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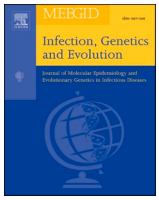

Review

Molecular insights into artemisinin resistance in *Plasmodium falciparum:* An updated review

Wihda Aisarul Azmi [a,b], Andita Fitri Mutiara Rizki [a,b], Yenny Djuardi [c], I. Made Artika [a,d], Josephine Elizabeth Siregar [a,*]

[a] *Eijkman Research Center for Molecular Biology, National Research and Innovation Agency, Cibinong, Bogor 16911, Indonesia*
[b] *Master's Programme in Biomedical Sciences, Faculty of Medicine Universitas Indonesia, Jakarta 10430, Indonesia*
[c] *Department of Parasitology, Faculty of Medicine Universitas Indonesia, Jakarta 10430, Indonesia*
[d] *Department of Biochemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor 16680, Indonesia*

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ABSTRACT

Malaria still poses a major burden on human health around the world, especially in endemic areas. *Plasmodium* resistance to several antimalarial drugs has been one of the major hindrances in control of malaria. Thus, the World Health Organization recommended artemisinin-based combination therapy (ACT) as a front-line treatment for malaria. The emergence of parasites resistant to artemisinin, along with resistant to ACT partner drugs, has led to ACT treatment failure. The artemisinin resistance is mostly related to the mutations in the propeller domain of the kelch13 (k13) gene that encodes protein Kelch13 (K13). The K13 protein has an important role in parasite reaction to oxidative stress. The most widely spread mutation in K13, with the highest degree of resistance, is a C580Y mutation. Other mutations, which are already identified as markers of artemisinin resistance, are R539T, I543T, and Y493H. The objective of this review is to provide current molecular insights into artemisinin resistance in *Plasmodium falciparum*. The trending use of artemisinin beyond its antimalarial effect is described. Immediate challenges and future research directions are discussed. Better understanding of the molecular mechanisms underlying artemisinin resistance will accelerate implementation of scientific findings to solve problems with malarial infection.

1. Introduction

For more than a century, malaria continues to cause an undesirable amount of morbidity and mortality among humans around the world despite the recent improvement in chemotherapeutics and vector control measures (Hanboonkunupakarn and White, 2022). Specifically, the malaria-endemic countries are currently struggling to eliminate malaria on account of the presence of several hindrances, such as parasite resistance, zoonotic cases, and the emergence of the COVID-19 pandemic. According to the latest report from WHO in World Malaria Report 2021, malaria incidence has climbed from 221 million cases in 2019 to 247 million cases in 2020, a considerably higher case load than in 2015, when cases were falling remarkably from 2000. In addition, there has been no significant decline in malaria cases and deaths since 2015 (WHO, 2021). Indonesia, the fourth most populated country, has about 130 million people living in malaria-high-risk areas. In 2021, total malaria cases in Indonesia were estimated at 800,000 (Sugiarto et al., 2022).

Malaria is an infectious disease caused by a protozoan parasite *Plasmodium* spp., which is transmitted to the host by a *Plasmodium*-infected female *Anopheles* mosquito during bloodmeal. The typical symptoms of malaria are a repeated cycle of fever paroxysm, shivering, body aches, abdominal pain, and other flu-like symptoms (Noreen et al., 2021). Among other clinical manifestations which are related to severe malaria are cerebral malaria, acute respiratory distress syndrome (ARDS), placental malaria, anemia, liver failure, and kidney failure. These symptoms can be lethal if not diagnosed and treated quickly, especially in high-risk individuals such as infants, young children, pregnant women and their unborn children, older adults, and travelers from non-endemic malaria countries (Fried and Duffy, 2017; Nureye and Assefa, 2020; Graça et al., 2020).

The five species of *Plasmodium* sp. known to cause malaria in humans

* Corresponding author at: Eijkman Research Center for Molecular Biology, Cibinong Science Center BRIN, Jalan Raya Jakarta-Bogor Km.46, Cibinong, Bogor 16911, Indonesia.
E-mail address: jose001@brin.go.id (J.E. Siregar).

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are *Plasmodium falciparum, P. vivax, P. malariae, P. ovale,* and *P. knowlesi*. The human malaria parasite causing the highest death rate in malaria-endemic areas is *P. falciparum*. Infection by this parasite leads to severe clinical manifestations such as cerebral malaria, pulmonary edema, jaundice, respiratory distress or acidosis, and other lethal symptoms (Kotepui et al., 2020). The major obstacle in malaria control is antimalarial resistance. In the late 1950s, the malaria control program was hit by chloroquine resistance. This was followed by resistance issues to drugs such as sulfadoxine-pyrimethamine and mefloquine (Goodman et al., 2016; Eyles et al., 1963; Hurwitz et al., 1981; Smithuis et al., 1993). Artemisinin was then used to reduce malaria associated morbidity and mortality worldwide. However, artemisinin resistant *Plasmodium falciparum* had then emerged, and this has been threatening malaria control and elimination efforts (Feng et al., 2019). Parasites resistant to antimalarial drugs are currently spreading even after the WHO introduced Artemisinin-based Combination Therapy (ACT), a combination of artemisinin and its partner drugs, as an alternative way to overcome antimalarial drug resistance. Low coverage and poor quality of malaria treatments have been suggested as factors which trigger the emergence of drug-resistant parasites (WHO, 2020).

Artemisinin resistance is associated with a reduction in the drug efficacy in clearing the parasite (Wicht et al., 2020). Many studies have been conducted in order to understand the mechanisms of artemisinin resistance in the *Plasmodium falciparum*, however the exact mechanisms remain unknown. At present, several mechanisms have been proposed (Mbengue et al., 2015; Wicht et al., 2020). Better understanding of detailed mechanism of artemisinin resistance will facilitate identification of new antimalarial drug targets. Then, the discovery and development of new antimalarial drugs will be more focused. The objective of this review is to update and summarize current molecular understanding of the artemisinin resistance phenomenon in *Plasmodium falciparum*, focusing on molecular mechanisms underlying artemisinin resistance. Mutations and molecular markers of artemisinin resistance are discussed. Challenges, directions for future studies and progress to control the spread of artemisinin resistance are described.

2. Artemisinin as antimalarial drug

Artemisinin is a sesquiterpene lactone ($C_{15}H_{22}O_5$), a secondary metabolite compound isolated from Qinghao, otherwise known as

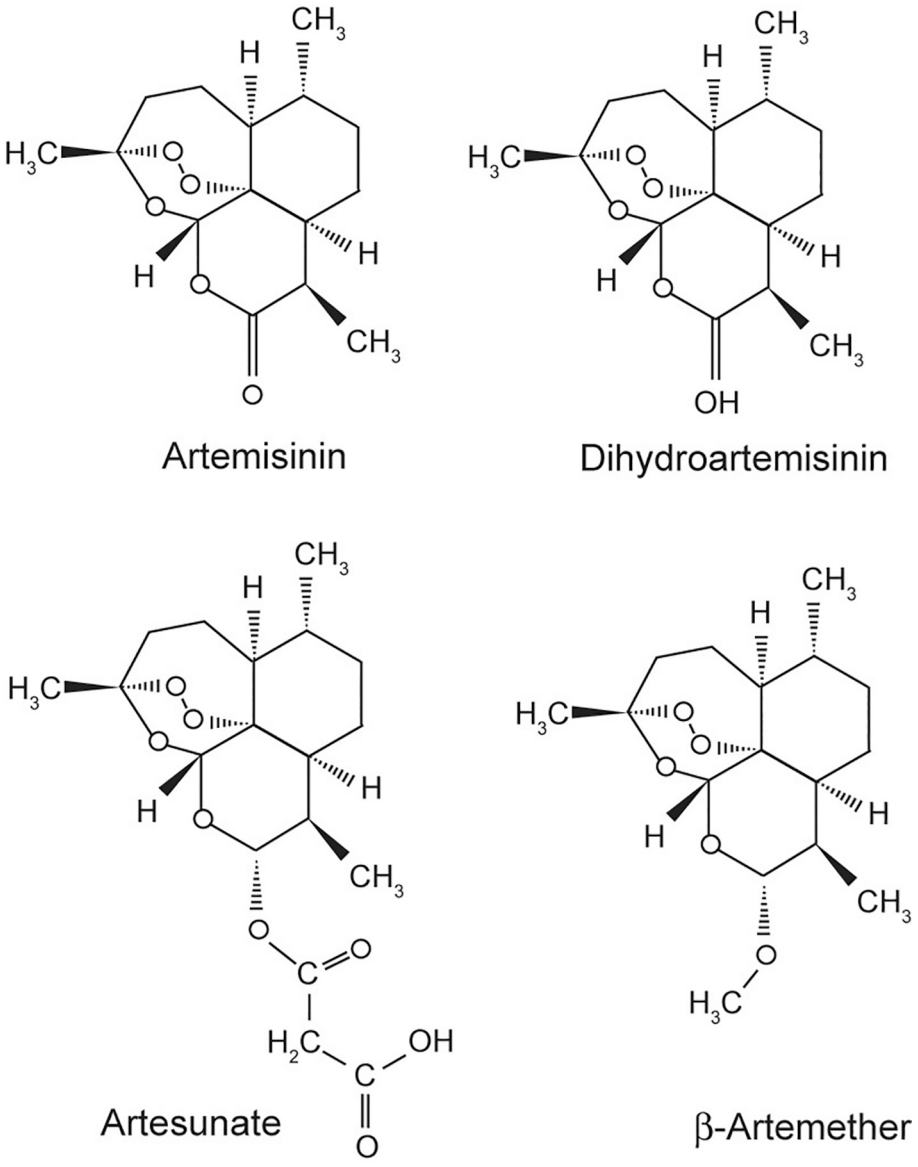

Fig. 1. Chemical structure of artemisinin and its derivatives.
Dihydroartemisinin, artesunate and β-artemether are the most common artemisinin derivatives used in the artemisinin-based combination therapy (ACT).

Artemisia annua L. (Tu, 2011). The special trait of artemisinin that distinguishes it with other antimalarial drugs is the peroxide bridge on its chemical structure. This peroxide bridge is found to have a significant role in its antimalarial activity based on several proposed mechanisms of artemisinin efficacy (Ma et al., 2020).

Artemisinin is a fast-acting antimalaria agent which is able to kill almost all forms of the parasites in blood stages. However, considering the low solubility of artemisinin in water or oil, and its low bioavailability, the derivates such as dihydroartemisinin, artesunate, and artemether, are mostly used rather than in its native structure (Yang et al., 2020; Desrosiers et al., 2020). The derivatives are also effective against malaria parasites in their asexual blood stages and male gametocytes forms (Rosenthal and Ng, 2020). The chemical structure of artemisinin and its derivatives is shown in Fig. 1.

The World Health Organization (WHO) recommends artemisinin and its derivatives for malaria treatment. Artemisinin-based combination therapies (ACTs) are now the core of the first-line antimalarial drugs for severe and uncomplicated *P. falciparum* malaria due to their rapid activity in clearing the parasite (Talman et al., 2019). The ACT regimens that last for three-days of treatment consist of artemisinin (or its derivatives) and a partner drug having a longer half-life than artemisinin (Rosenthal and Ng, 2020). Aside from its potential activity, this combination of artemisinin and its partner drug in ACT was expected to reduce the emergence and spreading of parasites' resistance to antimalarial drugs. The ACTs employ different partner drugs such as lumefantrine, piperaquine, mefloquine, amodiaquine, or pyronaridine (Wicht et al., 2020). The WHO recommends six drug combinations: artemether + lumefantrine, artesunate + amodiaquine, artesunate + mefloquine, artesunate + pyronaridine, artesunate + sulfadoxine-pyrimethamine and dihydroartemisinin + piperaquine (WHO, 2020). The most common drug combinations used in Southeast Asia are artesunate + mefloquine and dihydroartemisinin + piperaquine. In Africa, the main drug combinations are artemether + lumefantrine and artesunate + amodiaquine (Wicht et al., 2020).

Unlike chloroquine, sulfadoxine, pyrimethamine, and lumefantrine, the mechanism of action of artemisinin and its derivatives (collectively referred to as ARTs) has yet to be clearly defined (Tandoh et al., 2021). To exert their impact, ARTs need to be activated (Talman et al., 2019). ART-activation is triggered by Fe^{2+} heme generated as a result of hemoglobin digestion. Notably, during the erythrocytic phase of the parasitic life cycle, *P. falciparum* invades the host red blood cells and then hemoglobin endocytosis ensues (Noreen et al., 2021; Ma et al., 2021). The ARTs are likely to have a complex mechanism of action which may affect various structures and functions of the parasite. ARTs affect multiple metabolic machineries and processes such as proteasome protein polyubiquitination, phosphatidylinositol 3-kinase (PI3K), unfolded protein response (UPR), and the eukaryotic translation initiation factor 2a (eIF2α) (Talman et al., 2019).

ARTs cause parasite death by damaging proteins and inhibiting proteasome functionality (Bridgford et al., 2018). The accumulation of damaged proteins and proteasome-substrates stimulates an endoplasmic reticulum stress response. Inhibition of proteasome activity leads to accumulation of polyubiquitinated proteins which mediates parasite death (Bridgford et al., 2018). Furthermore, it was also reported that prevention of protein synthesis or protein ubiquitination reduces the level of polyubiquitinated proteins and so alleviates the stress response (Bridgford et al., 2018). ARTs also disturb the parasite UPR which is essential to increase folding capacity and therefore restore protein homeostasis (Talman et al., 2019).

ARTs are also thought to increase production of free radical species, which are probably carbon-centered or reactive oxygen species, as a consequence of the endoperoxide bridge cleavage after the activation of artemisinin. The cleavage of artemisinin peroxide bridge proceeds when it forms a bond with hematin (Fe(II)), the byproduct of hemoglobin degradation. This reactive oxygen species attacks the nucleophilic group of proteins and lipids of the parasites. This reaction takes place inside the digestive vacuole of the parasites in the erythrocytic stage and mainly destroys the parasites in their ring and trophozoite forms (Gopalakrishnan and Kumar, 2015; Yang et al., 2020). Artemisinin and artesunate, in particular, have also been indicated to potentially induce *P. falciparum* death by inhibiting hematin crystallization and heme detoxification following the drug's activation. This newly discovered pathway complements the deadly effects of free radical generation through drug activation and may open a new strategy to improve drug efficacy (Ma et al., 2021).

In addition, ARTs have been shown to have DNA damaging activity (Gupta et al., 2016). Accumulation of reactive oxygen species (ROS) has also been suggested to contribute to increased DNA damage, and both ROS and DNA damage were found to cause parasite death (Gopalakrishnan and Kumar, 2015). Moreover, ARTs were also found to promote *P. falciparum* PI3K ubiquitination causing reduction of its lipid product phosphatidylinositol 3-phosphate (PI3P). PI3P, which localizes into the parasite food vacuole membrane and apicoplast, is an essential lipid for vesicular transport such as in protein export (Tawk et al., 2010; Mbengue et al., 2015).

The world demand for artemisinin is high, approximately 119 metric tons every year (Badshah et al., 2018). To meet this demand, expansion of *A. annua* cultivation areas is needed. In addition, better *A. annua* cultivars having higher artemisinin content, and efficient extraction methods are critical (Babacan et al., 2022). Artemisinin can be extracted from various parts of *A. annua*, such as leaves, small green stems, buds, flowers and seeds. An efficient extraction step is critical to improve artemisinin yield and purity. Artemisinin is usually extracted using organic solvents such as chloroform, ethyl acetate, hexane, and petroleum ether at elevated temperature (Babacan et al., 2022). An alternative method to manufacture artemisinin is to produce artemisinic acid using yeast fermentation followed by chemical conversion of artemisinic acid to artemisinin. This procedure is termed the semisynthetic method and the artemisinin produced is called semisynthetic artemisinin. Semisynthetic artemisinin entered commercial production from 2013 (Kung et al., 2018). Because the synthesis of artemisinin is costly, different strategies have been developed to increase native artemisinin content in *A. annua*. One approach used metabolic engineering techniques so that genes which are involved in the artemisinin biosynthetic pathway are overexpressed. In other approach, metabolic pathways which compete with the artemisinin biosynthetic pathway are blocked (Tang et al., 2014; Badshah et al., 2018).

3. Artemisinin resistance and molecular marker

As a result of using artemisinin as an antimalarial drug, the emergence of *P. falciparum* resistance to artemisinin alone, ACTs and ACTs partner drugs is inevitable. The clinical manifestation of artemisinin resistance is the reduction of drug efficacy and a prolonged period of parasite clearance half-life after treatment of monotherapy artemisinin or its derivatives or ACT (Tilley et al., 2016). This usually leads to treatment failure and is implicated by gametocytemia and lengthening of parasite clearance to over 5 hours. Delayed parasite clearance half-life can be indicated as an increase of parasite half-life which can be determined through *in vitro* ring-stage assay (RSA) (Amaratunga et al., 2014; Davis et al., 2020). It should be noted, however, that in addition to intrinsic parasite susceptibility, treatment outcomes are also affected by other factors such as patient acquired immunity, initial parasite biomass, treatment adherence, dosing, drug quality and pharmacokinetics (WHO, 2020). Cases of both artemisinin resistance and ACTs resistance were reported in Southeast Asia, especially in Cambodia, Thailand, Laos and Vietnam (WHO, 2020; Rogers et al., 2009; Thanh et al., 2017; Imwong et al., 2017). The independent and local spread of clinically artemisinin-resistant parasites has also been identified in Africa (Balikagala et al., 2021). Failure using a combination of dihydroartemisinin and piperaquine was prevalent in the Pursat Province of Cambodia and the resistant parasites already existed in Thailand and

Vietnam (Amaratunga et al., 2016; Thanh et al., 2017; Imwong et al., 2017).

Artemisinin resistance in *P. falciparum* is associated with mutations in several genes which alter their resultant protein functionality. One of the genetic markers reported as a dominant factor in artemisinin resistance is a mutation in the propeller region of the kelch13 (k13) gene. In *P. faciparum*, the k13 gene (*Pfk13*) encodes protein K13, a 726-amino-acid protein that consists of a non-conserved *Apicomplexa*-specific initial N-terminal region and three annotated highly conserved domains (Coppée et al., 2019). The k13 gene was reported to be essential for the asexual intraerythrocytic stage of *P. falciparum* (Coppée et al., 2019). However, the defined function and suggested role of the K13 protein requires further elucidation. The propeller domain of K13 protein displays multiple protein-protein interaction sites and facilitates different cellular functions, such as degradation of ubiquitin-regulated protein and oxidative stress responses (Straimer et al., 2015; Xie et al., 2020). The mutations in the k13 genes linked to artemisinin resistance are nonsynonymous mutations which involve a change in the amino acid sequence of the protein. One mutation is sufficient to initiate parasite resistance to artemisinin (Straimer et al., 2015; Sá et al., 2018; Raman et al., 2019; Khammanee et al., 2019; Mok et al., 2021).

ARTs-resistant parasites with a mutation in their K13 protein showed a decline of hemoglobin endocytosis process and consequently less hemoglobin digestion. This lowered the drug activator Fe^{2+} heme, disturbed the activation of ARTs and in turn reduced efficacy of ARTs (Xie et al., 2020; Noreen et al., 2021). Molecular studies showed that mutation in the *P. falciparum* K13 protein is associated with several key features such as lowered level of K13 protein which can be measured by quantitative dimethyl-based proteomics analysis, increased expression of unfolded protein response pathways which can be detected by an *in vivo* transcriptomics analysis, and lowered levels of ubiquitinated proteins (Coppée et al., 2019). Indeed, the K13 protein has been implicated to play a role in facilitating protein ubiquitination and mutation in ubiquitination machinery is associated with reduced protein ubiquitination. Lower levels of ubiquitinated proteins lead to ARTs resistance (Bridgford et al., 2018). Molecular studies have also shown that treatment with ARTs inhibits proteasome function detected using proteasome-Glo assays (Bridgford et al., 2018). It is hypothesized, therefore, that the lowered ARTs activity due to mutation in protein K13 improves proteasome activity and contributes to ARTs resistance.

Another proposed mechanism linking artemisinin resistance and mutation of the K13 protein is based on its role in the parasite response to oxidative stress. Artemisinin resistance is suggested to be due to a reduction of parasite oxidative stress as a result of a lower activation of artemisinin (Siddiqui et al., 2021). It was also recently indicated that artemisinin resistance phenomenon involves redox mechanisms (Egwu et al., 2022). As mentioned previously, one of the mechanisms of ARTs antimalarial activity is by producing chemically reactive species. It was found that reactive oxygen production in artemisinin resistant parasites is lower than that in artemisinin sensitive parasites (Egwu et al., 2022). A newly discovered protein, named Kelch13 interacting candidate 5 (KIC5) has been implicated in playing a role as a regulator of artemisinin stress response in the malaria parasite *P. falciparum* (Simmons et al., 2023). Other crucial factors, such as improved ability for DNA repair, were suggested to attribute to ARTs resistance. Mutations in six out of the seven known DNA repair genes were reported to associate with K13 mutations (Xiong et al., 2020).

Mutation C508Y in K13 protein can change its binding capacity to PI3K, leading to decreased ubiquitination and increased levels of PI3K and its product PI3P. This was found to contribute to ARTs resistance *in vitro* (Mbengue et al., 2015; Bhattacharjee et al., 2018; Talman et al., 2019). It was found that mutation C508Y in K13 protein increases the PI3P vesicle levels which may play a role in widespread dissemination of protestatic capacity which neutralizes the artemisinin toxic effects and leads to artemisinin resistance. Alteration of export of the virulence adhesin, *Plasmodium falciparum* erythrocyte membrane protein-1 PfEMP1 to red blood cells was also observed (Bhattacharjee et al., 2018). Of note, treatment with dihydroartemisinin suppresses the expression of the *var* gene, the gene encoding the PfEMP1 protein. Down regulation of PfEMP1 increases parasite susceptibility to artemisinin indicating the involvement of PfEMP1 in resistance mechanism to antimalarial drugs (Chen et al., 2021). In addition, it was recently reported that mutations in the K13 protein are associated with increased metabolic plasticity indicated by drastic changes in the tricarboxylic acid (TCA) cycle, glycolysis and amino acid metabolism. This metabolic remodeling was found to stimulate artemisinin resistance (Yu et al., 2022). Recent findings suggested that overexpression of cyclophilin 19B in *P falciparum* triggers resistance to artemisinin. Of note, cyclophilin 19B plays a critical role in protein folding. The increased expression of the parasite cyclophilin 19B was found to be maintained by polymorphism in the short tandem repeat (SRT) sequence of its gene the promoter region of its gene (Kucharski et al., 2023). Translational suppression mediated by phosphorylation of eIF2α, can also lead to artemisinin resistance. Phosphorylation of eIF2α reduces general protein synthesis and promotes specific synthesis of proteins important for recovery from the stress. It was reported that *Plasmodium* having phosphorylated eIF2α are less sensitive to artemisinin (Zhang et al., 2017). Predicted molecular mechanisms of artemisinin resistance in *P. falciparum* are illustrated in Fig. 2.

Mutations other than those found in gene encoding the K13 protein have been indicated to be involved in conferring artemisinin resistance in *P. falciparum* (Mukherjee et al., 2017). A molecular genotyping study involving 68 *P. falciparum* isolates from Cambodia showed that several parasites exhibit artemisinin resistance without *pfkelch13* mutations (Mukherjee et al., 2017). Whole genome sequence analysis of *P. falciparum* isolates from West Africa showed that mutations in the gene encoding for the actinbinding protein coronin confer reduced artemisinin sensitivity (Demas et al., 2018). Similarly, whole genome sequencing of artemisinin-resistant parasites obtained using *in vitro* selection method indicated that mutations, or single nucleotide polymorphisms, in the parasite genes and intragenic regions may contribute to artemisinin resistance independent of K13 mutations. Those alterations may lead malaria parasites to enhanced adaptive responses against oxidative stress and protein damage (Rocamora et al., 2018). Another study showed that mutations of *pfap2* and *pfubp1* significantly reduce the ring stage susceptibility of *Plasmodium falciparum* to artemisinin. The *pfap2* gene encodes for the μ-subunit of the AP2 trafficking complex and the *pfubp1* gene encodes for the ubiquitin hydrolase UBP1. These findings suggested the involvement of multiple pathways leading to artemisinin resistance (Henrici et al., 2019). A report on *pfk13*-independent treatment failure in imported cases of *Plasmodium falciparum* infection treated with artemether-lumefantrine in the United Kingdom further suggested that factors other than K13 mutations play a role in parasite tolerance to ACTs. It was observed that none of the patients carried mutations in the propeller-encoding domain of the *pfk13* locus (Sutherland et al., 2017). While most attention is paid to the early stage of the parasite development, studies suggested that artemisinin resistance is not limited to the ring stage, but also occurred in trophozoites and schizonts (Cui et al., 2012; Lee et al., 2021). A study showed that in the late stage of dihydroartemisinin-resistant parasites a number of changes occurred such as *pfmdr1* amplification, elevation of the antioxidant defence network, increased expression of chaperones, all of which may be linked to antimalarial drug resistance (Cui et al., 2012). In addition, a study has demonstrated that *P. falciparum* rosetting, in that spontaneous binding of infected erythrocytes to uninfected erythrocytes, protects schizonts against artemisinin. In the late stages of artemisinin-resistant *P. falciparum*, upregulation of expression of proteins essential for proteins cytoadherence, such as PfEMP1, was also observed (Lee et al., 2021). Moreover, parasite sequestration, in that attachment of infected erythrocytes harboring late developmental stages of the parasite (trophozoites and schizonts) to the endothelium of capillaries, was also shown to prolong parasite clearance half-life in patients with

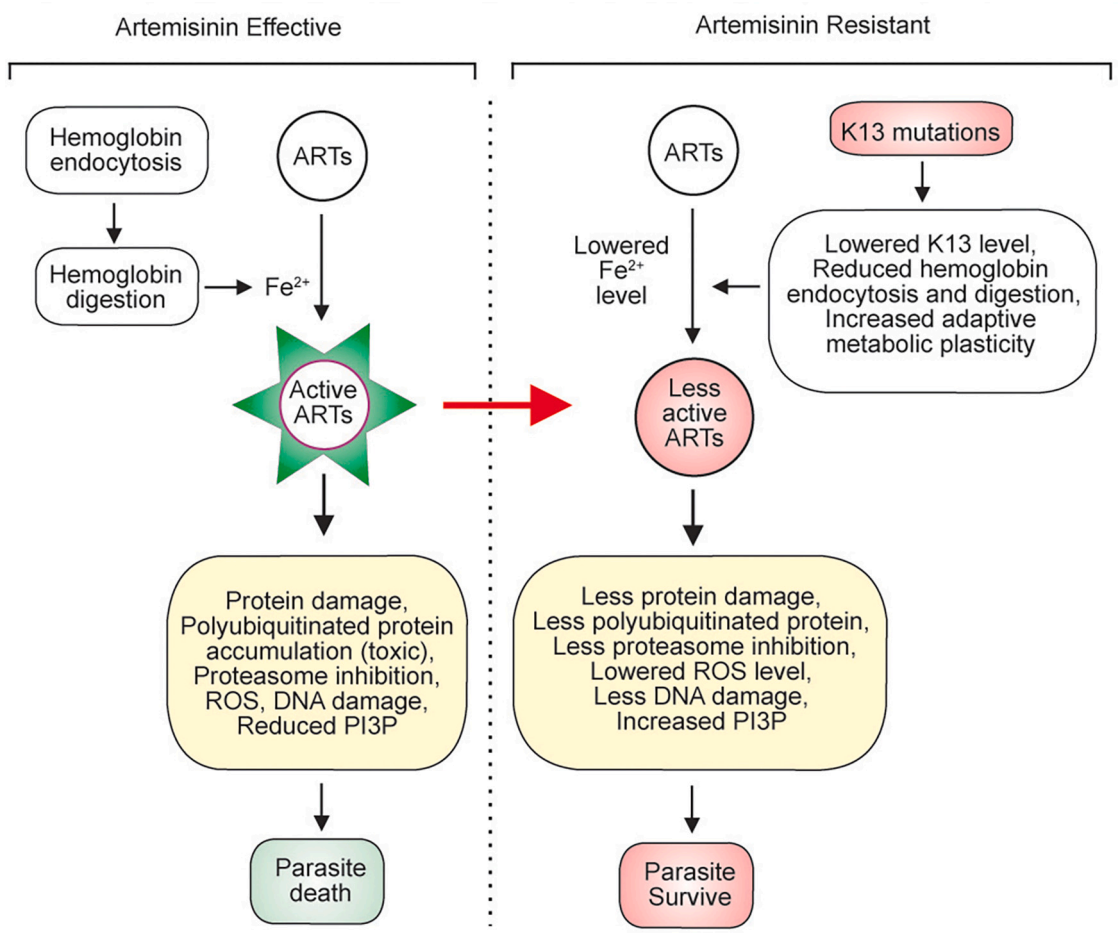

Fig. 2. Predicted molecular mechanisms of artemisinin resistance in *Plasmodium falciparum*.
During the erythrocytic phase of *P. falciparum* life cycle, the parasite invades the host red blood cells and hemoglobin endocytosis takes place. Within the parasite, the hemoglobin is enzymatically digested. The digestion process liberates Fe^{2+} which activates artemisinin and its derivatives (ARTs). The active ARTs affect the function of different components of the parasite including the unfolded protein response (UPR), protein polyubiquitination, proteasome, phosphatidylinositol-3-kinase (PI3K) and the eukaryotic translation initiation factor 2a (eIF2α). Accumulation of damaged proteins, proteasome substrates, polyubiquitinated proteins and stimulation of endoplasmic reticulum stress response lead to parasite death. Artemisinin resistance is associated with mutations of K13 protein which result in lower K13 level, reduced hemoglobin endocytosis, reduce hemoglobin digestion and reduced free Fe^{2+}. These conditions lead to less active ACTs which lead to lower protein damage, less polyubiquitinated proteins, less proteasome inhibition, lowered ROS level, less DNA damage and increased PI3P. These conditions allow parasite survival.

P. falciparum malaria without artemisinin-resistant mutations (Fukuda et al., 2023).

It should be noted that in malaria treatment with ACTs, the partner drugs contribute to a lesser extent in the clearance of parasites. In addition to ineffectiveness of artemisinin and its derivatives, ACT failure can also be due to failure of the partner drugs (Nsanzabana, 2019). Emergence and mechanism of *P. falciparum* resistance to ACT partner drugs have previously been reviewed (Martin et al., 2018; Nsanzabana, 2019). Related to ACT deployment, recent modeling studies to evaluate the roles of pre-existing partner-drug resistance on the evolution of artemisinin resistance showed that the presence of a higher level of pre-existing partner-drug resistant genotypes lead to the earlier emergence of artemisinin resistance. This indicated that partner-drug resistance to ACTs mediates the early emergence of artemisinin resistance (Watson et al., 2022).

Molecular markers of artemisinin resistance are defined as mutations identified as being associated with a change in parasite susceptibility to artemisinin and its derivatives. The molecular markers are useful for confirming artemisinin resistance, and the marker prevalence in a geographic area can be used as an early warning signal (WHO, 2020). It is currently well accepted that single nucleotide changes within the propeller region of the gene encoding K13 are linked to *P. falciparum* resistance to artemisinin and its derivatives. The artemisinin resistance was first detected in Pailin Province, Western Cambodia, in 2009. The drug resistance later spread to China, Thailand, Vietnam, Lao PDR, Myanmar, India, Papua New Guinea, Rwanda, Ethiopia, Tanzania, Mali, Ghana, Angola, Pakistan (Huang et al., 2015; Miotto et al., 2020; Owoloye et al., 2021; Ghanchi et al., 2021; Zhu et al., 2022a). Identification of molecular markers is intended to facilitate detection and monitoring of the spread of artemisinin resistance. More than 260 non-synonymous mutations in the *P. falciparum* gene encoding the K13 protein of have been identified. However, not all these mutations are associated with artemisinin resistance. Different mutations have different effects on the clearance phenotype. Currently, there are 10 validated and 11 associated (or candidate) molecular markers listed by the WHO (WHO, 2020). Types of K13 protein mutation linked to artemisinin resistance in *P. falciparum* and their geographic distribution is shown in Table 1.

The majority of the validated molecular markers for artemisinin resistance are found in Asia. The C580Y mutation is the most frequently identified (WHO, 2020). A systematic review and spatiotemporal analysis recently revealed that the prevalence of the C580Y mutation increased from 48•9% in 2002 to 84•9% in 2018. Similarly, the prevalence and geographic distribution of validated molecular markers increased steadily in the same period of time (Kagoro et al., 2022). The C580Y mutation was suggested to have emerged in Western Cambodia

Table 1
Types of K13 protein mutation associated with artemisinin resistance in *Plasmodium falciparum* and their geographic distribution*.

Mutation	Country/Region	Classification	Reference
F446I	Myanmar, China; Thailand, Mali	validated	WHO, 2020; Ménard et al., 2016; Huang et al., 2015; Ouattara et al., 2015; Imwong et al., 2017; Wang et al., 2018; Owoloye et al., 2021
N458Y	Cambodia, Thailand, Myanmar, China	validated	WHO, 2020; Ariey et al., 2014; Ménard et al., 2016; Boullé et al., 2016
M476I	Myanmar, Tanzania	validated	WHO, 2020; Bonnington et al., 2017; Owoloye et al., 2021; Kayiba et al., 2021
Y493H	Cambodia, Vietnam Laos	validated	WHO, 2020; Ariey et al., 2014; Ménard et al., 2016; Imwong et al., 2017
R539T	Cambodia, Vietnam, China-Myanmar border, Laos, Thailand	validated	WHO, 2020; Ariey et al., 2014; Ménard et al., 2016; Imwong et al., 2017
I543T	Cambodia, Vietnam, Laos	validated	WHO, 2020; Ariey et al., 2014; Ménard et al., 2016
P553L	Cambodia, Thailand, Angola	validated	WHO, 2020; Ariey et al., 2014; Ménard et al., 2016; Owoloye et al., 2021
R561H	Thailand, Myanmar, China, Rwanda, Tanzania	validated	WHO, 2020; Ménard et al., 2016; Bonnington et al., 2017; Imwong et al., 2017; Owoloye et al., 2021
P574L	Thailand, Myanmar, China, Rwanda	validated	WHO, 2020; Ménard et al., 2016; Tacoli et al., 2016
C580Y	Cambodia, Vietnam, Laos, Myanmar, China-Myanmar border, Guyana, Thailand-Myanmar border; Ghana, New Guinea	validated	WHO, 2020; Ariey et al., 2014; Ménard et al., 2016; Chenet et al., 2016; Boullé et al., 2016; Bonnington et al., 2017; WWARN, 2019; Owoloye et al., 2021; Imwong et al., 2017; Miotto et al., 2020
P441L	Thailand-Myanmar border	associated	WHO, 2020; Boullé et al., 2016
G449A	Myanmar	associated	WHO, 2020; Imwong et al., 2017
C469F/Y	Southern Pakistan, Uganda	associated	WHO, 2020; Ghanchi et al., 2021; Kayiba et al., 2021; Balikagala et al., 2021
A481V	Cambodia	associated	WHO, 2020; Ariey et al., 2014
R515K	South East Asia, Zambia	associated	WHO, 2020; WWARN, 2019; Kayiba et al., 2021
P527H	Myanmar	associated	WHO, 2020; Imwong et al., 2017
N537I/D	Myanmar	associated	WHO, 2020; Imwong et al., 2017
G538V	Thailand-Myanmar border	candidate or associated	WHO, 2020; Boullé et al., 2016
V568G	Cambodia,	associated	WHO, 2020; Ariey et al., 2014
R622I	Ethiopia	associated	WHO, 2020; Bayih et al., 2016
A675V	Thailand-Myanmar border, Rwanda, Uganda	associated	WHO, 2020; Boullé et al., 2016; Tacoli et al., 2016; Balikagala et al., 2021

* Less frequent variants have also been identified. However, the sample sizes were too small to determine statistical significance. These variants include K479I, G533A, R575K, M579I, D584V, P667T, F673I and H719N (WHO, 2020).

and then spread to Thailand and Laos (Imwong et al., 2017). By now, this mutation has been detected in many other countries such as Myanmar, Vietnam, Laos, China, Papua New Guinea, Guyana (South America) and Ghana (Western Africa) (WHO, 2020; Ariey et al., 2014; Ménard et al., 2016; Chenet et al., 2016; Boullé et al., 2016; Imwong et al., 2017; Miotto et al., 2017; Bonnington et al., 2017; WWARN, 2019; Owoloye et al., 2021). The C580Y mutation occurred in all Greater Mekong subregion (GMS) countries (Myanmar, Cambodia, Thailand, China and Laos). Furthermore, it was observed that C580Y mutation is mostly detected in the Southeastern GMS such as Cambodia, Laos, Thailand, and Vietnam (Kagoro et al., 2022).

In 2017, three *P. falciparum* mutants were identified to carry the C580Y mutation in Papua New Guinea (Miotto et al., 2020). The proportion of *P. falciparum* with K13 C580Y mutation in Papua New Guinea tended to increase from 2.2% in 2017, 5.7% in 2018, and 6.4% in 2020 (Yoshida et al., 2021). Of note, the eastern part of the New Guinea Island is the independent nation of Papua New Guinea and the western part is part of Indonesia (Indonesian Papua). It was found that multiple portions of the mutant genomes share a common origin with *P. falciparum* from Indonesian Papua. Furthermore, molecular analysis indicated an independent origin of the C580Y mutation (Miotto et al., 2020). Similarly, the C580Y allele identified in five samples in Guyana was suggested to arise from independent emergence as indicated by its flanking microsatellite profiles which are different from those observed in Southeast Asia (Chenet et al., 2016). Independent emergence of C580Y mutation and other artemisinin mutations have also been indicated to have arisen in different geographic regions in Southeast Asia (Takala-Harrison et al., 2015). This implies that programs to eliminate artemisinin-resistant parasites in one region may have little impact on the emergence of artemisinin resistance in another (Takala-Harrison et al., 2015). Reports on molecular surveillance of K13 mutations in Indonesia are limited. A study conducted in Pesawaran District, Lampung Province, Indonesia, using polymerase chain reaction (PCR) followed by DNA sequencing showed that there were no *P. falciparum* K13 mutations (Y493H, R539T, C580Y or M476L) detected, indicating that the ACT drug was still effective (Kurniawan et al., 2018). Another study involving 231 isolates from Northwestern Indonesia observed 9 isolates with nonsynonymous gene variants in the propeller domain of *pfk13*. This study identified K13 C580Y mutation with low confidence in only a single isolate (Lubis et al., 2020). Similarly, no K13 mutations were detected in Sabah area, Malaysia (Norahmad et al., 2016). Although in general artemisinin and its derivatives are still effective in treating malaria in Indonesia, the presence of several changes in *pfk13* in the parasite population indicates the need of further assessment of the parasite susceptibility to artemisinin in Indonesia (Rahmasari et al., 2022).

The F446I mutation was mainly identified in Northern GMS including Thailand, Myanmar and the border between China and Myanmar (Bonnington et al., 2017; Kagoro et al., 2022). It was reported that the F446I mutation was the most common mutation in Northern Kayin State, Myanmar (Bonnington et al., 2017). The F446I mutation has also been detected in Mali, Africa (Ouattara et al., 2015; Kayiba et al., 2021).

As shown in Table 1, the validated N458Y molecular marker was detected in Cambodia (Ariey et al., 2014), Thailand, Myanmar, and China (Ménard et al., 2016; Boullé et al., 2016). Apart from C580Y and N458Y, other validated molecular markers found in Cambodia include: R539T, Y493H, I543T, and P553L. In addition, the associated molecular markers A481V and V568G were also identified in Cambodia (Ariey et al., 2014). It was observed that the validated molecular markers C580Y, R539T, Y493H, and I543T were frequently detected in Cambodia, Vietnam and Laos, while the F446I, N458Y, P574L, and R561H were often found in Thailand, Myanmar and China (Ménard et al., 2016). The associated molecular markers P441L, G449A, P527H and N537I/D were found in Myanmar (Imwong et al., 2017). The G538V mutation, classified as associated molecular marker, was reported

present in Thailand-Myanmar border (Boullé et al., 2016).

In Africa, as many as five validated molecular markers conferring artemisinin resistance were found. The F446I mutation was detected in Mali, M476I in Tanzania, R561H in Rwanda and Tanzania, P553L in Angola and C580Y in Ghana (Owoloye et al., 2021). The associated molecular marker A675V was reported in Southern Rwanda from two *P. falciparum* isolates collected in 2015 (Tacoli et al., 2016). In Northwest Ethiopia, three isolates harboring the associated molecular marker R622I was reported (Bayih et al., 2016). Some other associated molecular markers were also found in Africa such as C469Y (Uganda), R515K (Zambia) and V568G (Kenya) (Kayiba et al., 2021). The R515 was also detected in Southeast Asia (WWARN, 2019). In Kenya, a non-synonymous mutation (W611S) was identified in the K13 protein of *P. falciparum* and predicted to cause structural changes on the K13 protein. The impact of this mutation on artemisinin resistance has yet to be elucidated (Musyoka et al., 2020).

In addition to molecular markers of artemisinin resistance, molecular markers of resistance to partner drugs have also been identified, although some of them have yet to be validated (WHO, 2020). Recently, two new drug resistance genes, the ubiquitin-specific protease 1 (*pfubp1*) gene and the adaptor protein complex 2 mu subunit (*pfap2mu*) gene, have been identified to associate with ACTs-resistant *P. falciparum*. The mutations are D1525E and E1528D in the ubiquitin-specific protease 1 and S160N in the adaptor protein complex 2 mu subunit. The identification of these mutations further proved the risk of ACTs resistance. More detailed studies are needed to validate the mutations as genetic markers (Cheng et al., 2022).

4. Trend use of artemisinin and development of resistance

Considering their remarkable potency and safety, the antimalarial artemisinin and its derivatives have been explored for possible repurposing to treat various diseases including cancer, inflammation, immunoregulation-related diseases, and coronavirus disease 2019 (COVID-19) (Shi et al., 2022). A growing number of reports showing the activity of artemisinin-type compounds against broad diseases further justify the idea of artemisinin repurposing (Lu and Efferth, 2021). Drug repurposing has been considered as a promising strategy to accelerate the discovery process of novel therapies (Krishna et al., 2021). Recent reports indicated the trend use of artemisinin and its derivatives as anticancer (Gong et al., 2022; Shi et al., 2023), anti-inflammation (Yang et al., 2021), anti-atherosclerosis (Cen et al., 2023; Yoon et al., 2022), anti-diabetes (Han et al., 2019; Bai et al., 2022) anti-SARS-CoV-2 (Badraoui et al., 2022; Krishna et al., 2021), antibacterial agent (Lin et al., 2018; Qian et al., 2021), antifungal agent (Zhou et al., 2021; Zhu et al., 2022a, 2022b). The trend use of artemisinin beyond antimalarial drug and its proposed mechanism is summarized in Table 2.

It was reported that combination of artesunate and WNT974 inhibits proliferation of colorectal cancer by inducing KRAS protein degradation. Notably, the KRAS protein is a product of an oncogene, KRAS, which is susceptible to mutations in colorectal cancer (Gong et al., 2022). Dihydroartemisinin was demonstrated to inhibit growth of cervical cancer cells in a time- and dose-dependent manner by promoting the cervical cancer cells to undergo ferroptosis (Shi et al., 2023). Ferroptosis is a type of programmed cell death characterized by the accumulation of lipid peroxidation and iron (Hu et al., 2022). Similarly, studies using two human breast cancer cell lines, MDA-MB-231 and MCF-7 cells, demonstrated that dihydroartemisinin inhibit growth and trigger pyroptosis in breast cancer cells (Li et al., 2021). Pyroptosis induction by dihydroartemisinin also causes growth inhibition of esophageal squamous cell carcinoma (Jiang et al., 2021). Pyroptosis refers to a form of programmed cell death involving high inflammation and plays a critical role in cancer and other diseases (Jiang et al., 2021). Novel artemisinin-isatin hybrids have been developed for anti-leukemic agents. It was found that the artemisinin-isatin hybrids exhibit promising effects against a human acute lymphoblastic leukemia cell line. Some of the hybrids were also found to be active against human myeloid leukemia cell lines (Wang et al., 2023). Artemisinin also inhibits thyroid cancer by suppressing aerobic glycolysis, a series of reactions used by cancer cells to generate a large amount of metabolites and ATP for faster growth (Yang et al., 2023). In addition, artemisinin was reported to drastically reduce the migration and invasion of uveal melanoma cells. Uveal melanoma is a type of eye cancer within the uveal tract, arises from melanocytes, the cells producing the dark pigment melanin (Farhan et al., 2021).

The potential anti-inflammatory bioactivities of artemisinin and its derivatives have also been investigated. For these purposes, a number of studies using animal models have recently been carried out. Using rosacea-like mice, artemisinin was shown to inhibit inflammation and markedly ameliorate the elevated pro-inflammatory factors. Noteworthy, rosacea is a common chronic facial inflammatory disease worldwide which diminishes the patient quality of life (Yuan et al., 2019). An artemisinin derivative, β-aminoarteether maleate, was suggested to have the potential to be used for treatment of inflammatory disorder, especially dry eye disease. This was based on experimental results using rodent and mice models which showed that topical application of β-aminoarteether maleate drastically reduces the levels inflammatory mediators and provides other benefit effects which alleviate dry eye disease (Yang et al., 2021). Similarly, dihydroartemisinin was demonstrated to attenuate pulmonary inflammation in rats by inhibiting expression of Janus activated kinase 2 (JAK2) and signal transducer and activator 3 (STAT3) which associate with pulmonary inflammation, the initial cause of pulmonary fibrosis. Therefore, dihydroartemisinin has the potential to be developed for therapeutic candidate to treat pulmonary fibrosis. It is important to note that pulmonary fibrosis is associated with COVID-19 and currently there are no effective treatments available for pulmonary fibrosis (You et al., 2022). A study employing mouse models with colitis, the inflammation in the lining of the colon, showed that artemisinin decreases inflammation-associated lymphangiogenesis, the formation of new lymphatic vessels which mediates inflammation under particular disease conditions. In addition, artemisinin also reduced the colitis symptoms. This suggested that artemisinin

Table 2
Potential use of artemisinin and its derivatives beyond an antimalarial drug.

Potential use	Proposed mechanism of action	Reference(s)
Anticancer	Inhibits cell growth, triggers ferroptosis and pyroptosis	Gong et al., 2022; Shi et al., 2023; Li et al., 2021
Anti-inflammation	Reduces inflammatory mediators, induces apoptosis	Yang et al., 2021; Zhao et al., 2007
Anti-atherosclerosis	Upregulates lipoprotein lipase expression, inhibits monocyte adhesion, lipid deposition and platelet aggregation	He et al., 2020; Wang et al., 2016; Cen et al., 2023; Yoon et al., 2022
Anti-diabetes	Reduces triglycerides, increases mitochondrial pyruvate carriers, prevents hyperglycemia, modulates amino acid metabolism	Li et al., 2017; Han et al., 2019; Bai et al., 2022; Rong et al., 2022
Anti-COVID-19	Inhibits viral replication, prevents cytokine storm	Badraoui et al., 2022; Krishna et al., 2021
Antibacterial agent	Increases membrane permeability, inhibits respiratory metabolism, inhibits biofilm formation, generates reactive oxygen species	Lin et al., 2018; Qian et al., 2021; Sisto et al., 2022; Chung et al., 2022
Antifungal agent	Inhibits cell growth, increases ergosterol level, disrupts mitochondrial function; induces apoptosis-like process	Zhou et al., 2021; Zhu et al., 2021; Zhu et al., 2022b; Das et al., 2020

can be developed for a new therapy for inflammatory lymphangiogenesis which is related to inflammatory bowel disease (Lee et al., 2020). Moreover, artemisinin was also found to inhibit neuroinflammation the important feature Alzheimer's disease, the major form of dementia in old individuals. Hence, artemisinin is considered as a potential therapeutic agent for Alzheimer's disease (Zhao et al., 2022). Dehydroartemisinin has recently been shown to suppress inflammation and proliferation of vascular smooth muscle cells (VSMCs) which play a critical role in the development of hypertension, restenosis, and atherosclerosis. The protective effects of dehydroartemisinin were shown to involve inhibition of expression of fat mass and obesity-associated (FTO) gene. More studies are needed for clinical use of dehydroartemisinin to treat diseases related to inflammation and proliferation of VSMCs (Huo et al., 2022). Previously, a study using rat models showed that artemisinin relieves the severity of tissue inflamation of rats with acute pancreatitis by inducing apoptosis. Data indicated that apoptosis induction reduces infiltration of inflammatory cells and production of inflammatory cytokines (Zhao et al., 2007).

The use artesunate as an anti-atherosclerosis agent has been proposed. In mice, artesunate was shown to prevent atherosclerosis by upregulating expression of lipoprotein lipase. It is important to note that lipoprotein lipase plays a critical role in hydrolyzing triglyceride and deficiency of this enzyme leads to atherosclerosis (He et al., 2020). Similarly, in rats, artesunate was found to be able to ameliorate atherosclerosis by inhibiting arterial lipid deposition, excessive arterial inflammatory responses and other cellular processes which promote atherosclerosis. Of note, atherosclerosis is known as the major cause of cardiovascular diseases (Cen et al., 2023). In a study using human platelet-rich plasma, artesunate was shown to inhibit platelet aggregation which is associated to cardiovascular disorders such as atherosclerosis and stroke (Yoon et al., 2022). In addition, artesunate may be beneficial to patients with systemic lupus erythematosus. A study involving 35 systemic lupus erythematosus patients showed that artesunate inhibits type I interferon-induced production of macrophage migration inhibitory factor which modulates both atherosclerosis and systemic lupus erythematosus. Of note, activation of type I IFN system triggers atherosclerosis in systemic lupus erythematosus patients (Feng et al., 2017). Earlier, artemisinin was demonstrated to have the ability to prevent monocytes adhesion to human umbilical vein endothelial cells, a process crucial for initiation of atherosclerosis. Therefore, artemisinin has the potential to be used to block initial development of atherosclerotic lesions (Wang et al., 2016). Recently, artemisinin was also shown to alleviate myocardial ischemia-reperfusion, the main cause of death in patients with cardiovascular diseases due to lack of blood supply to the heart. The mechanism involves increased viability of cardiomyocyte cells. Interestingly, in this study using myocardial ischemia-reperfusion rat models and cell models artemisinin was found to decrease apoptosis and oxidative stress (Han et al., 2022). Dihydroartemisinin has been considered as a potential novel therapy for pulmonary hypertension, a disorder associated with abnormal proliferation of pulmonary artery smooth muscle cells. Dihydroartemisinin was shown to inhibit the proliferation and migration of pulmonary artery smooth muscle cells in a study using mouse models (Cai et al., 2022). Similar results were also obtained in a study using rodent models which showed that artemisinin and dihydroartemisinin relieve pulmonary hypertension (Bao et al., 2022).

Artemether has been proposed a putative candidate for therapy of diabetes and diabetic kidney disease (Han et al., 2019). A study employing mouse models revealed that artemether alleviates type 2 diabetic kidney disease by increasing mitochondrial pyruvate carrier content, preventing hyperglycemia, improving diabetic symptoms, reducing urinary albumin excretion and restoring other conditions which provide renal protection. Notably, diabetic kidney disease is the fundamental cause of kidney disorder (Han et al., 2019). Similarly, Rong and colleagues recently demonstrated that artemether ameliorates diabetic kidney disease by modulating amino acid metabolism. Artemether was shown to have the ability to normalize the upregulated level of the branched-chain amino acids and citrulline as well as the downregulated level of glutamine, glutamic acid, aspartic acid, ornithine, glycine,

histidine, phenylalanine and threonine in diabetic mice (Rong et al., 2022). The possible use of artemisinin to treat diabetic nephropathy, a common complication of diabetes, and to provide protective effects against renal injury has been systematically reviewed (Feng et al., 2022). In diabetic rat models, artesunate was found to significantly alleviate hyperglycemia, blood lipid level and liver function. More pronounced benefit effects were observed when artesunate was combined with insulin for treatment (Bai et al., 2022). In another study using rat models, artesunate was shown to reduce elevated plasma triglycerides and other adverse effects induced by using clozapine, an antipsychotic drug for schizophrenia treatment. It was concluded that artesunate has the potential use to ameliorate clozapine-induced adverse metabolic effects in schizophrenia patients (Li et al., 2017).

The absence of effective therapy for the globally devastating COVID-19 pandemic caused by the newly emerging severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has also led to exploration of the potential efficacy of artemisinin as anti-SARS-CoV-2. It was found that in combination with piperaquine, artemisinin exhibits ability to shorten the time the SARS-CoV-2 remains in the body of COVID-19 patients (Li et al., 2021). Similarly, as many as five different ACTs (mefloquine-dihydroartemisinin, desethylamodiaquine-dihydroartemisinin, pyronaridine-dihydroartemisinin, lumefantrine-dihydroartemisinin, and piperaquine-dihydroartemisinin) were assessed in Africa for their effects on a clinical isolate of SARS-CoV-2. Results showed that all artemisinin-based combinations exert anti-SARS-CoV-2 activity and the mefloquine-artesunate combination was found to be the most effective (Gendrot et al., 2020). Other studies to evaluate the activities of artemisinin related compounds against SARS-CoV-2 showed that artesunate and dihydroartemisinin exhibit anti-SARS-CoV-2 effects and may be used as candidates for SARS-CoV-2 drug development (Cao et al., 2020). The *in silico* approach also predicted that artemisinin has anti- SARS-CoV-2 activity by binding to the virus main protein (Mpro), specifically to its cysteine 145 residue and hence inhibits viral replication (Badraoui et al., 2022). Artemisinin and its derivatives have been suggested to potentially provide benefit effects to COVID-19 patients by preventing proinflammatory cytokines and cytokine release syndrome or cytokine storm (Uzun and Toptas, 2020; Krishna et al., 2021). Recently, the efficacy of hot water extracts of *Artemisia annua* (the plant source of artemisinin) against SARS-CoV-2 and its variants has also been demonstrated (Nair et al., 2023). Of note, there is sufficient evidence for the anti-SARS-CoV-2 effects of artemisinin and its derivatives to support further clinical therapeutic studies to combat the COVID-19 pandemic (Krishna et al., 2021). The potential use of artemisinin to combat other pathogenic viruses such as flaviviruses (Japanese encephalitis virus, dengue virus, Zika virus) (Wang et al., 2020) and human cytomegalovirus (Çapcı et al., 2020) have also been assessed.

The repurposing of artemisinin and its derivatives as antibacterial agents has been attempted (Patel et al., 2019). In particular, it is of urgency to discover effective compounds to tackle the emergence and spread of drug-resistant bacteria and artemisinin repurposing may offer an efficient way (Lin et al., 2018). Artemisinin encapsulated with beta-cyclodextrin was found to exhibit inhibitory activity against methicillin-resistant *Staphylococcus aureus* (MRSA). In this strategy, the molecular encapsulation of artemisinin was intended to improve its solubility in order to enhance its antibacterial effect on MRSA. The hypothesized antibacterial mechanisms involved include increased MRSA membrane permeability and inhibition of MRSA respiratory metabolism. The beta-cyclodextrin-encapsulated artemisinin was suggested to have the potential use to treat bacterial infection (Lin et al., 2018). Artesunate was demonstrated to have ability to inhibit formation of *S. aureus* biofilm by preventing bacterial adhesion and inhibiting expression of bacterial virulence and adhesion genes. It is worthy to note that biofilm formation increases *S. aureus* resistance to various drugs and permits the bacteria

to avoid human immune system which results in chronic infection. Hence, artesunate also has clinical application potential as a therapy for bacterial infection (Qian et al., 2021). Artemisinin has been suggested to have the potential to be repurposed as an antibacterial agent. Artemisinin was shown to have ability to kill *Vibrio cholerae* via reactive oxygen species (ROS) generation and copper-mediated DNA damage (Chung et al., 2022). A combination of artemisinin and rifampicin was found to be synergistic against *Mycobacterium bovis* and *Mycobacterium tuberculosis* in an *in vitro* study (Patel et al., 2019). The efficacy of arylaminoartemisinin GC012 against clinical strains of *Helicobacter pylori* has been evaluated. It was found that arylaminoartemisinin GC012 inhibits growth and kills *H. pylori* by preventing biofilm formation and altering bacterial membrane structure. The antibacterial activity of the compound was four times higher than that of metronidazole and clarithromycin, the antibiotics currently applied to treat *H. pylori* infection. Enhanced antibacterial activity was observed when the compound is combined with an antibiotic metronidazole, clarithromycin, or amoxicillin. Arylaminoartemisinin GC012 was therefore suggested as a potential efficacious drug candidate against *H. pylori*. Of note, *H. pylori* is the main cause of gastric ulcer and is linked to gastric carcinoma (Sisto et al., 2022).

Development of new antifungal agents is of paramount important due to drug resistance and a limited repertoire of antifungals. Artemisinin repurposing is regarded to hold great potential in novel antifungal discovery (Zhou et al., 2021). The antifungal activity of artemisinin, dihydroartemisinin, artesunate and artemether has been tested against *Candida albicans* the main cause of morbidity and mortality in fungal infection. Results showed that all of the four artemisinins exhibit antifungal activity and can improve the activity of the widely used antifungals, amphotericin B, micafungin and fluconazole. Importantly, in combination with fluconazole, a commonly used antifungal agent, artemether exhibits the most potent antifungal activity with ability to inhibit growth of fluconazole-resistant clinical isolates. It was suggested that artemether inhibits the *C. albicans* pleiotropic drug resistance 5 (PDR5), a protein playing a role in drug efflux, leading to intracellular accumulation of fluconazole (Zhou et al., 2021). Another study also demonstrated that artemisinin acts as a potentiator to amphotericin B, causing enhanced activity against a wildtype strain and clinical isolates of *C. albicans*. In this synergistic phenomenon, artemisinin was found to upregulate expression of genes involved in the ergosterol biosynthesis, thus increasing the ergosterol level of *C. albicans*. This markedly strengthens the binding of fungal cells to the antifungal drug amphotericin B causing the *C. albicans* sensitive to amphotericin B. Therefore, artemisinin can potentially be developed for therapy to treat oral cadidiasis, an infection of the oral cavity by *C. albicans* (Zhu et al., 2021). Recently, artemisinin was found to inhibit growth of *C. glabrata*, another important causative agent of cadidiasis, by targeting the fungal transcription factor pleiotropic drug resistance 1 (PDR1) which leads to mitochondrial dysfunction. The PDR1 plays a critical role to regulate the drug efflux pump and ergosterol biosynthesis pathway. It should be noted that ergosterol is an essential component of fungal mitochondrial membrane, hence, deletion or overexpression of genes involved in ergosterol biosynthetic pathway disrupts mitochondria function (Zhu et al., 2022b). Artemisinin was also shown to exert cytotoxic effects on several clinical isolates of *Candida* species, *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. krusei*, *C. glabrata*, *C. guilliermondii* and *C. parapsilosis*. It was indicated that artemisinin promotes accumulation of intracellular ROS which leads to apoptosis-like processes. *C. glabrata*, *C. guilliermondii* and *C. parapsilosis* were found to be the most susceptible species to artemisinin treatment (Das et al., 2020).

The extended use of artemisinin and its derivatives has raised concern of the potential increased risk of artemisinin resistance. Increased use of artemisinin may promote artemisinin resistance. It is known that drug pressure contributes to emergence of drug resistance (Ataba et al., 2022). In particular, the situation in Africa, where more than 90% of malaria cases are detected, is dramatic and worrying because of the uncontrolled use of *Artemisia annua* (a plant containing artemisinin) for malaria and COVID-19 medication. In facing the COVID-19 pandemic, many people illegally use *A. annua* for preventing and curing COVID-19. This is mainly because herbal remedies are more readily accessible, low cost and have been used traditionally to treat diseases (Ataba et al., 2022; Agrawal et al., 2022). Of note, WHO disagrees with the use of *A. annua* for COVID-19 treatment without approval based on scientific evidence (Ataba et al., 2022). Apart from *A. annua*, other *Artemisia* species such as *A. afra* also reported to exhibit benefit effects against COVID-19. However, the effectiveness of using of *A. afra* as a tea to treat COVID-19 has been questioned as *A. afra* does not contain artemisinin. Moreover, extensive use of *Artemisia* extracts without precise quantification of artemisinin and other constituents, over a large population, has generated fears of malarial parasites developing resistance (Agrawal et al., 2022). Apart from increase use, other factors such as the use of defraud drugs, inadequate controls on prescription, poor adherence to treatment regimens and incorrect dose, may increase the chance of emergence of artemisinin resistance (Ataba et al., 2022).

5. Challenges, future research directions and progress

The increasing prevalence of *P. falciparum* resistance to artemisinin and its derivatives (the key compounds in ACTs) poses substantial and immediate challenges to global malaria control and elimination efforts (Liu et al., 2022). Moreover, the development of resistance to the partner drugs employed in the ACTs exacerbates the problem (Montenegro et al., 2021). Inadequate information on artemisinin resistance-related genes and molecular mechanisms underlying the artemisinin resistance phenomenon hampers rapid development of effective countermeasures and control strategies (Iwanaga et al., 2022). Although the use of artemisinin monotherapy and poor-quality antimalarials have been linked to artemisinin resistance development, other factors which may trigger the emergence of the resistance have yet to be fully identified (Ouji et al., 2018). Development of highly effective and durable vaccines against such complex eukaryotic *Plasmodium* parasites has been very challenging (Frimpong et al., 2018). So it remains of paramount urgency that novel effective antimalarial drugs be searched for to combat ACT-resistant parasites (Le Bihan et al., 2016). Limited access to malaria diagnostics can cause delays in case-identification and treatment. The availability of portable, sensitive and accurate diagnostic tools is critical (Rahi and Sharma, 2022). The current geographic distribution of resistant *P. falciparum* needs to be more precisely mapped for effective containment (Dondorp et al., 2017).

Future research and development should be directed towards: molecular surveillance of artemisinin-resistant *Plasmodium*, identification of mutations and molecular markers, elucidation of molecular mechanisms underlying artemisinin-resistance, discovery of novel antimalarial drugs, further development of vaccines, and development of molecular diagnostic tools. Molecular surveillance studies are critical in order to determine the intensity and distribution of the artemisinin-resistant parasites. Molecular surveillance is also important for monitoring imported artemisinin-resistant parasites and assessing their local risk and impact (L'Episcopia et al., 2021; Liu et al., 2022). Importantly, with the aid of genetic engineering techniques, the association of K13 mutations with the artemisinin resistance phenotype has been confirmed *in vitro* and *in vivo*, rationalizing the use of molecular surveillance through K13-sequencing to track the spread of artemisinin resistance in attempt to mitigate its impacts (Ariey et al., 2014; Straimer et al., 2015). Continued monitoring of treatment failures and decreased parasite susceptibility to ACTs is critical for evaluation of treatment guidelines (L'Episcopia et al., 2021; Liu et al., 2022). Similarly, a genetic characterization program, which includes whole genome sequencing, will uncover the genetic features and mutations linked to the artemisinin-resistance which may lead to identification of molecular markers and drug-resistance genes (Iwanaga et al., 2022). Molecular markers of artemisinin resistance are

essential for detecting artemisinin-resistant parasites and monitoring their spread (Liu et al., 2022). It should be noted that many mutational markers may be associated with artemisinin resistance. It is therefore of paramount importance to conduct molecular and genetic investigations in different parts of the world (Rawat et al., 2022). The proposed future research and development direction to combat artemisinin-resistant *Plasmodium falciparum* is schematically illustrated in Fig. 3.

Comprehensive studies which include multiomics analysis need to be prioritized to elucidate molecular mechanisms underlying the artemisinin-resistance in *Plasmodium*. Understanding the detailed mechanism of artemisinin resistance is very important for accelerating design of effective vaccines and discovery of novel antimalarial drugs (Rawat et al., 2022). New genes and the metabolic pathways which may be linked to the emergence of artemisinin-resistant *Plasmodium* need to be identified. The role of glycolytic and penthose phosphate pathway regulation in the artemisinin resistance phenomenon needs to be confirmed (Rawat et al., 2022). Comprehensive molecular analysis is critical to elucidate the evolution of *P. falciparum* genes and the corresponding encoded proteins associated to artemisinin resistance. Evolutionary data provide a powerful way to understand the functionality of the protein domains (Coppée et al., 2019). It was identified that the *P. falciparum* K13 protein contains one poorly-conserved domain and three conserved domains: a coiled-coil-containing (CCC) domain, a Broad-complex, tramtrack and bric-à-brac (BTB) domain and a Kelch-repeat propeller (KREP) domain. The BTB and KREP domains were predicted to be essential for protein-protein interaction. Moreover, it was predicted that the artemisinin resistance mutations (C580Y and R539T) of the K13 protein cause destabilization of KREP domain structure (Coppée et al., 2019). Previous genomics analysis using the whole chromosome shotgun-sequencing strategy revealed that a large proportion of *P. falciparum* genes are devoted to immune evasion and host-parasite interactions (Gardner et al., 2002). Recent genomics studies of *P. falciparum* populations in Ethiopia showed that the parasite population in the central region of Ethiopia was structurally divergent from both Southeast Asian and other East African populations. No validated mutations of PfKelch13 were identified except for an uncharacterized mutation K189T (Abera et al., 2021).

Novel antimalarial compounds with new modes of action and potency against multiple life-cycle stages of *Plasmodium* are urgently needed. Ideally, the new antimalarial compounds need to be active against all strains of *Plasmodium*, including the strains already resistant to artemisinin and other antimalarial drugs. *In vitro* and *ex vivo* drug susceptibility testing needs to be conducted to determine efficacious doses and modes of action (Le Bihan et al., 2016). Of particular note, the generation of artemisinin-resistant parasite models would be very beneficial for effective screening of new antimalarial drug candidates. The emergence of parasite strains resistant to antimalarial drugs may be caused by inadequate subtherapeutic dose treatment and by incomplete therapeutic treatment (Nuralitha et al., 2017a). Therefore, parasite models to investigate resistance to artemisinin can be generated by repeated treatment with a subtherapeutic dose of artemisinin and by repeated incomplete treatment with a therapeutic dose of artemisinin (Nuralitha et al., 2017a). These models could be very useful to elucidate the mechanisms of artemisinin resistance and to screen new drug candidates for artemisinin-resistant *P. falciparum* (Nuralitha et al., 2017b). The powerful genome editing techniques such as CRISPR/Cas9 can be applied to accelerate development of parasite models (Simwela et al., 2020). One of the strategies to control malaria is prompt treatment of infected individuals. Targeting the host's pathways involved in *P. falciparum* susceptibility may be an effective approach to discover novel treatments for *P. falciparum* infection (Asih et al., 2022). The human plasma membrane calcium-ATPase 4 (PMCA4) was proposed as a new potential target for novel antiplasmodial drugs. This protein is a transport protein which functions to extrude calcium (Ca^{2+}) from the cell (Asih et al., 2022). Inhibitors of PMC4, aurintricarboxylic acid (ATA) and resveratrol were found to inhibit growth of *Plasmodium* in a dose-dependent fashion (Asih et al., 2022). A novel ubiquitin-specific protease of *P. falciparum* has also recently been identified as a

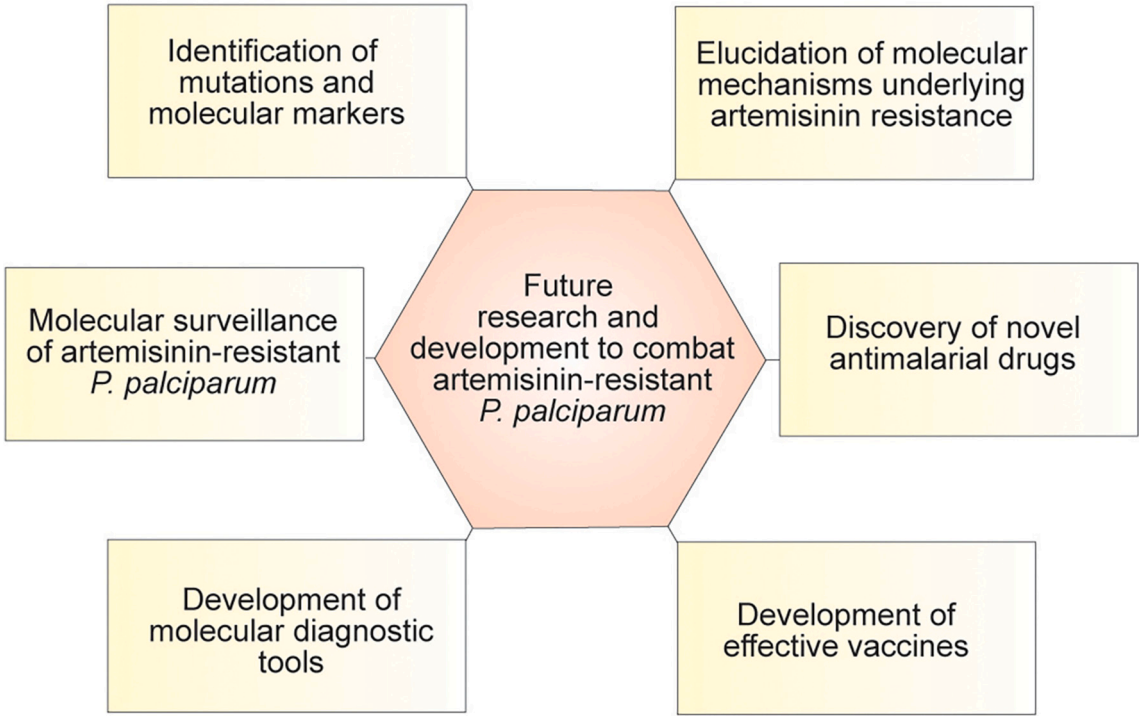

Fig. 3. Proposed future research and development direction to combat artemisinin-resistant *Plasmodium falciparum*.
Research and development activity should be directed towards molecular surveillance of artemisinin-resistant *P. falciparum*, identification of mutations and molecular markers associated with artemisinin resistance, elucidation of molecular mechanism underlying artemisinin resistance phenomenon, discovery of novel antimalarial drugs, development of effective vaccines and development of molecular diagnostic tools.

potential target of artemisinin-based drugs based on the fact that this protease is essential for parasite survival (Arora et al., 2023).

In the lack of sufficiently efficacious vaccines, the antimalarial drugs remain the most effective measure to treat and contain the spread of artemisinin-resistant *P. falciparum* (Noreen et al., 2021). Due to their efficacy, safety, availability and low cost, plant natural products have been used to treat malaria for many decades (Wicht et al., 2020; Noreen et al., 2021). Quinine and artemisinin are two very successful antimalarial drugs derived from plant natural products (Budiarti et al., 2020; Noreen et al., 2021). Presently, medicinal plants are still considered as a potential source of new antimalarial drugs. In Indonesian Papua, as many as 72 medicinal plant species are traditionally used to treat malaria. The most commonly used are *Alstonia scholaris* (L.) R. Br., *Carica papaya* L., *Andrographis paniculata* (Burm. f.) Nees, and *Physalis minima* L. (Budiarti et al., 2020). Ethanolic extracts of *Macaranga* gigantea were shown to have potent activity against *Plasmodium berghei*, a parasite model used in that study. Phytochemical analysis showed that the bioactive compound having potent antiplasmodial activity was apigenin (Muhaimin et al., 2019). As to whether this compound is active against artemisinin-resistant *P. falciparum*, further studies are required. In Chinese traditional medicine, the roots and the leaves of the herb *Dichroa febrifuga* have been used to treat malarial fever. The crude extract of the root was reported to be effective on clinical cases of tertian malaria (Jang et al., 1948). Similarly, in Africa, the water and water-acetone extracts of *Vernonia amygdalina* Del (Compositae) were shown to have antimalarial activities (Masaba, 2000). In Ghana, Africa, the roots of *Cryptolepis sanguinolenta* have been used to treat malaria for many generations. An herbal tea based on this plant has been manufactured and marketed. The roots were reported to contain bioactive compounds, indoquinoline alkaloids, which have been shown to be active against *P. falciparum*, including chloroquine-resistant strains (Tempesta, 2010). Of note, in an ACT, an artemisinin derivative is used to reduce the number of parasites and the partner drug is intended to effect total clearance (WHO, 2020). Considering the occasional recrudescence of *P. falciparum*, with or without K13 mutations following an ACT treatment, the discovery of better partner drugs is essential to effectively eliminate infecting parasites (Sá et al., 2018; Mumtaz et al., 2020).

A new comprehensive approach involving genomics, transcriptomics and proteomics analysis is required to design effective vaccines against malaria. Many technical and biological obstacles are faced in developing malaria vaccines because these parasites have various evasion mechanisms. The advances of reverse vaccinology, structural vaccinology and immunoinformatics should contribute to solving of these challenges and improving malaria vaccines (Frimpong et al., 2018). Following more than 40 years of research and development, the WHO recommended the use of the first malaria vaccine, RTS,S, for children in a malarial-endemic region. It has been shown that this vaccine induces a protective immunity which neutralizes sporozoite infection or alleviates clinical severity of infection (Zavala, 2022). Of note, however, is that the immune responses generated by this vaccine do not reduce the ability of *Plasmodium* gametocytes to infect mosquitoes. Therefore, better and broader acting vaccines are needed to control malaria including artemisinin resistant parasites (Frimpong et al., 2018). In order to increase the supply of malaria vaccine and to improve efficacy, a malaria vaccine named R21/Matrix-M has been developed. This vaccine shows efficacy of 75% or greater and currently is under clinical evaluation (Datoo et al., 2022).

Accurate and sensitive molecular diagnostic tools are indispensable to monitor the spatial and temporal spread of artemisinin-resistant *P. falciparum* and to evaluate malaria-control programs. Molecular assays are also useful for identifying genetic markers linked to artemisinin resistance (Nsanzabana et al., 2018; Guirou et al., 2020). Future research should also be directed towards the development of molecular diagnostic tools which are sensitive, accurate, portable, easy to use and inexpensive, suitable for rapid detection of artemisinin resistance in resource-limited settings. Although therapeutic efficacy studies remain the gold standard for detecting antimalarial drug resistance, these platforms are expensive and require extensive logistics to run. Similarly, the useful *in vivo/ex vivo* methods also need substantial infrastructure (Nsanzabana et al., 2018). Various molecular methods have been developed for detecting antimalarial drug resistance, such as mutation specific PCR, PCR-RFLP, real-time PCR, Sanger sequencing, next generation sequencing, microarray, *etc*. (Nsanzabana et al., 2018). Rapid diagnostic tests (RDTs) are widely used to measure malaria rates in endemic countries. These are fast, low cost and easy to use. However, RDTs show low sensitivity which can lead to detection failure. A molecular technique based on nucleic acid amplification using RDTs as a source of nucleic acids has been developed and evaluated. This technique is powerful for analyzing nucleic acids from large number of RDTs and can also be used to identify single nucleotide polymorphisms which occur in the kelch 13 gene of *P. falciparum* so as to obtain data on the circulation of artemisinin-resistant *P. falciparum* strains (Guirou et al., 2020).

Noninvasive assays using saliva or urine samples are preferred for detecting malaria (Mbanefo and Kumar, 2020). Development of non-invasive surveillance platforms is essential as they are considered to be safe, hence, they may improve patient care (Siregar et al., 2015; Al-Shehri et al., 2019). A non-invasive malaria detection method using ethanol-preserved faeces was reported to have been adequate for estimating the prevalence of *Plasmodium* infection in school children in Uganda (Al-Shehri et al., 2019).

6. Conclusion

As the cause of the majority of malaria infections and deaths, *P. falciparum* is the most virulent of all of malaria species. The emergence and spread of malaria parasite resistance to artemisinin are principally observed in Southeast Asia and Africa. The artemisinin resistance is currently jeopardizing malaria front-line treatment (ACT), especially in Asian and African regions, and forcing the need for alternative strategies for treating and controlling malaria. The WHO's strategies to eliminate malaria has been setback by the development of resistance of the parasite to several antimalarial drugs including artemisinin. The extended use of artemisinin for other diseases may further increase the risk of emergence of artemisinin resistance. Molecular analysis of the artemisinin-resistant parasites has been undertaken to better understand this resistance, so that the emergence and spread of artemisinin-resistant variants can be controlled. It is thought that the molecular mechanism of artemisinin resistance is substantially complex, involving multiple genes and biochemical pathways. Better understanding of the molecular mechanisms and genetic markers for artemisinin resistance will help surveillance and the development of new antimalarial drug-based strategies. The dominant gene marker for artemisinin resistance has been identified and studies to understand its mechanisms are underway. The mutations in the K13 gene in the artemisinin-resistant parasites are being analyzed to clarify and verify the exact mechanism of artemisinin resistance. In-depth molecular evolutionary and population genetic studies are critical for understanding the evolution and geographic distribution of artemisinin resistance, hence, such studies will contribute to development of strategies for malaria control and elimination. Advances of molecular techniques and strategies implemented to control COVID-19 should be a contingent for controlling and eliminating artemisinin-resistant *P. falciparum*. To relieve the human population from the effects of *P. falciparum* infection, the development of vector control strategies is also critical.

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Wihda Aisarul Azmi: Conceptualization, Writing – original draft. **Andita Fitri Mutiara Rizki:** Conceptualization, Writing – review & editing. **Yenny Djuardi:** Conceptualization, Writing – review & editing. **I. Made Artika:** Conceptualization, Visualization, Writing – review & editing. **Josephine Elizabeth Siregar:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

No data was used for the research described in the article.

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