UNIVERSITY OF RWANDA

GENETIC ARCHITECTURE OF *PLASMODIUM FALCIPARUM* AND IMPACTS ON PROPHYLAXIES IN RWANDA AND CAMEROON

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GENETIC ARCHITECTURE OF *PLASMODIUM FALCIPARUM* AND IMPACTS ON PROPHYLAXIES IN RWANDA AND CAMEROON

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- Prof. Antoine NSABIMANA

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DECLARATION

I, **Emelyne UWASE** hereby declare that this research project submitted to the University of Rwanda, for the degree Master of Science in Biotechnology is my own original work and has not been submitted before to any Institution by myself or any other person in fulfilment of the requirements to the award of any degree or any other qualification.

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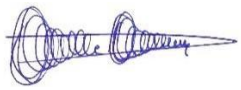

Date: 31st July,2025

DEDICATION

I dedicate this Thesis to Almighty God, as well as all of those who have assisted, supported, and inspired us, including King Faisal Hospital Rwanda research team, our friends and parents, who have never stopped supporting us in our educational endeavors. This thesis is also dedicated to my supervisors, Prof. Jacob SOUOPGUI, Dr. Edgar MUTEBWA KALIMBA, Prof. Antoine NSABIMANA.

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Lastly, I remain deeply indebted to my colleagues, friends, and family for their encouragement, patience, and unwavering support during this journey. Their presence and belief in me have been instrumental in achieving this milestone, both academically and personally.

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LIST OF ABBREVIATIONS

ACTs	Artemisinin-based Combination Therapies
CDC	Centers for Disease Control and Prevention
CNERSH	Comité National d'Éthique de la Recherche en Santé
CSP	Circumsporozoite Protein
CYP450	Cytochrome P450 (enzyme family)
CYT B	Cytochrome B (gene)
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
G6PD	Glucose-6-Phosphate Dehydrogenase
gVCF	Genomic Variant Call Format
IGV	Integrative Genomics Viewer
IPTi	Intermittent Preventive Treatment in Infants
IPTp	Intermittent Preventive Treatment in Pregnancy
IRS	Indoor Residual Spraying
ITNs	Insecticide-Treated Nets
KFH	King Faisal Hospital
MDR1 / pfmdr1	Plasmodium falciparum Multidrug Resistance Gene 1
MINISANTE	Ministry of Health (Rwanda)
NC	Negative Control
NGS	Next-Generation Sequencing (implied through ONT context)
ONT	Oxford Nanopore Technology

PCR	Polymerase Chain Reaction
RNEC	Rwanda National Ethics Committee
SMC	Seasonal Malaria Chemoprevention
SNP	Single Nucleotide Polymorphism
SO / SOs	Specific Objective(s)
SP	Sulfadoxine-Pyrimethamine
VCF	Variant Call Format
WHO	World Health Organization
WP / WPs	Work Package(s)

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ABSTRACT

Malaria remains a major public health challenge in sub-Saharan Africa. Increasing resistance to antimalarial drugs such as Malarone (atovaquone–proguanil) and Lariam (mefloquine) threatens the effectiveness of chemoprophylaxis, particularly in mobile populations.

This study aimed to assess molecular markers associated with resistance to proguanil, atovaquone, and mefloquine by analyzing mutations in the *dhfr*, *cytb*, and *pfmdr1* genes of *Plasmodium falciparum* isolates from Rwanda and Cameroon.

A total of **339 samples** were included in this study. Among them, **170 isolates from Rwanda** were analyzed for mutations in the *dhfr*, *cytb*, and *pfmdr1* genes, while **169 isolates from Cameroon** were analyzed exclusively for *cytb* mutations. Targeted PCR amplification and Sanger sequencing combined to Oxford Nanopore Technology were used to detect key point mutations: N51I, C59R, S108N, and I164L in *dhfr*; Y268S in *cytb*; and Y184F in *pfmdr1*.

In Rwanda, *dhfr* mutations were highly prevalent: N51I (97.1%), C59R (94.7%), S108N (98.2%), and I164L (30.6%). Cameroonian samples showed a similar triple mutant profile based on previous data, though I164L was not detected. No Y268S mutation was found in *cytb* from either country, indicating continued atovaquone efficacy. The Y184F mutation in *pfmdr1* was observed in 48.8% of Rwandan isolates, while recent studies indicate a prevalence of approximately 60% in Cameroon.

These findings suggest that proguanil resistance is near fixation, while atovaquone remains effective but at risk. The growing presence of *pfmdr1* mutations raises concerns about declining mefloquine efficacy. The study highlights the urgent need for region-specific prophylaxis policies and continuous molecular surveillance.

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CHAPTER 1. INTRODUCTION

Malaria remains a major public health concern, particularly in tropical and subtropical regions. According to the World Health Organization (WHO), there were an estimated **241 million** malaria cases in 2020, an increase of 14 million from 2019, with around 627,000 deaths, a 12% rise and **249 million malaria cases and 608,000 deaths** globally in 2022, In 2023, the WHO African Region was home to 94% of malaria cases (246 million) and 95% (569 000) of malaria deaths. Children under 5 accounted for about 76% of all malaria deaths in the Region. (WHO, 2023)

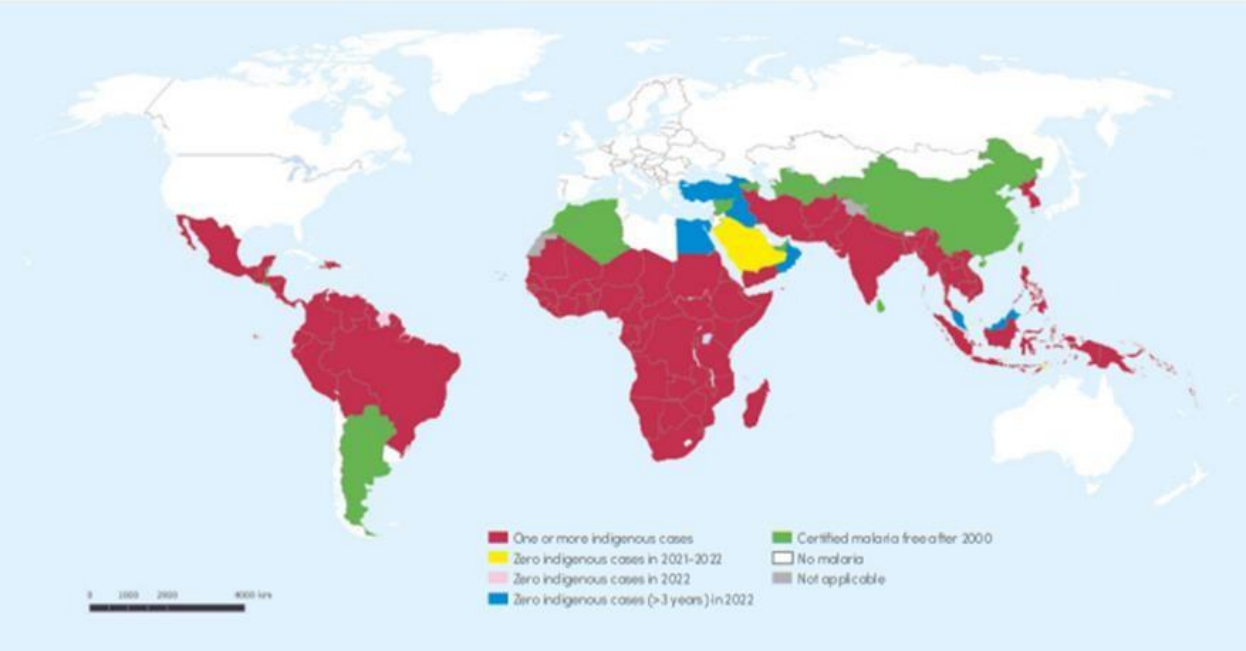

Figure 1: ***Countries and areas with indigenous cases in 2000 and their status by 2023***

The burden is heaviest among children under five and pregnant women. Despite global control efforts, progress has plateaued in many endemic regions due to insecticide and drug resistance, gaps in healthcare access, and climate-related shifts in transmission patterns. (Autino et al., 2012)

Rwanda is a sub-Saharan African nation whose entire population is at risk of contracting malaria. In 2012, Rwanda was classified as a malaria-endemic country by the World Health Organization (WHO)

In Rwanda Malaria transmission remains seasonal, peaking during *May–June* and *November–December*, with over **518,000 confirmed cases** reported from January to September 2024, a notable increase compared to the same period in 2023. (Guillaume, 2024)

Despite a historic 88% reduction in malaria incidence since 2016, recent trends suggest resurging cases due to expanded healthcare access, insecticide resistance, and climate variability (Umugwaneza et al., 2025)

In **Cameroon**, malaria remains hyperendemic, with over **2 million cases annually**, including more than **2.9 million confirmed cases and 1,756 deaths** in 2023. (Antonio-Nkondjio et al., 2019) Cameroon also became one of the first African nations to introduce the RTS,S/AS01 and R21M vaccine in 2024, covering thousands of children despite logistical challenges (More, 2024).

Human malaria is primarily caused by five species of *Plasmodium*: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlesi* but *P. falciparum* is the most virulent and predominant species in Africa, responsible for nearly all severe malaria cases and deaths. Transmission occurs through the bite of an infected **female *Anopheles* mosquito**, which introduces **sporozoites** into the human bloodstream. These travel to the liver, where they undergo asexual replication during the **exoerythrocytic stage**. Merozoites are then released into the bloodstream, infecting red blood cells and initiating the **erythrocytic cycle**, which causes the clinical symptoms of malaria include; Fever, chills, sweating, headache, exhaustion, nausea, vomiting, and stomach discomfort. In serious situations, anemia, organ failure, and cerebral malaria can happen. This can induce confusion, convulsions, and even coma. These symptoms usually appear 10 days to 4 weeks after being bitten by an infected mosquito, depending on the species of Plasmodium. Some parasites differentiate into **gametocytes**, which are taken up by mosquitoes during a blood meal, completing the transmission cycle. (Matteelli & Castelli, 2018)

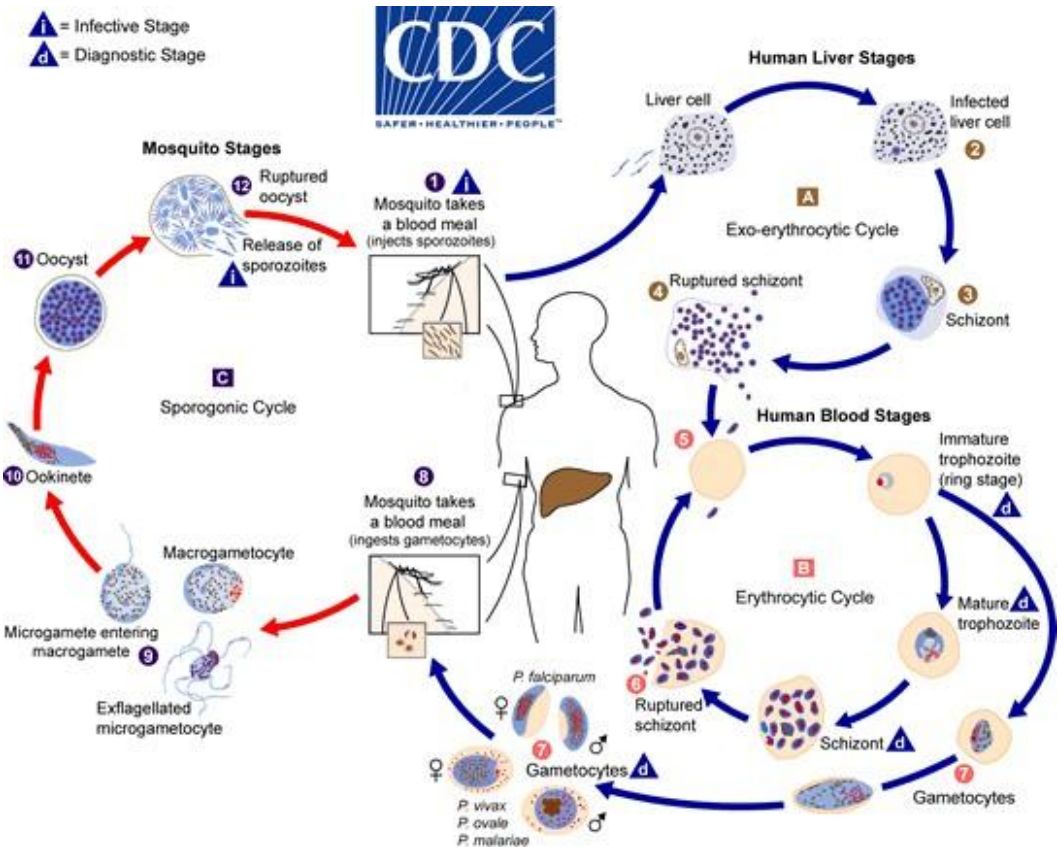

Figure 2: Life cycle of Plasmodium species showing human liver and blood stages, and mosquito stages. (CDC, 2015)

To manage malaria, you need to have a strategy that includes both treating people who already have it and stopping new cases from happening. Artemisinin-based combination therapies (ACTs) are usually used to treat uncomplicated Plasmodium falciparum malaria. ACTs are still the first-line treatment in most endemic countries, such as Rwanda and Cameroon. Intravenous artesunate is used to treat severe malaria, and then oral ACTs are given. However, rising concerns about artemisinin partial resistance in some areas has led to enhanced surveillance and efforts to keep the drug being effective.

Beyond epidemiology, the molecular mechanisms of parasite genetic variation play a central role in shaping malaria control outcomes. *P. falciparum* evolves rapidly through antigenic variation, whereby multigene families such as *var*, *rifin*, and *stevor* generate extensive surface protein diversity to evade host immunity (Kyes et al., 2007) . Homologous recombination and meiotic crossing-over during the sexual stage in mosquitoes further enhance genetic diversity In addition, copy number variation (CNV**)** in genes such as *pfmdr1* is a key mechanism underlying multidrug

resistance (Argyropoulos et al., 2023). These processes create a highly dynamic parasite population structure, complicating both treatment and prophylaxis.

In terms of prevention, malaria control programs are built upon three pillars: vector control, immunoprevention, and chemoprevention. Vector control is still the most important way for preventing the spreading of disease. This is done with insecticide-treated nets (ITNs) and indoor residual spraying (IRS). The **RTS, S/AS01** malaria vaccine, which offers moderate protection against clinical malaria in children, has recently been added to immunoprevention. It has been available in Cameroon since March,2024. (Amani et al., 2024) and Rwanda is planning to test it out. Additionally, the **R21/Matrix-M** vaccine, which has shown higher efficacy in early trials, is another promising addition to malaria immunization.(Datoo et al., 2024) Both vaccines aim to reduce the burden of malaria, but it is essential to address genetic polymorphisms in the **circumsporozoite protein (CSP)** gene, as variations in this gene may impact the vaccines' efficacy and their ability to confer long-term protection. These genetic differences could influence vaccine response and warrant further investigation to optimize malaria immunization strategies in different populations.

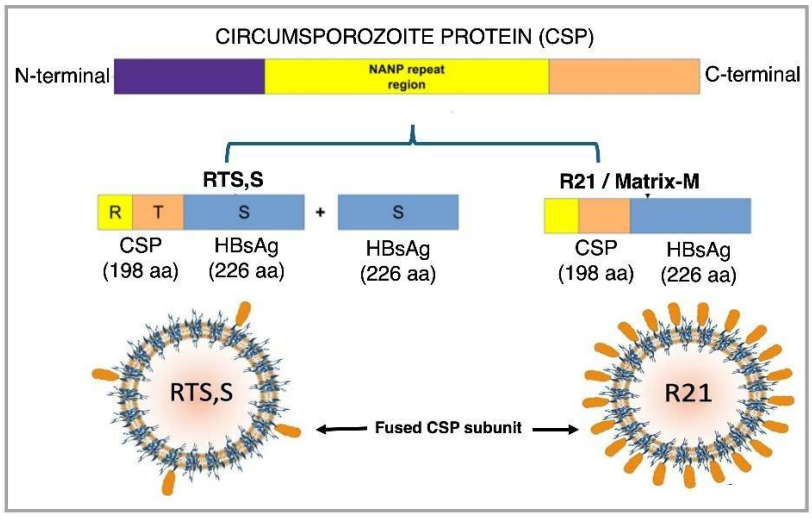

Figure 3: This image illustrates two malaria vaccine candidates targeting the Plasmodium falciparum circumsporozoite protein (CSP): RTS,S and R21/Matrix-M. Modified from (Lyimo et al., 2024)

Chemoprevention, it is vitally crucial to keep high-risk groups safe by utilizing antimalarial medications to keep them from getting sick, especially for vulnerable populations such as pregnant women, children under five. Some of the strategies it contains are intermittent preventive therapy in pregnancy (IPTp), intermittent preventive treatment in infants (IPT_i), seasonal malaria chemoprevention (SMC), and traveler's prophylaxis.

The process of giving pregnant women sulfadoxine-pyrimethamine (Fansidar; It block parasite's ability to replicate and survive in human bloodstream by inhibiting the parasite's folate synthesis and dihydrofolate reductase) is **IPTp**, during the second and third trimesters to stop them from getting malaria. Rwanda stopped using IPTp-SP in 2008 because SP(Fansidar) was becoming less effective; In some areas, more than 90% of P. falciparum isolates have mutations in the *DHFR* and *DHPS* genes that make them resistant. These mutations make the drug much less effective, which is why it was taken out of the national malaria control guidelines (Alruwaili et al., 2023).

In Cameroon, Fansidar also known as sulfadoxine-pyrimethamine (SP) is still used as an intermittent preventive treatment during pregnancy (IPTp). The Cameroonian Ministry of Health continues to use it in spite of resistance worries, advising expectant mothers to get at least three doses in the second and third trimesters in order to avoid complications from malaria.(Guimsop et al., 2024). Similarly, seasonal malaria chemoprevention (SMC) is also used for children between the ages of 3 and 59 months during the time of year when malaria spreads the most. From June to October 2023, SMC took place in 16 districts, encompassing about 96% of the area(Nikiema et al., 2022). **Rwanda** has also implemented SMC in certain regions but has not yet expanded it nationwide.

For travelers to malaria-endemic areas, chemoprophylaxis with antimalarial drugs is essential to prevent infection. Commonly prescribed drugs include Malarone (atovaquone-proguanil), Lariam (mefloquine), and doxycycline. However, the effectiveness of these drugs is increasingly compromised by the emergence of drug-resistant *Plasmodium falciparum* strains, which remain a major challenge to malaria control. Genetic mutations in critical parasite genes, such as *dhfr* and *pfmdr1*, have been linked to resistance, allowing the parasite to survive in the presence of antimalarial drugs.

In Rwanda and Cameroon, where malaria remains endemic, understanding these genetic resistance markers is crucial to maintaining the efficacy of chemoprophylactic and therapeutic interventions. Monitoring the prevalence of these mutations is essential for adapting treatment strategies and ensuring that the progress made in malaria prevention continues.

CHAPTER 2. STATE OF ARTS

Malaria caused by Plasmodium falciparum is a major concern in endemic regions, where it remains a leading cause of morbidity and mortality (Daily & Parikh, 2025). Chemoprophylaxis plays a critical role in the prevention and control of malaria, especially in high-risk populations, through the use of antimalarial drugs such as Malarone (atovaquone-proguanil), Lariam (mefloquine), Doxycycline and Fansidar (sulfadoxine-pyrimethamine).

However, the development of drug resistance by *P. falciparum* threatens the effectiveness of these treatments, underscoring the need for better understanding of the molecular mechanisms of resistance. Moreover, genetic variability in both the parasite and the host population further complicates the effectiveness of these drugs, necessitating region-specific surveillance and more targeted interventions.

2.1. MECHANISM OF ACTION AND DRUG RESISTANCE IN MALARIA PROPHYLAXIS

Antimalarial drugs target various stages of the P. falciparum life cycle, aiming to inhibit the parasite's ability to survive and reproduce. However, as these drugs are used over time, the parasite can develop resistance through genetic mutations in key genes. Below are the primary mutations associated with resistance to some widely used antimalarial drugs:

2.1.1. Dihydrofolate Reductase (DHFR) Gene and Resistance to *Proguanil* and *Sulfadoxine-Pyrimethamine*

DHFR is an essential enzyme in *Plasmodium falciparum* involved in the folate biosynthesis pathway, which is crucial for DNA replication and cell division. It reduces dihydrofolate **(DHF)** to tetrahydrofolate **(THF)**, which is required for purine and thymidylate synthesis. Antimalarial drugs like *Proguanil* inhibit DHFR, blocking folate synthesis and preventing parasite growth.

However, *P. falciparum* can develop resistance through mutations in the *DHFR* gene, such as **N51I**, **C59R**, and **S108N**, which decrease the drug's ability to bind to the enzyme. These mutations allow the parasite to continue synthesizing folate despite drug presence, leading to treatment failure. Resistance is particularly concerning for drugs like **proguanil** and **sulfadoxine-pyrimethamine**.

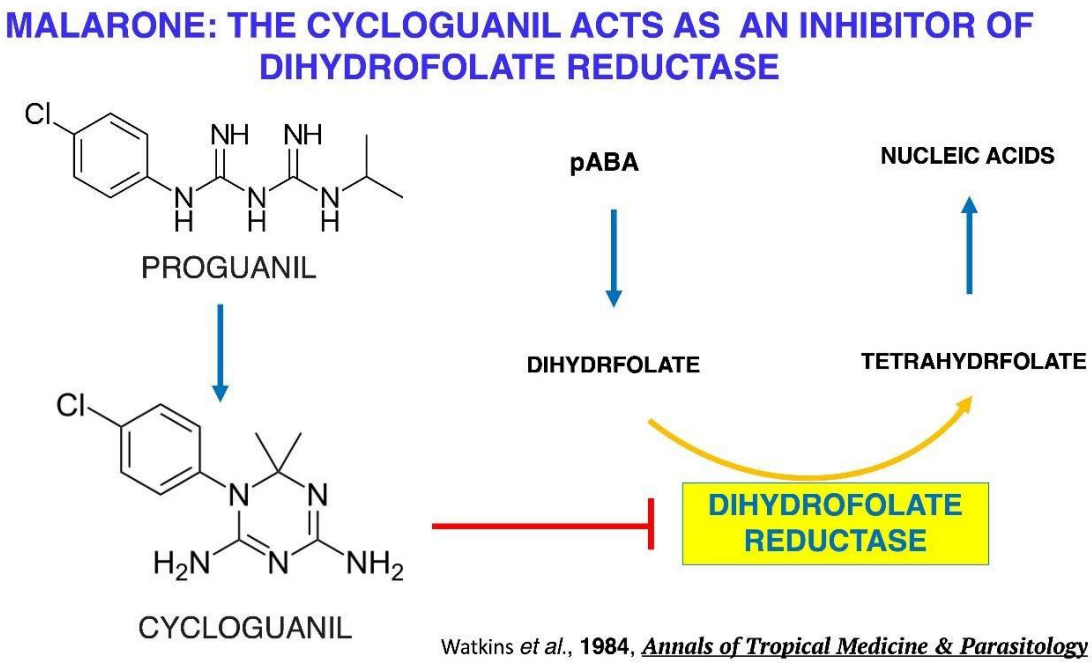

Figure 4: Mechanism action of DHFR (Brooks et al., 1994)

2.1.2. Cytochrome B (cytb) Gene and Resistance to *Atovaquone*

Cytochrome B (cytb) is a mitochondrial protein involved in the electron transport chain of *Plasmodium falciparum*, essential for ATP production. Atovaquone, an antimalarial drug, inhibits cytb by blocking electron transfer, disrupting mitochondrial function and depleting the parasite's energy, leading to its death.

Resistance to atovaquone arises through mutations in the *cytb* gene, particularly at **Y268S/C/N**, which alter the drug's binding site, reducing its effectiveness. These mutations allow the parasite to maintain mitochondrial function and survive despite the presence of the drug.

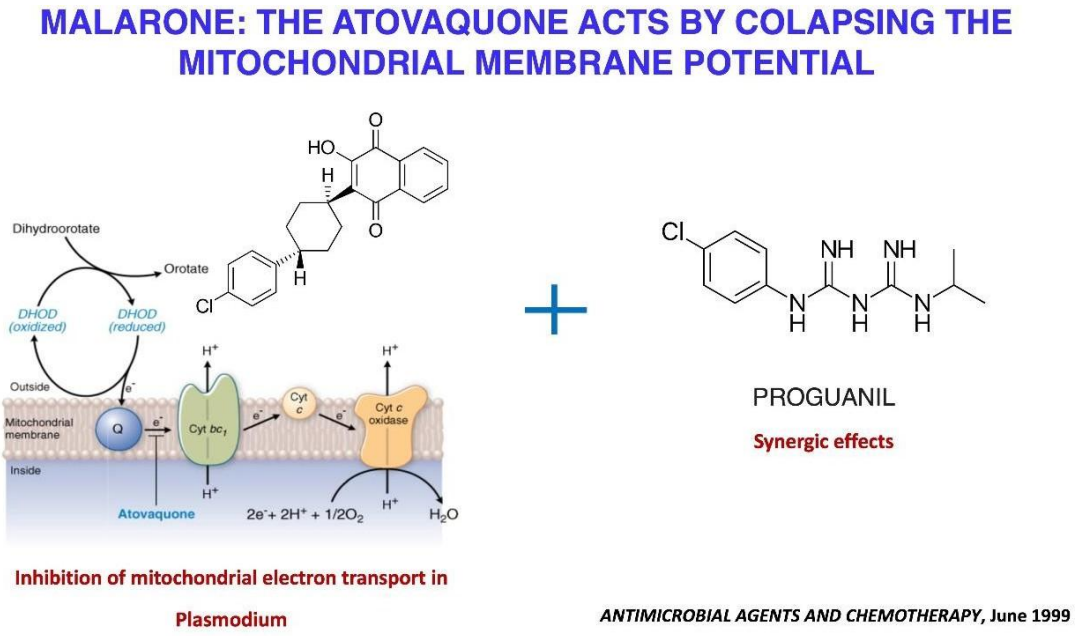

Figure 5: Atovaquone disrupts mitochondrial function in Plasmodium, while Proguanil inhibits DNA replication. Together, they work synergistically to effectively kill the parasite.

2.1.3. pfmdr1 Mechanism of Action and Resistance

The **pfmdr1** gene encodes a transporter protein that pumps antimalarial drugs like mefloquine, chloroquine, and quinine out of *Plasmodium falciparum* cells, reducing drug efficacy. Mutations in *pfmdr1* at positions 86, **184**, and 1042 increase transporter activity, enabling the parasite to expel drugs more efficiently. This reduces drug accumulation inside the parasite, contributing to resistance and diminishing the effectiveness of treatments, including **artemisinin-based combination therapies (ACTs)**.

2.2. IMPACT OF GENETIC VARIABILITY ON PROPHYLAXIS WITHIN ENDEMIC REGIONS

Genetic variability within *Plasmodium falciparum* and the human host significantly influences the effectiveness of malaria prophylaxis in endemic regions. The presence of genetic mutations in key resistance genes, such as **DHFR**, **cytb**, and **pfmdr1**, directly impacts the success of antimalarial prophylactic drugs, challenging malaria control efforts.

2.2.1. Parasite Genetic Variability and Resistance to Prophylactic Drugs

The genetic diversity of *P. falciparum* in endemic regions allows the parasite to adapt rapidly to drug pressure, leading to the selection of resistant strains. Mutations in genes reduce the efficacy of commonly used prophylactic drugs, such as **proguanil**, **atovaquone**, and **mefloquine**.

In high-transmission areas, mutations in these genes enable the parasite to survive despite drug treatment, rendering standard prophylactic regimens less effective. Studies (Lu et al., 2023) have shown that these mutations vary across regions, complicating the use of standard prophylactic regimens. The resistance profiles differ geographically, making continuous surveillance essential for adapting treatment strategies to local drug resistance patterns.

2.2.2. Host Genetic Variability and Prophylactic Drug Response

Host genetic factors, such as immune traits and variations in drug-metabolizing enzymes, also play a critical role in the success of malaria prophylaxis.

- **Immune Response Variability**: Genetic traits like sickle cell trait and G6PD deficiency offer partial protection against malaria, but they can also influence the effectiveness of certain prophylactic drugs. For example, individuals with G6PD deficiency may experience hemolytic reactions to drugs like **primaquine**, which complicates malaria prevention in affected populations (Nascimento et al., 2022).
- **Drug Metabolism Variability**: Host genetic differences in enzymes such as the cytochromeP450(**CYP450)** family affect the metabolism of drugs like **mefloquine** and **atovaquone**, altering their clearance rates and ultimately influencing drug efficacy. These variations may require adjustments in drug dosing to ensure optimal prophylaxis (Marwa et al., 2023).

2.2.3. Implications for Malaria Prophylaxis Strategies in Endemic Regions

The genetic variability of both the parasite and the host have direct implications for malaria prevention efforts.

- **Customized Treatments:** It's important to keep track of genetic changes in the parasite that make drugs less effective. By doing this, health officials can change treatment plans when new

drug-resistant strains appear, ensuring that treatments still work.

- **Personalized Malaria Prevention:** Testing people for genetic traits that affect how they process drugs can help choose the best drug and dosage. This way, treatments are more effective and have fewer side effects, because they are tailored to the individual's genetic makeup.
- **Using Multiple Drugs and New Approaches:** With growing drug resistance, it's important to use combinations of different drugs and create new ones. These new drugs can target different stages of the parasite's life cycle or work in new ways, helping to stay ahead of resistance and ensuring long-term success in malaria prevention.

2.3. MALARIA PROPHYLAXIS AND RATIONAL OF THE STUDY

Over the past few decades, chemoprophylaxis with drugs like Malarone (atovaquone-proguanil), Lariam (mefloquine), and Fansidar (sulfadoxine-pyrimethamine) has played a pivotal role in preventing malaria, especially in high-risk populations such as pregnant women, children under five, and travelers. However, the continued evolution of drug-resistant *P. falciparum* strains poses a growing threat to the efficacy of these treatments, leading to the failure of prophylactic strategies and complicating treatment regimens.

Particularly, WHO recommends malarone, lariam or doxycycline as prophylaxis for travelers going from malaria free-countries to endemic regions. However, recommendation for people travelling within endemic zone is not included in most of malaria prevention policies disconsititutes a gap in the overall malaria elimination strategies

Moreover, Malaria prevention in globally and chemoprophylaxis in particular face the following key issues:

- Limited Genetic Surveillance: Although resistance markers have been identified in both countries, molecular surveillance remains limited, particularly in rural areas. Comprehensive studies on the prevalence and spread of dhfr, cyt b, and pfmdr1 mutations are necessary to monitor resistance in real-time.

- Lack of Long-term Data: Most studies in Rwanda and Cameroon are cross-sectional, and long-term monitoring of resistance trends is essential to understand how drug resistance evolves over time.
- Limited research on prophylaxies: In endemic countries most of the studies focus on disease treatment and this create a gap in promoting prophylactic health care
- Limited capacity building: In most of malaria endemic area qualified human resources associated to limited functional state of the arts platforms represents a gap to support research in malaria elimination strategies

The following objectives contributed to address some of these gaps

2.4.OBJECTIVE

The overall objective of this study is to investigate the Genetic architecture of *Plasmodium falciparum* and its impacts on prophylaxis in the context of mobility across malaria endemic regions and from malaria free countries to endemic regions

2.4.1. SPECIFIC OBJECTIVES(SO)

SO_1: To determine the prevalence of Single Nucleotide Polymorphism (SNP) in the *dhfr* and *cyt B* of *Plasmodium falciparum* isolates from Rwanda and Cameroon.

Malarone is one of the most prescribed drugs to travelers going to malaria endemic regions. This drug comprises atovaquone and proguanil. The first act on the plasmodium respiratory chain targeting the cytochrome b in the mitochondria internal membrane, mutation Y268S/C/N is known to induce resistant to atovaquone. The partner drug targets the DHFR enzyme in the folate pathway three SNPs (N51I, C59R, and S108N) are known to induce resistant to proguanil. Hence, to determine the prevalence of this mutation the following work packages (WP) were implemented

WP_1: Blood Sample collection from symptomatic malaria patients who visited health care facilities of our study sites in Rwanda and Cameroon.

WP_2: Extraction and purification of genomic DNA from blood sample of interests.

WP_3: Targeted PCR amplification and purification of *CYT B* and *DHFR*

WP_4: Sequencing of amplicons of interest using both Oxford Nanopore Technology (ONT) and Sanger sequencing methods

WP_5: Bioinformatic and data analysis

SO2: To analyze the genetic mutations in the *pfmdr1* gene.

Lariam (mefloquine) is a widely used antimalarial drug, particularly for the prevention and treatment of malaria in regions with chloroquine-resistant *Plasmodium falciparum*. Key mutations, such as **Y184F**, alter drug transporter function of *Pfmdr1*, increasing the activity of this receptor leading to rapid influx of the drug into parasite digestive vacuole where it is not harmful to the parasite. To determine the prevalence of these resistance-associated mutation, the following work packages (WP) were implemented in this study.

$WP_{1:}$ Blood Sample collection from symptomatic malaria patients who visited health care facilities of our study sites in Rwanda.

WP_2: Extraction and purification of genomic DNA from blood sample of interests.

WP_3: Targeted PCR amplification and purification of *MDR1*

WP_4: Sequencing of amplicons of interest using both Oxford Nanopore Technology (ONT) and Sanger sequencing methods

WP_5: Bioinformatic and data analysis

Following the completion of the outlined work packages, the study will proceed with the methodology and the analysis of the collected data, focusing on the identification of genetic mutations associated with resistance to key antimalarial drugs. The results will provide valuable insights into the genetic landscape of *Plasmodium falciparum* and contribute to improving malaria control strategies in endemic regions.

CHAPTER 3. METHODOLOGY

This chapter explains the research design, sampling methods, data collection, lab procedures and analysis techniques used to study the genetic structure of Plasmodium falciparum and its implications for antimalarial prophylaxis. Laboratory work focused on molecular markers associated with Malarone and Lariam resistance, particularly the **DHFR, CYT B** and **MDR1** genes respectively.

3.1. Study Design

A molecular cross-sectional study was employed to analyze the prevalence and distribution of genetic mutations in *P. falciparum* isolates from Rwanda and Cameroon. This design allowed for identification of regional differences in genetic markers that influence resistance to prophylactic antimalarials, particularly Malarone and Lariam.

3.2. Study Area and Population

The study was conducted in health centers located in:

- **Rwanda** (East Africa)
- **Cameroon** (Central Africa)

Participants included patients of all ages with confirmed *P. falciparum* malaria. The population was selected from outpatient and inpatient departments in both countries during malaria transmission seasons.

3.3. Ethical Considerations

Ethical approval was obtained from **RNEC/MINISANTE** (120/RNEC/2022) in Rwanda and **CNERSH** in Cameroon. Informed consent was obtained from all participants, and health center staff were informed about the study. Privacy and confidentiality were ensured through sample anonymization, and all procedures followed national ethical and biosafety standards.

3.4. Sample Size and Collection

A total of 339 blood samples (170 from Rwanda and 169 from Cameroon) were collected from *Plasmodium falciparum*-positive patients confirmed by microscopy at various health centers.

A **convenience sampling method** was employed, whereby samples were obtained from patients who presented to healthcare facilities during the study period and met the inclusion criteria. In Rwanda, some samples were collected and processed directly at King Faisal Hospital (KFH), while others from peripheral sites were transported to KFH under cold chain conditions.

All samples were drawn into EDTA tubes and either processed on-site or stored at –80°C for later DNA extraction. Strict sample handling procedures were followed to maintain specimen integrity during transport and storage.

3.5. Inclusion Criteria

- Patients of all ages and genders presenting with malaria-like symptoms.
- Laboratory confirmation of *Plasmodium falciparum* infection by microscopy or rapid diagnostic test (RDT).
- Patients who provided informed consent (or whose guardians provided consent in the case of minors).
- Individuals who had not taken antimalarial drugs within the preceding 14 days.

3.6. Exclusion Criteria

- Patients with negative malaria test results or infection with non-*P. falciparum* species.
- Individuals who refused or were unable to give informed consent.
- Patients diagnosed with severe malaria, including cerebral malaria, due to differences in disease dynamics and treatment requirements.
- Patients who had received antimalarial treatment within the previous 14 days.
- Cases with insufficient or poor-quality blood samples unsuitable for molecular analysis.

3.7. LABORATORY PROCEDURE

Blood samples were collected from symptomatic *Plasmodium falciparum* malaria patients into EDTA tubes. Thick and thin blood smears were prepared and stained with Giemsa stain. The slides were examined under a microscope at 100x magnification to confirm the presence of *Plasmodium* parasites and assess parasitemia levels. The following step is genomic DNA extraction.

3.7.1. DNA EXCTRACTION

Genomic DNA was extracted from whole blood using the **Zymo Research DNA extraction kit**, following the manufacturer's protocol. DNA purity and concentration were assessed using a Nanodrop spectrophotometer. After quality assessment and quantify extracts, the DNA was immediately used for **PCR amplification** of target genes (*DHFR, CYTOCHROME B and MDR1*).

3.7.2. PCR AMPLIFICATION OF GENES

PCR was performed to amplify three target genes associated with antimalarial drug resistance: Lariam and Malarone, **DHFR** (proguanil/malarone resistance), **cytochrome b** (*cyt b*, associated with atovaquone resistance in Malarone) and **pfmdr1** (mefloquine/Lariam resistance).

Each **uniplex PCR reaction** was prepared in a total volume of **X µL**, consisting of the following components: **GoTaq Master Mix**, **nuclease-free water**, **gene-specific primers** (forward and reverse combined) **genomic DNA template**

PCR reactions (primer dilution and master mix preparation) were prepared on ice and in Biosafety cabinet.

3.7.2.1. Thermal cycling condition

PCR amplification was performed using a Bio-Rad thermocycler with the following thermal profiles optimized for each gene:

For dhfr and pfmdr1: Initial denaturation at 94°C for 5 minutes, **followed by** 35 cycles **of: Denaturation at** 94°C 30 seconds, **annealing at** 58°C for 1 minute, **Extension at** 72°C for 1 minute and 30 seconds, **Final extension at** 72°C for 5 minutes, **Hold at** 4°C indefinitely

For cytochrome b: There are the same except on Initial denaturation the times is **3 minutes** and on Annealing at **61°C for 1 minute and 30 seconds**

These optimized conditions ensured specific amplification of target regions in each gene. In cases of poor or failed amplification, reactions were repeated using **GoTaq® Endure qPCR** Master Mix (Promega, A6220) to improve yield and specificity.

Table 1: Gene-specific primers

Primer name	Primer size	Size of the target
Pfdhfr-Forward	5'-GTTTTCGATATTTATGCCATATGTG-3'	490bp
Pfdhfr-Reverse	5'-TGATAAACAACGGAACCTCCB-3'	
Pfcytb-Forward	5'-GATACATGCACGCAACAGGTG-3'	811bp
Pfcytb-Reverse	5'-GGAGCTGTAATCATAATCTGT-3'	
Pfmdr1-Forward	5'-TGTGTTTGGTGTAATAAAGAACA-3'	363bp
Pfmdr1-Reverse	5'-ACATAAGTCAAACGTGCATTT-3'	

3.7.3. Gel Electrophoresis

PCR amplicons were separated on an 2% agarose gel using electrophoresis and visualized under UV light, following established protocols. The gel was prepared by dissolving 3 g of agarose in 150mL of 0.5 TAE buffer in a glass beaker. After melting the mixture, 1.5µL of **Invitrogen™ SYBR™ Safe DNA Gel Stain** was added. The molten gel was then poured into casting trays with combs to form wells and allowed to solidify before loading the samples.

4 µL of each PCR product was loaded into individual wells and electrophoresed at 100 volts for 30 minutes, in accordance with standard procedures. DNA fragment sizes were estimated using a DNA ladder containing fragments ranging from 100 to 1000 base pairs.

Successful amplification was confirmed by the presence of distinct bands corresponding to the expected sizes of the target gene fragments: DHFR (490 bp), Pfmdr1 (363 bp), and Cyt B (~900 bp).

Following electrophoresis, the amplicons were pooled to reconstruct the complete gene sets for each sample. These pooled PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System, following the manufacturer's protocol, to remove residual primers and unincorporated nucleotides.

Purified DNA was eluted in **nuclease-free water**, then quantified using both a **Nanodrop spectrophotometer** (for purity assessment) and a **Qubit fluorometer** (for accurate DNA concentration measurement). The cleaned PCR products were subsequently stored at **–20°C** until they were submitted for **Sanger sequencing**.

3.7.4. Library Preparation and Sequencing

Library preparation was carried out according to the protocols provided by **Oxford Nanopore Technologies (ONT)** for native barcoded amplicon sequencing. The Native Barcoding Kits 96 V14 (SQK-NBD114.96) and 24 V14 (SQK-NBD114.24) were used in combination with the NEBNext® Ultra™ II End Repair/dA-Tailing Module (NEB, E7546), the Blunt/TA Ligase Master Mix (NEB, M0367), and the NEBNext® Quick Ligation Module (NEB, E6056), following the manufacturers' guidelines.

Four sequencing libraries were prepared: one using the Native Barcoding Kit 96 V14 protocol, multiplexing 96 samples, and three additional libraries prepared with the Native Barcoding Kit 24 V14 protocol, multiplexing 24, 24, and 19 samples respectively. In total, 163 samples were successfully sequenced. To assess the reproducibility and robustness of the ONT sequencing platform, a subset of samples was intentionally included in duplicate.

3.7.5. Bioinformatics Workflow and Variant Detection

The raw FASTQ files generated by the MinION were first assessed for quality using pycoQC (v 2.5.2) and NanoPlot (v 1.43.0) to evaluate the distribution of read lengths and overall data quality. Adapter sequences and low-quality termini were subsequently trimmed using Porechop (v 0.2.4),

with a follow-up check using Nano Plot to confirm the removal of reads with Q scores < 10 and any residual adapter sequences.

High-quality reads were then aligned to the *Plasmodium falciparum* 3D7 reference genome (version 3) using Minimap2 (v 2.28 r1209, map ont preset). Alignment statistics, filtering, sorting, and indexing were performed using the appropriate functions in Sam tools (v 1.21). Additional quality control at the BAM level was conducted using Qualimap (v 2.2.2), and visual inspection of the alignments was done in IGV (v 2.16.0). Depth and coverage metrics were calculated using samtools depth/coverage.

Single nucleotide polymorphisms (SNPs) were identified using Clair3 (v 1.0.10) in haploid mode (--include_all_ctgs --haploid_sensitive --gvcf). The resulting gVCFs were filtered for quality (≥ Q10), depth (> 5), and minor allele frequency (> 0.01, suitable for the highly polymorphic *Plasmodium* genome) using bcftools (v 1.21). These filtered gVCFs were then merged using GATK CombineGVCFs and converted into VCF format using GenotypeGVCFs.

Variants were annotated against the 3D7 genome using SnpEff (v 4.10). Gene-specific VCFs for pfdhfr, pfcyt b, and pfmdr1 were extracted using bcftools, and pfmsp2 genotypes were further parsed for subsequent analysis. SNPs were cross-referenced with the *PlasmoDB* database to identify known mutations.

All statistical analyses and visualizations were conducted in R (v 4.3.1) with packages such as ape, vcfR, tidyverse, ComplexHeatmap, adegenet, and ctc. Phylogenetic trees were generated using the hc2Newick function and formatted in iTOL (https://itol.embl.de/)**.**

The final dataset was compiled to assess: Mutation frequencies per gene and region (Rwanda vs. Cameroon), Prevalence of key resistance markers, Agreement between ONT and Sanger sequencing.

CHAPTER 4: RESULTS

The results presented in this report are based on 170 samples collected from Rwanda and 169 from Cameroon. These samples were analyzed for the Genetic architecture of *Plasmodium falciparum* and its impacts on prophylaxis in the context of mobility across malaria endemic regions and from malaria free countries to endemic regions

The samples were collected from healthcare facilities in the Nyarugenge, Gasabo, Kicukiro, Kirehe, Gisagara, and Kibuye districts in Rwanda, and Douala and Buea in Cameroon. Some samples were transported to King Faisal Hospital Rwanda for processing.

DNA extraction, PCR amplification, and gel electrophoresis, along with the quantification and normalization of DNA, were carried out at King Faisal Hospital in Rwanda. The purified PCR products, along with extracted DNA samples for both PCR and Sanger sequencing, were subsequently sent to Brussels for additional analysis. Furthermore, Oxford Nanopore Technology (ONT) for sequencing the dhfr gene was performed at both Kigali and the ULB Biopark in Belgium.

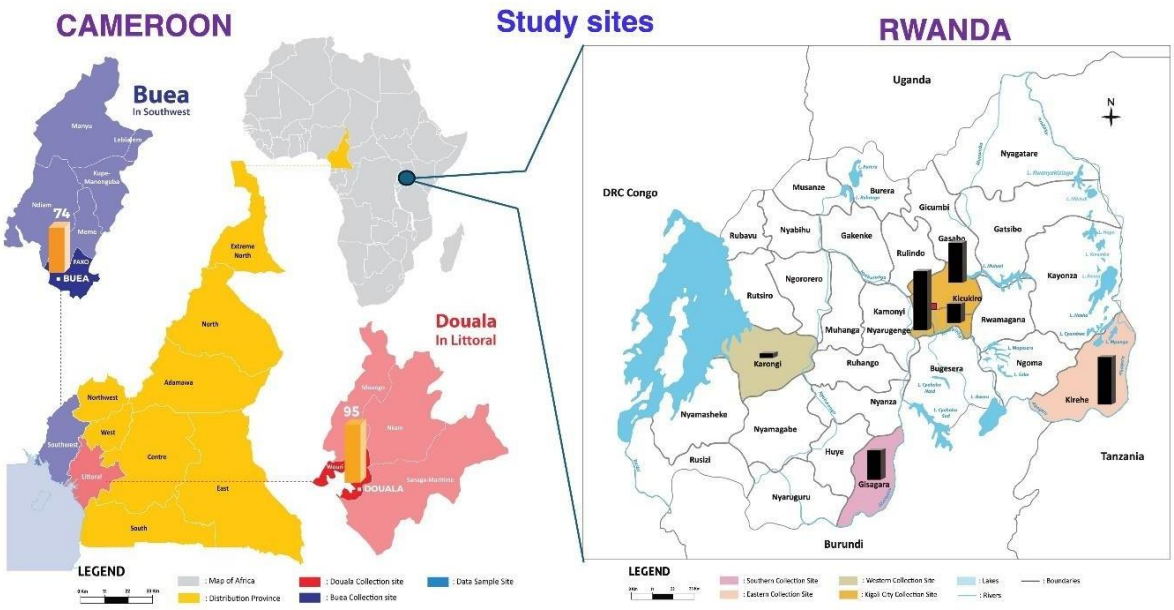

Figure 6: Map of Rwanda and Cameroon collection site

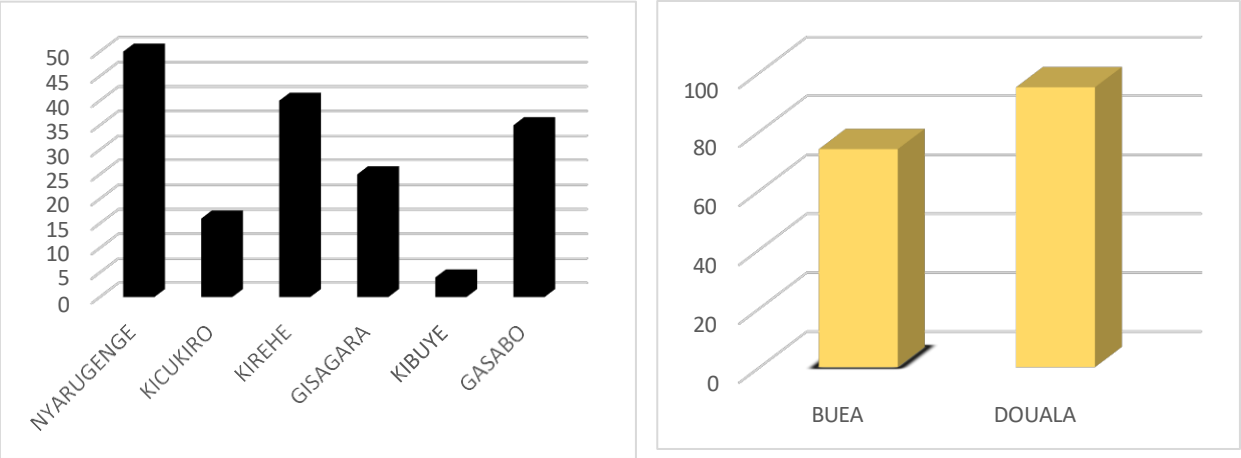

Figure 7: Sample collection from districts in Rwanda and Cameroon

*(**Left**) Samples collection from districts in **Rwanda**, Nyarugenge having the most (50), followed by Kirehe (40), Gasabo (35), Gisagara (25), Kibuye (4), and Kicukiro (16). (**Right**) **Cameroon**, Doula has (95) and Buea (74). These districts were key sites for studying **genetic resistance markers** in Plasmodium falciparum.*

4.1. THE PREVALENCE OF SINGLE NUCLEOTIDE POLYMORPHISM (SNP) IN THE *DHFR* AND *CYT B* OF *PLASMODIUM FALCIPARUM* ISOLATES FROM RWANDA AND CAMEROON.

A total of 339 blood samples were collected from confirmed Plasmodium falciparum malaria patients across Rwanda(n=170) and Cameroon (=169), as described in the Methodology. The prevalence of SNPs in the **Dihydrofolate reductase** (**DHFR)** and **Cytochrome B (CYT B)** genes was analyzed using PCR amplification, gel electrophoresis and sequencing.

- **DHFR Gene**: Mutations in the *dhfr* gene, including N51I, C59R, S108N and I164L, were observed in a significant proportion of isolates from Rwanda. These mutations are known to confer resistance to proguanil in Malarone. The frequency of each mutation varied by region, with the highest prevalence observed in the Rwandan samples.
- **CYT B Gene**: Mutations in the Cyt B gene (Y268S) associated with atovaquone resistance were identified with 0 mutation.

Gel electrophoresis confirmed successful amplification (see representative gel in **Fig below**) with distinct bands corresponding to expected fragment sizes. No amplification was observed in the negative controls (NC), confirming the specificity of the reactions.

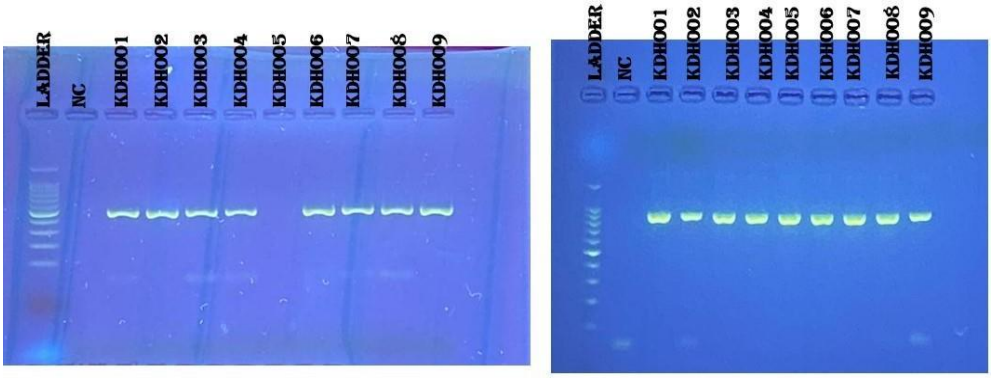

Figure 8: Gel electrophoresis images of amplification of DHFR (left) with band size 490bp and cyt B(right)with size of 811bp

4.1.1. MUTATION ANALYSIS OF DIHYDROFOLATE REDUCTASE (DHFR) GENE

The dihydrofolate reductase (DHFR) gene was analyzed to assess mutations associated with resistance to **proguanil**, a key component of the antimalarial drug Malarone. Four key point mutations—**N51I, C59R, S108N**, and **I164L**—were screened in *Plasmodium falciparum* isolates from Rwanda.

As shown in **Figure 9** the **N51I (97.1%)**, **C59R (94.7%)**, and **S108N (98.2%)** mutations were detected in nearly all isolates, indicating a widespread prevalence of the classical triple DHFR mutant haplotype. This combination is strongly associated with reduced sensitivity to proguanil and suggests that resistance to this drug is nearly fixed in the local parasite populations.

The **I164L** mutation was observed in **30.6%** of samples. While less common, its presence is clinically important, as it often amplifies drug resistance when combined with the triple mutant background. The emergence of this quadruple mutant profile signals evolving drug pressure and the potential for high-level treatment failure in areas where it is used extensively.

In contrast, recent data from Cameroon demonstrate near-fixation of the DHFR triple-mutant haplotype, with N51I present in ~99.5%, C59R in ~99.5%, and S108N in 100% of samples but I164L was completely absent (0%) indicating that while baseline antifolate resistance is widespread in Cameroon, high-level resistance involving the I164L substitution has not yet emerged in all surveyed isolates, even across studies from 2014 through 2020(Niba et al., 2023)

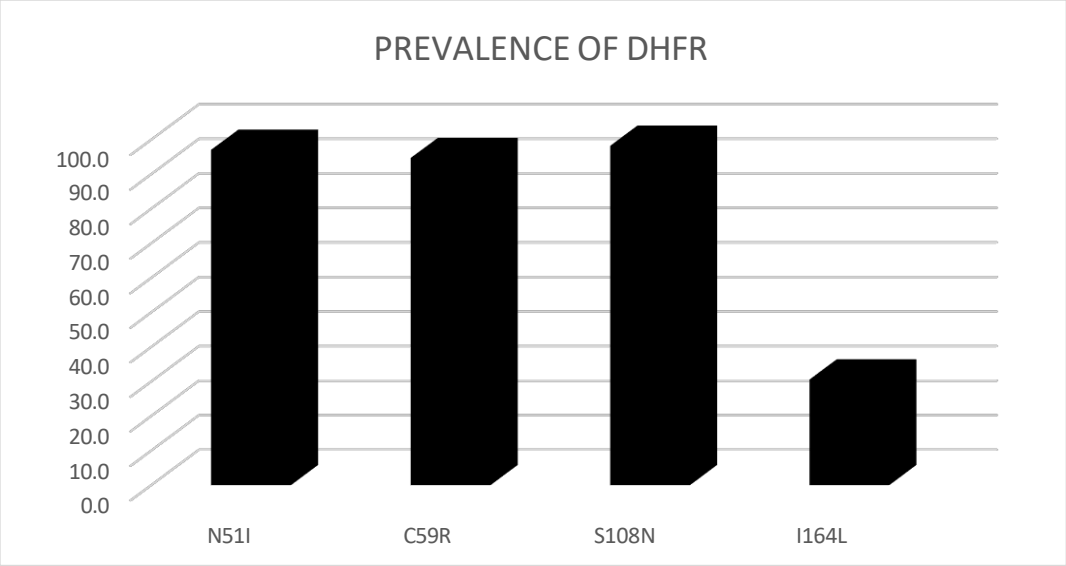

Figure 9: Prevalence of DHFR Mutations in Plasmodium falciparum Isolates

4.1.2. Mutation Analysis in the CYT B Gene (Y268S)

The cytochrome B (CYT B) gene was analyzed to identify the Y268S mutation, which is a known marker of resistance to atovaquone, one of the active compounds in Malarone. This mutation alters the mitochondrial electron transport chain, reducing the drug's ability to inhibit parasite respiration.

As illustrated in **Figure 10** none of the 170 in Rwanda and 169 in cameroon isolates from the study population harbored the Y268S mutation. All samples were wild-type (non-mutated) at this position.

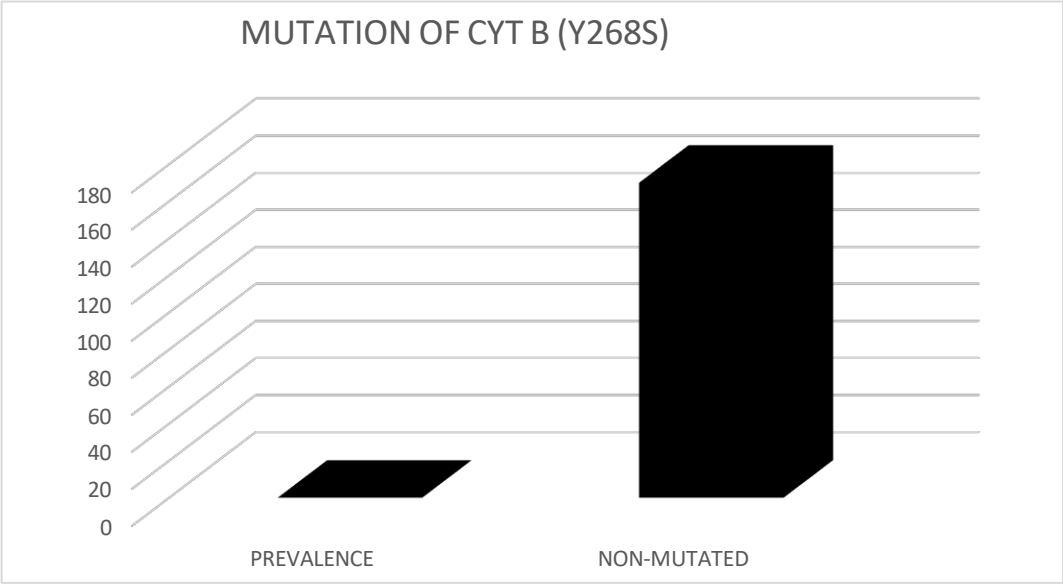

Figure 10: Mutation of cyt b(Y268S) is 0% in both countries 170,169 Rwanda and Cameroon, respectively

Validation of Mutations by Sanger Sequencing

To confirm the presence or absence of SNPs identified by Nanopore sequencing, representative samples were validated using Sanger sequencing, the gold standard for mutation detection. The chromatograms and multiple sequence alignments below illustrate the accuracy of the mutation calls for both DHFR and CYT B genes.

These validated substitutions support the earlier findings that the **triple mutant haplotype (N51I, C59R, S108N)** is highly prevalent, and **I164L**, while less common, is present in a significant subset of isolates.

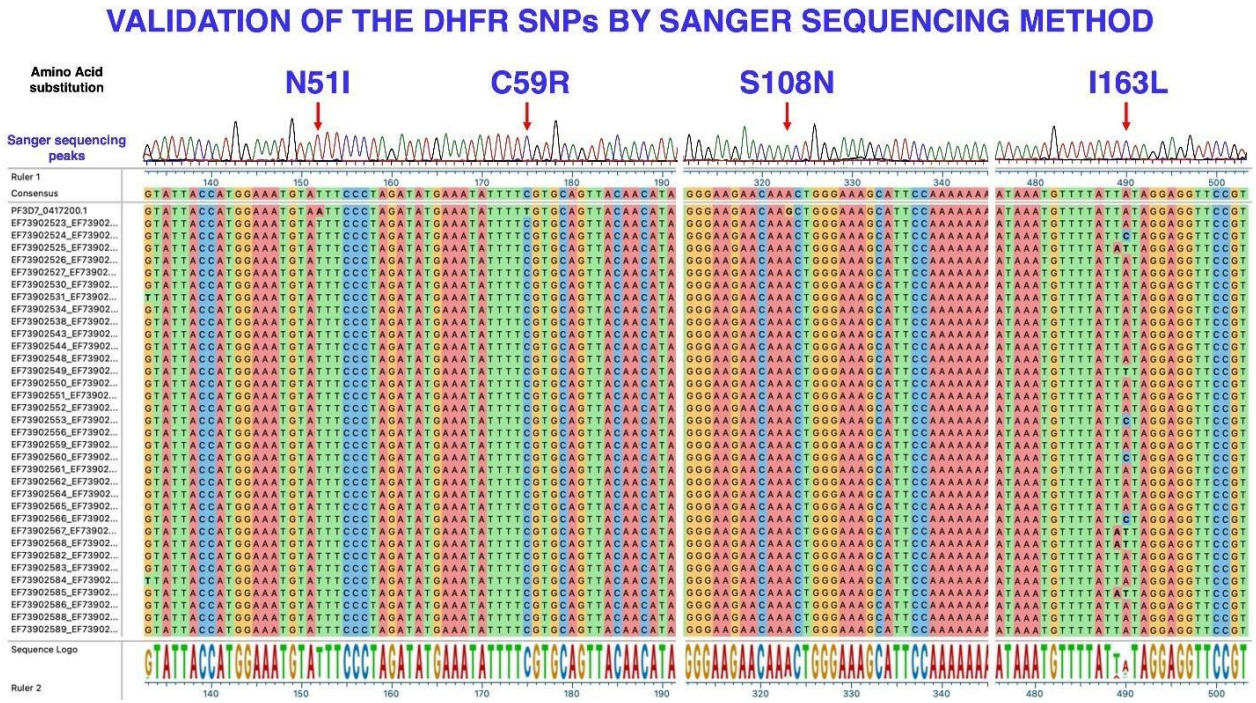

Figure 11: Validation of DHFR Mutations (N51I, C59R, S108N, I164L)

This Sanger sequencing result confirms the presence of the four key mutations in the *Plasmodium falciparum* DHFR gene. The chromatograms show clear base substitutions corresponding to:

N51I (AAT → ATT), **C59R** (TGT → CGT), **S108N** (AGC → AAC), and **I164L** (ATA → CTA).

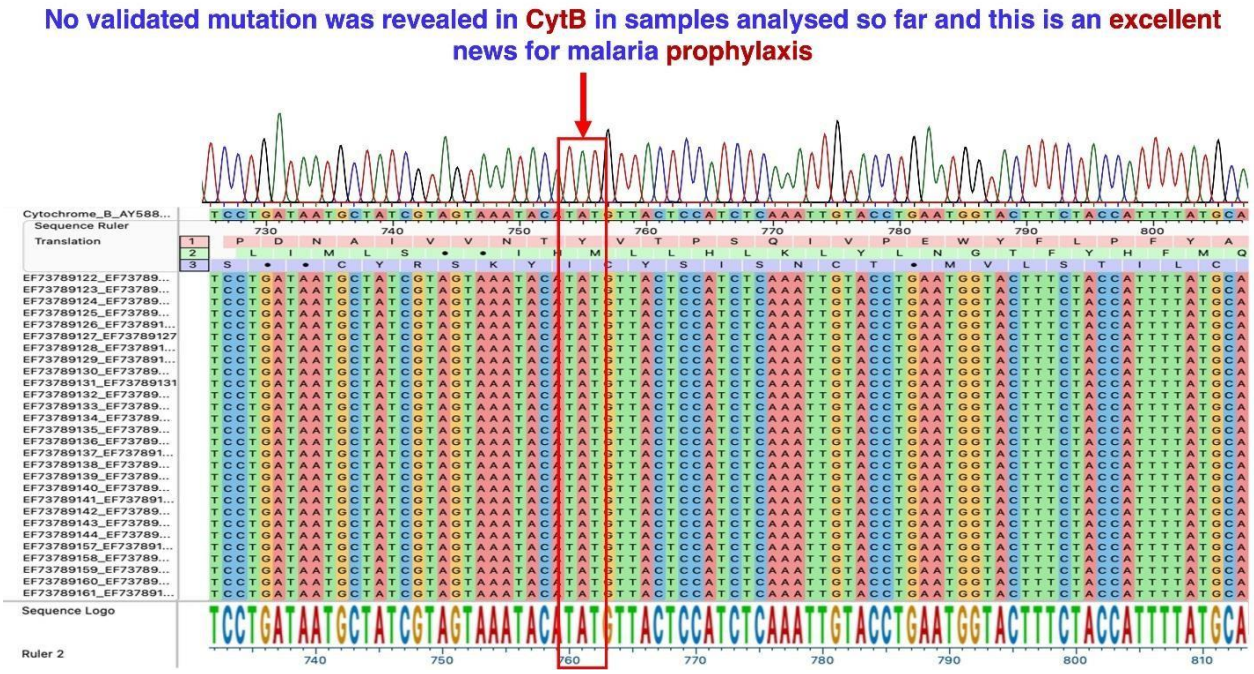

Figure 12: Validation of CYT B Wild-Type Sequence at Position Y268

This figure confirms the **absence of the Y268S mutation** in the CYT B gene. All analyzed samples showed wild-type sequences at this locus, supporting the data from fig12 that **no atovaquone resistance was detected** in the study population. This result is encouraging for malaria prophylaxis, indicating continued sensitivity to atovaquone.

4.2. ANALYSIS OF THE GENETIC MUTATIONS IN THE *PFMDR1* GENE, WITH A SPECIFIC FOCUS ON THE Y184F MUTATION ASSOCIATED WITH RESISTANCE TO MEFLOQUINE (LARIAM).

4.2.1. Gel Electrophoresis for Mutation Detection

Gel electrophoresis was used to confirm the presence and size of PCR-amplified fragments corresponding to specific gene regions. Amplified products of the *pfmdr1* and *dhfr* genes were loaded onto a 2% agarose gel stained with **Invitrogen™ SYBR™ Safe DNA** and bands were visualized under UV light. The expected fragment sizes were compared to a molecular ladder to validate successful amplification prior to sequencing or restriction enzyme digestion. This step ensured the accuracy of downstream mutation analysis

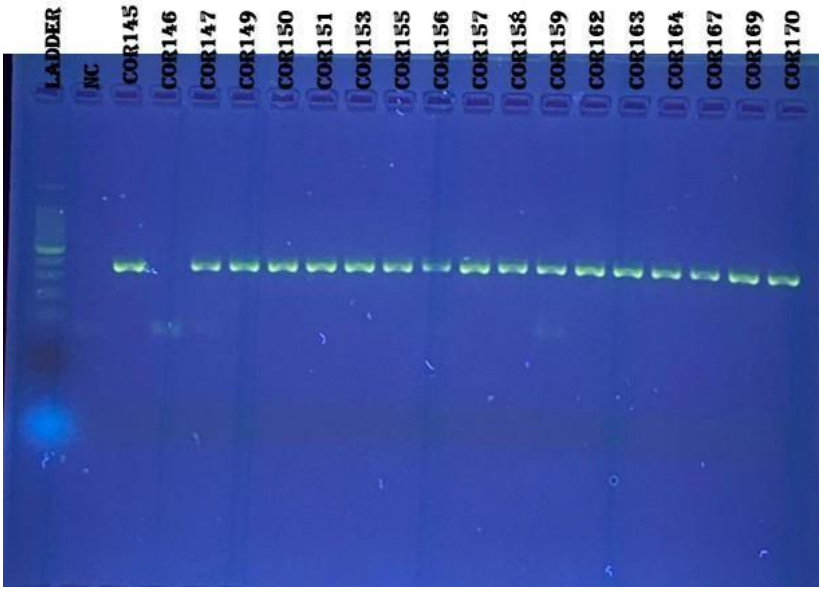

Figure 13: Gel electrophoresis images of amplification of pfMDR1 with band size 363bp

The **pfmdr1** gene encodes a transporter protein involved in drug transport across the parasite's digestive vacuole membrane. The **Y184F mutation** (a tyrosine-to-phenylalanine substitution at codon 184) is commonly associated with increased drug efflux, leading to reduced intracellular drug concentration and decreased mefloquine efficacy.

Analysis of the **170 samples** from Rwanda revealed the following:

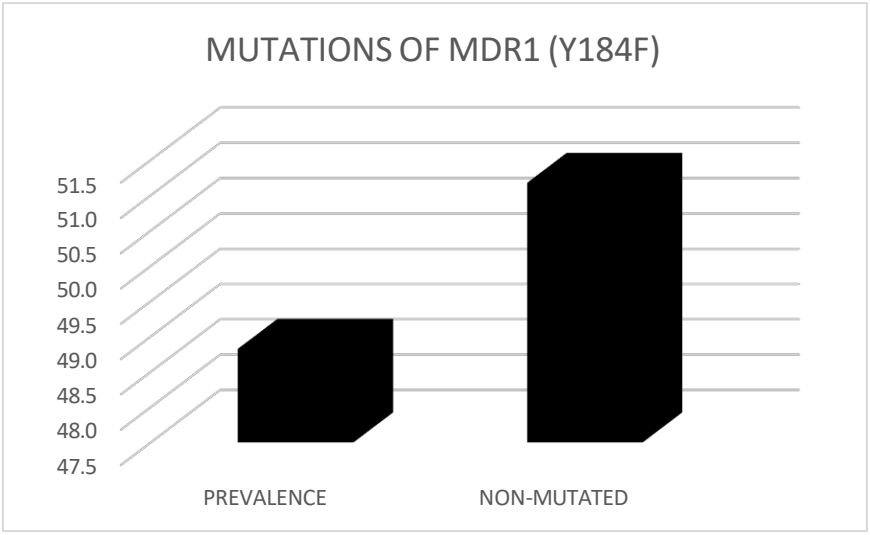

Figure 14: Prevalence of Y184F Mutation,

This bar graph illustrates the distribution of the Y184F mutation in the studied *P. falciparum* samples. 48.8% of isolates were found to carry the mutation, while 51.2% were wild-type at codon 184.

The Y184F mutation was detected in **48.8%** of the *P. falciparum* isolates, suggesting a moderate but significant prevalence of this resistance-associated marker in the Rwandan population. Although not yet dominant, this frequency points to ongoing selection pressure, potentially driven by mefloquine use or other ACTs sharing similar resistance pathways.

Recent studies in Cameroon also report a high prevalence of the Y184F mutation. A 2023 multi-region survey showed that Y184F was present in approximately 60% of *P. falciparum* isolates. The most common haplotype identified was N86/F184/D1246 (NFD), observed in 32.8% of samples. This haplotype is frequently associated with reduced susceptibility to lumefantrine and mefloquine. In contrast, the wild-type haplotype N86/Y184/D1246 was found in 43.7% of samples, indicating that both mutant and wild-type alleles are still co-circulating in the Cameroonian population (Niba et al., 2023).

These findings suggest that, like Rwanda, Cameroon is experiencing selective pressure favoring pfmdr1 polymorphisms associated with drug resistance, likely due to widespread use of ACTs.

CHAPTER 5: DISCUSSION

This chapter discusses the findings in light of existing literature, their implications for malaria prophylaxis in endemic regions, and the importance of molecular surveillance for drug resistance. It is framed within the study's overall objective: to investigate the genetic architecture of *Plasmodium falciparum* and its impacts on chemoprophylaxis in the context of human mobility across and within malaria-endemic areas. The discussion is structured around the two specific objectives, addressing both the molecular markers associated with Malarone and Lariam resistance.

5.1. DHFR and CYT B Mutations: Implications for Malarone Prophylaxis

5.1.1. High Prevalence of DHFR Mutations: A Red Flag for Proguanil Efficacy

Our data reveal an alarmingly high prevalence of classical *dhfr* mutations—**N51I (97.1%)**, **C59R (94.7%)**, and **S108N (98.2%)**—in *P. falciparum* isolates from Rwanda. These mutations are known to confer resistance to **proguanil**, the antifolate component of Malarone. The detection of the **I164L** mutation in **30.6%** of Rwandan samples adds a critical dimension: this mutation, when present alongside the triple mutant haplotype, is associated with high-level resistance and potential treatment failure.

While proguanil is not part of Rwanda's national first-line malaria treatment policy, it is still available in the form of Malarone (atovaquone–proguanil) and is commonly used for traveler prophylaxis or by non-immune individuals. Our findings suggest that Malarone's effectiveness in Rwanda may already be compromised, due to the fixed resistance to proguanil.

In contrast, **Cameroon** shows a similar resistance pattern for the *dhfr* triple mutant haplotype, with N51I, C59R, and S108N detected in **>99%** of isolates. However, the I164L mutation was not detected in Cameroonian samples, indicating that high-grade proguanil resistance has not yet emerged. This difference underscores the importance of regional surveillance and targeted policy: Cameroon still has an opportunity to delay or prevent the spread of I164L, but only if proactive monitoring and resistance-informed prophylaxis strategies are implemented.

5.1.2. Absence of CYT B Mutation: Temporary Reassurance for Atovaquone

In stark contrast to the widespread *dhfr* mutations observed in both Rwanda and Cameroon, **no Y268S mutation** was detected in the *cytb* gene across the 339 *P. falciparum* isolates analyzed. This strongly suggests that **atovaquone remains fully effective at least for now**.

This is encouraging, as **Y268S is a high-level resistance marker** and has been reported in Southeast Asia and parts of South America but remains rare in Africa (Lu et al., 2023).

Important note: Although *cytB* mutation is currently absent, relying solely on atovaquone (with proguanil resistance fixed) risks driving mono-therapy selection pressure, potentially accelerating the emergence of resistance.

This study is among the first to report *cytb* genotyping results from Rwanda and Cameroon, filling a notable gap in the literature. It should not only reassure public health officials of current efficacy but also serve as a strategic signal to prioritize *cytb* surveillance in national drug-resistance monitoring programs.

5.1.3. Combined Threat to Malarone Efficacy

Our study confirms that **Malarone is partially compromised**:

- **Proguanil** is likely ineffective due to fixed *dhfr* mutations.
- **Atovaquone** is still effective, but at risk.

This partial failure reflects trends seen in Kenya and Uganda, where atovaquone treatment failures were linked to de novo mutations . The drug's long half-life further enhances resistance selection when not paired with an effective partner.

Clinical implication: Relying on Malarone for prophylaxis in these regions may offer **incomplete protection**, especially during extended stays or in high-transmission areas.

5.2. Y184F Mutation in pfmdr1 and Resistance to Mefloquine (Lariam)

The pfmdr1 Y184F mutation was detected in **48.8%** of the Rwandan isolates, indicating a **moderate but concerning prevalence**. This SNP has been associated with **reduced mefloquine sensitivity**, though its role is often secondary to other pfmdr1 mutations like N86Y and D1246Y.

While Y184F alone may not fully confer resistance, its combination with other mutations (not assessed in this study) can significantly compromise drug efficacy. Additionally, **Y184F is increasingly recognized as a compensatory mutation** that can modulate parasite fitness and response to multiple drugs, including lumefantrine and artesunate.

Interpretation: Lariam's efficacy is already under strain, and its use in prophylaxis for travelers or residents in Rwanda should be re-evaluated. Importantly, ACT partner drug resistance may also be increasing, indirectly driven by pfmdr1 selection.

5.3. Surveillance Gaps and Emerging Resistance Risks

One of the most important findings of this study is not just the mutation frequency but what it implies for surveillance and future preparedness:

- **I164L is present in 30.6% of isolates** — a much higher rate than reported in earlier studies in Rwanda. This may indicate the beginning of **high-grade antifolate resistance**.
- **Sanger and Nanopore sequencing both confirmed these mutations**, strengthening confidence in the results and proving the feasibility of using ONT in low-resource settings.

Additionally, the absence of cytb mutations should not lead to complacency. Atovaquone resistance often arises rapidly when proguanil is no longer effective, particularly with **poor adherence to prophylaxis or underdosing**.

5.4. Broader Context: Prophylaxis, Mobility, and Regional Policy Gaps

This study confirms the **critical need to update malaria prophylaxis recommendations**, especially for **intra-endemic travelers** — an overlooked group in many national strategies.

- While WHO provides clear guidelines for travelers from non-endemic to endemic regions, there is **no coherent guidance** for people **moving within endemic zones**, such as seasonal workers, refugees, or rural–urban migrants.
- As resistance patterns vary geographically, even short-range movement can expose individuals to **different parasite strains** with **distinct resistance profiles**.

This study provides compelling evidence to advocate for **region-specific prophylaxis strategies**, guided by **molecular data** and **mobility trends**.

5.5. Strengths and Limitations

5.5.1. Strengths

- Dual-platform validation (Nanopore + Sanger)
- Inclusion of both East (Rwanda) and Central (Cameroon) African samples
- High-resolution gene-specific mutation analysis
- First report to link I164L prevalence with emerging resistance in Rwanda

5.5.2. Limitations

- ***pfmdr1* mutation data were limited to Y184F only**, excluding other clinically relevant mutations such as N86Y and D1246Y.
- **Samples from Cameroon could not be analyzed for pfmdr1 and dhfr mutations** due to challenges in accessing sufficient and intact DNA material for this gene.
- The **cross-sectional design** of the study limits the ability to assess temporal trends or resistance evolution over time.
- No clinical outcome data were available, preventing direct correlation of genetic profiles with treatment or prophylaxis failure.

Table 2: Summary and Implications

Drug	Target Gene	Key Mutation(s)	Prevalence	Implication
Proguanil	*dhfr*	N51I, C59R, S108N, I164L	Nearly 100%, 30.6%	Drug likely ineffective
Atovaquone	*cytB*	Y268S	0%	Still effective, but vulnerable
Mefloquine	*pfmdr1*	Y184F	48.8%	Reduced efficacy likely

5.6. Final Remarks

This study highlights the **fragility of current chemoprophylaxis tools** in malaria-endemic settings. With **proguanil resistance nearly fixed**, **Lariam under pressure**, and **atovaquone standing alone**, there is an urgent need to:

- Monitor *cytB* and *pfmdr1* mutations continuously
- Reconsider prophylaxis protocols in endemic zones
- Promote the development and deployment of **new prophylactic strategies**, such as long-acting injectables or multi-target combinations

Without adaptive, mutation-informed prevention strategies, both travelers and residents may soon be **unprotected against malaria** — despite taking standard prophylaxis.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1. CONCLUSION

This study provides compelling molecular evidence that malaria chemoprophylaxis in endemic regions like Rwanda and Cameroon is under significant threat due to evolving resistance patterns in *Plasmodium falciparum*. The nearly fixed prevalence of triple *dhfr* mutations (N51I, C59R, S108N) in both Rwanda and Cameroon, coupled with the emergence of the I164L mutation in 30.6% of Rwandan isolates, suggests that proguanil a key component of Malarone is likely ineffective in large portions of the population. In contrast, no Y268S mutation was detected in the *cytB* gene, indicating continued efficacy of atovaquone, though this may be temporary given the selective pressure when used in isolation.

In the case of mefloquine (Lariam), the Y184F mutation in *pfmdr1* was found in 48.8% of Rwandan samples, with recent data showing 60% prevalence in Cameroon. This reflects ongoing selection pressure driven by ACT use and suggests that Lariam's protective effectiveness may be compromised, particularly when prophylaxis is prolonged.

The absence of *pfmdr1* and dhfr data (2025) in Cameroonian samples limits direct comparisons, and the cross-sectional design restricts our ability to assess temporal resistance trends. Nevertheless, these results clearly expose the fragile efficacy of Malarone and Lariam in high-transmission areas and reinforce the urgent need to revise prophylactic strategies.

6.2. PERSPECTIVES

This study provided important insights into the genetic architecture of *Plasmodium falciparum* in Rwanda and Cameroon by identifying key mutations associated with resistance to antimalarial prophylactics, including *dhfr*, *cytb*, and *pfmdr1*. It confirmed that proguanil resistance is nearly fixed in both countries, atovaquone remains effective but vulnerable, and mefloquine resistance is emerging. These findings offer valuable evidence to inform national prophylaxis strategies and emphasize the importance of molecular surveillance in endemic settings.

Despite these contributions, several important gaps remain. This study focused on a limited number of gene markers and used a cross-sectional design, which restricts the understanding of resistance evolution over time. It also did not examine host genetic factors, such as CYP450 enzyme polymorphisms or G6PD deficiency, which could significantly influence drug efficacy and safety. Additionally, vaccine-related parasite diversity, longitudinal surveillance, and the role of mobility in spreading resistant strains remain underexplored.

These findings open multiple avenues for future research. A PhD-level study could build on this work by integrating whole-genome sequencing to uncover novel resistance mechanisms, exploring host–parasite genetic interactions to support personalized prophylaxis, or linking molecular resistance data with vaccine rollout outcomes. Moreover, incorporating geospatial analysis and human mobility data could improve predictive models for resistance spread and inform targeted interventions. Strengthening local capacity for real-time sequencing and bioinformatics would also enhance regional preparedness and contribute to global malaria elimination efforts.

Ultimately, this research highlights the need for adaptive, mutation-informed prevention strategies and paves the way for more personalized and regionally tailored approaches to malaria control.

6.3. RECOMMENDATIONS

This study provides a basis for future academic investigations into antimalarial drug resistance, with the following research-oriented recommendations:

1. **Deepen Genetic Surveillance Research**
   - Conduct longitudinal studies to track the evolution of mutations in *dhfr*, *cytB*, and *pfmdr1* in both high- and low-transmission areas.
   - Extend genotyping to include additional *pfmdr1* codons (e.g., N86Y, D1246Y) and investigate their role in drug tolerance or treatment failure.
2. **Investigate Efficacy of Current Prophylaxis in the Context of Resistance**
   - Design follow-up studies that evaluate the clinical outcomes of Malarone and other prophylactic regimens in populations where *dhfr* mutations are fixed but *cytB* mutations are absent.
   - Assess whether proguanil resistance alone compromises prophylactic efficacy or if atovaquone activity remains sufficient.
3. **Explore New Prophylactic Options Through Experimental Research**
   - Investigate the pharmacodynamics and molecular targets of alternative prophylactic compounds, including long-acting injectables and combination therapies.
   - Conduct in vitro and in vivo experiments to test efficacy against known resistance genotypes.
4. **Address Gaps in Regional Data Through Collaborative Research**
   - Prioritize sample collection and full molecular analysis from underrepresented regions, especially Cameroon, where *pfmdr1* data was lacking in this study.
   - Build regional academic partnerships to facilitate shared protocols, data harmonization, and resource pooling for broader surveillance.
5. **Expand the Scope of Future Research Beyond the Current Genes**
   - Explore other resistance-associated genes and potential compensatory mechanisms not covered in this study.
   - Integrate host genetic factors and pharmacogenomic profiling to understand individual variations in drug response and resistance risk.

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REPUBLIC OF RWANDA/REPUBLIQUE DU RWANDA

NATIONAL ETHICS COMMITTEE / COMITE NATIONAL D'ETHIQUE

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FWA Assurance No. 00001973
IRB 00001497 of IORG0001100

21 November 2024.

Principal Investigator: Prof Jacob Souopgui

ANNUAL RENEWAL APPROVAL NOTICE: RNEC597/2024

Protocol Title: "Addressing both naturally occurring and ACT induced Plasmodium reservoirs using Artemisia infusions and develop a family registry for malaria to accelerate malaria elimination and eradication in Rwanda: A proof of concept Randomized Controlled Trial."

After review of the protocol, progress report consent forms requested during the RNEC meeting of 09th November 2024 where quorum was met , **the requested annual renewal was approved.**

Please note that approval of the protocol and consent form both English and Kinyarwanda version is valid for **12 months**.

You are responsible for fulfilling the following requirements:

1. Changes, amendments, and addenda to the protocol or consent form must be submitted to the committee for review and approval, prior to activation of the changes.
2. Only approved consent forms are to be used in the enrollment of participant
3. All consent forms signed by subjects should be retained on file. The RNEC may conduct audits of all study records, and consent documentation may be part of such audits.

RWANDA NATIONAL ETHICS COMMITTEE R.N.E.C P.O BOX:84 KIGALI

REPUBLIQUE DU CAMEROUN
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NATIONAL ETHICAL COMMITTEE FOR RESEARCH ON HUMAN HEALTH

TECHNICAL SECRETARIAT

N° 2023/10/1602/CE/CNERSH/SP

Yaoundé, le 30 OCT 2023

CLAIRANCE ETHIQUE

Le Comité National d'Ethique de la Recherche pour la Santé Humaine (CNERSH), en sa session extraordinaire du 27 octobre 2023, a examiné le projet de recherche intitulé : **«Contributions in emptying *plasmodium* reservoirs to accelerate malaria elimination in high transmission settings using Artemisia afra infusion : case study in Cameroon»** soumis par le **Professeur Stephen M. GHOGOMU,** Investigateur Principal, Université de Buéa.

Le projet est d'un grand intérêt scientifique et social. L'objectif général de cette étude est de faire face à l'émergence de la résistance à l'ACT chez *P. falciparum* dans les régions de l'Ouest, du Sud-Ouest, du Littoral et du Centre - Cameroun. La procédure de l'étude est bien documentée et claire. Cette étude se déroulera dans quatre régions du Cameroun à savoir l'Ouest, le Sud-Ouest, le Littoral et le Centre. Les risques liés à l'étude sont précisés ainsi que les mesures pour les éviter et les minimiser. La notice d'information et le formulaire de consentement éclairé, en français et en anglais, sont bien élaborés et simples à comprendre. Les mesures prises pour garantir la confidentialité des données collectées sont présentes dans le document. Les CVs des Investigateurs les décrivent comme des personnes compétentes, capables de mener à bien cette étude. Pour toutes ces raisons, le Comité National d'Ethique approuve pour une durée d'un an, la mise en œuvre de la présente version du protocole.

Les Investigateurs sont responsables du respect scrupuleux du protocole approuvé et ne devraient y apporter aucun amendement aussi mineur soit-il, sans avis favorable du CNERSH. Les investigateurs sont appelés à collaborer pour toute descente du CNERSH pour le suivi de la mise en œuvre du protocole approuvé. Le rapport final du projet devra être soumis au CNERSH et aux autorités sanitaires du Cameroun.

La présente clairance peut être retirée en cas de non-respect de la réglementation en vigueur et des recommandations susmentionnées.

En foi de quoi, la présente clairance éthique est délivrée pour servir et valoir ce que de droit.

Ampliations

MINSANTE

Le Président

Pr MBACHAM Wilfred

Wilfred Fon Mbacham, ScD
Molecular Biology and Biotechnology

N.B : cette clairance éthique ne vous dispense pas de l'autorisation administrative de recherche (AAR), exigée pour mener cette étude sur le territoire camerounais. Cette dernière vous sera délivrée par le Ministère de la Santé Publique.

- N° d'enregistrement : IORG0007861-IRB00009439-FWA00016054 - setcominae@gmail.com
- Arrêté N° 0977/A/MSP/SESP/SG/DROS du 18 avril 2012 portant création, organisation et fonctionnement des Comités d'Ethique de la Recherche pour la Santé Humaine au sein des structures relevant du ministère en charge de la santé publique.