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.