PARASITE GENE EXPRESSION AND EVIDENCE OF EMERGING DRUG RESISTANT FALCIPARUM MALARIA INFECTION IN HEALTH FACILITIES IN BENIN METROPOLIS

BY

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CERTIFICATION

We the undersigned certify that NWALI MARY CHINENYE carried out this work, in the Department of Medical Biochemistry, University of Benin, Benin City and we approve same as adequate in scope and quality for the award of Masters of Science Degree (M.Sc) in Medical Biochemistry.

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DEDICATION

This work is dedicated to God Almighty, my Parent and my lovely husband.

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My sincere gratitude goes to God Almighty, the God who always comes through for me. I appreciate God for the grace to commence my postgraduate education and for His sustenance all through my studies.

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ABSTRACT

Malaria remains a significant public health challenge, particularly in tropical and subtropical regions such as Nigeria, prompting global efforts for a definitive cure and complete eradication. The search for a lasting cure and total eradication of malaria has become a major concern of the World Health Organization (WHO). However, antimalarial drug resistance has emerged as one of the most serious challenges to malaria control today surpassing the pace of new antimalaria drug development.Several concerns have been raised regarding the resistance observed in artemisinin combination therapy (ACT), the predominant treatment for malaria. The efficacy of ACT is been threatened due to reported cases of resistance, and if this resistance spreads, it could pose a significant threat to Africa, where malaria is prevalent. Hence, monitoring the emergence and spread of this resistance is crucial, particularly in malaria-prone regions like Nigeria. This study investigates the possible emergence of *Plasmodium falciparum* malaria infection resistance to artemisinin combination therapies (ACTs) drugs, currently used in the treatment of malaria in Benin City. Additionally, it examines the expression of the PFK13 gene, which is known for its association with artemisinin resistance. Two categories of artemisinin combination therapy, Lonart (artemether and lumefantrine) and Artequin (artemether and mefloquine), were employed. A cross sectional study of subjects with Lonart resistant malaria and artequin resistant malaria in Benin metropolis was carried out and the resulting resistant effects were documented. Clinical investigation of the artemisinin resistant malaria patients alongside with full blood count parameters such as packed cell volume, white blood cell count, neutrophils, eosinophils and lymphocytes were assessed and compared to a control group. Subsequently, subjects received quinine infusion and injections, and the same parameters were evaluated post-treatment and compared with the control. Gene expression analysis of the PFK13 gene was performed for both Lonart and Artequin-resistant malaria parasite in the blood of the patients with malaria parasite,

and the findings were compared with control groups. Results indicated lower packed cell volume and white blood cell values in subjects compared to the control group. However, after treatment with quinine infusion and injection, no significant differences were observed compared to the control group. PFK13 gene expression was higher in Lonart and Artequin-resistant malaria parasite subjects compared to the control groups. The study suggests the potential emergence of artemisinin resistance in Benin City. Nevertheless, further investigations, particularly into PFK13 gene mutations, will provide accurate marker for potential artemisinin resistance.

CHAPTER ONE

1.0 INTRODUCTION

Malaria has been recognized as a severe health concern since ancient times, and it continues to be one of the world's most dangerous illnesses, with high morbidity and mortality rates in people and other animals (Andrews *et al*., 2014). This disease spreads through mosquito bites caused by five protozoan parasites viz; *Plasmodium falciparum*, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, and Plasmodium knowlesi of the Plasmodium genus (Hedrick, 2011). Human infection occurs in the entire parasite (WHO 2021). Nevertheless, the most prevalent species of malaria are *Plasmodium falciparum* and Plasmodium vivax, with *Plasmodium falciparum* being the most virulent (WHO 2015). In fact, *Plasmodium falciparum* infections contribute to over 90% of global malaria-related deaths, representing a significant public health burden (Snow, 2015).

World Malaria report 2019 stated that the global incidence of malaria in 2018 reached 228 million cases, leading to 405,000 fatalities, with children under the age of five having the highest proportion (WHO 2019). Malaria is prevalent in over 90 countries, impacting over 40% of the global population (WHO 2019). In 2020, there are expected to be 241 million malaria cases and 627,000 global malaria deaths, up from 227 million cases in 2019—a 14 million case and 69,000 death rise from the previous year. Approximately 95% of global malaria incidence and mortality occur in Africa followed by Southeast Asia (7%), and the Eastern Mediterranean (2%), (Alonso and Noor, 2017, WHO 2017, WHO 2021).Despite an overall reduction in the prevalence of the disease worldwide, *Plasmodium falciparum* malaria continues to claim around 500,000 lives annually. Concerningly, the decline in malaria-related deaths has come to a halt since 2015, with

a notable increase attributed to the impact of the Covid-19 outbreak (WHO 2021). While malaria is a global public health concern, the tropics and subtropics bear the brunt of the disease, housing the majority of cases and fatalities (WHO, 2017).

An infected female Anopheles mosquito is the primary vector for humans, infecting them with sporozoites while feeding on blood, the same species of anopheles mosquitoes also act as a vector for transmission. After inoculation, parasites circulate in the blood and develop in the liver after undergoing a cycle of development. The typical signs and symptoms, such as fever and headache are as a result of parasites infecting and multiplying inside red blood cells (Getu *et al.,* 2022). Malaria could be fatal if untreated due to the disruption of the blood supply to vital organs. The disease is classified as fatal primarily due to the sequestration of P. falciparum in tissues, the upregulation of cytokines and other toxic substances, and the lack of access to effective antimalarial therapy (Milner, 2018). Human Plasmodium species share a common life cycle, commencing with liver development and concluding with subsequent proliferation in the host's bloodstream. Moreover, they demonstrate analogous susceptibility to certain antimalarial drugs such as quinine, chloroquine, and artemisinin, along with the emergence of drug resistance to these medications (Haldar *et al.,* 2018). The resistance to antimalarial drugs has become a significant obstacle in the efforts to control malaria, and its prevalence is surpassing the rate at which new antimalarial drugs are being developed for practical clinical application. Antimalarial drug resistance refers to the capacity of a particular strain of parasites to persist or reproduce even when a drug is given at dosages that are equivalent to or greater than the typically recommended amounts, but still within the patient's tolerance (WHO 2020). The development of resistance to antimalarial drugs can significantly compromise the efficacy of chemotherapy, result in a recurrence of malaria, and disrupt efforts to eliminate the disease. Consequently,

there has been significant focus on resistance surveillance to safeguard the recent progress. Drug resistance develops as a result of genetic mutations that occur spontaneously with a probability of 1 in 1012 parasites, an estimate that can happen easily in a patient who is hyperparasitemic (White, 2004). Artemisinin resistance in clinical settings, characterized by delayed parasite clearance, was initially identified in Pailin, Western Cambodia, in 2009 (Dondorp *et al.,* 2017). This resistance is predominantly influenced by mutations in the propeller domain of P. falciparum chromosome 13 (K13-propeller) in Southeast Asia, featuring 5 validated single nucleotide polymorphisms (SNPs) to date (N458Y, Y493H, R539T, I543T, and C580Y) (WHO 2017, Straimer *et al.,* 2017). The C580Y SNP has also been documented in Africa (WHO 2017). It is advisable to use molecular surveillance of K13-propeller polymorphism as an additional approach to determine the existence of artemisinin resistance in areas impacted by the malaria epidemic. The development of resistance to artemisinin-based combination therapy (ACT) partner drugs has led to treatment failures with various ACTs, including artemether-lumefantrine, artesunate-mefloquine, and artesunate-amodiaquine (Menard and Dondorp 2017, Leang *et al.,* 2013). Therefore, it is essential to systematically monitor the emergence and spread of resistance to artemisinin and partner drugs. This surveillance is crucial for informing public health interventions and ensuring appropriate administration at the local government level.

1.2 STAEMENT OF THE PROBLEM

The success of malaria prevention, control and cure is contingent on the sustained clinical efficacy of first-line artemisinin based combination therapy, for which the emergence and spread of drug resistance poses a constant threat (Blasco *et al.,* 2017). Treatment of falciparum malaria is heavily reliant on artemisinins based combination therapy. At present, No replacement antimalarial drugs with safety and efficacy profiles comparable with ACTs are available, and we

are unaware of any alternatives that are likely to be registered soon. Thus, a major health crisis is feared if the current decreased susceptibility phenotype progresses to full-blown resistance and/or becomes widespread in Africa, where most deaths occur. Even a modest (30%) increase in the failure rate of ACTs in Africa is predicted to increase the mortality rate by about 20% (Lubell *et al.,* 2014). Unfortunately, decreased susceptibility to the artemisinin drug class and resistance-associated mutations is now widespread across Southeast Asia. Resistance is currently limited in geographic spread and results in only partial loss of efficacy. Most cases of resistances were reported in Thailand-Cambodia border and other regions in Southeast Asia. Reports of reduced susceptibility to artemisinin and artemisinins based combination therapy and limited mutations in the Kelch 13 gene failure are also increasing in Africa. Several studies have been conducted in African countries such as Uganda, Niger republic, Ethopia, Kenya, Malawi, Nigeria among others to establish the presence of the kelch13 mutations (Abubakar *et al.,* 2020)

Nigeria accounts for 25% of global cases of malaria and an estimated 50% of the country's population suffer at least one episode of malaria every year (WHO 2018). Studies indicate that malaria transmission is still high with recent studies from regions in Nigeria reporting high mutations in genes that modulate response to nonartemisinin drugs by P. falciparum (Dokunmu *et al.,* 2018). Thus, any potential impact of artemisinin resistance on the ongoing control programme that relies on artemisinin-based combination therapies would have catastrophic consequences on the global malaria elimination target. Despite the need to regularly survey for the emergence of these pfk13 mutant alleles in different malaria-endemic regions of Nigeria, only a small number of systematic molecular epidemiological studies on field P. falciparum isolates have so far been conducted. A few studies have reported cases with delayed response to ACTs, and a sporadic scan for amino acid mutations in the pfk13 gene identified three

nonsynonymous mutations (G592R, Q613H, and G665S) and other synonymous mutations (Ajogbsile *et al.,* 2022). Surveillance of resistance marker genes is very important so as to promptly detect/identify and respond to emerging resistance, thus continuous monitoring of parasite responses to individual components of the recommended ACTs should indicate any early emergence of resistance and serve to preserve the efficiency of available anti-malarials in endemic areas. This study aims to investigate the emergence of artemisinin resistant P.falciparum malaria and evaluate the expression of PFK13 gene in Benin metropolis.

1.3 JUSTIFICATION OF STUDY.

Since the introduction and recommendation of Artemisinin based combination therapy for treatment of malaria infection by WHO in the year 2005 (Abubakar *etal.,* 2020), there has been remarkable reduction in global malaria incidence, mortality and morbidity, but a worrisome slowing-down trend has been observed since 2015. In 2019, malaria accounted for an estimated 229 million cases and 409,000 deaths globally (WHO, 2020) and Plasmodium falciparum is responsible for 97% of the global malaria incidence. Since an effective vaccine is not yet available, Artemisinin-based combination therapies (ACTs) have become the mainstay for malaria treatment in almost all malaria endemic settings. However, drug resistance is a recurrent problem, and the introduction of this new antimalarial treatment has been followed by reports of resistance development. Currently, Plasmodium falciparum has developed clinical resistance to the frontline antimalarial drug- artemisinin-based combination therapies (ACTs) leading to malaria resurgence and a major threat to the malaria elimination campaign running across the

globe. Therefore, there is an urgent need to strengthen and expand current resistance surveillance systems to track the emergence or spread of artemisinin resistance (Siddiqui *et al.,*2021).

The genetic background of the parasite may be a predisposing factor among several other factors accelerating the development of resistance to new drugs. Mutations in six DNA repair genes were identified in Artemisinin-resistant parasites from south east Asia, which may enable the parasites to deal with DNA damage more effectively, allowing these parasites to acquire resistance-conferring mutations (Miotto *et al.,* 2013, Xiong *et al.,* 2020). Since the first report of artemisinin resistance in western Cambodia and Thialand border in 2006 and 2015 respectively, artemisinin resistance has spread to other countries including Africa which is the epicenter of malaria pandemic (Siddiqui *et al.,*2021). The systematic identifcation and characterization of Plasmodium falciparum drug-resistant genes as part of surveillance intervention strategy is, therefore, very important for the eradication of resistance to aretemisinin based combination therapy (Ikegbunam *et al.,* 2019). In this present study, we investigate the emergence of artemisinin resistant P.falciparum malaria and evaluated the gene expression of the PFK13 gene that had been recognized to confer resistance to Plasmodium falciparum.

1.4 AIM OF RESEARCH

The aim of this study was to evaluate the possible emergence of *Plasmodium falciparum* malaria resistance to Artemisisnin-based combination therapy (ACT) and the evidence of parasite gene expression of the PFK13 gene that confers resistance to ACT drugs in the Benin metropolis, in the South-South region of Nigeria.

1.5 OBJECTIVE OF THE RESEARCH

- To determine the existance of Artemisinin-based combination (ACT) malaria, via positive malaria parasite test after treatment with ACTs
- To examine the status of their full blood count (FBC) after treatment with ACTs and after a follow-up treatment with quinine.
- To evaluate the parasite gene expression of PFK13 gene in the blood of patients with Lonart and artequin resistant malaria parasite.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Malaria Parasite

From time immemorial, medical professionals have been conducting research on malaria. It is considered as an important parasitic disease because it has significant health issues that have prompted global concern (Abigail *et al.,* 2021). The disease was formerly linked to swampy regions, thus the term malaria, derived from the Italian "mal'aria," which translates to "bad air." Dr. Ronald Ross, a British medical officer stationed in Hyderabad, India, made the significant finding that malaria is transmitted by mosquitoes towards the close of the 19th century. Charles Louis Alphonse Laveran, a French military surgeon, previously saw parasites in the bloodstream of a patient with malaria. Afterward, it was demonstrated by the Italian professor Giovanni Battista Grassi that only Anopheles mosquitoes could transmit human malaria (Tuteja, 2007). The malaria parasite is classified under the genus Plasmodium, which is the sole genus in the family Plasmodidae. It belongs to the order Haemosporida, class Coccidae, and phylum Sporozoa. There are more than 125 species in the genus that are responsible for causing malaria in mammals (Garba *et al.,* 2015). Several Plasmodium species, such as *Plasmodium falciparum*, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, and Plasmodium knowlesi, represent a risk to human health (WHO 2021). These species exhibit differences in their physical structure, immune system, geographical range, recurrence patterns, and reactions to drugs. *Plasmodium falciparum* is the primary cause of malaria-related deaths in young children in Africa and is responsible for severe and potentially deadly cases of malaria (Snow *et al.,* 2004). Plasmodium ovale, exclusively present in West Africa, has the lowest prevalence among malaria

parasites. On the other hand, Plasmodium malariae is more widely distributed but still occurs seldom. Plasmodium vivax is the most widespread malaria parasite, yet infections caused by this type are less lethal. While both Plasmodium vivax and *Plasmodium falciparum* can lead to anemia, Plasmodium vivax infections tend to induce mild anemia, whereas severe anemia caused by *Plasmodium falciparum* malaria is a significant cause of mortality in Africa.

2.2 Risk factors and Burden of Malaria parasite

The primary risk factors that contribute to the spread of malaria include demographic, environmental, and socioeconomic factors. Demographic elements encompass age and gender, whilst environmental factors encompass the presence or lack of vegetation such as bushes and forests, which contribute to the proliferation of mosquitoes. Temperature, humidity, and rainfall are climatic factors that might facilitate the swift proliferation and maturation of mosquito vectors. Finally, socioeconomic factors that directly impact human exposure and treatment patterns include education, occupation, and income (Awoselu *et al*., 2019). Other determining factors include population growth, urbanization, economic development, changes in migration patterns, etc (Cella, 2011). Malaria is most prevalent in areas of poverty and where disease detection, documentation, and reporting methods are inadequate (WHO 2014).

According to the World Health Organization (WHO), pregnant women, children, and people with compromised immune systems have the highest morbidity and mortality rates, and Africans bear the heaviest burden of malaria infection (WHO 2018). *Plasmodium falciparum*, the most common and pathogenic malaria parasite, is closely associated with severe illness and death, particularly in the WHO African region, where it constitutes 99.7% of malaria cases (Mace *et al.,* 2018). There were an estimated 228 million cases of malaria worldwide in 2018, according to the

WHO World Malaria Report 2019. *Plasmodium falciparum* is widespread in South-East Asia, the Eastern Mediterranean, and the Western Pacific. Children under five are the most vulnerable to malaria in high-transmission areas; they also account for 67% of all malaria-related deaths worldwide, along with primigravidae (Zekar and Sharman, 2022). The majority of malaria cases diagnosed in the United States of America are brought in from malaria-endemic countries (Gerstenlauer, 2019). The risk of infection is proportional to the length of exposure and the level of malaria transmission in the geographical region (Genton and D'Acremont, 2012). The Centers for Disease Control and Prevention (CDC) received 1517 cases of confirmed malaria in the United States in 2015, one of which was congenital, 1485 of which were imported from endemic countries, and 31 of which had an incomplete travel history. The 12.1% decline in imported malaria cases in 2015 aligns with the reduction in the number of cases imported from West Africa, a trend that may be attributed to changes in travel patterns due to the Ebola epidemic. Malaria transmission is intense in Nigeria; the disease is responsible for 60% of outpatient visits to health facilities, 30% of childhood deaths, 25% of death in children under one year, and 11% of maternal death. Malaria causes an estimated 132 billion naira in financial losses each year due to treatment costs, prevention, lost man hours, and so on (Kolawole *et al*., 2017).

2.3 Life cycle of Plasmodium Falciparum

Plasmodium species all have the same life cycle (Votpka *et al*., 2016). The two steps of the malaria transmission process are the initial infection of a person or vertebrate host and the subsequent transmission of the parasite to another host by an insect vector from the infected vertebrate host. Mosquitoes belonging to Anopheles genus are vectors responsible for transmitting the five Plasmodium species that naturally infect humans. Additionally, these

mosquitoes transmit other Plasmodium species infecting other mammals, while those infecting birds are transmitted by mosquitoes of different genera or other blood-sucking insects (Perkins, 2014). The onset of malaria infection begins with the sporozoite stage of the parasite, residing in the mosquito's salivary gland (Frischknecht *et al.,* 2017). This phenomenon takes place when an infected female mosquito bites an uninfected person while feeding on blood, introducing a small quantity of saliva into the wound on the skin. Male mosquitoes do not consume blood, therefore only female mosquitoes act as carriers. Saliva contains enzymes that possess antihemostatic and anti-inflammatory characteristics, which interrupt the process of blood clotting and suppress the sensation of pain. Usually, a single infected bite introduces between 5 and 200 sporozoites into the human host. Sporozoites spend a considerable amount of time in the skin before entering the bloodstream. Only the parasites that evade phagocytes can access the human bloodstream through a blood vessel, circulating for a brief period before infecting liver cells (Ejigiri and Sinnis, 2009).

2.3.1 Liver Stage in Man

The liver stage in humans involves the circulation of sporozoites in the bloodstream, their migration to the liver, and subsequent infection of a hepatocyte after traversing Kupffer cells and hepatocytes (Mota *et al.,* 2001). Upon reaching the liver, sporozoites undergo rapid expansion as they assimilate nutrients, eventually forming a large, spherical schizont. This schizont reproduces asexually through schizogony, a process characterized by multiple fissions and the generation of numerous small, spindle-shaped uninucleate cells known as merozoites (Sato, 2021). The preerythrocytic schizogony stage is identified by the release of merozoites into the venous channels or sinusoids of the liver following the rupture of schizonts. Merozoites are notable for their inherent immunity and medication resistance. Merozoites reach the erythrocytic stage of

their life cycle after being released into the bloodstream, at which time they do not exhibit any outward signs of the disease. Asexual reproduction allows a single schizont to produce thousands of merozoites. The development and rupture of schizonts in *Plasmodium falciparum* and Plasmodium malariae happen quickly, and merozoites can start erythrocyte invasion one to two weeks after hepatocyte invasion. On the other hand, Plasmodium ovale and P. vivax can remain as dormant cells (hypnozoites) in the liver for months or years before reverting to the schizont form and causing a relapse of the initial infection (Markus, 2015). Merozoites infiltrate erythrocytes using proteins derived from both parasites and red blood cells (RBCs). Notably, erythrocyte band 3 protein binds to major merozoite surface proteins (MSP)-1 and -9. Merozoites direct their apical membrane antigen-1 transmembrane protein towards the erythrocyte surface and penetrate the RBC via erythrocyte binding antigens and P. falciparum reticulocyte-binding homologs, which bind glycophorins and other unknown receptors. Merozoites initiate the blood stage of the asexual cycle by invading erythrocytes, a process that takes approximately 48 hours to complete. Throughout this period, the parasites undergo distinct stages, including ring, trophozoite, and schizont forms, each characterized by a unique structure and stage-specific features (Giribaldi *et al.,* 2014).

2.3.2 Erythrocytic Stage in Man

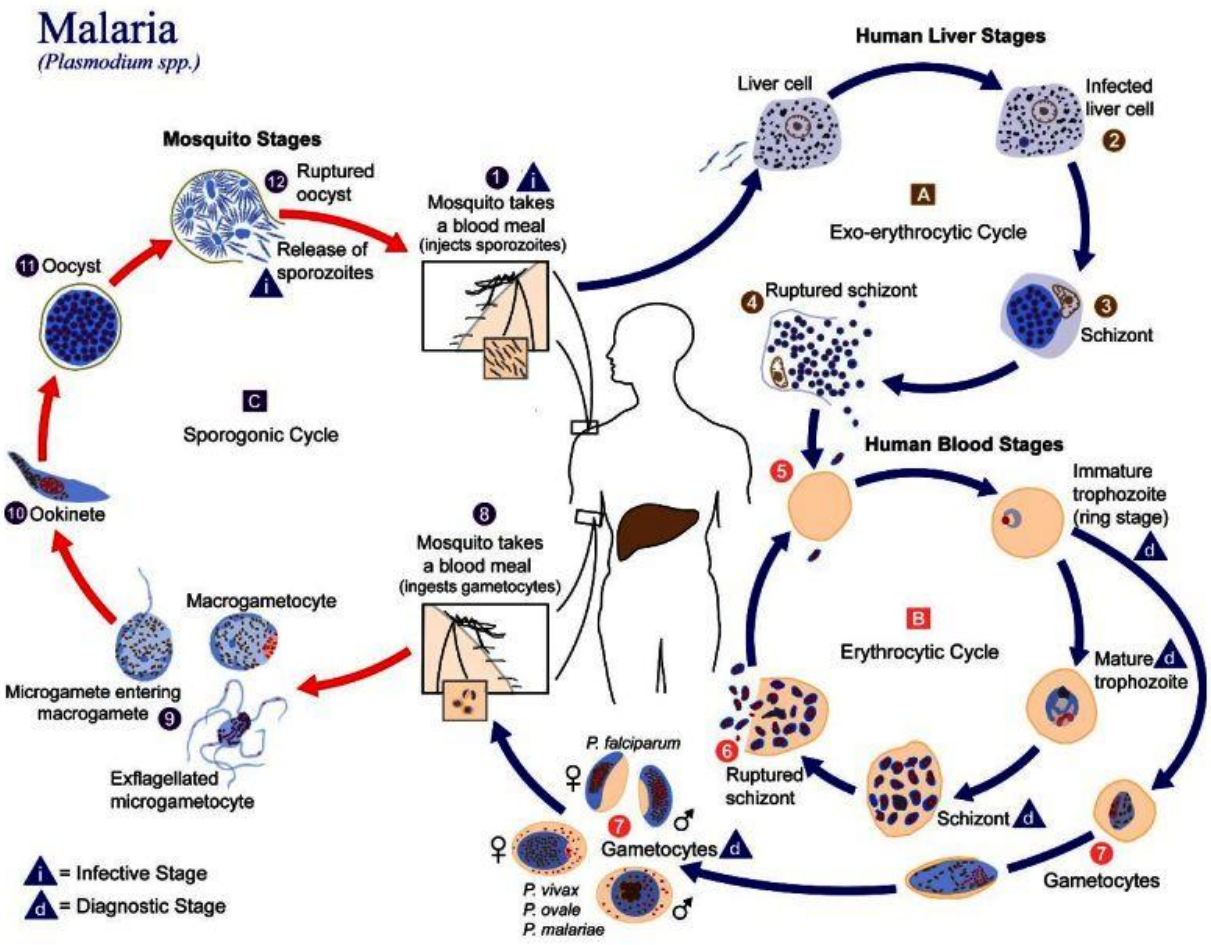
The sequence of events during the blood stage involves merozoites feeding on erythrocytes, rounding out, and gradually transforming into trophozoites. Trophozoites, in turn, undergo a transition into schizonts. Fully developed trophozoites are represented by schizonts, which emerge after a growth period of approximately 36–40 hours. Schizonts facilitate the invasion of red blood cells (RBCs) through nuclear divisions and an intensive synthesis and assembly of molecules. Over the next 6 to 8 hours, the schizont's nucleus divides, giving rise to 12 to 24

daughter nuclei that will ultimately develop into new merozoite cells within the erythrocyte. This stage of asexual reproduction is referred to as erythrocytic schizogony. The protease-dependent process finally leads to the lysis of both the parasitophorous vacuolar membrane and the RBC membrane. This causes the merozoites to break out of the RBC and disperse to additional erythrocytes. There are little merozoites, which are free and measure about 1.2 mm in length. As described by Bannister and Mitchell, these merozoites possess all the necessary tools for invading new red blood cells (RBCs). The completion of one erythrocytic cycle takes 48 hours. After spending about 60 seconds in the bloodstream, the parasite enters another erythrocyte, initiating the process anew (Giribaldi *et al.,* 2014).

2.3.3 Life Cycle in Mosquito

Gametocytes migrate with blood when a female Anopheles mosquito feeds on an infected person's blood, arriving at the mosquito's stomach where gametes are produced. Only gametocytes survive in the stomach, while other parasite forms and erythrocytes are digested. Microgametocytes (male) and macrogametocytes (female) are produced. Active microgametocytes undergo division of their nucleus, generating 6-8 haploid daughter nuclei arranged around the periphery. The cytoplasm produces flagella-like projections, each containing a daughter nucleus, independently of the cytoplasm. This process, known as exflagellation, results in the formation of 6-8 active microgametes from each microgametocyte. Meanwhile, the megagametocyte undergoes reorganization to give rise to megagametes. The female and male gametes fertilize quickly, producing a zygote that is dormant for a while before growing into an ookinete or vermicule, which resembles a worm. There are only two diploid stages: the zygote and the ookinete. The ookinete enters the stomach through the wall and settles behind the outer

layer of epithelium. There, it becomes encased in a cyst that is partially made of the zygote and partially made of the stomach of the mosquito; this encysted zygote is known as an oocyst. The oocyst absorbs nutrients and grows five times its original size, appearing as transparent rounded structures protruding from the stomach's surface. During the maturation process, which takes place over a period of 1-3 weeks, there are several nuclear divisions that occur. As a result of these divisions, the oocyst is divided into several haploid sporozoites. Every oocyst can potentially hold a multitude of sporozoites, which congregate in the vicinity of vacuoles. The process of asexual reproduction at this particular stage is referred to as sporogony. The complete sexual cycle in mosquitoes lasts for a duration of 10-21 days. Ultimately, the oocyst ruptures, liberating sporozoites into the mosquito's hemolymph. The sporozoites disperse throughout the hemolymph, ultimately reaching the salivary glands and infiltrating the hypopharyngeal duct. When the mosquito bites, the inoculation or injection of sporozoites into human blood initiates a new life cycle. A single infected mosquito may contain up to 200,000 sporozoites (Giribaldi *et al.,* 2014).



Life cycle of malaria parasite: source: MSD Manual 2020

2.4Hematological profile in malaria parasite

Haematological changes associated with malaria infection exhibit variability influenced by factors such as the level of malaria endemicity, background haemoglobinopathy, demographic variables, and the individual's malaria immunity. The ability to predict these haematological alterations is crucial for clinicians to implement effective and early therapeutic interventions, thereby preventing the occurrence of major complications. Haematology parameters play a crucial role in guiding presumptive treatment, particularly when the results of parasitological

examinations are delayed or uncertain, aiding in intensive patient care and preventing potential fatalities resulting from complications (Haruna *et al.,* 2013).The hematological changes that occur during the asexual stage of the malaria parasite's life cycle are strongly associated with the metabolic changes that take place. Malaria-infected individuals typically display a marked decrease in platelet, leukocyte, lymphocyte, eosinophil, red blood cell, and hemoglobin (Hb) levels, while the counts of monocytes and neutrophils are noticeably elevated in comparison to non-malaria-infected patients. Anaemia, leukopenia, and thrombocytopenia are frequently observed in P. falciparum infection, likely due to the higher levels of parasitemia found in these cases (Latif and Jamal 2015, Chianura *et al.,* 2012).

2.5. **Effect of malaria infection on full blood count**

White blood cell counts during malaria typically range from low to normal, reaching their minimum levels around the onset of fever and when the infection becomes detectable through microscopy. An increase in white blood cell count, known as leukocytosis, particularly in the neutrophil count, may indicate concurrent infections or a poor prognosis, although this is not always the case. The apparent decrease in neutrophil count (neutropenia), with absolute neutrophil counts falling below 1000 cells/μL, can be attributed to various factors such as a shift of neutrophils from circulation to sites of inflammation, the presence of serum lymphotoxic factors, localization of neutrophils in the spleen, and intercurrent bacterial infections. Studies on P. vivax infection suggest that neutropenia may result from the rapid release of granulocytes from the bone marrow into the blood (left shift), along with a shift of neutrophils from circulation to enlarged marginal cell pools (McKenzie *et al.,* 2005). Apoptosis of lymphocytes due to malaria infection has also been suggested. IL-12 has been implicated in the development

of malarial pancytopenia, including leukopenia. Malarial toxins, such as glycosylphosphatidylinositol, directly affect monocytes and macrophages, leading to the release of pro-inflammatory cytokines that can suppress hematopoiesis and cause dyserythropoiesis. Macrophage function is also altered by the ingestion of malaria pigment released during schizogony, which inhibits macrophage and dendritic cell functions and can suppress erythropoiesis (Giribaldi *et al.,* 2004).

White blood cell counts have been used to estimate parasitemia microscopically, and automated detection of malaria pigments in white blood cells has been employed for malaria diagnosis. Regardless of the method, monitoring the trend of white blood cell counts over time and with treatment can provide valuable guidance for managing the disease (Giribaldi *et al.,* 2019).

2.6 Antimalarial drugs

In 2019, Ross and Fidock explained that antimalarial medicines used to clinically treat human malaria are classified into five categories based on their structural backbone and apparent effect. The classes of drugs are as follows: (1) endoperoxides (such as artemisinin and its derivatives), (2) 4-aminoquinolines (chloroquine), aryl-amino alcohols (quinine, mefloquine), (3) antifolates (pyrimethamine, proguanil, sulfadoxine), (4) naphthoquinones (atovaquone), and (5) 8-aminoquinolines (primaquine, tafenoquine). The main targets of inhibition by these categories are different. 4-Aminoquinolines, antifolates, and naphthoquinones specifically inhibit the process of haem detoxification from digested hemoglobin, pyrimidine biosynthesis, and the oxidoreduction function of mitochondrial cytochrome b, respectively. Endoperoxides, like artemisinin, seem to block the same metabolic pathways as inhibitors of the aryl amino alcohol

class, whereas inhibitors of the aryl amino alcohol class tend to hinder the same metabolism as 4-aminoquinolines. Quinine, the initial pharmaceutical employed for treating malaria, was unearthed throughout the seventeenth century from the bark of South American quina-quina trees (Cinchona spp.), which harbors an antimalarial substance. Subsequently, quinine, a compound, has been employed for the treatment of malaria (Achan *et al.,* 2011). Efforts to chemically synthesize pure compounds with antimalarial activity date back to the nineteenth century. Notably, some clinically effective synthetic antimalarials, including chloroquine, emerged as available options in the 1930s. Following the discovery of quinine, a number of other natural and synthetic compounds such as chloroquine, mefloquine, sulfadoxine, and pyrimethamine were developed. However, as time passed, parasite strains developed resistance to these drugs, rendering them less effective. Drug resistance first appeared in Asia and South America, and then spread to Africa. As resistance worsened, morbidity and mortality increased. It became necessary to substitute effective drugs for the failing chloroquine and sulfadoxine-pyrimethamine. Indeed, the pursuit of antimalarial compounds led to the discovery of artemisinin and its derivatives. The efficacy of these drugs has been established, leading to their endorsement by the World Health Organization (WHO) for the management of uncomplicated falciparum malaria since 2006.

2.7 Artemisinin

The synthesis of artemisinin from Artemisia annua, a plant frequently employed in traditional Chinese medicine, was first performed in 1971 by Tu Youyou. She received the Nobel Prize in Medicine in 2015 for her significant role in the discovery of artemisinin. Artemisinin, which was originally isolated from the plant Artemisia annua, as well as its semi-synthetic derivatives such

as artemether, artesunate, and dihydroartemisinin, belong to this class of potent medications known for their rapid reduction of Plasmodium parasites in the blood of malaria patients (Ouji *et al.,* 2018). It is worth noting that all kinds of multi-drug-resistant *Plasmodium falciparum* are susceptible to artemisinin.Arteether, artesunate, and artemether are the most widely used derivatives of artemisinin. Prodrugs are these semi-synthetic derivatives which are converted to the active metabolite dihydroartemisinin. Artemisinins have been instrumental in combating malaria, especially via Artemisinin-based Combination Therapies (ACT), which comprise the vast majority of current treatments. These compounds are distinct from other antimalarials due to their ability to eliminate parasitemia rapidly. Their effectiveness stems from their unique capability to target both early and late erythrocytic parasite stages. Unlike many antimalarial medications, artemisinins act on the ring stage forms, preventing their maturation and sequestration in blood vessels. This mechanism allows the parasites to be expelled from host red blood cells and removed from circulation, inhibiting the parasites' capacity to cling to endothelium cells,, blood cells, and platelets, which is important in malaria pathogenesis.Adducts of haem and artemisinin are formed when artemisinins undergo a chemical reaction with the hazardous haem substance produced when hemoglobin is degraded in the vacuole of the parasite that is feeding. The aforementioned adducts inhibit haem detoxification and haemozoin polymerization by interacting with the proteins of *Plasmodium falciparum*; consequently, haem accumulates. Artemisinins also cause parasite protein alkylation, inducing oxidative stress and resulting in irreversible damage and parasite death. Artemisinins play a vital role in decreasing the quantity of gametocytes, which are the sexual-stage parasites responsible for transmitting the parasite to the Anopheles mosquito vector. This reduction occurs directly and indirectly through the decrease in the asexual parasite population, the source of new gametocytes. These

combinations, which include artemisinins and another antimalarial drug with a longer plasma half-life, enhance efficacy. Currently, five ACTs are in use globally. In 2016, 409 million ACT-based treatments were administered worldwide, making them the most effective antimalarial medicines available. The efficacy of artemisinins is not well established during the initial trimester of pregnancy; therefore, the World Health Organization (WHO) does not advise their utilization during this time. However, studies have demonstrated that its administration throughout the second and third trimesters is deemed safe and does not entail higher risks compared to other antimalarial medications in these periods.

2.7.1 Pharmacology of Artemisinin-based combination therapy: Artemether and lumenfathrine (Lonart).

The antimalarial properties of artemether, an artemisinin derivative, result from disruption of the mitochondrial function of the parasite, inhibition of angiogenesis, and manipulation of host immune function (WHO 2011). Artemether absorbs very quickly when taken orally and reaches peak plasma concentrations in just two hours. It has a half-life of 1–3 hours. It is quickly converted to the more effective antimalarial metabolite DHA by the enzymes CYP450 2B6, CYP450 3A4, and possibly CYP450 2A6. DHA is then primarily glucuronidated by the enzymes UGT1A1, 1A8/9, and 2B7 to produce inactive metabolites (WHO 2011), Within two to three hours of administration, DHA reaches its peak plasma concentration. Artemether swiftly eliminates malaria parasites from the bloodstream. Artemether and DHA has potent antimalarial activities, leading to a substantial reduction in the amount of asexual parasites by at least 10,000 times (4 log) every reproductive cycle. Additionally, they prompt a rapid disappearance of symptoms. Its short -elimination half-life is caused by the CYP3A system, which is the main metabolic pathway acting on artemether. This system causes rapid clearance and elimination of

artemether and DHA from the serum. The bile/fecal pathways and the urine are both used for the excretion of the metabolic byproducts of artemether and DHA (WHO 2011). While artemether causes rapid defervescence, parasite clearance, and clinical improvement when used alone (WHO 2011, Pauline *et al*., 2011), it also has a relatively high recrudescence rate, necessitating a combination therapy. Lumefantrine is an aryl-amino alcohol that inhibits the detoxification of haem, leading to the accumulation of toxic haem and free radicals, which are detrimental to the parasite. Lumefantrine is very slowly absorbed; it begins to absorb 2 hours after oral administration and reaches peak plasma concentration 3–4 hours later. It prevents reoccurring malaria parasitemia and has a half life of 3-6 days. Following the elimination of artemether and DHA from the body, their absorption and clearance occur gradually, acting to get rid of any remaining parasites and prevent recrudescence. After ingestion, serum high-density lipoproteins bind to lumefantrine to a degree of 99%. Lumefantrine has a significantly longer half-life of elimination than artemether, which enables it to stay in the body longer and lessen the chance of parasite regrowth following the rapid parasite biomass reduction brought on by artemether (WHO, 2011). Lumefantrine is metabolized primarily by CYP450 3A4 to desbutyl-lumefantrine, which has a 5-8-fold higher antiparasitic effect than lumefantrine (Pauline *et al*., 2011)

2.7.2 Pharmacology of Artemisinin-based combination therapy: Artesunate and Mefloquin (Artequin)

Artequin is a combination of artesunate and mefloquin. Artesunate is a derivative of artemisinin obtained from Artemisia annua. Mefloquine is a 4quinoline methanol antimalarial medication that is effective against treatment-resistant *Plasmodium falciparum* (Airton *et al.,* 2021). Mefloquine's mechanism of action is not totally known. According to some research, mefloquine

particularly targets the *Plasmodium falciparum* 80S ribosome, reducing protein synthesis and generating schizonticidal effects (Wong *et al.,* 2017). The combination of artesunate and mefloquin improves parasite clearance early on, reducing the likelihood of any remaining parasites surviving the residual action of mefloquine. The spread of parasite resistance to mefloquine may be halted by preventing the first high load of parasites from being exposed to mefloquine alone. The mefloquine-resistant malaria strains may be less spread due to the gametocidal effects of artesunate. When mefloquine is combined with artesunate, a practical short dose of artesunate can be used, lasting only three days as opposed to five or seven days when artesunate is used alone. Recrudescence rates of up to 37% have been observed with a five-day treatment of artesunate, compared to 7% when artesunate is combined with mefloquine. This suggests that the combination may also lower recrudescence rates (Bukirwa and Orton 2005).

2.8 Artemisinin resistance

It is well known that *Plasmodium falciparum* has a high potential for developing drug resistance. From the first reports of quinine resistance surfaced more than a century ago (Silva *et al.,* 2022), this parasite has demonstrated its ability to avoid the effects of practically every widely used antimalarial treatment. Since their widespread adoption during the first decade of the twenty-first century, artemisinin combination therapies (ACTs) have been the mainstay for the treatment of uncomplicated malaria. ACTs were developed to combat the phenomenon of drug resistance. The ACT combines an artemisinin (a fast-acting but rapidly-cleared) derivative with a long-acting partner drug like lumefantrine, amodiaquine, or mefloquine (WHO 2015). But despite the

fact that these treatments are typically very effective, reports point to an ongoing decline in effectiveness. This was first recognized for artesunate-mefloquine and artesunate-amodiaquine (Silva *et al.,* 2022). Furthermore, the recent failure of dihydroartemisinin-piperaquine (DHA-PPQ) therapy in Cambodia, just a few years after its formal implementation, demonstrates the parasite's remarkable resistance to drug pressure (Amato *et al*., 2017, Hamilton *et al*., 2019). Artemether-lumefantrine (AL) is the primary ACT in Africa, the worldwide malaria epicenter, and is included in virtually every national malaria program on the continent. It is the backbone of malaria clinical therapy, and it has shown remarkable resilience until lately. As a result, recent reports of decreased artemether-lumefantrine effectiveness have been met with a great deal of concern (Plucinski *et al*., 2017). Artemisinin resistance was identified along the Thailand-Cambodia border in 2008, subsequent to the administration of artesunate monotherapy (Ouji *et al.,* 2018). However, it appears that artemisinin resistance began to develop in 2001, long before the extensive implementation of ACTs in Cambodia (WHO 2015). Initially, research by Ouji et al. (2018) established a correlation between artemisinin resistances and diminished parasitic clearance following the initial three days of treatment with artemisinin monotherapy or ACTs. Additionally, resistance was associated with elevated rates of clinical failures due to heightened parasitic recrudescence. The observed correlation between an extended clearance half-life and heightened rates of in vivo recrudescence subsequent to artemisinin clearance provides strong evidence that the parasites are resistant to treatment with artemisinin and can persist in growth even after the drug is eliminated from the body. The parasite clearance half-life refers to the physiological time required for the level of parasitemia to decrease by 50% following the administration of an antimalarial dose. However, in the majority of cases where a delay in parasite clearance occurred following an ACT treatment, recovery from the ailment is possible so

long as the complementing medication remains effective. This explains why, despite the fact that resistance to artemisinin can facilitate the emergence of concurrent resistance to the companion drug; treatment failure is not always the consequence of delayed parasite clearance.Artemisinin resistance makes it impossible for parasites to replicate and multiply when being treated with other malaria treatments. Artemisinin resistance is predicated on the mechanism of entrance into quiescence, which is unique to the ring stage.The F32-ART5 parasite line, an extremely artemisinin-resistant strain that developed in vitro after five years of exposure to successively increasing concentrations of artemisinin and that eventually reached 7000-fold the IC50 value of the parental and sensitive F32-Tanzania strain, was used as an experimental model for this discovery. Later, isolates of *Plasmodium falciparum* from Cambodia confirmed this finding (Witkowski *et al.,* 2013, Ouji *et al.,* 2018).WHO-recommended standard in vitro chemosensitivity assays, which assess the effectiveness of antimalarial drugs in inhibiting parasite growth, are unsuitable for monitoring artemisinin resistance. This is due to the fact that artemisinin resistance results from the parasites entering a quiescent state when exposed to artemisinins (Paloque *et al.,* 2016). Ex vivo or in vitro artemisinin resistance can be demonstrated using the Ring stage Survival Assay (RSA0-3h), which is based on highly synchronized *Plasmodium falciparum* parasites exposed to 700 nM dihydroartemisinin for 6 hours at the ring stage, followed by 72 hours of culture under drug-free conditions until the microscopic read-out (Witkowski *et al.,* 2013).The entire genome sequences of the parasite strains F32-ART5 and F32-TEM revealed the existence of a mutation in the propeller domain of the Kelch protein 13 (K13) gene, which is linked to artemisinin resistance. While the precise function of this protein is unknown, it resembles the human Keap1 protein, which is involved in the physiological response to oxidative stress. K13 is identified in the parasite's reticulum

endoplasmic (Bhattacharjee *et al.,* 2018).In conclusion, P. falciparum Kelch 13 (PfK13) has been identified as the primary cause of artemisinin reduced susceptibility, which is clinically defined as an infection with a clearance halftime of >5h (Ouji *et al.,* 2018). Other factors may be enhancing the susceptibility (Veiga *et al*., 2014). According to the World Health Organization (WHO), artemisinin resistance is currently defined by three criteria: a significant delay in the time it takes for parasites to be cleared from the body during treatment, a high survival rate of parasites in the Ring stage Survival Assay (RSA0-3h) conducted either outside or inside the body, and genetic variations in the pfk13 gene (WHO 2018). Hence, it is crucial and imperative to conduct a survey on the occurrence and spread of artemisinin and partner medication resistance to inform public health interventions and ensure appropriate administration.

2.8.1 Causes of drug resistance

One of the biggest risks to public health worldwide is drug resistance, which has hampered the development of new pharmaceutical drugs and compromised the way diseases are treated (Holmes *et al.,* 2016). The causes of drug resistance are complex and may be rooted in practices of drug manufacturing process, health care professionals, and patients' behavior towards the use of drug as well as supply chains of drugs in the population (Ayukekbong *et al.,* 2017). Some of these factors may include:

1. Genetic factors: Microbes can change their genetic makeup spontaneously or as a result of exposure to certain medications, which allows them to alter or evade antimicrobial targets. Bacteria have adapted to their surroundings by random mutation across trillions of microbial generations, including the development of genes that code for mechanisms

of resistance to different medications and chemicals. (D'Costa *et al.,* 2011, Holmes *et al.,*2016). For example, viruses can mutate to evade antiviral agents designed to target their replication cycle, and parasites can change their metabolic pathways to avoid antiparasitic agents. Bacteria can mutate to change their cell walls or the enzymes that break down antibiotics (Naveed, 2020). Additionally, certain bacteria have the capacity to horizontally transfer to other organisms genes encoding for resistance mechanisms (Holmes *et al.,* 2016, Chang *et al.,*2015). Strong evolutionary selection pressures have been applied to microbes as a result of the dramatic, unprecedented rise in human interventions in the microbial world (particularly the widespread and excessive use of antimicrobial agents), which has led to the emergence, increased frequency, and persistence of resistant microbes in humans, animals, and the environment (Holmes *et al.,* 2016).

2. Inappropriate prescription practices: Healthcare providers are crucial for disease treatment and prevention, but their effectiveness can be compromised if their practices lack evidence-based approaches. In developing countries, limited access to current information due to ineffective surveillance systems and inadequate research dissemination can lead to outdated knowledge among health professionals. Consequently, they often resort to using broad-spectrum antibiotics for treating infections caused by various bacteria or when identifying the exact cause is challenging or time-consuming.This reliance on broad-spectrum antibiotics contributes to antibiotic resistance development by exerting selective pressure not only on the disease-causing agent but also on a significant portion of the patient's microbiota. Additionally, some healthcare

providers issue prescriptions without evidence-based support, relying instead on a syndromic approach. This involves treating infections based on recognizable signs, symptoms, and the microorganisms commonly associated with each syndrome. Unfortunately, this practice is becoming more common due to the lack of legal consequences for improper antibiotic prescriptions. Addressing these issues is vital for preserving antibiotic effectiveness and combating the increasing threat of antibiotic resistance (Ayukekbong *et al.,* 2017).

2.9 Artemisinin resistant gene

2.9.1Plasmodium falciparum Kelch 13 (PFK13) gene

In both laboratory trials and natural infections, point mutations in *Plasmodium falciparum* K13 (Kelch13) are the key genetic determinants linked with artemisinin resistance. These mutations predominantly, though not exclusively, occur in the beta-propeller domain of the K13 protein (Siddiqui *et al.,* 2021, Paloque *et al.,* 2022). The presence of these mutations allows a subset of early ring-stage parasites to survive the cell-cycle arrest induced by exposure to artemisinin, enabling these parasites to resume transcription and complete their intraerythrocytic developmental cycle once artemisinin is no longer present at inhibitory concentrations (Mok *et al.,* 2015, Barrett *et al.,* 2019, Mok *et al.,* 2021). In vitro resistance is typically defined by the survival of more than 1% of early ring-stage parasites exposed to 700 nM dihydroartemisinin (DHA), the primary active metabolite of artemisinin, for 6 hours, followed by drug-free culture incubation for an additional 66 hours (known as the RSA0–3 h assay) (Witkowski *et al.,* 2013). Resistant parasites display transcriptional diversity and varying stress-response gene expression (Nötzel and Kafsack 2022). The resistance mechanism entails an intricate interaction between

the amount of K13 protein, the process of hemoglobin endocytosis, and the stress response of the parasite. Mutations in the K13-propeller gene result in decreased levels of this crucial protein, most likely as a result of changes in its protein-folding properties and solubility. This decrease varies depending on background and developmental stage (Mok *et al.,* 2021). Downregulation of K13 protein levels results in reduced artemisinin sensitivity, while overexpression of either mutant or wild-type K13 resensitizes resistant parasites (Schumann *et al.,* 2021, Gnädig *et al.,* 2020, Liang *et al.,* 2022). K13 has been detected in multiple cellular compartments, such as the parasite plasma membrane, vesicular compartments, and the endoplasmic reticulum. K13 is localized at the hemoglobin-filled cytostomes' neck in the plasma membrane. These cytostomes are important for transferring host hemoglobin from the cytosol of red blood cells to the parasite's lysosome-like digesting vacuole. The genetic misplacement of K13 disrupts the movement of cytostomes and lowers the levels of peptides generated from hemoglobin. The interactome of K13-related proteins comprises many endocytosis proteins, including AP-2μ and the ubiquitin hydrolase UBP1, both of which are associated with artemisinin resistance. When interactome proteins are conditionally deactivated, the susceptibility to artemisinin is diminished, indicating that K13 is involved in clathrin-independent endocytosis and the uptake of hemoglobin. The resistance observed in the ring stage may be attributed to a reduction in the transportation of hemoglobin to the digestive vacuole, resulting in lower amounts of the pharmacological activator Fe2+ haem. Mutations in the K13 gene seem to facilitate artemisinin resistance by enhancing the inherent stress response, so enabling better management or restoration of cell damage caused by artemisininas have others that are thought to play a role in vesicular trafficking (coronin, falcipain 2) (Mok *et al*., 2021 Sharma *et al*., 2020). These findings highlight the importance of studying K13 and other potential artemisinin-resistance mediators,

especially given the recent emergence of resistance in Africa (Uwimana *et al*., 2021 Straimer *et al*., 2021).

2.9.2 Artemisinin partner drug resistant gene (PfMDR1 and PfCRT)

The main contributor to reduced susceptibility to artemisinin, clinically characterized as an infection with a clearance halftime of >5 hours, has been identified as the P. falciparum Kelch 13 (PfK13), with other factors possibly augmenting susceptibility (Straimer *et al.,* 2015; Silva *et al.,* 2022). Treatment failure, on the other hand, is more likely to be caused by the failure of the extended half-life companion medicine. As a result, it is critical to track and identify new signs of partner medication resistance. While the complete molecular basis of resistance is unknown, two transporters, P. falciparum multidrug resistance protein 1 (PfMDR1) and P. falciparum chloroquine resistance transporter (PfCRT), have been shown to play critical roles in the parasite's response to ACT partner drugs. PfMDR1, also known as the P-glycoprotein homologue (Pgh), is a 1419 amino acid protein with 12 transmembrane domains that belongs to the ATP-binding cassette (ABC) superfamily. PfMDR1 is oriented toward the digestive vacuole (DV) lumen and functions as an importer of solutes into this organelle, including antimalarials (Silva *et al.,* 2022). PfMDR1 single nucleotide polymorphisms (SNPs), particularly the N86Y allele, have been associated with in vivo and in vitro sensitivity to various quinoline antimalarials (e.g., mefloquine, lumefantrine, amodiaquine) and artemisinin derivatives (Veiga *et al.,* 2016; Gil and Krishna, 2017). The key factor responsible for chloroquine resistance has been found as PfCRT. PfCRT is a protein consisting of 424 amino acids and including 10 transmembrane domains. It belongs to the drug metabolite transport superfamily. It localizes in the DV membrane of the parasite and can pump antimalarial drugs out of this organelle (Silva *et*

al., 2022). The proposed physiological function of PfCRT is related to the export of host-derived peptides to the cytoplasm (Shafik *et al.,* 2020). The critical chloroquine resistance mutation K76T and other polymorphisms have been documented to influence parasite sensitivity to quinolines and artemisinin compounds (Silva *et al.,* 2022).In Southeast Asia and South America, several point mutations in the pfk13 gene (F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L, and C580Y) have been validated to correlate with clinical ART resistance and confirmed to confer elevated survival rates based on Ring-stage Survival Assays (RSA)0-3 h. Also, mutations and increased copy numbers of genes like P. falciparum multidrug resistant gene-1 (pfmdr1) and P. falciparum chloroquine resistance transporter (pfcrt) have been linked to resistance in artemisinin (and derivatives) partner drugs like lumefantrine, amodiaquine, mefloquine, and piperaquine (Kayode *et al.,* 2021). The effectiveness of ACTs may be jeopardized as a result of these alterations. Two confirmed mutations in the pfk13 gene (R561H and P574L) have been associated with laboratory resistance to ACTs in Rwanda, marking the first occurrence of such variants in Africa. Although it is necessary to regularly monitor the emergence of these pfk13 mutant alleles in various malaria-endemic regions of Nigeria, only a limited number of detailed molecular epidemiology studies have been carried out on field P. falciparum isolates so far. A sporadic screening for amino acid changes in the pfk13 gene discovered three nonsynonymous variants (G592R, Q613H, and G665S) and several synonymous mutations (Ajogbasile *et al.,* 2022).

The purpose of this study is to describe the potential emergence of artemisinin combination therapies resistance in the pfk13 gene of plasmodium falciparum parasite the blood of patients with malaria infection in in healthcare facilities in Benin metropolis.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 **Study Design and Setting**.

A facility-based cross-sectional study was conducted at Water-gate medical center, GRA, Faith Mediplex hospital, GRA and Sama poly clinic 1st East road both in Benin city, which is located in Edo state in the south-south region of Nigeria

3.2 **Sample size determination**

Sample size was calculated from the Cochran's formulae (Cochran, 1977)

$$N = \frac{Z^2pq}{d^2}$$

Where N = sample size

Z = Standard deviation set at 1.96

p = Prevalence of malaria

q = 1-p (Prevalence of malaria)

d = Precision level = 0.05

The prevalence of artemisinin resistance 3.5-8%

q = 1-p = 1-8%

q = 1-0.08 = 0.92

$$N = \frac{(1.96)^2 \times 0.08 \times 0.92}{(0.05)^2} = 113.1$$

Sample size (N) =113

10% Attrition ratio = 11.3

Sample size = 113+11= 124

Sample size = 124 samples per studies

No of studies = 4

124x4 = 496

Total sample size = 496

3.2 **Study population.**

The sample size was calculated using Cochrian formulae (1977), standard deviation set at 1.96, prevalence rate of 8%, precision level of 5%. Accordingly, the calculated sample size was 124, of the 124 sample size recruited for this study; only 100 met the inclusion criteria. Informed consent and the presence of P. falciparum malaria, as confirmed by laboratory data (parasite count ranging from 2,000 to 200,000 per µl blood) two weeks following treatment with

artemisinin-based combination therapies (lonart or artequin), are prerequisites for study participation.

Other inclusion criteria were:

1. The ability to be monitored for 7 days following treatment with artemisinin-based combination therapy.
2. The presence of febrile symptoms induced by *Plasmodium falciparum* malaria.
3. Lack of general risk signs or symptoms of severe and complicated P. falciparum malaria.
4. The fact that the patient had artemisinin-based combination therapy and is still experiencing malaria symptoms two weeks later.

The study excluded patients who presented with clinical symptoms consistent with severe or complicated malaria, as well as those who displayed any other symptoms or signs that were not caused by malaria. Patients who met the following criteria were also precluded from the study: those who experienced recurrent vomiting, diarrhea, malnutrition, pregnancy, resistance to medication, or failed to attend the required follow-up appointments on days 7 and 14. Patients who satisfied the eligibility criteria and presented at the health center laboratory for blood film examination were selected using a systematic random sampling technique. Included were febrile patients with clinical suspicion of malaria who were at least 5 years old and consented to take part in the research.

3.3 Ethical clearance

Ethical clearance (CMS/REC/2023/351) was approved by the research ethics committee, College of Medical Sciences, University of Benin, Benin City, Nigeria.

3.4 Materials

3.4.1 Apparatus and Equipment

Syringe, tourniquet, slides, microscope, capillary tube, haematocrit reader, EDTA bottles, cotton wool, counting chamber, cover slip, Centrifuge, haematology analyzer, hand gloves, vortex machine, blue light transilluminator, biofuge, pippete, water bath, UV/VIS spectrophotometer, Eppendof thermocycler.

Chemicals and Reagents

Giemsa stain**,** Lens cleaning fluid, Turk's solution, Leishman stain, Zymo Quick-DNA Miniprep plus kit, PCR master mix, PCR microtube, safeview classic, 100bp DNA ladder, Agarose gel, loading dye, TAE buffer.

3.5 Sample collection.

Blood sample for malaria parasite and full blood count was collected in an EDTA sample bottle.. 2ml of venous blood was utilized for these procedures. Blood sample for PFK13 gene expression was collected in a plain sample bottle containing DNA/RNA shield in the ratio of 0.5ml: 0.5ml and left to stand at room temperature until further analysis

3.6 Laboratory procedure

Malaria parasite test was carried out microscopically to confirm the presence of malaria using the thin and thick blood smear method. Blood slides were prepared and stained with 10% Giemsa,

microscopic abnormality of blood in smear and presence or absence of malaria was determined.All malaria positive blood samples and those of the control group were analyzed for blood cell counts. Blood counts were performed using an automated hematology analyzer, the Beckman Coulter counter (URIT-3300), following the manufacturer's instructions. The following hematological parameters were considered for this study:percentage of packed cell volume (PCV%), white blood cell counts (WBC), percentage of lymphocytes (LYM%) eosinophils (EOS cm^3), and percentage of neutrophils (NEUT%).

3.6.1 Malaria parasite test

The thick and thin blood films stained with Giemsa's stain which the gold standard for diagnosing malaria (WHO 2009, Norgan*et al.,* 2013) was used for this procedure. A small amount of peripheral blood is drawn from the patient's finger, placed on a glass slide, a thick and thin blood film was made using another slide as spreader the slide was the air dried for 15 minutes. The thick blood smears are used to detect the presence of plasmodium parasite while the thin blood smears are used to identify the Plasmodium species causing the infection and the developmental stages that are currently circulating in the patient's blood. The film was fixed by dipping into methanol and then stained with Giemsa’s stain. The slide was washed using clean water and was allowed to dry. The dried slide was then viewed under the microscope for the presence of malaria parasite and also for the stage of the parasite

3.6.2 Full blood count Procedure

3.6.2.1 Procedure for Packed Cell Volume (PCV)

A plain non heparinized capillary was used to collected blood sample while applying pressure on one end of the capillary tube to prevent the blood from flowing out, after which it was sealed with plasticine. The capillary tube placed inside the centrifuge machine and another capillary tube was placed in the opposite direction to balance the centrifuge, the sample was allowed to spin inside the centrifuge at 10,000 revolutions per minutes for 10 minutes causing the blood to separate into serum and red blood cell, the readings were taken thereafter.

Hematocrit= (height of RBC in mm/Height of RBC and plasma) x100

3.6.2.2 Procedure for White Blood Cell count (WBC)

A drop of blood and 19 drops of Turk's solution was added to a test tube and mixed for about 1 minute. The cover slip was then placed over the counting chamber and mixed solution is drawn with a pipette and placed on both side of the chamber and viewed under the microscope with X10 and X40 objective to count the white cells present in the 4 corner square ($1mm^2$) areas

3.6.2.2 Procedure for White Blood Cell Differntials

A thin of blood was made on a slide and was allowed to dry. The slide was thereafter flooded with leishman's stain for 2 minutes and distilled water for 8 minutes. It was then rinsed gently with water and was left to air dry for about 2 minutes. A drop of immersion oil was added and viewed under the microscope for the presence of Eosinophil, lymphocytes and neutrophils.

3. 7 Treatment and Post laboratory analysis

On the day of the first sample collection, Patients were treated with Quinine infusion and tablets for a period of 5days. Patient returned for follow-up on day 7. Blood sample were collected for post treatment malaria parasite test and analysis of full blood count parameters on day 14, after treatment with quinine

3.7 Parasite genomic DNA extraction

Blood samples were analyzed for parasite genomic DNA extraction in accordance with the manufacturer's protocol using the Zymo Quick-DNA Miniprep Plus kit (17062 Murphy Avenue, Irvine, California 192614, United States of America).

3.8Amplification of pfk13 gene

The pfk13 propeller domain was amplified by nested PCR. The primers utilized for the primary PCR were kelch-outer-F 50-gggaatctggtggtaacagc-30 and kelch-outer-R 50-cggagtgaccaaatctggga-30. For the nested PCR, the primers were kelch-inner-F 50-gccaagctgttgaaagaagcaga-30 and kelch-inner-R 50-gccaagctgccattcatttg-30. The 849-bp nested PCR product will correspond to codons 427–709 of the PF3D7_1343700 K13 propeller domain, which contain mutations associated with delayed parasite clearance (representing nucleotide sequence 1279–2127). In the primary reaction, the PCR solution was reconstituted to a final volume of 20 µL. For amplification, 2 µL of DNA was subjected to 0.5 µM of each primer. The cycling conditions consisted of 95˚C for duration of one minute, followed by 35 cycles at 60˚C for one minute, 58˚C for 20 seconds, and 95˚C for 20 seconds; the final extension lasted for three minutes at 60˚C. An additional 0.5 M of each primer was utilized to amplify one microlitre of the

primary reaction in the nested PCR procedure. The cycling regimen consisted of an initial 5 minutes at 95 ˚C, followed by 35 cycles at 56 ˚C for 20 seconds, 95 ˚C for 20 seconds, and 60 C for 1 minute; the exercise concluded with a final extension at 60 C for 3 minutes. To validate amplification, nested amplicons were subjected to electrophoresis on a 2% agarose gel.

3.9 Statistical analysis.

Statistical package SPSS version 20(IBM corporation, NY) for windows was used to analyze the data obtained from this study. Results obtained were expressed as mean ± SEM (Standard Error of Mean). Differences among the means were determined by one-way analysis of variance (ANOVA). Values were considered statistically significant if P-value is less than 0.05 ($P< 0.05$). LSD Post Hoc test was used to determine where the significance lay.

CHAPTER FOUR

RESULT

4.0 Analysis of full blood count status of patients with artemisinin drug resistant malaria

The results of the full blood count status of artemisinin drug resistant malaria patients who attended some healthcare facilities in Benin City after treatment with ACTs (Lonart and Artequin) and after a follow-up treatment with quinine are presented below. Out of the 123 patients who were recruited for each of the studies, 100 of them tested positive for malaria after the initial treatment with Lonart or Artequin. A follow up treatment with quinine infusion and tablets was given to these patients for a period of 5 days and their blood samples were collected for post treatment malaria parasite test and analysis of full blood count parameters on day 14, after treatment with quinine. The results of their full blood count status by gender and age were also presented in the tables below. Table 4.7 shows the level of expression of the plasmodium falciparum parasite PFK13 gene in the blood of patients with artemisinin drug resistance malaria.

4.1 Full blood count of patients with lonart resistant malaria

In table 4.1, there was difference in the PCV value before treatment compared to the control group although this difference was not statistically significant, but no significant difference in the PCV value after treatment compared to the control group. There was difference in the WBC value before and after treatment compared to the control group although this difference was not statistically significant. There was no significant difference in the values of lymphoctyes, eosinophils, before treatment and after treatment, when compared to the control groups. The values of neutrophils before and after treatment showed statistically significant difference compared to the control group at ($P<0.05$).

In table 4.2, for the male gender, there was difference in the PCV value before treatment compared to the control group, although this difference was not statistically significant, but there was no significant difference in the PCV value after treatment compared to the control group. There was a significant difference in the WBC value after treatment compared to the control group. There was no significant difference in the values of Neutrophils, lymphocytes, Eosinophils, before treatment and after treatment.

For the female gender, there was difference in the PCV value before treatment compared to the control group, although this difference was not statistically significant, but there was no significant difference in the PCV value after treatment compared to the control group. There was a difference in the WBC value before and treatment when compared to the control group. There was a significant difference in the neutrophils and eosinophils value when compared with the control

Across the three age groups, there was difference in the PCV value before treatment compared to the control group, although this difference was not statistically significant, but there was no

significant difference in the PCV value after treatment compared to the control group. There was a difference in the WBC value before and after treatment when compared to the control group. The value of neutrophils for age 21-39 and 40+ were statistically significant at $P<0.05$ compared to the control group (Table 4.3)

Table 4.1: Full blood count of patients with lonart resistant malaria before and after treatment

Groups	% PCV	WBC (cm^3)	% Neutrophils	% Lymphocytes	% Eosinophils
Control	39.86±0.41	7012.42±206.60	129.95±0.40	40.03±0.60	3.02±1.04
Before Treatment	30.26±0.171	6269.00±116.49	58.92±0.11*	39.09±0.10	1.99±0.08
After Treatment	41.89±0.37	6150.00±120.60	58.84±0.11*	39.11±0.09	2.03±0.08

*Values are expressed as Mean±SEM; means with superscript * are statistically significant at $p<0.05$.*

Table 4.2: Full blood count of patients with lonart resistant malaria before and after treatment according to gender

Gender	% PCV		WBC (cm^3)	% Neutrophils	% Lymphocytes	% Eosinophils
Male	Control	40.75±0.41	7000.00±504.14	58.18±1.66	41.31±1.60	1.68±0.17
	Before Treatment	30.22±0.24	6133.33±172.39	58.71±0.18	39.28±0.14	2.00±0.12
	After Treatment	41.24±0.30	5986.66±180.97*	59.00±0.15	39.04±0.14	1.95±0.11
Female	Control	39.69±0.49	7014.78±227.72	58.61±0.81	39.78±0.65	2.95±1.74
	Before Treatment	30.29±.24	6380.00±157.72	59.09±.14*	38.92±.15	1.98±.11*
	After Treatment	42.41±.61	6283.63±160.98	58.70±0.16*	39.16±0.29	2.09±0.12

*Values are expressed as Mean±SEM; means with superscript * are statistically significant at $p<0.05$.*

Table 4.3: Full blood count of patients with lonart resistant malaria before and after treatment according to age

Age		% PCV	WBC (cm^3)	% Neutrophil	% Lymphocytes	% Eosinophils
-20	Control	40.41±0.23	6806.52±273.43	59.30±0.91	40.00±0.86	1.76±0.18
	Before Treatment	30.55±0.58	6588.88±421.12	58.66±0.40	39.55±0.24	1.77±0.27
	After Treatment	43.44±2.74	6566.66±359.78	58.77±0.40	39.22±0.27	1.77±0.22
21-39	Control	39.38±0.736	7187.81±303.83	59.12±0.37	40.05±0.86	1.50±0.22
	Before Treatment	30.10±0.22	6419.29±141.67	58.89±0.15*	39.12±0.13	1.98±0.11
	After Treatment	41.92±0.45	6366.66±148.47	58.82±0.15	39.07±0.13	2.10±0.11
40+	Control	39.86±0.41	7012.4±2026.60	589.95±0.40	39.03±0.60	1.02±0.04
	Before Treatment	30.44±0.30	5932.35±214.148	59.02±0.176*	38.91±0.20	2.05±0.14
	After Treatment	41.41±0.31	5676.47±216.23	58.88±0.19*	39.14±0.15	1.97±0.14*

*Values are expressed as Mean±SEM; means with superscript * are statistically significant at $p<0.05$.*

4.2: Full blood count of patients with artequin resistant malaria

In table 4.4, there was difference in the PCV value before treatment compared to the control group although this difference was not statistically significant, but no significant difference in the PCV value after treatment compared to the control group. There was difference in the WBC value before and after treatment compared to the control group although this difference was not statistically significant. The values of neutrophils and eosinophils before and after treatment showed a statistically significant difference when compared to the control group at $P<0.05$

For the male gender in table 4.5, there was difference in the PCV value before treatment compared to the control group, although this difference was not statistically significant, but there was no significant difference in the PCV value after treatment compared to the control group. There was difference in the WBC value before and after treatment compared to the control group. There was no significant difference in the values of neutrophils, lymphocytes, eosinophils, before and after treatment.

For the female gender, there was difference in the PCV value before treatment compared to the control group, although this difference was not statistically significant, but there was no significant difference in the PCV value after treatment compared to the control group. There was a difference in the WBC value before and after treatment when compared to the control group. There was a significant difference in the neutrophils and eosinophils value when compared with the control

Across the three age groups in table 4.6, there was difference in the PCV value before treatment compared to the control group, although this difference was not statistically significant, but there was no significant difference in the PCV value after treatment compared to the control group. There was difference in the WBC value before and after treatment when compared to the control

group. The value of neutrophils for age 21-39 and 40+ as well as eosinophils were statistically significant at $P<0.05$ compared to the control group.

4.3 PFK13 gene expression of patients with Lonart and artequin resistant malaria

In table 4.7, there was a significant difference in the expression of PFK13 gene in patients with lonart and artequin resistant malaria compared to the control group with lonart having the highest value and statistically significant at ($P<0.05$)

Table 4.4: Full blood count of patients with artequin resistant malaria before and after treatment

Groups	% PCV	WBC (cm^3)	% Neutrophil	% Lymphocytes	% Eosinophils
Control	39.86±0.41	7012.42±206.60	58.95±0.40	40.03±0.60	2.02±1.04
Before Treatment	30.41±.19	6306.00±124.93	59.14±0.10*	39.05±0.1.10	1.81±0.07*
After Treatment	41.87±0.31	6142.00±119.40	58.85±0.10*	39.17±0.08	1.98±0.08*

*Values are expressed as Mean±SEM; means with superscript * are statistically significant at $p<0.05$.*

Table 4.5: Full blood count of patients with artequin resistant malaria before and after treatment according to gender

Gender		% PCV	WBC (cm^3)	% Neutrophil	% Lymphocytes	% Eosinophil
Male	Control	40.75±1.65	7000.00±2016.59	58.18±6.67	41.31±6.42	1.68±0.70
	Before Treatment	30.60±2.03	6660.37±1270.72	59.09±1.13	39.22±1.35	1.71±0.68
	After Treatment	41.82±3.73	6534.61±1157.05	58.80±1.02	39.28±0.82	1.90±0.86
Female	Control	39.69±4.44	7014.78±2087.18	58.61±0.17	39.78±6.03	2.95±0.38
	Before Treatment	30.19±1.76	5906.38±1107.17	59.19±0.94*	38.85±1.122	1.91±0.77*
	After Treatment	41.91±2.33	5716.66±1093.17	58.89±1.11*	39.04±0.87	2.06±0.83

*Values are expressed as Mean±SEM; means with superscript * are statistically significant at $p<0.05$.*

Table 4.6*:* Full blood count of patients with artequin resistant malaria before and after treatment according to age

Age		% PCV	WBC (cm^3)	% Neutrophil	%Lymphocytes	% Eosinophil
-20	Control	40.41±1.62	6806.52±1854.53	59.30±6.18	40.00±5.86	1.76±0.83
	Before Treatment	30.20±2.04	6000.00±1178.51	59.20±1.13	39.00±1.15	1.60±0.84
	After Treatment	42.72±2.64	5827.27±1204.23	58.72±1.19	39.18±0.75	2.09±0.83
21-39	Control	39.38±5.41	7187.81±2232.68	59.12±0.07	40.05±6.32	1.50±9.44
	Before Treatment	30.62±2.02	6225.49±1364.52	59.07±1.16*	39.03±1.46	1.92±0.77
	After Treatment	42.00±2.05	5903.84±1210.40*	58.86±1.08*	39.09±0.93	2.03±0.86
40+	Control	39.86±4.14	7012.42±2066.02	59.95±0.07	40.03±6.08	1.02±6.49
	Before Treatment	30.17±1.73	6489.74±1104.24	59.20±0.86*	39.07±0.98	1.71±0.64*
	After Treatment	41.43±4.29	6570.27±1068.45	58.86±1.03*	39.27±0.76	1.86±0.85*

*Values are expressed as Mean±SEM; means with superscript * are statistically significant at $p<0.05$.*

Table 4.7: PFK13 gene expression of patients with Lonart and artequin resistant malaria

Negative Control	Positive Control	Lonart	Artequin	F-Value	P-Value
109.78±18.38	118.32±17.13	143.94±6.85*	135.89±4.5	2.114	0.111

*Values are expressed as Mean±SEM; means with superscript * are statistically significant at $p<0.05$.*

CHAPTER FIVE

DISCUSSION

Malaria, a significant public health concern, often leads to changes in full blood count parameters, playing a crucial role in malaria pathology, data on full blood count parameters could serve as a marker for antimalaria drug resistance (Hänscheid *et al.,* 2008). *Plasmodium falciparum*, the causative agent of malaria, is associated with mutations in the PFK13 gene, particularly its propeller region, considered a molecular marker for artemisinin resistance (Ariey *et al.,* 2014). Proteins associated with increased gene expression have been linked to various drug-resistant traits in Plasmodium falciparum (Inoue *et al.,* 2018, Calcada *et al.,* 2020). Gene expression, which can influence protein levels, is a potential mechanism for modulating drug responses. However, there have been limited studies investigating the patterns of gene expression in Plasmodium falciparum during artemisinin-based combination therapy (ACT) for uncomplicated malaria cases. Notably, while artemisinin resistance is primarily linked to decreased levels of PfK13 due to specific genetic mutations (Birnbaum *et al.,* 2020), lower levels of pfk13 transcripts in vivo have also been associated with prolonged parasite clearance times during artemether-lumefantrine (AL) treatment (Silva *et al.,* 2019).

In this study, full blood count parameters and parasite's PFK13 gene expression were analyzed in subjects with resistance to Artemisinin Combination Therapy (ACT) in Benin metropolis. The subjects exhibited resistance to Lonart (Artemether and Lumefactrine) and Artequin (Artesunate and Mefloquine). The results highlighted changes in full blood count parameters and parasite's PFK13 gene expression before and after treatment with quinine infusion (an alternative antimalarial drug), providing valuable insights into artemisinin resistance.

Subjects with Lonart and Artequin resistance displayed similar variations in white blood cell (WBC) and packed cell volume (PCV) values. Before quinine treatment, PCV values were lower, indicative of excessive red blood cell destruction. After quinine infusion, values normalized, aligning with previous findings (Meraiyebu and Ajibola, 2012). WBC values were consistently lower, consistent with reports by McKenzie *et al.,* (2005), stating P. falciparum malaria patients' exhibit lower WBC counts.

In both gender, similar trends were observed in full blood count parameters, with notable variations in neutrophils and eosinophils among females with Artequin resistance. Across age groups, differences in PCV values were observed pre-treatment, but post-treatment values showed no significant differences. Neutrophil values for ages 21-39 and 40+, as well as eosinophils, were statistically significant compared to the control group.

The Greater Mekong Subregion is considered the focal point for the emergence of Plasmodium falciparum malaria resistance, with the potential for this resistance to spread to other malaria-endemic regions. Several factors contribute to this regional specificity, including varying levels of host immunity leading to frequent use of antimalarial drugs, genetic factors of the parasite stemming from its origins, limited access to effective drugs, and continued use of ineffective monotherapy. Additionally, the prevalence of low-quality and counterfeit antimalarial drugs is widespread, particularly in Sub-Saharan Africa, and is seen as a major factor driving the development and spread of resistance (Mita *et al.,* 2009). From a molecular perspective, the emergence of resistance to artemisinins, a key antimalarial drug, is heavily influenced by the genetic background of the parasite. Resistance to artemisinins is known to be heritable and therefore has a clear genetic basis (Phyo *et al.,* 2012). Studies on genome modification have

demonstrated that the impact of various mutations in the pfk13 gene on parasite clearance and survival rates depends on the genetic makeup of the parasites (Straimer *et al.,* 2015). Additionally, research by Lee and Fiddock (2016) suggests that mutations in certain DNA repair genes, such as mlh1, pms1, and exo1, are overexpressed in artemisinin-resistant parasites, further highlighting the role of genetics in resistance development.

Studies by Dahal *et al.,* (2016) propose that monitoring for artemisinin resistance in Sub-Saharan Africa should consider a threshold of 5% of cases still positive for parasites on day 3, reflecting the higher levels of acquired immunity in African populations, which contribute to faster parasite clearance. Moreover, various factors can influence parasite clearance times during artemisinin-based combination therapy, including initial parasite load, efficacy of partner drugs, patient age, health status, and artemisinin dosage. Recent research indicates that lower levels of immunity are associated with a higher prevalence of pfk13 mutations, suggesting that these factors should be integrated into monitoring efforts for artemisinin resistance (Thu *et al.,* 2017).

The gene expression studies showed that subjects with artemisinin drug resistant malaria exhibited increased level of PFK13 gene expression compared to the control group. The values for both lonart and artequin resistant subjects exhibited a statistically significant increase ($P< 0.05$) compared to the positive and negative control group. While mutations in the PFK13 gene are the primary genetic markers for artemisinin resistance, variations in gene expression could potentially influence the overall response of the parasite to artemisinin. However, high levels of PFK13 expression alone are not a direct indicator of artemisinin resistance. Specific mutations in the PFK13 gene provides more reliable indicator of artemisinin resistance. Therefore, molecular surveillance of these mutations would give a more accurate indication of artemisinin resistance

rather than relying solely on the level of gene expression (She *et al.,* 2020). Several studies on mutations in K13 gene have been described in Africa and South-East Asia (WHO, 2016), Studies by She *et al.,* (2020) showed that PFK13 mutation have been detected in many studies in Africa. However, no resistance-associated mutations have been found outside the Greater Mekong subregion (WHO, 2016). Studies carried out by Igbasi *et al.,* in Lagos state, Nigeria did not detect any of the validated mutations associated with artemisinin resistance (Igbasi *et al.,* 2019). Dokumbo *et al.,* (2018) reported limited artemisinin resistance in Africa stating that it is pertinent however, to monitor possible emergence in Africa from detected Kelch 13 SNP on codon A578S reported in some African countries (e.g. Kenya). As part of strategies devised to contain the spread of artemisinin resistance, active monitoring of parasite responses and markers of artemisinin resistance in different areas is advocated as regional differences in parasite response may exist in Nigeria (Ikeda *et al.,* 2018). A potential challenge would be to identify and validate resistance markers if unique markers exist in other areas. (Dokumu *et al.,* 2018)

This study contributes to understanding artemisinin resistance dynamics and emphasizes the need for continuous surveillance, including molecular analysis of resistance markers. Limitations include the inability to identify PFK13 mutations; future studies should address this to validate artemisinin resistance emergence in Benin metropolis.

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FINDINGS

The findings from the study are as follows:

1. the study reveals a difference in the value of PCV and WBC of subjects who showed resistance to artemisinin drugs. Both lonart and artequin resistance subject showed similar pattern of alteration in PCV and WBC value.
2. the study of the gene expression showed that subjects with artemisinin drug (lonart and artequin) resistant malaria have a higher level of PFK13 gene expression compared to the control group.

CONTRIBUTION TO KNOWLEDGE

This study contributes to understanding artemisinin resistance dynamics and emphasizes the need for continuous surveillance, including haematological and molecular analysis of resistance markers. Ensuring the deployment of efficacious artemisinin-based combination therapies (ACTs) is crucial in the fight against artemisinin resistance. Moreover, sustaining haematological and molecular surveillance of artemisinin drugs is of paramount importance to monitor potential artemisinin resistance. Continuous monitoring allows for the early detection of changes in drug efficacy, facilitating prompt interventions to combat emerging resistance and prevent its further spread.

CONCLUSION

Plasmodium resistance to antimalarial drugs remains one of the most persistent issues in the effort to eradicate malaria. Regular monitoring of antimalarial medication efficacy is critical for detecting changes in treatment effectiveness and permitting rapid responses to combat resistance and prevent its spread.This study aims to evaluate the prevalence of drug-resistant malaria infection in Benin City by examining the present state of the PFK13 gene expression, which has been recognized as a molecular indicator of artemisinin-resistant malaria. The investigation focused on changes in full blood count parameters and PFK13 gene expression associated with artemisinin resistance. This study revealed a positive alteration in the full blood count parameters of subjects with lonart and artequin resistant *Plasmodium falciparum* malaria despite having been treated earlier with antimalaria drugs. Furthermore, PFK13 gene expression was found to be significantly elevated in people who were resistant to artemisinin, which may indicate the emergence of resistance to currently available antimalarial medications. To establish the functional role of PK13 mutation as molecular markers of artemisinin resistance, however, a specific mutation in the PFK13 gene is required. Therefore, a comprehensive examination of the kelch13 gene mutation in P. falciparum is imperative to establish its epidemiological impact on artemisinin resistance in Benin City. This research will deepen our understanding of the dynamics of artemisinin resistance and help devise practical solutions to the ever-changing problems associated with treating malaria.

RECOMMENDATION

Presently, the emergence of artemisinin resistance poses a significant challange to the eradication and control of malaria. Ensuring the deployment of efficacious artemisinin-based combination therapies (ACTs) is crucial in the fight against artemisinin resistance. To address this, effective regulation of drug manufacturing companies becomes imperative to mitigate the production and distribution of counterfeit drugs, a primary contributor to drug resistance. Patients' education and public awareness of the danger of drug misuse, as well as educating the people on the proper use of artemisinin combination therapies will further reduce the spread of emergence of resistance. Moreover, sustaining molecular surveillance of artemisinin drugs is of paramount importance to monitor potential artemisinin resistance. Continuous monitoring allows for the early detection of changes in drug efficacy, facilitating prompt interventions to combat emerging resistance and prevent its further spread. By regulating drug manufacturing, ensuring the authenticity of medications, and maintaining vigilant molecular surveillance, we can bolster our efforts in the global fight against malaria and the threat of artemisinin resistance.

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APPENDIX

Appendix I

TABLE SHOWING THE FULL BLOOD COUNT AND MALARIA PARASITE TEST OF SUBJECTS WITH LONART RESISTANT MALARIA BEFORE TREATMENT WITH QUININE

S/N	SEX	AGE	MP	PCV(%)	WBC	N(%)	L(%)	E(%)
01	M	22	+	32	6100	58	40	2
02	M	24	++	30	5700	59	40	1
03	M	25	+	30	7200	60	38	2
04	M	26	++	28	8400	58	40	2
05	M	33	+	29	5300	57	40	3
06	F	30	++	28	5400	60	37	3
07	F	31	+	30	7100	58	40	2
08	F	34	++	33	5800	60	39	1
09	F	40	+	32	5700	59	39	2
10	F	44	++	33	6900	60	37	3
11	M	45	+	30	7100	57	40	3
12	M	18	++	30	7000	60	39	1
13	M	19	++	32	6900	57	40	3
14	M	22	+	30	7200	60	38	2
15	M	25	+	28	8100	59	40	1
16	M	27	++	29	7400	60	37	3
17	M	28	+	30	5200	57	40	3
18	M	32	++	32	7100	59	40	1
19	M	37	+	30	5400	60	37	3

20	M	25	+	30	6100	57	40	3
21	M	27	++	29	4800	60	38	2
22	M	37	+	28	5000	60	39	1
23	M	38	+	30	5500	58	40	2
24	M	40	+	33	4300	60	39	1
25	M	39	++	30	4500	57	40	3
26	F	39	+	32	6100	58	40	2
27	F	38	+	33	5800	60	39	1
28	F	40	+	28	4200	60	38	2
29	F	41	++	30	4300	58	39	3
30	F	44	++	33	6500	60	37	3
31	F	50	++	30	4800	59	40	1
32	F	49	+	28	4900	60	38	2
33	F	50	++	32	5100	59	40	1
34	F	38	+	33	7200	60	38	2
35	F	40	++	30	7800	58	40	2
36	F	41	++	29	6900	60	37	3
37	F	42	+	28	7500	60	39	1
38	F	50	+	29	6900	59	40	1
39	F	47	+	30	7100	60	37	3
40	F	48	++	32	7600	59	39	2
41	F	50	++	30	8100	60	37	3
42	F	21	+	29	6900	60	39	1
43	F	22	+	30	7100	59	40	1

44	F	25	++	28	6500	60	38	2
45	F	19	+	30	8100	58	40	2
46	F	18	+	33	4200	60	39	1
47	F	21	++	28	7500	58	40	2
48	F	24	+	29	6100	57	40	3
49	F	23	+	33	7400	58	39	3
50	F	27	+	30	5400	60	37	3
51	M	37	+	32	6100	60	39	1
52	M	38	++	30	5700	57	40	3
53	M	39	++	29	4500	60	39	1
54	M	40	+	28	4600	59	40	1
55	M	38	++	30	7100	60	39	1
56	M	37	++	33	8200	57	40	3
57	M	44	+	28	5800	60	37	3
58	M	18	+	30	5600	57	40	3
59	M	19	++	30	6700	58	40	2
60	M	20	++	29	7100	59	40	1
61	F	26	+	28	7800	60	37	3
62	F	18	+	33	8100	60	38	2
63	F	27	+	30	6800	59	40	1
64	F	28	++	32	4800	58	40	2
65	F	29	++	29	7100	60	38	2
66	M	36	++	28	7600	57	40	3
67	M	40	++	30	7500	60	39	1

68	M	48	+	31	5600	59	39	2
69	M	50	+	33	5400	58	40	2
70	M	39	+	33	4600	60	39	1
71	F	40	++	31	4500	58	40	2
72	F	33	+	28	7100	60	37	3
73	F	28	++	29	7200	59	40	1
74	F	27	++	28	8100	57	40	3
75	F	26	++	30	6300	60	39	1
76	F	22	++	32	6100	60	39	1
77	F	25	+	33	6200	59	40	1
78	F	40	++	30	4500	60	39	1
79	F	41	++	29	6100	60	37	3
80	F	44	++	32	6200	57	40	3
81	M	45	++	33	5700	59	40	1
82	M	39	+	30	6200	58	40	2
83	M	38	+	29	6800	60	39	1
84	M	25	+	28	7100	59	39	2
85	M	27	++	29	8100	60	38	2
86	M	28	++	33	5700	57	40	3
87	F	29	++	30	6200	58	40	2
88	F	27	+	33	8100	60	39	1
89	F	38	+	31	7200	60	38	2
90	F	39	+	29	5800	57	40	3
91	F	40	+	32	4800	59	40	1

92	F	41	++	28	7900	57	40	3
93	F	44	++	29	7400	58	40	2
94	M	46	+	30	4500	59	39	2
95	M	50	++	33	4600	60	37	3
96	M	49	++	32	5100	59	40	1
97	M	48	+	29	5800	57	40	3
98	F	28	+	30	5600	58	39	3
99	F	25	++	29	4500	60	39	1
100	F	18	++	28	5600	59	40	1

TABLE SHOWING THE FULL BLOOD COUNT AND MALARIA PARASITE TEST OF SUBJECTS WITH LONART RESISTANT MALARIA AFTER TREATMENT WITH QUININE

S/N	SEX	AGE	MP	PCV(%)	WBC	N(%)	L(%)	E(%)
1	M	22	Scanty	39	5900	60	39	1
2	M	24	Np	40	5600	60	38	2
3	M	25	Np	41	7100	60	37	3
4	M	26	Scanty	44	8300	59	39	2
5	M	33	Np	39	5200	58	40	2
6	F	30	Np	39	5300	57	40	3
7	F	31	Scanty	39	7000	57	40	3
8	F	34	Scanty	41	5700	58	40	2
9	F	40	Np	41	5600	60	39	1
10	F	44	Scanty	41	6800	59	39	2
11	M	45	Np	44	6800	59	40	1
12	M	18	Scanty	40	7000	60	38	2
13	M	19	Scanty	40	6900	59	39	2
14	M	22	Np	40	6800	60	39	1
15	M	25	Np	41	7100	59	40	1
16	M	27	Scanty	45	8000	58	39	3
17	M	28	Np	43	7500	57	40	3
18	M	32	Scanty	43	5200	60	38	2
19	M	37	Scanty	43	5300	60	39	1

20	M	25	Scanty	41	6000	57	40	3
21	M	27	Np	39	4700	59	40	1
22	M	37	Np	39	4900	60	37	3
23	M	38	Np	39	5400	60	38	2
24	M	40	Scanty	40	4200	60	39	1
25	M	39	Scanty	41	4400	58	40	2
26	F	39	Np	39	6000	60	39	1
27	F	38	Scanty	40	5700	58	40	2
28	F	40	Scanty	42	4100	59	40	1
29	F	41	Scanty	45	4200	57	40	3
30	F	44	Np	43	6400	60	39	1
31	F	50	Np	43	4700	60	37	3
32	F	49	Scanty	44	4800	58	39	3
33	F	50	Scanty	41	5000	60	39	1
34	F	38	Np	39	7100	59	40	1
35	F	40	Np	39	7700	57	40	3
36	F	41	Np	41	6800	59	39	2
37	F	42	Scanty	41	7400	58	39	3
38	F	50	Scanty	39	6700	60	37	3
39	F	47	Np	44	7500	57	40	3
40	F	48	Np	41	8000	59	39	2
41	F	50	Np	44	6800	60	37	3
42	F	21	Np	40	6900	57	40	3
43	F	22	Scanty	40	6400	60	37	3

44	F	25	Np	40	8000	57	40	3
45	F	19	Np	41	4100	58	40	2
46	F	18	Np	42	7400	57	40	1
47	F	21	Np	43	6000	60	38	2
48	F	24	Np	44	7700	60	37	3
49	F	23	Scanty	41	8000	60	38	2
50	F	27	Np	42	6200	60	38	2
51	M	37	Np	39	5900	60	39	1
52	M	38	Scanty	40	6000	59	38	3
53	M	39	Scanty	43	4400	58	40	2
54	M	40	Scanty	44	4500	59	40	1
55	M	38	Scanty	45	7000	60	37	3
56	M	37	Np	45	8100	57	40	3
57	M	44	Np	44	4900	57	40	3
58	M	18	Np	39	6600	59	40	1
59	M	19	Np	39	7000	60	39	1
60	M	20	Np	41	7700	60	38	2
61	F	26	Scanty	45	8000	60	39	1
62	F	18	Scanty	44	6700	59	39	2
63	F	27	Scanty	43	4700	57	40	3
64	F	28	Np	44	4800	58	40	2
65	F	29	Np	42	7500	58	39	3
66	M	36	Np	43	7400	57	40	3
67	M	40	Scanty	41	5500	59	39	2

68	M	48	Scanty	41	5300	60	38	2
69	M	50	Scanty	39	4200	57	40	3
70	M	39	Np	39	4500	59	38	3
71	F	40	Np	40	4400	60	39	1
72	F	33	Np	41	7000	57	40	3
73	F	28	Np	42	7100	58	39	3
74	F	27	Np	44	8000	59	40	1
75	F	26	Np	43	6200	60	38	2
76	F	22	Scanty	39	5700	60	39	1
77	F	23	Np	40	6000	57	40	3
78	F	40	Np	41	4400	59	39	2
79	F	41	Np	44	6000	60	38	2
80	F	44	Scanty	39	6100	57	40	3
81	M	45	Np	39	5600	60	38	2
82	M	39	Np	41	6100	59	39	2
83	M	38	Np	45	6700	60	38	2
84	M	25	Np	44	7000	60	39	1
85	M	27	Np	41	8000	59	40	1
86	M	28	Np	41	5600	58	39	3
87	F	29	Scanty	42	6100	60	39	1
88	F	27	Scanty	44	8000	60	39	1
89	F	38	Scanty	41	7100	59	40	1
90	F	39	Np	43	4700	57	40	3
91	F	40	Scanty	41	7800	58	40	2

92	F	41	Np	40	7300	59	40	1
93	F	44	Np	40	4400	60	39	1
94	M	46	Scanty	39	4500	58	40	2
95	M	50	Scanty	40	5000	59	40	1
96	M	49	Scanty	41	5500	60	39	1
97	M	48	Np	42	4100	58	40	2
98	F	28	Np	63	6400	59	40	1
99	F	25	Np	44	5500	60	38	2
100	F	18	Scanty	65	5700	57	40	3

TABLE SHOWING THE FULL BLOOD COUNT AND MALARIA PARASITE TEST OF SUBJECTS WITH ARTEQUIN RESISTANT MALARIA BEFORE TREATMENT WITH QUININE

S/N	GENDER	AGE	PARASITAEMIA	PCV (%)	WBC	N (%)	L (%)	E (%)
01	M	22	++	30	5,700	58	40	2
02	M	30	+	29	6,100	60	38	2
03	M	28	++	32	6,400	59	40	1
04	M	18	++	28	5,200	60	39	1
05	M	29	+	30	7,100	58	39	3
06	F	44	++	28	4,200	60	37	3
07	F	40	+	32	5,400	58	40	2
08	F	18	++	29	4,500	60	39	1
09	F	24	++	30	5,700	59	38	3
10	F	25	+	32	6,200	60	37	3
11	M	26	++	29	4,500	58	40	2

12	M	31	++	33	7,100	60	39	1
13	M	32	+	30	6,300	59	40	1
14	M	40	++	28	7,100	60	39	1
15	M	44	+	29	5,400	58	40	2
16	M	25	++	28	8,100	59	38	3
17	M	26	++	33	4500	60	39	1
18	M	23	++	30	7,100	59	40	1
19	M	22	+	29	4,500	60	38	2
20	F	19	++	30	6,200	58	40	2
21	F	18	+	29	7,400	60	39	1
22	F	19	++	33	4,800	60	37	1
23	F	28	+	30	4,500	59	40	1
24	F	29	++	33	6,800	60	38	2
25	F	35	++	30	4,900	59	40	1

26	F	44	+	32	4,800	58	40	2
27	M	41	++	30	5,700	60	38	2
28	M	40	+	29	6,400	59	40	1
29	M	39	++	33	6,700	60	38	2
30	M	18	+	30	6,200	58	40	2
31	M	36	++	28	4,200	60	37	3
32	M	39	+	30	8,700	59	40	1
33	M	40	++	29	6,100	58	40	2
34	M	44	+	33	7,000	60	39	1
35	M	31	++	30	6,100	59	40	1
36	F	40	++	29	5,200	60	37	3
37	F	41	+	28	4,800	58	40	2
38	F	28	++	30	7,600	57	40	3
39	F	45	+	30	5,400	60	39	1

40	F	49	++	29	6,400	59	39	2
41	F	50	+	28	5,700	60	38	2
42	F	45	++	29	6,200	58	40	2
43	F	25	+	33	5,400	60	37	3
44	F	26	++	28	4,200	59	38	3
45	F	27	++	30	5,200	58	40	2
46	M	28	+	29	8,700	60	39	1
47	M	39	++	30	8,100	60	38	2
48	M	33	+	33	6,500	58	40	2
49	M	48	++	30	6,200	60	39	1
50	M	50	+	32	5,400	58	40	2
51	M	50	+	30	6,400	60	39	1
52	M	49	++	29	5,900	59	40	1
53	M	44	+	33	6,000	60	38	2

54	M	45	++	30	5,400	58	40	2
55	M	38	+	28	5,200	60	39	1
56	F	28	++	29	6,500	59	40	1
57	F	30	+	33	6,800	60	39	1
58	F	33	++	30	7,200	58	40	2
59	F	29	++	29	4,800	60	38	2
60	F	19	+	33	5,900	57	40	3
61	F	20	+	28	4,800	60	39	1
62	F	23	+	29	5,100	59	40	1
63	F	24	+	33	6,700	60	37	3
64	F	26	++	30	7,100	57	40	3
65	F	28	++	30	6,800	60	38	2
66	F	29	++	33	6,700	59	40	1
67	F	34	++	30	5,200	60	37	3

68	F	35	+	33	7,200	59	39	2
69	F	31	++	30	7,200	60	38	2
70	F	44	++	28	8,100	60	39	1
71	M	46	++	29	7,900	58	40	2
72	M	50	+	28	8,400	60	37	3
73	M	45	+	30	7,500	60	39	1
74	M	46	+	32	6,400	60	38	2
75	M	47	+	33	7,900	58	40	2
76	M	49	++	30	6,200	58	40	2
77	M	50	++	32	8,100	60	38	2
78	M	19	+	29	7,200	59	40	1
79	M	20	+	33	7,800	60	37	3
80	M	21	+	30	8,100	58	40	2
81	F	22	+	33	5,400	60	39	1

82	F	24	++	28	6,100	59	40	1
83	F	27	++	29	5,800	60	38	2
84	F	29	+	30	4,200	59	39	2
85	F	33	++	29	4,500	60	37	3
86	F	35	+	28	4,700	58	40	2
87	F	39	++	33	7,200	60	38	2
88	F	40	++	30	6,800	59	39	2
89	F	44	+	29	7,200	60	39	1
90	F	46	++	30	8,100	59	40	1
91	M	47	++	30	7,900	60	38	2
92	M	49	+	33	8,100	59	40	1
93	M	50	++	30	6,900	60	39	1
94	M	44	+	28	8,100	59	40	1
95	M	45	+	33	6,400	60	38	2

96	M	45	++	32	5,900	59	40	1
97	M	39	+	33	6,100	60	38	2
98	M	38	+	32	5,400	57	40	3
99	M	40	++	33	6,100	59	38	3
100	M	50	+	20	7,200	60	39	1

TABLE SHOWING THE FULL BLOOD COUNT AND MALARIA PARASITE TEST OF SUBJECTS WITH ARTEQUIN RESISTANT MALARIA AFTER TREATMENT WITH QUININE

S/N	GENDER	AGE	PARASITAEMIA	PCV (%)	WBC	N (%)	L (%)	E (%)
01	M	22	SCANTY	39	5,600	58	40	2
02	M	30	NP	40	6,000	59	40	1
03	M	28	NP	41	6,300	60	39	1
04	M	18	SCANTY	39	5,100	57	40	3
05	M	29	NP	44	7,000	60	37	3
06	F	44	NP	45	4,100	59	38	3
07	F	40	SCANTY	41	5,300	60	39	1
08	F	18	SCANTY	45	4400	58	39	3
09	F	24	NP	39	5600	59	39	2
10	F	25	NP	39	6100	60	37	3
11	M	26	NP	41	4400	59	38	3
12	M	31	NP	44	7000	60	39	1

13	M	32	SCANTY	45	6200	58	40	2
14	M	40	SCANTY	40	7100	57	40	3
15	M	44	SCANTY	40	5300	59	40	1
16	M	25	SCANTY	41	8000	57	40	3
17	M	26	SCANTY	43	4400	60	37	3
18	M	23	SCANTY	43	7000	58	39	3
19	M	22	SCANTY	41	4400	58	39	3
20	F	19	NP	39	6100	60	39	1
21	F	18	NP	44	7500	60	38	2
22	F	19	SCANTY	41	4700	60	38	2
23	F	28	SCANTY	42	4400	57	40	3
24	F	29	NP	43	6700	58	39	3
25	F	35	NP	44	4800	59	40	1
26	F	44	SCANTY	39	4700	57	40	3

27	M	41	NP	40	5600	58	39	3
28	M	40	NP	41	6300	60	39	1
29	M	39	SCANTY	44	6600	59	40	1
30	M	18	NP	45	6100	60	39	1
31	M	36	NP	44	4100	60	38	2
32	M	39	SCANTY	45	8600	59	40	1
33	M	40	NP	41	6000	59	39	2
34	M	44	NP	39	6900	58	39	3
35	M	31	NP	41	6000	57	40	3
36	F	40	SCANTY	40	5100	60	39	1
37	F	28	NP	41	4700	59	40	1
38	F	45	NP	42	7500	60	38	2
39	F	49	SCANTY	43	5300	60	37	3
40	F	50	NP	44	6300	58	40	2

41	F	45	SCANTY	45	5600	59	40	1
42	F	25	NP	41	6100	57	40	3
43	F	26	NP	40	5300	59	39	2
44	F	27	NP	45	4100	58	39	3
45	F	28	NP	44	5100	60	37	3
46	M	39	SCANTY	43	8600	60	38	2
47	M	33	NP	44	8000	58	40	2
48	M	48	NP	45	6400	59	40	1
49	M	39	SCANTY	42	6100	60	39	1
50	M	50	SCANTY	43	5300	58	40	2
51	M	50	SCANTY	40	6300	58	39	3
52	M	49	SCANTY	41	5800	59	40	1
53	M	44	SCANTY	39	6000	57	40	3
54	M	45	SCANTY	44	5300	58	39	3

55	M	38	NP	45	5100	60	38	2
56	F	28	NP	40	6400	60	39	1
57	F	30	NP	39	6700	59	39	2
58	F	33	SCANTY	41	7100	60	38	2
59	F	29	SCANTY	45	4700	57	40	3
60	F	19	SCANTY	44	4800	58	39	3
61	F	20	NP	45	5800	59	39	2
62	F	23	NP	39	4700	60	38	2
63	F	24	NP	40	4900	58	39	3
64	F	26	SCANTY	44	6600	59	40	1
65	F	28	SCANTY	45	7000	60	39	1
66	F	29	NP	44	6600	59	40	1
67	F	34	NP	41	4700	60	38	2
68	F	35	NP	40	5100	57	40	3

69	F	31	NP	39	7100	60	38	2
70	F	44	NP	39	8000	59	40	1
71	M	46	NP	40	7800	60	38	2
72	M	50	SCANTY	44	8300	58	40	2
73	M	45	SCANTY	43	7400	59	40	1
74	M	46	NP	45	6800	60	38	2
75	M	42	NP	44	7800	60	39	1
76	M	49	SCANTY	39	6100	60	39	1
77	M	50	NP	42	8000	58	39	3
78	M	19	NP	44	7200	59	40	1
79	M	20	SCANTY	45	7700	58	40	2
80	M	21	SCANTY	44	8000	59	40	1
81	F	22	SCANTY	41	5300	60	39	1
82	F	24	NP	43	6000	57	40	3

83	F	27	NP	39	5700	58	39	3
84	F	29	SCANTY	40	4100	59	39	2
85	F	33	NP	41	4400	60	39	1
86	F	35	SCANTY	39	4600	57	40	3
87	F	39	SCANTY	44	7100	60	39	1
88	F	40	SCANTY	45	6400	58	40	2
89	F	44	SCANTY	41	7100	59	39	2
90	F	46	NP	47	8000	60	39	1
91	M	47	NP	39	7800	57	40	3
92	M	49	NP	44	8000	60	39	1
93	M	50	SCANTY	39	6800	59	40	1
94	M	44	SCANTY	40	8000	60	39	1
95	M	45	SCANTY	44	6300	57	40	3
96	M	45	NP	45	5,800	58	40	2

97	M	39	NP	41	6000	59	40	1
98	M	38	SCANTY	43	5300	58	39	3
99	M	40	NP	44	6000	59	39	2
100	M	50	SCANTY	45	7100	60	38	2

TABLE SHOWING THE FULL BLOOD COUNT AND MALARIA PARASITE TEST OF NORMAL SUBJECTS GROUP (CONTROL GROUP)

PATIENT	AGE (YRS)	GENDER	MP	PCV(%)	WBC(MM^3)	N(%)	L(%)	E(%)
1.	21	MALE	SCANTY	42	5000	57	42	1
2.	23	MALE	NO MP SEEN	40	7100	60	38	2
3.	29	MALE	NO MP SEEN	38	5300	58	42	1
4.	25	MALE	SCANTY	40	6300	64	35	1
5.	24	MALE	NO MP SEEN	40	5000	53	43	3
6.	30	MALE	NO MP SEEN	39	9800	50	49	1
7.	24	MALE	NO MP SEEN	45	8400	56	42	2
8.	23	MALE	SCANTY	40	6200	52	47	1
9.	29	MALE	NO MP SEEN	40	10700	59	41	2
10.	28	MALE	SCANTY	40	7100	66	35	1
11.	30	MALE	NO MP SEEN	39	5000	50	49	2
12.	32	MALE	NO MP SEEN	41	6900	52	47	1
13.	38	MALE	NO MP SEEN	42	9200	67	31	2
14.	39	MALE	SCANTY	42	5600	50	49	1
15.	33	MALE	NO MP SEEN	38	5400	60	39	1
16.	34	MALE	NO MP SEEN	40	7400	63	36	1
17.	37	MALE	NO MP SEEN	41	4900	68	32	
18.	36	MALE	SCANTY	39	6100	63	37	
19.	32	MALE	NO MP SEEN	40	8400	54	46	
20.	38	MALE	NO MP	40	4900	59	31	

			SEEN					
21.	21	MALE	SCANTY	41	8100	67	33	
22.	29	MALE	NO MP SEEN	39	8000	60	40	
23.	24	MALE	SCANTY	39	4800	62	38	
24.	23	MALE	NO MP SEEN	42	5100	66	34	
25.	21	MALE	SCANTY	39	4900	55	45	
26.	30	MALE	NO MP SEEN	38	8400	68	31	1
27.	35	MALE	NO MP SEEN	38	8400	68	32	
28.	33	MALE	SCANTY	38	10100	70	30	
29.	36	MALE	NO MP SEEN	41	9800	59	41	
30.	39	MALE	SCANTY	42	6200	61	39	
31.	21	FEMALE	NO MP SEEN	39	5700	58	42	
32.	25	FEMALE	NO MP SEEN	38	8000	66	33	1
33.	23	FEMALE	SCANTY	38	10600	70	30	
34.	30	FEMALE	NO MP SEEN	40	8900	58	42	
35.	29	FEMALE	NO MP SEEN	43	6700	59	39	2
36.	26	FEMALE	SCANTY	39	5200	66	34	
37.	30	FEMALE	NO MP SEEN	40	5400	58	42	
38.	26	FEMALE	SCANTY	39	10700	50	48	2
39.	24	FEMALE	NO MP SEEN	39	7300	52	48	
40.	23	FEMALE	NO MP SEEN	40	5200	61	39	
41.	30	FEMALE	SCANTY	40	6400	65	35	
42.	39	FEMALE	NO MP SEEN	39	9200	58	40	2
43.	36	FEMALE	NO MP SEEN	39	6800	51	49	
44.	35	FEMALE	SCANTY	42	10600	60	40	

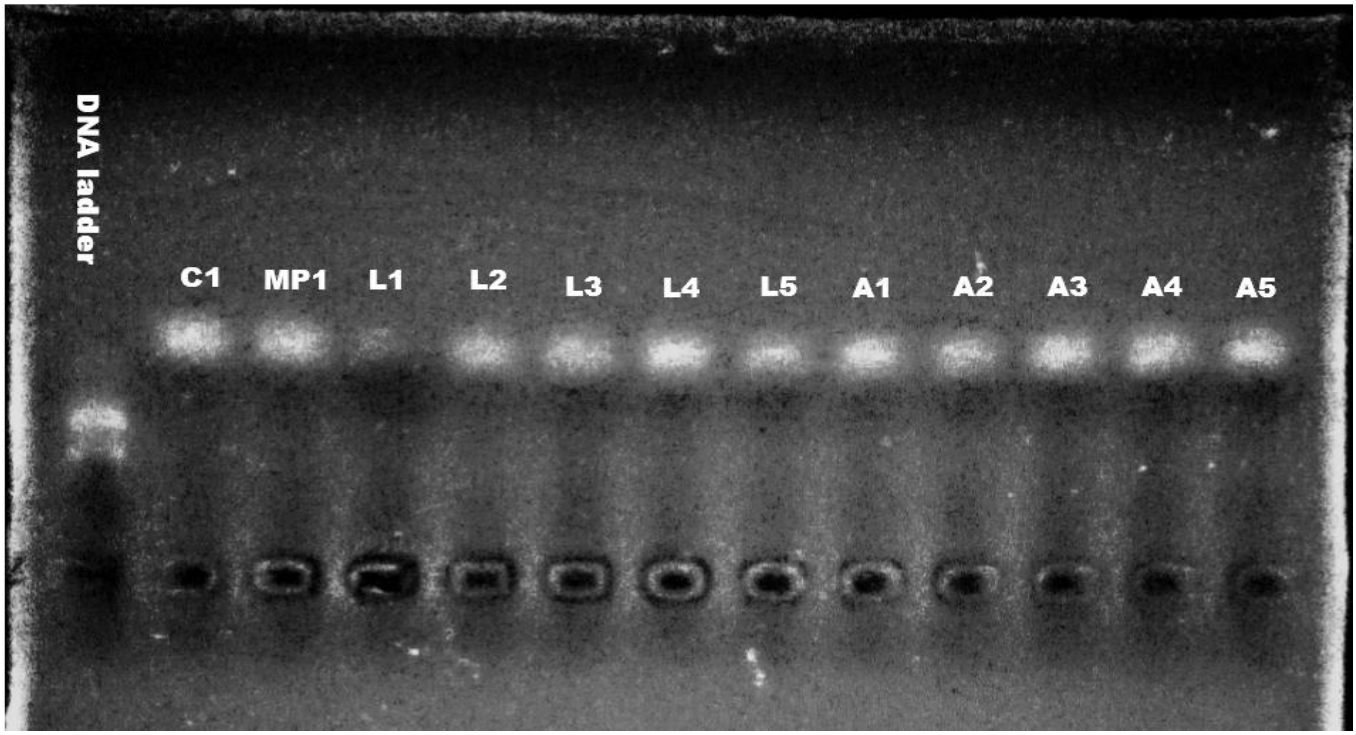
45.	38	FEMALE	NO MP SEEN	39	9800	58	37	5
46.	36	FEMALE	NO MP SEEN	37	5800	53	42	
47.	39	FEMALE	SCANTY	40	10100	62	38	
48.	37	FEMALE	NO MP SEEN	39	5100	69	31	
49.	39	FEMALE	SCANTY	42	8400	70	30	
50.	36	FEMALE	NO MP SEEN	40	9400	58	42	
51.	40	FEMALE	NO MP SEEN	42	9100	52	48	
52.	39	FEMALE	SCANTY	39	4900	64	36	
53.	37	FEMALE	NO MP SEEN	41	8500	61	38	1
54.	38	FEMALE	SCANTY	39	10300	69	31	
55.	35	FEMALE	NO MP SEEN	38	6600	55	45	
56.	22	FEMALE	NO MP SEEN	40	8100	61	39	
57.	24	FEMALE	SCANTY	39	4900	58	42	
58.	28	FEMALE	NO MP SEEN	40	5000	68	30	2
59.	30	FEMALE	SCANTY	40	10700	65	35	
60.	29	FEMALE	NO MP SEEN	39	6300	54	45	
61.	40	FEMALE	NO MP SEEN	38	5200	52	48	
62.	43	FEMALE	NO MP SEEN	42	7100	58	42	
63.	45	FEMALE	SCANTY	42	6400	61	39	
64.	44	FEMALE	NO MP SEEN	40	7900	57	43	
65.	41	FEMALE	NO MP SEEN	39	5900	58	40	2
66.	42	FEMALE	SCANTY	43	10100	52	48	
67.	40	FEMALE	NO MP SEEN	41	7300	62	38	
68.	40	FEMALE	NO MP	39	6100	68	30	2

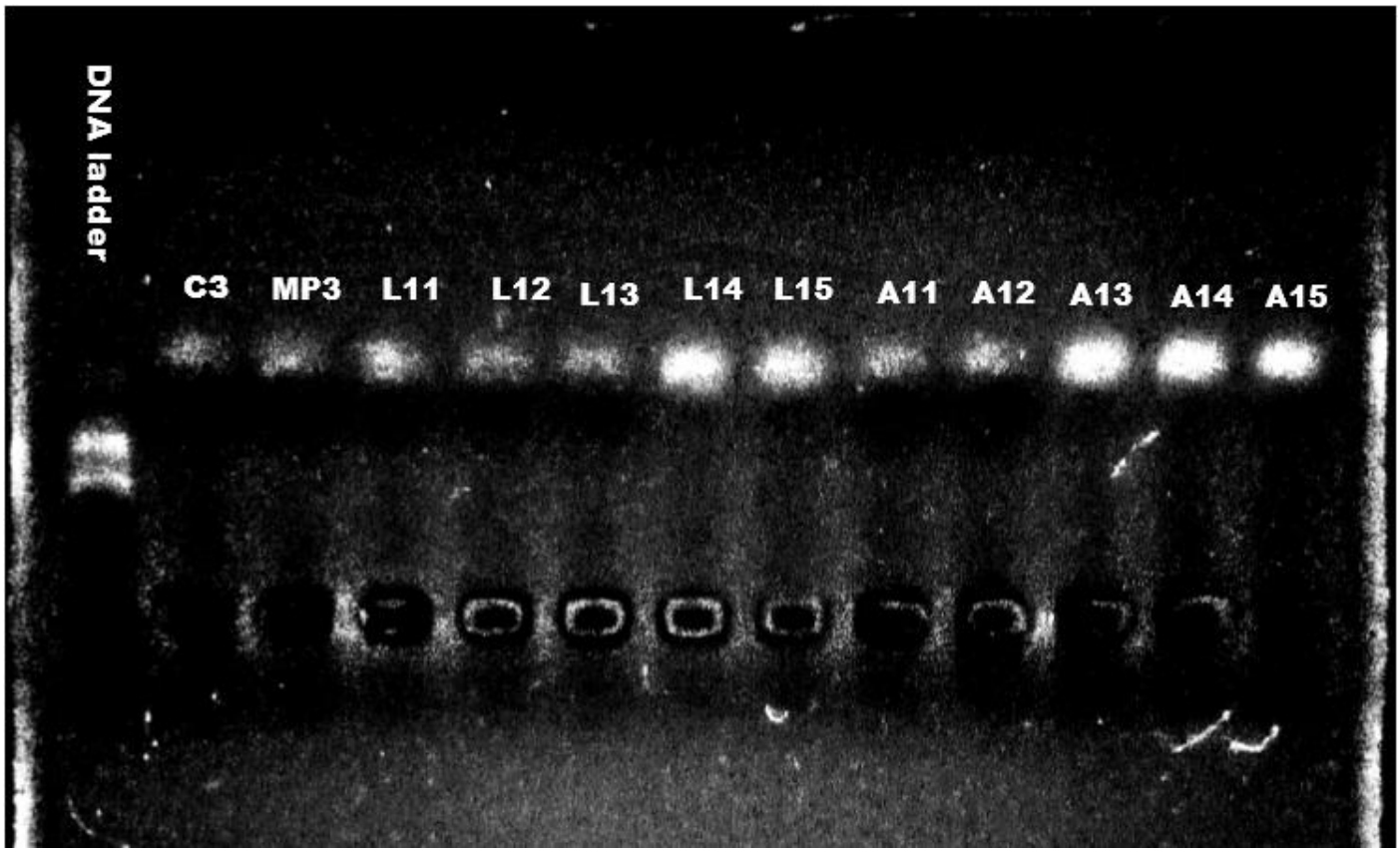
			SEEN					
69.	42	FEMALE	SCANTY	42	4800	62	38	
70.	46	FEMALE	NO MP SEEN	40	5100	50	44	3
71.	46	MALE	NO MP SEEN	42	4900	48	46	4
72.	40	MALE	SCANTY	42	10300	61	39	
73.	43	MALE	NO MP SEEN	39	4900	56	44	
74.	47	MALE	SCANTY	41	5100	67	33	
75.	45	MALE	NO MP SEEN	41	4800	61	39	
76.	43	MALE	SCANTY	39	8600	57	43	
77.	45	MALE	NO MP SEEN	41	10400	65	34	1
78.	46	MALE	NO MP SEEN	44	7200	52	46	2
79.	48	MALE	SCANTY	41	6700	66	31	3
80.	40	MALE	NO MP SEEN	44	7000	58	40	2
81.	43	MALE	NO MP SEEN	40	5700	59	40	1
82.	45	MALE	SCANTY	41	5000	60	38	2
83.	16	MALE	NO MP SEEN	40	7800	57	42	1
84.	42	MALE	SCANTY	39	4500	51	48	2
85.	13	MALE	NO MP SEEN	42	6600	53	46	1
86.	18	MALE	NO MP SEEN	41	4000	61	37	2
87.			SCANTY	40	8200	56	43	1
88.	20	FEMALE	NO MP SEEN	42	7400	60	37	3
89.	19	FEMALE	SCANTY	39	10100	51	47	2
90.	18	FEMALE	NO MP SEEN	40	7100	62	37	1
91.	16	FEMALE	NO MP SEEN	43	6000	54	44	2
92.	13	FEMALE	SCANTY	38	7900	56	39	1

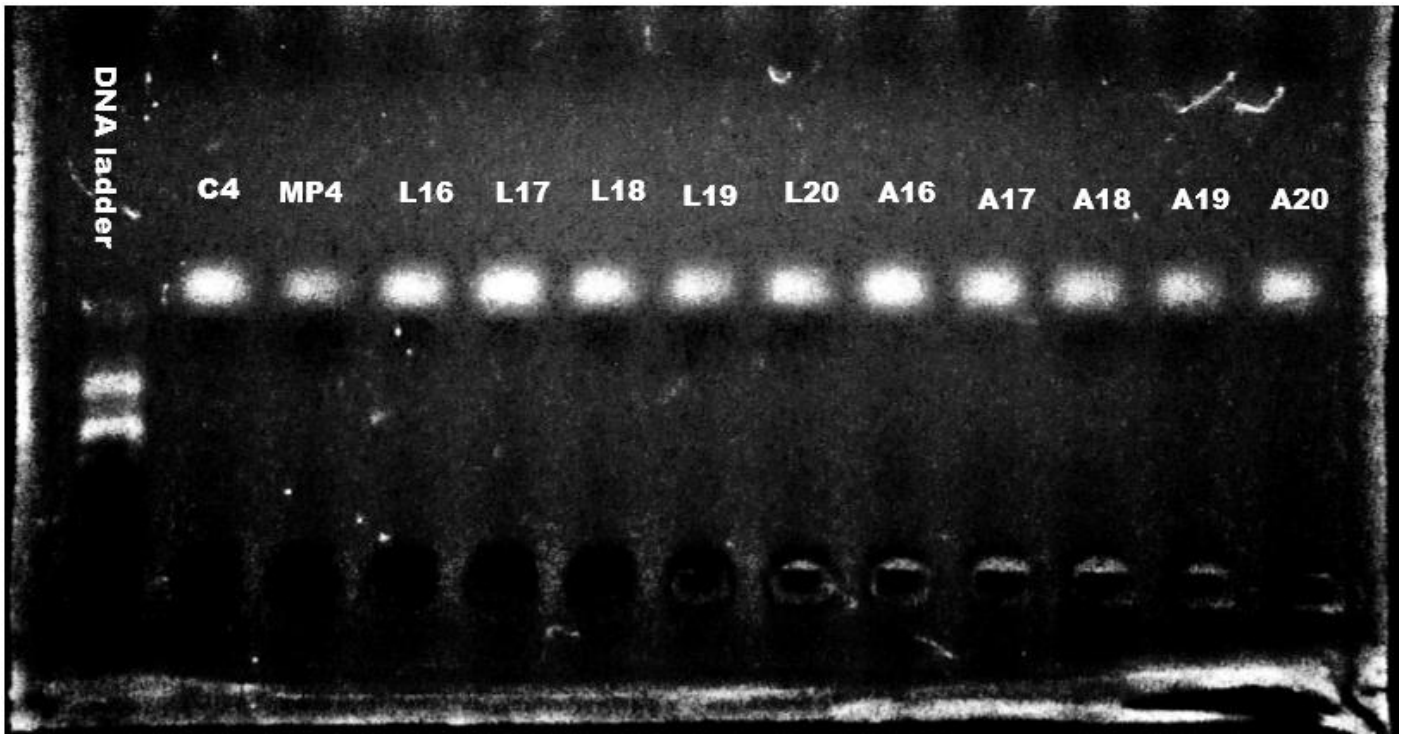
93.	14	FEMALE	NO MP SEEN	40	4100	54	44	2
94.	17	FEMALE	NO MP SEEN	41	5800	66	32	2
95.	18	FEMALE	SCANTY	39	6400	50	47	3
96.	16	FEMALE	NO MP SEEN	42	9300	60	38	2
97.	18	FEMALE	SCANTY	42	10900	50	49	1
98.	17	FEMALE	NO MP SEEN	39	4500	53	45	2
99.	20	FEMALE	NO MP SEEN	44	5900	51	48	1
100.	16	FEMALE	NO MP SEEN	38	10600	54	44	2

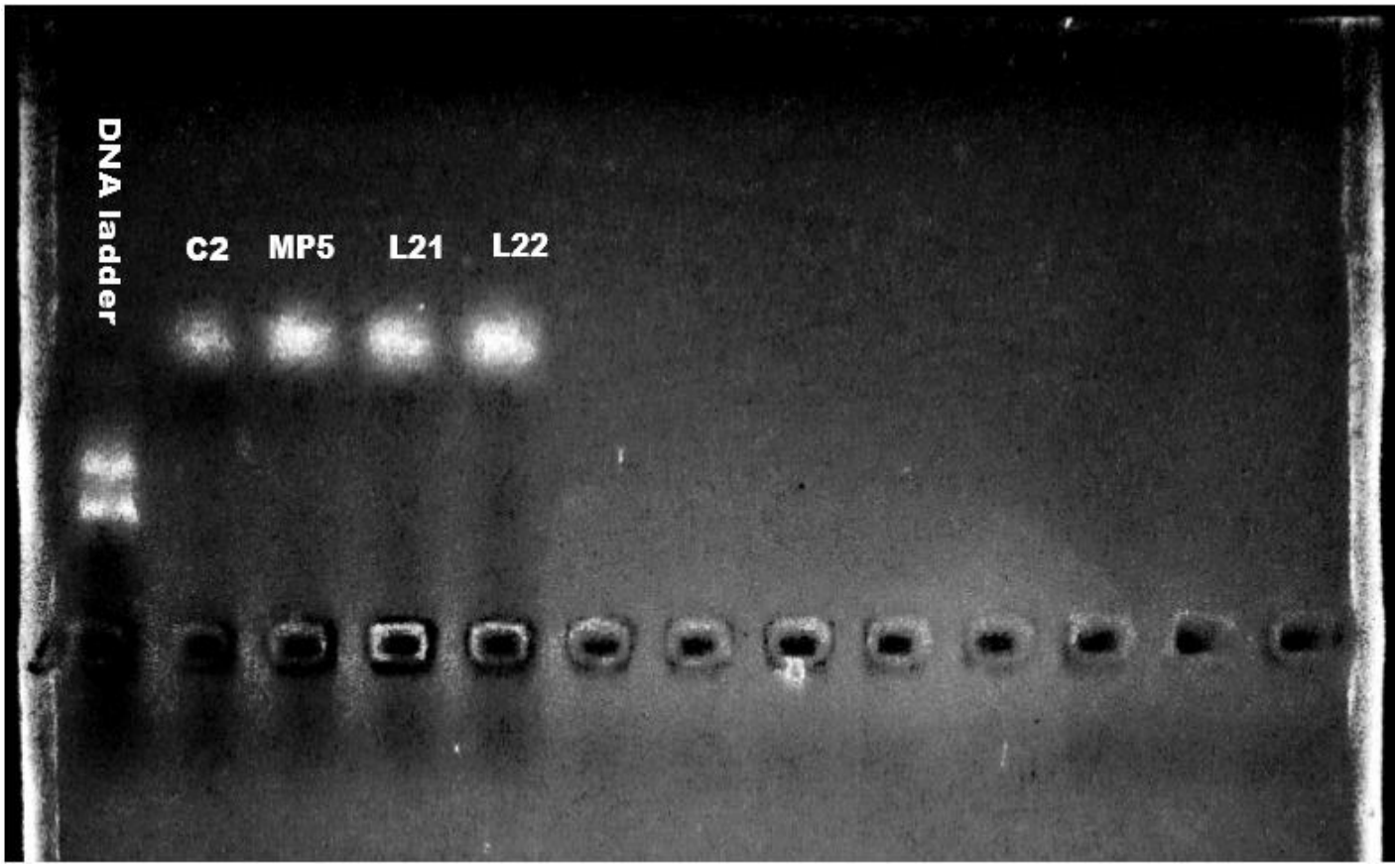
Appendix II

Gene expression for Lonart and Artequin resistant subjects




DNA ladder
C3
MP3
L11
L12
L13
L14
L15
A11
A12
A13
A14
A15


DNA ladder
C4
MP4
L16
L17
L18
L19
L20
A16
A17
A18
A19
A20


DNA ladder
C2
MP5
L21
L22

Appendix III

TABLE SHOWING RESULT FOR THE BAND INTENSITY OF SUBJECTS WITH LONART AND ARTEQUIN RESISTANT ALONG WITH THE CONTROL GROUP

	SLIDE 1		SLIDE 2		SLIDE 3
SAMPLE LABEL	BAND INTENSITY	SAMPLE LABEL	BAND INTENSITY	SAMPLE LABEL	BAND INTENSITY
C1	130.96	C2	147.133	C3	46.212
MP2	129.852	MP2	153.058	MP3	63.642
L1	83.802	L6	163.117	L11	100.52
L2	124.31	L7	167.782	L12	79.447
L3	133.004	L8	181.357	L13	85.328
L4	150.926	L9	174.072	L14	181.14
L5	132.292	L10	171.003	L15	163.465
A1	143.905	A6	159.817	A11	92.983
A2	125.802	A7	159.991	A12	95.586
A3	134.66	A8	149.123	A13	193.041
A4	129.911	A9	153.639	A14	182.31
A5	120.032	A10	135.449	A15	150.98

	SLIDE 4		SLIDE 5
SAMPLE LABEL	BAND INTENSITY	SAMPLE LABEL	BAND INTENSITY
C4	133.004	C2	91.615
MP4	95.122	MP5	149.946
L16	139.578	L21	157.441
L17	169.158	L22	179.189
L18	150.591		
L19	136.254		
L20	142.993		
A16	162.174		
A17	145.953		
A18	123.158		
A19	100.871		
A20	100.043		

KEY

C=CONTROL

L=LONART

A=ARTEQUIN