RNA sequencing analysis of *Plasmodium falciparum* extracellular vesicles

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Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

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Abstract

Plasmodium falciparum is the most lethal malaria parasite. Blood stage *P. falciparum* parasites secrete extracellular vesicles (EVs), which contain a variety of bioactive molecules and can transfer them to host and/or parasite recipient cells. The RNA content of *P. falciparum* EVs, which could potentially affect biological processes within the host or mediate communication within the parasite population, has not been previously characterised. In my MPhil project, I described transcripts that are preferentially enriched in PfEVs relative to the whole parasite transcriptome.

P. falciparum culture supernatants were collected at multiple developmental time windows from both a long-term laboratory isolate and two short-term lab-adapted clinical isolates. EVs were purified using preparative ultracentrifugation and imaged using sectioned transmission electron microscopy. PfEV-cDNA libraries were generated using the dUTP method and sequenced. Read mapping was done using HISAT2, while differential expression analysis and gene ontology were performed using the R/Bioconductor packages '*edgeR*' and '*ClusterProfiler*' respectively.

Sectioned transmission electron microscopy imaging showed PfEVs to have a dense lumen surrounded by a lipid bilayer. The size range of the PfEVs was 40 - 200 nm with median size of 100 nm. Analysis of the EV-RNA using an Agilent Bioanalyzer pico RNA chip showed that EV-RNA was in the size range of 25 - 4000bp, peaking at 500bp with little or no traces of ribosomal RNA. I identified 958 transcripts enriched in PfEVs secreted by late stage parasites relative to the total parasite RNA profile as a whole , and fewer than 300 for the other parasite time windows. Gene ontology showed that the transcripts enriched in PfEVs released by mature parasites expressed proteins involved in regulation of cellular processes. There were no overrepresented gene ontology terms for the early and mid-parasite time windows.

Conclusively, PfEVs are enriched in transcripts that express regulatory proteins such as RNA binding proteins and transcription factors, as well as the exported family of P. falciparum proteins. In other systems, EV-RNA is translated in recipient cells and can activate cytosolic immune sensors. Therefore, further studies are required to shed light on the role of PfEV-RNA in host-parasite interaction.

Acronyms and Abbreviations

ABCA	ATP-binding cassette transporter 1
AMA1	Apical Membrane Antigen 1
AP2	Apetala 2 domain
APiAP2	Apicomplexa Apetala 2 domain
BAM	Binary alignment/Map
BED	Browser Extensible Data
Cav-1	Caveolin-1
CCM	Culture conditioned medium
cDNA	complementary deoxyribonucleic acid
CGNP	Category gene network plot
CSP	Circumsporozoite protein
dUTP	deoxy-uridine triphosphate
edgeR	Empirical Analysis of Digital Gene Expression Data in R
ELV	exosome like vesicles
ENA	European Nucleotide Archive
ESCRT	Endosomal sorting complex required for transport
ETRAMP	Early transcribed membrane protein
evDEGs	extracellular vesicle differentially enriched genes
EVs	extracellular vesicles
EXP	exported proten

FPKM	fragments per kilobase of exons per million reads
gDNA	genomic deoxyribonucleic acid
GECO	gametocyte erythocyte cytosolic protein
GEST	gamete egress and traversal protein
GEXP	gametocyte exported protein
GFF3	General Feature Format 3
GIG	gametocytogenesis implicated protein
GM3	monosialodihexosylganglioside
GO	Gene Ontology
GRA	dense granule
GTF	Gene transfer format
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HISAT	Hierarchical Indexing for Spliced alignment of Transcripts
HSP	heat schock protein
IFN	interferron
IL	interleukin
IMC	inner membrane complex
iRBC	infected-red blood cell
ISEV	International Society of Extracellular Vesicles
KAHRP	knob associated histidine rich protein
KEMRI	Kenya Medical Research Institute

LacCer	lactosylceramide
MAF1	repressor of RNA polymerase III transcription
MAHRP1	membrane associated histidine rich protein 1
MC-2TM	Maurer's Cleft two transmembrane proteins
MCs	Maurer's Cleft
mRNA	messenger ribonucleic acid
MSP1	Merozoite Surface protein1
MVs	Microvesicles
MVBs	Multi-vesicular bodies
ncRNA	non-coding RNA
NHSBT	National Health Insurance Blood and Transplant
PBS	Phosphate buffered saline
PC	Phosphatidylserine
PEG	Polyethylene glycol
PfEVs	*Plasmodium falciparum* extracellular vesicles
PfPTP1	PfEMP1 trafficking protein1
PHIST	*Plasmodium* helical interspersed subtelomeric protein
PI	Phosphatidylinositol
PIC	Pre-initiation complex
PLGA	poly-lactic-co-glycolic acid
PS	Phosphatidylserine

PTEX	*Plasmodium* translocon of exported proteins
PUF1	gene encoding a mRNA binding protein
PV	Parasitophorous vacoule
PVM	Parasitophorous Vacoule Membrane
Rap2	Rhoptry associated protein 2
RBCs	red blood cells
REXP1	ring exported protein 1
RhopH2	Rhoptry high molecular weight protein 2
RIFIN	repeatitive interspersed family of proteins
RIN	RNA integrity number
ROS	reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute 1640 culture medium
rRNA	ribosomal ribonucleic acid
RSEM	RNA-Seq by Expectation Maximization
RTS,S,	a malaria vaccine
SAM	Sequence Alignment/Map
SBP1	Spectrin binding protein 1
SMN	Survival motor neuron like protein
SNARE	Soluble NSF Attachment Protein REceptor"
STEVOR	Subtelomeric variable open reading frame protein family
STING	Stimulator of interferon genes

TEM	Transmission electron microscopy
TNFα	Tumor necrosis factor alpha
TSG101	Tumor susceptibility gene 101
TVN	Tubulovesicular network
VCAM-1	Vascular cell adhesion protein 1
VPS	Vacoular protein sorting complex
WSI	Wellcome Sanger Institute

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CHAPTER ONE: INTRODUCTION

1.1 Background

One of the ways through which cells communicate is through the release of extracellular vesicles (EVs) into their environment. *Plasmodium falciparum* derived extracellular vesicles (PfEVs) mediate cell-cell communication within the parasite population, and with the host [1, 2]. PfEVs contain both parasite proteins and RNA and can transfer these to recipient cells. Several groups have shown that PfEVs have the potency to activate cells of the immune system and endothelium. Interestingly, when added to parasite cultures, PfEVs promote the formation of sexual stages that are transmitted from humans to the mosquito vector [1, 3-5]. However, the key parasite transcripts and pathways that are selectively included in PfEVs are poorly understood.

1.2 Research questions

1) Do the breadth and quantity of the parasite RNA content of EVs secreted by *P. falciparum* change over the intraerythrocytic life cycle?

2) Does the parasite RNA profile of *Pf*EVs secreted by two *P. falciparum* Kenyan clinical isolates differ to those secreted by NF54, a long-term adapted laboratory parasite line?

1.3 Objectives

1.3.1 General objective

To generate a dense map of *Pf*EV-RNA content across the intraerythrocytic life cycle, and establish the difference of this content between *P. falciparum* NF54 strain and two Kenyan clinical isolates.

1.3.2 Specific objectives

1) To determine the parasite RNA contained in *Pf*EVs released during the asexual life cycle of *P. falciparum*.

2) To determine whether the parasite RNA selectively included into *Pf*EVs secreted by two *P. falciparum* clinical isolates differs to *P. falciparum* NF54 strain.

1.4 Significance of the study

Specific transcripts could potentially be selectively packaged into PfEVs and transferred to recipient cells to impact function. However, this has not been determined before, and to the best of my knowledge, this is the first study to explore such a possibility. Analysing the content of PfEVs will help us understand their role in host-parasite interaction, and could guide novel malaria interventions.

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.

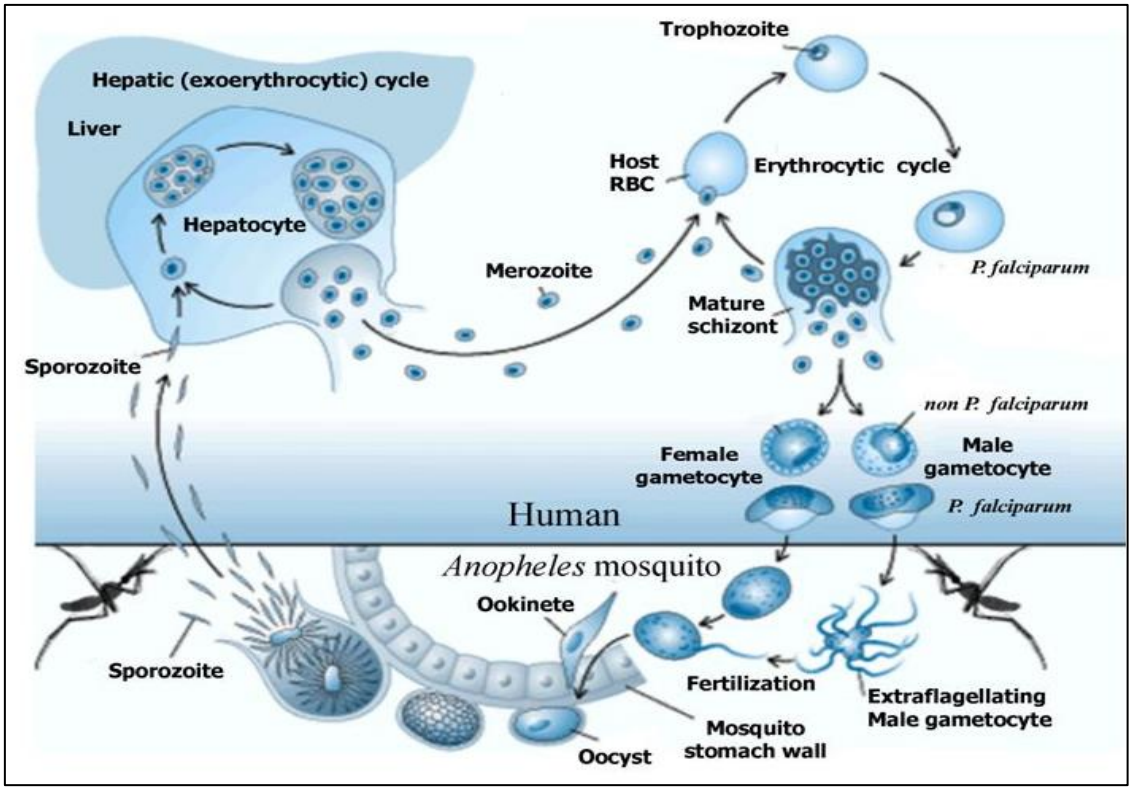

Figure 1: The life cycle of *Plasmodium falciparum*.
Copied from: http://slideplayer.com/slide/9776812/

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].

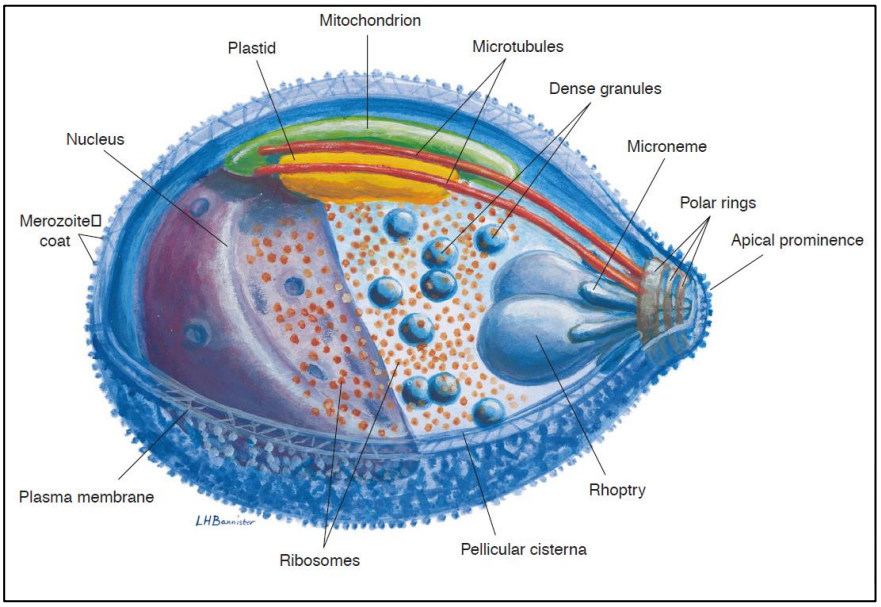

Figure 2: Schematic diagram of a merozoite.

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-

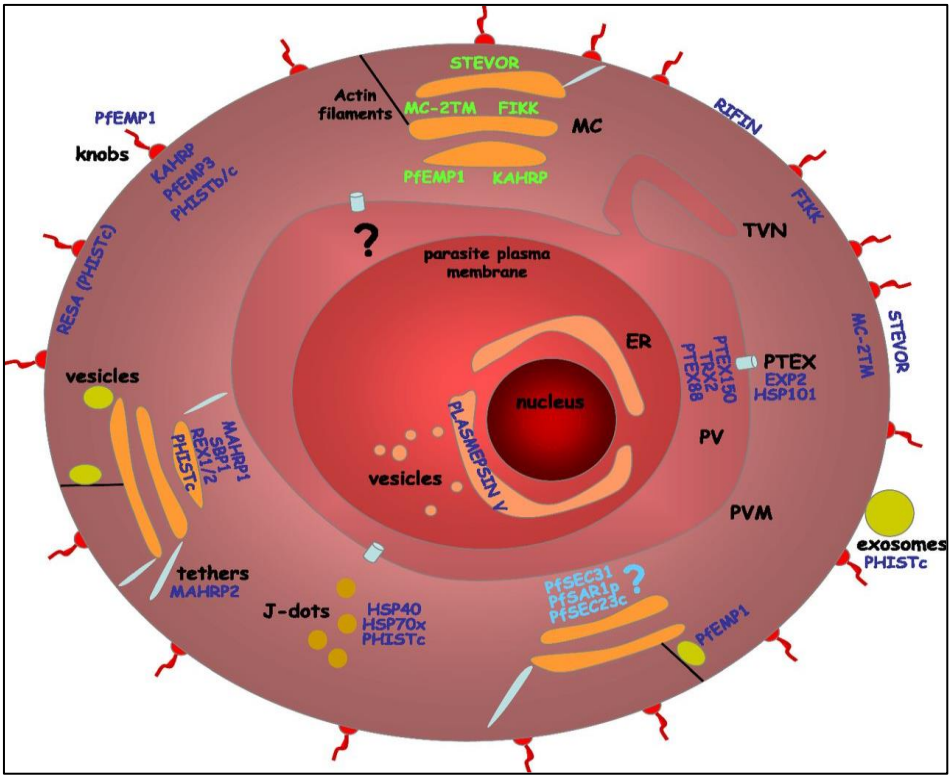

Figure 3: Schematic representation of parasite infected red blood cell

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].

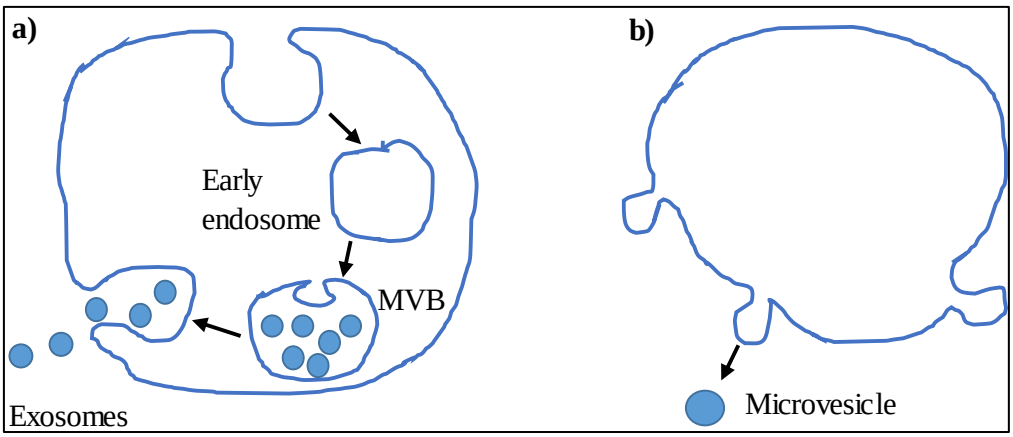

Figure 4: A diagram showing biogenesis of **a)** Exosomes **b)** Microvesicles

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.

CHAPTER THREE: METHODS

3.1 *Plasmodium falciparum* culture

3.1.1 Preparation of EV-depleted culture medium

Five percent (w/v) Albumax II (Life Technologies) was dissolved in water and depleted of vesicles by centrifugation at 150,000 x g for 2 h at 4 ℃ (Beckmann Coulter, 70 Ti rotor). The EV-depleted Albumax was sterilised by filtration through 0.22 µM (Millipore). Depleted Albumax was aliquoted into 12.5 ml portions and stored at – 20 ℃ until use. A litre of incomplete medium was prepared as follows: 10.43 g RPMI 1640 Life Technologies 51800019 (with L-Gln, no NaHCO3), 7.15g HEPES (Sigma), 2 g glucose (Sigma), 1 ml of 50 mg/ml hypoxanthine (Sigma), 0.5 ml of 50 mg/ml gentamicin (Sigma) and 900 ml Milli-Q H_2O and pH adjusted to 7.2. The incomplete medium was filtered using a 0.22 µM filter and stored at 4 ℃. To 1 L incomplete medium, 7.5% NaHCO3 (Sigma) and 5 g/l (12.5 ml) of EV-depleted Albumax II (both pre-filtered) were added to make up the complete culture medium, which was stored at 4 ℃ until use.

3.1.2 *P. falciparum* isolates used in this study

Three *Plasmodium falciparum* strains were used for this study. These included *P. falciparum* NF54, which is a long term-adapted laboratory strain that is known to produce gametocytes, and two Kenyan clinical isolates that have more recently been adapted to culture (approximately <100 cycles). The two Kenyan isolates are named as 9775 and 11019 based on KEMRI-Wellcome Trust parasite isolate naming system. The genomes of the two isolates have been sequenced at the Wellcome Sanger Institute and have been re-named as PfKE11 and PfKE10 respectively.

3.1.3 *Plasmodium falciparum culturing for* EV isolation

The parasites were maintained at 2% parasitaemia and 2% haematocrit in fresh (less than two weeks old) human O^+ RBCs (NHS Blood and Transplant, Cambridge, UK; informed consent from donors was obtained by NHSBT) using complete medium as prepared above, gassed using a mixture of 5% O2, 5% CO2 and 90% N2, and incubated at 37 ℃. Giemsa smears were performed on a daily basis to monitor parasitaemia. When required, the parasites were synchronized using 5% sorbitol, which kills mature stages (trophozoites and schizonts) and expanded into three 50 ml culture flasks per parasite strain for EV production.

3.1.4 Production of PfEVs

Once parasites reached optimal growth, the three 50 ml flasks were synchronized for the second time using 5% sorbitol at around 12 h post-invasion (small rings) and expanded to six 50 ml flasks per strain (5% parasitaemia, 2% haematocrit). After 12 hours (24 hrs after invasion), a Giemsa – stained smear was prepared to confirm the asexual parasite stage of the cell culture, which was predominantly early trophozoites. The cultures were suspended by pipetting up and down and transferred to 50 ml tubes. The 50 ml tubes were centrifuged (Centrifuge 5810, eppendorf) for 5 min at 600 x g with a low brake. The supernatant, referred to as culture conditioned medium (CCM), which contained PfEVs released between 12 - 24 h after invasion, was collected into 50 ml Falcon tubes and stored temporary at 4 ℃. The iRBC pellets were resuspended in fresh medium and returned to incubation at 37 ℃. The processing of the CCM was done on the same day once iRBCs pellets had been resuspended in fresh media. This process of 12 - hourly collection of CCM was repeated twice more, resulting in collection of conditioned

medium from three successive parasite development time windows: 12 - 24 h, 24 - 36 h, 36 - 48 h (in the same cycle as 12 - 24 h) (**Figure 5**). After collection of the 36 - 48 h sample, the cultures were diluted to 2% parasitaemia and the 0 -12 h sample was collected in the next cycle. The entire process was later repeated once to obtain a duplicate CCM samples for PfEV isolation from each strain.

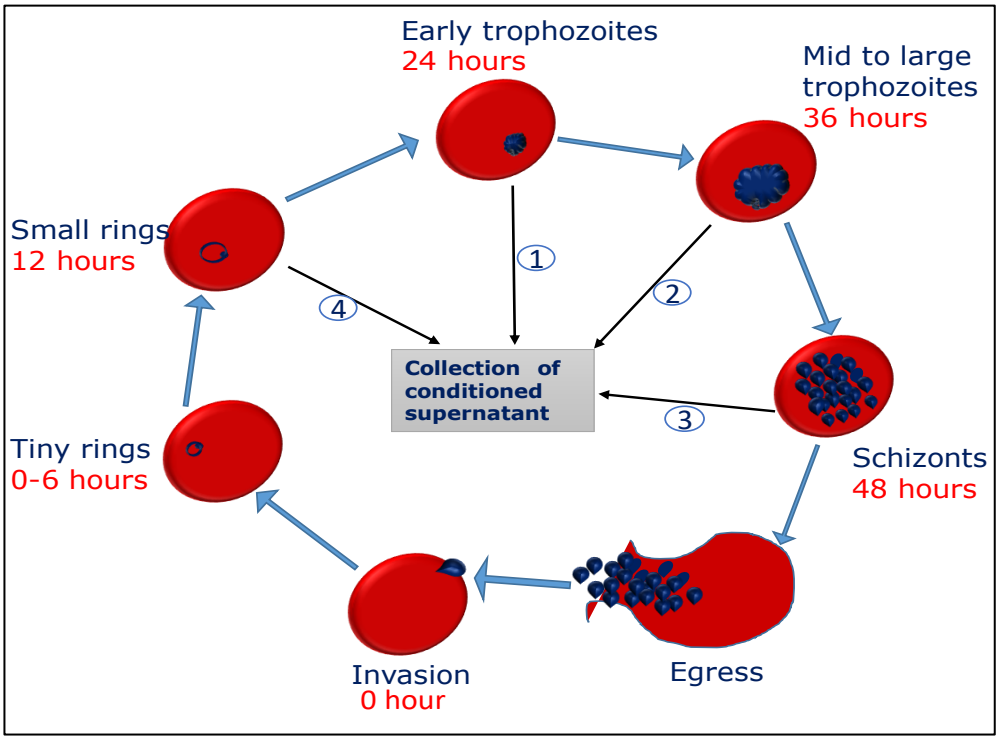

Figure 5: Illustration of time series collection of parasite conditioned medium. Parasite were synchronized and suspended in fresh medium at "small ring-stage". Samples were collected as indicated: 1; (12-24 h), 2; (24-36 h), 3; (36-48 h) and 4; (0-12 h).

3.2 Processing of CCM and purification of PfEVs

Purification of PfEVs was done as previously described [3]. The collected CCM samples briefly stored at 4°C (**Section 2.1.3**) were centrifuged at 2000 x g in 50 ml tubes for 10 min at 4 °C to remove traces of cells and debris. The supernatant was transferred to a new 50 ml tube and spun at 3200 x g for 30 min, 4 °C with low brake, with the pellet being again discarded. The supernatants from each time point of the parasite life cycle

were pooled together and passed through 0.22 µM (Millipore) to exclude vesicles larger than 220 nm majority of which are microvesicles. The collected sterile filtrate was aliquoted into 50 ml tubes, temporarily stored at -20 ℃ and later transferred to -80 ℃ until use.

The processed culture supernatants were thawed and centrifuged for 20 min at 15000 x g, 4 ℃. The supernatant was transferred to 15 ml concentration columns (50kDa cut-off, Millipore) to decrease the supernatant volume by a factor of 3 - 4. The concentrate was transferred to 13.5 ml Quick seal ultracentrifuge tubes (Beckmann) and spun for 2 h at 150,000 x g, 4 ℃ in a preparative ultracentrifuge (Beckmann Coulter, 70 Ti rotor). The supernatant was discarded and the pellets from the same time course pooled, washed once with 13.5 ml of PBS and centrifuged for 2 h at 150,000 x g, 4 ℃. The presence of extracellular vesicles in both the pellet and the PfEV suspension was confirmed by sectioned and negative stained transmission electron microscopy respectively.

3.3 PfEV-RNA isolation

The PfEV pellet was suspended in 400 µl of PBS and treated with 2 units (1 µl) of RNase A (Biolab) for 15 min at room temperature to remove non-vesicular RNA. The treated PfEV suspension was digested with 500 µl of lysis buffer (Isolate II RNA Min Kit, Bioline) and stored at -80 ℃. RNA was isolated using the Isolate II RNA Min Kit, (Bioline) following manufacturer's instructions and eluted in 100 µl of nuclease free water. Purified RNA was quantified using Bioanalyzer Pico RNA chips (make) and stored at -80 ℃, until library preparation.

3.3 Preparation of cDNA libraries

An established protocol [111] was used to prepare the cDNA libraries with some minor modifications. Succinctly, RNAcleanXP beads (Biolabs) solution was used to concentrate the total PfEV-RNA five times. The concentrated RNA was eluted from the beads using a Qiagen elution buffer (10 mM Tris-HCl pH 8.0). All RNA elution in downstream steps was done using this buffer. First strand cDNA was synthesised using Superscript III reverse transcriptase (Invitrogen) with the following cycling parameters: 25 ℃ for 10 minutes, 42 ℃ for 60 minutes and a final hold of 4 ℃. Both oligo d(T) and random hexamers from Qiagen were used in the first strand cDNA synthesis reaction. The first strand reaction was cleaned using 1.8 volumes of RNAcleanXP beads solution followed by two successive washes with 80% ethanol while on the magnetic stand. RNA was eluted from the beads with 41 µl of Tris-HCl, but the beads were left in the wells for later use in cleaning the second strand reaction. Second strand cDNA was synthesised using NEBNext RNA Second Strand Synthesis Module (Biolabs) (parameters: 16 ℃ for 2.5 hours and a final hold of 4 ℃) replacing dTTP with dUTP. Double stranded cDNA was cleaned using the RNAcleanXP beads already in the wells. The binding properties of the second strand cDNA to the beads was rejuvenated by addition of 1.8 volumes of 20 % polyethylene glycol (PEG), 2.5 M NaCl solution. Two successive washes of the beads were performed using 80% ethanol while on the magnetic stand. The cDNA was eluted, quantified using a Bioanalyzer high sensitivity DNA chip and then the beads were discarded. Cleaned cDNA in 50 µl elution buffer was processed in AFA Covaris for 30 sec (parameters: duty factor 5, peak power 175, cycles 200) to obtain cDNA fragments. Quality control of the fragmented cDNA was performed using the Agilent Bioanalyzer high sensivity DNA chip. End repair and dA tailing of the cDNA fragments were done using NEBNext Ultra II DNA Library Prep Kit. The blunt-ended, polyA-tailed cDNA

was purified using 1.8 volumes of AMPureXP beads in 20% PEG, 2.5M NaCl solution, washed twice using 80% ethanol while still in the magnetic stand and eluted in 50 µl elution buffer. The end-repaired cDNA was ligated to 5 µl of 0.1 µM NETflex adapter oligo mix using Quick DNA ligase and incubation at 20 ℃ in a thermal cycler for 15 min. The ligated cDNA was purified using 0.8 volumes of 20% PEG, 2.5 M NaCl AMPureXp bead solution, washed twice using 80% ethanol while still in the magnetic stand. The clean ligated cDNA was eluted in 24 µl elution buffer (10 mM Tris-HCl pH 8.0) and quality control was performed using Agilent Bioanalyzer HS DNA chip. The libraries were digested with 0.1 volumes of Uracil-Specific Excision Reagent (USER enzymes, Biolabs) at 37 ℃ for 15 min followed by a DNA denaturation step at 95 ℃ for 10 min. The single strand ligated cDNA was amplified (in 10 cycles) using KAPA HiFi HotStart ReadyMix PCR kit (KAPABIOSYSTEMS) and the PCR primers P5 and P7 (Illumina) following the manufacturer's recommended cycling parameters. PCR products were quantified using qRT-PCR and again cleaned using 0.8 volumes AMPureXP beads in 20% PEG, 2.5 M NaCl solution. All the cDNA library samples were pooled in equimolar concentrations into one tube and sequenced using the Illumina HS2500 genome analyser in 158 cycles.

3.4 PfEV-RNA data analysis

3.4.1 Read processing and mapping

CRAM files for data generated in this study were obtained from the WSI sequencing pipeline and converted to fastq format using *samtools -1.3 view*. The tool FastQC [112] was used to confirm quality details of the data such as average Phred score, GC content, distribution of read length and overrepresented sequences. FastQC provides relatively

conservative cut-offs even for data generated using the state of the art sequencing facilities. However, attention was paid to details to ensure that the data did not have obvious flaws like a high number of duplicates or below average read quality. Next, *bowtie2* [113] was used to eliminate rRNA sequences from the data by mapping the short reads against an artificial *P. falciparum* ribosomal RNA sequence file and only reads not mapping to these rRNA sequences were used in downstream steps.

The super-fast splice aware mapping tool called HISAT2 [114] was used to align the processed reads to the parasite genome as described earlier. Succinctly, a HISAT2 index was produced from the *P. falciparum* genome sequences downloaded from GeneDB [115], using default parameters. Paired reads were aligned to the genome sequences using the following HISAT2 parameters: *-max-intron 5000 --rna-strandness RF --dta –I 75 –X 500.* These parameters specified a maximum intron size of 5000 for *P. falciparum*, a strand specific PfEV-cDNA library generated using the dUTP method and an expected fragment length of 75 – 500 bp. The *--dta* option commands HISAT2 to produce SAM files that are compatible with **d**ownstream **t**ranscript **a**ssembly tools.

For control comparisons, whole parasite data generated by Lopez-Barragan and others [116] was downloaded from GenBank (accession number SRP009370), processed and mapped to the reference genome as described above. This is a time series data collected at 8 h, 19 h, 30 h and 42 h of the *P. falciparum* asexual life cycle. This data is in sync with the time points of sample collection of the present study, and was therefore used as a background to predict transcripts highly enriched in PfEVs relative to the whole parasite transcriptome.

3.4.2 Mapping quality control and quantification of expression

Each SAM file was sorted and converted to a BAM file in a single step using *samtools-1.3 sort,* while *samtools-1.3 flagstat* was used to determine the unique alignment rate per sample [117]. Subsequently, *samtools-1.3 merge* [117] was used to merge BAM files originating from different sequencing lanes but belonging to the same sample. A *P. falciparum* GFF3 file was downloaded from GeneDB [115], and converted to GTF and BED formats using RSEM tools [118] for use in downstream steps. Next, RSeQC tool kit [119] was used to determine read distributions within exons, introns and intergenic regions, as well as strand specificity of the PfEV-cDNA library. Visualization of BAM files was done using the integrated genome browser (IGV) [120]. Assembling and summation of expression was done using *stringtie* [121] with the following parameters: *stringtie -G –e -A.* These parameters instructed *stringtie* to use the provided GTF file as a guide during assembly, to only output transcript alignments matching the GTF file (not to predict novel transcripts), and to output a file containing gene expression in FPKM (**f**ragments **p**er **k**ilobase of exons per **m**illion reads) values.

3.4.3 Data normalisation and expression analysis

Data normalisation was performed prior to linear modelling in subsequent steps. This was done to not only reduce technical variation within the PfEV data, but to also make it more comparable to the whole parasite data. First, the expression data from individual samples were pooled together to form a single data matrix. Subsequently, a R package called *Linnorm* [122] was used to perform library size/batch effect correction. *Linnorm* has a function (*linnorm.norm*) that transforms the FPKM values into log2FPKM values, estimates the mean variance relationship and uses this to calculate appropriate

observation-level weights (in this case raw counts). Expression analysis was done by modelling in *edgeR* [123] using the quasi-likelihood F test. A false discovery rate (FDR) adjusted P value of <0.05 was chosen as a cut-off for significant genes while the Log2 (Fold Change) threshold was set at absolute 5.

3.5 Gene ontology analysis of transcripts differentially enriched in PfEVs

Gene ontology [124], network analysis and visualization were performed using a R/bioconductor package called *clusterProfiler* [125]**.** The *Plasmodium falciparum* organism specific R package (*org.Pf.plasmo.db*) [126] was used to provide the mappings between gene symbols and gene ontology terms. The hypergeometric test (an over-representation test) option of *clusterProfiler* was used, with adjusted P value of 0.05 set as a cut-off. The results of gene ontology test were visualized by bar plots, enrichment maps and category-gene-network plots.

CHAPTER FOUR: RESULTS

4.1 *Plasmodium falciparum* infected RBCS release exosome-like vesicles

Extracellular vesicles were isolated from processed parasite conditioned medium using a combination of ultrafiltration and preparative ultracentrifugation, as described in the Methods section. An examination (by David Goulding, Wellcome Sanger Institute) of the purified material using sectioned transmission electron microscopy (TEM) revealed that they had a size range of 40 – 200 nm and a dense lumen surrounded by a lipid bilayer as shown by **Figure 6**. This morphology matches that described for cell exosomes reported in literature [127], but these vesicles can only be referred to as exosome-like vesicles (ELV) because their biogenesis has not been proven. The sample examined microscopically contained a mixture of vesicles isolated from the entire intraerythrocytic parasite cycle, in recognition that the parasite stage in which PfEVs are released is not yet resolved.

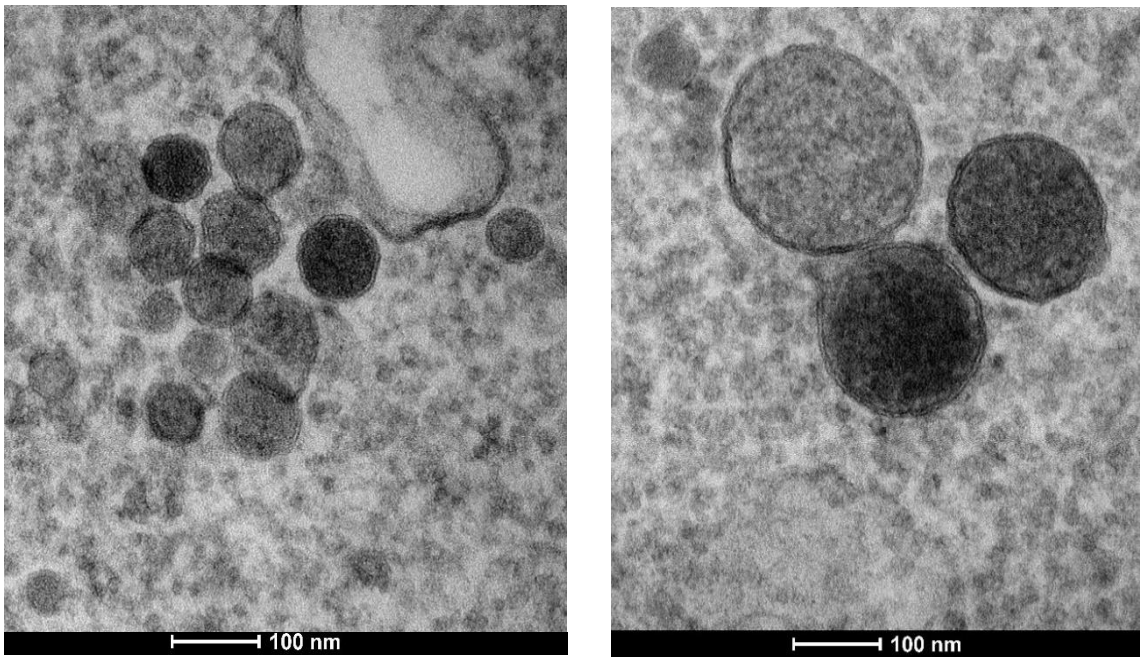

Figure 6: Sectioned transmission electron microscopy images of *Plasmodium falciparum* extracellular vesicles.

4.2 Sequencing of asexual parasite PfEV-RNA time course samples

In order to characterise transcripts enriched within PfEVs, three parasite strains comprising of the long-term adapted laboratory isolate, NF54, and two recent clinical isolates (named 9775 and 11019 as per the KEMRI-Wellcome Trust Research Programme in-house naming system) were cultured, and samples of 300 ml medium collected in four different time windows across the intraerythocytic development cycle. Parasites were synchronised at 12 h post-invasion and conditioned medium collected after 24 h, 36 h, 48 h and 60 h post-invasion in real time. As the intraerythocytic cycle is around 48 h, the 60h sample would in fact represent a time-point 12 h after a second round of invasion. It was therefore termed as the 12 h time point. The time course was repeated for all three strains to provide biological duplicate samples; in total therefore 24 samples were collected, comprising 2x4 time points from each of the three strains.

After purification of PfEVs, the pellets were treated with RNase A and DNAse I to remove non-vesicular RNA and genomic DNA respectively. The RNA content of PfEVs was subsequently purified using the guanidium lysis method. Bioanalyzer analysis revealed that PfEVs contain RNA in the size range of 25 - 4000 bp, peaking at 500 bp (**Figure 7**). The RNA integrity number (RIN) was generally less than 3 while that of the whole parasite RNA ranged between 6 and 8.5. This was expected because RIN is based on the rRNA content of the sample and PfEV-RNA contain little or no traces of ribosomal RNA. The observation was in line with a previous report of PfEV-RNA [128].

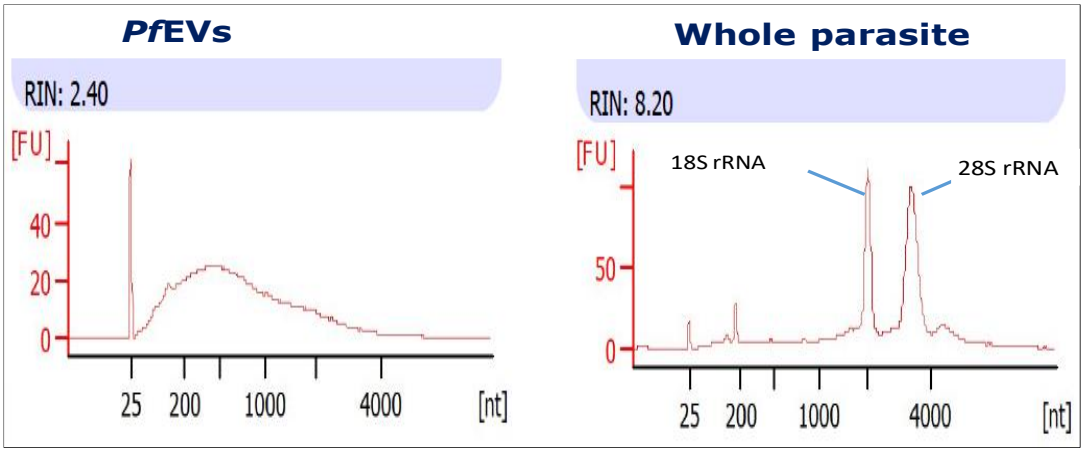

Figure 7: Examples of Bioanalyser traces of both PfEV and whole parasite RNA. The whole parasite RNA was diluted 10 fold before quantification to make its concentration within the measurement range of the Bioanalyser Pico RNA chip

Next, PfEV-cDNA libraries were prepared using the dUTP method to preserve strand specificity, and the KAPA Hifi polymerase was used to amplify the libraries in 10 cycles to boost cDNA concentration. Out of the total 24 samples collected in two batches, libraries could only be prepared from 13 of them as the remaining samples yielded too little RNA for library generation (**Table 1**). Nevertheless, at the end of the library preparation exercise, there was at least one successful library for each time window from NF54 and Pf9775. However, there were libraries for only three time points of Pf11019, as all the samples for the 48 hr time point had to be excluded due to low yield.

Strain	Parasite time window	Batch sample sequenced
Nf54	0-12h	first
	12-24h	first
	24-36h	second
	36-48h	first and second
Pf11019	0-12h	first and second
	12-24h	first
	24-36h	first
	36-48h	none
Pf9775	0-12h	first
	12-24h	first
	24-36h	first
	36-48h	first

Table 1: Table showing PfEV-RNA samples taken for sequencing

The thirteen samples were sequenced on two lanes in an Illumina HS2500 genome analyser in 158 cycles. Sequencing depth ranged between 10 to 16 million reads and a high base quality and zero overrepresented sequences was noted for 12 out of the 13 samples. However, the sample for the second time window of Pf9775 (12-24 h) had a noticeably low yield of 5 million reads per lane and a high number of overrepresented sequences.

4.3 Mapping, quantification of expression and data normalisation

Although the input PfEV-RNA contained little or no traces of rRNA as detected by Bioanalyzer (**Figure 7**), experience has it that even samples treated with ribosomal depletion kits before library preparation still contain some traces of rRNA reads. Cellular

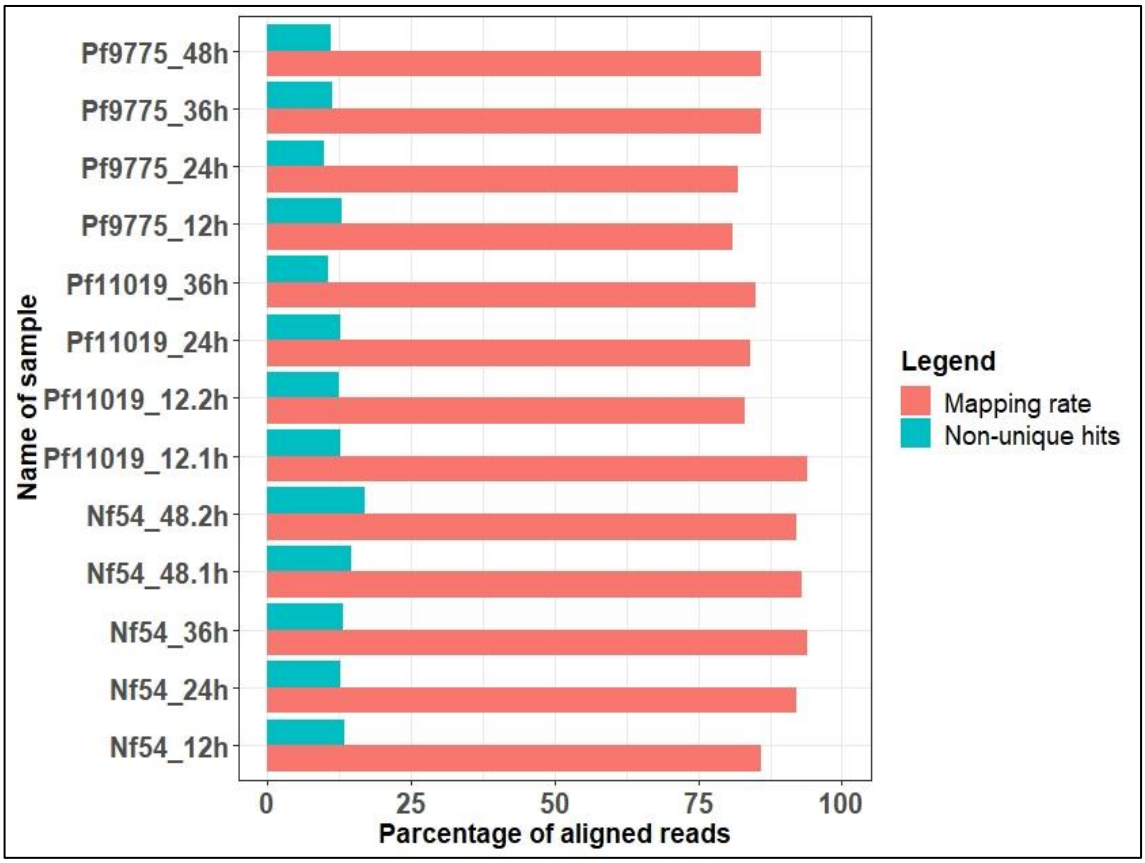

Figure 8: An overview of the alignment rate. Red bars correspond to the overall alignment rate while green bars correspond to non-primary hits

rRNA is more abundant relative to other species and may confound the results of mRNA expression analysis. To remove any traces of rRNA sequences, *bowtie2* [113] was employed to map all reads from each sample against an artificial *P. falciparum* rRNA fasta file, and only reads not mapping to these rRNA were used in subsequent steps. Approximately 0.07 – 2% of reads in each sample mapped uniquely to rRNA sequences. Non-rRNA reads were aligned to the *P. falciparum* genome using HISAT2 [114] and mapping quality assessed using metrics first published by Wang et.al [119]. Briefly, quality metrics calculated include strand specificity, overall alignment rate, exonic rate, intronic rate and intergenic rate. The overall alignment rate ranged between 81 – 94 % as shown by **Figure 8.**

Despite a strand-specific protocol being used to prepare the cDNA libraries, strand specificity was very low as only about 55 -72% of reads mapped to the reference strand in the correct orientation. This was expected because a final amplification of the cDNA libraries was included to increase yield, but which also made the libraries unstranded. Although majority of the reads mapped uniquely to exons, about 2.5 - 5% of reads in each sample aligned to non-coding regions. Exonic reads mapped to the full length of transcripts as shown by **Figure 9** (IGV snapshot), indicating that PfEVs contain full mRNA rather than random fragments.

A popular transcriptome assembly tool called *stringtie* [121] was used to assemble the uniquely mapped reads into transcripts. *Stringtie* was given two instructions: 1) to not assemble novel transcripts as the strand specificity was not preserved during library preparation, and 2) to normalise the reads by transcript length in order to output expression data in FPKM values. To make the Lopez-Barragan et.al [116] whole parasite data comparable to the PfEV data, normalisation by sequencing depth was done by first converting the FPKM values into log2FPKM, and then using this to estimate raw counts.

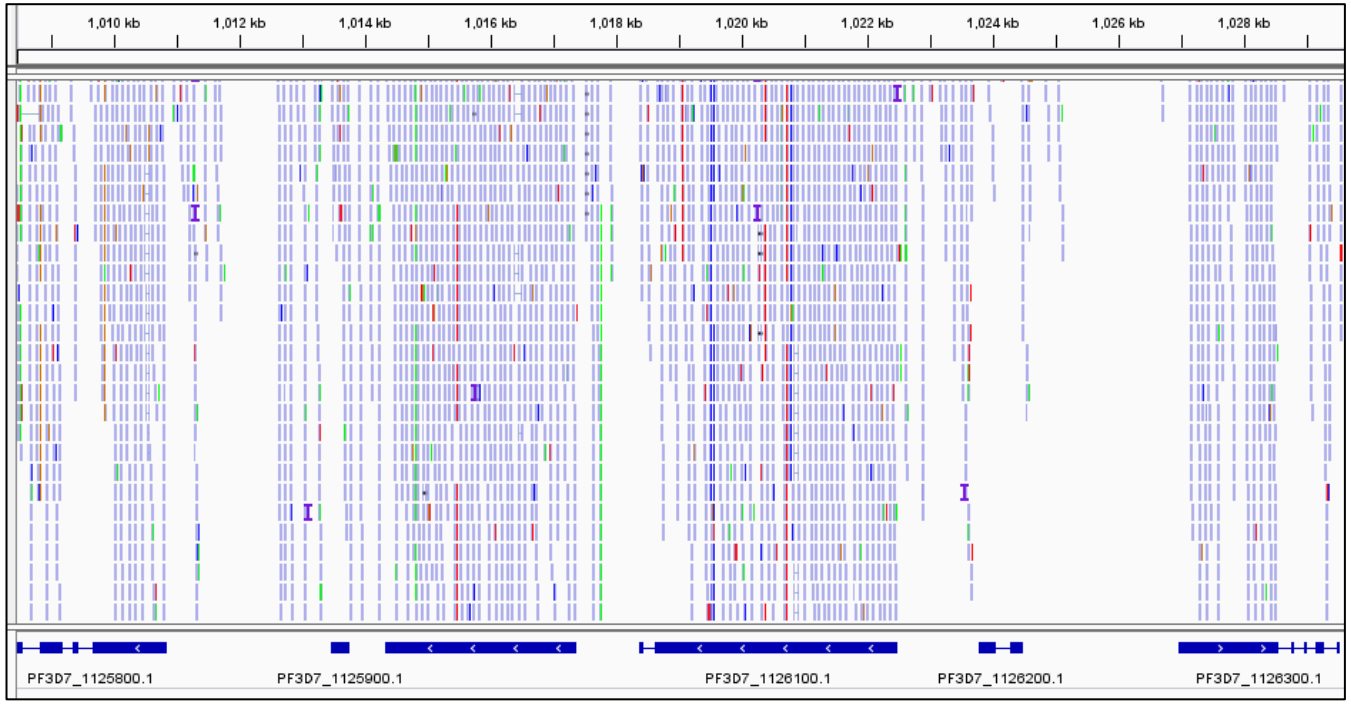

Figure 9: An example of a snapshot showing the read coverage. Reads mapped to the entire lengths of the transcripts.

The calibrated top line indicates a section of chromosome 11 of the *P. falciparum* genome. The majority light blue bars correspond to the reads that mapped correctly to the genome in a BAM file. Mismatched bases are shown in different colours: blue (cytosine), green (adenine), brown (guanine) and red (thymine). This purple bar I is the symbol for insertions. Deletions are indicated by a blank space crossed by a line. The bottom bars correspond to *P. falciparum* genes in the annotation GTF file, while spaces in between are the intergenic regions. A gap in a gene crossed by a line indicates an intron.

4.4 *P. falciparum* extracellular vesicle contain differentially enriched genes

To achieve the experimental goal of this thesis, the quasi-likelihood F test of *edgeR* [123] was applied to identify genes that are enriched in PfEVs relative to the parasite. This test was chosen because it reflects the uncertainty of estimating dispersion for each gene especially when the number of replicates is small. The 0 -12 h and 36 - 48 h time points were first individually compared with the respective Lopez-Barragan et.al., [116] whole

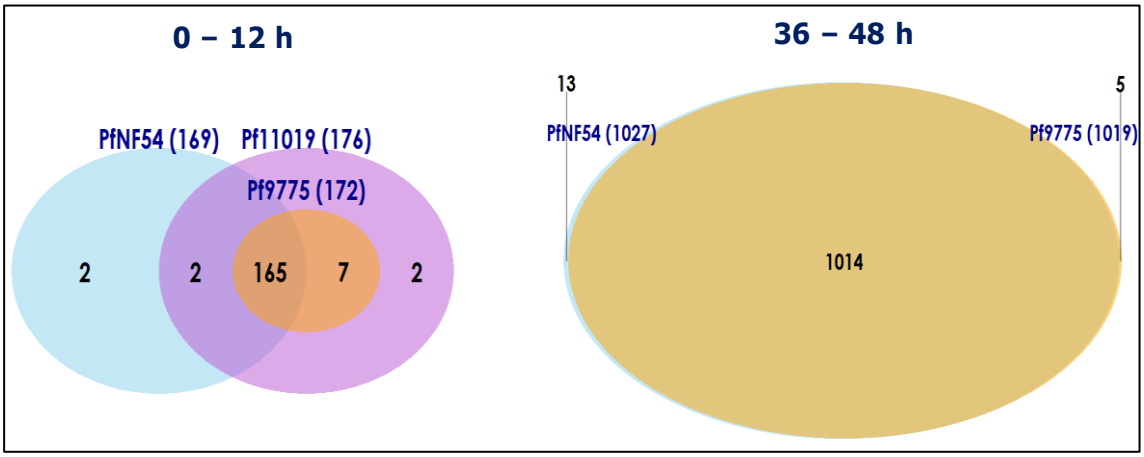

Figure 10: Parasite strain comparison of PfEV enriched genes (evDEGs) **a)** Comparison of 0 - 12 h evDEGs between strain PfNF54, Pf11019 and Pf9775 identified an overlap of 165 genes. **b)** Comparison of 36 -48 h evDEGs between PfNF54, and Pf9775 identified an overlap of 1014 genes. For each comparison, only genes with a P.adj < 0.05 and log2FC>5 were considered as evDEGs. The total number of evDEGs in each strain appears in parentheses

parasite transcriptome data using *edgeR*. This was done to provide some insights as to whether there is a difference between the transcriptome of PfEVs released by a long-term laboratory strain and more recent clinical isolates. These two time points were chosen due to the availability of at least two samples belonging to at least one strain, which is a requirement of *edgeR*. In this comparison, there was a substantial overlap of PfEV differentially enriched genes (evDEGs) across all the three isolates, as shown by the high number of transcripts within the intersection of the samples in **Figure 10**. The number of overlapping DEGs was higher in the 36 - 48 h PfEVs (1014 genes) than 0 -12 h (165 genes).

Reliable estimation of biological coefficient of variation (BCV) between conditions requires replicates [123]. Therefore, the observation that the RNA content of PfEVs from recent clinical isolates behave similarly to that of NF54, prompted the combination of samples from all strains for each individual parasite time window. This allowed me to treat the samples from a given time point as replicates, which allowed for a more for a more robust comparison. In the combined comparison, the following number of evDEGs were significantly enriched in PfEVs as compared to the parasite: 0 - 12 h (173 evDEGs), 12 -24 h (291 evDEGs), 24 - 36 h (135 evDEGs) and 36 - 48 h (958 evDEGs) as shown by **Figure 11** and **Figure 12**. This supports the notion that PfEVs released by late stage parasites contain a higher number of transcripts than those released by early stage parasites, just as late stage parasites contain more RNA than early stage parasites do. Although, there was an overlap of genes between the time points, a large proportion of evDEGs were time specific. Interestingly, only 56 genes (1014 minus 958) (**Figure 10** and **Figure 11**) detected as evDEGs during the individual comparison were not

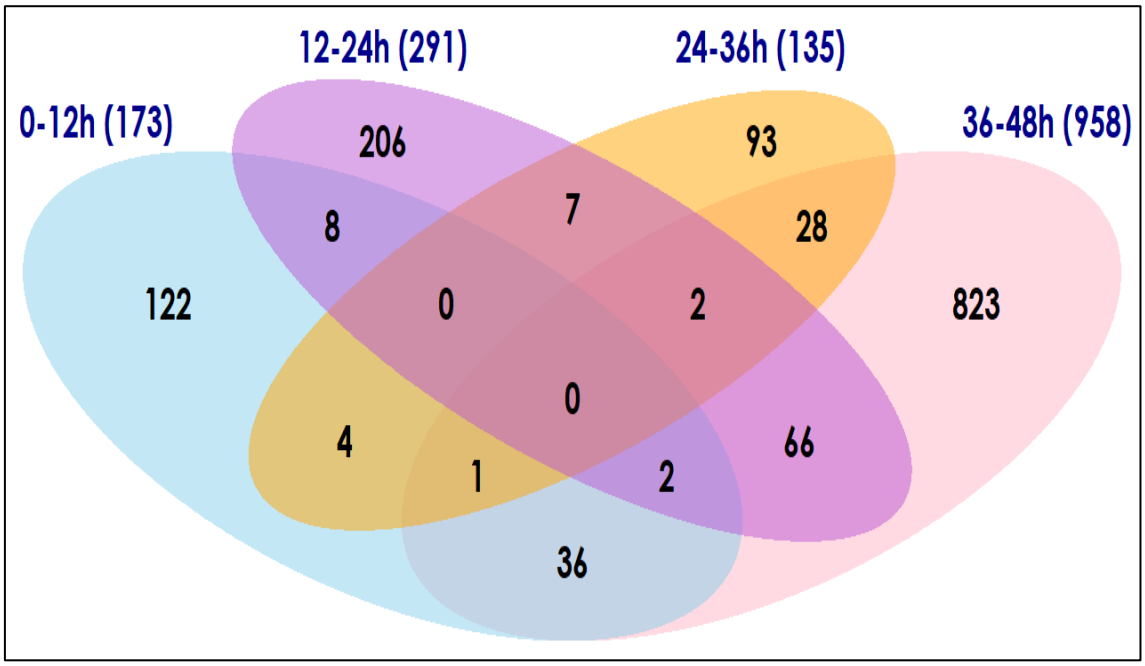

Figure 11: Overlapping of evDEGs across the asexual parasite cycle. Only genes with an adjusted P-value <0.05 and log2 (Fold Change)>5 were considered to be upregulated in PfEVs. The total number of evDEGs at each parasite time window appears in parentheses

differentially expressed when the 36 – 48 h samples were treated as replicates. This supports the theory that vesiculation could be a conserved phenomenon that may not vary much between parasite isolates.

Generally, transcripts expressing members of multi-copy families of proteins were highly upregulated in PfEVs in all time points. These include transcripts encoded by the *var, rif, phist, stevor and surfin* genes. However, there were also stage specific transcripts. For example, interesting genes enriched in 0 - 12 h PfEVs include a member of APiAP2 transcription factors (Pf3D7_1456000), sortilin and syntaxin (Qa-SNARE family). Gene products enriched in 12-24 h PfEVs include TATA-box binding protein (PF3D7_0506200), karyopherin, DNA repair protein RAD2, putative (PF3D7_0206000), kelch 13, WD repeat-containing protein 82 putative (PF3D7_1243800), and histone-lysine N-methyltransferase, H3 lysine-4 specific (PF3D7_1221000). Others include kelch 13, ABC transporter B, SNARE (SYN6) and cop-coated vesicle membrane protein p24 precursor (Pf3D7_1314500).

The PfEVs harvested between 24-36 h are rich in transcripts encoding early transcribed membrane proteins (ETRAMPs), the highly variable *Plasmodium* proteins: PfEMP1, SURFIN, RIFIN and PHISTb, as well as Maurer's cleft resident proteins (Pfmc-2TM Maurer's cleft two transmembrane proteins). Interestingly, an mRNA for a protein expressed by early sexual stages, gametocyte erythrocyte cytosolic (GECO) protein, HAD domain ookinete protein and gametocyte exported protein 20 (GEXP 20) had a higher fold change in 24 -36 h PfEVs relative to the parasite. Other interesting mRNA enriched in 24-36 h PfEVs are two non-coding RNA; signal recognition particle RNA

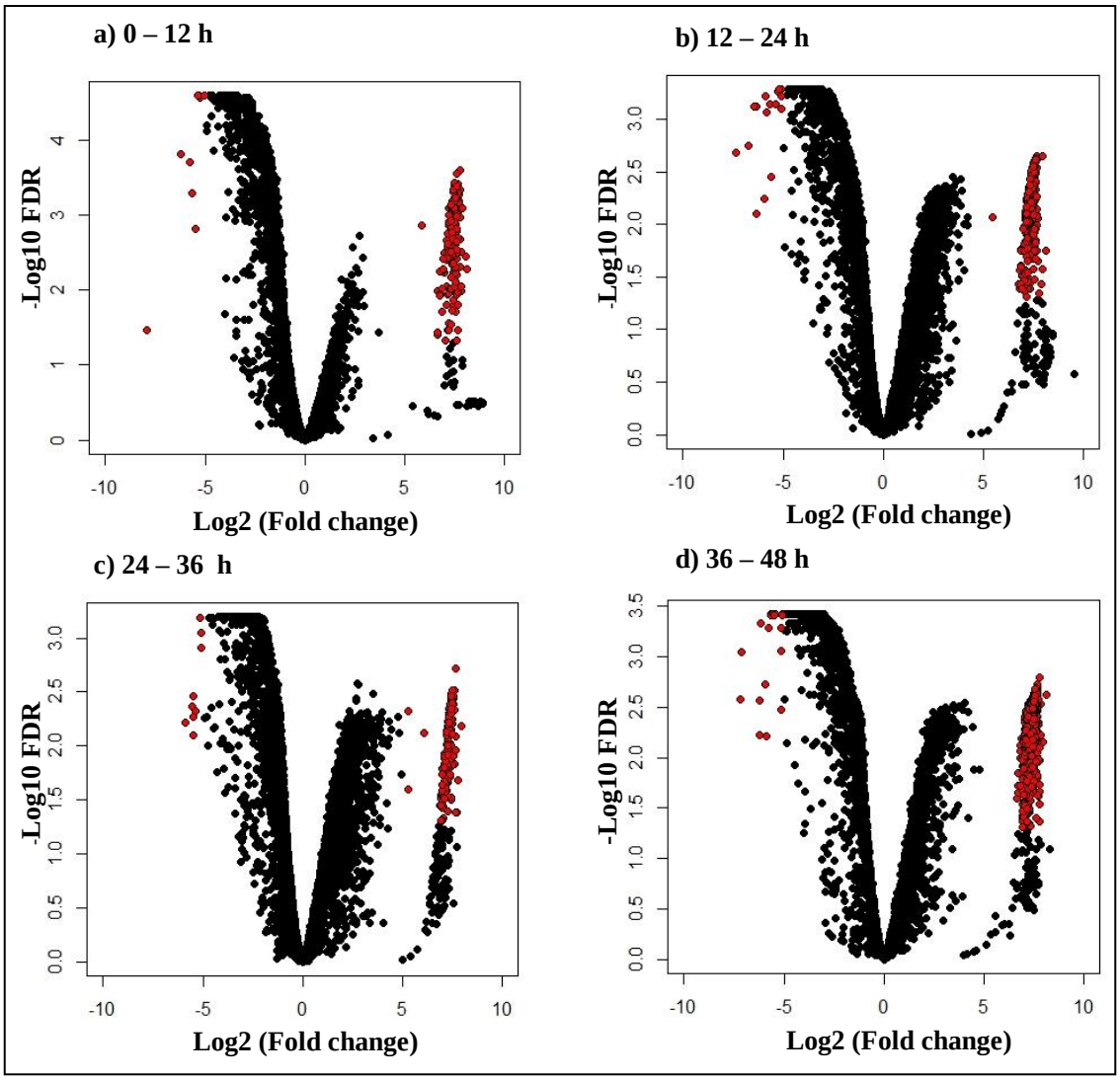

Figure 12: Volcano plots showing differentially enriched genes across the asexual parasite cycle. y-axis: negative log10 of FDR adjusted P value. x-axis: log2 (Fold change). Red shows significant genes while black shows non-significant genes. Genes with adjusted P value < 0.05 and an absolute log2Fold change threshold of 5 were termed significant.

and a member of *Plasmodium* RNA of unknown function (Pf3D7_0420800) as well as members of the exported protein family. Also enriched in 24-36 h PfEVs was multidrug resistance protein 1 (Pf3D7_0112200).

The gene products enriched in the 36-48 h PfEVs were mainly transcription factors (and associated enzymes) such as AP2 domain containing transcription factors (AP2-G, PF3D7_0420300, PF3D7_0802100, PF3D7_1222400, PF3D7_1456000, PF3D7_1449500), histone deacetylase (PF3D7_1472200), histone acetyltransferase

(PF3D7_0416400), repressor of RNA polymerase III transcription MAF1 (PF3D7_0416500), mRNA-binding protein PUF1 (PF3D7_0518700), translation initiation factors (PF3D7_0111800 and PF3D7_0716800), HCNGP-like protein, cyclins, zinc finger proteins and conserved *Plasmodium* proteins whose exact functions are not yet known. Other gametocyte-associated transcripts included: gamete antigen 27/25, GEXP10, GEXP17, gamete egress and traversal protein (GEST) and gametocytogenesis implicated protein (GIG). Another class of abundant gene products in 36-48 h PfEVs were transcripts that express the retromer complex commonly referred to as vacuolar protein sorting associated (VPS) proteins. Specifically, VPS2, VPS3 VPS4 and VPS9 were identified as highly abundant transcripts in 36-48 h PfEVs. Small nucleolar RNAs (snoR3, snoR6, snoR23, snoR24 and snoR27) and survival motor neuron-like protein (SMN) were also detected as 36-48 h evDEGs.

4.5 Gene ontology and network analysis

The 36-48 h PfEV transcriptome was highly enriched in mRNA encoding products involved in regulation of cellular processes and were assigned GO terms such as “regulation of macromolecule biosynthesis”, “regulation of gene expression”, “regulation of RNA biosynthesis”, among others (**Figure 13**). There were no overrepresented GO terms for the other three time windows. To explore the interaction between the GO terms, an enrichment map (**Figure 14**) and a category gene network plot (CGNP) (**Figure 15)** were constructed. These enrichment functional visualizations enabled mutually overlapping GO terms to cluster together. The upregulated GO terms for the 36 - 48 h comparison were organized in an intricate network involving all the 21 GO terms identified (**Figure 14**). This points out that parasites seem to pack functionally related gene products into PfEVs to perform a particular task.

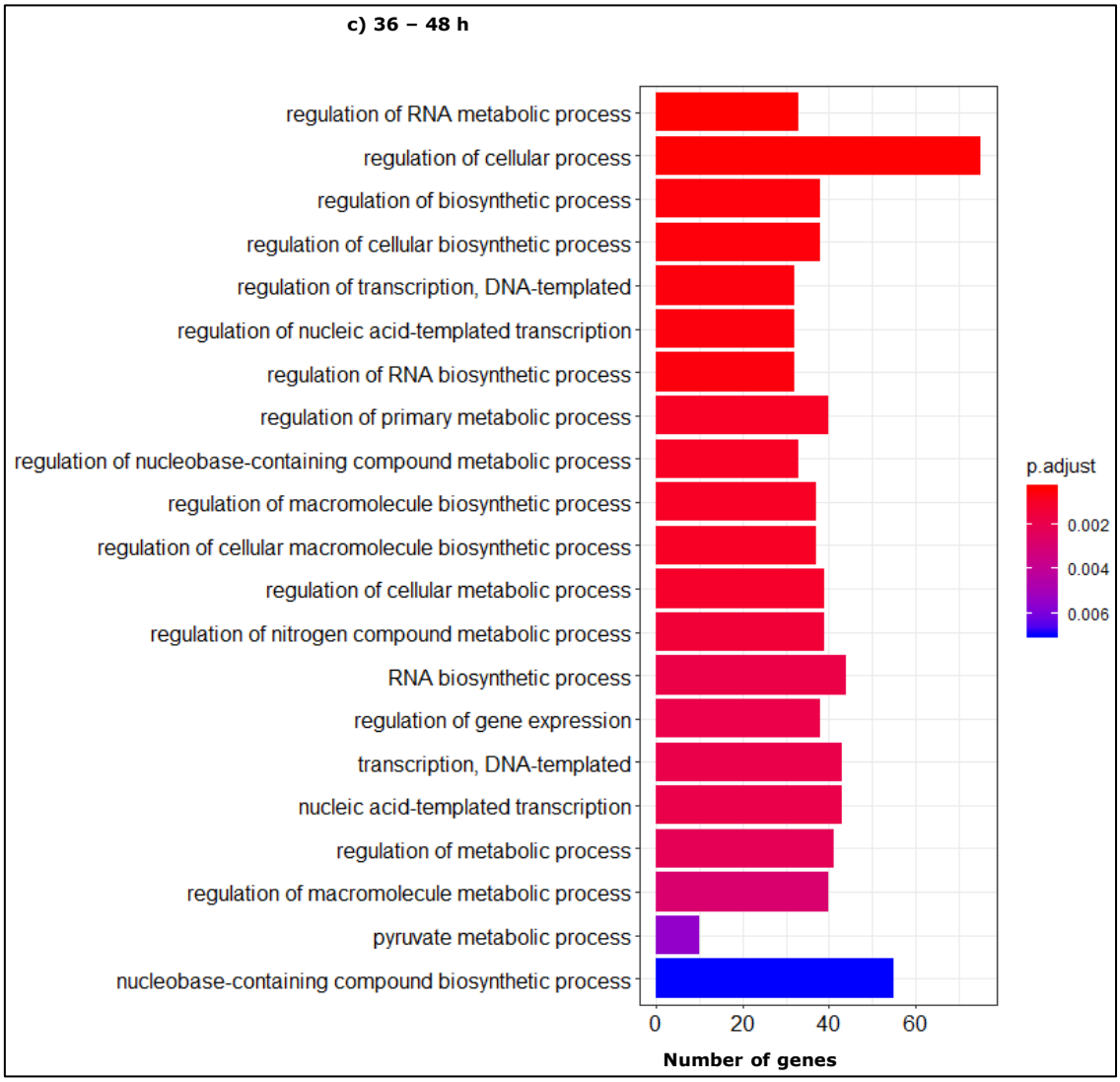

Figure 13: Enriched GO term analyses for the 36-48 h time window. GO terms with a P.adj value < 0.05 were considered significant. The red colour indicates GO terms with the smallest P.adj value with the increasing blue colour corresponding to larger adjusted P values

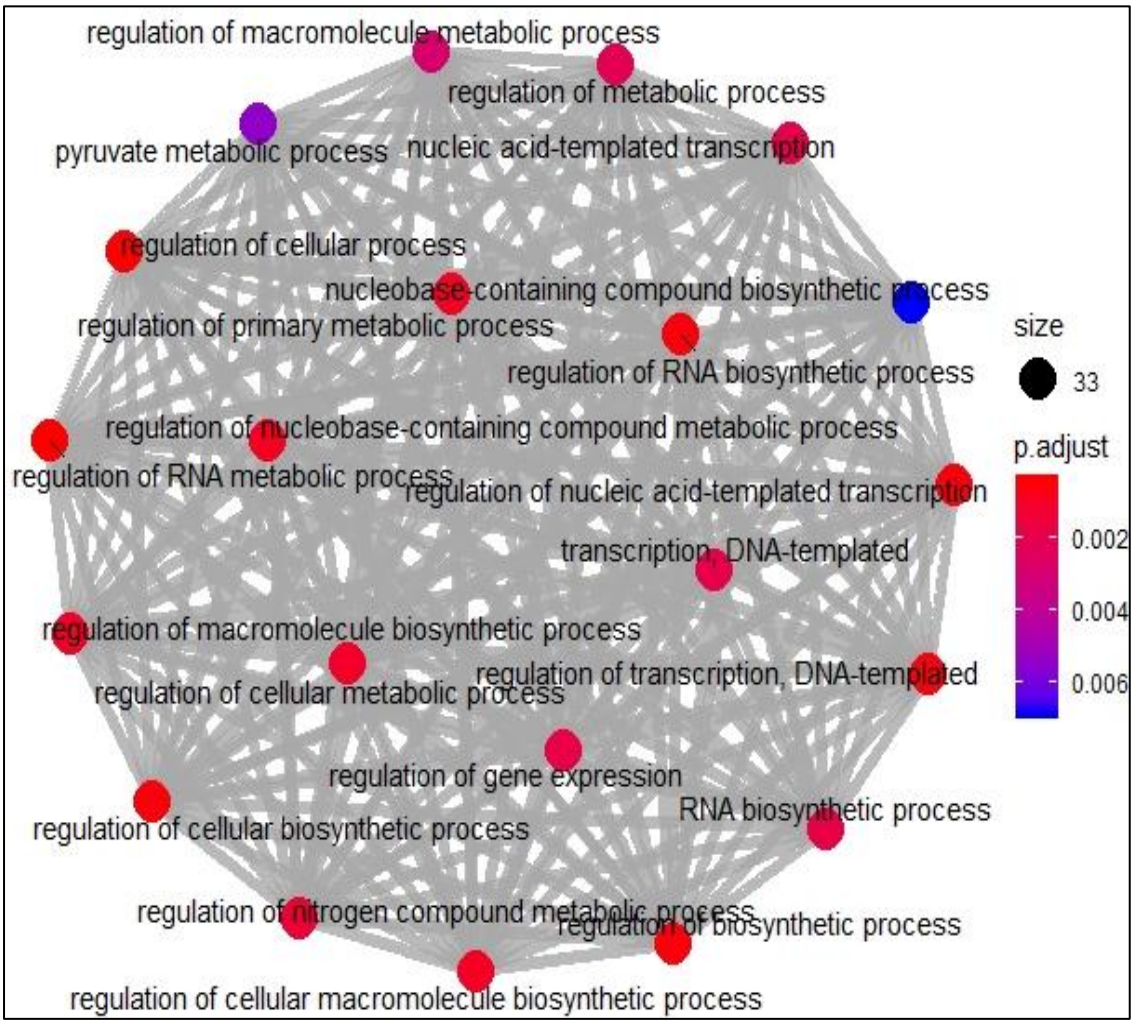

Figure 14: An enrichment map showing the interaction of GO terms in 36 - 48 h PfEVs.

The enrichment plot overcomes the problem of *P. falciparum* gene-set redundancy by grouping related GO terms into a similarity network, where nodes represent gene-sets, links represent the overlap of member genes, and node colour corresponds to the enrichment score.

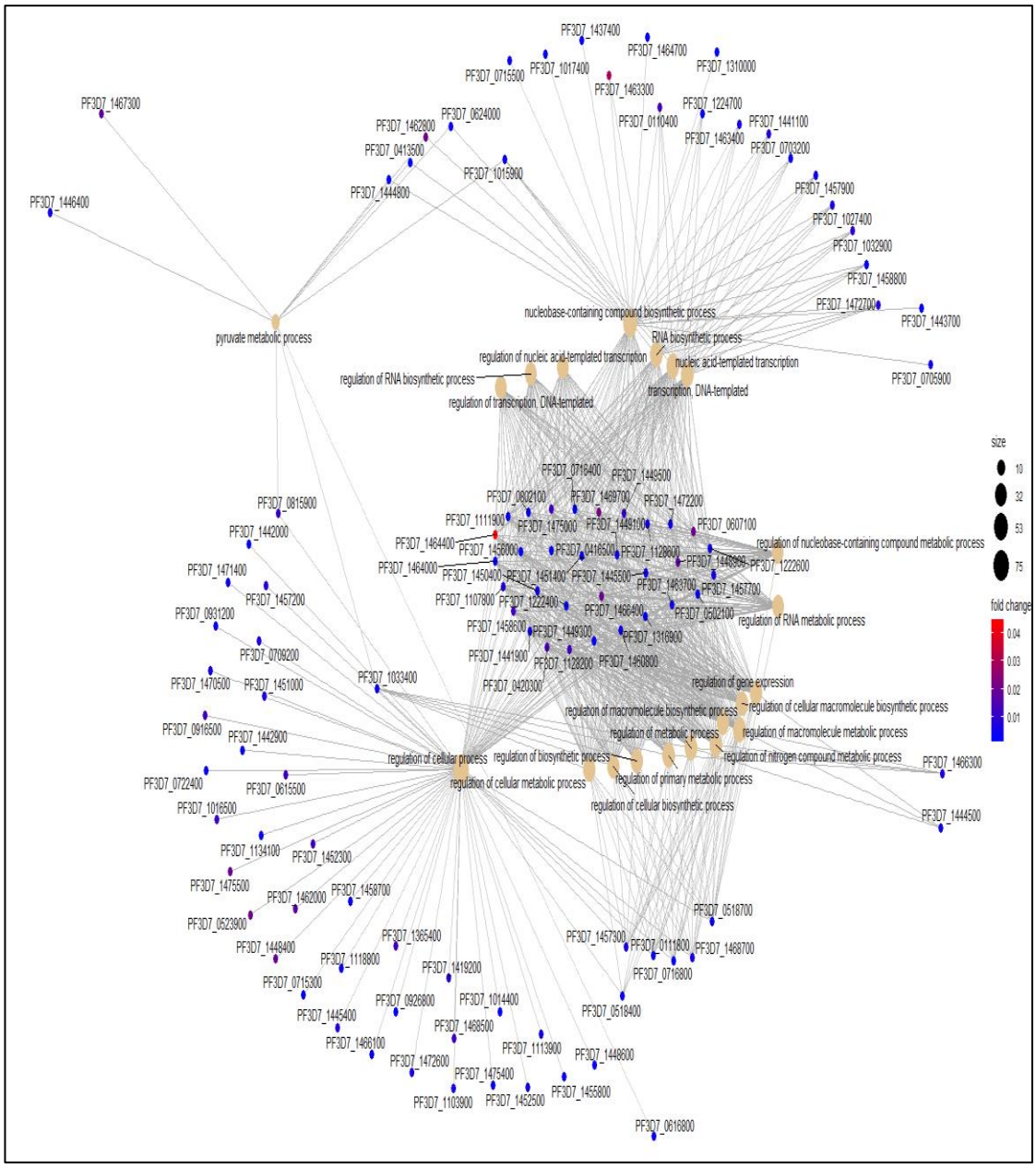

Figure 15: Category gene network for the 36-48 h comparison. The network shows that evDEGs released between the 24-36 h of the asexual parasite cycle can be grouped into three classes: **(i)**. genes exclusively involved in "Regulation of cellular processes", **(ii)**. genes exclusively involved in "nucleic acid biosynthesis" **(iii)**. and genes involved in both processes. Blue dots indicate genes assigned the smallest adjusted P-values while red dots correspond to larger adjusted P values. Yellow circles correspond to the GO terms.

CHAPTER FIVE: DISCUSSION AND CONCLUSION

5.1 Discussion

Emerging evidence supports that *P. falciparum* is an avid secretor of nano-sized particles called extracellular vesicles (EVs) [1, 2]. EVs are mediators of cell-cell communication that possess the intriguing ability to exchange their contents within the cell population [127]. In other systems, EVs have been demonstrated to transport functional mRNAs that can be translated and induce phenotypic effects in recipient cells [129]. The contents of PfEVs such as proteins, lipids and small non-coding RNA are well characterized [1, 3, 5]. However, the biological pathways whose mRNAs are preferentially packed into PfEVs are incompletely known. Therefore, this study aimed to establish gene products differentially enriched in PfEVs, and whether specific pathways were over-represented, by comparing RNAseq data in PfEVs to that of the whole parasite. The study also sought to identify the point of extracellular RNA release during the intraerythrocytic parasite cycle.

Analysis of a previously generated PfEV RNAseq data (provided by Dr. Abdirahman Abdi; generated with only one strain and two time points, with no replicates and with a different RNAseq and sequencing methodology) had revealed that genes involved in malaria pathogenesis [130] were selectively upregulated in PfEVs while mRNAs involved in core metabolic processes such as chromatin organization were secluded from PfEVs (data not shown). Similarly, this previous data showed that RNAs preferentially excluded from PfEVs are those sequestered in membrane-bound organelles such as the nucleus, mitochondrion, ribosome and endoplasmic reticulum, thus effectively preventing their packaging into PfEVs.

The current study examined the RNA content of PfEVs released by the asexual parasites in four time windows, using samples from three different strains and sequencing with the well-established Illumina platform. The earliest parasite time window that encompasses parasite egress and invasion was included to understand the biological content of vesicular RNA released during the end and onset of the parasite life cycle. The data shows dynamic changes in the RNA content of PfEVs at different stages of the asexual parasite life cycle. It was also found out that PfEVs are preferentially enriched in transcripts encoding transcription factors and proteins involved in host-parasite interaction.

Specifically, PfEVs released by ring stages (12-24 h) are more enriched in mRNA coding for the basal transcription factors that form the preinitiation complex (PIC) with RNA polymerase II [131]. These included the TATA-box binding protein (TBP), general transcription factor 3C (Pf3D7_1210400) and origin recognition complex subunit 5 (Pf3D7_0215800). Other gene products enriched in the 12-24 h PfEVs include an importin involved in shuttling of molecules from the cytoplasm into nucleus called karyopherin [132]. Although, the presence of mRNA for nuclear importins in PfEVs is puzzling, it is known that upon stress, importins stop transporting molecules into the nucleus and are sequestered in “cytoplasmic granules” [132]. Since cellular stress induces the release of EVs, the presence of nuclear transporters’ transcripts in malaria-derived vesicles suggests a second pathway through which nuclear shuttling is regulated. Similarly, transcript for a nuclear exporter of mRNA (Pf3D7_1223200) was enriched in 12-24 h PfEVs. Two gene products for regulatory components of the SET1 complex, the WD repeat-containing protein 82 and the associated histone-lysine N-methyltransferase are also preferentially sorted into the 12-24 h PfEVs.

The 24 - 36 h PfEVs were enriched in mRNA that express malaria multi-copy family of proteins such as PfEMP1, RIFIN, PHISTb and STEVOR. These proteins are key malaria antigenic ligands responsible for antigenic variation and adherence to vascular endothelium. Cytoadherence of infected RBCs to the placenta and brain is associated to life threatening clinical diseases [133]. Although PHISTb transcript has been detected in PfEVs previously [110], this is the first study to establish preferential packing of highly variable *P. falciparum* gene products into PfEVs. Similarly, ETRAMP 11.2, which was identified as a PfEV enriched transcript recently [101] was also detected in the current study. Surprisingly, a few mRNA that are known to be expressed in sexual parasite stages were also highly abundant in 24 -36 h PfEVs. A transcript for a well-characterized marker for sexually committed rings-stage parasites, GEXP5, was also enriched in 24 -36 h PfEVs [134, 135]. A third gametocyte specific mRNA identified in the 24-36 h PfEVs expresses a protein folding chaperone called gametocyte erythrocyte cytosolic protein (GECO) [134]. Although it is not yet proved that mRNA transferred to recipient parasites by PfEVs is translated into proteins, the enrichment of gametocyte-specific mRNA in malaria vesicles is in keeping with previous evidence that PfEVs promote sexual commitment [1, 2].

Apart from transcripts that express proteins involved in host-parasite interaction, ATP dependent helicases also showed a higher fold change in 24-36 h PfEVs as compared to the parasite. Human ATP dependent helicases are essential in DNA repair, maintenance of telomeres and replication of GC- rich regions [136] and *Plasmodium* homologs may have related functions. Some of the GC-rich non-coding RNA (ncRNA) positioned adjacent to the *var* genes were also detected in PfEVs. The GC-rich ncRNA, currently annotated as *Plasmodium* RNA of unknown function 6 (RUF6) are involved in *trans* regulation of *var* gene expression [137]. Therefore, the co-upregulation of ATP

dependent helicases, the subtelomeric *var* genes and the GC-rich ncRNA, supports PfEVs as authentic molecular products rather than just random membrane-bound cell debris.

It was noted that there is an enrichment of transcripts involved in regulation of cellular processes in PfEVs released by late parasite stages (36-48 h). Specifically, 6 members of the 27 Apicomplexan AP2 (APiAP2) domain containing proteins [138] that act as parasite development regulators were highly abundant in 36 - 48 h PfEVs . One of these APiAP2 proteins is termed AP2-G and is widely accepted as the master regulator of parasite sexual differentiation [139]. Also of note is the upregulation of histone deacetylase, histone acetyl transferase, HCNGP-like protein (Pf3D7_0502100) and mRNA binding PUF1 protein, which have been proposed to play roles in gametocytogenesis together with AP2-G [138, 140, 141]. The presence of these transcription factors again supports previous reports of the ability of PfEVs to reprogram recipient parasites to sexually commit. Similarly, MAF1 (Pf3D7_0416500) is another transcription factor upregulated in 36-48 h PfEVs. MAF1 is a canonical repressor of RNA polymerase III that confers survival to *P. falciparum* during starvation and stress [142]. Late stage PfEVs are also rich in transcripts for a conserved family of transcription factors called cyclins that control parasite cell cycle. In addition to histone acetyltransferase, two other nucleic acid binding zinc-finger proteins are highly upregulated in PfEVs released by mature parasites. More than two thirds of transcripts enriched in PfEVs are conserved *Plasmodium* proteins of unknown function, and therefore more studies are required to reveal their exact functions.

It was also observed that PfEVs generally contain transcripts that express extracellular vesicle protein markers. These include vacuolar protein sorting-associated protein (VPS), sortilin, SNARE proteins and ADP-ribosylation factor, putative (Pf3D7_1442000) which play a role in vesiculation [143]. An accurate mapping of PfEV transcriptome to PfEV

proteome will provide a list of genes expressed in PfEVs in form of both mRNA and proteins.

5.2 Limitations of the study

The present study had several limitations, with the most important one being a relatively lack of replication due to the challenging nature of the project (it took some time to perfect the PfEV purification process), the limited time available, and the extremely low RNA content of purified PfEVs, which meant that more than half of the samples collected lacked sufficient material for RNAseq. While samples taken at the same point from different strains were pooled bioinformatically to act as technical replicates, clearly a larger study with multiple biological and technical replicates is required for a definitive assessment of PfEV RNA content. Plans are underway to scale up the project for just such a purpose.

Second, although normalisation theoretically levelled the observational weights of the PfEV-data to that of Lopez-Barragan et.al. whole transcriptome, the ideal design would have been to sequence the parasites from which PfEVs were isolated for use as a comparator. This will be considered in the next experiment design. Finally, despite being the current “gold standard” in EV isolation, preparative ultracentrifugation is only an enrichment method and PfEVs are potentially co-isolated with other protein-bound RNA. PfEV treatment with RNase A may not completely digest all the protein-bound RNA, and this may confound results of differential expression analysis. Exploration of additional purification techniques would be recommended. The observation that the PfEV transcriptome of Nf54 does behaves similarly to that of more recent clinical isolates need to be confirmed. This is because the isolates have been cultured by several people since

they left KEMRI-Wellcome Trust premises, and therefore it is not exactly clear for how long they have been in culture.

5.3 Conclusion

The present study aimed to identify *Plasmodium falciparum* transcripts that are enriched in PfEVs by comparing a time series comparison of PfEV-RNA data to previously generated whole parasite data. Transcripts that express members of the parasite's multi-copy families of proteins such as PfEMP1, STEVOR, and RIFIN were highly abundant in PfEVs as compared to the parasite. Other PfEV enriched mRNA include that of gametocyte specific proteins, transcription factors and EV protein markers. PfEVs collected between 36 - 48 h had 958 differentially enriched genes - almost five fold higher than that of other parasite time windows. Gene ontology revealed that transcripts enriched 36-48 h PfEVs are mainly involved in regulation of cellular processes. Further study of PfEV-RNA content is clearly required.

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