COPY NUMBER VARIATION OF *GTP CYCLOHYDROLASE 1* (*GCH1*) GENE AND ITS IMPACT ON ANTIFOLATE DRUG RESISTANCE OF *PLASMODIUM FALCIPARUM* IN GHANA

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DECLARATION

I, Musah Osei, do hereby declare that, except for the references to other people's work duly cited, the experimental work reported here is the result of my own work conducted at the Noguchi Memorial Institute for Medical Research (NMIMR) and the Department of Biochemistry, Cell and Molecular Biology (BCMB) laboratories, University of Ghana, Legon, under the supervision of Prof. Neils B. Quashie, Prof. Gordon A. Awandare and Dr. Nancy O. Quashie.

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DEDICATION

I dedicate this work to the Almighty Allah for His grace and granting me the wisdom, knowledge, understanding and strength to carry out this project.

Again, I dedicate this work to my wife, Aisha Dufie, and especially to my two lovely kids, Haroon and Shamaila for their love, support and understanding.

Also, I dedicate this work to my parents, Mr. & Mrs. Mohammed Osei and siblings for their support and prayers.

Finally, I dedicate this work to all children who participated in the studies from which I selected my samples and all the good scientists committed to eradicating malaria.

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LIST OF ABBREVIATIONS

ACT	Artemisinin Combination Therapy
Ala (A)	Alanine
Arg (R)	Arginine
Art	Artemisinin
Asn (N)	Asparagine
CDC	Centre for Disease Control
CNV	Copy Number Variation
Cys (C)	Cysteine
DHFR	Dihydrofolate reductase
DHNTP	Dihydroneopterin triphosphate
DHPS	Dihydropteroate Synthase
cDNA	Complementary DNA
gDNA	Genomic DNA
GCH1	GTP-cyclohydrolase 1
GTP	Guanosine triphospate

Glu (E)	Glutamic acid
Gly (G)	Glycine
Ile (I)	Isoleucine
IRS	Indoor residual spraying
IPTp	Intermittent Preventive Treatment in Pregnancy
Leu (L)	Leucine
LLIN	Long lasting insecticide treated net
Lys (K)	Lysine
MCBI	Molecular Cell Biology of Infectious Diseases
NMIMR	Noguchi Memorial Institute of Medical Research
*p*ABA	*para*-aminobenzoic acid
Pf	*Plasmodium falciparum*
Phe (F)	Phenylalanine
Ser (S)	Serine
SMC	Seasonal Malaria Chemoprevention
SNP	Single Nucleotide Polymorphism

SP	Sulfadoxine-Pyrimethamine
Thr (T)	Threonine
THF	Tetrahydrofolate
WACCBIP	West African Centre for Cell Biology of Infectious Pathogens
WHO	World Health Organisation

ABSTRACT

As part of the malaria control measures in Ghana, sulfadoxine-pyrimethamine (SP) is used as an Intermittent Preventive Treatment (IPTp) among pregnant women and now as a seasonal chemoprophylaxis (SMC) in children on pilot basis. However, parasite resistance to the drug has been reported in the country and this has been linked to point mutations in the *dhps* (dihydroptoreate synthase) and *dhfr* (dihydrofolate reductase) genes, the targets of SP. There is also an evidence of amplification of GTP cyclohydrolase 1 (*gch1*) gene, which codes for the first enzyme in the parasite *denovo* folate pathway, amongst parasites which harbor the highest SP resistance point mutation (164L) in South East Asia. These point mutations make the parasites less fit, but the acquisition of multiple copies of the *gch1* gene may compensate for this fitness cost. This study sought to determine the prevalence and effects of *Pfgch1* copy number variations (CNV) on SP resistance amongst clinical isolates in Ghana. Two hundred and two (202) blood samples collected from children aged 14 years and below, with uncomplicated malaria presenting at health centres in Accra, Kintampo, Cape coast, and Navrongo were used in this study. Quantitative real-time PCR (qPCR) and RT qPCR were used to estimate the copy numbers and expression levels respectively. The *pfdhps* and *pfdhfr* regions were PCR amplified and directly sequenced. Almost ninety-three percent (92.6%) and 7.4% of the parasite isolates harbored single and double copies of the *gch1 g*ene respectively. Duplication of *gch1* gene was independent of the different study sites (P=0.696). Point mutations at *dhfr*108N (P<0.001) and *dhfr*108T (P<0.001) were found to be associated with the study sites. Mutations at *dhfr*108 appear to be fixed in the parasite population and mutations at *dhfr*S108T and *dhps*A581G were observed for the first time in Ghana. However, there were no point mutations observed at codons 164L, 50R and 163T as

reported elsewhere. For correlation between the mutations and *gch1* CNV, only mutations at *dhps*540E (P=0.001), and *dhps*581G (P=0.002) were found to be significant. The relative expression between parasites with *gch1* CN of 1 and 2 was about 3-folds. The findings from this study revealed that mutations at *dhps*540E and *dhps*581G correlated with double *gch1* gene, implying that *gch1* may compensate for the fitness cost in parasites harboring these mutations. Continuous monitoring of *gch1, dhfr & dhps* genes and also further studies to discover component drugs that can inhibit the *gch1* gene product are required.

CHAPTER ONE

1.0 INTRODUCTION

Malaria is a life-threatening infectious disease caused by *Plasmodium* parasite and majorly transmitted from persons to persons by the female *anopheles* mosquitoes. Although there have been a global reduction in the incidence of morbidity and mortality of malaria from 2001 to 2015, (Cibulskis et al., 2016) there still remains the enormous burden of the disease which can never be underestimated. According to the WHO malaria report in 2016, an estimated 3.2 billion people worldwide are at risk of malaria. In the same report, 212 million cases were recorded which led to 429, 000 deaths. Out of these, 90% and 92% of cases and deaths respectively occurred in Africa (WHO, 2016). In sub-Saharan Africa, most severe cases and deaths occur in children younger than 5 years and in pregnant women. As per the WHO 2016 malaria report, 71% of the global mortality occurred in children under 5 years and a child dies of malaria every 2 minutes (WHO, 2016).

In Ghana, malaria transmission is perennial and endemic with seasonal and geographical variations. It is the most important cause of mortality and morbidity, especially among children under five years and pregnant women. In 2015, the National Malaria Control Program (NMCP) reported that 38.1% (10,169,829) of outpatient department (OPD) cases and 7% (2,133) of deaths were due to malaria. Of these, 31.2% (3,169,512) cases and 48.4% (1,033) deaths were recorded among children under five. Also, among pregnant women presenting at the various health centres in the country, malaria accounted for 17.6% of OPD attendance, 13.7% of admissions and 3.4% of maternal deaths (NMCP annual report, 2015).

Control measures adopted in the country include promoting home-based care, use of Insecticide Treated Nets (ITN) /Long Lasting Insecticide Nets (LLIN), improving case management in health facilities and use of appropriate chemoprophylaxis in pregnancy and children. Several efforts are under way to curb and eradicate the menace. However, a major setback is the acquisition of drug tolerance by the malaria parasite given the absence of an effective malaria vaccine (Arama & Troye-Blomberg, 2014).

The emergence and spread of *Plasmodium* parasite resistance to antimalarial drugs continue to be a major public health problem especially in endemic areas. Resistance of *Plasmodium* parasites, notably *falciparum*, to antimalarial drugs after a period of usage has become difficult to deal with in both high and low transmission areas (Hyde, 2007). Drug resistance is one of the main reasons for failures in malaria treatment and control strategies in sub-Saharan Africa. Resistance has already developed to all the antimalarial drug classes with one notable exception, the artemisinins, for which resistance has only been recorded in South-East Asia (Olliaro & Yuthavong, 1999; Witkowski et al., 2013).

In Ghana, sulphadoxine-pyrimethamine (SP) and artemisinin combination therapy (ACT) replaced chloroquine in the National Malaria Control Program guidelines as Intermittent Preventive Treatment in pregnancy (IPTp) and the first-line drug for treatment respectively in 2005, because of the high parasite resistance rates reported for the chloroquine drug (Quashie NB, 2007). However, there are reports of more than 60% prevalence of parasites harboring resistant mutations to the antifolate drugs in Ghana (Duah et al., 2012; F. Marks et al., 2005; Mockenhaupt FP & Otchwemah RN, 2005; Owusu-Agyei et al., 2009).

Antifolates against *Plasmodium falciparum* (*Pf*) has both treatment and prophylaxis benefits (Müller & Hyde, 2010). The antifolates kill parasite by targeting the enzymes in its *de novo* folate pathway and are active against all the growing stages in the liver, erythrocytic stages and growing stages in the mosquito (sporogonic stages). Among these targeted enzymes are the dihydrofolate reductase (dhfr) and dihydropteroate synthase (dhps) which are inhibited by pyrimethamine and sulfadoxine drugs respectively (Hyde, 2007). The inhibition of this pathway, limits the availability of folate derivatives to the parasite that serve as one-carbon carriers during nucleotide biosynthesis and amino acid metabolism. Mutations in these genes, however, decrease the binding affinity of the drugs to the targeted enzymes (Yuvaniyama et al., 2003).

At the molecular level, resistance of *P. falciparum* to SP has been shown to be due to point mutations in the *dihydrofolate reductase (dhfr)* and *dihydropteroate synthase (dhps)* genes (of the parasite), encoding the parasite enzyme dihydrofolate reductase (*Pf*dhfr) and dihydropteroate synthase (*Pf*dhps) respectively. In *Pfdhfr*, point mutations involving changes in Asn 51 to Ile (N51I), Cys 59 to Arg (C59R), Ser 108 to Asn (S108N), and Ile 164 to Leu (I164L) confer resistance to pyrimethamine (A. F. Cowman et al., 1988; Gregson & Plowe, 2005b). Parasites which carry *Pfdhfr* alleles with mutations at N51I and/or C59R and I164L, resulting in double, triple, or quadruple mutations, are increasingly resistant to pyrimethamine (Peterson et al., 1988). Resistance to sulfadoxine on the other hand depends on point mutations in the *Pfdhps* gene at codons 436 (S436A/F), 437 (A437G), 540 (K540E), 581 (A581G), and 613 (A613S/T) (Brooks et al., 1994; T. Triglia & Cowman, 1994; Wang et al., 1997). The level of the parasite resistance is linked to the number of point mutations in the *dhps* and *dhfr* genes. Therefore, multiple mutations in the two genes are considered to be most responsible for SP resistance (Kublin et al., 2002).

Reports of increasing prevalence of these mutations and its combinations in the *dhfr* and *dhps* genes have been described in Africa (Andriantsoanirina et al., 2010; Kiara et al., 2009). With the exception of some few *dhfr* and *dhps* mutants, most of the other mutants have been identified in Ghanaian isolates (Duah et al., 2012; Florian Marks et al., 2005; Mockenhaupt et al., 2005).

Although the use of SP for malaria treatment and prevention has fallen dramatically, surveys of circulating parasites in field settings demonstrate the continued presence of resistant haplotypes (Mbogo, 2014). A number of factors are likely to play roles in the maintenance of an SP-resistant parasite population. This may be due to the ongoing antifolate pressures in cases of IPT, antifolate containing antibiotics and seasonal malaria chemoprevention. Additional factors could be mosquito preferences for mutant alleles selection, modification of flux through the folate pathway as well as host factors.

A systematic analysis of the parasite's genome revealed a number of genes in multiple copies (T. J. Anderson et al., 2009). These are structural variations defined as two-fold or more multiplications of DNA segments larger than 1 kb. One of them is the *GTP-cyclohydrolase I* (*gch1*) which codes for the first enzyme in the folate pathway, but had not previously been identified as mediating resistance to antifolate drugs except the downstream enzymes, dhfr and dhps. This *gch1* gene is located on chromosome 12 and it is in functional linkage with folate biosynthesis, with *dhfr* and *dhps* located on chromosomes 4 and 8 respectively.Amplifications of *gch1* were detected in a study looking at geographically distinct parasites with known drug resistance profiles (Nair et al., 2008). The genomic amplifications in *gch1* resulted in an increased expression level of the corresponding mRNA. However, another study by Heinberg *et al* did not show a linear correlation

between multiple copies of *gch1* and its expression (Heinberg et al., 2013). It was also shown that the presence of multiple copies of the *gch1* was associated with the highest grade of resistance conferring point mutations in the dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*) genes (Heinberg et al., 2013; Nair et al., 2008; M. Ravenhall et al., 2016).

Amplifications of *gch1* may be vital to compensate for the putatively fitness-reducing mutations in *dhfr* and *dhps* by providing higher concentrations of downstream substrates in its folate-biosynthetic pathway (Heinberg & Kirkman, 2015). A joint analysis of Copy Number Variations (CNVs), Single Nucleotide Polymorphisms (SNPs) and transcriptomics may shed light on the molecular markers involved in drug resistance and its persistence, in order to guide policy, monitoring and ways to design drugs that will restore the efficacy of this antifolate.

1.1 Justification

The antifolate, SP, is still used in Ghana as a policy drug for IPTp during pregnancy and as seasonal malaria chemoprevention (SMC) in children on pilot basis, even though reports by Duah *et al* in 2012 and others indicate increasingly high circulating drug resistant parasite rates (Duah et al., 2012; Mockenhaupt FP & Otchwemah RN, 2005). The unapproved usage of SP (fansidar) by non-pregnant individuals and the administration of cotrimoxazole, an antifolate containing antibiotic, against patients co-infected with bacteria and malaria parasites, among other factors, may have also led to high drug pressure and possibly the selection of the antifolate drug resistant parasites (Duah et al., 2012).

Work done by Nair *et al* in 2008 provided compelling evidence of *gch 1* CNVs as an adaptive consequence of selection by antifolate drug pressures. This *gch1* CNV has also been shown to have a direct association with single nucleotide polymorphisms in *dhps* and *dhfr* which are targets of the SP drug. This led to the proposal that increased CNV of *gch 1* may potentially contribute to fixation of resistant parasites even if the drug pressure is dramatically reduced (Heinberg & Kirkman, 2015; Heinberg et al., 2013). These findings clearly show the importance of *Pfgch1* CNVs in maintaining the drug resistant isolates. However, data on CNVs of *Pfgch 1* in Africa where the disease is most prevalent is scanty.

It is therefore important to assess the prevalence of *gch1* CNVs of circulating parasites and the corresponding antifolate susceptibility profiles. This will reveal the proposed compensatory role of the *gch 1* amplifications for the fitness cost and fixation of resistant parasites. The role and validation of *pfgch1* CNVs in the persistence of resistant parasites when elucidated will inform policy makers on appropriate IPT drug interventions and also the consequences of using SP as a partner drug in ACTs.

1.2 Hypothesis

The *gch1* CNV plays a compensatory role for the fitness cost of SP-resistant parasites, and thus maintains these mutants in the population

1.3 Aim

To determine the prevalence and effects of *Pfgch1* copy number variations on SP resistance amongst clinical isolates in Ghana.

1.3.1 Specific objectives

1: To determine *gch1* copy number variations, *Pfdhfr* /*Pfdhps* single nucleotide polymorphisms (SNPs) and haplotypes in clinical isolates

2: To investigate the associations between the *gch1* copy number variations and the *Pfdhfr* /*Pfdhps* SNPs and haplotypes

3: To determine the relationship between *gch1* copy number variations and expression levels of this gene

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 MALARIA BURDEN

Malaria is a mosquito-borne infectious disease caused by unicellular protozoan parasites of the genus *Plasmodium* and phylum *apicomplexa*. Malaria affects primates, reptiles, birds and mammals, including humans (Arrow et al., 2004). For humans, it mostly affects people in tropical and subtropical areas of Africa, South America, Asia, and Oceania (Arrow et al., 2004). The disease is normally associated with flu-like symptoms such as fever, vomiting, chills, sweats, fatigue, malaise, dry cough and the severe form may result in diminished consciousness, convulsions, respiratory distress, hyperparasitemia, severe anaemia, hypoglycemia, jaundice, renal insufficiency, hemoglobinuria, shock, cessation of eating and drinking, repetitive vomiting, hyperpyrexia or death (Perkins et al., 2011).

The malaria parasite is commonly transmitted to the human host through the bite of an infected female *Anopheles* mosquito. It can as well be transmitted through blood transfusion and mother to child transmission may also occur during delivery (Menendez & Mayor, 2007). There are about 200 known species of the parasites that infect different animals. However, five of these species are known to infect humans, namely *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi*. Among these five species, *Plasmodium falciparum* is the most pathogenic and causes the most severe form of malaria predominantly in the African setting. The other four species are relatively less pathogenic and normally cause a milder form of the disease (Perkins et al., 2011).

2.2 BIOLOGY OF MALARIA PARASITE

The biology of the malaria parasite has been well studied although certain aspects are still unclear and need to be explored eg genetic mechanisms of drug resistance to certain drugs (T. Anderson et al., 2011). In the following sessions, insights into the biology of the parasite's life cycle and its folate metabolism are reviewed.

2.2.1 Life Cycle

As illustrated in figure 2-1, the life cycle of *P. falciparum* occurs principally at three different stages, the human hepatcocyte also termed as exo-erythrocytic schizogony which lasts for 9-14 days (varies depending on the infecting species), the human erythrocytes also known as erythrocytic schizogony within a 48 hour period, and a final cycle in the female *Anopheles* spp. mosquitoes, the definite host, called the sporogonic cycle for 10-18 days depending on the specie and geographical temperature.

During a blood meal of the female *Anopheles* mosquito on an infected person, the mosquito must ingest both microgametocyte (male gametocyte) and macrogametocyte (female) to initiate the parasite's life cycle. A diploid zygote is formed when the male gametocytes ex-flagellate and fertilize the female gametocyte. The zygotes are then elongated and become motile termed as the ookinets. They move to the midgut wall of the mosquito and transform into oocysts. The oocysts mature into several sporozoites, which travels to the salivary gland of the mosquito.

Upon a blood meal, the malaria-infected mosquito injects the sporozoites into the human host. These sporozoites migrate through various blood tissues to the hepatocytes. They then penetrate

the liver cells, preferentially the Kupffer cells (resident macrophages in liver) as shown with *P. yoelli* (Baer et al., 2007). The sporozoites has the circumsporozoite (CSP) protein which help them to attach and invade the liver cells. In these liver cells, a sporozoite develops into schizonts containing thousands of merozoites. Upon rapture of the liver cells and schizonts, these merozoites are released into the blood stream where they invade red blood cells. For ovale and vivax however, the parasite may remain dormant in the liver described as hypnozoites and may live for several weeks to years, known to be responsible for relapsing malaria.

Specifically to *P. falciparum* and some other species, merozoites enter the bloodstream and invade erythrocytes using the parasites' ligand and host RBCs receptor interactions (Alan F Cowman & Crabb, 2006; Gaur et al., 2004). The merozoites use ligands such as merozoite surface proteins 1 (MSP-1 to 9), apical membrane antigen-1(AMA-1), erythrocyte binding antigen-175 (EBA-175), and others, to bind specific host receptors such as erythrocyte binding-like proteins, sialic acids, and others. This initial interaction helps the parasite to re-orient itself and invade the RBC with its apical end. Unlike *P. falciparum which* uses various receptors to invade human erythrocytes, erythrocyte invasion by *P. vivax* and *P. knowlesi* use the Duffy receptors which is a blood group antigen on young erythrocytes (reticulocytes) (Grimberg et al., 2007). Therefore, individuals negative for the Duffy antigen are not susceptible to these infections as in the case of most people in West Africa.

A parasitophorous vacuole (PV) is created as the parasite forces its way into the erythrocyte. Part of the erythrocyte membrane pinches off to form the PV membrane. The parasite then develops inside the PV. Inside the RBC, the parasite undergoes a stage-wise development through early

trophozoites (ring stage, 0-24hrs) to young trophozoites, late trophozoites (24-36 hours) and schizonts (40-48 hours). The time for completion of the erythrocytic life cycle may be strain-dependent, ranging from 44-48 hours (Arnot & Gull, 1998).At the late ring stage, the parasites start to carry across its proteins to modify the surface of the erythrocyte. At this stage, the parasites begin to feed from the contents of the RBC. Hemoglobin is broken down into the moiety ferriprotoporphyrin IX (free heme) (Bannister et al., 2000). This molecule is toxic to the parasite and has to convert it to less toxic compound called hemozoin.

The active stages of the parasites development are the trophozites and schizonts. At these stages, more hemoglobin are degraded and the parasite create pores at the erythrocyte membrane known as the new permeability pathways for influx or efflux of nutrients and wastes (Ginsburg et al., 1983). Some early ring-stage trophozoites develop and mature into gametocytes without going through the normal intraerythrocytic cycle. These gametocytes will be picked by a mosquito upon taking a blood meal, to start the cycle all over again.

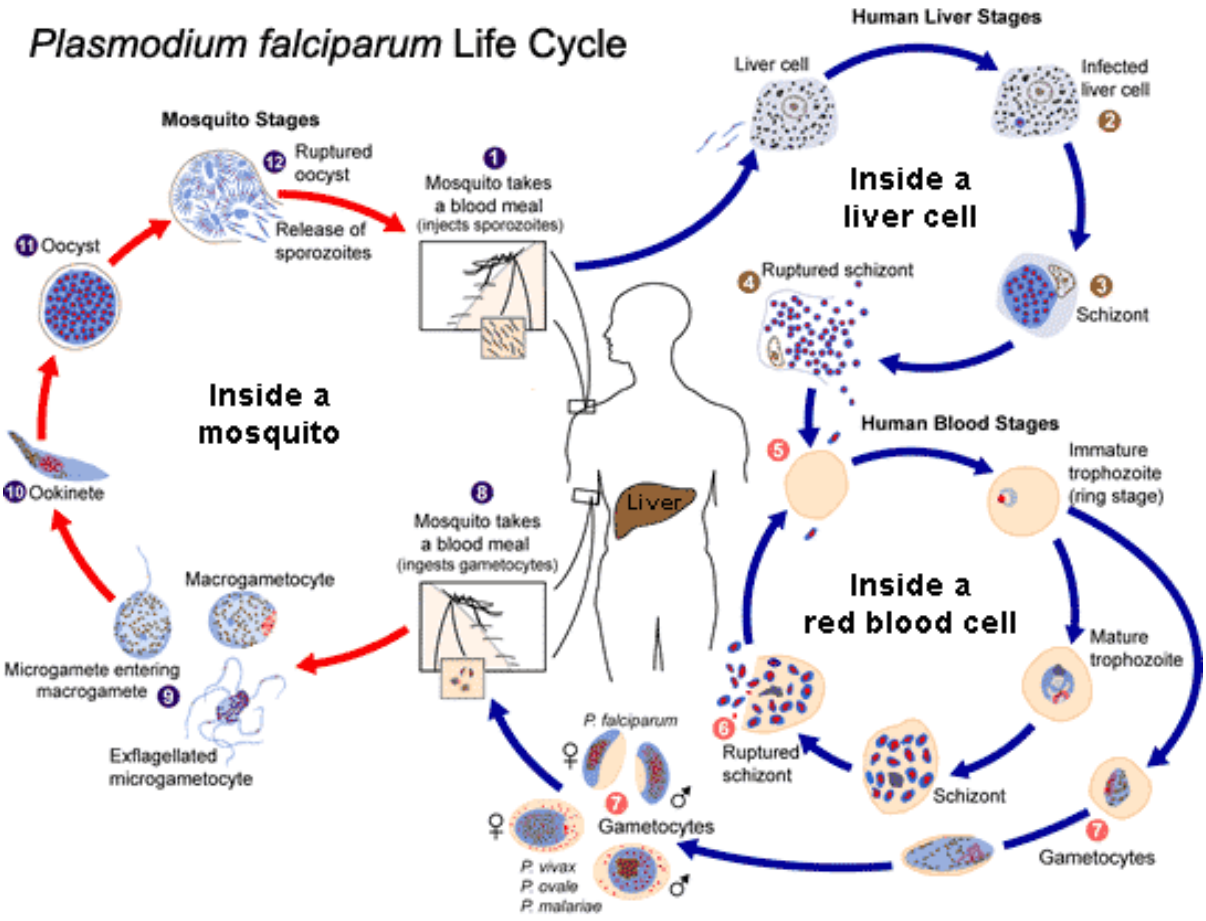


Figure 2-1: The life cycle of *P. falciparum*

(Source: adapted from CDC, https://www.pinterest.com/pin/2462974769116945/)

2.2.2 Folate biosynthesis in *Plasmodium*

Despite the fact that the parasite can salvage preformed folates from host and *in-vitro* cultures, the folate metabolism is essentially critical to the survival of a highly replicating malaria parasites. The predominant folate derivative circulating in the mammalian host is 5-CH_3-THF, which represents 80–90% of the total folate pool (Belz & Nau, 1998). However, this form of folate was found to be a poor substrate for the two Plasmodium folate transporters (Salcedo-Sora et al., 2011).

Folate is required for nucleotide and amino acid synthesis and serves as precursor for other folate-dependent reaction. The folate pathway in the parasite presents key enzymes which are targets of the antifolate drugs. The most common antifolates in use are pyrimethamine, sulfadoxine, proguanil, and dapsone, which have a history of effectiveness and relatively cheaper in middle-income countries (Sibley et al., 2001).

The folate metabolism yield cofactors which act as a source of one-carbon (C1) units in many cellular metabolic reactions. The coenzymes and their derivatives are always in their reduced state, tetrahydrofolate (THF). This need of THF by the parasite makes the antifolates attractive choice as an antimalarial. However, drugs in use as antifolates target only two of the pathway enzymes. There is, therefore, the need to explore other enzymes in the pathway as potential targets (Hyde, 2005).

Earlier works have shown the importance of folate in pyrimidine biosynthesis. In this biosynthesis, dUMP is converted to dTMP by the addition of methyl group from 5, 10-methylenetetrahydrofolate (methyleneTHF). This is subsequently phosphorylated to dTTP and incorporated into the growing DNA chain by the DNA polymerase.

2.2.2.1 Principal components of the folate pathway

In the de novo biosynthesis of folate as shown in fig. 2-2, the starting precursor GTP is converted to DHNTP by the gch1 enzyme. The enzyme catalyses the breakages of guanine and ribose rings in GTP and rearranges them to form a pterin ring system. This step is rate limiting in the pathway as seen in other microorganisms (Kumpornsin et al., 2014). The gch1 enzyme in *Plasmodium* differs from the orthologues in the human host. Although at the C-terminal the catalytic domain is

significantly conserved, it differs considerable at the N-terminal. The parasite has a long gch1 N-terminal extension of about 135 residues which is absent from the human gch1 (Kumpornsin et al., 2014). The DHNTP product is processed downstream by enzymes in the folate pathway including DHPS and DHFR, targets of SP. DHPS catalyses the conversion of pABA and 6-hydroxymethyl dihydropterin pyrophosphate to DHP. Sulfadoxine, as an antimalarial, which is a structural analog of pABA inhibit the activity of the DHPS enzyme. The downstream product, 7, 8 dihydrofolate (DHF) is acted upon by the pyrimethamine inhibitor, DHFR, which converts it to the reduced form, tetrahydrofolate (THF). The production of each molecule of dTMP is due to the oxidation of the THF molecule to DHF, which must be recycled by dihydrofolate reductase (DHFR) back to the THF form. Biosynthesis of the folate moiety itself is mediated by the action of five key enzymes, whereas interconversion of folate among the various forms required for C_1 transfer reactions is principally carried out by a further four activities. One of the latter, folylpolyglutamate synthase (FPGS), converts reduced folates to their polyglutamated forms, in which extra glutamate residues are conjugated to the single glutamate whose addition to dihydropteroate produces DHF. It has been shown in other systems that this process is critical for the cellular retention of folates as well as enhancing their affinity for other folate-dependent enzymes (Salcedo-Sora & Ward, 2013)

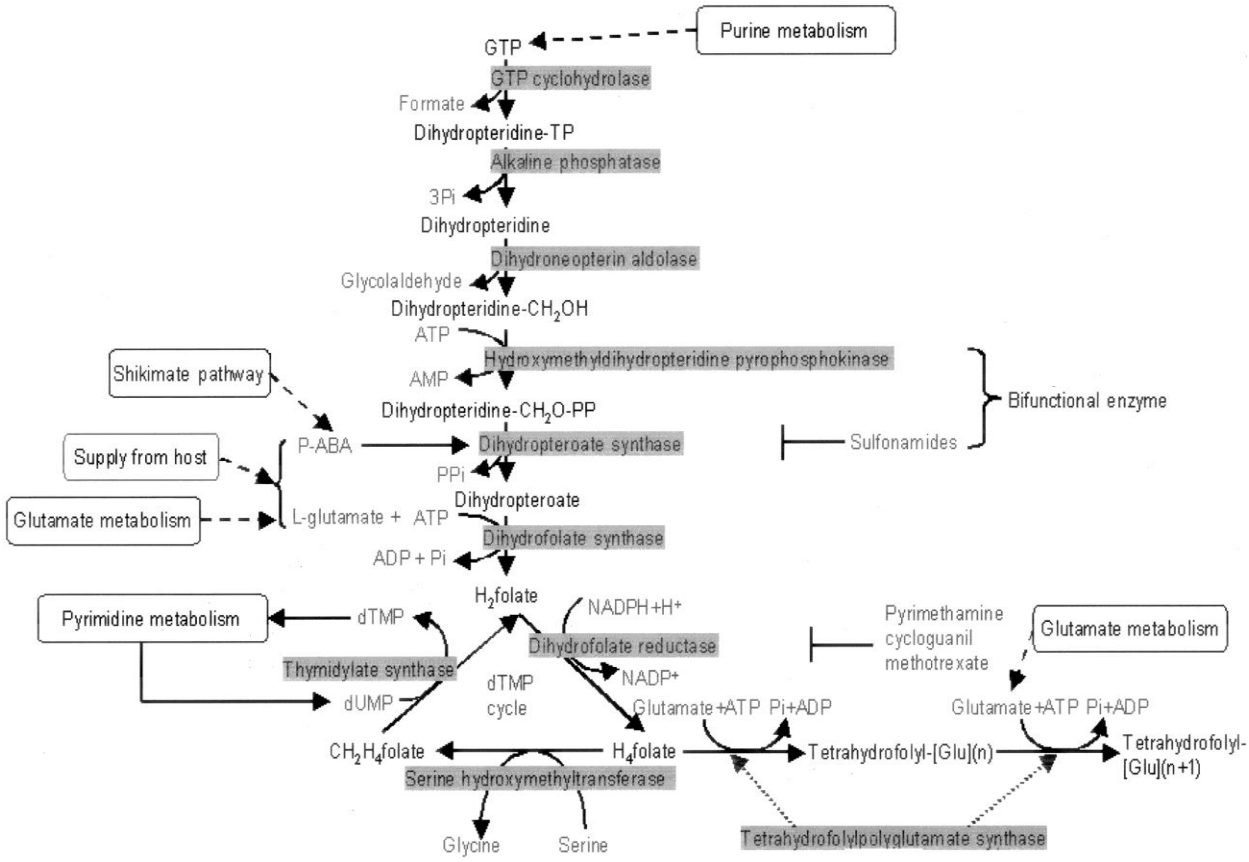


Figure 2-2: Folate metabolism pathway of the malaria parasite

(Source: http://malaria.atcc.org/metabolic_pathways/maps/folatebiopath.html).

2.3 CLINICAL SIGNS AND SYMPTOMS

The signs and symptoms of malaria normally initiate 8–25 days after infective mosquito bite (Tuteja, 2007), however, symptoms may arise later in those on antimalarial prophylaxis (Nadjm & Behrens, 2012). Irrespective of the infecting species, the disease presents an initial flu-like signs and symptoms (Bartoloni & Zammarchi, 2012) and can resemble other conditions such as sepsis, gastroenteritis, and viral diseases. The manifestation may include headache, fever, shivering, joint

pain, vomiting, hemolytic anaemia, jaundice, haemoglobin in the urine, retinal damage, and convulsions (Beare et al., 2006).

The classic symptoms of malaria are a periodic change in body temperature in a cyclical fashion which results in sudden coldness followed by shivering and then fever and sweating. Depending on the infecting species this cycle may take a different time period. *Plasmodium. vivax* and *P. ovale* infect reticulocytes and have a 48-hour cycle (tertian) whereas *P. malariae* infects old RBCs with a 72-hour cycle (quartan). The most severe form, *P. falciparum*, infects all RBC types, blood stages and cause recurrent fever in a 48 hour cycle (tertian malaria) (Arrow et al., 2004).

Among all the species that infect humans, *P. falciparum* causes severe complications such as severe anaemia, cerebral malaria, acute renal failure, hypoglycaemia and pulmonary infection. The two features that actually separate *P. falciparum* from the other human malaria are the ability to attack erythrocytes of all ages, causing high parasitaemia and enhanced growth and the capability to adhere to vascular endothelium through sequestration (Okwa, 2003) (Bartoloni & Zammarchi, 2012).

Some of the well-studied severe complications are cerebral malaria, placental malaria and anaemia due to malaria. Cerebral malaria caused by *P. falciparum* is when infected blood cells obstruct the blood vessels in the brain; other vital organs can also be damaged often leading to death of the patient.

Malaria in pregnancy is widespread. Pregnant women are especially vulnerable because of iron deficiency, a special problem in malaria endemic areas. It endangers the health of women and

prospects for the new born. Pregnancy exacerbates malaria through a nonspecific hormone-dependent depression of the immune system. Malaria infection in pregnancy is significant in sub -Saharan Africa, where its fatality as a result of virulent *P. falciparum* is a far greater problem than in most parts of the world (Mason, 2003).

2.4 MALARIA CONTROL

Effective control measures, policies, funding and research have led to the decline of malaria in the past decade. According to WHO 2016 report, there have been a mortality reductions of 62% globally between 2000 and 2015 and by 29% between 2010 and 2015 (WHO, 2016).

Among the control measures that led to the decline are enhanced vector control, rapid diagnostic methods and easy access and effective antimalarial drugs (O'Meara et al., 2010) (WHO, 2012).

2.4.1 Vector control

Vector control can be achieved by the use of long lasting insecticide treated nets (LLINs), larval control or indoor residual spraying (IRS). Different chemicals are used in LLINs. However, pyrethroid is highly recommended by WHO (WHO, 2015). They have a minimum lifespan of 3 years which is also dependent on field settings. The net protects individuals by repelling and killing the mosquitoes that come into contact with it thereby reducing the infective bites.

Indoor residual spraying employs the application of long-lasting chemicals as insecticide against the mosquito vector on the walls, ceilings and eaves, and kills the parasite upon resting or feeding.

This reduces the malaria transmission intensity at the community at large. The vector takes lethal doses of the insecticide when it comes into contact with the sprayed surfaces, thereby quickening its death. From 2005 to 2010, the coverage of people protected by IRS rose from 10 million to 78 million in the WHO African region. A total of 6% and 4% of people at risk globally were protected by IRS in 2010 and 2013 respectively (WHO, 2011, 2015).

Larval control and general environmental management may be used to augment the existing vector control (WHO, 2006).

2.4.2 Vaccines

It is obvious that the development of malaria vaccines have had lots of setbacks in the previous years. In recent times, however, a new malaria vaccine (RTS'S) has proven to be efficacious to an extent (Rts et al., 2012). This is the only vaccine so far advanced in clinical trials with an efficacy that reduces the incidence of clinical and severe malaria by 39% and 31.5% respectively among children of 5 to 17 months old (WHO, 2016). In view of this finding, the WHO has recommended five countries in sub-Saharan Africa, where the trials occurred, to implement the RTS'S vaccine on pilot bases, in 2018. The low percentage of protection by the vaccine presupposes that there is the need to augment it with the existing chemo preventive and treatment measures.

2.4.3 Chemoprevention and therapy

As mentioned earlier, the use of drugs for treatment or prophylaxis cannot be underemphasized so long as effective vaccine(s) are not available. Most of these antimalarials are out of use in certain geographical areas due to resistance. The major antimalarials and their mechanisms of actions are discussed in the next sessions.

2.4.3.1 Quinine

Quinine (QN) as an antimalarial drug was isolated from the bark of the cinchona tree, found in Peru. Although there are reports of resistance to the quinine drug, it is still used for the treatment of acute cases of severe malaria although replacement with artesunate is being recommended (Dondorp et al., 2010). It is also suitable in areas where there is high level of resistance to mefloquine, sulfadoxine-pyrimethamine and chloroquine.

Quinine is a blood schizonticidal alkaloid that accumulates in the food vacuoles of *Plasmodium* species, especially *falciparum* and acts by inhibiting hemozoin biocrystallization and the activity of heme catalase (Slater, 1993).

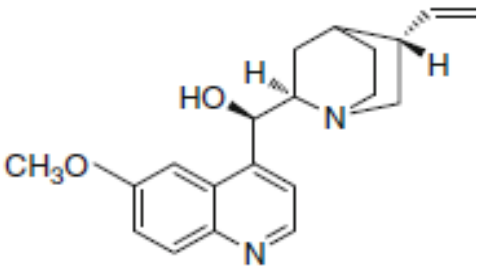


Figure 2-3: Structure of quinine (Melnyk et al., 2015)

2.4.3.2 Chloroquine

Chloroquine is a 4-aminoquinolone compound which was known to be highly effective, cost effective and safe for several years until the onset of resistance. Chloroquine is known to act by inhibiting the polymerization of harmful haem moieties produced as a by-product of haemoglobin

breakdown by the parasite as a source of its amino acids. This process occurs in a lysosome-like acidic digestive vacuole of pH 4.5–5.0, in which chloroquine accumulates and gets protonated. In this way the chloroquine does not diffuse out and prevents the polymerization of toxic heme to the less toxic hemozoin, thereby killing the parasite.

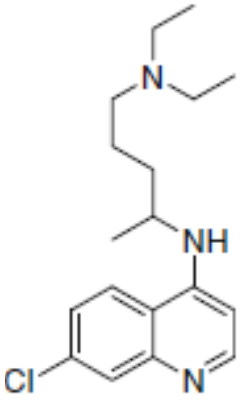


Figure 2-4: Structure of chloroquine (Melnyk et al., 2015)

2.4.3.3 Piperaquine

Piperaquine is a 4- amino quinolone which is majorly used for prophylaxis and as partner drug in artemisinin combination drugs (Biagini et al., 2012). Piperaquine has shown to be safe, relatively cheap, long half-life and also effective against chloroquine resistant parasites, thus its use in dihydroartemisinin-piperaquine by WHO (WHO, 2011).

Piperaquine in known to have structural similarity to chloroquine and thus a similar mode of action (Warhurst et al., 2007). However, the exact mode of action is still not clear although studies have shown its concentration in the digestive vacuole and a possible inhibitor of heam polymerization.

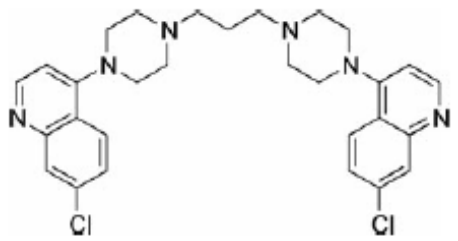


Figure 2-5: Structure of piperaquine (Melnyk et al., 2015)

2.4.3.4 Mefloquine

The U.S. Experimental Therapeutics Division of the Walter Reed Army discovered mefloquine in the 1970's as prophylaxis due to its long half-life (Kitchen et al., 2006). Mefloquine is active against the erythrocytic stage of the parasite. Studies have shown that mefloquine interfers with the transport of hemoglobin from the host erythrocyte to the food vacuole of the parasite which causes swelling and cytotoxicity of the vacuole (Hoppe et al., 2004). However, emergence of resistance and fears about neurotoxicity are limiting its use (Croft, 2007). Resistance has been shown to be mediated by an increase in copy number of *P. falciparum* multi-drug resistance (MDR) gene-1 (pfmdr1), a gene which codes for the parasite –transport protein (Price et al., 2004).

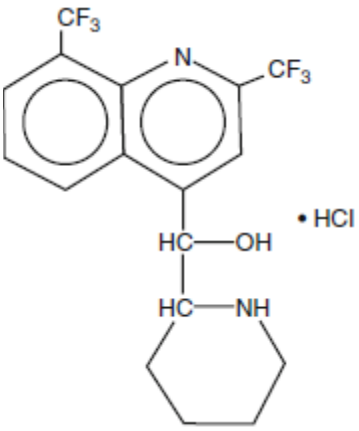


Figure 2-6: Structure of mefloquine hydrochloride (Aminake & Pradel, 2013)

2.4.3.5 Lumefantrine

Lumefantrine was synthesized by the Beijing Academy of Military Medical Sciences (Basco et al., 1998) in the 1980s. It is used in combination with artemether under brand name Coartem. The artemether-lumefantrine (AL) combination was the first fixed dose artemisinin-based combination therapy recommended and pre-qualified by WHO for the treatment of *P. falciparum* uncomplicated malaria (Nosten & White, 2007). Although the drug has never been used as monotherapy, *in vitro* studies implicate single nucleotide polymorphisms and increase copy number in *pfmdr-1* to increase IC_{50} values (Mwai et al., 2009).

It is active against the asexual stage of the parasite excluding the pre-erythrocytic liver and gametocytes stages. The mechanism of action is not clearly known but is assumed to inhibit heam polymerization due to its class of antimalarial.

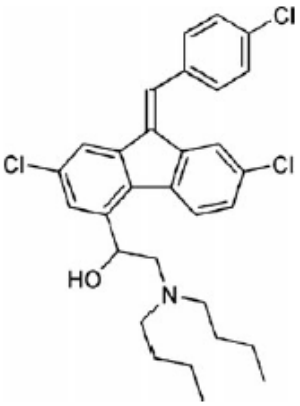


Figure 2-7: Structure of lumefantrine (Aminake & Pradel, 2013)

2.4.3.6 Primaquine

It is an 8-aminoquinoline class of antimalarial. Primaquine is an important class of compound due to its ability to act against the liver stages of *Plasmodium* infection. It is highly effective against asexual blood-stages of *P. vivax* than *P. falciparum.* In addition to being effective at the liver stage, it also acts as gametocidal and thus blocks malaria transmission. Primaquine accumulates in the parasite's mitochondria and interferes with its function (Vale et al., 2009).

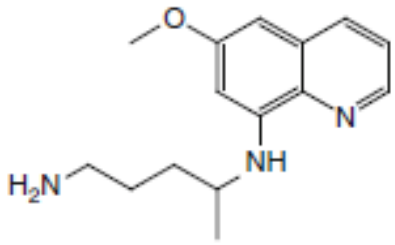


Figure 2-8: Structure of primaquine (Aminake & Pradel, 2013)

2.4.3.7 Artemisinin compounds

Artemisinin comes from the *qinghao* plant (*Artemisia annua*), used in ancient China as herbal treatment for relapsing fever. The active compound, sesquiterpene lactone artemisinin, was rediscovered from the plant in the 1970's for malarial illness (Cui & Su, 2009).

For the Artemisinin (ART) to be pharmacologically advantageous, derivatives such as water soluble artesunate and the more active oil-soluble dihydroartemisinin (DHA) and artemether (Cui & Su, 2009) were synthesized. Artemisinin and its derivatives are currently used in most endemic areas as the first-line treatment. The advantages of using ART and its derivatives include many possible routes of administration, suitability for treating severe malaria, thus substituting QN and avoiding its side effects (Dondorp et al., 2010) Additionally, ARTs are active against early ring forms as well as the usual targets of chloroquine and quinine, trophozoites, blood schizonts, early gametocytes and later ring forms (Krishna et al., 2004). This latter property may help diminish transmission rates at a population level (Price et al., 1996) since attacking early ring forms, can impair gametocyte development. However, the use of ART as monotherapy over short periods (less than 5 days) is associated with high treatment failure rates (recrudescence) because of the short plasma half-lives of these drugs (Giao et al., 2001). For this reason, and to avoid resistance development, ART is only used in combination therapy.

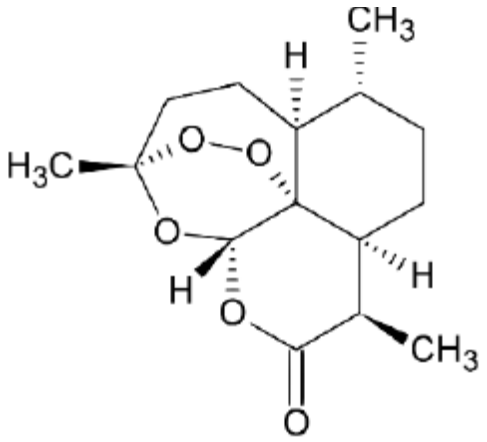


Figure 2-9: Structure of artemisinin(Aminake & Pradel, 2013)

2.4.3.8 Antifolates

The inhibition of folate metabolism remains very useful for discovery of new antimalarials. Targets in this pathway date back to the 1940's with the discovery of triazine proguanil. Proguanil is still used in combination with atovaquone (Malarone®) as prophylaxis for non-immune travelers. Pyrimethamine was then discovered in the early 1950s based on chemical similarity to cycloguanil, the active metabolite of proguanil, and has been used in synergy with sulfadoxine as Sulfadoxine-Pyrimethamine (SP) also known as FansidarTM (A. Nzila, 2006). SP is also used in combination with amodiaquine as seasonal malaria chemoprevention (SMC) in children to reduce morbidity and mortality of malaria in Sahel sub-Region of Africa (WHO, 2013). SMC is not recommended elsewhere, due to known high levels of resistance to amodiaquine and SP in eastern and southern Africa.

Sulfadoxine-pyrimethamine (SP) is safe and effective, relatively cheap, has a long half-life and is considered a drug with good compliance due to its single dose administration (Basco et al., 1998). After its introduction as a new antimalarial drug, SP replaced CQ in many countries, due to the emergence and spread of CQ resistance (White, 2004).However, resistance to SP has restricted its use as prophylaxis to pregnant women and infants in high transmission settings (WHO, 2013). SP resistance emerged mostly in the Amazon Basin of South America and most areas of South East Asia which led countries to adopt the more effective artemisinin combination therapies (Gregson & Plowe, 2005a). The structures of commonly used antifolates are shown in fig. 2-10

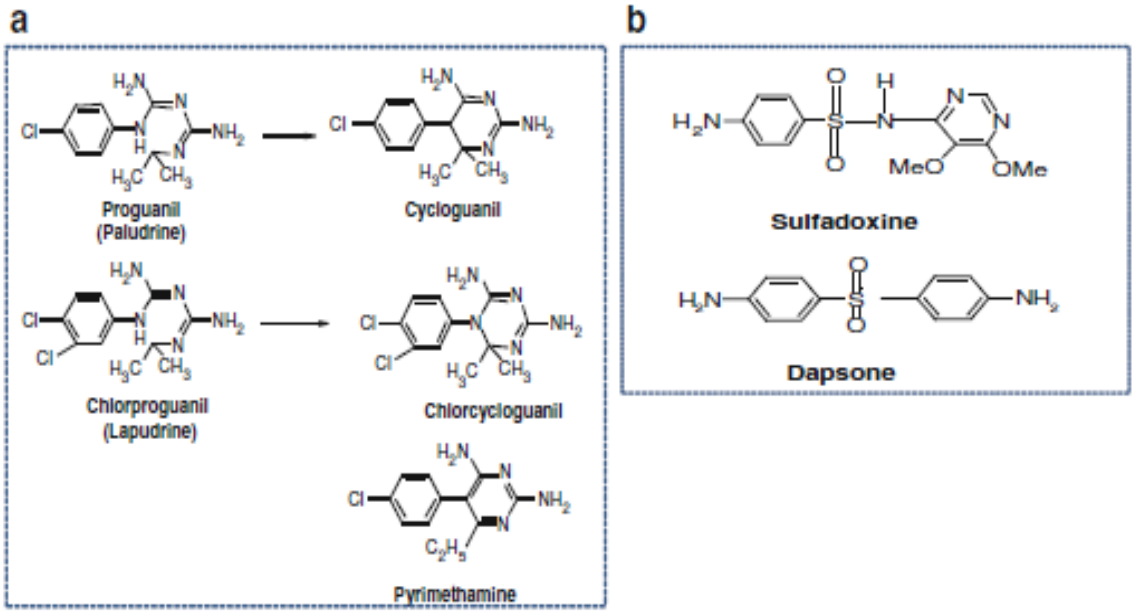


Figure 2-10: Structure of antifolates (Aminake & Pradel, 2013)

a: Inhibitors of DHFR enzyme b: Inhibitors of DHPS enzyme

2.5 EMERGENCE OF ANTIMALARIAL DRUG RESISTANCE

Currently, the success of malaria control depends majorly on effective antimalarial drugs. However, resistance of Plasmodia, specifically *falciparum* specie, to all the antimalarial drugs have been reported in different settings and their toll effect on morbidity and mortality can never be under estimated. In the 80's and 90's, Africa recorded an increase in morbidity and mortality due to chloroquine resistance (Trape, 2001) at the time. The situation is not different from today, where resistance to available antimalarial drugs leads to increase morbidity and mortality.

The introduction of ACT has made a tremendous impact in reducing malaria morbidity and mortality over the last decade. Also, the use of SP for preventive treatment has contributed to the overall success chopped in the last decade especially in endemic settings. The high rate of SP resistance may thwart the drug preventive measures against the disease in high transmission areas.Antimalarial drugs remain as one of the most powerful tools in the fight against malaria and the development of resistance to these compounds is probably the greatest problem faced by malaria control programmes.

In the last decade, first line treatment with ACTs has played a major role in reducing malaria burden (Dondorp et al., 2011). Currently, no alternative antimalarial treatment offers the same level of efficacy and tolerability as ACTs (WHO, 2013). Therefore, the emergence and spread of ART resistance would be a serious threat to the elimination/eradication efforts.The presence of drug pressure is known to select for resistant-parasite genotypes. Although the emergence of *de novo* mutation is independent of drug pressure, the drug pressure provides a selective advantage

to the mutants. The spread of such resistant genotypes by mosquitoes increases their frequencies in the population (Hastings, 2006).

The emergence of genetically resistant parasites can be as a result of point mutations, gene copy number variations and may also occur through epigenetics (Glasspool et al., 2006). One of these factors or combinations of them may be the causal effect of the resistance. In the first two scenarios, the cause is normally as results of errors of DNA repairs and replications, genetic recombination processes, chromosome segregation, irradiations and chemicals that induce mutations. The parasites carrying the mutations are selected if antimalarial drug dose is enough to inhibit the growth of susceptible parasites but inadequate to prevent the development of the newly emerged mutants, a process described as "drug selection". In the subsequent sessions, the role and emergence of these types of mutations are described in the context of SP resistance.

2.6 MECHANISM OF SULFADOXINE-PYRIMETHAMINE DRUG RESISTANCE

Well-studied point mutations in the dihydrofolate reductase (*dhfr)* and dihydropteroate synthetase (*dhps)* genes, that code for the drug target enzymes in the parasite's folate biosynthetic pathway, have been shown to confer resistance by decreasing the binding affinity of the enzyme. Host factors play vital role in the eventual treatment outcome of a drug intervention. In *in vitro* culture, parasite resistant to a drug due to their mutational background may be cleared in natural infections. This emphasizes the important role of host immunity that confounds the true effectiveness of a drug intervention (Sibley et al., 2001). In view of this, WHO still recommends the use of SP among pregnant women as IPTp and seasonal malaria chemoprevention (SMC) in children, even in high transmission settings with proven resistance markers (WHO, 2016).

2.6.1 Mutations in *dhfr*

Point mutations in dhfr underlie resistance to pyrimethamine. The level of resistance is associated with the stepwise accumulation of point mutations, normally described as haplotypes, within the gene encoding the dhfr enzyme at codons 50, 51, 59, 108, and 164 (Plowe et al., 1997) The more mutations accumulate in the gene, the higher the level of resistance conferred to the parasite. For example, the S108N mutation confers some pyrimethamine tolerance to the parasite, but not as high as in the triple mutant, N51I/C59R/S108N(White, 2004). High-grade pyrimethamine resistance correlates with the presence of I164L mutation (Peterson et al., 1988) which always occur together with the triple N51I/C59R/S108N mutant in Southeast Asia and the Americas (Cortese et al., 2002). The I164L mutation, which is shown to cause rapid spread of SP resistance, has been observed in parts of East Africa (Juma et al., 2014; McCollum et al., 2006). Also, *dhfr* mutations, A16V and S108T, have been shown to confer reduced parasite susceptibility to cycloguanil than pyrimethamine. These mutations are highly prevalent in areas where proguanil is widely used as a prophylactic agent (Gregson & Plowe, 2005a).

2.6.2 Mutations in *dhps*

Five point mutations in dhps are well known to mediate sulfadoxine resistance and, as with dhfr; these occur in a stepwise fashion following the introduction of treatment with Sulfadoxine/Pyrimethamine (SP) (Hyde, 2007; Roper et al., 2003). These mutations are at codons S436A/F, A437G, K540E, A581G, and A613T/S. The most common forms of mutation in dhps that compromise the effectiveness of sulfadoxine include the codon changes A437G/K540E in

Africa and A437G/A581G or A437G/K540E/A581G in South East Asia, with the latter often observed in parts of South America (Hamour et al., 2005). The A437G mutation is highly prevalent in areas of SP resistance, suggesting that this may be the first mutation in response to the drug pressure. The mutation at S436A/F had been debated to be an alternative wild type form (A. M. Nzila et al., 2000). However, the median IC_{50} for parasites with S436A/F and A437G were higher than only A437G (Tony Triglia et al., 1997). Following these changes, mutations at codons 540, 581 and 613 are sequentially acquired and confer increasing resistance to sulfadoxine. The dhfr triple mutant (Asn-108 + Ile-51 +Arg-59) and dhps double mutant (Gly-437 + Glu-540) have been strongly associated with potential resistance in sub- Saharan Africa (Eltayeb, 2015) and a strong indicator of likely clinical outcome. The 581 and 613 mutations are most common in SEA. Recent studies have shown that the presence of A581G more than 10% in a population can compromise the efficacy of SP as IPTp intervention (R Matthew Chico et al., 2015)

2.6.3 Mutations in *gch1*

Recent genomic analyses of the malaria parasites showed multiple copies of GTP cyclohydrolase I (GCH1), the first and the rate-limiting enzyme of the de novo folate biosynthesis, which also influence SP resistance (Figure 2-2) (Hamour et al., 2005; Kidgell et al., 2006). In Southeast Asia, multiple copies of the *gch1* have been shown to have a direct association with point mutations in the *dhfr* and *dhps* genes, and confirmed in a separate study which used genetic manipulations of parasite lines (Heinberg et al., 2013; Nair et al., 2008). The *gch1* copy number was found to correlate with the highest level of *dhfr* resistance, I164L (Nair et al., 2008) in Thailand where there was long standing use of SP. In Africa however, a recent study in Malawi reported a novel *gch1*

promoter duplication in parasites with quintuple (3*dhfr* + 2*dhps*) mutation background. This promoter duplication differs from the whole gene duplication found in Southeast Asia (M. Ravenhall et al., 2016).

Multiple copies of the gene has been shown to reduce pyrimethamine sensitivity slightly, but most importantly, the amplification was thought to optimize the fitness of SP drug-resistant parasites (Heinberg et al., 2013; Kümpornsin, Modchang, et al., 2014). The increase in the GCH1 enzyme was found to improve the folate metabolic flux by several orders of magnitude through the folate pathway (Hossain et al., 2004). The drug-resistant mutations at the dhps and dhfr, though advantageous under SP pressure, makes the parasite less fit due to the changes at the active site (Brown et al., 2010; Chookajorn & Kümpornsin, 2011; Lozovsky et al., 2009). It was therefore hypothesized that the *gch1* CNV plays a compensatory role for the fitness cost of SP-resistant parasites, and thus maintaining the SP-resistant parasites in the population.

Studies on the function and biochemical characterization of the gene have shown that the GTP analogue inhibitor, 8-oxo GTP, has an inhibitory effect against the gch1 enzyme (Kumpornsin et al., 2014). The inhibition of *P. falciparum* gch1 could become a novel approach for combating the emerging threat of SP resistance. The gch1 could also serve as a molecular marker for surveillance of SP malaria drug resistance.

2.7 METHODS OF DETECTING DRUG RESISTANCE IN *PLASMODIUM* PARASITE

2.7.1 *In vivo*/Clinical studies

An *in vivo* test is the assessment of the treatment outcome of an antimalarial drug in the living after administering a standard dose of the drug (WHO, 2003). The main characteristics of this method are the evaluation of the clinical and parasitological responses. The process involves parasitological examination of blood film on day 0 and subsequent follow-ups on day 3, 7, 14, 28 or even 42 after the standard treatment (Stepniewska et al., 2004). Although set to be the gold standard for resistance studies, treatment outcome incorporates the complex interactions of the host, parasite and the drug. Therefore, in addition to other drawbacks, reduced drug efficacy can be masked by immune clearance of parasites among patients with high titers of acquired immunity (Marfurt et al., 2010).

2.7.2 *In vitro* test of parasite susceptibility

This method relies on culturing of *P. falciparum* isolates in the presence of a range of antimalarial drug concentrations for one cycle or part of intraerythrocytic asexual replication. The efficacy of the drug is based on the minimum concentration required to inhibit parasite schizogony by 50% (IC_{50}). This test removes confounding factors such as acquired immunity, re-infection and pharmacokinetics. The method allows different drugs to be tested simultaneously (WHO 2003). However, the test has certain significant setbacks. Prodrugs, such as proguanil, which require to be metabolized within the host into its active form cannot be tested. Neither can drugs that need some level of synergism with the host's immune system be tested. Equally, it may give false

suggestions if the *in vitro* susceptibilities do not eventually translate into parasitological or clinical resistance.

2.7.3 Detection of molecular markers

Detection of molecular markers for resistance offer valuable tools for public health surveillance. Molecular tests use polymerase chain reaction (PCR) to detect the presence of mutations that lead to phenotypic resistance to antimalarial drugs. The method allows for determining resistance in a relatively larger sample size within a short period. Also, only filter paper blood spot is needed and it is far easier as opposed to *in vitro* and *in vivo* studies. It is also appropriate to use this method to track the rates of resistance after the withdrawal of an intervention in a population. However, highly skilled personal is required. Also, some of the reported resistance markers in certain geographical areas may not be applicable for the same drug in other settings.

All the above methods should be considered as complementary rather than competing sources of information about drug resistance (WHO, 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study sites

Archived clinical samples from four sentinel designated sites located in three different ecological zones in Ghana with different malaria transmission rates were used in this study. Samples from three of these sites, Kintampo, Navrongo and Accra were collected during an Erythrocyte Invasion Mechanism (EIM) Projects led by Prof. Gordon A. Awandare. Also, samples collected by Dr. Nancy O. Quashie and Prof. Neils B. Quashie from two sentinel sites, Navrongo and Cape Coast, designated as part of a surveillance program for monitoring malaria drug resistance in Ghana were used. The sites, among others, were established by the NMIMR in collaboration with the National Malaria Control Program (NMCP).

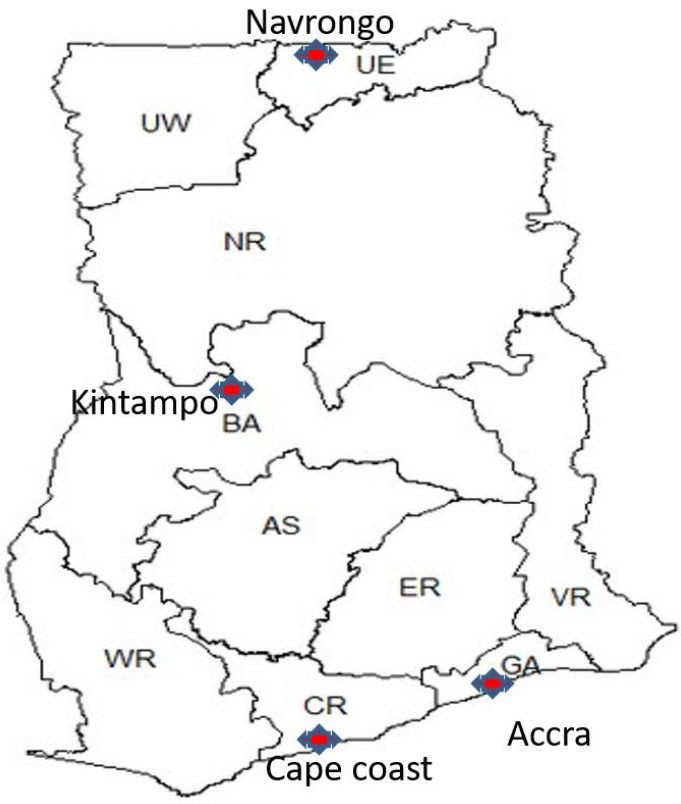


Figure 3-1: Map of Ghana showing the four study sites

3.2 Study population

Samples from the EIM study were collected from children aged 2-14 years who presented with fever and tested positive for RDT in the Kintampo, Navrongo hospitals in 2013/2014 and Accra Lekma hospital in 2016. Part of each blood sample was processed and cryo-preserved in liquid nitrogen which was used for this study.

For the malaria drug resistance monitoring program by NMIMR, blood samples were collected from children 6–59 months of age presenting at health centres in Ghana with uncomplicated

malaria in 2015/2016. Filter paper blood blots were prepared for each patient, air dried, and placed in a zip-locked bag containing a few crystals of desiccant. Bags were stored at room temperature in a clean and dry environment.

A total of two hundred and two (202) blood samples (Table 3-1) were selected from the two previous studies for this project.

Table 3-1: Number of samples selected from each site

Site	Sample Size (n)
Cape Coast	51
Accra	38
Kintampo	40
Navrongo	73
Total	**202**

3.3 Ethical consideration

Ethical approval was sought from the ethics committees and Institutional Review Boards (IRB) of the Ghana health service, the Noguchi Memorial Institute of Medical Research, the Kintampo Health Research Centre and the Navrongo Health Research Centre prior to the conduct of these studies. The proposals for ethical clearance stated the categorical use of the samples for molecular analysis.

3.4 DNA extraction

Genomic DNA was extracted from all the 202 dried blood filter paper samples using the QIAamp DNA Mini kit manufacturer's protocol (www.qiagen.com/handbooks/DNA_mini_blood).

3.5 PCR of *pfdhfr* and *pfdhps*

The determination of single nucleotide polymorphisms (SNPs), require PCR amplification of the gene segment expected to contain the SNPs as shown in Table 3-2.

For this work, SNPs were determined using a nested PCR approach followed by sequencing. The *pfdhfr* and *pfdhps* genes with fragment sizes of 616bp and 647bp, respectively, were PCR amplified using the Tetrad PTC-225 Thermal Cycler. The outer PCR was carried out in a total reaction volume of 25 µl with primer sets and conditions described in Table 3-2 below. The component of the reaction mixture for each PCR contained 1× PCR buffer, 1.5mM $MgCl_2$, 0.25 µM of each primer, 200 µM deoxynucleotides (dNTPs), 1 unit high fidelity *Taq* Polymerase (Roche, UK), 5 µl (5ng/ µl) of target DNA and nuclease free water added to make the final volume of 25 µl.

In the second round PCR, the inner primer sets and conditions as indicated in Table 3-2 were used and 1 µl of the outer PCR amplicon served as the target DNA. The same reaction concentrations were used as in the outer reaction. However, the final volume for the secondary PCR was in 50 µl.

Table 3-2: Primer sequences and PCR conditions for amplification of dhfr and dhps genes

	Primers	Sequence	Size/bp	PCR Conditions
		DHFR		
OUTER PCR	F3	5'-TCCTTTTTATGATGGAACAAG-3'	653	94^{o}C -5min,
	M5	5'-AGTATATAC ATCCTAACAGA-3'		[95^{o}C -30 secs, 50^{o}C -30secs, 72^{o}C -1min] 35X
				72^{o}C -5min, 4^{o}C -Hold
INNER PCR	M1	5'-TTTATGATGATGGAACAAGT-3'	616	94^{o}C, 5min,
	dhfr R2	5'-ACTCATTTTCATTTATTTCTGG-3'		[95^{o}C -30 secs, 52^{o}C -30secs, 72^{o}C -1min] 30X
				72^{o}C -3min, 4^{o}C -Hold
		DHPS		
OUTER PCR	DHPS-F1	5'-AACCTAAACGTGCTGTTCAA-3'	711	95^{o}C -5min,
	DHPS-R1	5'-AATTGTGTGATTTGTCCACAA-3'		[95^{o}C -30 secs, 50^{o}C -30secs, 72^{o}C -1min] 35X
				72^{o}C -5min, 4^{o}C -Hold
INNER PCR	DHPS-F2	5'-ATG ATA AATGAAGGTGCTAG-3'	647	95^{o}C -5min,
	DHPS-R2	5'-TCA TTT TGT TGT TCA TCA TGT-3'		[95^{o}C -30 secs, 52^{o}C -30secs, 72^{o}C -1min] 30X
				72^{o}C -5min, 4^{o}C -Hold

3.6 Resolution of PCR products on agarose gel

A 2% W/V agarose gel was made in 1× TAE buffer. The mixture was melted and 2 µl of ethidium bromide added to the 100µl gel volume. The solution was casted onto the gel cast (Amersham Biosciences, UK) and allowed to solidify for about 30 to 45 minutes. The combs were removed and the gel was assembled into the electrophoresis tank containing 1× TAE buffer. A volume of 5 µl of the amplified PCR product mixed with 2 µl of loading dye was loaded onto the gel and allowed to run at 100V until better resolution was achieved. A molecular weight marker (100bp, Roche Biosciences, Germany), PCR amplified positive (3D7) and negative (non-template) controls were run alongside. After the electrophoretic run, the gel was visualized using the

Amersham Imager 600 (General Electric, USA) and electronically photographed as shown in Fig. 3-2. This was done to determine the specificity and success of the PCR for sequencing

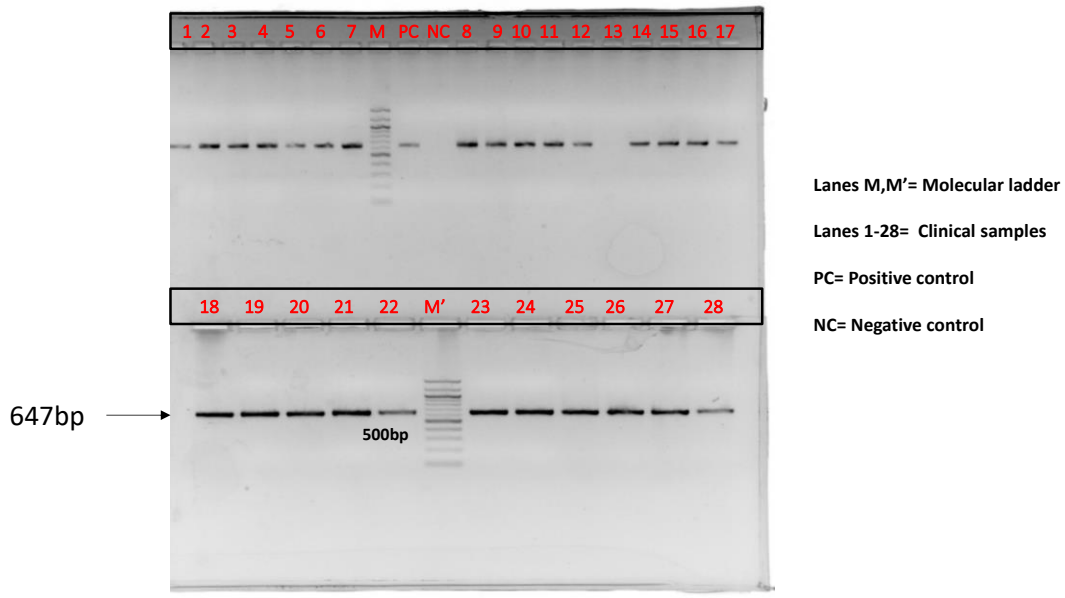


Figure 3-2: A representative gel for pfdhps amplification

3.7 Editing and SNP detection from sequenced data

One hundred and ninety-two out of the 202 total samples were sequenced using the Sanger method by the Macrogen® Company. The PCR products were purified prior to sequencing by Macrogen®. The sequenced results in the ABI file format were analysed using Qiagen CLC Main Workbench analysis software (version 7.8.1) and the Codon Code Aligner software (version 7.0.1). The peaks were trimmed in the CLC Main Workbench and only high-quality peaks were called and included in the analysis. This process was double checked with the Codon Code Aligner software. Detection

of SNPs was done by aligning the sample sequences with the reference 3D7 (Wild-type) sequences retrieved from plasmodb.org (http://plasmodb.org/plasmo/app/record/genomic-sequence/download/Pf3D7_04_v3)

Conflicts with the reference and their positions were noted. The codon containing the conflict(s) were translated into amino acid and compared with the wild-type. SNPs and their combinations (haplotypes) were generated. A representative chromatogram of the sequenced samples is attached at the appendix (Fig. A 2).

3.8 Maintenance of continuous parasite culture

To obtain an intact and maximum yield of RNA for the *gch1* expression levels, cryo-preserved parasites were cultured for the RNA extraction. Clinical isolates harboring different copies of the *gch1* gene and laboratory strain Dd2 (control) were thawed and put into continuous culture. The thawed parasites were suspended in a complete parasite medium (CPM) at 5% hematocrit using un-infected human group O+ erythrocytes. The CPM contains RPMI 1640 (Sigma Aldrich) with 5 mg/ml Albumax II (Gibco), 0.2 µg/mL hypoxanthine, 2 mM L-glutamine, 25 mM HEPES, 23.8 mM $NaHCO_3$, 10 µg/mL gentamycin and 2% normal human serum. The culture was gassed with a mixture of 5% CO_2, 1% O_2 and 94% nitrogen for 30 seconds to 1 minute. The flask was immediately placed in the incubator at 37^{o}C. The media were changed daily and blood smear prepared and stained with 10% Giemsa to determine parasitaemia.

At a parasitaemia of 2 to 5%, the parasites were synchronized using sorbitol and put back into culture until they reach late trophozoites, a stage where the *gch1* has been shown to be highly expressed (Kümpornsin, Kotanan, et al., 2014). At this stage, 5 ml of the culture was centrifuged

and the supernatant discarded. An equal amount of TRIzol was added to the pellet and stored at -80^{o}C until RNA extraction.

3.9 RNA extraction and cDNA synthesis

RNA extraction was done using the AllPrep DNA/RNA Mini kit protocol by Qiagen. The TRIzol lysates were thawed at 37^{o}C. A volume of 0.15 ml chloroform was added and mixed by vigorous vortexing followed by an addition of 350 µl Buffer RLT containing 1% β-mercaptoethanol. The mixture was transferred to an RNeasy spin column placed in a 2 ml collection tube and then centrifuged at full speed (8000 rpm) for 15secs. The flow through was discarded and 700 µl of wash Buffer RW1 added to the spin column, followed by full speed centrifugation for 15 secs. A volume of 500 µl of another wash Buffer RPE was added to the spin column and centrifuged for 15 secs at full speed. The step was repeated again with the Buffer RPE and 30 µl of RNase-free water was added to the column and spun to finally elute the RNA.

Prior to cDNA synthesis, the RNA samples were treated with DNase to remove residue DNA. cDNA synthesis was then carried out using the Superscript III first-strand protocol by ThermoFisher Scientific. The buffer/enzyme and the primer/RNA mixes were prepared according to the manufacturer's protocol. The reaction master mix was prepared and 8 µl of RNA added into each test reaction in a 20 µl final volume. The cDNA synthesis reaction condition include 25^{o}C for 10 min, 50^{o}C for 50 min and 85^{o}C for 5 min. Following the cDNA synthesis, 1 µl of RNase H was added to each reaction, mixed and incubated at 37^{o}C for 20 min and 95^{o}C for 10 min. The resulting cDNA was stored at -20^{o}C until use.

3.10 Quantitative-PCR to determine *gch1* Copy Number and Expression Levels

Changes in gene copy numbers are often associated with varying levels of the gene expression. Copy number and associated gene expression were determined using SYBR-Green based qPCR.

All reactions were performed in a final volume of 10 µl containing primer concentrations of 0.5 µM, 2 µl of target DNA (gDNA and cDNA for copy number an expression levels, respectively) and 1X PerfeCTa SYBR Green SUPERMIX® (Quantabio). Each sample was run in triplicate using the QuantStudio5 (Applied Biosystems) real-time PCR machine as seen in Fig. 3-3. The real-time PCR reaction conditions and primers are indicated in Table 3-3. The seryl-tRNA synthetase primer set with a validated amplification efficiency as the target gene primer set, was used as the endogenous control. All runs with *gch1* copy number more than one, were repeated two more times and results of at least two were used.

Reference samples (Dd2 and 3D7) with known *gch1* copy numbers and a non-template negative controls were included in each run. The melting curve analysis was performed for each run and experiments with non-specific products, Ct > 32 or standard deviation (SD) ≥ 0.5 were repeated.

The delta delta Ct formula ($2^{-\Delta\Delta Ct}$) was used to estimate the relative copy numbers and expression.

Table 3-3: Primer sequences and real-time PCR conditions

Gene	Forward primer	Reverse primer
GTP cyclohydrolase 1	5'-ATGAAACACATAATATGGAAGAAAAA-3'	5'-TCCTTTTCATCTATCACAACAAGG-3'
Seryl-tRNA synthetase	5'-AAGTAGCAGGTCATCGTGGTT-3'	5'-TTCGGCACATTCTTCCATAA-3'

Cycling conditions:

50°C - 2 min
95°C 10 min
95°C - 15 secs
54°C - 40 secs
60°C - 1 min
} 40X

95°C - 15 secs
60°C - 1 min
95°C - 1 secs
} melt curve stage

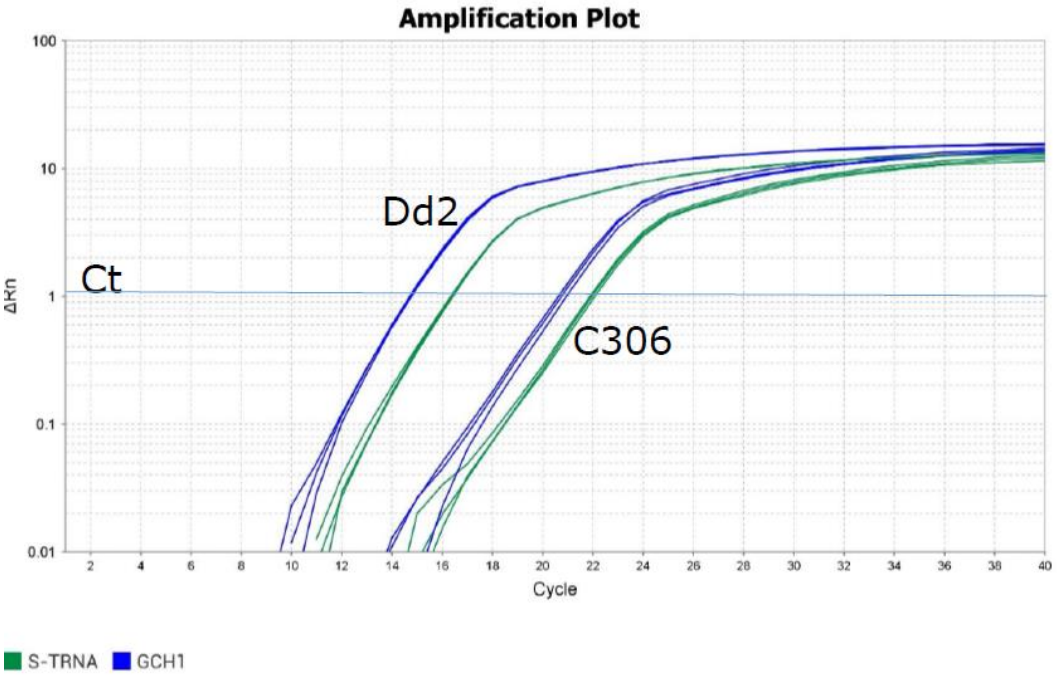


Figure 3-3: A real-time qPCR plot for a representative test and reference sample

Ct= Threshold cycle, **Dd2**=Reference sample, **C306** = Test sample, **S-TRNA**= Endogenous control, **GCH1**= Target gene

3.11 STATISTICAL ANALYSIS

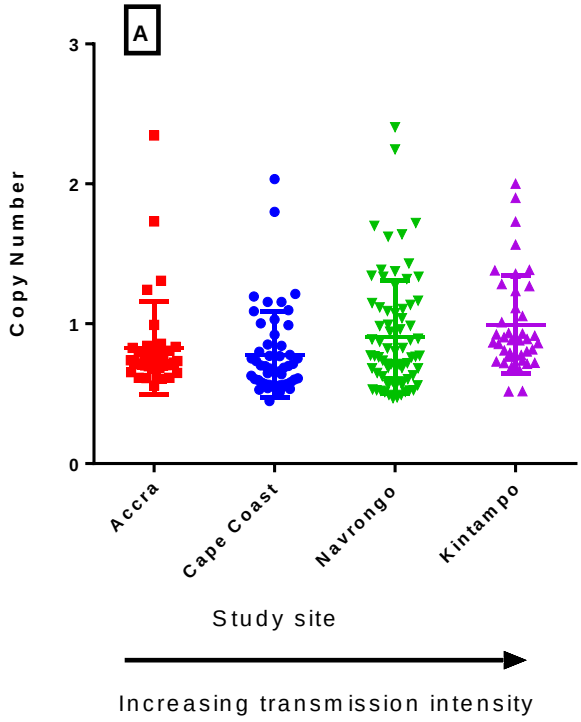
Data were analysed using SPSS software (version 20) and GraphPad Prism version 6. Descriptive analyses for mutations were performed. Chi-square and multiple comparison tests were used to compare the difference between groups. All tests were considered statistically significant with $P<0.005$.

CHAPTER FOUR

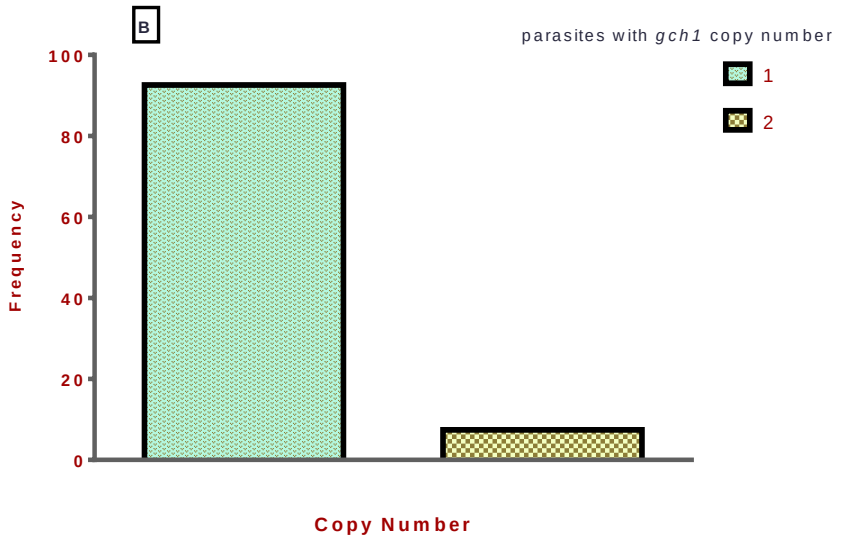
4.0 RESULTS

4.1 *Gch1* copy numbers across site

Gch1 copy number in 202 samples of Ghanaian *Plasmodium* clinical isolates was determined. Samples from Accra, Cape Coast, Navrongo and Kintampo had proportions of 5.26%, 3.92%, 9.59% and 10.00% respectively multiple *gch1* gene (Fig. 4-1, A). However, there was no statistical difference across the study sites. In total, 92.57% (187/202) and 7.43% (15/202) harbored single and double copies of the gene respectively (Fig. 4-1, B).



A. Copy number variations per site. Each dot represents estimated copy number

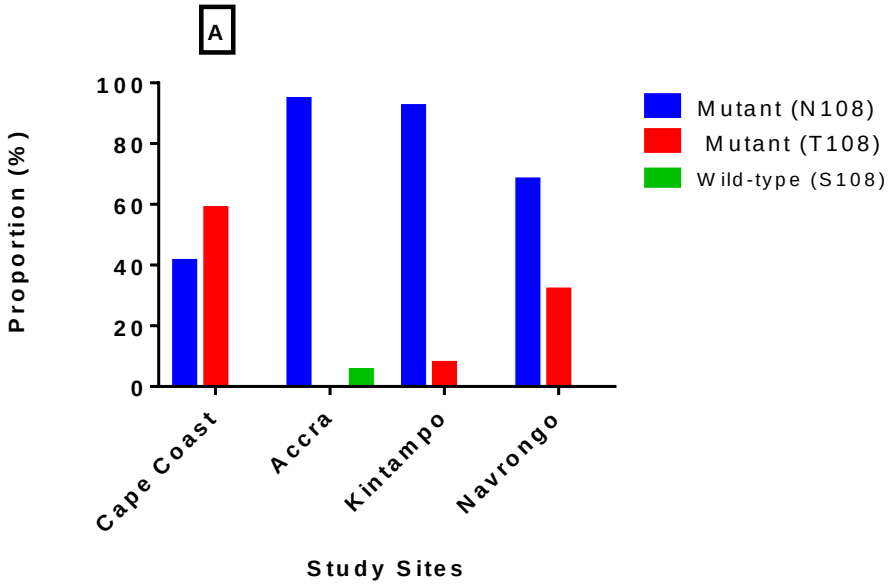


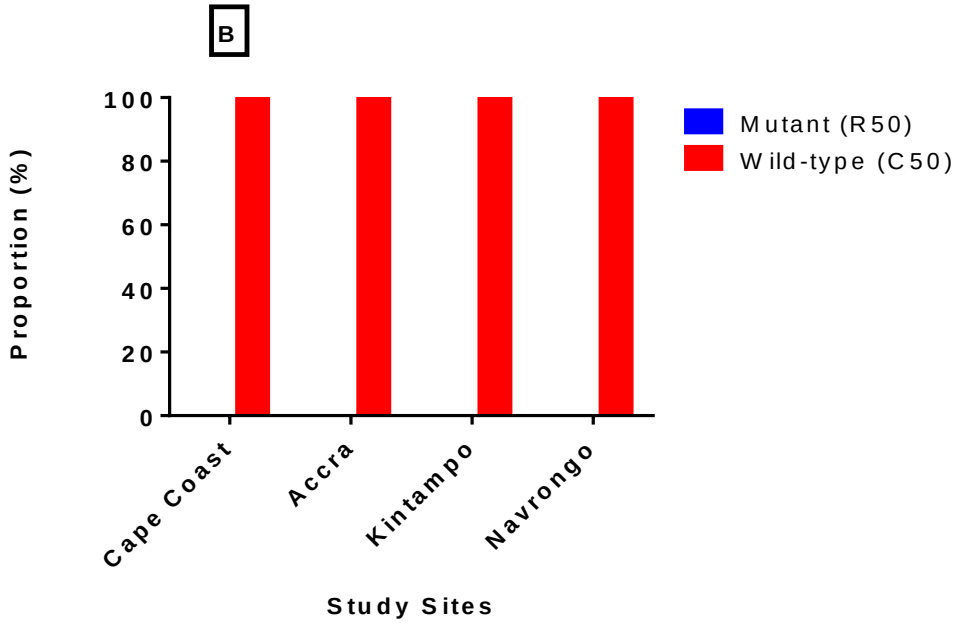
B. The chart shows the total frequencies of *gch1* copies amongst the two hundred and two (202) parasites isolates.

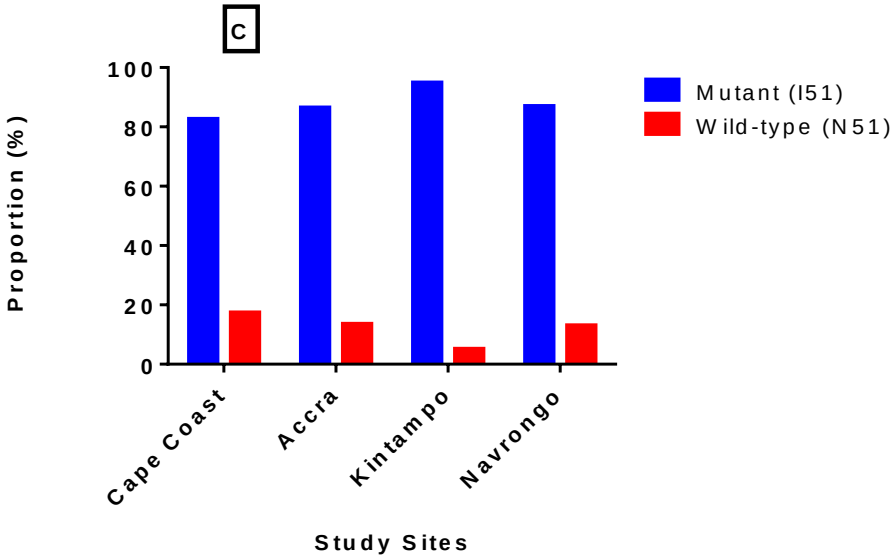
Figure 4-1: Distribution of estimated copy number variations

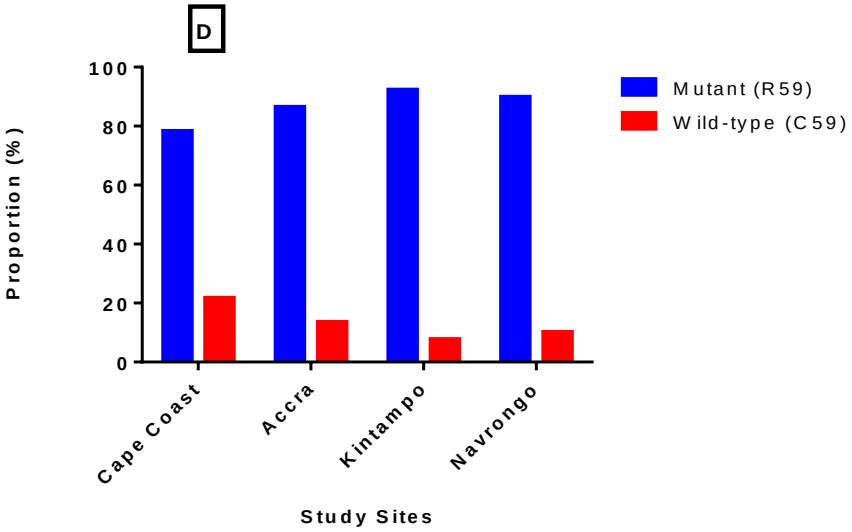
4.2 Prevalence of *dhfr* SNPs

Single nucleotide polymorphisms in the *dhfr* gene conferring pyrimethamine resistance were assessed per site. The *dhfr* allele mutation S108N/T occurred in almost all the parasite isolates. The mutation was either from S to N or S to T. The prevalence of N108 mutation was 71.7% and that of T108 was 32.5% but only 1% had the wild-type, S108 (Fig 4-2 'A'). Parasites with mixed mutations at this codon were observed. The mutations at this position, *dhfr* 108, strongly correlated with study site (Table 4-1). Also, mutations at positions 51 and 59 were 87.4% and 86.9% respectively (Fig. 4-2 'C' & 'D'). However, no mutations were detected at codons 164 and 50 as seen in Fig. 4-2 'B' & 'E'.


A
100
80
60
40
20
0
Proportion (%)
Mutant (N108)
Mutant (T108)
Wild-type (S108)
Cape Coast
Accra
Kintampo
Navrongo
Study Sites


B
100
80
60
40
20
0
Proportion (%)
Mutant (R50)
Wild-type (C50)
Cape Coast
Accra
Kintampo
Navrongo
Study Sites


C
100
80
60
40
20
0
Proportion (%)
Cape Coast
Accra
Kintampo
Navrongo
Study Sites
Mutant (I51)
Wild-type (N51)


D
100
80
60
40
20
0
Proportion (%)
Cape Coast
Accra
Kintampo
Navrongo
Study Sites
Mutant (R59)
Wild-type (C59)

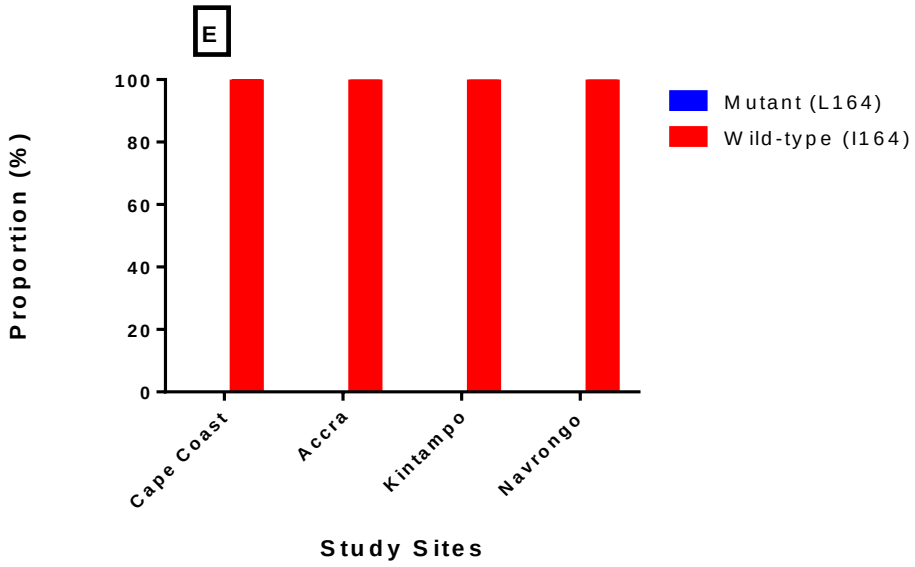


Figure 4-2: Proportions of mutant and wild-type dhfr alleles in each site

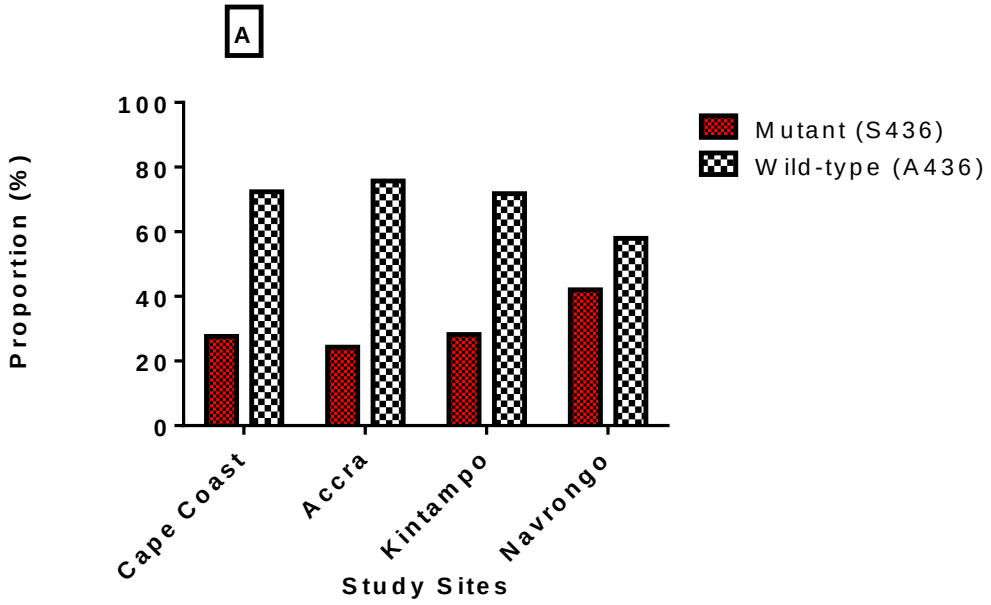
A. S108N, **B.** C50R, **C.** N51I, **D.** C59R and **E.** I164L mutant alleles

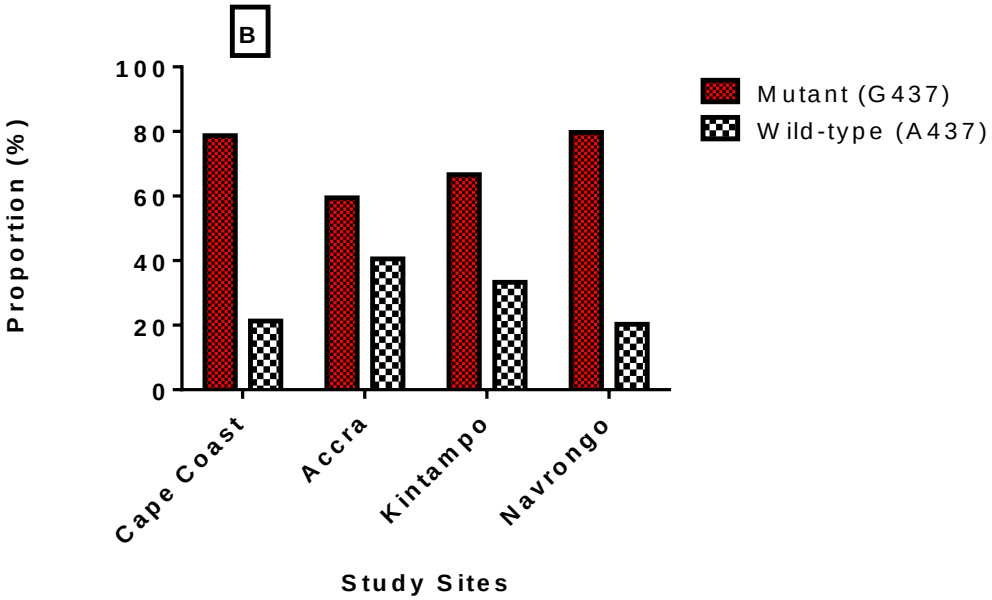
Table 4-1: Association of dhfr 108 mutant alleles with sites

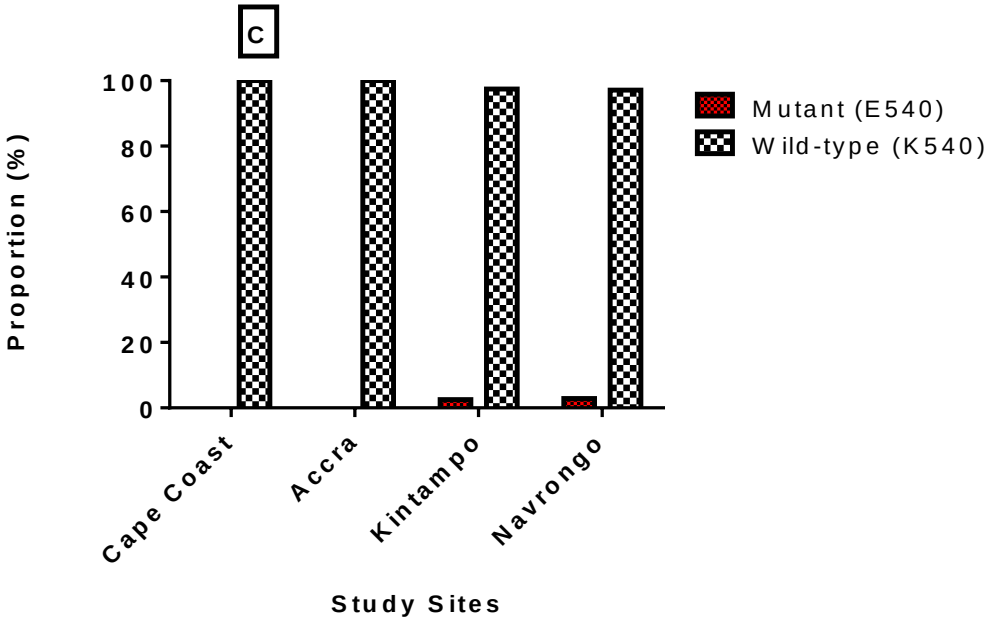
	Site, n (%)					
Mutant Allele	Cape Coast	Accra	Kintampo	Navrongo	Pearson χ^2	P-value
108N	41.3	97.2	92.3	68.11	59.673	<0.001
108T	58.7	0	7.7	46.4	64.049	<0.001

4.3 Prevalence of *dhps* SNPs

The proportion of *dhps* mutants, implicated in sulfadoxine resistance, in each transmission site were evaluated. Among the *dhps* mutant alleles, G437 was the highest with a pooled prevalence of 72.9%, followed by S436 at 32.3% (Fig. 4-3 'A'). The overall polymorphism rate at E540 and G581 were 1.5% and 2.1% respectively (Fig. 4-3 'C' and 'D'). The prevalence of parasites with S613 mutant allele was 8.4% but no mutation was detected for the T613 allele (Fig. 4-3 'E'). The results of a sequence analysis in the Qiagen CLC Main Workbench is shown in Fig. 4-4, where a rare mutation, K540E, in West Africa was observed.




B
100
80
60
40
20
0
Proportion (%)
Mutant (G437)
Wild-type (A437)
Cape Coast
Accra
Kintampo
Navrongo
Study Sites


C
100
80
60
40
20
0
Proportion (%)
Mutant (E540)
Wild-type (K540)
Cape Coast
Accra
Kintampo
Navrongo
Study Sites

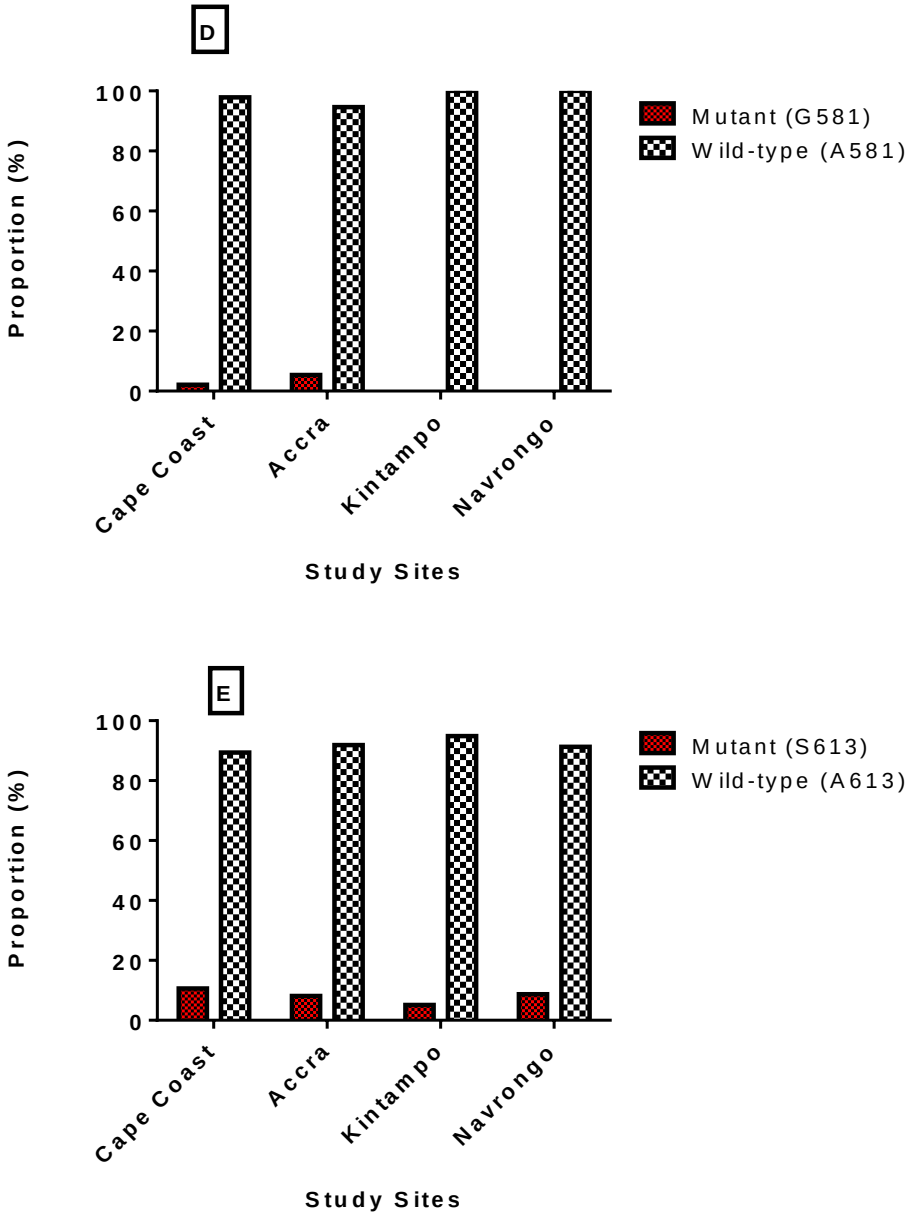


Figure 4-3: Prevalence of mutant and wild-type dhps alleles at the different sites

A. S436A, **B.** A437G, **C.** K540E, **D.** A581G and **E.** A613S

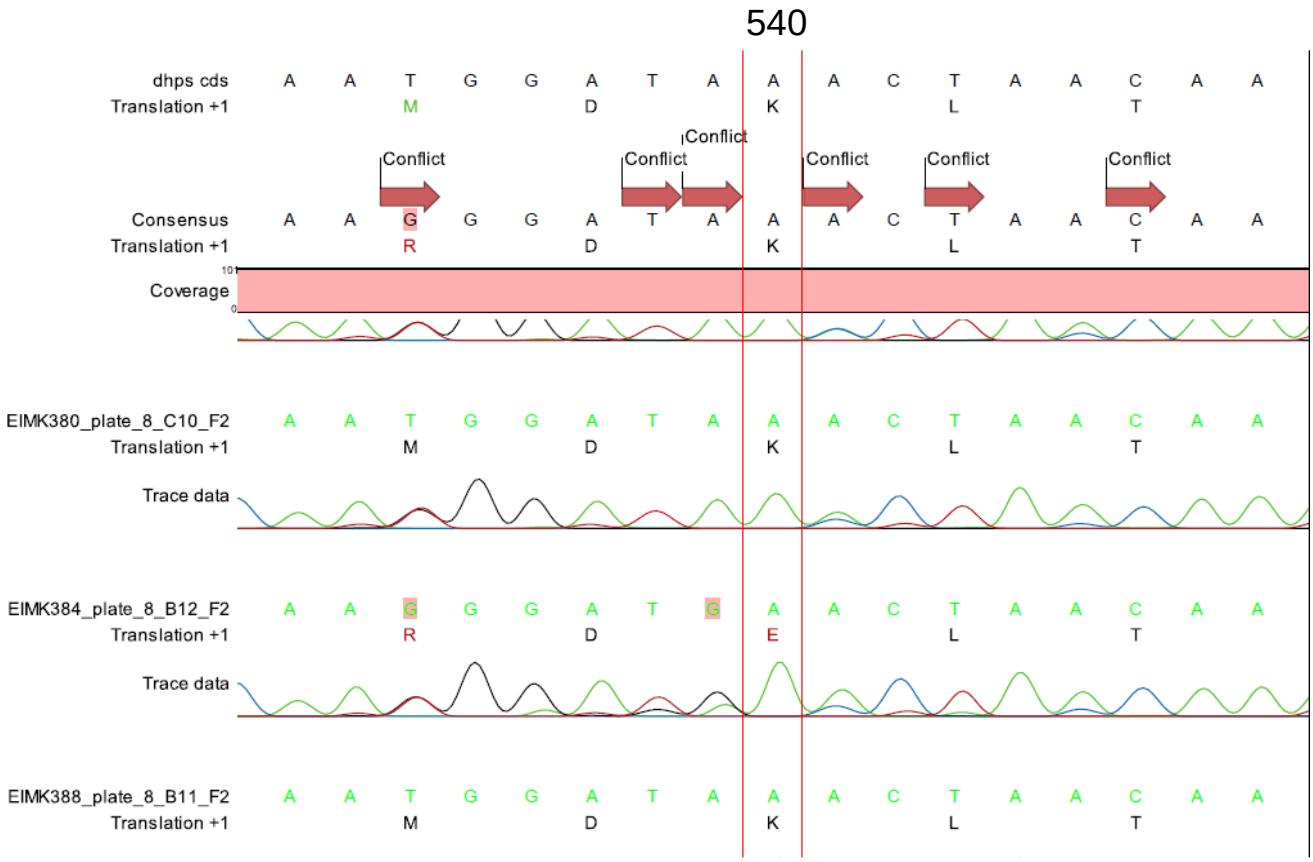


Figure 4-4: Graphical view of a single nucleotide polymorphisms in a clinical sample coded 'EIMK 384'.

A mutation from A to G nucleotide led to a non-synonymous amino acid change from K (AAA codon) to E (GAA codon) at position 540.

4.4 Prevalence of circulating haplotypes

The prevalence of *dhfr* triple mutant (bar highlighted in red in fig 4-5.) implicated in pyrimethamine resistance was relatively higher, with a prevalence of 76.6% than the other haplotypes. Also, the quintuple mutant highlighted in blue (Fig 4-5) shown to be a very important marker of SP treatment outcome in Africa was 2.1%. The prevalence of other haplotypes which increasingly confer resistance to SP is as shown in Fig 4-5.

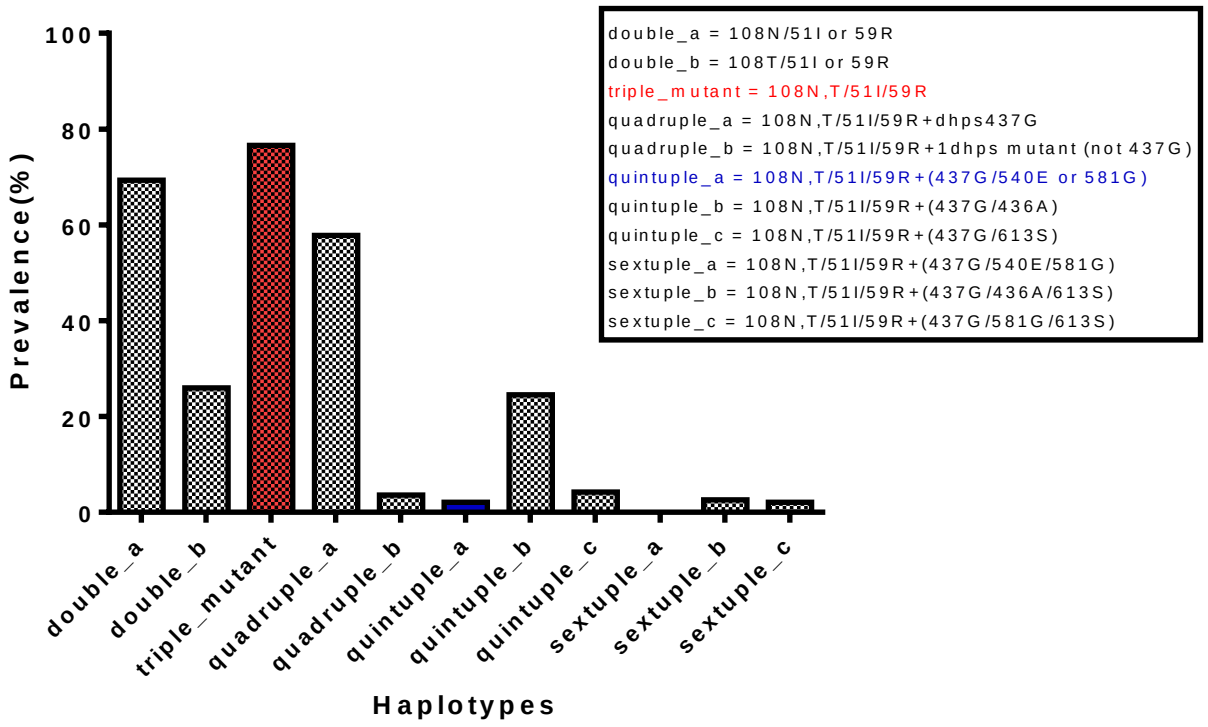


Figure 4-5: Haplotypes that increase the prediction of resistance to SP.

4.5 Association between SNPs/Haplotypes and *gch1* CNV

The correlation between *dhfr/dhps* SNPs or haplotypes and increased *gch1* copy number were drawn to assess their dependence. Although most of the SNPs positively correlated with the increased *gch1* gene, only the SNPs at dhps540E and dhps581G (highlighted in red in Fig. 4-6 'A') showed statistical significance. However, there was no statistically significant correlation between any haplotype and increased *gch1* gene (Fig 4-6 'B').

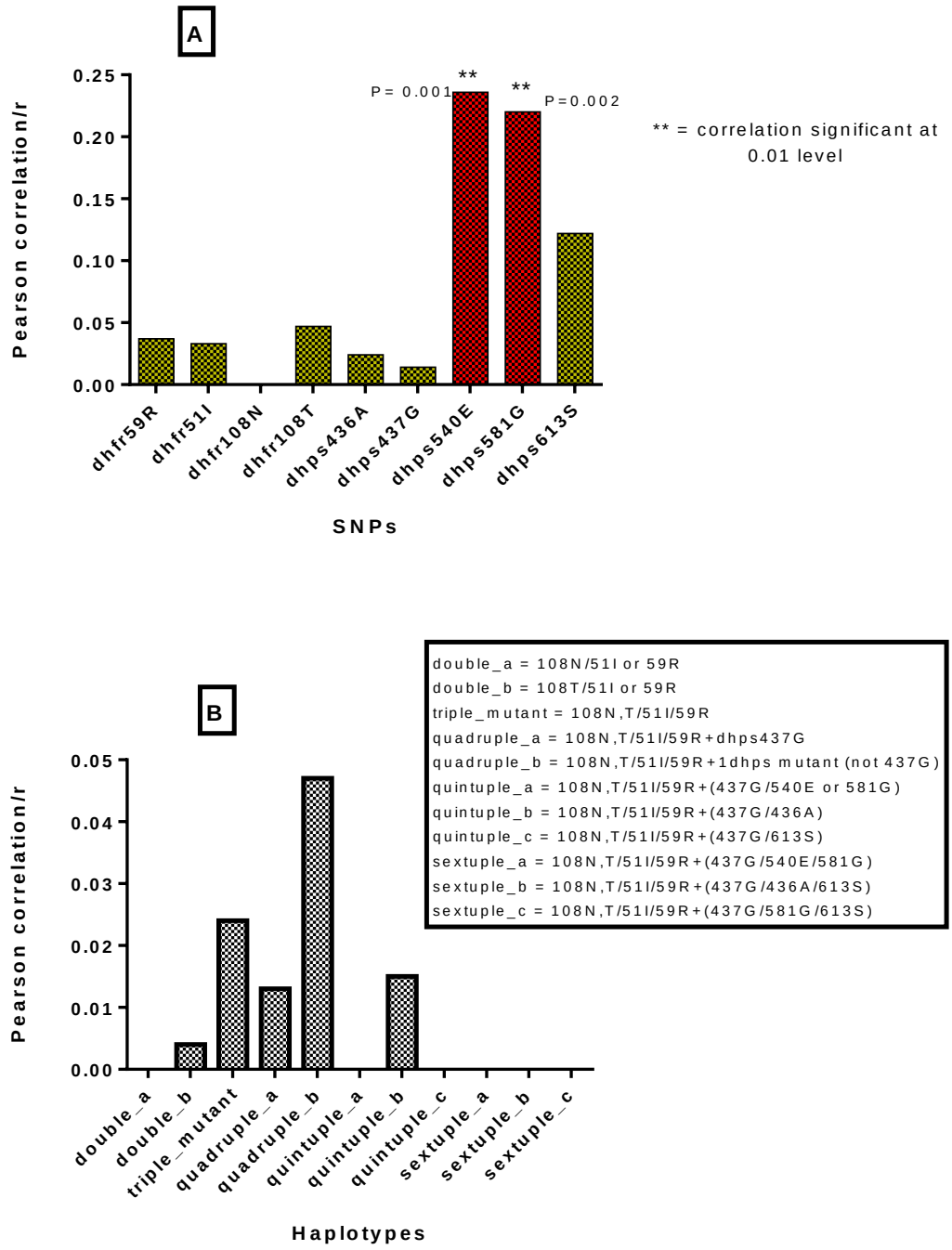


Figure 4-6: Association of dhps/dhfr SNP/haplotypes with duplicate gch1 copies

A. Association between SNPs and *gch1* CNV **B.** Association between haplotypes and g*ch1* CNV

4.6 *Gch1* expression levels

To ascertain the transcriptional importance of *gch1* gene dosage, the expression levels of cultured parasites with differing *gch1* gene copies were determined. Isolates with a single copy had a mean relative expression of 0.44 as compared to 1.52 expression level amongst parasites with double *gch1* gene (Fig. 4-7)

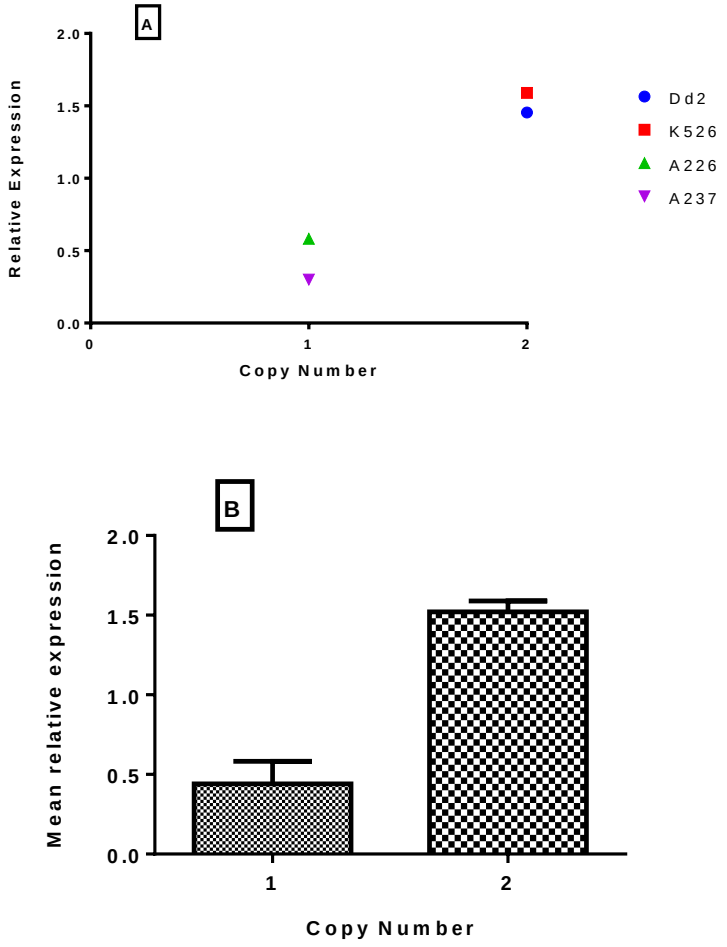


Figure 4-7: Expression levels of gch1 gene in cultured parasites with differing gch1 copy numbers relative to the endogenous control gene (S-TRNA) by RT-qPCR.

A. A plot of single and double *gch1* copy number(s) against the expression levels of four individual isolates
B. Mean expression of *gch1 gene* with single and double copy numbers.

CHAPTER FIVE

5.0 DISCUSSION

GTP cyclohydrolase 1 gene (*gch1*) encodes the GTP gch1 enzyme that catalysis the first reaction in the folate pathway in *Plasmodium falciparum.* Copy number variation of this gene has been reported to be associated with resistance conferring-SNPs in the *dhfr/dhps* genes (Kidgell et al., 2006; Nair et al., 2008). It has been postulated that multiple copies of this gene plays a major role in SP resistance by two mechanisms; either by (1) optimizing parasite fitness and or (2) directly increasing resistance (Heinberg & Kirkman, 2015; Kümpornsin, Modchang, et al., 2014). For parasites with few or no *dhfr* mutations, multiple copies of the *gch1* gene have been shown to directly increase pyrimethamine resistance. However, similar studies reveal that in parasites with increased *dhfr* mutations, duplication of the *gch1* gene provided fitness advantage rather than directly increasing pyrimethamine resistance (Heinberg et al., 2013; Kümpornsin, Modchang, et al., 2014).

The role of *gch1* in compensating for the fitness cost may stem from the fact that increased copies of the gene may lead to high transcriptional and enzyme levels by many folds (Hossain et al., 2004; Nair et al., 2008). This will in-turn increase the downstream metabolites thereby enhancing enzyme activity including the DHR and DHPS enzymes. Thus, in less fit parasites; due to their acquired SP-resistant *dhfr/dhps* SNPs, such an adaptation may compensate for their fitness cost. Therefore, in this study, we sought to determine the association between the *dhfr/dhps* SNPs and CNV of the *gch1* gene amongst clinical isolates of *P. falciparum* in Ghana. Also, the role of *gch1* gene duplication and its transcriptional levels were investigated.

Our data showed that about 7.4% of the parasites in Ghana harbor double copies of the *gch1* gene. There was no statistical significance difference observed between double *gch1* gene and transmission sites. Nevertheless, the high transmission areas seemed to have higher proportions of parasites with double *gch1* compared to the lower transmission sites. The prevalence of *gch1* gene duplication reported in our work is slightly higher than the rate, 2.3%, found in West Africa, as reported in the global dataset of *Plasmodium* genome (M. Ravenhall et al., 2016). However, in Malawi with a prolonged use of SP (1993 to 2007), a novel *gch1* promoter duplication was observed amongst 96.8% of Malawian parasites (M. Ravenhall et al., 2016). This promoter duplication may act in a similar fashion like the whole gene duplication but further investigation is required to confirm its role. It, therefore, suggests that the CNV is an adaptive consequence of selective drug pressure. This assertion is consistent with work done by Nair *et al* in 2008, which reported that 72% of parasites carry multiple copies of the *gch1* gene in Thailand where SP was used as the first line treatment for ten (10) years compared to just 2% in parasites from Laos where the drug was used for only two (2) years as first line treatment (Nair et al., 2008).

If the *gch1* copy number evolution is as a result of an adaptation to drug pressure, then the observation made in Ghana indicate a high use of antifolate drug in the country. This appears to exceed that in other West Africa countries, where the use of SP was introduced around the same time (WHO, 2004). It must be emphasized that although the use of SP in Ghana is restricted to pregnant women and children as prophylaxis, most chemical and pharmacy shops still stocks and sell the drug to individuals outside these groups (Freeman et al., 2017). Another reason for the relatively high drug pressure may be the use of antifolate-containing antibiotics prescribed in the country (Duah et al., 2012). A sulfonamide antifolate-containing antibiotics known as

cotrimoxazole which is a combination of trimethoprim and sulfamethoxazole is often prescribed to treat pneumonia, bronchitis, infections of the urinary tract, ears and intestine, and as prophylaxis in HIV infected individuals. The administration of this drug in patients with bacterial and malaria co-infection may, therefore, lead to high drug pressure with a consequent selection for drug resistant parasites.

Several independent studies also found a strong correlation between *gch1* CNV and *dhps/dhfr* SNPs (Heinberg et al., 2013; Nair et al., 2008; M. Ravenhall et al., 2016). A study conducted by Nair *et al*, 2008, found a strong link between multiple copies of *gch1* gene and the highest conferring SNP to pyrimethamine resistance, I164L (Nair et al., 2008). Another study by Ravenhall *et al*, 2016, also observed an association of the *gch1* promoter duplication in parasites with the quintuple haplotype (*dhfr* N51I/C59R/S10N plus *dhps* A437G/K540E) (Matt Ravenhall et al., 2016) known to be the highest SP resistance marker in Africa (Eltayeb, 2015; Hyde, 2007). Our data also showed a correlation of *dhps* K540E and *dhps* A581G SNPs with the duplication of *gch1*. The K540E and A581G are the key markers in conferring high-level resistance to sulfadoxine drug (Hamour et al., 2005). This, therefore, suggests that there is a co-evolution of high-level SP resistant SNPs and duplication of the *gch1* gene. The association may help the less fit resistant parasites to survive in the presence or absence of drug pressures.

Whereas the *gch1* duplication was associated with SNPs in the *dhfr* or both *dhfr* and *dhps* in the previous studies (Heinberg & Kirkman, 2015; Matt Ravenhall et al., 2016), our work showed the association with only the *dhps* gene. This gives the indication that mutational markers in the *dhps* gene in our setting may lead to treatment failure and hence such markers should be closely

monitored. Also, if the duplication acts to reduce the fitness cost of the SP resistance variants, then 7% of circulating parasites with their associated SP resistant point mutations may forever persist amongst circulating parasites in Ghana.

The most critical scenario with this new parasite adaption is the ability of mutants to equally or favorably compete with the wild type in the absence or reduced SP drug pressure and thus cause the expansion and persistence of the resistant type. If this speculated mechanism holds, then it may jeopardize the use of SP drug and other antifolate-containing antibiotics in Ghana. This phenomenon may be different from that of chloroquine resistance, where there have been reports of reemergence of chloroquine sensitive parasites in areas where the drug was disused (Farham, 2007; Kateera et al., 2016). However, it is interesting to note that there are still several reports of high cases of SP resistant parasites in areas with no or restricted use of the SP drug (Cisse et al., 2017; Kateera et al., 2016; Tahita et al., 2015)

After the change of drug policy in Ghana in 2005, SP was used as an IPTp and there have been reports of increased SP resistance markers over the years (Alam et al., 2011; Duah et al., 2012). Studies by Mockenhaupt in 2005 reported a 47% *dhfr* triple and 0.8% quintuple mutants (Mockenhaupt et al., 2005). The above frequencies are even higher amongst parasites collected in 2007/2008 by Alam *et al*, where the triple *dhfr* and quintuple mutants were reported as 58.7% and 1.8% respectively (Alam et al., 2011). Duah and colleagues also reported the prevalence of the triple *dhfr* and quintuple mutants in parasites collected in 2010 as 55% and 1.12% respectively (Duah et al., 2012). The relatively high prevalence of the triple (76.6%) and quintuple (2.1%) mutants observed in the current study is therefore alarming since these mutant markers are strongly

linked to pyrimethamine and SP treatment outcome. The increased prevalence of these resistant markers in our study may be attributed to drug pressures and possibly, the duplication of the *gch1* gene maintaining these resistant parasites.

In addition to the increased prevalence of resistance markers observed in our study, we also found for the first time in Ghana, the S108T and A518G mutations. The *dhfr* S108T mutation has been shown to confer a relatively higher resistance to cycloguanil than pyrimethamine (Gregson & Plowe, 2005a). This mutation was, however, found only in Cape Coast and Navrongo. This observation may be as a result of an increased use of cycloguanil in these areas or possible cross-resistance which can occur with the use of other antifolates (Iyer et al., 2001). It is important to note that the S108T mutation has been reported to be a gain-of-function mutant where the specific activity of its dhfr enzyme is twice the wild type (Mharakurwa et al., 2011). However it is worth noting that this phenomenon occurs mostly in the mosquito host which help the parasite to develop and multiply faster (Mharakurwa et al., 2011). There is therefore the possibility that any drug pressure that selects for this mutation may trigger this occurrence rapidly in such settings.

The A581G mutation has been shown to reduce the effectiveness of SP preventive therapy. In areas where the prevalence of this mutation was more than 10%, there was an associated increased parasitemia and decreased infant birth weight among pregnant women receiving SP as IPTp (R. M. Chico et al., 2015). Although the prevalence reported in our study is not up to the 10%, there is still the need to monitor the prevalence of this marker in order to inform policy makers promptly for an appropriate action.

An interesting observation made in this study is that all the parasites analysed had mutations at the *dhfr* 108 codon. Since the initial mutation in the *dhfr* gene is likely to occur at this position

(Sirawaraporn et al., 1997), it can be conjunctured that continuous resistant selection might have fixed the S108N/T mutant in the parasites.

Regardless of reports of parasites resistance to SP, the WHO still recommends it use as IPTp in high transmission areas. This recommendation is based on the successful maternal outcomes even with increased resistant markers (WHO, 2016).

The expression profiles reported in this study confirms the functional impact of the duplicated *gch1* gene and a possible increase in metabolites downstream of the folate pathway. There is, however, the need to confirm this increase in metabolites in future studies as it has been confirmed in plants by Hossain (Hossain et al., 2004).

5.1 Conclusion

This study reports about 7% of parasites circulating in Ghana have a double *gch1* gene copy. This relatively high prevalence as compared to the overall rate in West Africa may be as a result of high antifolate drug pressures due to increased use of the drug and folate-containing antibiotics in the country.

The outcome also shows that mutations at *dhps*540E and *dhps*581G correlated with the double *gch1* gene, implying that the duplicated *gch1* may compensate for the fitness cost in parasites harboring these high-level mutations.

The relative expression between parasites with *gch1* CN of 1 and 2 was observed to be about 3-folds. This could be a reflection of the functional impact of the gene dosage which might support the proposed mechanism of possible reduction in fitness cost through increased metabolites.

Mutations at *dhfr*S108T and *dhps*A581G were also observed for the first time in Ghana and it indicates the use of antifolate drugs that select for 108T mutant. Again increase in SP drug pressure

leading to selection of the parasites carrying the high-level sulfadoxine conferring SNP, A581G may account for this.

It was also demonstrated that almost all the parasites analysed in this study had mutations at codon 108. This suggest a possible fixation of the S108N/T mutant in Ghana.

5.2 Recommendation

The effect of the duplication of *gch1* gene in clinical isolates on antifolate drug susceptibility needs to be established.

Continuous surveillance of *gch1, dhfr & dhps* genes is required to monitor SP resistance.

Further studies to discover component drugs that can inhibit the *gch1* gene product are required.

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APPENDIX

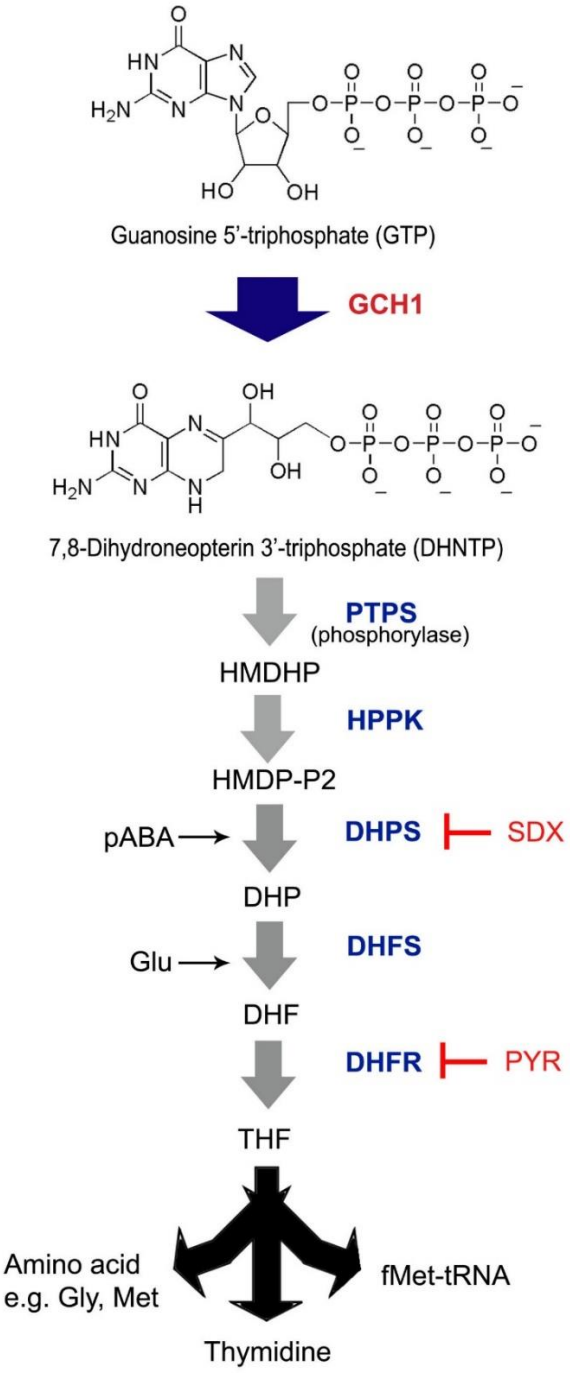

Figure A 1: Targets of Sulfadoxine-Pyrimethamine

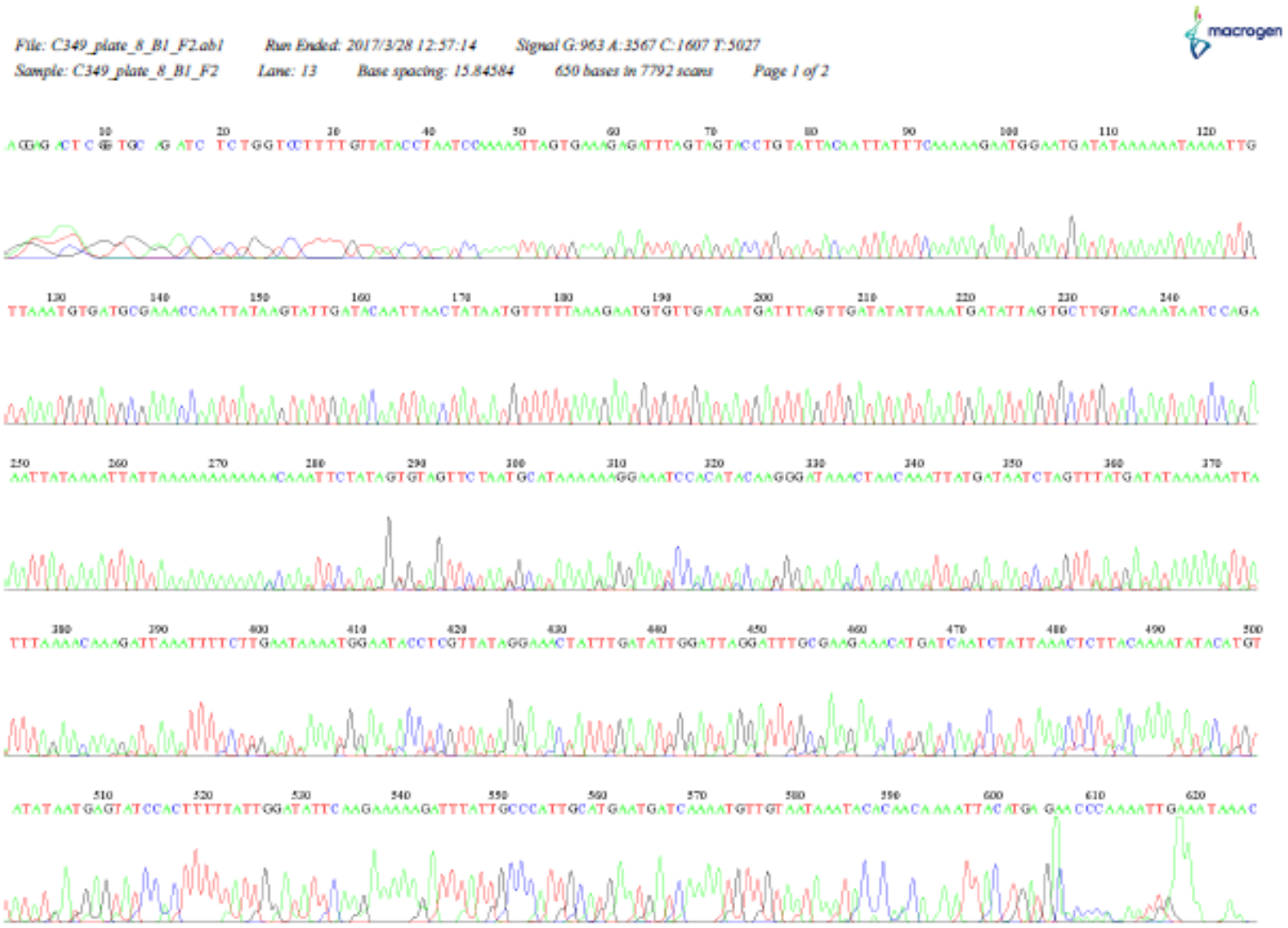

Figure A 2: Dhps representative chromatogram of a sequenced sample coded 'C349'

Table A 1: Sample IDs and their estimated gch1 copy numbers

Sample Name	Estimated *gch1* CN	Sample Name	Estimated *gch1* CN	Sample Name	Estimated *gch1* CN	Sample Name	Estimated *gch1* CN	Sample Name	Estimated *gch1* CN
C314	0.578437388	C445	0.563772082	EIMK097	0.517132163	EIMN120	0.823811691	N111	0.684739053
C315	0.567967355	C445	0.563772082	EIMK119	1.385387778	EIMN124	0.492332432	N115	0.631115556
C316	0.604781389	C446	0.777934253	EIMK139	0.882036705	EIMN131	0.984955311	N117	0.585466206
C319	0.54873991	C449	0.851350307	EIMK150	0.775300126	EIMN134	1.335912228	N119	1.090934992
C321	0.540218592	EIMA184	0.611673072	EIMK152	1.357212067	EIMN164	0.987006187	N122	0.60388869
C322	0.528039992	EIMA185	0.681648254	EIMK156	0.808125238	EIMN166	0.769350886	N124	0.624538839
C323	0.685081542	EIMA186	0.785442978	EIMK163	0.86615356	EIMN175	1.720319152	N126	0.761262953
C329	1.030973792	EIMA211	0.733106285	EIMK173	1.900290489	EIMN180	0.682663445	N127	0.514130831
C332	0.752189755	EIMA212	0.760461807	EIMK177	1.268779993	EIMN187	0.523978178	N129	0.524599671
C337	1.213485122	EIMA213	2.34508878	EIMK179	1.110875249	EIMN221	0.521073987	N132	0.99413687
C344	0.752034956	EIMA214	0.712695524	EIMK200	0.668443976	EIMN223	1.273564935	N134	1.039867878
C347	0.680741489	EIMA215	0.647777319	EIMK201	0.71771949	EIMN226	2.247125626	C430	0.559673309
C348	0.770941297	EIMA216	0.739826083	EIMK210	1.380312324	EIMN353	1.3752352	C434	1.003462315
C349	0.920767595	EIMA217	0.715228915	EIMK214	0.693421722	N010	0.872480929	C437	0.659248948
C352	0.799561501	EIMA219	0.992388189	EIMK220	0.890335499	N012	1.639871359	C440	0.627648294
C354	0.53382349	EIMA220	0.786523223	EIMK248	0.933552444	N013	1.700889826	C442	0.599649668
C355	0.732993245	EIMA221	0.835615396	EIMK249	0.790435274	N014	0.959915102	EIMK001	1.565965176
C356	1.089057595	EIMA222	0.725225806	EIMK252	0.855039204	N016	0.774420917	EIMK041	1.232734203
C358	0.842496872	EIMA223	0.799248576	EIMK356	0.861244279	N017	1.076498628	EIMK047	0.923319386
C359	0.702613432	EIMA224	0.857913256	EIMK359	0.737814557	N020	0.894785285	EIMK076	1.010891959
C360	1.155324697	EIMA225	0.680185854	EIMK380	0.764853077	N021	0.758511782	EIMK087	0.516536568
C362	1.193922043	EIMA226	0.72141923	EIMK388	0.720475248	N023	0.943865478	EIMN051	0.588362342
C363	1.155063033	EIMA227	0.695784569	EIMK431	1.055084745	N024	0.692259431	EIMN056	0.468089931
C364	0.697094142	EIMA228	0.685447991	EIMK450	0.928617292	N025	0.472579092	EIMN060	1.336613536
C368	1.097411156	EIMA230	0.841977775	EIMK465	1.282372355	N027	0.764752209	EIMN080	1.136415005
C369	0.447342515	EIMA231	1.307929158	EIMK466	0.913634181	N028	0.563333392	EIMN114	1.431615472
C377	0.549948394	EIMA232	0.557522774	EIMK470	0.881894256	N029	1.106560588	N096	0.524608314
C379	0.768101156	EIMA233	0.613216579	EIMK478	0.730232551	N030	1.116526127	N097	0.647893012
C382	0.596374929	EIMA234	0.653351367	EIMK479	0.712656665	N031	1.622572303	N106	0.56615591
C383	0.582362652	EIMA235	0.707551986	EIMK481	1.730126858	N049	0.491961002	N108	0.706944585
C384	0.653139234	EIMA236	0.700966537	EIMK484	2.543853045	N050	0.718278825	N109	0.52965039
C388	2.034221888	EIMA237	0.828141153	EIMK492	0.802313328	N052	0.713564813	C423	0.638830066
C392	0.541103858	EIMA238	0.74137871	EIMK506	0.774084231	N053	0.773017466	C427	1.800593615
C394	0.646746457	EIMA239	0.807023674	EIMK508	0.902995288	N055	0.681187391	EIMA246	0.606220424
C395	0.56915158	EIMA240	0.702384174	EIMK510	0.815926903	N060	0.715474904	EIMA247	1.242758632
C401	0.61052072	EIMA241	0.613999963	EIMN001	0.882134676	N061	0.588958561	EIMN042	0.587197487
C408	0.713860851	EIMA242	0.704295039	EIMN008	0.804368951	N063	0.737926364	EIMN043	1.164358735
C420	0.990420163	EIMA243	0.785436332	EIMN011	0.826646505	N072	2.404252529	N084	0.512700319
C421	0.510347724	EIMA244	1.732130051	EIMN014	1.147772551	N078	0.888775647	N095	0.559087038
C422	0.697562933	EIMA245	0.782559156	EIMN035	1.344495058	N082	0.711474001		

Table A 2: Sample IDs and their associated SNPs

Id	dhfr 51I	dhfr 59R	dhfr 108N	dhfr 108T	dhfr 164L	dhps436A	dhps437G	dhps540E	dhps581G	dhps613S	dhps613T	double_a	double_b	triple_mutant	quadruple_a	quadruple_b	quintuple_a	quintuple_b	quintuple_c	sextuple_b	sextuple_c
C314	Yes	Yes	No	Yes	No	Yes	Yes	No	No		No	No	Yes	Yes	Yes	No	No	Yes	No	No	No
C315	Yes	No	No	Yes	No	Yes	Yes	No	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No
C316	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
C319	Yes	No	No	Yes	No	No	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No
C321	No	No	No	Yes	No	Other	Other	No	No	No	No	No	No	No	No	No	No	No	No	No	No
C323	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
C329	Yes	Yes	No	Yes	No	Yes	Yes	No	No	Yes	No	No	Yes	Yes	Yes	No	No	Yes	No	No	No
C337	Yes	Yes	Yes	No	No	No	Yes	No	No	Yes	No	Yes	No	Yes	Yes	No	No	Yes	Yes	Yes	No
C344	No	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
C347	Yes	Yes	Yes	No	No	Yes	Yes	No	Yes	Yes	No	Yes	No	Yes	Yes	No	Yes	Yes	Yes	No	Yes
C348	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
C349	No	Yes	No	Yes	No	No	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No
C352	Yes	Yes	No	Yes	No	No	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	No	No	No	No
C354	Yes	No	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
C355	Yes	Yes	No	Yes	No	No	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No
C356	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	No	No	No	No
C359	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
C360	Yes	No	No	Yes	No	Yes	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No
C362	No	Yes	No	Yes	No	Other	Other	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No
C363	Yes	Yes	No	Yes	No	Other	Other	No	No	No	No	No	Yes	Yes	No	No	No	No	No	No	No
C364	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
C368	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
C369	No	No	No	Yes	No	No	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No
C377	Yes	Yes	Yes	No	No	Yes	No	No	No	No	No	Yes	No	Yes	No	Yes	No	No	No	No	No
C379	Yes	Yes	No	Yes	No	Other	Other	No	No	No	No	No	Yes	Yes	No	No	No	No	No	No	No
C382	Yes	Yes	No	Yes	No	Yes	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	Yes	No	No	No
C383	Yes	Yes	Yes	No	No	Other	Other	No	No	Yes	No	Yes	No	Yes	No	Yes	No	No	No	No	No
C384	Yes	Yes	No	Yes	No	No	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	No	No	No	No
C388	Yes	Yes	No	Yes	No	Other	Other	No	No	No	No	No	Yes	Yes	No	No	No	No	No	No	No
C392	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
C394	Yes	No	No	Yes	No	No	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	No	No	No	No
C395	Yes	Yes	No	Yes	No	No	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No
C401						No	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No
C408	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	Yes	Yes	Yes	No	No	No	No	No	No
C420	Yes	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No
C421	No	Yes	No	Yes	No	Yes	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No
C422	Yes	Yes	No	Yes	No	Yes	Yes	No	No	Yes	No	No	Yes	Yes	Yes	No	No	Yes	No	No	No
C423	Yes	Yes	No	Yes	No	No	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	No	No	No	No
C427	Yes	Yes	No	Yes	No	Other	Other	No	No	No	No	No	Yes	Yes	No	No	No	No	No	No	No

Table A 3: Sample IDs and their associated SNPs

Id	dhfr51I	dhfr59R	dhfr108N	dhfr108T	dhfr164L	dhps436A	dhps437G	dhps540E	dhps581G	dhps613S	dhps613T	double_a	double_b	triple_mutant	quadruple_a	quadruple_b	quintuple_a	quintuple_b	quintuple_c	sextuple_b	sextuple_c
C430	No	Yes	No	Yes	No	No	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No
C434	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
C437	Yes	Yes	No	Yes	No	No	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	No	No	No	No
C440	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
C442	Yes	No	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
C445	No	Yes	No	Yes	No	No	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No
C446	Yes	No	No	Yes	No	No	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No
C449	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMA185	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMA186	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
EIMA211	No	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
EIMA212	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
EIMA213	Other	Other	Yes	No	Other	Yes	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No
EIMA214	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMA215	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
EIMA216	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
EIMA217	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
EIMA219	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
EIMA220	Yes	Yes	Other	No	No	Yes	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No
EIMA221	No	Yes	Yes	No	No	Other	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
EIMA222	Yes	Yes	Yes	No	No	Other	Other	No	Yes	Yes	No	Yes	No	Yes	No	Yes	Yes	No	Yes	No	Yes
EIMA223	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMA224	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
EIMA225	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
EIMA226	Yes	Yes	Yes	No	No	Yes	Yes	No	Yes	Yes	No	Yes	No	Yes	Yes	No	Yes	Yes	No	No	Yes
EIMA227	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
EIMA228	Yes	Yes	Yes	No	No	Other	Other	No	No	Yes	No	Yes	No	Yes	No	Yes	No	No	No	No	No
EIMA230	Other	Other	Yes	No	Other	Other	Other	No	No	No	No	No	No	No	No	No	No	No	No	No	No
EIMA231	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMA232	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMA233	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMA234	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMA235	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
EIMA236	Yes	No	Yes	No	No	Other	Other	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
EIMA237	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMA238	Yes	Yes	Yes	No	No	Yes	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
EIMA239	Yes	No	Yes	No	No	No	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
EIMA240	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
EIMA241	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No

Table A 4: Sample IDs and their associated SNPs

Id	dhfr51I	dhfr59R	dhfr108N	dhfr108T	dhfr164L	dhps436A	dhps437G	dhps540E	dhps581G	dhps613S	dhps613T	double_a	double_b	triple_mutant	quadruple_a	quadruple_b	quintuple_a	quintuple_b	quintuple_c	sextuple_b	sextuple_c
ElMA242	No	No	No	No	No	Other	Other	No	No	No	No	No	No	No	No	No	No	No	No	No	No
ElMA243	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
ElMA244	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
ElMA245	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
ElMA246	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
ElMA247	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
ElMK001	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
ElMK047	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	No	Yes	Yes	No	No	No	No	No	No	No
ElMK076	Yes	Yes	No	Yes	No	Other	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	No	No	No	No
ElMK087	Yes	Yes	No	Yes	No	No	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	No	No	No	No
ElMK097	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
ElMK119	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
ElMK139	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
ElMK150	Yes	Yes	Yes	No	No	No	No	No	No	Yes	No	Yes	No	Yes	No	Yes	No	No	No	No	No
ElMK152	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
ElMK156	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
ElMK163	Yes	Yes	Yes	No	No	No	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
ElMK173	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
ElMK177	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
ElMK179	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
ElMK200	Yes	Yes	Yes	No	No	Yes	Yes	No	No	Yes	No	Yes	No	Yes	Yes	No	No	Yes	Yes	Yes	No
ElMK201	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
ElMK210	Yes	Yes	Yes	No	No	Other	No	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
ElMK214	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
ElMK220	Yes	No	Yes	No	No	No	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
ElMK248	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
ElMK249	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
ElMK252	Yes	No	Yes	No	No	No	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
ElMK356	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
ElMK359	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
ElMK380	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
ElMK384	Yes	Yes	Yes	No	No	Other	Other	Yes	No	No	No	Yes	No	Yes	No	Yes	No	No	No	No	No
ElMK388	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
ElMK431	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
ElMK450	No	Yes	Yes	No	No	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
ElMK465	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
ElMK466	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
ElMK470	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
ElMK478	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
ElMK479	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No

Table A 5: Sample IDs and their associated SNPs

Id	dhfr 51I	dhfr 59R	dhfr 108N	dhfr 108T	dhfr 164L	dhps436A	dhps437G	dhps540E	dhps581G	dhps613S	dhps613T	double_a	double _b	triple_mutant	quadruple_a	quadruple_b	quintuple_a	quintuple_b	quintuple_c	sextuple_b	sextuple_c
EIMK481	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMK492	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
EIMK506	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
EIMK508	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
EIMK510	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMN001	Yes	Yes	Yes	No	No	Other	Yes	Other	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMN008	Yes	Yes	Yes	Yes	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMN011	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
EIMN035	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMN042	Yes	Yes	Yes	No	No	No	No	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
EIMN043	Yes	Yes	Yes	No	Other	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
EIMN051	No	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
EIMN056	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
EIMN060	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMN080	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
EIMN084	Yes	Yes	Yes	No	No	No	No	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
EIMN088	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMN094	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
EIMN114	Yes	Yes	Yes	Yes	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
EIMN120	No	Yes	Yes	No	No	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
EIMN124	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
EIMN131	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
EIMN166	Yes	Yes	No	Yes	No	No	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	No	No	No	No
EIMN175	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
EIMN180	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
EIMN187	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
EIMN221	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
EIMN223	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
EIMN226	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
N010	Yes	Yes	No	Yes	No	Yes	Yes	No	Yes	Yes	No	No	Yes	Yes	Yes	No	Yes	Yes	Yes	No	Yes
N016	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
N018	Yes	Yes	No	Yes	No	No	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	No	No	No	No
N020	No	Yes	Yes	Yes	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
N021	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
N023	Yes	No	No	Yes	No	Other	Other	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No
N024	Yes	Yes	No	Yes	No	Yes	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	Yes	No	No	No
N025	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
N027	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
N028	Yes	No	No	Yes	No	Yes	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No

Table A 6: Sample IDs and their associated SNPs

Id	dhfr 51I	dhfr 59R	dhfr 108N	dhfr 108T	dhfr 164L	dhps436A	dhps437G	dhps540E	dhps581G	dhps613S	dhps613T	double_a	double _b	triple_mutant	quadruple_a	quadruple_b	quintuple_a	quintuple_b	quintuple_c	sextuple_b	sextuple_c
N029	Yes	No	No	Yes	No	No	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No
N030	Yes	Yes	No	Yes	No	Yes	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	Yes	No	No	No
N031	Yes	Yes	No	Yes	No	No	Yes	No	Other	Other	No	No	Yes	Yes	Yes	No	No	No	No	No	No
N049	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
N050	Yes	Yes	No	Yes	No	Yes	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	Yes	No	No	No
N052	Yes	Yes	Yes	No	No	Other	Other	No	No	Yes	No	Yes	No	Yes	No	Yes	No	No	No	No	No
N053	Yes	Yes	No	Yes	No	Yes	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	Yes	No	No	No
N055	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
N060	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
N061	No	Yes	Yes	No	No	Other	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
N063	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
N072	No	No	No	Yes	No	No	Yes	No	Other	Other	No	No	No	No	No	No	No	No	No	No	No
N078	Yes	Yes	No	Yes	No	No	Yes	Other	Other	Other	No	No	Yes	Yes	Yes	No	No	No	No	No	No
N082	Yes	Yes	No	Yes	No	No	Other	No	No	No	No	No	Yes	Yes	No	No	No	No	No	No	No
N084	Yes	Yes	Yes	No	No	Other	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
N095	No	Yes	No	Yes	No	Yes	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No
N096	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
N097	Yes	No	No	Yes	No	Other	Other	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No
N102	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
N103	No	No	No	Yes	No	No	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No
N104	Yes	Yes	No	Yes	No	Yes	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	Yes	No	No	No
N106	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
N107	No	Yes	Yes	Yes	No	Yes	Yes	No	No	Yes	No	Yes	No	No	No	No	No	No	No	No	No
N109	Yes	Yes	No	Yes	No	No	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	No	No	No	No
N111	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
N115	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No	Yes	No	Yes	No	Yes	Yes	No	No	Yes	Yes	Yes	No
N117	Yes	Yes	Yes	No	No	Yes	Yes	No	No	Yes	No	Yes	No	Yes	Yes	No	No	Yes	Yes	Yes	No
N119	No	Yes	Yes	Yes	No	Yes	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No
N122	Yes	Yes	No	Yes	No	No	Yes	No	No	No	No	No	Yes	Yes	Yes	No	No	No	No	No	No
N124	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No
N126	Yes	Yes	No	Yes	No	Yes	No	No	No	No	No	No	Yes	No	Yes	No	No	Yes	No	No	No
N127	Yes	No	No	Yes	No	Other	Other	No	No	No	No	No	Yes	Yes	No	No	No	No	No	No	No
N129	Yes	Yes	Yes	No	No	Other	Other	No	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No
N132	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	No
N134	Yes	Yes	Yes	No	No	Yes	Yes	No	No	Yes	No	Yes	No	Yes	Yes	No	No	Yes	Yes	Yes	No

Table A 7: Correlations between SNPs and gch1 CNV

Correlations

		dhfr50R	dhfr59R	dhfr51I	dhfr108N	dhfr164L	dhfr108T	dhps436A	dhps437G	dhps540E	dhps581G	dhps613S	dhps613T	*gch1* CNV
dhfr50R	Pearson Correlation	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]
	Sig. (2-tailed)													
	N	191	191	191	191	191	191	191	191	191	191	191	191	191
dhfr59R	Pearson Correlation	.[a]	1	.260**	.375**	.285**	-.298**	.018	-.008	.039	-.048	.017	.[a]	.037
	Sig. (2-tailed)			.000	.000	.000	.000	.808	.911	.595	.509	.811		.615
	N	191	191	191	191	191	191	191	191	191	191	191	191	191
dhfr51I	Pearson Correlation	.[a]	.260**	1	.222**	.288**	-.248**	.022	.144*	.038	-.052	-.029	.[a]	.033
	Sig. (2-tailed)		.000		.002	.000	.001	.765	.047	.606	.477	.686		.652
	N	191	191	191	191	191	191	191	191	191	191	191	191	191
dhfr108N	Pearson Correlation	.[a]	.375**	.222**	1	.075	-.860**	.042	.006	-.034	-.172*	-.095	.[a]	-.039
	Sig. (2-tailed)		.000	.002		.301	.000	.564	.934	.641	.017	.193		.592
	N	191	191	191	191	191	191	191	191	191	191	191	191	191
dhfr164L	Pearson Correlation	.[a]	.285**	.288**	.075	1	-.088	.083	.040	-.015	-.023	-.040	.[a]	.120
	Sig. (2-tailed)		.000	.000	.301		.228	.252	.583	.833	.750	.584		.099
	N	191	191	191	191	191	191	191	191	191	191	191	191	191
dhfr108T	Pearson Correlation	.[a]	-.298**	-.248**	-.860**	-.088	1	-.056	-.069	.020	.147*	.118	.[a]	.047
	Sig. (2-tailed)		.000	.001	.000	.228		.443	.341	.788	.042	.104		.518
	N	191	191	191	191	191	191	191	191	191	191	191	191	191
dhps436A	Pearson Correlation	.[a]	.018	.022	.042	.083	-.056	1	.623**	.060	-.069	.006	.[a]	.024
	Sig. (2-tailed)		.808	.765	.564	.252	.443		.000	.406	.339	.930		.746
	N	191	191	191	191	191	191	192	192	192	192	192	192	192
dhps437G	Pearson Correlation	.[a]	-.008	.144*	.006	.040	-.069	.623**	1	.006	-.029	-.026	.[a]	.014
	Sig. (2-tailed)		.911	.047	.934	.583	.341	.000		.938	.691	.719		.851
	N	191	191	191	191	191	191	192	192	192	192	192	192	192
dhps540E	Pearson Correlation	.[a]	.039	.038	-.034	-.015	.020	.060	.006	1	.319**	.228**	.[a]	.236**
	Sig. (2-tailed)		.595	.606	.641	.833	.788	.406	.938		.000	.001		.001
	N	191	191	191	191	191	191	192	192	192	192	192	192	192
dhps581G	Pearson Correlation	.[a]	-.048	-.052	-.172*	-.023	.147*	-.069	-.029	.319**	1	.748**	.[a]	.220**
	Sig. (2-tailed)		.509	.477	.017	.750	.042	.339	.691	.000		.000		.002
	N	191	191	191	191	191	191	192	192	192	192	192	192	192
dhps613S	Pearson Correlation	.[a]	.017	-.029	-.095	-.040	.118	.006	-.026	.228**	.748**	1	.[a]	.122
	Sig. (2-tailed)		.811	.686	.193	.584	.104	.930	.719	.001	.000			.093
	N	191	191	191	191	191	191	192	192	192	192	192	192	192
dhps613T	Pearson Correlation	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]
	Sig. (2-tailed)													
	N	191	191	191	191	191	191	192	192	192	192	192	192	192
gch1 CNV	Pearson Correlation	.[a]	.037	.033	-.039	.120	.047	.024	.014	.236**	.220**	.122	.[a]	1
	Sig. (2-tailed)		.615	.652	.592	.099	.518	.746	.851	.001	.002	.093		
	N	191	191	191	191	191	191	192	192	192	192	192	192	192

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

a. Cannot be computed because at least one of the variables is constant.