Chapter 1

An overview of malaria parasite biology
1.1 Introduction

Malaria is one the most important tropical diseases causing significant morbidity and mortality in the world. Malaria is transmitted through the bite of infected female *Anopheles* mosquito. Five species of *Plasmodium* namely *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* infect humans. Although *P. falciparum* is responsible for a majority of infections and mortality, *P. vivax* has similar infection rates as well with less mortality (Bousema and Drakeley, 2011). An estimated 198 million cases of malaria occurred globally in the year 2013 and led to 584 000 malaria deaths (World Malaria Report, 2014). Emerging resistance to the artemisinin-based combination therapies (Dondorp et al., 2010) and spread of artemisinin resistance (Ashely et al., 2014) alongside, the absence of an effective vaccine highlight an urgent need to develop new drug targets and vaccine candidates [Hobbs et al., 2011; Vanderberg, 2009]. Globally, the total population at high risk of infection has been depicted in Figure 1.1

![Global Malaria map of the total population at high risk](image)

**Figure 1.1:** Global Malaria map of the total population at high risk, created by Global Malaria Mapper, 2014
1.2 A brief history of malaria

Malaria is one of the most ancient human infectious diseases with its early description found in a Chinese document from about 2700 B.C., clay tablets of Mesopotamia from 2000 BC, the Egyptian papyri from 1570 B.C. and in Hindu texts from the sixth century B.C. (reviewed by Cox, 2010). A recent study based on molecular methods, has established the endemicity of malaria co-occurrence with human tuberculosis in Fayum Depression, Lower Egypt dates back to 800 B.C. (Lalremruata et al., 2013). Enlarged spleen, presumably due to malaria, has been found in Egyptian Mummies more than 3000 years old and malaria antigen has been detected in skin and lung samples of mummies from 3200 and 1304 B.C. (Miller et al., 1994). The records describing enlargement of spleen has been founded in the inscriptions of the Vedic period (1500 to 800 B.C.) suggesting that, malaria was prevalent in India at that time (Sherman, 1998). The association of tertian (every third day) and quartan (every fourth day) fevers with enlarged spleen has also been mentioned in the Chinese medical classic Nei Ching (2700 B.C.), in which the symptoms of headache, chills, and fever has been represented to three demons: one carrying a hammer, another with a pail of water and the third with a stove (Bruce-Chwatt, 1988). The early Greeks, including Homer (850 BC), Empedocles of Agrigentum (550 BC) and Hippocrates (400 BC), were familiar with the relatedness of poor health, malarial fevers and enlarged spleens seen in people living in marshy places. There was a persistent thought for over 2500 years that malaria, fever was caused by miasmas rising from swamps (reviewed by Cox 2010). In the Eighteenth century, these specific fevers, known in England as ‘agues’, received the Italian name ‘malaria’ (bad air), because it was then widely believed that their cause was related to the foul air common near the marshy areas (Gilles, 2002).

The discovery of microbial cause of many diseases by Louis Pasteur (1822-1895) and Robert Koch (1843-1910), firmly established that microbe, but not miasmas caused the diseases. This paved the way to search the microbial cause of malaria. In 1879 Edwin Klebs and Carrado Tomassi-Crudeli, isolated a rod-shaped bacterium, which they named ‘Bacillus malariae’ from the mud of malarious marshes and from the urine of a patient with malaria. Further, injecting the cultured bacterium into the rabbits caused the febrile infections accompanied by enlarging spleens similar to the characteristics of malaria. However, many investigators including Patrick Manson tried to cultivate B. malariae from the blood of patients with tertian malaria, but all of their attempts to link bacterial cause of malaria ended in failure. The focus of malaria research then shifted from bacteria to malaria pigment as the
cause of the disease and Heinrich Mechkel in 1847 was the first to propose that, the hemozoin pigment caused malaria (Sherman 1998; Cox, 2010). Although many researchers have detected black-pigmented bodies in the blood, spleen and other tissues of patients, it was Charles Louis Alphonse Laveran, who was looking for pigment in the fresh blood samples of patients identified a tiny, wriggling, crescent-shaped organism in fresh blood samples under a high power microscope and named it as Oscillaria malariae. His descriptions included crescents (gametocytes), pigmented trophozoites and the process of exflagellation (Kean et al., 1978). Under the influence of bacteriology, the scientific community remained sceptical about this discovery till the observations from several other workers confirmed Laveran’s findings (Boyd, 1949). In 1890, Camillo Golgi demonstrated the asexual development of this parasite, its reproduction by multiple-fission and the coincidence of fever with the rupture of red blood cells (RBCs) and release of merozoites into the bloodstream. A year later, Dimitri Romanowsky demonstrated the use of eosin and methylene blue for staining parasite nucleus and cytoplasm, which helped in the identification of different species of this parasite (Kean et al., 1978). The genus name Plasmodium was maintained for all these species. In 1907, Laveran received the Nobel Prize for his discovery of the causative agent of malaria, the protozoan parasite belonging to the genus Plasmodium.

Ronald Ross, a Surgeon-Major in British Indian Medical service, fed by the experience of Patrick Manson, who detected microfilariae in bloodsucking mosquitoes, eventually identified the Anopheles mosquito as the vector for human malaria. He then used avian malaria parasite P. relictum and its vector Culex fatigans to show that the sporozoites, which developed within the oocysts, travelled from the gut to the salivary glands of these mosquitoes. He received the Nobel Prize in 1902 for this breakthrough discovery (Foster, 1965; Ross, 1923; Harrison, 1978). Meanwhile, researchers observed a delay in the appearance of blood stage parasites following the bite of an infected mosquito in comparison to infections induced by the inoculation of infected blood. In 1935, Huff and Bloom from United States described the development of P. elongatum in lymphoid and myeloid cells of birds inoculated with sporozoites before their appearance in the blood. These parasite stages which develop outside RBCs are called Exo-erythrocytic or pre-erythrocytic forms and are found to be responsible for the delay in mosquito-transmitted infections (Huff, 1949). Finally, Shortt, Granham and their collaborators at the Ross Institute at the London School of Hygiene and Tropical medicine identified the developmental stages for P. vivax and P. falciparum in the liver biopsies of human volunteers and described their complete life cycle.
In the 1900s, larvicides alongside the drainage were introduced to limit mosquito breeding sites with water. This was very successful in reducing malaria transmission in some parts of the world. In 1939, work by the Swiss chemist Paul Hermann Müller lead to the synthesis of the pesticide dichlorodiphenyltrichloroethane (DDT), and it was introduced as part of a malaria eradication campaign. The work Paul Hermann Müller was recognized 1948 for the insecticidal use of DDT for vector control and was awarded the Nobel Prize. The use of DDT was very successful and led to malaria elimination in many island areas. However, the use of DDT was interrupted due to the emergence of DDT-resistant mosquitoes and the negative environmental side effect of the pesticide (Gilles, 2002).

1.3 Classification
The causative organisms of the disease malaria are protozoa of the genus *Plasmodium*, which invade and multiply within erythrocytes of vertebrates, and are transmitted by mosquitoes. The motile invasive stages (merozoite, ookinete and sporozoite) are elongate, uninucleate cells able to enter cells or pass through tissues, using specialized secretory and locomotory organelles. The intracellular stages live in a membrane-lined cavity (parasitophorous vacuole) within the host cell cytoplasm. The genus is currently classified on the basis of molecular and other evidence as: Kingdom Protozoa, Subkingdom Biciliata, Infra kingdom Alveolata, Phylum Myzozoa, Subphylum Apicomplexa, Class Aconoidasida, Order Haemosporina, and Genus *Plasmodium*. The Subphylum Apicomplexa comprises nearly 5000 described species, all parasitic including several genera of medical and economic importance, including besides *Plasmodium, Babesia, Toxoplasma, Cryptosporidium, Theileria, Eimeria* and *Isospora*. They all lack cilia and flagella except for the microgametes, but possess invasive organelles (rhoptries, micronemes and polar rings) constituting the apical complex, structures diagnostic for this group (hence the name Apicomplexa). They also typically contain one or more mitochondria, and an elongate membranous organelle known as the apicoplast, and move by a unique form of gliding locomotion. Evolutionarily, the nearest relatives of this group are the ciliates and dinoflagellates (Cavalier-Smith, 2003; Bannister et al., 2009).
1.4 Life cycle of Plasmodium

Malaria parasites undergo a complex series of invasive and replicative stages requiring a vertebrate and invertebrate host to complete its life cycle (Figure 1.2). The life cycle of malaria parasites requires five different cell types for invasion, namely Kupffer cells, hepatocytes, and erythrocytes in the human host and midgut and salivary gland epithelial cells in the mosquito (Vega-Rodriguez et.al, 2014). The infection is initiated when sporozoites are injected with saliva into the skin of a vertebrate, by a feeding Anopheles mosquito. These sporozoites enter the bloodstream or lymphatic and circulate to infect the liver in mammals. However, the sporozoites infect the spleen, endothelial cells and macrophages in birds and lizards (Bannister et.al, 2009). There they invade hepatocytes via Kupffer cells and become intracellular to proliferate and form hundreds of invasive merozoites which are then released into the bloodstream. This phase of liver stage infection is known as pre-erythrocytic or Exo-erythrocytic phase. In *P. vivax* and *P. ovale* infection, some sporozoites convert to dormant forms called hypnozoites, which can cause relapses after weeks, months or even years. However, these resting stages do not appear in *P. falciparum*, *P. malariae* or *P. knowlesi*. Once released into the blood stream, the merozoites immediately invade the erythrocytes and develop to a ring stage and then to a trophozite which replicates to form a multinucleated schizont containing merozoites (Greenwood et al., 2008). The mature schizont raptures releasing merozoites that infect new erythrocytes. This phase of blood stage infection is known as the asexual blood stage or the intraerythrocytic stage of *Plasmodium* development. The characteristic periodic fevers of malaria are caused by synchronous parasite development and erythrocyte rupture which releases new merozoites, malaria antigens and toxic metabolites. In continuation with the asexual stage cycle, a small number of asexual stage parasites converts to form male and female sexual forms of parasites known as gametocytes (Carter and Miller, 1979). These gametocytes are ingested as part of the blood meal by female mosquitoes thereby initiating the sexual phase development in the mosquito midgut. Male gametocytes divide rapidly into a number of motile flagellated microgametocytes each of which can fertilize a female macrogamete to form a zygote. The parasite then becomes a motile ookinete, penetrating the mosquito gut wall and encysting as a rounded oocyst. The parasite multiplies asexually within this to form many hundreds of motile sporozoites (a process known as sporogony). Mature sporozoites escape through the oocyst wall into the insect’s blood cavity (haemocoel) and thence to the salivary glands, penetrating their walls to reach the mosquito’s stored saliva in readiness for transmission to a vertebrate at another blood meal (Bannister et al., 2009).
**Figure 1.2**: Life cycle of *Plasmodium falciparum*. The different stages of parasite development in the life cycle of *Plasmodium* have been detailed in text. (Adopted with modification from CDC).

### 1.5 Developmental stages of Plasmodium

#### 1.5.1 The Pre-erythrocytic Stage of Plasmodium: The Journey of Sporozoite into mammalian host

The beginning of asexual phase in the vertebrate host starts when the infectious mosquito probes the skin for a blood meal and consumes the blood by directly cannulating from a subcutaneous blood vessel or from a pool of blood generated by capillary damage by depositing saliva to prevent blood from coagulating (Frevert 2004; Beier, 1998). A small number of sporozoites (an average of 100 sporozoites injected by single infected mosquito) present in the mosquito saliva is inoculated into the skin during probing (Medica and Sinnis, 2005; Jin *et al.*, 2007) and these sporozoites remains motile for 1-3h (Yamauchi *et al.*, 2007). Once into the skin, a few actively motile sporozoites find a blood capillary and successfully invade it (Amino *et al.*, 2006a; Vanderberg and Frevert, 2004) however, around 20% of the sporozoites enter a lymphatic vessel and are drained to the lymph node, mostly being internalized inside dendritic cells (Sinnis and Zavala, 2008; Amino *et al.*, 2006b). The
sporozoites, which were unable to migrate out of the skin and those that are ending up in draining lymph node induce strong cell-mediated immune response. Evidences suggest that this immune response may be the basis of protection from subsequent pre-erythrocytic stage infection (Sinnis and Zavala, 2008). Sporozoites that successfully enter the blood stream will migrate to liver sinusoid.

The liver sinusoid having a unique feature, mainly composed of endothelial and Kupffer cells, have open fenestrations allowing for small lipoproteins, such as heparan sulphate proteoglycans (HSPGs) to extend from the Disse space to the sinusoidal lumen of the blood vessels and directly interact with Circumsporozoite Surface Protein (CSP) from circulating parasites (Sinnis et al., 1996; Frevert, and Crisanti, 1998). Although HSPGs are present in most tissues in the mammalian body, liver HSPGs are known to be more highly sulphated than in all other tissues (Lyon et al., 1994). The endothelial cells having a low sulfated level of HSPGs induce migration of sporozoites, whereas highly sulphated liver HSPGs activates sporozoites invasion (Coppi et al., 2007). After the sporozoites recognizes the HSPGs in the liver sinusoid, the CSP is processed to expose the C-terminal cell-adhesive thrombospondin repeat (TSR) domain, which allows attachment of the sporozoites to the endothelium (Coppi et al., 2011). At the same time, the successful invasion of sporozoites into the host hepatocyte requires, scavenger receptor B1 (SR-B1) which mediates the selective uptake of cholesteryl esters from both high and low density lipoprotein, together with host molecules such as CD81 and CD9 which are important for recognition and invasion (Zheng et al., 2014). A recent study in the rodent malaria parasite has shown that sporozoites can enter and fully develop and can also differentiate into merozoites in the skin (Gueirard et al., 2010) however, whether the same phenomenon occurs in other Plasmodium species has to be studied with great detail.

In order to reach the final destination the hepatocytes, sporozoites traverses through Kupffer cells to enter the Disse space (Baer et al., 2007; Frevert et al., 2006) where they rest shortly and invades by traversing a series of hepatocytes, a process known as glideosome machinery actively controlled by the parasite to force its way into the host cell before choosing the final one and form a parasitophorous vacuole (PV) (Mota et al., 2001; Keeley and Soldati, 2004). The traversal of sporozoites through hepatocytes causes wounding of cells and results in the release of hepatocyte growth factor (HGF) by the wounded cells, which subsequently promotes hepatocytes survival (Carrolo et al., 2003; Leiriao et al., 2005). Several parasite proteins have been shown to be essential for cell traversal and gliding motility in the hepatic
stage infection (Figure 1.3). Sporozoites microneme protein essential for cell traversal 2 (SPECT2), the cell traversal protein for ookinete and sporozoites (CelTOS) and the phospholipase (PbPL) has been reported to be associated with cell traverse (Bhanot et al., 2005; Ishino et al., 2005a; Ishino et al., 2004; Kariu et al., 2006). Further, CSP has been shown to immediate sporozoites attachment to the hepatocytes, while TRAP is required for internalization. The apical membrane antigen 1 (AMA1) along with Pb36p and Pb36 is reported to play a role in recognition and (or) invasion of hepatocytes (Pinzon-Ortiz et al., 2001; Matuschewski et al., 2002a; Ishino et al., 2005b; Silvie et al., 2004).

Figure 1.3: Proteins involved in sporozoites migration and gliding to invade hepatocyte and subsequent liver stage infection. The various stages of parasite development inside the mammalian host are depicted. Both parasite and host proteins known to be important for each stage of development are listed. EC: Endothelial Cells; LE: Lymphoid Endothelium; SE: Fenestrated Endothelia KC: Kupffer Cell; PVM: Parasitophorous Vacuole Membrane; PI: Post Infection. Image adapted from Vaughan et al, 2008.

After successfully establishing itself within a hepatocyte, the parasite cleverly blocks its host cell from undergoing programmed cell death by secreting cysteine protease inhibitors (Rennenberg et al., 2010). Within 24h of hepatocyte invasion, the parasite remodels its
parasitophorous vascular membrane (PVM) and structurally transforms into trophozoite and often localizes close to the host cell nucleus (Graewe et al., 2011; 2012). Several of the parasite genes have been reported to be up-regulated in infective sporozoites namely UIS3, UIS4 and Pb36p and gene disruption studies of the same has proved its essentiality in the early liver stage development (Matuschewski et al., 2002b; Mueller et al., 2005a; Mueller et al., 2005b; van Dijk et al., 2005). The parasite initiates many rounds of nuclear division, succeeding the trophozoite stage with in ~ 30h resulting in the generation of up to 30,000 new nuclei, ensuing increase in the size of parasitophorus vacuole (PV) (Graewe et al., 2012). This tremendous replication rate clearly indicates the existence of various host parasite interactions that could facilitate the parasite with necessary nutrients, thereby giving a glimpse into the dynamic interactions between parasite and host proteins, which occurs primarily at PVM (Bano et al., 2007).

To cope with this rapid growth and synthesis of additional membranes, the parasite needs a significant amount of nutrients e.g. Lipids for which, the parasite has evolved with a type II fatty acid synthesis (FASII) pathway, distinct from mammalian type 1 pathway. Knockouts for the parasite enzymes in this pathway namely FabB/F, FabZ and Fab1, were unable to form merozites in the liver (Yu et al., 2008; Vaughan et al., 2009). In spite of synthesizing fatty acid de novo, the parasite also sequesters host cell fatty acids (Albuquerque et al., 2009). Furthermore, cholesterol from the host hepatocyte has been shown to be utilized until the release of merozoites in the liver stage development (Labaied et al., 2007). As a result of rapid division, the parasite transforms to a multinucleated developmental stage known as schizont. Once the division is complete, the cytokinesis begins by repeated invagination of parasite membrane resulting in the formation of daughter cells known as merozoites (Graewe et al., 2012). Parasite proteins are activated by proteases to disrupt the PVM soon after the formation of merozoites (Sturm et al., 2009) which releases the merozoites into the host hepatocytes. At the same time, host cell death is induced (Heussler et al., 2010; Sturm et al., 2006) and the merozoites exit the liver in host cell derived vesicles called mersomes, which protects the hepatic merozoites from being phagocytised by Kupffer cells. Finally the mersomes accumulate within the pulmonary capillaries of the lung, and enters the bloodstream, thereby initiating the beginning of erythrocytic stage infection (Sturm et al., 2006; Baer et al., 2007).
1.5.2 The erythrocytic stage of Plasmodium

The pathogenic manifestations and all of the clinical symptoms associated with malaria infection in mammals are caused by the asexual erythrocytic phase of the *Plasmodium* life cycle. This phase is initiated when thousands of invasive merozoites which are released into the bloodstream from mature hepatic schizonts (Barnwell and Galinski, 1998).

1.5.2.1 Invasion of merozoite into erythrocyte

The erythrocyte invasion by merozoites is a complex process comprising of several distinct phases (i) initial recognition and reversible attachment of merozoite to the erythrocyte membrane (ii) reorientation followed by junction formation between the merozoite apical and erythrocyte membrane (irreversible attachment) (iii) release of substances from rhoptry and microneme organelles, leading to the formation of PV (iv) movement of the junction and invagination of erythrocyte membrane accompanied by the removal of merozoite surface coat and (v) merozoite internalization followed by membrane closure (Figure 1.4). A large number of molecular interactions have been found to be associated between erythrocyte and the merozoites during this complex and rapid invasion process. The parasite has three organelles on the apical end, the rhoptries, micronemes and the dense granules. (Chitnis and Blackman, 2000; Miller *et al.*, 2002; Baum *et al.*, 2005; Tuteja, 2007) which facilitates the invasion. The initial reversible adhesion occurs by a surface coat of proteins that is largely comprised of glycosylphosphatidylinositol (GPI)-anchored membrane proteins. There are at least nine potential erythrocyte ligands predicted which are GPI-anchored proteins (Cowman and Crabb, 2006). By means of multiple weak interactions, Merozoite surface proteins (MSPs) are likely to be involved in initial contact with receptors on the surface of erythrocytes (Boyle *et al.*, 2014). Several other proteins also share similar role other than from GPI-anchor, such as erythrocyte binding–like (EBL) or Duffy binding–like (DBL) domains that are specific to *Plasmodium* spp. are involved in pre- and post-invasion process together with their role in cytoadherence, whereas Epidermal Growth Factor (EGF) and six-cysteine (6-Cys) domains are involved in protein-protein interactions (reviewed by Cowman *et al.*, 2012). After the initial adhesion with the erythrocyte, the merozoite re-orient such that its apical end points towards and interacts with the erythrocyte membrane allowing the formation of a tight irreversible junction between merozoite and the erythrocyte membrane. MSP1 is likely to be involved in this initial attachment (Bannister and Mitchell, 2003; Goel *et al.*, 2003). The secretory organelles from the parasite insert microneme protein, Apical membrane antigen 1 (AMA1) in the parasite plasma membrane and a complex of four rhoptry
neck proteins (RON-2, -4, -5, -8) at the erythrocyte membrane (Lamarque et al., 2011; Srinivasan et al., 2011).

The strong interaction between AMA1 and a C-terminal region of RON-2 is critical for invasion (Lamarque et al., 2011). Other interactions between the merozoites and the erythrocytes includes the erythrocyte binding antigen-175 (EBA-175), EBA-140 and EBA-181 found on the merozoites which bind to glycoporin A, glycoporin C, and band 4.1 respectively, in the erythrocytes (reviewed in Cowman et al., 2012). The invasion of erythrocytes is mediated by merozoites through internal molecular motor based on actin and myosin to drive itself into the erythrocyte (Baum et al., 2008; Soldati-Favre, 2008). Once the junction is successfully formed, the release of rhoptry bulb is activated facilitating the release of proteins and lipids required for PVM and PV to establish the space into which the merozoite can accommodate as it enters (reviewed by Cowman et al., 2012). The PVM remains through the erythrocytic cycle and enlarges as the intraerythrocytic parasite grows (Bannister and Mitchell, 2003). The rhoptry associated proteins (RAP) which aid in the invasion process is RAP-1, -2, -3, high molecular weight rhoptry proteins RhopH1/1/3 and protease gp76 (Etzion et al., 1991). The invasion force is driven by myosin attached to flattened double membrane cisternae known as Inner Membrane Complex (IMC) through a multiplex of proteins forming a substrate with which it can hold (reviewed in Cowman et al., 2012). The glideosome (a motor complex required for gliding motility) is anchored to IMC (Agop-Nersesian et al., 2009) via a complex interactions between MyoA, Myosin Tail Interacting Protein (MTIP), Glideosome associated protein 45 (GAP45) and GAP50 (Pinder et al., 1998; Webb et al., 1996; Jones et al., 2006). At this site the actin filaments also concentrate forming a ring like distribution at the tight junction of invading merozoite trailing the RON complex (reviewed by Cowman et al., 2012). The moving junction is now pulled along the surface of merozoite together with erythrocyte membrane until the PVM is sealed (Reviewed by Cowman et al., 2012). Meanwhile the dense granules move to the parasite surface and release Ring Infected Erythrocyte Surface Antigen (RESA), Ring Membrane Antigen (RIMA), and the subtilisin-like proteases which may fuse with PV and interact with erythrocyte membrane lipid bilayer and erythrocyte membrane skeletal proteins (Howell et al., 2003). These results in further enlargement of PVM as parasite derived material are integrated into its structure. Following the invasion of erythrocyte by merozoite, certain parasite surface proteins and micronemal proteins are efficiently shed by proteolytic process which includes shedding of MSP1 and AMA1 from the parasite surface which is mediated by
subtilisin-like serine protease called *Pf*SUB2 as merozoites invade erythrocytes (Olivieri et al., 2011). Yet another set of serine proteases known as the Rhomboids is also involved in shedding process, namely *Pf*ROM-1,-4 cleaves AMA1, merozoite TRAP homologue (MTRAP), Reticulocyte-binding homologues (RH 1, -2a, -2b, -4) and EBA-175, EBA-140/BAEBL, EBA-181/JESEBL and MAEBL (Baker et al., 2006). The parasite is now called as ring stage, which actively feed on haemoglobin via endocytosis process (Figure 1.4)

1.5.2.2 Food vacuole formation and haemoglobin degradation.

Once the merozoite invades the erythrocyte, parasite within the PV progresses towards intraerythrocytic maturation and develop into the trophozoite stage. During this maturation process the early ring stage parasites undergo a drastic morphological transformation by folding them into a cup shape and while doing so engulfs a large portion of erythrocyte cytoplasm thereby originating the parasite’s lysosomal compartment known as the food vacuole (FV) (Elliott et al., 2008). The maturation of parasite is associated with a rapid increase in metabolic and biosynthetic activity of proteins and nucleic acid. The main source of amino acids for parasite’s protein synthesis comes from the degradation of haemoglobin in FV (Roth, 1990; Sherman, 1977). This is achieved by a specialized structure called cytostome from which vesicles bud’s off and transport erythrocyte cytosol (hemoglobin) to the FV where the hemoglobin is hydrolysed (Rosenthal and Meshnick, 1998; Francis et al., 1997). During the process of haemoglobin degradation, free heme is released as a by-product in the FV which is extremely toxic to the parasite. To neutralize this, several proteins have been implicated in the conversion of heme to hemozoin namely Heme detoxification protein (HDP) (Jani et al., 2008), Histidine rich proteins (HRPs) in *P. falciparum* (Sullivan et al., 1996) and also the involvement of lipids (Fitch et al., 1999). Several proteases are involved in the degradation of hemoglobin within the FV. The digestion of hemoglobin is initiated by aspartic proteases namely Plasmepsin-I,-II,-IV at the hinge region of the α-globin chain (Gluzman et al., 1994; Banerjee et al., 2002; Wyatt and Berry, 2002) resulting in the release of globin chain. Further proteolysis of globin chain is mediated by the same enzymes along with three cysteine proteases, falcipain-2,-2’,-3 (Shenai et al., 2000; 2003; Sijwali and Rosenthal 2004). The resultant short globin polypeptide is converted into oligopeptides consisting of 5-10 amino acids by a metalloprotease enzyme known as falcilysin (Eggleson et al., 1999) followed by hydrolyses of these oligopeptides by calpain-like dipeptidyl peptidase I (DPAP1) into dipeptides (Klemba et al., 2004). There are evidences that these dipeptides are converted into amino acids inside food vacuole by DPAPs (Dalal and Klemba, 2007),
alternately the peptides are exported to cytoplasm for the generation of amino acids by aminopeptidase activity (Curley et al., 1994). The amino acids obtained as a result of globin hydrolysis are utilized by the parasite for energy metabolism and incorporated into its proteins. However, with the limited capacity of de novo amino acid synthesis by Plasmodium and its poor source of methionine, cysteine, glutamine and glutamate and complete absence of Isoleucine indicates that, the haemoglobin degradation alone is not sufficient for the metabolic needs of the parasite (Francis et al., 1997). To overcome these problems the parasite activates the New Permeability Pathway (NPP) wherein, the parasite up-regulates the transporters and channels within the erythrocyte plasma membrane by phosphorylation and at the same time also integrates its own transporters and channels in the erythrocyte membrane to facilitate uptake of nutrients (Kirk, 2001; Decherf et al., 2004; Saliba et al., 1998) and to eliminate toxic waste products produced during the growth of the parasite.

**Figure 1.4:** The intraerythrocytic stages of *Plasmodium falciparum*. (A) Merozoite invasion (B) The ring form (C) Trophozoite stage (D) Schizont stage (E) Release of merozoites from ruptured erythrocytes. The various steps involved in this process and the associated molecular events are discussed in the text. (Adapted with modification from Bannister and Mitchell, 2003).
1.5.2.3 Schizont maturation and egress

The metabolically active parasites at the trophozoite stage now progress towards the next developmental stage known as the schizont (Figure 1.4). During erythrocytic schizogony, the parasite undergoes multiple rounds of nuclear division and initiates the biogenesis of specialized apical secretory organelles that promote invasion of daughter merozoites into a new host cell. Eventually, each mature schizont containing ~16 daughter merozoites that ruptures in a process known as egress mediated by parasite secreted proteases in a two-step exit process, initially cysteine protease are secreted into the PV followed by aspartic protease and subtilisin-like serine proteases that degrade the membrane skeleton of the erythrocyte (Salmon et al., 2001; Wickham et al., 2003; Yeoh et al., 2007). Since most of the haemoglobin is digested by the parasite (Lew, 2005), the membrane degradation and rearrangement of newly formed daughter merozoites inside the schizont will gives rise to the irregular short-lived distinctive flower shaped structure (Trager, 1956; Glushakova et al., 2005) which is associated with an influx of fluid into the erythrocyte due to the excess colloid-osmotic pressure of intracellular proteins. This causes the PVM and the erythrocyte plasma membrane to blow apart, thereby scattering the merozoites. The force of the merozoite release is strong enough to bring the merozoites into contact with new erythrocytes even in places where the parasites have been sequestered. The parasite therefore does not need to be motile and only becomes motile when a host cell is encountered and that the parasite has committed itself to invasion. This repetitive intra-erythrocytic cycle continues and occurs quite synchronously, taking about 48 h in *P. falciparum*, *P. vivax* and *P. ovale* infections and 72h in *P. malariae* infection (Tuteja, 2007).

1.5.3 Gametocytogenesis and sexual stages development in Plasmodium

The sexual phase of malarial parasite begins with the differentiation of male and female gametocytes (pre-gametes) from sexually committed asexual stage parasites. This process has been termed as Gametocytogenesis (Baker, 2010; Kuehn and Pradel, 2010; Talman et al., 2004). Once committed, the gametocyte undergoes morphological changes and develops through five distinctive stages (I–V Stages). Although these stages do not directly cause any clinical symptoms in vertebrate host, they are the principal stages responsible for continued transmission of parasites from one vertebrate hosts, to another via a complex sexual life cycle in the mosquito vector. The stage V gametocytes represent the exclusive stage that can undergo sexual reproduction when taken up by the feeding mosquito (Dixon et al., 2008; Kuehn and Pradel, 2010). The sexual stage commitment always occurs one cycle before the
manifestation of gametocytes and all the merozoites from a single committed schizont will only produce either male or female gametocytes (Talman et al., 2004; Dixon et al., 2008). The sex ratio among gametocytes is female-biased, with one mature male gametocyte for about five mature female (reviewed in Paul et al., 2002; Kuehn and Pradel, 2010; Talman et al., 2004). Various factors such as environmental conditions, immunological stress, parasite density, chemotherapy and impact of host response have been shown to induce and modulate the rate of gametocytogenesis (Sinden, 1998). Commitment to sexual differentiation in malarial parasites involves a number of sexual stage specific genes and their protein products based on a coordinated cascade of gene expressions (Lobo and Kumar, 1998). The extracellular vesicles (EVs) in Plasmodium namely exosome-like vesicles and microvesicles derived from the infected red blood cells (RMVs) has been shown to promote differentiation of parasites into sexual forms and PfPTP2 is identified as the key P. falciparum protein involved in the transfer of genetic information between the parasites (Regev-Ruddzki et al., 2013; Mantel et al., 2013). The master regulator of gametocytogenesis has been identified as AP2-G belonging to a conserved member of Apicomplexan AP (ApiAP) family of transcription factors in two Plasmodium species, PfAP2-G in P. falciparum and PbAP2-G, -G2 in P. berghei (Kafsack et al., 2014; Sinha et al., 2014).

A group of P. falciparum early gametocyte genes (Pfge) have been identified that are mediated through P. falciparum gametocyte development 1 gene (Pfgdv1) as crucial for early sexual differentiation. The Pfge includes known gametocyte specific genes Pf16, Pf27, Pf14.744, Pf14.748, Pfpeg3 or Pfmdv1, Pf47 and Pfgeco (Eksi et al., 2012). Pf16 gene known to be the first molecular marker expressed within 24h of merozoite invasion and localized in PV of early gametocytes (Bruce et al., 1994; Baker et al., 1994), Pf27 gene encoded a dimeric cytosolic phosphoprotein expressed during early sexual differential process and maintains cellular integrity of gametocytes (Lobo et al., 1999; Olivieri et al., 2009), Pf14.744 and Pf14.748 genes expressed during early gametocytogenesis prior to stage II (Eksi et al., 2005; Silvestrini et al., 2010), Pf47 mediates immune evasion and is vital for successful transmission from a vertebrate host to mosquito (Molina-cruz et al., 2013), Pfpeg3 or Pfmdv1 are early gametocyte proteins (Florens et al., 2002; Furuya et al., 2005) and P. falciparum gametocyte erythrocyte cytosolic protein (Pfgeco) encodes for type IV heat shock protein 40 (HSP40) expressed from stage I-IV gametocytes and are exported to the RBC cytoplasm (Morahan et al., 2011). Some of the other early gametocyte markers include PfPuf2 an RNA binding protein that has been proposed to regulate
gametocytogenesis and sexual differentiation (Miao et al., 2010), *P. falciparum* Nima-related kinase (*Pfnek*-4) expressed in stage II-V gametocytes and in sexually committed asexual parasites at the schizont stage (Reininger et al., 2012), *Pfpeg4* a gametocyte export protein (Silvestrini et al., 2005), *P. falciparum* gene implicated in gametocytogenesis (*Pfgig*) involved in transition to early gametocytogenesis (Gardiner et al., 2005), *PfRex*-3 and *P. falciparum* gametocyte exported proteins 10 (*PfGEXP10*) are proteins expressed in early gametocytogenesis and exported to the infected erythrocyte cytosol (Silvestrini et al., 2010).

1.5.3.1 Gamete to ookinete transition

There is a considerable difference between mature male and female gametocytes, both at the molecular and cellular levels owning towards fertilization. The cellular structure of male gametocyte has a reduced ribosome and endoplasmic reticulum, whereas the female gametocyte has well-developed endoplasmic reticulum, mitochondria and apicoplast. This drastic change in molecular cell biology of gametocytes is in preparation towards rapid development as a zygote which is mediated by DOZI-mediated translationally repressed/silent transcripts (Paton et al., 1993; Mair et al., 2006). The secretory organelles known as osmiophilic bodies are found abundantly in female and also minimally in male and the *Pfg377* is known to be involved in the formation of osmiophilic bodies which help in gametocyte egress during gametogenesis (Lal et al., 2009; Ponzi et al., 2009; de Koning-Ward et al., 2008). At the proteome level, only 69 proteins are shared between the male and female gametocytes, however sex-specific proteome data shows that 19% of proteins are unique to female gametocytes in comparison to male having around 36% of unique proteins (Khan et al., 2005). Thus, with all these pre-requisite preparations, the gametocyte gets arrested at the *G0* phase of Cell cycle in the vertebrate host (Sinden, 1996) awaiting the transmission to the mosquito vector for gametogenesis (Figure 1.5).

When the mosquito feeds on an infected vertebrate host, gametocytes are taken up along with blood meal and only the mature gametocytes (arrested at *G0* phase of cell cycle) can undergo further sexual stage development. Once in the midgut of the mosquito, the parasite experiences a sudden drop in temperature and encounters the mosquito-derived molecule xantherenic acid (XA) which activates the gametocyte escape from erythrocyte membrane and form male and female gametes, the process referred to as gametogenesis (Billker et al., 1997; 1998; Garcia et al., 1998). The male gametocyte undergo further differentiation with three
rounds of DNA replication and axoneme assembly resulting in the release of eight flagella (microgamete) by a process termed as exflagellation promoted by cGMP (Kawamoto et al., 1990) stimulated by Guanylyl cyclase (GC) which is in turn mediated by XA (Muhia et al., 2001) whereas the female gametocyte produces a single extracellular non-motile macrogamete. The microgamete freely swims until it encounters macrogamete. The micro and macrogametes binds together by cell-cell adhesion possibly mediated by Pfs48/45 and P230 (on male gamete) and P47 (on female gamete) and play an essential role in the process of fertilization (van Dijk et al., 2010) and produces zygote. The zygote which is spherical in shape transforms into a banana-shape motile ookinete within a period of 18-24h. NIMA-related kinase 4 (Nek-4) is known to be crucial for the transformation of zygote into ookinete (Reininger et al., 2005) (Figure 1.5).

1.5.3.2 Ookinetes to oocyst transition

The Plasmodium ookinetes are unique when compared to other invasive stages in the life cycle of malaria parasite, because the ookinete can develop extracellularly and lacks rhoptries and dense granules- the organelles associated with cell invasion and vacuole formation and are known to exhibit actin-myosin-based gliding motility (Patzewitz et al., 2013; Guttery et al., 2012; Baum et al., 2008; Matuschewski and Schüler, 2008). The ookinete has to efficiently navigate through several barriers before becoming an oocyst. The chitin-rich peritrophic matrix is the first barrier which forms, 16-30h post-feeding, around the ingested blood meal bolus (Shahabuddin and Kaslow, 1994). To penetrate this, the ookinete secretes an enzyme known as micronemal chitinases that cleaves the chitin present in the peritrophic matrix (Huber et al., 1991), and makes its way through the peritrophic matrix to reach the midgut epithelium of the mosquito (Shahabuddin et al., 1993). The ookinete tolerates the proteolytic activity within the mosquito stomach, with the aid of its surface proteins, particularly the glycosyl-phosphatidylinositol (GPI) anchored epidermal growth factor domain containing proteins P25 and P28 (Tomas et al., 2001). The efficient invasion and traversal by ookinete is achieved by its specific molecular interactions with the epithelial cells of midgut. Several ookinete protein have been suggested to be involved in this process, namely enolase (Ghosh et al., 2011), circumsporozoite TRAP-related protein (CTRP), von Willebrand factor A domain-related protein (WARP) (Yuda et al., 2001), membrane-attack ookinete protein (MAOP) (Katoda et al., 2004), Plasmodium perforin-like protein 5 (PPLP5) (Ecker et al., 2007), subtilisin-like protease (SUB2) (Han et al., 2000), cell-traversal protein for ookinetes and sporozoites (CelTOS) (Kariu et al., 2006), secreted ookinete adhesive
protein (SOAP) (Dessens et al., 2003) together with mosquito factors namely enolase-binding protein (EBP) (Vega-Rodríguez et al., 2014), aminopeptidase 1 (APN1) (Dinglasan et al., 2007), annexin-like proteins (Kotsyfakis et al., 2005), carboxypeptidase B (Lavazec et al., 2007), croquemort scavenger receptor homolog (González-Lázaro et al., 2009) and calreticulin (Rodríguez-Mdel et al., 2007). Based on these specific molecular interactions, the ookinete initiates the cell traversal into the epithelium and settles between the basement cell membrane and the basal lamina of the midgut wall (Han et al., 2000; Sinden and Billingsley, 2001). The epithelial cells get severely damaged during the ookinete invasion process and undergo apoptosis (Han et al., 2000; Vlachou et al., 2004). Meanwhile, the ookinete traversal leads to the activation of mosquito immune response that causes parasite lysis and melanisation mediated by Thioester containing protein 1 (TEP1), Leucine rich repeat immune protein 1 (LRIM1) or Anopheles Plasmodium-responsive leucine-rich repeat 1 (APL1) (Whitten et al., 2006; Blandin et al., 2004; Osta et al., 2004; Riehle et al., 2006). Those ookinetes that overcome the challenges of traversal through peritrophic matrix and the epithelium followed by immune evasion reach the basal sub-epithelial space to form oocyst (Angrisano et al., 2012) (Figure 1.5).

### 1.5.3.3 Oocyst Differentiation and sporozoite formation

Once the ookinete emerges out from the epithelial cell, it interacts with the basal lamina components, namely laminin and collagen IV, which seems to trigger the differentiation of parasite into a replicative form the oocyst (Adini and Warburg, 1999; Arrighi et al., 2005; Sinden, 2002). The transformation into oocyst involves the rapid expansion of the cytoplasm as the parasite begins to grow and mature. The oocyst is surrounded by a dense fibrous capsule in the basal lamina of the midgut epithelium. The oocyst capsule is shown to be made up of Plasmodium oocyst capsule protein (PbCap380) which allows the uptake of nutrients and exit of metabolite together with protecting the parasite from the mosquito immune response (Srinivasan et al., 2008). The apical complex, inner membrane complex and its subpellicular microtubules are resorbed into the oocyst cytoplasm and the developing oocyst undergoes multiple rounds of endomitosis to produce polyploid nucleus. Concomitantly, the oocyst plasma membrane is folded and extended inwards, thereby partitioning the oocyst cytoplasm into sporoblasts. Within the sporoblasts, the sporozoite buds containing nascent apical complexes are assembled, followed by the mobilization of nucleus and other cellular organelles into each budding sporozoite. Unlike ookinetes, sporozoites lack apical collar and contain less number of subpellicular microtubules. Micronemes and (or) rhoptries develop
within the apical region of the emergent sporozoites and each sporozoite probably contains one mitochondrion and apicoplast. The time duration of this entire sporogony varies from 1-2 weeks and the final outcome is the formation of a mature oocyst that contains numerous haploid sporozoites (Figure 1.5). During sporogony, the oocysts undergo massive growth that leads to the stretching and shredding of the basal lamina. Also, the oocyst capsule becomes progressively thinner and ruptures, eventually releasing the mature sporozoites into the hemolymph of the mosquito (Baton and Ranford-Cartwright, 2005; Vlachou et al., 2006).

**Figure 1.5:** The *Plasmodium* life cycle stages within the mosquito. The three major transformation processes during sexual stage development in mosquitoes are (1) gametogenesis (2), midgut traversal and (3) salivary gland infection. The various steps involved in this process and the associated molecular events are discussed in the text (Adapted from Angrisano et al., 2012).
1.5.3.4 Sporozoite motility and invasion of Salivary gland

Once the sporogony is complete, the sporozoites are released into the mosquito haemococel by an active process involving parasite Egress cysteine protease (ECP1) which breach the oocyst capsule in conjunction with region II-plus of CS protein, thereby resulting the exit of sporozoites from oocyst (Aly and Matuschewski, 2005; Wang et al., 2005). A well-defined sporozoites surface protein, the circumsporozoite (CS) protein, is observed on oocyst sporozoites and continues to be expressed by sporozoites after their invasion of the mosquito salivary gland (Beier and Vanderberg, 1998). Upon release into the haemocoel, the sporozoites freely flow along the entire mosquito body and only a small proportion of sporozoites seem to reach the salivary gland (Hillyer et al., 2006). Unlike ookinetes, sporozoites traverse the salivary glands from basal to apical surface and undergo parasitophorous vacuole (PV) formation. Despite these differences, the molecular mechanisms associated with midgut and salivary gland invasions are similar. The invasion of salivary glands, thought to be mediated by receptor-ligand interactions resulting from the binding of parasite surface ligands to specific receptors on the salivary glands, requires the gliding motility of sporozoites (Schuler and Matuschewski, 2006). Several micronemal proteins has been hypothesized and shown to be associated with the sporozoite invasion of the mosquito salivary glands, including the circumsporozoite protein (CSP), the apical membrane antigen/erythrocyte binding-like protein (MAEBL), the thrombospondin-related anonymous protein (TRAP), and the up-regulated-in-oocysts sporozoites protein 3 (UOS3) also called S6/TREP. One of the salivary gland proteins secreted by the distal lobes of the female salivary glands and whose expression is induced by blood feeding, Saglin, is believed to act as one of the sporozoite receptors (reviewed by Ghosh and Jacobs-lorena, 2009). The successful invasion of salivary glands by the sporozoites marks the end of the Plasmodium cycle in the mosquito (Figure 1.5). This complex multiple step process of invasion is not yet fully understood, however, only superficially defined. The sporozoites invade through the basal side of salivary gland epithelial cell and then exits from the apical side to reach the lumen of the acinus where the sporozoites associate with each other to form bundles (Ghosh and Jacobs-Lorena, 2009) ready to be released along with the saliva of the mosquito.
1.6 Antimalarial drugs

Antimalarial drugs have selective actions on the different phases of the parasite life cycle. The antimalarials are categorised as, casual prophylactic drugs that prevent the establishment of the parasite in the liver, tissue schizontocidal drugs that acts on Exo-erythrocytic forms in the liver, the blood schizontocidal drugs that kills the parasite in the red blood cells (RBCs) thereby prevents or abolishes the clinical attack, gametocytocidal drugs that eliminates the sexual forms of the parasite in the blood. Some of these drugs are also hypnozoitocidal as they also can kill the dormant hypnozoites in the liver (responsible for relapses in *P. vivax* and *P. ovale*), and sporontocidal drugs that inhibits the development of oocyst on the stomach wall of the mosquito that has fed on the human gametocyte carrier so that the mosquito can’t transmit the disease (Figure 1.6). These categories of antimalarial drugs mainly include the Artemisinin derivatives (artesunate, artemether, dihydroartemisinin), the Aminoquinoline (Chloroquine, Amodiaquine, Primaquine), the Arylaminoalcohols (Quinine, Mefloquine, Lumefantrine,) the Antifolates (Sulfadoxine-Primethamine, Proguanil and Chlorproguanil-Dapsone), Atovaquone and Antibiotics (Tetracycline, Clindamycin, Doxycycline) (Bruce-Chwatt, 1962; Warrell *et al.*, 2002; Milhous and Kyle, 1998; WHO, 2010).

1.6.1 Artemisinin derivatives

Artemisinin (a sesquiterpene lactone) is the principle antimalarial derived from the ancient Chinese medicinal herb qinghao (*Artemisia annua*) and was isolated and characterized by Chinese scientists in 1972 (Klayman, 1985). The highly lipophilic nature of artemisinin makes it poorly soluble in water and oil, thereby restricting its development as an oral formulation in chemotherapeutic use for malaria. To overcome these problems, several semi-synthetic derivatives, namely artesunate, artemether and dihydroartemisinin was developed, which can be administered orally, intramuscular or intravenous and also through rectal mode (Navaratnam *et al.*, 2000; Bégué *et al.*, 2005). Dihydroartemisinin is the main active metabolite of artemisinin and its derivatives, which is responsible for the overall antimalarial activity (Nosten *et al.*, 2007; Titulaer *et al.*, 1991; Suputtamongkol *et al.*, 2001). The conversion of artesunate to dihydroartemisinin can be through a pH-dependent chemical hydrolysis and (or) enzymatic de-esterification catalysed by blood esterases depending upon the route of administration whereas, the demethylation of artemether is catalysed by the cytochrome P450 enzyme system (CYP1A2, 2B6, 3A4) (Navaratnam *et al.*, 2000; Erissons *et al.*, 2014).
Figure 1.6: Stages of *P. falciparum* and effect of antimalarial drugs. When parasites are sensitive to the drug unless otherwise stated. Positive and negative arrows indicate the effect of the drug, enhancement (+) and suppression (−), respectively, on the parasite stage or its development. (Adapted with modification from WHOa, 2010).

The artemisinin derivatives actively kill all the *Plasmodium* parasites throughout the asexual phase of intraerythrocytic stages, including multidrug resistant *P. falciparum* and also in the early gametocytes (sexual stages) more rapidly than other conventional antimalarial drugs currently in use (German and Aweeka, 2008; Bégué *et al.*, 2005; Kumar and Zheng, 1990; Wilairatana *et al.*, 2010) however, they have no activity on the Exo-erythrocytic stage (Liver stages) (Hommel, 2008). The Endoperoxide Bridge is common among artemisinin derivative which is essential for its antimalarial activity. The mechanism of action of artemisinin
derivative is not completely understood, however, *P. falciparum* sarcoplasmic-endoplasmic reticulum ATPase (PfATP6 or PfSERCA) has been proposed as possible targets (Eckstein-Ludwig *et al*., 2003; Krishna *et al*., 2004). The breakdown of Endoperoxide Bridge generates free radicals that rapidly undergo alkylation reactions. Parasite membranes are particularly sensitive to this oxidative damage ultimately leading to parasite death (Bégué *et al*., 2005). The major stumbling block of artemisinin derivatives is their short half-life due to which and also to prevent the development of resistance, artemisinin derivations are used in combination with one or two long acting antimalarial drugs, namely sulfadoxine/pyrimethamine or lumefantrine, mefloquine and or amodiaquine as artemisinin-based combination therapy (ACT) (Kerb *et al*., 2009). Artemisinin-based combination therapies are the present first line treatment for falciparum malaria globally (Dondorp *et al*., 2009).

### 1.6.2 Aminoquinolines

#### 1.6.2.1 Chloroquine

Chloroquine (CQ), a 4-aminoquinoline drug is a cheap, relatively well tolerated drug initially developed for the treatment of malaria in the 1930s (Cooper and Magwere, 2008; Sáenz *et al*., 2012). CQ is the only antimalarial drug known to be safe for children and pregnant women. The CQ usage has been severely compromised by the spread of CQ-resistant strains of *P. falciparum* (Krogstad and De, 1998) and *P. vivax* in some part of the world (Price *et al*., 2014), however *P. malariae, P. ovale* and *P. knowlesi* are fully susceptible to CQ (WHOa, 2010; Faith *et al*., 2013). CQ still remains the choice of drug for all *P. vivax* and *P. ovale* and CQ sensitive *P. falciparum* infection, particularly among pregnant women diagnosed for uncomplicated malaria except for regions were CQ resistances are prevalent (WHOa, 2010; CDC, 2013). Although CQ has played an important role as a main stay antimalarial drug over the years, its mode of action still remains controversial. The CQ being a weak base gets accumulated within the acidic food vacuole of the parasites, where it is protonated and binds to toxic free heme thereby inhibiting the detoxification into hemozoin which ultimately become lethal for parasite survival (Slater, 1993; Sullivan Jr *et al*., 1996; Bray *et al*., 2005). CQ has a very long half-life (1-2months) (Talisuna *et al*., 2004) which allows the prolonged exposure of the drug to the parasites which may be one of the reasons for the emergence of CQ resistance. Mutation in two different transporter genes has been associated with CQ resistance, namely *P. falciparum* chloroquine resistant transporter (PfCRT) that bears a point mutation K76T which enhances the efflux of CQ from the digestive vacuole where it localise and *P. falciparum* multidrug resistance reporter protein-1 (Pfmdr1) also known as the P-
glycoprotein homolog 1 (Pgh-1) (Fidock et al., 2000, Cooper et al., 2005; Griffin et al., 2012; Duraising and Cowman, 2005).

1.6.2.2 Amodiaquine
Amodiaquine (AQ) is a 4-aminoquinoline, similar to CQ that has been used widely for the treatment and prevention of malaria (Olliaro and Mussano, 2003). AQ was initially introduced as a cheap alternative to CQ and it also showed activity against CQ resistant P. falciparum, gaining widespread use in regions where CQ resistance was prevalent (Johansson et al., 2009). AQ is metabolized to its primary metabolite, N-desethylamodiaquine (DEAQ), by the enzyme cytochrome P450 (CPY) 2C8 (Parikh et al., 2007) and DEAQ is known to be responsible for the observed antimalarial activity (Churchill et al., 1985). The mechanism of drug action of AQ is similar to that of CQ (Gil, 2008; Famin & Ginsburg, 2002). AQ also inhibits the glutathione-dependent destruction of ferriprotoporphyrin IX (FP-IX) in Plasmodium, which results in high levels of membrane FP-IX that irreversibly disturbs the ion homeostasis of the parasite and cause parasite death (Famin et al., 1999). The long-term use of AQ had rare but serious adverse effects of hepatitis and agranulocytosis, which resulted in the withdrawal of this drug from clinical use (Gil, 2008; Winstanley et al., 1990; Rhodes et al., 1986). However, the AQ was reintroduced in combination with artesunate, as one of the ACT along with four more combinations recommended by WHO for malaria control programmes (WHOa, 2010). Several drug efficacy studies have shown that Artesunate-AQ combination are effective in some regions (Nosten and White, 2007; Anvikar et al., 2012; Coulibaly et al., 2014).

1.6.2.3 Primaquine
Primaquine (PQ), is an antimalarial drug of the class 8-aminoquinoline. PQ is the only transmission-blocking drug generally available and in clinical use that shows a potent gametocytocidal activity against all the species of Plasmodium that infects human, including multidrug resistant P. falciparum strains. PQ is also a very effective antimalarial against all exoerythrocytic forms of the Plasmodium including hypnozoites (dormant forms in vivax and ovale malaria) and used as prophylactic and radical curative drug together with other antimalarials in P. vivax and P. ovale treatment (Tekwani and Walker, 2006; Vale et al., 2009; Recht et al., 2014). The prise mechanism of action of PQ is still unresolved but, PQ is thought to interfere with the electron transport chain in the parasite. PQ treated P. falciparum gametocytes shows enlarged mitochondria as a result of damage in the internal mitochondrial
structure (Lanners, 1991) and also cells treated with PQ inhibits the generation of functional transport vesicles (Hiebsch et al., 1991) suggesting the possible mode of drug action. Recent studies have shown that the principal metabolite of PQ, the Carboxyprimaquine (Mjhaly et al., 1984) is mediated by enzymes Cytochrome P450 (CYP 2D6) and monoamine oxidase A (MAO-A) (Pybus et al., 2013, 2012). In spite of its unique therapeutic effect as antimalarial, PQ induces hemolysis among people with reduced defences against oxidant compounds mainly glucose-6-phosphate dehydrogenase (G6PD) deficiency (X-linked genetic defect) which poses a significant sole obstacle which negates the use of PQ among populations with G6PD deficiency that are relatively common in malaria-endemic countries (Recht et al., 2014).

1.6.3 Aryl amino alcohol

1.6.3.1 Quinine

Quinine (QN) is a chemical compound which naturally occurs in the bark of the cinchona tree (quinquina) and was used to treat all sorts of fevers in the early 16th and 17th century. QN is the first successful chemical compound used to treat an infectious disease, malaria and an important antimalarial still in use even after 400 years after its first documentation (Meshnick, 1997; Achan et al., 2011). QN was first isolated in 1820 and belongs to the aryl amino alcohol group of drugs. QN remains the only therapeautic option for the treatment of severe malaria due its availability as intravenous antimalarial preparation (Achan et al., 2011; Pasvol, 2005). QN rapidly destroys the intraerythrocytic stage of malaria parasite having a potent schizonticidal activity. The QN has no activity against the Exo-erythrocytic forms of malaria parasites in the liver but, it has gametocytocidal activity against P. vivax and P. malariae, however, it is inactive against P. falciparum gametocytes (Achan et al., 2011; Peatey et al., 2012). The mechanism of action of QN is still not deciphered however, like CQ it is thought to interfere with the polymerization of heme into hemozoin (Slater and Cerami, 1992; Egan et al., 1994). A recent study has identified the enzyme involved in metabolism of quinine as CYP 3A4 for 3-hydroxyquinine and 2'-oxoquininone and CYP 2D6 for O-desmethylquinine (Marcssisin et al., 2013). QN resistance is usually low grade characterized by delayed drug action, but, there is no convincing evidence of high grade QN resistance reported so far in the treatment of severe malaria (Achan et al., 2011; Aminake and Pradel, 2013). QN is relatively a toxic drug which is associated with several adverse events followed by treatment, particularly in patients with severe malaria where it induces hyperinsulinemic hypoglycaemia and hemoglobinuria, commonly known as Blackwater fever (BWF) (Day and
Dondorp, 2007; Marcsisin et al., 2013; Lon et al., 2014). As per the World Health organization (WHO) 2010 guideline for treatment of malaria, QN is recommended to be given in combination with doxycycline, tetracycline or clindamycin as second line treatment in the case of the treatment failure with the first line drug for uncomplicated malaria and QN in combination with clindamycin for the treatment of malaria during early pregnancy (first trimester) (WHOa, 2010).

1.6.3.2 Mefloquine

Mefloquine (MQ) is an aryl amino alcohol group of drug, which is used for the prophylaxis and treatment of CQ resistant malaria and also as malaria chemoprophylaxis among non-immune travellers for several decades (Croft and Herxheimer, 2002; Janowsky et al., 2014; González et al., 2014). MQ first became available in 1985 as Lariam for the chemoprophylaxis of malaria by European travellers (Croft and Garner, 1997). MQ is an orally administered blood schizontocidal drug having a relatively long half-life (Palmer et al., 1993; Desjardins et al., 1979). MQ is effective against the intraerythrocytic stages of all Plasmodium spp. that infect humans, including P. knowlesi but does not kill the gametocytes of P. falciparum and has no activity against the Exo-erythrocytic stages of P. vivax and P. ovale (Schlagenhauf et al., 2010; Lelie`vre et al., 2012; Warrell et al., 2002). So, treating vivax malaria with MQ should be followed by a course of PQ (which acts on sexual and liver stage forms) to reduce the frequency of relapse as well as to block the continued transmission (Vinetz, 2006; Baird, 2009). Like many other antimalarial drugs, the mechanism of action of MQ is also unresolved, however, studies have shown MQ inhibits the accumulation of hemozoin in the infected cells (Sullivan et al., 1998; Zhang et al., 1999) suggesting the similarity in the mode of action like 4-aminoquinoline by interfering with polymerization with heme into hemozoin (Slater and Cerami, 1992). Further, MQ is also shown to bind with phospholipids with good affinity suggesting the possible interaction between MQ and phosphatidylinositol that are found enriched in malaria parasites (Chevli and Fitch, 1982). Yet another study showed that the volume-regulated anion channel (VRAC) a transmembrane pathway which is used by the parasite for influx necessary substrates and efflux of toxic compounds is affected by MQ (Maertens et al., 2000) suggesting that VRAC pathway may be the MQ target. The resistances towards MQ is associated with increased expression and multi drug resistance (MDR) gene that is reflected in reduced Cytosolic concentration of (Wilson et al., 1993; Price et al., 1999). Several adverse effects have been associated with the use of MQ among individuals who have experienced neuropsychiatric reactions, gastrointestinal
disturbances, nervousness, fatigue, sleeping (Minei-Rachmilewitz, 1999; Croft and Herxheimer, 2002; Peterson et al., 2011). The MQ is given in combination with artesunate, as one of the ACT along with four more ACT combinations recommended by WHO for malaria control programmes (WHOa, 2010).

1.6.3.3 Lumefantrine
Lumefantrine (LM) also known as Benflumetol is an aryl amino alcohol group of drug which is commonly used for the treatment of uncomplicated malaria (Schlitzer, 2007). LM was developed in 1970s by Academy of Military Medical Science, in Beijing and registered as an antimalarial drug in china. LM is now commercially available as a fixed dose combination with artemether with the name Riamet® and Coartem®. Riamet® is used as a standby treatment among travellers in non-endemic countries for malaria whilst Coartem® is used in malarial endemic countries for the treatment of uncomplicated falciparum malaria (Olliaro and Taylor, 2003). This ACT combination Artemether-lumefantrine (AL) attains its antimalarial effect of sequential actions initially by rapid reduction of parasite burden by artemether which is responsible for clinical parasitological response followed by the action of LM which finally eliminates the remaining viable parasites by its rather slow action helping in radical cure. The overall cure depends on the removal of residual parasites which in turn depends on the bioavailability of LM (Ezzet et al., 2000; Wernsdorfer, 2004). The mechanism of action of LM is unknown, but it is thought to accumulate in the food vacuole of the parasite and bind to heme forming toxic complex (Warrell et al., 2002; Combrinck et al., 2013). Recent studies have shown that exposure to LM triggers eryptosis of infected erythrocytes and over-representation of genes associated with fatty acid metabolism, suggesting that LM may interfere with membrane phospholipids to exert its antimalarial action (Alzoubi et al., 2014; Mwai et al., 2012). The metabolism of LM is mediated by CYP 3A4 to desbutyl-lumefantrine that exerts blood schizontocidal activity against falciparum and vivax malaria (Wong et al., 2011; Byakika-Kibwika et al., 2011; Verbeken et al., 2011). Resistances to LM and in combination drug AL have been linked to multiple copies of Pfmdr1 gene with mutations have shown to modulate the therapeutic efficacy of LM (Price et al., 2006; Sisowath et al., 2005; Hastings and Ward, 2005; Sisowath et al., 2007). Treatment with LM seems to be well tolerated by individuals and some of the reported side effects LM are difficult to distinguish from that of the symptoms of acute malarial which include headache, mild nausea, abdominal discomfort and dizziness. The LM is given in combination
with artemether, as one of the ACT along with four more ACT combinations recommended by WHO for malaria control programmes (WHOa, 2010).

1.6.4 Antifolate drugs

1.6.4.1 Sulfadoxine/pyrimethamine

Sulfadoxine/pyrimethamine (SP) is a blood schizonticidal combination drug marketed under the trade name Fansidar®. SP is a fixed dose combination of 20 parts of sulfadoxine with 1 part of pyrimethamine that may be administered orally or by the intravenous route. As a replacement drug for CQ, SP has become one of the most widely used antimalarial drug globally since it has an advantage as a single dose in the form of a tablet which is well tolerated, and highly efficacious as an ideal first line drug for the treatment of uncomplicated malaria (Hyde, 2002; WHOa, 2010; Barnes et al., 2006; Bacon et al., 2009). Both sulfadoxine and pyrimethamine are antifolate drugs that interfere with the synthesis of thymidylate in the malaria parasite. Sulfadoxine competes with para-aminobenzoic acid (PABA) to bind with dihydropteroate synthase (dhrs) in the synthesis of folic acid (Hyde, 2002; WHOa, 2010). Pyrimethamine inhibits the action of dihydrofolate reductase (dhfr) which is necessary for folic acid synthesis, thereby indirectly blocking the nucleic acid synthesis in malaria parasite (Black et al., 1986; WHOa, 2010). A combination of both the drugs leads to synergistic action, making SP more efficacious than the monotherapy drugs (Chulay et al., 1984; Bacon et al., 2009). The widespread use of the SP has led to the rapid emergence and spread of SP resistant parasite in areas where it has been deployed limiting its effectiveness (Barnes et al., 2006; Bacon et al., 2009). The mutations within dhfr with changes in amino acid at four positions, namely Ser-436, Gly-437, Ala-581, Ala-613 confers the resistance to sulfadoxine (Triglia and Cowman, 1994; Brooks et al., 1994; Triglia et al., 1997; 1998) whereas, pyrimethamine resistance results from the accumulation of point mutations in dhfr which first commences with the codon 108N followed by 50R, 51I, 59R and 164L (Cowman et al., 1988; Peterson et al., 1988; Zolg et al., 1989; Gebru-Woldearegai et al., 2005). The SP is given in combination with artesunate, as one of the ACT along with four more ACT combinations recommended by WHO for malaria control programmes (WHOa, 2010).
1.6.4.2 Proguanil and Chlorproguanil

Proguanil and Chlorproguanil are both biguanide compounds that are metabolised in the body via the polymorphic cytochrome P450 enzyme CYP2C19 to their active metabolites, cycloguanil and chlorcycloguanil. Both the cycloguanil and chlorcycloguanil competitively inhibits *Plasmodium* DHFR, an enzyme that regenerates folate cofactors, which are essential for 2-carbon transfer reactions and the synthesis of parasite nucleic acids (Warrell *et al.*, 2002; Wright *et al.*, 1995; Nduati *et al.*, 2005; Canfield *et al.*, 1995) thereby effecting the developmental stages of malaria parasites. Though used as chemotherapeutic drug, both proguanil and chlorproguanil have been used as casual prophylactics and also as sporontocidal drug that makes the gametocytes non-infective to the mosquito vector (Warrell *et al.*, 2002; WHOa, 2010). Proguanil is generally not used as a monotherapeutic drug as the resistance develops very quickly so, proguanil is given in combination with atovaquone. Chlorproguanil is used in combination with Dapsone mainly as a replacement for the SP. Since both chlorproguanil and Dapsone are rapidly eliminated from the body, thereby exerts low selection pressure for development of resistance (WHOa, 2010; Curtis *et al.*, 2002). Some of the adverse effects associated with these drugs include mild to moderate gastric intolerance, nausea, mouth ulceration, hair loss and megalobastic anaemia (Warrell *et al.*, 2002; WHOa, 2010). Chlorproguanil-Dapsone (LapDap) has been approved for general use by United Kingdom Medicine and Healthcare products, regulatory Agency and has been launched in Kenya, Ghana, Nigeria, Zambia and Cameroon (Bukirwa *et al.*, 2004) whereas proguanil-atovaquone (Malarone) is recommended for use by travellers returning from malarious region to non-endemic countries (WHOa, 2010).

1.6.5 Atovaquone

Atovaquone is a hydroxynapthoquinone antimalarial drug used as a fixed-dose combination together with proguanil (Malarone) for treating uncomplicated malaria infections among children and adults and or also used as chemoprophylaxis for preventing malaria among travellers (Nixon *et al.*, 2013a; WHOa, 2010). Atovaquone is active against all *Plasmodium* species and targets the developmental stages of parasite in the liver and on sexual stage in the mosquito in addition to asexual blood stages (Delves *et al.*, 2012). Atovaquone interferes with cytochromes of mitochondrial electron transport and disintegrates the membrane potential of mitochondria. Atovaquone is a competitive inhibitor of ubiquinone (Coenzyme Q), by specifically inhibiting *Plasmodium* mitochondrial bc₁ activity that reflects in the disintegration of transmembrane electrochemical potential, resulting in loss of mitochondrial
function (Fry et al., 1992; Srivastava et al., 1997; Warrell et al., 2002; Biagini et al., 2006; Birth et al., 2014). The major role of mitochondria in malaria parasite is to provide the orotate for pyrimidine biosynthesis via the activity of dihydroorotate dehydrogenase (DHOH). The inhibition of cytochrome bc\textsubscript{1} complex by atovaquone drastically reduces the level of metabolites in the pyrimidine biosynthesis of the parasite, meanwhile cycloguanil an active metabolite of proguanil acts synergistically by inhibiting Plasmodium DHFR in pyrimidine biosynthesis thereby suggesting that the atovaquone-proguanil combination would act as a site specific uncoupler of parasite mitochondria in a selective manner (Warrell et al., 2002; Fisher et al., 2012; WHOa, 2010; Birth et al., 2014; Srivastava and Vaidya, 1999). The monotherapy using atovaquone leads to rapid emergence of resistance caused by mutations in cytochrome bc\textsubscript{1} complex (Vaidya and Mather, 2000; Birth et al., 2014). Atovaquone is generally a well-tolerated drug. Some of the adverse effects associated with atovaquone usage are headache, skin rashes, nausea and vomiting, increased levels of liver enzymes and rarely leads to haematological disturbance. Atovaquone-proguanil is recommended for use by travellers returning from malarious region to non-endemic countries (WHOa, 2010).

1.6.6 Antibiotics used for treating malaria

The use of some selected antibiotics for treatment of malaria was recognized only in the mid of last century. The presence of two important organelles in Plasmodium namely the mitochondria and the apicoplast that are involved in important metabolic functions of heme and fatty acid biosynthesis that makes them indispensable for parasite survival and offers as a potential target for the use of antibiotics in treating malaria. (Pradel and Schlitzer, 2010; Kiatfuengfoo et al., 1989). Some of the selected antibiotics used for the treatment of malaria as per the recommendation of WHO are tetracycline, doxycycline and clindamycin (WHOa, 2010). The tetracyclines are basically derived from certain Streptomyces species and are used as the synthetic form for treatment. The antimalarial activity of tetracycline was first recognized in 1949. Tetracyclines are slow acting antimalarial with a very short half-life and are effective when used in combination with a more rapidly acting curative drug and also used as a chemoprophylaxis drug for malaria treatment (Warrell et al., 2002; WHOa, 2010; Briolant et al., 2008). Tetracycline are inhibitors that prevents the binding of aminoacyl transfer RNA during protein synthesis, however knowledge of its mode of action in Plasmodium remains uncertain. It has been shown that Plasmodium exposed to tetracycline give rise to progeny of parasite that lacks functional apicoplast thereby exerting its slow, albeit potent antimalarial activity (WHOa, 2010; Briolant et al., 2008; Dahl et al., 2006).
Tetracyclines are contraindicated in pregnant women and in children less than eight years of age because of the risk of interference with the development of bone and teeth and the discoloration of teeth. High doses of tetracycline can damage the liver and kidney. Common side-effects are gastrointestinal symptoms, notably nausea, vomiting and diarrhoea, dry mouth, stomatitis, oesophageal ulceration and itching of the skin have also been reported (Warrell et al., 2002; WHOa, 2010). The tetracycline is used in combination with arteusnate or quinine as second-line treatment recommended by WHO for the malaria control programme (WHOa, 2010).

Another antibiotic that is preferred over tetracycline is its synthetic derivative doxycycline mainly because of its longer half-life, partially efficacious as causal prophylactic drug active against liver stage development of Plasmodium and a slow acting blood stage antimalarial that when used in combination with a rapid acting schizontocidal drug can be used to treat malaria (WHOa, 2010; Tan et al., 2011). The mechanisms of action of doxycycline are not clearly defined, however, studies have shown that exposure to doxycycline affect the mitochondria and apicoplast of Plasmodium thereby inhibiting the protein synthesis and follows the similar mode of action as tetracycline (Briolant et al., 2010; Tan et al., 2011). Compared to the toxicity of tetracycline, doxycycline displays only a few gastrointestinal effects, however if sufficient water is not taken with tablet or capsule will result in oesophageal ulceration. Doxycycline should not be given to children up to eight years old and particularly among pregnant and lactating women. The doxycycline is used in combination with Arteusnate or Quinine as second-line treatment and quinine plus doxycycline for traveller from malarious areas, retuning to non-endemic countries as recommended by WHO for the malaria control programme (WHOa, 2010).

Next, yet another antibiotic used for the management of uncomplicated malaria is a lincosamide antibiotic known as Clindamycin which has similar properties of antimalarial activity and possible mode of drug action like other antibiotics in use for malaria therapy (WHOa, 2010; Obonya and Juma, 2012). The major advantage when compared to tetracycline and doxycycline is, clindamycin in combination with a rapid acting schizontocidal drug quinine, can safely be administered to both children’s and pregnant women, irrespective of the trimester during pregnancy, making Quinine-Clindamycin combination as extremely useful antimalarial combination for management of malaria among the target population of childerns and pregnant women (Lell and Kremsner, 2002; Obonya.
and Juma, 2012, WHOa, 2010). Clindamycin produces gastrointestinal symptoms, notably diarrhoea, in 2-20 percent of patients, vomiting, abdominal pain and unpleasant taste in the mouth. *Clostridium difficile* toxin pseudomembranous colitis is a particular problem, come in women and in elderly patients. Toxicity of the quinine-clindamycin combination is reduced if the drugs are given sequentially rather than together (Warrell et al., 2002; WHOa, 2010). The clindamycin is used in combination with artesunate or quinine as second line treatment for uncomplicated malaria and or for treatment of malaria among pregnant women in first, second and third trimester and also quinine-clindamycin combination drug for traveller’s from malarious areas, retuning to non-endemic countries as recommended by WHO for the malaria control programme (WHOa, 2010).

### 1.7 Malaria Vaccine

The malaria parasites are the one among the most biologically sophisticated and wilier organisms afflicting humans for many centuries, which the mankind still remains unsuccessful in conquering the full control over their widespread prevalence causing significant morbidity and mortality. Although malaria can be diagnosed and treated, the combat against emerging drug resistances towards first-line antimalarial drugs (Ashley et al., 2014; WHO, 2010b) has increased the urgency to develop a vaccine against malaria. Despite decades of research, the goal of an effective malaria vaccine has remained elusive due to the complex nature of malaria parasites that rapidly evolve by quickly adapting to its ever-changing environment and effectively evading host immune response (Thera and Plowe, 2012; Hoffman and Epstein, 2002; Barry et al., 2012). Several problems such as multistage life cycle with stage-specific expression of proteins with high degree of genetic diversity of parasite surface antigens and the allelic-specificity of the immune response with limited understanding on the mechanisms of naturally acquired immunity to malaria have hampered the progress towards developing a long lasting and broadly efficacious malaria vaccine (Hoffman and Epstein, 2002; Takala and Plowe, 2009; Flück et al., 2004; Crompton et al., 2010; Barry and Arnott, 2014). With the array of polymorphic antigens expressed at various developmental stages of *Plasmodium* life cycle indicates that, an effective vaccine may have to be comprised of several target antigens expressed at various stages in its life cycle. So, the malaria vaccine currently developed targets one of the three stages, i.e., the pre-erythrocytic, the erythrocytic or the transmissible stages of the life cycle (Birkett et al., 2013; Schwartz et al., 2012; Targett et al., 2013).
1.7.1 Pre-erythrocytic vaccines (Liver-stage Vaccine)

During the last decade, considerable progress has been made towards the development of pre-erythrocytic vaccines and new approaches are in the pipeline at various phases of clinical trials (reviewed by Schwartz et al., 2012; Targett et al., 2013; Barry and Arnott, 2014). Studies have shown that individuals born in endemic areas with prior exposure to infection gradually develop resistant initially to severe infection and then to clinical manifestation of malaria by harbouring parasites in a low density, but are known to lacks immunological memory, however subsequent re-exposures are necessary to maintain this level of acquired immunity towards infection (Cohen et al., 1961; Bouharoun-Tayoun et al., 1990; Struik and Riley., 2004). This indicates that protection from malaria via a vaccine would be possible and that will be short-lived and may require several boosters. But, one drawback is, the vaccines directed against the erythrocytic stage has a very limited window of time to act when compared to liver stage infection. Further, the number of parasitized host cells is low in liver stage compared to blood stage and also been clinically silent stage offers a relatively large time window for an effective immune response against the parasites (Crompton et al., 2010; Vaughan et al., 2010; Takala and Plowe, 2009) making the pre-erythrocytic stage a better vaccine target. The pre-erythrocytic stage vaccines are designed to prevent malaria infection in the human host. The liver stage vaccine is mainly composed of specific antigens of sporozoites or of whole cell preparations of sporozoites and or merozoite stages that represent pre-erythrocytic vaccine candidates targeting the invasive sporozoites stage by means of neutralising antibodies to block the invasion of hepatocytes or by activating cell mediated immune response via Interferon gamma (IFNγ) secreting effector T-cells, CD8+ and CD4+ cytotoxic T cells or natural killer (NK) T cells after the successful invasion of the hepatocytes. This approach is expected to either prevent liver stage infection or block the progression towards blood stage infection and finally disease transmission (reviewed by Riley and Stewart, 2013; Girard et al., 2007).

One of the key antigen highly expressed on the surface of sporozoites as it migrates to the liver and are involved in the sporozoites adhesion and invasion of the hepatocyte is known as CSP which is known to trigger anti-CSP antibody response that are shown to be associated with a reduced risk of clinical malaria (Coppi et al., 2011; Cohen et al., 2010; Zavala et al., 1985; Nussenzweig and Nussenzweig, 1989; John et al., 2008). Additionally, CSP has been determined to be important for the transfer of sterile immunity upon infection of genetic or radiation attenuated sporozoites (Kumar et al., 2009), making it as a lead pre-erythrocytic
vaccine candidate. The efficacy induced by a CSP specific vaccine against malaria morbidity is stronger than other antigens (Schwartz et al., 2012 that paved the way for development of CS based subunit malaria vaccines RTS, S. The RTS,S consists of a recombinant protein that fuses a part of the repeat region of CSP with hepatitis B virus surface antigen and expressed as virus-like particle, combined with an Adjuvant systems (AS) AS01 or AS02 that are shown to induce both humoral and cellular immune response through their immunostimulants namely monophosphoryl lipid A molecule (MPL), a chemically detoxified form of parent lipopolysaccharides (LPS) from Salmonella minnesota and QS21, a natural saponin molecule purified from the bark of Quillaja saponaria (Gordon et al., 1995; Vaughan and Kappe, 2012; Vekemans et al., 2009; Regules et al., 2011). With no licenced vaccine available currently for malaria, RTS, S is the most advanced subunit vaccine for malaria and is the first and only vaccine to undergo phase III clinical evaluation (Schwartz et al., 2012; Barry and Arnott, 2014) and has shown initially around 30-50% clinical protection (Bejon et al., 2008; Sacarlal et al., 2009; Agnandji et al., 2011; Olotu et al., 2011) however, over time with increasing exposure to malaria, the efficacy of RTS, S declines making it as only a partially efficacious malaria vaccine (The RTS, S Clinical Trials Partnership (2014), 2014; Olotu et al., 2013; Barry and Arnott, 2014).

Potent cell mediated immunity to subunit vaccination has been difficult to achieve, but recently a different strategy for vaccination utilizing virally vectored platforms and a prime boost vaccination approach has produced promising results (Limbach and Richie, 2009). Clinical evaluation of non-replicating adenovirus 35 vector encoding the CS protein (Ad35 CS) has shown to be modestly immunogenic among the study population, further clinical evaluation of Ad35 CS in prime boost with RTS, S AS01E is ongoing at various phases (Ouédraogo et al., 2013; Schwartz et al., 2012). Another potential liver stage vaccine candidate is TRAP which triggers the host immune response similar to that of CSP and mediate immunity by CD8+ T cells against Plasmodium liver stage infection (John et al., 2005; Hafalla et al., 2013). To elicit a stronger antibody and T cell response, TRAP has been combined with multiple epitopes (ME), resulting as a ME-TRAP construct, that has been tested in combination with several different vectors for delivery and expression, namely fowl pox vector, DNA vector, modified vaccinia Ankara (MVA) and chimpanzee adenovirus vector 63 (ChAd63). Since these constructs have presented with an insufficient T cell response among individual in malaria endemic regions, a prime-boost immunization approach was further evaluated for vectors MVA ME-TRAP and ChAd63 ME-TRAP and
was shown to induce high level of T cell responses (Schwartz et al., 2012; Vaughan and Stefan, 2012; Ogwang et al., 2013). Additionally, the other vaccines under clinical evaluation includes, a pre-erythrocytic DNA vaccine known as Polyepitope DNA EP1300 that consists multiple epitopes with linker sequences from four different liver stage antigens namely sporozoites surface protein 2 (SSP2)/ TRAP, CSP, Liver-stage antigen 1 (LSA-1) and exported protein 1 (Exp1) and PfCelTos FMP012 being a potentially interesting vaccine candidate. Due to various limitations, research on developing a vaccine against \textit{P. vivax} lags considerably when compared to \textit{P. falciparum} however, \textit{P. vivax} CSP (PvCSP) and \textit{P. vivax} Duffy binding protein (PvDBP) have undergone Pre-clinical studies of which PvCSP with the adjuvant system AS01B known as VMPOO1/AS01B in under clinical trial (reviewed by Schwartz et al., 2012; Arama and Troye-Blomberg, 2014; Barry and Arnott, 2014; Arévalo-Herrera et al., 2010; García-Basteiro et al., 2012).

1.7.2 Attenuated sporozoites: a promising whole organisms approach

The use of sporozoites based whole cell vaccine approach was first demonstrated more than a century ago in 1910 using inactivated sporozoites in avian malaria that ensue studies in 1941-42 and 1967-69 showing immunization with irradiated sporozoites could prevent malaria infection by immunological protection (Sergent and Sergent, 1914; Mulligan et al., 1941; Russell and Mohan, 1942; Nussenzweig and Vanderberg, 1967; 1969). These landmark findings has now revisited after several decades when human volunteers were immunized by exposure to around 1000 mosquitoes harbouring radiation attenuated \textit{P. falciparum} sporozoites confer protection from subsequent challenge (Hoffman et al., 2002) proving it as a promising approach that has led to the development of metabolically active, non-replicating \textit{P. falciparum} sporozoites vaccine (PfSPZ) showing around 90% protection achieved among human volunteers only when relative sufficient numbers of immunization done by adequate number of PfSPZ infected mosquitoes (Hoffman et al., 2010). In spite of its potential, some of the consequences such as the fate of under-radiated sporozoites that may complete liver stage development and move on to establish blood stage infection and (or) over-radiation will inactivate the sporozoites that may ultimately fail to invade the hepatocytes resulting in poor protection against malaria infection (Silvie et al., 2002) has to be studied in great detail. Presently, PfSPZ is under clinical evaluation to assess its safety, immunogenicity and the route of administration in naïve volunteers (Epstein et al., 2011; Targett et al., 2013). Further, the use of chemically attenuated sporozoites (Purcell et al., 2008a; 2008b) and use of prophylactic chloroquine treatment along with whole sporozoites immunization (Belnoue
al., 2004; 2008; Roestenberg et al., 2009) has also been attempted and evaluated, however some of the drawbacks namely the random DNA breaks within the genome induced by radiation or chemical treatment would result in the generation of a highly heterogenous population of sporozoites and issues pertaining to correct doses required for both irradiation and chemical/drug treatment to ensure efficient attenuation without killing the parasites (Annoura et al., 2012) negates the full implementation of these approaches.

1.7.3 Genetically attenuated sporozoites

The Genetically attenuated Parasite (GAP) is a novel immunization approach that does not rely on the external attenuation of sporozoites or the metabolic killing of parasites, but, are generated through the targeted gene deletion resulting in live sporozoites forms of homogenous mutation population with defined genetic identity and attenuation phenotype that can be designed to induce optimal protective immunity making them superior and advantageous over irradiated and chemically attenuated sporozoites immunization (Khan et al., 2012; Annoura et al., 2012; Arama and Troye-Blomberg, 2014). It has been shown that the GAP, when deleted with specific genes the mutant parasite gets arrested specifically in the liver stage (Khan et al., 2012; Vaughan and Kappe, 2013). A considerable number of GAP with specific gene deletions have been described, namely highly up-regulated in Infectious sporozoites 3 (UIS3) known to be essential for liver stage development showed complete arrest upon deletion of UIS3 and UIS4 (Mueller et al., 2005a; 2005b). Further, the other genes that are known to be vital for liver stage development and are known to get arrested at the late liver stage upon gene deletion includes P52 gene encoding a putative GPI anchored protein and membrane of 6-Cys-protein super family, P36 gene encoding a putative secreted protein (van Dijk et al., 2005; Ishino et al., 2005), Sporozoite and Liver stage Asparagine-Rich Protein (SLARP) a specific regulator of gene expressions involved in the replication process of liver stage (Silvie et al., 2008) and Sporozoites Asparagine-rich Protein 1 (SAP1) involved in the post transcriptional regulation of sporozoites and early liver stage development (Aly et al., 2008; 2011).

Yet another gene known to be critical for liver stage development is Fab B/F, an enzyme involved in type II fatty acid synthesis and gene disruption of the same lead to parasite arrests in the late liver stage (Vaughan et al., 2009). Immunization experiments in mice with rodent malaria GAP’s using UIS3⁺, UIS4⁺ and P52⁻ parasites induces long-lasting protection against subsequent wild type sporozoite challenge (Mueller et al., 2005a; 2005b; Tarun et al.,
known to be mediated by CD8+ T cell responses (Tarun et al., Jobe et al., Mueller et al., 2007; Douradinha et al., 2011). Clinical evaluation of *P. falciparum* GAP (PfGAP) that harbour individual or simultaneous deletions of P52 and P36 genes (*P. falciparum* orthologues of *P. yoelii* P52 and P36) marks the first in-human, proof-of-concept trial to assess its safety and immunogenicity (vanBuskirk et al., 2009; Spring et al., 2013) and has shown to be efficiently blocking the hepatocyte invasion by sporozoites (Finney et al., 2014). Despite of this unambiguous progress, various setbacks in our present knowledge and available resource towards developing a long-lasting efficacious malaria vaccine necessitates a continuous quest to identify new vaccine candidate that may serve as superior or as a replacement and or backup. In this thesis, we have also focused on studying the gene involved in de novo heme biosynthetic pathway of *Plasmodium* for its potential as a genetically attenuated sporozoites vaccine candidate.

1.7.4 *Erythrocytic stage vaccine (Blood stage vaccine)*

The erythrocytic stage vaccines are designed primarily to target the asexual stage parasites and are aimed to prevent invasion and multiplication of merozoites in the blood cycles thereby reducing the overall parasite burden. Since all the clinical manifestations associated with malaria infection occur during this stage, most of the *Plasmodium* antigens expressed during the blood stage are targeted as vaccine candidate that can prevent the malaria disease. However, the presence of the highly polymorphic gene in *Plasmodium* blood stage parasites may reduce efficacy of blood stage vaccine and also promote adaptive strategies by the parasite to survive, resulting into potentially more pathogenic forms than the existing ones (Moorthy et al., 2004; Barry and Arnott, 2014; Riley and Stewart, 2013). Presently, several blood stage vaccine candidates are under clinical evaluation, namely apical membrane antigen 1 (AMA1), merozoite surface protein-1 (MSP1), MSP3, erythrocyte-binding antigen-175 (EBA175), Glutamate-rich protein (GLURP), serine repeat antigen 5 (SERA5) expressed in *E.coli* known as SE36 and SR11.1 and antigen corresponding to a unique subregion of *Pf*11.1 megaprotein. Due to the high polymorphic nature of the MSP1 and AMA1, considerable efforts have been made to enhance their vaccine efficacy using viral vector prime-boost approach along with novel adjuvants such as AdCh63/MVA MSP1 and AdCh63 AMA1/MVA AMA1.

Further, FMP010/AS01B consists of *E.coli* expressed MSP1 (based on 3D7 allele of MSP1) together with the adjuvant with AS01B, FMP2.1/AS02A consists of *E.coli* expressed AMA1
(3D7 Strain) with the adjuvant AS02A and FMP2.1/AS01B consisting the same AMA1 antigen (3D7 strain) expressed in E. coli but adjuvanted with AS01B. The use of recombination fusion protein of GLURP and MSP3 expressed in L. lactis known as GMZ2 with the adjuvant aluminium hydroxide is under clinical evaluation (Ambrosino et al., 2010; reviewed by Schwartz et al., 2012; Arama and Troye-Blomberg, 2014; Barry and Arnott, 2014). Some of the other blood stage vaccine candidates, include AMA1 (3D7 strain) expressed in Pichia pastoris termed as AMA-C1 with a combination of adjuvants Alhydrogel plus CpG 7909, a toll like receptor 9 agonist triggers potentially more antibody response. BSAM-2/Alhydrogel plus CPG is another blood stage vaccine that consists of the combination of AMA1 expressed in P. pastoris and MSP1 expressed in E. coli with adjuvant combinations Alhydrogel plus CPG whereas JAIVAC made of MSP1 and EBA175 both expressed in E.coli is evaluated with the adjuvant Montanide ISA 720 (Philippe et al., 2012).

1.7.5 Sexual stage vaccine (Transmission-blocking vaccine)

The sexual stage vaccine are aimed to target the precursor sexual stage, the gametocytes in the human host through the further sexual stages development in the mosquito by inducing the antibody response, thereby either effecting the progression of gametes towards fertilization and subsequently prevention of parasite development in the mosquito midgut or by blocking the sporogony thereby preventing the development of infectious sporozoites that are constitutively termed as transmission-blocking vaccines (TBVs). TBVs are meant to reduce the overall malaria burden on entire or selected populations in a given specific geographic region, but not among the individuals who are vaccinated (Carter et al., 2000; Girard et al., 2007; Barry and Arnott, 2014; Arama and Troye-Blomberg, 2014) which has a tremendous potential that may be utilized by policy makers in malaria control and eradication program (Vannice et al., 2012; Targett et al., 2013). Although the logistic challenges make it extremely difficult to conduct large population based randomized proof-of-principle efficacy trials, the standard membrane feeding assay would become a valuable tool that enables the evaluation of transmission blocking interventions (Schwartz et al., 2012; Bousema et al., 2012; Miura et al., 2013). Some of the leading vaccine candidates under the category TBVs include P. falciparum ookinete surface antigens Pf$s25$ and Pf$s28$ and their homologs in P. vivax Pvs25 and Pvs28, Pfs45/48 and Pfs230 are major gametocyte and gamete surface antigens of which only two of the vaccine projects based on Pfs25 are under clinical evaluation wherein the recombinant Pfs25 protein is chemically crosslinked to Pseudomonas aeruginosa Exo-protein A (EPA) and delivered as an nanoparticle Pfs25-EPA that enhances
the immunogenicity. *Pfs*25 is also conjugated with a Virus Like Particles (VLP) known as *Pfs*25-VLP is presently under clinical evaluation (Barry and Arnott, 2014; Arama and Troye-Blomberg, 2014; Hisaeda et al., 2002; Ouédraogo et al., 2011; Schwartz et al., 2012; Shimp et al., 2013).

1.8 Metabolic pathways as drug target in *Plasmodium*

Over the past two decades, considerable progress has been made in our understanding of malaria parasite biochemistry that has allowed the identification of many potential targets for new drugs (Philippe et al., 2012). This was made possible by the landmark exploration projects through the genomics, proteomics and transcriptomics approaches that enabled the availability of the genomes of several *Plasmodium* species and other Apicomplexans (Gardner, et al., 2002; Carlton et al., 2002; 2008; Pain et al., 2008; Florens, et al., Lasonder et al., 2002; Le Roch et al., 2004; Hall et al., 2005; Kissinger et al., 2003; Abrahamsen et al., 2004; Florent et al., 2010). These databases allowed the whole genome comparison studies, namely the prediction of gene functions, gene polymorphisms and biochemical pathways and metabolism involved in subcellular compartments in *Plasmodium* (Frech and Chen, 2001; Otto et al., 2014; Hall et al., 2005; Ngwa et al., 2013; Prieto et al.; Ganesan et al., 2008; Briolant et al., 2010; Sims and Hyde, 2006; Huthmacher et al., 2010; Tymoshenko et al., 2013). The use of powerful Bioinformatics tools to interpret post-omics data (Aurrecoechea et al., 2009; Bréhélin et al., 2008; Kumar et al., 2014) has given a constitutive lead for rapid identification of putative targets that are homologous to validated targets in other organisms.

In the traditional validation approach called as chemical-validation, a specific inhibitor that is designed or identified as being active against specific target will indeed show antimalarial activity *in vitro* and or *in vivo*, but fails to demonstrate that the inhibitor kills the parasite by truly acting specifically on this target and not by inhibiting unrelated biochemical mechanisms (Philippe et al., 2012). Thus, it is of utmost importance to study in parallel, the essentially of the target gene by the genetic-validation approach wherein the deletion of the corresponding gene must seriously impair the parasite’s growth or ideally prevent its survival. Recent advances in genetic understanding of *Plasmodium* have greatly increased the ability to genetically validate potential drug targets (Limenitakis and Soldati-Favre, 2011; Chan et al., 2013; Qidwai et al., 2014). In this thesis, we have focused on studying the significance and essentiality of genes involved in *de novo* heme biosynthetic pathway in the entire life cycle of *Plasmodium* by generating gene knockouts for parasite encoded enzymes.
1.8.1 Hemoglobin Metabolisms in Plasmodium as drug target

Hemoglobin is a vital source of nutrient for intraerythrocytic stages of malaria parasites. During asexual development within the infected RBC, the *Plasmodium* parasite imports up to 80% of host haemoglobin into the acidic digestive vacuole, where the collective action of multiple aspartic, cysteine and metal-dependent proteases hydrolyzes the globin polypeptide into diverse small peptides and amino acids that are utilized by the parasite for energy metabolism and incorporated into its proteins (Goldberg, 2013; Sigala and Goldberg, 2014; Francis *et al.*, 1997) thus making the haemoglobin metabolisms as central for its survival. The hemoglobin degradation pathway in malaria parasite has been explained under the section (1.5.2.2). The process of haemoglobin catabolism within the digestive vacuole of the malaria parasite liberates vast quantities of potentially cytotoxic heme as a by-product that are neutralized by parasite derived enzymes by polymerization into an inert crystalline pigment, the hemozoin (Sigala and Goldberg, 2014; Jani *et al.*, 2008; Sullivan *et al.*, 1996). Inhibition of hemozoin formation is considered as an attractive target for antimalarial drugs (Pandey *et al.*, 2001; Tekwani and Walker, 2005; Weissbuch and Leiserowitz, 2008). The 4-aminoquinolines such as chloroquine and amodiaquine act by interfering with this polymerization process. Cysteine and aspartic parasite proteases (falcipains and plasmepsins, respectively) involved in the degradation of haemoglobin are also potential targets (Wegscheid-Gerlach *et al.*, 2010; Lindner *et al.*, 2013).

The proteases of digestive vacuole are attractive targets for novel antimalarial drugs and have been the subject of intense investigations for the last decades (Wegsheid-Gerlach *et al.*, 2010). Among the proteases involved in the pathway of haemoglobin degradation, falcipain-2, 2’ and -3 and plasmepsin-I, -II, -III (or HAP, for histo-aspartyl protease) and –IV belongs to cysteine and aspartyl endoproteases and have emerged as highly promising antimalarial targets, with specific inhibitors active with nM concentration levels on native or recombinant enzymes, inhibiting parasite growth in culture from the nM to μM concentration levels and is capable to cure *Plasmodium*-infected mice (Teixeira *et al.*, 2011; McKerrow *et al.*, 2008, Rosenthal, 2010). The malaria parasites treated with protease inhibitors namely pepstatin A, leupeptin and E-64 has shown accumulation of un-degraded globin in the food vacuole (Bailly *et al.*, 1992; Liu *et al.*, 2005; Rosenthal *et al.*, 1988; 1995) thereby effecting the growth of parasite by deprival of amino acids. However, gene disruption studies have clearly shown that all of these enzymes except for falcipain-3 are highly redundant and falcipain-3 is the only enzyme for which gene disruption couldn’t be achieved suggesting its essentiality of
parasite survival in the erythrocytic stage (Omara-Opyene et al., 2004; Liu et al., 2006; Sijwali and Rosenthal, 2004; Sijwali et al., 2006). Some of the other proteases namely cathepsin C-like cysteine protease dipeptidyl aminopeptidase 1 (DPAP1), falcilysin, a metallo-aminopeptidases 1 (PfA-M1) and PfA-M17 working downstream on the peptide produced by globin degradation may represent a better and improved drug target over falcipains and or plasmepsins and has been validated as genetically essential during erythrocytic stages of the parasite (Klemba et al., 2004; McGowan et al., 2009, Skinner-Adams et al., 2009, Trenholme et al., 2010; Tanaka et al., 2013). Other proteases for which essential roles during the parasite asexual development have been demonstrated include Plasmepsin V, involved in maturation of proteins exported to the infected-red blood cell (Boddey et al., 2010, Russo et al., 2010).

### 1.8.2 Apicoplast as drug target in Plasmodium

The apicoplast is a non-photosynthetic, vestigial plastid-like organelle present in most of the Apicomplexan parasites, including *Plasmodium* that are inherited by secondary endosymbiosis from the prokaryotes (Lim and McFadden, 2010; Sato, 2011; Philippe et al., 2012). The apicoplast organelle of *P. falciparum* maintains a 35-kb circular genome and unique metabolic pathways that are vital for the development of liver stage, erythrocytic stages and sporozoite development of *Plasmodium* together with several housekeeping processes distinct from humans, thus providing many attractive targets for drug development (Wilson et al., 1996; Gisselberg et al., 2013; van Schaijk et al., 2014; Lim and Mcfadden, 2010; Sato, 2011). The prominent pathways in the apicoplast include Fatty acid synthesis (FAS), isoprenoid, Iron-sulphur (Fe-S) clusters, DNA transactions and heme biosynthesis, which is a unique pathway is split between apicoplast and the mitochondria as well as in the cytosol (Seeber and Soldati-Favre, 2010; Lim and McFadden, 2010; Qidwai et al., 2014). Of these, the most important metabolic functions that are operated inside the apicoplast are the type II fatty acid synthesis (FAS) pathway which involves 6 distinct enzymes in *Plasmodium* that are both essential and fundamentally different from the cytosolic Type I FAS pathway of the human host (Goodman and McFadden, 2007). Further, the mevalonate independent 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway of isoprenoid synthesis in *Plasmodium* is contrary to humans (Grawert et al., 2011) and the apicoplast genome replication, transcription and translation which involves enzymes that are of bacterial origin (Dahl and Rosenthal, 2008).
The two triose-phosphate transporters present on the apicoplast membrane namely PfiTPT/PfAPT1 and PfоІТPT/PfATP2 have been characterized that are believed to import the crucial metabolites from the cytosol into the apicoplast to fuel the DOXP pathway are considered as putative drug targets (Lim and McFadden, 2010). Some of the main promising antimalarials targeting apicoplast are triclosan that are believed to target the NADH dependent enoyl Acyl Carrier Protein (ACP) reductase or FabI enzyme, thiolactomycin which targets FabH and FabB enzymes and fosmidomycin acting on targeting the DOXP reductoisomerase. Concurrently, antibiotics such as rifampicin and quinolone antibiotics that target the prokaryotic DNA and RNA machinery and tetracycline and clindamycin targeting prokaryotic protein synthesis were shown to inhibit *Plasmodium* growth *in vitro* culture and *in vivo* (reviewed by Sato and Wilson, 2005; Seeber and Soldati-Favre, 2010; Goodman and McFadden, 2013). The uncertainty of outcomes associated with genetic validation of target genes in the type II FAS pathway raises the question on the actual target of triclosan in the blood stage parasites (Yu et al., 2008; Baschong et al., 2011), however the failure to delete the DOXP reductoisomerase gene confirmed the relevance of DOXP non-mevalonate isoprenoid pathway as a potential target for antimalarial drug development (Odom and Van Voorhis, 2009).

1.8.3 Mitochondria as drug target in *Plasmodium*

The mitochondria of *Plasmodium* play a vital role in parasite physiology and are essential in the life cycle stages of the parasite with the presence of non-proton pumping, rotenone-insensitive, alternative type-II NAD(P)H dehydrogenase, which does not have a human counterpart together with several molecular and functional differences between the parasite’s mitochondria and the mitochondria of human cells makes it a unique drug target (Vaidya, 1998; MacRae et al., 2013; van Dooren et al., 2006; Nixon et al., 2013). The mitochondria of *Plasmodium* are closely associated with the apicoplast at all stages of parasite development probably to facilitate metabolite exchange and for the metabolic interactions of the unique heme biosynthetic pathway that is split between mitochondria and the apicoplast as well as in the cytosol (reviewed by Padmanaban et al., 2007; Torrentino-Madamet et al., 2010; Ralph et al., 2004). *Plasmodium* mitochondria have a small genome of 6kb, encoding the genes for cytochrome b, cytochrome oxidase I and III, besides fragment ribosomal RNAs and acquire several nuclear gene-coded proteins through import mechanisms involving Tim-Tom complexes (van Dooren et al., 2006; Feagin et al., 2012; Padmanaban et al., 2007). The *Plasmodium* mitochondrial electron transport chain (ETC) is believed to contain five
dehydrogenases, namely NADH: ubiquinone oxidoreductase (PfNDH2), succinate: ubiquinone oxidoreductase complex II, glycerol-3 phosphate dehydrogenase, the malate quinone oxidoreductase and dihydroorotate dehydrogenase (DHODH). The dehydrogenase activity serves to provide electrons to the downstream complexes, namely Ubiquinol: cytochrome c oxidoreductase (complex III or cytochrome bc1 and cytochrome c oxidase (complex IV) with ubiquinone (coenzyme Q) and cytochrome c functioning as electron carriers between the complexes. The ATP synthase (complex V) is not reported to generate ATP (unlike its mammalian counterpart), but is nevertheless proposed as an essential component, possibly acting as a proton escape for the ETC (reviewed by Mather et al., 2007; Torrentino-Madamet et al., 2010; Nixon et al., 2013).

The inhibition of *P. falciparum* bc1 complex is currently the only component of the ETC with a clinically used antimalarial drug atovaquone associated with it, however the rapid emergence of resistance to atovaquone has resulted in it being used as a combination therapy with proguanil. This has led the drug development programmes to explore the new and low cost inhibitors such as hydroxynapthoquinones, pyridines, acridinediones, acridones and quinolones (ELQ300). Further, Triazolopyrimidine based DHODH inhibitors have been identified as preclinical drug development candidates alongside other classes of inhibitors under development include benzimidazoyl thiophene-2-carboxamides, S-benzyltriazolopyrimidines, biaryl carboxamides, brequinar analogs, N-substituted salicylamides and leflunomide analogs (reviewed by Nixon et al., 2013b). A recent study has shown a unique generation of quinolone lead antimalarial inhibitor known as hydroxyl-2-dodecyl-4-(1H)-quinolone (HDQ) with a dual mechanism of action against two respiratory enzymes, NADH: Ubiquinone oxidoreductase (*Pf*NDH2) and cytochrome bc1 (Biagini et al., 2012) which may provide an advantage in the fight against drug resistance (Nixon et al., 2013b). The parasite mitochondria also have possible roles to play in folate, Fe-S cluster, CoQ and heme biosynthesis and with experimental validation many steps in these pathways could be explored as drug targets.

1.8.4 Kinases as drug target in Plasmodium

The search for new antimalarial drugs has led to explore the novel metabolic pathways that are governed by tightly regulated signal transductions controlled by phosphorylation and de-phosphorylation reactions of numerous proteins by different protein kinases (PK) and phosphatases that are essential for various developmental processes in the entire life cycle
stages of malaria parasites (McNamara et al., 2013; Anamika et al., 2005). A number of *Plasmodium* kinases have been shown to be essential across various stages in the parasite life cycle by reverse genetics approach, thereby genetically validating it as potential targets (Lucet et al., 2012). The malarial parasite, *P. falciparum* has a relatively small kinome of approximately 90 protein kinases (Leroy and Doerig, 2008; Zhang et al., 2012) with members classified into one of the following groups, namely Casein kinase group that includes *Pj*CK1 (Barik et al., 1997), the Cyclin-dependent, mitogen-activated and glycogen-synthase. The cyclin-dependent kinase-like kinases (CMGC) is one of the major groups of parasite kinome that includes five cycline-dependent kinases (CDKs) which are major regulators of cell-cycle progression (Doerig et al., 2002; 2008). Further, two mitogen-activated protein kinases (MAPKs) namely *Pf*map-1 and *Pf*map-2 are important transducers of intra- and extracellular signals to effectors such as cell cycle control element or transcription factors (Lin et al., 1996; Doerig et al., 1996; Graeser et al., 1997; Dorin et al., 1999; Dorin-Semblat et al., 2007; Doerig et al., 2008; Zhang et al., 2012). Next, the three glycogen-synthase kinases 3 (GSK3) family which are also the major regulators of cell proliferation (Kappes et al., 1995; Droucheau et al., 2004; Doerig et al., 2008; Zhang et al., 2012) and cyclin-dependent kinase-like kinases (CLKs) which plays an vital role in RNA metabolism (Doerig et al., 2008; Zhang et al., 2012). The AGC group includes *Pj*PKA is the only known cyclic adenosine monophosphate (cAMP) effector kinase in *Plasmodium* (Syin et al., 2001; Doerig et al., 2008; Zhang et al., 2012).

The *Pj*PKG is a cyclic guanosine monophosphate (cGMP) known to be essential for asexual stages, schizont maturation and gametogenesis (Deng and Baker, 2002; Diaz et al., 2006; Taylor et al., 2010; McRobert et al., 2008; Doerig et al., 2008; Zhang et al., 2012) and *Pj*PKB which is known to play a role in Calcium-calmodulin-dependent kinases mediated signalling pathway (Vaid et al., 2006; 2008; Doerig et al., 2008; Zhang et al., 2012). The calcium-calmodium dependent kinase (Ca2+/camKs) includes mainly six important *Pj*CDPKs which are known to be essential for microgamete formation, ookinete development and motility and the sporozoite invasion into hepatocyte. All these processes requires calcium as a second messenger and calcium is known to regulate CDPK activity (Billker et al., 2004; Silva-Neto et al., 2002; Ishino et al., 2006; Siden-Kiamos et al., 2006; Doerig et al., 2008; Zhang et al., 2012). The never in mitosis/Aspergillus (NIMA) kinase family, which plays a central role in the eukaryotic cell division and regulation of centromere replication constitutes four Nek kinases of which, *Plasmodium* Nek-1 is essential for completion of the asexual
stage whereas, *Plasmodium* Nek-2, Nek-3 and Nek-4 is known to be essential for sexual stage development (Dorin-Semblat *et al.*, 2011; Reininger *et al.*, 2005; 2009; Le Roch *et al.*, 2003; Doerig *et al.*, 2008; Zhang *et al.*, 2012). The Phosphoinositide kinases (PIKs) family are key cell cycle regulators that include *Plasmodium* PI3P which is crucial for haemoglobin endocytosis and serves as a co-factor for the formation of the parasite digestive vacuole and PI4,5-bisphophate are known to trigger the exflagellation of microgametocytes (Vaid *et al.*, 2010; McIntosh *et al.*, 2007; Martin *et al.*, 1994; Zhang *et al.*, 2012). Recent studies using reverse genetics approach have identified several PKs that have been assigned to specific aspects of parasite development; belong to orphan PKs that do not align with a specific kinase family, suggesting the non-conserved functions that are unique for the growth and development of the malaria parasite (Doerig *et al.*, 2010; Zhang *et al.*, 2012). Thus, the protein kinase regulated pathway in *Plasmodium* offers potential drug targets.

**1.8.5 Transporters as drug targets in Plasmodium**

During the developmental processes within the erythrocyte of its host, the malaria parasite takes up nutrients from the extracellular medium, exports metabolite and maintains a tight control over its internal ionic composition and disposes the toxic wastes. This is facilitated via membrane transport proteins, carrier proteins, channels or pumps and integral membrane proteins that mediate the passage of solutes across the various membranes that separate the biochemical machinery of the parasite from the extracellular environment. The host cell transporters modified by the internalized parasite may also be involved in various biological functions playing a vital role in antimalarial drug resistance. Thus, the proteins of this type as such offer a potential drug target (Philippe *et al.*, 2012; Kirk and Lehane, 2014). *Plasmodium* permeome (permeome refers to the total complement of proteins involved in membrane permeability in a given organism which included the complete range of channels and transporters encoded in the genome) indicates that at least 2.5% of the parasite genome encodes transporters, channels and pumps of which, 120 membrane transport proteins are thought to be encoded by the malaria parasite (Martin *et al.*, 2005; 2009; Kirk *et al.*, 2005; Kirk and Lehane, 2014). Experimental approaches by the use of either specific antibodies or epitope-tagged and or parasites transfected with fluorescent recombinant fusion protein has shed the knowledge on the localization of few of these transporters to parasite surface, namely *Pf* hexose transporter 1 (*Pf*HT1), *Pf* equilibrative nucleoside transporter 1 (*Pf*ENT1) or *Pf* nucleoside transporter 1 (*Pf*NT1), *Pf* multidrug resistance-associated protein (*Pf*MRP), *Pf* aquaporins (*Pf*AQP), ATP/ADP transporter to the mitochondrial membrane and the
transporters of food vacuole membrane comprising of \( Pf \) chloroquine resistance transporter \((PfCRT)\), P-glycoprotein homologue \((Pgh1)\), V-type H+ ATPase and ATP-driven H+ pump thereby giving such a diverse source of potential targets, however extensive experimental work is needed to validate these transporters as targets (Slavic et al., 2010, 2011; Saliba et al., 2008; Riegelhaupt et al., 2010; Raj et al., 2009; Cui and Bastien, 2012; Sanchez et al., 2010; Philippe et al., 2012; Kirk and Lehane, 2014; Staines et al., 2010).

Gene disruption studies for a limited number of candidate transporter genes such as those encoding \( Pf\)ENT1, \( Pf\)MRP, the \( P. berghei \) orthologue of the \( Pf\)AQP, \( Pf\)Kch1 or \( Pf\)K1 and \( Pf\)ACα, have shown none or limited impact on the asexual stage growth of the parasite, whereas the deletion of genes could not be achieved for \( Pf\)HT1, \( Pf\)CRT and \( Pgh1 \) suggesting their essentiality in parasite survival (reviewed by Sanchez et al., 2010). Some of the transporter gene that is dispensable for the asexual development turned out important for the development of other parasite stages in insects or liver (reviewed by Martin et al., 2009; Staines et al., 2004). Recently, ZIP-domin-containing protein (ZIPCO), a putative metal ion transporter has been shown to be vital for \( Plasmodium \) liver stage development (Sahu et al., 2014). Among all these candidates, \( Pf\)HT1 becomes the only malaria transporter to be validated both chemically and genetically confirming the essential role of the hexose transporters in life cycle stages of malaria parasites (Blume et al., 2010; Slavic et al., 2010, 2011).