CHAPTER TWO: LITERATURE REVIEW

2.1 Summary and Aims

Plasmodium falciparum is the protozoan parasite species that causes the overwhelming majority of human malaria mortality [6]. It has recently been established that blood stage *P. falciparum* parasites secrete extracellular vesicles (EVs), which can mediate interaction within the parasite population and with the host. In other systems, EVs contain a wealth of bioactive molecules such as proteins, lipids and nucleic acids and can transfer these molecules to recipient cells, impacting function. To reflect the scope of the work, this introductory chapter has two parts. In the first part, I provide a general background to the current knowledge of *Plasmodium falciparum* cell biology, focussing on blood stages. In the second part, I describe extracellular vesicles, with special emphasis on the role of *Plasmodium falciparum* extracellular vesicles in malaria biology and pathogenesis.

2.2 Malaria is still a significant public health problem

Protozoan parasites of the genus *Plasmodium* cause malaria especially in low and middle income tropical countries [6]. In 2016 alone, there were approximately 216 million new malaria cases and 445 000 malaria fatalities. Africa bears the highest malaria burden, accounting for approximately 90% of malaria cases and 91% of malaria deaths in 2016. Most malaria related deaths occur in vulnerable populations of children under the age of five years and pregnant women [7, 8]. The only malaria vaccine candidate to reach Phase III testing, RTS,S, has performed poorly in clinical trials (30-50% effectiveness) and induces immunity that wanes with time [9-11]. This lack of an effective vaccine, coupled with reports that *Plasmodium falciparum* could be developing

resistance to the currently frontline antimalarial drug artemisinin [12-15], together constitute a significant challenge to the fight against malaria.

2.2.1 Five *Plasmodium* species cause human malaria

Plasmodium species belong to the phylum *Apicomplexa* that is named after the specialised apical complex of organelles involved in host cell invasion [6]. There are five species of *Plasmodium* that infect humans: *P. falciparum, P.vivax, P. ovale, P. malariae* and *P. knowlesi* [6, 16, 17]. Of the five *Plasmodium* species, *P. falciparum* causes the majority of severe malaria cases and deaths, primarily in resource-limited areas in Africa and Asia [8, 18]. *P. vivax* is mostly present in temperate climate areas and accounts for most of the malaria cases in Asia and Latin America [19]. An explanation for this distribution is that most Africans lack the Duffy antigen. *P. vivax* was long thought to be completely dependent on the Duffy antigen for erythrocyte invasion [20-22] , although the recent description of *P. vivax* in Duffy-negative people in Africa, which could either be a recent parasite adaptation or a long-term problem that has been undetected [23], complicates this simple view of *Plasmodium* species distribution. *P. ovale* and *P. malariae* are less studied *Plasmodium* species found in Africa and Asia, but most prevalent in West Africa [24, 25]. Previously, scientists considered *P. knowlesi* as a non-human primate parasite but it is now well established that it can cause zoonotic infections of humans, primarily in Malaysia [26, 27]. The work reported in this thesis focuses on *P. falciparum*, the most lethal of all the *Plasmodium* species, and the most well studied.

2.2.2 *Plasmodium falciparum* has a multistage lifecycle

Plasmodium falciparum has an intricate life cycle involving a human host and a mosquito vector. At each stage the parasite has evolved mechanisms to survive and exploit different environments, including human hepatocytes and erythrocytes as well as the mosquito midgut lumen, epithelial basal lamina and salivary glands (**Figure 1**) [28]. Human *P. falciparum* malaria begins when a female mosquito of the *Anopheles* genus injects thread-like forms called sporozoites acquired from a previous blood meal. Within 30-60 min after inoculation into the bloodstream, the majority of the sporozoites journey through the venous circulation to the liver, where they invade hepatocytes [29] and reproduce by asexual binary fission to generate thousands of daughter parasites, contained in a cyst like structure called hepatic schizont [30]. During this hepatic schizogony process, the patient remains malaria symptom-free.

The hepatic schizonts rupture after about 72 h, releasing the daughter merozoites into the bloodstream where most of them invade circulating erythrocytes within 30-60 sec [31]. Inside the erythrocytes, the parasites transform within a span of 48 hours from rings to larger forms called trophozoites, and then to multinucleated erythrocytic schizonts containing daughter parasites. The erythrocytic schizonts finally rupture releasing merozoites into the circulation. Each of these blood stage merozoites invades a new erythrocyte and continues the asexual life cycle of the parasite [32]. In non-immune individuals, the release of merozoites from blood schizonts induces recurring fevers, sweats and chills [33]. A fraction of sexually committed parasites differentiates to male and female gametocytes, the *Plasmodium* form transmitted from human to the mosquito vector [34, 35]. The male (microgametocytes) and female (macrogametocytes) gametocytes concentrate in skin capillaries from where a female *Anopheles* mosquito siphons them during a blood meal. While inside the mosquito midgut, the

microgametocyte and macrogametocyte fuse to form a diploid zygote, which differentiates to an elongated motile form called ookinete. This motile form invades the midgut wall epithelial cells and exits the mosquito gut lumen into the basal lamina where it transforms into an oocyst. An oocyst undergoes replication cycles and differentiation to form sporozoites, which migrate from the abdomen to the salivary glands of the mosquito [36]. In her next blood meal, the infected mosquito inoculates sporozoites into a new human host, thus sustaining the *P. falciparum* life cycle.

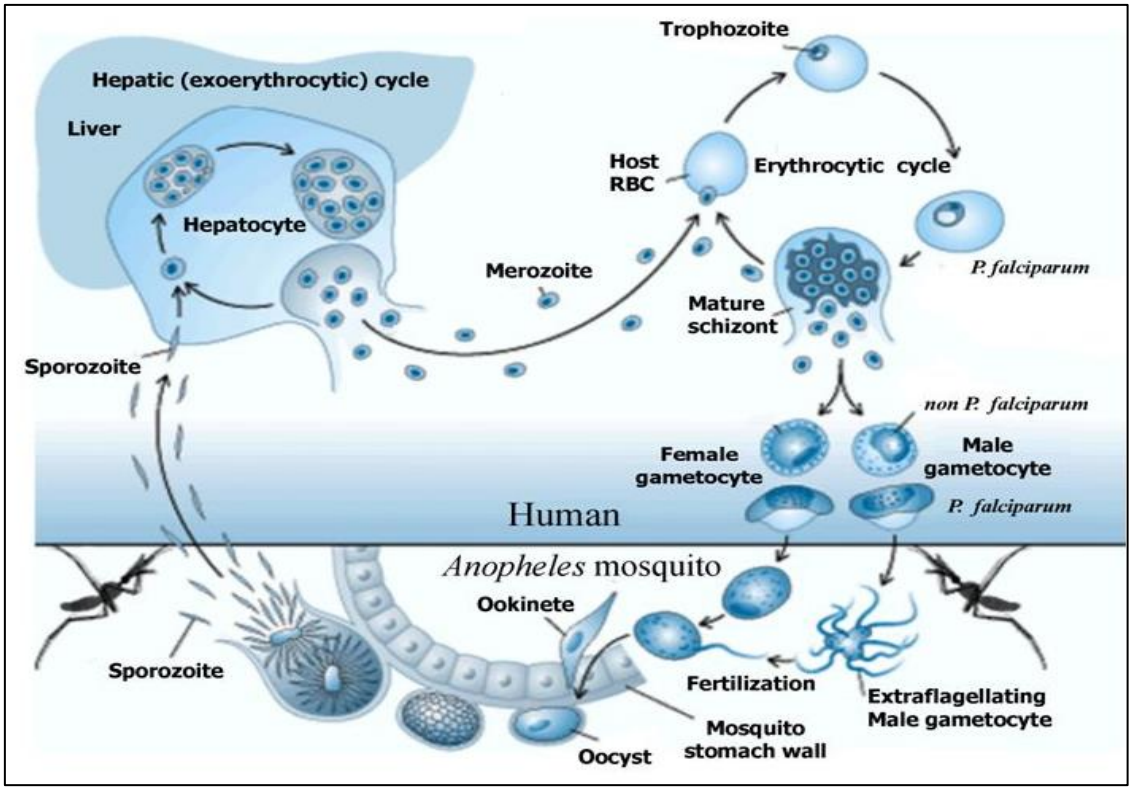

Figure 1: The life cycle of *Plasmodium falciparum*.
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2.2.3 The structure of the merozoite

The asexual stage of *P. falciparum* is an obligate intracellular parasite. It cyclically invades, feeds and asexually replicates inside RBCs to produce 8-36 invasive forms (merozoites) per schizont that then invade fresh RBCs [32, 37]. This cycle of invasion, differentiation, multiplication, and egress largely drives malaria clinical disease [33]

The *Plasmodium falciparum* merozoite (**Figure 2)** is an ellipsoid unicellular organism that measures approximately 1.2 µm in length and 1 µm in width. It has a tapered apical end and a round basal end [38], and is uniquely designed to invade erythrocytes. At the rear end of the merozoite are the classic eukaryotic cell organelles including the nucleus, mitochondrion [39, 40] and a vestigial plastid called apicoplast [41], which carry out genetic and metabolic functions [42]. Excluding the apical end, a trilaminar pellicle composed of a cell membrane and two inner membranes surrounds the rest of the merozoite. Underneath the inner membrane complex (IMC) are subpellicular microtubules that originate from the anterior end and extend posteriorly [43]. The IMC and the subpellicular microtubules act like a cytoskeleton by providing mechanical support to the merozoite and potentially play a role in erythrocyte invasion. Additional cytoskeletal structures called polar rings are located at the apex of the merozoite [44, 45].

The apical end of the merozoite has three membrane-bound secretory organelles, namely, rhoptries, micronemes and dense granules, which differ in size, shape and contents [45]. The protein content of these secretory organelles is involved in the adherence and entry of the merozoite into the RBC. The largest of the three secretory organelles are the rhoptries, which are composed of a pair of pear-shaped domains called bulbs and a narrow duct called neck. The contents of the rhoptry bulbs and neck are distinct and discharged at different time points during RBC invasion [46, 47]. Micronemes are smaller, elongated, numerous bodies that are clustered around the

rhoptries [38]. Dense granules are spheroidal bodies located less apically compared to the other two types of secretory organelles [48]. Rhoptries and micronemes release their contents through the apical prominence, while the dense granules secrete onto the sides of the parasite after it enters an erythrocyte [49].

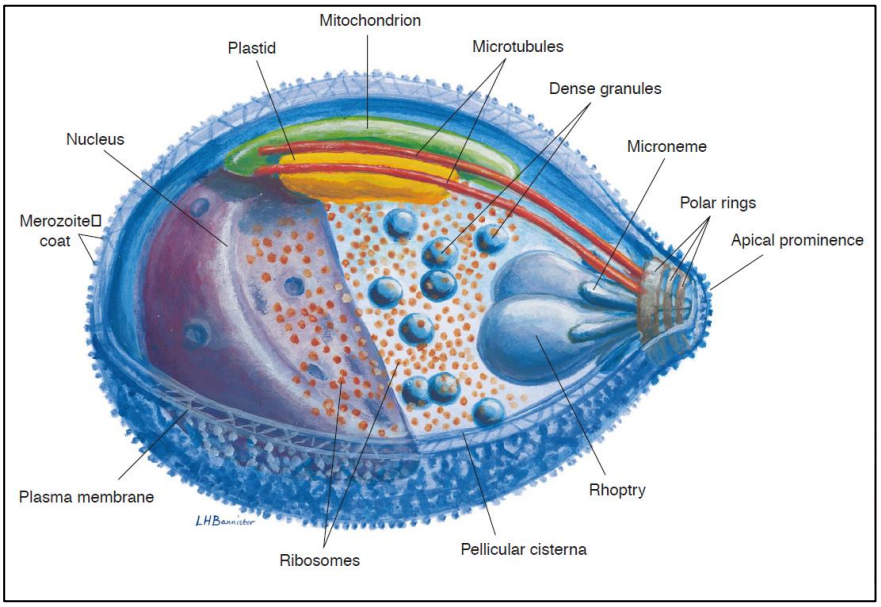

Figure 2: Schematic diagram of a merozoite.

Diagram was originally drawn by LH Bannister [50]

2.3 *P. falciparum* modifies the erythrocyte to make a suitable habitat

A mature RBC lacks internal organelles, in order to accommodate adequate amounts of haemoglobin for oxygen transport in the body [51]. In addition, its plasma membrane is highly deformable, a property that allows the cell to squeeze through narrow blood capillaries [51]. Once inside the erythrocyte, *P. falciparum* induces alterations in RBC physiology, morphology and function to enhance parasite survival [52-54]. There are two types of parasite-induced host cell modifications: 1) formation of parasite-derived membrane structures in the erythrocyte cytosol [55] and 2) insertion of *Plasmodium* proteins into the host cell membrane [52].

As it is most relevant to the topic of this thesis, I will concentrate primarily on the formation of *Plasmodium*-derived structures in infected cells. The exomembrane system is a collection of membrane-bound structures within the infected erythrocyte cytosol that are absent in non-infected cells [55]. These membranous structures play a role in the transport of parasite material to the RBC cytosol and beyond and include the parasitophorous vacuole membrane (PVM), the tubovesicular network (TVN) [56] and the Maurer's clefts (MCs) [57].

The PVM is a continuous membrane that surrounds the parasite at all stages [58]. The PVM is formed during invasion and contains parasite proteins such as heat shock protein 101 (HSP101) [59], exported protein 1 (EXP1) [60], exported protein 2 (EXP2) [61], early transcribed membrane protein (ETRAMP) [62], rhoptry-derived proteins [63] and dense granule proteins (GRA) . Of these, only HSP101, EXP2 and GRA have a well-

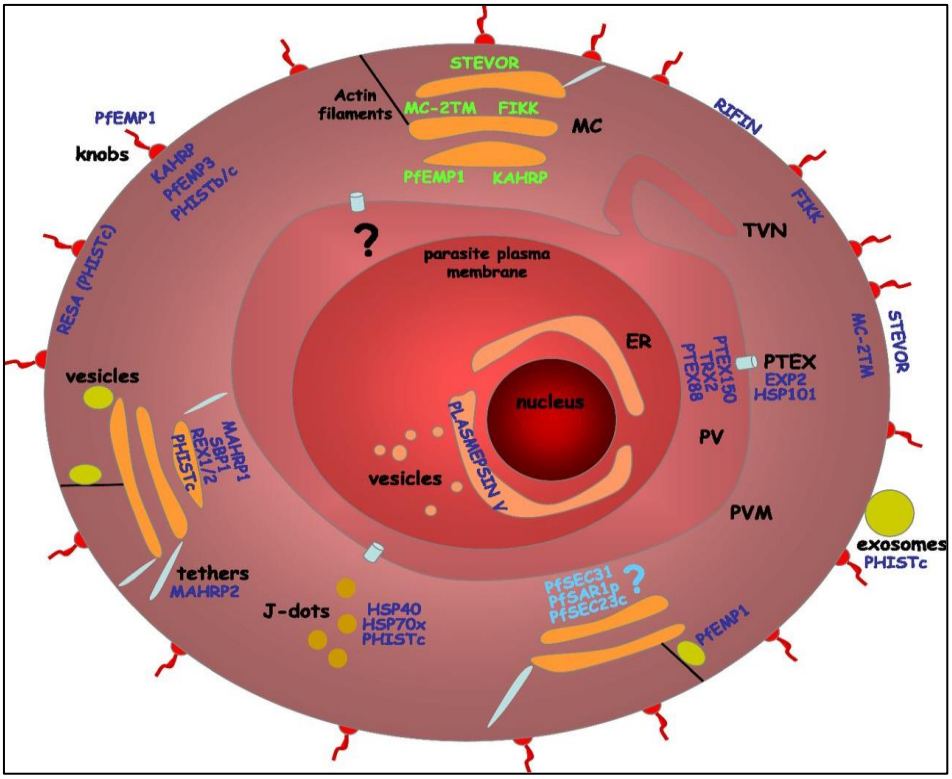

Figure 3: Schematic representation of parasite infected red blood cell

Mundwiler-Pachlatko and Beck, National Academy of Sciences, 2013

known function. EXP2 and GRA proteins form pores within the PVM that allow diffusion of solutes from the erythrocyte cytosol into the PV. In fact, HSP101 and EXP2 are components of *Plasmodium* translocon of exported protein complex (PTEX), an ATP-powered channel that mediates protein transport from the PV to the RBC cytosol [59, 61, 64, 65]. Related apicomplexans such as *Theileria* spp and *Babesia* spp exit the PV into the host cell cytosol immediately after invasion [66, 67]. The reason why, unlike these related parasites, the malaria parasite remains within the PV in order to create a more hospitable home for growth and survival is unknown.

The TVN and the MCs are the most striking parasite-derived compartments in the infected erythrocyte cytosol [52, 55]. The TVN is a network of whorl-like membranous structures that are continuous with the PVM. They have a double membrane and a lumen. The exact function of the TVN remains unresolved because TVN proteins such as EXP1 and EXP2 also locate to the PVM [56]. The TVN could play a role in protein trafficking and/or nutrient uptake [68]. MCs are single membranous compartments that derive from the PV and lie close to the erythrocyte plasma membrane [57, 69]. Both early and mature MCs contain *Plasmodium* ring-exported protein 1 (REXP1) indicating that the parasite plays an essential role in MCs formation [70]. Other MCs resident proteins include membrane-associated histidine-rich protein-1 (MAHRP1) [71], spectrin binding protein 1 (SBP1) [72], Maurer's cleft-two transmembrane proteins (MC-2TM) [73], Pf322 (a Maurer's cleft marker) [74, 75] and PfEMP1 trafficking protein 1 (PfPTP1) [76]. MCs proteins play a role in the transport of erythrocyte membrane protein 1 (PfEMP1), the best characterized virulence protein in malaria pathology [70-72, 77]. PfEMP1 is exported to the erythrocyte surface to facilitate sequestration of iRBCs via binding to the endothelia. Other proteins transported by the MCs include the erythrocyte membrane protein 3 (PfEMP3) [78], the knob-associated histidine-rich protein (KAHRP) [77],

members of subtelomeric variable open reading frame (STEVOR) protein family [79] and the repetitive interspersed family of proteins (RIFIN) [73, 79]. It is very likely that the MCs [80]) also transport nucleic acids and lipids from the parasite to the erythrocyte cytosol and beyond, but reports to prove the theory are lacking.

Above, I have illustrated how the parasite interacts with the outside environment through secretory organelles established inside the cytosol of the infected erythrocytes through which export of proteins such as PfEMP1 to the surface of the infected erythrocytes occurs. At the merozoite stage, the parasite also uses proteins contained in secretory organelles to invade erythrocytes. Apart from the physical interaction with the host through the cytoadhesive proteins expressed on the surface of the infected erythrocytes or released from the apical organelles of the merozoite, the parasite also releases extracellular vesicles that are important for interaction within the parasite population and with the host [1-3]. Below, I will review the literature on EVs in with particular focus on that of *Plasmodium* parasite.

2.4 Extracellular vesicles - general background

Extracellular vesicles (EVs) are nanoscale-sized particles shed by all cells into their extracellular milieu. They have a phospholipid bilayer that surrounds a lumen containing a wealth of biologically active molecules including proteins, nucleic acids and lipids. Recipient cells can internalize EVs, and as a result these provide a form of cell-to-cell communication. EVs have recently attracted attention as potential targets for therapies and diagnostics in both cancer and infectious diseases [81].

2.4.1 The history of cell-derived vesicles

The first study on EVs dates to 1946 when a prolonged high-speed centrifugation (31000 g for 150 min) of cell-free plasma extended the coagulation time of the supernatant. Reintroduction of the pellet to the supernatant shortened the coagulation time of the plasma indicating that subcellular factors promote blood clotting [82]. More than twenty years later, observation by electron microscopy revealed that this subcellular pellet consists of nanovesicles ranging from 20 to 50 nm and a density of 1.020 to 1.025 g/ml. These vesicles originated from platelets and were hence named “platelet dust” [83]. A decade later, Dalton identified “numerous microvesicles” of a diameter between 30 to 60 nm in foetal calf serum [84]. The term “exosome” was coined after the isolation of similar sized vesicles released by reticulocytes. These vesicles contained the transferrin receptor and hence proved to be a protein disposal route in cells [85]. Cumulatively, these early studies indicate that cells utilize extracellular vesicles (EVs) to externalize intracellular biomolecules.

2.4.2 The nomenclature and biogenesis of extracellular vesicles

Currently, there is no consensus in the nomenclature of EVs, due to a combination of detection difficulties and the multidisciplinary nature of the field. A wide range of terminology is used including dexosomes (EVs from dendritic cells) [86], oncosomes (EVs derived from tumour cells) [87], prostasomes (EVs from prostate cancer) [88], exosomes, exosome-like particles, nanoparticles, microparticles and microvesicles [89]. Recently, members of the International Society for Extracellular Vesicles (ISEV) tentatively proposed two main types of EVs based on their biogenesis, that is, exosomes and microvesicles [89, 90].

Exosomes are 30 to 150 nm in diameter and their biogenesis begins with the formation of multi-vesicular bodies (MVBs) through the inward budding of late endosomes . MVBs fuse with the cell membrane and discharge exosomes into their environment [89, 91]. (**Figure 4**). Conventional exosome surface markers include heat shock proteins (HSP), tetraspanins (such as CD9, CD63, and CD81) and members of the endosomal sorting complex required for transport (ESCRT) including Alix and tumour susceptibility gene 101 (TSG101) [89]

Exosomes differ from microvesicles (also called microparticles or ectosomes), which are larger in size (100-1000 nm) and shed directly from the outward invagination of the plasma membrane [89-91] (**Figure 4**). Microvesicles (MVs) form due to a disturbance in the asymmetrical balance of phospholipids between the inner and outer layers of the cell membrane. Under normal conditions, phosphatidylserine (PS) reposes almost exclusively in the inner leaf let of the lipid bilayer. When cells undergo activation (such as apoptosis), PS flips to the outer monolayer of the plasma membrane leading to the shedding of microvesicles [92]. The current isolation protocols do not clearly distinguish between exosomes and MVs, and therefore the term extracellular vesicles (EVs) accommodates all types of cell-derived vesicles [90].

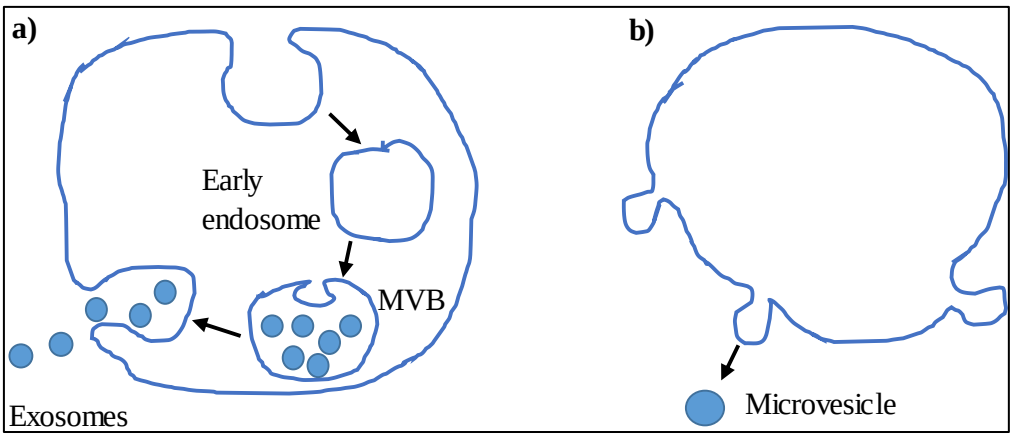

Figure 4: A diagram showing biogenesis of **a)** Exosomes **b)** Microvesicles

2.5 *Plasmodium* infected erythrocytes secrete extracellular vesicles

2.5.1 Animal models provide insights into the *Plasmodium*- EVs

Mouse models of malaria have shed light on the role of parasitized cell-derived vesicles in severe malaria. Plasma of mice infected with *Plasmodium berghei* contain submicron-sized MVs that express parasite antigens and phosphatidylserine (PS) on their surface. Interestingly, the production of MVs correlates with the onset of malaria clinical signs [93]. Similarly, reticulocytes from mice infected with *Plasmodium yoelii* secrete exosome-like vesicles that have parasite proteins and modulate the immune system [94]. These two rodent malaria studies infer that *Plasmodium*-infected cells release EVs into the blood circulation and might play a role in clinical disease.

2.5.2 Human-infective malaria parasites secrete extracellular vesicles

Erythrocytes infected with human-specific *Plasmodium* species secrete 10-13 times more MVs (100-400 nm) compared to uninfected erythrocytes [1, 95] and high MV concentrations are associated with higher parasitaemia and severe malaria [95]. *In vitro* studies showed that the release of MV by infected cells increases as the parasite matures within the erythrocyte and peaks at schizogony [1, 95]. However, Regev-Rudzik *et.al.* conducted a well-controlled study that established that ring-stage *P. falciparum*-infected RBCs (iRBC) also secrete exosome-like vesicles ranging from 80-120 nm in diameter [2].

2.5.3 Biogenesis of *Plasmodium falciparum* extracellular vesicles

The biogenesis of extracellular vesicles in *Plasmodium* is partially a mystery, given that infected RBCs theoretically lack what would be classic endosomes or multi-vesicular

bodies (MVBs). The current literature, although scanty, suggests that *Plasmodium* secretes a mixture of both exosomes and MVs. The size of ring-stage PfEVs (80 -120 nm) [2] and the presence of *Pf*Flotilin1, an exosome marker [5], suggests they could be exosomes originating from the parasite's exocytotic pathway, which is poorly described. On the other hand, PfEVs released by mature parasites are MVs as indicated by their size (100-400 nm) and the presence of microvesicle markers such as ADP-ribosylation factor 6 (ARF6) and PS [1, 95]. This supports the notion that *Plasmodium falciparum* may possess the machinery required for conventional EV biogenesis.

2.5.4 Application of extracellular vesicles in vaccine development

Many *Plasmodium falciparum* antigens have been evaluated as vaccine candidates with limited success. Therefore, efforts are still in progress to develop an effective antimalarial vaccine or improve the delivery of the well-characterized antigens. There is interest to use new technologies for large-scale production of EVs, and the utilization of EVs as vaccine delivery agents could provide novel approaches to malaria vaccine development. Immunization of mice with parasite-derived exosomes protected them from sub-lethal doses of *P. yoelii* [94]. Immunization of mice with poly-lactic-co-glycolic acid (PLGA) vesicles loaded with *P. vivax* antigens (that is, AMA1, CSP and MSP1) improved their immune response relative to conventional adjuvants [96, 97]. Similarly, the use of synthetic EVs loaded with transmission stage antigens as vaccines elicited long-lasting antibody titres through a controlled gradual release of the antigens [98]. Thus, the use of EVs, whether synthetic or of biological origin, might improve the delivery of frontline vaccine candidates. These approaches are nearing clinical application in other systems - *bexsero* is a vesicle-based vaccine used to immunize against sero-group B meningococcal disease and is licenced for use in Europe [99, 100]. This shows that the incorporation of

EVs in malaria vaccine development is practically possible, and motivates further investigation of parasite-derived vesicles.

2.6 The role of PfEVs in malaria pathology

2.6.1 PfEVs modulate the immune system

The interaction of immune cells with pathogen products largely drives clinical disease. *In vitro* studies have provided several insights into the potential impact of PfEVs on the immune system, and hence pathology. Late-stage PfEVs activate monocytes to secrete pro-inflammatory cytokines interleukin 6 (IL6) and tumour necrosis factor α (TNFα) as well the anti-inflammatory cytokine interleukin 10 (IL10) [1]. The balance between pro-inflammatory and anti-inflammatory mediators may be beneficial for parasite survival. Remarkably, ring-stage PfEVs contain gDNA that activates an immune sensor in monocytes called stimulator of interferon genes (STING). Activated STING triggers a signalling cascade that results to increased production of type I interferon by monocytes [101]. Patients with severe malaria have augmented levels of type I interferon compared to healthy subjects. Therefore, type I interferon (IFN) most likely dampens the immune system thus promoting parasite proliferation. Type I IFN also induces inflammation, and therefore damage to tissues, which favours the onset of severe malaria [102].

There have also been reports of the effect of PfEVs on neutrophils. The first published study used microfluidic devices to demonstrate that PfEVs activate neutrophils by inducing increased migration and probably exhaustion of these innate immune cells [1]. Conversely, a second study shows that PfEVs inhibit neutrophils by suppressing their ability to produce cytokines and reactive oxygen species (ROS) [103]. This negatively affects the bactericidal activity of neutrophils and may contribute to the increased risk to

bacterial infections reported among malaria patients [104]. In addition, EVs also influence cells of the adaptive immunity. *Plasmodium vivax* derived EVs isolated from plasma of patients stimulate differentiation of T cells [105]. All these findings provide evidence that PfEVs interact with and influence the biology of host immune cells.

2.6.2 PfEVs manipulate endothelial cells

PfEVs contain human miR451a-Argonaute 2 complex as part of their functional cargo [4, 106]. Uptake of PfEVs by endothelial cells leads to downregulation of Caveolin-1 (Cav-1) gene by miR451a. Cav-1 regulates expression of proteins involved in maintaining the integrity of the endothelial barrier, and therefore its downregulation compromises the normal function of recipient endothelial cells. This increases the permeability of the endothelial cells and may promote localized inflammation in blood vessels [4]. Unidentified PfEV components also upregulate expression of vascular cell adhesion protein 1 (VCAM-1) and secretion of cytokines by recipient endothelial cells. Increased expression of VCAM-1 (a PfEMP1 receptor) may augment iRBC sequestration while the increased cytokine secretion by recipient cells could promote local inflammation and partially drive malaria [4].

2.6.3 PfEVs can transfer drug resistance genes between transgenic parasite lines

Experiments using transgenic parasite lines showed that exchange of genetic material within the parasite population can also occur via PfEVs [2]. Two parasite lines containing either blasticidin deaminase or dihydrofolate reductase enzymes were used to confer resistance to blasticidin or WR99210 respectively. When cultured separately in presence of the two drugs, neither strain survived but when co-cultured in the presence of the two

drugs, both parasites survived. *In vitro* assays demonstrated that PfEVs purified from the culture of a drug resistant parasite mediate the transfer of resistance genes to the drug sensitive strains. This gene transfer occurred via PfEVs secreted during the early ring-stage of the asexual lifecycle of the parasite. [2]. However, it is not yet known whether clinical parasites would use a similar mechanism to transfer drug resistance genes, as these experiments were carried out using episomes containing the drug resistance markers, and *Plasmodium* parasites do not naturally harbour plasmids.

2.6.4 PfEVs promote parasite sexual commitment

EVs can also act as signals that regulate pathogen population and differentiation. Two groups independently showed that PfEVs promote the formation of gametocytes, the sexual form of the parasite transmitted from humans to mosquitoes [1, 2]. Interestingly, the use of parasite-conditioned medium as a stimulus for gametogenesis is a common practice among malaria researchers. Since PfEVs are isolated from the same conditioned medium, they may be the active components that trigger parasites to switch to sexual stages. However, this is highly speculative as the conditioned medium also contains a whole load of other secreted molecules and the exact mechanism through which PfEVs promote gametogenesis is unclear.

2.7 *Plasmodium falciparum* extracellular vesicles contain parasite biomolecules

2.7.1 Protein content of *Plasmodium falciparum* extracellular vesicles

Infected erythrocyte-derived PfEVs contain both parasite and host proteins. The parasite protein composition of PfEVs is related to the intraerythrocytic parasite stage, with the proteomic content of PfEVs released within the first 12 hours after invasion

(early-stage PfEVs) differing significantly from those of mature stage parasites. Proteomic analysis of early stage PfEVs detected 178 host proteins and 101 *P. falciparum* proteins, including the main virulence factor PfEMP1 [5]. In contrast, PfEVs released by late stages of long-term laboratory strains contain more than 450 human proteins and 78 parasite proteins. The parasite proteins detected in mature stage PfEVs include Maurer's cleft resident proteins (PfMC-TM, SPB1, MARHP1 and REX) and iRBC surface proteins such as RESA and Clag3.1. PVM proteins (Exp2 and Etramp2) as well as rhoptry proteins (Rap2 and RhopH2) are also part of the mature stage PfEV proteome, but surprisingly, KAHRP and PfEMP1 are absent [1]. DNA binding proteins such as human argonaute 2 [4] and parasite histones H3 and H4 [101] are also present in PfEVs. Importantly, proteomic analysis of PfEVs secreted by a clinical isolate (adapted to short term *in vitro* culture) shows 100 proteins that are absent from PfEVs secreted by long term laboratory parasite lines [1, 3]. However, in all these PfEV proteomic studies, the number of biological and technical replicates was limited, and differences in protein number and abundance needs to be interpreted with caution.

2.7.2 The lipid composition of PfEVs is characteristic of the parent cell

The lipid profile of EVs resembles that of the parent cell. EVs have a phospholipid bilayer that is similar to a classical plasma membrane. In 2015, Gulati et.al., used lipidomic analysis to establish the lipid profile of PfEVs relative to the parasite [107]. They found that phosphatidylserine (PS) and phosphatidylinositol (PI) are enriched in PfEVs at the expense of phosphatidylcholine (PC). Both PS and PI play a role in exocytosis, and hence their abundance in PfEVs support their host cell origin. Other lipids enriched in PfEVs include monosialodihexosylganglioside (GM3), ceramide and

lactosylceramide (LacCer) [107], which are potent immune modulatory molecules [108, 109].

2.7.3 PfEVs contain parasite nucleic acids in their lumen

Two recent studies independently explored the nucleic acid content of PfEVs [101, 110]. In the first study, small RNA deep sequencing established that PfEVs secreted by ring stage parasites contain small RNA species. The most enriched non-coding RNA was PF13TR011, which is found on chromosome 13 next to a gene encoding a hypothetical protein (PF3D7_1371500). In addition, staining with a DNA stain and a membrane dye confirmed the co-localization of PfEVs with DNA, hence corroborating the argument that DNA is part of the luminal cargo of vesicles. Extensive treatment of PfEVs with DNase I prior to extraction further validated that DNA was indeed protected within vesicles. Whole genome sequencing analysis of PfEVs-DNA revealed sequences for parasite genes such as *msp2*, *rop14* and *gap40*. In addition, mRNA for a protein called early transcribed membrane protein 11.2 (Etramp11.2) was also detected, confirming that intact parasite mRNAs are part of the PfEVs cargo [101].

In the second study, RNA sequencing identified small RNA species and mRNA in PfEVs secreted by mature-stage parasites. mRNA detected in late-stage PfEVs includes that of drug resistance genes (mdr1, kelch13, and ABCA1 transporter) as well as exported proteins (e.g. PHISTb, ETRAMPs) [110]. Each of these two studies mainly focused on PfEVs-small RNA secreted by a single parasite stage rather than the whole intraerythrocytic cycle. The RNA content of PfEVs is not only a representative of the parent parasite, but could also be enriched for certain species reflective of EV biogenesis

and target function. In the present study, I embarked on sequencing and analysing the total RNA content secreted in PfEVs over the asexual parasite life cycle.