CHAPTER 1

LITERATURE REVIEW

1.1 HISTORY OF MALARIA

Symptoms and characteristics of malaria have been documented in historical writings from ancient times [1], such as the Ebers Papyrus from 1570 before Christ (B.C.) [2] and the Chinese medical book Nei Ching (2700 B.C.) [3]. These records mentioned splenomegaly, periodical fevers and headaches. The prevalence of the disease in early civilizations was confirmed with modern methods, which detected malaria antigens in the skin and lungs of Egyptian mummies dating back to 3200 and 1304 B.C. [2]. In the Roman Republic (200 B.C.) the disease was prominent in the marshes of the Roman Campagna region and temples were dedicated to the goddess Febris, pictured with a prominent belly and swollen veins, in ancient Rome [3]. The condition was eventually known as Roman fever and gave rise to the Italian word mal'aria meaning "bad air", regarded as the cause of the disease at the time [1].

In 1880, Laveran (1845-1922) examined the blood of a soldier in Algeria suffering from intermittent fever and noticed crescent-shaped bodies within red blood cells. He subsequently realised that the bodies were alive and named them *Oscillaria malariae*. He could detect these life forms in 148 blood specimens from malaria patients, but not in those of controls [4]. Laveran reported his findings, but Italian scientists that also observed the motile parasites within erythrocytes subsequently named them *Plasmodium malariae* without considering Laveran's reports [1]. However, 26 years later in 1906, Laveran received a Nobel prize for discovering the causative agent of malaria [5]. Seventeen years after Laveran's discovery (1897), Ronald Ross (1857-1932) demonstrated that the dapple-winged, brown *Anopheles* mosquito transmits malaria [6]. In 1898 he postulated that human malaria goes through the same developmental stages as bird malaria [7, 8]. He received a Nobel prize for his work in 1902 [5].

Almost 60 years after the erythrocytic stages of malaria were discovered (1948), the tissue stages of primate and human malaria parasites were detected in the livers of rhesus monkeys infected with *P. cynomolgi* sporozoites by Shortt, Garnham and colleagues at the Ross Institute in London. Shortt and colleagues later also described the complete life cycle of *Plasmodium falciparum* [9].

1.2 MALARIA AS GLOBAL HEALTH PROBLEM

Most of Europe and the United States were cleared from malaria in the first half of the twentieth century by changed land use and vector control. A global malaria eradication programme was initiated in the 1950s and

1960s after the development of the insecticide dichlorodiphenyltrichloroethane (DDT), and was successful in countries of the former Soviet Union, Sri Lanka and India. However, due to the costs of the programme and problems such as the resistance of communities to repeated spraying of their homes and the emergence of resistance to the insecticide, global eradication was not achieved. Unfortunately, the failure of the programme and elimination of the disease from the western world (Europe and North America) led to a loss of interest in malaria for ~25 years (1970 - 1996), with regard to drug and insecticide development. The development of resistance of *P. falciparum* to the only affordable antimalarials, chloroquine and sulphadoxine/pyrimethamine, worsened the situation and the morbidity and mortality due to malaria increased [10]. Antimalarial drug resistance is "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended, but within the limits of tolerance of the subject" [11]. The varying response of individual parasites to the available antimalarial drugs has been one of the major limiting factors in the prophylaxis and treatment of malaria [12].

Accurate estimation of the extent of the morbidity and mortality caused by malaria is difficult [13], but the current consensus is that there are annually about 500 million clinical cases of malaria, 2-3 million severe attacks and 1 million deaths, which equal in the order or 3000 deaths a day [13]. These numbers are probably an underestimation in view of the weakness of reporting systems for infectious diseases in Africa, where the majority of these cases are concentrated due to the presence of the *A. gambiae* mosquito in this region [10, 13, 14]. Most deaths occur in sub-Saharan Africa where children and pregnant women are affected worst. It is estimated that a child dies from malaria every 40 s on this continent [15]. The worldwide distribution of malaria and chloroquine resistance is shown in Fig. 1.1.

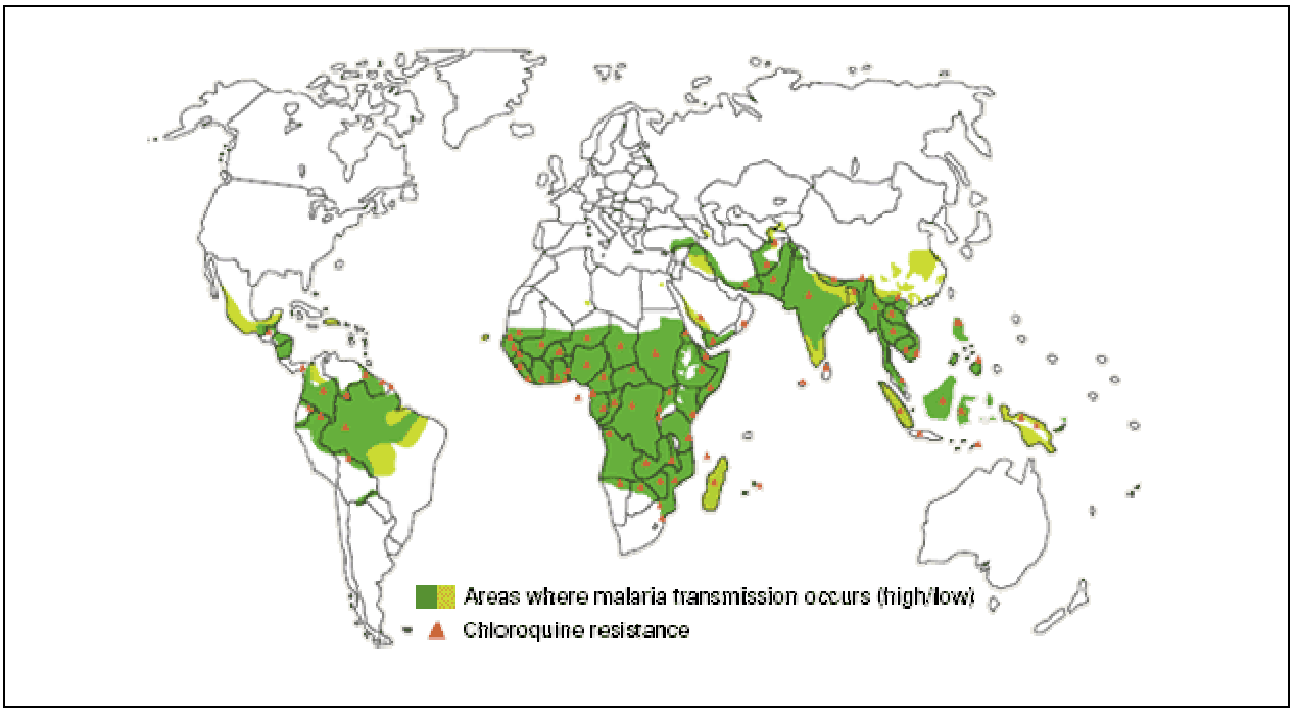

Fig. 1.1 Malaria geographical distribution and chloroquine resistance (WHO/UNICEF, World Malaria Report 2005, Geneva).

The potential influence of global warming on the transmission of malaria due to the changed habitat of the vectors is currently under debate [16-18]. Initial reports expressed the fear that the increased temperature in the 1980s and 1990s in areas of high altitude such as the East African highlands, where *P. falciparum* transmission was previously limited by low temperature, caused the increased number of cases witnessed in these areas in recent years [16, 19]. The rise in the numbers was subsequently attributed to factors other than meteorological patterns since a direct correlation could not be demonstrated [16], but fears still exist that climate change may result in the emergence of vector-borne diseases such as malaria, leishmaniasis, West Nile fever etc. in Europe and North America where these diseases are not endemic [18, 19].

The implications of malaria extend far beyond the morbidity and mortality of the disease. The economic effect on the affected communities is immense and it is estimated that the yearly gross national product of countries with endemic malaria is 2% less than in countries with similar backgrounds where the disease does not occur and that malaria costs Africa about US$12 billion every year [13]. As a result, there is an unmistakable correlation between malaria and poverty. Poverty is concentrated in the tropical and subtropical areas, which closely coincides with the distribution of malaria transmission [20]. Adults in endemic areas generally develop partial immunity, but young children, especially at the pre-school stage, bear a considerable burden. School-age children also suffer symptoms resulting in reduced school attendance and loss of productivity and evidence suggests that the disease can impair intellectual development, with cerebral malaria potentially resulting in permanent developmental abnormalities [13]. The global burden of disease can be calculated by using a standard unit of health measurement, namely disability adjusted life years (DALYs). By using this single measure for morbidity, disability and mortality, the costs and the effects of intervention strategies to reduce the disease burden can be compared across diseases and debilitating risks, e.g. malaria resulted in 46 million DALYs compared to 84 million due to AIDS and 39 million due to road traffic accidents in 2002 [21]. The natural selection of malaria-protective genetic polymorphisms such as glucose-6-phosphate dehydrogenase deficiency and sickle cell disease, despite the reduced life expectancy resulting from homozygous inheritance of the latter, illustrates the enormous burden on communities living with malaria on a daily basis [20, 22].

1.3 THE PARASITE'S LIFE CYCLE

All malaria parasites are obligate intracellular protozoa of the genus *Plasmodium* with a complex life cycle consisting of sexual reproduction (sporogonic phase) in invertebrates, e.g. mosquitoes, and asexual reproduction (schizogonic phase) in vertebrates, e.g. mammals, birds and reptiles [23, 24].

Malaria is transmitted to humans by the intravenous inoculation of sporozoites by the bite of an infected female *Anopheline* mosquito (in Africa mainly *A. gambiae*), but in rare cases transmission occurs through exposure to

infected blood products or congenitally [25]. The sporozoites invade hepatocytes and transform, multiply and develop into tissue schizonts. This asymptomatic, pre- or exoerythrocytic stage lasts for ca. 7 to 30 days, depending on the *Plasmodium* species [25]. The tissue schizonts rupture and release thousands of merozoites into the bloodstream that invade erythrocytes, initiating the intraerythrocytic developmental cycle (IDC, Fig. 1.2). In *P. falciparum* and *P. malariae* infections, no parasites remain in the liver but with *P. vivax* and *P. ovale*, tissue parasites (hypnozoites) persist and can produce relapses months to years after the primary infection. Once the parasites enter the IDC they cannot invade tissues, therefore malaria contracted by transfusion does not have a tissue stage [24].

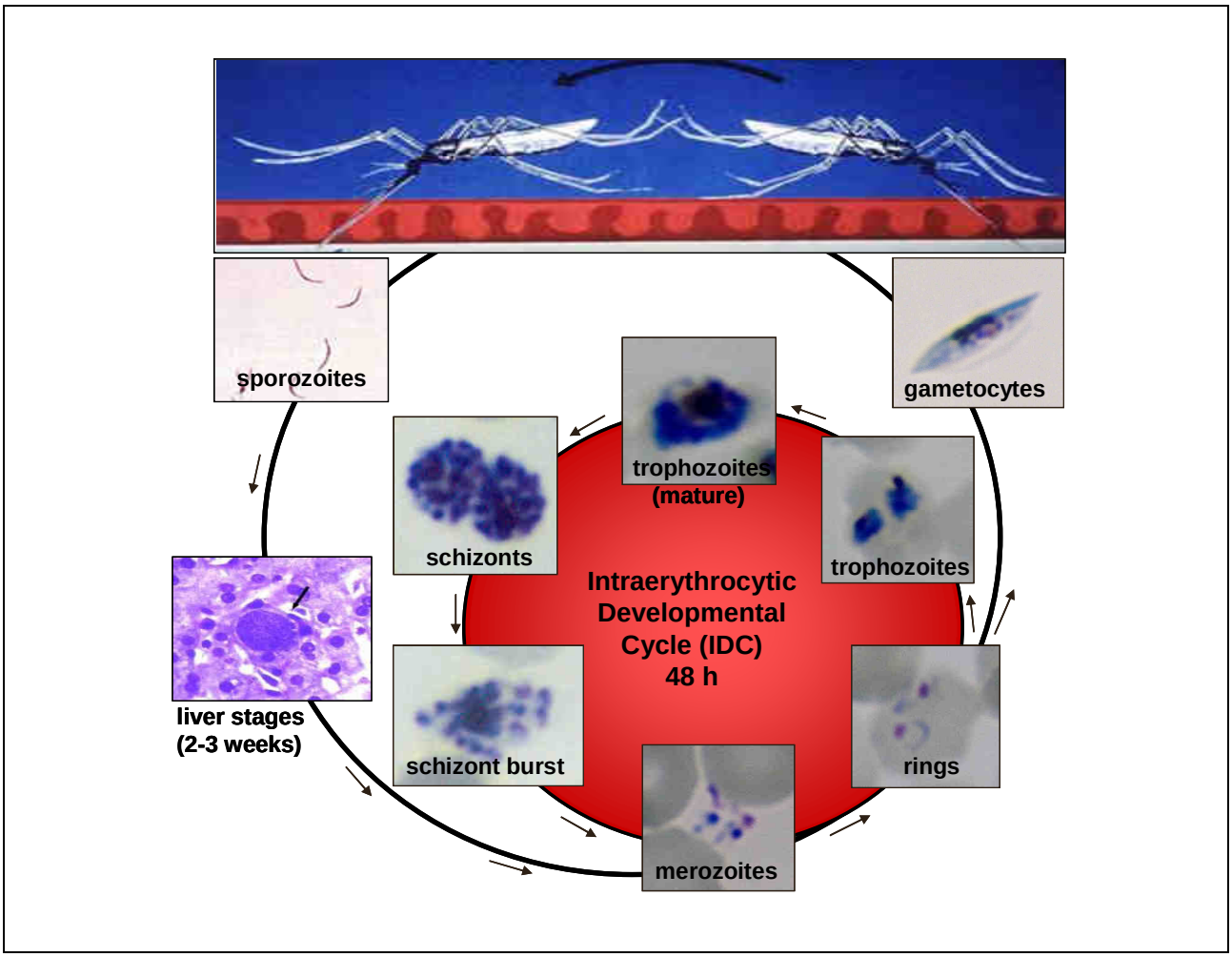

Fig. 1.2 Giemsa-stained thin smears depicting the life cycle of 3D7 *P. falciparum* (compiled from own photographs, sporozoites and liver stages from http://images.google.com)

During the IDC most parasites undergo asexual development from rings to trophozoites to schizonts (Fig. 1.2). The asexual parasites digest the host haemoglobin in their acidic food vacuoles to provide in their amino acid requirements [26], but this process is also necessary to provide room for parasite growth within the erythrocyte [27]. The haemoglobin degradation results in the generation of free radicals and haem, which is polymerised with the aid of lipids to form an insoluble pigment, haemozoin [28].

The IDC of *P. falciparum* lasts ~48 h. Blood schizonts release up to 32 merozoites [29]. The synchronous merozoite release causes the typical febrile attacks on days 1 and 3 in falciparum malaria, hence named "tertian malaria". More erythrocytes are invaded by the released merozoites and the next IDC commences. The cycle continues until the death of the host or death of the parasites due to drug treatment or acquired partial immunity [24]. A few erythrocytic parasites differentiate into sexual forms, named gametocytes. When infected blood containing gametocytes is ingested by a female *A. gambiae* mosquito, the male gametocyte exflagellates and male gametogenesis and fertilisation of the female gamete occurs in the mosquito gut. The zygote develops into an oocyst in the gut wall of the mosquito and infective sporozoites eventually invade the insect salivary glands to be released during the next human blood meal [24].

1.4 HUMAN MALARIA SPECIES

Humans have been regarded as the natural hosts of four species of malaria, namely *P. falciparum, P. malariae, P. vivax* and *P. ovale* [24], but there are more than 100 *Plasmodium* species that infect a variety of hosts such as reptiles, birds, rodents, primates and other mammals [9]. Each species causes a characteristic illness and has unique morphological features in blood smears under the microscope [24]. *P. vivax* is the most prevalent world-wide, and *P. falciparum* is the most dangerous and virulent species that causes malignant malaria, which is associated with severe complications such as cerebral malaria, renal failure and pulmonary affection [24, 30]. *P. falciparum* infection is potentially lethal due to its ability to invade erythrocytes of all ages (compared to *P. vivax* that invades only a subpopulation, i.e. the reticulocytes [31]), resulting in overwhelming parasitaemias and enhanced growth rate. Moreover, it has the capacity to adhere (cytoadherence) to the peripheral microvasculature (capillaries and venules) through sequestration. The parasitised erythrocytes attach to the venular endothelium via parasite-derived proteins that are expressed on the erythrocytic surface, e.g. erythrocyte membrane protein 1 (PfEMP1) [32], and remain attached until rupture and merozoite release [33]. Thus, the ring stage is the predominant form seen in the peripheral circulation [33]. By hiding in the microvasculature, the falciparum parasites avoid clearance by the immune system through the spleen [32], whereas *P. vivax, P. ovale* and *P. malariae* infected erythrocytes are not sequestered and are more successfully removed, therefore causing only benign human malaria without the danger of fatal complications [24]. Infected erythrocytes are also able to adhere to uninfected erythrocytes (i.e. rosetting), which can cause thrombus formation, resulting in tissue oxygen deprivation [34, 35].

The widely held view of four human malaria species was recently challenged by reports of *P. knowlesi* infection in humans [36]. The first naturally acquired case of *P. knowlesi* in a human male was documented in 1965 [37], but the vector, *A. hackeri*, was afterwards demonstrated to be predominantly zoophagic and the threat was dismissed [38]. However, recent surveillance detected a large focus (120/208 patients) of this simian malaria parasite in the human population of Malaysian Borneo, indicating the significance of zoonotic malaria transmission [36]. The natural hosts of *P. knowlesi* are the long-tailed and pig-tailed macaques and

banded leaf monkeys, but it has been shown to be able to infect a wide range of other primates, including man. These diverse primate groups diverged millions of years ago and it is unlikely that the parasite's ability to infect such a variety of hosts is a recent adaptation or that human susceptibility to *P. knowlesis* is new [38]. Human-to-human transmission in South-East Asia was probably prevented by the restriction of the vector to a jungle habitat, which overlaps with the natural environment of the macaques and due to cross-species competition with the other *Plasmodium* species, already established in human populations. The recent success of malaria-control programmes in this region, combined with human population expansion and habitat destruction of the natural hosts, could have provided the opportunity leading to the observed host-switching [38]. The significant threat of a fifth species of human malaria should be recognised and it should be included in current malaria eradication and drug discovery programmes.

1.5 THE PATHOGENESIS AND CLINICAL PRESENTATION OF MALARIA

Falciparum malaria causes an acute illness with initially non-specific symptoms including fever, headache, malaise, mild jaundice, hyperventilation, hepatosplenomegaly, myalgia etc. [25]. The fever peaks occur at the time of erythrocyte rupture with the release of merozoites and malaria toxins [e.g. glycosylphosphatidylinositol (GPI)]. These toxins induce the secretion of pro-infammatory cytokines by the macrophages and parasite antigens stimulate T-cells to directly secrete or induce cytokine production by other cells [33, 35]. The tertian episodes of fever and erythrocyte destruction often lead to severe aneamia and other complications specific to *P. falciparum* infection, such as cerebral malaria, anaemia, hypoglycemia, renal failure and noncardiac pulmonary oedema. In the non-immune patient, these complications may occur in isolation or in combination, resulting in an often complex clinical syndrome [33]. However, the clinical presentation of severe disease in the previously exposed African child differs and renal failure and noncardiac pulmonary oedema do not occur [33].

Severe malaria is one of the potentially fatal complications of *P. falciparum* infection. It was previously regarded as either severe anaemia (due to erythrocyte destruction) or cerebral malaria (due to small blood vessel obstruction of the brain), but nowadays it is recognised to be a complex multi-system disorder with many similarities to sepsis syndromes [35]. Metabolic acidosis leading to the clinical picture of respiratory distress is currently acknowledged as the strongest predictor of death in severe malaria. Hypovolaemia, exacerbated by anaemia and microvascular obstruction due to parasite sequestration, results in decreased oxygen delivery to tissues, anaerobic metabolism and lactic acidosis, but hyperlactataemia is not always present [35]. As with sepsis, cytokine-induced failure of oxygen utilisation [39] has an important role in the pathogenesis but the major influence of immunopathogenic processes, such as proinflammatory cytokine cascades resulting in complex metabolic changes and vascular physiological changes, are now recognised [35, 40]. Pro-inflammatory mediators such as the interleukins (IL), tumour necrosis factor (TNF), interferons (IFN), prostaglandins, as well as molecules such as nitric oxide (NO) and indoleamin 2,3-deaminase, are

biomarkers of severe malaria [35]. The balance and timing of secretion of both the pro-infammatory and anti-inflammatory cytokines may be important in disease and parasite clearance, with IL-4 and IL-10 apparently protecting against severe disease, whereas increased TNF is associated with severe pathology [35]. The role of NO is controversial, but recent evidence indicates that low rather than high NO bioavailability contributes to the genesis of cerebral malaria in animals [40] and that expression of haem oxygenase-1 prevents the development thereof [41]. Haem oxygenase-1 produces carbon monoxide (CO), which prevents blood-brain barrier disruption, brain microvasculature congestion and neuron inflammation including $CD8^+$ -T-cell brain sequestration. The protective effect of CO may be NO-dependent, as NO is a potent inducer of haem-oxygenase-1. CO binds to haemoglobin, preventing haemoglobin oxidation and free haem generation, which triggers the cerebral malaria pathogenesis [41].

Pregnant woman are particularly vulnerable to malaria and are more likely to become infected than non-pregnant women with *P. falciparum*, resulting in severe disease. This is partially due to the transient depression of cell-mediated immunity that occurs during pregnancy. Furthermore, the enhanced function of pancreatic β-cells in pregnant women resulting in a tendency to hypoglycaemia is further aggravated by the parasite's glucose requirements and decreased liver glycogen stores from decreased oral intake due to emesis and anorexia. Pregnancy-associated malaria is characterised by placental sequestration of malaria parasites in the intervillious space of the placenta, causing histological changes including leukocyte-induced damage to the trophoblastic basement membrane. The sequestration results from parasite binding to chondroitin sulphate A (CSA) receptors in the placenta and disrupts oxygen and nutrient transport across this membrane. Anti-adhesion immunoglobulin G antibodies against CSA-binding parasites are associated with protection from maternal malaria, but it only develops over successive pregnancies. Pregnancy-associated malaria can occur without clinical symptoms and the resulting placental sequestration can cause the malaria to be missed when based purely on peripheral blood smears. The effects during pregnancy differ depending on the woman's immunity, her gravidity, the trimester of pregnancy and the presence or absence of other disease. Adverse consequences of placental malaria and maternal anaemia may include spontaneous abortion, preterm delivery, low birth weight due to intrauterine growth retardation, congenital infection and a 2-fold increased risk of stillbirth [25].

Despite persistent malarial infections, neutralising antibodies that block erythrocyte invasion (as with many virus infections) do not occur because of the high degree of antigenic diversity of the malaria surface proteins [33]. Similar to other unicellular protozoa (African trypanosomes and *Babesia* sp.), bacterial pathogens (*Borrelia* sp. and *Neisseria* sp) and pathogenic fungi (*Candida* sp.), *Plasmodium* has the ability to vary surface protein expression to alter the profile of antigens exposed to the host immune system [34]. Antigenic variation involves the ability of the parasite to tightly regulate the expression of individual genes within large, hypervariable gene families, thus exposing only a small portion of the parasites' antigenic repertoire to the host

at any given time. These hypervariable gene families are the *var* (variant, 59 genes), *rifin* (repetitive interspersed family, 149 genes), *stevor* (subtelomeric variable open reading frame, ~30-40 genes) and *Pfmc-2TM* (two transmembrane protein domains, 13 genes) genes that are predominantly found in subtelomeric chromosomal regions. It appears that the degree of sequence variability within these families is almost limitless in natural parasite populations [34, 42]. In contrast to the *var* genes, the *rifin*, *stevor* and *Pfmc-2TM* genes all have a PEXEL (*Plasmodium* export element)/VTS (vacuolar transport signal) motif that is responsible for the transport of these proteins to the erythrocyte cytoplasm. The var and rifin proteins are ultimately targeted to erythrocyte surface, but the stevor and Pfmc-2TM proteins remain in the flat vesicular membranous Maurer's clefts [34]. These are parasite-derived structures within the erythrocyte cytoplasm that are postulated to function as protein-sorting compartments between the parasite and the erythrocyte membrane [43]. The most extensively studied variant antigens are the *var* multigene family, which expresses the cytoadhesive protein, PfEMP1. PfEMP1 is displayed within the knobs on the infected erythrocyte surface and binds to several host endothelial cell surface receptors, e.g. cluster determinant 36 (CD36), intercellular adhesion molecule 1, thromobospondin, complement receptor 1 and CSA. A single PfEMP1 protein is expressed at any given time and, depending on the variant, the cytoadherence and antigenic phenotype will vary dramatically. Therefore, PfEMP1 is a major virulence factor in falciparum malaria [34].

1.6 ANTIMALARIAL VACCINES

There have been more than 40 clinical trials of antimalarial vaccines in the last 25 years [44]. These preliminary vaccines generally followed three strategies of immunisation, namely to target the pre-erythrocytic liver stage, the blood stage or the sexual stage (transmission-blocking vaccines). Pre-erythrocytic stage vaccines aim to prevent sporozoite invasion of hepatocytes and/or to eliminate those already infected. Blood stage or asexual vaccines prevent merozoite invasion of erythrocytes and prevent clinical symptoms, whereas transmission blocking vaccines are designed to break the cycle of infection [45].

Currently, the focus of vaccine discovery programmes is on species-specific vaccines for *P. falciparum* and *P. vivax* in response to results of sequential heterologous infections from the 1950 - 1960s and cross-species challenge experiments from the 1970s, which determined that multi-species protection would be difficult to achieve with a single vaccine. However, combinations of antigens could provide broad protection once the successful species-specific vaccines have been developed [44]. Since *Plasmodium* is a multistage organism, a good vaccine should furthermore contain antigens from different stages (multistage vaccine) and should include several antigens from each stage (multivalent) to circumvent antigenic variation. Finally, vaccines should be simple and elicit the correct type of immune response [44]. Yet, despite many years of effort, an effective antimalarial vaccine remains elusive. Currently, there is reason for optimism due to evidence of partial human protection provided after immunisation with irradiated sporozoites, development of naturally acquired immunity after repeated malaria infections, the efficacy of vaccines based on recombinant

circumsporozoite protein and the successful protection of mosquitoes against *P. falciparum* and *P. vivax* by preliminary transmission-blocking vaccines [44].

Most vaccines tested up to now have been pre-erythrocytic stage vaccines against sporozoites or liver stage parasites consisting of synthetic peptides or recombinant proteins based on malarial antigens. However, a commonly experienced problem is difficulty in obtaining a strong and long-lasting immune response in humans [44]. Collaboration between the US Army Walter Reed Institute of Research and GlaxoSmithKline produced the pre-erythrocytic stage vaccines RTS,S and TRAP/SSP2 and the blood-stage vaccines MSP1 (merozoite surface protein 1)-3D7 and AMA1 (apical membrane antigen 1)-3D7 [44]. The RTS,S vaccine is a chimaeric fusion protein between circumsporoizoite protein and the hepatitis B surface antigen, which was tested in several clinical trials. It caused a significant reduction of infection rate in Gambian adult males, but the effect was short-lived [46]. However, a phase IIb trial in Mozambican children demonstrated 35.3% efficacy against risk of clinical malaria and 48.6% against severe malaria with a good safety profile [47]. A phase IIa trial in malaria-naïve adults at the US Army Walter Reed Institute of Research provided 42 – 47% protection [48]. Based on these promising results it is hoped that a malaria vaccine will become available within the next decade.

1.7 ANTIMALARIAL THERAPEUTICS

Due to the current lack of an approved vaccine strategy, antimalarial intervention consists of drug treatment with the primary objective of eradicating malaria parasites completely from the body, i.e. to provide a cure for the disease [49]. Quinine's history of medicinal use dates back 350 years and artemisinin (qinghaosu) has been used in China for over 2000 years [24]. Most other drugs currently used in the treatment of malaria were discovered as long ago as the 1940s, e.g. chloroquine (1943), proguanil (1945), primaquine (1946) pyrimethamine (1951), halofantrine (1960s) and mefloquine (1963). Of the 1223 new drugs developed from 1970 to 1996, only three were antimalarials [50]. Most of the antimalarials have closely related structures and modes of action (resulting in cross-resistance), which underscores the urgency to progress antimalarial drug discovery. The most important antimalarial drugs, their modes of action and limitations are presented in Table 1.1.

Table 1.1 Antimalarial therapeutics and combinations

Pharmacological class	Mode of action	Limitations
Quinolines and related compounds		
Quinine	Inhibits haem detoxification, but exact mechanism still debated	Poor compliance, toxicity, resistance
Chloroquine		Resistance
Amodiaquine		Side-effects, resistance
Mefloquine		Side-effects, resistance
Primaquine		Resistance
Halofantrine		Side-effects, resistance, cost
Lumefantrine		Cost
Artemisinins		
Arteether	Free-radical-induced damage or inhibition of sarcoplasmic reticulum calcium-dependent ATPase 6 (SERCA), but exact mechanism still debated	Compliance, side-effects, cost
Artemether		
Artesunate		
Antifolates		
Sulphadoxine	Inhibits dihydroopteroate synthase (DHPS)	Resistance
Dapsone		
Proguanil	Inhibits dihydrofolate reductase (DHFR)	
Pyrimethamine		
Naphtaquinones		
Atovaquone	Mimics ubiquinone and interferes with mitochondrial electron transport	Resistance potential, cost
Antibiotics		
Tetracycline	Inhibits prokaryotic-like protein synthesis in the apicoplast (plastid)	Side-effects (children)
Doxycycline		
Clindamycin		
Antimalarial combinations		
Chloroquine/Proguanil	Combination of above	Resistance
Atovaquine/Proguanil		Resistance, cost
Artemether/Lumefantrine		Resistance potential, cost
Artesunate/Mefloquine		Resistance potential, cost
Pyrimethamine/Sulphadoxine		Resistance
Chlorproguanil/Dapsone		Resistance
Pyrimethamine/Dapsone		Resistance
Chlorproguanil/Dapsone/Artesunate		Resistance potential, cost

Compiled from [24, 51, 52].

1.7.1 Quinoline and related antimalarials

The quinoline antimalarials were originally derived from quinine (Fig. 1.3). Quinine is a 4-aminoquinoline and the chief alkaloid from the bark of the South American cinchona tree that was imported to Europe from Peru around 1633-1640 [51]. The quinoline drugs are blood schizontocides with high activity against the erythrocytic forms of susceptible Plasmodia as well as gametocytes of *P. vivax*, *P. ovale* and *P. malariae*, but not *P. falciparum.* They are weak bases and accumulate in the food vacuoles of susceptible parasites. As result, the pH of the acidic food vacuole increases, haem peroxidase activity is inhibited and the non-enzymatic polymerisation of haem to haemozoin is thought to be disrupted [28]. The toxic haem accumulates and kills the parasite via oxidative damage to cell membranes, proteases and other critical molecules, but the exact mode of action is not completely elucidated [53, 54].

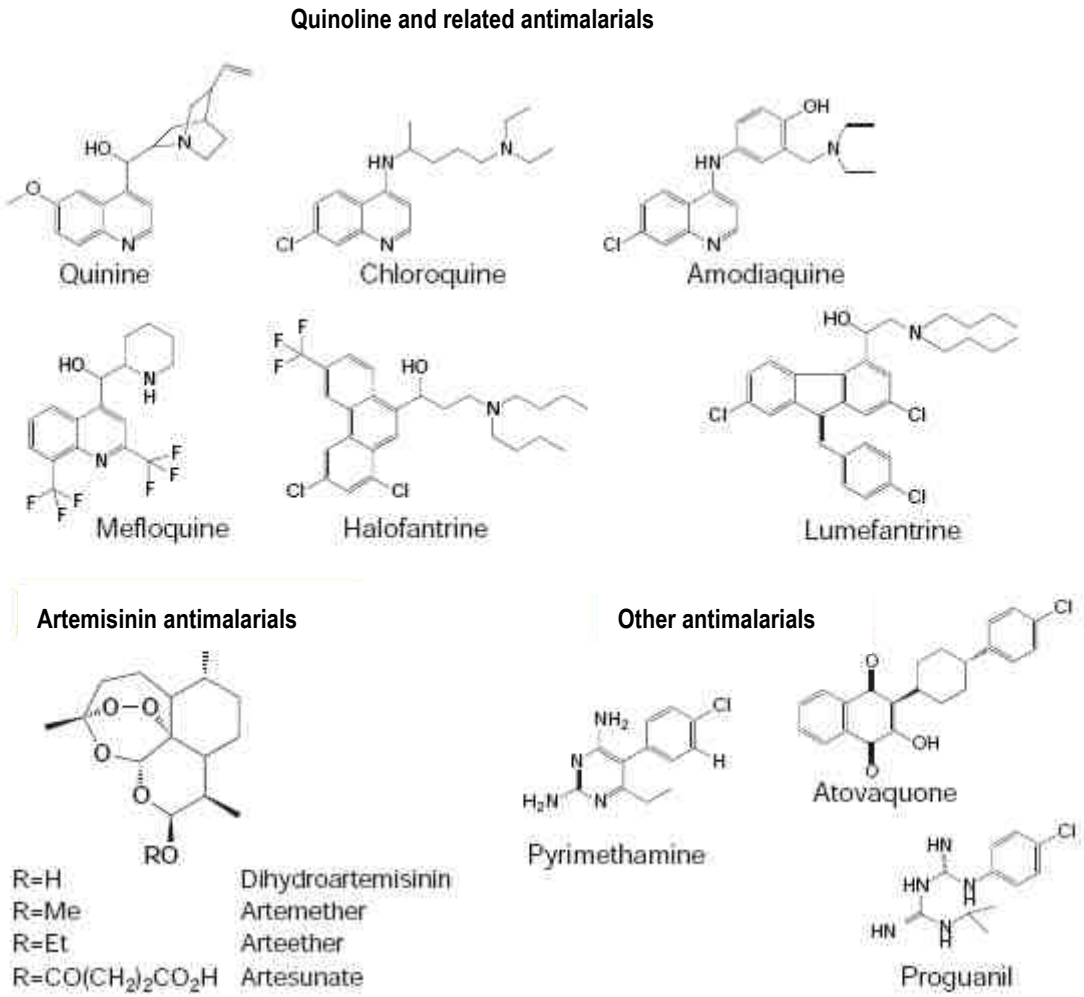

Figure 1.3 Currently used antimalarial drugs [51]

The toxicity of quinine (side-effects such as tinnitus), inconvenient dosage interval (three times daily resulting in poor compliance) and dependence on plant material for extraction motivated the development of the fully synthetic 4-aminoquinolines i.e. chloroquine and amodiaquine (Fig. 1.3) [51]. Chloroquine was already discovered in 1934 in Germany, but was not known elsewhere and was rediscovered by American scientists during World War II (1943). It has fewer side-effects than quinine and higher efficacy against malarial parasites susceptible to both drugs. Chloroquine is actually an extraordinarily safe drug for prophylaxis and cure of susceptible *P. falciparum* infections [24], but unfortunately, chloroquine resistance against *P. falciparum* is now almost global (Fig. 1.1) [51]. The use of amodiaquine has been limited since the mid-1980s due to an association with agranulocytosis, but it has retained a high degree of efficacy against the most highly chloroquine-resistant strains [51].

Mefloquine is a 4-quinoline-methanol that was first used to treat chloroquine-resistant falciparum malaria in Thailand and is currently reserved for the prevention and treatment of chloroquine-resistant and multidrug-resistant *P. falciparum* infections (Fig. 1.3). Halofantrine is a phenanthrene-methanol with blood schizontocidal properties similar to the quinoline antimalarials (Fig. 1.3). It was initially developed as an alternative to quinine

and mefloquine in the treatment of acute malarial infections of chloroquine-resistant or multidrug-resistant *P. falciparum* strains [24]. However, resistance against both mefloquine and halofantrine can develop rapidly [51] and both have contra-indications e.g. mefloquine in patients with a history of seizures or neuropsychiatric disturbances and both drugs in patients with a history of heart disease [24, 51]. The aryl-alcohol, lumefantrine, is similar to mefloquine and halofantrine, but it has a better safety profile (no neurotoxicity) and is one of the most recently approved antimalarials [51].

Drug resistance usually develops within 10 years after an antimalarial was introduced [55] and *P. falciparum* chloroquine resistance was observed for the first time about 50 years ago. Currently chloroquine resistance is the result of polymorphisms in the *pfcrt* gene located on chromosome 7 or in the *pfmdr1* gene on chromosome 5 of the *P. falciparum* genome. The *pfcrt* gene codes for the chloroquine resistance transporter (PfCRT), a vacuolar membrane transporter protein and *pfmdr1* codes for the multidrug resistance type 1 protein (MDR1), a P-glycoprotein homologue and well-characterised ABC-transporter [55]. These mutations in parasite-encoded drug transporters lead to reduced drug accumulation and therefore reduced susceptibility. The exact mechanism is controversial, but one hypothesis is that these mutations significantly change the pH of the food vacuole, resulting in reduced chloroquine accumulation. Another hypothesis is that PfCRT transports chloroquine directly out of the food vacuole. Recent evidence indicates that the chloroquine resistance conferring mutations in PfCRT enable the protein to transport chloroquine together with H^+ ions from the food vacuole [56]. Mutations of the *pfmdr1* gene have also been implicated in *P. falciparum* resistance against quinine, mefloquine and artemisinin and resistance to atovaquone has been ascribed to point mutations in the gene encoding cytochrome bc_1 of the parasite electron transport chain [55, 57]. Gene polymorphisms that lead to non-synonymous amino acid substitutions can cause complete loss of function of the proteins involved [55].

1.7.2 Artemisinin and derivatives

Artemisinin or qinahaosu is a sesquiterpene lactone endoperoxide that is extracted from the weed *Artemisia annua* (sweet wormwood). Chinese scientists synthesised three artemisinin derivatives, two oil-soluble methyl esters named artemether and arteether and a water-soluble hemisuccinate salt of dihydroartemisinin named artesunate (Fig. 1.3) [24, 51]. The three derivatives are metabolised *in vivo* to dihidroartemisinin, the main active agent [51]. The Chinese reported rapid, safe and efficient treatment of malaria with the artemisinins in 1979 and since then more than 2 million patients have been treated successfully in China, Southeast Asia and Africa [24].

The endoperoxide moiety is essential for antimalarial activity, but substitutions of the lactone carbonyl increase potency significantly. Apparently, cleavage of the drug's endoperoxide bridge is catalysed by intraparasitic haem iron of infected erythrocytes, which is followed by intramolecular rearrangement to produce carbon-centered radicals that modify and damage specific malarial proteins by covalent interactions [24]. However,

the exact mode of action is under debate and the inhibition of the sarco/endoplasmic reticulum calcium-dependent ATPase 6 (SERCA, PfATPase6) has also been proposed [58].

Artesunate has been demonstrated to act primarily on young ring-form parasites, preventing their development to mature trophozoites [59]. Artemisinin and its derivatives are very effective in the treatment of these asexual parasites of chloroquine-sensitive, chloroquine-resistant and multidrug-resistant *P. falciparum.* The compounds also have gametocytocidal activity but do not affect liver stage parasites and can therefore not be used for chemoprophylaxis or to prevent relapses of vivax/ovale malaria. *In vitro* activity against other protozoa, e.g. *Leishmania major* and *Toxoplasma gondii,* has also been demonstrated [24].

On the negative side, the artemisinins have short half lives, necessitating treatment of 5 - 7 days, and are increasingly used in combination with drugs with longer half lives to reduce the treatment time [51] e.g. artemether/lumefantrine and artesunate/mefloquine. Futhermore, the cost of artemisinin derivatives is significantly more than that of traditional antimalarials such as chloroquine and the observation of selective brain stem neuronopathy in laboratory animals treated with a high dose of artemisinins parenterally also had a negative impact [51]. The decrease in *in vitro* susceptibility of *P. falciparum* against artemether in French Guiana [58] due to a single polymorphic mutation of the *PfATPase6* gene demanded the immediate deployment of drug combinations [58]. However, the declining efficacy of the artesunate/mefloquine combination on the Cambodia/Thailand border was recently reported, but this is most likely due to mefloquine resistance rather than artemisinin resistance, as increased copy numbers of *pfmdr1* were detected [52].

1.7.3 Antifolates

In contrast with the quinolines and artemisinins, the antifolates were not derived from plants. These drugs were mostly generated through knowledge of cell biology and synthetic medicinal chemistry [51]. Nucleotide biosynthesis and amino-acid metabolism require one-carbon transfer reactions, which are dependent on completely reduced folate co-factors. The antifolates target one of two subsequent enzymes in folate metabolism, either dihydrofolate reductase (DHFR) or dihydroopteroate synthase (DHPS) [51]. However, the effects of inhibition are only visible late in the life cycle due to failure of nuclear division during schizont formation [24].

The commonly used antimalarial combination pyrimethamine/sulphadoxine inhibits both DHFR and DHPS [51]. The success of the combination lies in the synergism of the two components. However, resistance against pyrimethamine/sulphadoxine has increased substantially and its use is nowadays restricted to suppressive treatment of chloroquine-resistant falciparum malaria in regions where antifolate resistance has not yet developed [24]. The molecular basis behind the resistance to pyrimethamine/sulphadoxine is characterised best of all antimalarial resistance. Point mutations at five codons (codon 16, 51, 59, 108, 164) of the *dhfr* gene

are implicated in conferring resistance to pyrimethamine by decreasing its binding affinity to the enzyme. In the same way specific point mutations (codon 436, 437, 540, 581 and 613) in the *dhps* gene have been identified to result in a decreased binding affinity of DHPS for sulphadoxine, other sulphonamides and the sulfone dapsone [55, 60]. The selection for point mutations in *dhfr* and *dhps* are not equal, but appear to occur in *dhfr* first and mutations in *dhps* are selected only if most parasites in a population carry a double or triple mutant allele of *dhfr*. The reason for this asymmetry may be due to importance of DHFR to enable *P. falciparum* to salvage exogenous folate, thus bypassing the *de novo* biosynthesis via DHPS. However, inhibition of DHFR via pyrimethamine hampers the use of exogenous and synthesised folate by the parasite [60]. Apart from point mutations, another reason for the rapid selection for antifolate resistance is the long elimination half lives of both pyrimethamine (81 h) and sulphadoxine (116 h), resulting in the selection of resistance against these drugs when blood levels decrease below therapeutic concentrations [60].

In an effort to overcome pyrimethamine resistance and to restore the antifolates in regions of drug resistance, medicinal chemistry efforts have identified improved DHFR inhibitors, e.g. the biguanides. Chlorproguanil retains activity against most pyrimethamine-resistant parasite strains and is administered in combination with dapsone and artesunate to prevent further development of resistance (Table 1.1) [51]. These three drugs all have half lives of less than a day, resulting in reduced selection pressure for resistance [60].

1.8 ANTIMALARIAL DRUG TARGETS

One of the initial steps in rational drug development is the identification and subsequent validation of drug targets in *Plasmodium* [61]. The efficacy and specificity of anti-infective drugs depend on their ability to interfere with metabolic pathways or proteins of pathogens that are essential for survival but also significantly different from those of the host to enable selective toxicity [51]. Developmental stages of the IDC are specifically targeted, since the asexual parasites are responsible for all the clinical symptoms of malaria and are therefore the focus of most antimalarial drugs and potential vaccine strategies. Moreover, distinct organelles, such as the food vacuole, the apicoplast and the acrystate mitochondrion with its limited electron transport system, are also regarded as potential drug targets [51]. Both the apicoplast (plastid) and mitochondrion are prokaryote-like organelles inside the eukaryotic parasite. The apicoplast houses enzymes involved in type II fatty acid biosynthesis, a non-mevalonate pathway for isoprenoid biosynthesis and haem biosynthetic pathways, whereas the mitochondrion contains a complete citric acid cycle [62]. A post-genome survey of *Plasmodium* identified about 50 proteins as potential antimalarial targets [54]. These potential targets belong to the following broad biological functions: energy metabolism, coenzyme and prosthetic group metabolism, protein modification, lipid metabolism, deoxyribonucleic acid (DNA) replication and transcription, haemoglobin digestion and antioxidant defence. The targets include a bifunctional, key-regulatory and biosynthetic enzyme of polyamine and methionine metabolism, S-adenosylmethionine decarboxylase/ornithine decarboxylase (PfAdoMetDC/ODC) [54], which will be discussed in more detail in the following paragraphs.

1.9 POLYAMINE METABOLISM

1.9.1 The biological importance of polyamines

The polyamines are small, aliphatic compounds containing two or more amino groups, which in eukaryotes mainly include the diamine putrescine (1,4-diaminopropane), the triamine spermidine [N-(3-aminopropyl)-1,4-diaminobutane] and the tetra-amine spermine [N,N^1-bis(3-aminopropyl)-1,4-butanediamine] (Fig. 1.4). At physiological pH these polycations interact electrostatically with numerous anionic macromolecules, thereby stabilising DNA, ribonucleic acid (RNA), nucleotide triphosphates [e.g. adenosine triphosphate (ATP)], phospholipids and proteins [63, 64]. These interactions with polyamines can alter DNA conformation, regulate replication and transcription, strengthen membranes, regulate ion channels and protect DNA and phospholipids from oxidative stress [63-67]. However, polyamines are also implicated in apoptosis [67]. Polyamine depletion generally causes cytostasis or growth arrest, which implies that these molecules are involved in cell cycle progression and regulation and it is speculated that polyamines regulate cyclin degradation [63, 68]. Therefore, polyamines are essential for cellular growth, differentiation and macromolecular synthesis and are ubiquitous components of all living cells, except two orders of Archaea [63].

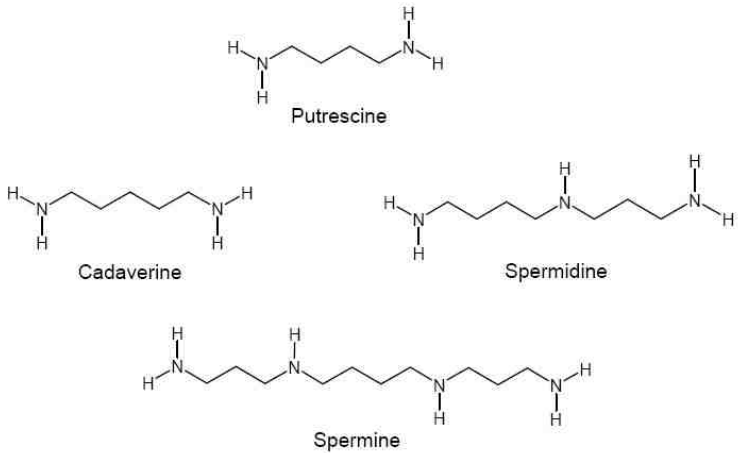

Figure 1.4 Chemical structures of the natural polyamines

A fourth polyamine, cadaverine, is actually a diamine and structural analogue of putrescine, with only an additional methylene group (Fig. 1.4) [69]. Most research on cadaverine has been performed in prokaryotes [70-73], but the importance thereof in plants has also been demonstrated [74, 75]. Cadaverine was shown to have functions similar to the other polyamines, including protection against oxidative stress [76] and involvement in root development [75]. Recently, the first cadaverine aminopropyl transferase, with functions similar to spermidine synthase, was identified in *Pyrococcus furiosus* [72]. In protozoa, cadaverine sustained growth of a *L. donovani* ornithine decarboxylase (ODC)-deletion mutant [77], but almost nothing is known about the biological role of cadaverine in Plasmodia.

Polyamine metabolism is particularly important in rapidly proliferating cells and has been exploited in the treatment of cancer [63] and parasitic diseases [78]. It is also a potential target for antimalarial therapeutic intervention [54, 79]. The various inhibitors and effects of inhibition will be discussed in Chapter 2.

1.9.2 The biosynthesis of polyamines

The natural polyamines (excluding cadaverine) are synthesised by means of six interdependent enzyme reactions in eukaryotes, starting from L-arginine (via arginase to L-ornithine) and L-methionine [via S-adenosylmethionine (AdoMet) synthetase to AdoMet]. Ornithine decarboxylation via ODC forms putrescine, which combines with decarboxylated AdoMet (dcAdoMet) from AdoMet decarboxylase (AdoMetDC), to produce spermidine through the aminopropyltransferase action of spermidine synthase. In most organisms a second aminopropyltransferase reaction involving dcAdoMet occurs via spermine synthase and leads to the formation of spermine [80]. Polyamine metabolism of mammalian cells is shown in Fig. 1.5.

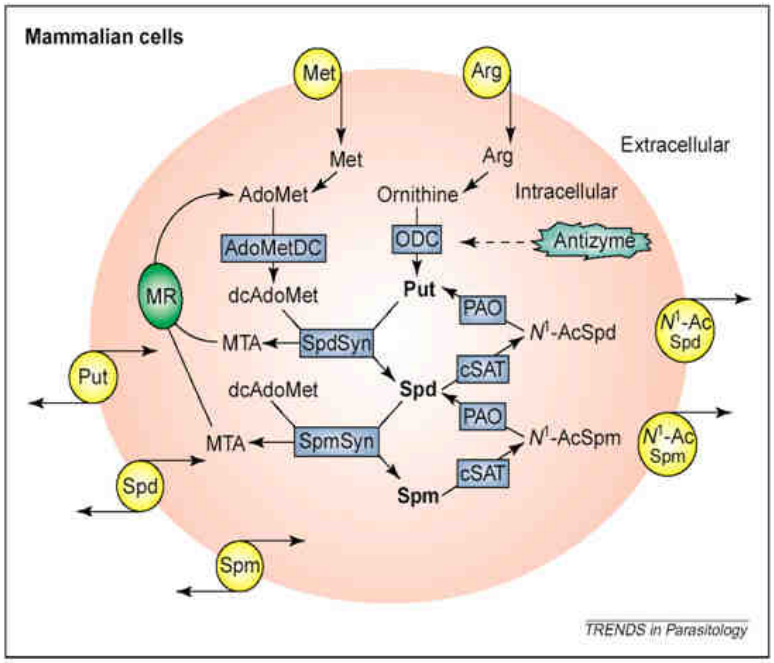

Figure 1.5 Polyamine metabolism in mammalian cells [81].
AdoMet, S-adenosymethionine; AdoMetDC, S-adenosylmethionine decarboxylase; cSAT, cytosolic N1-acetyltransferase specific for spermidine and spermine; dcAdoMet, decarboxylated S-adenosylmethionine; MR, methionine recycling pathway; MTA, methylthioadenosine; N1-AcSpd, N1-acetyl spermidine; N1-AcSpm, N1-acetyl spermine; ODC, ornithine decarboxylase; PAO, polyamine oxidase; Put, putrescine; Spd, spermidine; SpdSyn, spermidine synthase; Spm, spermine; SpmSyn, spermine synthase.

In mammalian cells, the regulation of ODC and AdoMetDC (and thus polyamine levels) occurs at the transcriptional, translational and post-translational levels [81]. ODC is positively and negatively feedback-regulated by the polyamines, resulting in increased biosynthesis when polyamine concentrations are low and decreased synthesis when concentrations are high. The feedback regulation is a combination of post-transcriptional regulation and the induced expression of antizyme, which is a unique ODC-specific inhibitor. ODC can also be released from antizyme upon growth stimuli by another unique protein, named antizyme

inhibitor, which has higher affinity for antizyme than ODC [63]. As a result of quick degradation, ODC and AdoMetDC have short half lives, i.e. 15 min and 35 min, respectively [81]. Apart from the regulation of biosynthesis, polyamine levels are also regulated by transport, excretion and interconversion, i.e. spermine can be converted back to spermidine and spermidine to putrescine (Fig. 1.5) [81]. The interconversion pathway is mediated by cytosolic N_1-acetyltransferases (specific for spermidine and spermine) and polyamine oxidase [81]. The intricate regulation of polyamines further underscores their importance. Low polyamine concentrations or depletion generally impair growth and cellular differentiation, resulting in growth arrest (cytostasis). However, both polyamine depletion and accumulation can also stimulate programmed cell death or apoptosis. Thus, the effects of polyamines and their regulation are generally very complex [63].

In *P. falciparum*, ODC and AdoMetDC are transcribed as a single transcript and are translated into one hinge-linked bifunctional protein, PfAdoMetDC/ODC [82]. Up to date, only monofunctional ODC and AdoMetDC has been detected in other organisms [83], but the enzyme complex is bifunctional in at least three Plasmodia (*P. falciparum, P. berghei* and *P. yoelii*) [84]. Plasmodial polyamine biosynthesis occurs as described for mammalian cells, but spermine synthesis is now believed to be catalysed by spermidine synthase as well (Fig. 1.6) [85]. The diamine, cadaverine, is synthesised by decarboxylation of lysine via lysine decarboxylase (LDC) [69], the function of which in Plasmodia is currently unknown [79]. Plasmodia do not have an antizyme homologue and in contrast with the short half lives of the mammalian enzymes, PfAdoMetDC/ODC has a half life of ~2 h. Similar differences in the intracellular turn-over rate of ODC in Trypanosoma compared to mammals (hours versus 15 min) is partially responsible for the selective toxicity and relative safety of ODC inhibitors such as DL-α-difluoromethylornithine (DFMO) against the causative agent of African sleeping sickness, *Trypanosoma brucei gambiense* [81]. The longer half lives of the parasite enzymes result in a longer-lasting inhibitory effect of DFMO on the parasite than on the host. Recently, it was shown that trypanosomal AdoMetDC uniquely requires a protein called prozyme, which dimerises with the parasite's AdoMetDC and is an essential allosteric activator thereof [86]. *Plasmodium* does not have a polyamine interconversion pathway and there are differences in the regulation of ODC and AdoMetDC in comparison to mammals and trypanosomes. *P. falciparum* ODC activity is feedback-regulated to a greater extent by its product, putrescine, than mammalian ODC, putrescine does not stimulate the parasite's AdoMetDC, whereas it does stimulate the mammalian enzyme [81] and from recombinant AdoMetDC expression studies it was concluded that *Plasmodium* does not have a prozyme homologue [81, 87].

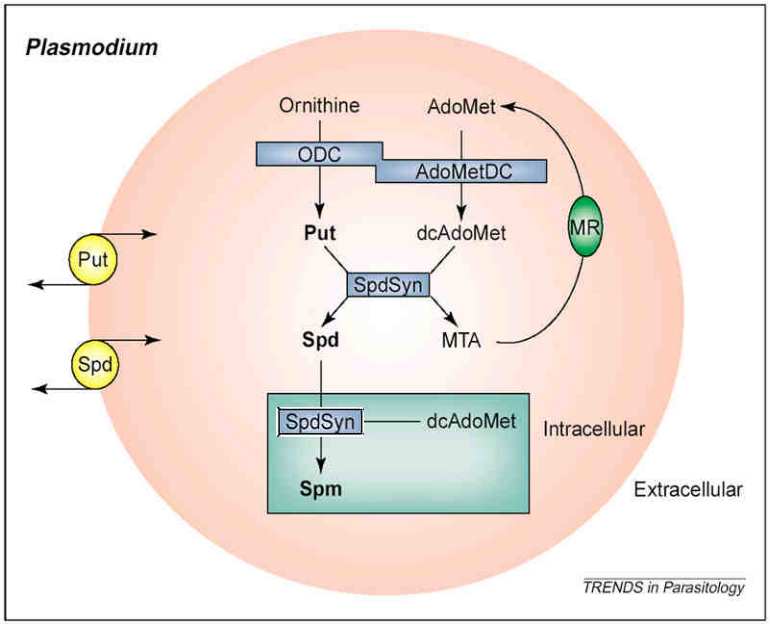

Figure 1.6 Polyamine metabolism in *Plasmodium* (slightly modified from [81]).
AdoMet, S-adenosymethionine; AdoMetDC, S-adenosylmethionine decarboxylase; dcAdoMet, decarboxylated S-adenosylmethionine; MR, methionine recycling pathway; MTA, methylthioadenosine; ODC, ornithine decarboxylase; Put, putrescine; Spd, spermidine; SpdSyn, spermidine synthase; Spm, spermine.

Human erythrocytes are anuclear and do not contain ODC or AdoMetDC. However, ODC and AdoMetDC activity and, therefore polyamine concentrations, are markedly increased in *P. falciparum*-infected erythrocytes [88]. This elevated decarboxylase activity is maximal in the early trophozoite stage [88] when the major macromolecular synthesis occurs [89] and consequently polyamine levels increase with the development from rings to schizonts (Fig. 1.7) [90].

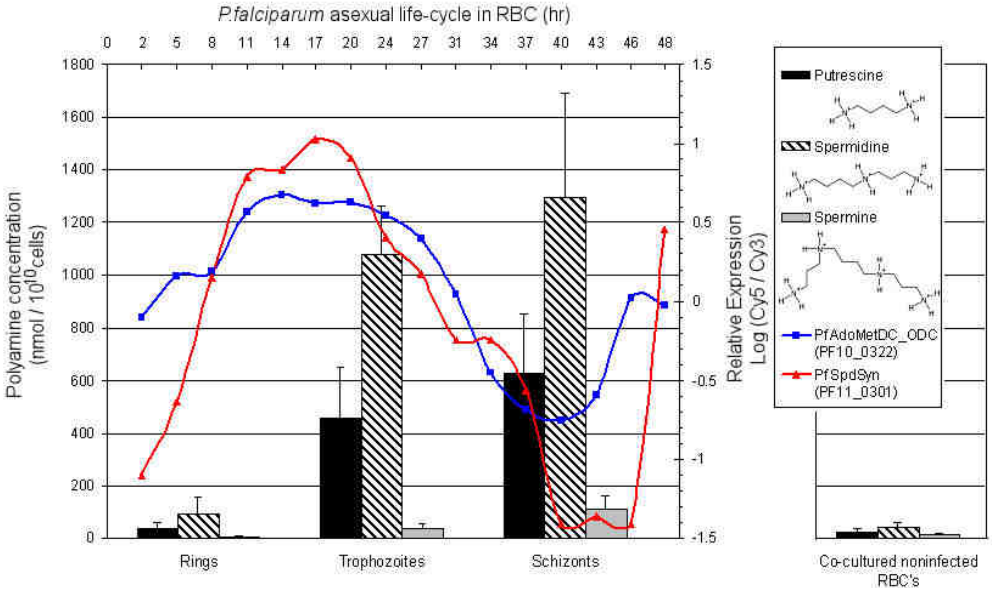

Figure 1.7 Composite diagram of polyamine levels [90] and biosynthetic enzyme transcript levels (PfAdoMetDC/ODC and spermidine synthase) [91] during the IDC of *P. falciparum* (Williams *et al.*, manuscript in preparation).

In addition to PfAdoMetDC/ODC, *P. falciparum* has other bifunctional proteins such as dihydrofolate reductase/thymidylate synthase (DHFR/TS) [92, 93] and dihydroopteroate synthase/dihydroxymethylpterin pyrophosphokinase (DHPS/PPPK) [94]. Organisation into bifunctional proteins may have biological advantages such as metabolic channelling and domain-domain interactions, which could facilitate synthesis of the respective products or regulation of the partner-domain's activity [87]. However, substrate channelling is unlikely in the case of PfAdoMetDC/ODC since another enzyme, spermidine synthase, is required to metabolise the putrescine and dcAdoMet to spermidine [82]. Furthermore, when both active sites of PfAdoMetDC/ODC were inhibited separately, it was concluded that they function independently and that domain-domain interaction is not required [87]. It is advantageous that only one bifunctional protein needs to be regulated to control polyamine synthesis in *P. falciparum* [87], but this advantage to the parasite also provides unique drug-targeting opportunities. PfAdoMetDC/ODC is furthermore unique due to the presence of parasite-specific inserted amino acids that almost double the size of the protein (330 kDa) compared to homologues in other organisms [84]. Parasite-specific inserts have also been reported for other *P. falciparum* proteins [84] and from experimental data these inserts appear to have a regulatory role [95]. These unique regions provide additional opportunities for selective inhibition of PfAdoMetDC/ODC as an antimalarial drug target [84].

1.10 MOLECULAR ASPECTS OF MALARIA

1.10.1 Sequenced *Plasmodium* genome data

The genome sequences of six *Plasmodium* species have now been published. The complete sequences of the *P. falciparum* 3D7 strain and the rodent malaria *P. y. yoelii* 17XNL clone [96, 97] appeared in 2002 and the genomic data of two more rodent malaria species, the *P. berghei* ANKA clone and *P. chabaudi* AS clone, were published in 2005 [98]. Recently, the genome sequences of the human malaria *P. vivax* Salvador 1 strain and the human/simian malaria *P. knowlesi* H strain, along with a comparative analysis with *P. falciparum,* were released [99, 100]. Thus, this genus has the highest number of sequenced species of any eukaryotic organism yet [61].

Comparative analysis of the publicly available *Plasmodium* genomes revealed that they are all haploid with a standard size of 23 – 27 Mb, which is distributed among 14 linear chromosomes between 0.5 – 3.0 Mb in size. The base composition varies among the different species, with the rodent and *P. falciparum* genomes being extremely A+T-rich (80.6% on average and close to 90% in introns and intergenic regions in *P. falciparum*) in contrast with the more G+C-rich *P. knowlesi* and *P. vivax* genomes (37.5% and 42.3%, respectively) [30, 96, 99, 101]. Each *Plasmodium* genome has in the order of 5000 - 6000 predicted genes, most of which (51%) contain at least one intron and ~60% are orthologous among the different species [30, 98]. The difference in gene number is the result of differential gene expansion in distinct lineages and the presence of large variant gene families that are involved with antigenic variation [30]. The unique genes of the different species are

often localised within the subtelomeric regions and code for immunodominant antigens [30]. The mean gene length of the three sequenced human malarias (including *P. knowlesi*) is ~2.2 to 2.3 kb, compared to the average of 1.3 to 1.6 kb in other organisms [96, 101]. The reason for these long gene lengths is not known and this is compounded by the fact that these long genes usually encode hypothetical proteins with unknown function [96]. Gene-mapping studies of conserved genes have shown that gene location, order and even exon-intron boundaries have been preserved over large regions across the three sequenced rodent *Plasmodium* species and *P. falciparum* [30].

In addition to the nuclear genome, the parasites also have a linear mitochondrial genome of ~6 kb in the case of *P. falciparum*, which is the smallest mitochondrial genome known [102], and a ~35 kb circular apicoplast genome [96]. The *P. falciparum* nuclear genome exhibits minimal redundancy in transfer RNA (tRNA) and encodes 43 tRNAs [96] compared to the ~30 of *Homo sapiens* [103]. The parasite tRNAs bind all 64 possible codons except TGT and TGC that both specify cysteine (Cys). As no other codons specify Cys, it is possible that these tRNA genes are located within the currently unsequenced regions, since Cys is incorporated into *P. falciparum* proteins [96]. The small *P. falciparum* mitochondrial genome does not encode any tRNAs [104] compared to the 22 tRNA of the circular 16.6 kb human mitochondrial genome [105]. The *P. falciparum* mitochondrion therefore imports tRNAs from the cytoplasm, whereas the apicoplast genome encodes sufficient tRNAs for protein synthesis within the organelle [106].

The *P. falciparum* genome does not contain tandemly repeated ribosomal RNA (rRNA) gene clusters as seen in many other eukaryotes, but it contains individual 18S-5.8S-28S rRNA units at loci on seven of the chromosomes [96]. The sequence of the particular rRNA genes is distinct in the different units and the expression of each unit is developmentally regulated, depending on the stage of the parasite life cycle [107]. It is anticipated that by transcribing different rRNAs at different life stages, the parasite could change its ribosomal properties and the translation rate of all or specific messenger RNA (mRNA), which could alter the cell growth rate or cell development pattern. Previously, the rRNA expressed in the mosquito was described as S (sexual)-type and that expressed in the human host as A (asexual)-type [96]. Parasite rRNA is also species-specific and can be assessed for diagnostic purposes [36].

More than 60% of the predicted 5268 open reading frames (ORFs) of *P. falciparum* have no sequence similarity to genes from other sequenced organisms [96]. The absence of sequence similarity complicates characterisation of the unknown ORFs, but might hold the answer to finding selective drug targets [29]. There is currently a dedicated initiative aimed at improving the annotation status of *P. falciparum* led by the *Plasmodium* database, PlasmoDB (www.plasmodb.org).

1.10.2 *Plasmodium* transcriptome data

Whole genome transcriptional profiling of the *P. falciparum* life cycle was performed in two concurrent studies in 2003 [29, 108]. Bozdech and colleagues published a high-resolution transcriptome analysis of the IDC of highly synchronised HB3 parasites over 48 h at 1 h intervals [29]. Le Roch and colleagues presented the transcriptome of nine parasite stages, including the mosquito salivary gland sporozoites, seven periodic asexual stage parasites (free merozoites and different stages spanning from early ring forms up to mature schizonts) and the sexual stage gametocytes, but excluding liver stage parasites [108]. However, recently a combined transcriptome and proteome survey of *P. yoelii* liver stage parasites [109] and the IDC transcriptome of *P. vivax* appeared [110].

By ordering the transcripts according to phase and frequency of gene expression (i.e. order according to expression peaks) as determined with fast Fourier transformation, Bozdech *et al.* demonstrated the unprecedented mode of transcriptional regulation of the malaria parasite with more than 75% of the transcripts produced only once per cycle just before they are required (Fig. 1.8) [29]. There is a clear relationship between transcriptional regulation and developmental progression of *P. falciparum* through the IDC. In contrast with the cell cycle of *Saccharomyces cerevisea* and human HeLa cells, where only 15% of the genome is periodically regulated, the IDC resembles early development of *Drosophila melanogaster* when 80% of the genome is expressed. The continuous cascade of *P. falciparum* expression starts with genes involved with generalised cellular processes such as protein synthesis, followed by DNA replication and ending with genes encoding proteins required for invasion [29]. In contrast with the polycistronic gene expression of related organisms such as *Leishmania*, contiguous genes along the nuclear chromosomes are rarely co-regulated in *P. falciparum*, whereas expression from the apicoplast genome is polycistronic and highly co-regulated [29]. Both the mitochondrion and apicoplast are thought to have a prokaryotic origin via evolutionary endosymbiotic events [111] and the maturation and protein expression of these organelles appear to be synchronised to the second half of the IDC [29].

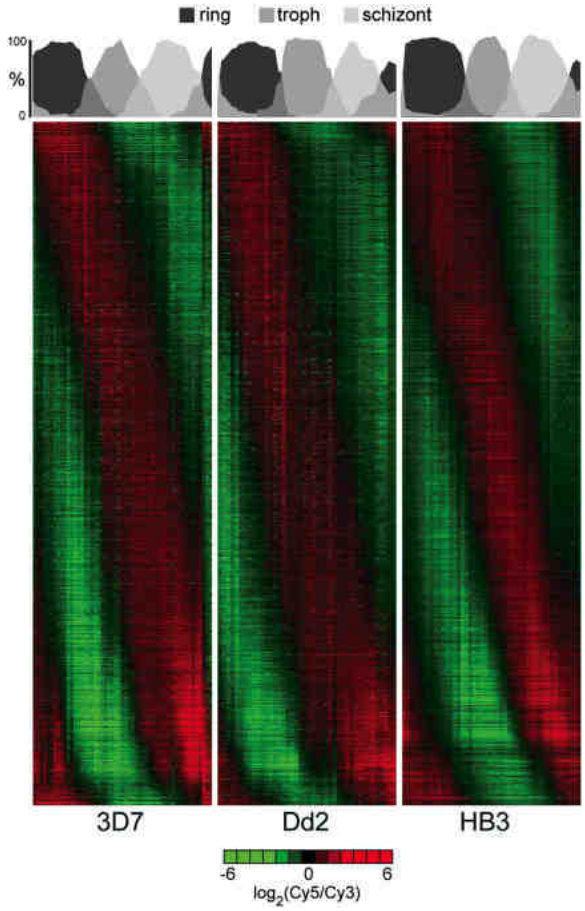

Fig. 1.8 The 48 h IDC transcriptomes of 3D7, Dd2 and HBR *P. falciparum* demonstrating the “”just in time” mode of transcription across the three strains with differential expression mainly in subtelomeric regions [91].

Le Roch and colleagues demonstrated the stage-specific gene expression of different parasite stages and that the transcripts of genes with similar functions had similar expression profiles, therefore clustering together. Based on this observation they claimed to identify the potential roles of more than 1000 hypothetical proteins. They also found that genes that were involved in similar processes, such as growth and maintenance, were localised in specific chromosomal regions [108], in contrast to Bozdech and colleagues who found that contiguous genes are rarely co-regulated [29].

The IDC transcriptome of strain HB3 was subsequently followed by a comparative analysis of the IDC transcriptomes of strains HB3, Dd2 and 3D7, each of which was originally derived from different geographical regions and with distinct drug sensitivity phenotypes (Fig. 1.8) [91]. Surprisingly, there was little difference between the transcriptional profiles obtained for the three strains and the main differences were in the genes coding for surface antigens [91].

Similar to *P. falciparum*, the IDC of *P. vivax* is characterised by extensive transcriptional control and each biological function is timed to a specific period of the IDC. The IDC expression profiles of most of the *P. vivax* genes are identical to those of their *P. falciparum* syntenic orthologues, but there are partial shifts in the transcriptional profiles of 22% of genes and dramatic differences in those of 11% of genes. These changes

result in significant alteration in the timing of specific biological functions such as haemoglobin degradation, host-parasite interaction, protein export and DNA replication. Moreover, the non-syntenic *P. vivax* genes are predominantly activated at the schizont/ring transition, which indicates that the inter-species differences may have derived from events during invasion and early intraerythrocytic development [110].

Since publication, the transcriptome data of the 2003 *P. falciparum* studies have proven indispensable as reference of the expected gene expression during normal parasite development and as baseline in comparative studies of environmental perturbation and genetic alteration.

1.10.3 The *P. falciparum* proteome and interactome

A comprehensive study of the *P. falciparum* sporozoite, merozoite, trophozoite and gametocyte proteomes appeared in the same year as the *P. falciparum* genome sequence [96, 112]. Over 2415 proteins were confidently identified with multidimensional protein identification technology (MudPIT) among these stages, which is almost half of the predicted ~5300 proteins of the parasite. The majority of the proteins expressed in each stage correlated well with the stage physiology, but surprisingly several of the antigenically variant *var* and *rif* genes, known to be expressed on the erythrocyte surface, were also detected in sporozoites [112].

The sporozoite proteome is markedly different from the other stages with approximately 49% unique proteins, mainly including cell surface (e.g. host cell invasion peptides from *var* and *rifin* genes) and organellar proteins. Only 25% of the sporozoite proteins are also expressed in other parasite stages. Merozoites, trophozoites and gametocytes all have between 20% and 33% unique and between 39% and 56% common proteins. The shared proteins are mostly involved with housekeeping functions, e.g. transcription factors, histones, ribosomal proteins and cytoskeletal proteins [112]. It appears as if the specific stage proteomes include those proteins that are required to survive the circumstances to which a particular stage is exposed, e.g. the merozoite proteome includes abundant proteins for cell recognition and invasion (via active actin-myosin processes), the trophozoites express proteins to mediate cytoadherence (knob-associated HRP) and haemoglobin digestion, the gametocyte proteome includes enzymes from the mitochondrial tricarboxylic acid cycle and oxidative phosphorylation as an adaptation to life within the mosquito, whereas the asexual blood stages are mostly dependent on anaerobic respiration (glycolysis and pyruvate-lactate conversion). Survival of *P. falciparum* parasites under a variety of complex circumstances, such as vertebrate/invertebrate and intracellular/extracellular environments, thus requires specialised protein expression at specific stages [112].

In total, 46% of the predicted gene products were successfully identified in one of the four *P. falciparum* stages examined. In an attempt to infer protein function on a global scale, including many of the unidentified proteins, the *P. falciparum* interactome was modelled by integrating *in silico* and functional genomics data within a

Bayesian framework [113]. The resulting network covered 68% of the genome and protein function for more than 2000 unidentified proteins could be inferred, based on association [113].

In another investigation, comparative analysis of protein network topologies and the controlling, highly connected nodes indicated an evolutionary conservancy and homogeneity of biological networks. The *P. falciparum* interactome network was analysed by combining experimental results (*P. falciparum* interactome with genomic and proteomic data from other well-studied model organisms) and those of computational methods [114]. Functional clustering of the combined network of protein interactions revealed clusters of proteosomal, ribosomal and spliceosomal activities that were previously reported lacking in the experimental interactome dataset [115]. Moreover, the controlling nodes in the *P. falciparum* interactome were shown to feature an oligoarchy of highly interacting proteins, indicating the presence of the so-called 'rich-club' phenomenon, where controlling nodes are well connected to one another [114]. These results signify a topological signature in the parasites' interactome, which covers parasite-specific biological features mainly revolving around invasion [61].

1.10.4 The *P. falciparum* metabolome

Very little is known regarding global *Plasmodium* metabolism or the metabolome, but recently two-dimensional nuclear magnetic resonance (2D-NMR) was used to identify and quantitate more than 50 metabolites using four different extractants with varying degrees of hydrophylicity/lipophylicity from isolated mature *P. falciparum* trophozoites [116]. The metabolite profiles obtained were generally similar, but perchloric acid was found to provide the most comprehensive metabolite profile. The isolated trophozoites contained significant amounts of various amino acids (from haemoglobin digestion), including millimolar levels of 4-aminobutyrate (GABA). Malate and most of the other tricarboxylic acid cycle intermediates were also detected, which indicated a role of oxidative energy metabolism in the trophozoite [116] in contrast with the postulation of ubiquinone regeneration and pyrimidine synthesis being the only function of the mitochondrion during the asexual stage [102]. Furthermore, glutathione, phospholipid precursors (phosphocholine and phosphoethanolamine) and polyamines were found to be major intracellular metabolites [116].

Despite the limited information on the *P. falciparum* metabolome, the integration of parasite genomic data (i.e. enzyme homologues) with known metabolic processes from the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) enabled the construction a parasite-specific website of metabolic processes, Malaria Parasite Metabolic Pathways (MPMP, http://sites.huji.ac.il/malarial/). KEGG maps were regarded as valid if homologues for three to four consecutive enzymes could be identified in the *P. falciparum* genome or if biochemical evidence indicated enzyme functionality. However, for most pathways the enzymes involved have not yet been tested independently. The metabolic maps also indicate the stage-dependent

transcription of the genes involved as transcriptomics clocks, which reveals whether genes that code for enzymes from the same biological process are coordinated in their expression or not [117].

1.10.5 Gene regulation in *P. falciparum*

Characteristic eukaryotic transcription requires the assembly of a multisubunit pre-initiation complex at the site of the promoter. RNA polymerase II is not able to locate and bind promoters in itself, but combines with general transcription factors (TFIIA, B, D, E, F and H) to form a pre-initiation complex that can bind to the DNA [118]. Initial searches with similarity-based clustering identified all 12 subunits of RNA polymerase II in the *P. falciparum* genome, but these initial experiments failed to find the general transcription factors except for the TATA-binding protein [119]. More recent analyses using search algorithms based on secondary structure overcame many of the computational challenges presented by the numerous AT-stretches resulting in low-amino-acid complexity regions, and identified homologues to many of the outstanding general transcription factors [120]. The extreme A+T-richness of the *P. falciparum* genome, which is more than 90% in intergenic regions, may indicate a unique set of binding interactions that underlie transcriptional control in the parasite, e.g. there is a positive correlation to the degree to which nuclear factors bind specific regions of the calmodulin promoter and the length of the poly(dA)poly(dT) strethes within that region [121].

Recently an expanded family of Apicomplexan Apetala2 (ApiAP2) transcription factors were discovered *in silico* in the genomes of four *Plasmodium* species [122], followed by experimental evidence of the DNA-binding specificities of 2 of the 26 ApiAP2 members in *P. falciparum* [123]. Subsets of these proteins are expressed throughout the IDC i.e. in the ring, trophozoite, early-schizont and late-schizont stages, and this cascade was proposed to regulate stage-specific transcription within the parasite [123].

Similar to other eukaryotic organisms, *P. falciparum* uses epigenetic mechanisms to control phenotypic states of inheritable expression, e.g. in the absence of DNA modification, the active or silenced state of *var* gene expression is inherited by daughter parasites in subsequent IDC cycles [124]. Epigenetic machinery is generally well conserved in *Plasmodium* and all four core histones required for nucleosome assembly (H2A, H2B, H3 and H4), as well as the variant histones (H3.3, CenH3, H2AZ and H2Bv), have been identified in the genome [125]. The dynamic nature of nucleosome-associated chromatin is attributed to various post-translational modifications on the N-terminal tails of the different histones. These modifications affect chromatin structure by altering interactions between histones and DNA or by affecting the recruitment of chromatin-associated proteins. Acetylation of lysines and methylation of lysines and arginines on histones H3 and H4 have been studied best and all of these modifications are found on critical residues of *Plasmodium* histones. Modified nucleosomes can mediate transcriptional control by altering the physical interactions between histones and DNA or by binding other regulatory proteins. However, the mere presence of

nucleosomes within the genome is an indication of decreased transcriptional activity and active promoters are usually located in "nucleosome-free" regions [118].

Reversible modification of DNA provides another mechanism for epigenetic control of gene expression, which typically involves methylated bases e.g. 6-methyl-2-deoxyadenine (6mA) or 5-methyl-2-deoxycytosine (5mC). Previously, partial methylation of a CpG dinucleotide was demonstrated in the DHFR/TS gene during the IDC [126] and *in silico* analysis revealed a greater than expected incidence of CpG dinucleotides in intergenic regions less than 500 bp from genes [127]. However, in contrast to these results, liquid chromatography/electron spray ionisation mass spectrometry (LC-ESI/MS) could not detect any methylation of 2-deoxycytosine bases within *P. falciparum* genomic DNA (gDNA) [124].

In recent years there have been controversial reports regarding the role of transcriptional regulation versus post-transcriptional control in *Plasmodium* [128]. The evidence supporting both sides of the debate will be discussed in detail in Chapters 3 and 4.

1.10.6 Manipulation of the *Plasmodium* genome

The –omics technologies (transcriptomics, proteomics, metabolomics etc.) form part of the functional genomics platform, which also includes gene manipulation. Genetic manipulation via transfection is a powerful method to establish gene function and enables the identification of genes responsible for specific phenotypes (forward functional genomics) [129]. Initial attempts at *P. falciparum* transfections were unsuccessful because of technical difficulties [130], but current transfection technology of *Plasmodium* has improved significantly and several papers reporting success have been published in recent years [129, 131, 132]. RNA interference (RNAi) is still a highly controversial issue, since there is currently no evidence for the homologues of the RNAi machinery within the parasite [133].

1.11 FUNCTIONAL GENOMICS FOR DRUG DISCOVERY AND TARGET VALIDATION

The availability of full genome sequencing and microarrays late in the 1990s was accompanied by enthusiasm that these technologies would aid in identifying drug targets of small molecules. This anticipation was based on early experiments in which it was demonstrated that treatment with particular drugs resulted in the altered transcription of the pathways containing these drugs' targets due to feedback inhibition [134, 135]. However, deciphering the significance of the large amount of data obtained from microarray studies is the biggest challenge of this method [136] and many of these early studies simply downplayed the fact that the transcription of many other pathways were also affected [135]. Yet, despite scepticism, the application of such whole genome approaches has been useful in elucidating the mechanism of drug action and resistance for a number of unicellular organisms [137-139]. Antibacterial drug discovery in particular has benefited greatly from the application of functional genomics techniques (especially transcriptomics), which improved knowledge of

gene function, bacterial physiology, the effects of antibiotics on bacterial metabolism, *in vitro* target identification and the mode of action of novel antibacterial compounds [140]. Therefore, functional genomics has now become an indispensable tool in the drug discovery process [141, 142] due to its capacity to monitor the effects of perturbations at a global level, as opposed to the molecular level of the more traditional methods. In the urgent search for novel antimalarials, the introduction of functional and structural genomics and bioinformatics at the very early stages of the discovery pipeline can accelerate the discovery of new and robust antimalarial drugs and novel targets and serve to validate these targets (Fig. 1.9) [61, 143, 144].

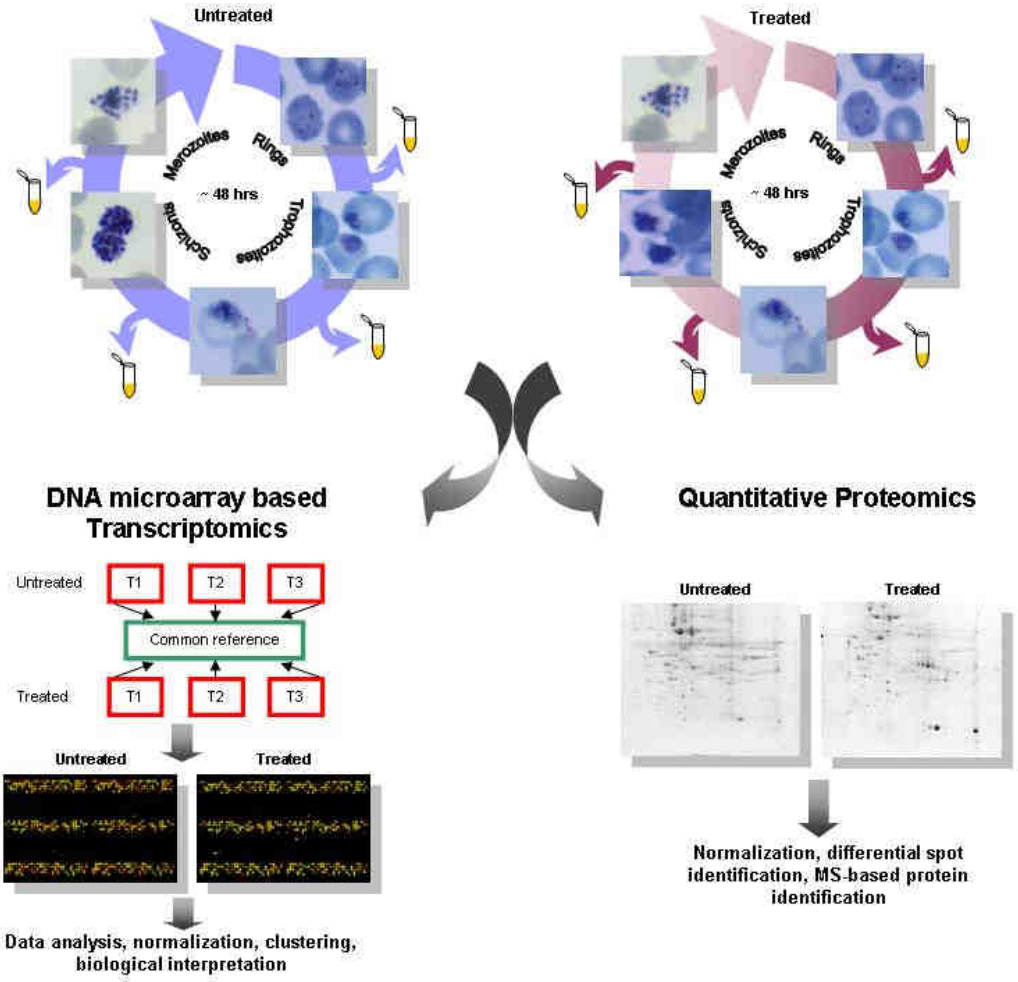

Fig. 1.9 The proposed experimental layout for the application of transcriptome and proteome analysis to drug-challenged malaria parasites. Duplicate, synchronised *P. falciparum* asexual cultures should be maintained in parallel for untreated and drug treated cultures. Simultaneous RNA and protein isolation should be performed at the highest frequency possible. Changes in transcript levels can be compared to proteome changes with e.g. quantitative 2D-gel electrophoresis [61].

1.12 RESEARCH OBJECTIVES

The study presented here was aimed at elucidating the physiological response of the malaria parasite during cytostasis as a result of polyamine depletion induced by the co-inhibition of both catalytic sites of the bifunctional protein PfAdoMetDC/ODC. To achieve this goal, polyamine biosynthesis inhibitors were carefully selected and characterised to ensure complete inhibition of PfAdoMetDC/ODC and a functional genomics investigation was conducted, which included the global profiling of the transcriptional, proteomic and

metabolomic response of the perturbed 3D7 *P. falciparum* parasites. The ultimate objective of the investigation was to validate PfAdoMetDC/ODC as a potential drug target for antimalarial therapeutic intervention.

Chapter 2 describes the determination of the median inhibitory concentrations (IC_{50}) of various inhibitors of PfAdoMetDC/ODC. The results obtained were used to determine the treatment dosages and most appropriate inhibitors to be used for co-inhibition during the functional genomics investigations.

Chapter 3 presents the transcriptional response of *P. falciparum* after co-inhibition of both catalytic sites of PfAdoMetDC/ODC with specific inhibitors and cytostatic drugs, as determined via oligonucleotide microarray analysis.

Chapter 4 describes the proteomic and metabolomic analyses of PfAdoMetDC/ODC co-inhibited parasites and completes the functional genomics investigation by examining specific hypotheses resulting from the study with focussed biochemical assays.

Chapter 5 presents a concluding discussion of the scientific contribution of the study, the most important research highlights and the future perspectives.

The knowledge gained from this study has led to the following contributions in scientific journals and at conference proceedings:

Papers:

Van Brummelen A.C., Birkholtz L-M and Louw, A.I. (2008) A critical evaluation of antimalarial drug sensitivity methods for testing cytostatic drugs (Manuscript in preparation).

Williams M., Niemand J., van Brummelen A.C., Clark K., Wells G., Burger P., Reeksting S., Birkholtz L-M and Louw A.I. (2008) Polyamines in *Plasmodium*: peculiarities and possibilities (Manuscript in preparation).

Van Brummelen A.C., Olszewski K.L., Wilinksi D., Llinás M., Louw A.I and Birkholtz L-M. (2008) Co-inhibition of *Plasmodium falciparum* S-adenosylmethionine decarboxylase/ornithine decarboxylase reveals perturbation-specific compensatory mechanisms by transcriptome, proteome and metabolome analyses (*J. Biol. Chem, 10.1074/jbc.M807085200, In Press*).

Birkholtz L-M., van Brummelen A.C., Clark K., Niemand J., Maréchal E., Llinás M., Louw A.I. (2008) Exploring functional genomics for drug target and therapeutics discovery in *Plasmodia*. Acta Trop. **105**, 113-123.

Conference proceedings:

Birkholtz L-M., van Brummelen A.C., Clark K. and Louw A.I. (2008) Functional genomics investigations of polyamine depleted *Plasmodium falciparum* reveal compensatory responses and novel metabolic activities (poster). Polyamines: Forty years of mammalian ornithine decarboxylase, Kuopio, Finland.

Van Brummelen A.C., Wilinski D., Llinás M., Louw A.I. and Birkholtz L-M. (2008) Co-inhibition of S-adenosylmethionine decarboxylase/ornithine decarboxylase of *Plasmodium falciparum* reveals compensatory mechanisms in the transcriptome (poster). Molecular Approaches to Malaria, Lorne, Australia.

Van Brummelen A.C., Llinás M., Wilinski D., Louw A.I. and Birkholtz L-M. (2007) Transcriptional profiling of polyamine depletion in *Plasmodium falciparum* (paper). Molecular and Cell Biology Group Symposium (MCBG), Pretoria, South Africa. Best oral presentation award.

Van Brummelen A.C., Birkholtz, L-M and Louw, A.I. (2006) Comparative transcriptomics for target validation of plasmodial AdoMetDC/ODC (poster). The 11th International Congress of Parasitology, Glasgow, Scotland.

Van Brummelen A.C., Birkholtz, L-M and Louw, A.I. (2006) Critical evaluation of antimalarial drug sensitivity methods for cytostatic compounds (poster). 20th Meeting of the South African Society for Biochemistry and Molecular Biology (SASBMB), Pietermaritzburg, South Africa.