INSIGHTS INTO *PLASMODIUM* GENETIC CHANGES RELATED TO MALARIA INTERVENTIONS

by

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ABSTRACT

CHEIKH CAMBEL DIENG. Insights into *Plasmodium* Genetic Changes related to Malaria Interventions. (Under the direction of DR. EUGENIA LO)

Over 80% of all malaria deaths occur in children under 5 years of age. Malaria control strategies have been progressively shifted to specific populations and/or areas to maximize effectiveness. Malaria is a significant public health problem in Ghana. Seasonal Malaria Chemoprevention (SMC) using a combination of sulfadoxine-pyrimethamine and amodiaquine has been implemented since 2015 in northern Ghana where malaria transmission is intense and seasonal. In this study, we estimated the prevalence of asymptomatic P. falciparum carriers in three ecological zones of Ghana, and compared the sensitivity and specificity of different molecular methods in identifying asymptomatic infections. Moreover, we examined the frequency of mutations in pfcrt, pfmdr1, pfdhfr, and pfdhps that relate to the ongoing SMC. A total of 535 asymptomatic schoolchildren were screened by microscopy and PCR (18s rRNA and TARE-2) methods. Among all samples, 28.6% were detected as positive by 18S nested PCR, whereas 19.6% were detected by microscopy. A high PCR-based asymptomatic prevalence was observed in the north (51%) compared to in the central (27.8%) and south (16.9%). The prevalence of pfdhfr-N51I/C59R/S108N/pfdhps-A437G quadruple mutant associated with sulfadoxine-pyrimethamine resistance was significantly higher in the north where SMC was implemented. Compared to 18S rRNA, TARE-2 serves as a more sensitive molecular marker for detecting submicroscopic asymptomatic infections in high and low transmission settings. These findings establish a baseline for monitoring P. falciparum prevalence and resistance in response to SMC over time.

Ghana is also one of the three African countries where the world's first malaria vaccine, RTS, S, was launched recently. The vaccine contains part of the central repeat region and the complete C-terminal of the circumsporozoite protein (CSP)

gene of the 3D7 strain. Polymorphism in the PfCSP protein has been reported from several parts of the world. However, whether RTS, S-induced immunity is PfCSP allele-dependent and if selection favors non-3D7 strains are unclear. This study aims to examine the genetic polymorphism of the PfCSP genes in clinical P. falciparum cases and provide a baseline of parasite diversity prior to vaccine implementation in Ghana. A total of 212 clinical samples were collected from Seikwa located in the Brong Afrong region where the vaccine is currently being deployed. Preliminary data indicated a high rate of polyclonal infections, with some samples harboring up to 3 clones based on the allele frequency among mapped reads. Parasite clones detected within the same host were not genetically similar to one another. Instead, they were distributed in various subclades and closely related to clones identified from other hosts. It is yet to be investigated if the high PfCSP haplotype diversity and low resemblance to the 3D7 strain have an impact on the anti-CSP immune response and thereby the efficacy of RTS,S.

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ABSTRACT

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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.

1.1 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, diagnos-

tic 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.

1.2 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]. Al-

though 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 con-

tribute 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.

1.3 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 parasitesμl of blood (approximately 0.001% parasitemia, assuming an erythrocyte count of 5 $\times$ 106 cellsμl) [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 *18s* 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 *18s* 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.

1.3.1 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, 40, 41, 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.

1.3.2 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

Table 1.1: Existing malaria diagnostic tests and their respective performance.

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	*18s* rRNA	64	92	Laboratory	0.5–5 parasites /μL of blood	[50]
PCR	*COX3*	-	-	Laboratory	0.6–2 parasites /μL blood	[50]
PCR	*TARE-2*	81	49	Laboratory	6–24 parasites in 200μL whole blood	[51]
PCR	*var*ATS	-	-	Laboratory	12–30 parasites in 200μL whole blood	[52]
PCR	*Pfs25*	-	-	Laboratory	0.3 mature females /μL blood	[53]
ELISA	*LDH*	69.9	100	Laboratory	3 parasites /μL blood	[54, 55]
LFIA	*Pfs25*	-	-	Laboratory	0.02 gametocytes /μl blood	[56]
SERS	Hemozoin	-	-	Laboratory	30 parasites /μL of blood	[57]

- *Denotes that the sensitivity or specificity is not reported.*

bonding affinity to proteins on a solid surface [58]. Samples, including and positive and negative controls are inserted on a 96 well plate which enables the possibility to analyse multiple samples simultaneously, then samples get incubated and detection of the signal is generated directly or indirectly via secondary tag on the specific antibody [59]. 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 [60]. 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) [54]. However, ELISA has a lower sensitivity (69.9%) to antigens than RDT (88%) [54], and can be expensive and require a trained technician to operate and interpret the results [54].

1.3.3 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 [61]. 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% [62] of the infectious reservoirs. Previous studies have shown that infections with parasitemia as low as 4 gametocytes/μl can sustain transmission [63]. 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 [64]. 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 [56], [35]. 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 [56]. 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 [65]. Although convenient, *pfhrp2*-based LFIA could be less useful in detecting asymptomatic infections.

1.3.4 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 1.1). 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 1.1).

1.3.4.1 *18s* rRNA

The *18s* rRNA genes have been the main gene target for molecular screening as it contains 5–8 copies per genome [52] in *Plasmodium*, but recent studies indicated a relatively high level of misdiagnosed infections [66]. The advantage of using the *18s* 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 *18s* rRNA.

1.3.4.2 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]. More importantly, there are around 20 to 150 copies of the *COX3* gene in the *Plasmodium* genome [52] and PCR analysis of this gene indicated a detection limit of 0.6–2 parasites/μL, much more sensitive than the *18s*-rRNA [50].

1.3.4.3 Telomere Associated Repetitive Element 2 (*TARE-2*)

The recently discovered Telomere Associated Repetitive Element 2 (*TARE-2*) has demonstrated better performance than the conventional *18s* 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 [51]. A recent study in Ghana showed a slightly higher sensitivity of *TARE-2* compared to *18s* 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.

1.3.4.4 var gene acidic terminal sequence (*var*ATS)

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) [51]. The *var*ATS has a detection limit of 12–30 para-

sites in 200 μL whole blood. Though more sensitive *18s* rRNA, *var*ATS employs a qPCR approach that could be less feasible for routine case detection in rural areas or developing countries [52].

1.3.5 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 [57]. 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.

1.4 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].

1.4.1 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 [80] and discrimination of major from minor clones [81]. It also provides an increased capability to detect the genetic relatedness among clones within and between hosts as well as minor *P. falciparum* variants [82]. 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 [80].

1.4.2 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 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.

Table 1.2: Gene mutations associated with antimalarial drug resistance.

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

1.5 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.

1.5.1 Pre-erythrocytic vaccines

Pre-erythrocytic vaccines aim to kill infected hepatocytes and prevent sporozoites from reaching the liver [91].There are various gene targets currently being investigated and in clinical trials.

1.5.1.1 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 Organization (WHO) in Malawi, Ghana, and Kenya to further investigate vaccine efficacy [92]. 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 [93]. 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 [94]. After vaccination, the host immune system will response to *PfCSP* antigen by producing anti-CSP antibodies and activating CD4+ T cells [95]. RTS,S/AS01E is given on a three-dose schedule within three months followed by a fourth dose at 20 months [94]. 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 [96] [97], [98]. 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 [94]. 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 [92]. 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 [92]. Another similar vaccine R21 is currently testing in Phase 1/2a clinical trials [99]. 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 [99]. It has been shown to increase the production of T cells and is still in the process of development [92]. 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.

1.5.1.2 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 [100]. *PfSPZ* vaccine prevents the sporozoites reaching the liver and infecting hepatocytes [101], and is currently undergoing phase III clinical trial [102]. *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 [103]. The vaccine induced interferon gamma (IFN-γ) that can recruit and activate CD8+ and CD4+ T cells against homologous and heterologous parasitic strains [103]. Subjects who received the *PfSPZ* vaccine also developed IgM antibod-

ies that can help inhibit proliferation of the parasites [104]. However, the vaccine requires specific storage in ultra-cold condition and trained medical workers to inject the vaccine intravenously [92]. 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 [102]. Further studies are needed to compare efficacy among different geographical or transmission settings.

1.5.1.3 *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 [105]. 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 [106], [105]. LSA-1 can induce IgG and IgM antibodies as well as CD4+ T cell production [107], [105]. 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 [108]. LSA-3 appears to play a role in the parasitic growth in infected hepatocytes and erythrocytes [108]. A recent study indicated that LSA-3 provided full protect to chimpanzees from heterologous *P. falciparum* sporozoites [105]. Like LSA-1, LSA-3 is also in phase I clinical trial and its efficacy and immunogenicity in humans remain unclear.

1.5.2 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.

1.5.2.1 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 [109]. MSP1 plays an important role in the invasion of erythrocytes as it binds and recruits other peripheral merozoite surface proteins to form a complex [109]. MSP1 starts off as a precursor of about 196 kDa and then cleave into four subunits before invading the erythrocytes [110]. The four subunits are held non-covalently forming a complex attached to the merozoite's GPI anchor [110]. Once the MSP1 complex is formed, it binds with the receptors on erythrocytes and activates a spectrin-binding function to enter the erythrocytes [110]. 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 [110]. 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 [110]. To date, MSP1 vaccine is in phase I clinical trial. Further investigation is needed to evaluate efficacy.

1.5.2.2 PfAMA-1

The erythrocytic vaccine based on Apical Membrane Antigen 1 (AMA1) is currently in phase II clinical trial [111]. The AMA1 protein is approximately 83 kDa and can be found in both the merozoite and sporozoite stages of *P. falciparum* [111]. 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 [112]. 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 [112]. 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 [113]. To increase efficacy of the AMA1 vaccine, AMA1 was paired with the RON2 receptor and vaccinated in eight *Aotus* monkeys [112]. Half of the monkeys were able to achieve complete immunity from the *P. falciparum* infection when vaccinated with AMA1 and RON2 [112]. 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.

1.5.2.3 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 [114]. 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 [115]. 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 [115]. 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* [115].

1.5.2.4 *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 [116]. *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 [116]. Monoclonal antibodies against *PfRH5* has been found to disrupt the binding between basigin and *PfRH5* [116]. *P. falciparum* Cysteine-rich Protective Antigen (*PfCyRPA*) is a highly conserved 43 kDa protein [116]. It plays an important role in erythrocyte invasion by interacting with *PfRH5* to bind to the receptor basigin [63]. Although *PfCyRPA* is not immunogenic compared to *PfRipr* and *PfRH5*, monoclonal antibodies produced against this protein can cause cross strain neutralization [116]. *P. falciparum* RH5-interacting Protein (*PfRipr*) is a highly conserved 120 kDa protein found in the schizont stage [116]. It is composed of 87 cysteines and 10 epidermal growth factor-like (EGF) domains [116]. 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 [116]. Antibodies against *PfRipr* EGF domains 6-8 have been shown to neutralize the parasites [116]. 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* [117].

1.5.3 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.

1.5.3.1 *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 [118]. The parasites require *Pfs25* to survive in the *Anopheles*'s midgut and develop into oocysts [119]. *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 [120]. 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 [121]. 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 [122]. Further studies should examine the formulation of this vaccine for better efficacy.

1.5.3.2 *Pfs230* and *Pfs48/45*

Pfs230 and *Pfs48/45*-based vaccines are antigens rich in cysteine produced by the sexual stage gametocytes [123]. *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* [124]. *Pfs230* is a 230 kDa protein that contains 14 6-cysteine rich domains [123]. *Pfs48/45*, on the other hand, contains three 6-cysteine rich domains [124]. The 6-cysteine rich domains of these antigens are essential for the formation of disulfide bonds on epitopes needed for antibody elicitation [124]. A recent study showed that mice injected with fragments of *Pfs48/45* and *Pfs230* prodomain produced higher levels of antibodies that induced complement fixation [124]. *Pfs230*

and *Pfs48/45* combined with a FAB fragment of a monoclonal antibody 4F12 have been shown to further increase vaccine efficacy [123]. This vaccine is currently in early clinical development phase [124], [123].

1.6 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 [125]. 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.

CHAPTER 2: Contrasting Asymptomatic and Drug Resistance Gene Prevalence of *Plasmodium falciparum* in Ghana: Implications on Seasonal Malaria Chemoprevention

2.1 Abstract

Malaria is a significant public health problem in Ghana. Seasonal Malaria Chemoprevention (SMC) using a combination of sulfadoxine-pyrimethamine and amodiaquine has been implemented since 2015 in northern Ghana where malaria transmission is intense and seasonal. In this study, we estimated the prevalence of asymptomatic *P. falciparum* carriers in three ecological zones of Ghana, and compared the sensitivity and specificity of different molecular methods in identifying asymptomatic infections. Moreover, we examined the frequency of mutations in *Pfcrt*, *Pfmdr1*, *Pfdhfr*, and *Pfdhps* that relate to the ongoing SMC. A total of 535 asymptomatic schoolchildren were screened by microscopy and PCR (18s rRNA and TARE-2) methods. Among all samples, 28.6% were detected as positive by 18S nested PCR, whereas 19.6% were detected by microscopy. A high PCR-based asymptomatic prevalence was observed in the north (51%) compared to in the central (27.8%) and south (16.9%). The prevalence of *Pfdhfr*-N51I/C59R/S108N/*Pfdhps*-A437G quadruple mutant associated with sulfadoxine-pyrimethamine resistance was significantly higher in the north where SMC was implemented. Compared to 18S rRNA, TARE-2 serves as a more sensitive molecular marker for detecting submicroscopic asymptomatic infections in high and low transmission settings. These findings establish a baseline for monitoring *P. falciparum* prevalence and resistance in response to SMC over time.

2.2 Introduction

In 2018, an estimated 219 million cases of malaria occurred worldwide. Among them, 435,000 malaria-related deaths and 92% of the global malaria burden occurred in sub-Saharan Africa 1,[126]. *Plasmodium falciparum* is responsible for the majority of malaria morbidity and mortality. In the last decades, a number of chemotherapeutic agents and insecticides have been used in the control of transmission. However, malaria still remains endemic in many parts of Africa. The rapidly emerging resistance of both the vector mosquitoes and *P. falciparum* has severely impacted the effectiveness of control measures. In addition, socio-economic factors such as poverty and poor healthcare infrastructure, as well as insufficient educational and monitoring systems, make malaria elimination challenging especially in remote areas of many African countries.

In Ghana, malaria is a significant public health problem, especially in rural areas. The President's Malaria Initiative, Ghana has employed various control strategies to reduce malaria infection. These include free malaria diagnosis and treatment across the country, as well as routine indoor sprays of insecticides against mosquito vectors and distribution of free long-lasting insecticidal bednets in the north [3]. These strategies have resulted in striking reductions in a number of clinical cases. The percentage of outpatient attendance in public health facilities decreased sharply from 48% in 2008 to 28% in 2016 [4]. However, malaria prevalence still remains high in some parts of the country. A multiple cluster survey conducted in 2011 has shown a wide variation in parasite prevalence between the north (51%) and south (4%) of Ghana [127]. Such a variation could be explained by a combination of factors such as vector abundance and distribution, climate, environment, land use change, socioeconomic difference, and human movement. For instance, in the coastal (south) and forest (central) areas, malaria transmission is perennial, whereas in the savannah zone (north), malaria transmission is highly seasonal and intense during/after the rainy

season (June–October) [128]. The contrasting pattern of malaria transmission plays a key role in determining and implementing malaria control measures in Ghana.

In highly malaria-endemic areas of Ghana, individuals are frequently exposed to malaria parasites and acquire protective immunity. These individuals provide a parasite reservoir and can initiate transmission [129]. Due to low parasite density, asymptomatic infections are usually less detectable by microscopy or rapid diagnostic tests. Though less sensitive, microscopy is the first-line diagnostic tool for malaria in many developing countries because of its convenience and affordability. Previous studies have shown that over 20% of microscopic-negative samples were positive when diagnosed by PCR [130]. In most cases, asymptomatic individuals are left undiagnosed and untreated, which severely impedes malaria control and elimination. A conventional molecular method such as 18S rRNA nested PCR has been shown to increase sensitivity of detecting submicroscopic infections [131]. Recently, an ultra-sensitive marker Telomere Associated Repetitive Element 2 (TARE-2) has demonstrated better performance than the conventional 18S rRNA marker in detecting low density parasite infections, especially in active case surveillance [51]. TARE-2 is a high-copy telomere-associated repetitive element that has 250 copies per genome [69], considerably more abundant than the 18S rRNA with only 5–8 copies per genome in *P. falciparum* [132]. This marker has been previously shown to be useful for detecting ultra-low-density *P. falciparum* infections in Papua New Guinea and Tanzania [51]. It was our goal to determine the most sensitive method for detecting asymptomatic infections in high transmission areas of Ghana in this study.

For decades, chloroquine (CQ) had been used as the first-line treatment for malaria in Ghana. However, the emergence and spread of CQ resistance led to the introduction of artemisinin-based combination therapy (ACT) [133]. Currently, the first line of drug for the treatment of non-severe *P. falciparum* malaria in Ghana is the combination of artesunate and amodiaquine [14]. In March 2012, the World

Health Organization (WHO) recommended a new intervention against *P. falciparum* malaria in children during the peak transmission season known as Seasonal Malaria Chemoprevention (SMC), where children under five-years-old are given a single dose of sulfadoxine-pyrimethamine (SP) combined with a 3-day course of amodiaquine (AQ) once a month for up to 4 months [15]. SMC has been introduced in 12 African countries since its first announcement. In Burkina Faso, SMC have been shown to be effective in reducing the prevalence of malaria and anemia among children, as well as the occurrence of fever episodes [134]. In Ghana, considering the variation in transmission pattern between the northern savannah and the southern coastal region, the authorities implemented SMC in the north in July 2015 [127],17. A previous report on sulfadoxine-pyrimethamine resistance in Ghana was documented in 1988, long before the introduction of SMC [135]. It is yet unclear as to whether the SMC has imposed any impact or selective pressure on the parasite genomes.

It has been well documented that antimalarial resistance is tightly associated with specific mutations in the *P. falciparum* genome. For instance, mutation at *P. falciparum* chloroquine resistance transporter (*Pfcrt*) codon 76T is associated with CQ resistance [136]. Mutation at multidrug resistance transporter (*Pfmdr1*) codons 86Y and 184F is associated with AQ resistance [133]. Mutations in *P. falciparum* dihydrofolate reductase (*Pfdhfr*) have been known for several years to decrease *in vitro* and clinical *P. falciparum* susceptibility to pyrimethamine [137]. The three mutations N51I, C59R, and S108N in combination are referred to as *Pfdhfr* triplet. Sulfadoxine is associated with mutations at codons N51I, C59R, S108N in *Pfdhfr*, and pyrimethamine is associated with mutations at A437G and K540E mutations in the dihydropteroate synthase (*Pfdhps*) gene. The latter two mutations together are referred to as *Pfdhps* double [138]. The combination of *Pfdhfr* triple and *Pfdhps* double are known as the quintuple mutation. In vivo and in vitro experiments have shown that SP resistance is tightly correlated to *Pfdhfr* triple and *Pfdhps* double mutations

[139].

In this study, we (1) determined the asymptomatic prevalence of *P. falciparum* across the three ecological zones, i.e., the northern savannah, central forest, and southern coast of Ghana; (2) compared the sensitivity and specificity of different molecular methods in order to provide an accurate estimate of asymptomatic prevalence especially in low transmission settings and; (3) examined the frequency of *Pfcrt*, *Pfmdr1*, *Pfdhfr*, and *Pfdhps* mutations that relates to the antimalarial drugs used in the ongoing SMC. These findings will establish a baseline for monitoring *P. falciparum* prevalence and resistance in response to SMC over time.

2.3 Materials and Methods

2.3.1 Ethics Statement

Scientific and ethical approval was given by the Institutional Scientific and Ethical Review boards of the Noguchi Memorial Institute of Medical Research, University of Ghana, Ghana and the University of North Carolina at Charlotte, USA (IRB00001276). Written informed consent/assent for study participation was obtained from all consenting parents/guardians (for minors under the age of 18), and each individual who was willing to participate in the study. All methods were reviewed and approved by the institutional review board (IRB) and performed in accordance with the relevant guidelines and regulations stated in the IRB protocols.

2.3.2 Sampling and Study Sites

Five sites were selected from three ecological zones of Ghana. These included Pagaza (PZ) in Tamale Municipality and Kpalsogou (KG) in Kumbungu district in the northern savannah region; Duase (KD) in Konongo district in the central forest region, and Ada (AD) and Dodowa (DO) in the southern coastal region. Sample collection was conducted during June-September of 2017. Finger-prick blood samples were collected from 535 schoolchildren aged 5 to 14 years old who present no fever

or malaria-related symptoms at the time of collection. Thick and thin blood smears were prepared for microscopic examination and 30–50 µL of blood was blotted on Whatman 3MM filter papers. Filter papers were air-dried and stored in zip-sealed plastic bags with silica gel absorbent at room temperature until DNA extraction.

2.3.3 Microscopic and Molecular Screening

Blood smears were examined at a magnification of 100x under microscopes. Parasites were counted against 200 leukocytes and a smear was considered negative when no parasites were observed after counting at least 100 microscopic fields. The density of parasites (parasitaemia) was expressed as the number of asexual *P. falciparum* per microliter of blood, assuming a leukocyte count of 8000/µL.

Parasite DNA was extracted from dried blood spots by the Saponin/Chelex method [140]. The final extracted volume was 200 µL. Molecular screening of *P. falciparum* was diagnosed by three PCR assays. A nested PCR approach of the *P. falciparum* 18S rRNA gene using previously published primers was used (Supplementary Table S1). In addition, the SYBR Green quantitative real-time PCR (qPCR) assays of the 18S rRNA (5–8 copies per genome) [132] and the high-copy telomere-associated repetitive element 2 TARE-2 (250 copies per genome) [69] were used to screen all the samples following the published protocols. DNA from *P. falciparum* isolates 7G8 (MRA-926) and HB3 (MRA-155) were used as positive controls in all amplifications, and water and uninfected samples were used as a negative control to ensure a lack of contamination. Samples yielding a threshold cycle (Ct) higher or equal to 40 were considered negative for *P. falciparum*. The parasite density in a sample was quantified based on the threshold cycle using the following equation: $2\ \mathrm{E}\times(40\text{-Ct sample})$, where Ct represents the threshold cycle of the sample and E, the amplification efficiency. The parasite density of samples in each site was reported as geometric mean and range values.

2.3.4 Assessing Sensitivity and Specificity

To evaluate the sensitivity and specificity of the different diagnostic methods in detecting positive asymptomatic samples, a set of 135 samples from the northern savannah sites (KG and PZ) were used. These samples were selected for marker sensitivity comparisons because they were collected from a highly endemic region where asymptomatic infections were expected to be high. The 135 samples were first screened by microscopy, and then 18S rRNA nested PCR followed by 18S rRNA qPCR and TARE-2 qPCR methods. The percentages of positive and negative infections were recorded and compared among these methods. Sensitivity and specificity tests were performed with MedCalc software (version 12; Mariakerke, Belgium). TARE-2 was used as the gold standard given that this gene contains 250 copies in the *P. falciparum* genome and has been previously shown to be an ultra-sensitive marker in detecting low density *P. falciparum* infections [69]. The sensitivity values were calculated as true positives/(true positive + false negatives), and specificity values were calculated as true negatives/(true negatives + false positives).

2.3.5 Resistance gene Mutation Frequency

Polymorphisms in the *Pfcrt* and *Pfmdr1* were assessed by nested PCR using previously published primers and protocols [141]. A PCR-restriction fragment length polymorphism assay was then used to assess the mutations at codon 76 of *Pfcrt*. Digestion fragments were visualized on a 2% agarose gel. For codons 86 and 184 of *Pfmdr1*, PCR amplicons (603 bp) were purified and sequenced in an ABI 3730xl DNA analyzer. Likewise, the *Pfdhfr* and *Pfdhps* genes were amplified and sequenced using previously published protocols [142]. Mutations at *Pfdhfr* codons 57, 59, and 108, as well as *Pfdhps* codons 437 and 540 were assessed. All sequences were aligned against the *P. falciparum* reference 3D7 strain using Bioedit Sequence Alignment Editor [**?**]. Chi-square test was used to test for significant differences in mutation prevalence for

each of the gene codons among study sites. All statistical analyses were performed in GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

2.4 Results

2.4.1 Asymptomic Prevalence across Ecological Zones

Of the 535 total samples, 105 (19.6%) were found positive by microscopy, 153 (28.6%) by 18S nested PCR, and 169 (31.6%) by 18S qPCR methods. In the north (sites KG and PZ), 24 out of 112 (21.4%) samples were microscopic-positive. The number of positive infections increased to 58 (51.8%) when screened by 18S nested PCR method (Figure 2.1; Supplementary Table S2). In the central (site KD), 52 of 216 (24%) samples were microscopic-positive and the number of positive infections increased to 60 (27.8%) by nested PCR. In the south (sites (DO and ADA), 29 of 207 (14%) samples were microscopic-positive. The number of positive infections increased to 35 (16.9%) when screened by nested PCR (Figure 2.1). The asymptomatic prevalence of infections was markedly different among the three zones, with the north being the highest and the south being the lowest. However, there was no significant difference in the parasite density measured by 18S qPCR method among the positive infections from all sites ($p = 0.43$; Figure 2.2).

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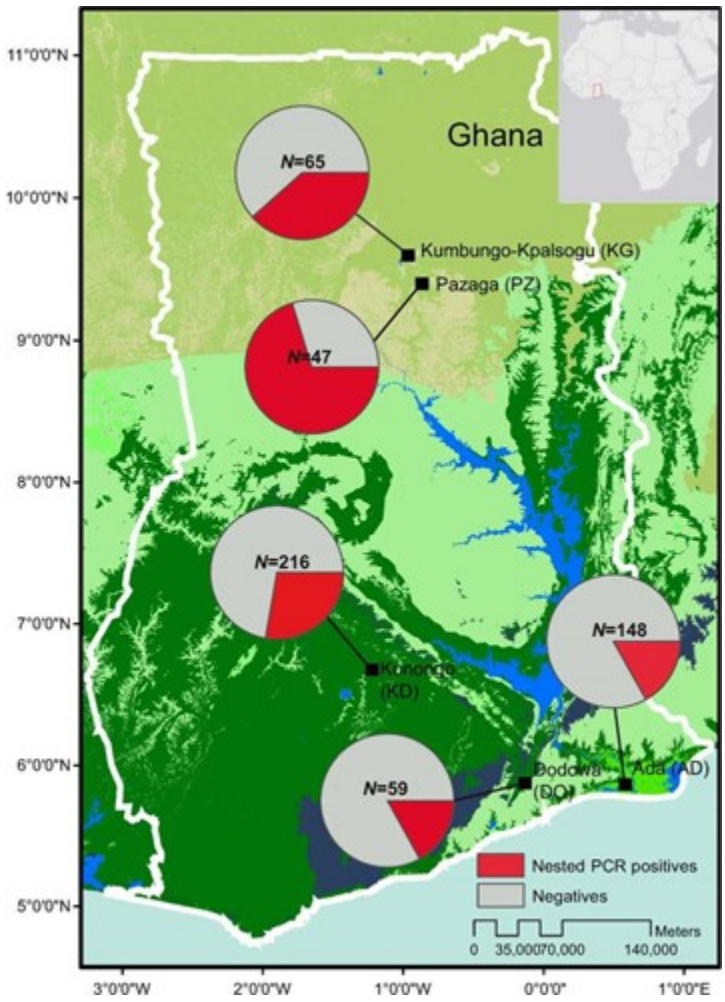

Figure 2.1: Map showing the study sites in Ghana and the prevalence of asymptomatic *P. falciparum* infections based on 18S rRNA nested PCR (pie charts). N indicated the total number of samples collected and screened in each of the study sites.

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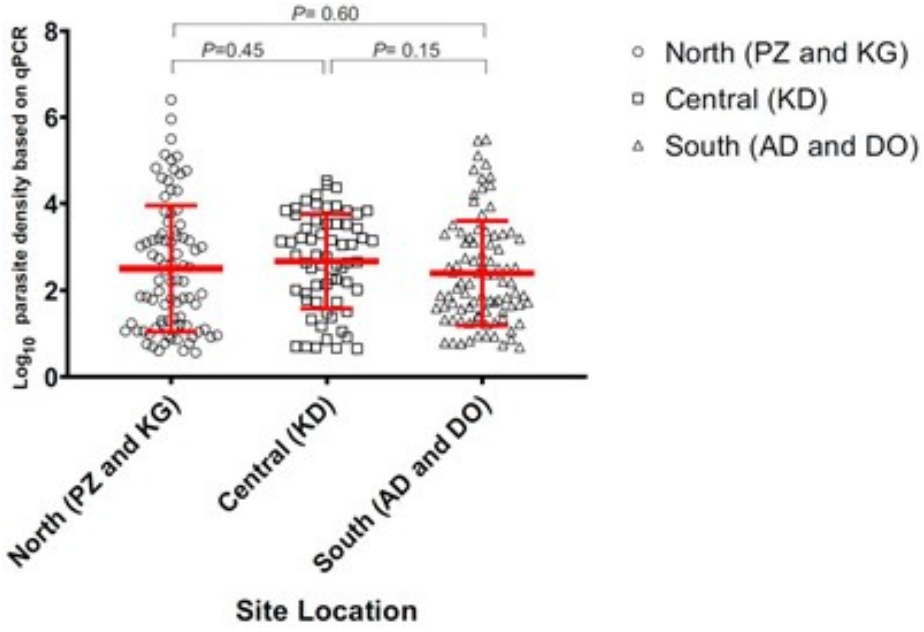

Figure 2.2: Estimated parasite density of samples collected in the north, central, and south of Ghana.

2.5.2 Sensitivity and Specificity of Diagnostic Methods

Among the 135 samples collected from the north (sites KG and PZ), 35 of the samples were detected as positive by microscopy, 62 by 18s nested PCR, 76 by 18s qPCR, and 89 by TARE-2 qPCR (Table 2.1). Samples screened with 18s nested PCR performed with a sensitivity of 63.8% (95% confidence interval [CI]; range, 53.3

Table 2.1: Sensitivity and specificity table showing the percentage of positive infections detected by the different methods, sensitivity, and specificity of PCR against microscopy for the 135 samples collected from the north (sites KG and PZ).
(* Microscopy was used as gold standard.)

Method	**Positive**	**Negative**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
Microscopy *	35 (18.5%)	100 (81.5%)	-	-
18s Nested PCR	62 (45.9%)	73 (54.1%)	63.8% (53.3–73.5%)	91.3% (71.9–98.9%)
18s qPCR	76 (56.3%)	59 (43.7%)	80.9% (71.4–88.2%)	75.5% (60.4–87.1%)
TARE-2 qPCR	89 (65.9%)	46 (34.1%)	81.2% ((74.4–86.8%)	48.3% (30.1–66.9%)

to 73.5%) and a specificity of 91.30% (95%CI; range 71.9 to 98.9%) compared to microscopy. The 18s qPCR and TARE-2 qPCR indicated a considerably higher level of sensitivity (80.9 and 81.2%, respectively), though both methods also yielded a slightly lower specificity than the 18s nested PCR (Table 2.1).

2.5.3 Frequency of Resistance Gene Mutations

Out of all the 165 samples that were successfully genotyped for *Pfcrt* codon K76T, four (1%) were found to contain the mutant allele 76T. The remaining samples had the wild type (Table 2.2, Supplementary Figure S1). No significant difference was detected in the frequency of the *Pfcrt* K76T mutation among the three ecological zones. For *Pfmdr1*, a total of 399 samples were successfully genotyped. Of the 120 samples in the northern savannah (sites PZ, KG), 86Y and 184F mutations were found in four (3.3%) and 32 samples (26.7%), respectively. In the central (KD), there were no 86Y mutations and 34 out of 47 samples (72%) harbored the 184F mutation. In the south (DO and AD), five of the 51 samples (10%) had the 86Y mutation and 16 (50%) had the 184F mutation. There was no significant difference in the 86Y and 184F mutations across the three ecological zones. For *Pfdhps* the 437G mutant allele was highly dominant in all regions (100% in the south and central; 91–100% in the north; Table 2.2), with an overall prevalence of 97% across the country. By contrast, the 540E mutation was only found in a single sample in the north (PZ). For *Pfdhfr*, the 51I mutant was found in 89% of the samples from the south, 88% in the central

region and 90% of the samples in the north. The 59R mutation was found in all the samples from the south and 92% of the samples from the central region. By contrast, only 75% of the samples from the north harbored this mutation. The 108N mutation was found in all the samples from the south, 94% of the samples in the central region, and 98% of samples in the north, making it the most prevalent (98%) mutation across the country (Table 2.2).

Across Africa, the prevalence of mutations related to anti-malarial drug resistance also indicated a wide range of variation (Table 2.3). For *Pfcrt*, the prevalence of the 76T mutation was apparently higher in other parts of Africa (ranged from 43 to 80%; Table 2.3) compared to our findings in Ghana (Table 2.2; 1%). For *Pfmdr1*, the prevalence of 86Y mutation was relatively low in Ghana (4%), Gambia (17%), and Ethiopia (7%), as compared to Kenya (69%) and Zimbabwe (67%). The mutation rate of 184F varied widely from 23–90% across the different countries. For *Pfdhps*, the 437G mutant allele prevalence in Ghana (97%) and Kenya (99%) were very high compared to the others (13–26%). By contrast, while the 540E mutation was dominant in Kenya (90%) and Gambia (100%), it was only found in 1% of the samples in Ghana and 15% in Zimbabwe. For *Pfdhps*, the 51I, 59R, and 108N mutant alleles were highly prevalent in Ghana and Kenya compared to Gambia, Ethiopia, and Zimbabwe where a lower percentage of samples showed mutation (Table 2.3).

The frequencies of single, triple, quadruple, and quintuple mutations were compared among regions (Tables 2 and 4; Supplementary Figure S2). The mutation at *Pfdhfr* S108N was defined as a single mutant, *Pfdhfr* N51I, C59R, and S108N as triple mutants, *Pfdhfr* N51I, C59R, S108N and dhps A437G as quadruple mutants, and the combination of *Pfdhfr* N51I, C59R, S108N plus *Pfdhps* A437G and K540E as quintuple mutants. In the northern savannah (KG and PZ), 97 out of 99 samples (98%) had the *Pfdhps* single mutant, 70 (71%) had the *Pfdhps* triple mutants, and 45 (45%) had the *Pfdhfr*/dhps quadruple mutants (Table 2.4). In the central region

Table 2.2: Prevalence of the *Pfcrt*, *Pfmdr1*, *Pfdhfr*, and *Pfdhps* point mutations in isolates from the three geographical regions (five different sites) in Ghana.

N	Gene	Variant	Total (%)					
			Region					
			North		Central	South		
			KG	PZ	KD	AD	DO	
	Pfcrt							
165	K76T	K	38 (97)	36 (100)	45 (98)	26 (96)	16 (94)	161 (99)
		T	1 (3)	0	1 (2)	1 (4)	1 (6)	4 (1)
	Pfmdr1							
216	N86Y	N	55 (100)	61 (94)	45 (100)	29 (88)	17 (94)	207 (96)
		Y	0	4 (6)	0	4 (12)	1 (6)	9 (4)
183	Y184F	Y	40 (74)	32 (64)	13 (28)	6 (43)	10 (56)	101 (55)
		F	14 (26)	18 (36)	34 (72)	8 (57)	8 (44)	82 (45)
	Pfdhps							
109	A437G	A	3 (9)	0	0	0	0	3 (3)
		G	30 (91)	33 (100)	45 (100)	24 (100)	13 (100)	106 (97)
155	K540E	K	32 (100)	40 (98)	45 (100)	24 (100)	13 (100)	154 (99)
		E	0	1 (2)	0	0	0	1 (1)
	Pfdhfr							
189	N51I	N	6 (12)	4 (8)	6 (12)	4 (11)	0	20 (11)
		I	43 (88)	47 (92)	43 (88)	33 (89)	0	169 (89)
209	C59R	C	13 (28)	12 (23)	4 (8)	0	0	29 (14)
		R	34 (72)	41 (77)	47 (92)	35 (100)	23 (100)	180 (86)
205	S108N	S	2 (4)	0	3 (6)	0	0	5 (2)
		N	45 (94)	52 (100)	49 (94)	35 (100)	23 (100)	204 (98)

Table 2.3: Comparison of prevalence of the *Pfcrt*, *Pfmdr1*, *Pfdhfr*, and *Pfdhps* point mutations in *P. falciparum* from West (Ghana from this study; Gambia [27]), East (Ethiopia [28]; Kenya [29], and Southern Africa (Zimbabwe [30]).
(N denoted the total number of successfully amplified samples. Percentages (%) were presented in parentheses. '-'indicated data not available.)

Gene	Variant						
			West Africa			East Africa	Southern Africa
			Ghana	Gambia	Ethiopia	Kenya	Zimbabwe
Pfcrt							
	K76T	K	161 (99)	138 (57)	116 (39)	69 (27)	22 (20)
		T	4 (1)	105 (43)	189 (61)	185 (73)	90 (80)
Pfmdr1							
	N86Y	N	207 (96)	202 (83)	142 (93)	78 (31)	37 (33)
		Y	9 (4)	43 (17)	10 (7)	174 (69)	75 (67)
	Y184F	Y	101 (55)	88 (36)	18 (10)	195 (77)	-
		F	82 (45)	157 (64)	181 (90)	58 (23)	-
Pfdhps							
	A437G	A	3 (3)	180 (74)	152 (85)	3 (1)	98 (87)
		G	106 (97)	63 (26)	27 (15)	250 (99)	14 (13)
	K540E	K	154 (99)	23 (10)	-	0 (0)	96 (85)
		E	1 (1)	222 (90)	-	253 (100)	16 (15)
Pfdhfr							
	N51I	N	20 (11)	240 (98)	45 (23)	8 (3)	100 (89)
		I	169 (89)	5 (2)	154 (77)	245 (97)	12 (11)
	C59R	C	29 (14)	241 (98)	100 (50)	26 (10)	102 (91)
		R	180 (86)	6 (2)	99 (50)	227 (90)	10 (9)
	S108N	S	5 (2)	243 (99)	44 (22)	0 (0)	72 (64)
		N	204 (98)	2(1)	155 (78)	253 (100)	40 (36)

Table 2.4: Haplotype prevalence of mutations in the *Pfdhfr* codons N51I, C59R, S108N, and *Pfdhps* A437G and K540E present among the *P. falciparum* isolates from three regions in Ghana. N denoted the total number of successfully amplified samples. Percentages (%) were presented in parentheses.

Region	Study Site		N (%)			
			Pfdhps	*Pfdhfr*	*Pfdhfr*/*Pfdhps*	*Pfdhfr*/*Pfdhps*
		Total	N108	I51R59N108	I51R59N108/G437	I51R59N109/G437E540
North						
	KG	47	45 (96)	33 (70)	17 (36)	0
	PZ	52	52 (100)	37 (71)	28 (54)	0
Central						
	KD	52	49 (94)	43 (83)	3 (6)	0
South						
	DO	23	23 (100)	0	0	0
	AD	36	35 (97)	3 (8)	1 (3)	0

(KD), 49 out of 52 samples (94%) had the *Pfdhps* single mutant, 43 (83%) had the *Pfdhps* triple mutants, and three (6%) had the *Pfdhfr*/dhps quadruple mutants. In the southern coastal region (AD and DO), 58 out of 59 samples (98%) had the *Pfdhps* single mutant, three (5%) had the *Pfdhps* triple mutants, and one (2%) had the *Pfdhfr*/dhps quadruple mutants. Among sites, there was no significant difference for the *Pfdhps* single mutant S108N (one-tailed t-test; p-value > 0.05). However, the prevalence of the *Pfdhfr* N51I, C59R, S108N triple mutant was significantly higher in the north and central regions compared to the south ($p < 0.001$; Supplementary Figure S1). Likewise, a significant difference was observed in the prevalence of the *Pfdhfr* N51I, C59R, S108N and *Pfdhps* A437G quadruple mutants between the north and other regions ($p < 0.001$; Figure 3.3). No quintuple mutants were observed in all samples.

2.6 Discussion

Successful malaria control and elimination rely on accurate and sensitive methods to measure parasite prevalence. This study specifically compared the sensitivity and specificity of different molecular methods in order to assess the true asymptomatic malaria prevalence and determine the most sensitive test. Microscopy remains the gold standard in malaria case management despite a detection limit of 10–50 para-

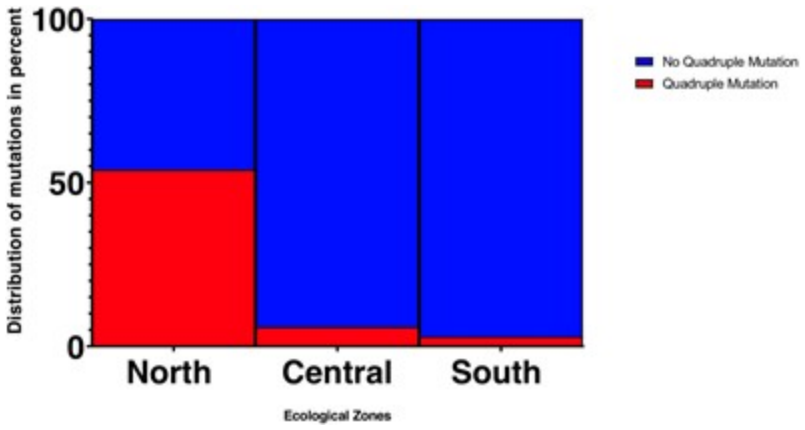

Figure 2.3: Frequency of the quadruple mutation *Pfdhfr* N51I/C59R/S108N, *Pfdhps* A437G across the different ecological zones.

sites/µL [31,[143] . By contrast, PCR methods can detect $\leq$5 parasites/μL and are known to be markedly more sensitive than microscopy [144, 145]. Consistent with previous studies, our findings indicated that the sensitivity of 18S rRNA nested PCR was the lowest (63.8%) given the low parasite density in asymptomatic infections. Compared to 18S rRNA nested PCR, qPCR methods detected a larger number of positive samples. Almost all samples detected positive by TARE-2 qPCR were also positive using 18S rRNA qPCR, with the exception of four samples that were detected as positive by TARE-2 and negative by 18S rRNA qPCR. Because of the high-copy nature of the TARE-2 gene, it offers a more sensitive gene marker than 18S rRNA in detecting low-density *P. falciparum* infections among asymptomatic samples in this study [51]. This study reinforces the need of novel gene target for parasite screening of low-density infections, and the TARE-2 gene has so far produced very promising results.

Asymptomatic prevalence in the northern savannah was found to be relatively high compared to that in the south. Land use and socio-economic factors might explain such differences. In the north, agricultural-related activities such as irrigation throughout the year could increase mosquito habitats and allow high malaria transmission [146]. Previous studies indicated that the entomological inoculation

rate (EIR) in the north (e.g., Navrongo) was 643 infective bites per person per year (ib/p/y) [147], about 30-times higher than in the south (e.g., Dodowa; 21.9 [**?**]) where urban communities predominate. The fewer mosquito habitats available in the south may be associated with low transmission. A national survey on key socio-economic factors among Ghanaians conducted in 2016 showed 25% of the population in the Northern Region did not seek medical treatment in the six months duration of the survey. Comparatively, in the Central and the Greater Accra Region, 7–9% of the population did not seek for medicine or medical care in the six months of the survey [38]. Such a contrast indicates that people living in rural areas may have poor access to hospitals or are unable to afford medical diagnosis or treatment. Apart from insufficient healthcare infrastructure in the north, human movements from the north to the south due to resource endowments and labor demand could enhance the spread of malaria [39]. In fact, the southern regions of Ghana are the destinations for 88 percent of all internal migrants, while the northern and two upper regions together account for only 5 percent of the total [148]. These socio-economic factors and migration patterns might play a role in the spread of drug resistance and contribute to the overall malaria burden. In this study, we did not observe a significant difference in parasitemia among sites, although the study sites in the north have a much higher prevalence rate. These disparities could be due to the fact that in high transmission areas, children are routinely exposed to the parasite and develop immunity that enables them to remain asymptomatic. In contrast, individuals who live in low transmission areas have less exposure to the parasites, and this might result in a weak immune system [149]. While immunity offers protection against malaria at the individual level, the asymptomatic population provides a reservoir for the parasites and could have a profound effect to the transmission of the disease.

The low prevalence of *Pfcrt* mutant allele across the country corroborates with the withdrawal of CQ as first-line treatment for *P. falciparum* since 2004 [137]. Apart

from *Pfcrt* codon 76, the overall low prevalence of *Pfmdr1* Y86 among our study sites is consistent with the pattern seen in *Pfcrt*, suggesting the absence of strong selection pressure against CQ. Previous studies conducted between 2012 and 2016 in Ghana comparing the *Pfcrt* K76T and *Pfmdr1* N86Y prevalence also showed similar trends with the decline of both mutant alleles [141]. It is likely that reduced selection of CQ-resistance strains has allowed the wild-type phenotype to predominate across Ghana. On the other hand, *Pfmdr1* F184 mutation has been shown to be strongly associated with amodiaquine resistance [133],[146]. The relatively high prevalence of *Pfmdr1* F184 mutation in the central (KD: 72%) and south (AD: 57% and DO: 44%) is concerning with regard to the efficacy of amodiaquine. Nevertheless, the relatively low prevalence of *Pfmdr1* F184 mutation in the north (KG: 26% and PZ: 36%) suggested that the use of amodiaquine for both chemoprevention and first-line treatments has not yet imposed strong selection in the north.

The frequency of mutation in both *Pfdhfr* and *Pfdhps* were considerably high ranging from 91% to 100% across all three ecological zones. When single, double, triple, and quadruple mutants were examined, we observed striking differences in the triple and quadruple mutants among regions. The prevalence of triple mutant (*Pfdhfr* I51/R59/N108) was significantly higher in the north and central regions than in the south. The quadruple mutant (*Pfdhfr* I51/R59/N108 + *Pfdhps* G437) was significantly higher in the north (36–54%) than the central and south regions (0–6%). Similar trends have been described in Senegal where a significant increase of the quadruple mutation was reported in a region under SMC between 2003 and 2011 [139]. Our findings suggested that the SMC may have a greater impact on a set of linked mutations at the different gene codons that confer a high level of SP resistance than on a single mutation [139]. It is noteworthy that the SMC may not be the only driver of *Pfdhfr* and *Pfdhps* polymorphisms in our study sites. In 2003, Ghana adopted the WHO recommendation and implemented the Intermittent Pre-

ventive Treatment in pregnancy women (IPTp) with SP as the standard of care in malaria-endemic regions [142]. The IPTp may in part explain the overall high prevalence of *Pfdhfr* and *Pfdhps* mutations across Ghana, as well as the similar trend in mutation prevalence between Ghana and Kenya [150, 151]. High prevalence of the *Pfdhfr* or *Pfdhps* resistance alleles among *P. falciparum* in Ghana was reported as early as 2003 [152]. It is not surprising to see such a pattern prevails in our samples after more than a decade. While the majority of our samples are known to be polyclonal based on microsatellite data (unpublished), we did not detect a large number of mixed nucleotides at the targeted positions within samples. Thus, it is plausible that different clones within the host have undergone the same mutational changes by similar selection. It is yet to be tested whether parasite gene flow might also play a role in the wide distribution of the *Pfdhfr* and *Pfdhps* mutations.

2.7 Conclusion

In conclusion, this study highlights the observations of high asymptomatic prevalence and quadruple *Pfdhfr*/*Pfdhps* mutants in the north. These findings have important implications for the efficacy of ongoing SMC and IPT interventions in Ghana. Although this study did not assess the efficacy of chloroquine in treating clinical falciparum malaria, the resurgence of CQ susceptible genotype poses the possibility of reintroducing CQ as a first-line falciparum malaria treatment, as well as part of the SMC regime. We are currently investigating copy number variation for *Pfmdr1*, as well other gene markers to further explore how the parasite genomes alter susceptibility to various antimalarial drugs. Our findings emphasize the need for highly sensitive methods to assess the accurate parasite prevalence especially in high malaria-endemic areas for effective disease control and management.

The following are available online at http://www.mdpi.com/2073-4425/10/7/538/s1. Figure S1: Percentage of single, triple, and quadruple mutations of *Pfdhfr* and *Pfdhps* among *P. falciparum* samples from respective sites in Ghana, Figure S2: Percentage

of *P. falciparum* samples that showed mutations in the respective resistance gene codons, Table S1: Primers and PCR conditions for *P. falciparum* resistance gene amplifications, Table S2: Information of *P. falciparum* samples collected in each of the study sites in Ghana.

CHAPTER 3: Comparing *K13* gene polymorphisms of Plasmodium falciparum isolates across transmission settings in Ghana

3.1 Introduction

Malaria is a significant global public health concern, causing around 200 million cases and over 600,000 deaths worldwide [153]. More than 90% of global malaria cases are from countries in Sub-Saharan Africa where malaria is endemic [154]. Malaria transmission is determined by various factors in Ghana. For example, in the northern savannah and central forest regions, irrigation and gold mining activities create breeding grounds and/or nesting sites for malaria transmitting mosquitoes Anopheles, contributing to higher transmission rates; whereas in the southern coastal region with more urban setting and fewer mosquito habitats, transmission rate is relatively low [155] [156]. In addition, socioeconomic factors such as inadequate healthcare access in the rural remote areas of the northern savannah region also increase malaria incidence [156] [157]. Prior studies have addressed the relation between the rate at which antimalarial drug resistance evolves and transmission intensity [158]. Yet most concur that these two variables do not directly associate with one another [7-9]. Instead, the level of host immunity [159], population size [160], and/or the number of parasite clones co-infecting the same host [161] are more likely to affect drug resistance. The antimalarial drug treatment regime has been changing ever so often in the last few decades due to the emergence of resistance. Chloroquine (CQ) has been the frontline antimalarial drug widely used in the 1950s [162]. In 2012, WHO recommended chemoprevention with a monthly course of amodiaquine and sulfadoxine-pyrethamine (SP) to combat malaria during high transmission seasons [163]. Resistance of P. falciparum to CQ and SP became widespread in the 1950s and 1960s, with the first

reported CQ resistance in Ghana in 1987 [164]. In response to the increased malaria related morbidity and mortality, artemisinin-based combination therapy (ACT) was recommended as the first line treatment [165]. Artemisinin has been shown to be effective against CQ sensitive and resistant P. falciparum malaria[166]. However, this drug has a short half-life and is ineffective against latent forms of primary and hepatic malaria [166]. Due to poor outcomes of artemisinin monotherapies, artemisinin is used in combination with other long-lasting antimalarials against multiple blood stages of the parasite, especially in early stages [167]. Selective pressure has driven to resistance against artemisinin as seen in South America and Southeast Asia in the late 1990's [168], and is especially prevalent in high transmission areas of Thailand and Cambodia [169]. Studies over the last decade have shown that ART resistance in P. falciparum is attributed to selection for mutations in genes that govern the physiological responses of the parasites and determine parasite clearance. Specifically, mutations within the highly conserved kelch propeller domain on chromosome 13 (pfk13) inactivate proteins needed for endocytosis of hemoglobin. Given ART is activated by the products from hemoglobin degradation, the lack of these products prevents ART activation and results in parasitic resistance [170]. Several codon mutations in pfk13 gene associated with artemisinin resistance such as C580Y, Y493H, R539T, I543T, and N458Y have been identified in the field isolates from Southeast Asia and parts of Africa (Supplementary Table 3) [171, 172]. In Ghana, several non-synonymous as well as few synonymous pfk13 mutations have been reported in the central forest region [173, 174, 175]. The Ministry of Health (MOH) in Ghana changed its policy to follow the recommendations of WHO for artemisinin combination therapy since 2005 [155]. Though malaria transmission occurs all year round, different regions vary in transmission intensity due to climatic and landscape variations, population density, and urbanization [176]. Our previous study has shown that mutations in Pfcrt, Pfmdr1, Pfdhfr, and Pfdhps associated with CQ and SP resistance were not signifi-

cantly different across the three ecological zones of Ghana, and that the majority of the isolates had the wildtype codons, with no sign of selection pressure [157]. However, the mutation frequency of the Pfk13 gene in Ghana and if such frequency differs among ecological zones remain unclear. This study determined and compared Pfk13 non-synonymous and synonymous mutations among the three ecological regions of Ghana and examined the phylogenetic relatedness among the mutant and wildtype isolates to understand the evolution of P. falciparum across the three regions. These findings will help improve the surveillance of artemisinin resistance in West Africa.

3.2 Materials and Methods

3.2.1 Study sites and sample collection

Samples were collected from five sites including Pagaza (PZ) in Tamale Municipality and Kpalsogou (KG) in Kumbungu district in the northern savannah region; Duase (KD) in Konongo in the central forest region; and Ada (AD) and Dodowa (DO) in the southern coastal region of Ghana during June-July of 2018 [157]. In the northern savannah zone, malaria transmission is highly seasonal and intense during/after the rainy season (June–October), whereas in the south coastal (low elevation) and central forest areas, malaria transmission is perennial [177] (Figure 1). Finger-prick blood samples were collected from 172 asymptomatic individuals, showing no fever or malaria-related symptoms at the time of collection. Thus, these individuals did not receive any antimalarial treatment prior to blood sample collection. Thick and thin blood smears were prepared for microscopic examination. Blood samples (30-50 µL) were blotted on Whatman 3MM filter papers. The filter papers were air-dried and stored in sealed plastic bags with silica gel absorbent at room temperature until DNA extraction. Parasitic DNA was extracted from the dried blood spots by the Saponin/Chelex method [178]. The SYBR Green quantitative real-time PCR (qPCR) assay of the 18S rRNA was conducted to screen for P. falciparum using the published protocols [179]. For the P.

falciparum-confirmed samples, a 849-bp amplicon of the Pfk13 gene was amplified using the published primers (Forward: GTAAAGTGAAGCCTTGTTG-3'; Reverse: 5'-TTCATTTGTATCTGGTGAAAAG -3') spanning nucleotide positions 1139- 1979 [180]. Briefly, PCR was conducted in a 20ul reaction mixture containing 2ul of genomic DNA, 10ul of 2xDreamTaq Green PCR Master Mix (Thermo Fisher) and 0.5uM primer. Reaction was performed with an initial denaturation at 94°C for 2 min, followed by 30 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, with a final 5 min extension at 72°C. PCR products were purified prior to Sanger sequencing.

3.2.2 Pfk13 sequence analyses

All sequences were aligned against the P. falciparum reference 3D7 K13 gene using BioEdit Sequence Alignment Editor [181]. We specifically focused on previously identified Pfk13 mutations that have been validated for artemisinin resistance in vitro [173], in addition to other synonymous and non-synonymous mutations (Supplementary Table 1). To determine and compare sequence polymorphisms among regions, haplotype diversity (Hd; the probability that two randomly sampled alleles are different), nucleotide diversity (Pi; the average number of nucleotide differences per site in pairwise comparisons), and Tajima's D value were estimated using DNASP [182]. The Tajima's D and Fu's FS tests were conducted to distinguish between evolving neutrally and one evolving under a non-random process, including directional or balancing selection. To infer the genetic relatedness among the Pfk13 sequences from different transmission zones, we employed the maximum likelihood method using the Randomized Accelerated Maximum Likelihood (RAxML) for phylogenetic inferences with 500 iterations of bootstrapping for assessing confidence [183]. The resulted tree was visualized in FigTree v1.4.2 [184].

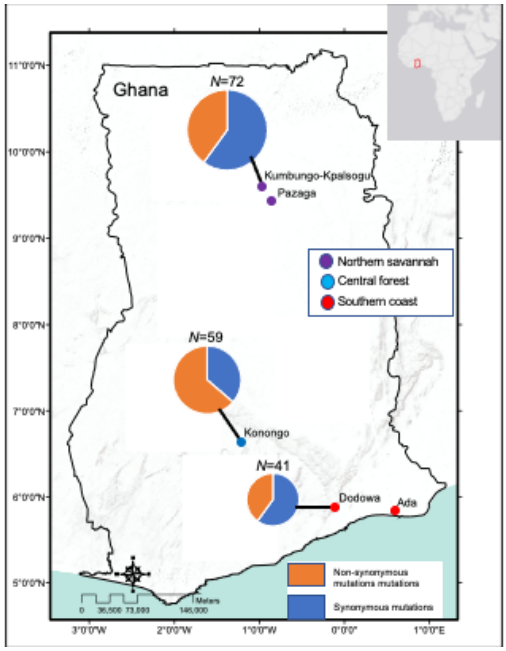

Figure 3.1: Map of Ghana topography and sampling. The total number of samples in the north was N=72 with 64% (46) of the samples being wild types (WT) and 36% (25) of mutants (MT). In the central region, N=59 with 75% (44) of the samples being WT and 25% (15) MT. And in the south, N=41 with 49% (20) of the samples being WT and 51% (21) MT

3.3 Results

3.3.1 Pfk13 mutations

A total of 172 Pfk13 sequences (north=72; central=59; south=41) were analysed. In the north (PZ and KG), 11 out of the 72 samples (15%) were detected with non-synonymous mutations at 11 codon positions including I418M, S423G, R471C, P475L, S477F, Y493H, V494P, N499Y, Y500F, D501N (Figure 2). Each of these mutations was found in a single sample except D501N found in two samples. Among all codons found in the north, only Y493H has been validated in vitro that confers to ART resistance [185] (Table 1). In the central region (KD), 10 out of the 59 samples (17%) were detected with non-synonymous mutations at six codon positions including G449A, N458K, A481T, I543L, N548I, and C580Y. Mutations G449A and C580Y were each found in two different samples; and mutation I543L was detected in three

Table 3.1: Codon mutations distribution and function based on literature in north, central, and south Ghana. C580Y is the most prevalent non-synonymous mutation in Southeast Asia that mediates ART resistance both in vitro and in vivo. Y493H has also been validated in vitro that confers to ART resistance.

Region	Codon mutation	Validation for ART resistance	Other malaria endemic areas
North	I418M	No	Yes
	S423G	No	No
	P475L	No	No
	R471C	No	No
	S477F	No	Yes
	Y493H	Yes	Yes
	V494P	No	No
	N499Y	No	No
	Y500F	No	Yes
	D501N	No	No
Central	G449A	No	No
	N458K	No	No
	A481T	No	No
	I543L	No	No
	N548I	No	No
	C580Y	Yes	No
South	N408D	No	No
	M460T	No	No
	M472T	No	No
	P475L	No	No
	Y482F	No	No
	D501G	No	Yes

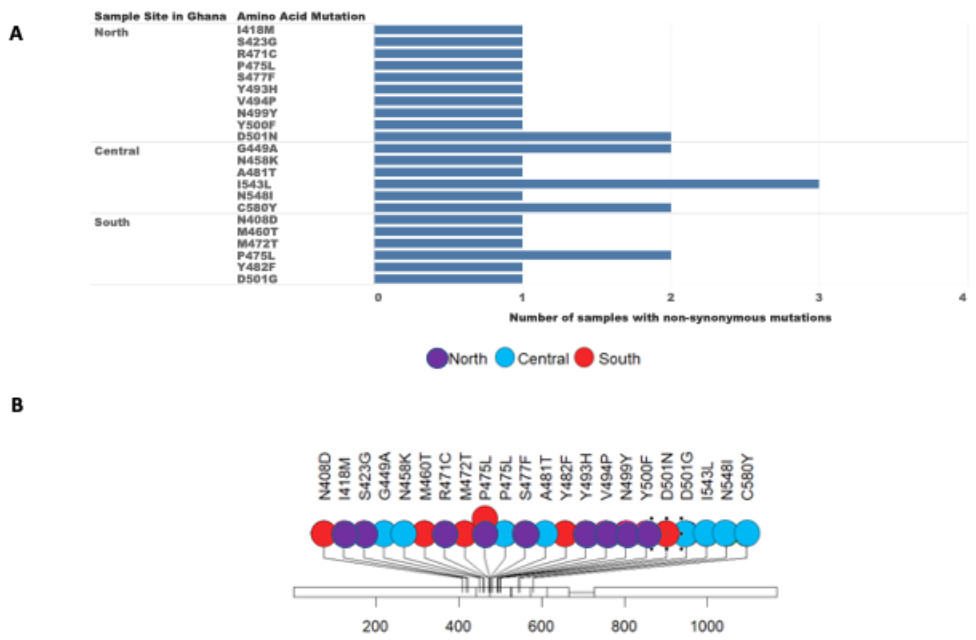

Figure 3.2: A) Chart of the number of samples with non-synonymous mutations. In the north, 11 out of the 72 samples (15%) were detected with non-synonymous mutations at 11 codon positions including I418M, S423G, R471C, P475L, S477F, Y493H, V494P, N499Y, Y500F, D501N. Each of these mutations was found in a single sample except D501N found in two samples. In the central region, 10 out of the 59 samples (17%) were detected with non-synonymous mutations at six codon positions including G449A, N458K, A481T, I543L, N548I, and C580Y. Mutations G449A and C580Y were each found in two different samples; and mutation I543L was detected in three. Other mutations including A481T, N458K, N548I were found only in a single sample, respectively. In the south, 7 of the 41 samples (17%) were detected with non-synonymous mutations at six codon positions including N408D, M460T, M472T, Y482F, P475L, and D501G. B) Structure of the kelch13 propeller domain, showing the position of mutations found in this study.

(**??**). C580Y is the most prevalent non-synonymous mutation in Southeast Asia that mediates ART resistance both in vitro and in vivo [171, 185]. Other mutations including A481T, N458K, N548I were found only in a single sample, respectively. In the south (AD and DO), 7 of the 41 samples (17%) were detected with non-synonymous mutations at six codon positions including N408D, M460T, M472T, Y482F, P475L, and D501G (Figure 2). None of these mutations has been shown to be linked to ART resistance based on prior studies (Table 1). Each of these mutations was found in a single sample, except for P475L that was also detected in a sample from the north.

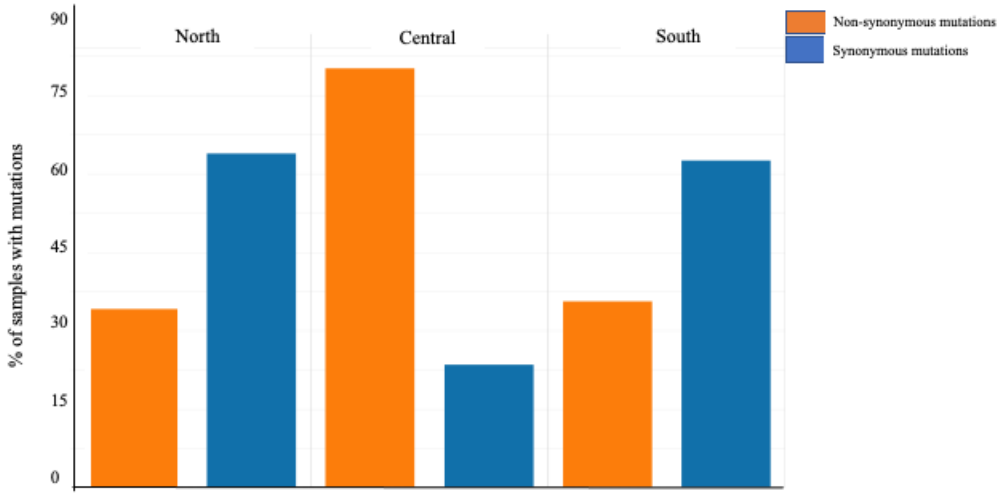

Figure 3.3: Chart of the percentage of synonymous vs non-synonymous mutations by region. In the north, 68% of the mutations were synonymous and 32% were non-synonymous. Similar proportion was observed in the south where 67% of the mutations were synonymous and 33% were non-synonymous. In the central region, 75% of the mutations were non-synonymous and 25% were synonymous.

Apart from non-synonymous mutations, several synonymous mutations were also found in the Pfk13 gene among samples. In the north, there were 46 non-wildtype samples, of which 68% of the mutations were synonymous and 32% were non-synonymous. Similar proportion was observed in the south where 67% of the mutations were synonymous and 33% were non-synonymous among 21 non-wildtype samples (Figure 3). By contrast, in the central region, 75% of the mutations were non-synonymous and 25% were synonymous among the 15 non-wildtype samples (Figure 3).

3.3.2 Phylogenetic and diversity analyses

Three small clades (I-III) were observed among the 172 Pfk13 sequences (bootstrap >95%; Figure 4). There was no clear distinction between samples from the north, central, or south as they were found in all well-supported clades (Figure 4).

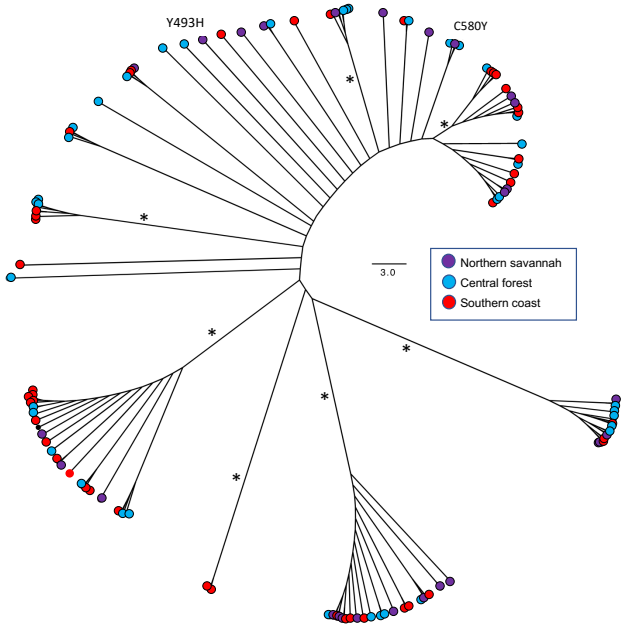

Figure 3.4: Haplotype diversity (Hd), nucleotide diversity (Pi), and neutrality test statistics of PfK13 gene among samples in north, central, and south Ghana. Both haplotype and nucleotide diversity were higher in the north (Hd=0.8; Pi=0.003) and central regions (Hd=0.9; Pi=0.004) than the southern region (Hd=0.2; Pi=0.0012). Negative Tajima's D values were observed in the three regions, suggesting that the Pfk13 gene is under selection pressure. The results of Fu's FS test statistics were negative for all regions, indicating that the presence of a higher number of rare/unique Pfk13 haplotypes than what would be expected under neutrality.

A total of 28 different Pfk13 haplotypes were detected among the samples. Samples containing the 580Y and 493H mutations showed only one amino acid change (Supplementary Table 2). Both haplotype and nucleotide diversity were higher in the north (Hd=0.8; Pi=0.003) and central regions (Hd=0.9; Pi=0.004) than the southern region (Hd=0.2; Pi=0.0012; Table 2). Negative Tajima's D values were observed in the three regions, suggesting that the Pfk13 gene is under selection pressure. The results of Fu's FS test statistics were negative for all regions, indicating that the presence of a higher number of rare/unique Pfk13 haplotypes than what would be expected under neutrality (p-value = 0.005; Table 2).

Table 3.2: Haplotype diversity (Hd), nucleotide diversity (Pi), and neutrality test statistics of *Pfk13* gene among samples in north, central, and south Ghana. Both haplotype and nucleotide diversity were higher in the north (Hd=0.8; Pi=0.003) and central regions (Hd=0.9; Pi=0.004) than the southern region (Hd=0.2; Pi=0.0012). Negative Tajima's D values were observed in the three regions, suggesting that the *Pfk13* gene is under selection pressure. The results of Fu's FS test statistics were negative for all regions, indicating that the presence of a higher number of rare/unique *Pfk13* haplotypes than what would be expected under neutrality.

Ghana	N	Pi	Hd	Hap	Neutrality test		
					Fu&Li D*	Fu&Li F*	Tajima D
North	72	0.003	0.8	55	-3.4	-2.3	-0.3
Central	59	0.004	0.9	47	-1.3	-1.9	-1.9
South	41	0.001	0.2	6	-3.4	-3.5	-2.1

3.4 Discussion

Considering the trends of growing ART resistance in Southeast Asia and evidence of ART resistance in Sub-Saharan Africa [186], monitoring the spread of P. falciparum malaria and identification of Pfk13 mutations is crucial to controlling the disease. This study indicated in Ghana, that P. falciparum from regions of higher transmission intensity had a greater number of Pfk13 mutations than those from low transmission regions, even though parasites were phylogenetically close to one another. There are multiple biomarkers of ART resistance in Southeast Asia and Africa. In Southeast Asia, isolates with mutations C580Y, Y493H, R539T, I543T, and N458Y were showed with delayed clearance [171]. Of the 27 non-synonymous mutations identified in the Ghanaian samples, C580Y and Y493H has been previously validated for ART resistance and are widespread across Southeast Asia. The northern savannah zone of Ghana has the highest number of Pfk13 mutations. This region has a high rate of poverty, lack of access to healthcare services, and almost half of the regional population comprises children under the age of 14, who are more vulnerable to malaria infection than adults [187]. In the north, malaria transmission is seasonal and significantly higher during and after the rainy season. Consistent to other studies, parasites from the southern region have low prevalence of Pfk13 mutations [177].

Despite the humid and warmer weather near the coast climate that is optimal for mosquito development, the southern region is densely populated, highly urbanized, higher socio-economic status, and greater access to healthcare services [188]. These factors likely contribute to low incidence of malaria, as people living in this area are more likely to be diagnosed and treated promptly for malaria.

The central forest region serves as a transitional zone between the north savannah and urban south; and reflects how the parasitic prevalence and variability may change as urbanization occurs. The central region of Ghana has the country's major mineral reserves. Large-scale mining activity leads to landscape damage and pockets of stagnant water favor mosquito development [189]. In this region, we found relatively low frequency of Pfk13 mutations. One of the mutations G449A was previously identified as a candidate mutation for ART surveillance in Southeast Asia [171]. The type of amino acid substitution is also important when inferring the impact on resistance. For example, while I543L has not been yet confirmed as a mutation resulting in ART resistance, I543T was validated as a key marker for ART resistance [171]. Further analysis by CCF53_62 matrices that determines the effect of amino acid changes to protein function could offer deeper insights into the significance of the type of substitutions [190]. Other codon mutations associated with delayed ART clearance among African P. falciparum parasites include Q613H reported in Senegal [191], P574L in Rwanda [192] and Henan Province of China [193]), A675V in Rwanda [192] and Uganda [194], P553L in Kenya [195], and K189T in Senegal [191] and Uganda [196]. Previous study has shown that the mutation 580Y was observed in Chinese migrant workers returning from Ghana [197]. A study conducted between 2007-2016 in Ghana have reported a high number of mutations of which 77% was nonsynonymous [173], though their functional significance still remains unclear and warrants further investigations. Though not validated in function, A578S is the most commonly reported non-synonymous mutation in different parts of Africa including

Ghana [198, 199] Equatorial Guinea [200], Mali [201], Kenya [202], Togo [203], but this mutation was not found in this study.

Based on neutrality tests, the Pfk13 gene was detected with significance selection pressure, particularly in the central region where there is a greater ratio of nonsynonymous to synonymous substitutions. It is plausible that easy access to antimalarial drugs in this region accelerates the development of selection pressure to ART resistance. With more than 10,000 licensed chemical seller (LCS) shops in Ghana, these shops are often the first point of care for febrile illnesses such as malaria, particularly in hard-to-reach areas [204]. Due to the increased accessibility of antimalarials, patients tend to opt for self-treatment, which can result in inadequate and inappropriate usage of non-prescribed drugs [204, 205]. As there are more LCS shops relative to health facilities in rural areas, artemisinin combination therapy could become more available in the northern savannah region and increase the Pfk13 mutation frequency. Many mutant Pfk13 codons in Ghana were found to be zone specific, implying that intra-country gene flow could be limited in spreading parasites through human migration [173]. One limitation of this study is lack of clinical efficacy data. Therefore, novel candidate mutations should be examined for both in vivo and in vitro resistance to artemisinin to clarify the significance of the mutations.

3.5 Conclusion

The global surveillance of artemisinin resistance is and will continue to be crucial to understanding how antimalarial drug resistance evolves and spreads. This study confirmed generally low rates of Pfk13 mutations between high and low transmission zones in Ghana. Most of the observed mutations have not been validated for ART resistance nor previously reported elsewhere in Africa. Our findings emphasize the need for further clinical and/or in vitro testing of the functional significance of novel Pfk13 codon mutations. Future studies should investigate the association of transmission intensity with pfk13 mutation prevalence at a broader geographical

scale. Understanding how factors such as intra- and inter-country migration patterns and urbanization affect parasitic diversity and antimalarial resistance will have great implications on controlling malaria spread in Africa.

CHAPTER 4: Genetic variations of *plasmodium falciparum* circumsporozoite protein and the impact on interactions with human immunoproteins and malaria vaccine efficacy

4.1 Abstract

In October 2021, the world's first malaria vaccine RTS,S was endorsed by WHO for broad use in children, despite its low efficacy. This study examined polyclonal infections and the associations of parasite genetic variations with binding affinity to human leukocyte antigen (HLA). Multiplicity of infection was determined by amplicon deep sequencing of *PfMSP1*1. Genetic variations in *PfCSP* were examined across 88 samples from Ghana and analyzed together with 1,655 *PfCSP* sequences from other African and non-African isolates. Binding interactions of *PfCSP* peptide variants and HLA were predicted using NetChop and HADDOCK. High polyclonality was detected among infections, with each infection harboring multiple non-3D7 *PfCSP* variants. Twenty-seven *PfCSP* haplotypes were detected in the Ghanaian samples, and they broadly represented *PfCSP* diversity across Africa. The number of genetic differences between 3D7 and non-3D7 *PfCSP* variants does not influence binding to HLA. However, CSP peptide length significantly affects its molecular weight and binding affinity to HLA. Despite the high diversity of HLA, the majority of the HLAI and II alleles interacted/bound with all Ghana CSP peptides. Multiple non-3D7 strains among *p. falciparum* infections could impact the effectiveness of RTS,S. Longer CSP peptides should be considered in future versions of RTS,S.

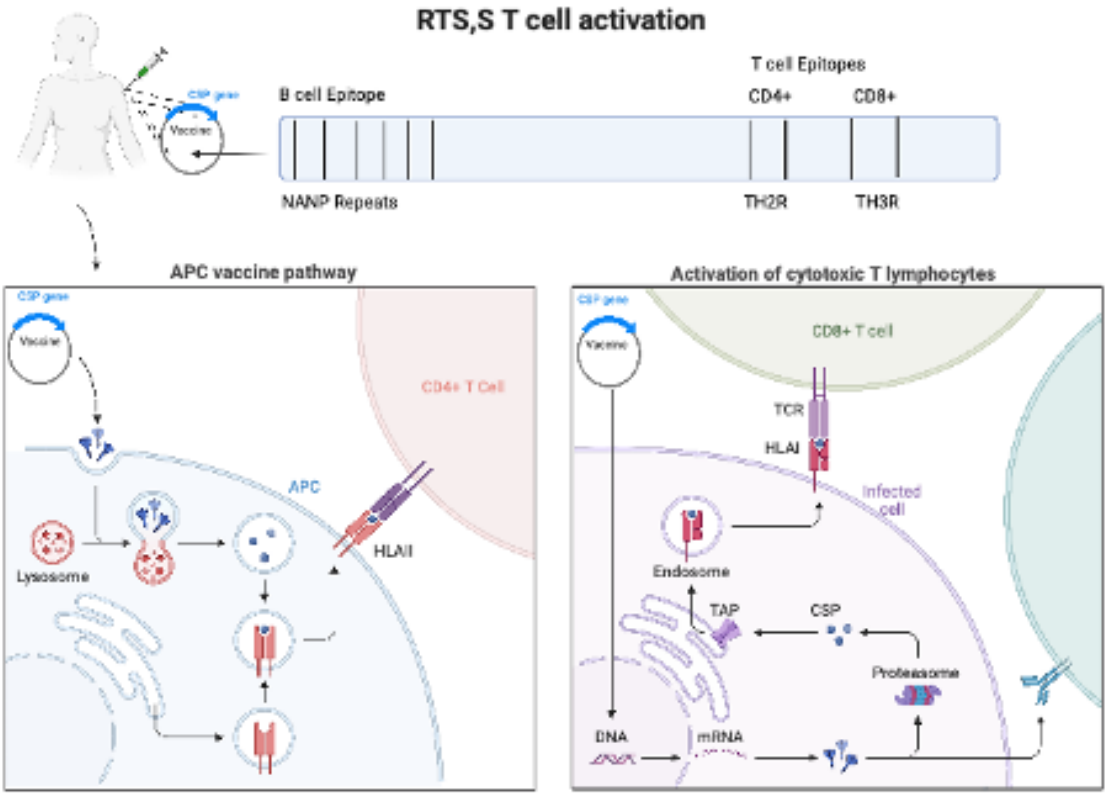

Figure 4.1: CD4+ and CD8+ T cell activation in response to the RTS,S. The C-terminal, specifically the Th2R and Th3R regions, encode epitopes recognized by the CD8+ and CD4+ T-cells. TCR protein recognizes the *PfCSP* antigen peptide presented by an HLA molecule. CD4+ T-cells recognize antigen peptides bound to MHC class II molecules, whereas CD8+ T-cells recognize antigen peptides bound to MHC class I molecules.

4.2 Introduction

In 2020, malaria caused over 219 million clinical cases and 435,000 deaths, with more than 80% in children and 90% deaths in sub-Saharan Africa [206]. In October 2021, the world's first malaria vaccine RTS,S/AS01 (referred to as RTS,S hereafter) was endorsed by WHO for broad use in children, despite its low efficacy against *plasmodium falciparum* infection. RTS,S is a pre-erythrocytic vaccine that aims to trigger the human immune system to defend against *p. falciparum* infection of the liver. RTS,S construct is based on a portion of the central NANP repeats (B-cell epitopes) and the C-terminal (T-cell epitopes) of the circumsporozoite protein (CSP) of the 3D7

lab strain [207, 208]. The C-terminal, precisely the Th2R and Th3R regions, encode epitopes recognized by the CD8+ and CD4+ T-cells (Figure 1). The Th2R and Th3R portions (RTS) fuse to the native hepatitis B surface antigen (S) and self-assemble into virus-like particles exposed on the surface of the CSP protein [209, 210, 211]. Phase III trials conducted at 11 sites across seven African countries indicated 36-55% efficacy against clinical malaria in 5-17-month-old children who received three primary doses of RTS,S plus a booster at 20 months (RTS,S Clinical Trials Partnership) [212, 213]. The explanation for such low efficacy is unclear. High genetic diversity was reported in the *PfCSP* C-terminal as malaria transmission increases across geographic areas [214]. In high transmission areas, multiple genetically diverse parasite strains are common within the same host and in the community [215]. Diverse parasite strains could harbor different CSP genotypes contributing to variations in the invasion and/or evasion mechanisms of the parasites. Antibody-mediated immunity against blood-stage *p. falciparum* is strain-specific [216]. Antibody responses elicited against one strain do not produce the same levels of inhibition against heterologous strains [217]. RTS,S efficacy against non-3D7 parasite strains was lower than that against 3D7 strain [218, 219], and vaccine efficacy reduced from 50.3% for parasites with a perfect match to *PfCSP* 3D7 C-terminal sequence to 33.4% for parasites with any amino acid mismatch [220] and 26% for severe malaria [209]. Besides, vaccine efficacy declined as the number of amino acid differences increased [221]. Mutational changes in amino acid sequence could alter CSP protein conformation and the binding reaction of the CSP peptide with human leukocyte antigens (HLA), which is vital to the induction of T cell immune response, such as production and secretion of cytokines and the capacity to mediate cytotoxicity [211]. The recognition of *p. falciparum* by CD8+ and CD4+ T-cell requires interactions with the antigen-presenting cells (APCs) that display polypeptide fragments of *PfCSP* on their surfaces [222] (Figure 1). The interaction between T-cell and the CSP peptide is mediated by the

HLA, which is expressed on the surface of APCs [223, 224]. It is unclear if non-3D7 *PfCSP* variants exhibit different binding affinities to HLA that could impact T-cell recognition. Computational analyses using a machine learning-based model to predict protein structure, folding, macromolecule interactions and/or binding affinities have been at the forefront of protein-ligand complexes studies [225]. Such mechanistic insights would shed light on the low RTS,S efficacy. In Ghana, malaria accounts for 30% of outpatient attendances and 23% of inpatient admissions [226]. Ghana is one of the three African countries where RTS,S is being implemented [227]. In this study, we (1) determined the multiplicity of infection (MOI) and CSP diversity of *p. falciparum* across different transmission settings of Ghana and compared CSP diversity with other African isolates; (2) examined HLA allelic diversity of the African populations; (3) employed protein predicted structure to compare binding affinity of CSP peptide variants to HLA. Our findings have important implications for the development of next-generation RTS,S.

4.3 Methods

4.3.1 Study sites and sample collection

Samples were collected from five sites including Pagaza (PZ) in Tamale Municipality and Kpalsogou (KG) in Kumbungu district in the northern savannah region; Duase (KD) in Konongo and Seikwa district in the central forest region; and Ada (AD) and Dodowa (DO) in the southern coastal region of Ghana during June-July of 2019 [228]. About 200µL capillary blood samples were collected from 88 subjects who were diagnosed as malaria-positive by rapid diagnostic tests. DNA was extracted using Quick-DNA kit (Zymo Research) following the manufacturer's protocol and stored in -20°C. Plasmodium species identification and DNA quantification was performed by real-time quantitative PCR of the 18S rRNA gene based on published protocols [229].

Table 4.1: Comparison of multiplicity of infections (MOI) and percentage of polyclonal samples based on *PfMSP1*1, as well as nucleotide (Pi) and haplotype (Hd) diversity based on PfCSP among samples from north, central, and south Ghana.

	Total samples	*PfMSP1*1		PfCSP	
		% polyclonalty	MOI (mean, range)	Pi	Hd
North	27	80	2.9, [1-6]	0.01662	0.951 -0.011
Central	27	100	3.1, [1-6]	0.01738	0.945 -0.009
South	29	100	2, [1-4]	0.01808	0.938 -0.009

4.3.2 Amplicon deep sequencing and data analyses

The 395-bp *PfMSP1*1 and the 321-bp C-terminal of *PfCSP* containing the Th2R and Th3R regions were amplified using previously published primers [230, 231] (Supplementary Table 1). The Illumina partial adapters[232] were added to the purified PCR products prior to library constructions. Multiple samples were pooled and sequenced in pair-end on the Illumina Hiseq 2500 [233]. Coverage of at least 50,000 reads were obtained for each sample amplicon (sequences and sample information are available in NCBI.BioProject PRJNA783000: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA783000) The HaplotypR [234] package was used for SNP and haplotype detection based on *PfCSP* and *PfMSP1*1 sequences (Supplementary Figure 1). Given that *PfMSP1*1 is length polymorphic, haplotypes were generated based on the size of the amplicon (Table 1), which were determined by iterative testing of various thresholds to distinguish between real haplotypes and random noise. MOI was calculated as the number of genetically distinct clones co-infecting a single host with cut-off settings for haplotype calling to be supported by more than three reads in more than two samples (including independent replicates of the same sample) [234].

4.3.3 *PfCSP* sequence analyses

A haplotype network was generated with the TCS for multiple sequence alignment evaluation and phylogenetic reconstruction. The construction of the haplotype network involves extracting the sequences two by two to build a library for T-Coffee. The library was then used to obtain a TCS score of every pair of aligned residues by averaging the PairTCS score over all pairs of aligned residues in each target from which the SequenceTCS and the AlignmentTCS are derived [235, 236]. The network was visualized using a TCS web-based program tcsBU. In addition, 1,655 published *PfCSP* sequences including 774 from Africa and 881 elsewhere outside Africa were obtained from NCBI. These sequences were analyzed together with the 27 haplotype sequences from Ghana. A phylogenetic tree was reconstructed using a maximum likelihood (ML) method in DECIPHER and visualized using the R package ggtree v2.2.2 [237].

4.3.3.1 CSP peptide prediction and binding to HLA alleles

To predict the resulting polypeptide expressed after proteolytic degradation of the CSP protein, the complete *PfCSP* haplotype sequences were scored using NetChop 3.0 [238]. Residues with an expression probability >50% in the Th2R and Th3R regions of the CSP protein were selected. These short peptide sequences were then assembled as 3D structures using the Peptide Builder package in Biopython [239]. A PDB file of each peptide was produced and used in protein interaction modeling (Supplementary File 1). A total of 59 HLA class I and 200 HLA class II alleles/sequences that represent the African populations from broad geographic regions were identified using the Common Intermediate Well-Documented (CIWD) version 3.0.0 [240]. We analyzed the binding affinity of these HLA sequences with the Th2R and Th3R peptides by positions, respectively, through NetMHCpan-4.0/4.1 [241]. Binding affinities were defined as non-binders, strong binders, and weak binders based on HLA affinity, TAP

transport efficiency, and C-terminal proteasomal cleavage [241]. We further predicted the interactions of the generated CSP peptide structures and the reference structure with HLA-2A PDB: 6TRN (chain A) using HADDOCK 2.4 [242]. The HADDOCK score quantifies changes in protein-protein interactions derived from various biophysical factors, including van der Waals energy, electrostatic energy, desolvation energy, and restraints violation energy (see details in Supplementary File). Plasmas from participant blood samples were examined for antibody titers against CSP by standardized enzyme-linked immunosorbent assays [216] using the full-lengh recombinant CSP[243] .

4.3.4 Measurement of anti-CSP antibody levels

Plasma from participant blood samples were examined for antibody titters against CSP by standardized enzyme-linked immuno-absorbent assays (ELISA) following previous protocol [216]. Recombinant IgG (BP055) was used as a standard calibrator for total IgG measurements. The CSP antigen was diluted to a concentration of 1μg/mL in carbonate buffer (pH 9.0) and coated at 100μL/well onto ELISA plates at 4°C overnight, followed by washing with 1×PBS/Tween20 for four times. Plasma from malaria naïve individuals from Denmark was used as negative control samples. Absorbance was measured using a ELx808 Absorbance Reader (Biotek, USA) and resulting optical density values were converted to IgG concentration using ADAMSEL (Ed Remarque).

4.4 Results

4.4.1 Multiplicity of infection (MOI) and haplotype variations

For the 88 samples in Ghana, the proportion of polyclonal infections was 76% with a mean MOI of 2.63 based on *PfMSP1*1 (Table 1). A total of 30 haplotypes were detected, ranging from 1 to 6 distinct haplotypes/clones within a single host (Figure 2). Considerable size polymorphisms were observed among different *PfMSP1*1

haplotypes. Polyclonal infections were detected in 80% of the samples from the north and all samples in the central and southern regions. The mean MOI was highest in central, followed by the north and south Ghana, but the difference was not significant (Table 1). For *PfCSP*, 27 haplotypes were detected among samples, ranging from 1 to 8 haplotypes/clones within a host (Figure 2). Nucleotide (Pi) and haplotype diversity (Hd) were similar among the three studied regions (Table 1). Though the Pi values were found to be less than 0.02 in each region, Hd values ranged from 0.938–0.951, indicating a high clonal diversity in the parasite populations. For all samples, MOI values estimated from the *PfMSP1*1 and *PfCSP* were significantly correlated with one another (r= 0.33; P=0.005). When samples were analyzed by regions, only those in the north showed a significant correlation (r=0.41; P=0.039; Figure 2). The northern, central, and southern populations shared similar *PfCSP* haplotypes, and no clear clustering of haplotypes was observed by geographic regions (Figure 3A). Of the 27 *PfCSP* haplotypes, haplotypes with ID CSP-10 and CSP-31 were most closely related to the 3D7 haplotype with only one SNP difference (Figure 3A). By contrast, haplotypes CSP15, CSP-117, and CSP-22 were the most distant from 3D7, each with 11-12 nucleotide differences. For the haplotype with ID CSP-117, mutations observed at the Th2R and Th3R regions were synonymous, and thus, this haplotype has the identical amino acid sequence as 3D7 (Supplementary Figure 2).

The 27 *PfCSP* haplotypes from Ghana were representative of the *PfCSP* diversity across Africa (Figure 3B). The majority of the non-African *PfCSP* sequences were clustered in a single large clade (purple branches; Figure 3B); whereas the African *PfCSP* sequences were scattered throughout the phylogenetic tree and found in several smaller subclades (yellow branches), some of which were mixed with the non-African lineages. About 93% (25 out of 27; green branches) of the Ghana *PfCSP* haplotype sequences were found to be closely related to the other African *PfCSP*, except for CSP-31 and CSP-99, which were more closely related to the non-African *PfCSP*.

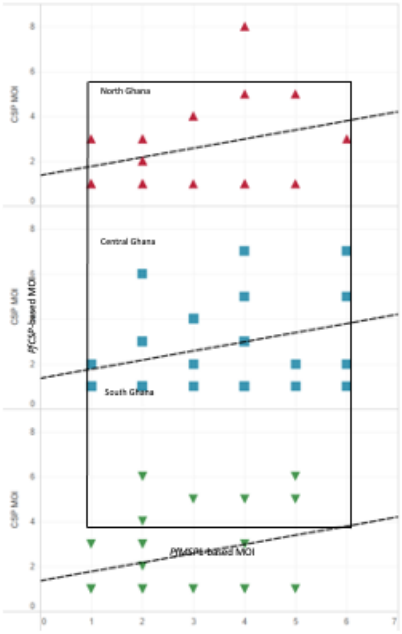

Figure 4.2: Comparison of the number of unique parasite clones based on *PfMSP1*1 and *PfCSP* sequences in north, central, and south Ghana. A significant correlation was detected between the CSP and MSP MOI (r= 0.33; P=0.005). When samples were analyzed by regions, only those in the north showed a significant correlation (r=0.41; P=0.039).

4.4.2 Interactions of HLA alleles and *PfCSP* peptides by NetMHC

HLA class I molecules including HLA-A, HLA-B, and HLA-C are expressed on all nucleated cells and contain two noncovalently bound polypeptide chains (i.e., alpha and beta-2 microglobulin chains). HLA class II molecules, expressed by antigen-presenting cells, are heterodimers composed of an alpha and a beta chains. In Africa, for HLA I, HLA-C alleles 06:02 and 07:01 were most frequent across the continent, followed by HLA-A alleles 31:02, 31:01, 03:01 and 01:01. For HLA II, allele HLA-DPB1 alleles 40:01, 11:01 were the most frequent (Supplementary Figure 4). For each HLA allele, we calculated the number of CSP peptides after proteolytic degradation with increased (strong binders) and decreased (weak binders) affinity (Figure 4A). A peptide was identified as a strong binder if the percentage rank was below the default threshold of 0.5% (orange; Figure 4A), and as a weak binder (blue) if the

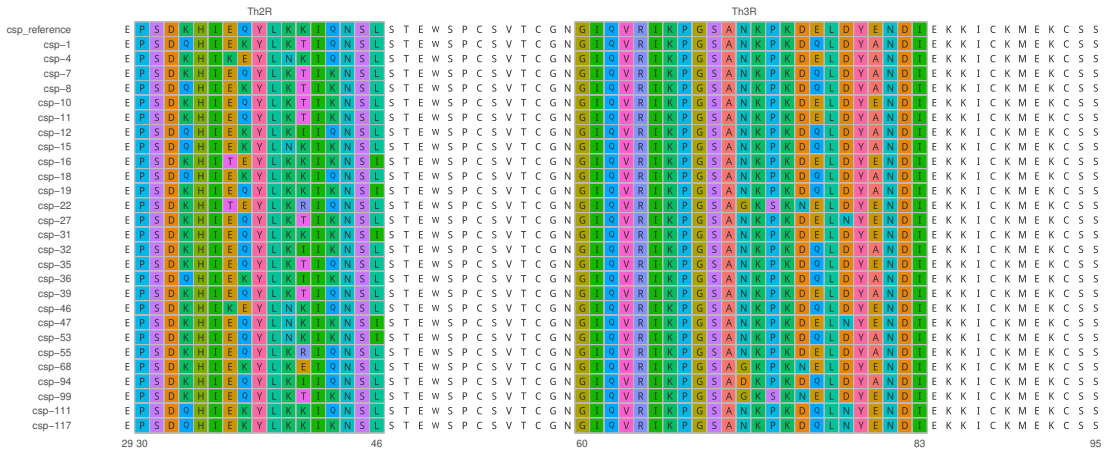

Figure 4.3: Sequences of the 27 CSP haplotypes found in this study. The Th2R and Th3R regions are highlighted to show variations from the 3D7 reference sequence.

percentage rank was above the threshold of the strong binders but below the default threshold of 2%. For Class HLAI-A, allele 30:01 has the strongest binding (SB) with 104 different CSP peptides, followed by allele 03:01 with 83 SB and 03:02 with 56 SB. For HLA class II, alleles DPA10401-DPB10501 and DPA10103-DPB10501 have the strongest binding with 26 CSP peptides, followed by DPA10301-10501 with 18 SB (Figure 4A). The top 30 best-performing HLA class I and II molecules were further analyzed with the 27 *PfCSP* haplotypes identified from Ghana that broadly represent Africa. All 30 HLA class I molecules were able to bind most of the *PfCSP* haplotypes; 16 out of the 27 *PfCSP* haplotypes (55%) showed higher binding affinity than the *PfCSP* reference 3D7. For HLA class I, alleles HLA-A 03:01, 30:01, 01:01, and HLA-C 03:03, 03:04, 08:01, 12:02, 16:01 showed mostly strong binding with all Ghana CSP peptides (Figure 4B). Conversely, alleles HLA-A 30:02, 33:03, 68:01, HLA-B 27:05, and HLA-C 07:01, 07:02 showed mostly weak binding (Figure 4B). For HLA II, only alleles DPA10103-DPB10501 and DPA10401-DPB10501 were shown to interact with all Ghana *PfCSP* haplotypes and the reference (Figure 4C). Over 90% of the HLA II alleles interacted/bound with only 60% of the *PfCSP* haplotypes. Haplotypes CSP-4, 18, 19, 31, 46, 47, 53, 55, 111, and 117 had the worst binding performance with HLA

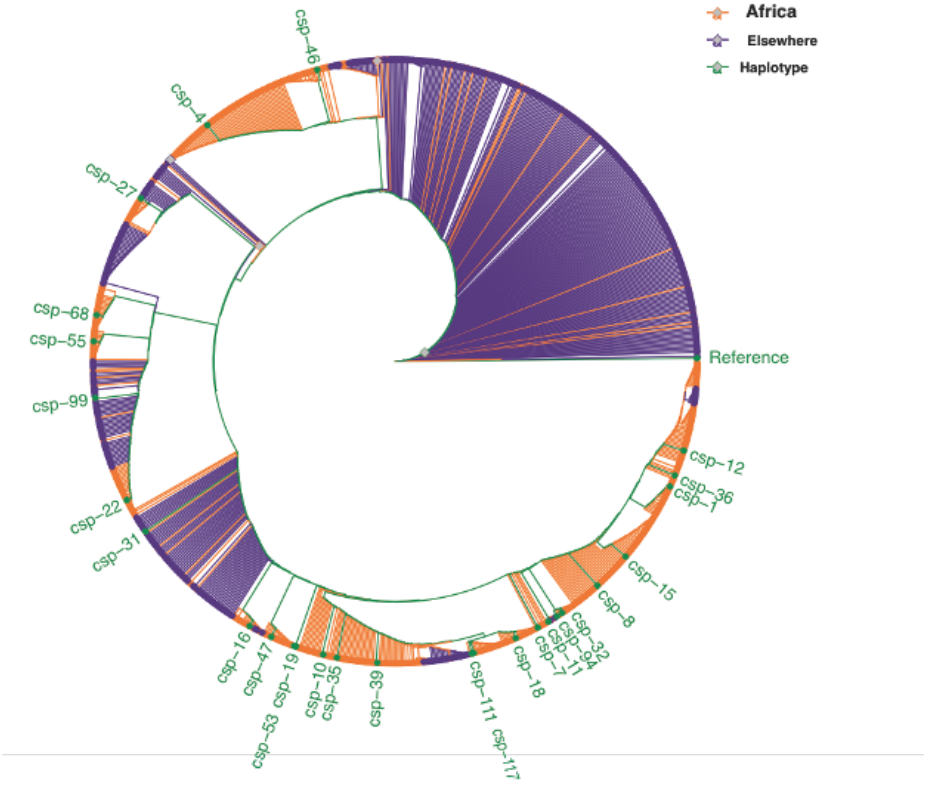

Figure 4.4: Comparison of the number of unique parasite clones based on *PfMSP1*1 and *PfCSP* sequences in north, central, and south Ghana. A significant correlation was detected between the CSP and MSP MOI (r= 0.33; P=0.005). When samples were analyzed by regions, only those in the north showed a significant correlation (r=0.41; P=0.039).

II (Figure 4C). It is worth noting that only CSP-94 bind well with both HLA I and II (Figure 4).

4.4.3 Binding affinity of *PfCSP* variants based on HADDOCK

The C-terminal of the *PfCSP* sequences contained the Th2R and Th3R regions (Figure 1). The Th2R region was more variable and contained 17 distinct peptide sequences (Table 2). The binding peptides of Th2R predicted by NetChop ranged from 4 to 9 residues in length and all begin with a glutamate (D) residue. A significant correlation was detected between the peptide molecular weight (or length) and its interaction with HLA (Figure 5). Longer peptides showed a higher binding affinity within the HLA groove (r=-0.94; P<0.001). Based on the HADDOCK scores, the

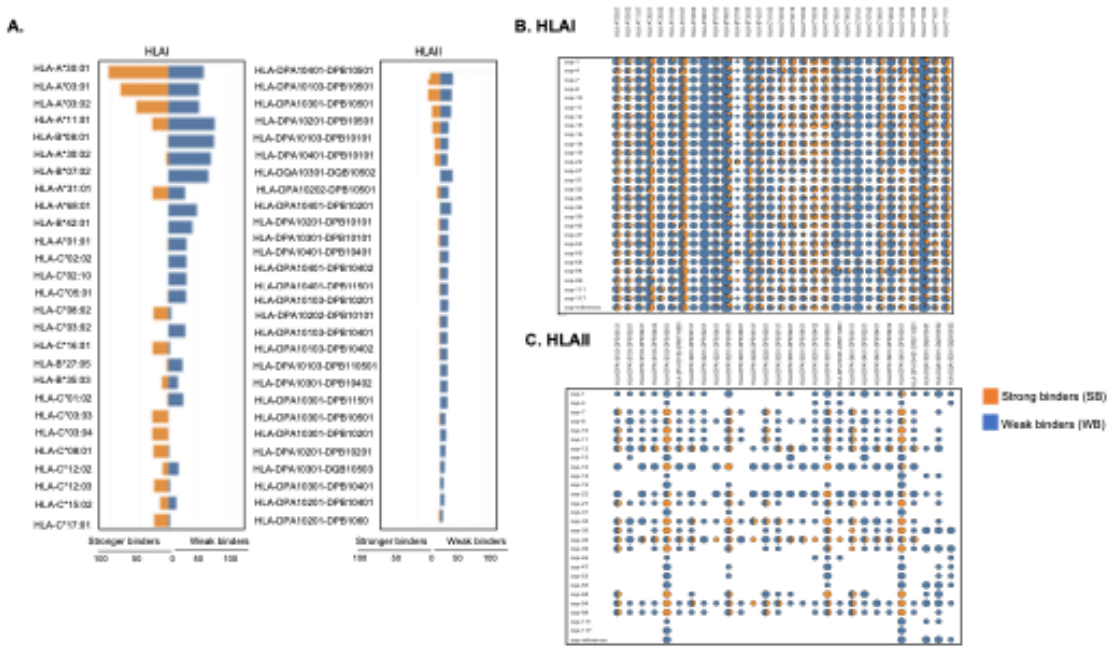

Figure 4.5: Comparison of the number of unique parasite clones based on *PfMSP1* 1 and *PfCSP* sequences in north, central, and south Ghana. A significant correlation was detected between the CSP and MSP MOI (r= 0.33; P=0.005). When samples were analyzed by regions, only those in the north showed a significant correlation (r=0.41; P=0.039).

binding affinity of the Th2R peptides with HLA did not correlate with the genetic relatedness with the 3D7 *PfCSP* haplotype. For instance, CSP-15, one of the most distant haplotypes containing a 5-residue peptide, showed the lowest HADDOCK score of -132.1 (highest binding affinity; 6.1% higher than 3D7; Table 2; Supplementary Table 2). Similarly, CSP117 that contains a 4-residue peptide showed a HADDOCK score of -128.6 (3.3% better than 3D7), the second-best interaction with the HLA (Table 2). While CSP10 and CSP-31 were the two most closely related haplotypes to 3D7, their HADDOCK scores were markedly different (Table 2). CSP10 containing a 6-residue peptide (DIELIN) has a similar HADDOCK score as 3D7, but CSP-31 with a 4-residue peptide (DIEI) has one of the lowest HADDOCK scores (Table 2; Supplementary Table 2). For the Th3R region, four peptides (VIELYE, VIQLYA, VIELYA, VIQLYE) were detected based on six amino acid residues (Table 2). HADDOCK scores of these peptides with the HLA were very similar without clear differences.To

Table 4.2: Correlations of peptide features against docking metrics by interaction and T-cell type and PfCSP gene region. Significant correlations at the α= 0.05 level are asterisked (*P<0.05; **P<0.01; ***P<0.001).

T-Cell Type and CSP Region	Interaction	Peptide Feature	Metric	Correlation	p-value
CD4+ / Th2R	HLA-CSP peptide	Molecular Weight	HADDOCK score	0.941	<0.001***
			Electrostatic energy	0.097	0.711
			Desolvation energy	0.681	0.003**
			Restraint violation energy	0.614	0.009**
			Van der Waals energy	0.907	<0.001***
CD8+ / Th3R	HLA-CSP peptide	Molecular Weight	HADDOCK score	-0.736	0.264
			Electrostatic energy	-0.767	0.233
			Desolvation energy	0.428	0.572
			Restraint violation energy	0.661	0.339
			Van der Waals energy	0.757	0.243

further elucidate if residues with larger side chains including tyrosine or glutamine may cause the CSP peptide to block the HLA groove, thereby reducing the binding interaction, we compared the HADDOCK scores and other evaluation matrices for peptides with and without tyrosine or glutamine. The χ^2-statistic indicated no significant difference among these peptides (Supplementary Table 3; Supplementary Figure 3). These findings indicated that the number of genetic variations from 3D7 does not correlate with the binding affinity with the HLA molecules.

4.4.4 Association of *PfCSP* antibody levels with ligand-peptide binding affinity

For a subset of samples, the CSP antibody level was measured to examine its correlation with the binding affinity between CSP peptide and HLA. No significant correlation was found between the CSP antibody level and binding affinity of CSP peptide with HLA (r=0.61; P=0.059). The antibody level was also not associated with qPCR-based parasitemia among the infected samples (r=0.58; P=0.082; Figure 5).

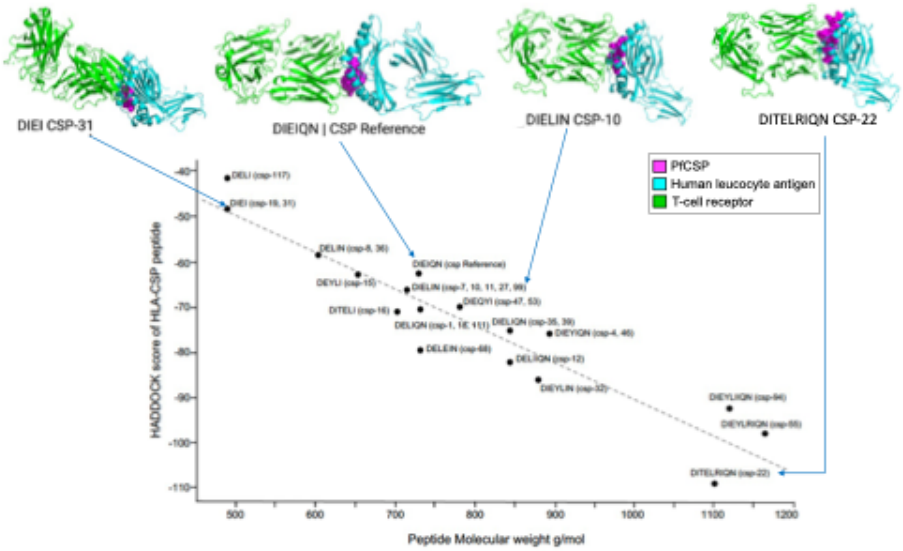

Figure 4.6: Comparison of the number of unique parasite clones based on *PfMSP1*1 and *PfCSP* sequences in north, central, and south Ghana. A significant correlation was detected between the CSP and MSP MOI (r= 0.33; P=0.005). When samples were analyzed by regions, only those in the north showed a significant correlation (r=0.41; P=0.039).

4.5 Discussion

plasmodium falciparum infections in highly endemic areas are usually genetically diverse and polyclonal. Such diversity could impact antimalarial interventions such as the emergence of resistant parasites and breakthrough infections of malaria vaccines (RTS,S Clinical Trials Partnership) [212, 244]. As RTS,S is widely implemented in Ghana and other parts of Africa, the high prevalence of multiclonal infections and non-3D7 *PfCSP* variants may allow the parasites to escape RTS,S-induced immunity, explaining the relatively low efficacy in phase 3 trial [224]. Consistent with other studies, *PfMSP1*1 is more variable than *PfCSP* in distinguishing parasite clones within hosts [224]. The positive correlation of *PfCSP* MOI and *PfMSP1* MOI values suggested that individuals who were infected with a higher number of parasite clones harbored multiple *PfCSP* variants that could impact immune responses induced by RTS,S. Among the Ghanaian samples, the genetic heterogeneity of the *PfCSP* was relatively high, with up to six distinct haplotypes within a sample. While three sam-

ples had identical Th3R sequences as the 3D7 reference, no samples had the 3D7-type Th2R sequences. This result agrees with a previous study that found no polymorphism in Th3R, suggesting that this region may be under balancing selection [245]. The four Th3R peptides (VIELYE, VIQLYA, VIELYA, VIQLYE) had similar HADDOCK scores and conceivably similar binding affinity. Previous studies have shown that vaccine efficacy declined as the number of amino acid differences increased, suggesting that allele-specific immunity is important in eliciting protection [221]. In Ghana, multiple *PfCSP* haplotypes are present within a polyclonal sample and most CSP haplotypes are markedly different from the 3D7 strain at the Th2R region. The 27 *PfCSP* haplotypes from Ghana revealed a great range of genetic variations comparable to the 1,655 *p. falciparum* isolates from other African countries. Most of the Ghana *PfCSP* haplotypes were clustered together with the other African *PfCSP*, except for CSP-99 and CSP-31. The CSP-reference is more closely related to the non-African *PfCSP* sequences. This is in accordance with previous studies that showed a greater genetic diversity amongst the African *p. falciparum* isolates than the Asian ones and that only 3% of the African isolates were similar to the 3D7 reference [246], [247]. Based on our findings, haplotypes that were most closely related to 3D7 could have peptide length and/or molecular weight different from 3D7 after proteolysis and exhibit completely different binding affinity with HLA. This finding suggests that the CSP peptide length rather than amino acid differences alone determine binding affinity and downstream immune responses. Further, residues with larger side chains such as tyrosine or glutamine in the CSP peptide have been shown to block the HLA groove [248], thereby reducing binding interaction. Nevertheless, our analyses showed no significant effect of tyrosine or glutamine on the docking of the CSP peptides. Across ethnic groups, several variants of HLA I and HLA II molecules [34] could exhibit different binding affinities [249]. High levels of HLA polymorphism within a population could confer a greater chance of survival against a wide array of pathogens. However,

such high diversity could also impact the host's ability to present foreign peptides to T-cell receptors [250]. In this study, we assessed the importance of the HLA diversity in relation to binding affinity with CSP peptides. Over 20 HLA-I and HLA-II alleles could bind strongly with all the CSP peptides detected in Ghana isolates. However, for HLA-II, the 3D7 reference and other CSP peptides did not show clear binding, suggesting that additional T-epitopes identified from broad CSP variants as well as longer epitopes should be considered in the vaccine construct to elicit a strong T-cell response. Similar studies on coronaviruses have shown that many SARS-Cov-2 epitopes for CD8 T-cells are HLA specific [251, 252], though studies on the association and significance of HLA diversity and binding affinity to T-cells across various diseases are still lacking. A clear correlation was detected between the molecular weight or length of CSP peptides and binding affinity when the peptide was docked in the protein-binding groove between the α1- and β1-subunits of HLA. The peptide-binding groove of HLA-I is known to contain deep binding pockets surrounded by polymorphic sidechains, which impose tight constraints on the residues and overall peptide length to achieve binding [253]. By contrast, the binding groove of HLA-II is open, allowing peptides of various lengths to fit into the binding groove [254]. This explains the challenge in predicting interactions of the HLAII-peptide compared to HLAI-peptide complexes. It is possible that longer peptides with exposed side chains allow for better interactions with HLA [255, 253]. Peptide length has been shown to affect the interactions with HLA molecules. For instance, a study of CD8+ T-cell response to the BZLF1 protein of the Epstein-Barr virus showed that individuals with HLA-B*18:01 responded strongly and exclusively to an octamer peptide and those with HLA-B*44:03+ responded to a dodecamer peptide [254]. Other biophysical factors such as the nature of the amino acids and peptide conformations can also affect peptide docking and binding affinity [256]. However, the interactions of this CSP-HLA complex with T-cell receptors as well as the impact on RTS,S responses

in vivo require further investigations. Our results indicated no significant correlation between anti-CSP antibodies and binding affinity for the HLA-CSP complex. However, only the most prevalent haplotype within the sample was investigated and the presence of other variants may affect antibody production. A recent study showed that high proportions of strain-specific antibody responses are likely to be elicited during high transmission season due to increased MOI and in turn, a higher preponderance of cross-reactive antibodies [217]. Our samples may represent only a portion of CSP variants present in Africa and none of the haplotypes are identical to the 3D7 reference. Expanding field isolates with a high prevalence of 3D7-matching haplotypes at the Th2R and Th3R regions would allow us to compare docking results and vaccine efficacy with samples of mostly non-3D7 haplotypes as in the present study. Also, only Th2R and Th3R regions of *PfCSP* were examined but epitopes in the N-terminal and NANP repeat regions may also affect binding interactions with HLA and other immunoproteins. The impact of *PfCSP* diversity in the N-terminal and central repeat regions on B-epitope recognition and vaccine efficacy remains unclear. Future studies should validate the in silico-derived candidate sequences in vitro using custom-designed assays to investigate the HLA as well as T-cell receptor interactions.

4.6 Conclusion

This study reveals a high level of polyclonality in *p. falciparum* infections across different transmission settings in Ghana. Mutations were found predominantly in the Th2R region of *PfCSP* and the most prevalent *PfCSP* haplotypes are not closely related to the 3D7 reference. The length of the Th2R peptide is significantly correlated with binding affinity with the HLA molecules, but genetic relatedness to 3D7 does not impact binding interactions. These findings provide new insights into low vaccine efficacy and inform the development of next-generation RTS,S.

The following are available here https://pubmed.ncbi.nlm.nih.gov/36841398/

CHAPTER 5: CONCLUSIONS

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 [125]. study highlights the observations of high asymptomatic prevalence and quadruple *pfdhfr*/*pfdhps* mutants in the north that have have important implications for the efficacy of ongoing SMC and IPT interventions in Ghana. Moreover, high level of polyclonality in *P. falciparum* were found predominantly in the Th2R region of *PfCSP*. The most prevalent

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