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Breaking the Cycle of Malaria

Molecular Innovations, Diagnostics, and Integrated Control Strategies

Edited by Yash Gupta, Surendra Kumar Prajapati and Raja Babu Singh Kushwah

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Published in London, United Kingdom

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http://dx.doi.org/10.5772/intechopen.1006417
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First published in London, United Kingdom, 2025 by IntechOpen
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British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Breaking the Cycle of Malaria - Molecular Innovations, Diagnostics, and Integrated Control Strategies
Edited by Yash Gupta, Surendra Kumar Prajapati and Raja Babu Singh Kushwah
p. cm.

This title is part of the Infectious Diseases Book Series, Volume 40
Topic: Parasitic Infectious Diseases
Series Editor: Alfonso J. Rodriguez-Morales
Topic Editor: Amidou Samie

Print ISBN 978-1-83635-268-6
Online ISBN 978-1-83635-267-9
eBook (PDF) ISBN 978-1-83635-269-3
ISSN 2631-6188

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IntechOpen Book Series

Infectious Diseases

Volume 40

Aims and Scope of the Series

This series will provide a comprehensive overview of recent research trends in various Infectious Diseases (as per the most recent Baltimore classification). Topics will include general overviews of infections, immunopathology, diagnosis, treatment, epidemiology, etiology, and current clinical recommendations for managing infectious diseases. Ongoing issues, recent advances, and future diagnostic approaches and therapeutic strategies will also be discussed. This book series will focus on various aspects and properties of infectious diseases whose deep understanding is essential for safeguarding the human race from losing resources and economies due to pathogens.

Meet the Series Editor

Dr. Alfonso J. Rodriguez-Morales received his MD from Universidad Central de Venezuela, Caracas, and his MSc in Protozoology/Parasitology from Universidad de Los Andes, Trujillo, Venezuela. He received his Diploma in Tropical Medicine & Hygiene (DTM&H) from Universidad Peruana Cayetano Heredia, Lima, Peru, and the University of Alabama at Birmingham, Alabama, USA. He also holds a DipEd. Dr. Rodriguez-Morales is a fellow of the Royal Society for Tropical Medicine & Hygiene (FRSTMH), London, United Kingdom; of the Faculty of Travel Medicine (FFTM) of the Royal College of Physicians and Surgeons of Glasgow (RCPSG), Glasgow, Scotland, United Kingdom; of the American College of Epidemiology (FACE), USA; and the International Society for Antimicrobial Chemotherapy (FISAC). He has a HonDSc from Universidad Privada Franz Tamayo (UniFranz), Cochabamba, Bolivia. He is the President of the Latin American Society for Travel Medicine (SLAMVI) (2023-2025) and the Past President of the Colombian Infectious Diseases Association (2021–2023). He is a member of the Council (2020-2026) of the International Society for Infectious Diseases (ISID). He is a senior researcher of Colciencias (2015–2027), and a professor at the Fundación Universitaria Autónoma de las Américas, Pereira, Risaralda, Colombia, and the Universidad Científica del Sur (UCSUR), Lima, Peru. He is a visiting professor at multiple national and international universities.

Meet the Volume Editor

Dr. Yash Gupta is a biomedical scientist specializing in parasitology and infectious disease therapeutics. His research has significantly advanced the understanding of malaria pathogenesis and drug discovery. Dr. Gupta has identified novel antimalarial compounds such as Calxinin, a multistage inhibitor that disrupts Plasmodium falciparum calcium homeostasis, and has contributed to the development of HDAC and GAPDH-targeting therapeutics. His work integrates *in silico* modeling with *in vitro* and *in vivo* validation, resulting in high-impact publications and patents. With over 35 peer-reviewed articles and editorial roles in leading journals, Dr. Gupta's contributions are internationally recognized. He has collaborated across institutions, including the Mayo Clinic and Penn State, and his discoveries are being translated into clinical applications through biotech partnerships.

Dr. Surendra K. Prajapati is a molecular parasitologist and research faculty at the Uniformed Services University of the Health Sciences, USA, with over 14 years of postdoctoral experience in malaria parasite biology (*Plasmodium falciparum* and *P. vivax*). His research focuses on the molecular mechanisms of sexual differentiation, transmission, invasion, and virulence. He has developed prognostic biomarkers for malaria transmission and pioneered assays to study parasite invasion and gametocyte biology. Dr. Prajapati has authored focused peer-reviewed publications, including those in Nature Communications, and contributed to research on malaria transmission, genomics, vaccine targets, and drug resistance surveillance. His work spans global collaborations across India, Belgium, Peru, Ghana, and the U.S., and he is a recognized reviewer for leading journals and funding agencies in infectious disease research.

Dr. Raja Babu Singh Kushwah is a vector biologist with over 15 years of experience in infectious disease research, specializing in mosquito-parasite interactions and vector control strategies. His work has contributed to understanding insecticide resistance and the development of innovative mosquito management tools, including a patented synergistic repellent formulation. Dr. Kushwah has held research positions at the National Institute of Malaria Research, Tata Institute for Genetics and Society (TIGS), Texas A&M AgriLife Research, and Texas A&M University, where he continues to explore translational approaches to innovative vector control. He has received international recognition, including a Global Health Travel Award from the Bill & Melinda Gates Foundation, and has been honored for his mentorship and scientific outreach efforts. His expertise bridges entomology, public health, and molecular parasitology.

Contents

Preface

Malaria continues to be one of the most formidable global health challenges, affecting over 240 million people annually and causing hundreds of thousands of deaths, particularly in sub-Saharan Africa [1]. Despite decades of control efforts, the emergence of drug and insecticide resistance, diagnostic limitations, and the complex biology of Plasmodium parasites have hindered efforts to eradicate the disease. This book, *Breaking the Cycle of Malaria – Molecular Innovations, Diagnostics, and Integrated Control Strategies*, brings together a diverse collection of chapters that reflect the latest scientific advances and interdisciplinary strategies aimed at overcoming these barriers.

Malaria control and elimination efforts are increasingly enhanced through the integration of molecular biology, computational tools, and public health strategies, with a strong emphasis on innovation and future directions [2–4]. Molecular tools are now indispensable for identifying symptomatic, asymptomatic, and submicroscopic malaria cases—cases that traditional diagnostics often miss. These tools also enable genotyping of parasites, which is critical for understanding transmission dynamics and resistance patterns [5].

In parallel, computational biology has emerged as a beacon of hope for accelerating the discovery of new drugs. Techniques such as virtual screening, molecular docking, artificial intelligence (AI), and machine learning (ML) are being used to identify novel therapeutic candidates and optimize existing treatments [4]. These approaches are complemented by vector control innovations, including the use of genetically modified insects and integrated vector management (IVM), which combine evidence-based decision-making with community engagement and capacity-building [6, 7].

The book also examines public health strategies that are crucial for translating scientific advances into tangible, real-world impact. These include integrated service delivery, family and community health education, and cross-sector collaboration between clinical and public health services [8–10]. Tailoring interventions to local epidemiological contexts remains a cornerstone of effective malaria control [11].

Looking ahead, the future of malaria elimination will depend on [12, 13]:

- Identifying molecular markers and mutations associated with drug resistance.
- Elucidating the biochemical mechanisms of resistance.
- Discovering new chemical entities for treatment.
- Advancing vaccine development and implementation.
- Expanding molecular surveillance systems.
- Engaging stakeholders in the evaluation of gene drive-modified mosquitoes.

However, these efforts must also contend with emerging challenges such as artemisinin and insecticide resistance, global health security threats from international travel, and the ecological impacts of climate change on vector distribution and medicinal plant biodiversity [14–16].

To support strategic planning and implementation, this volume includes visual frameworks illustrating malaria control strategies in fragile and conflict-affected settings, vaccine feedback loops, and the roles of stakeholders across health systems [17–19].

By integrating these innovations, strategies, and considerations, this book aims to serve as a comprehensive resource for researchers, clinicians, public health professionals, and policymakers. It not only highlights current breakthroughs but also charts a path forward for the global effort to break the cycle of malaria.

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Section 1

Innovations in Malaria Diagnostics and Surveillance

Chapter 1

Advancements and Challenges in Malaria Diagnostics

Vinit Chauhan, Rajesh Anand, Anisha Thalor and Agam Prasad Singh

Abstract

Diagnostic methods are vital for dealing with the global malaria burden and decreasing malaria incidence. The diagnosis by microscopy is considered a gold standard; however, rapid diagnostic tests (RDTs) have become a primary diagnostic test in many malaria-endemic areas. RDTs have many advantages; however, gene deletion, poor sensitivity with low parasite levels, cross-reactivity, and prozone effect are certain disadvantages. The quantitative buffy coat (QBC), polymerase chain reaction (PCR), flow cytometry, loop-mediated isothermal amplification (LAMP), and mass spectrometry have certain disadvantages that limit their mass scale implications in endemic areas. Recently, malaria diagnosis based on artificial intelligence and smartphone-based applications for malaria diagnosis have been developed, which can be implemented in the fields once high sensitivity and specificity are achieved. In the current scenario, gene deletion events in *Plasmodium falciparum* have created a vacuum that can be filled with the development of more advanced RDT.

Keywords: malaria, diagnostics, RDT, LAMP, PCR, QBC

1. Introduction

Malaria is the most common parasitic infection caused by the protozoan parasite *Plasmodium* and transmitted through the bite of female *Anopheles* mosquitoes [1]. Out of 200 *Plasmodium* species, majorly 5 species have been found to show significant infection in humans (*Plasmodium vivax, Plasmodium knowlesi, Plasmodium falciparum, Plasmodium ovale, Plasmodium malariae*) [2].

According to the global malaria report 2023 by the World Health Organization (WHO), there were approximately 249 million incidences of malaria in 85 malaria-endemic countries and regions with an increment of 5 million malaria cases [3]. Malaria is also prevalent in India, which accounts for approximately 1.4% of the global burden [3]. Around 70% of malaria cases in India come from five states that include Odisha (36%), Chhattisgarh (12%), Jharkhand (9%), Madhya Pradesh (9%), and Maharashtra (5%) [4].

Malaria remains a leading cause of mortality and morbidity around the world. Timely and precise diagnosis plays a critical role in effectively managing the disease. The diagnosis of malaria can be achieved through various diagnostic methods such

as light microscopy, PCR-based tests, Rapid diagnostic tests (RDTs), etc. [5–7]. Emerging advanced technologies like Loop-mediated Isothermal Amplification (LAMP), mass spectrometry, and enzyme-linked immunosorbent assay are showing promising results for diagnosing the malaria parasite [8, 9]. Since 2010, it has been recommended by WHO that every suspected case of malaria should be verified either with a rapid diagnostic test or light microscopy [10].

The development of novel technologies based on artificial intelligence and image analysis is revolutionizing malaria diagnostics by automating the diagnostic process and reducing reliance on expert microscopists. Convolutional neural network (CNN) models can now analyze microscopic images to detect parasitic infection and quantify parasitemia levels. Additionally, the development of smartphone-based applications such as Malaria Screener and PVF-Net for the diagnosis of malaria is also emerging and potentially useful in endemic areas to screen larger populations efficiently with minimal resources [11, 12].

Light microscopy has been in use for over a century and is considered the standard method for malaria diagnosis [13]. However, it does have some limitations, like it is time-consuming, requires skilled staff and infrastructure. The RDTs overcome all the major problems associated with microscopy and have been found to be very useful in endemic areas, but recently, problems have been observed with the currently available RDTs as well [11]. Recently, deletion of HRP2 and HRP3 genes in *Plasmodium falciparum* have been observed in South America, African regions, and in some parts of India as well [14–16]. The *Plasmodium* lactate dehydrogenase (*p*LDH)-based RDT cannot detect parasites in low-density parasitemia, and the sensitivity of the aldolase-based RDT also completely depends on the parasite density [11]. Consequently, there is still a need for a reliable diagnostic test for the effective management and treatment of the disease.

The global malaria diagnostic market plays a critical role in the fight against malaria, and the market has grown significantly due to the growing demand for early and accurate diagnosis. This rise has fueled the development of diagnostic technologies like RDTs, PCR, LAMP, etc. The global malaria market was valued at approximately $819 million and is anticipated to expand at a 5% compound annual growth rate (CAGR) by 2024–2032 [17].

2. Diagnosis of malaria

The malarial infection symptoms are unspecific and thus unreliable to distinguish it from other tropical diseases (Health Organization, 2023). The effective diagnosis of the disease depends on several factors, such as different species of the *Plasmodium*, different stages, parasitemia, immunity, population movement, etc. [1].

Diagnostic methods for infectious diseases should be rapid, easy, accurate, and cost-effective. There are various techniques currently available for malaria diagnosis, ranging from the earliest conventional light microscopy using blood smears to cutting-edge techniques using rapid molecular methods. In the past decade, there has been growing interest in immunoassay-based RDTs, flow cytometry, ELISA, and molecular methods, which include real-time PCR, nested PCR, and LAMP [8, 9].

2.1 Traditional diagnostics methods

Malaria is an age-old disease, and the traditional diagnostic methods of malaria predominantly involve clinical diagnosis on the basis of symptoms such as fever,

headache, chills, etc. The light microscopy-based examination of blood smears is considered the gold standard method to identify malarial parasites efficiently. In addition, serology testing that detects the antibodies produced in response to the infection was found to be ineffective in diagnosing malaria, as antibodies could persist even after clearance of infection.

2.2 Clinical diagnosis of malaria

Clinical diagnosis is the most widely used method by doctors around the world, despite the early symptoms of malaria being indistinguishable. The initial malarial symptoms or indications are non-specific (flu-like) and include higher body temperature, headache, shivering, vomiting, nausea, wooziness, and anorexia (**Figure 1**), which makes it challenging to differentiate from other tropical illnesses [18, 19].

The recent study in Ghana assessing different diagnostic methods suggested that the sensitivity and specificity of clinical diagnosis are much lower as compared to the conventional microscopy and immunochromatographic assays [20].

2.3 Light microscopy-based diagnosis of malaria

The microscopic detection of malaria involves the identification of *Plasmodium* species in the Giemsa-stained smears of peripheral blood films. The diagnosis of malaria by light microscopy (**Figure 2**) involves the preparation of thin and thick blood films (thin film for species confirmation and thick for parasite detection) on a clean glass slide followed by staining with 10% Giemsa stain and incubation for 10 minutes at RT, then removal of the stain by washing with water and its examination under the microscope using a high-power immersion oil lens (100X) [21].

The percentage of parasitemia is used to determine malaria parasite load [22], and it is calculated by the following formula: % Parasitemia = No. of infected RBCs/Total no. of red blood cells × 100. Parasite density is categorized as high parasitemia (>10%), moderate parasitemia (1–10%), and low parasitemia (<1%) [23]. Expert microscopy makes it possible to differentiate all four major species of malaria parasite that are competent to cause infection in humans [24].

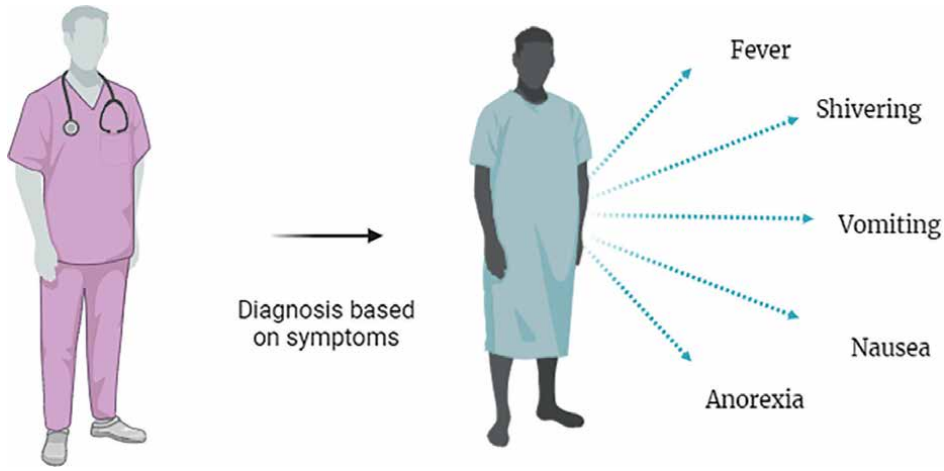

Figure 1.
Clinical diagnosis based on symptoms.

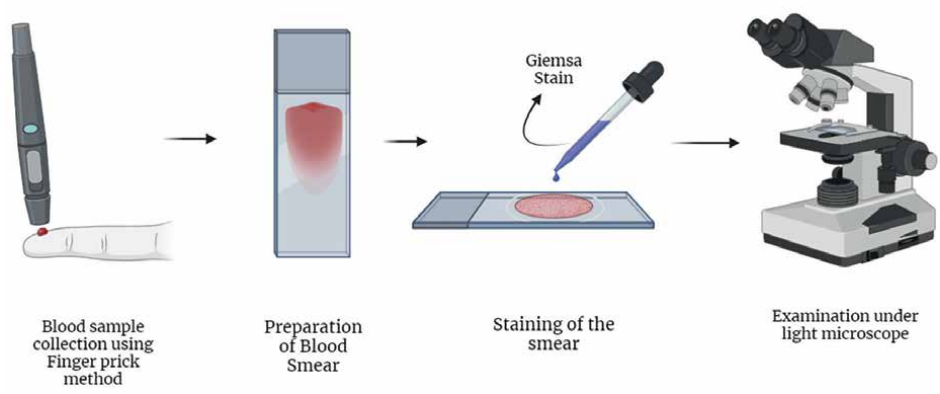

Figure 2.
Diagnosis of malaria using light microscopy.

The light microscopy-based methods have certain advantages, like low-cost diagnosis, parasite-level calculations, and species identification [25–32]. The conventional method of diagnosing malaria by microscopic analysis of stained blood films has a number of disadvantages. Because of its labor-intensive nature and need for specialized infrastructure and well-trained personnel, it is less viable in environments with low resources. In endemic locations, its reliability declines, and when parasitemia levels are low, it has difficulty in correctly detecting infections, particularly in areas where malaria is most common (**Table 1**) [25–32].

2.4 Serological test

Serological testing is commonly based on the detection of antibodies generated against the *Plasmodium* parasite. However, these tests are not considered to be

Diagnostic technique	Advantages	Disadvantages	Sensitivity	Specificity
Traditional diagnostics methods				
Clinical diagnosis	(i) No instrument or specific facility required [18, 19] (ii) Only symptoms-based [18, 19]	(i) Challenging to differentiate from other tropical illnesses [18, 19]	17.2% [20]	86.5% [20]
Microscopic examination	(i) Availability [1, 25] (ii) Low-cost diagnosis [1, 25] (iii) Parasite-level calculations [29, 30, 32] (iv) Species identification [1, 27, 29–32]	(i) Requires expert personnel [1, 26, 30] (ii) Results are expert-dependent [1, 26, 30] (iii) Thin vs. thick blood film variations [1, 28–33]	56% [34]	100% [34]
Serology	(i) Seroprevalence study [1, 35] (ii) Malaria transmission [1, 36] (iii) Screening of potential blood donors [1, 35]	(i) Non-reliable diagnostic technique [36] (ii) Not indicative of active infection [36]		

Diagnostic technique	Advantages	Disadvantages	Sensitivity	Specificity
Advanced diagnostic methods				
Rapid diagnostic tests (RDTs)	(i) Fast preparation and diagnosis results [1, 37–39] (ii) Easy handling [1, 37–39] (iii) Low-cost diagnosis [1, 37, 39] (iv) Species identification [37, 39, 40] (usually *P. falciparum* from non-*P. falciparum* species)	(i)pfHRP2/3 gene deletions [37, 39, 41, 42] (ii) Low sensitivity with low parasite levels [37, 39] (iii) Low sensitivity with *P. ovale* and *P. malariae* species [38, 40]. (iv) Cross-reactivity [40, 42] (v) Prozone effect [37, 43]	84.2% [44] 63.4–100% [45] 84.2% [46] 37–88% [34] 95% (HRP2) [11] 93.2% (pLDH) [11]	99.8% [44] 53.4–99.9% [45] 95.2% [46] 93–100% [34] 95.2% (HRP2) [11] 98.5% (pLDH) [11]
Quantitative buffy coat (QBC)	(i) Simple, reliable, and user-friendly [1, 6, 47–49] (ii) Rapid and sensitive [1, 47–50] (iii) High specificity [50, 51] (iv) Less training time [50]	(i) Requires expert personnel [1, 47] (ii) Requires fluorescent microscopy setup [1, 47] (iii) Low sensitivity in field [49]	70.5% [46] 55.9% [47] 93% [52] 97.7% [49] 70.9% [49] (field)	92.1% [46] 88.8% [47] 99% [52] 99.7% [49] 97.4% [49] (field)
PCR	(i) High sensitivity and specificity [30, 53–60] (ii) Accurate species identification [53–57, 60] (iii) Reference tool for comparative studies [53, 59, 60] (iv) Works in low parasite density [56–58, 60]	(i) Specialized instrumentation [1, 30, 53, 54, 60] (ii) Difficult implementation in endemic areas [30, 53, 54, 56, 60] (iii) Expensive diagnosis [1, 30, 53, 54, 57, 60]	100% [53, 59, 60]	100% [53, 59, 60]
LAMP	(i) High sensitivity and specificity [61–65] (ii) Species identification [62, 64, 65] (iii) Inexpensive, no thermocyclers needed [61–63] (iv) Less turnaround time, comparable to RDT [62]	(i) Less sensitive for other species (other than *P. falciparum* and *P. vivax*) [65]	99% (Pan) [64] 90% (*P. falciparum*) [64] 95% [61] 98.89% [62] 100% [34] 95–98% [63]	100% (Pan) [64] 93% (*P. falciparum*) [64] 99% [61] 100% [62] 86–99% [34] 91–99% [63]
Mass spectrometry	(i) High specificity [66, 67] (ii) Early detection of infection [68]	(i) Low sensitivity [66, 67] (ii) Specialized and costly instrumentation [66, 67]	52% [69] 80.2% [66]	92% [69] >95% [66]
Flow cytometry	(i) Quantification of infected erythrocytes [66, 67, 70–73] (ii) Automated parasite-level calculations [70–73]	(i) Low sensitivity than PCR [71] (ii) Difficult implementation in endemic areas [70–73]	100% [72]	98.39% [72]

Table 1.
Advantages, disadvantages, sensitivity, and specificity of different malaria diagnostic techniques.

appropriate as they are not capable of determining whether the antibodies are produced due to past or ongoing infection, but they are usually utilized for epidemiological studies and for screening of potential blood donors [74]. There are several assays, such as IFA, ELISA, hemagglutination tests, etc. Despite being time-consuming, the immunofluorescence antibody testing (IFA) is a reliable test for detecting antimalarial antibodies. It is specifically useful for screening prospective blood donors to avoid transfusion-transmitted malaria (**Table 1**) [1, 35, 36].

3. Advancements in malaria diagnosis

Recent advancements focus on the identification of disease-specific biomarkers that allow for more precise detection and characterization of malarial infections. While traditional diagnostic methods of malaria primarily rely on blood or its products for the detection of the disease, recent developments in non-invasive techniques using other bodily fluids such as saliva and urine have shown promising results. Techniques like nested PCR targeting malarial small-subunit rRNA (18S PCR), quantitative PCR, chemiluminescent ELISA, and UMT (Urine malaria test), which detects *Pf*-HRP2 in urine samples, are being used to diagnose malaria. In addition, there have been improvements in blood-based assays as well that include methods like loop-mediated isothermal amplification, Photo induced electron transfer (PET)-PCR, biosensor-based quartz crystal microbalance technology, and SELEX (Systematic Evolution of Ligands by Exponential Enrichment), which holds the potential to revolutionize the field of malaria diagnostics [75–78].

In order to reduce the dependency on conventional microscopy, several software applications and tools are also being developed to detect malaria parasites in thick and thin blood smear microscopic images [32]. These approaches aim to automate the detection of the disease using deep learning Artificial Intelligence (AI) and advanced image analysis to streamline the procedure of diagnosis. Convolutional neural networks are used as trained classifier models to identify specific objects or features in images and videos using deep learning algorithms; it is specifically used in healthcare for medical diagnosis of various diseases [79].

3.1 Rapid diagnostic test

RDT is a lateral flow device that is based on the principle of immunochromatography, which involves the movement of liquid across the nitrocellulose membrane (**Figure 3**). During testing, the parasitic antigen from the patient's blood is captured by a monoclonal antibody that is attached to a gold particle or a liposome with dye; these monoclonal antibodies are raised against the target malaria antigen. Another antibody captures the antigen-antibody complex as it moves across the nitrocellulose membrane in the immobile phase, which results in generating a clear line. A labeled goat anti-mouse antibody is used as a control to ensure that the test works properly or not [80, 81].

Currently, there are several RDT kits available that target HRP2 (Histidine-rich protein2), aldolase, and pLDH antigen for the detection of malaria. HRP2 is a surface membrane protein in RBC that is produced in abundance during the asexual stage of the *Plasmodium* parasite, and it has been found to be highly specific for *Plasmodium falciparum* infection [40, 82–86]. LDH is a soluble glycolytic enzyme that is expressed at high levels in the blood stage of *Plasmodium*. *p*LDH-based RDTs effectively detect two species of *Plasmodium*, i.e., *P. vivax* and *P. falciparum* [85, 86]. Aldolase is also an

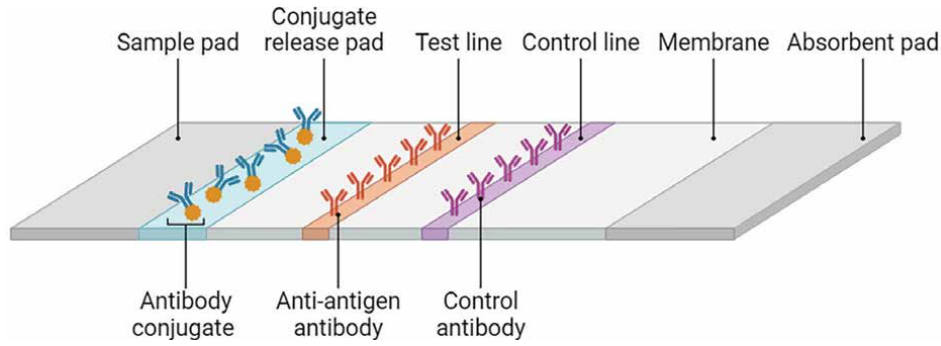

Figure 3.
Typical lateral flow device used in RDT.

enzyme produced during the glycolytic pathway of the malaria parasite, and its presence has been observed in all *Plasmodium species*, which makes it capable of detecting all infections of malaria [37, 86].

The RDT test involves the collection of blood samples and placing the sample on the pad specified for samples. Then a few drops of buffers are placed in the sample pad, and the results are obtained within minutes (**Figure 4**).

Many studies have demonstrated that RDT-based diagnosis of malaria shows robust performance in terms of the sensitivity and specificity of the test [87–90]. Although RDTs cannot determine the parasite burden of patients and their sensitivity also decreases with low parasitemia (<100 parasites/uL), still it is thought to be the most reliable method for screening large populations in resource-limited areas [37].

The development of RDTs around the 1990s resolved some of the issues linked with conventional microscopy and PCR-based diagnosis, it is rapid and easy to use and requires minimal understanding [38]. Also, it was found very reliable in endemic areas to screen large populations in a short period of time. In a comparative study, the sensitivity and specificity for *P. falciparum* using HRP2-based RDT have been found to be 95% and 95.2%, respectively. Similarly, for pLDH-based RDT, 93.2% and 98.5% sensitivity and specificity were respectively obtained [11, 34, 44–46]. But, recently, deletion of the HRP2 gene in *Plasmodium falciparum* has been observed in South America, African regions, and some parts of India as well [14–16]. The *p*LDH-based RDT cannot detect parasites in low-density parasitemia, and parasite-released aldolase enzyme in

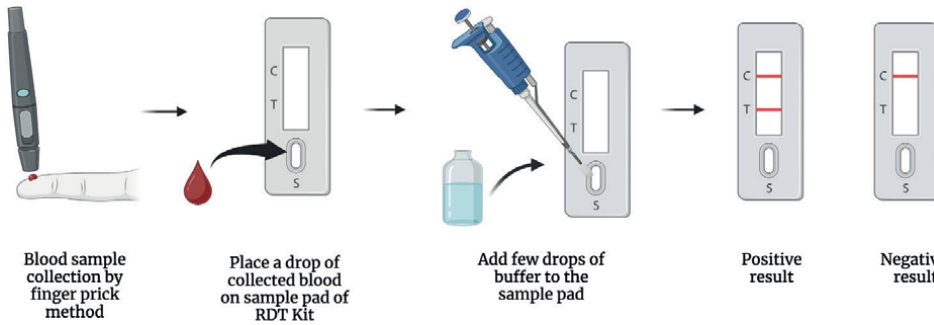

Figure 4.
Malaria diagnosis using an RDT kit.

low concentrations decreases the sensitivity of the aldolase-based RDT [11]. A large number of studies have highlighted issues with the currently available antigen-based RDTs [33, 37, 39, 41–43, 91]. Thus there is a critical need to find a novel species-specific target that can efficiently detect parasite infection and is free from all the issues.

3.2 Quantitative buffy coat (QBC) test

The quantitative buffy coat analysis is a well-known and highly sensitive test that was developed to enhance the detection of malaria in peripheral blood [51]. The QBC method relies on fluorescence and density-gradient centrifugation of malaria-infected erythrocytes [50]. In the QBC technique, fluorescent dyes are used (acridine orange) to stain the parasitic DNA in infected blood specimens, followed by centrifugation at high speed for 5 minutes and then the detection of the malaria-causing parasite under a fluorescent microscope [1]. Within non-fluorescing red blood cells, the parasite is spotted as green under a fluorescence microscope [47].

In addition, the QBC test has been found to be highly specific in identifying malaria in the Indian setup, where two species of *Plasmodium*, i.e., *P. falciparum* and *P. vivax*, predominantly cause malaria [49]. The QBC test is also a helpful technique in correctly diagnosing filariasis and visceral leishmaniasis [51]. Studies demonstrated that the QBC test is a reliable diagnostic tool for malaria. It shows excellent specificity and sensitivity and possesses good accuracy in identifying both positive and negative results [46–48, 52]. The field sensitivity of the QBC test has been reported to decrease significantly under field conditions [49].

Despite its high specificity and sensitivity, QBC is not a suitable diagnostic test in resource-limited endemic areas because it requires specialized equipment, trained lab staff, and a good infrastructure to carry out the testing operations (**Table 1**). The quantitative buffy coat test that involves centrifugation and use of acridine orange dye to visualize parasites under a fluorescent microscope, the studies demonstrated that QBC possesses better sensitivity compared to the microscopic examination; however, it requires skilled lab staff, infrastructure to carry out testing, and high costs (**Table 1**) [1, 6, 31, 51, 92].

3.3 Molecular diagnosis of malaria

The molecular amplification of 18S rRNA, which is conserved in all *Plasmodium* species, was first implemented by scientists using the nested PCR technique [93]. Since then, molecular-based diagnosis of malaria has constantly evolved with the development of RT-PCR, Nucleic Acid Sequence-Based Amplification (NASBA), reverse-transcriptase PCR, and LAMP. These PCR-based diagnoses can detect multiple gene targets, and it has been shown to be more sensitive than microscopy [94].

RDT and microscopy can detect 100–200 parasites/μl, whereas molecular diagnostic methods such as PCR, LAMP, mass spectrometry, and flow cytometry can detect 5–50 parasites/μl [95, 96]. Thus, studies have demonstrated the higher sensitivity of molecular methods as compared to other methods.

3.3.1 Polymerase chain reaction (PCR)

The polymerase chain reaction is attributed as a reliable test for identifying mixed infections with better sensitivity as compared to the other laboratory tests. It involves the use of two sets of primers, from which one set of primers is used

to detect the presence of the *Plasmodium* and the other set is species-specific that will only amplify when the particular species is present in the sample, such as *Plasmodium falciparum* [97, 98].

Real-time PCR uses fluorescence-based technology and can efficiently detect the target amplicons in real time, and the melting curve analysis is done to precisely differentiate the *Plasmodium* species [10, 99–102]. Whereas the multiplex PCR is capable of amplifying multiple target sequences in a single reaction using different pairs of primers for each target but it is also associated with several problems, such as non-specific amplification and mispriming artifacts. However, the nested PCR performs two steps of amplification, the product from the first step amplification serves as the template for secondary amplification and uses a different set of primers internal to those used in primary PCR to avoid mispriming artifacts and primer dimers. Nested PCR can detect submicroscopic malaria parasites with a low density of 10 parasites/μl of blood [103].

Diverse studies suggest that PCR-based diagnosis of malaria possesses better sensitivity and specificity in comparison with the other two most widely used diagnostics and is also able to identify missed cases by these tests [29, 53–60]. PCR-based molecular diagnosis of malaria is considered a more suitable technique compared to other conventional methods as it possesses better sensitivity and specificity [57]. While it is more sensitive and capable of identifying the parasite at the species level, it is linked to multiple challenges, such as it is not very reliable in remote areas; diagnosis takes 2–3 hours and is associated with high costs [29, 53].

3.3.2 Loop-mediated isothermal amplification (LAMP)

The LAMP technique is considered to be a more simplified and affordable technique. It identifies the *Plasmodium falciparum*'s conserved 18s ribosomal RNA gene and is also able to detect other *Plasmodium* species as well [61]. In LAMP assay amplification is done at a constant temperature, i.e., around 62–65°C. DNA is amplified 10^9–10^{10} times in 15–60 minutes, and the amplification is monitored using SYBR Green dye and automated monitoring using a turbidity meter (**Figure 5**) [75].

A meta-analysis of multiple studies demonstrated the LAMP method's sensitivity is between 93.9% and 100%, and specificity ranges between 93.8% and 100%, which makes it comparable with PCR-based diagnostics [34, 62, 63, 104]. Further, it is less time-consuming and capable of detecting all *Plasmodium* species efficiently [63–65, 105].

The molecular technique known as loop-mediated isothermal amplification (LAMP) has been released into the market to detect malaria. LAMP satisfies the

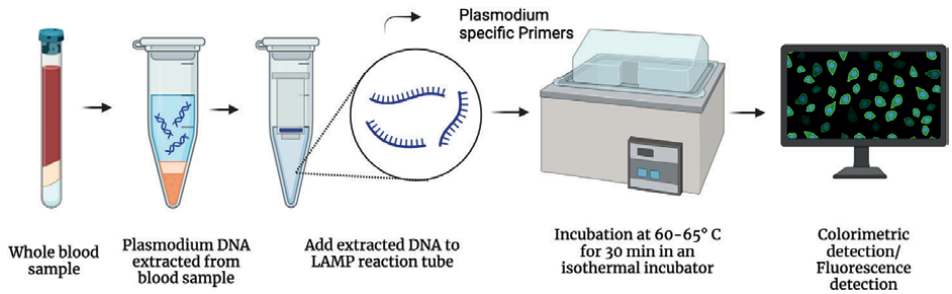

Figure 5.
Basic workflow of LAMP technique.

requirements for a point-of-care diagnostic screening test since it is easy to use and does not require sophisticated equipment or training [63–65]. So far, the LAMP technique is producing better results as compared to other methods but more research and field studies have to be done to validate its results (**Table 1**).

3.3.3 Mass spectrometry

The laser desorption mass spectrometry-based diagnostic for malaria was developed two decades ago. It is based on the principle of identifying a heme group of hemozoin in malaria-infected samples and does not detect heme bound to hemoglobin or other proteins in uninfected samples [106]. Based on a study, Liquid Chromatography-Mass Spectrometry (LC-MS) was found to be a rapid and sensitive test and could detect <10 parasites/μl (*Plasmodium falciparum*) in infected blood samples. The comparative study demonstrated the detection of *Plasmodium* infection within 2 days post-infection in 0.3 μl of blood sample, whereas the microscopy and colorimetric hemozoin detect the infection between 2 and 5 days [68]. Samples from pregnant women with submicroscopic parasitemia, hemozoin screening using Laser desorption-time of flight (LD-TOF) showed sensitivity of 52% and specificity of 92% when compared with PCR [69]. Recently, a study has shown a high specificity (>95%) but insufficient sensitivity (80.2%) of LD-TOF-based detection of hemozoin for malaria screening [66]. In yet another study more than 10 peaks specific for *P. falciparum* were identified in the blood of infected patients by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) suggesting the potential of the technique for the diagnosis of malaria. However, the sensitivity was not sufficient in this study also [67].

3.3.4 Flow cytometry

Flow cytometry is a high-throughput technique to count and separate viable cells in a stream of fluid using a laser device that reveals the fluorescent markers on the cells [107]. Flow cytometry provides more insights about the growth and development of malaria parasites compared to any other currently available techniques [8]. The flow cytometry-based investigation of malaria parasites in the blood stage relies on the absence of DNA in normal RBCs. The ratio of RBCs that stain positive for DNA to the total number of RBCs analyzed can be used to accurately determine the parasitemia in blood samples (**Figure 6**).

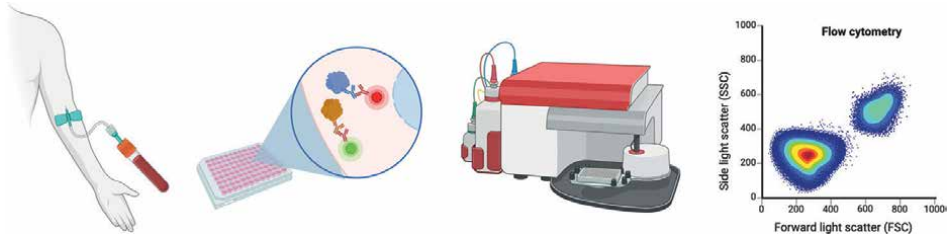

Figure 6.
Flow-cytometry-based detection of malaria parasite.

In the first use of flow cytometry to detect malaria parasite, the test blood samples were mixed with saponin that lysed the RBCs without affecting the parasite and WBCs. Cells were then stained using two fluorescent dyes, i.e., Hoechst 33258 and a FACStar, to analyze the parasitic DNA. Parasite and WBCs were then differentiated by blue Hoechst 33258 fluorescence and forward scattering [70]. A simpler and more rapid tricolor flow cytometry assay was developed in 2011, which helps to quantify, characterize, and differentiate different *Plasmodium* species. Leukocytes were detected using an antibody against CD45, and the parasites were stained with dihydroethidium, Hoechst 33342, or SYBR Green I [71]. In a recent study, microscopy sensitivity of 100% (95% CI: 97.13–100) and specificity of 98.39% (95% CI: 95.56–100) have been reported for the diagnosis of imported malaria cases. Moreover, the study showed the determination of parasitemia in a very short time compared to other methods [72]. Flow cytometry-based diagnosis of malaria has significant disadvantages, like it is expensive, labor-intensive, and requires trained laboratory staff, though it is incredibly useful in malaria studies and for malaria screening [29, 73].

4. Artificial intelligence and image analysis techniques

Artificial intelligence is a transformative technology that has the potential to revolutionize the field of malaria diagnosis. Convolutional neural networks (CNNs) are AI models (such as YOLOv5, R-CNN, SSD, and RetinaNet) that can help in analyzing microscopic images of thick and thin blood smear samples using deep learning tools. Deep Malaria convolutional neural network classifier model (Deep MCNN) is capable of estimating parasitemia in malarial parasite MP/ul as recommended by WHO. It can calculate total malaria parasite and WBC count and achieves a sensitivity and specificity ratio of 0.92/0.90 compared to expert-level microscopy [108]. Additionally, the development of handheld microscopes or smartphone microscopes also has great potential in the field of diagnosis. Several studies have been done on the use of Cyscope which is a portable fluorescent microscope. It is considered a rapid detection test for malaria and provides results within 10 minutes. This technique has the capability to quantify parasitemia from blood specimens [109–115].

4.1 Artificial intelligence based object detection system (AIDMAN)

AIDMAN is an AI tool that uses deep learning algorithms to detect *Plasmodium* parasites in thin blood smear images with an accuracy of 98.4%, comparable to expert microscopists. It uses a combination of YOLOv5 and transformer model for image analysis for the detection of malaria parasites [116]. It may be utilized to screen more samples in limited-resource areas in order to reduce cost and time. Other AI systems for Plasmodium detection using smartphones have been reported [117–119].

4.2 Automated AI-based microscopy (EasyScan GO)

A fully automated microscopy that scans the blood film sample of patients and uses an AI software to detect and quantify malaria parasites through image processing with an accuracy of 88% has been developed by UK scientists. The system called EasyScan GO detects malaria parasites in field-prepared Giemsa-stained blood films [120].

For clinical validation, researchers have sampled around 1200 blood samples of people who traveled to the UK from malaria-endemic areas; out of 113 positive samples for malaria, the automated microscope was able to detect 99 positive samples with an accuracy of 88%. AI software cannot be considered as good as expert microscopists as it has also given 122 false positive samples, but it can be improved further and trained on large datasets to provide more outstanding results [120].

4.3 Smartphone-based application for malaria diagnosis (Malaria Screener and PVF-net)

Malaria Screener is the first smartphone-based application that can detect *Plasmodium falciparum* infection from Giemsa-stained blood smears with an accuracy of 74% compared to the expert microscopy and 71% when compared with the nested PCR as a reference. During microscopy, the Malaria Screener app requires mounting a smartphone onto the microscope's eyepiece; then the app will automatically capture an image, process it instantly, and display the result on the screen. Its accuracy reached 91.8% when the threshold of parasite count (number of parasites used to determine whether a patient was infected or uninfected) was shifted [12]. Plasmodium VF-Net was developed as a more improved algorithm that can detect both *P. falciparum* and *P. vivax* infections. It is trained using sample datasets from Bangladesh [121] and provides an accuracy of 83.1% when compared with microscopy and 81% when compared with the nested PCR. Smartphone-based applications show immense potential to be used in limited-resource settings for routine screening of malaria in endemic areas. It will help to reduce the reliance on expert microscopists and save both time and cost.

5. Malaria diagnostic market

Malaria remains the prevalent, life-threatening disease worldwide. The malaria diagnostic market is becoming a dynamic and evolving sector that plays a crucial role in the global fight against malaria. The list of available malarial RDTs shows worldwide existence [122]. The malaria RDTs that detect *Pf*-HRP2 continue to be the leading product in the donor-funded market, accounting for 70–80% of the total volume [84]. WHO estimates show that more than 312 million RDTs were distributed worldwide in the year 2016 [123]. At present, there are multiple prequalified RDT options that are available for each standard case management test type (such as HRP2-detecting tests for *Plasmodium falciparum*, *Pf*/pan, and *Pf*/*Pv*). However, there are still very few options for regions experiencing *Pf*-HRP2/3 gene deletions.

The primary factor that drives the market is the elevating incidences of malaria, which leads to the increasing demand for effective malaria diagnostics. In 2023, the global malaria diagnostic market was valued at approximately USD 819.1 million, and it is anticipated that it will grow at 5% CAGR during the projection period from 2024 to 2032 (**Figure 7**) [17]. The major factor behind this enormous growth is the ongoing advancements in diagnostic technologies, increasing investment in global health initiatives, and increased emphasis on enhancing diagnostic accessibility and accuracy in endemic areas [17].

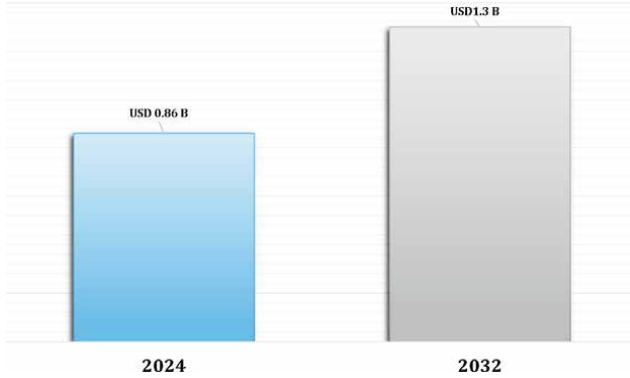

Figure 7.
Forecast period from 2024 to 2032.

6. Conclusions and future directions

The precise and timely detection of malaria infection is indeed critical for effective management and treatment. In many regions, delays in diagnosis and treatment often lead to severe complications and fatalities. The technique of microscopy is considered a benchmark in malaria diagnosis. On the other hand, RDTs with many advantages, like low-cost diagnosis, easy handling, fast preparation and diagnosis results, and species identification, are primarily implied in many malaria-endemic areas. However, the gene deletion event of pfHRP2/3, low sensitivity with low parasite levels, cross-reactivity, and the prozone effect are certain disadvantages of RDTs. Additionally, many molecular methods have also been developed. The quantitative buffy coat has fast preparation and results with high specificity; however, the requirement of a fluorescent microscope and expert personnel, along with the low sensitivity under field conditions, are the disadvantages. The molecular technique, such as loop-mediated isothermal amplification and PCR, provides results with higher specificity and sensitivity but has drawbacks in terms of requirements of specialized instrumentation and difficult implementation in endemic areas. On the other hand, mass spectrometry-based diagnostics have certain drawbacks, like low sensitivity and the requirement of specialized, costly instruments and reagents. The molecular method based on flow cytometry has advantages like quantification of infected erythrocytes and automated parasite-level calculations. Nonetheless, low sensitivity, specialized instrumentation, and difficult implementation in endemic areas are the driving forces behind the large-scale implications of flow cytometry in the diagnosis of malaria. Recently, malaria diagnosis based on artificial intelligence systems such as AIDMAN, automated AI-based microscopy (Easy Go scan), and smartphone-based applications for malaria diagnosis (Malaria Screener and PVF-Net) have been developed, which can be implemented in the fields once the high sensitivity and specificity are achieved.

Incentives to drive innovation in malaria diagnostics are constrained due to narrow profit margins, and most innovations are donor-funded, leading to slow progress. Ongoing innovations and the development of new diagnostic tools present significant opportunities for early detection, particularly in resource-limited areas, where advanced diagnostics can play a key role in effectively managing and treating malaria.

The gene deletion events in *Plasmodium falciparum* have created a vacuum that can be filled with more advanced RDT. Recently we have shown the effectiveness of ELISA in detecting the T-cell immunomodulatory protein of *P. berghei* in the sera of infected mice [124]. Presently we are working on the development of an RDT diagnostic kit based on the T-cell immunomodulatory protein of *P. falciparum*.

Acknowledgements

The authors thank NII for funding.

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Chapter 2

The Role of GIS in Designing Timely and Targeted Malaria Intervention Allocation in Africa

Abdulrahman Bello, Adesola Hassan, Kabir Popoola, Sunday Oladejo and Dauda Awoniran

Abstract

Geographic Information System (GIS) has demonstrated its potential in improving the understanding of the previously incomprehensive and rather obscured epidemiological picture of malaria, by elucidating the association between the disease, its anopheline mosquito vector and the environment. The tremendous progress made in malaria transmission modelling is being facilitated by GIS. Model-based geostatistics and spatiotemporal risk models have been used to generate malaria risk maps, including; the famous global map of malaria endemicity, Global Malaria Atlas Project (MAP) and Continental MARA/ARMA Map of Africa. Numerous studies have reported the potential of GIS in malaria mapping. Adoption of these maps will guide in the planning of timely and targeted allocation of malaria intervention resources which will in turn promote cost-effectiveness and optimal outcome. However, despite these several reports of the significance of this GIS, its full potential may yet to be fully explored, particularly in high malaria burdened countries such as Nigeria. The risk of malaria was reported to be associated with water body presence, while other studies reported that perennial rainfall declined malaria risk by washing away and causing high mortality of Anopheles mosquito vectors. Georeferencing of participants residences to ascertain the actual geospatial data and entomological indices, and hence, the association between malaria risk and Anopheles mosquito abundance and distribution, for improving predictive performance of malaria risk maps were often not investigated. This may cause hindrance to the maximum exploration of the potential of GIS in accurately mapping the spatial and temporal distribution patterns of malaria.

Keywords: malaria, cost-effectiveness, spatiotemporal distribution, geospatial analysis, GIS

1. Introduction

The WHO African region continues to bear the highest burden of malaria, accounting for an estimated 94% of the global malaria cases and 95% of global

deaths reported in 2023, with only five countries, namely Nigeria DR Congo, Uganda, Ethiopia and Mozambique accounting for 52% (**Table 1**). The people and communities living in poverty are the most affected, thereby further impoverishing the vulnerability conditions of the families and households. An effective approach of combating the disease should therefore involve targeting the most affected and vulnerable populations and communities, as emphasised in the 2024 WHO World Malaria Report with the theme, of addressing inequity in the global malaria response [1]. However, targeting these vulnerable population, will rely on accurate data on the

S/N	WHO African county	Malaria cases (%)	Malaria deaths (%)
1	Nigeria	25.9	30.9
2	DR Congo	12.6	11.3
3	Uganda	4.8	2.7
4	Ethiopia	3.6	3.1
5	Mozambique	3.5	3.0
6	Tanzania	3.3	4.3
7	Angola	3.1	2.7
8	Burkina Faso	3.1	2.7
9	Mali	3.1	2.4
10	Cameroon	3.0	1.9
11	Niger	3.0	5.9
12	Cote d'Ivoire	2.8	1.8
13	Ghana	2.5	1.9
14	Madagascar	2.4	2.7
15	Benin	2.0	1.7
16	Malawi	1.8	1.2
17	Guinea	1.7	1.7
18	Chad	1.5	2.3
19	Zambia	1.4	1.4
20	Burundi	1.3	1.1
21	Kenya	1.3	1.9
22	Sudan	1.3	1.3
23	South Sudan	1.1	1.1
24	Sierra Leone	0.9	1.1
25	Togo	0.8	0.6
26	Central African Republic	0.6	0.8
27	Other African countries	1.1	1.8
	TOTAL	93.5	95.3

Source: Compiled from WHO [1].

Table 1.
Malaria cases and deaths that WHO African region accounted for in the 2023 global malaria report.

environmental, biological, social, structural and economical determinants influencing the risk of malaria and the barriers hindering accessibility to services and malaria interventions. The effective harnessing of these parameters for decision-making and objective planning, implementing, and monitoring are feasible options for malaria control. However, accurate and well-organised epidemiological data on malaria determinants can only be obtained in the context of GIS-based malaria mapping studies [2].

The application of survey maps and other field data and epidemiological intelligence in routine intervention programmes in many African countries dates back to the mid-1950s during the era of the Global Malaria Eradication Programme (GMEP). However, the skills and expertise required in the design of malaria intervention and control programmes on the basis of optimum comprehension of the spatial epidemiology of the disease were disused in the 1970s when malaria control agenda was put under the control of a less coordinated integrated mandate of the primary healthcare that focuses on the management of fevers. However, a renewed entreaty for optimum malaria mapping to manage malaria in Africa came up in 1996 [3], and since that time, there has been improvement on malaria-related spatial data and populations that were not available to the malaria epidemiologist several decades ago. The introduction of technological advancement that led to the emergence of Geographic Information Systems (GIS), Remote Sensing (RS) and Geographic Positioning System (GPS) in the health field and their application from simple automated epidemiological mapping to advanced satellite image analysis has further improved the understanding of malaria epidemiology in terms of the association between the disease, its vector and the environment [4]. This has even made it possible to model and map malaria risk and access to intervention in time and space *via* the use of Model-Based Geostatistics (MBG) [4] and spatiotemporal risk models.

The applications of GIS in health and disease epidemiology have been reviewed by several authors [5]. The tool has been used for rapid investigation of geographic distribution patterns and processes, as it has the potential to accept and process repetitive multiple operations and rapidly make comparison between several sources of spatial data and various spatial aspects, including environmental and geographic epidemiology or spatial health research. While the former entails the analysis of spatial patterns of disease and exposure to environment health risk (disease geography), the latter focuses on the spatial requirement and delivery of disease prevention intervention (health promotion), health services and health inequalities (geography of health).

Natural science approaches dominate epidemiological research, while research on health services has been under the control of social science approaches [5]. The use of overlay technique in GIS provides timely information to health experts based on early warning reports [6], such as malaria epidemic reports. The employment of GIS in the timely and targeted allocation of malaria intervention has especially become inevitable in the Sub-Saharan African countries which is home to 95% of the estimated global malaria mortality reported in 2023 [3].

2. Evolution of spatial modelling and malaria mapping

In togetherness with quality epidemiological data for evaluation, and in view of the spatial and temporal variations of malaria, there has been a substantial premise to evolve new modelling systems to fit in the prevailing heterogeneity and apply these

models for evaluation. Over the past 20 years, tremendous progress has been made with respect to malaria transmission modelling based on data generated from entomological, climatic/environmental, topographic and the pattern of human settlement studies [7–10].

The development of the MARA/ARMA map and the Malaria Atlas Project (MAP) are evidences of the renewed efforts towards the mapping of malaria [9]. This initiative is based on models that are generated from studies on malaria epidemiology, patterns of entomology, appropriate climatic features as well as human settlement patterns and geographic terrains. One major pitfall of the MARA/ARMA map is that the data used for mapping malaria transmission was based almost entirely on climatic variability [11, 12]. However, the MAP initiative came with some advancement over the MARA/ARMA map in that the initiative is based on the integration of survey data generated from multiple area with climatic features, thereby producing a more robust and empirical malaria risk maps and epidemiological data [9, 13]. Based on the initiative, the global map of endemicity of malaria was developed in 2007 [10]. These maps were also not without some limitations then. For instance, they were developed from a combination of several smaller-scale studies that used varying sampling techniques, time frames, and population types. In addition, there may be the appearance of selection bias, which may result when a significant proportion of the surveys selected are studies that are carried out in places where malaria endemicity is high. And lastly, huge cross-section surveys are traditionally designed with the aim of generating robust malaria estimates at the national and regional level, while district- or other microscale-level data are less common [14].

2.1 Geostatistic modelling of malaria endemicity mapping

A number of strategies have been used to develop continuous maps of malaria endemicity using data from malariometric studies. However, these data will require the application of a geostatistical model for the prediction of malaria endemicity values at geographic locations where there is unavailability of survey data [15]. The malaria maps generated from such models are prone to intrinsic unreliability and its evaluation remains a major issue in mapping disease.

Past surveys employed a predictive Bayesian Geostatistic framework [4] for spatially predicting the endemicity of malaria [16], due to the model's capacity to generate a valid statistical explanation of the classical geostatistical tool for spatial malariometric data analysis while also giving room for the accommodating the MBG methods of inferential statistics [17, 18]. The generated maps can thus provide valid contemporary global maps of malaria endemicity, when it is based on the assessment of confidence in the prediction using MBG and provide a clear description of the reliability of the prediction for the users of such maps [4]. One main underlying principle of the geostatic model is that the uncertainty of mapped predictions increases with decreasing quantity of and increasing distance away from close data points. This principle can be applied through different times and spaces, if the data are obtained at varying times and locations.

Bayesian geostatistics approach of generating malaria risk maps using climatic variables has promoted the adoption of these survey data in advanced geostatistics and epidemiological modelling techniques [18]. Unlike the traditional classical statistical methods, Bayesian modelling allows for the embracing and progressively updating of prior knowledge relating to the parameters of the models [19], thereby promoting greater pliability in modelling parameters that are not known and

evaluating the probability distribution in a specified estimate. With this advantage, Bayesian modelling has proven to be highly relevant in malaria prevalence mapping when there are vast areas to sample or when there are certain unsampled periods and when some observed covariates are not precise or well defined [20]. By applying spatial and temporal autocorrelation, data can be extrapolated from neighbouring places or from sampled time frames to generate estimates from unsampled places and time frames, respectively [20].

The Bayesian geostatistics techniques use statistical inference from Bayesian analysis to accommodate distinct environmental variables, spatially referenced point data sets, and corresponding spatial autocorrelation to be concurrently modelled and predicted on an uninterrupted smooth surface and for the determination of uncertainty [21]. There is available evidence that this method has successfully predicted the spatial distribution of malaria transmission, thereby guiding in targeting intervention efforts [14, 22]. For instance, spatial distribution patterns of malaria were evaluated, and a Bayesian predictive model was generated in Zambia based on the data from the Malaria Indicator Survey (MIS) for the year 2006 [22]. With the current renewed effort to eliminate malaria, mapping malaria foci has proposed a new rationale for targeting surveillance and control of malaria [23]. However, a major challenge then was that no geostatistical models were applied for malaria control programme evaluation with survey data that are routinely obtained. Thus, there is a need for improved integration of these approaches into vast programme evaluation impact as this will facilitate adequate control for confounding environmental and climatic factors and spatial autocorrelation.

Previously developed routine cross-sectional surveys at the national scale have been used to generate spatial risk patterns of malaria in several settings; however, routine cross-sectional malaria surveys are yet to be applied in the evaluation of the spatial patterns of distribution of malaria at different time frames with regard to the non-static level of intervention coverage and changing weather patterns. The application of these methods in this form of evaluation process generates vital information that enhances the understanding of the relative influence of the climatic factors and intervention intensification (or decline) with respect to geographically specific varying prevalence rates of malaria. Additionally, the specific parasite survey timings may substantially result in overreporting of the outcome of the prevalence of malaria, which is dependent on rainfall peak periods. Conduction of malaria prevalence survey few months sequel to the peak of the rains, may confound the comparison of the outcome with other years [24]. A large proportion of national cross-sectional data used for spatial analysis studies are based on only one survey to map malaria risk or on myriads of surveys aggregated into a single risk map. Consequently, such risk maps may not depict the real patterns of distribution in years owing to the changing annual climatic parameters. Furthermore, there was a dearth of Bayesian mapping data that incorporates temporal aspects of malaria [24], particularly for malaria prevalence surveys.

2.2 Role of malaria cartography in planning malaria interventions

The correct knowledge regarding the patterns of malaria distribution is highly essential in the planning and evaluation of malaria control interventions [3]. Malaria mapping is highly relevant in all aspects of malaria control efforts and coordination [9]. For instance, the summit meeting of the Sub-Saharan African region held in Nigeria in 2000 with respect to the malaria situation in the country reported a

complete lack of detailed and comparable malaria data and, hence, advocated, among other things, for the need to carry out more researches regarding malaria incidence and prevalence, clinical malaria epidemiology and the disease epidemics [25].

In an environment made up of international policymakers, in which the control of malaria has been forced to re-strategise malaria elimination plausibility, malaria cartography can be considered as a highly important and imperative strategy to plan, implement and evaluate the impact of intervention efforts [26]. One integral part of the Spatial Decision Support System (SDSS) is malaria prevalence and incidence mapping. For instance, following the review of the existing SDSS for malaria elimination by Kelly et al. [27], improved planning, implementation of the intervention programmes and monitoring and evaluation of the intervention programmes was suggested. The implementation of trial SDSS applications has been implemented in a number of malaria interventions in Southern Africa [28]. In the same vein, Diallo et al. [29] used an innovative subnational tailoring approach to identify the most appropriate interventions in Guinea, considering the resources that were available. The use of local data to inform eligibility and prioritisation fostered the identification of the optimal mix of interventions.

2.2.1 Global and continental malaria cartography: Pros and cons

Malaria model risk maps at the global and continental scales have previously been developed in accordance with expert opinions and option rules [30] and later on climate favourability [11]. The foremost *P. falciparum* malaria endemicity digitised risk map at the global scale was developed by Lysenko and Semashko [31]. This represented the best traditions of manual cartography that placed emphasis on the acceptance of a wide range of various sources of data and integrating them into a single synthesised map without any form of formal underlying quantitative system [9]. The map provided a comprehensive description of the classic state of malaria endemicity, each of which relates to the prevalence of malaria in children.

The map provided impressive information regarding the state of knowledge of global malaria endemicity during that period; however, the speculative nature of the approach was not without some setbacks, the most important of which is the less likely accuracy of the map and its variability from place to place, which is not measurable and hence, cannot be communicated to end-users. These important deficiencies have placed a basic limitation on the application of this map for making decisions regarding critical public health interventions [32].

Over the five decades that the Lysenko map came into existence, there was very little effort to make improvements on the map during that period. However, the development of the continental-scale MARA/ARMA map in the year 1997 and the global MAP developed in 2005, led to a rebirth which facilitated the successful transformation of the science of malaria mapping studies and its application in the control, elimination and ultimate eradication of malaria [32].

2.2.2 Contemporary maps of malaria endemicity

Malariometric data-based empirical maps have hitherto been published, which ranged from large spatial scales, such as national- and regional [3, 12, 33], to small spatial scales, such as village and settlement [34, 35]. National- and regional-scale malariometric maps are advantageous in that it has approximate homogeneity of variables relating to malaria intervention and control. However, these maps tend

to disregard the broader view of the effects that are not within the political borders of the country under investigation [32], as geographic variability also occurs, even within smaller-scale entities such as within settlements [35].

The previous global distribution maps of malaria witnessed a number of setbacks [36, 37], the most important of which are the use of a partial description of the data, basing the definition of the risk contours on irrational and insufficiently explained expert-opinion rules and estimations of the uncertainty surrounding the predictions were not given. However, the development of contemporary maps of malaria endemicity at the county/regional level has overcome the deficiencies with the global scale malaria maps

2.3 GIS technology in malaria prevalence mapping

The main significance of adopting GIS technology in the epidemiological mapping of malaria is that such maps give an additional spatial context in relation to the analysis of data, which aids in the visualisation of the complex patterns of the disease. For instance, accurate malaria mapping was identified as a vital tool that can be used to promote malaria vector intervention efforts [4]. There are arrays of studies that have utilised spatial technologies in mapping malaria prevalence (**Table 2**).

However, the majority of these maps were developed at unrefined spatial resolutions, thereby limiting their operational application in malaria control programmes at lower administrative spatial scales. Small spatial scale maps, some of which were based on specific georeferenced point malaria prevalence data, have also been developed by various authors [16, 29, 34, 38–48]. For example, GIS was used to map malaria prevalence in Botswana based on reported cases of malaria at the local health centres [39].

A regional map of malaria showing the spatial patterns of distribution of *P. falciparum* malaria (**Figure 1**) was also developed in Ethiopia [49]. In the same vein, malaria prevalence map across Somalia, which was based on malaria data that were obtained from previous routine cluster and malaria cluster surveys, was also developed [16]. According to the map, the transmission pattern of malaria ranged from hypo-endemic to meso-endemic in Somalia. The map identified locations in proximity to the Shabella River and Juba as the primary malaria transmission foci in Somalia. The incidence of malaria was developed at a smaller scale in a village in Central Ethiopia, where malaria transmission is unstable [34].

Structured GPS survey questionnaires were developed by these authors to record the geographic location of participants along with relevant demography. A spatial clustering method was adopted in mapping malaria incidence in addition to estimating the malaria incidence density of 1000 people per year for each of the villages that were georeferenced. Malaria prevalence mapping has fostered a growing interest in the modelling of the endemicity of malaria in relation to environmental risk variables. The adoption of GPS-based malaria prevalence and entomological parameters in malaria mapping will aid in the generation of the true epidemiological picture of the association between malaria and related environmental indices, and hence, the development of an accurate small spatial scale malaria map. This was especially demonstrated in a study by Bello and Hassan [50]. The authors used malaria-related data collected from participants whose households were georeferenced to generate risk maps of malaria showing the spatial distribution patterns of the disease and also identified areas where malaria clustering occurred. In the same vein, Kibibi [51] explored the use of GIS for mapping malaria transmission hotspots in West and East

Country of study	Research objective	Research method	Key findings	References
Somalia	Mapping malaria distribution in low transmission regions	Spatial clustering analysis	Malaria transmission varied from hypo- to meso-endemic	[16]
Guinea	predicting the impact of different intervention mix scenarios	Subnational tailoring (SNT) approach	SNT fostered adaptation of intervention strategy at the district level	[29]
Ethiopia	Mapping the local distribution pattern malaria	Spatial cluster analysis of malaria incidence	Local clustering of malaria incidence occurred between pairs of villages	[34]
Malawi	Malaria risk mapping	Interpolation analysis	Malaria variation occurred at local level	[38]
Botswana	Historical malaria prevalence mapping	Logistic regression (univariate)	Malaria prevalence was significantly associated with the environment	[39]
Nigeria	Examination of malaria evolution	spatial autocorrelation, and hotspot analysis	Occurrence of environmentally-mediated spatial variation of malaria	[40]
Kagera, Tanzania	Assessment of local malaria burden	GIS and local spatial statistic method	Malaria exhibited high temporal and spatial heterogeneity	[41]
Nigeria	Mapping malaria prevalence	Systematic grid-point sampling	Presence of spatial variations of malaria	[42]
Nigeria	Developing an operational risk map for malaria control	Weighted overlay analysis	Risk of malaria varied from very low to very high in the study area	[43]
Ethiopia	Assessment of malaria risk areas	Weighted overlay technique	Malaria risk varied from low- to very high-suitability	[44]
Nigeria	Identification of vector-proliferating environmental factors	GIS and Remote Sensing tool	Environmental factors influenced Anopheline malaria vector proliferation	[45]
Mozambique	Mapping and modelling malaria risk maps	GIS-based spatial modelling technique	All of the study population were at risk of contracting malaria	[46]
Zimbabwe	Identification of seasonal hotspot of malaria cases	GIS and spatial statistic methods	*Anopheles arabiensis* habitat suitability predicted malaria hotspots	[47]
Ethiopia	Assessment of malaria risk	Weighted overlay analysis	Malaria risk varied from high to very high	[48]

Table 2.
GIS-based malaria incidence and prevalence mapping studies in Africa.

Africa. The authors found that recent GIS underscore the association between malaria transmission and environmental and socioeconomic factors.

For instance, epidemiological surveys that extends spatial geostatistical model to accommodate time are not common, however, a full spatial and temporal geostatistical modelling techniques has evolved [24, 49]. By incorporating the dimension

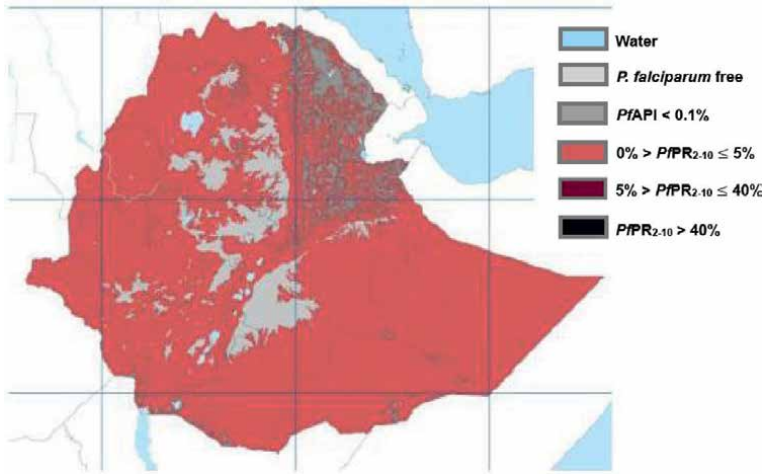

Figure 1.
*Spatial heterogeneity of Malaria (*P. falciparum*) in Ethiopia. Source: Federal Democratic Republic of Ethiopia Ministry of Health [49].*

of time, unambiguous comparison between future malaria map iterations and the benchmark is guaranteed. The map will hence produce an explicit description of the geographic framework, which can be used to survey, monitor and evaluate the effectiveness of global malaria intervention efforts.

Nigeria is not left out in the studies on malaria epidemiology using GIS spatial technology tools. However, there are few such studies at the country level [52, 53] while the other researches are confined to a few states [54–59]. Malariometric data spanning a period of 15 years, from 1993 to 2007, for 23 states in Nigeria, were obtained from the Data Bank of WHO and the Epidemiological Unit/Roll Back Malaria of both the Federal and State Health Ministries. The sum of monthly malaria cases for the 15 years period were aggregated by months, which represented the study variables. Principal Component Analysis (PCA) analysis was then used to analyse the spatial distribution patterns of malaria, after which the map showing the spatial distribution patterns of malaria in Nigeria was developed [52]. The main components were recognised on the basis of seasonality, namely, component 1 (dry season), component 2 (rainy season) and component 3 (transition from rainy to dry season).

For the purpose of deriving comprehensive observations and accurate spatial distribution patterns of malaria in Nigeria, the authors grouped Nigeria states into three categories on the basis of the level of malaria transmission intensity, namely: high-level (>1.00), medium-level (1.00–0.01) and low-level (<0.01) malaria transmission zones (**Figure 2**). According to the map, Lagos state was located within the low-level malaria transmission zone; however, following the dry season (October to March), Lagos migrated from the low-level to the high-level malaria transmission zone (1.222), which highlights the significance of seasonal variation in malaria transmission [52].

According to Akpan [53], based on environmental suitability, *An. gambiae* s.s., *An. gambiae* s.l. and *An. arabiensis* is most widespread in the Derived savannah and humid forest, moderately distributed in the Northern and Southern Guinea savannahs, and limited distribution in the Mid-Altitude zones and Sahel savannah. *An. arabiensis* is widespread throughout all the Nigerian states, with the highest

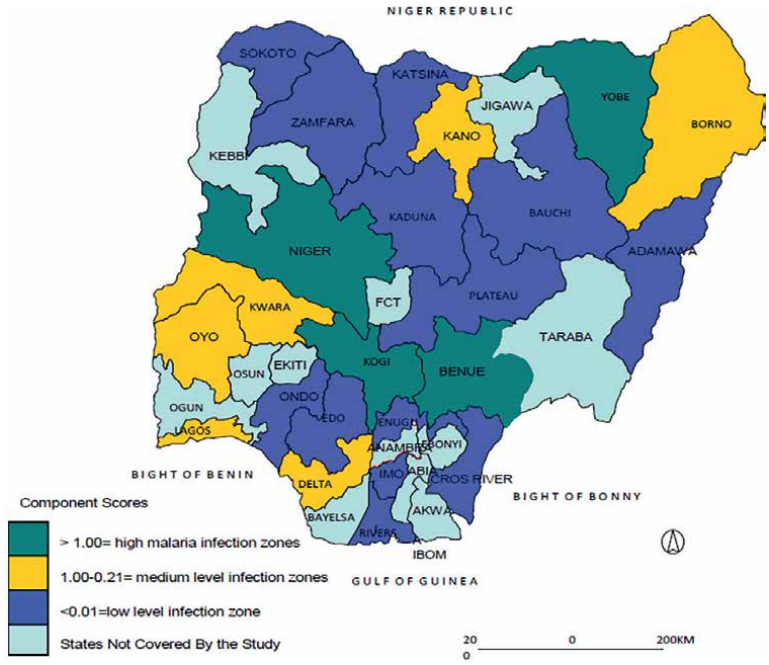

Figure 2.
Spatial variation in malaria infestation in Nigeria. Source: Onwuemele [54].

recorded in a Southwestern geopolitical region state (Lagos) and lowest in South-South geopolitical region state (Bayelsa). The mean abundance of *An. gambiae* s.l. was also highest in Lagos state, while the least was recorded in Yobe state. Omogunloye et al. [59] used GIS technology to analyse the influence of environmental risk parameters, namely temperature, rainfall, and relative humidity, on malaria cases in Lagos state.

A risk map of malaria, showing all the 20 local government areas in the state and the corresponding level of malaria cases (high and low), was generated. In addition, a significant association was observed between malaria cases and the corresponding environmental features; hence, a spatial clustering of malaria and a predictive model was developed.

2.4 Application of GIS in the assessment of environmental risk variables

The dynamics of transmission of endemic malaria have since been related to the abundance and distribution patterns of the Anopheles mosquito vectors, which in turn is related to the availability of favourable and preferred breeding habitats as well as to favourable environmental features. A lot of studies have reported associations between the abundance and patterns of distribution of Anopheles mosquitoes and climatic variables, mostly concentrated on country or regional scales [47]. The primary objectives of these studies were to investigate the statistical associations between malaria and environmental risk variables. For example, Gething et al. [60] used the technique in their study and found an estimated 2.6 billion who live in the tropics to be at risk of *P. falciparum* malaria, while a relatively smaller number of humans (2.5 billion) were found to be at risk of *P. vivax* malaria. In the same vein, map showing the spatial heterogeneity of the endemicity of *P. falciparum* malaria has also been developed [10].

The significance of environment on malaria distribution was demonstrated in Epe and Orimedu in a study by Bello and Hassan [50]. The characteristic distinct terrain explained the significant spatial heterogeneity and clustering of malaria in Epe while the very high malaria risk terrain in Orimedu was attributed to the fairly evenly distributed high malaria risk terrain. Topographic terrain and perennial water body presence have also been incriminated in determining the patterns of the distribution of malaria.

Steep topographic terrain was characterised by distinct altitude zones. Service [61] reported that inhabitants in upper altitude zones where there is absence of perennial water bodies such as river were forced to remain outside in the late evening to get fresh breeze before going to bed. This was substantiated in another study where malaria prevalence was significantly lower in the lower altitude zone in Epe, where the river present in the area provides a relatively cooler environment and fresher air. Consequently, eliminating or at least limiting the need to stay outside in the late evening to get fresh breeze [50]. In a similar study, villages that were classified as hotspots of malaria transmission were attributed to environmental parameters characterised by abundant water bodies [62].

3. Conclusion

The allocation of timely and targeted malaria intervention has fostered cost-effectiveness, hence, increasing the likelihood of the successful elimination of the disease. This approach is informed by accurate data on the epidemiological picture and distribution patterns of the diseases, in which GIS has proven to be a potential tool in its acquisition. However, the available small-scale predictive risk maps of malaria still suffer from inaccuracies due to lack of georeferenced data on malaria source. This has limited the full exploration of the potential of GIS in providing accurate maps of the spatial and temporal distribution patterns of malaria.

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Section 2

Antimalarial Therapeutics and Resistance

Chapter 3

Artemisinin: A Revolutionary Antimalarial Agent

Mohan Tiwari and Saman Pathan

Abstract

The treatment of malaria has significantly improved with the groundbreaking antimalarial drug artemisinin, which is derived from *Artemisia annua.* It was found in the 1970s and damages Plasmodium parasites by producing reactive oxygen species. Because of its quick action and effectiveness against drug-resistant strains, artemisinin-based combination treatments (ACTs) are the gold standard. However, long-term efficacy is threatened by resistance, particularly in Southeast Asia. The history, mechanism, and clinical application of artemisinin are examined in this chapter, along with its function in the prevention of malaria and current studies aimed at overcoming resistance and creating novel derivatives. In the worldwide battle against malaria, artemisinin is still essential.

Keywords: artemisinin, malaria, *Plasmodium*, ACTs, resistance, *Artemisia annua*, therapeutic innovations

1. Introduction

In the fight against malaria, artemisinin and its derivatives are an essential and potent class of medications. Extensive worldwide study has improved our knowledge of this extraordinary phytochemical, including its distinct chemical and pharmacological characteristics, since its discovery in the early 1970s.

Today, while artemisinin remains the cornerstone of antimalarial therapy, several challenges have emerged in its ongoing use and development. These include the rise of delayed treatment responses to artemisinin in malaria patients and attempts to repurpose these drugs for non-malaria applications.

A crippling illness that has affected people all over the world since ancient times, malaria remains one of the most pervasive, destructive, and deadly epidemics in human history [1]. Malaria, which frequently emerged and spread in the humid regions surrounding marshes and swamps, was once thought to be caused by the "bad air" preventing it in these areas [2]. This misconception gave rise to the term "malaria", derived from the medieval Italian words ma (bad) and aria (air). For centuries, the disease was wrongly attributed to foul air, delaying the discovery of its true transmissible and parasitic nature. It was not until the late 1800s that the groundbreaking discoveries of Charles Louis Alphonse Laveran and Roland Ross uncovered the true cause of malaria. Their discoveries revealed that malaria is caused

by protozoa of the *Plasmodium* genus and is primarily transmitted by *Anopheles* mosquitoes. These groundbreaking findings earned Laveran and Ross recognition as among the earliest recipients of the Nobel Prize in Physiology or Medicine [3].

Only five of the more than 100 Plasmodium species can affect humans. The main causes of malaria are *Plasmodium malariae, Plasmodium ovale, Plasmodium vivax,* and the extremely deadly *Plasmodium falciparum*, but *Plasmodium knowlesi* is not very dangerous to people.

Malaria treatment depends on chemotherapy with medication that targets various stages of the *Plasmodium* parasite's life cycle. These consist of mefloquine (Larium), lumefantrine, doxycycline, quinoline compounds, sulfadoxine/pyrimethamine, and artemisinin-based combination treatment (ACTs). Mefloquine (ASMQ), lumefantrine (Coatem), amodiaquine (ASAQ), piperaquine (Duo-Cotecxin), pyronaridine (Pyramax), and other antimalarial medications are often combined with an artemisinin derivative in the most widely used ACTs. Additionally, preventative medicines like chloroquine, doxycycline, mefloquine (Lariam), primaquine, and the atovaquone-proguanil combination (Malarone) may be used for prophylaxis when necessary [4].

The World Health Organization (WHO) started the Emergency Response to Artemisinin Resistance in the Greater Mekong Subregion and the Global Plan for Artemisinin Resistance Containment (GPARC) to combat the growing threat of artemisinin-tolerant and resistant malaria. These programs support a systemic, multitiered strategy aimed at preventing, reducing, and eventually eradicating the emergence and transmission of malaria that is resistant to artemisinin [5]. Significantly lowering the worldwide malaria burden has been made possible by increasing access to artemisinin-based combination treatments (ACTs) in areas where malaria is endemic. At present, no alternative antimalarial treatment matches the efficacy and tolerability of ACTs.

2. Artemisinin

A naturally occurring sesquiterpene lactone substance with a distinct chemical structure is artemisinin, and its derivatives, which are collectively referred to as 'artemisinin' unless otherwise noted. It comes from the *Asteraceae* family's sweet wormwood plant (*Artemisia annua* L.). Artemisinin has been proven effective against different forms of *Plasmodium* parasites, making it a focal point of significant scientific and medical interest [6, 7]. The discovery of artemisinin traces back to a Chinese government initiative in the late 1960s aimed at finding a cure for malaria. *Tu Youyou* won the 2015 Nobel Prize in Physiology or Medicine for her revolutionary discovery in 1972, which was the result of her hard work. The current global standard for treating malaria caused by *Plasmodium falciparum* and other *Plasmodium* species is artemisinin-based combination treatments (ACTs).

Since the 1980s, artemisinin derivatives have increasingly become the focus of research due to their affordability, effectiveness, and ease of use [8–10]. In 2006, the World Health Organization (WHO) officially recommended artemisinin-based combination therapies (ACTs) as the first-line treatment for *Plasmodium falciparum* malaria [11, 12]. Artemisinin and its derivatives remain the most crucial and effective drugs for malaria treatment [13]. However, the emergence of drug-resistant *Plasmodium falciparum* has driven continuous advancements in antimalarial drug research, with particular focus on enhancing artemisinin-based combination therapies (ACTs) [13–15].

3. Chemical structure of artemisinin and its derivatives

Artemisinin is a sesquiterpene trioxane lactone distinguished by the presence of a unique peroxide bridge (-O-O-) within a seven-membered ring; when its peroxide bridge interacts with iron in the parasite, leading to the destruction of the parasite's cellular structure, which is crucial for its antimalarial activity (**Figure 1**) [16]. The lactone group in artemisinin can be readily reduced using sodium borohydride, yielding dihydroartemisinin. This derivative demonstrates even greater antimalarial activity *in vitro* compared to artemisinin itself [17].

To enhance its effectiveness, numerous derivatives (**Figures 2–6**) have been synthesized from dihydroartemisinin. Among them, artemether, arteether, artesunate, and artelinic acid are either currently in use or under evaluation. These derivatives play a crucial role in artemisinin-based combination therapies (ACTs), which the WHO recommends as the first-line treatment for *Plasmodium falciparum* malaria [18].

a. *Dihydroartemisinin (DHA):* It is a semisynthetic derivative or a reduced form of artemisinin where the lactone is converted to a hydroxy group. Known for enhanced solubility in water and greater antimalarial potency compared to artemisinin. It kills parasites by damaging their membranes and disrupting their mitochondrial function. DHA is low in toxicity and has saved many lives [19].

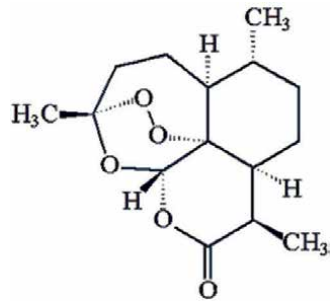

Figure 1.
Artemisinin.

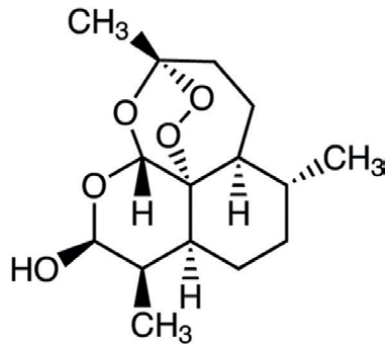

Figure 2.
Dihydroartemisinin (DHA).

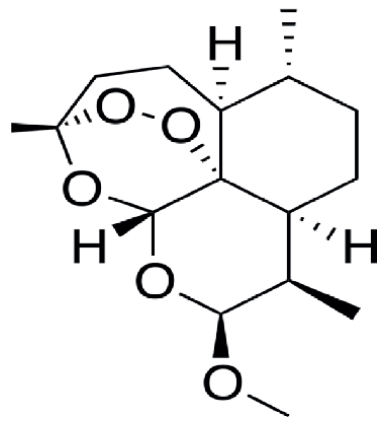

Figure 3.
Artemether.

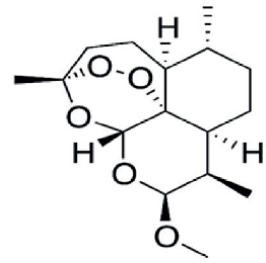

Figure 4.
Arteether.

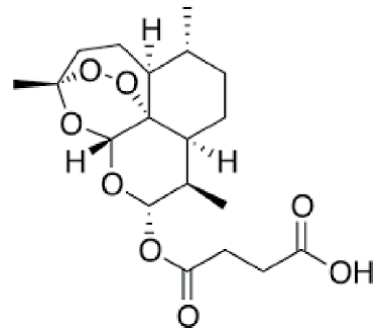

Figure 5.
Artesunate.

b. *Artemether:* A peroxide-containing lactone, a derivative of dihydroartemisinin formed by methylation of the hydroxy group. Lipophilic, making it suitable for intramuscular injection or oral use. It is used to treat uncomplicated malaria.

c. *Arteether:* Arteether is an ethyl ether derivative of dihydroartemisinin. Its lipophilic nature allows for intramuscular administration in long-acting formulations. Being oil soluble, it has an extended elimination half-life (greater than

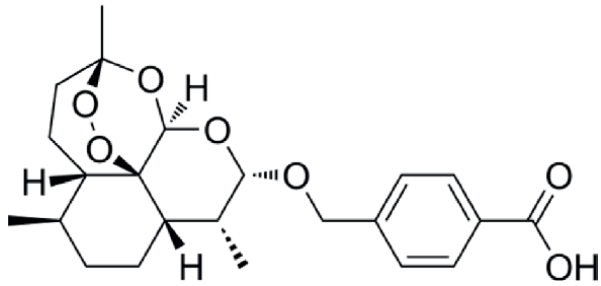

Figure 6.
Artelinic acid.

20 hours), enhanced chemical stability, and greater overall stability compared to other artemisinin compounds [20].

d. *Artesunate:* A water-soluble derivative of dihydroartemisinin achieved through hemisuccinate esterification. Commonly used in severe malaria cases due to its rapid action and intravenous or intramuscular administration.

e. *Artelinic acid:* It is a semisynthetic derivative of artemisinin, specifically a triaxone dicarboxylic acid derivative of dihydroartemisinin (DHA). It was developed to improve the pharmacokinetic properties and stability of artemisinin derivatives for antimalarial therapy. Artelinic acid retains the core sesquiterpene trioxane lactone structure of artemisinin but introduces a dicarboxylic acid group, which enhances its water solubility and chemical stability compared to other derivatives like artesunate. The peroxide bridge (-O-O-) critical for antimalarial activity is preserved.

Each of these derivatives retains the critical peroxide bridge, which is activated upon interaction with iron from the parasite's heme, generating free radicals that inflict damage on the parasite. These structural modifications enhance solubility, bioavailability, and pharmacokinetics, optimizing the derivatives for specific clinical applications.

4. Extraction of artemisinin

In 1969, Professor Youyou Tu was appointed to lead a research team as part of a project dedicated to screening Traditional Chinese Medicine (TCM) for novel antimalarial drugs. The China Academy of Chinese Medical Sciences' Institute of Chinese Materia Medica is where this study was carried out. A list of more than 2000 herbal medicines was developed by Tu and her team using a wealth of TCM knowledge, including folklore, ancient literature, and practitioner interviews. They then reduced this list to about 640 viable options. From this refined selection, they tested more than 380 extracts from around 200 herbs, including Qinghao (*Artemisia annua*), though most failed to produce satisfactory results [21, 22]. The Qinghao extract, however, gained significant attention around 1971 due to its promising yet inconsistent results. This inconsistency prompted a meticulous re-examination of the traditional literature, ultimately leading to a pivotal breakthrough in the discovery process.

Drawing from classical literature, particularly Ge Hong's *Zhouhou Beiji Fang* (Handbook of Prescriptions for Emergency), and her deep knowledge of Traditional

Chinese Medicine (TCM), *Tu* proposed a crucial modification to the extraction process using low-temperature conditions to preserve the active compounds. The extracts obtained through this refined method were further purified by separating the acidic and neutral phases, a technique designed to retain the active components while reducing toxicity. This strategy produced a breakthrough: in tests carried out in or around October 1971, the resultant material demonstrated 100% efficacy against rodent malaria. In late December of the same year, this astounding discovery was confirmed in full in monkey malaria trials, conclusively demonstrating the effectiveness of the Qinghao extract [21].

This success confirmed the safety profile of the Qinghao extract, allowing clinical trials to proceed without delay in the latter half of 1972. The extract's effectiveness in treating malaria was further confirmed by the extremely positive outcomes of the studies, which were carried out in Hainan Province and at the 302 Hospital PLA (now a component of the fifth Medical Center of the Chinese PLA General Hospital) in Beijing. These results helped propel Qinghao's research to the national level, driving further investigation and development. A concerted effort by the Chinese scientific community further propelled the research and development of Qinghao. In November 1972, Tu's team at the Institute of Chinese Materia Medica successfully isolated the active component from the Qinghao extract; artemisinin (also known as Qinghaosu).building on this discovery the team later developed dihydroartemisinin (DHA), a derivative that remains one of the most pharmacologically significant antimalarial agents in use today.

Subsequently, artemisinin-based therapies significantly improved parasite clearance and quickly reduced symptoms in both mild and severe *Plasmodium falciparum* malaria infections, particularly when paired with slower-acting antimalarials like mefloquine or piperaquine. Additionally, these treatments demonstrated excellent tolerability with minimal reports of toxicity or safety concerns [22].

The exceptional effectiveness and safety of artemisinin-based treatments became more evident after more than 10 years of independent randomized clinical trials and meta-analyses. In 2006, the WHO officially revised its treatment guidelines to fully implement artemisinin-based combination therapies (ACTs) as the first-line treatment for malaria [23]. To this day, ACTs remain the most effective and widely recommended antimalarial treatments [24].

5. Biosynthesis of artemisinin

Two C_5 isoprenoid units from the cytosolic mevalonate (MVA) pathway and one isoprenoid unit from the non-mevalonate (MEP or DXP) pathway [25] are used to manufacture Farnesyl pyrophosphate (FPP, C_{15}), a crucial precursor of artemisinin [19, 20, 26]. FPP then undergoes cyclization through the enzymatic action of amorpha-4,11-diene synthase (ADS), leading to the formation of amorpha-4,11-diene [27–29]. This process involves the generation of bisabolyl and 4-amorphenyl cation intermediates (**Figure 7**) [25, 30, 31].

Amorpha-4,11-diene 12-monooxygenase (CYP71AV1) then oxidizes amorpha-4,11-diene to artemisinin alcohol [32]. Additionally, this enzyme makes it easier for artemisinic alcohol to continue oxidizing into artemisinic aldehyde and subsequently artemisinic acid. Additionally, alcohol dehydrogenase 1 (ADH1) catalyzes the oxidation of artemisinic alcohol to artemisinic aldehyde, while aldehyde dehydrogenase 1 (ALDH1) converts artemisinic aldehyde into artemisinic acid [33, 34]. Initially, artemisinic acid was thought to be the final precursor of artemisinin [25].

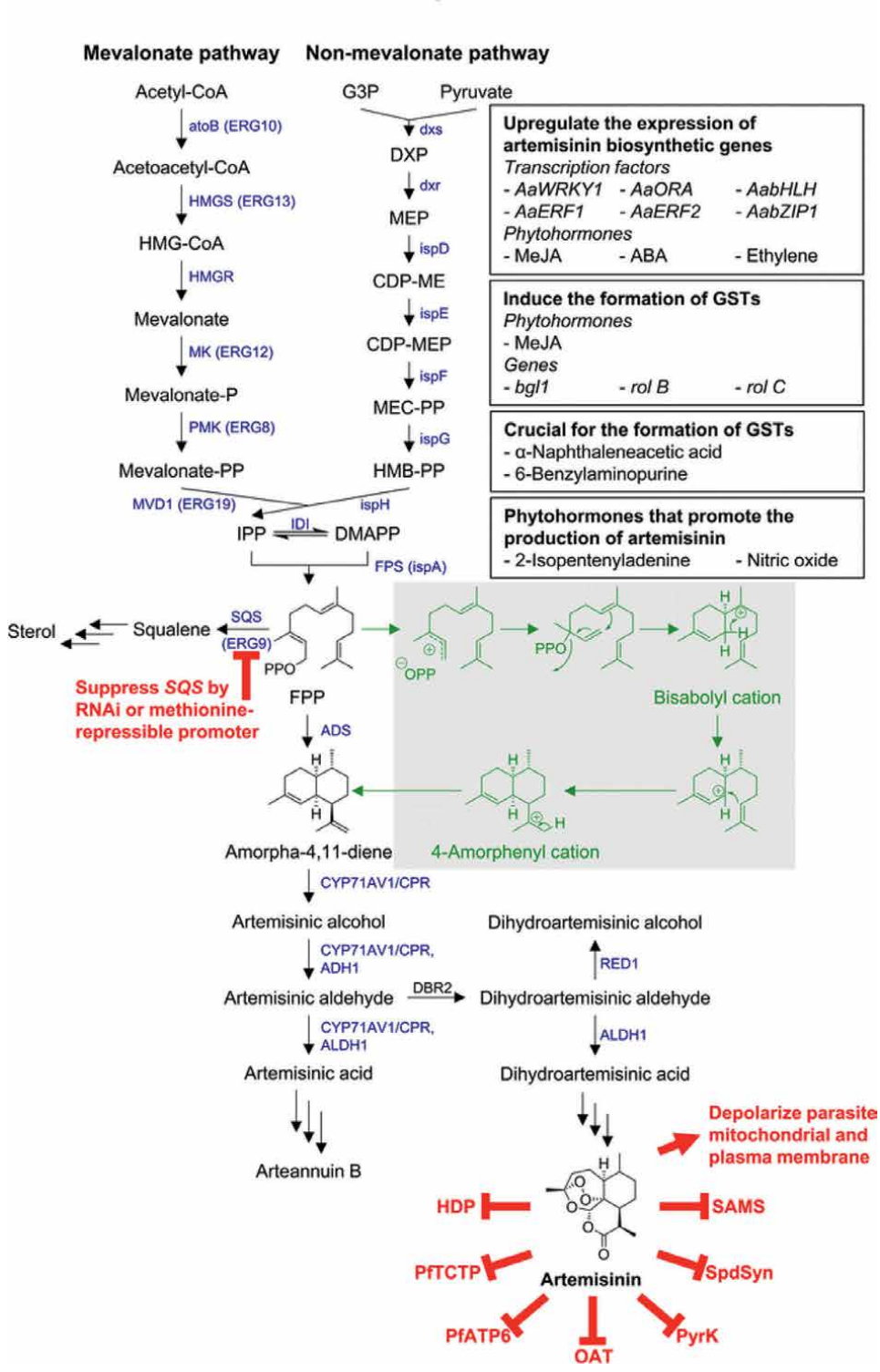

Figure 7.
Biosynthesis of artemisinin. Source: *Ref. [25]. [updated 2025 Apr 03]. Available from: https://link.springer.com/article/10.1007/s11418-016-1008-y CC BY 4.0.*

However, studies have revealed that it undergoes non-enzymatic conversion into arteannuin B and related compounds rather than directly forming artemisinin [25, 35].

Artemisinic aldehyde Δ11(13) reductase (DBR2) catalyzes the reduction of artemisinic aldehyde to dihydroartemisinic aldehyde [25], which is the subsequent stage in the production of artemisinin [36]. As shown in **Figure 7**, aldehyde dehydrogenase 1 (ALDH1) subsequently oxidizes dihydroartemisinic aldehyde to dihydroartemisinic acid, which subsequently goes through a non-enzymatic conversion to artemisinin [33, 36]. The enzyme dihydroartemisinic aldehyde reductase 1 (RED1), which converts dihydroartemisinic aldehyde to dihydroartemisinic alcohol, was discovered by Rydén et al. [37]. Although the exact function of RED1 in the biosynthesis of artemisinin is yet unknown, research indicates that *Artemisia annua* may produce more artemisinin if RED1 is silenced.

Artemisinin is primarily synthesized in glandular secretory trichomes (GSTs), and as the plant ages, its accumulation decreases. According to Olofsson et al. [38], *Artemisia anna* has GSTs in all aerial tissues but not in roots or hairy roots. The highest density of GSTs is seen in flower buds and young leaves, progressive decreasing as the leaves age [25].

6. Antimalarial mechanism of artemisinin/mechanism of action of artemisinin

Artemisinin and its derivatives exhibit potent and rapid antimalarial activity by reducing malaria parasite levels and alleviating symptoms. They act by disrupting the erythrocytic stage of the parasite's life cycle, particularly inhibiting merozoite formation in red blood cells. The activation of artemisinin relies on the cleavage of its endoperoxide bridge, leading to the generation of reactive free radical species. Two primary activation pathways have been proposed: the mitochondrial pathway and the heme-mediated degradation pathway [39, 40].

6.1 Bioactivation of artemisinin in parasite

A number of protease enzymes in the malaria parasite's host break down hemoglobin, releasing peptides and amino acids necessary for the parasite's growth and freeing up room in the digesting vacuole. Hematine accumulates during this process, which can be quite harmful to the parasite, as shown in **Figure 8** [42]. To mitigate this threat, the parasite has developed a detoxification mechanism wherein hematin undergoes biomineralization, forming insoluble and non-toxic hemozoin, commonly referred to as malaria pigment.

Detoxification of hemoglobin: The malaria parasite transforms toxic hematin, which is created when heme monomers hydrogen bond [42], into hemozoin, an insoluble, non-toxic substance. Recent studies suggest that during hemozoin formation, the propionate group of each Fe(III) PPIX molecule coordinates with the Fe(III) center of an adjacent molecule, stabilizing the crystalline structure and reducing toxicity [43].

6.2 Mitochondria-activated artemisinin

It induces cytotoxicity by triggering lipid peroxidation, generating reactive oxygen species (ROS), and causing depolarization of the parasite's mitochondrial and plasma membranes [40, 44–47]. Superoxide anion, hydroxyl radical, peroxy radical, hydrogen peroxide, and lipid hydroperoxide are examples of radical or pro-radical compounds

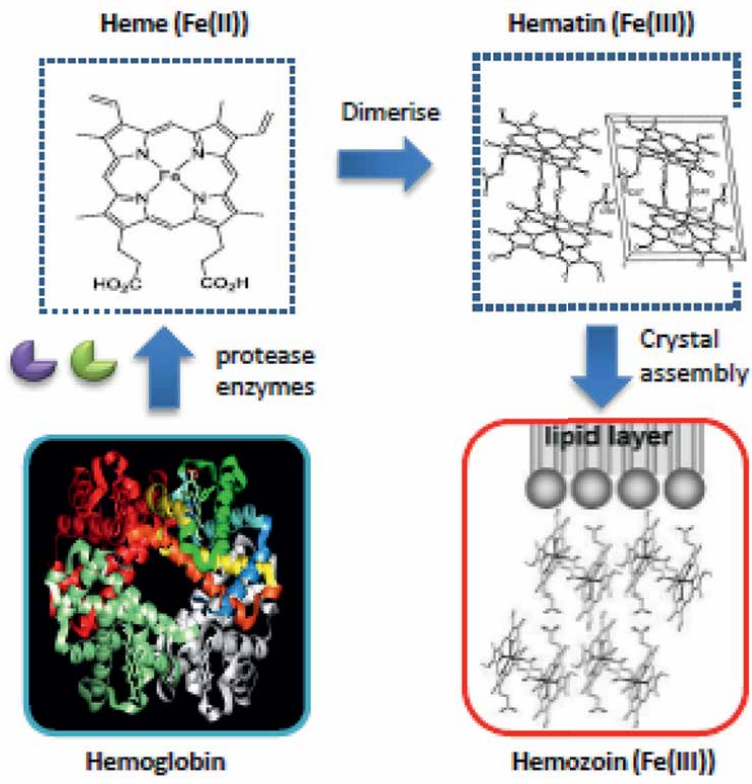

Figure 8.
Detoxification of hemoglobin. Source: *Ref. [41]. [updated 2025 Apr 03]. Available from: https://pubmed.ncbi.nlm.nih.gov/20336009/ CC BY 4.0.*

that contain oxygen. These highly reactive molecules inhibit malaria parasite survival by damaging critical biomolecules, such as lipids, proteins, and nucleic acids, ultimately disrupting cellular integrity and function [47]. Reducing iron from the unstable iron pool in the cytoplasm of Plasmodium, along with iron released from heme decomposition, activates artemisinin to generate free radicals, which play a crucial role in its antimalarial action. Two primary types of free radicals contribute to this effect: oxygen free radicals and carbon free radicals. However, their formation is sequential rather than simultaneous. Under the influence of iron, the peroxy bridge in artemisinin is cleaved, leading to the production of oxygen-free radicals, which subsequently facilitate the formation of carbon-free radicals through electron rearrangement [48].

6.3 Heme-mediated pathway

In the heme-mediated activation pathway, two models have been proposed: the reductive scission model and the open peroxide model, both of which lead to the formation of an active carbon-centered radical [41, 49]. Additionally, while some studies suggest that heme plays a major role in artemisinin activation, outweighing the contribution of Fe^{2+} ions, despite some studies suggesting that non-heme Fe^{2+} ions can bind to and activate artemisinin [50, 51]. As shown in **Figure 9**, heme is generated in *Plasmodium* species by hemoglobin breakdown at the trophozoite stage and endogenous production during the early ring stage. However, the amount of heme produced

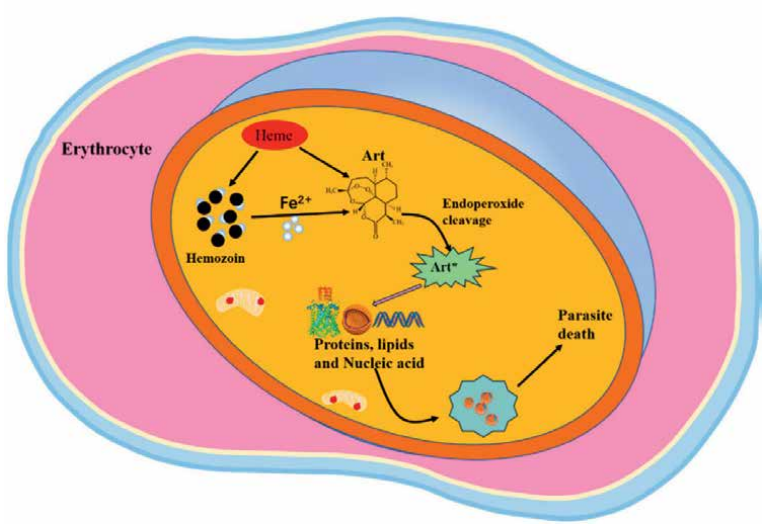

Figure 9.
Antimalarial mechanism of artemisinin. Source: *Ref. [52]. [updated 2025 Apr 03]. Available from: https://www.mdpi.com/2414-6366/9/9/223# CC By 4.0.*

by hemoglobin digestion is much greater than that produced by endogenous heme, indicating that hemoglobin-derived heme plays a dominating role in artemisinin activation [51, 53].

Hemozoin, a parasite pigment, is deposited within the food vacuole following hemoglobin digestion. Although the plasmodium stages are most vulnerable to artemisinin action, which happens too early in development to show visible pigment, despite the fact that it has long been suggested that artemisinins target it [54, 55]. In infected erythrocytes, excess heme is converted into hematin, a toxic molecule capable of inducing oxidative damage and lysing cell membranes [56]. To neutralize hematin toxicity, malarial parasites employ a detoxification mechanism that converts hematin into inert, crystallized hemozoin through a biocrystallization process [43]. Activated artemisinin has been reported to inhibit hemozoin formation by alkylating heme, functioning similarly to other antimalarial drugs that target hemozoin synthesis, such as chloroquine (CQ) [57–60]. Consequently, free heme from hemoglobin digestion serves as both the activator and target of artemisinin, reinforcing its potent antimalarial effect [57].

In *in vitro* studies, the endoperoxide bridge of artemisinin is proposed to be activated by ferrous iron, generating oxygen- or carbon-centered free radicals, which subsequently alkylate heme. Since iron is a key component of hemozoin, the digestion of hemoglobin by the parasite is believed to make them particularly vulnerable to locally activated artemisinins. Heme and iron produced by hemozoin can activate artemisinin to produce free radicals. The activated artemisinin disrupts the physiological functions of *Plasmodium* by targeting proteins, lipids, and nucleic acids, leading to the parasites' death. The asterisk signifies the activated form of ART.

6.4 The intra-erythrocytic parasite and proposed targets of artemisinins

Plasmodiun falciparum replicates within red blood cells, relying on hemoglobin digestion for survival during its 48-hour asexual life cycle (**Figure 10**) [61].

Artemisinin have long been proposed to target the parasite's hemoglobin digestion process within the 'food vacuole' (**Figure 10b**). Additionally, studies suggest that artemisinins may also act on the parasite mitochondrion, the translationally controlled tumor protein (TCTP), and PfATP6, parasite-encoded sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA).

The intricate life cycles of parasites that cause human malaria involve three cycles of asexual reproduction and one cycle of sexual reproduction, and they depend on both human hosts and mosquito vectors. Within the host's red blood cells, one of

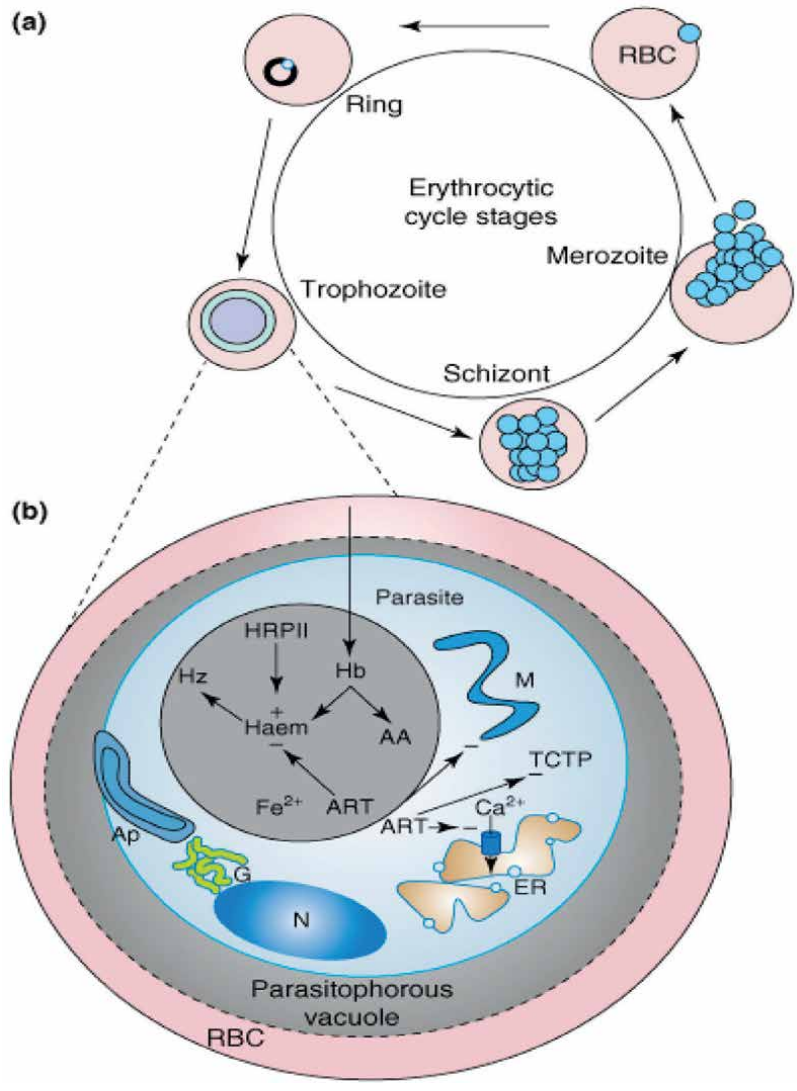

Figure 10.
Complex life cycle of Plasmodium falciparum*.* Source: *Krishna et al. [61] [Updated 2025 Apr 03]. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC2758403/ CC BY 3.0. DV, digestive vacuole; ER, endoplasmic reticulum; AA for amino acids; Ap for apicoplast; ART for artemisinins; G, the Golgi apparatus; M stands for mitochondria; N for the nucleus; Hb for hemoglobin; Hz for hemoglobin; Red blood cells, or RBCs; translationally controlled tumor proteins, or TCTPs.*

the asexual stages takes place (**Figure 10a**). Invasive forms of the parasite, known as merozoites, enter red blood cells and remain relatively metabolically inactive for 10–15 hours during the ring stage. This is followed by a rapid growth phase over the next 25 hours, forming the trophozoite stage, during which the parasite digests most of the host cell's hemoglobin and expands to occupy more than 50% of the cell's volume. Hemoglobin digestion occurs within a specialized organelle called the food vacuole (**Figure 10b**), leading to the formation of heme. As heme is generated, it associates through one of its peripheral carboxyl groups with the Fe^{3+} of an adjacent heme molecule, forming insoluble hemozoin. It has been suggested that a protein called histidine-rich protein II aids in this process, albeit this has not been proved. The parasite goes through several rounds of division during the schizont stage after the trophozoite stage. The cycle is continued when the host cell lyses 48 hours after invasion, releasing freshly produced merozoites.

Over the years, it has been suggested that artemisinins target a variety of pathways, some of which may not require activation by Fe2+ (**Figure 10b**). These include the endoplasmic reticulum-localized calcium pump known as PfATP6, the mitochondrion, the translational controlled tumor protein (TCTP), and the heme detoxification route.

7. Artemisinin resistance

Clinically, artemisinin resistance is characterized by the delayed clearance of malaria parasites from the bloodstream following treatment with artemisinin derivatives or artemisinin-based combination therapies (ACTs) [62]. The delayed clearance of malaria parasites following artemisinin treatment is more precisely termed "partial

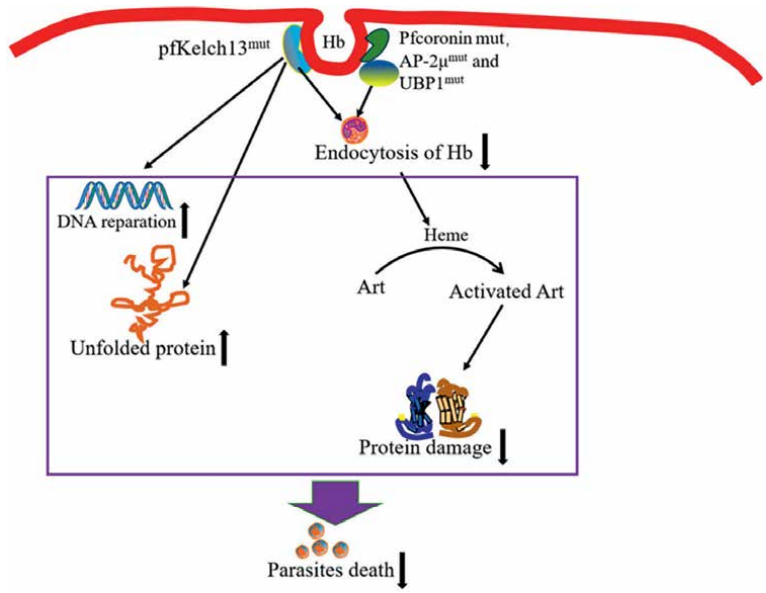

Figure 11.
The mechanism of artemisinin resistance. Source: *Ref. [52]. [updated 2025 Apr 03]. Available from: https://www.mdpi.com/2414-6366/9/9/223# CC By 4.0.*

resistance", as it is restricted to specific timeframes and stages within the parasite's life cycle. Research indicates that resistance mechanisms predominantly impact the ring stage, allowing parasites to temporarily withstand drug exposure before progressing through later developmental stages [63]. The emergence of partial artemisinin resistance is worrisome, as it could progressively extend to other stages of the parasite's life cycle. If this evolution continues, it may ultimately result in complete resistance, compromising the efficacy of artemisinin-based treatments.

To mitigate the risk of artemisinin resistance, it is essential to use a potent partner drug in artemisinin-based combination therapies (ACTs). Furthermore, careful evaluation of the ACT partner drug's efficacy is necessary to minimize the likelihood of treatment failure [49, 50]. Resistance to ACT partner drugs has been detected in the Greater Mekong Subregion (GMS) [51]. Effective management of drug resistance necessitates continuous monitoring of the efficacy of both artemisinins and their partner drugs, alongside a thorough investigation of the underlying resistance mechanisms. Mutations in the K13 gene, along with alterations in other associated genes, have been identified as key contributors to the emergence of artemisinin resistance in Plasmodium parasites (**Figure 11**).

The PfK13 mutation decreases the endocytosis of host hemoglobin, which lowers hemoglobin catabolism levels and inhibits the activation of artemisinin medications. Furthermore, the K13 mutation improves DNA repair processes and reduces protein damage, increasing resistance to artemisinin. Other mutations, including Pfcoronin, AP-2μ, and UBP1, may further inhibit artemisinin activation, thereby reducing its parasiticidal effect.

8. Therapeutic properties of artemisinin and its derivatives

Ongoing research has led to the widespread clinical adoption of artemisinin compounds, extending their use beyond malaria treatment. These compounds have shown promising potential in treating other parasitic diseases, as well as in combating tumors, inflammation, and various other medical conditions [64–67].

8.1 Anti-schistosomiasis effect

Artemisinin and its derivatives can deplete cellular iron and selectively target iron-dependent cells [68, 69]. Artemisinin compounds have been shown to effectively kill both malaria ring-stage parasites and schistosomula, independent of hemozoin formation. This suggests that both *Plasmodium* parasites and schistosomes rely on iron for survival. Additionally, studies indicate that parasitized erythrocytes contain higher iron levels than uninfected red blood cells, further supporting the role of iron in the action of artemisinins [70]. Furthermore, iron levels increase as the malaria parasite progresses from the early ring stage to the late schizont stage [70]. Artemisinin and its derivatives exhibit anti-schistosomiasis effects by inducing oxidative stress within the schistosome parasite, leading to cellular damage and death. This effect primarily occurs through interactions with iron within the parasite's body. Clinical studies have shown that ACTs can specifically reduce transmission and contribute to the elimination of schistosomiasis [71]. Additionally, their use in treating both urinary schistosomiasis [72] and intestinal schistosomiasis [73] has been found to be safe and effective.

8.2 Anti-toxoplasma gondii effect

Toxoplasma gondii can be effectively killed by nanomolar concentrations of artemisone in *in vitro* models. It is a sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) orthologue, TgSERCA, which shares similarities with PfATP6 in Plasmodium and has been shown to be susceptible to inhibition by artemisinin when expressed in yeast [74]. Additionally, artemisinins interfere with calcium metabolism in parasites, disrupting their invasion mechanisms. The extent of these effects may vary depending on whether the parasites are residing within host cells or exist as free-living organisms [75].

8.3 Activity against pathogenic picomplexan parasites

Babesia species are tick-borne intra-erythrocytic parasites capable of infecting humans and various domestic animals, depending on the specific parasite species. Unlike *Plasmodium* infections, *Babesia* does not form a parasitophorous vacuole and does not digest hemoglobin to produce hemozoin [76]. These studies further confirm that neither hemozoin nor hemoglobin is essential for the antiparasitic activity of artemisinins. This raises interest in evaluating the SERCA hypothesis as a potential mechanism of action of artemisinins in these related pathogenic parasites.

8.4 Antitumour properties of artemisinins

Artemisinins, particularly artesunate, have exhibited potent activity against various tumor cell lines, including those associated with colon, breast, and lung cancer, as well as leukemia and pancreatic cancer [77, 78]. Artemisinins inhibit human umbilical vein endothelial cells' (HUVECs') migration, proliferation, and tube formation. Furthermore, they suppress the production of VEGF receptors and prevent vascular endothelial growth factor (VEGF) from attaching to surface receptors on HUVECs. KDR/flk-1 and Flt-1 [79, 80]. In cancer cells, artemisinins downregulate the VEGF receptor KDR/flk-1 in both tumor and endothelial cells, leading to the inhibition of angiogenesis and tumor progression. This effect has been demonstrated in studies where artemisinins slowed the growth of human ovarian cancer HO-8910 xenografts in nude mice [80, 81].

8.5 Cardioprotective effect of artemisinins

Preclinical studies have shown that artemisinin and its derivatives hold promising potential for treating various diseases, including cardiovascular diseases (CVDs) [80]. *In vitro* studies, conducted in a controlled environment outside the physiological system, help elucidate the precise molecular mechanisms by which these compounds act on target cells. Artemisinin and its derivatives have demonstrated the ability to attenuate CVD progression by targeting specific cellular components.

9. Conclusions

Adequate knowledge and further research on the biological activities of *Artemisia annua*, particularly its key constituents such as artemisinin and other crucial metabolites, will provide deeper insights into their therapeutic applications and potential

medical benefits. Alongside improved diagnostics and effective vector control measures, artemisinin-based combination therapies (ACTs) have the potential to significantly reduce the burden of malaria in tropical regions. However, to maximize their impact, it is crucial to ensure greater affordability and accessibility. Despite its widespread use, the precise antimalarial mechanism of artemisinin and its derivatives remains unclear, creating challenges for drug development, clinical applications, and malaria control efforts. Further research is crucial to fully understand how artemisinin exerts its effects against malaria. Notably, artemisinin has shown superior antimalarial efficacy compared to many other plant-derived secondary metabolites, highlighting its unique pharmacological properties. Furthermore, extensive research has revealed the potential of artemisinin and its derivatives in managing metabolic disorders and obesity-related diseases. Beyond their well-established antimalarial effects, these compounds exhibit diverse biological activities, including anti-proliferative, anti-angiogenic, antifungal, anti-helminthic, anti-protozoal, anti-tumor, and antibacterial properties. Their broad therapeutic potential continues to drive interest in their application across various medical fields. Key derivatives of artemisinin, including dihydroartemisinin (DHA), artesunate, artemisone, artemisinin, artemiside, artemether, and arteether, have all been identified in *Artemisia annua*. Future research should prioritize the development of novel pharmaceutical formulations incorporating targeted transport systems. These advancements could enhance the anticancer efficacy of artemisinin and its derivatives, opening new avenues for therapeutic applications and treatment strategies across various medical fields.

Acknowledgements

SP wants to thank MT for the concept of the content. Both the authors are grateful to IntechOpen and Dr. Yash Gupta for giving us the opportunity.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

HMD-CoA 3	hydroxy-3-methylglutaryl-coenzyme A
G3P	glycerol-3-phosphate
DXP	1-deoxy-D-xylulose 5-phosphate
MEP	2C-methyl-D-erythritol 4-phosphate
CDP-ME	4-diphosphocytidyl-2C-methyl D-erythritol
CDP-MEP	CDP-ME 2-phosphate
MEC-PP	2C-methyl-D-erythritol 2,4-cyclodiphosphate
HMB-PP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
IPP	isopentenyl pyrophosphate
DMAPP	dimethylallyl pyrophosphate
atoB	(ERG10) acetoacetyl-CoA thiolase
HMGS	(ERG13) HMG-CoA synthase
HMGR	HMG-CoA reductase

MK	(EGR12) mevalonate kinase
PMK	(ERG8) phosphomevalonate kinase
MVD1	(ERG19) mevalonate pyrophosphate decarboxylase
dxs	DXP synthase
dxr	DXP reductase
ispD	CDP-ME synthase
ispE	CDP-ME kinase
ispF	MEC-PP synthase
ispG	HMB-PP synthase
ispH	HMB-PP reductase
IDI	IPP isomerase
FPS (ispA)	farnesyl pyrophosphate (FPP) synthase
SQS	(ERG9) squalene synthase
ADS	amorpha-4,11-diene synthase
CYP71AV1	amorpha-4,11-diene 12-monooxygenase
CPR	cytochrome P450 reductase
ADH1	alcohol dehydrogenase 1
ALDH1	aldehyde dehydrogenase 1
DBR2	artemisinic aldehyde Δ11(13) reductase
RED1	dihydroartemisinic aldehyde reductase 1
ART	artemisinin
Hb	hemoglobin
Pfcoronin	*Plasmodium falciparum* actin-binding protein coronin
AP-2μ	adaptor protein 2μ
UBP1	upstream binding protein 1

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Chapter 4

Pondering *Plasmodium*: Revealing the Parasites Driving Human Malaria and Their Core Biology in Context of Antimalarial Medications

Ankur Kumar, Priyanka Singh, Ganesh Kumar Verma, Avinash Bairwa, Priyanka Naithani, Jitender Gairolla, Ashish Kothari, Kriti Mohan and Balram Ji Omar

Abstract

Malaria is one of the most severe infectious diseases, imposing significant clinical and financial burdens, particularly in underdeveloped regions, and hindering socio-economic development. The disease is caused by unicellular protozoan parasites of the genus *Plasmodium*, which infect not only humans but also various animals, including birds, mammals, and reptiles. Among over 200 recognized *Plasmodium* species, five—*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*—pose serious risks to human health. The first four are specific to humans, while *P. knowlesi*, primarily found in macaque monkeys, is responsible for zoonotic malaria in Southeast Asia. Malaria transmission relies on an intermediate insect vector, typically *Anopheles* mosquitoes, which act as both carriers and final hosts, facilitating the sexual reproduction of the parasite. This dependence on anopheline mosquitoes underscores the complex ecological dynamics influencing malaria epidemiology. *Plasmodium* species exhibit significant genetic plasticity, enabling rapid adaptation to external pressures such as changes in host specificity and the evolution of treatment resistance. This chapter explores the biology of human-infecting *Plasmodium* species and the significant threats they pose to humanity, highlighting their complex interactions with hosts and vectors.

Keywords: antimalarial, asymptomatic carrier, drug resistance, host specificity, host switch, malaria, mosquito, *Plasmodium*, recurrence, zoonosis

1. Introduction

The history of malaria extends from its prehistoric origin as a The history of malaria spans from its origins in primates in Africa to its impact on humans from the 1800s through the twenty-first century; malaria has remained a serious health threat, permanently altering the lives of people living in endemic areas [1]. This persistent scourge is caused by parasitic protozoa of the species *Plasmodium*. Malaria has exerted constant

pressure on human populations in impacted areas, shaping their evolutionary trajectory, and allowing for the formation and selection of unique genetic adaptations. Notably, there is some protection against malaria from genetic illnesses, such as sickle-cell disease and thalassemia, which are common in areas where malaria is endemic. Similar to this, communities in Central and West Africa have a high incidence of Duffy-negative blood type [2], which confers a particular resistance against infection by the *Plasmodium* parasite *P. vivax* [3, 4]. According to estimates, this genetic feature first appeared about 42,000 years ago [5]. The prevalence of *Plasmodium falciparum* malaria has decreased over time, while *Plasmodium vivax* malaria has increased in certain regions, particularly in sub-Saharan Africa [6]. The complex interactions between malaria's unrelenting drive and human genetics are highlighted by such evolutionary dynamics. Even with the availability of efficient antimalarials and measures, such as insecticide-treated bed nets (ITNs), malaria still plagues many parts of the world, especially developing nations where *P. falciparum* is the greatest concern. Financial limitations have impeded the ongoing fight against malaria, making it more difficult to execute comprehensive control programs. Acknowledging the gravity of the issue, the United Nations made the eradication of malaria a central goal in the 2000 Millennium Development Goals. Global investments in the fight against malaria increased over time, and by 2009, they had surpassed $2 billion. This increased financing has resulted in notable improvement, as evidenced by the notable declines in malaria-related mortality that have been observed globally, mainly in Africa [7, 8]. With numerous countries reaching the milestone of successive years with zero indigenous cases and receiving certification from the WHO as malaria-free zones, the number of countries reporting fewer than 1000 indigenous malaria cases has significantly increased as a result of the coordinated efforts [8–10]. The *Plasmodium* species that cause human malaria have similar life cycles, are susceptible to some antimalarial medications, and depend on particular insect vectors for transmission. Primarily, *P. vivax* malaria has distinct difficulties since there are episodes of relapse following the therapy; nevertheless, this can be successfully addressed by using medications, such as primaquine [11–13]. Even though zoonotic malaria cases are uncommon, the fact that *P. knowlesi* has become a major human infection highlights the possible danger that comes from *Plasmodium* species that inherently infect nonhuman primates [14–16]. Concerns have been expressed about *P. knowlesi* instances found in humans in Malaysia, especially in areas where malaria from other human-infective species has all but disappeared [17–19]. Similar to this, it has been determined that certain *Plasmodium* species, including *P. cynomolgi* and *P. inui*, that are carried by nonhuman primates may be susceptible to zoonotic transmission [20–22]. This emphasizes the importance of ongoing surveillance and investigation into newly developing infectious illnesses. The complexity of malaria epidemiology and the need for an all-encompassing approach to disease surveillance [23–25] and control is further highlighted by zoonotic malaria cases that have been documented in South America and are caused by organisms that are closely related to *P. vivax* and *P. malariae* [26–30].

2. Malaria and *Plasmodiu*m biology

2.1 Life cycle of Plasmodium

Every *Plasmodium* species has a similar life cycle that consists of two key stages: the transfer of the parasite from a vertebrate host to an insect vector [31]. Anopheles mosquitoes are the primary vector of transmission for humans among the five species that

infect them. On the other hand, species of *Plasmodium* that infect birds and reptiles spread their infection through different genera of mosquitoes or other insects that feed on blood [32–34]. The life cycle begins when sporozoites—produced by the insect vector—enter the vertebrate host's circulation after a mosquito bite, as shown in **Figure 1** [35, 36]. The life cycle of Plasmodium falciparum begins when sporozoites from the salivary gland of a mosquito enter the human host's circulation after a mosquito bite. The sporozoites mature in the liver and then infect hepatocytes, where they undergo asexual multiplication in a process called exo-erythrocytic schizogony [37–39]. This stage is crucial in the parasite's life cycle, as it allows for the continuous infection and replication of the parasite within the host. The resulting merozoites are liberated and proceed to invade red blood cells, initiating a pivotal stage in the parasite's life cycle known as erythrocytic schizogony. During the intraerythrocytic phase, spanning from 24 to 72 hours, contingent upon the specific species, merozoites proliferate within red blood cells, perpetuating the cycle of infection by invading fresh erythrocytes [40, 41]. The onset of sexual reproduction occurs as particular merozoites differentiate into gametocytes, initiating the next phase of the parasite's life cycle [42, 43].

Different species differentiate their gametocytes differently. For example, *P. falciparum* needs multiple intraerythrocytic cycles to begin gametocytogenesis, but *P. vivax* makes gametocytes continually [44, 45]. Gametocytes in the midgut of mosquitoes undergo differentiation into macrogametes and microgametes after ingesting contaminated blood [46, 47]. After these gametes fuse, a zygote is created, which goes through meiosis to become an ookinete, a motile form. After penetrating the mosquito's midgut wall, the ookinete emerges as an oocyst on the exterior [48–50]. Sporogony takes place inside the oocyst, producing a large number of sporozoites. As the oocyst rips open during maturity, sporozoites are released into the hemolymph of the mosquito and go to the salivary glands [51–53]. The transmission cycle is completed when infected mosquitoes inject sporozoites into vertebrate hosts during successive blood meals [54, 55]. Apart from the nucleus, two unique organelles found in *Plasmodium* cells are the mitochondrion and the apicoplast, each of which has its own genetic material [56–58].

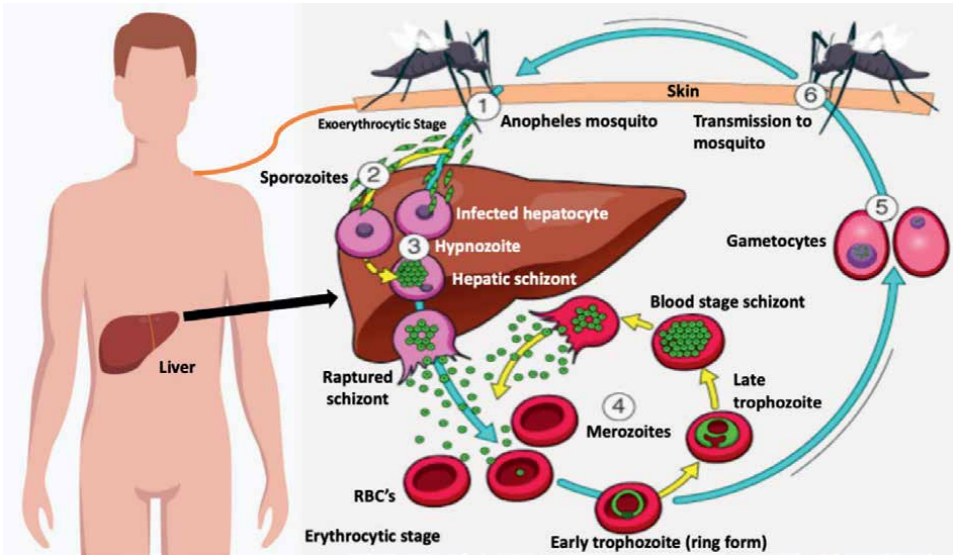

Figure 1.
Life cycle of Plasmodium.

Research shows that mitochondrial DNA from the female gamete is inherited uniparentally, emphasizing the role of the macrogamete in the transmission of organelles [59, 60]. These discoveries broaden our knowledge of *Plasmodium* biology by illuminating important facets of the parasite's life cycle and inheritance mechanisms [61, 62].

2.2 Recurrence of malaria and the hypnozoite

Vivax malaria is a chronic hazard because it can return, frequently presenting as recrudescence or relapse, even after parasites appear to have been removed from the patient's system [63]. A little pool of parasites that avoid detection and carry on multiplying in the host's bloodstream is the source of recrudescence [64, 65]. On the other hand, relapse is brought on by dormant hypnozoites, which are cryptic cells found in the liver. Interestingly, hypnozoites never originate from circulating merozoites in the bloodstream; instead, they only ever arise from sporozoites. *P. vivax* is unique among human malaria parasites in the fact that it may generate hypnozoites, which can cause relapses even after antimalarial medication, such as quinine or chloroquine [66, 67]. Interestingly, research indicates that the genotype of parasites responsible for relapses could be different from those during the original acute episode. This could be because the host is infected by several sporozoite genotypes or because extrinsic cues, such as concurrent infections, activate particular hypnozoites [67, 68]. Although *P. ovale* has long been suspected of developing hypnozoites, a lack of sufficient clinical and experimental evidence has led to a recent review that casts doubt on this idea. On the other hand, neither *P. falciparum* nor *P. malariae* are linked to the creation of hypnozoites, although they can linger in the bloodstream of the host for long stretches of time, sometimes even without exhibiting any symptoms [69, 70]. Cases of chronic infections that linger for years highlight how host immunity and parasite development must coexist in a delicate equilibrium. The fact that certain *P. vivax* recurrences may originate from non-hypnozoite sources, despite the fact that hypnozoites are well-established in *P. vivax* malaria, highlights the complexity of malaria recurrence mechanisms. Interestingly, studies of *P. vivax* recurrence in neurosyphilis patients getting blood from malaria patients point to non-hypnozoite alternate routes for recurrence, that adds to our understanding of the mysterious recurrence patterns of malaria [71, 72].

2.3 Gametocytes

The haploid genome of *Plasmodium* parasites is preserved during their growth in vertebrate hosts. Surprisingly, a cloned lineage of *Plasmodium* that started from a single cell can produce both male and female gametocytes, indicating that chromosomal elements are probably not the only variables influencing gametocyte sex determination [73, 74]. The details of this mechanism are still being investigated, but mounting data suggests that some environmental factors may facilitate the spread of parasites. Asexual intraerythrocytic development is the primary target of artemisinin and chloroquine, two antimalarial medications; however, gametocytes of *Plasmodium* species that infect people are resistant to both drugs [75–77]. As a result, gametocytes continue to exist in the circulation and may spread malaria to other individuals, even after asexual parasites are eliminated as a result of therapy with these antimalarials [77, 78]. According to research, *P. falciparum* gametocytes can survive for weeks after asexual blood-stage parasites are eliminated, and some cells can survive for up to 2 months. The existence of asymptomatic infections, host immunity, and treatment techniques all have an impact on the clearance rates and infectivity reduction of

gametocytes [79–81]. In order to stop the parasite from spreading further, it is crucial to treat both symptomatic malaria patients and asymptomatic carriers with gametocytocidal medication [82, 83]. This is because people who harbor asymptomatic infections may carry significant quantities of gametocytes.

2.4 Asymptomatic carriers

Due to acquired immunity, a sizable fraction of the populace in malaria-endemic areas carries *Plasmodium* parasites but does not exhibit any symptoms [84, 85]. Frequently, more sensitivity procedures, such as molecular detection by PCR and LAMP or ultrasensitive versions of RDTs, are required for correct diagnosis because traditional diagnostic methods, such as microscopy or rapid detection tests (RDTs), fail to identify parasites in these asymptomatic carriers [86–88]. Even though asymptomatic carriers have the potential to spread infectious gametocytes to mosquitoes, with low gametocyte levels, their influence on local malaria transmission is usually negligible. But as their immunity weakens, as when they move to malaria-free areas where immunity is not maintained, their propensity to spread the disease increases [89, 90]. As a result, carriers who do not exhibit symptoms may unintentionally aid in the spread of malaria by means of organ or blood transplants [91, 92]. In the globalized world of today, where economic growth and transportation have led to a greater movement, managing asymptomatic carriers becomes critical. In addition, the increase in the number of refugees from malaria-endemic areas plagued by conflict highlights how critical it is to identify and treat asymptomatic carriers [93–95]. Similar to symptomatic malaria patients, these carriers must be properly identified and cared for in order to stop the illness from spreading or re-emerging in areas that are malaria-free. Thus, to maintain malaria control efforts and prevent future outbreaks in susceptible groups, comprehensive interventions aimed at asymptomatic carriers are crucial [95].

2.5 Apicoplast and plant-like metabolism

Within the superphylum Alveolata, the genus Plasmodium is related to creatures, such as dinoflagellates and ciliates [96]. It is a member of the varied group of protozoans known as apicomplexa. Almost all apicomplexans, including all species of Plasmodium, are obligatory parasites that include an apicoplast—a non-photosynthetic and vestigial plastid—inside their cells [97–99]. Encased in four layers of membrane, this secondary plastid has the smallest known plastid genome, a minute genome [100, 101]. Remarkably, recent findings have revealed remarkable apicomplexan species known as chromerids, which have photosynthetic plastids that allow for phototrophic growth without the assistance of other organisms [102–104]. The apicoplasts of parasitic apicomplexans, including *Plasmodium*, are not photosynthetic in contrast to their photosynthetic counterparts. Most of the gene products encoded in the organellar genome are linked to transcription or translation [105–107]. When genetic information became available, the presence of a non-photosynthetic plastid in parasitic apicomplexans—which had previously been perplexing—became more evident [108, 109]. It became clear that the *Plasmodium* apicoplast is involved in key metabolic processes that are similar to those in plants, including as the manufacture of haem, type II fatty acids, and isoprenoid compounds [109–111]. The apicoplast-derived pathways are essential for *Plasmodium* survival and are especially important at specific phases of development, such as in mosquitoes and the liver [112–114]. Interestingly, though, research has revealed that *Plasmodium* may thrive in culture

even in the absence of the apicoplast if it is given enough of the essential precursor chemical isopentenyl pyrophosphate (IPP). The only source of IPP in *Plasmodium* is the plant-like methylerythritol phosphate (MEP) pathway found in the apicoplast, which is vital to the parasite's survival [115–117]. Targeting the apicoplast metabolism has therapeutic potential, as demonstrated by the notable antimalarial effect of the medication fosmidomycin against *P. falciparum*, which targets the enzyme of the MEP pathway [118–120]. In contrast, certain apicomplexan species—such as *Gregarina* and *Cryptosporidium*—completely lack the apicoplast, while others—such as *P. falciparum* and *T. gondii*—cannot survive without it [121–123]. Species without the organelles usually lack the genes encoding the enzymes needed in plant-like metabolism within the apicoplast [124, 125]. Species of cryptosporidium that lack an apicoplast have a large number of putative amino acid transporters, indicating a different method of obtaining necessary metabolites from the host [126, 127]. Consequently, the complicated relationship between host-parasite interactions and apicoplast-dependent metabolism highlights the essential function of this organelle in the survival and pathophysiology of Plasmodium and other apicomplexan parasites [128–130].

2.6 Antimalarial drugs and resistance

Traditional medicine has been using a variety of plant compounds to treat malaria since ancient times. The South American quina-quina tree's bark, which contains the antimalarial drug quinine, was found and used to cure malaria in the seventeenth century [131]. The 1930s witnessed the development of therapeutically effective synthetic antimalarials, such as chloroquine, as a result of centuries-long efforts to synthesize pure chemicals with antimalarial characteristics [132]. A natural substance that has long been utilized in China; artemisinin was discovered to have strong antimalarial properties in 1972. Currently used in clinical settings, antimalarial medications fall into five structural classes, each with unique modes of action. These classes include endoperoxides (like derivatives of artemisinin), 4-aminoquinolines (like chloroquine), aryl-amino alcohols (like quinine, mefloquine), naphthoquinones (like atovaquone), antifolates (like pyrimethamine, proguanil, sulfadoxine), and 8-aminoquinolines (like primaquine, tafenoquine). These medications target a number of metabolic pathways that are essential to the parasite's survival, including mitochondrial oxidoreduction, pyrimidine biosynthesis, and hemoglobin detoxification. The parasites that cause human malaria, especially *P. falciparum*, have grown resistant to each class of medication over time, despite the fact that these treatments are effective [133]. Point mutations in target transporters or enzymes frequently cause this resistance, making the medications useless. Moreover, medication resistance may also be exacerbated by processes, such as gene amplification or modifications to regulatory networks [134, 135]. Furthermore, resistance strains' deficiencies in DNA mismatch repair enable a higher pace of genetic alterations, which may help parasites endure strong pharmacological pressure [133, 136, 137]. Small genetic alterations that lead to treatment resistance are also made easier by the high A + T content of the *Plasmodium* species' genome [138–140]. Thus, creating successful malaria prevention methods requires an understanding of the mechanisms underlying antimalarial drug resistance.

2.7 Host specificity

The natural host range of *Plasmodium* exhibits both narrow and large ranges, contingent upon the species. For example, although African apes and humans are closely

related phylogenetically, *P. falciparum*, which only infects humans, does not affect them [141]. On the other hand, *P. relictum* affects more than 100 bird species globally, belonging to different families and orders [142]. *Plasmodium* species are traditionally divided into discrete subgenera according to their morphology, vertebrate hosts, and vectors. Every subgenus of *Plasmodium* appears to be monophyletic, despite the genus itself appearing to be polyphyletic. The three subgenera into which most mammalian *Plasmodium* species fall are Laverania, *Plasmodium*, and Vinckeia; apes are infected by Laverania, monkeys by *Plasmodium*, and rodents by Vinckeia [143, 144]. Mammalian malaria parasites are mainly spread by anopheline mosquitoes, in contrast to avian *Plasmodium* species, which are spread by a wide variety of mosquitoes, such as Culex and Aedes. Some non-anopheline mosquitoes, however, are capable of supporting a little amount of parasite growth [145]. For example, *P. falciparum* gametocytes in non-anopheline mosquitoes may develop into ookinetes, but they die before oocysts form. The environment, feeding habits, and host choice of different Anopheles species vary, which affects the dynamics of *Plasmodium* species' transmission and host specificity [146]. The subgenus Laverania's *P. falciparum*, which is the most common human *Plasmodium* species, can infect chimpanzees by laboratory adaptation, but it primarily infects humans. Only infecting gorillas, *P. praefalciparum* is phylogenetically related to *P. falciparum*. Strong host specificity is exhibited by species in the subgenus Laverania, which is probably controlled by gene families, such as stevor and Rh5, which are involved in host-parasite interactions. However, other species of *Plasmodium* found in the subgenus *Plasmodium*, such as *P. vivax* and *P. malariae*, exhibit a higher capacity for host change [147, 148]. For instance, isolates similar to *P. vivax* from Gabonese mosquitoes grouped together with *P. simium*, indicating a tendency for host flipping. In a similar vein, *P. brasilianum*, another zoonotic species that infects humans as well as simian hosts, most likely originated from *P. malariae* [149, 150]. Despite these findings, nothing is known about the mechanisms that allow *Plasmodium* subgenus hosts to swap hosts.

3. Conclusion

Malaria, which is brought on by *Plasmodium*, has plagued people throughout history. Malaria is now curable due to the development of both synthetic and natural antimalarial medications [151, 152]. When combined with insecticide-based mosquito control strategies, the use of synthetic antimalarials, such as artemisinin and chloroquine, has dramatically decreased the worldwide malaria load in comparison to earlier periods. Nevertheless, hundreds of thousands of people die from malaria every year. Drug-resistant parasite development and transmission is a major obstacle to controlling malaria [153, 154]. To tackle this problem, scientists are looking for new antimalarial substances that have different targets from those of current medications. By combining these novel inhibitors with existing antimalarials, the likelihood of parasite resistance can be reduced. Inhibitors directed toward the parasite's metabolic pathways, such as plants, for example, exhibit potential. The ease with which *Plasmodium* can spread from endemic to non-endemic areas as a result of greater human movement is another barrier [155–157]. Although areas free of malaria are growing, imported malaria still poses a serious hazard to public health in many nations. Asymptomatic carriers may inadvertently start malaria epidemics in areas where the disease is not common by contracting the disease spontaneously or through organ and blood transplantation. *Plasmodium* species that infect humans have evolved to share certain traits that allow them to do so.

Even while the number of human malaria species in existence is currently restricted, more *Plasmodium* species may spontaneously become human infectious, which could result in fresh outbreaks of zoonotic malaria [158, 159]. Depending on the proximity between humans and nonhuman primates—which is determined by local development—the probability of interspecies transmission may differ. Different mosquito hosts can also be adapted to by *Plasmodium* species. Avian malaria parasites can grow in non-anopheline mosquitoes, in contrast to mammalian *Plasmodium* [160, 161]. This raises the possibility that mammalian *Plasmodium* may develop immunity against non-anopheline mosquitoes and use them as vectors. Some kinds of non-anopheline mosquitoes are able to survive in a variety of settings, including cities, and if they spread malaria, they might pose a threat to the entire world [162, 163]. Effective malaria management measures require a thorough understanding of parasite biology due to the constant interplay between humans and *Plasmodium*. The realization of a healthier global society can be aided by the use of this information.

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Section 3

Malaria Vaccine Development and Immunological Insights

Chapter 5

Malaria Vaccine Development and Associated Challenges

Selorme Adukpo

Abstract

Malaria is a deadly disease that can be managed with an efficacious vaccine. However, developing anti-malarial vaccines has been a complex and challenging process due to certain inherent characteristics of the *Plasmodium* parasite, the complexity of the parasite biology, and the disease. These notwithstanding, anti-malarial vaccine development efforts have been ongoing for several decades, with various innovative approaches that target multiple stages of the parasite's life cycle, either in the secondary human or the primary mosquito host being explored. These efforts have so far led to the development of RTS,S/AS01, a subunit vaccine based on the circumsporozoite protein of the parasite through the GlaxoSmithKline and PATH Malaria Vaccine Initiative partnership. Following extensive clinical trials with RTS,S/AS01, the results revealed only moderate efficacy in reducing malaria episodes in young children and infants in Africa. This, notwithstanding, the World Health Organization approved RTS,S/AS01 as the first malaria vaccine followed by R21, developed by the University of Oxford, and marketed as R21/Matrix-M. Both vaccines work to prevent infection and are, therefore, being viewed as complementary tools to existing malaria control measures. Beyond these two, several other vaccine candidates are in various stages of development for improved efficacy, durability, and ease of administration.

Keywords: vaccine, malaria, plasmodium, antibodies, T cells, immunity

1. Introduction

Immune response to malaria involves both innate and adaptive immune responses which are mediated by cells and molecules of innate and adaptive immune systems, respectively. There is a crosstalk between them. Development of immunity to malaria is, however, a complex and varied process involving the host immune system as well as the *Plasmodium* species which are the causative agent of malaria. The anti-malarial immunity is, therefore, acquired over time through repeated exposure to the parasite with the level of immunity varying among individuals and populations. There are two main types of immunity associated with malaria: innate immunity and acquired immunity.

1.1 Innate immunity

The first line of defense against malaria parasites like any other infectious agents involves physical and chemical barriers that prevent entry of the malaria parasite

into the body. These barriers include the skin and mucous membranes. However, once these anatomical barriers are bridged through the inoculation of the parasite into the human body by the female Anopheles mosquito *via* its proboscis and saliva, innate immune cells, such as macrophages, neutrophils, and natural killer cells, play a crucial role in recognizing and eliminating the malaria parasite as their pattern recognition receptors (PRRs) interact with the pathogen-associated molecular patterns (PAMPs) of the parasite. In addition to this, there are several pieces of evidence from epidemiological studies that haemoglobinopathies, Southeast Asian ovalcytosis, hemoglobin C, hemoglobin S, glucose-6-phosphate dehydrogenase deficiency, and thalassemia offer a varying degree of resistance to malaria, especially the severe form of the disease that kills through limiting the growth, maturation, and multiplication of the parasite [1–4].

1.2 Acquired immunity

Acquired immunity to malaria, defined as anti-disease immunity, offering protection against clinical disease, which affects the risk and degree of morbidity associated with a given parasite density; anti-parasite immunity, conferring protection against parasitaemia, which influences the density of parasites; and premunition, protecting against new infections by maintaining a low-grade parasitaemia that exists in equilibrium with immune response, is mediated by lymphocytes and their products [5]. It thus develops after repeated exposure to the parasite during a period in which the immune system learns to recognize and respond more effectively to specific antigens associated with the parasite.

Except for the liver stage that resides in the hepatocytes and merozoite stage, the parasite is directly inaccessible to CD8+ T cells producing interferon-γ that are mainly involved in killing intrahepatic parasites since the parasite resides in erythrocytes which lack major histocompatibility complex (MHC) I as the matured erythrocytes are not nucleated and possess neither MHC I nor MHC II molecules. Other cells like natural killer cells [6, 7] and gamma delta T cells (γδT) also kill intrahepatic parasites through secretion of type I interferons and IFN-γ. Thus, the T cells also kill intrahepatic parasites through the secretion of type I interferons and IFN-γ. Thus, the T-cell or T lymphocytes recognize and destroy hepatocytes infected with the malaria parasite, contributing to the clearance of the infection at the liver stage.

During the blood stage, the immune system becomes exposed to not only the free merozoites but also the infected erythrocytes as the merozoites invade and develop in the erythrocytes. Immune response at this stage, therefore, involves both cellular and antibody or humoral immune responses, respectively, to control free merozoites and the intraerythrocytic stage. CD4+ T cells play a crucial role in coordinating the immune response while B cells produce antibodies that target the Plasmodium parasites and its associated toxins to protect us from the disease [5]. The immune protection is believed to be mediated through:

1.2.1 Blocking of invasion

Antibodies bind to a specific protein on the sporozoites, and merozoites and interfere with the invasion and subsequent multiplication of the parasite within hepatocytes or erythrocytes. For instance, antibodies to CSP, AMA1, MSP1, 3, EBA175, *P. falciparum* reticulocyte-binding protein homologs (PfRhs), and erythrocyte-binding like proteins prevent infection to appreciable levels [8–10]. The PfRhs

include PfRh1, PfRh2a, PfRh2b, PfRh3, PfRh4, and Rh5. Antibodies to Rh5 or C-terminal region of PfRipr block the parasites from entering RBCs multiplication of the parasite in the erythrocytes to perpetuate infection and hence disease [9, 10].

1.2.2 Opsonization

Antibodies can opsonize or coat and mark the parasites at any stage of the parasite development for destruction by immune cells. Once antibodies bind to the surface of the parasite, they mark it for recognition by immune cells such as phagocytes or natural killer cells, which then phagocytose and destroy them.

1.2.3 Complement system activation

Activation of the complement system through the classical pathway to either trigger the complement cascade leading to the formation of pores in the merozoites and free sporozoites and subsequent destruction by membrane attack complex or through enhancement of phagocytosis as C3b generated from complement molecule C3 cleavage is an excellent opsonin, interacts with the phagocytes [11] *via* its receptors, including complement receptor 1 (CR1) or CD35 which are expressed on the phagocytes.

1.2.4 Antibody-dependent cellular inhibition (ADCI)

This mechanism involves antibodies binding to VSA on the infected erythrocytes to form an immune complex. The complex then interacts with immune cells such as monocytes, macrophages, and neutrophils to kill the parasites preventing further multiplication and spread of the parasite.

1.2.5 Antibody-dependent respiratory burst (ADRB)

Antibodies can induce a respiratory burst in immune cells, such as neutrophils or macrophages, leading to the release of a large amount of reactive oxygen species that are toxic to the parasite.

1.2.6 Antibody-mediated agglutination

Through the formation of immune complexes with infected erythrocytes, cells can clump together in a process known as agglutination. This clumping can hinder the movement of infected erythrocytes and make them more susceptible to immune attacks. Neoantigens at the surface of infected hepatocytes may also be recognized and bound by these antibodies, and in cooperation with phagocytes, Kupffer cells and natural killer cells kill through an antibody-dependent cell-mediated mechanism.

1.2.7 Neutralization of parasite and parasite-associated toxin

Antibodies may bind to malaria toxins like glycosylphosphatidylinositol (GPI), a potent inflammation mediator, to neutralize it and prevent its excessive inflammatory action and hence fever or at the earliest stage of infection, bind to sporozoite to impede its motility [12]. These antibodies may bind to free sporozoites and CSP

protein to block its invasion of a hepatocyte or to neutralize proteins required for cell traversal and invasion of hepatocytes.

1.3 Immune evasion by Plasmodium falciparum

Due to immune pressure, malaria parasites have developed several strategies to evade the human immune system, allowing them to persist in the host and cause disease in some cases. Some of these immune evasion mechanisms include:

Sporozoites: Free sporozoites and intrahepatic parasites must overcome the host's immune response to enter the erythrocytic stage. Sporozoites actively pass through Kupffer and endothelial cells; some sporozoites cross the gaps between an endothelial cell and Kupffer cells. It is however puzzling how sporozoites safely pass through Kupffer cells that kill other microorganisms except that evidence from experimental malaria suggests that the parasite not only traverses the barriers but also suppresses the killing function of these cells among others by inhibiting respiratory burst activity in the Kupffer cells [13].

Intraerythrocytic immune evasion: The success of evasion depends on merozoites and infected erythrocytes surface proteins. Mostly, immune evasion by intraerythrocytic parasites is the result of antigenic diversity and sequestration [14]. Development and survival in erythrocytes help the parasite escape by avoiding direct interaction with immune cells, including CD8+ T cells as erythrocytes lack MHC-I or MHC-II molecules necessary for antigen presentation to CD8+ and CD4+ cells, respectively.

Expression of variable antigenic surface proteins from multicopy gene families and polymorphic alleles on infected erythrocytes helps parasites evade host immune recognition and attack [14]. The *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) is one of the most highly polymorphic proteins, encoded by approximately 60 copies of var. genes, with only one of them being expressed at a time. It has different variable domains that interact with and promote adherence to endothelial cells [14]. For example, VAR2CSA encoded by a member of the var. gene family mediates the cytoadherence of infected erythrocyte to placenta syncytiotrophoblasts only [14]. The other variant antigens, such as repetitive interspersed family (RIFIN) and telomeric variable open reading frame (STEVOR) proteins, are coded for by their respective sets of multigene [14].

The second immune evasion mechanism at this stage is sequestration, mediated by parasite adhesins, *Pf*EMP1, STEVOR, and RIFIN proteins encoded by multigene families and exported to the surface of infected RBC to form variant surface antigens. This allows antigens or adhesins, especially PfEMP-1 on the infected erythrocytes to bind to non-immune IgM to create rosettes [15] that may facilitate sequestration to prevent splenic clearance. Furthermore, these adhesins allow infected erythrocytes to stick to vascular endothelium that excludes the infected cells hence the mature trophozoite and schizonts stages of the parasites from circulation and protect them from clearance by the spleen [15]. However, the parasite re-enter circulation again, first as erythrocytic stage merozoites with its own sets of antigens, some of which may be different from those of the other stages to infect other erythrocytes before transforming into trophozoites and then mature trophozoite/schizont with the capacity to adhere to the vascular endothelium again when the adhesins are expressed and placed on the infected erythrocytes. Endothelial receptors such as the endothelial cell protein C receptor, chondroitin sulfate A (CSA), cluster of differentiation (CD) 36, and intracellular adhesion molecules (ICAM) among others are also important for sequestration.

1.4 Suppression of immune response by the parasite

Malaria parasites suppress both innate and adaptive immune responses in the host as functions of the immune cells, such as phagocytes and T cells, are suppressed, thereby weakening the host's ability to contain infection effectively. The phagocytic functions of phagocytic cells, including macrophages and monocytes are also impeded by *P. falciparum* malaria pigment or haemozoin such that the repetition of phagocytic activity by monocytes/macrophages is drastically reduced after the ingestion of haemozoin, a byproduct of hemoglobin digestion by the parasite, leading to reduced production of oxygen radicals or its intermediates with plasmocidial effects. *Plasmodium falciparum* infection also upregulates such immune response inhibitory molecules as Programmed Cell Death Protein 1 (PD-1), T cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domains (TIGIT), T-cell immunoglobulin and mucin domain 3 (TIM-3) or CD366 and lymphocyte activation gene 3 (LAG3) or CD223 [16] to dampen inflammation which affords the parasite escape route to perpetuate infection. Most *P. falciparum* parasite isolates use independent sialic acid (SA-) pathways, including complement receptor 1, and gain access to erythrocytes and thereby evade antibody immune response [17]. Polymorphic tandem repeats in antigens mask critical epitopes or divert immune response from possibly critical epitopes or targets to another, most likely to the biologically irrelevant antigens or epitopes to hinder the development of effective adaptive immune response to such immune-relevant antigens of the parasite [18].

1.4.1 Immune evasion by merozoites

Merozoites express several variable antigens on the surface that help them evade the immune response. These merozoite surface proteins (MSPs), *P. falciparum* apical membrane antigen 1 (AMA1), *P. falciparum* erythrocyte binding antigen (EBA)-75, and *P. falciparum* reticulocyte-binding protein homologs (PfRHs) among others, are not only highly polymorphic but also show strong similarity with or homology to host proteins to obstruct effective and quicker detection [18], which offer an escape route from immune attack to perpetuate invasion of new RBC. Expression of RIFINs, STEVORs, and SURFINs is important for evasion [14]. Free merozoites bind to complement factor H (FH) and FH-like protein 1 (FHL-1), which are both regulatory proteins of the complement system to inactivate C3b and to abrogate the formation of membrane attack complex and lysis of the parasite or detection by phagocytes and subsequent phagocytosis.

1.4.2 Escape mechanisms of gametocytes

Gametocytes express RIF, VAR, and STEVOR proteins that provide an immune evasion mechanism akin to the asexual blood stage [19]. Rather than blocking transmission to mosquitoes, some proteins like human complement factor H enhance or promote the infectiousness of the parasites to the mosquitoes through inhibition of complement-mediated destruction of the parasites in the mosquito [20].

These evasion mechanisms contribute to the establishment of chronic infections and cause recurrent disease. In general, antigenic polymorphism is a difficult hurdle for the development of effective *P. falciparum* malaria vaccines, and understanding these mechanisms is important for the development of effective vaccines and treatments against malaria.

2. History of malaria vaccine development

Development of the malaria vaccine has been a complex and challenging process due to some inherent characteristics of the Plasmodium parasite, the complexity of the parasite biology, and the nature of the disease. The search for the elusive effective malaria vaccine has, however, undergone marked advancement punctuated with numerous milestones over some decades.

Tracing it back, the earliest efforts to develop a vaccine for malaria were started in the 1940s–1960s when attempts were made to use weakened or dead whole parasites or crude extracts of the parasite antigens for vaccine development, a process which was faced with safety and efficacy issues besides being a sluggish one. Additional challenges were posed by the complexity of the life cycle of the parasite, the asexual stage in the intermediate human host, and the sexual stage, possibly associated with genetic recombination in the definitive host, the female anopheline mosquito (**Figure 1**). Considering the parasite genome is made up of about 5400 coding genes, genetic recombination is possible which most likely will lead to the development of several traits to confer a survival advantage on the parasites that emerge later in the definitive host [21].

Malaria infection starts when an infected female Anopheles mosquito bites a person, injecting the sporozoites form of the Plasmodium parasite into the bloodstream. 2. The sporozoites quickly travel to the human liver. 3. The sporozoites multiply asexually in the liver cells over the next 7–10 days, a stage that is associated with no clinical symptoms. 4. In an animal model, the parasites, in the form of merozoites, are released from the liver cells in vesicles, journey through the heart, and arrive in the lungs, where they settle within lung capillaries. The vesicles eventually disintegrate to release merozoites into the bloodstream that invade erythrocytes to initiate the blood stage of infection. 5. Upon invading erythrocytes, each merozoite transforms into ring stage, trophozoite, and then schizont to give rise to more merozoites which are then released into the bloodstream when the erythrocytes burst to initiate another round of this asexual reproduction. It takes *P. knowlesi* and *P. malariae* approximately 24 and 72 hours, respectively to complete this cycle, while *P. falciparum, P. vivax,* and *P. ovale* take 48 hours to do the same. 6. Following an indeterminate number of multiplications, some of the infected red blood cell parasites leave the cycle of asexual multiplication cell stage due to physiological stress and unknown factors. and develop into sexual forms of the parasite, called gametocytes, that circulate in the bloodstream. 7. When a mosquito bites an infected human, it ingests the gametocytes, which develop further into mature female and male sex cells called macrogamete and microgamete, respectively, or collectively called gametes. 8. Through the process of exflagellation, the male gametes emerge from the erythrocyte to fertilize the female gametes, which then develop into actively moving ookinetes that burrow through the mosquito's midgut wall and form oocysts. 9. Inside the oocyst, thousands of active sporozoites develop. The oocyst eventually bursts to release sporozoites that travel to the salivary gland of the mosquito ready to be released alongside the saliva of the mosquito. 10. The cycle of human infection begins again when the mosquito bites another person [21].

Furthermore, it was also soon realized that the parasite displays several life cycle stage-specific antigens with the 1970s–1980s seeing the identification of antigenic circumsporozoite protein (CSP), a sporozoite stage-specific antigen. The CSP antigen was first cloned in 1987 which raised the hope of scientists for more targeted and antigen-specific approaches to malaria vaccine development. In 1987, the CSP vaccine went into clinical trials, revealing only a limited amount of protection. A significant breakthrough was however made in the 1990s when Glaxo Smith Kline (GSK) in

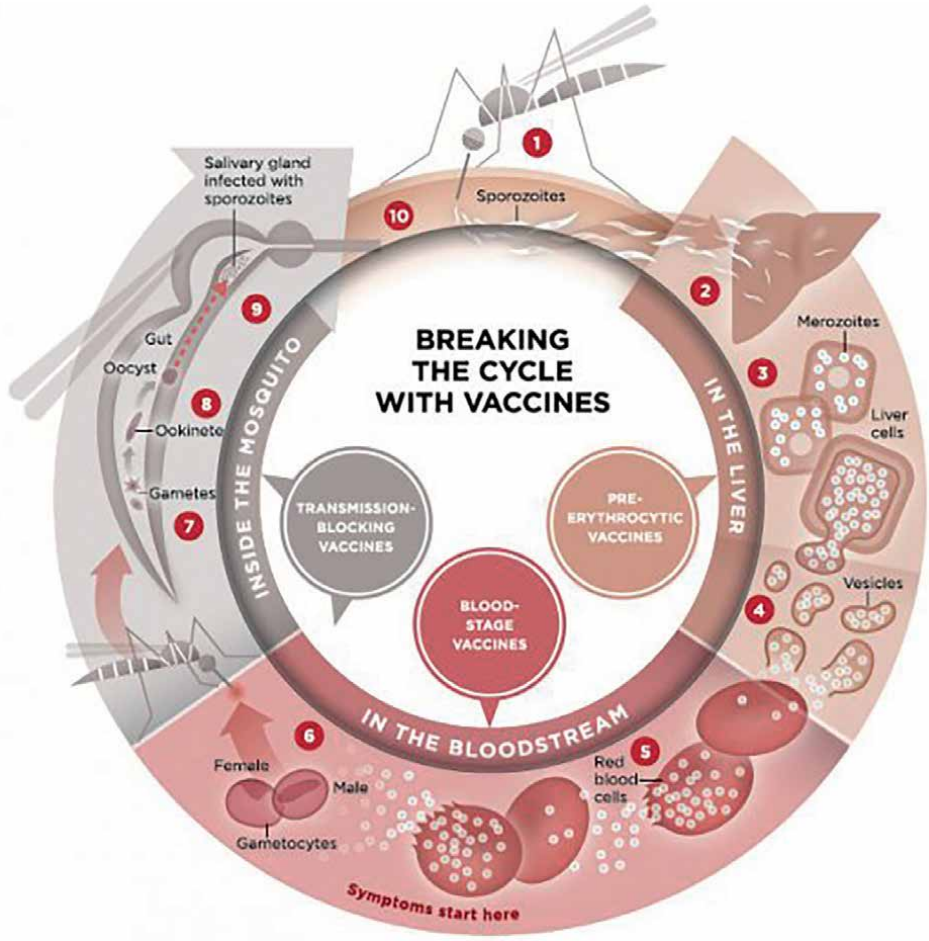

Figure 1.
The life cycle of the malaria parasite. This was courtesy of the PATH malaria vaccine initiative, and the details can be found at http://www.malariavaccine.org/malaria-and-vaccines/vaccine-development/life-cycle-malaria-parasite (accessed on 22 March 2024). The image was adapted from the malaria vaccine initiative website to show the various vaccine target stages.

collaboration with the Walter Reed Army Institute of Research, USA developed RTS, S. The RTS, S, marketed as Mosquirix, is a subunit vaccine based on the central repeat and C-terminal epitopes of CSP protein [22, 23]. The vaccine went into clinical trial first in 1996 followed by several phase II and III clinical trials with revelation of provision of partial protection against malaria, especially in children between 5 and 17 months of age. However, as the first malaria vaccine to show efficacy in a large-scale clinical trial, the European Medicines Agency issued a positive scientific opinion for it in 2015, thereby making it the first malaria vaccine to receive regulatory approval. Despite this, the World Health Organization (WHO) issued approval for its large-scale use in children in October 2021 after piloting its implementation in Ghana, Kenya, and Malawi in 2019–2020 to further evaluate its effectiveness and feasibility in real-world settings, malaria-endemic region. Other antigens, including whole parasite vaccines and alternative antigen targets, are also undergoing development for improved efficacy and broader protection to be considered as a malaria vaccine shortly [24].

3. Phases of vaccine and malaria vaccine clinical trials

A vaccine trial is a clinical trial that establishes the safety and efficacy of a vaccine before it is licensed for use. The candidate vaccine antigen is first identified through preclinical evaluations that could involve screening and selecting the proper antigen to elicit an immune response. Depending upon the time needed for study participants to react to the vaccine and develop the appropriate antibodies, some clinical trials last for months or even a year. Thus, the vaccine goes through phases 1–IV of clinical trials as explained below to assess its suitability for use.

Phase I is a small-scale trial conducted in adults to establish the safety of antigens and to determine the dosages of the vaccine and what immune response it elicits. This phase is normally divided into Phase Ia and 1b during which the vaccine is tested in healthy adults, and a relevant and more appropriate target population like in the malaria endemic region for malaria vaccine, respectively. This small group is then scaled up in Phase II to a larger group to generate data on the safety and efficacy of the vaccine in a target group for whom the vaccine is meant which may be infants, children, or adults. Phase III study finds out whether the vaccine induces immunity to a level capable of preventing disease and provides evidence under the natural condition of the disease that the vaccine can reduce disease cases in a particular population. This is done in a larger group involving hundreds of trial participants and in some cases at different study sites to assess statistically significant safety and efficacy data for the Summary of Product Characteristics. If the vaccine shows safety and efficacy over a defined period, the manufacturer could apply to the relevant regulatory authorities for product licensure. Following the licensure, data is collected in Phase IV across a wide range of populations that are using the vaccine to detect rare adverse effects and to evaluate any long-term efficacy.

4. Development of a vaccine for malaria

A couple of platforms or approaches exist that are being explored to develop vaccines including the malaria vaccine. One of the platforms, the protein subunit vaccine approach, involves the use of specific immunogenic proteins or antigens from the malaria parasite that are critical for the survival of the parasite or its invasion of human cells, RTS, S/AS01, expressed as a virus-like particle and administered with an adjuvant (AS01), which is used to enhance the immune response to vaccination for instance was developed with this approach.

Viral vector vaccines: Viral vectors, such as adenoviruses or poxviruses, carry genes that encode malaria antigens. When administered, these antigen gene-carrying vectors, including adenovirus type 5 (AdHu5) and adeno-associated virus serotype 1 (AAV1) are to infect cells and express the malaria immunogens to trigger an immune response. The ChAd63-MVA ME-TRAP vaccine is an example of a viral vector vaccine for malaria [25].

Live Attenuated Vaccines: These vaccines use weakened forms of the malaria parasite that are unable to cause disease but can still stimulate an immune response. This approach aims to mimic natural infection without causing illness. However, safety concerns and the need for careful attenuation make this approach challenging.

Whole Parasite Vaccines: Whole parasite vaccines use either live, attenuated malaria parasites or killed parasites, irradiated sporozoites, or chemically attenuated malaria parasites whereby people are treated just before they develop clinical malaria following inoculation with the viable parasites, to induce an immune response.

These vaccines aim to mimic the natural infection process more closely than other approaches. However, challenges include ensuring safety and scalability.

Nucleic Acid Vaccines: Nucleic acid vaccines, such as DNA vaccines or RNA vaccines, involve delivering genes encoding malaria antigens directly into cells. The cells then produce the antigens, stimulating an immune response. This approach offers potential advantages in terms of ease of production and stability.

Based upon the life cycle of the parasite, three main stages of the parasite are being targeted for the development of malaria vaccine [26] as depicted in **Figure 2**.

A morphologically distinct form of the parasite at various stages; sporozoite, merozoite, gametocyte forms of parasite life cycle stages, and the vaccine strategy for targeting each stage. Label A shows merozoites in the schizont stage while B shows merozoites that have just attached to the erythrocyte vaccines that target multiple life cycle stages are also in development.

4.1 Pre-erythrocytic

Developing an effective malaria vaccine has been challenging due to the complexity of the Plasmodium life cycle and the ability of the parasite to evade the human immune system as the parasite passes through distinct stages in both humans and mosquitoes, making it crucial to target various stages to achieve effective immunity.

The life-threatening stage of malaria infection is the asexual stage in the bloodstream and an effective pre-erythrocytic stage vaccine may arrest the parasite development before the infection is truly established. *Plasmodium falciparum* (Pf) sporozoites (SPZ) in PfSPZ vaccine, targeting the pre-erythrocytic stage of the parasite, involves the use of live but weakened sporozoites, the infectious stage of the parasite that is normally inoculated into the human host through infective female anopheles mosquito bites of the malaria parasite to elicit an immune response. The PfSPZ vaccines, therefore, expose individuals to live and/or weakened sporozoites, including radiation-attenuated sporozoites, for their immune system to recognize and mount a response.

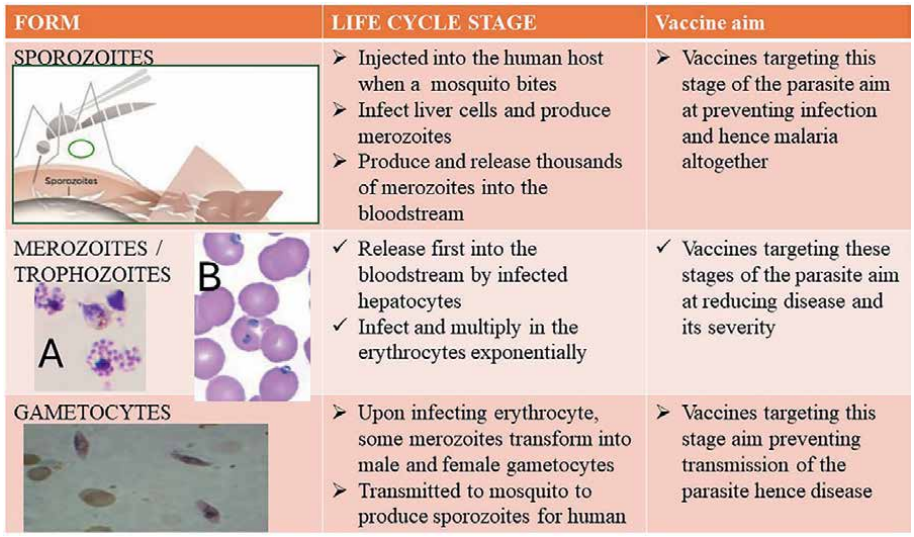

Figure 2.
Vaccine strategies against different life cycle stages of malaria parasites.

This includes activation of B lymphocytes to produce PfSPZ-specific antibodies and the activation of other immune cells [27]. To achieve this, several of them are in clinical trials which mostly include controlled human malaria infection or challenge studies, radiation *via* mosquitoes, intradermal administration, or intravenous administration to assess the protective efficacy of the vaccine. In a first clinical trial with *P. falciparum* radiation-attenuated sporozoites in humans, immunization through mosquito bites was utilized, which resulted in suboptimal immune responses and protection in comparison to the intravenous route that elicited strong *P. falciparum* specific CD8+ T cell response. Overall, the immunization reduced the risk of malaria infection in malaria-endemic regions. The robustness of the response, therefore, seems to depend on the route of vaccine administration. Sadly, however, there are reported breakthrough infections in some cases due to inconsistency or incomplete attenuation of the sporozoites. In similar studies, however, participants or volunteers are deliberately exposed to the malaria parasites using sporozoite from *P. falciparum* strain NF54, followed by treatment as soon as the parasites are detected in the bloodstream of study participants, but before the manifestation of overt clinical symptoms [27, 28].

It is important to note that the parasites express several antigens, including several liver stage ones, which in most cases are too large to be used as a vaccine. So, the development of peptide-based vaccines, through recombinant DNA technology targeting immune-relevant sections of the parasite antigen, is also being attempted. Liver stage antigens such as liver stage antigen 1 (LSA1), cell-traversal protein for ookinetes and sporozoites (CelTOS), and recombinant protein of *P. falciparum* circumsporozoite protein (CSP) regions known to induce humoral (R region or central repeat region of the *P. falciparum* CS protein) and cellular (T region or T-cell epitope of the CSP antigen) immune responses, linked covalently to the hepatitis B virus surface antigen (S), RTS, S, and R21 vaccine, which is a protein antigen from the CSP among others, are therefore being explored to prevent the establishment of infection in the liver [29].

RTS, S elicits both humoral and cellular immune responses as manifested in significant levels of antibodies and CD4 T cell responses in vaccines. Frequencies of CSP- and HBsAg-specific CD4+ T cells producing such inflammatory cytokines as IL-2, TNF-α, and CD40L and HBsAg-specific CD4+ T producing IFN-γ and IL-17 were observed in comparison to baseline and the control group. During a clinical trial, RTS, S/AS01 vaccine-induced T cells of higher functional heterogeneity and polyfunctionality with detectable central and effector memory [30]. It averted many cases of clinical and severe malaria cases over the one and half years after vaccine dose 3, with the most profound impact of efficacy observed in areas with the highest malaria incidence, and mostly among children than in infants [31]. However, its effectiveness decreases with time as RTS, S is about 56% effective over a year and 36% effective over four years suggesting the need for revaccination [31]. R21 is also a subunit vaccine like RTS, S that incorporates a higher proportion of *P. falciparum* CSP C-terminus bound to HBsAg N-terminus display of more epitopes on the vaccine particle surface than RTS, S for enhanced B cell activation and stronger anti-CSP humoral immune responses [32]. The R21 vaccine developed by Oxford University with an increased level of efficacy compared to RTS, S/AS01 in their current form, and marketed under the trade name R21/Matrix-M™, is the second malaria vaccine recommended by WHO for the prevention of the disease in children and together, the two are expected to be implemented widely to offer a high public health impact [33, 34]. CelTOS is a 25-kDa protein whose sequence is highly conserved among the various *Plasmodium* species. It is vital for the ookinete traversal in the mosquito midgut, as well as for the sporozoite infection of liver cells in the human host to elicit a protective immune response capable of preventing

clinical infection in the human as well as preventing infectivity of the parasite to mosquitos. Despite its potency demonstrated so far, the CelTOS vaccine seems to need more refinement before being considered a potential malaria vaccine [32, 35].

4.2 Erythrocytic stage

The blood stage of the *Plasmodium* parasite life cycle is one of the critical malaria vaccine targets. During this stage, the parasites establish infection in the erythrocytes, and the stage is associated with such pathological symptoms as fever, chills, fatigue, and anemia. During this stage, the host is exposed to both free merozoites and infected erythrocytes with the parasite expressing antigenic proteins, which facilitate invasion of growth and survival within erythrocytes. Some of these antigens have been focal points for the development of malaria vaccine: Merozoite surface proteins (MSPs) are proteins present on the surface of the merozoite, the form of the parasite with the ability to invade erythrocytes. MSPs mediate the attachment of the merozoite stage of the parasites to the erythrocytes and subsequent invasion. MSP1, MSP2, and MSP3 are among the most studied MSPs as vaccine candidates, with MSP3 being a standalone vaccine candidate as well as being fused with glutamine-rich protein (GLURP), another antigen of the parasite that is expressed at both the merozoite and the sporozoite stages, to form the GMZ2 malaria vaccine candidate [36]. GMZ2, which is based on the conserved region of the MSP3 and GLURP, has been clinically trialed in malaria-endemic regions where individuals most at risk of natural exposure to the parasite are. The clinical trials have recorded variable efficacy, with some reporting modest protection against malaria infection and disease which altogether call for a need to develop it further by possibly combining it with other antigens or adjuvants to improve its efficacy and induction of immune responses.

Apical membrane antigen 1 (AMA1) is another protein present on the surface of the merozoite that is critically important for the invasion of erythrocytes by the parasite. AMA1-based vaccines have been extensively studied in preclinical and clinical trials. Erythrocyte-binding antigens (EBAs) are a family of proteins that interact with receptors on the surface of erythrocytes, facilitating parasite invasion. Examples of these proteins attracting immense attention for vaccine development include EBA175 and EBA140. Other antigens important for red blood cell invasion and under consideration for vaccine development include *P. falciparum* reticulocyte-binding protein homologs (PfRh) proteins. Among these, PfRh2a and PfRh5 are being explored with Rh5 showing a lot of promise in preclinical studies [37].

The erythrocytic stage vaccine development also calls for a specific vaccine for pregnancy-associated malaria (PAM). This has been faced with several challenges, including sequence polymorphism, the number of variants of VAR2CSA that need to be used for optimal vaccine efficacy, and appropriate surrogate of protection to assess when evaluating the efficacy of the vaccine. Generally, the VAR2CSA is structurally conserved between *P. falciparum* isolates and is mostly over-expressed by placental isolates [38, 39]. However, multiple alleles of VAR2CSA exist in the field [40] that pose the challenge of allele-specific immunity. PRIMVAC and PAMVAC, based on the CSA-binding region of VAR2CSA from the 3D7 and FCR3 strains respectively, which are currently the leading PAM vaccine candidates have been assessed in Phase I clinical trials in Europe and Africa after extensive preclinical assessment. Analysis of the results showed the limited ability of the antibodies produced against VAR2CSA that originated from heterologous parasite strains to highlight the difficulty of producing a PAM vaccine with the highly polymorphic protein when designing a vaccine for PAM [41–43].

4.3 Transmission blocking vaccine

Except for transmission through blood transfusion, malaria infection is restricted to only those who are currently infected unless the sexual stages of the parasite, the micro- and macro gametocytes, get to the mosquito to re-enter the same person or another one *via* infective female anopheles mosquito bite as sporozoites. This stage therefore has attracted the attention of researchers to develop a vaccine that blocks transmission of the parasite to the arthropod finite host and thereby breaks the transmission of the parasite in a population. This also stems from the fact that atovaquone, atovaquone/proguanil combination, and several other drugs reduce mosquito infectivity when ingested during blood meals, hence stopping malaria transmission through inhibition of ookinete formation and oocyst maturation [44, 45] is possible if blood factors like antibodies able to interfere with fertilization in the mosquito or proper development of the parasite in the mosquito. Thus, the malaria transmission-blocking vaccine (TBV) is a critical component in the global effort to control and finally eliminate malaria as it aims at the induction of immune response in humans that targets and impedes the proper development of the sexual stages of the parasite in the mosquito when ingested by arthropod host during a blood meal to break the transmission cycle. The antibodies that are ingested alongside the gametocytes bind to the ookinetes and interfere with fertilization and subsequent oocyst formation (**Figure 1**). Recently, it was demonstrated that they were effective at inhibiting fertilization in mosquito midgut, resulting in a decrease in the density of oocysts hence the development of the parasite in the mosquito vector. Several protein molecules are targeted as TBV candidates including the six-cysteine motif (6-CM) family of proteins with Pfs48/45 and Pfs230. These candidates are the most studied ones [46] as experimental evidence indicates that these proteins are essential for fertilization of the macrogamete in the mosquito by the microgametes [47, 48]. Despite the effectiveness of antibodies to Pfs230 in inhibiting oocyst formation in mosquitoes studies have reported that liposome vaccine adjuvant mixed with Pfs230 fragments and administered could trigger a stronger blocking effect [45]. Despite all these, it faces a lot of challenges being developed into an effective vaccine. Pfs230 is a large protein, >300 kDa that contains 14 cysteine motif domains. It is produced *in vivo* only. as its large size and cysteine-rich nature hinder its production as an intact protein [49]. A smaller and more convenient molecule is Pfs48/45. Despite the relatively smaller molecular size of Pfs48/45 being appropriate, its correct and efficient production into recombinant vaccines is still posing a challenge. This is however being tackled with DNA plasmid encoding the antigen after codon optimization or endo H enzymatic deglycosylation in plants. Both approaches have significantly enhanced the immunogenicity of the recombinant Pfs48/45 and improved its transmission-blocking capability [50–52]. Additionally, Pfs230–Pfs48/45 chimera post a better transmission-blocking vaccine effect than a single protein [53, 54].

5. Challenges associated with the development of a vaccine

The journey of malaria vaccine development shows a persistent effort to combat a disease with a significant impact on global health. Despite the great strides that have culminated in the approval of RTS, S, the development of effective malaria vaccines is still a complex task, and researchers continue to adopt innovative approaches. It faces challenges such as financial considerations, as financial constraints in advancing the

science for the vaccine development, the transformation of *P. vivax* and *P. ovale* into the dormant stage, hypnozoite, in the liver, which is not tackled by the blood-stage vaccines, antigen polymorphism, antigenic variation, and immune evasion mechanisms employed by the parasite. Therefore, many vaccine candidates are targeting multiple blood-stage antigens or combine blood-stage antigens with antigens from other stages of the parasite's life cycle to enhance efficacy and broaden protection. Additionally, innovative vaccine platforms and delivery systems are being explored to improve the immunogenicity and efficacy of malaria vaccines that target blood-stage antigens.

Maintenance of cold chain until the sporozoite, be it genetically or radiation attenuated, vaccine is injected into the targeted population least its stability and/or viability is compromised in the impoverished malaria-endemic regions, which poses a challenge to this type of vaccine as one may not easily distinguish between a viable and non-viable sporozoite in a vial at the point of inoculation.

6. Way forward

Continued research is needed to identify more biologically relevant and conserved epitopes across strains and species to overcome antigenic variation and diversity (**Figure 3**). Like GMZ2 and RTS, S, there is a need to refine the formulation of the current vaccine candidates to enhance the efficacy and durability of the protection the vaccine offers. Studies may focus on understanding immune correlates of protection and identifying strategies to overcome vaccine limitations. RTS, S and the various potent malaria vaccines may be combined to form a cocktail or multivalent vaccine to maximize their potential. They may also have to be combined with other malaria control measures such as vector control and antimalarial drugs, as part of a comprehensive malaria control strategy. In conclusion, there is hope for an improved version of RTS, S and all other leading candidates as a malaria vaccine to combat the age-old enemy, malaria.

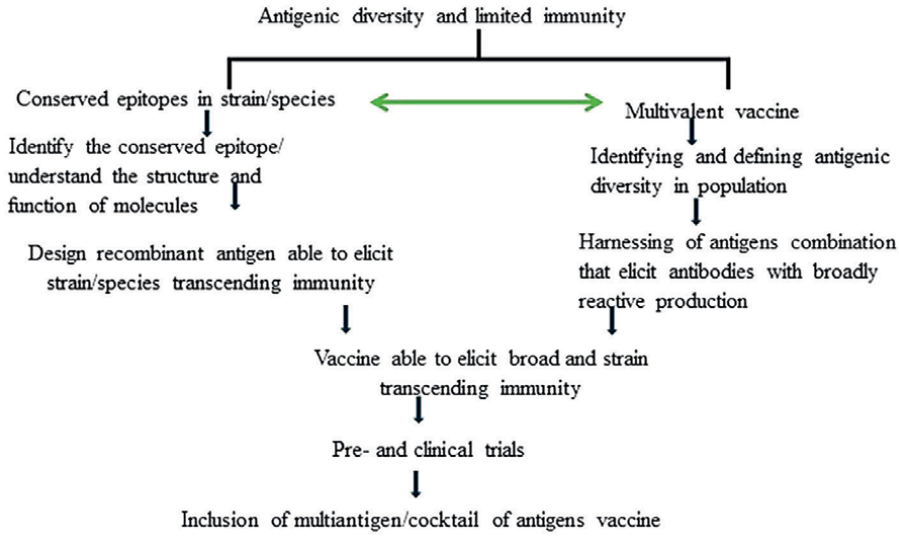

Figure 3.
Strategies for the development of a potent malaria vaccine. Flow chart showing steps to be taken in the effort to develop an efficacious and more potent vaccine to control malaria.

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Section 4

Genetic and Vector-Based Control Strategies

Chapter 6

Translational Usage of Genetically Modified Arthropods in Fighting Malaria and Other Tropical Diseases

Christian Ogaugwu, Sangeeta Singh, Peeyush Mittal, Hari Shankar, Yash Gupta and Raja Babu Singh Kushwah

Abstract

Myriads of tropical diseases plague the tropical areas of the world. Many of these tropical diseases are transmitted by the arthropods that are abundant in these areas. Control of these diseases has been challenging for several decades, but recent advances in genetic biotechnology have enabled the engineering and development of genetically modified arthropods for use in novel disease control strategies. This chapter focuses on the different arthropod-borne diseases found in different tropical parts of the world, the genetically modified arthropod strains that have been engineered and deployed for their management, and the challenges or limitations of these GM arthropods for tropical disease control.

Keywords: GMO, arthropods, disease control, tropical diseases, vectors

1. Introduction

Within the tropics lie a realm of unparalleled natural beauty, where lush ecosystems teem with life in dazzling arrays. Yet amidst this splendor lurks a persistent menace: tropical diseases. These afflictions, borne by a myriad of pathogens encompassing viruses, bacteria, and parasites, thrive within the warm, humid confines of tropical regions [1]. Regrettably, they disproportionately afflict nations with lower economic means, exacting a toll on public health, economic progress, and societal well-being. Although traditionally associated with tropical climates, these diseases are beginning to defy geographical confines. In an era marked by heightened mobility and global travels, the reach of tropical diseases has extended far beyond their traditional boundaries as they have found new footholds in regions as diverse as the United States, United Kingdom, and Europe, presenting a novel challenge for public health authorities.

The prevalence of tropical diseases in these regions not only affects human health but also hampers socioeconomic development. High disease burdens contribute to decreased productivity, increased healthcare expenditures, and loss of life,

Arthropod vectors and disease transmission	
Mosquitoes (*Anopheles spp.)*	Malaria (*Plasmodium spp*.), Lymphatic filariasis (*Wuchereria bancrofti, Brugia malayi, Brugia timori*)
Mosquitoes (*Culex spp.)*	Arbovirus encephalitis (Japanese B encephalitis, St Louis encephalitis, West Nile virus), Lymphatic filariasis (*Wuchereria bancrofti, Brugia malayi*)
Mosquitoes (*Aedes spp.)*	Yellow fever, dengue, chikungunya, Zika, Lymphatic filariasis (*Wuchereria bancrofti, Brugia malayi*),
Sandflies (*Phlebotomus spp., Lutzomyia spp.*)	Leishmaniasis (*Leishmania donovani*), sandfly fever (Pappataci 3-day fever, Toscana, Sicilian, and Naples virus infections), bartonellosis (*Bartonella bacciliformis*), Chandipura virus
Tsetse flies (*Glossina spp*.)	Sleeping sickness (*Trypanosoma brucei rhodesiense*, *Trypanosoma brucei gambiense*)
Black flies (*Simulium spp.*)	River blindness (Onchocerciasis) (*Onchocerca volvulus*)
Horse/deer flies (*Chrysops spp*.)	Filariasis (*Loa loa*), Tularemia (*Francisella tularensis*), Equine infectious anemia
Lice (*Pediculus spp., Pthirus spp.)*	Trench fever, Bacillary angiomatosis, Endocarditis (*Bartonella quintana*), Epidemic typhus (*Rickettsia prowazekii*), Louse-borne relapsing fever (*Borrelia recurrentis*), Vagabond's disease (*Pediculosis corporis*)
Fleas (*Ctenocephalides spp.*)	Plague (*Yersinia pestis*), Endemic/murine typhus (*Rickettsia typhi*), bartonellosis, cat scratch disease (*Bartonella henselae*), flea tapeworm (*Dipylidium caninum*)
Mites (*Neoschongastia americanum, Leptotrombidium sp.*)	Chiggers, Scrub typhus (*Orientia tsutsugamushi*) Mange—Scabies (*Sarcoptes scabiei*), Rickettsial pox (*Rickettsia akari*)
Ticks (*Ixodes spp., Amblyomma spp., Dermacentor spp., Rhipicephalus spp.)*	Lyme disease (*Borrelia spp.*), Rocky Mountain spotted fever (*Rickettsia rickettsii*), Ehrlichiosis (*Ehrlichia spp.*) (*Anaplasma phagocytophilum*), relapsing fever (*Borrelia spp.*), arboviruses (Crimean-Congo hemorrhagic fever, Omsk haemorrhagic fever, Powassan virus, Heartland virus, Bourbon virus, Tick-borne Encephalitis, Colorado Tick Fever), Babesiosis (*Babesia microti*)

Table 1.
Some tropical diseases and their arthropod vectors.

particularly among vulnerable populations such as children and the elderly. Effective disease control measures are essential to alleviate these burdens and promote sustainable development in tropical regions.

Vectors such as mosquitoes, fleas, lice, sandflies, and many other arthropods play a pivotal role in the transmission of tropical diseases. Traditional methods of controlling these diseases, while essential, face significant challenges. Overuse of chemical insecticides leads to widespread resistance in insect vector populations. Vaccines are available for some diseases, but they are not always effective. Additionally, access to clean water and sanitation, which are crucial for preventing diseases, remains inadequate in many tropical regions. These limitations highlight the need for innovative approaches to combating tropical diseases. **Table 1** provides an overview of some of the arthropod-borne tropical diseases:

2. Existing methods used to control vectors and arthropod populations

Controlling the spread of diseases by arthropods requires a multifaceted approach. Arthropod populations can be targeted through various methods, including manipulating their environment with traps and repellents, introducing natural enemies, or

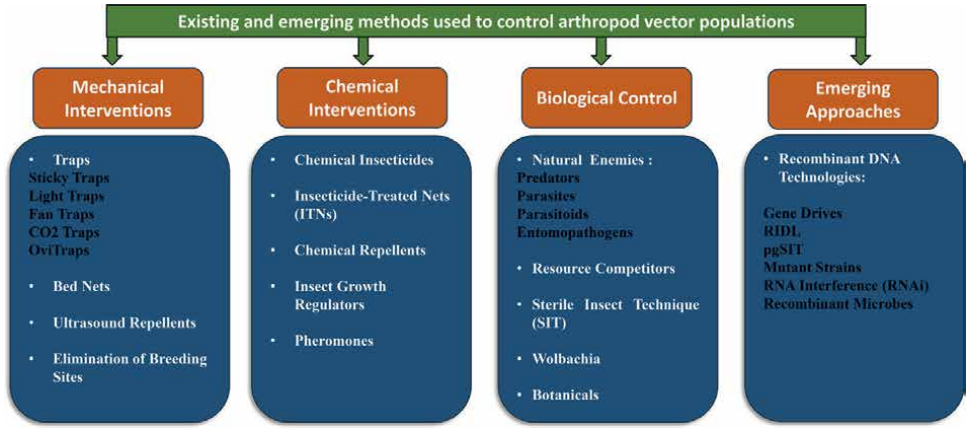

Figure 1.
Overview of existing and emerging methods for arthropod vector population control.

utilizing chemical interventions such as insecticides. This section explores the variety of old and existing approaches for managing arthropod populations, particularly those that transmit tropical diseases (**Figure 1**).

- Mechanical interventions: These methods manipulate the environment or utilize tools to directly affect arthropods.

  Sticky traps and light traps: Traps can be used to capture and kill insects, but require strategic placement [2].
  Barriers: Physical barriers such as bed nets can be used to stop insect disease vectors such as mosquitoes from having access to their host.
  Ultrasound repellents: These are devices that emit high-frequency sound waves, which are not audible to humans, and disrupt the communication and navigation of mosquitoes and flies [3].

- Chemical interventions: This approach relies on chemicals, primarily insecticides, to kill or repel arthropods directly.

  Insecticides: The common chemical method used in the form of spray on the wall and surface where vectors rest, known as Indoor Residual Spraying (IRS), is also used as a larvicide in breeding sites of vectors in the form of fog to target adult vectors. This method provides rapid solutions but raises concerns about the environmental impact and potential development of insecticide resistance [4, 5].
  Insecticide-treated nets (ITNs): Bed nets are impregnated with insecticides to kill or repel mosquitoes seeking to feed on humans, significantly reducing malaria transmission [6].
  Acaricides: These chemicals kill arachnids such as mites and ticks. They may sometimes include chemicals that also function as insecticides.
  Repellents: These are applied to the skin or clothing to deter insects from biting. They offer personal protection but require reapplication [7, 8].
  Insect growth regulators (IGRs): IGRs are chemical compounds that mimic the hormones that regulate insect growth by disrupting the normal development and reproduction of vector populations, which prevents the immature stage of the

vector, such as pupae and larvae, from developing into adults, by breaking the vector's lifecycle. For example, pyriproxyfen is used to control mosquito populations.
Pheromones: These are chemical signals released by insects that can be used for insect control by interfering with mating, attracting pests to traps, or enhancing insecticide effectiveness as synergists, thereby providing an alternative to traditional pest and vector control methods [8, 9].

- Biological control: This strategy uses biologically based approaches such as introducing natural enemies or manipulating the reproductive biology to suppress arthropod populations.

  Predators: Introduction of natural predators, such as fish, birds, and other arthropods, into the food chain to control the target vector population is a control method. For example, the *Gambusia* fish feeds on mosquito larvae in confined water bodies, demonstrating the potential of this approach [10]. Also, the mosquito species *Toxorhynchites splendens* feeds on eggs and larvae of other mosquito species and has potential for mosquito control [11–13].
  Parasites/Parasitoids: Parasites or parasitoids such as the mermithidae nematodes can be used against arthropods as they affect the growth or kill the host arthropods [14, 15].
  Competitors: Introducing non-disease-transmitting competitors that outcompete the target vectors for food and other resources, such as *Toxorhynchites* species that share similar habitat to vector mosquitoes [12].
  Entomopathogens: Entomopathogenic organisms such as *Bacillus thuringiensis*, *Beauveria bassiana*, *Microsporidia*, and *Metarhizium anisopliae* can be used to infect the insect population and kill them off. *Bacillus thuringiensis israelensis* (Bti), for example, is a soil-dwelling bacterium that is widely used to control vectors, particularly mosquitoes. Bti produces crystal proteins that are toxic to mosquito larvae. Bti became active in the alkaline environment of the larvae midgut; this disrupts the digestive system and leads to death [16].
  Wolbachia: Wolbachia bacteria are widely used as biological controls because they can infect mites, ticks, and mosquitoes and cause cytoplasmic incompatibility to prevent reproduction. Wolbachia can also reduce the ability of the vector to transmit certain human pathogens. This offers a self-propagating method of disease control, as mosquitoes carrying Wolbachia have a reproductive advantage over those without it, gradually increasing the proportion of the population with reduced disease transmission capability [17–20].
  Sterile insect technique (SIT): This genetic method involves mass release of sterilized males into the wild to disrupt reproduction in wild populations. Classical SIT uses radiation to render the male insects sterile, and these males compete with the wild males, thereby achieving infertile mating and reducing the target population.

3. Limitations of arthropod control methods

3.1 Mechanical and chemical methods

- *Limited Target Range*: These methods may not be effective against all insect species or life stages. For example, traps and repellents designed to attract adult

mosquitoes may not work on larvae, and some mosquito species, like *Aedes aegypti*, primarily feed during the day when light traps and nighttime repellents offer minimal protection [2, 21].

- *Behavioral Adaptations*: Insects can develop behavioral adaptations to avoid these methods. For instance, some mosquitoes have shifted their feeding times to avoid peak insecticide spraying periods.

- *Period of Usage*: The period when the method is applied may constrain its effectiveness. Insecticide treated bed net (ITN) may not be effective for day-biting mosquitoes like *Aedes aegypti* [2, 21].

- *Environmental Impact*: Overuse of chemical insecticides can harm nontarget organisms and pollute the environment. Additionally, improper disposal of used traps can contribute to plastic pollution.

- *Development of Resistance*: Continuous exposure to insecticides can lead to insect populations developing resistance, rendering the chemicals ineffective over time [4, 22].

3.2 Biological control

- *Habitat Specificity*: Introducing natural predators like fish is only effective in specific habitats with the resources and conditions necessary to sustain the predator population [10].

- *Limited Applicability*: The Sterile Insect Technique (SIT) has shown success against many agricultural pests, but achieving sufficient male sterility in some insect vectors, like mosquitoes, remains a challenge in some insect vectors like mosquitoes due to issues with achieving sufficient male sterility, competitiveness, and fitness concurrently [23–25].

- *Ethical Concerns*: The release of biological controls, especially Wolbachi and Bti, faces regulatory hurdles and public opposition regarding their long-term environmental impacts due to safety ethics and potential environmental risks.

- *Slow Impact*: Biological controls, such as predators, parasites, and pathogens, usually take time to establish and spread, which may create delays during outbreaks, unlike chemical pesticides that act fast.

- *Challenges in Predicting Interactions*: Despite Wolbachia showing effective results in vector control, its impact can vary greatly depending on the specific Wolbachia strain and mosquito species involved. For example, one study found that introducing Wolbachia (wAlbB strain) into *Culex tarsalis* mosquitoes surprisingly increased their susceptibility to West Nile Virus (WNV) [26]. This was correlated with the downregulation of the mosquito immune response. This highlights the potential risks associated with this specific Wolbachia strain in WNV control. In contrast, another study showed that a different Wolbachia strain increased WNV resistance, but only in a specific laboratory strain of *Culex quinquefasciatus* mosquitoes with a high Wolbachia density [18]. This effect was

not observed in other mosquito populations, suggesting the need for careful evaluation of Wolbachia-based interventions in each specific context to ensure effectiveness and avoid unintended consequences.

4. Emerging technologies for arthropod vector control

As the limitations of traditional methods become increasingly apparent, the development of new technologies for arthropod control is crucial for the control of these disease vector (**Figure 1**). Recombinant DNA technology has enabled the emergence of promising approaches for arthropod vector control (**Figure 2**). These

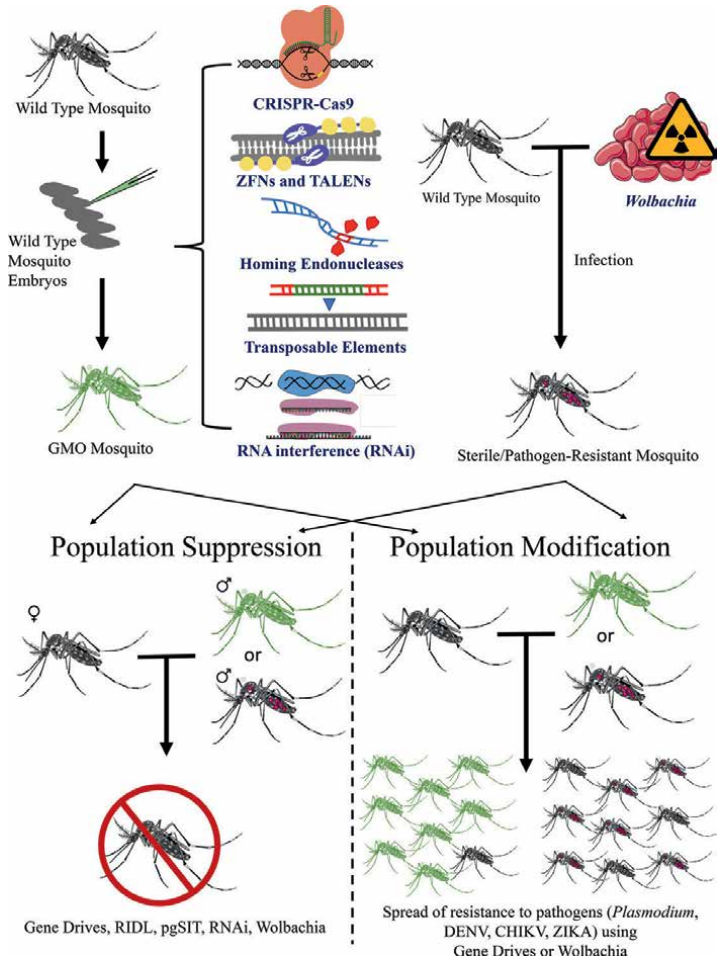

Figure 2.
Recombinant DNA technologies for vector control. The approaches for the control of mosquitoes (or other arthropod) vectors through population suppression and population modification using the genetic modification techniques including CRISPR-Cas9, Homing Endonucleases, Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), Transposable Elements, RNA interference, as well as Wolbachia bacterial infection. Wild-type mosquitoes are genetically engineered to produce individuals with desired traits such as sterility or resistance to pathogens such as CHIKV (Chikungunya virus), DENV (Dengue virus), and ZIKV (Zika virus). Also, wild mosquitoes can be infected with Wolbachia strains, resulting in cytoplasmic incompatibility-based sterility or pathogen-resistant phenotypes.

include the use of genetically engineered arthropod strains or microbes and through genetic interference.

Genetically engineered arthropod strains: Arthropod strains can be genetically engineered or altered to introduce desired traits or produce specific biological products. The modifications are achieved through various techniques such as the use of transposable elements, Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and homing endonucleases and CRISPR-Cas nucleases, with each offering unique advantages and applications. These have resulted in development of gene drive arthropods [27–29], other transgenic arthropod strains that could be used in Release of Insects carrying a Dominant Lethal (RIDL) [30] or precision-guided Sterile Insect Technique (pgSIT) [31], as well as mutant arthropod strains that exhibit desired phenotypes such as parasite suppression that are beneficial for disease control [32].

The use of genetically engineered arthropod strains has explored two main strategies: population suppression and population modification (**Figure 2**). Population suppression involves the release of genetically modified arthropods that disrupt the reproduction of wild populations through the use males that cannot produce viable offspring. This strategy aims to significantly reduce or even eliminate entire population. Population modification involves the introduction of genetically modified arthropods that may be less capable of transmitting diseases or resistant to infection, effectively reducing the disease. The goal of this strategy is to effectively manage arthropod-borne diseases while minimizing any unintended environmental consequences. Although generally still under development, advancements on the use of genetically engineered strains offer exciting possibilities for targeted and environmentally friendly arthropod control.

Genetically engineered microbes: Microorganisms such as bacteria and fungi can also be engineered using techniques similar to those used for arthropods. The engineered microbes express either desired products that are toxic to the target arthropod vector or molecules that act against the disease-causing parasites/pathogens transmitted by the arthropod vectors [33, 34].

Gene interference: This approach involves the interference or suppression of expression of genes that play important roles. RNA interference (RNAi) uses small RNA molecules, usually double-stranded RNA (dsRNA), that bind to the mRNA transcript to prevent the production of the protein coded for by a gene [35]. This technique offers a powerful tool for studying gene function as well as has been shown to have potentials for control of arthropod-borne diseases [36–40]. It is important to note that RNA interfering dsRNA can be engineered into an arthropod species to create a genetically modified RNAi strain, as well as can be transiently delivered *via* ingestion or prolonged skin contact to generate strains that are not considered as genetically modified.

5. Use of genetically modified arthropods in field vector control programs

Over the past two decades, many types of genetically modified strains have been developed and tested in the laboratory for the control of different arthropod vectors. Some of these have been successfully employed in the field for population control of the target species, while many are still in translation.

The first open-field release of a genetically engineered insect for disease control was performed by the British company Oxitec in Grand Cayman, the Cayman Islands

for the yellow fever mosquito *Aedes aegypti* [41]. This mosquito is responsible for the transmission of several tropical diseases (**Table 1**). The field control trial utilized the *Ae. aegypti* RIDL strain, OX513A, a self-limiting late-acting dominant lethal genetic system developed earlier on by Phuc and colleagues [30]. Successful suppression of the local *Ae. aegypti* population was achieved after the release of about 3.3 million of OX513A male individuals in a 23-week period, amounting to an overflooding ratio of about 5:1 GM to wild males [42]. This positive result validated the potential of genetically modified arthropods for disease vector control and encouraged further programs and implementations in other countries.

In Malaysia, field release of genetically engineered sterile males of *Ae. aegypti* mosquito (OX513A) was safely and successfully carried out [43]. The program was conducted in an uninhabited forest area lacking population of *Aedes aegypti* and originally designed to test survival and dispersal of different mosquito strains in a non-island setting, in this case a wild-type *Ae. aegypti* Malaysian strain and the GM OX513A strain. The released GM males had similar survival, but somewhat reduced dispersal compared to wild-type males, supporting the safety and potential effectiveness of genetic control methods for dengue vector management [43]. The results from this field program showed that GM mosquitoes are less likely to disperse to very far locations in open-field usage. In addition, it addressed concerns of inter-species crossing by showing that the *Ae. aegypti* strains did not cross with local *Aedes albopictus* present within the test area.

Sustained release of males of the GM OX513A *Ae. aegypti* strain was conducted for over a year in Juazeiro, Bahia, Brazil and led to about 95% suppression of the local *Ae. aegypti* mosquito population [44]. The results from this field control program highlighted the effectiveness of the self-limiting genetic approach for dengue vector control, with potential to prevent future epidemics and outbreaks in similar settings.

Similarly, field release and population suppression of *Ae. aegypti* was successfully achieved in West Panama. The genetically modified self-limiting *Ae. aegypti* (OX513A) was able to reduce the local population by up to 93% without affecting the abundance of the coexisting *Ae. albopictus* [45]. During the period of the program, no evidence of species replacement or environmental risks was found, further supporting the potential of this technology for effective and safe mosquito population control.

To investigate the possible outcomes during the post-release period of *Ae. aegypti* in a field control, two field pilot studies were conducted in Juazeiro and in Jacobina, Brazil. Both programs successfully suppressed the wild population by about 70% compared to the pre-release period and the wild populations began to recover 4–5 months after interruption of release, indicating that continuous or strategically reduced releases are necessary to maintain control and prevent re-establishment [46].

In addition to self-limiting RIDL strains, other types of genetically modified strains have also been applied in open field control of tropical disease vectors. For evaluation of the prospect of malaria control using genetically modified mosquitoes, a field trial was performed with the release of sterile GM *Anopheles coluzzii* males was in Bana, Burkina Faso. The trial that represents the first field release of a genetically modified strain in West Africa sought to determine potential fitness costs due to the transgene and utilized an *An. coluzzii* strain carrying a homing endonuclease (I-PpoI) that causes complete male sterility [47]. The trial found that GM *An. coluzzii* males were less fit than their wild counterparts, but offers valuable insights for improving future genetic mosquito control strategies.

More recently, genetically modified strains of the Indian malaria vector, *Anopheles stephensi*, were released in Ambouli, Djibouti by Oxitec, marking the first field release

of a GM in East Africa and the second field release on the African continent [48, 49]. Djibouti had been almost malaria free with fewer than 30 cases nationwide prior to 2013, but the invasion of the African continent by *Anopheles stephensi* exponentially increased malaria to about 73,000 cases with many deaths [49]. The new field application of a GM *An. stephensi* is yet to be assessed, but there is optimism from past field release programs in other countries, and it remains to be seen if the program will successfully suppress or eradicate malaria in Djibouti.

6. Challenges to using GM arthropods in field disease control programs

Despite the significant advancements in science medicine, the fight against malaria and other tropical diseases remains a constant battle. While vaccines and treatments have been developed for some diseases, controlling their spread often presents a complex challenge. The use of GM arthropod strains has shown huge potentials for curtailing the spread of tropical diseases. However, their field use faces several challenges and limitations, some of which have been revealed by a few of the field trials that have been conducted so far.

One of the major challenges observed came from the GM *Ae. aegypti* field program in Jacobina, Bahia, Brazil, where the unintended gene flow the transgenic OX513A *Ae. aegypti* strain and the local population was reported [50]. The original strain was developed using different strains form Cuban and Mexican populations. Genetic analyses of the Jacobina mosquito population about 27–30 months post-release of the sterile males showed that some parts of the genetically modified genome had incorporated into the local mosquito population, suggesting that a rare viable hybrid offspring had survived and reproduced [50]. While the new tri-hybrid will likely give more vigor to the population, this finding of an unintended gene flow underscores the need for deeper planning and genetic monitoring during such GM vector control efforts to track potential ecological and epidemiological consequences.

Besides challenges encountered by field trials, there are also concerns about potential challenges that future field GM programs could face. These include some of the following:

Off-target effects: One of the main concerns of the use of GM arthropods in open-field control is the possibility of modifying unintended sections of the genome of the target population. This can occur due to imprecise techniques or unexpected interactions within the organism's genome, potentially leading to unforeseen consequences [51]. For instance, if a gene editing technique aimed at a specific disease-related gene might accidentally modify a gene crucial for development, resulting in unintended defects or other issues like reduced fitness.

Unstable insertions: Another concern with the use of GM arthropods is the stability of the introduced genetic material. It might be possible for transposons or other elements around the genomic location of insertion to cause transgene instability. Unpredictable outcomes may result, with the genetic modification being lost in subsequent generations or even causing harm to the organism [51].

Unexpected biological interactions: Even if a genetic modification targets the intended gene precisely and integrates stably, unforeseen interactions with existing genetic pathways can occur. These interactions can disrupt the organism's delicate biological balance, leading to unintended consequences like reduced lifespan, fertility issues, or altered behavior [51]. Modifying a gene to disrupt a pathogen's life cycle, for

example, may inadvertently affect another gene involved in the arthropod's immune system, potentially leaving it more susceptible to different pathogens.

Gene transfer and species specificity: There are also concerns that genetic elements from a genetically modified species may transfer to another species. In 2015, Sharma et al. reported evidence of horizontal gene transfer from plants to the mosquito *Anopheles culicifacies* [52]. This has opened discussion on the possibility of transferring transgenes from released GM arthropods to other non-targeted species.

Unknown effects of environmental factors: The effectiveness of the use of GM arthropods in field disease control interventions can be significantly influenced by environmental factors. These factors can include changes in temperature, humidity, and even the presence of competing organisms or harmful incompatible substances in the ecosystem.

Unforeseen ecological consequences: The introduction of genetically modified organisms (GMOs) for disease control raises concerns about potential disruption to ecological balance. Unforeseen consequences like unintended harm to non-target organisms or disruption of food webs necessitate thorough environmental risk assessments before field deployment of GM arthropods [53].

Efforts have been made to develop self-eliminating transgene systems and anti-drives that could prevent the persistence of transgenes or block functionality of a GM system [54–57]. These strategies provide solutions that address concerns of potential unforeseen consequences.

7. Limitations of GM arthropod field use

Field programs usually require production and release of millions of males [41, 44]. A major limitation facing the use of GM arthropods in field control of disease vectors is the technological and financial difficulty of producing such large numbers of males for release [58]. In addition, an efficient and accurate method of sex separation will be needed to prevent accidental release of biting females as minor errors when producing large numbers of the GM strains could result in release of thousands of females. Sex-separation or sex distorting systems can be incorporated into future GM mosquito control programs to address or reduce this limitation [59, 60].

8. Conclusion

Genetically modified (GM) arthropods have emerged as a promising weapon in the fight against tropical diseases transmitted by insects. These modified organisms have the potential to significantly reduce disease burden and even pave the way for eradication in some cases. However, the use of GM arthropods is not without its peculiar challenges and limitations such as unintended gene flow, breakdown of transgenes, among others. Some of the challenges can be addressed through measures like careful design of future GM arthropod strains and better planning and genetic monitoring. Computational modeling and machine learning are crucial for designing, evaluating, and optimizing GM insect interventions. These tools could enable simulation of complex ecological interactions, prediction of epidemiological outcomes, and calibration of intervention strategies for specific geographical and environmental contexts. Self-eliminating transgenes, anti-drives, or other suitable safety approaches could be integrated into GM arthropod control systems for enhanced biosafety and

regulatory compliance. Taken together, this chapter underscores the transformative potential of GM arthropods in global health while also advocating for a cautious, data-driven, and ethically grounded approach to their implementation. As the field of arthropod vector control using GM strains advances, interdisciplinary collaboration and robust community engagement will be essential to ensure that the technologies fulfill their potential of reducing the burden of tropical diseases while safeguarding the environment for future generations.

Acknowledgements

The authors acknowledge the use of Grammarly and ChatGPT for language refinement.

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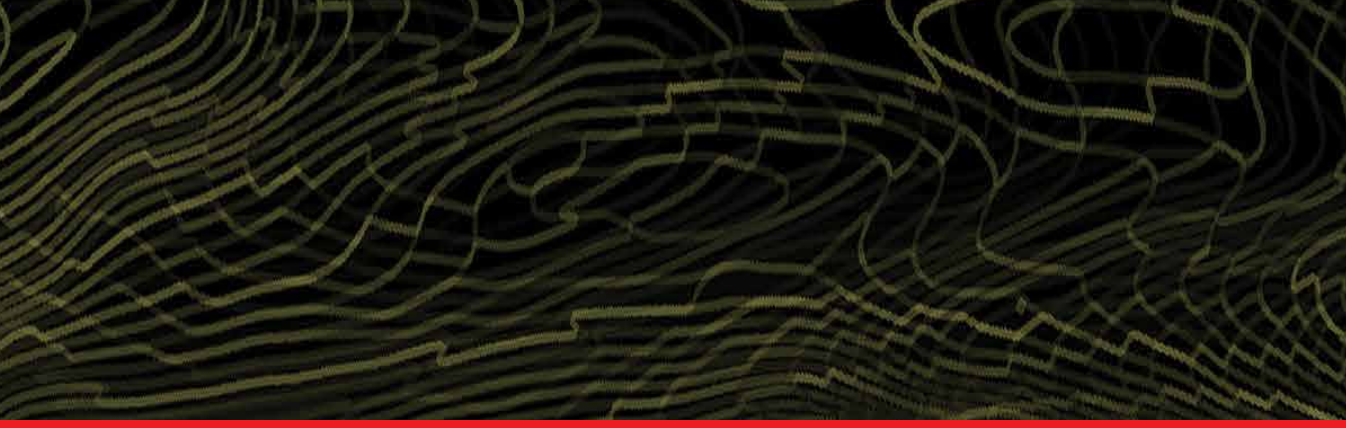

Edited by Yash Gupta, Surendra Kumar Prajapati and Raja Babu Singh Kushwah

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ISSN 2631-6188
ISBN 978-1-83635-269-3
9 781836 352693

Published in London, UK

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