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EFFECTS OF DNA DAMAGE AND VESICULAR EXCHANGE IN P. FALCIPARUM

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Stockholm 2019

Cover illustration: Single-cell technology, Antimalarial drugs activity and exosomes

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Published by Karolinska Institutet.
Printed by Eprint AB 2019

ISBN 978-91-7831-284-9

EFFECTS OF DNA DAMAGE AND VESICULAR EXCHANGE IN P. FALCIPARUM

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”**— Marie Curie**

ABSTRACT

Plasmodium falciparum causes human malaria and is a global leading cause of mortality from parasitic infections. While decades of concerted effort has yielded significant result in reducing the endemicity, there is evidence of a recent resurgence in transmission intensity.

Notably, *Plasmodium falciparum* parasites are master adapter to various environmental pressures, including stresses mounted by the host immune system as well as assaults from an arsenal of anti-malarial drugs. Intuitively, this adept adaptability constitutes a major impedance to a continued success in the control program. The mechanisms that confer such a high adaptability to parasite is currently unclear, but the plastic AT-rich genome, a versatile but enigmatic transcription network as well as the recently characterized cell-cell communication module via extracellular vesicles are likely contributors to such cause. Through experimental designs that sought to investigate the dynamics of these aforementioned molecular and cellular programs on both population and single-cell level, the works presented in this thesis aim to further the current understandings on how the parasites respond and adapt to various environmental pressures.

In paper I, we investigated the short-term effect of anti-malarial drugs on the genomic plasticity of parasite. We revealed that all the tested anti-malarial drugs can cause acute DNA damages and trigger a robust response to the damages by the upregulation of specific DNA repair pathways. We further demonstrated that the DNA damages elicited by the drug action can be erroneously repaired to incorporate random mutations. Therefore, due to the random nature of DNA damages and the subsequent error-prone repair, this can serve as a mechanism to rapidly diversify the genetic pools of the exposed parasite population and pre-deposit it to a rapid selection of resistant genotypes.

In paper II, we described the role of extra-cellular vesicles (EVs) during malaria infection. Infected RBCs are previously characterized for their enhanced capacity to generate EVs. In this study, we discovered that EVs originated from infected cell contain a subset of host miRNAs and the Ago2 proteins. These EVs are readily internalized by endothelial cells and that the miRNAs trafficked within these EVs can accumulate in the endothelial cells and exert a global effect on the post-transcriptional gene regulatory network. We show that this unilateral cellular communication can contribute to vascular dysfunction, local and systemic immunological modulation as well as endothelial activation. In particular, we note that endothelial activation can promote sequestration of infected RBCs and, thereby, serve to avoid splenic clearance.

In paper III, we developed and detailed a technical platform that enables single-cell transcriptomic analysis of individual *Plasmodium falciparum* parasites. We then utilized the method to decipher the transcriptional cascade underlying the process of gametogenesis, which is triggered by yet undetermined environmental cues. Interestingly, we revealed huge heterogeneity even within a highly synchronous parasite population, supporting the presence of a versatile transcriptional network. Moreover, we identified a distinct gene signature that is

associated with sexually committed and differentiating parasites. This work has generated important knowledge that can be exploited for the design of transmission blocking drugs.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers:

I. **Hjelmqvist D,** Hedberg P,Talavera López C, Md Idris Z, Ankarklev J, Kirkman L, Diestch K, Ribacke U, Chan S, and Wahlgren M. "Antimalarial drugs and DNA damage response in *Plasmodium falciparum."*
Manuscript

II. Mantel P-Y, **Hjelmqvist D**, Walch M, Kharoubi-Hess S, Nilsson S, Ravel D, Ribeiro M, Gruring C, Ma S, Padmanabhan P, Trachtenberg A, Ankarklev J, Brancucci NM, Huttenhower C, Duraisingh MT, Ghiran I, Kuo WP, Filgueira L, Martinelli, Marti M (2016). "Infected erythrocyte-derived extracellular vesicles alter vascular function via regulatory Ago2-miRNA complexes in malaria." Nature Communications 7 (1): 12727.

III. Ngara M, Palmkvist M, Sagasser S, **Hjelmqvist D**, Björklund P K, Wahlgren M, Ankarklev J, Sandberg R (2018). "Exploring parasite heterogeneity using single-cell RNA-seq reveals a gene signature among sexual stage Plasmodium falciparum parasites." Exp Cell Res **371**(1): 130-138.

The following publications were also obtained during the course of the PhD studies but are not included in this thesis:

I. Pellé KG, Jiang RH, Mantel PY1, Xiao YP, **Hjelmqvist D**, Gallego-Lopez GM, O T Lau A, Kang BH, Allred DR, Marti M. Shared elements of host-targeting pathways among apicomplexan parasites of differing lifestyles. Cell Microbiol. 2015 Nov;17 (11): 1618-39.

II. Gulati S, Ekland EH, Ruggles KV, Chan RB, Jayabalasingham B, Zhou B, Mantel PY, Lee MCS, Spottiswoode N, Coburn-Flynn O, **Hjelmqvist D**, Worgall TS, Marti M, Paolo GD, and Fidock DA. "Profiling the Essential Nature of Lipid Metabolism in Asexual Blood and Gametocyte Stages of Plasmodium falciparum." Cell Host Microbe. 2015 Sep 9; 18(3): 371–381.

INNEHÅLLSFÖRTECKNING

LIST OF ABBREVIATIONS

A-NHEJ	Alternative-non homologous end joining
AB	Apoptotic body
ADR	Adverse drug reaction
AQ	Amodiaquine
ARMD	Accelerated resistance to multiple drugs
ART	Artemisinin
ATS	Artesunate
BER	Base excision repair
BMEC	Bone marrow-derived endothelial cell
C-NHEJ	Canonical-non homologous end joining
CDC	Centre of disease control
CNV	Copy number variants
CQ	Chloroquine
DDI	Drug –drug interaction
DCF-DA	Dihydrodichlorofluorescein diacetate
DHA	Dihydroartemisinin
DSB	Double-strand break
EM	Electron microscopy
EV	Extracellular vesicle
EXP	Exported protein
GSH	Glutathione
HR	Homologous recombination
HRR	Homologous recombination repair
iRBC	infected red blood cell
IDC	Intraerythrocytic cycle
IFA	Immunofluorescence assay

INDEL	Small insertions and deletions
miRNA	Micro RNA
MAC	Magnetic-activated cell sorting
MDA	Mass drug administration
MMEJ	Micro-homology end joining
MMR	Mismatch repair
MP	Micro-particles
MV	Micro-vesicles
NER	Nucleotide excision repair
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
OTM	Olive tail moment
PPQ	Piperaquine
RBC	Red blood cell
RISC	RNA-induced silencing complex
RNAi	RNA interference
ROS	Reactive oxygen species
scRNA-seq	Single cell RNA sequencing
SNP	Single nucleotide polymorphism
SV	Structural variants
TCA	Tricarboxylic acid
TEER	Transepithelial electrical resistance
UPR	Unfolded protein response
WHO	World Health Organization

1 INTRODUCTION

1.1 MALARIA AND PUBLIC HEALTH

Malaria remains one of the most devastating infectious diseases in the tropics, although global eradication efforts in the past decade have decreased the number of deaths. There is still an estimated 219 million cases and 435 000 related deaths of malaria in 2017 according to the WHO´s 11th World Malaria Report (WHO, 2018). These deaths are primarily cause by *Plasmodium falciparum* in African children under five years of age and pregnant women. Mortality among young children is due to severe malaria.

Cerebral malaria, hypoglycemia and severe anaemia are the common features of severe malaria. Malaria is a disease of poverty and the impact of malaria takes its toll mostly in poor, tropical and subtropical areas of the world. African countries bear the heaviest burden of malaria due to *Anopheles gambiae* mosquito which is responsible for high transmission according to the Centers for Disease Control and Prevention (CDC) and WHO.

Malaria is still one of the most important challenges to global public health and in order to be ultimately controlled and eliminated, the economic and social conditions need to be addressed. Moreover, the development of resistance to antimalarial drugs also compromises control and elimination strategy.

1.2 THE *PLASMODIUM* PARASITE AND THE LIFE CYCLE

Parasitic infections of the erythrocytes are among the most common and oldest infections in vertebrates. Malaria is caused by protozoan parasites called *Plasmodia* that belong to the apicomplexa parasites. *Plasmodium* is a genus of unicellular eukaryotes and has a complex and multistage life cycle occurring within two hosts, the vector mosquitoes and the human hosts. There are more than 250 species of *Plasmodium* of which five infect humans. *Plasmodium falciparum* is the predominant species in tropical and subtropical areas especially in sub-Saharan Africa and is responsible for the majority of deaths due to malaria (White, Pukrittayakamee et al. 2014).

P. falciparum can cause severe malaria as it quickly reproduces in the blood and cause anemia. Subsequently, the infected erythrocytes may coagulate in small blood vessels and when this happens in the brain the outcome may be deadly (CDC). *P. vivax* is predominant in South East Asia, Latin America and in some part of Africa (Autino, Noris et al. 2012). *P. ovale* is principally widespread in West Africa and the islands of western Pacific. Two distinct sub species of *P. ovale* have recently been described namely *P. ovale walikeri* and *curtisi* (Sutherland, Tanomsing et al. 2010). *P. malariae* is found worldwide but at low prevalence and is the only species that has three-day cycle. Most of the malaria parasite species have a two-day cycle. *P. malariae* can cause chronic infection and leads to serious complications if untreated. *P. knowlesi* is present in South East Asia particularly in Malaysia and causes malaria in long-tailed and pig-tailed macaques but it has also been shown to infect humans.

P. knowlesi has a 24-hour cycle and can rapidly progress from uncomplicated to a severe infection (White, Pukrittayakamee et al. 2014).

The life cycle of malaria parasites that infect humans is complex and involves two hosts, human and mosquito vector. The infected female *Anopheles* mosquito injects sporozoites into the human host during a blood meal. Subsequently, motile sporozoites travel to the liver and infect hepatocytes. Inside the hepatocytes each sporozoite matures into schizonts and multiplies to produce 8-32 merozoites. Upon rupture of the hepatocytes the merozoites infect erythrocytes. The parasites undergo asexual multiplication in the erythrocytes (Fig. 1).

The parasites go through several stages, starting as ring stage, developing into trophozoites and finally becoming mature schizonts, which then rupture the erythrocyte and release daughter merozoites. A small fraction of merozoites form sexual gametocytes, that is able to transmit from human to mosquito vector. Gametocyte maturation is measured in stages. Stages I-IV are referred to as immature gametocytes and are sequestered in the bone marrow. Only stage V mature gametocytes circulate in peripheral blood and are taken up by a feeding mosquito. Each gametocyte forms one female macrogamete and/or up to eight male microgametes (Cowman et al. 2002).

In the mosquito mid-gut the microgamete fuses with the macrogamete, producing a zygote that develops into a motile ookinete. The ookinete penetrates the mid-gut wall and forms an oocyst, which divides and bursts to release sporozoites that migrate to the mosquito salivary gland. The sporozoites can be injected into a new host during a blood meal (Cowman et al. 2002).

The life cycle between *Plasmodium* species is similar, with some distinguishing differences. In *P. vivax* and *P. ovale,* sporozoites may not follow the same multiplication rate as in *P. falciparum* and stay dormant (hypnozoites) in the liver, causing relapse of malaria between two weeks to more than one year after initial infection (CDC, White, Pukrittayakamee et al. 2014). The exoerythrocytic stage do not cause a disease and does not generate symptoms or signs of the disease. The pre-erythrocytic phase takes 5-7 days for *P. falciparum*, 6-8 days for *P. vivax*, 9 days for *P. ovale* and 14-16 days for *P. malariae.* The asexual replication cycle in the erythrocytes takes 48 hours for *P. falciparum* and *P. vivax*, 50 hours for *P. ovale* and 72 hours for *P. malariae* (Bousema, Okell et al. 2014).

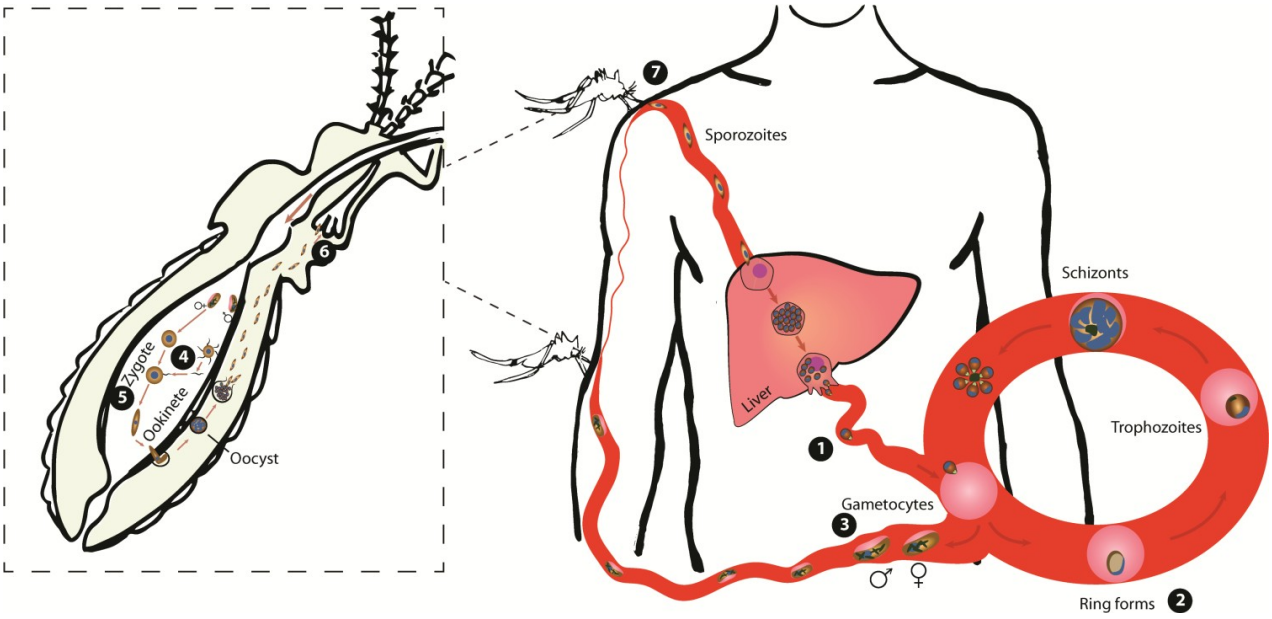


Fig. 1. The life cycle of *P. falciparum.* *Plasmodium* parasites life cycle involves two hosts, the mosquito vector (left) and the human hosts (right). Malaria infection starts with the transmission of *Plasmodium* parasites from a female *Anopheles* mosquito vector and to human host. Approximate number of parasites in each host at each stage (1=~10^4-10^5 (Merozoites), 2=~10^{10}-10^{12} (ring stage), 3=~10^8-10^{10} (gametogenesis), 4=~10^2-10^4 (female & male gametes), 5=~1-10 (Oocyst), 6=~10^4-10^5) (sporozoites in mosquito) and 7=~1-10^2 (sporozoites in human host) are shown to emphasize the enormous expansion that occur during the parasite life cycle. In the mosquito, during the sporogony process in an oocyst, *Plasmodium* parasites undergo several rounds of DNA replication (meiotic and mitotic replication) to produce thousands of haploid sporozoites that infect human host. In the human host, parasite DNA replication occurs in hepatic schizogony, erythrocytic schizogony and gametogenesis. *Plasmodium* parasites DNA replication is asynchronous and can generate an array of sister chromatids up to ~24n. Illustration by Madle Sirel.

This thesis will focus on the deadliest form of the malaria parasite, P. falciparum, which is responsible for the vast majority of the morbidity and death especially among children under the age of five. P. falciparum is also most closely associated with drug resistance and hence responsible for the majority of the global malaria burden.

1.3 PATHOGENESIS OF *PLASMODIUM FALCIPARUM* MALARIA

The pathogenesis of *P. falciparum* infection is a multifaceted interaction of parasite-induced erythrocyte alterations and microcirculatory abnormalities. Clinical manifestations of *P. falciparum* infection are generated by the asexual erythrocytic of blood stage parasites. During the asexual and sexual intraerythrocytic development of *P. falciparum*, numerous molecular processes cause the alteration of uninfected and infected erythrocytes, but how these alterations become pathogenic is not completely understood. Splenomegaly is a common feature of endemicity in *P. falciparum* transmission areas and it correlates with a decrease hemoglobin level (Snow et al. 1997). Patients who died from severe malaria have

larger spleens than patients who died from sepsis (Urban et al. 2005). However, splenic rupture due to malarial splenomegaly is uncommon (Imbert et al 2010). In uncomplicated malaria patients with splenomegaly, the clearance of heat-stiffened autologous red blood cells (RBCs) was increased before antimalarial drug treatment (White et al. 2017, Looareesuwan et al. 1987). The destruction of labeled autologous RBCs was markedly increased in patients with severe malaria. Other malaria infection symptoms are severe anemia due to recurrent infections, premature delivery or delivery of a low-birth-weight infant during pregnancy, neurologic defects such as ataxia, palsies, blindness, deafness and speech difficulties, which may sometimes persist especially in children who have cerebral malaria (cdc.gov).

1.3.1 Uncomplicated malaria

Malaria disease can be classified as uncomplicated and complicated malaria. Uncomplicated malaria can be caused by all *Plasmodium* species and patients have circulating parasites but no severe symptoms. Generally, symptoms arise 7-10 days after initial mosquito bite. Clinical manifestation of uncomplicated malaria includes sensation of cold, shivering, fever, headaches, nausea, vomiting, seizures in young children, sweating and general malaise. Diagnosis of malaria depends on the confirmation of parasites in the erythrocyte, typically by microscopy. Other laboratory finding might include mild decrease in blood platelets, bilirubin elevation, mild anemia and aminotransferases elevation (CDC.gov., Mawson et al. 2013).

1.3.2 Severe malaria

Clinical manifestations of severe *P. falciparum* infections depend on the nature and severity and differential diagnosis from other conditions. Severe malaria is often linked to hyperparasitemia and is associated with increased mortality. Clinical manifestations of severe malaria include cerebral malaria, severe anemia and hemoglobinuria due to hemolysis, acute respiratory distress syndrome, abnormalities in blood coagulation, acute kidney injury, cardiovascular collapse, hyperparasitemia, metabolic acidosis, and hypoglycemia (low blood glucose). Cerebral malaria is a medical emergency that demands urgent treatment. Pathogenesis of cerebral malaria is caused by vascular endothelium damage through parasite sequestration, generation of inflammatory cytokine and vascular leakage, which results in brain hypoxia (Beeson et al. 2002, Yusuf et al. 2017). In children who have cerebral malaria, neurologic defects such as ataxia, palsies, blindness, deafness, and speech difficulties may sometimes persist. Patients with impaired immunity including pregnant women and HIV positive patients are most susceptible to severe malaria. However, if these patients with impaired immunity survive the repeated infections, more or less semi-immunity to malaria is developed over the years. Such immunity can neutralize the parasites through various mechanisms. Severe malaria should be treated quickly and aggressively, if left untreated the outcome can be fatal (Beeson et al. 2002, Buffet et al. 2011, cdc).

1.4 MALARIA TREATMENT – ANTIMALARIAL DRUGS

Generally, malaria is a curable and preventable disease if diagnosed early and treated properly and promptly with effective antimalarial drugs. The length of treatment and types of drugs

vary, depending on the species of malaria parasite involved and severity of the disease. There are three major classes of antimalarial drugs: quinolines, folate antagonists and artemisinin compounds. Quinoline, quinine, mefloquine and artemisinin are effective antimalarial drugs, however, there is a serious concern that *P. falciparum* has developed resistance to almost all antimalarial drugs. There is growing pressure in maintaining the efficacy of current treatments and developing alternative treatments. Several approaches have been evaluated to overcome drug resistance, including combination therapy which involves the combination of two or more antimalarial drugs and hybridization approach which involves the combination of two distinctive pharmacophoric features from known and new antimalarial drugs to create a compound with increased efficacy against most parasite stages (Capela et al. 2011, Murugan et al. 2015, Rajendran et al. 2015, Fivelman et al. 2007). Hybrid approach is more effective compared to the combination therapy because of the lower occurrence of adverse drug reactions (ADRs) induced by drug-drug interactions (DDIs). Antimalarial drugs studied in this thesis are presented in Figure 2. In the first part of this thesis, we apply the tools of whole genome sequencing to study the factors contributing to the acquisition of drug resistance in *P. falciparum*.

1.4.1 Amodiaquine (AQ), Chloroquine (CQ) and Piperaquine (PPQ)

The importance of antimalarial drugs with the 4-aminoquinoline scaffold including AQ and CQ has inspired research centers and pharmaceutical companies to focus on designing and synthesizing new analogues of these quinoline-based antimalarial drugs (Parhizgar et al. 2017). AQ and CQ are 4-aminoquinoline derivatives and widely used as antimalarial drugs. AQ, CQ and other quinoline analogues are fast-acting and highly effective blood schizonticidal drugs against *P. falciparum*. AQ has been associated with severe cases of acute hepatitis and agranulocytosis which can be deadly. It has been used extensively to treat and prevent malaria since the 1940s. Although AQ is effective against CQ-resistant *P. falciparum* strains, its usage has been restricted due to hepatic and hematological toxicities (Parhizgar et al. 2017).

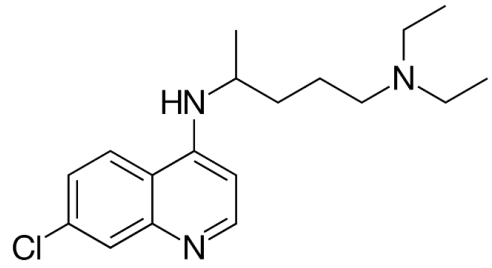
Like other quinoline compounds, AQ mechanism of action has not been fully studied, AQ seems to inhibit heme polymerase activity in the body, resulting in accumulation of free heme which is toxic to the malaria parasites (Hasugian et al. 2009). AQ is used in combination with artesunate for the treatment of infection by CQ resistant *P. falciparum* (Adjuik et al. 2002). CQ-resistant *P. falciparum* first developed in the late 1950s and 1960s in Southeast Asia, South America and Oceania (Jensen et al. 2009). The spread of malaria parasites resistant to CQ and their cross-resistance to other analogues have decreased their usage in many malaria endemic areas. Although CQ is more effective and safer than quinine as a blood schizonticidal drug, it is not active against the liver stage and mature gametocytes (Parhizgar et al. 2017). PPQ is a dimer of CQ analogue and was widely used for the treatment of CQ-resistant *P. falciparum* strains in China (Wells et al. 2010, Davis et al. 2005). PPQ consists of two 4-aminoquinoline groups which have been coupled to each other by dipiperazine-propyl linker (Parhizgar et a. 2017). PPQ activity against the resistant *P. falciparum* parasites is attributed to its bulky structure that inhibits its connection to the PfCRT (CQ resistance transporter) site.

It is known that PPQ is trapped in the food vacuole of the parasite with its positive charges and inhibits detoxification of heme in the malaria parasites. The pharmacokinetic properties of PPQ show high lipid-solubility, tolerability, good bioavailability, fast clearance and long elimination half-life. Recently, the occurrence *P. falciparum* resistance to PPQ has reduced monotherapy usage (Hung et al. 2004).

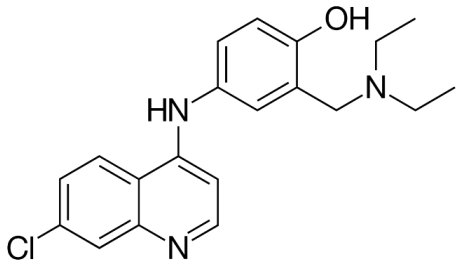
1.4.2 Artemisinin (ART), Artesunate (ATS) and Dihydroartemisinin (DHA)

ART and its derivatives are very important class of antimalarial drugs. ART or qinghaosu is a sesquiterpene lactone and is a natural compound originated from Chinese herb qinghao or *Artemisia annua* (Sweet wormwood) (Hyde et al. 2007, Tu et al. 2016). ART is effective against multi-resistant *P. falciparum* and has broad stage specificity throughout the asexual blood stages (Price et al. 1996, ter Kuile et al. 1993). ART has also been used to treat fever and hemorrhoids (Ciu et al. 2009, Maude et al. 2010) and recent studies have shown that ART and its derivatives can potentially inhibit the growth and proliferation of cancer cells (Konstat-Korzenny et l. 2018, Hou et al. 2008, Lai et al. 2013). ATS, dihydroartemisinin and artemether are the most important ART derivatives and show better efficacy, tolerability, minimal side effects and oral bioavailability than ART due to poor solubility of ART (Chen et al. 1994, Meshnick et al. 2002). ATS is the most effective ART derivative compound that can reduce parasitemia rapidly in a single 48-h erythrocytic cycle (Chen et al. 1994) and in animal experiments ATS showed anti-allergic properties by effecting mast cell degranulation which makes ATS a promising candidate for allergic asthma treatment (Cheng et al. 2013, Cheng et al. 2011). Although there are less data on DHA, but studies suggested that DHA showed rapid parasite clearance with single high-dose regimens (Djimde et al. 2009, Guo et al. 2018).

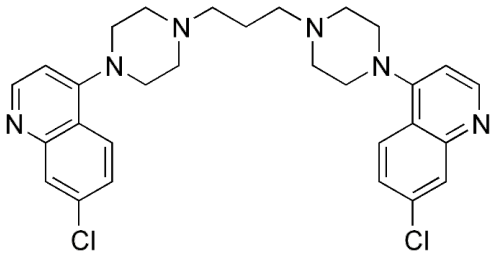
ART and its derivatives are known to act rapidly but are also eliminated rapidly. Their rapid action makes these drugs especially effective against severe malaria. Their rapid clearance could be an important factor why resistance to ART has been slow to develop, and may also explain why failures in treatment are so common when these drugs are used in monotherapy (Meshnick et al. 2002). It has been recommended that ART and its derivatives should not be used as oral monotherapies for uncomplicated malaria treatment due to poor adherence to the required 7 days of treatment, which results in partial parasite clearance that can promote resistance to this important class of antimalarial drugs (Castelli et al. 2012, Eastman et al. 2009, Tilley et al. 2016). The emergence of antimalarial drug resistance has led to an augmentation of efficiency monitoring to permit early detection of resistance. In 2015, malaria control programs are implementing ACTs (artemisinin-combination therapies) as standard treatment for *P. falciparum* malaria worldwide (WHO,2015).



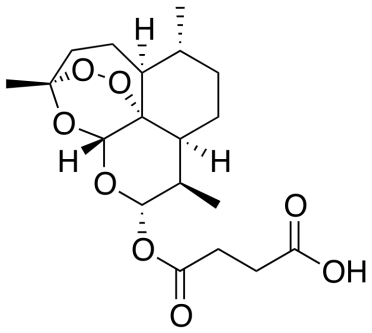
Chloroquine, C18H26ClN3



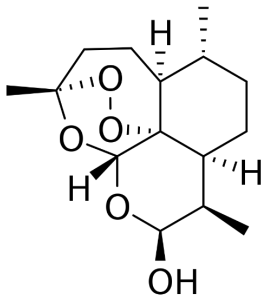
Amodiaquine, C20H22ClN30



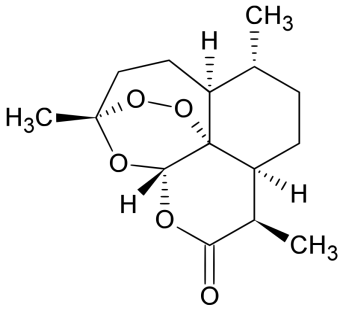
Piperaquine, C29H32Cl2N6



Artesunate, C19H28O8



Dihydroartemisinin, C15H24O5



Artemisinin, C15H22O5

Fig. 2. Molecular structures of AQ, ART, ATS, DHA AND DHA.

1.5 RESISTANCE TO ANTIMALARIAL DRUGS

Antimalarial drugs such as quinine, CQ and its analogues compounds, ART and its derivatives have been used to treat and prevent malaria. Although the use of these antimalarial drugs has long history, the occurrence of resistance to these antimalarial drugs is a somewhat new phenomenon. Resistance to antimalarial drug is defined as the capability of malaria parasites to survive and/or multiply regardless of the administration and absorption of a drug given in doses at least equal to those recommended, but within the tolerance limits of the subject (WHO, 1986). In an effort to fight resistant strains, a number of alternative synthetic antimalarial drugs were implemented to both treat and prevent malaria (Packard et al. 2014, Ashley et al. 2018, Flannery et al. 2013). The drawbacks of the new therapies were their increased cost, which made them less available to the populations at the highest risk. To prevent the development of resistance to artemisinin-based drugs, the World Health Organization (WHO) recommended the usage of ACTs. The combination of artemether along

with lumefantrine (long-acting antibiotic) proved to be 97% effective in curing the deadliest forms of *P. falciparum* malaria. WHO initially restricted the usage of these products to the treatment of complicated *P. falciparum* malaria due to the fear that wider use would contribute to drug resistance, but in 2010 the WHO reversed its policy due to the pressure from national health programs and consequently ACTs have become the first-line treatment for *P. falciparum* malaria in many endemic countries (Packard et al. 2014). However, the WHO's fear that the widespread use of ACTs would accelerate the resistance development is not unjustified. It has been reported that resistance to artemisinin-based drugs has occurred in several locations in Southeast Asia (Ashley et al. 2014). There is a tendency to view antimalarial drug resistance development as an unavoidable outcome of the drugs´ extensive use (White et al. 2004). Nevertheless, resistance to antimalarial drugs has been accelerated by the way the drugs are used and also by the economic and social circumstances in which they are used (Packard et al. 2014). It has been reported that mass drug-administration (MDA) programs may have also contributed to the increase of drug resistance and there is a strong connection between the geographic areas where MDA programs were implemented and the places where drug resistance developed (Packard et al 2014). It has been suggested that the emergence of artemisinin resistance is due to malaria parasites prone to mutation (Dondorp et al. 2010). Furthermore, an intensive campaign to eliminate ART resistance in the greater Mekong Delta region and prevent further spread of ART resistance focuses on identifying and treating to cure all cases of malaria in the region. Whether these efforts will be successful remain uncertain. In order to prevent ART-based drugs from following CQ path, efforts to address the social and economic circumstances that contribute to the spread of resistance to antimalarial drugs such as the marketing of monotherapies and cheap counterfeit drugs containing subclinical quantities of artemisinin are crucial. It is also important that investments continue to flow in the development and production of new antimalarial drugs.

1.5.1 Antimalarial drugs mode of action and resistance mechanism

Malaria parasite inherent resistance is one constituent that defines the efficacy of antimalarial drug *in vivo*. Antimalarial pharmacological properties and human immune responses play crucial roles in determining the clinical outcome of antimalarial drugs treatment.

CQ acts by interfering with the detoxification of hematin, a toxic byproduct of hemoglobin degradation, in the food vacuole of *P. falciparum*. Although CQ is no longer recommended for the treatment of *P. falciparum* parasites, it is still the drug of choice for the treatment of *P. vivax* and *P. ovale*, less severe forms of malaria which can cause recurrent infections (Travassos & Laufer 2010).

AQ plasmodial action is not completely clear. Like other quinoline analogues, it is thought to inhibit heme polymerase activity, which results in free heme accumulation that is toxic to the parasites. AQ binds to the free heme and prevent the malaria parasite from converting heme to an inactive form. AQ-heme complex disrupts membrane function (Jewell et al. 1995, Harrison et al. 1992). PPQ mechanism of action is expected to be quite similar to that of CQ (Davis et al. 2005)

ART and DHA mechanism of action has not been fully studied, but they may act via a common mechanism (White et al. 1994). ART and DHA are believed to bind to heme within the *P. falciparum* parasites and the source of this heme may vary with the intraerythrocytic developmental stage of the parasites (Wang et al. 2015, Paitayatat et al. 1997). In earlyring stage ART and DHA are thought to bind to heme produced by the parasite´s heme biosynthesis pathway and in the later stages they possibly bind to heme release by hemoglobin digestion (Olliaro et al. 2001). When bound to heme, ART and DHA are believed to undergo activation involving ferrous iron via reductive scission that splits the endoperoxide bridge to produce a reactive oxygen species (ROS) (Olliaro et al. 2001, Haynes et al 1996). This reactive oxygen is believed to undergo a subsequent intramolecular hydrogen abstraction to produce reactive carbon radical which is thought to be the source of the drugs potent activity against *P. falciparum* by alkylating a wide range of proteins targets (Olliaro et al. 2001, Ravindra et al. 2015, Wang et al 2015). The degree to which this alkylation affects specific protein function is still not known. Proteomics studies show that ART and its derivatives alkylate hundreds of proteins, suggesting that the drugs may kill the parasite by inducing global degradation of its cytoplasm (Wang et al. 2015, Ismail et al. 2016). Other studies suggested that ART inhibits the sacro/endoplasmic reticulum Ca^{2+} ATPase found in *Plasmodium* parasites (Asawamahasakda et al. 1994, Eckstein-Ludwig et al. 2003). ART and DHA appear to specially accumulate in the infected red blood cells, where drug concentrations are higher by several hundred folds compared to uninfected red blood cells (Gu et al. 1984). ATS mechanism of action is believed to involve cleavage of the endoperoxide bond via reaction with heme (Artesunate product Information).

This generates free radicals that alkylate parasitic proteins. ATS has been demonstrated to inhibit an important parasite calcium adenosine triphosphate enzyme. ATS is thought to inhibit malaria protein EXP1, a glutathione S-transferase, and accounting for the breakdown of cytotoxic hematin (Lisewski et al. 2014), but it is still not clear to what extent this inhibition contributes to the antimalarial action of ATS.

The occurrence of resistance in *Plasmodium* is determined by several factors such as parasite mutation rate, the fitness costs correlated with the resistance mutations, total parasite load, the strength of selection of drug and the compliance of treatment (Petersen et al. 2011). The rate of mutation in the parasites has a direct effect on the frequency at which resistance can occur. Though higher rates of mutations allow for a faster occurrence of resistance, they may also lead to an accumulation of harmful mutations (Petersen et al. 2011). The mutation rate in *P. falciparum* was determined to be approximately 10^{-9} from experiments measuring spontaneous mutations in the *Pfdhfr* (*P. falciparum* dihydrofolate reductase) gene, which is quite low (Paget-McNicol et al. 2001). An increased rate of mutation is beneficial for the parasite's adaptation to quickly changing environments (Sniegowski et al. 2000). Some studies reported an accelerated resistance to multiple drugs (ARMD) phenotype present in parasite isolates from Southeast Asia. *In vitro* experiments showed that parasites with the ARMD phenotype developed resistance to drugs at a higher rate than other geographically distinct parasite strains, and such ARMD parasites might explain the observation that

resistance to new antimalarial drugs often occurs first in Southeast Asia (Rathod et al. 1997). Transmission is additional crucial step for the spread of antimalarial drug resistant parasites and the intensity of transmission has a significant role in the determining if parasites are efficiently spread during a mosquito blood meal (Petersen et al. 2011). Moreover, antimalarial drug resistance may increase transmission if drug selection pressure reduces the viability of sensitive gametocytes in a multiple-strain infection, enhancing the tendency for transmitting antimalarial drug-resistant parasites (Hastings et al. 2006).

Since mutations are associated with drug resistance, several studies have identified the cause of resistance by sequencing of full *P. falciparum* genome, which revealed approximately 5300 genes distributed among 14 chromosomes (Gardner et al. 2002). *P. falciparum* parasite could alter its response to drug through regulation of gene expression. Most mutations in relation to antimalarial drug resistance are either single nucleotide polymorphisms (SNPs) in the regulatory areas surrounding the genes, which could change the gene expression, insertion/deletion in a coding area of gene that might alter protein structure and functions, or gene amplifications (Gardner et al. 2002).

The mechanism of resistance to CQ is thought to be associated with polymorphisms in *PfCRT* (*P. falciparum chloroquine resistance transporter*) (Sidhu et al. 2002). *PfMDR1* (*P. falciparum multidrug resistance 1*) also modulates the degree of CQ resistance in some parasite strains, signifying that some alleles and overexpression of PfMDR1 can enhance the CQ concentration in the food vacuole by active transport (Barnes et al. 1992). Remarkably, studies have showed linkage disequilibrium between PfCRT and PfMDR1 alleles in CQ resistant parasites in Southeast Asia and Africa, signifying a functional interaction between these proteins (Menard and Dondorp et al. 2017, Osman et al. 2007). Cross-resistance between AQ and CQ has been described and mutations in PfCRT and PfMDR1 are linked with reduced susceptibility to both AQ and CQ, but this cross-resistance is inadequate since some CQ resistant parasites remain susceptible to AQ (Sá et al. 2009). It has been established that modulation of PPQ susceptibility by mutations in PfCRT, however the alteration in PPQ response was unclear and clinical significance of this discovery remains uncertain (Muangnoicharoen et al. 2009).

Resistance to ART and its derivatives is seen in the ring stage of parasites that show delayed clearance from circulation after direct administration of ARTs to patients (Noedl et al. 2008, Dondorp e al. 2009, Witkowski et al. 2013). *Pfkelch13* gene in parasites is the main marker of ART resistance as demonstrated both clinically and in *in vitro* studies using the ring stage survival assay (Witskowski et al. 2013). Mutations in *Pfatp6* (*P. falciparum Ca2+ transporting ATPase 6*) has been linked to decreased artemether sensitivity in field parasite isolates from French Guyana (Jambou et al. 2005) and polymorphisms in *ubp1* (encoding for deubiquitination enzyme) are linked to increased resistance to ATS in the rodent *P. chabaudi* parasites (Hunt et al. 2007). However, artemether and ATS resistance does not demonstrate consistent association with malaria parasites with delayed clearance, the presently described phenotype of *P. falciparum* parasites with decreased ART susceptibility (Imwong et al. 2010). Though not mediating complete ART derivative resistance, PfMDR1 amplification may

considerably decrease parasite susceptibility (Sidhu et al 2006, Imwong et al. 2010, Chavchich et al. 2010). Global transcriptomic profiling of over a thousand clinical strains led to the discovery of upregulation of parasite oxidative stress and protein damage pathways via unfolded protein response (UPR) as a mechanism of ART resistance in parasites with *Pfkelch13* mutations (Mok et al. 2015).

1.6 PLASMODIUM FALCIPARUM GENOME

P. falciparum 3D7 clone was the first *Plasmodium* genome to be sequenced (Gardner et al. 2002). The genome consists of ~23.3-megabase (Mb) of nuclear genome distributed among 14 linear chromosomes (with telomeres and centromeres) ranging from 0.643 to 3.29 Mb in size, about 6-kilobase (kb) of mitochondrial and a 35-kb circular apicoplast genome. The apicoplast genome encodes only 30 proteins including housekeeping enzymes involved in DNA replication, transcription, translation and post-translation modifications, protein export and turnover, cofactor synthesis and specific metabolic and transport activities. Approximately 60% of the apicoplast genome is of unknown function. About 60 genes are identified as of mitochondrial origin. Most of the proteins encoded by these mitochondrial genes have known functions such as tricarboxylic acid (TCA), oxidative damage protection, synthesis of heam, and ubiquinone.

The *P. falciparum* genome is extremely AT-rich (~80%), and increases to ~90% in non-coding regions. The genome encodes approximately 5300 genes. The average gene density is 1 gene per 4,338 base pairs (bp) and the average length was 2.3 kb without introns. 15.5% of genes are more than 4 kb in length which is much longer compared to other organisms. Many of these large genes encode proteins of unknown function. Notably, *P. falciparum* genome encodes a small proportion of enzymes and transport proteins and a large proportion of genes that are involved in immune evasion and host-parasite interactions. Genes involved in antigenic variation (e.g. *var*, *rif* and *stevor*) are located in the subtelomeric regions of the chromosomes.

Plasmodium also encodes DNA replicative machinery such as DNA polymerases (Gardner et al. 2002), proliferating cell nuclear antigen (PCNA) (White et al. 1996, Mitra et al. 2015, Kilbey et al. 1993), and minichromosomes maintenance proteins (MCMs) (Ansari et al. 2012). *P. falciparum* genome also encodes at least some major components of DNA repair processes that are also found in other eukaryotes including genes involved in nucleotide excision repair (RAD25, RAD2, RAD1, RAD3 and ERCC1) and homologous recombination repair (MRE11, RAD50, RAD51, RAD54 and DMC1), although some of the accessory proteins such as XRS2, RAD4, XPC that are found in eukaryotes have not yet been found in *P. falciparum*. Other genes involved in DNA repair processes that are also found in eukaryotes are genes involved in post-replication mismatch repair (MSH6, MLH1, MSH2-1, MSH2-2 and PMS1) and base excision repair (glycosylase homologues and apurinic/apyrimidinic (AP) endonucleases). Interestingly, homologues of the genes encoding enzymes involved in non-homologous end joining (NHEJ) repair in eukaryotes such as Ku70, Ku86, Ligase IV and XRCC1 are apparently absent in *P. falciparum.* NHEJ repair genes are

important in repairing DNA double strand breaks (DSBs) and other cellular processes that create DSBs. In other organisms, NHEJ plays an important role in regulating telomere stability (Eisen et al. 1999, Dudásová et al. 2004).

1.7 DNA DAMAGE AND REPAIR

Plasmodium falciparum DNA is subjected to damage from immune response, DNA damaging agents such as reactive oxygen species produced by the parasite's metabolic processes, free radicals generated after uptake of antimalarial drugs including ART, ATS or CQ, genotoxic agents and DNA replication errors when it reproduce asexually within mammalian host (Berdelle et al. 2011, Li et al. 2008). This damage may affect individual bases or may lead to the production of DSBs. DNA damage together with damage in the protein is believed to be a mode of action for ART, mediated via free radicals (Gopalakrishnan et al. 2015). *Plasmodium* parasites enter dormancy at G0/G1 phase and upregulate DNA repair machinery particularly PfRAD51, PfRAD54, PfRPAIl and PfRPAIS in response to ART (Gupta et al. 2016, Gopalakrishnan et al. 2013). This dormancy may be a key mechanism of ART resistance.

DNA damage from transcription, collapse of replication fork or by-product of cellular processes including hemoglobin degradation, has not been well studied in *P. falciparum*. However, it has been reported that when parasites grow in the RBCs, they degrade hemoglobin in their digestive vacuoles and release heme. This heme is oxidized from ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron which leads to the generation of hydroxyl radicals, a powerful DNA damaging agent (Atamna et al. 1993). Thus, it is feasible that in *P. falciparum* parasites, cellular processes including degradation of hemoglobin in the digestive vacuole and the release of free radicals, together with the many rounds of DNA replication may lead to DSBs, which need to be repaired in order to maintain DNA integrity that is crucial to genomic stability and parasite viability.

Like all other pathogens, malaria parasites must rely on DNA repair mechanism to correct damaged nucleotides. DNA damage repair is a process by which a cell identifies and corrects damage to the DNA molecule. DNA damage repair is closely tied to DNA replication to ensure precise copying of the genome. DNA damage may lead to multiple lesions such as deletions, insertions, mutations, translocations, and loss of chromosomes and genetic information. In the *Plasmodium* genome, DNA repair machinery is largely conserved, and parasites respond to DNA damage by upregulation of the DNA repair machinery and alteration of chromosome structure (Gupta et al. 2016).

In mammalian cells, the signaling system of DNA damage is complex and involves proteins that can be classified into four groups: sensors, transducers, mediators and effectors. DNA damage response mechanism includes DNA damage detection, multiple DNA repair pathways, damage tolerance processes and cell-cycle checkpoints to maintain genomic integrity (Gaglia-Mari 2011). In *Plasmodium*, the existence of cell cycle checkpoints remains unclear and genes encoding checkpoint proteins such as ATM, ATR and p53 have not yet identified (Matthews et al. 2018).

DNA damage response is a chromatin-associated process that is usually closely controlled in time and space (Giglia-Mari et al 2001). In eukaryotes, chromatin plays a crucial role in regulating the DNA damage response. Histone modification and chromatin remodeling complexes alter the chromatin structure to give access to the damage site for the DNA repair genes during DNA damage. Phosphorylation of H2A/H2A-X and acetylation of H4 play vital roles in the recruitment DNA repair genes includes ATM and ATR kinase, RAD51, BRCA1, TIP60 HAT, TP53. In *P. falciparum* RAD51 performs homologous recombination repair to change the expression of genes encoding surface membrane glycoproteins and create antigenic variation (Freitas-Junior et al. 2000). In addition, recombinational rearrangements are accountable for the multidrug resistance gene (*pfmdr1*) amplification (Triglia et al. 1991) indicating the importance of homologous recombination repair to create genomic adaptability and plasticity in *Plasmodium* parasites. The majority of histone modifications and chromatin remodeling complexes have been identified in *P. falciparum* but the role of chromatin dynamics in the DNA response is still to be explored. Recent studies have showed that chromatin structure is altered during DNA repair (Gupta et al. 2016).

DSBs are one of the most severe types of DNA damage as they affect both strands of DNA and lead to loss of genetic information. DBS has been extensively studied in *Saccharomyces cerevisiae* (Paques and Haber 1999, Krogh and Symington 2004). DSBs have to be repaired to prevent chromosomal rearrangements and accumulation of further mutations (Lee et al. 2014).

Other DNA lesions consist of single-strand breaks, inter- and intra-strand crosslinks, adjacent nucleotides crosslinking and base modifications, base mismatches during replication as well as non-bulky lesion produced by alkylation, deamination, and oxidation of bases. After DNA is damaged, sensors such as MRN/X (Mre11/Rad50/NBS1 (XRS2) and Ku70/Ku80) bind to damaged sites and recruit transducers that help amplify and maintain the DNA damage signal. These sensors play a vital role in DNA double-strand breaks repair. In addition, transducers such as Ataxia telangiectasia mutated (ATM), DNA-PKcs are key factors involved in several pathways in the DNA damage response. The interaction between the transducers and the mediator proteins leads to the signal dispersal all over the cell nucleus and effectors kinases are activated. (Hoeijmakers et al. 2009).

The variety of mutations observed in *P. falciparum* is highly uncommon. SNP mutation rate is approximately 2.45×10^{-10} mutations per base pair per life cycle (Matthews et al. 2018), due to the presence of effective DNA repair system. Recent studies have shown that parasites respond to DNA damage through upregulation of specific DNA repair genes and at the same time alter its chromatin structure (Gupta et al. 2016). Studies from Cambodian isolates identified mutations in a number of DNA repair genes, including MLH1, PMS1 and EXO1 in MMR pathway (Lee et al. 2014). It has been proposed that accelerated resistance to multiple drugs (ARMD) parasites have reduced DNA repair efficiency as compared to non-ARMD parasites, which may trigger their increase mutational features. Furthermore, whole genome sequencing identified point mutations of 18 DNA repair genes, which is believed to

contribute to the ARMD phenotype, due inefficient repair in the DNA damage (Gupta et al. 2016).

1.8 DNA REPAIR PATHWAYS

DNA repair pathways function to defend against genomic damage imposed by various sources, including host immune response, replication errors, and metabolic byproducts. *P. falciparum* has a complex life cycle and probably needs refined global and local regulators, including refined DNA repair mechanisms and checkpoint, however these are currently not fully understood. Other infectious organisms including bacteria may possibly profit from mistakes in DNA repair, as such errors usually cause variation that can contribute to the development of drug resistance or other means of accelerated adaptation to the host (Sinha et al. 2007).

DNA repair can be divided into six pathways: homologous recombination repair (HRR), non-homologous end-joining (NHEJ), base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), mitotic recombination and methyltransferase repair. Homologous recombination (HR) and canonical nonhomologous end joining (C-NHEJ) are the two well-characterized mechanisms for DNA DSB repair in both eukaryotes and prokaryotes (Moynahan and Jasin 2010).

HR-based repair is considered to be an error free repair and uses a template to guide repair while CNHEJ repair is more likely to introduce mutations. HRR pathway is a slow repair process and can only take place in late S phase and G2 phase, when DNA has been replicated and the sister chromatid is available as undamaged template, promoting high fidelity repair of DSBs. It involves strand resection and invasion of DNA followed by synthesis and ligation of DNA. After the DNA damage, the sensors (MRN/X complex) bind to DNA ends, activate the transducers, recruit nucleases and are involved in the end-trimming process (Shibata et al. 2011, Fleck and Nielsen et al. 2004). The *P. falciparum* genome encodes a functional homologous recombination pathway (Gardner et al. 2002). HRR pathway plays an important role for the completion of the parasite life-cycle (Fox et al 2009) and homologous recombination is the main source of DSB repair in the parasites (Kirkman et al. 2019, Lee et al. 2014).

NHEJ repair pathway does not require a template and is available throughout the entire cell cycle. Compare to HR, NHEJ is a fast but error prone repair process. During DNA end processing, loss or changes of a few nucleotides may occur. The heterodimer Ku70/Ku80 binds to the DNA ends at the site of DNA DSB and recruits' transducers, which tether the loose ends and form a bridge. Subsequently, the DNA ends are trimmed by Artemis nuclease and MRN/X protein complex. Damaged bases are removed to prepare for synthesis and then ligation is carried out by the LigIV/XRCC4 complex (Weterings and van Gent 2004, van Gent and van der Burg 2007). In *Plasmodium* the core genes of the NHEJ pathway appear to be missing across the genus (Aravind et al. 2003).

Damage to individual bases is repaired by excision repair pathways, such as base excision repair (BER) in which the damaged bases are removed by DNA glycosylase and replaced with the correct nucleotide by DNA polymerase β in a localized burst of DNA synthesis, nucleotide excision repair (NER) and mismatch repair (MMR) (Matthews et al. 2018). Single strand breaks are usually repaired by BER mechanism (Hegde et al. 2008) (Almeida and Sobol 2007). *P. falciparum* lacks orthologs for short-patch BER, however homologs for a long-patch BER pathway are present (Haltiwanger et al. 2000).NER differs BER in that NER uses different enzymes such as Transcription Factor IIH and removes a large spectrum of single strand lesions that cause local helix destabilization. NER is a multi-step process and involves at least 25 different polypeptides and consists of two different damage detection modes. Transcription-coupled NER senses the damage by RNA polymerase, removes transcription-stalling lesions and quickly permits resumption of transcription. Global genome NER localizes lesions anywhere in the genome (Hoeijmakers 2009) (Gillet and Scharer 2006, Krokan, et al. 1997, Prakash and Prakash 2000). Bioinformatically, the orthologs of the majority of genes involved in the excision repair pathways in *Plasmodium* have been identified except for p62 and XPC(Tajedin et al. 2015).

Mismatch repair mechanism corrects base-base mismatches and generates insertion and deletion loops during DNA replication and recombination. It is an immediate post replicative correction mechanism and the proteins involved are up-regulated during cell cycle. MSH2 and MLH1 proteins are key factors in MMR and were reported to be up-regulated when quiescent cells were stimulated to proliferate. DNA polymerases, Exo1, PCNA and RFC are replication factors, which are also required in MMR. (Marti et al. 2002). Orthologs of the majority of the MMR pathway are present in *Plasmodium* however there are prominent differences from other eukaryotes.

Throughout all haploid growth phases, *Plasmodium* parasites must depend upon alternative end joining pathways such as microhomology-mediated end joining (MMEJ) to repair DSBs in the core genome, because there is no repair template to allow for HR repair (Singer et al. 2015). MMEJ repair is inefficient or used infrequently and that *Plasmodium* parasites with 1N genome content are incapable to repair DNA damage as efficiently as trophozoites with 2N or more genome content (Oakley et al. 2013, Waki et al. 1983, Waki et al. 1985). In eukaryotes, MMEJ involves initial sensing and resection of double-strand break. DSB is first sensed by the sensor MRN/X complex (Mre11-Rad50-NBS1/Xrs2), which then binds to the double stranded ends, positions both ends in close proximity and initiates 5' - to 3' resection together with Sae2, creating short 3' terminated single-stranded DNA (ssDNA) tails. This is followed by 5'-to 3' extensive resection by redundant factors such as Exo1 (exonuclease 1), Sgs1, Top3, Rm1 and Dna2 complex that commits DSBR to HR and produces long 3' ssDNA tails (Lee, Symington et al. 2014). Alternative non-homologous end-joining (A-NHEJ) pathway is a backup repair pathway for abrogated NHEJ and HR repair pathways. The DNA repair proteins that are involved in A-NHEJ include PARP1, WRN, Mre11, CtIP, Polymerase δ, Histone1, Lig3 and XRCC1 or Lig1 (Iliakis, et al. 2015).

1.9 EXTRACELLULAR VESICLES (EVS)

In the last decade, the discovery of extracellular vesicles (EVs) as cellular communicators and potential biomarkers has led to an unexpected growth of publications in the field of EV research. The first experimental visual evidence of EVs was published by Peter Wolf in 1967 using electron microscopy (EM) and was called platelet dust (Wolf et al. 1967). A couple of years later, other studies illustrated the release of submicron vesicles via a direct blebbing of particles of the plasma membrane from activated platelets using EM (Webber and Johnson 1970). In later years, other work helped to define the role of vesicles in the secretory pathway of intracellular protein transport, especially in relation to the secretion of hormones, enzymes and neurotransmitters (Palade et al. 1975, Fries et al. 1980, Rothman et al. 2002).

These studies provided significant evidence for vesicles' role in the transfer of cellular substances, vesicles fusion with the plasma membrane and discharge of intravesicular components into the extracellular space (Rothman et al. 2002). Intercellular communication is important in maintaining homeostasis, tissue and organ integrity. Several types of cell-to-cell communication mechanisms have been investigated, such as electrical stimuli, extracellular matrix interactions, direct cell-cell connections and release of different substances. EVs such as exosomes, microvesicles, microparticles and oncosomes are a heterogenous collection of membrane-bound carriers with complex cargos and have emerged due to their role in intercellular communication that are important between cells. Like all other protozoans and microorganisms, malaria parasites use several pathways to communicate within their own populations and to influence their outside environments with the aim of harmonizing the growth rate and transmission (Mantel et al. 2014).

Other pathogens such as bacteria use extracellular vesicles to export virulence effectors into target host cells (Kuipers et al. 2018, Szempruch et al. 2016). Cells release vesicles of different sizes via endosomal pathway and by budding from plasma membrane. EVs are small spherical vesicles, ranging from 0.1-1μm in size, which contain biomolecules including bioactive lipids, proteins, and genetic material such as DNA and non-coding RNA, which can be transferred to specific recipient cells (Jones et al. 2018, Mantel et al 2014). It has been suggested that EVs play an important role in cellular communication and used as biomarkers (Jones et al 2018). Recent studies suggest that EVs derived from pathogen or host are released during infection (Schorey et al. 2015). In malaria, recent evidence suggests that *Plasmodium* parasites secrete EVs to evade host immune system and to promote growth and transmission (Mantel et al. 2014). Cells used EVs to communicate and organize social activities and several types of vesicles have been identified based on their size, biogenesis and source of cellular compartment (Mantel et al. 2014).

1.10 DIFFERENT TYPES OF EVS

Although interests in EV research field have grown in recent years, it has been accompanied by a disparate use of terminology that causes misperception within the field. Researchers have named the vesicles based on their origin, such as oncosomes for cancer cells, tolerosomes for vesicles from intestinal epithelial cells and matrix vesicles for vesicles from bone cells and

cartilage (van der Pol et al 2015, Ostman et al. 2005, Di Vizio et al. 2009, Ronquist et al. 2015, Schmidt et al. 2016). Other terms referring to the biogenesis mechanism including exosomes, microvesicles, apoptotic bodies and microparticles are often used indistinguishably. Hence, the International Society of Extracellular Vesicles (ISEV) published a paper in 2014 to encourage the use of EVs as a collective term to incorporate all secreted vesicles and request that authors in the EV field give complete lucidity in reporting the defined EV populations of study (Gould et al. 2013, Lötvall et al. 2014). Several markers are used to identify exosomes including annexin V (phospholipid) TSG101, heat shock proteins, Alix and tetraspanins such as CD9, CD63 and CD81 (Bobrie et al. 2011, Théry et al. 2009). Figure 4 shows the typical characteristics ofEVs.

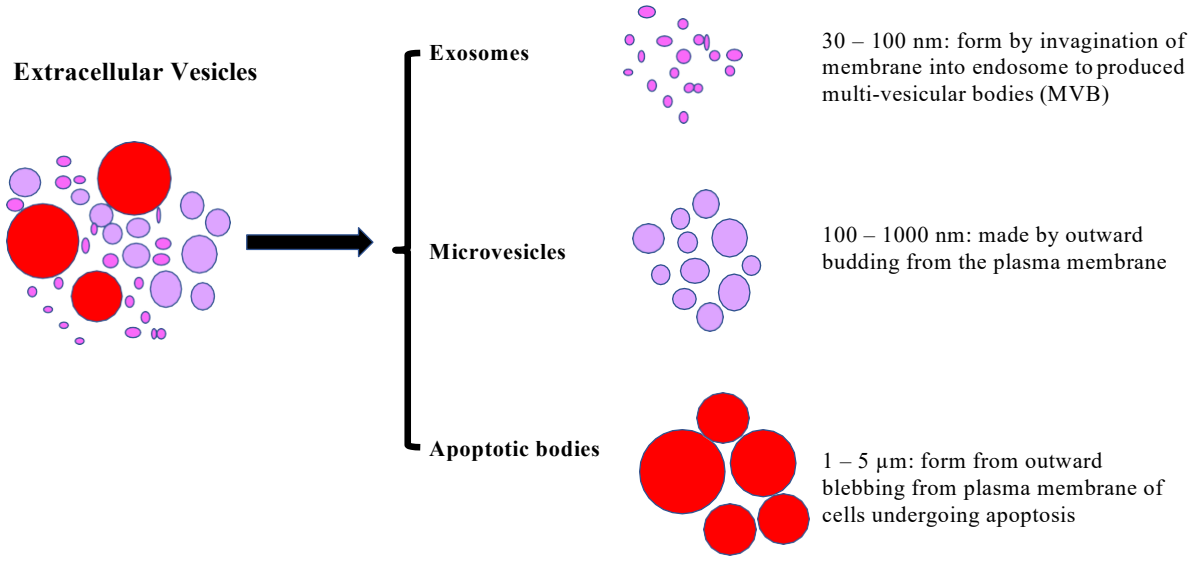


Fig. 4. Different types of extracellular vesicles. EV is the collective term used to comprise all cell-derived secretory vesicles such as exosomes, microvesicles and apoptotic bodies.

1.10.1 Exosomes

Exosome is the term often used for nano-scale subpopulations of EVs. Exosomes are endosome-derived membrane vesicles and are the smallest types of EVs, approximately 30-100nm in diameter (Bhatnagar et al. 2007, Moldovan et al. 2013). These exosomes have the ability to carry and deliver their cargos such as proteins, non-coding RNA and microRNA via direct cell-to- cell contact as well as distant signaling (Cheng et al. 2014, Mittelbrunn et al. 2011, Moldovan et al. 2013, Ridder et al. 2014, Valadi et al. 2007). During infection, exosomes facilitate cell to cell information transfer and immune response regulation (Valadi et al. 2007). Exosomes have the capacity to be released from antigen-presenting cells to induce immune responses *in vivo*, making exosomes ideal therapeutics and biomarkers (Schenoda et al. 2016). Exosomes are present in cerebrospinal fluids and blood and play an important role in intercellular communication by transporting their cargo between source and target cells (Schorey et al. 2015). Several studies have suggested that exosomes might be used as biomarkers of infectious diseases with the potential to preventinfection.

1.10.2 Microvesicles (MVs)

MVs or ectosomes or membrane vesicles are larger than exosomes, approximately 100-1000 nm in diameter. Their release mechanism and biogenesis may differ from exosomes. MVs are shedded from blebbing of stressed or apoptotic cells or via fission of membrane vesicles from the plasma membrane which resembles the abscission step in cytokinesis (Lee et al. 2012, Rackov et al. 2018, Stahl et al. 2018, D´Souza et al 2018, Tiberti et al. 2016). Like all other EVs, MVs also carry an array of inducible bioactive substances which allow their composition to be often augmented via induction in response to specific stimulus (Lee et al. 2012, Rackov et al. 2018, Stahl et al. 2018, D´Souza et al 2018). The release of MVs also shares a common feature with the mechanism of virus budding (Jones et al. 2018).

1.10.3 Apoptotic Bodies (ABs)

ABs are another type of extracellular vesicles which are released by apoptotic cells during the later stages of programmed cell death as blebs. They are the largest EVs, approximately 1-5μm in diameter and serve as a signal stimulating phagocytosis of apoptotic cells before induction of necrosis (Caruso et al 2018, Christiakov et al. 2016, Rackov et al. 2018). Like exosomes, apoptotic bodies can also carry and transfer cargoes including oncogene, cellular organelles and DNA between cells, and have been shown to be crucial in presentation of antigen and immunosuppression (Hsu et al. 2010, Thery et al. 2001, Holmgren et al. 1999, Cocca et al. 2002, Bellone et al. 1997, Bergsmedh et al. 2001). Apoptotic bodies are augmented with different types of damage-related molecular pattern proteins, which may induce inflammation (Caruso et al. 2018, Christiakov et al. 2016).

1.11 BIOGENESIS, COMPOSITION AND FUNCTION OF EXTRACELLULAR VESICLES

The biogenesis of EVs is a highly conserved phenomenon in living organisms. The majority of reports on the EVs biogenesis has been elucidated in mammalian cells therefore the mechanisms and pathways of the generation of EVs in parasites could differ. EV generation pathways include the classical pathway (endocytic pathway) for the formation of exosomes and the direct pathway (alterations in plasma membrane asymmetry) for the formation of microvesicles budding. The endocytic pathway is the inward budding of plasma membrane, resulting in intracellular vesicles whose main function is to regulate the cell surface receptor expression that can be internalized and degraded or recycled depending on the cell's needs (Vanlandingham et al. 2009). The direct pathway is the modifications in the asymmetry of the plasma membrane causing an outward budding of the membrane directly into the extracellular space, which leads to the production of microvesicles that contain surface proteins of the cell of origin (Manno et al. 2002).

Exosomes can be found in plasma, urine, cerebrospinal fluid, saliva, amniotic fluid, bronchoalveolar fluid and breast milk, making exosomes the ideal candidates to serve as biomarkers (Corrado et al. 2013, Zhou et al. 2008). Exosome biogenesis starts after the formation of intraluminal vesicles as the late endosome membrane buds inward

(Frydrychowicz et al. 2015). The multivesicular bodies transit to the surface of the cell and fuse with the cell membrane and subsequently release intraluminal vesicles outside of the cell and into the extracellular environment (Meckes et al. 2011, Gyorgy et al. 2011). Exosomes are the only secreted cellular vesicles which are produced from internal membranes. Exosome compositions are rich in lipids and proteins, and can contain molecules involved in the presentation of antigens as well as numerous surface molecules that can activate different cell receptors and allow them to exchange materials (e.g. miRNA, carbohydrates and pathogens) between cells (Lee at al. 2012, Meckes et al. 2011). The recipient cell is able to take up or receive this cargo from the exosomes via receptor-mediated endocytosis, direct fusion with plasma membrane or via phagocytosis (Schorey et al. 2015). Receptor-mediated endocytosis comprises the direct binding of exosomes to receptors on the plasma membrane or the membrane of endocytic organelle of the recipient cell (Schorey et al. 2015).

MVs biogenesis comprises vertical trafficking of different molecular cargo to the plasma membrane, membrane lipids redistribution and the use of contractile machinery at the surface allowing vesicle pinching (D'Souza-Schorey et al. 2012). MVs secreted via outward budding and fission of plasma membrane are the result of interplay between redistribution of phospholipids and cytoskeletal protein contraction (Akers et al. 2013). MV content appears highly enriched for protein subset. For example integrin receptors are enriched in melanoma cells while transferrin receptors are highly enriched in exosomes but missing in MVs (Muralidharan-Chari et al. 2009 & 2010). Unlike exosomes, MVs could transfer reporter function to recipient cells in the form of plasmid DNA (Kanada et al. 2015).

Apoptotic bodies are formed only during apoptosis, which is a major cell death mechanism for both normal and cancer cells (Kerr et al. 1972, Elmore et al. 2007). Apoptotic bodies are characterized by the presence of organelles within the vesicles. Apoptotic body clearance by macrophages through phagocytosis is mediated by interactions between the phagocytes' recognition receptors and the specific changes in the apoptotic cell membrane composition (Akers et al. 2013, Elmore et al. 2007, Taylor et l. 2008).

EVs can be secreted by an array of cells including endothelial cells, epithelial cells, blood leukocytes, red blood cells (RBCs), infected RBCs (pRBCs), platelets, tumor cells, T cells, B cells, mast cells, astrocytes and stem cells (Corrado et al. 2013, Eldh et al. 2010, Mantel et al. 2014, Zhou et al. 2008). EVs may contain different forms of DNA such as genomic DNA fragments and mtDNA (Cai et al. 2013, Waldenstrom et al. 2012, Guescini et al. 2010). EVs have different surface molecules and can activate several receptors that allow EVs to participate in the exchange of materials between cells (Lee et al. 2012). Several studies have shown the importance of extracellular vesicle functions as well as their unique characteristics such as packaging and targeting abilities (Jones et al. 2018). Numerous pathogenic organisms including *Leishmania major*, *Trypanosoma cruzi*, and *Plasmodium* species are capable of releasing exosomes that may play a role in the host-cell invasion and infection modulation (Bayer-Santos et al. 2013, Borges et al. 2016, Martin-Jaular et al. 2011, Schorey et al. 2015, Silverman et al. 2010).

1.12 THE ROLE OF EVS IN MALARIA DISEASE

EVs have been proposed to play an important role in the transportation of molecules between cells for transcriptional regulation during immune and inflammatory responses and for signal transduction (Robbins et al. 2014, Buzas et al. 2014). It has been reported that during malaria infections, EVs plays an important role in disease pathogenesis and susceptibility as well as immune responses and cell-to-cell communication (Guimaraes Sampaio et al. 2017). Several studies have also shown that EV generation is elevated in different diseases including inflammation, cancer, and cardiovascular diseases (Kim et al. 2003, Distler et al. 2005, Jy et al. 1992, VanWijk et al 2003).

In recent years, studies have indicated that circulating EVs or microparticles (MPs) were elevated in patients with malaria and these EVs or MPs seemed to be associated with the progress of immunopathological lesions in cerebral malaria (Campos et al. 2010, Combes et al. 2004, Nantakomol et al. 2011). Other studies found that patients infected with *P. falciparum* had the highest concentrations of RBC-derived EVs compared to heathy controls (Mfonkeu et al. 2010). In *P. falciparum*-infected patients in Cameroon, (Mfonkeu et al. 2010) found a significant increase in circulating MVs, in particular platelet-derived EVs, in patients with complicated or cerebral malaria but not in uncomplicated malaria or controls. Furthermore, similar results were shown in a field study in India, where EVs or MVs secreted from endothelial cells, platelets and erythrocytes were elevated during malaria infection (Sahu et al. 2013). Intriguingly, an in vivo study showed that EVs from platelet bind only to pRBCs in a *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) dependent manner and transfer platelet antigens into the infected cells, but not to uninfected RBCs. These platelet-EVs dramatically enhance the binding of pRBCs to the endothelial cells (Faille et al. 2009).

This study provided mechanistic understandings into the association of MVs in cerebral malaria, signifying that EVs can promote cerebral pathology through stimulation of iRBC cytoadhesion in the brain. It has also been suggested that *P. falciparum* EVs can mediate cell-to-cell communications between parasites (Mantel et al. 2013, Regev-Rudzki et al. 2013). Recently, a study of *P. vivax* infected patients in Brazil found that active infection was also correlated with increased MV secretion, but these MVs came from platelets, erythrocytes and leukocytes and not from endothelial cells (Campos et al. 2010). It has been proposed that these platelet-derived EVs might play an important role in the inflammatory symptoms of *P. vivax* infection (Campos et al. 2010). It is important to note that MVs were consistently found to be augmented with *P. falciparum* infection but not with *P. vivax* and the presence of endothelial derived MVs in *P. falciparum* infection probably reflects the main involvement of this type of cell in the development of cytoadherent-dependent severe malaria disease symptoms. EVs can induce the innate immune system from cells both in human and rodent malaria system and can also facilitate cellular communication between pRBCs through transfer of signaling molecules from donor to a recipient iRBC (Mantel et al 2013, Couper et al. 2010). Furthermore, this pathway of communication is associated with the development of malaria transmission stages (gametocytes) and the ability to transfer episomal DNA

(Mantel et al. 2013, Regev-Rudzki et al. 2013). In addition, it has been demonstrated that parasites are capable of exchanging genetic materials via EVs.

In this study, different parasite strains, each containing separate drug resistance genes (WR99210 or blasticidin) and plasmids encoding different fluorescent markers were co-cultured. Interestingly, parasites were able to survive after exposure to both drugs and the next generation of parasites harbored both drug resistance genes and fluorescent marker. This transfer of plasmid was detected to occur through exosome-like vesicles secreted from the pRBCs. Moreover, this transfer of plasmid was seen to happen most effectively during early stage of the asexual life cycle (Regev-Rudzki et al. 2013).

The result of this study suggested that drug resistance could be transferred to drug-sensitive iRBC thru EVs. Recently, data from *in vitro* studies have reported that EVs from *P. falciparum* infected RBCs act as messenger, create communication between parasites during asexual stages as well as directing the parasite generation into gametocytes. These data suggested that EVs seemed to be an important factor in the regulation of gametocytes commitment in *P. falciparum* (Mantel et al. 2013). Additionally, stimulation of cell-to-cell communication mediated by EVs also led to increased gametocytogenesis, suggesting that EVs from parasites might give a means of quorum sensing to activate gametocytogenesis (Guimaraes Sampao et al. 2017, Mantel et al. 2013, Regev-Rudzki et al. 2013). All these studies provide evidence that EVs or MPs secreted from pRBCs are involved in parasite-to-parasite and parasite to host cell communications and host immunoregulation (Campos et al. 2010, Mantel et al. 2013, Wang et al. 2017).

1.13 ARGONAUTE2 (AgO2) PROTEIN AND miRNA

It has been reported that EVs derived from pRBCs have the ability to transfer cargo including protein and nucleic acids. Further *in vitro* studies found that EVs from *P. falciparum* pRBCs could affect host endothelial cells via transfer of functional microRNA, suggesting that *Plasmodium* might actively manipulate the host immune system through EVs or target host RBC and alter EVs for parasite invasion (Mantel et al. 2014). In other pathogens such as Epstein-Barr virus, the virus activates secretion of EVs that contain viral microRNAs from pRBCs, which can downregulate the expression of cytokine in uninfected monocyte-derived dendritic cells (Pegtel et al. 2010).

In other systems, nucleic acid including messenger RNA and microRNA were found in the EVs which may be transported to and function in recipient cells. Recent studies have reported that human argonaute 2 (hAgo2) was found to bind to hundreds of miRNAs and these hAgo2-miRNA complexes were transferred into the parasites via EVs. PfEMP1 expression was downregulated by miR-451/140 through the latter's binding to the A and B subgroups of *var* genes (a family of genes encoding PfEMP1) (Wang et al. 2017). The recent study demonstrated that hAgo2 in both EVs and parasites inhibits parasite development and that hAgo2-miRNA (miR-451/miR-140) complexes target several important *P. falciparum* genes that are involved in the pathogenesis of malaria (Wang et al. 2017).

The result of this study also revealed that the level of hAgo2 decreases when the parasitemia was higher than 1.5% and that human miR-451 and miR-140 can recognize the 5′or 3′ UTR of *var* genes at the transcriptional level, efficiently downregulating the expression of the luciferase reporter (Wang et al. 2017). These data implied that both miRNAs could be involved in the broad transcriptional regulation of all A and B subgroups of *var* genes. Furthermore, depletion of hAgo2 from RBCs can accelerate the development of *P. falciparum* parasites in culture, suggesting that miRNAs play an important role in the inhibition of parasite activities. miRNAs are noncoding RNAs of 19-24 nucleotides that originate from larger primary transcipts encoded in the genome. Argonaute-2 protein plays a crucial role in RNA-mediated silencing (RNAi) processes. Ago-2 binds to a short guide RNA including miRNA and short interfering RNA (siRNA) and are a vital component of the RNA-induced silencing complex (RISC) (Meister et al. 2004, Meister & Tuschl 2004).

1.14 SINGLE CELL TECHNOLOGY

Single-cell technologies allow for analyses at the individual cell level and are being used in different areas of biology and medicine. In recent years, single-cell RNA sequencing techniques have undergone rapid development and have become an important area of biomedical science (Nakamura et al. 2015). Traditional RNA sequencing usually involves a heterogenous population of cells, which mask the difference among individual cells (Geschwind and Konopka 2009), whereas single-cell RNA sequencing analyses will provide a finer-grained picture of biology complex and unmask heterogeneity that is present in cells. Furthermore, single-cell resolution is needed to discover more complicated mechanism in cellular development, confirmation of the distinct gene expression signature across different cell types, identification of the functional differences among the same cell type, evaluate effectiveness of the drug and identify markers of resistance to targeted drugs (Ho et al. 2018, Wang and Bodovitz 2010). Single-cell profiling methods will also allow researcher to uncover new and possibly unforeseen biological findings compare to traditional methods that assess bulk population.

1.15 SINGLE-CELL GENE EXPRESSION ANALYSIS

Recent advancements in single-cell gene expression in particular single-cell transcriptomics have contributed to the way researchers addressing unanswered questions concerning intracellular gene regulatory networks from a cell-centric viewpoint and bridging to the characterization of intercellular pathways which is masked in bulk analysis. Bulk RNA-seq expression profiles are the results of averaging over millions of cells that might differ widely (Figure 6). Populations of cells that have very similar cellular phenotypes may have different transcriptome profiles at the single cell level due to inherent biological heterogeneity, unsynchronized cell-cycle stages or stochastic transcription events (Grün et al. 2015). Single cell transcriptomics provide information on heterogeneity, prevalence and co-expression of a gene at the single cell level (Dalerba et al. 2011, Hashimshony et al. 2016, Grün et al. 2015). The first single-cell transcriptome analysis paper was published in 2009 demonstrating the possibility of scRNA-seq and the advantage of single cell microarrays in a mouse blastomere

study (Tang et al. 2009). High-impact studies using scRNA-seq have been growing rapidly in several fields and there has been rapid development in single cell technologies such as reducing noise, increasing sensitivity and specificity and in particular the throughput for single-cell transcriptomics (Kolodziejczyk et al. 2015, Islam et al. 2014, Picelli et al. 2013, Ramskold et al. 2012, Picelli et al. 2014,Picelli et al. 2014, Wilson et al. 2015). Several important features can be defined at the single cell level for the expression of each gene and more importantly gene expression patterns predicting a specific cell type including heterogeneity, prevalence and co-expression (Dalerba et al. 2011, Hashimshony et al. 2016).

1.16 SINGLE CELL RNA SEQUENCING (scrRNA-seq)

In the past decade, scRNA-seq has emerged following next generation sequencing (NGS) and since then it has revolutionized biological research. Typically, RNA-seq is performed in bulk and the data represent the averaged patterns of gene expression across millions of cells, which might mask biologically relevant differences in gene expression among cells (Olsen et al. 2018). Single-Cell RNA-seq can overcome this problem. Numerous protocols have been developed for scRNA-seq including STRT (Islam et al. 2011), Smart-Seq (Ramsköld et al. 2012), CEL-Seq and CEL-Seq2 (Hashimshony et al. 2012 & 2016), Smart-seq2 (Picelli et al. 2013), Quartz-Seq and Quartz-Seq2 (Sasagawa et al. 2013 & 2018) MARS-Seq (Jaitinet al. 2014), SCRB-Seq (Soumillon et al. 2014), Drop-Seq (Mackosko et al. 2015) and SPLiT-seq (Rosenberg et al. 2018).

scRNA-Seq provides many valuable insights into biological complex system, ranging from cancer genomics to diverse microbial groups. Single-cell RNA sequencing enables researcher to address questions about heterogeneity that are inaccessible using methods that average over bulk cell extracts. It can uncover rare and complex cell populations and reveal regulatory relationships among genes and it makes possible to measure the absolute numbers of RNAs including short RNA molecules in a cell. It can also detect large numbers of small RNAs such as miRNA as well as tRNA and snoRNA whose function is still unknown (Segerstolpe et al. 2016). Single-cell RNA/DNA sequencing provides a higher resolution of cellular differences and understanding of the function of individual cells.

The main shortcomings in scRNA-seq include the increased levels of noise in the measured transcript abundances, unnecessary transcript dropout rates, stochastic bursting events in scRNA-seq data generating abundant nondetections, high variability, and complex expression data distribution (Ho et al. 2018). Hence, it is crucial to differentiate high noise and low-quality samples, which are degraded during preparation of the library or poorly, amplified (Ho et al. 2018). Bulk sequencing can only capture 1% of the cell population. The main disadvantage of single cell sequencing is that it is more challenging to perform in comparison with bulk sequencing (Shintaku et al. 2014). Although the single-cell technology has been expanding rapidly, there is still a number of issues that deserve careful consideration such as inefficient synthesis of full-length cDNAs by reverse transcription (Kalisky and Quake 2011, Kolodziejczyk et al. 2015).

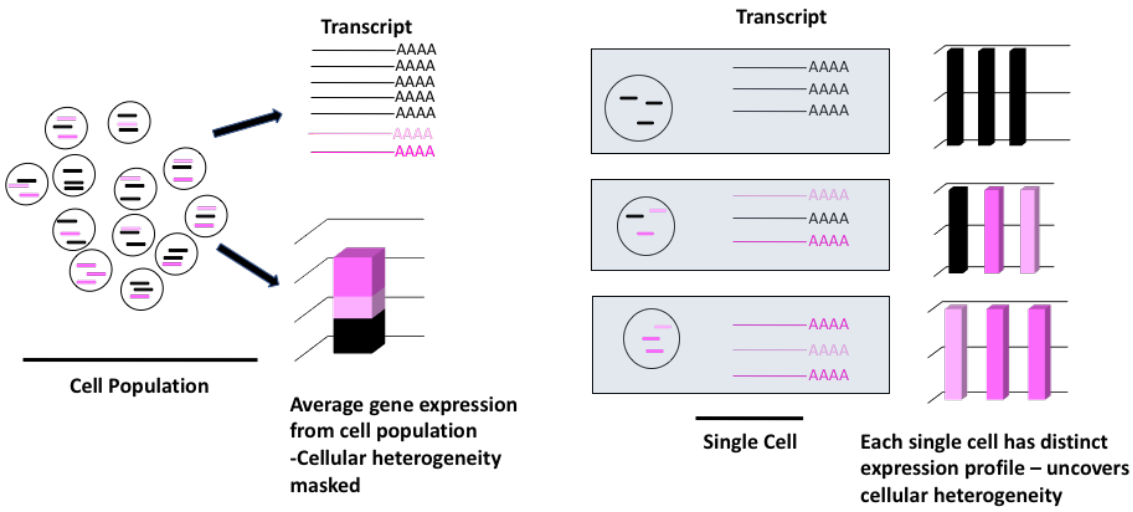


Fig. 6. The illustration of the averaging effect. Transcriptomes of mRNA from population of cells (left) that includes several types of cell is subjected to an averaging effect in which mRNA data from multiple cells are averaged. This averaging effect results in the dilution of mRNA species which are only present in a subset of cell transcripts (pink and black). In contrast, transcriptomes of mRNA from single cells (right) exactly report the abundances of each mRNA specie relative to other mRNA species in that particularcell.

2 AIM

Specific Aims:

Paper I

To investigate if antimalarial drugs such as AQ, CQ, DHA and PPQ could cause random DNA damages in the malaria parasites and that the gene pools of a parasite population can be rapidly diversified through an erroneous repair mechanism.

Paper II

To elucidate if EVs derived from iRBC can traffick miRNAs to target cells and regulate their gene expression.

Paper III

- To develop and enhance workflow for individual pRBCs isolation and Single - Cell RNA-seq library preparation from intra-erythrocytic developmental cycle stages of *P. falciparum* parasites.

- To elucidate Single-Cell RNA-sequencing for molecular analyses of rare *P. falciparum* phenotypes as well as characterization of sub-stages of the intraerythrocytic developmental cycle and gene marker that distinguish between asexual and sexual stages of individual *P. falciparum* parasites.

- Identification and differentiation of genes from variable genes families at single cell level.

3 RESULTS AND DISCUSSION

Paper I

Antimalarial drugs and DNA damage response in *Plasmodium falciparum*

The emergence of highly drug-resistant parasites poses a central problem in malaria eradication. *P. falciparum* DNA is subject to high levels of genotoxic insults during its complex life cycle within the human host and mosquito vector. DNA damage response in *P. falciparum* is thought to contribute to many crucial biological functions of malaria parasites. Since the DNA repair machinery are essential in the maintenance of genome integrity and affect organismal genome mutation rates, they may serve as a potential source for the generation of drug resistance. Most of the components of the DNA repair system are conserved in the parasite genome.

In this study, we investigated if antimalarial drugs such as AQ, ATS, DHA, CQ, and PPQ cause random DNA damages in the parasites and that the gene pool of a parasite population can be diversified via incorrect repair mechanism.

We initiated the study by determining the IC_{50} of these drugs and assessed if these drugs can cause random DNA damage in the parasite. We then treated the parasites with these drugs at different doses and incubated for 12h and 24h. To evaluate DNA damage, we used a comet assay based single-cell electrophoresis to quantify the extent of DNA damage expressed as Olive Tail Moment values, with higher values implying greater DNA damage. ATS and DHA were used as positive controls. All parasites showed evidence of nuclear DNA damage after 12h and 24h post- exposure to drugs, suggesting that all drugs tested can induce DNA breaks. Noteworthy was the high level of DNA damages detected after 12h of exposure to AD, ATS, and DHA and after 24h of exposure to CQ and PPQ, suggesting that CQ and PPQ may confer a delayed effect. This observation cannot be generalized since AQ, which is also a quinoline analog, causes a similar effect to ART derivatives such as ATS and DHA. To confirm our results, parasites were also evaluated in the comet assay after gamma irradiation exposure at 65Gy, which is known to cause DNA double-strand breaks. As we predicted, parasites exposed to gamma irradiation also displayed comet-tails like with OTM value comparable to parasites exposed to drugs. Our results showed that both quinoline and artemisinin-derived compounds could induce DNA damage in the parasite genome.

It has been reported that ART and ATS induced DNA damage by increased free radical formation via the generation of ROS (Gopalakrishnan et al. 2015, Cumming et al. 1997). We next investigated if DNA damage caused by these drugs is mediated by intracellular ROS. We measured the amount of intracellular ROS using DCF-DA fluorescence assay. We found that all tested drugs resulted in the increase of intracellular ROS level after six hours of exposure. Furthermore, most drugs caused the maximum peak of ROS production after 12 hours. Remarkably, the levels of intracellular ROS declined from the peak levels between 12- and 24-hours post-exposure except for CQ and PPQ, in which further increase in intracellular ROS

levels was observed, suggesting that CQ and PPQ had delayed effect in the parasites. This observation was coherent with the results obtained in the comet assay. The decreased intracellular ROS levels at 24-hour post-treatment may also indicate that most parasites were killed after exposure to drugs. In addition, dose-dependent increases in ROS levels were only seen in CQ and PPQ treated parasites but not in AQ, ATS, and DHA. The results from this assay gave an association between drugs-mediated DNA damage and the induction of ROS.

Glutathione (GSH) pathway plays a crucial role in the detoxification of intracellular ROS and ferriprotoporphyrin IX in *Plasmodium*. We then investigated if drug treatment could affect the GSH levels in the parasites. Indeed, total GSH levels were decreased in all drug-treated parasites and a parallel increase in GSSG (oxidized form of GSH) levels was observed. However, the increases in oxidized GSH were less obvious than the reduction in GSH levels. This observation indicated that the amount of the free GSH may also be reduced via formation of GSH-protein adducts. Low GSH: GSSG ratio is believed to be a marker of oxidative stress. The GSH: GSSG ratio in all drug-treated parasites was lowered compared to non-treated parasites, suggesting that these drugs induce oxidative stress in the parasites.

Next, we sought to investigate whether a transcriptional cascade receptive to DNA damage would be upregulated by exposure to these drugs. We selected a group of DNA repair genes involved in different DNA repair pathways. This selection was based on previous reports as well as their sequence homology to known eukaryotic and yeast model organisms. Indeed, our results showed up-regulation of the DNA repair gene expression involved in different DNA repair pathways 12 and 24 hours post-treatment. Interestingly, some of the DNA repair gene expression levels differed among drugs, likely attributed to the type and the quantity of DNA damage induced by these drugs. Up-regulation of DNA repair genes involved in the HR pathway implied that these drugs induced DNA DSBs in the parasites, as DSBs are usually repaired via HR, MMEJ/A-NHEJ, and NHEJ. Orthologs of DNA repair genes involved in the NHEJ pathway are absent in the parasite genome. Mismatch repair genes encode proteins accountable for repairing errors that occurs during DNA replication (Mulligan et al. 2001). MMR proteins identify these errors and form a protein complex and eliminates the mismatched bases and subsequently corrects the sequence. Interestingly, we observed an up-regulation of DNA repair genes involved in mismatch repair pathways, suggesting that there was a mismatch resulting from faulty DNA repair. This error in the repair implied that drugs might induce DSBs in the parasites, which use an error-prone A-EJ/MMEJ pathway to repair DNA DSBs.

All of the results supported our hypothesis that antimalarial drugs induce DNA damage in malaria parasites. It has been reported that defective DNA repair is associated with increased mutation rates and rapid development of drug resistance in *P. falciparum* (Trotta et al. 2004). We therefore tested further our hypothesis that antimalarial drug-induced DNA damages and subsequent erroneous repairs can accelerate the rates of mutation and hence the generation of drug resistant trait. We performed whole genome shotgun sequencing to search for functional consequences in the parasite genome after short exposure to antimalarial drugs. 27 parasite clones were successfully sequenced yielding a mean coverage of 56X. The genetic variation

background between the parental 3D7S21 clone (four untreated control parasite cultures) and the reference 3D7 were first assessed. Our results revealed that 3D7S21, which was cloned from 3D7 population cultured for a long period of time, seems to harbor > 3000 genetic variations from the reference 3D7 genome. Although the total numbers of called variants differed among control cultures, they were sufficiently comparable to each other since variant calling by false discovery rate can result in an increased type 1-error rate. Interestingly, the number of total variants found among clonal parasites originated from the same drug treatment can quickly result in an increase in variance in the genome in a parasite population. Provided that the period of the drug exposure was relatively short, the observed genome variance is unlikely to be a selection process driven by drug pressure but that each individual clone parasites could be subjected to random DNA damage and defective DNA repair. To further support the potential clonal variance driven by drug treatment, a principal component analysis of the genome sequences was done. Our results revealed that control parasites were tightly clustered while most drug-treated parasites did not cluster with their counterparts originated from the same drug treatment condition. Additionally, recovery cloned parasites were more divergent from the control parasites when comparing with the populations. We then calculated the proportion of variants located in the subtelomeric regions to further elucidate these individualized effects of the drugs. We found that drug-treated parasites showed wider range of the calculated proportions, confirming our observation that drug treatment had pushed genome divergence as seen by the altered variants distribution in the genome. A higher indel to SNP ratio were also seen in the parasite genome after exposure to drugs compared to the untreated parasites, except for PPQ. The manifestation of an indel is more likely to have resulted from defects in DNA repair of DSB site, thus higher indel to SNP ratios may imply that these drugs induced DSB in the parasites. This result was consistent to our results obtained from qRT-PCR experiments. Finally, we identified all indel variants that resulted in frameshift. In particular we saw at least one frameshift that occurred in a predicted DNA repair gene, which may result in inefficient in repair the DNA damage, destabilization of the parasite genome, and increased mutation rate in the parasite clone.

Paper II

Extracellular vesicles are important for cell-to-cell communications between parasites but EV's effect on host cells still remains largely undetermined. In rodent and human malaria system, EVs can induce innate immune system of the cells (Mantel et al. 2013, Couper et al. 2010). It was also showed that EVs enable cell- to-cell communication among pRBCs through the transfer of the signaling substances from a donor to a recipient iRBC. This type of cellular communication is thought to be associated with the development of gametocytes (Mantel et al. 2013). Moreover, it was also showed that EVs derived from pRBCs are effectively engulfed by macrophages and produce a strong inflammatory response. pro-inflammatory cytokines and chemokines generation leads to activation of endothelial cells and consequently recruit and infiltrating inflammatory cells, which then cause additional activation of endothelial cells and parasite sequestration. In addition, the analysis of pRBCs-derived EVs content discovered a

set of specific proteins from RBC and parasite and RNA such as small RNA species (Mantel et al. 2013).

In this study, we aimed to elucidate the theory that EVs derived from iRBC contain microRNAs that can modify the recipient cells gene of target. We previously showed that purified EVs contained specific proteins from the parasite and host such as hAgo2 and RNA from EVs derived from pRBCs, even though the nature and function of these RNA species in malaria are not clear (Mantel et al. 2013). In order to confirm our results, we then elucidate the nucleic acid composition in EVs by analyzing the size distribution of RNA from purified EVs derived from pRBCs. We found small RNA species approximately 21-25 nucleotides which signify the occurrence of mature microRNAs. Although *Plasmodium* parasites don't have functional RNA interference machinery that can produce miRNAs (Baum et al. 2009), we sought to investigate if these miRNAs came from the host. It was previously illustrated that RBCs from humans express a panel of miRNA with different function upon maturation of the RBCs (Chen et al. 2008). We then defined the content of human miRNAs in pRBCs-derived EVs using nanostring expression array that contain probes for all 800 miRNAs species derived from human and compared the contents and the number of human miRNAs from RBCs, pRBCs as well as EVs. Indeed, we detected a total of 21 RBC-specific miRNA in RBC and we also found several of these miRNA in pRBCs and pRBCs-derive EVs. We found out that all the miRNA were reduced in pRBCs and EVs except for miR-451 and let-7b as compared with RBCs. miR-452 is abundantly expressed in RBCs and plays a significant role in erythroid homeostasis upon maturation of RBC (Patrick et al. 2010). We also confirmed the presence of mature miR-452a, let-7b, *RNU6,* and miR-106b in RBCs, pRBCs and EVs using northern analysis. All four miRNA were detected in RBCs and EVs derived from pRBCs. Overall, our results revealed the presence of RBC-derived miRNAs, specifically miR-45a, in both EVs and pRBCs.

There is growing evidence that shows that EV-mediated miRNAs transfer among cells. We then postulated that this subset of miRNA found in both pRBCs and EVs may bind to Ago2. Although the function of in erythroid cells has been described (Lawrie et al. 2010), there is still no evidence that shows if the RNAi machinery still functional when erythroid cells differentiate to normocytes or mature erythrocytes. To determine further if these functional miRNAs were present in mature erythrocytes, we investigated the expression of the RNAi machinery components from hematopoietic stem cells to normocytes during *in vitro* RBC development (Bei et al. 2010). Ago2 was detected in mature erythrocytes, whereas Drosha, as well as Dicer were only observable in erythroid cells, before anucleation. We further investigated the Ago2 localization in mature erythrocytes by immunofluorescence assay and FACS. In mature erythrocytes, Ago2 was localized to the membrane peripheral. Similar results were found in pRBCs that contain ring stage of parasites (up to 20 hpi), but, the signal disappears during the late cycle of pRBCs. We revealed that miR-451a and let-7b formed an active complex with Ago2 in EVs. Our results also confirmed the presence of Ago2 in the soluble fractions that represent RBC cytosol and food vacuole by western blot analysis. The protein was detected in the pellet fraction during the 24 h of parasite development which also confirmed the

observation in IFA localization assay in the parasite and at peripheral RBC membrane and/or cytoskeleton.

Human Ago2 and stomatin were also found in EV-derived from pRBCs by western blot analysis, confirming our previous proteomic results (Mantel et al. 2013). Our study revealed that Ago2 is present in EVs through immuno electron microscopy. Recent studies have shown that almost all miRNAs detected in the healthy donor's plasma are usually linked with the protein-lipid complexes and not with EVs (Arroyo et al. 2011). We used size exclusion chromatography and Proteinase K protection assays in order to eliminate the probability that Ago2 and miRNA are serum-derived residual complexes. Our results showed that serum from human certainly has a subpopulation of miRNAs and proteins as those from EV preparations. Ago2 resisted to Proteinase K treatment with the purified EVs suggesting they are protected inside the EVs from protease (Mantel et al. 2013). Our results confirmed that human Ago2 and miRNA are present inside EVs.

In order for miRNAs to be functional, it has to complex with Ago2 to form RISC. RISC binds to the targeted mRNA via the seeding sequence of the miRNA. We further investigate if this complex form a functional silencing complex. We tested the sequence-specific endonucleolytic activity of RISC by incubating purified pRBCs-derived EV with a radioactively labeled RNA sensor probe that contain a complementary sequence to either miR-451a or let-7b. This preliminary experiment revealed a time-dependent cleavage activity. We further quantify the activity of EV lysate and determined the concentration of the probe during of specific cleavage of the miR-452a probe. We found that the probe was fully degraded at higher concentrations, indicating the nonspecific RNases in the EV lysate. We also showed the importance of active enzyme by treating the EV lysate with Proteinase K or EDTA. To further corroborate the Ago2-miRNA complex (RISC) functionality in EVs, HEK293 cells were transiently transfected with a constructed reporter gene. We found that the luciferase assay with the miR-451a target sequence in the 3′-UTR of a reporter cassette showed that the activity of the reporter gene was substantially downregulated in transfected HEK293 cells upon incubation with EVs. Supplementation of either let-7b or miR-15a of miR-106a responsive element showed a substantially depleted the luciferase reporter gene activity, even in the absence of EVs, suggesting that HEK cells express a substantial amount of those miRNAs. Our results revealed that miRNAs in EVs are functional and Ago2 association is vital for activity.

Confirmation of the occurrence of functional miRNA-Ago2 complexes in EVs, we set to elucidate the miRNA-Ago2 complexes physiological function during infection. We previously reported that EVs were internalized in pRBCs and macrophages (Mantel et al. 2013). To investigate if EVs are able to internalize in endothelial cells which may contribute to the changes in the function of endothelial cells during infection, we labeled the membrane of purified EVs and subsequently co-incubated with BMEC (Schweitzer et al. 1997). We detected an increase in the uptake of labeled EVs and confocal microscopy analysis also verified the increase of labeled EVs in the perinuclear region of endothelial cells. We further determine if the uptake of pRBCs-derived Evs into BMEC is through endocytosis. We also tested with

known endocytosis inhibitors (Mulcahy et al. 2014) to measure uptake of EVs over time. Our results confirmed that EVs were internalization in endothelial cells via endocytosis. We also found that EVs are capable of activating pro-inflammatory cytokines including IL-6 and IL-1 which lead to increase expression of adhesion molecules (Rabb et al. 2002), therefore contributed to the vascular dysfunction by activating an excess leukocyte adhesion and transmigration. Additionally, the internalization of EVs directly activates VCAM-1 expression possibly intensifying vascular dysfunction.

We further investigated if EVs derived from pRBCs may transport regulatory miRNAs to the endothelial cell. We determined miR-451a levels in endothelial cells upon EV internalization using qRT-PCR. Our results showed an accumulation of miRNA in a dose-dependent manner approximately up to the 50-fold above background. We also identify and quantify copy number of miR-451a in endothelial cells during uptake of EV through RNA fluorescent *in situ* hybridization (RNA FISH). We further treated BMEC with α-amanitin before addition of EVs to eliminate the probability that miR-452a expression is activated in endothelial cells upon uptake of EV instead of transferred via EVs and demonstrated that the increase in the expression of miR-451a was not affected by α-amanitin. Overall our experiments revealed that miR-451a can be transferred to endothelial cells through EVs, and it was not expressed in endothelial cells.

We then investigated if internalized EVs can target the gene expression in endothelial cells via RNA interference of EV-derived miRNA complexes on target genes. We used miR-451a target gene prediction search tools. Our result revealed multiple targets either within coding regions of genes or within the 3′-UTR of genes. We subsequently established the expression of these putative targets in the BMEC line through microarray expression analysis. In order to evaluate possible expression regulation by EV-derived miRNAs in BMEC, we focused on the previously validated miR-451a targets including *CAV-1*, *ATF2*, *GRSF1* and *PSMB8* (Orom et al. 2012, Trajkovski et al. 2011, Lv et al. 2014, Lopotova et al. 2011), all of which are expressed above the median of all genes in this BMEC line. miR-451a has been shown to inhibit *ATF2* expression through binding to its 3′-UTR (Lv et al. 2014). ATF2 is a cAMP-response element-binding protein with a basic leucine zipper (bZIP) domain, through interactions with other bZIP proteins (Lv et al. 2014). This protein activates an array of genes that are involved in anti-apoptotic signaling (Bhoumik et al. 2004), and inhibition of this protein through siRNA causes apoptosis in a melanoma model (Bhoumik et al. 2002). ATF2 downregulation by miR-451a might consequently add to the detected apoptosis of endothelial cells upon infection (Hemmer et al. 2005). Quantification of transcript levels by qRT-PCR in endothelial cells upon EV incubation compared to control showed significant downregulation of two markers, *CAV- 1* and *ATF2* (both with partial miR-451a target sites in their 3′-UTR) after 24h of EV incubation compared with untreated control cells. *GRSF1* and *PSMB8* gene expression were not affected during EVs incubation. Caveolin-1 (*CAV-1*), a scaffolding protein and key component of caveolae, particular lipid rafts in the plasma membrane of many cell types with a function in signal transduction and endocytosis (Bastiani et al. 2010). Western blot analysis showed the downregulation of both CAV-1 and ATF2 on the protein level after EVs incubation. Our

studies have shown that pRBCs-derived EVs be able to regulate gene expression in the recipient endothelial cells. Particularly, we showed that miR-451a derived from EVs targets endothelial cell expression markers including *ATF2* and *CAV-1*.

To confirm further that *CAV-1* and *ATF2* downregulation is facilitated by miR-451a derived from EVs, two different approaches were used. BMEC was transduced with lentiviral expression vector containing miR-451a or GFP for control. Our results showed that in the BMEC/miR-451a line miR-451a was highly activated while *CAV-1* and *ATF2* expression on both the transcript and protein levels were decreased, compared to the GFP control (BMEC/GFP) and BMEC. We then transiently expressed Sponge miR-451a in endothelial cells before incubation with EVs to confirm the targets of miR-451a derived EVs. We found that Sponge miR-451a binds and sequesters cellular target miRNAs and thus neutralizes them. We also revealed that the expression of Sponge miR-451a restored the expression of CAV-1 and ATF2 on the mRNA and protein levels and verifies that they are the target of miR-451a-derived Evs. We were able to repeat the detected effect of EVs on endothelial barrier properties in the BMEC/miR-451a line thru permeability assays using rhodamine-labeled dextran. Our experiments verified a regulatory function miRNA-derived EVs-complexes in endothelial gene expression and barrier function.

In summary, data obtained from this study showed that EVs derived from pRBCs contain host miRNAs that form a functional RISC complex with Ago2. We also demonstrated that EVs were effectively internalized in endothelial cells, where the miRNA-Ago2 complexes modify target gene expression and barrier functions. The data gained from different *in vitro* experiments support a model where EV release adds to both local and systemic pro-inflammatory cytokines and chemokines generation and to vascular dysfunction which stimulates endothelial induction, leakage, and sequestration of parasites including pathology upon malaria infection. Moreover, our results give a mechanistic association between EVs and vascular dysfunction upon malaria infection.

Paper III

The ability to measure gene expression of individual *P. falciparum* parasites gives us an opportunity to answer key questions that involve small sub-populations of *P. falciparum* parasites, including mechanism triggering the switch from asexual to sexual development which may help to identify targets for transmission-blocking agents, artemisinin-induced dormancy in resistant parasites and mechanism underlying chloroquine resistance, with important implications for malaria treatment. *P. falciparum* parasites display cellular plasticity during their complex life cycle. Malaria clinical symptoms result from asexual replication within erythrocytes, while transmission to new hosts depends on replication in the mosquito vector. Both transmission and development of malaria disease are therefore supported by the malaria parasite´s capability to successively differentiate into morphologically distinct forms, such as invasive, replicative and gametocytogenesis (sexual stages). This adaptability is orchestrated by tight regulation of the *P. falciparum* genome, in which the functions of most genes are still not well understood (Otto et al. 2014). Insight into gene use and gene function

during the *P. falciparum* parasite´s complex life cycle will help to identify targets for new drugs and transmission-blocking agents.

In this study, we investigated the capacity of Single-Cell RNA-seq for molecular analyses of rare *P. falciparum* parasites phenotypes. We sought to establish a capillary-based iRBC method in isolating individual pRBCs followed by Single-Cell RNA-seq using Smart-Seq2. We picked individual pRBCs at six different time points during the 48h intraerythrocytic development cycle over three separate experiments. Our result showed that although we tightly synchronized the parasites, the clustering of individual-parasite transcriptomes is not completely aligned. This may be due to difficulties in visually distinguishing the intermediate IDC stages using 10X magnification when picking single parasites. Higher magnification is indeed needed in order to specifically verify the morphological differences of cells. However, when we performed computational analyses, we could designate a group of parasites to distinct IDC stages.

It was demonstrated that scRNA-seq lacked sensitivity with regard to gene detection (Ramsköld et al. 2012) and population-level analyses of the ring stage of the 48h IDC have revealed the small amount of RNA present in the ring stage and also inadequate transcriptional activity (Sims et al. 2009). In our single-cell result, we were able to identify the sub-stages of IDC of *P. falciparum* especially during the early stages of the IDC and a gene marker that distinguishes among asexual and sexual parasite stages by transcriptionally profile the 165 individual pRBCs at different time points during IDC. We identified a minor population of putative gametocytes based on a single genetic marker. We also established numerous RNA-seq libraries from populations at each time points as control. The average numbers of *P. falciparum* genes detected were 300-800 in every single pRBCs, similar to two current published Single-Cell RNA-seq data sets (Poran et al. 2017, Reid et al. 2018).

Assessment of the overall structures in individual *P. falciparum* parasite transcriptomes using principal component analyses and t-distributed stochastic neighbourhood embedding shown that individual pRBCs displayed heterogeneity. Furthermore, we investigated the transcriptomes of each single pRBCs and concentrated on their expression of a previously known data set of gene during IDC in *P. falciparum* (Bozdech et al. 2003). We identified genes with significant differential expression in most sub-populations. For example, SP1 and SP6 showed the difference in expression of 42 genes that involved in cytoplasmic translation machinery in SP1 on the other hand SP6 early rings and merozoite invasion transcripts and also actin myosin related genes were up-regulated. Though we observed a large number of dropouts in single-cell data due to little amount RNA presence, we still found that the pattern of gene expression followed the temporal pattern of expression as described previously (Bozdech et al. 2003).

In order to assess the detection capacity of rare parasite phenotypes in cell population, we then investigated the RFP expression (164-tdTom express RFP under the PF10_0164 promoter) (Buchholz et al. 2011) in the transcriptome profiles. This promoter is induced when parasites become gametocytes stages. We discovered expression of RFP in three single pRBCs and

identified several known gametocyte markers. Interestingly, characterization of the putative gametocytes found novel putative gametocyte genetic markers with substantial increased expression in the RFP expressing cells. Validation of ten gametocyte specific genes with high expression in RFP-expressing single cells using qRT-PCR showed that five of ten consistently had higher levels of expression among stage I and mid-stage gametocytes compared to schizont stage parasites. Our results demonstrated that it is possible to molecularly identify rare parasite phenotypes using Single-Cell RNA-seq and functional study of these candidate gametocyte markers will determine if they are crucial to *P. falciparum* gametocyte development or sexual stage commitment, which will also help to design a transmission-blocking strategy.

P. falciparum contains *var*, *stevor* and *rif* gene families that code for proteins known as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), sub-telomeric variable open reading frame (stevor) and repetitive interspersed family (rifin), respectively (5, 124-130). Variable gene families (*var* genes) are clustered towards the telomeres and code for proteins that are transported to the surface of pRBCs where they mediate adherence to host endothelial receptors (131), resulting in the sequestration of infected cells in a variety of organs. These properties are important virulence factors which contribute to the development of severe malaria (132-135). Transcriptional switching among *var* genes enables antigenic variation and is a means of immune evasion, enabling chronic infection and transmission (136). Thus, *var* gene family products play a crucial role to the pathogenesis of malaria and to the production of protective immunity. We therefore investigated the feasibility of analyzing expression of the *var* gene families at single cell-level, we carried out targeted analysis of *var* and *clag* genes which are involved in erythrocyte invasion and cytoadhesion. We confirmed that *clag 3.1* and *clag 3.2* genes are dominantly expressed in a considerably larger number of parasites and dual-expression of these genes in a few individual parasites. We also verified the dominant expression of *var* gene in three single cells including UPSC1, UPSB1 and UPSB6 and UPSB7. *Var2csa* gene, which is involved in pregnancy-associated malaria, was found expressed in one single cell.

Moreover, we showed that our technique has the capacity of facilitating differential gene expression analyses from the *va*r gene families in *P. falciparum* parasites at the single cell level. Recent study have showed that smart-seq2 oligo-dT modification (Reid et al. 2018), yielded a positive effect on the total coverage of genes expressed in late intraerythrocytic developmental stage. It is interesting to further scrutinize if this modification of poly-T oligo will also increase yields in the ring stage of *P. falciparum* IDC parasites. Lastly, we have developed and evaluated a strategy for global transcriptional profiling of *P. falciparum* at single-cell level. This strategy can be useful in elucidating at the cellular level the molecular basis of malaria disease. It could also be used for other *Plasmodium species* and other members of the Apicomplexa including *Babesia*, *Cryptosporidium parvum* and *Toxoplasma gondii*.

4 CONCLUSION AND FUTURE PERSPECTIVES

The development of drug resistance in *P. falciparum* has compromised the efficacy of antimalarial drugs. Emerging multidrug resistance to current drugs poses a central problem in global malaria control. Identifying the causes of drug-resistant malaria seems to be more complex than perhaps originally predicted. The lack of clear understanding of the molecular mechanisms underlying antimalarial drug action and drug resistance, commitment to gametocytogenesis, and parasite-to-parasite communications hinders efforts to eradicate malaria. This thesis attempted to answer some of those questions stated above.

I) There are still a lot of unanswered questions concerning the DNA repair mechanism and the important of different DNA repair genes involved in these pathways in *P. falciparum*. One major factor that contributes to the resistance development is the high plasticity of the parasite genome that permits the rapid acquisition and fixation of adaptive mutation, gene-duplication and deletion upon exposure to antimalarial drugs. Although the causes of high plasticity in the parasite genome are not well understood, DNA repair mechanisms as well as the overall DNA damage response are believed to contribute to the maintenance of the genome integrity and genome mutation rate. In Paper I, we tried to investigate the effect of antimalarial drug on the genome integrity in *P. falciparum* and the possible role of DNA repair mechanism in the development of resistance to antimalarial drug. We revealed direct and indirect evidence of DNA damages in parasites after short exposure to all the tested antimalarial drugs. Genome sequencing revealed that some of these DNA damages were likely repaired by an error-prone DNA repair mechanism. Thus, even short exposure to antimalarial drugs could lead to novel mutations and diversify the gene pool in a population, potentially allowing for selection of drug resistant parasites. Further studies are needed to understand the mechanisms affecting mutation rate, the role of DNA repair processes as well as the presence of indels in DNA repair genes. These studies may provide insights on drug design for malaria treatment and development of new strategies to effectively prevent drug resistance.

II) Cell to cell communication is believed to be essential for cell survival, differentiation, and control of population density. *P. falciparum* infected erythrocytes directly communicate with one another using extracellular vesicles (EVs) that are capable of delivering protein, lipid, messenger RNA and microRNA, as well as pathogenic molecules and antigens. EVs are also involved in several physiological processes such as immune regulation. Human Argonaute 2 (hAgo2) is an effector protein that binds to microRNAs to form RISC. The sequence-specific miRNA directs this RISC complex by targeting the coding region sites of target mRNAs for endonucleolytic cleavage and/or to the 3′-untranslated regions (UTR), resulting in repression in translation. In this study, we detected several miRNA from the host in EVs derived from pRBCs and revealed that these miRNA were

bound to human Ago2 and formed functional complexes. Furthermore, the internalization of iRBC-derived EVs into endothelial cells was observed. These Ago2-miRNA complex-containing EVs were shown to modify target gene expression and alter endothelial barrier properties. Overall, our discoveries give a mechanistic link between EVs and vascular dysfunction that promote endothelial activation and parasite sequestration during malaria infection. In the future, it will be important to determine the role of EVs in gametogenesis and also the spread of resistant traits.

III) Single-cell genomic is a powerful technique that has helped unravel the dynamic of genetic changes within the population and the genetic structure of unicellular organisms. Single-cell RNA sequencing of *P. falciparum* could be key to understanding the complexity of malaria infection. We developed and assessed a technical platform that enables single-cell transcriptomic analysis of individual *P. falciparum* parasites. In this study, we identified a minor population of gametocytes with distinct gene expression signatures, a sexual stage parasite based on a single genetic marker. We also showed huge heterogeneity in transcript profiles among highly synchronized parasite populations. Our method showed the capacity to elucidate the transcriptional programs underpinning rare phenotypes from single parasite level. Furthermore, we proved that the coverage of the sequence was adequate to generate lists of important genes based on global differential expression analyses. This platform will allow future studies to further explore the mechanism by which the parasites switch and commit to sexual development.

5 ACKNOWLEDGEMENTS

There are several people whom in different ways have contributed to this thesis and I want to express my sincere gratitude to:

All my colleagues that helped me out during these four full years. Special thanks to my main supervisor **Mats Wahlgren** for giving me the opportunity to join your group and many thanks for your guidance and support over these years. I am extremely grateful to my second main supervisor (co-supervisor) **Sherwin Chan**, for your endless help and never ending devotion in helping me and for making me go further and for making me think twice and also for keeping me alert, you're a brilliant co-supervisor! Thank you also so much for your relentless support, understanding, kindness, patience and for all the laughter and fun, my partner in Ping-Pong and lunch partner, and for letting me barrow Mochi often to keep my husband company while I was doing my time course experiments and of course for being sincere friend. I will miss working together with you and I am sure you will succeed in whatever you do. Many thanks to my co-supervisors **Ulf Ribacke** for your help, especially during the last year of my PhD and for all the fun and laughter, **Kirsten Moll** for your help and support in the lab and **Johan Ankarklev** for helping me to join Mats Wahlgren´s group and to work with Laura Kirkman, Kirk Deitsch and also to work in Mattias Marti lab.

I would like to extend my deepest gratitude to **Pierre-Yves Mantel** for your supervision, help, valuable advice, profound belief in my abilities and enrelenting support while I was a working in Harvard University, School of Public Health, Boston, USA. Thanks for introducing me to extracellular vesicles, and all the experimental techniques, I really learned a lot. You're a good colleague, supervisor and friend. Thanks also to **Matthias Marti** for allowing me to work in your lab. Thanks to the former lab members of **Marti´s group**, **Kassie Dantzler, Deepali Ravel, Karell Pelle, Nicolas, Gabriel** especially thanks to **Justin Zelin** and **Marina Ribeiro** for all your help and also for the trips and fun and laughter. Thanks to **Daniel Irimia** and **Anh Hoang** at Massachusetts General Hospitals, Boston, USA for letting me work with microfluidics.

I also wish to thank the people at Weill-Cornell Medical College, New York, USA. **Laura Kirkman**, thank you for sharing your great knowledge and helping me with my PhD project and **Kirk Deitsch** for allowing me to work in your lab and for inspiring scientific discussions.

Many thanks to **Carlos Talavera López** at Wellcome Sanger Institute and Björn Andersson at KI for helping me with the whole genome sequencing.

I also wish to thank **Rickard Sandberg** for giving me the opportunity to be part of the single cell paper and for scientific discussions. I also had a great pleasure of working with **Mtakai Ngara,** thanks for inspiring scientific minds and friendship.

I would like to express my deepest appreciation to my opponent, **Dr. Lena Åslund**, each one of my examination board members: **Prof. Ingrid Faye**, **Prof. Mikael Rhen** and **Associate Prof. Eleni Aklillu**.

To the senior members: Thanks to **Anders Björkman**, **Akira Kaneko, Pedro Gil** and **Susanne Nylén** for stimulating scientific discussion during Friday meetings.

I am also very grateful to my colleague and friend **Berit Schmidt** for your guidance, encouragement and also for giving me inspiration.

I´d also like to extend my sincere gratitude to **Manuel Patarroyo**, you're always there when I needed someone to talk to. Thank you so much for all the good conversation both scientifically and personally, for your guidance, for all the fun conversation and endless moral support and encouragement.

Special thanks to **Pontus Hedberg**, for all the fun times, encouragement, helpful advice, insightful suggestions and for being a friend. I very much appreciate **Madle Sirel**, for helping me with the cover illustration, *P. falciparum* life cycle illustration and two other illustrations for my thesis. I appreciate it a lot. You would also be an excellent graphic designer!

My former colleagues and friends **Chim Chan**, thank you so much for all your help reviewing each topic of my thesis, and for being such a wonderful friend. **Zulkarnian Md Idris**, **Oppa**, many thank for all the laughter and fun, for introducing me to Korean drama and for moral support. I appreciate it a lot.

To my Columbian and Honduran friends and former colleagues, **Pilar Quintana Varón**, **Irina Jovel** and **Paola Martinez** thank you so much for all your support and it has been great to spend time with you guys.

To my wonderful, loving and caring friend **Caterina Scasso,** thank you so much for your friendship, for bringing happiness in the lab and for accompanying me sometimes when I had my time course experiments.

To all my former colleagues, **Wataru, Viktor, Shuhan, Alejandra, Junhong, Alan, Atiqul, Reetesh, Suchi, Maryam, Mia.**

Xiaogang Feng, I still remember the first day I met you, when we had our registration seminar. You made me really nervous before my presentation haha. But I am really glad that we got registered and started out PhD journey together. Thank you for all the laughter and fun. I also had great pleasure of working with **Cajsa Classon**, thanks for being there when I need someone to talk to. **Ulrika Morris**, it was a great pleasure for me to meet you and thank you so much for you encouragement. I appreciate it a lot!

Many thanks to **Martina Löfstedt Jalava**, for all the help with orders and administrative paper work and for being a good friend.

I am also grateful to **Velmurugesan Arulampalam**, for all your help, guidance and advice during my PhD.

I very much appreciate my mentor **Ulf Sandström,** for your guidance and moral support.

Åsa Belin, I can´t thank you enough for all the support, encouragement, patience that cannot be underestimated and guidance you have given me during my PhD, thank you for being there when I needed someone to talk to. Thanks, should also go to **Vivian Saucedo Hildebrand**, **Mia Sjöblom**, **Kristina Gullsby,** for all the administrative help and moral support.

I am also grateful to my wonderful friends, **Falk Saupe** for unwavering support, encouragement, profound belief in my abilities, **Oana Tudoran** for believing in me and unwavering moral support, **Svitlana Vasylovska** for helpful advice and moral support, **Alan Shaw, Eric Benson, Fernce Fördös, Andrew Blair and Zhou Peng.**

Hodan Ahmed Ismail, thanks for your friendship, support and encouragement and for all the fun times outside the lab, my movie companion.

I would like to express my deepest appreciation to my close friends, **Linda & Christian Buján**, **Lena & Micke Sundquist**, **Lill-Anita & Jan-Ove Sönnerbo** for all the support, encouragement and for believing in me**.**

I am also grateful to my family, especially my brother **Charlie Torino**, my sister-in law **Lourdes Torino** and my nephew **Wilhelm Torino** for giving me love, inspiration and encouragement. Special Thanks to **Kimverly Villamor** for your profound belief in my abilities and for unwavering moral support. I am also grateful to my father-in law **Bertil Hjelmqvist**, my brother-in law **Johan Hjelmqvist**.

Fredrik Hjelmqvist, my husband, my life companion, my driver. Thanks for your understanding, support and help during all these years, especially when I have been working hard late nights until dawn and week-ends. Your guidance and love have made my studies possible. I am grateful for all the experiences we have made together, looking forward to continuing explore life and see the world with you.

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