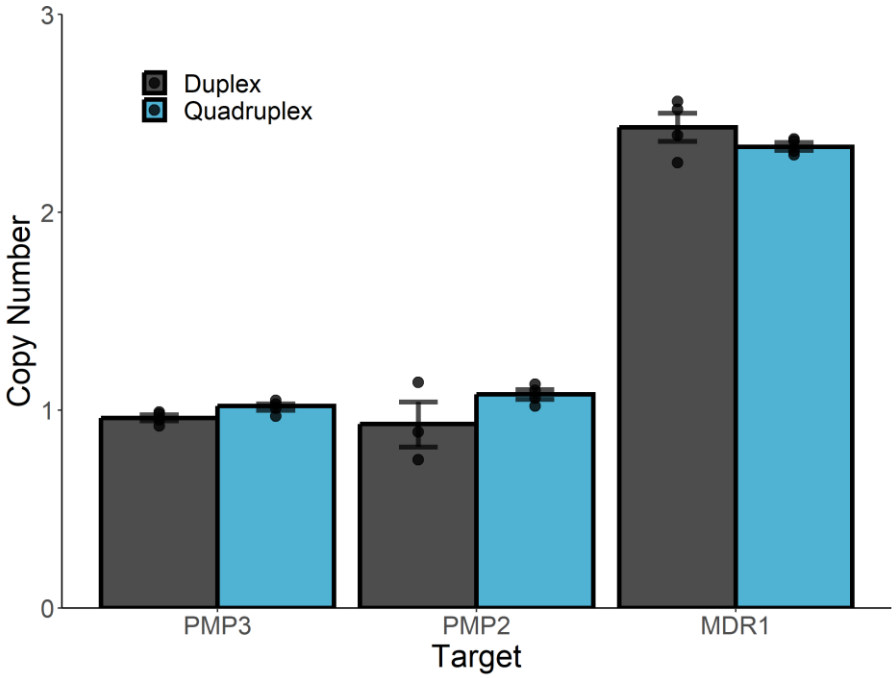

Figure S4: Quadruplex and duplex assays yield consistent copy number quantification. Sample: *P. falciparum Dd2* genomic DNA, 0.05ng total. Duplex assays used the same primer/probe concentrations as listed for the quadruplex assay and are as follows: *pfpmp3*-FAM/*pfhsp70*-HEX; *pfpmp2*-FAM/*pfhsp70*-HEX; *pfmdr1*-FAM/*pfhsp70*-HEX. Error bars, SEM. Statistical test: student's unpaired t-test. p= 0.0575; p= 0.1889; p= 0.2430 for *pfpmp3, pfpmp2, and pfmdr1*, respectively.

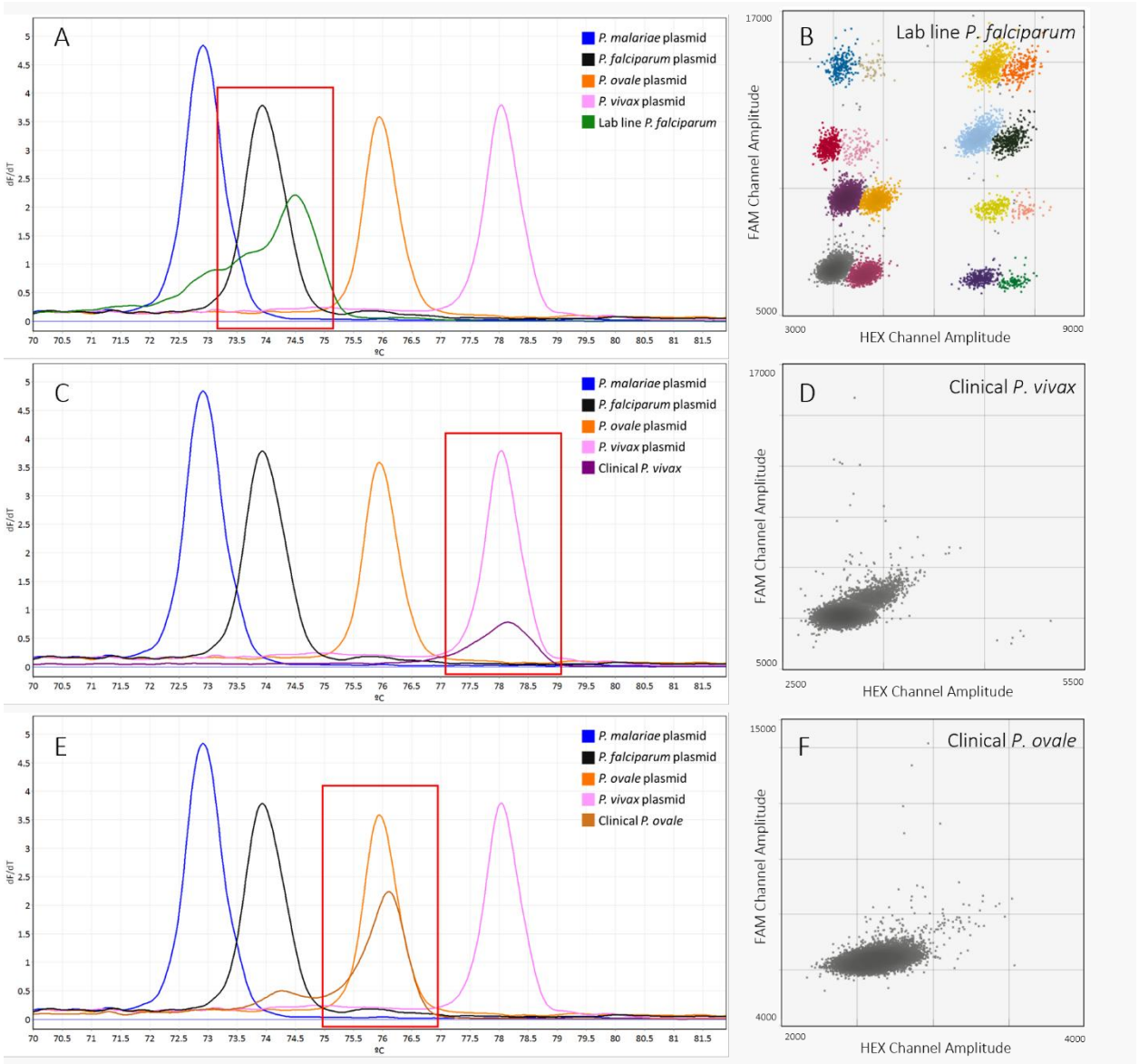

Figure S5. Non-*Plasmodium falciparum* species are not amplified by the quadruplex assay. A, C, E. Species confirmation using high resolution melt (HRM) analysis of 18s ribosomal RNA genes from various *Plasmodium* species. Control plasmids, which contain single copies of species-specific 18s ribosomal RNA genes, are presented on each graph for comparison to genomic DNA from the test parasite lines or isolates (**A:** Laboratory cultured *Dd2 P. falciparum* genomic DNA; **C:** Genomic DNA from clinical isolate positive for *P. vivax*; and **E:** Genomic DNA from clinical isolate testing for *P. ovale*). Red boxes highlight relevant comparison for each sample. Multiple peaks are diagnostic of genomic DNA samples from specific species due to amplification of distinct 18s rRNA gene copies present in the genomes (# of total 18s rRNA genes in genome/# of 18s rRNA genes with primer homology: *P. falciparum* 5/5; *P. vivax* 3/2; and *P. ovale wallikeri* 2/2). **B, D, E.** Representative quadruplex ddPCR performed on each sample, with HRM- confirmed species (**B:** *Dd2 P. falciparum* laboratory line, 15988 total droplets; **D:** *P. vivax* clinical

sample, 16284 total droplets); and **F:** *P. ovale* clinical sample, 13856 total droplets). Grey droplets: droplets containing no target DNA (negative population). Positive droplets colored as in FIG 1.

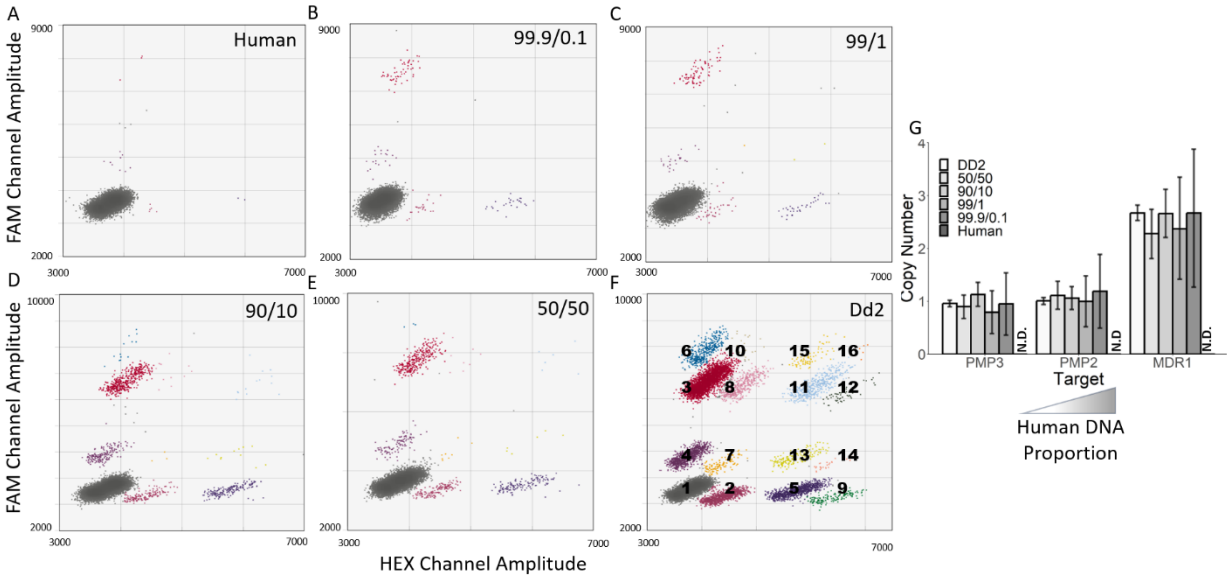

Figure S6: Quadruplex clustering and copy number determination is unaffected by contaminating human DNA. Sample: laboratory cultured *Dd2 P. falciparum* DNA (*pfmdr1* CNV+) mixed with different proportions of human DNA. **A-F.** Representative ddPCR plots. **A.** 100% Human DNA (input 0.05ng, 15847 total droplets). **B.** 99.9% human DNA (7.92ng) with 0.1% parasite DNA (0.008ng), 17043 total droplets. **C.** 99% human DNA (0.792ng) with 1% parasite DNA (0.008ng), 16837 total droplets. **D.** 90% human DNA (0.792ng) with 10% parasite DNA (0.08ng), 17068 total droplets. **E.** 50% human DNA (0.08ng) with 50% parasite DNA (0.08ng), 14804 total droplets. **F.** 100% *Dd2 P. falciparum* DNA (0.05ng, 15025 total droplets). (1) droplets containing no target DNA (negative population), (2) droplets containing at least one copy of *pfhsp70*, (3) droplets containing at least one copy of *pfmdr1*, (4) droplets containing at least one copy of *pfpmp3*, (5) droplets containing at least one copy of *pfpmp2*, (6) droplets with both *pfpmp3* and *pfmdr1*, (7) droplets with both *pfmdr1* and *pfhsp70*, (8) droplets with both *pfpmp3* and *pfhsp70*, (9) droplets with both *pfhsp70* and *pfpmp2*, (10) droplets with *pfmdr1*, *pfhsp70*, and *pfpmp3*, (11) droplets with *pfpmp2* and *pfpmp3*, (12) droplets with *pfhsp70*, *pfpmp3*, and *pfpmp2*, (13) droplets with *pfpmp2* and *pfmdr1*, (14) droplets with *pfhsp70*, *pfpmp2*, and *pfmdr1*, (15) droplets with *pfmdr1*, *pfpmp2*, and *pfpmp3*, (16) and droplets with *pfmdr1*, *pfpmp2*, *pfpmp3*, and *pfhsp70* **G.** Determination of copy number for each target throughout experiments performed in A-F. *Dd2 P. falciparum* is single copy at *pfpmp2* and *pfpmp3* and has 3 copies for *pfmdr1*. N.D., not able to be determined due to too few droplets to quantify. Error bars, 95% CI.

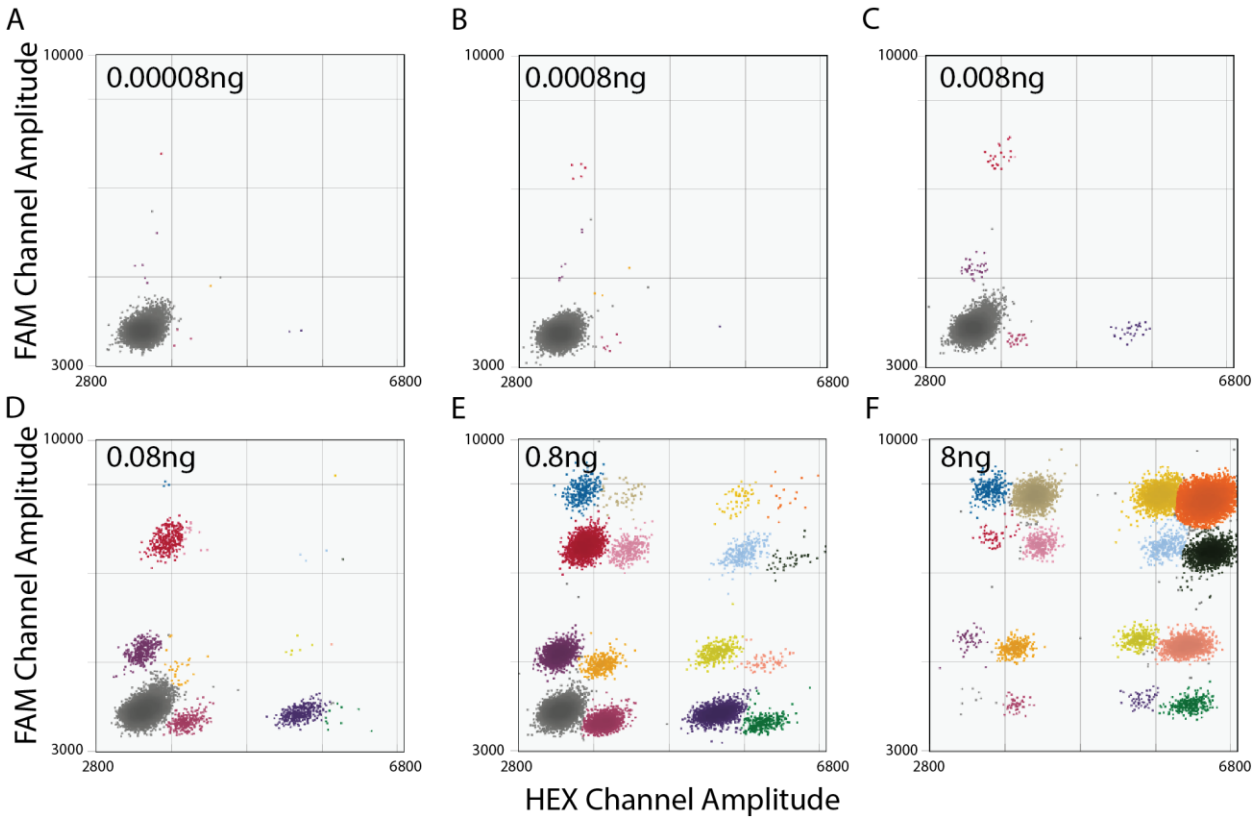

Figure S7: Laboratory parasite DNA dilutions show quadruplex ddPCR assay sensitivity. Sample: laboratory cultured *NF54 P. falciparum* DNA, 10-fold DNA dilutions. Cycling condition: 60 cycles. Clusters of droplets are colored as in Figure 1. **A.** 0.00008ng, 17349 total droplets. **B.** 0.0008ng, 18266 total droplets. **C.** 0.008ng, 16969 total droplets. **D.** 0.08ng, 17739 total droplets. **E.** 0.8ng, 18262 total droplets. **F.** 8ng, 18180 total droplets.

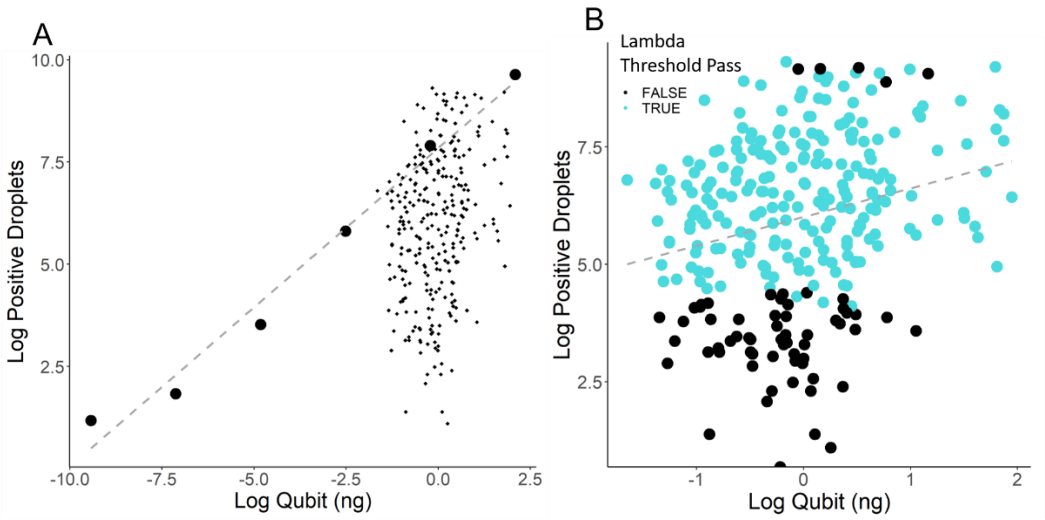

Figure S8: Diluted parasite DNA displays sensitivity of quadruplex ddPCR assay and clinical sample range. Quantification of positive droplets by ddPCR compared to DNA concentration using Qubit fluorimeter. Cycling condition: 60 cycles. **A.** Large black circles, 10-fold dilutions of DNA from laboratory cultured *NF54 P. falciparum*, R_2 = 0.9766, p = 1.3E-4 (0.00008 to 8ng, as in **FIG S7**); small black diamonds, Mozambique clinical samples with measurable DNA concentrations. **B.** Clinical samples with measurable DNA concentrations (n = 283), R_2 = 0.0599. Blue circles, samples in range of λ (0.005 – 1.1); black circles, samples outside of λ range.

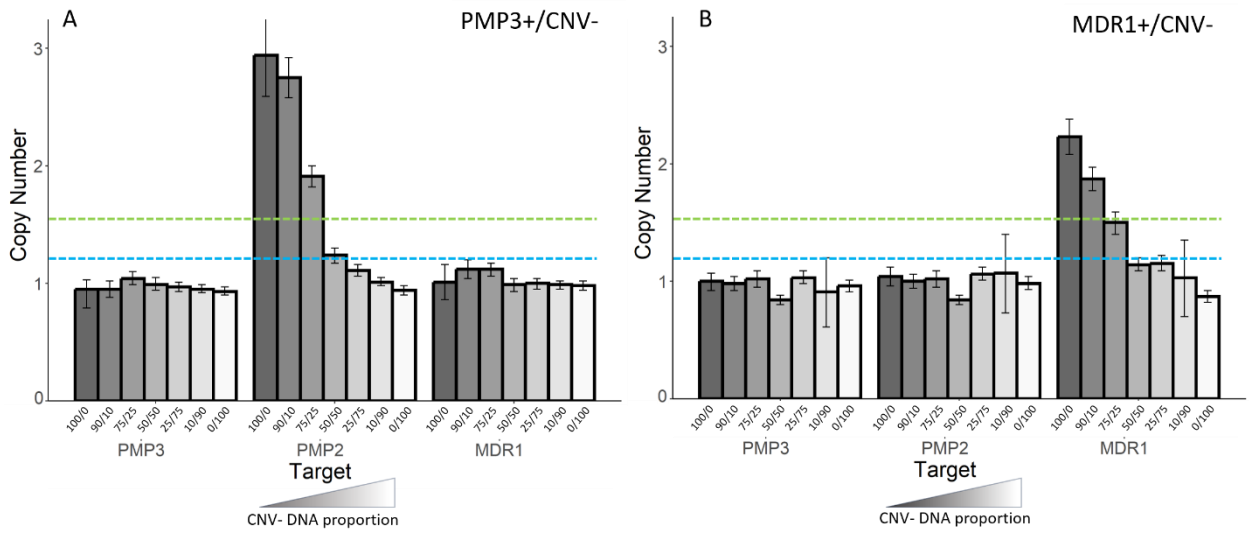

Figure S9: Mixed CNV genotypes impact copy number determination in the quadruplex ddPCR assay. Samples: **A.** PM2GT clone F4 (*pfpmp3* CNV+) mixed with *3D7* (CNV-), 0.05ng total; **B.** *Dd2* (*pfmdr1* CNV+) mixed with *3D7* (CNV-), 0.05ng total. Proportions (CNV+/CNV-) from left to right: 100/0, 90/10, 75/25, 50/50, 25/75, 10/90, and 0/100. Error bars, 95% CI. Blue dotted line: copy number of 1.2; green dotted line: copy number of 1.5.

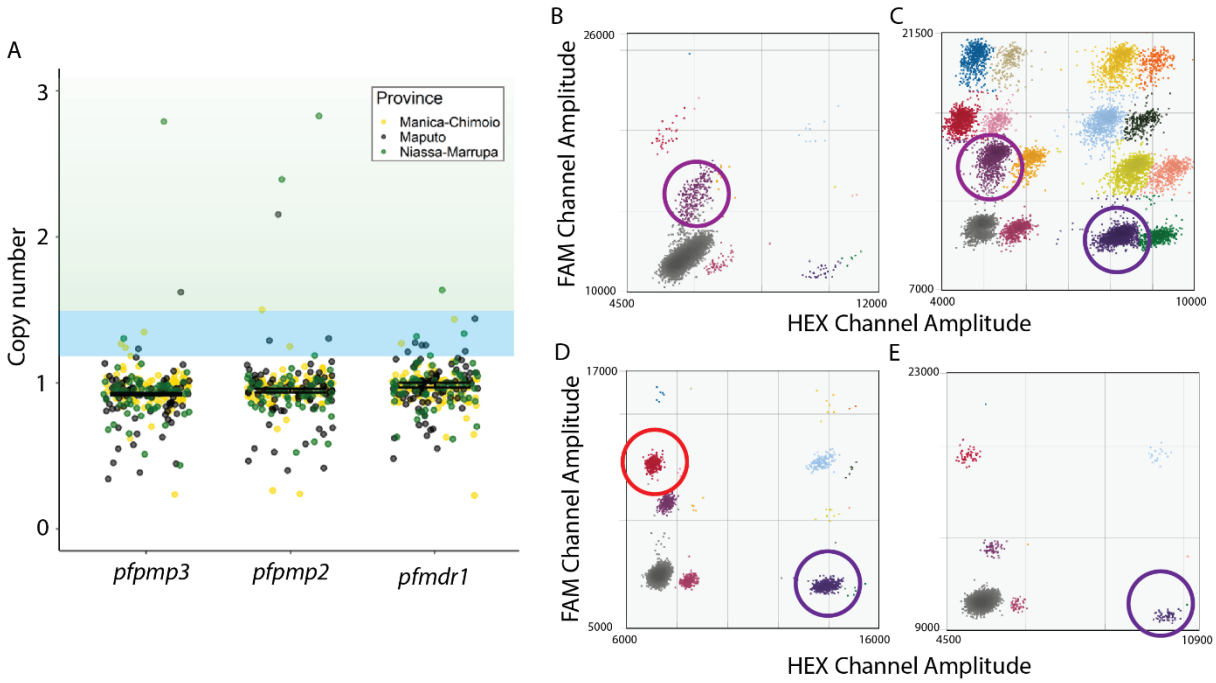

Figure S10. **Quadruplex ddPCR assay detects CNVs in high quality samples from Mozambique. A.** Copy number for each target from high quality clinical samples from the three Mozambique provinces. Error bars, SEM. Blue region; potential CNV samples (copy number of 1.2-1.5), Green region; CNV positive samples (copy number of >1.5). **B-E**. Quadruplex ddPCR assay with clinical sample DNA. All samples were run at 60 cycles at dilutions described in Table S1. Clusters of droplets are colored as in Figure 3. **B.** Sample: 014PC (Manica), *pfmdr1* CNV (copy number =2.6) circled in purple (15083 total droplets). Note: this sample only passed λ= 0.005-1.1 for the *pfmdr1* locus. **C.** Sample: 124PN (Niassa), *pfmdr1* (CN= 1.6) and *pfpmp2* (CN=2.4) CNVs circled in purple and navy, respectively (12104 total droplets). Note: *pfpmp3* CN= 1.3 indicating possible tandem CNV in this sample. **D.** Sample: 012A (Maputo), *pfpmp2* (CN= 2.2) and *pfpmp3* (CN=1.6) CNVs circled in navy and red, respectively (18332 total droplets). **E.** Sample: PC7048 (Manica), *pfpmp2* CNV (CN=1.5) circled in navy (15500 total droplets). Note: *pfmdr1* CN= 1.2 in this sample but it did not pass λ= 0.005-1.1.

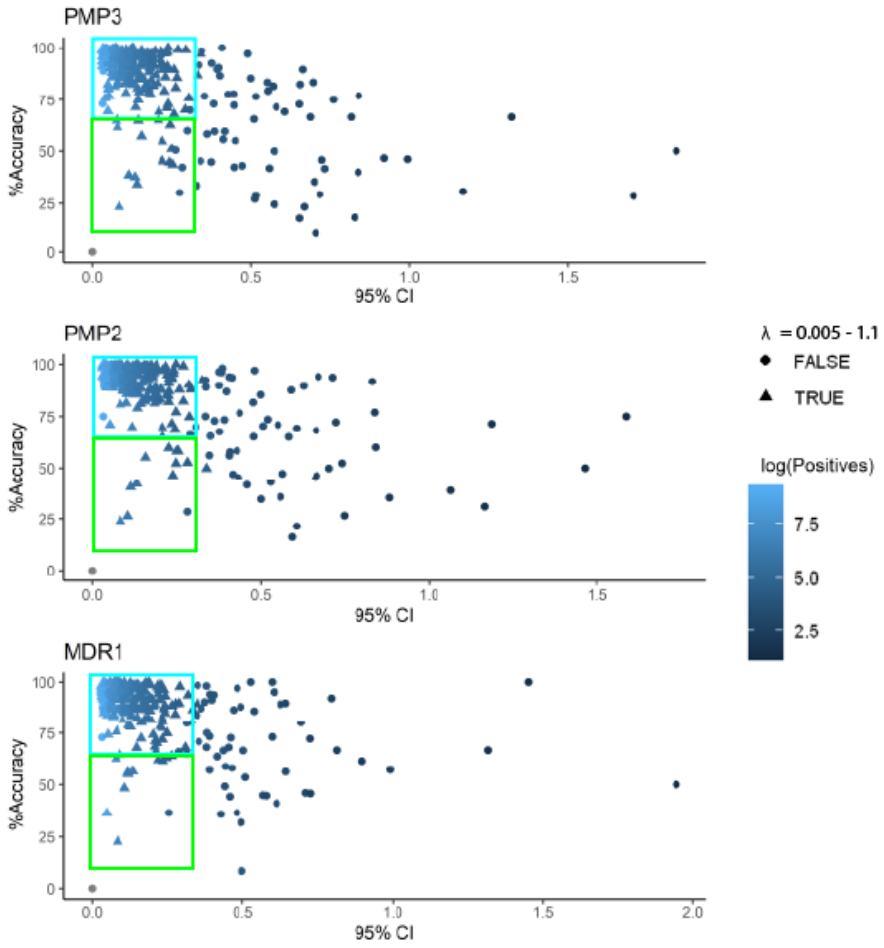

Figure S11: Application of λ threshold identifies high quality samples and predicts samples with potential CNVs. 95% CI from ddPCR analysis; Accuracy = 100 * |1 – Copy Number| for the *pfpmp3* (top), *pfpmp2* (middle), and *pfmdr1* (bottom) loci. Blue and green boxes indicate high quality samples within a λ range (λ = 0.005-1.1) used for further analysis. Blue heat map indicates the log of positive droplets.

Table S1: Clinical sample ddPCR assessment including dilutions and repeats

https://docs.google.com/spreadsheets/d/1VYOwIyQtv6_35WbD4JfTmmg_-SzgwYRv/edit?usp=sharing&ouid=106317560360502654502&rtpof=true&sd=true

Table S2: Clinical sample CNV calls and quality control

https://docs.google.com/spreadsheets/d/1OVjo4MfXD1l9XkPv80Pl890OY2MF6qIN/edit?usp=sharing&ouid=106317560360502654502&rtpof=true&sd=true

4.3. Chapter 3: Assessment of ex vivo susceptibility of P. falciparum to antimalarial drugs in use in Maputo, Mozambique.

4.3.1. Paper IV: Surveillance of *Plasmodium falciparum* susceptibility to Antimalarial Drugs: From *ex vivo* assays to genotyping of Maputo Province Clinical Isolates, 2022

Reference: Denise Duarte[1#], Clemente da Silva[1,5#], Daniela Matias[1], Ana Dias[3], Agostinho Teófilo[2], Sofia Narina[2], Augusto Francisco[2], Teobaldo Mazango[2], Isabel Nhavoto[2], Crizolgo Salvador[2], Bernardete Rafael[4], Paulo Arnaldo[2], Sonia Enosse[3], Fatima Nogueira[1*]

In this manuscript, where I shared the first co-authorship with Denise, I had the following roles:

- Participated in study design,
- Collecting samples from health facilities
- Analysis of the results
- Writing an initial draft and review of the manuscript

Denise Duarte[1#], Clemente da Silva[1,5#], Daniela Matias[1], Ana Dias[3], Agostinho Teófilo[2], Sofia Narina[2], Augusto Francisco[2], Teobaldo Mazango[2], Isabel Nhavoto[2], Crizolgo Salvador[2], Bernardete Rafael[4], Paulo Arnaldo[2], Sonia Enosse[3], Fatima Nogueira[1*]

[1]Global Health and Tropical Medicine, GHTM, Associate Laboratory in Translation and Innovation Towards Global Health, LA-REAL, Instituto de Higiene e Medicina Tropical, IHMT, Universidade NOVA de Lisboa, UNL, Rua da Junqueira 100, 1349-008 Lisboa, Portugal.
[2]Instituto Nacional de Saúde, Maputo (INS), Av. Eduardo Mondlane Nº 1008, Caixa Postal 264, Maputo, Mozambique.
[3]Malaria Consortium, Maputo, Mozambique
[4]Programa Nacional do Controlo da Malária (PNCM)
[5]Centro de Investigação em Saúde de Manhiça, Maputo, Mozambique

[#] **Contributed equally**

*** Corresponding author:** fnogueira@ihmt.unl.pt

Abstract:

Background: Malaria places a high socioeconomic burden on Mozambique. The country is amongst the 5 countries in sub-Saharan Africa with the highest incidence of the disease. The last global malaria report published by the World Health Organization in 2023 estimated 249 million cases, of which Mozambique contributed with 10.4 million. We have conducted this study with the aim of informing decision makers on the current malaria *Plasmodium falciparum* phenotypic and genotypic profile in Mozambique.

Methods: *Ex vivo P. falciparum* susceptibility assays, namely, ring-stage survival assays (RSA), piperaquine survival assays (PSA) and amodiaquine survival assays (AQSA) were performed in 43 non-complicated positive malaria samples from the Maputo City province collected between May and July 2022. Additionally, the profile of *pfk13* and *pfmdr1* single-nucleotide polymorphisms (SNPs), as well as *pfpm2 and pfmdr1* copy number variation (CNV) from isolates phenotypically susceptible to antimalarials was assessed.

Results: Survival rates revealed the absence of surviving parasites when exposed to dihydroartemisinin, piperaquine and amodiaquine. The survival rate was less than the thresholds for RSA, PSA and AQSA, respectively. As for molecular characterization of resistance markers, no *pfk13*-mediated artemisinin resistance gene mutation was detected in all 29 isolates phenotypically susceptible to antimalarial drugs. However, we reported the presence of 2 nonsynonymous mutations: namely, R575S and S576T in two isolates. These SNPs were predicted to be deleterious. In addition, our study identified one isolate (3.45%; 1/29, >1.5 copies) carrying *pfpm2 C*NV. As for *pfmdr1*, none of the isolates presented SNPs in the positions N86 and D1246. Polymorphisms were observed only at codon 184, in 51.72% (15/29) of the samples. NFD and NYD haplotypes (codons 86, 184, and 1246) were present in 51.72% and 48.28% of the samples, respectively.

Conclusions: These results suggests that *P. falciparum* isolates from the studied settings still show high sensitivity to antimalarial drugs, with no mutations associated with artemisinin resistance. Continued monitoring of parasite susceptibility to Artemisinin-based combination therapies (ACTs) and molecular surveillance should be intensified to reduce chances of artemisinin resistance in the near future.

Keywords: *Plasmodium falciparum*; *ex vivo* susceptibility assays, RSA, PSA, AQSA, CNV, Mozambique

Introduction

Malaria places a high socioeconomic burden on Mozambique. The country is amongst the 5 countries in sub-Saharan Africa with the highest incidence of the disease (WHO, 2023d). The World Health Organization's (WHO) *World malaria report 2023* estimated 249 million cases in 2022, 10.4 million of which in Mozambique (WHO, 2016). The incorrect use of mosquito nets and resistance to indoor residual spraying by the local population is thought to play a part in the increase of malaria cases (WHO, 2016, 2023d). Furthermore, malaria-related morbidity in sub-Saharan Africa increased from 2020 onwards due to the COVID-19 pandemic, which lead to interruptions in control strategies aimed at the diagnosis, prevention and treatment of malaria (Macicame et al., 2023).

Since 2006, the WHO has advocated for the utilization of artemisinin-based combination therapies (ACTs) as the primary approach for malaria treatment. Over the last 15 years, the use of ACTs has significantly decreased the number of new cases and deaths related to malaria in endemic countries (Benoit-Vical et al., 2016). ACTs are an association of a fast-acting drug, an artemisinin derivative that clears circulating parasites, and a slow-acting partner drug, which acts on the remaining parasites. However, *Plasmodium falciparum* one of the most virulent *Plasmodium* species and the cause of cerebral malaria (Feachem et al., 2019)has the ability to develop drug resistance mechanisms to certain medications(Balikagala et al., 2020; Bergmann et al., 2021). Tendentially, these resistance mechanisms develop in regions with low endemicity, great population influx, and poor access to medication, slow diagnosis, inadequate care, drug pressure, and incorrect use of antimalarials (Rasmussen et al., 2022). This prompts worldwide concern over the management of malaria, since artemisinin derivatives are part of the first-line treatments for uncomplicated *P. falciparum* malaria, and their effectiveness is crucial in preventing the spread of resistant malaria strains. In addition, during ACT treatment of artemisinin-resistant *P. falciparum* malaria, the parasitic burden left for the partner drug to eliminate becomes excessive, increasing the risk that the parasite becomes resistant to said partner drug (Si et al., 2023)

Mozambique introduced ACT first-line treatments artemether–lumefantrine (AL) and artesunate–amodiaquine (ASAQ) for the treatment of uncomplicated malaria in 2009 (Nhama et al., 2021). Despite no evidence of therapeutic failures nor of an increase in the frequency of molecular markers associated with resistance to the antimalarial drugs currently used in the clinic in Mozambique (Gupta et al., 2020), continuous monitoring of the efficacy of these drugs is critical, in order to guarantee the early discovery and prompt reaction against emerging resistance to first-line malaria treatments strategies.

The identification of molecular markers associated with drug resistance is a crucial step in epidemiological surveillance of malaria. It has been relevant in several drug efficacy studies and to monitor the spread of antimalarial resistance (Diakité et al., 2019; Iwanaga et al., 2022). These markers include single nucleotide polymorphisms (SNPs) as well as copy number variation (CNV) in genes that encode for drug target proteins in the parasite's biochemical pathways.

The presence of SNPs in the *Kelch13* gene (*pfk13*, PF3D7_1343700) on chromosome 13, particularly in the region, which encodes for the kelch13 propeller domain, has been associated with artemisinin resistance that responds to oxidative stress (Benoit-Vical et al., 2016). Molecular markers in this region were first identified in Cambodia, but have spread across the surrounding region and into the African continent (Kong et al., 2022; Lu et al., 2017; Straimer et al., 2015). Ten *pfk13* SNPs (F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L and C580Y) are validated by the WHO (WHO, 2019; World Health Organization, 2018). These SNPs in *pfk13* configure delayed parasite clearance (a half-life of 5 hours or more) and parasitemia relapse 3 days after treatment with ACTs (Ashley). In addition, partial resistance leads to therapeutic failure of the drugs dihydroartimisin (DHA), piperaquine (PPQ) and amodiaquine (AQ) (Mbye et al., 2020), as the parasite survival rates are higher than the thresholds in the ring-stage survival assays (RSA), piperaquine survival assays (PSA) and amodiaquine survival assays (AQSA), respectively (Ariey et al., 2014; Ouattara et al., 2015a)

The *multidrug resistance protein 1* gene (*pfmdr1*, PF3D7_0523000) on chromosome 5, which is responsible for drug distribution between the parasite’s digestive vacuole and cytosol, has been reported as a mediator of resistance to artemisinin partner drugs of the

quinoline class, such as lumefantrine (LUM) and AQ (Pirahmadi et al., 2013; Shafik et al., 2022). Moreover, CNV in *pfmdr1* has been implicated in resistance to LUM (Ronan Jambou, 2020). The *plasmepsin II* gene (*pfpm2,* PF3D7_1408000) on chromosome 14, gene encoding digestive proteases CNV in *pfpm2*. has been linked to parasite resistance to piperaquine(Boonyalai et al., 2021; WHO, 2016).

This study aimed to assess multiple components of *P. falciparum* antimalarial resistance in Mozambique and contribute to surveillance and decision-making regarding malaria treatment in the country. We examined the *ex vivo* susceptibility of *P. falciparum*-positive isolates collected in the Maputo province to DHA, as well as to the partner drugs PPQ and AQ. Furthermore, we characterized the prevalence of resistance-associated SNPs in *pfk13* and *pfmdr1*, as well as amplifications in both *pfpm2* and *pfmdr1*.

Materials and Methods

Ethics statement

This study was submitted and approved by the National Bioethics Committee for Health of Mozambique (CNBS—IRB00002657) (Ref: 131/CNBS/2021) dated: March 2021.

Study site, sample collection and clinical isolate preparation

Recruitment took place at Matola Provincial Hospital, Maputo province (Figure1), between May and July 2022. In Maputo, the peak of malaria incidence is between November and Abril. Patients with positive diagnosis for malaria through the *P. falciparum* histidine-rich protein 2 (PfHRP2) based rapid diagnostic test (RDT) (SD Bioline Malaria Ag P.f) and who fit the inclusion criteria (over a year old, with *P. falciparum* mono-infection confirmed by laboratory examination of thick drops and peripheral blood smears, parasite density between 2000 and 80000 parasites/μL blood, no history of antimalarials in the previous month) were included in the study after signing their informed consent. Patients with severe malaria as well as pregnant women were excluded. The age of the participants ranged from 1 to 40 years, with a mean age range of 17,7 years. Clinical isolates were obtained by collecting 2mL of venous blood into EDTA tubes **(**SARSTEDT S-Monovette®**)** by authorized biomedical personnel. 43 patients enrolled in this study. Samples were sent to

the molecular parasitology laboratory of Maputo's National Institute of Health, shipped in a sample transport cooler, and afterwards processed for drug susceptibility testing *in vitro* within 3 hours. Furthermore, blood spot samples were made on Whatman® FTA® cards, air-dried, and individually stored in sample bags with silica gel desiccants at -20°C for further genotypic molecular characterization of the parasites. Samples with *P. falciparum* monoinfection were identified and directed for further processing. To estimate parasitemia percentage, thin blood smears stained with 20% Giemsa (Merck KGaA™) were observed by light microscopy. Blood was washed three times with RPMI-1640 (Sigma-Aldrich®) to completely remove plasma and leucocytes before the survival assays. Parasitemia was adjusted to 0.5-0.8%. For samples exhibiting parasitemia levels greater than 1%, uninfected type-0 positive (0+) was added to dilute parasitemia to 1%. A 2% hematocrit mixture was then prepared for the survival assays.

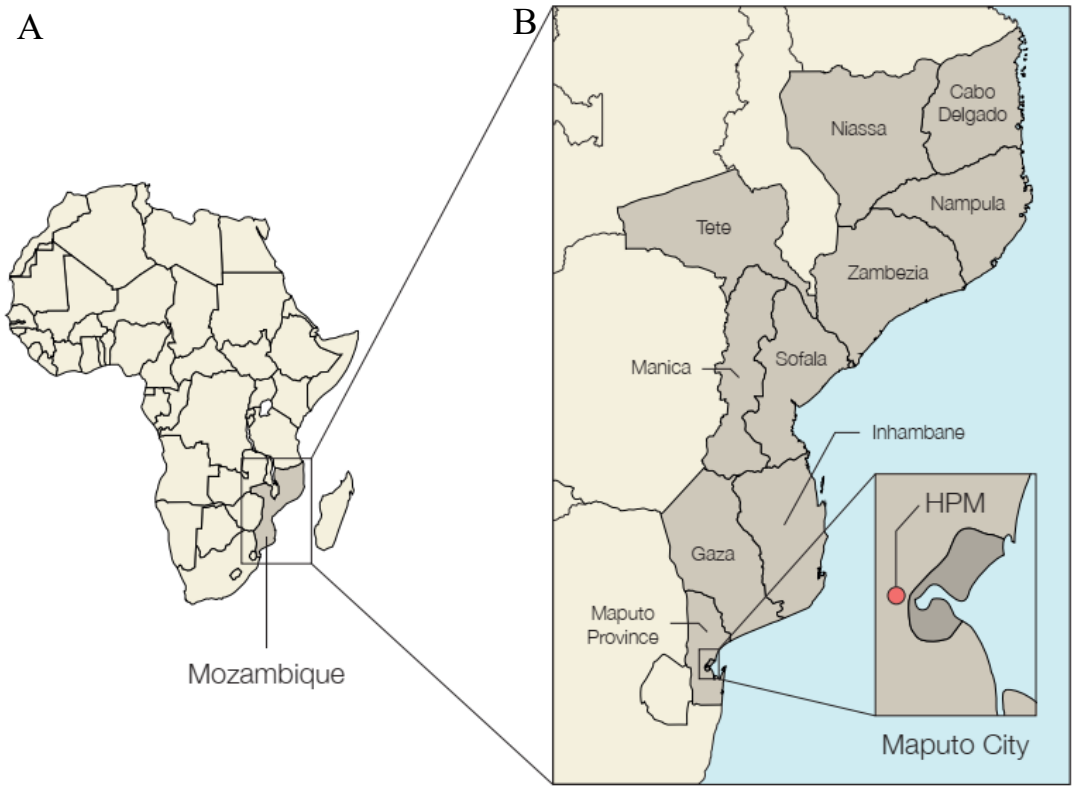

Figure 1: Map of Africa and Mozambique with study site. (A) Map of Africa with Mozambique highlighted in grey. (B) Map of Mozambique, with *Hospital Provincial da Matola* (*HPM*) red doted.

Chemicals and reagents

The antimalarial drugs AQ, PPQ and DHA were purchased from Sigma-Aldrich and dissolved in sterile DMSO (Sigma-Aldrich®) at a concentration of 5mM. The culture medium for the drug susceptibility assays was made by dissolving 1.044% (w/v) RPMI-1640 with L-glutamine (Biowest™), 0.59% (w/v) HEPES buffer (VWR™), 0.5% (w/v) AlbuMAXII (Gibco™), 0.2% of sodium bicarbonate (Merck KGaA™) and 0.005% (w/v) Hypoxanthine (Sigma-Aldrich®) in Milli-Q water. The culture medium was then sterilized through filtration, supplemented with 50 µg/mL of gentamycin (Sigma-Aldrich®), and stored at 4°C until use.

P. falciparum ex vivo **susceptibility assays** Drug

All procedures were performed in a Class II biosafety laminar flow chamber. Essays were performed as previously described with some modifications (REF). Briefly, a 2% hematocrit suspension was added to 96-well microplates. Synchronized ring-stage parasites (0–3 hours post-invasion) were exposed to 700 nM DHA for RSA. Simultaneously, 200 nM of PPQ and AQ were added to the respective wells for PSA and AQSA. A control well without drug exposure was also included. The plate was incubated at 37°C in a desiccator using the candle jar technique.

Cultures were treated for 6 hours for RSA and 48 hours for both PSA and AQSA. After incubation, the drugs were removed and the parasites were resuspended in RPMI-1640 culture medium and reincubated for an additional 66 hours (RSA), 48 hours (AQSA), and 24 hours (PSA) under the same conditions. After incubation, methanol-fixed smears stained with 20% Giemsa were prepared to estimate ring-stage survival rates. The estimations were performed by certified microscopist. Of the 43 total samples, 14 were unable to adapt to culture conditions. The remaining 29 samples (16 males and 13 females) were used for parasitemia calculations and molecular characterization.

In each assay, three independent microscopists assessed parasitemia in approximately 10,000 erythrocytes by counting viable parasites that developed into second-generation rings or trophozoites, using the equation below.

$$Survival\ rate\ (\%) = \frac{number\ of\ viable\ parasites\ in\ \ treated\ wells\ \ after\ 72h}{number\ of\ \ viable\ parasite\ in\ control\ wells\ after\ 72h} X100$$

A survival rate above the defined threshold for each assay indicates resistance to the drug, while a survival rate below the threshold indicates susceptibility. The survival rate thresholds for RSA, PSA and AQSA are 1%, 10% and 45%, respectively (Macicame et al., 2023; Plowe et al., 2007; World Health Organization, 2018).

Molecular characterization

DNA extraction

Parasite genomic DNA was extracted from dried blood spots on Whatman® FTA® cards using the Chelex-100 extraction method (Bereczky et al., 2005).

Determination of the *pfk13* and *pfmdr1 s*ingle-nucleotide polymorphism profile

P. falciparum isolates were genotyped *pfmdr1* and *pfk13* as described by (Serrano et al., 2021; da Silva et al., 2023). Primers were designed for the amplification of a 452bp fragment containing *pfmdr1* codons 86 and 184, a 508bp fragment containing *pfmdr1* codon 1246 and an 856bp fragment containing *pfk13* codons 436 to 706. Primer sequences are listed in Table 1. *P. falciparum* strain 3D7 DNA was used as positive control in all reactions. For *pfmdr1* amplifications, the reaction included 12.5 µL of NZYTaq II 2x Green Master Mix (NZYTech, Portugal), 0.5 µL of forward and reverse primer each, 9.5 µL of MilliQ H_2O and 2 µL of sample DNA. For *pfk13* amplifications, the reaction included 15 µL of the same master mix, 0.25 µL of forward and reverse primer each, 12.5 µL of MilliQ H_2O and 2 µL of sample DNA. PCR conditions were uniform for the amplification of all fragments: 94°C for 2 min; [94°C for 1 min, 56°C for 1 min, 72°C for 1 min] 10X; [94°C for 1 min, 50°C for 1 min, 72°C for1 min] 30X; 72°C for 3 min. 10 µL of the PCR product was analyzed by electrophoresis on a 2% agarose (NZYTech, Portugal) gel stained with

GreenSafe Premium (NZYTech, Portugal) to confirm single band amplification. PCR products were then purified using ADS Exo-Alp PCR Cleanup MIX (AdvancedSeq, USA) and shipped to Eurofins Genomics (GAC services, Germany) for Sanger sequencing. Successfully sequenced samples were aligned to the PF3D7_1343700 (*pfk13*) sequence using Multalign software (http://multalin.toulouse.inra.fr/; free online) and/or BioEdit version 7.2 for mutation detection. In order to predict the potential impact of non-synonymous SNPs on protein function, we used SIFT software (Sorting Intolerant From Tolerant, free online, https://sift.bii.a-star.edu.sg/index.html). This software takes into account the position at which the variation takes place and the type of amino acid change, then chooses related proteins and obtains an alignment of these proteins with the query (Ng & Henikoff, 2003). Finally, it calculates the probability that this particular amino acid change will be tolerated (Ng & Henikoff, 2003). If the calculated value is less than a cut-off value of 0.05, the substitution is predicted to be deleterious, and the opposite is considered not deleterious (Hu & Ng, 2012, 2013; Kobayashi et al., 2019; Ng & Henikoff, 2003).

Determination of the *pfpm2 and pfmdr1* copy number variation profile

The copy number of *pfpm2* and *pfmdr1* was determined using a SYBR-green–based quantitative PCR using an ABI 7500 Fast Real-Time PCR System equipment (Applied Biosystems) in a protocol modified from(Benoit Witkowski et al., 2017b). PCR was performed in a total reaction volume of 15 µL, with 7.5 µL of SYBR™ Green PCR Master Mix (NZYTech, Portugal), 0.3 µL of each primer, 5.4 µL of MilliQ water and 1.5 µL of sample DNA. PCR conditions were as follows: 95°C for 5 min; [95°C for 15 s, 60°C for 1 min] 40X. Copy number of *pfpm2* and *pfmdr1* was calculated using the *tubulin beta chain* gene (*pfβ-tubulin*, PF3D7_1008700) as the endogenous control in the 2 -ΔΔCt method. *P. falciparum* strain 3D7 (MRA-1029) DNA was used as a single-copy calibrator (Livak et al, 2001), while IPC_6261 (MR4-1284) with 3 copies of *pfpm2* [42] was used as an internal plate control and Dd2 (MRA-150) as a reference for 3 or 4 copies of *pfmdr*1 (Duah et al., 2013). Primer sequences are listed in Table 1. Primers and all experiments were validated using serial dilutions of *P. falciparum* strain 3D7 DNA to verify linearity of concentrations used. PCR efficiency was between 90-110% for *pfpm2, pfmdr1*, and for *pfβ-tubulin* genes.

All procedures were performed in triplicate. Relative copy number was calculated using the $2^{-\Delta\Delta Ct}$ method for relative quantification. ΔCt was calculated as ΔΔCt was calculated as ΔΔCt =ΔCt (Ct target gene – Ct, *pfβtubuli*n)χ – ΔCt (Ct, target gene – Ct, pfβtubulin)y, where χ is unknown sample and y refers to *P. falciparum* 3D7 (Livak & Schmittgen, 2001), where 3D7 is the calibration control of genomic 3D7 (MRA-102) DNA, with one copy of all genes (*pfBtub*, *pfpm2* and *pfmdr1*) (Price et al., 2004). Specificities of *pfpm2* and *pfmdr1* amplification curves were evaluated by visualising the respective melting curves. For both *pfmdr1* and *pfpm2*, single copies were defined as a 2−ΔΔCt result of <1.499 and multiple copies as a result of ≥1.500 (Leroy et al., 2019; Benoit Witkowski et al., 2010)

Table 1. Primers used for PCR of *pfmdr1* and *pfk13* SNPs detection and quantitative PCR (qPCR) of *pfpm2* and *pfmdr1* CNVs.

Genes	Primer	Sequence (5'-3')
pfmdr1* *PF3D7_0523000	**Codon 86/184**	
	MDR1_Fw	GTATGTGCTGTATTATCAGGAGGA
	MDR1_Rv	TTAATTTATGTTTGTGGTGTCATATG
	Codon 1246	
	MDR1_Fw	GAAAGAAGCAGAATTTTATGG
	MDR1_Rv	GAGAATAGCTATAGCTAGAGC
pfk13* *PF3D7_1343700	*pfk13*_Fw	GAAAGAAGCAGAATTTTATGG
	*pfk13*_Rv	GCTTGGCCCATCTTTATTAGTTCCC
	CNV	
pfpmp2* *PF3D7_1408000	*pfpmp2* CNV_Fw	GGATTCGAACCAACTTATACTGC
	pfpmp2 CNV_Rv	AATTGGATCTACTGAACCTATTGATAA
Pfβtub* *PF3D7_1008700	*pfβtub*_Fw	AAAAATATGATGTGCGCAAGTGA
	*pfβtub*_Rv	AACTTCCTTTGTGGACATTCTTCC
***pfmdr1* Pf3D7_0523000**	*pfmdr1*CNV_Fw	TGCATCTATAAAACGATCAGACAAA
	***pfmdr1*CNV_Rv**	ACAGTCACATGGAACACACGA

Results

P. falciparum ex vivo susceptibility to antimalarials in use in Maputo

Of the 43 initial patient samples included in the study and subsequent establishment of the study criteria, 14 were excluded for inability to adapt to the culture conditions. Therefore, we selected the remaining 29 pre-treated field isolates from day 0 for showing a successful phenotypical description. To ensure an effective artemisinin susceptibility assay, we took into consideration the developmental stage of the parasite in ring-stage from 0 to 3 hours since, in this tight window, the parasites show higher susceptibility to artemisinin. After 72 hours, we evaluated both treated and control wells. The treated group showed a marked decline in predicted survival. No significant variations were identified in the parasites´ phenotype resistance within this group after observation through conventional microscopy.

The majority of samples showed no surviving parasites after RSA, except for eight isolates. Samples S03, S010, S011, S014, S026, S36, and S045 had survival rates lower than the 1% threshold associated with artemisinin resistance (0.2%, 0.04%, 0.06%, 0.02%, 0.04%, 0.17%, and 0.02%, respectively). Sample S034 exhibited a survival rate discreetly higher than 1%. .

After PSA, 4 samples, S034, S035, S036 and S046 showed survival rates of 0.34%, 0.36%, 0.34% and 0.37%, respectively, far from the 10% threshold which indicates piperaquine resistance. Similarly, after AQSA, only 2 samples, S10 and S45 showed survival rates of 0.02% and 0.27%, also much lower than the 45% threshold which indicates amodiaquine resistance. Our results suggest that artemisinin-resistant *P. falciparum* has not yet emerged in Maputo (Mozambique).

pfk13 **and** ***pfmdr1*** **single-nucleotide polymorphism profile of isolates phenotipically susceptible to antimalarials**

All 29 samples were genotyped in order to confirm that the phenotype presented in the *ex vivo* assays are aligned with the genotype. No *pfk13*-validated polymorphisms for artemisinin resistance were detected in these samples. However, 6.89% (2/29) of samples harbored two non-synonymous mutations; namely, R575S and S576T (Supplementary table 1). Using SIFT software as described in [8], it was predicted that mutations S576**T** would be R575**S** tolerated and may affect the protein function, with a score of 0.07 and 0.02, respectively. As for *pfmdr1*, none of the isolates presented SNPs in positions N86

and D1246, with polymorphisms being observed only at codon 184, in 51.72% (15/29) of the samples. The NFD and NYD haplotypes (codons 86, 184 and 1246) were present in 51.72% and 48.28% of samples, respectively (Supplementary table 1).

pfmdr1 and *pfpm2* copy number variation profile

All 29 samples successfully passed the initial quality control check: a cycle threshold (Ct) value over 40 or no Ct. All samples were simultaneously analyzed for both *pfpm2* and *pfmdr1*. The qPCR assay's accuracy was confirmed using established *P. falciparum* strains with known copy numbers. As expected, strain 3D7 displayed a single copy for *pfpm2* and *pfmdr1*, while IPC_6261 and IPC_5202 displayed three copies of *pfpm2* and *pfmdr1*, respectively. One isolate harbored definitive *pfpm2* CNV (>1.5 copies). No *pfmdr1* CNV was observed (**Figure 3**).

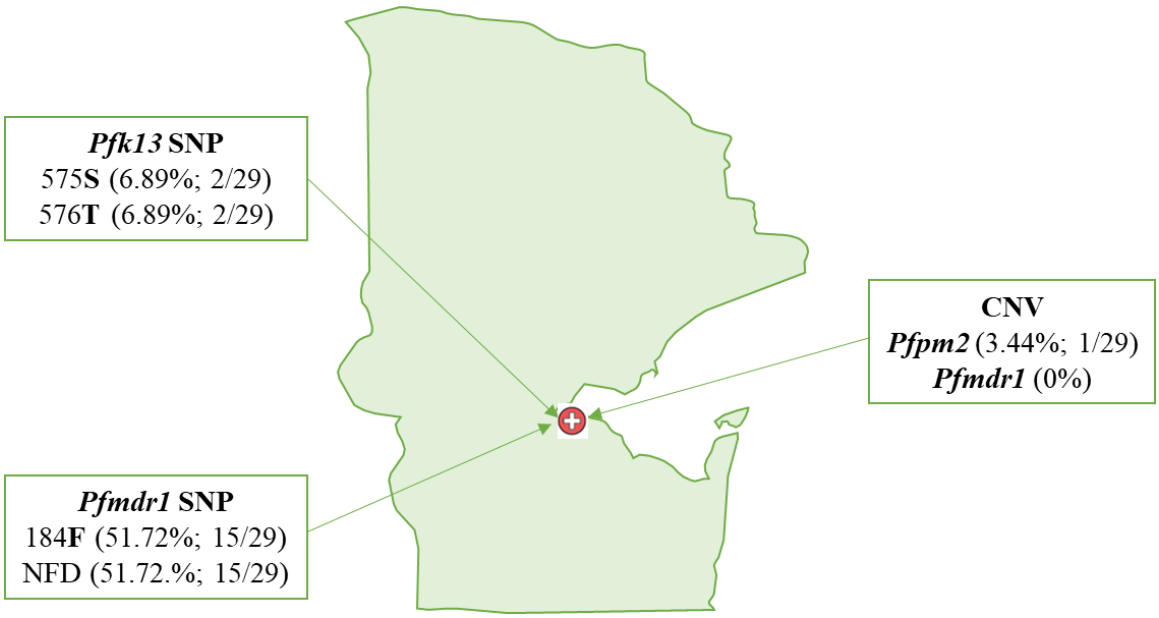

Figure 3: Prevalence of single-nucleotide polymorphism (SNP) and copy number variations (CNV) of isolates phenotipically susceptible to antimalarials. *Pfk13- Plasmodium falciparum* kelch protein K13. *pfmdr1- Plasmodium falciparum multidrug resistance gene*. *pfpm2- Plasmodium falciparum* plasmepsin 2 gene.

Discussion

P. falciparum is the most frequent causeof malaria in Mozambique, accounting for over 90% of all malaria infectionsin the country, while *Plasmodium malarie* and *Plasmodium ovale* infections are observed in 9% and 1% cases, respectively (INE, 2019, 2023; MISAU, 2013).

Our unprecedented study in the Maputo province phenotypically and genotipically characterized *P. falciparum* parasites from field isolates. These results described the rates of *P. falciparum* survival following exposure to DHA and ACT partner drugs such as PPQ and AQ by PSA and AQSA, respectively, to search for resistance (Mbye et al., 2020). We combined *ex vivo* RSA, PSA and AQSA with relevant molecular characterization of the samples, such as SNPs in *pfk13 and pfmdr1* and copy number variation in *pfpm2* and *pfmdr1*. This proof-of-concept study only included 29 samples from Maputo, which prevented the results from being extrapolated nationwide.

Recently, therapeutic efficacy studies in sub-Saharan Africa, showed parasites survival rates over 10% after DHA treatment by RSA, indicating that the decline of artemisinin derivatives' efficacy is an imminent threat in Africa (Ahorhorlu et al., 2023). We did not observe significant resistance rates in our samples, and none exceeded the specified thresholds of 1%, 10%, and 45% for DHA, PPQ, and AQ, respectively, as established by the WHO (Duru et al., 2015; Mairet-khedim et al., 2021; B Witkowski et al., 2013; Benoit Witkowski et al., 2013). These results are in accordance with previous studies performed in Senegal combining *ex vivo* RSA phenotype and *pfk13* genotyping, in which resistance was also not detected in field samples (Yade et al., 2023).

To complement the phenotypic approach, sample genotyping was performed, where no *pfk13* validated or candidate mutations [5] for artemisinin partial resistance was detected. However, we reported the presence of 2 nonsynonymous mutations in *pfk13*; 575S and 576T in two isolates. 576T was previously observed in samples collected from the Republic of Congo, specificaly in Katana in 2019 (Yobi et al., 2022). Although its association with resistance of P. falciparum to artemisinin is still unknown.

As for the *pfmdr1* gene, genotyping focused on codons 86, 184, and 1246. Our study assessed the polymorphisms N86Y, Y184F, and D1246Y associated with modulating

susceptibility to CQ, LUM and AQ (WHO, 2019). None of the samples showed the 86Y and D1246 mutations, while 51.72% (15/29) of samples exhibited the 184F mutation. 86Y is associated with chloroquine and amodiaquine resistance (L. et al., 2003; Martin et al., 2018; NAGESHA et al., 2003). Our results may be explained by the fact that cloroquine was withdrawn from use in Mozambique in 2013 and amodiaquine is currently only being used in seasonal malaria chemoprevention with sulphadoxine-pyrimethamine (SPAQ) (Al-Mekhlafi et al., 2022; WHO, 2023b). Furthermore, the results are in accordance with what was previously reported in Mozambique (Chidimatembue et al., 2021; Gupta et al., 2018; Serrano et al., 2021; Silva et al., 2023), where no mutations were detected in codons 86 and 1246, as opposed to codon 184. Our results showed high prevalence of the 184F allele and, consequently, of the NFD haplotype. This haplotype was previously associated with increased tolerance to lumefantrine and/or artemisinin (Rosenthal et al., 2024; Siddiqui et al., 2021). Moreover, the high prevalence of the NFD haplotype might be indicative of the emergence of artemether-lumefantrine-resistant *P. falciparum* strains (Al-Mekhlafi et al., 2022) [58] in Mozambique in the near future. Thus, molecular surveillance of these markers is extremely important and should be extended nationwide.

We detected multiple copies of *pfpm2* in 3.45% (1/29, > 1.5 copies) of our samples. Contrarily, no *pfmdr1*multiple copies were detected. These results are not in accordance those recently reported by (Brown et al., 2024), where 13 total amplifications were found in 229 (5.7%, >1.2 copies) clinical samples from Mozambique, in *pfpm2*, *pfmdr1* and *pfpm3*. We were unable to detect *pfmdr1* amplifications due to the sample size of our study. The low prevalence of *pfpm2* amplification that we report is consistent with the fact that piperaquine has only been used in malaria near-elimination zones Maputo (Cuinhane et al., 2023; Galatas et al., 2020) and Gaza provinces (Brown et al., 2024) and in the emergency context in Cabo Delgado.

Conclusion

DHA, PPQ, AQ are still effective against *P. falciparum* malaria in Maputo Province. Continuous surveillance of *P. falciparum*'s ex vivo susceptibility to ACTs and molecular

surveillance should be intensified in order to minimize the risks of possible emergence of resistant parasites to artemisinin and partner drugs in the near future.

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Author contributions

C.d.S., and F.N.: Designed the study protocol; D.D., C.d.S., A.T., S.N., A.F., T.M., I.N., C.S., B.R., P.A., S.E.: Conducted the field activities (sample collection); D.D., D.M.: Conducted laboratory activities; C.d.S., D.D., A.D. and D.M.: Analysed the data; C.d.S., D.D., D.M., and F.N.: Wrote the first draft of the manuscript. All authors read, reviewed and approved the final manuscript.

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Availability of data and materials

The data related to this study are included in the manuscript or its supplementary data and are available upon request to the corresponding author.

Declarations

Competing Interests: The authors declare no conflict of interest.

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Supplementary table:

Table 1: *Ex vivo* susceptibility assay and genotyping of *P. falciparum* isolates to antimalarial drugs in use in Maputo. DHA-dihydroartemisinin, **PPQ-** Piperaquine, **AQ-**Amodiaquine. RSA-Ring stage survival assay, PSA- Piperaquine Survival Assay, AQSA-

Amodiaquine Survival Assay. **Non Syn-** non-synonymous mutations, **Syn-**synonymous mutations.

			RSA	PSA	AQSA	*pfk13*		*pfmdr1*		*Pfpm2*
Sample ID	**Age**	**SEX**	**DHA (%)**	**PPQ (%)**	**AQ (%)**	***pfk13***	**N86**	**Y184**	**D1246**	**>1.5 CNV**
S03	11	M	0.2	0	0		N	F	D	
S05	13	F	0	0	0		N	F	D	
S06	23	M	0	0	0		N	F	D	
S07	40	F	0	0	0		N	Y	D	
S10	9	F	0.04	0	0.02		N	F	D	
S11	41	F	0.06	0	0		N	F	D	
S12	1	F	0	0	0		N	Y	D	
S13	18	F	0	0	0		N	F	D	
S14	19	F	0.02	0	0		N	F	D	
S15	36	M	0	0	0		N	Y	D	
S16	10	M	0	0	0	**R**575**S** **S**576**T**	N	F	D	
S17	8	M	0	0	0		N	F	D	
S18	19	M	0	0	0		N	Y	D	**X**
S19	5	M	0	0	0	**R**575**S** **S**576**T**	N	F	D	
S20	23	M	0	0	0		N	F	D	
S22	19	F	0	0	0		N	F	D	
S23	40	M	0	0	0		N	F	D	
S24	10	F	0	0	0		N	Y	D	
S25	8	F	0	0	0		N	Y	D	
S26	20	F	0.04	0	0		N	Y	D	
S34	25	F	1.05	0.34	0		N	Y	D	
S35	19	M	0	0.33	0		N	Y	D	
S36	21	F	0.17	0.34	0		N	Y	D	
S37	34	F	0	0	0		N	Y	D	
S39	4	M	0	0	0		N	Y	D	
S40	7	M	0	0	0		N	Y	D	
S43	13	F	0	0	0		N	Y	D	
S45	7	F	0.02	0	0.27		N	Y	D	
S46	9	M	0	0.37	0		N	F	D	

4.4. Chapter 4: Other results in the context of PhD thesis

4.4.1. Methodology

4.4.1.1. Study Profile

To assess the prevalence of mutations in genes associated with the response to ACTs, namely *pfK13, pfmdr1* and *pfpm2/3*.

4.4.1.2. Study area

Sampling process was carried out in seven provinces of Mozambique, namely Maputo, Gaza, Inhambane, Sofala, Manica, Tete and Niassa (**Figure 7**).

To characterize the molecular markers of *P. falciparum* resistance to ACTs, the study was carried out in Maputo, Gaza, Inhambane, Sofala, Manica, Tete and Niassa. The selection of the provinces was based on the prevalence of malaria according to malaria indicator survey (*IIM*) 2018, since with the exception of Maputo; the other provinces have high levels of prevalence. Another factor was the existence of few molecular characterization studies of resistance markers in the selected areas. In each province, health facilities were selected for sample and data collection between April 2021 and April 2022.

4.4.1.3. DNA Extraction

Parasite genomic DNA from dried blood spots was extracted throughusing the Chelex method described by (Bereczky et al., 2005). Briefly, dried blood spot (DBS) were punched and transferred to a sterile 1.5mL tube marked earlier. This process was repeated between each sample. To each tube 1ml of 0.5 % saponin in PBS was added and tubes were inverted several times and placed at 4ºC overnight. Supernatant was removed and 1ml of 1xPBS was added to each tube and place at 4 ºC for 15-30 minutes. The PBS was discarded and 50µl of 20% Chelex-100 and 150µL of sterile water were added to each tube. Each tube was vigorously vortex (Genie 2, Scientific Industries) for 30 seconds and placed in a heat block at 100ºC for ± 10-15 minutes, with the tubes opened. The tubes were centrifuged at 14000rpm for 2 minutes and the supernatant was transferred to a new identified tube (without carrying the Chelex). The last step was repeated until supernatant presented a translucent colour. All the samples were stored at -20ºC or -70ºC for genotyping.

4.4.1.4. Confirmation of infection with *P. falciparum* by Real-Time PCR

Real-time PCR was used for *P. falciparum* confirmation. PCR reactions targeting the 18S rRNA gene were conducted as described in Rosanas-Urgell et al 2010, with modifications. Briefly, forward primer 5'-TATTGCTTTTGAGAGGTTTTGTTACTTTG-3' and reverse primer ACCTCTGACATCTGAATACGAATGC and the probe FAM-ACGGGTAGTCATGATTGAGTT-MGB-BHQ were used. PCR reaction mixture consisted of 7.5 µL of 2X (NZYTECH, Portugal), 600 nM of each primer and 200 nM of FAM™- labeled probe (IDT Integrated DNA Technologies, USA), 1 µL of genomic DNA and water up to 15 µL. PCR conditions: 50 ºC for 2 minutes and 95 ºC for 10 minutes; these were followed by 40 cycles at 94 ºC for 30 seconds and a final cycle at 60 ºC for 1 minute. Triplicate samples were assayed in the Bio-Rad 500 Real Time PCR System™ (Applied Biosystems, USA). All reactions were performed with positive controls (DNA from 3D7 strain of *P. falciparum* culture).

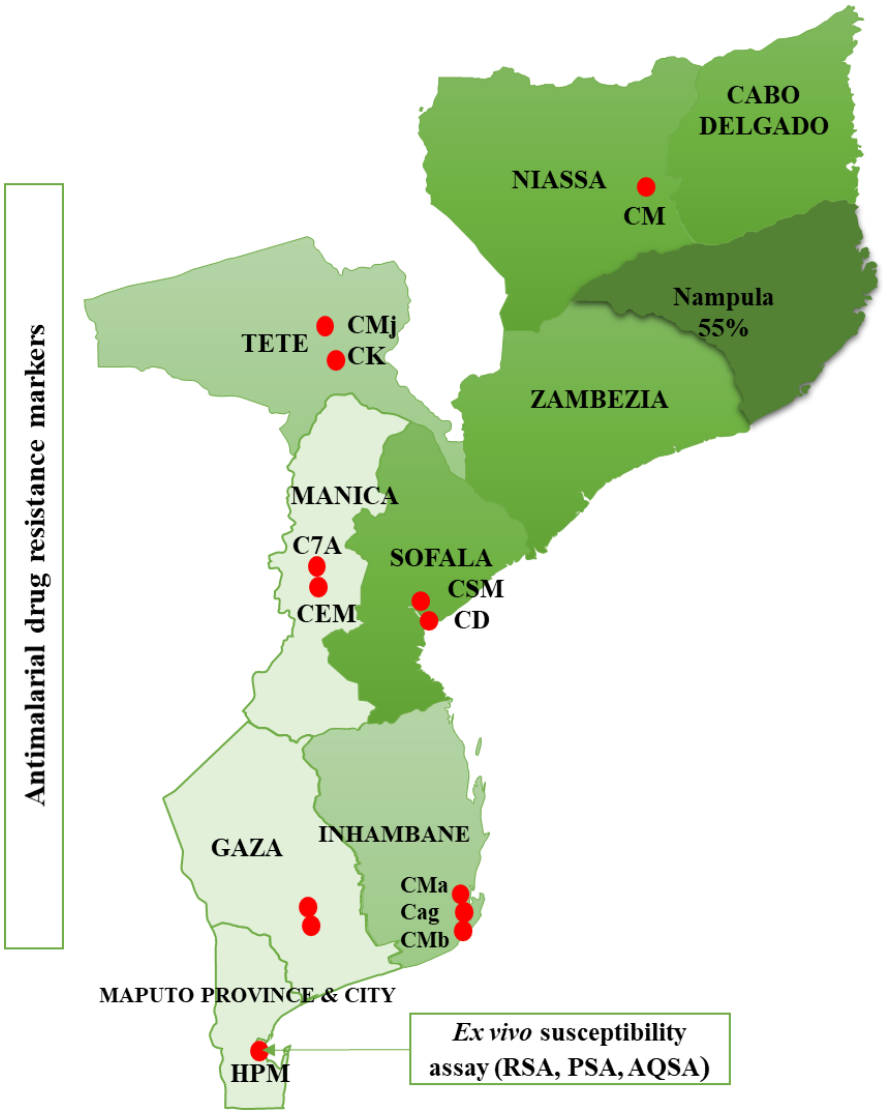


Figure 7: **Map of Mozambique with study sites**. Red spots represent Helth centres (HC). **CM**- Marrupa HC, **CMj**- Manje HC, **CK**- Kaunda HC, **C7A**- 7 de Abril HC, **CEM**- Eduardo Mondlane HC, **CSM**- Mafambisse HC, **CD**- Dondo HC, **CMa**- Maxixe HC, **CAg**- Agostinho Neto HC, **CMb**- Mabil HC, **CCh**- Chibuto HC, **CMi**- Milenium HC, **HPM**- Matola Provincial Hospital,.

4.4.2. Target amplicon based sequencing

4.4.2.1. Primer design

To amplify, construct libraries and sequence *P. falciparum* genes associated to ACTs resistance, *pfmdr1* and *pfk13*, primers were designed using Primer3 version 4 software (https://bioinfo.ut.ee/primer3-0.4.0/) (Nag et al., 2017) using the 3D7 reference assembly from PlasmoDB (http://PlasmoDB.org). The melting temperature (Tm) was estimated using the version 3.27 of Oligonucleotide Properties Calculator software (Oligo Calc).

All designed primers were confirmed using SnapGene Viewer, version 5.3.2 as it could be seen in the image bellow (**figure 8**). Example of designed primer and the genetic marker 86 of the *pfmdr1* gene is represented in figure 8. All oligonucleotides were synthetized by Eurofins Genomics (Kissinger et al., 2002; PN et al., 2016).

Figure 8- Example of the confirmation of an accurate primer design with one of the primer generated to flank the position N86 and Y184 of the *pfmdr1* gene. **BC**- barcode, **Fw**- Primer forward, **MDR**- multidrug resistance. Figure generated using SnapGene Viewer Version 5.3.2.

4.4.2.2. Pooling and barcoding of the samples

After the design of primers, DNA extraction and confirmation of infection by *P. falciparum*, the next step was the library preparation. This step started with multiplexed PCR (mPCR) for this drug resistance module. This module covers 4 fragments, 2 for each gene. For *pfmdr1* gene, one of the fragments (*pfmdr1*-A) comprehends 453 base pair (bp) lenght (nucleotide positions 184-636) covering 61-212 amino acid codons, and a second one (*pfmdr1*-B) was a fragment with 441bp (nucleotide positions 3481-3921), within 1160-1307 amino acid codons. As for the *pfk13* gene, one of the fragments (*pfk13*-A)

comprehends 499bp length (nucleotide positions 1236-1734) covering 412-578 amino acid codons and, and a second one (*pfk13*-B) was a fragment with a 530bp fragment (*pfk13*-B) (nucleotide positions 1640-2169) covering 546-723 amino acid codons. The primes to amplify these fragments are shown in the supplementary table 3. All primers included a unique barcode used to differentiate each sample during the genome sequencing analysis, and also include an Illumina tail that will be used in a secondary PCR (provided by Eurofins Genomics) to insert Illumina adaptors, which are necessary for the Illumina sequencing platforms (**table 4).**

4.4.2.3. Multiplexed and Indexing PCR

We amplified the extracted parasite DNAs by PCR, targeting the four aforementioned fragments. The PCR was performed using a MyCycler BIORAD thermal cycler. Once optimised, the PCR was performed using the following reagents to achieve a final volume of 50 µL (25 µL of Green Master Mix (NZYTaq II 2x), 0.5 µL of each of the 8 primers (10 µM) and 19 µL of DNAse-free water and 2 µL of each DNA template sample). The primers were combined as follows: forward BC1 with reverse BC7, forward BC2 with reverse BC8 and forward BC3 with reverse BC9. It was performed on all eight samples. The samples were then amplified under the following conditions: initial denaturation for 2 minutes at 95°C, then 45 cycles of 15 seconds denaturation at 95°C, 30 seconds annealing at 57°C, 90 seconds extension at 68°C and an extra 5 minutes of final extension at 72°C. The PCR products were stored at -20°C. This step of the PCR used gene-targeted primers containing barcodes to identify each sample and partial Illumina tails (see **Figure 8 and Table 4**). After this step, the amplicons from different samples were pooled into one 1.5 ul tube, as each sample contains a unique barcode. A second PCR was used to incorporate the remaining Illumina tails into the amplicon pool, which had been prepared at Eurofins Genomics.

4.4.2.4. Library purification

Pooled library was purified using SureClean Plus (meridian Bioscience) protocol, followining manufactures procedures. Briefly, the Pink co-precipitant and library pool were mixed in equal volumes, then the same volume of SureClean plus was added and vortexed (Genie 2, Scientific Industries) and incubated at room temperature for 10 min.

The solution was centrifuged in a bench-top centrifuge (5417 C by Eppendorf) at 14000rpm for 10 min and the supernatant was discarded. Ethanol (70%) was added with twice the volume of the initial sample volume and vortexed. The solution was centrifuged under the same conditions and the supernatant removed and air-dry. The pellet was eluted in 20µL of DNAse free water.

The purification process was confirmed by electrophorese. Gel mold was prepared and the electrophoresis buffer 1x TAE was added. A solution of agarose was prepared in electrophoresis buffer (1x TAE) at a concentration of 2%. When the molten gel has cooled, the GreenSafe Premium (NZYTech) was added. The hot gel agarose solution was mixed by gentle swirling and layed into the mold. While the agarose solution is cooling, the slots were placed in position to form a perfect well. The gel was mounted in the electrophoresis tank, and it was added just enough electrophoresis buffer to cover the gel. The NZYTech Ladder VI (5 µL) and the samples (5 µL) were loaded. The gel run at a voltage of 110V, 400 mA during 30 minutes (PowerPac Basic, BIO-RAD). The gel tray was removed and placed directly on a transilluminator and examined using a UV light and an image of the gel was captured through MiniBIS Pro, Bio-Imaging Systems (Green & Sambrook, 2019). Target DNA amplified by PCR was identified by visualization of the appropriate size band (ME et al., 1991)

Once the experimental design was complete, the amplicons of 8 patients from Mozambique (6 amplicons x 8 samples = 48 amplicons in total) were pooled equally and the concentration was measured using a NanoDrop 1000 (Spectrophotometer, Thermo Scientific). 35 µL of pooled library at 100.6 ng/µL was shipped to Eurofins Genomics, in Germany, to be sequenced with the Illumina MiSeq. The Sequencing output was analysed by Tablet software, version 1.21.02.08 (https://ics.hutton.ac.uk/tablet/) (Milne et al., 2009)

Table 4 - Identification of the Illumina tails and barcodes that were added to the primers and its work condition.

Fragment ID	Tail	Barcode	Primer Adapter	Primer Sequence	TM	GC (%)	Length (bp)
pfmdr1 86_184 Fw BC1	5'ACACTCTTTCCC TACACGACGCTCT TCCGATCT-3'	5'ATCA CG-3'	GTATGTGCTGTAT TATCAGGAGGA	5'ACACTCTTTCCCTACACGACGCTCTTCCGAT CTATCACGGTATGTGCTGTATTATCAGGAGGA -3'	54	47	63
pfmdr1 86_184 Rv BC7	5'GACTGGAGTTC AGACGTGTGCTCT TCCGATCT-3'	5'CAGA TC-3'	ACATATGACACC ACAAACATAAAT TAAC	5'GACTGGAGTTCAGACGTGTGCTCTTCCGATC TCAGATCACATATGACACCACAAACATAAAT TAAC-3'	52.6	42.4	66
pfmdr1 1246 Fw BC1	5'ACACTCTTTCCC TACACGACGCTCT TCCGATCT-3'	5'ATCA CG-3'	GGAGAAACAGGT AGTGGAAAATC	5'ACACTCTTTCCCTACACGACGCTCTTCCGAT CTATCACGGGAGAAACAGGTAGTGGAAAATC -3'	53.5	48	62
pfmdr1 1246 Rv BC7	5'GACTGGAGTTC AGACGTGTGCTCT TCCGATCT-3'	5'CAGA TC-3'	TGGTCCAACATTT GTATCATATTTAT TTGG	5'GACTGGAGTTCAGACGTGTGCTCTTCCGATC TCAGATCTGGTCCAACATTTGTATCATATTTA TTTGG-3'	54.8	42.6	68
pfk13 A Fw BC1	5'ACACTCTTTCCC TACACGACGCTCT TCCGATCT-3'	5'ATCA CG-3'	TCCGTTAACTATA CCCATACCAAAA G	5'ACACTCTTTCCCTACACGACGCTCTTCCGAT CTATCACGTCCGTTAACTATACCCATACCAAA AG-3'	54.8	46.2	65
pfk13 A Rv BC7	5'GACTGGAGTTC AGACGTGTGCTCT TCCGATCT-3'	5'CAGA TC-3'	AGCTGATGATCTA GGGGTATTCA	5'GACTGGAGTTCAGACGTGTGCTCTTCCGATC TCAGATCAGCTGATGATCTAGGGGTATTCA-3'	53.5	49.2	61
pfk13 B Fw BC1	5'ACACTCTTTCCC TACACGACGCTCT TCCGATCT-3'	5'ATCA CG-3'	ATGGCTCTTCTAT TATACCGAATG	5'ACACTCTTTCCCTACACGACGCTCTTCCGAT CTATCACGATGGCTCTTCTATTATACCGAATG-3'	52.3	46	63
pfk13 B Rv BC7	5'GACTGGAGTTC AGACGTGTGCTCT TCCGATCT-3'	5'CAGA TC-3'	TATTAAAACGGA GTGACCAAATCT G	5'GACTGGAGTTCAGACGTGTGCTCTTCCGATC TCAGATCTATTAAAACGGAGTGACCAAATCT G-3'	52.8	46	63
pfmdr1 86_184 Fw BC2	5'ACACTCTTTCCC TACACGACGCTCT TCCGATCT-3'	5'CGAT GT-3'	GTATGTGCTGTAT TATCAGGAGGA	5'ACACTCTTTCCCTACACGACGCTCTTCCGAT CTCGATGTGTATGTGCTGTATTATCAGGAGGA -3'	54	47.6	63

pfmdr1 86_184 Rv BC8	5'GACTGGAGTTC AGACGTGTGCTCT TCCGATCT-3'	5'ACTT GA-3'	ACATATGACACC ACAAACATAAAT TAAC	5'GACTGGAGTTCAGACGTGTGCTCTTCCGATC TACTTGAACATATGACACCACAAACATAAAT TAAC-3'	52.6	40.9	66
pfmdr1 1246 Fw BC2	5'ACACTCTTTCCC TACACGACGCTCT TCCGATCT-3'	5'CGAT GT-3'	GGAGAAACAGGT AGTGGAAAATC	5'ACACTCTTTCCCTACACGACGCTCTTCCGAT CTCGATGTGGAGAAACAGGTAGTGGAAAATC -3'	53.5	48.4	62
pfmdr1 1246 Rv BC8	5'GACTGGAGTTC AGACGTGTGCTCT TCCGATCT-3'	5'ACTT GA-3'	TGGTCCAACATTT GTATCATATTTAT TTGG	5'GACTGGAGTTCAGACGTGTGCTCTTCCGATC TACTTGATGGTCCAACATTTGTATCATATTTA TTTGG-3'	54.8	41.2	68
pfk13 A Fw BC2	5'ACACTCTTTCCC TACACGACGCTCT TCCGATCT-3'	5'CGAT GT-3'	TCCGTTAACTATA CCCATACCAAAA G	5'ACACTCTTTCCCTACACGACGCTCTTCCGAT CTCGATGTTCCGTTAACTATACCCATACCAAA AG-3'	54.8	46.2	65
pfk13 A Rv BC8	5'GACTGGAGTTC AGACGTGTGCTCT TCCGATCT-3'	5'ACTT GA-3'	AGCTGATGATCTA GGGGTATTCA	5'GACTGGAGTTCAGACGTGTGCTCTTCCGATC TACTTGAAGCTGATGATCTAGGGGTATTCA-3'	53.5	47.5	61
pfk13 B Fw BC2	5'ACACTCTTTCCC TACACGACGCTCT TCCGATCT-3'	5'CGAT GT-3'	ATGGCTCTTCTAT TATACCGAATG	5'ACACTCTTTCCCTACACGACGCTCTTCCGAT CTCGATGTATGGCTCTTCTATTATACCGAATG- 3'	52.3	46	63
pfk13 B Rv BC8	5'GACTGGAGTTC AGACGTGTGCTCT TCCGATCT-3'	5'ACTT GA-3'	TATTAAAACGGA GTGACCAAATCT G	5'GACTGGAGTTCAGACGTGTGCTCTTCCGATC TACTTGATATTAAAACGGAGTGACCAAATCT G-3'	52.8	44.4	63
pfmdr1 86_184 Fw BC3	5'ACACTCTTTCCC TACACGACGCTCT TCCGATCT-3'	5'TTAG GC-3'	GTATGTGCTGTAT TATCAGGAGGA	5'ACACTCTTTCCCTACACGACGCTCTTCCGAT CTTTAGGCGTATGTGCTGTATTATCAGGAGGA -3'	54	47.6	63
pfmdr1 86_184 Rv BC9	5'GACTGGAGTTC AGACGTGTGCTCT TCCGATCT-3'	5'GATC AG-3'	ACATATGACACC ACAAACATAAAT TAAC	5'GACTGGAGTTCAGACGTGTGCTCTTCCGATC TGATCAGACATATGACACCACAAACATAAAT TAAC-3'	52.6	42.4	66
pfmdr1 1246 Fw BC3	5'ACACTCTTTCCC TACACGACGCTCT TCCGATCT-3'	5'TTAG GC-3'	GGAGAAACAGGT AGTGGAAAATC	5'ACACTCTTTCCCTACACGACGCTCTTCCGAT CTTTAGGCGGAGAAACAGGTAGTGGAAAATC -3'	53.5	48.4	62

pfmdr1 1246 Rv BC9	5'GACTGGAGTTC AGACGTGTGCTCT TCCGATCT-3'	5'GATC AG-3'	TGGTCCAACATTT GTATCATATTTAT TTGG	5'GACTGGAGTTCAGACGTGTGCTCTTCCGATC TGATCAGTGGTCCAACATTTGTATCATATTTA TTTGG-3'	54.8	42.6	68
pfk13 A Fw BC3	5'ACACTCTTTCCC TACACGACGCTCT TCCGATCT-3'	5'TTAG GC-3'	TCCGTTAACTATA CCCATACCAAAA G	5'ACACTCTTTCCCTACACGACGCTCTTCCGAT CTTTAGGCTCCGTTAACTATACCCATACCAAA AG-3'	54.8	46.2	65
pfk13 A Rv BC9	5'GACTGGAGTTC AGACGTGTGCTCT TCCGATCT-3'	5'GATC AG-3'	AGCTGATGATCTA GGGGTATTCA	5'GACTGGAGTTCAGACGTGTGCTCTTCCGATC TGATCAGAGCTGATGATCTAGGGGTATTCA-3'	53.5	49.2	61
pfk13 B Fw BC3	5'ACACTCTTTCCC TACACGACGCTCT TCCGATCT-3'	5'TTAG GC-3'	ATGGCTCTTCTAT TATACCGAATG	5'ACACTCTTTCCCTACACGACGCTCTTCCGAT CTTTAGGCATGGCTCTTCTATTATACCGAATG-3'	52.3	46	63
pfk13 B Rv BC9	5'GACTGGAGTTC AGACGTGTGCTCT TCCGATCT-3'	5'GATC AG-3'	TATTAAAACGGA GTGACCAAATCT G	5'GACTGGAGTTCAGACGTGTGCTCTTCCGATC TGATCAGTATTAAAACGGAGTGACCAAATCT G-3'	52.8	46	63

TM-Melting temperature, GC (%) - percentage of guanine contents, BC- barcode.

4.4.3. Results

4.4.3.1. Characterisation of the study site and sample size

In the period under analysis, i.e. from April 2021 to August 2022, a total of 1,428 dried blood samples (DBS) were collected taken in 7 provinces, namely Maputo (Matola Provincial Hospital), Gaza (Chibuto Health Centre and Milenium Health Centre), Inhambane (Maxixe Health Centre, Agostinho Neto Health Centre and Mabil Health Centre), Sofala (*Mafambisse* Health Centre and *Dondo* Health Centre), Manica (*Eduardo Mondlane* Health Centre and *7 de Abril* Health Centre), Tete (*Manje* Health Centre and *Kaunda* Health Centre) and Niassa (*Marrupa* Health Centre). The goal was to reach 200 samples per site. Thus, an overall 1428 participants enrolled in the study and provided blood samples. 198, 217, 210, 203, 214, 186 e 200 participants belonged to Maputo, Gaza, Inhambane, Sofala, Manica, Tete and Niassa respectively (**figure 8**).

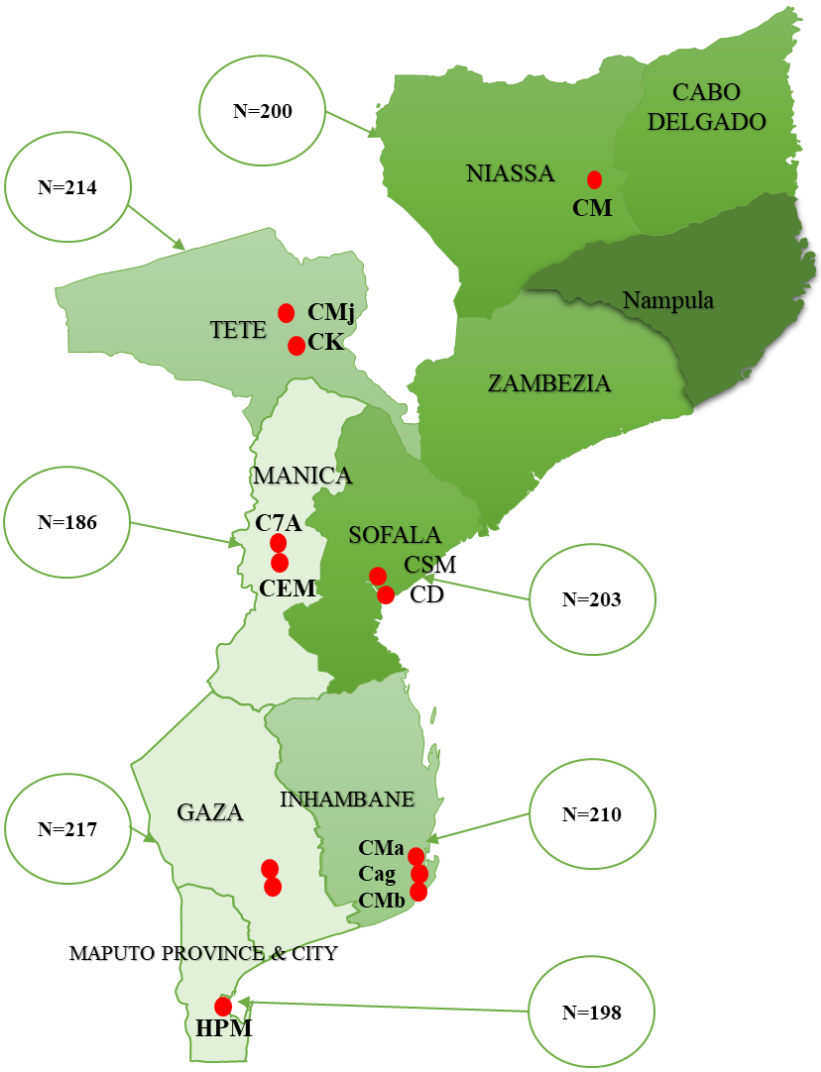


Figure 9: Characterisation of the study site and sample size. Number of red spots represents the number of health facillitie per site. **N**- Represented the sample size per province. **HPM**-Matola Provincial Hospital, **CCh**-Chibuto Health Centre, **CMi-**Milenium Health Centre, **CMa-**Maxixe Health Centre, **Cag-**Agostinho Neto Health Centre, **CMa-**Mabil Health Centre, **CSM-***Mafambisse* Health Centre, **CD-***Dondo* Health Centre), **CEM-***Eduardo Mondlane* Health Centre, **C7A-***7 de Abril* Health Centre, **CMj-***Manje* Health Centre, **CK-***Kaunda* Health Centre, **CM-***Marrupa* Health Centre. Figure was created using R software.

4.4.3.2. Sociodemographic characterisation of the study population

The age enrolled participants varied between 6 months and 86 years **(Table 3)**. Analysing province by province, Maputo, Gaza, Inhambane, Sofala, Manica, Tete and Niassa, included participants with an average age of 25.3 (CI: 23.3-27.3), 17.5 (CI: 15.6-19.5),

11.2 (CI: 9.89-12.5), 14.8 (CI: 13.2-16.4), 11.7 (CI: 10-13.3), 15 (CI: 12.9-17.1) and 7.85 (CI: 6.05-9.65) respectively.

O verall, 55% of these participants, (786/1428) were male and most of them were children accompanied by their legal representatives (**table 3**). Maputo, a low malaria transmission area, showed a low variability of ages (56.7%) when compared to the provinces of medium to high malaria transmission where the coefficient of variation ranged from 78-164% (**table 3)**.

Table 5: Epidemiological data of the study population. Std- standard, CI- confidence interval. * Pvalue=0.0435, 2-way ANOVA. M-male, F-female

Age		Maputo	Gaza	Inambane	Sofala	Tete	Manica	Niassa
Sample size (N)		198	217	210	203	214	186	200
Sex*	**M**	52% (103/198)	56.7% (116/210)	49% (103/217)	64% (130/203)	53.3% (114/214)	58.6% (109/186)	55% (110/200)
	F	48% (95/198)	43.3% (94/210)	51% (114/217)	36% (73/203)	46.7% (100/214)	41.4% (77/186)	45% (90/200)
Minimum (Age by year)		6	1	1	1	1	0.5	0.5
25% Percentile		15	6	5	7	3	2.8	1
Median		23	12	9	11	8	11	3
75% Percentile		31.3	25.5	13	19	15.3	22	7
Maximum (Age by year)		86	69	70	61	63	61	82
Range		80	68	69	60	62	60.5	81.5
Mean		25.3	17.5	11.2	14.8	11.7	15	7.9
Std. Deviation		14.3	14.7	9.6	11.5	12.1	14.4	12.9
Std. Error of Mean		1	1	0.7	0.8	0.8	1.1	0.9
Lower 95% CI of mean		23.3	15.6	9.9	13.2	10	12.9	6.1
Upper 95% CI of mean		27.3	19.5	12.5	16.4	13.3	17.1	9.7
Coefficient of variation		56.7%	83.9%	85.8%	78.0%	104%	96.4%	164%

4.4.3.3. Descrepances between *Plasmodium falciparum* positive samples by HRP2-based RDT and real time polymerase chain reaction (qPCR)

A total of 1214 samples belonging to 6 provinces namely, Maputo, Gaza, Inhambane, Sofala, Manica and Niassa were successfully DNA extracted. The samples from Tete, 214, have not been extracted yet, due to limited budget. A total of 1004 samples from all provinces except Tete and Inhambane underwent 18S qPCR, representing an 82.7% success rate.

67.43% (677/1004) of extracted samples and submitted to 18S qPCR were confirmed positive for *P. falciparum* infections. Below we describe the *P. falciparum* positivity rate by qPCR for each sampling site. 64.14% (127/198), 56.8% (105/185), 74.2% (138/186), 65.9% (143/217) and 85.50% (171/200) were confirmed positive for *P. falciparum* from isolates collected in Maputo, Gaza, Manica, Sofala and Niassa respectively (**table 4, figure 10)**.

Table 6: Discrepancies between routine RDT testing and molecular qPCR in samples from Maputo, Gaza, Sofala, Manica and Niassa: PCR- Polymarase chain reaction. PCR (+) - Positive samples for *P. falciparum*. PCR (-) - Negative samples for *P. falciparum*. PCR-/RDT+ -Sample positive by rapid diagnostic teste and negative by PCR. #- Samples pending confirmation by PCR.

Study site	Sample size	RDT+	PCR+	PCR -	PCR-/RDT+
Maputo	198	198	127	71	35.8% (71/198)
Gaza	196	196	116	80	40.8% (80/196)
Inhambane	210	210	#	#	#
Sofala	217	217	143	74	34.1% (74/217)
Manica	186	186	138	48	25.8% (48/186)
Niassa	200	200	171	29	14.5% (29/200)

Analysing the results, there were considerable differences between the samples that tested positive by TDR and by qPCR. These discrepancies tend to increase from north to south, where 14.5% (29/200), 25.8% (48/186), 34.1% (74/217), 40.8% (80/196) and 35.8% (71/198) were reported in Niassa, Manica (**table 4).**

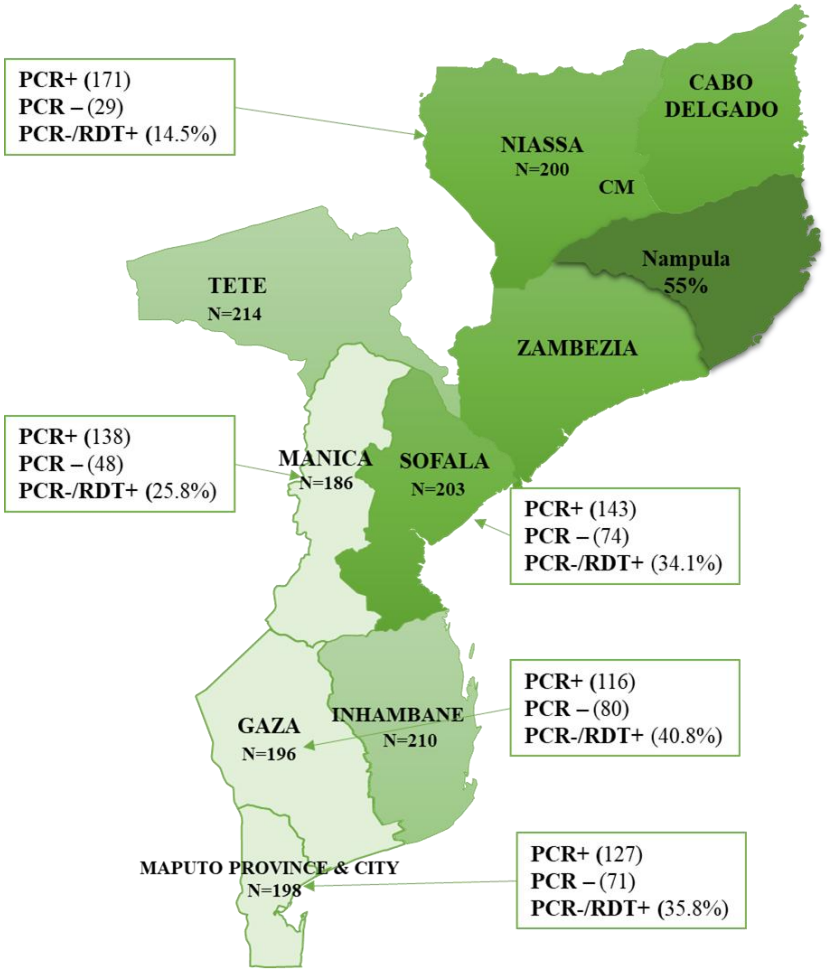


Figure 10: Discrepancies between routine RDT testing and molecular assay (PCR) in samples from Maputo, Gaza, Inhambane, Sofala, Manica Tete and Niassa: PCR-Polymarase chain reaction. PCR (+) - Positive samples for *P. falciparum*. PCR (-) - Negative samples for *P. falciparum*. PCR-/RDT+ -Sample positive for rapid diagnostic teste but negative for PCR.

4.4.4. Analyses of *P. falciparum* genetic diversity by targeted amplicon based Next Generation Sequencing (NGS)

We used targeted amplicon-based next-generation sequencing to assess the genetic diversity profile. We began with the antimalarial resistance modules, focusing on the *pfmdr1* and *pfk13* genes, to refine the technique in our laboratory. We will then include the genetic diversity module.

4.4.4.1. Preparation of fragments of interest for *pfmdr1* and *pfk13* genes

Targeted amplicon sequencing was used to investigate the presence of SNPs in the *pfmdr1* and *pfk13* genes in samples obtained Maputo as a starting point. A multiplex PCR containing the four fragments of the two genes studied, *pfmdr1* gene position N86/Y184, *pfmdr1* gene position D1246, *pfk13* A gene and *pfk13* B gene was performed. There were some unspecific PCR amplifications, together with the fragments of interest, observed around the 500 base pairs in electrophoresis agarose gel. Primer dimers were also observed. No amplification was detected in Negative controls (**Figure 11A**). The unspecific PCR did not interfere with the TADS analisis.

4.4.4.2. Profile of *pfmdr1* and *pfk13* SNPs data from *P. falciparum* parasites collected in Maputo, Mozambique

The library of eight samples from Maputo province was successfully prepared. The samples were then pooled in a single 1.5-mL eppendorf tube. The figure below shows the pfmdr1 and *pfk13* gene fragments amplified at around 500 bp. Subsequently, the samples were shipped to Eurofins Genomics for sequencing using MiSeq from Illumina.

Our group has successfully sequenced multiple fragments simultaneously per run for the first time. These eight samples did not contain any *pfk13*-validated mutations. However, two samples did show a synonymous mutation at position 571L. *pfmdr1* showed no SNP in the positions N86 and D1246. However, 62.5% of the samples displayed the well-known mutation at the codon Y184F. Among the eight samples, NFD (86, 184, 1246 codons) and NYD haplotypes were present in 62.5% and 37.5%, respectively. (**Figure 11B**).

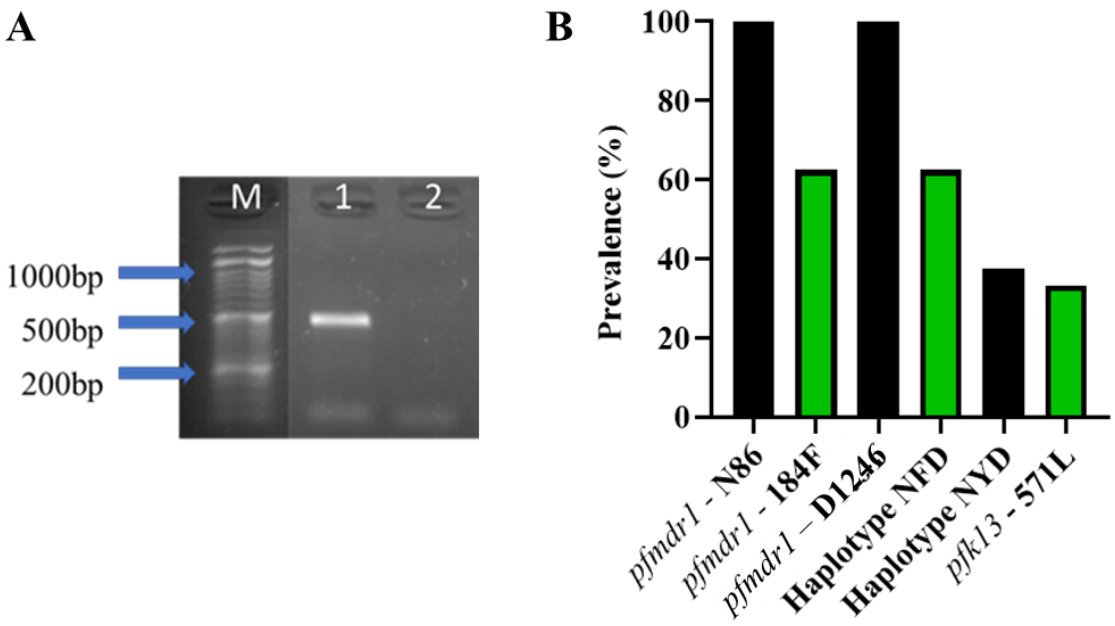


Figure 11: Profile of *pfmdr1* and *pfk13* SNPs from *P. falciparum* parasites collected in Maputo. **A:** Agarose gel electrophoresis**.** M – NZY DNA Ladder VI (MB089); 1 – Sample pool; 2 – Negative control. **B**: Prevalence of *pfmdr1* and *pfk13* SNPs by targeted amplicon based sequencing. Wildtype SNPs and haplotypes were represented in black and mutant in green. The graph was created using GraphPad Prism 8.01.

5. DISCUSSION AND CONCLUSIONS

The main objective of this project, which supported my PhD, was to study *P. falciparum* resistance to ACTs throughout the characterization of molecular markers and assessment of *ex vivo* susceptibility of *P. falciparum* to antimalarial in use in Mozambique. With the results of this study, we wanted to update the genotype profile of mutations in the *pfmdr1*, *pfk13* and *pfpm2/3* genes associated with the reduced susceptibility of *P. falciparum* to antimalarial drugs. In parallel, we introduced for the first time the phenotypic component of *ex vivo* susceptibility assays of *P. falciparum* to drugs in order to complement the molecular approach and the in vivo approach (therapeutic efficacy studies), which already existed. This thesis was splitted into 3 chapters that comprise three manuscripts. In addition, we included the fourth chapter in order to present other results that were not included in the manuscript. **The chapter 1** was dedicated to an in-depth examination of the prevailing circumstances surrounding antimalarial drug resistance in Mozambique and across the globe. This chapter served as a pivotal point of reference, providing a foundation upon which subsequent findings were built.

5.1. Chapter 2: Characterisation molecular markers of *P. falciparum* resistance to ACTs in Maputo, Sofala, Manica and Niassa provinces.

The activities conducted in the context of this chapter resulted in two published manuscriprt (Brown et al., 2024; da Silva et al., 2023). In the first manuscript, we estimated the prevalence of mutations in *pfK13* gene associated with *P.* falciparum resistance to ACTs. As for the second manuscript, we estimated the prevalence of mutations, copy number variation of *pfmdr1* and *pfpm2/3* associated with *P. falciparum* resistance to mefloquine and piperaquine respectively.

5.1.1. Paper II: Anti-malarial resistance in Mozambique: Absence of *Plasmodium falciparum* Kelch 13 (K13) propeller domain polymorphisms associated with resistance to artemisinins

Artemether-Lumenfantrine (Coartem®) is mostly dispersive antimalarial drugs used for the treatment of uncomplicated malaria in Mozambique, where in case of allergy or contraindication the combination Artesunate-Amodiaquine is adopted as the second line of

treatment (MISAU, 2017). The recommendation to include these drugs in Mozambique's national health system has brought tremendous benefits, given that previous therapeutic approaches were no longer effective due to cases of *P. falciparum* resistance to them that had reached higher prevalences by that time.

Recently, we have seen a growing record of parasite resistance to the ACTs mentioned above in Asia and sub-Saharan Africa (WHO, 2020). This phenomenon could jeopardise the efforts of the National Malaria Control Programme to manage malaria cases. Surveillance of *P. falciparum* validated and/or resistance-associated SNPs of the artemisinin is a powerful weapon to control the spread of parasite resistance through early detection of mutations (Ariey et al., 2014; Nsanzabana, 2021; Nsanzabana et al., 2018). Moreover, these mutations are being associated with the reduction in *pfkelch13* function, a protein required for parasite-mediated endocytosis of host haemoglobin in the newly invaded intra-erythrocytic ring stages (Ariey et al., 2014; Dondorp et al., 2009).

Recent study that performed a systematic review and spatiotemporal analysis reported that the prevalence of the most frequent validated mutation, C580Y, was around 70% in Asia (World Health Organization, 2018). Africa has been reporting several validated and candidate SNPs associated with *P. falciparum* partial resistance to artemisinin resistance (Bergmann et al., 2021; Bwire et al., 2020; Kirby et al., 2023; Ndwiga et al., 2021a; Straimer et al., 2022; Uwimana et al., 2020).

Ghana was the first African country to report validated mutation, associated with the majority of ART resistance in SE Asia, with the following contribuition in tems of prevalence C580Y (3.6%), P615L (4.8%), A578S (4.8%), I543V (2.4%) and A676S (1.2%), where the overall prevance is 17.7% (Aninagyei et al., 2020). In Ruanda 17.5% of 212 isolates showed validated mutations were 561H SNP contributed the most with 9% followed by 675V and 469F with a prevalence of 5.7% and 2.8% respectively (van Loon et al., 2024). In Tanzania, two studys presented two differents mutations, namely, 561H and 622**I**, showing 9% (Ishengoma et al., 2024) and 0.05% (Bakari et al., 2024) prevalence respectively. Finaly, in Uganda the mutations 469F, 561H, 441L and 675V reached a prevalence of 40%, 23% , 23% and 5% respectively (D. et al., 2023; Ndwiga et al., 2021b).

Mozambique, like some other African countries has not reported *pfk13* validated mutation (Chidimatembue et al., 2021; da Silva et al., 2023; K. et al., 2017; Serrano et al., 2021; Silva et al., 2023).

The absence of validated mutations in Mozambique shows that resistance has not yet emerged. This assumption is supported by therapeutic efficacy studies, which have not reported any therapeutic failure of the drugs in use in Mozambique (Nhama et al., 2014, 2021). A study in Tanzania, with samples from 2016, reported high efficacy, above 98%, of AL in treating uncomplicated malaria (Ishengoma et al., 2019). A slightly more recent study, also in Tanzania, revealed a decline in the efficacy of AL for treating uncomplicated *P. falciparum* malaria below the WHO 90% threshold in one of the study sites, Pwani. In the same district, no *pfk13*-validated mutation was observed. It was assumed that the partial resistance could be related to the failure of the partner drug, lumenfantrine (Laury et al., 2024). This shows that there is a growing trend towards a reduction in the effectiveness of the drug, which is proven by the recent emergence of two validated mutations in this neighbouring country of Mozambique (Ishengoma et al., 2024).

These results emphasise the need for constant genomic surveillance of circulating parasites in Mozambique, in order to detect the emergence of validated or candidate mutations as early as possible. Our study has reported that 27 participants harboured 21 not validated or candidate *pfk13* SNPs. 16 out of 21 SNPs were non-synonymous mutations, of which 4 have already been reported and 12 are novel SNPs. These mutations serve as a background for the candidates and then becomes validated. These types of mutations have been increasing in Mozambique as time goes by (Chidimatembue et al., 2021; da Silva et al., 2023; Silva et al., 2023).

5.1.2. Paper III. Antimalarial resistance risk in Mozambique detected by a novel quadruplex droplet digital PCR assay

To complement the list of mutations of extreme importance for the molecular surveillance of *P. falciparum*, we assessed the presence of molecular markers associated with resistance to Piperaquine and Mefloquine, CNVs of the *pfpm2/3* and *pfmdr1* genes respectively. Tracking and estimating the prevalence of these mutations is instrumental, since piperaraquine has recently been widely used in Mozambique for chemoprevention (Cirera et al., 2020; Cuinhane et al., 2023; Galatas et al., 2020).

In this study, we performed a quadruplex ddPCR, in steady of regular duplex ddPCR, in order to improve the limit of detection. This method was developed at SARS-CoV-2 pandemic period, where it was of great value for samples with low viral loads (Rong et al., 2024). Recently, it has been extremely important in the context of antimalarial resistance surveillance, as it is used to quantify parasite density, measure copy number variations (CNVs) of *pfmdr1* and *pfpm2/3* genes (Srisutham et al., 2021).

From our findings we have managed to determine the optimal ddPCR conditions, from which all the steps of the assay undergone successfully. The results confirmed that the developed assay is accurate, sensitive and specific. According to (Hindson et al., 2011; Srisutham et al., 2021; Z. Wang et al., 2019), the abovementioned ddPCR characteristics are granted by its ability to generate multiple data points in a single result, the fragmentation of genomic DNA into thousands of individual compartments (droplets) that do not compete with each other.

As for the CNVs, of the *pfmdr1* and *pfpm2/3* genes were detected in 5.7% (13/229) of the samples that passed the quality filter. These percentages were achieved thanks to the quality of the technique we used, which enabled us to create a gradient (>1-1.2; >1.2-1.5 and >1.5) over a wider range. Previous studies in Mozambique showed different trends to our study in terms of the prevalence of these CNVs, with the first study reporting a 1.1% prevalence of multiple *pfpm2 and pfmdr1* copies (Gupta et al., 2018) and the second study, which is multicentre, showing 12.5% of CNVs (Leroy et al., 2019). The second one showed higher percentage due to the lower sample size, which was 8 (12.5%=1/8). Neither study detected multiple copies of *pfpm3*, making our study the first to do so in Mozambique.

Much higher CNVs prevalence than those estimated for Mozambique have been reported in Asia and other African countries where CambodiaVietnam and other African countries reported 23% (Lim et al., 2009), 54.3% (Phuc et al., 2017) and (average of 21.1 % for *pfmdr1* and >30% for *pfpmp2*) (Leroy et al., 2019).

These low prevalences in Mozambique are related to the infrequent use of piperaquine and mefloquine in health system. However, it is important that surveillance for piperaquine resistance markers (*pfpm2/3*) continue, as this drug has been used in mass drug administration campaigns.

5.2. Chapter 3: To determine ex vivo susceptibility of *P. falciparum* to antimalarial drugs in use in Maputo, Mozambique.

In the second chapter, we looked at the first two published manuscripts, where we focused solely on the genotypic or molecular component of the resistance of *P. falciparum* to the antimalarial drugs used in Mozambique. This third chapter, focused on adding the phenotypic component. This is the first time that phenotypic testings, i.e. the determination of *ex vivo* susceptibility of *P. falciparum* to antimalarials, have been conducted in Mozambique. The objectives of this chapter were to assess the *ex vivo* susceptibility of *P. falciparum* isolates from Maputo to dihydroartemisinin (DHA), piperaquine (PPQ), and amodiaquine (AQ).

In Mozambique, the only studies that have included this component are the Therapeutic Efficacy Studies (TES) that have been conducted since 2001. The first was carried out in Maputo province, Manhiça district, between 2001-2002, in which children under the age of 5 were treated with CQ, SP, AS, AQ and the SP-AQ, SP-AS combinations. In the first days of follow-up, efficacy was 91.6 %, 82.7 % and 47.1 % for AQ, SP and CQ, respectively. The combinations of SP-AQ, SP-AS and AQ-AS showed 100% efficacy (Abacassamo et al., 2004). Two multicentric studies including Mozambique, also in children over 6 months and under 5 years old, reported 96.9% (Bassat et al., 2009) and 97.8% (Abdulla et al., 2008) efficacy of AL in 2006 and 2007 respectively.

In 2011, another study was carried out in 5 sentinel sites in Mozambique, namely in the districts of Montepuez, Dondo, Tete, Chokwe, and Manhiça, to assess the efficacy of AL

and AQ-AS in children aged between 6 months and 5 years, where the efficacy of AL and AQ-AS was 96% and 99.6%, respectively (Nhama et al., 2014). Similar study was carried out in 2018 where the efficacy of AL was 97.9% and that of AQ-AS was 99.6% (Nhama et al., 2021).

To complement these studies, the data from our article in preparation, revealed parasite survival rates below the thresholds established by the WHO, which showed that the parasites circulating in Maputo are still sensitive to DHA, PPQ and AQ. Our results reinforced the findings aforementioned by Nhama et al. Furthermore, these results were confirmed by genomic datas, where no *pfk13* validated mutation, and nore multiples copies of *pfpm2* and *pfmdr1* were oserved. As for *pfmdr1* SNPs, almost 50% of the samples showed 184**F** and N**F**D haplotypes. Similar outputs were observed by (Chidimatembue et al., 2021), when Genotyping samples collected in the context o TES of 2018 (Nhama et al., 2021).

The results of our AQSA are consistent with those of (Fukuda et al., 2021) in Uganda, which recommended the use of AQ in the event of lumefantrine failure. *Ex vivo* RSA revealed similar results to ours, since they reported parasites susceptibility to artemisinin in Senegal, with parasites survival rates below 1% (Yade et al., 2023). The results of the RSA tests were slightly different in Ghana, where survival rates above 1% were found in 4 of the 29 clinical parasite isolates. The same study showed the same results as we reported for the PSA component (survival rate <10%) and no validated *pfk13* mutations and *pfmdr1* 86**Y** (Zhao et al., 2022). Also in Ghana, a survival rate of more than 1% was recently observed in 7 of 29 isolates (Ahorhorlu et al., 2023). However, at that time the percentage was over 10%, 8 units higher than the 2.2% observed by (Zhao et al., 2022).

The results from this two chapters, reported that *P. falciparum* isolates from studied settings (Maputo, Manica and Niassa) are still showing high sensitivity to antimalarial, with no mutations associated to artemisinin and its partner drug resistance. Continued monitoring of parasite susceptibility to ACTs and molecular surveillance should be intensified to reduce chances of artemisinin resistance in the near future.

5.3. Chapter 4: Other results in the context of this PhD thesis

5.3.1. Descrepances between *P. falciparum* positive samples by HRP2-based RDT and real time polymerase chain reaction (qPCR)

When genotyping malaria parasite DNA samples, one of the critical steps is to use an additional method to confirm *P. falciparum* infection. In this study, real-time PCR (qPCR) was used to amplify the 18S rRNA gene. The *18S* rRNA gene is a conserved coding gene that is expressed by *P. falciparum* (Gunderson et al., 1987; Schneider et al., 2005), therefore, we targeted this gene to detect this parasite.

Table 4 and Figure 10 showed that there are significant differences between the samples that tested positive by HRP2 RDTs (Bioline™ Malaria Ag P.f (HRP2)) and those that tested positive by qPCR. These differences tend to decrease from south to north, ranging from 35.8% to 40.8% in the south, 25.8%-34.1% in the centre and 14.5% in the north. This decrease is in contrast to the prevalence of malaria in the regions, which, according to the latest survey, increases from south to north (INE, 2023).

In general, we reported 30.2% (302/997) of discrepancies. In order to clarify whether these results could be due to operator error, we had technical replicates.This leaves the second possibility, which is that some samples had a very low concentration of DNA and this was further reduced during DNA extraction. High levels of rheumatoid factor (RF), an autoantibody that can bind to the trapping antibody on the RDT strip and buffer sustitution may also be one of the high level of discrepancies. Finally, these false-positive results may be due to the detection of HRP2 antigens still present in the bloodstream despite recent effective parasite clearance (Das et al., 2017). As these discrepancies may represent the number of patients attending the health facility who were treated with AL but did not have malaria, they are extremely important.

5.3.2. Analyses of *P. falciparum* genetic diversity by targeted amplicon based Next Generation Sequencing (NGS)

For the first time in our laboratory, we carried out genotyping of *P. falciparum* using amplicon-based targeted sequencing approach. This technique and the adaptations made in

our laboratory, such as the design of primers with barcodes, adaptors and tails incorporated, allowed us to cover all the fragments of interest in the *pfmdr1* and *pfk13* genes in a single run, something we could not do with the usual sanger sequencing. According to (Bybee et al., 2011) targeted amplicon based sequencing is usually a two-step PCR process that first amplify a targeted gene region (amplicon) using traditional PCR, followed by an additional PCR that attaches a known tag, barcode to identify amplicons from different samples. In our adaptations, the library preparation process was relatively quick, one-step PCR reaction. The following processes (library pooling and sequencing) are similar to stated by (Bybee et al., 2011; Hung et al., 2018).

Of the 8 samples genotyped for this study, we obtained the same prevalence rates as those reported by us in chapters 5.1 and 5.2. We did not observe any validated mutations for the *pfk13* gene. Likewise, we had over 50% prevalence of the 184F mutation and the NFD haplotype in *pfmdr1* gene.

5.4. LIMITATIONS

This thesis described the prevalence of mutations associated with resistance to antimalarial drugs in use in Mozambique and, for the first time, the ex vivo parasite's susceptibility to antimalarial drugs. However, there were some limitations to the study:

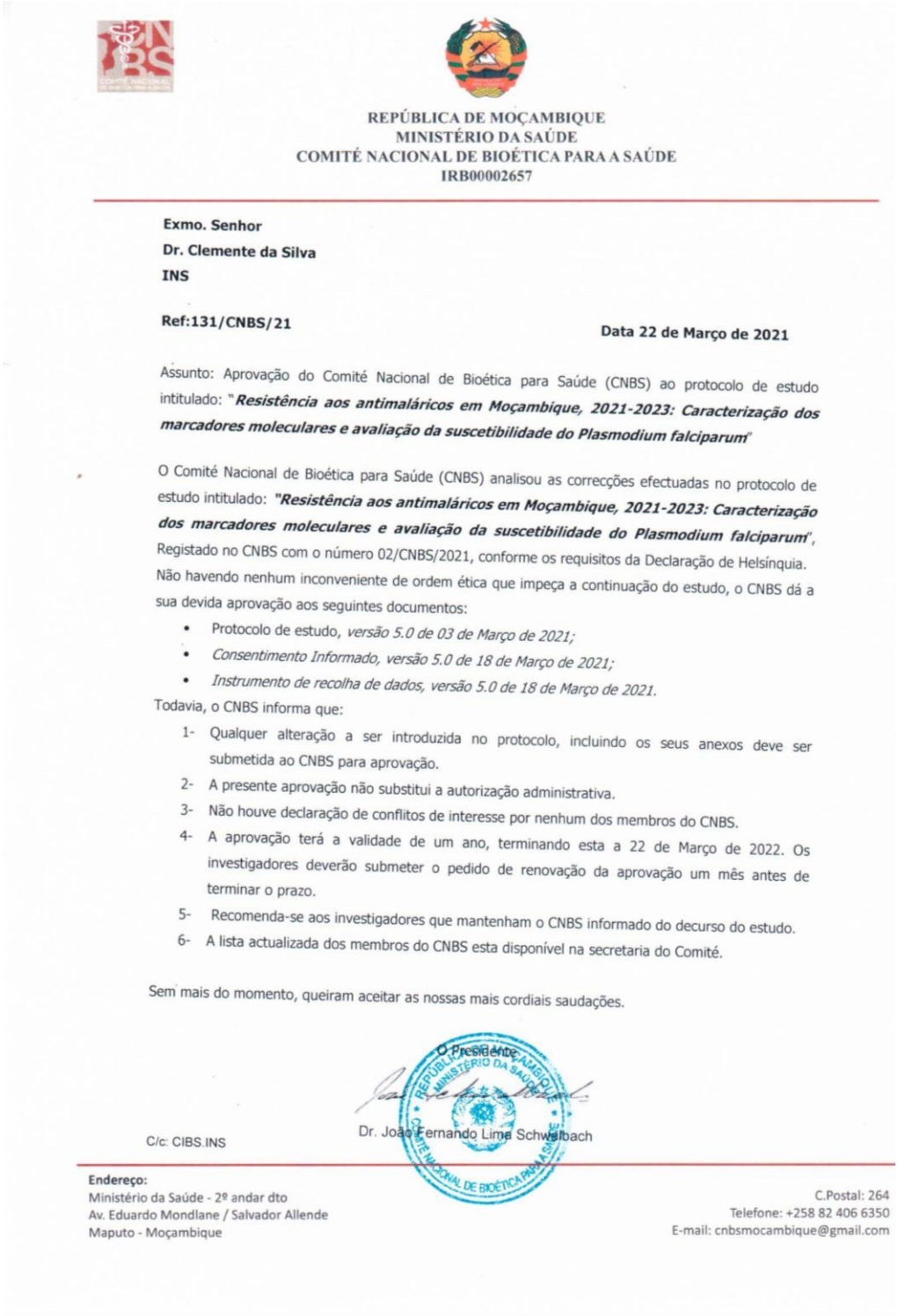
- This study followed convenience sampling, i.e. the health facilities were not selected randomly.
- The emergence of the coronavirus pandemic, which restricted the study team's access to health which limited the number of patients included in the study

5.5. FUTURE WORK

- Genotyping of 196, 210 and 214 samples collected in Gaza, Inhambane and Tete respectively.

6. THESIS SUPPLEMENTARY INFORMATION

6.1. Ethical clearance of the study by the National Bioethics Committee for Health in Mozambique



REPÚBLICA DE MOÇAMBIQUE
MINISTÉRIO DA SAÚDE
COMITÉ NACIONAL DE BIOÉTICA PARA A SAÚDE
IRB00002657

Exmo. Senhor
Dr. Clemente da Silva
INS

Ref:131/CNBS/21 **Data 22 de Março de 2021**

Assunto: Aprovação do Comité Nacional de Bioética para Saúde (CNBS) ao protocolo de estudo intitulado: "***Resistência aos antimaláricos em Moçambique, 2021-2023: Caracterização dos marcadores moleculares e avaliação da suscetibilidade do Plasmodium falciparum***"

O Comité Nacional de Bioética para Saúde (CNBS) analisou as correcções efectuadas no protocolo de estudo intitulado: "***Resistência aos antimaláricos em Moçambique, 2021-2023: Caracterização dos marcadores moleculares e avaliação da suscetibilidade do Plasmodium falciparum***", Registado no CNBS com o número 02/CNBS/2021, conforme os requisitos da Declaração de Helsínquia. Não havendo nenhum inconveniente de ordem ética que impeça a continuação do estudo, o CNBS dá a sua devida aprovação aos seguintes documentos:

- Protocolo de estudo, *versão 5.0 de 03 de Março de 2021;*
- *Consentimento Informado, versão 5.0 de 18 de Março de 2021;*
- *Instrumento de recolha de dados, versão 5.0 de 18 de Março de 2021.*

Todavia, o CNBS informa que:

1- Qualquer alteração a ser introduzida no protocolo, incluindo os seus anexos deve ser submetida ao CNBS para aprovação.
2- A presente aprovação não substitui a autorização administrativa.
3- Não houve declaração de conflitos de interesse por nenhum dos membros do CNBS.
4- A aprovação terá a validade de um ano, terminando esta a 22 de Março de 2022. Os investigadores deverão submeter o pedido de renovação da aprovação um mês antes de terminar o prazo.
5- Recomenda-se aos investigadores que mantenham o CNBS informado do decurso do estudo.
6- A lista actualizada dos membros do CNBS esta disponível na secretaria do Comité.

Sem mais do momento, queiram aceitar as nossas mais cordiais saudações.

O Presidente

Dr. João Fernando Lima Schwalbach

C/c: CIBS.INS

Endereço:
Ministério da Saúde - 2º andar dto
Av. Eduardo Mondlane / Salvador Allende
Maputo - Moçambique

C.Postal: 264
Telefone: +258 82 406 6350
E-mail: cnbsmocambique@gmail.com

6.2. Authorisation from the Mozambican Ministry of Health to carry out the study

República de Moçambique
MINISTÉRIO DA SAÚDE
Gabinete do Ministro

Exmo. Senhor
Dr. Ilesh V. Janni MD PhD
Director Geral do Instituto Nacional de Saúde
MAPUTO

Nota nº 437/GMS/290/**020**

Assunto: Pedido de Autorização de Realização do Estudo

Incumbe-me Sua Excelência o Ministro da Saúde Dr. Armindo Daniel Tiago, de acusar a recepção da vossa nota com a referência nº536, na qual solicita autorização de um estudo intitulado:" **Resistência aos antomaáricos em Moçambique, 2021-2023: Caracterização dos mercados moleculares e avaliação da suscetibilidade do Plasmodium falciparum "**

Neste âmbito, vimos por meio desta informar o despacho de Sua Excelência Ministro da Saúde cujo teor é o seguinte:

"Autorizo"

Assinado: Dr. Armindo Daniel Tiago
(12/04/2021)

Sem mais do momento, subscrevo-me com elevada estima e consideração

Maputo, 13 de Abril de 2021

A Chefe do Gabinete

Fátima Soulo

MINISTÉRIO DA SAÚDE
INSTITUTO NACIONAL DE SAÚDE
ENTRADA 295
DATA 14 / 04 / 2021
ASS

Ministério da Saúde
Av. Eduardo Mondlane Nº 1008 – C. Postal 264
Tel.: 258 (1) 21314488/ Fax: 258 (1) 306621
Email: gm.misau@gov.mz

6.3. Registration of the protocol with the IHMT Ethical Board

https://www.ihmt.unl.pt/organizacao/conselho-de-etica/ > Projetos

7. DISCUSSION REFERENCES

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