Chapter

Progress in Parasite Genomics and Its Application to Current Challenges in Malaria Control

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Abstract

A wide deployment of malaria control tools have significantly reduced malaria morbidity and mortality across Africa. However, in the last five to seven years, there has been a resurgence of malaria in several African countries, raising the questions of whether and why current control mechanisms are failing. Since the first *Plasmodium falciparum* reference genome was published in 2002, few thousands more representing a broad range of geographical isolates have been sequenced. These advances in parasite genomics have improved our understanding of mutational changes, molecular structure, and genetic mechanisms associated with diagnostic testing, antimalarial resistance, and preventive measures such as vaccine development. In this chapter, we summarize the current progress on: (1) genomic characteristics of *P. falciparum*; (2) novel biomarkers and revolutionary techniques for diagnosing malaria infections; and (3) current vaccine targets and challenges for developing efficacious and long-lasting malaria vaccines.

Keywords: genomics, *Plasmodium falciparum*, malaria diagnosis, bioinformatics, antimalarial resistance, malaria vaccines, biomarkers, genetic diversity

1. Introduction

Malaria remains a serious public health problem in several developing countries. Globally, there are about 3.2 billion people at risk of malaria and 435,000 malaria-related deaths, most of which happening in West Africa [1, 2]. Malaria is a complicated disease caused by the genus *Plasmodium* in the protozoan phylum Apicomplexa. While *P. falciparum* is the most prevalent form of malaria, *P. vivax* is most widespread around the world. *Plasmodium* has a complicated life cycle that reproduces asexually in human and sexually in mosquito hosts. The parasite is transmitted by the female *Anopheles* mosquitoes. *Anopheles* mosquitoes thrive in warm, tropical climates as the temperature allows for quicker breeding and hatching [3]. The abundance of vectors is positively correlated with the transmission rate of malaria [4]. People infected with malaria normally experience fever, chills, diarrhea, vomiting, and anemia [5]. If remain untreated, the disease can progress to a severe form and result in death [6].

A wide deployment of malaria control tools in the past few decades have significantly reduced malaria morbidity and mortality worldwide. The number of countries with fewer than 100 clinical malaria cases increased from six to 27. Countries including Iran, Malaysia, Timor-Leste Belize, Cabo Verde, China, and El Salvador reported zero malaria cases in 2019 and malaria cases dropped by 90% in the Greater Mekong subregion (GMS) [7]. However, in the last five to seven years, there has been a resurgence of malaria in several African countries, raising the questions of whether and why current control mechanisms are failing. A number of factors has limited malaria control and elimination efforts. First, *Plasmodium* isolates may respond differently to antimalarial drugs, with some evolved to become more resistant than the others due to prolonged drug use. Second, multiplicity of infection (MOI), i.e., the number of *Plasmodium* isolates co-infecting a single host, has made molecular characterization of the parasites and understanding of disease severity difficult [8]. Third, diagnostic inaccuracy related to false negative results by rapid diagnostic tests (RDTs) is becoming a more widespread phenomenon [9]. Other factors such as asymptomatic reservoirs leading to transmission, lack of effective vaccine, and warmer climates and changing environments caused by human activities [10] have also hampered malaria elimination efforts. Thus, it is critically important to create new tools that allow us to monitor parasite changes and use that information to improve existing control strategies. In this chapter, we will summarize the current progress on: (1) genomic characteristics of *P. falciparum*; (2) novel biomarkers and revolutionary techniques for diagnosing malaria infections; and (3) current vaccine targets and challenges for developing efficacious and long-lasting malaria vaccines.

2. Genomic characteristics of *Plasmodium falciparum*

Knowledge of the evolution and genetic variation of the *Plasmodium* genome offers incredible insights into novel means of malaria diagnosis and treatment. The advances in parasite genomics have improved our understanding of mutational changes, molecular structure, and genetic mechanisms associated with failure in diagnostic testing [11], antimalarial resistance [12], and preventive measures such as vaccine development [13]. Since the first reference genome of *P. falciparum* was published in 2002 [14], several thousand DNA sequences have been collected and deposited in public databases. *Plasmodium falciparum* genome is approximately 23.7 Mb with 14 chromosomes, a plasmid of about 35 kb, and lots of mitochondrial DNA copies of about 6 kb [15]. There are currently 5,438 genes that have been predicted/discovered within the genome with 33% uncertainty of their functions [16]. The genome contains many rich AT regions in both exons and introns (80% and 90%, respectively), which has some advantages and disadvantages when learning more about the genetic architecture of the parasites [17]. The advantage associated with the genomic data would be using its rich polymorphic AT content as biomarkers to map out the evolutionary structure of the parasites and correlating it with any drug resistant genes [15]. Although having a rich AT genome has contributed to a high yield in microsatellites or simple sequence repeats, there are some disadvantages when using the genome in genetic studies [15]. For example, within CRISPR-Cas9, the high AT content resulted in a decrease in the amount of gRNA target sites needed [18]. A deeper knowledge of the genome's polymorphic and conserved genes are therefore essential towards understanding the evolutionary timeline of various *P. falciparum* lineages.

Compared to other eukaryotic organisms, whole genome sequencing (WGS) showed that the genome of *P. falciparum* contains fewer genes for enzymes and

transporters, but more genes for immune evasion to support host–parasite interactions [14]. With an average length of 2.4 Kb, *P. falciparum* genes are considerably larger than many organisms. *P. falciparum*'s genome contains a full set of transfer RNA (tRNA) ligase genes with minimal redundancy. 43 tRNAs have been identified to bind all codons except TGT and TGC, which code for cysteine, thus giving *P. falciparum* a slightly different amino acid translation than is seen in humans and other eukaryotes. By contrast, the mitochondrial genome of *P. falciparum* is only about 6Kb and does not contain any genes that encode for tRNAs, implying that the mitochondrion must import tRNAs from elsewhere into the cells [19, 20]. Polymorphic genes in the *P. falciparum* genome are useful in creating linkage maps to monitor mutational changes and genetic diversity of the parasites in response to malaria interventions and control efforts [21]. Polymorphic genes are variations in genes at higher frequencies that can be advantageous, neutral, or disadvantageous [22]. The role of polymorphic genes is to influence coding regions, alter protein sequences and gene expression, and eventually the metabolic pathway and function [23]. For example, remarkable polymorphisms observed in the merozoite surface proteins (*MSP*), *PfAMA1*, *PfEBA*, and *PfRHs* genes that involved in merozoite evasion have been shown to increase the evasion ability of *P. falciparum* to the host immune system [24].

In comparison, conserved genes are genes that have not been altered. They contribute to important biological processes and fitness [25]. Information of conserved genes allow us to infer phylogenetic relatedness and trace the genetic origin of different lineages, determine new targets for therapeutic treatment, and serve as a guide when determining functions of unknown genes [18]. The processes that determine the polymorphisms of the parasitic genome include both selective pressures and recombination frequencies. Selective pressures on conserved genes allow certain important genes to remain unmodified for normal metabolic activities, whereas polymorphic genes diversify through frequent recombination allow for better evasion of antimalarial drug treatments and escape detection from diagnostic tests [26]. For example, the *PfHRP2* gene has shown with partial deletions and/or mutations over the past few years due to the usage of *HRP2*-specific Rapid Diagnostic tests (RDTs) [27, 28]. More specifically, the histidine-rich repeats in the *PfHRP2* gene drastically change the length of the gene and the ability of the parasites to evade RDTs. Therefore, novel genes that encode for parasite specific function might be potential new targets for malaria diagnosis and/or treatment [14]. It is important to distinguish metabolic pathways that the parasites use for invasion of the host cells as well as evasion of the host immune system.

P. falciparum is clearly genetically distinct by geographical regions [29, 30]. Given the complex life cycle of *Plasmodium*, genomic data coupled with Genome-Wide Association Studies can offer deep insights into the tangled relationships among humans, mosquitoes, and the parasites. To date, databases such as PlasmoDB have integrated sequence information, functional genomics, and annotation of data emerging from the *P. falciparum* genome sequencing consortium [31]. These databases provide an important platform to retrieve biological meaning from new 'omic' data and enhance diagnosis and treatment of infections caused by this dreadful malaria pathogen.

3. Conventional and novel methods for malaria diagnosis

One of the challenges to malaria elimination is the burden of submicroscopic asymptomatic infections that contribute to malaria transmission [7]. The gold standard for malaria diagnosis is microscopy [32]. Microscopy can differentiate malaria

species and quantify parasite density, but only has a detection threshold of 10 to 50 parasites μl of blood (approximately 0.001% parasitemia, assuming an erythrocyte count of 5 × 106 cells μl) [33]. However, it requires well trained microscopists and is labor-intensive and time-consuming [32]. Previous studies have reported higher prevalence of asymptomatic parasitemia (6–7 times higher) using PCR compared to microscopy [5]. Nested PCR of the *18 s* rRNA genes has been commonly used to detect submicroscopic infections. PCR-based methods are also more sensitive than microscopy at detecting gametocytes particularly in asymptomatic individuals with low-density infections [34]. However, recent studies indicated a relatively high number of misdiagnosed infections [9], possibly due to low parasite density being less detectable by conventional PCR. The *18 s* rRNA gene has a reported detection thresholds of 0.5–5 parasites μl [35], but parasite density of asymptomatic infections especially in low transmission settings could be well below this threshold. As a result, many of these infections remain undetected in the general populations of several malaria-endemic countries [10, 36, 37] and provide perfect reservoirs for transmission at any time. Therefore, it is crucial to identify new gene targets or novel tools that are convenient and affordable for detecting asymptomatic infections, particularly in countries approaching elimination phase. Below are comparisons of various conventional and novel detection methods.

3.1 Rapid diagnostic test (RDT)

Rapid Diagnostic Test (RDT) is a lateral flow immunochromatographic test that can detect the presence of *Plasmodium* parasites by using blood from patients. The blood samples collected from patients must be lysed before the *Plasmodium* antigens being stained. RDT will indicate if the patient has malaria or not [38]. This diagnostic method is useful in rural areas as it is inexpensive and does not require expertise to perform the test [38]. There are various types of RDTs that can detect different antigens of *Plasmodium* parasites including *P. falciparum* and *P. vivax*, namely *P. falciparum* histidine-rich protein 2 (*PfHRP2*) and lactate dehydrogenase (*PfLDH*). However, recent studies indicated that parasites lacking the *pfhrp2/pfhrp3* genes could result in false-negative *PfHRP2*-RDT results, and these *pfhrp2/pfhrp3* variants have been spreading in many East and West African countries [39–42]. Reports of deletion of *HRP2* and *HRP3* genes was first reported in 2010 within the Peruvian Amazon [43] and the number of false negative RDTs has substantially increased in late 2014 [44]. Prevalence of false negative cases related to *HRP2* deletions in South America (with the exception of Peru) is lower than Asia and Sub-Saharan Africa [45]. The highest prevalence (50% *pfhrp2* deletions among all positive cases) were reported in Cambodia, Peru, and Eritrea [46]. Such a high prevalence could be explained by technical errors, mutations, selection and spread of the *pfhrp2* and 3 variants [47] from South America to Africa and Asia [41]. Recently, a novel HRP2-based Alere™ Malaria Ag P.f RDT (uRDT) was developed specifically for the detection of asymptomatic infections. Compared to the existing RDTs, the uRDT showed a greater than 10-fold improvement in diagnosing clinical and asymptomatic cases [48]. However, the sensitivities of the uRDT were inconsistent among different transmission settings [49]. Thus, although very promising for the detection of asymptomatic infections, the performance of uRDT must be further evaluated especially in areas with a already high prevalence of *pfhrp2* and *pfhrp3* gene deletions.

3.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA is an enzyme-linked immunosorbent assay that is used to detect antigens and antibodies by utilizing a highly specific antibody–antigen interaction and their

bonding affinity to proteins on a solid surface [50]. Samples, including and positive and negative controls are inserted on a 96 well plate which enables the possibility to analyze multiple samples simultaneously, then samples get incubated and detection of the signal is generated directly or indirectly via secondary tag on the specific antibody [51]. ELISA can be very efficient diagnostic tool for *Plasmodium* because it is highly specific and sensitive and therefore can be used to screen blood donors and pregnant women who may or may not have been exposed to the parasite [1]. However there are some limitations associated with using ELISA, as it is unable to detect antibodies in patients with acute infections, patients with different *Plasmodium* species, and various antigens within the different life stages of the parasite [52]. A recent study showed a lower detection threshold (3 parasites/μl) by ELISA test of the *Plasmodium* lactate dehydrogenase (*pLDH*) as compared to RDT (50–60 parasites/μl) [53]. However, ELISA has a lower sensitivity (69.9%) to antigens than RDT (88%) [53], and can be expensive and require a trained technician to operate and interpret the results [53].

3.3 Lateral flow immunoassay (LFIA)

One of the current diagnostic tests in development is an antibody-based lateral flow immunoassay (LFIA). Unlike ELISA that requires repeated incubation and washing steps, LFIA is considered to be a simple, user-friendly and cost-effective method for front-line diagnosis [54]. LFIA is versatile enough to detect target genes in sample matrices including whole blood, saliva, and urine. This method is primarily applied to detecting gametocytes in malaria-infected samples. Gametocytes are the sexual form of the parasite that gets transmitted to the mosquito host. They play a very important role in malaria transmission, and can contribute to up to 80% [55] of the infectious reservoirs. Previous studies have shown that infections with parasitemia as low as 4 gametocytes/μl can sustain transmission [56]. LFIA targets *Pfs25* in infected blood samples, which is a glycophosphatidylinositol-linked protein expressed on the surface of *P. falciparum* zygotes and ookinetes but only found on female gametocytes in the human hosts [57]. The detection limit of *Pfs25* LFIA is 0.02 gametocytes/μl, much more sensitive than *pfhrp2* RDT with a detection limit of 50–100 parasites per μL of blood [35, 58]. Another protein at the surface of the female gametocyte *PSSP17*, which is presumably more abundant in saliva samples, was also investigated in Cameroon, Zambia and Sierra Leone with an estimated sensitivity of 83% (95% CI, 61 to 95) in symptomatic patients when compared to PCR as the gold standard [58]. Detection of *pfhrp2* in saliva have been reported recently using LFIA, but the sensitivity was only shown to be improved in severe to moderate form of infections with parasitemia >60,000 parasites/μL [59]. Although convenient, *pfhrp2*-based LFIA could be less useful in detecting asymptomatic infections.

3.4 PCR-based methods

The combination of PCR-based assays and sequencing technologies have revolutionized malaria diagnosis since their introduction in the early 2000s. Various gene targets have be used to monitor genetic and/or mutational changes in the parasites that cause the disease. PCR is a very common technique used in malaria diagnoses in laboratory settings. The main advantage of this technique is that it enables us to identify individuals with low parasitemia (**Table 1**). There are multiple versions of this technique including nested conventional PCR, real-time quantitative PCR and reverse transcriptase PCR. Nested PCR is the easiest and least expensive methods among the others. It only requires a thermocycler, set of primers, reagents and visualization after gel electrophoresis. It is mostly a

qualitative method as it is fairly difficult to estimate parasite density on a agarose gel. Unlike conventional PCR that uses gel electrophoresis to visualize PCR products, real-time PCR or qPCR detects and quantifies the amount of amplified DNA usually by SYBR green or a fluorogenic probe designed based on a target gene segment (TaqMan). The standard cycle threshold (Ct) value is inversely proportional to the amount of target DNA in the sample [66] and allows estimation of parasite density even in submicroscopic samples. Different from nested and qPCRs, reverse transcriptase PCR uses RNA as template and transcribes RNA into complementary DNA. This method has been widely used to detect and quantify gametocyte density in malaria samples. Though PCR-based methods are undoubtedly more sensitive than microscopic diagnosis, the level of sensitivity is highly dependent on the gene targets (**Table 1**).

3.4.1 18 s rRNA

The *18 s* rRNA genes have been the main gene target for molecular screening as it contains 5–8 copies per genome [62] in *Plasmodium*, but recent studies indicated a relatively high level of misdiagnosed infections [45]. The advantage of using the *18 s* rRNA is that it is highly specific compared to microscopy or RDT diagnoses. The primer sequences and protocols for both nested and qPCRs have been readily established. However, it fails to reveal infections with low parasite density (0.5–5 parasites/μL of blood) [35]. Thus, new target genes have been examined in the past few years with the goal to achieve an ultra-sensitive biomarker with higher sensitivity and specificity compared to the *18 s* rRNA.

3.4.2 Mitochondrial cytochrome c oxidase III (COX3)

The cytochrome c oxidase III (*COX3*) gene plays a very important role in cellular respiration [67]. It is a mitochondrial gene that inherited solely from the female gametocyte and less likely to undergo genetic recombination, making it an ideal candidate for identifying the origin and transmission of the parasites [68].

Type	Target	Sensitivity (%)	Specificity (%)	Application	Detection Limit	Refs
RDT	*HRP2/3*	57	99	Field	50–100 parasites/μL of blood	[35]
RDT	*LDH*	58	93	Field	50–100 parasites/μL of blood	[35]
PCR	*18 s* rRNA	64	92	Laboratory	0.5–5 parasites/μL of blood	[60]
PCR	*COX3*	—	—	Laboratory	0.6–2 parasites/μL blood	[60]
PCR	*TARE-2*	81	49	Laboratory	6–24 parasites in 200 μL whole blood	[61]
PCR	*var*ATS	—	—	Laboratory	12–30 parasites in 200 μL whole blood	[62]
PCR	*Pfs25*	—	—	Laboratory	0.3 mature females/μL blood	[63]
ELISA	*LDH*	69.9	100	Laboratory	3 parasites/μL blood	[53, 64]
LFIA	*Pfs25*	—	—	Laboratory	0.02 gametocytes/μl blood	[58]
SERS	Hemozoin	—	—	Laboratory	30 parasites/μL of blood	[65]

— Denotes that the sensitivity or specificity is not reported.

Table 1.
Existing malaria diagnostic tests and their respective performance.

More importantly, there are around 20 to 150 copies of the *COX3* gene in the *Plasmodium* genome [62] and PCR analysis of this gene indicated a detection limit of 0.6–2 parasites/μL, much more sensitive than the *18 s*-rRNA [60].

3.4.3 Telomere associated repetitive element 2 (TARE-2)

The recently discovered Telomere Associated Repetitive Element 2 (*TARE-2*) has demonstrated better performance than the conventional *18 s* rRNA marker in detecting low density parasite infections. There are about 250 copies of the *TARE-2* gene in the *Plasmodium* genome and this gene is highly specific to *P. falciparum* [69]. *TARE-2* has been previously shown to be useful for detecting ultra-low density *P. falciparum* infections in Papua New Guinea and Tanzania [62]. A recent study in Ghana showed a slightly higher sensitivity of *TARE-2* compared to *18 s* rRNA (81.2% vs. 80.9%) using microscopy as a gold standard, but with a lower specificity reduced by almost two-fold [37], making this gene less desirable for active case surveillance.

3.4.4 Var. gene acidic terminal sequence (varATS)

The *var* gene family is located primarily in the subtelomeric region of the *Plasmodium* genome. It is a family of genes known to be highly polymorphic. For instance, the genome of the 3D7 culture strain harbors 59 different *var* genes with an estimated 50–150 copies per genome [14]. One of the main gene in the *var* gene family encodes the *P. falciparum* erythrocyte membrane protein 1 (*PfEMP1*) that contains a highly variable extracellular part and a well conserved intracellular *var* gene acidic terminal sequence (*var*ATS) [61]. The *var*ATS has a detection limit of 12–30 parasites in 200 μL whole blood. Though more sensitive *18 s* rRNA, *var*ATS employs a qPCR approach that could be less feasible for routine case detection in rural areas or developing countries [62].

3.5 Raman spectroscopy

Raman spectroscopy is a relatively novel technique that can be used to diagnose the presence of *Plasmodium*. This technique measures the amount of wavelength and light intensity of scattered light reflected from the target molecule of *Plasmodium* present in an infected sample either in liquid, gas, or solid form [70]. Raman spectroscopy can be used specifically to characterize hemozoin, a pigment produced by infected erythrocytes and can be used to track the progression of the disease and efficacy of drug treatment [71]. A specific type of Raman spectroscopy known as Surface Enhanced Raman Spectroscopy (SERS) has been utilized to detect early signs of erythrocytic infections and was shown with a detection limit of 30 parasites/μL [65]. Tip-enhanced Raman spectroscopy (TERS), on the other hand, can enhance *Plasmodium*'s hemozoin vacuole from 10^6 to 10^7 [71]. While Raman spectroscopy is more cost effectiveness than PCR and microscopy [71], this technique cannot detect hemozoin in early ring stage nor differentiate hemozoin from active and previous infections [71]. Further investigations are needed to refine Raman signals in order to enhance the technique's detection abilities.

4. Revolutionized techniques and genomics tools for monitoring parasite changes

Extensive level of genetic diversity observed in *Plasmodium* is a major threat to the eradication of the disease. Previous studies used sanger sequencing of the merozoite surface protein (MSP) or microsatellite genotyping methods to assess parasite genetic

diversity. Though affordable, these methods are time-consuming and labor-intensive for large scale genetic studies. Moreover, differentiating or phasing clonal genotypes for samples with more than two clones can be difficult. Restriction enzyme cutting followed by gel electorphoresis of the MSP gene has been conventionally used to assess parasite diversity and define multiplicity of infections (MOI) based on the number of distinct bands present on a gel. MSP is the most abundant protein at the surface of the merozoite and play a critical role in the *Plasmodium* invasion mechanism to the erythrocyte [72]. It is an informative gene for resolving clonal relationships and depicting population structure of *Plasmodium* given its size polymorphisms [73]. However, in high transmission areas where parasites are highly variable and different by single nucleotide polymorphisms (SNPs), gel electorphoresis of the MSP gene would underestimate the levels of diversity and polyclonality [74]. Microsatellite genotyping has also extensively been used for *Plasmodium* population structure and genetic diversity study [75]. While this marker has the advantages of being polymorphic, evolutionary neutrally, and are abundant in the *Plasmodium* genome [76], polyclonal samples with more than two alleles detected from two or more genetic loci are usually discarded in the analyses. Thus, next-generation sequencing (NGS) technologies offer a novel, alternative approach to shed light on the polyclonal and complex nature of *Plasmodium* infections [77].

4.1 Amplicon deep sequencing

In regions in high malaria endemicity, individuals typically harbor multiple *Plasmodium falciparum* isolates due to repeated exposure to mosquitoes infected with multiple parasite isolates [78]. Polyclonal infections have become a growing concern as some parasite isolates may be resistant to antimalarial drugs and/or more pathogenic [79]. Identification of genetically distinct clones is necessary to critically evaluate the causation of resistance to drugs and any other therapeutic treatments. Recently, due to reduced cost of high throughput NGS technology, other types of molecular tools have emerged to address the complex issue related to MOI. MOI is defined as the number of parasite clones within an infected sample. Deep sequencing of a targeted gene amplicon coupled with bioinformatic analyses allow differentiation of various *P. falciparum* strains based on SNPs [75] and discrimination of major from minor clones [80]. It also provides an increased capability to detect the genetic relatedness among clones within and between hosts as well as minor *P. falciparum* variants [81]. This technique can be applied to different gene regions such as Circumsporozoite (CSP), MSP1/2, and Apical membrane antigen (AMA) to monitor changes as well as selection pressure acting on the parasite populations [82].

4.2 Molecular inversion probe

Molecular inversion probes (MIPs) are another deep sequencing technique that targets several short gene regions across the genome. MIPs are single stranded DNA molecules that contain flanking regions of the targeted gene regions up to several hundred base pairs long [83]. MIPs can hybridize with the target sequence and undergo gap filling ligation to form circular DNA. The target sequence will also contain adaptors and barcodes to be further amplified by PCR [83]. The advantages of this technique include low rate of errors, small amount of DNA samples, high throughput, and cost-effective for several hundreds of samples. It is scalable to the number of targeted gene regions and samples, requires minimal costs in terms of reagents and labor, and allows efficient capture of DNA extracted from dried blood spots [84]. The latter advantage would make large-scale population studies feasible. MIPs have been used for monitoring SNP mutations associated with drug

Anti-malarial	Chloroquine	Amodiaquine	Sulfadoxine	Pyrimethamine	Artemisinin
Gene	Crt	Mdr1	Dhps	Dhfr	K13
Mutation(s)	K76T	N86Y, Y184F	A437G, K540E	N51I, C59R, S108N	M476I, Y493H, R539T, I543T, C580Y

Table 2.
Gene mutations associated with antimalarial drug resistance.

resistance, such as *crt*, *mdr1*, *dhps*, *dhfr*, and *K13* (**Table 2**) [84]. For example, a recent study based on MIPs analysis found that the *PfDHPS* gene associated with Sulfadoxine resistance has been rapidly spread from east to west of the Democratic Republic of Congo [84]. Apart from drug-resistant mutations, a number of microsatellite loci have also been added to the MIP panel to estimate genetic structure and diversity of *P. falciparum*. This technique allows multiple loci to be genotyped simultaneously. However, the design of the MIP panel would require prior information of the gene regions of interest.

5. From parasite genes to malaria vaccines

Genetic information of *P. falciparum* has allowed a careful selection of gene targets for vaccine development [85]. Vaccines can offer protection against clinical malaria especially in young children and reduce transmission in a population. However, there are several challenges in developing a highly effective malaria vaccine, mostly due to the complexity in the parasite life cycle and host immune system. *Plasmodium* parasites reproduce asexually in human hosts. They can be found throughout the body's bloodstream and liver in various stages. There are numerous potential parasite antigens that elicit different levels of host immune response [86], but the protective response towards a particular antigen or one parasite life-stage is not effective in conferring protection against other stages [87]. One solution for that is to choose an immunogenic antigen/epitope that can elicit a strong immune response and potentially confer the highest efficacy. Conjugating the target antigen or epitope with an adjuvant or better drug delivery system can help elicit a stronger and safer immune response [88]. Alternatively, it is also possible to activate other immune cells such as natural killer cells and neutrophils that can elicit a long-lasting immune response [89]. Natural killer cells act faster than T cells while neutrophils can activate either a humoral or cell mediated immunity [90]. Both natural killer cells and neutrophils in response to *P. falciparum* invasion have not been studied in depth compared to antibodies and T cells [90]. To date, there are three types of malarial vaccines that are being studied and tested in clinical trials: pre-erythrocytic, erythrocytic, and transmission blocking vaccines (**Table 3**).

5.1 Pre-erythrocytic vaccines

Pre-erythrocytic vaccines aim to kill infected hepatocytes and prevent sporozoites from reaching the liver [101]. There are various gene targets currently being investigated and in clinical trials.

5.1.1 RTS,S/AS01E

RTS,S/AS01E is a well-known pre-erythrocytic vaccine that has successfully completed phase III of clinical testing. It is currently distributed by World Health

Name of Vaccine	Type of Vaccine	Target of Vaccine	Malarial Life Cycle Stage	Phase of Vaccine	Efficacy	Refs
RTS,S/ AS01E	Recombinant protein vaccine	Circumsporozoite protein (CSP)	Pre-Erythrocytic	Phase III	35.2% in Children 20.3% in Infants	[91]
PfSPZ	Live, radiation-attenuated vaccine	Sporozoite (SPZ)	Pre-Erythrocytic	Phase III	Homologous challenge: 64% Heterologous challenge: 83%	[92]
PfLSA Vaccine	Recombinant protein vaccine	Liver-stage antigen 1 (*LSA-1*)	Pre-Erythrocytic	Phase I	Efficacy has yet to be found in humans	[93]
MSP1 Vaccine	Subunit based Vaccine	MSP1	Erythrocytic	Phase I	Efficacy has yet to be found in humans	[94]
AMA-1 Vaccine	Recombinant protein vaccine?	*AMA-1*	Erythrocytic	Phase II	Efficacy has yet to be found in humans	[95]
R21 Vaccine	Recombinant protein vaccines	Illicit a higher anti-CSP antibody count	Erythrocytic	Phase I/IIa	Efficacy has yet to be found in humans	[96]
Pfs 230 & *Pfs48/45*	Potential new target	*Pfs230* and *Pfs48/45* combined with a FAB fragment of a monoclonal antibody, 4F12, can act on parasitic gametes by forming a membrane-bound protein complex and increase the vaccine activity	Transmission Blocking Vaccine	None	Efficacy has yet to be found in humans	[97]
Pfs25	Recombinant conjugated vaccines	Antibodies against the *Pfs25* antigen in the human host to stop the development and transmission of the parasite when the vector feeds on the host.	Transmission Blocking Vaccine	Phase I	Efficacy has yet to be found in humans	[98]
PfGARP	Potential new target	Glutamic acid rich protein	Erythrocytic	None	Efficacy has yet to be found in humans	[99]
PfRH5-PfCyRPA-PfRipr (RCR) complex	Potential new target	Induce strain-transcending neutralizing antibodies against blood-stage *P. falciparum*	Erythrocytic	Phase I/II	Efficacy has yet to be found in humans	[100]

Table 3.
Overview of current vaccines candidates against Plasmodium falciparum.

Organization (WHO) in Malawi, Ghana, and Kenya to further investigate vaccine efficacy [96]. RTS,S is a vaccine that targets circumsporozoite proteins (CSP) from

P. falciparum. CSP are surface proteins that is important in hepatocyte invasion. Interactions between CSP and heparin sulfate proteoglycans (HSPGs) allows the sporozoite to attach to the surface of hepatocytes and triggers a signaling cascade that allows for the sporozoite to invade liver cells [102]. Subsequent to invasion, the sporozoites will mature, multiply, and feed on the hepatocytes until the hepatocytes lyse and release merozoites into the bloodstream. Preventing the invasion of sporozoites will inhibit the progression and severity of the disease. Using the C-terminus and central tandem repeat (NANP) of *PfCSP*, Hepatitis B surface membrane antigen (HSbAg), and an AS01 adjuvant system, the vaccine will elicit a strong, stable immune response [91]. After vaccination, the host immune system will response to *PfCSP* antigen by producing anti-CSP antibodies and activating CD4+ T cells [103]. RTS,S/AS01E is given on a three-dose schedule within three months followed by a fourth dose at 20 months [91]. It has been shown across clinical malarial studies that the vaccine has an 39–50% efficacy in children ages 5–17 months and 23–30% efficacy in children ages 6–12 months [104–106]. Efficacy warned rapidly from 35.2% and 20.3% to 19.1% and 12.7%, respectively, in children and infants within 20–32 months without a booster [91]. Such a decay in vaccine efficacy could be due to reduced IgG and IgM antibodies against CSP antigen. Anti-CSP antibodies were shown to increase by almost 10-fold from 318.2 EU/mL to 34.2 EU/mL in children one month after a booster was given [96]. Apart from the short-lived nature of the vaccine, older children who were vaccinated showed an increased risk of malarial infections, likely due to RTS,S/AS01E interference with naturally acquired immunity [96]. Another similar vaccine R21 is currently testing in Phase 1/2a clinical trials [107]. This vaccine aims to elicit a high anti-CSP antibody content similar to the mechanisms of RTS,S vaccine. R21 comprises particles from CSP-HBsAg protein infused with an adjuvant, matrix-M [107]. It has been shown to increase the production of T cells and is still in the process of development [96]. Further studies should investigate alternative antigens that can elicit stronger and long-lasting efficacy as well as the mechanisms of the cell-mediated immune response against malaria in humans.

5.1.2 PfSPZ

Plasmodium falciparum sporozoites (PfSPZ) is another pre-erythrocytic vaccine target. As aforementioned, sporozoites transferred from the infected Anopheles mosquito to the human host. They enter the bloodstream and reach the liver before invading the hepatocytes using the sporozoite proteins P36 and P52. P36 interacts with hepatocyte's extracellular receptor EphA2 to create a protective parasitophorous vacuole that facilitates hepatocyte invasion [108]. *PfSPZ* vaccine prevents the sporozoites reaching the liver and infecting hepatocytes [109], and is currently undergoing phase III clinical trial [92]. *PfSPZ* vaccine was designed to have a live, whole sporozoite that is radiated-attenuated. It is injected intravenously and given in 3–5 doses. Recent studies using controlled human malaria infection (CHMI) showed that this vaccine provided about 33 weeks of stabilized protection in 50% of the vaccinated subjects [110]. The vaccine induced interferon gamma (IFN-γ) that can recruit and activate CD8+ and CD4+ T cells against homologous and heterologous parasitic strains [110]. Subjects who received the *PfSPZ* vaccine also developed IgM antibodies that can help inhibit proliferation of the parasites [111]. However, the vaccine requires specific storage in ultra-cold condition and trained medical workers to inject the vaccine intravenously [96]. Furthermore, efficacy varied by locations. For example, in Mali, 29% efficacy was reported in subject who were exposed to heterologous strain; whereas in CHMI, 83% of subjects were found to be protected from exposure to heterologous strains [92]. Further

studies are needed to compare efficacy among different geographical or transmission settings.

5.1.3 PfLSA-1 and PfLSA-3

Liver surface antigen (LSA) is another pre-erythrocytic vaccine target. LSA is essential for the survival of the parasites during the late liver schizogony stage [93]. Schizonts are asexual stage of *Plasmodium* that developed from sporozoites and matured in infected hepatocytes. Once the infected hepatocytes are filled up with mature schizonts, they rupture and release merozoites into the bloodstream to invade erythrocytes. LSA vaccine prevents the maturation of schizonts and rupture of infected hepatocytes. There are two liver stage antigens, LSA-1 and LSA-3, that are used as vaccine targets. *PfLSA-1* is high conserved in *P. falciparum* and is found in parasitophorous vacuole in the liver stage of the parasites. *PfLSA-1* contains 17 amino acids repeats and is associated with the late liver schizont stage [93, 112]. LSA-1 can induce IgG and IgM antibodies as well as CD4+ T cell production [93, 113]. LSA-1 vaccine is currently in phase I clinical trial and is still unclear its efficacy in humans. LSA-3, on the other hand, is found in dense granules in the blood stage of the parasites and the protein is about 175 kDa [114]. LSA-3 appears to play a role in the parasitic growth in infected hepatocytes and erythrocytes [114]. A recent study indicated that LSA-3 provided full protect to chimpanzees from heterologous *P. falciparum* sporozoites [93]. Like LSA-1, LSA-3 is also in phase I clinical trial and its efficacy and immunogenicity in humans remain unclear.

5.2 Erythrocytic vaccines

Erythrocytic vaccines aim to kill and terminate the asexual reproduction and invasion of the parasite within red blood cells (RBC). Infected individuals typically experience symptoms when the parasites invade a threshold number of RBCs and disrupt their normal functions. Preventing the parasites developed into blood stage will inhibit progression of malaria symptoms such as chills, aches, and fevers.

5.2.1 PfMSP1

Merozoite surface protein 1 (MSP1) is one of the targets used for erythrocytic vaccine. MSP1 is a glycosylphosphatidylinositol-anchored protein found in abundance on the surface of the merozoites [115]. MSP1 plays an important role in the invasion of erythrocytes as it binds and recruits other peripheral merozoite surface proteins to form a complex [115]. MSP1 starts off as a precursor of about 196 kDa and then cleave into four subunits before invading the erythrocytes [94]. The four subunits are held non-covalently forming a complex attached to the merozoite's GPI anchor [94]. Once the MSP1 complex is formed, it binds with the receptors on erythrocytes and activates a spectrin-binding function to enter the erythrocytes [94]. MSP1 has been shown to elicit both humoral (IgM and IgG antibodies) and cell-mediated immune responses (memory T cells) that lasted about 6 months after immunization [94]. These results are promising as MSP1 vaccine will also activate antibodies for complement fixation, induce opsonizing antibodies, and initiate secretion of reactive oxygen species by other immune cells [94]. To date, MSP1 vaccine is in phase I clinical trial. Further investigation is needed to evaluate efficacy.

5.2.2 PfAMA-1

The erythrocytic vaccine based on Apical Membrane Antigen 1 (AMA1) is currently in phase II clinical trial [116]. The AMA1 protein is approximately 83 kDa and can be found in both the merozoite and sporozoite stages of *P. falciparum* [116]. The complex AMA1 and another parasite protein namely the rhoptry neck protein 2 (RON2), is essential for merozoite invasion during the blood stage of infection, and initiates the parasite traversal into the RBCs [117]. The AMA1 vaccine has been shown to elicit high levels of antibodies that can block the invasion of the erythrocytes, despite high polymorphisms observed in the AMA1 protein [117]. The AMA1-Diversity Covering (DiCo) vaccine was thus designed to include three recombinant variants of AMA1 and this vaccine is currently testing in phase Ia/Ib clinical trial [118]. To increase efficacy of the AMA1 vaccine, AMA1 was paired with the RON2 receptor and vaccinated in eight *Aotus* monkeys [117]. Half of the monkeys were able to achieve complete immunity from the *P. falciparum* infection when vaccinated with AMA1 and RON2 [117]. The vaccine with AMA1 and RON2 induced a higher level of antibodies than the AMA1 vaccine. Further investigation is needed to examine other potential AMA1 variants or merozoite structures that can be paired up with the AMA1 protein to increase efficacy.

5.2.3 PfGARP

Plasmodium falciparum glutamic acid rich protein (PfGARP) is an 80 kDa antigen commonly expressed on the surface of infected erythrocytes during the late trophozoite stage [119]. The *PfGARP* gene is relatively conserved. Antibodies against the *PfGARP* antigen protein have been shown to confer protection against severe malaria and reduce parasite densities by 3.5 folds [99]. Further, anti-*PfGARP* antibodies were able to successfully induce apoptosis in ring-stage parasite cultures, resulting in full loss of their mitochondrial function within a 24 hour period [99]. In addition, the size of food vacuoles in the parasites was decreased or condensed tightly around the hemozoin crystals, making them inaccessible and parasite growth was reduced by 76–87% *in vitro* [99].

5.2.4 PfRH5-PfCyRPA-PfRipr (RCR) complex

The *PfRH5-PfCyRPA-PfRipr* (RCR) complex is a protein trimer composed of three different proteins *PfRH5*, *PfCyRPA*, and *PfRipr* that are found on the surface of merozoites [100]. *P. falciparum* Reticulocyte-binding Protein Homolog 5 (*PfRH5*) is a 63 kDa protein commonly expressed during the schizont stage. After *PfRH5* binds with basigin (a receptor found on human erythrocytes), a large amount of calcium is released to initiate invasion [100]. Monoclonal antibodies against *PfRH5* has been found to disrupt the binding between basigin and *PfRH5* [100]. *P. falciparum* Cysteine-rich Protective Antigen (*PfCyRPA*) is a highly conserved 43 kDa protein [100]. It plays an important role in erythrocyte invasion by interacting with *PfRH5* to bind to the receptor basigin [56]. Although *PfCyRPA* is not immunogenic compared to *PfRipr* and *PfRH5*, monoclonal antibodies produced against this protein can cause cross strain neutralization [100]. *P. falciparum* RH5-interacting Protein (*PfRipr*) is a highly conserved 120 kDa protein found in the schizont stage [100]. It is composed of 87 cysteines and 10 epidermal growth factor-like (EGF) domains [100]. Prior to erythrocyte invasion, *PfRipr* cleaves into two different fragments including the N-terminus and C-terminus. The N-terminus contains EGF domains 1 and 2 while the C-terminus contains EGF domains 3–10 [100]. Antibodies against *PfRipr* EGF domains 6–8 have been shown to neutralize

the parasites [100]. Combining the anti-PfCyRPA c12 mAb with anti-RH5 BS1.2 mAbr will inhibit parasite growth *in vitro* from 21–31% to 59%. Thus, this antigen protein complex is expected to elicit a strong immune response against blood stage *P. falciparum* [120].

5.3 Transmission blocking vaccine (TBV)

TBV aims to prevent and kill the sexual stages of the *Plasmodium* parasites before transferring into the mosquito hosts. This vaccine offers protection against infection and transmission.

5.3.1 Pfs25

Pfs25 is an important glycophosphatidylinositol-linked protein expressed on the surface of ookinetes. It is found only within the *Anopheles* host and is approximately 25 kDa with 11 disulfie bonds [121]. The parasites require *Pfs25* to survive in the *Anopheles*'s midgut and develop into oocysts [122]. *Pfs25* is a conserve protein with low diversity. *Pfs25* vaccine was designed to elicit antibodies against the *Pfs25* antigen in humans and prohibit the development and transmission of gametocytes [98]. However, a recent study based on an adjuvant of *Pfs25* and a non-enveloped virus like protein (VLP) indicated weak IgG antibody responses in healthy individuals [123]. In another study, the antibody response of *Pfs25* proteins combined with four different adjuvants including alum, Toll-like receptor 4 (TLR-4) agonist glucopyranosal lipid A (GLA) plus alum, squalene–oil-in-water emulsion, and GLA plus squalene–oil-in-water emulsion were compared in mice. *Pfs25* combined with GLA plus squalene–oil-in-water emulsion was shown to induce the highest amounts of IgG antibodies [124]. Further studies should examine the formulation of this vaccine for better efficacy.

5.3.2 Pfs230 and Pfs48/45

Pfs230 and *Pfs48/45*-based vaccines are antigens rich in cysteine produced by the sexual stage gametocytes [97]. *Pfs48/45* can be found on the plasma membrane surface of both male and female gametocytes and are bounded to a GPI anchor that form a complex with *Pfs230* [125]. *Pfs230* is a 230 kDa protein that contains 14 6-cysteine rich domains [97]. *Pfs48/45*, on the other hand, contains three 6-cysteine rich domains [125]. The 6-cysteine rich domains of these antigens are essential for the formation of disulfide bonds on epitopes needed for antibody elicitation [125]. A recent study showed that mice injected with fragments of *Pfs48/45* and *Pfs230* prodomain produced higher levels of antibodies that induced complement fixation [125]. *Pfs230* and *Pfs48/45* combined with a FAB fragment of a monoclonal antibody 4F12 have been shown to further increase vaccine efficacy [97]. This vaccine is currently in early clinical development phase [97, 125].

6. Conclusion

WHO aims to achieve malaria elimination in at least 35 countries, reduce incidence and mortality rates by 90%, and prevent resurgence in malaria-free countries by 2030. This ambitious goal has been challenged by the emergence and spread of antimalarial resistance, inaccurate diagnostic testing, asymptomatic transmission, and lack of effective vaccines [126]. Information of the *Plasmodium* genomes allow us to improve and reinvent tools/techniques for monitoring parasite changes as well

as tracking and stopping transmission of the disease. Several hundreds of *P. falciparum* genomes have been generated in the past two decades. Genetic variation and function of various genes have improved our understanding of mutational changes, molecular structure, and evolutionary mechanisms in *Plasmodium*. There is an urgent need to retrieve utmost biological meaning from the available genomic data and translate such into tools that help resolve epidemiological challenges. This includes the identification of novel antigens for accurate and affordable diagnostic assays and vaccines, informative biomarkers that can distinguish different isolates and pinpoint the source of infections at fine geographical scale, and sensitive tool(s) for large-scale screening of asymptomatic infections in both high and low transmission areas. Future studies should examine how climate/environmental changes and selective pressure from interventions mediate genetic changes in the parasites, how host immune system responses to parasite changes, and how to uncover hidden parasite reservoirs and effectively control transmission.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization, C.C.D. and E.L.; resources, D.A.J. and E.L.; writing—original draft preparation, C.C.D., C.T.F., J.H., and E.L.; writing—review and editing, C.C. D., C.T.F., L.E.A, Y.A.A., D.A.J., and and E.L.; funding acquisition, E.L. All authors have read and agreed to the published version of the manuscript.

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References

[1] Talapko J, Škrlec I, Alebić T, Jukić M, Včev A. Malaria: The Past and the Present. Microorganisms. 2019 Jun;7(6). [PubMed Central:PMC6617065] [DOI: 10.3390/microorganisms7060179] [PubMed:30760324].

[2] Abossie A, Yohanes T, Nedu A, Tafesse W, Damitie M. Prevalence of Malaria and Associated Risk Factors Among Febrile Children Under Five Years: A Cross-Sectional Study in Arba Minch Zuria District, South Ethiopia. Infect Drug Resist. 2020;13:363–372. [PubMed Central:PMC7012238] [DOI: 10.2147/IDR.S223873] [PubMed: 27137913].

[3] Ahmed T, Hyder MZ, Liaqat I, Scholz M. Climatic Conditions: Conventional and Nanotechnology-Based Methods for the Control of Mosquito Vectors Causing Human Health Issues. Int J Environ Res Public Health. 2019 08;16 (17). [PubMedCentral:PMC6747303] [DOI:10.3390/ijerph16173165] [PubMed:23688176].

[4] Castro MC. Malaria Transmission and Prospects for Malaria Eradication: The Role of the Environment. Cold Spring Harb Perspect Med. 2017 Oct;7 (10). [PubMedCentral:PMC5629986] [DOI:10.1101/cshperspect.a025601] [PubMed:7907685].

[5] Kimenyi KM, Wamae K, Ochola-Oyier LI. Understanding P. falciparum Asymptomatic Infections: A Proposition for a Transcriptomic Approach. Front Immunol. 2019;10:2398. [PubMed Central:PMC6803459] [DOI:10.3389/fimmu.2019.02398] [PubMed: 10450423].

[6] Milner DA. Malaria Pathogenesis. Cold Spring Harb Perspect Med. 2018 01;8(1). [PubMed Central: PMC5749143] [DOI:10.1101/cshperspect.a025569] [PubMed: 24854175].

[7] Organization WH. World malaria report 2020: 20 years of global progress and challenges. World Health Organization; 2020.

[8] Mahdi Abdel Hamid M, Elamin AF, Albsheer MM, Abdalla AA, Mahgoub NS, Mustafa SO, et al. Multiplicity of infection and genetic diversity of *Plasmodium falciparum* isolates from patients with uncomplicated and severe malaria in Gezira State, Sudan. Parasit Vectors. 2016 06;9(1):362. [PubMed Central:PMC4924276] [DOI:10.1186/s13071-016-1641-z] [PubMed:16879742].

[9] Koita OA, Doumbo OK, Ouattara A, Tall LK, Konar'e A, Diakité M, et al. False-negative rapid diagnostic tests for malaria and deletion of the histidine-rich repeat region of the hrp2 gene. Am J Trop Med Hyg. 2012 Feb;86(2):194–198. [PubMed Central:PMC3269266] [DOI: 10.4269/ajtmh.2012.10-0665] [PubMed: 20565816].

[10] Chaumeau V, Kajeechiwa L, Fustec B, Landier J, Naw Nyo S, Nay Hsel S, et al. Contribution of Asymptomatic Plasmodium Infections to the Transmission of Malaria in Kayin State, Myanmar. J Infect Dis. 2019 04;219(9): 1499–1509. [PubMed Central: PMC6467188] [DOI:10.1093/infdis/jiy686] [PubMed:3227404].

[11] von Seidlein L. The failure of screening and treating as a malaria elimination strategy. PLoS Med. 2014 Jan;11(1):e1001595. [PubMed Central: PMC3904824] [DOI:10.1371/journal.pmed.1001595] [PubMed:11075770].

[12] White NJ. Antimalarial drug resistance. J Clin Invest. 2004 Apr;113 (8):1084–1092. [PubMed Central: PMC385418] [DOI:10.1172/JCI21682] [PubMed:14688565].

[13] Hill AV. Vaccines against malaria. Philos Trans R Soc Lond B Biol Sci. 2011

Oct;366(1579):2806–2814. [PubMed Central:PMC3146776] [DOI:10.1098/rstb.2011.0091] [PubMed:21237715].

[14] Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature. 2002 Oct;419(6906):498–511. [PubMed Central:PMC3836256] [DOI:10.1038/nature01097] [PubMed:11069183].

[15] Su XZ, Lane KD, Xia L, Sá JM, Wellems TE. Plasmodium Genomics and Genetics: New Insights into Malaria Pathogenesis, Drug Resistance, Epidemiology, and Evolution. Clin Microbiol Rev. 2019 09;32(4). [PubMed Central:PMC6750138] [DOI:10.1128/CMR.00019-19] [PubMed:28213434].

[16] Bohme U, Otto TD, Sanders M, Newbold CI, Berriman M. Progression of the canonical reference malaria parasite genome from 2002–2019. Wellcome Open Res. 2019;4:58. [PubMed Central:PMC6484455] [DOI:10.12688/wellcomeopenres.15194.2] [PubMed:20562415].

[17] Hamilton WL, Claessens A, Otto TD, Kekre M, Fairhurst RM, Rayner JC, et al. Extreme mutation bias and high AT content in *Plasmodium falciparum*. Nucleic Acids Res. 2017 02;45(4):1889–1901. [PubMed Central:PMC5389722] [DOI:10.1093/nar/gkw1259] [PubMed:351412].

[18] Zhang M, Wang C, Otto TD, Oberstaller J, Liao X, Adapa SR, et al. Uncovering the essential genes of the human malaria parasite *Plasmodium falciparum* by saturation mutagenesis. Science. 2018 05;360(6388). [PubMed Central:PMC6360947] [DOI:10.1126/science.aap7847] [PubMed:9002517].

[19] Vaidya AB, Akella R, Suplick K. Sequences similar to genes for two mitochondrial proteins and portions of ribosomal RNA in tandemly arrayed 6-kilobase-pair DNA of a malarial parasite. Molecular and biochemical parasitology. 1989;35(2):97–107.

[20] Vaidya AB, Lashgari MS, Pologe LG, Morrisey J. Structural features of Plasmodium cytochrome b that may underlie susceptibility to 8-aminoquinolines and hydroxynaphthoquinones. Molecular and biochemical parasitology. 1993;58(1):33–42.

[21] Zhang CL, Zhou HN, Liu Q, Yang YM. Genetic polymorphism of merozoite surface proteins 1 and 2 of *Plasmodium falciparum* in the China-Myanmar border region. Malar J. 2019 Nov;18(1):367. [PubMed Central:PMC6862846] [DOI:10.1186/s12936-019-3003-8] [PubMed:19706922].

[22] Karki R, Pandya D, Elston RC, Ferlini C. Defining "mutation" and "polymorphism" in the era of personal genomics. BMC Med Genomics. 2015 Jul;8:37. [PubMed Central:PMC4502642] [DOI:10.1186/s12920-015-0115-z] [PubMed:11237011].

[23] Jin Y, Wang J, Bachtiar M, Chong SS, Lee CGL. Architecture of polymorphisms in the human genome reveals functionally important and positively selected variants in immune response and drug transporter genes. Hum Genomics. 2018 09;12(1):43. [PubMed Central:PMC6139121] [DOI:10.1186/s40246-018-0175-1] [PubMed:26888265].

[24] Belachew EB. Immune Response and Evasion Mechanisms of Plasmodium falciparum Parasites. J Immunol Res. 2018;2018:6529681.

[25] Dimonte S, Bruske EI, Enderes C, Otto TD, Turner L, Kremsner P, et al. Identification of a conserved var gene in different *Plasmodium falciparum* strains. Malar J. 2020 May;19(1):194. [PubMed Central:PMC7260770] [DOI:10.1186/s12936-020-03257-x] [PubMed:22004584].

[26] Miles A, Iqbal Z, Vauterin P, Pearson R, Campino S, Theron M, et al. Indels, structural variation, and recombination drive genomic diversity in *Plasmodium falciparum*. Genome Res. 2016 09;26(9):1288–1299. [PubMed Central:PMC5052046] [DOI:10.1101/gr.203711.115] [PubMed:17325376].

[27] Baker J, McCarthy J, Gatton M, Kyle DE, Belizario V, Luchavez J, et al. Genetic diversity of Plasmodium falciparum histidine-rich protein 2 (PfHRP2) and its effect on the performance of PfHRP2-based rapid diagnostic tests. J Infect Dis. 2005 Sep; 192(5):870–877.

[28] Baker J, Ho MF, Pelecanos A, Gatton M, Chen N, Abdullah S, et al. Global sequence variation in the histidine-rich proteins 2 and 3 of *Plasmodium falciparum*: implications for the performance of malaria rapid diagnostic tests. Malaria Journal. 2010 May;9(1):129. Available from: https: // doi.org/10.1186/1475-2875-9-129.

[29] Miotto O, Amato R, Ashley EA, MacInnis B, Almagro-Garcia J, Amaratunga C, et al. Genetic architecture of artemisinin-resistant Plasmodium falciparum. Nature genetics. 2015;47(3):226–234.

[30] Miotto O, Almagro-Garcia J, Manske M, MacInnis B, Campino S, Rockett KA, et al. Multiple populations of artemisinin-resistant Plasmodium falciparum in Cambodia. Nature genetics. 2013;45(6):648–655.

[31] Bahl A, Brunk B, Crabtree J, Fraunholz MJ, Gajria B, Grant GR, et al. PlasmoDB: the Plasmodium genome resource. A database integrating experimental and computational data. Nucleic Acids Res. 2003 Jan;31(1):212–215. [PubMed Central:PMC165528] [DOI:10.1093/nar/gkg081] [PubMed: 17040811].

[32] Ayogu EE, Ukwe CV, Nna EO. Assessing the reliability of microscopy and rapid diagnostic tests in malaria diagnosis in areas with varying parasite density among older children and adult patients in Nigeria. J Postgrad Med. 2016;62(3):150–156.

[33] Mathison BA, Pritt BS. Update on Malaria Diagnostics and Test Utilization. J Clin Microbiol. 2017 07;55 (7):2009–2017. [PubMed Central: PMC5483902] [DOI:10.1128/ JCM.02562-16] [PubMed:22550113].

[34] Ballard E, Wang CYT, Hien TT, Tong NT, Marquart L, Pava Z, et al. A validation study of microscopy versus quantitative PCR for measuring Plasmodium falciparum parasitemia. Trop Med Health. 2019;47:49.

[35] Mbanefo A, Kumar N. Evaluation of Malaria Diagnostic Methods as a Key for Successful Control and Elimination Programs. Trop Med Infect Dis. 2020 Jun;5(2). [PubMed Central: PMC7344938] [DOI:10.3390/ tropicalmed5020102] [PubMed: 29078786].

[36] Hassanpour G, Mohebali M, Zeraati H, Raeisi A, Keshavarz H. Asymptomatic Malaria and its Challenges in the Malaria Elimination Program in Iran: a Systematic Review. J Arthropod Borne Dis. 2017 Jun;11(2): 172–181.

[37] Dieng CC, Gonzalez L, Pestana K, Dhikrullahi SB, Amoah LE, Afrane YA, et al. Contrasting Asymptomatic and Drug Resistance Gene Prevalence of *Plasmodium falciparum* in Ghana: Implications on Seasonal Malaria Chemoprevention. Genes (Basel). 2019 07;10(7).

[38] Iwuafor AA, Ita OI, Ogban GI, Udoh UA, Amajor CA. Evaluation of Diagnostic Accuracy of Rapid Diagnostic Test for Malaria Diagnosis among Febrile Children in Calabar, Nigeria. Niger Med J. 2018;59(6):64–69. [PubMed Central:PMC6668302] [DOI:

10.4103/nmj.NMJ 165 18] [PubMed: 9988333].

[39] Golassa L, Messele A, Amambua-Ngwa A, Swedberg G. High prevalence and extended deletions in Plasmodium falciparum hrp2/3 genomic loci in Ethiopia. PLoS One. 2020;15(11): e0241807.

[40] Thomson R, Beshir KB, Cunningham J, Baiden F, Bharmal J, Bruxvoort KJ, et al. pfhrp2 and pfhrp3 Gene Deletions That Affect Malaria Rapid Diagnostic Tests for *Plasmodium falciparum*: Analysis of Archived Blood Samples From 3 African Countries. J Infect Dis. 2019 09;220(9):1444–1452.

[41] Watson OJ, Slater HC, Verity R, Parr JB, Mwandagalirwa MK, Tshefu A, et al. Modelling the drivers of the spread of Plasmodium falciparum hrp2 gene deletions in sub-Saharan Africa. Elife. 2017 08;6.

[42] Grignard L, Nolder D, Sepúlveda N, Berhane A, Mihreteab S, Kaaya R, et al. A novel multiplex qPCR assay for detection of *Plasmodium falciparum* with histidine-rich protein 2 and 3 (pfhrp2 and pfhrp3) deletions in polyclonal infections. EBioMedicine. 2020 May;55. Available from: https://doi.org/10.1016/j.ebiom.2020.102757.

[43] Li P, Xing H, Zhao Z, Yang Z, Cao Y, Li W, et al. Genetic diversity of *Plasmodium falciparum* histidine-rich protein 2 in the China-Myanmar border area. Acta Trop. 2015 Dec;152:26–31. [PubMed Central:PMC4918506] [DOI: 10.1016/j.actatropica.2015.08.003] [PubMed:24086328].

[44] Berhane A, Russom M, Bahta I, Hagos F, Ghirmai M, Uqubay S. Rapid diagnostic tests failing to detect *Plasmodium falciparum* infections in Eritrea: an investigation of reported false negative RDT results. Malar J. 2017 03;16(1):105. [PubMed Central: PMC5339986] [DOI:10.1186/s12936-017-1752-9] [PubMed:16407339].

[45] Kojom LP, Singh V. Prevalence of *Plasmodium falciparum* field isolates with deletions in histidine-rich protein 2 and 3 genes in context with sub-Saharan Africa and India: a systematic review and meta-analysis. Malar J. 2020 Jan;19 (1):46. [PubMed Central:PMC6986054] [DOI:10.1186/s12936-019-3090-6] [PubMed:30170596].

[46] Thomson R, Parr JB, Cheng Q, Chenet S, Perkins M, Cunningham J. Prevalence of Plasmodium falciparum lacking histidine-rich proteins 2 and 3: a systematic review. Bull World Health Organ. 2020 Aug;98(8):558–568.

[47] Amoah LE, Abuaku B, Bukari AH, Dickson D, Amoako EO, Asumah G, et al. Contribution of P. falciparum parasites with Pfhrp 2 gene deletions to false negative PfHRP 2 based malaria RDT results in Ghana: A nationwide study of symptomatic malaria patients. PLoS One. 2020;15(9):e0238749.

[48] Das S, Peck RB, Barney R, Jang IK, Kahn M, Zhu M, et al. Performance of an ultra-sensitive Plasmodium falciparum HRP2-based rapid diagnostic test with recombinant HRP2, culture parasites, and archived whole blood samples. Malar J. 2018 Mar;17(1):118.

[49] Unwin VT, Ahmed R, Noviyanti R, Puspitasari AM, Utami RAS, Trianty L, et al. Use of a highly-sensitive rapid diagnostic test to screen for malaria in pregnancy in Indonesia. Malar J. 2020 Jan;19(1):28.

[50] Waritani T, Chang J, McKinney B, Terato K. An ELISA protocol to improve the accuracy and reliability of serological antibody assays. MethodsX. 2017;4:153–165. [PubMed Central: PMC5387895] [DOI:10.1016/j.mex.2017.03.002] [PubMed:9013990].

[51] Dewasurendra RL, Dias JN, Sepulveda N, Gunawardena GS, Chandrasekharan N, Drakeley C, et al. Effectiveness of a serological tool to

predict malaria transmission intensity in an elimination setting. BMC Infect Dis. 2017 01;17(1):49. [PubMed Central: PMC5223541] [DOI:10.1186/s12879-016-2164-0] [PubMed:26770994].

[52] Ruiz Moreno Y, Donato ST, Nogueira F, Sousa Silva M. Comparative Analysis of the Serological Reactivity of Individuals with Clinical History of Malaria using Two Different ELISA Tests. Diagnostics (Basel). 2019 Oct;9 (4). [PubMed Central:PMC6963549] [DOI:10.3390/diagnostics9040168] [PubMed:22561398].

[53] Kwenti TE, Njunda LA, Tsamul B, Nsagha SD, Assob NJ, Tufon KA, et al. Comparative evaluation of a rapid diagnostic test, an antibody ELISA, and a pLDH ELISA in detecting asymptomatic malaria parasitaemia in blood donors in Buea, Cameroon. Infect Dis Poverty. 2017 Aug;6(1):103. [PubMed Central:PMC5537946] [DOI: 10.1186/s40249-017-0314-2] [PubMed: 22550113].

[54] Koczula KM, Gallotta A. Lateral flow assays. Essays Biochem. 2016 06;60 (1):111–120.

[55] Bousema T, Okell L, Felger I, Drakeley C. Asymptomatic malaria infections: detectability, transmissibility and public health relevance. Nat Rev Microbiol. 2014 12;12(12):833–840.

[56] Favuzza P, Blaser S, Dreyer AM, Riccio G, Tamborrini M, Thoma R, et al. Generation of Plasmodium falciparum parasite-inhibitory antibodies by immunization with recombinantly-expressed CyRPA. Malar J. 2016 Mar;15:161.

[57] Santolamazza F, Avellino P, Siciliano G, Yao FA, Lombardo F, Ouédraogo JB, et al. Detection of Plasmodium falciparum male and female gametocytes and determination of parasite sex ratio in human endemic populations by novel, cheap and robust RTqPCR assays. Malar J. 2017 Nov;16 (1):468.

[58] Tao D, McGill B, Hamerly T, Kobayashi T, Khare P, Dziedzic A, et al. A saliva-based rapid test to quantify the infectious subclinical malaria parasite reservoir. Sci Transl Med. 2019 01;11 (473). [PubMed Central:PMC6441545] [DOI:10.1126/scitranslmed.aan4479] [PubMed:21871864].

[59] Aninagyei E, Abraham J, Atiiga P, Antwi SD, Bamfo S, Acheampong DO. Evaluating the potential of using urine and saliva specimens for malaria diagnosis in suspected patients in Ghana. Malar J. 2020 Sep;19(1):349. [PubMed Central:PMC7526349] [DOI: 10.1186/s12936-020-03427-x] [PubMed: 24906879].

[60] Echeverry DF, Deason NA, Davidson J, Makuru V, Xiao H, Niedbalski J, et al. Human malaria diagnosis using a single-step direct-PCR based on the Plasmodium cytochrome oxidase III gene. Malar J. 2016 Feb;15: 128.

[61] Hofmann N, Mwingira F, Shekalaghe S, Robinson LJ, Mueller I, Felger I. Ultra-sensitive detection of *Plasmodium falciparum* by amplification of multi-copy subtelomeric targets. PLoS Med. 2015 Mar;12(3):e1001788. [PubMed Central:PMC4348198] [DOI: 10.1371/journal.pmed.1001788] [PubMed:15635001].

[62] Lloyd YM, Esemu LF, Antallan J, Thomas B, Tassi Yunga S, Obase B, et al. PCR-based detection of Plasmodium falciparum in saliva using mitochondrial cox3 and varATS primers. Trop Med Health. 2018;46:22.

[63] Schneider P, Reece SE, van Schaijk BC, Bousema T, Lanke KH, Meaden CS, et al. Quantification of female and male Plasmodium falciparum gametocytes by reverse transcriptase quantitative PCR. Mol Biochem Parasitol. 2015;199(1–2):

29–33. [DOI:10.1016/j.molbiopara.2015.03.006] [PubMed:25827756].

[64] Noedl H, Yingyuen K, Laoboonchai A, Fukuda M, Sirichaisinthop J, Miller RS. Sensitivity and specificity of an antigen detection ELISA for malaria diagnosis. Am J Trop Med Hyg. 2006 Dec;75(6):1205–1208.

[65] Chen F, Flaherty BR, Cohen CE, Peterson DS, Zhao Y. Direct detection of malaria infected red blood cells by surface enhanced Raman spectroscopy. Nanomedicine. 2016 08;12(6):1445–1451. [PubMed Central:PMC4955673] [DOI:10.1016/j.nano.2016.03.001] [PubMed:23563168].

[66] Haanshuus CG, Mørch K, Blomberg B, Strøm GEA, Langeland N, Hanevik K, et al. Assessment of malaria real-time PCR methods and application with focus on low-level parasitaemia. PLoS One. 2019;14(7):e0218982. [PubMed Central: PMC6611585] [DOI:10.1371/journal.pone.0218982] [PubMed:20925928].

[67] Fontanesi F, Soto IC, Barrientos A. Cytochrome c oxidase biogenesis: new levels of regulation. IUBMB Life. 2008 Sep;60(9):557–568.

[68] Schmedes SE, Patel D, Kelley J, Udhayakumar V, Talundzic E. Using the Plasmodium mitochondrial genome for classifying mixed-species infections and inferring the geographical origin of P. falciparum parasites imported to the U. S. PLoS One. 2019;14(4):e0215754.

[69] Figueiredo LM, Pirrit LA, Scherf A, Pirritt LA. Genomic organisation and chromatin structure of Plasmodium falciparum chromosome ends. Mol Biochem Parasitol. 2000 Feb;106(1):169–174.

[70] Gitta B, Kilian N. Diagnosis of Malaria Parasites Plasmodium spp. in Endemic Areas: Current Strategies for an Ancient Disease. Bioessays. 2020 01;42(1):e1900138. [DOI:10.1002/bies.201900138] [PubMed:31830324].

[71] Chen K, Yuen C, Aniweh Y, Preiser P, Liu Q. Towards ultrasensitive malaria diagnosis using surface enhanced Raman spectroscopy. Sci Rep. 2016 Feb;6:20177. [PubMed Central: PMC4746575] [DOI:10.1038/srep20177] [PubMed:18165483].

[72] Baldwin MR, Li X, Hanada T, Liu SC, Chishti AH. Merozoite surface protein 1 recognition of host glycophorin A mediates malaria parasite invasion of red blood cells. Blood. 2015 Apr;125(17):2704–2711.

[73] Zhong D, Afrane Y, Githeko A, Yang Z, Cui L, Menge DM, et al. Plasmodium falciparum genetic diversity in western Kenya highlands. Am J Trop Med Hyg. 2007 Dec;77(6):1043–1050.

[74] Somé AF, Bazié T, Zongo I, Yerbanga RS, Nikiéma F, Neya C, et al. Plasmodium falciparum msp1 and msp2 genetic diversity and allele frequencies in parasites isolated from symptomatic malaria patients in Bobo-Dioulasso, Burkina Faso. Parasit Vectors. 2018 May;11(1):323.

[75] Carter TE, Malloy H, Existe A, Memnon G, St Victor Y, Okech BA, et al. Genetic Diversity of Plasmodium falciparum in Haiti: Insights from Microsatellite Markers. PLoS One. 2015;10(10):e0140416.

[76] Fola AA, Kattenberg E, Razook Z, Lautu-Gumal D, Lee S, Mehra S, et al. SNP barcodes provide higher resolution than microsatellite markers to measure Plasmodium vivax population genetics. Malar J. 2020 Oct;19(1):375.

[77] Havryliuk T, Orjuela-Sánchez P, Ferreira MU. Plasmodium vivax: microsatellite analysis of multiple-clone infections. Exp Parasitol. 2008 Dec;120(4):330–336.

[78] Das S, Muleba M, Stevenson JC, Pringle JC, Norris DE. Beyond the entomological inoculation rate: characterizing multiple blood feeding behavior and *Plasmodium falciparum* multiplicity of infection in Anopheles mosquitoes in northern Zambia. Parasit Vectors. 2017 01;10(1):45. [PubMed Central:PMC5267472] [DOI:10.1186/s13071-017-1993-z] [PubMed:15619515].

[79] Abukari Z, Okonu R, Nyarko SB, Lo AC, Dieng CC, Salifu SP, et al. The Diversity, Multiplicity of Infection and Population Structure of P. falciparum Parasites Circulating in Asymptomatic Carriers Living in High and Low Malaria Transmission Settings of Ghana. Genes (Basel). 2019 06;10(6). [PubMed Central:PMC6628376] [DOI:10.3390/genes10060434] [PubMed:28070005].

[80] Lin JT, Hathaway NJ, Saunders DL, Lon C, Balasubramanian S, Kharabora O, et al. Using Amplicon Deep Sequencing to Detect Genetic Signatures of Plasmodium vivax Relapse. J Infect Dis. 2015 Sep;212(6):999–1008.

[81] Tessema SK, Raman J, Duffy CW, Ishengoma DS, Amambua-Ngwa A, Greenhouse B. Applying next-generation sequencing to track falciparum malaria in sub-Saharan Africa. Malar J. 2019 Sep;18(1):268. [PubMed Central:PMC6720407] [DOI:10.1186/s12936-019-2880-1] [PubMed:31298657].

[82] Miller RH, Hathaway NJ, Kharabora O, Mwandagalirwa K, Tshefu A, Meshnick SR, et al. A deep sequencing approach to estimate *Plasmodium falciparum* complexity of infection (COI) and explore apical membrane antigen 1 diversity. Malar J. 2017 Dec;16(1):490. [PubMed Central:PMC5732508] [DOI:10.1186/s12936-017-2137-9] [PubMed:21565059].

[83] Niedzicka M, Fijarczyk A, Dudek K, Stuglik M, Babik W. Molecular Inversion Probes for targeted resequencing in non-model organisms. Sci Rep. 2016 Apr;6:24051. [PubMed Central:PMC4820773] [DOI:10.1038/srep24051] [PubMed:21565059].

[84] Aydemir O, Janko M, Hathaway NJ, Verity R, Mwandagalirwa MK, Tshefu AK, et al. Drug-Resistance and Population Structure of *Plasmodium falciparum* Across the Democratic Republic of Congo Using High-Throughput Molecular Inversion Probes. J Infect Dis. 2018 08;218(6):946–955. [PubMed Central:PMC6093412] [DOI:10.1093/infdis/jiy223] [PubMed:25061906].

[85] Bettencourt P. Current Challenges in the Identification of Pre-Erythrocytic Malaria Vaccine Candidate Antigens. Front Immunol. 2020;11:190. [PubMed Central:PMC7046804] [DOI:10.3389/fimmu.2020.00190] [PubMed:9252123].

[86] Palacpac NMQ, Horii T. Malaria vaccines: facing unknowns. F1000Res. 2020;9. [PubMed Central:PMC7194457] [DOI:10.12688/f1000research.22143.1] [PubMed:23613845].

[87] Hoffman SL, Vekemans J, Richie TL, Duffy PE. The march toward malaria vaccines. Vaccine. 2015 Nov;33 Suppl 4:13–23. [PubMed Central:PMC5634526] [DOI:10.1016/j.vaccine.2015.07.091] [PubMed:24872326].

[88] Draper SJ, Sack BK, King CR, Nielsen CM, Rayner JC, Higgins MK, et al. Malaria Vaccines: Recent Advances and New Horizons. Cell Host Microbe. 2018 07;24(1):43–56.

[89] Tannous S, Ghanem E. A bite to fight: front-line innate immune defenses against malaria parasites. Pathog Glob Health. 2018 02;112(1):1–12.

[90] Beeson JG, Kurtovic L, Dobaño C, Opi DH, Chan JA, Feng G, et al. Challenges and strategies for developing efficacious and long-lasting malaria

vaccines. Sci Transl Med. 2019 01;11 (474). [DOI:10.1126/scitranslmed.aau1458] [PubMed:30626712].

[91] Sánchez L, Vidal M, Jairoce C, Aguilar R, Ubillos I, Cuamba I, et al. Erratum: Author Correction: Antibody responses to the RTS,S/AS01E vaccine and *Plasmodium falciparum* antigens after a booster dose within the phase 3 trial in Mozambique. NPJ Vaccines. 2020;5:63. [PubMed Central: PMC7371631] [DOI:10.1038/s41541-020-00213-3] [PubMed:32704386].

[92] Molina-Franky J, Cuy-Chaparro L, Camargo A, Reyes C, Gómez M, Salamanca DR, et al. *Plasmodium falciparum* pre-erythrocytic stage vaccine development. Malar J. 2020 Feb; 19(1):56. [PubMed Central: PMC6998842] [DOI:10.1186/s12936-020-3141-z] [PubMed:24992338].

[93] Skwarczynski M, Chandrudu S, Rigau-Planella B, Islam MT, Cheong YS, Liu G, et al. Progress in the Development of Subunit Vaccines against Malaria. Vaccines (Basel). 2020 Jul;8(3). [PubMed Central: PMC7563759] [DOI:10.3390/vaccines8030373] [PubMed:30219621].

[94] Blank A, Furle K, Jaschke A, Mikus G, Lehmann M, Husing J, et al. Immunization with full-length *Plasmodium falciparum* merozoite surface protein 1 is safe and elicits functional cytophilic antibodies in a randomized first-in-human trial. NPJ Vaccines. 2020;5:10. [PubMed Central: PMC6994672] [DOI:10.1038/s41541-020-0160-2] [PubMed:26468747].

[95] Duffy PE, Patrick Gorres J. Malaria vaccines since 2000: progress, priorities, products. NPJ Vaccines. 2020;5:48. [PubMed Central:PMC7283239] [DOI: 10.1038/s41541-020-0196-3] [PubMed: 24133188].

[96] Laurens MB. RTS,S/AS01 vaccine (Mosquirixâ¡U+0084¿¢): an overview. Hum Vaccin Immunother. 2020 03;16 (3):480–489. [PubMed Central: PMC7227679] [DOI:10.1080/21645515.2019.1669415] [PubMed: 30376866].

[97] Singh K, Burkhardt M, Nakuchima S, Herrera R, Muratova O, Gittis AG, et al. Structure and function of a malaria transmission blocking vaccine targeting Pfs230 and Pfs230-Pfs48/45 proteins. Commun Biol. 2020 Jul;3(1):395. [PubMed Central:PMC7381611] [DOI: 10.1038/s42003-020-01123-9] [PubMed:24179220].

[98] Scally SW, McLeod B, Bosch A, Miura K, Liang Q, Carroll S, et al. Molecular definition of multiple sites of antibody inhibition of malaria transmission-blocking vaccine antigen Pfs25. Nat Commun. 2017 11;8(1):1568.

[99] Raj DK, Das Mohapatra A, Jnawali A, Zuromski J, Jha A, Cham-Kpu G, et al. Anti-PfGARP activates programmed cell death of parasites and reduces severe malaria. Nature. 2020 06;582 (7810):104–108. [PubMed Central: PMC7372601] [DOI:10.1038/s41586-020-2220-1] [PubMed:32427965].

[100] Ragotte RJ, Higgins MK, Draper SJ. The RH5-CyRPA-Ripr Complex as a Malaria Vaccine Target. Trends Parasitol. 2020 06;36(6):545–559. [PubMed Central:PMC7246332] [DOI: 10.1016/j.pt.2020.04.003] [PubMed: 26458807].

[101] Zheng J, Pan H, Gu Y, Zuo X, Ran N, Yuan Y, et al. Prospects for Malaria Vaccines: Pre-Erythrocytic Stages, Blood Stages, and Transmission-Blocking Stages. Biomed Res Int. 2019; 2019:9751471. [PubMed Central: PMC6794966] [DOI:10.1155/2019/9751471] [PubMed:29700348].

[102] Zhao J, Bhanot P, Hu J, Wang Q. A Comprehensive Analysis of Plasmodium Circumsporozoite Protein Binding to Hepatocytes. PLoS One. 2016;11(8):

e0161607. [PubMed Central: PMC4999272] [DOI:10.1371/journal.pone.0161607] [PubMed:8876233].

[103] White MT, Verity R, Griffin JT, Asante KP, Owusu-Agyei S, Greenwood B, et al. Immunogenicity of the RTS,S/AS01 malaria vaccine and implications for duration of vaccine efficacy: secondary analysis of data from a phase 3 randomised controlled trial. Lancet Infect Dis. 2015 Dec;15(12):1450–1458. [PubMed Central:PMC4655306] [DOI:10.1016/S1473-3099(15)00239-X] [PubMed:25012228].

[104] Mahmoudi S, Keshavarz H. Efficacy of phase 3 trial of RTS, S/AS01 malaria vaccine: The need for an alternative development plan. Hum Vaccin Immunother. 2017 09;13(9):2098–2101.

[105] authors listed N. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. Lancet. 2015 Jul;386(9988):31–45. [PubMed Central: PMC5626001] [DOI:10.1016/S0140-6736(15)60721-8] [PubMed:21816029].

[106] van den Berg M, Ogutu B, Sewankambo NK, Biller-Andorno N, Tanner M. RTS,S malaria vaccine pilot studies: addressing the human realities in large-scale clinical trials. Trials. 2019 May;20(1):316. [PubMed Central: PMC6544944] [DOI:10.1186/s13063-019-3391-7] [PubMed:30035349].

[107] Collins KA, Snaith R, Cottingham MG, Gilbert SC, Hill AVS. Enhancing protective immunity to malaria with a highly immunogenic virus-like particle vaccine. Sci Rep. 2017 04;7:46621.

[108] Kaushansky A, Douglass AN, Arang N, Vigdorovich V, Dambrauskas N, Kain HS, et al. Malaria parasites target the hepatocyte receptor EphA2 for successful host infection. Science. 2015 Nov;350(6264):1089–1092. [PubMed Central:PMC4783171] [DOI:10.1126/science.aad3318] [PubMed:19625622].

[109] Richie TL, Billingsley PF, Sim BK, James ER, Chakravarty S, Epstein JE, et al. Progress with *Plasmodium falciparum* sporozoite (PfSPZ)-based malaria vaccines. Vaccine. 2015 Dec;33(52):7452–7461. [PubMed Central: PMC5077156] [DOI:10.1016/j.vaccine.2015.09.096] [PubMed:21514658].

[110] Lyke KE, Ishizuka AS, Berry AA, Chakravarty S, DeZure A, Enama ME, et al. Attenuated PfSPZ Vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection. Proc Natl Acad Sci U S A. 2017 03;114(10):2711–2716.

[111] Zenklusen I, Jongo S, Abdulla S, Ramadhani K, Lee Sim BK, Cardamone H, et al. Immunization of Malaria-Preexposed Volunteers With PfSPZ Vaccine Elicits Long-Lived IgM Invasion-Inhibitory and Complement-Fixing Antibodies. J Infect Dis. 2018 04;217(10):1569–1578.

[112] Halbroth BR, Sebastian S, Salman AM, Ulaszewska M, Gola A, Longley RJ, et al. Preclinical Development and Assessment of Viral Vectors Expressing a Fusion Antigen of *Plasmodium falciparum* LSA1 and LSAP2 for Efficacy against Liver-Stage Malaria. Infect Immun. 2020 01;88(2). [PubMed Central:PMC6977128] [DOI:10.1128/IAI.00573-19] [PubMed:18159254].

[113] Pratt-Riccio LR, De Souza Perce-Da-Silva D, Da Costa Lima-Junior J, Pratt Riccio EK, Ribeiro-Alves M, Santos F, et al. Synthetic Antigens Derived from *Plasmodium falciparum* Sporozoite, Liver, and Blood Stages: Naturally Acquired Immune Response and Human Leukocyte Antigen Associations in Individuals Living in a

Brazilian Endemic Area. Am J Trop Med Hyg. 2017 Nov;97(5):1581–1592. [PubMed Central:PMC5817778] [DOI: 10.4269/ajtmh.17-0359] [PubMed: 16111789].

[114] Morita M, Takashima E, Ito D, Miura K, Thongkukiatkul A, Diouf A, et al. Immunoscreening of *Plasmodium falciparum* proteins expressed in a wheat germ cell-free system reveals a novel malaria vaccine candidate. Sci Rep. 2017 04;7:46086. [PubMed Central: PMC5380959] [DOI:10.1038/ srep46086] [PubMed:19874576].

[115] Lin CS, Uboldi AD, Marapana D, Czabotar PE, Epp C, Bujard H, et al. The merozoite surface protein 1 complex is a platform for binding to human erythrocytes by Plasmodium falciparum. J Biol Chem. 2014 Sep;289 (37):25655–25669.

[116] Wang YN, Lin M, Liang XY, Chen JT, Xie DD, Wang YL, et al. Natural selection and genetic diversity of domain I of *Plasmodium falciparum* apical membrane antigen-1 on Bioko Island. Malar J. 2019 Sep;18(1):317. [PubMed Central:PMC6751645] [DOI: 10.1186/s12936-019-2948-y] [PubMed: 8910611].

[117] Srinivasan P, Baldeviano GC, Miura K, Diouf A, Ventocilla JA, Leiva KP, et al. A malaria vaccine protects Aotus monkeys against virulent Plasmodium falciparum infection. NPJ Vaccines. 2017;2.

[118] Sirima SB, Durier C, Kara L, Houard S, Gansane A, Loulergue P, et al. Safety and immunogenicity of a recombinant *Plasmodium falciparum* AMA1-DiCo malaria vaccine adjuvanted with GLA-SE or AlhydrogelÂ® in European and African adults: A phase 1a/1b, randomized, double-blind multi-centre trial. Vaccine. 2017 10;35(45): 6218–6227.

[119] Wrighton KH. A novel vaccine target for malaria. Nat Rev Drug Discov. 2020 06;19(6):386. [DOI:10.1038/ d41573-020-00082-4] [PubMed: 32355320].

[120] Favuzza P, Guffart E, Tamborrini M, Scherer B, Dreyer AM, Rufer AC, et al. Structure of the malaria vaccine candidate antigen CyRPA and its complex with a parasite invasion inhibitory antibody. Elife. 2017 02;6.

[121] Huang WC, Deng B, Mabrouk MT, Seffouh A, Ortega J, Long C, et al. Particle-based, Pfs230 and Pfs25 immunization is effective, but not improved by duplexing at fixed total antigen dose. Malar J. 2020 Aug;19(1): 309.

[122] McLeod B, Miura K, Scally SW, Bosch A, Nguyen N, Shin H, et al. Potent antibody lineage against malaria transmission elicited by human vaccination with Pfs25. Nat Commun. 2019 09;10(1):4328. [PubMed Central: PMC6760140] [DOI:10.1038/s41467-019-11980-6] [PubMed:31551421].

[123] Chichester JA, Green BJ, Jones RM, Shoji Y, Miura K, Long CA, et al. Safety and immunogenicity of a plant-produced Pfs25 virus-like particle as a transmission blocking vaccine against malaria: A Phase 1 dose-escalation study in healthy adults. Vaccine. 2018 09;36 (39):5865–5871.

[124] Patra KP, Li F, Carter D, Gregory JA, Baga S, Reed SG, et al. Alga-produced malaria transmission-blocking vaccine candidate Pfs25 formulated with a human use-compatible potent adjuvant induces high-affinity antibodies that block Plasmodium falciparum infection of mosquitoes. Infect Immun. 2015 May;83(5):1799–1808.

[125] Singh SK, Thrane S, Chourasia BK, Teelen K, Graumans W, Stoter R, et al.

Pfs230 and Pfs48/45 Fusion Proteins Elicit Strong Transmission-Blocking Antibody Responses Against Plasmodium falciparum. Front Immunol. 2019;10:1256.

[126] World Health Organization. Global targets; 2019. Available from: https://www.who.int/malaria/areas/global targets/en/.