RESEARCH ARTICLE

Proteomic analysis of extracellular vesicles from a *Plasmodium falciparum* Kenyan clinical isolate defines a core parasite secretome [version 1; referees: 1 approved, 2 approved with reservations]

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Abstract

Background: Many pathogens secrete effector molecules to subvert host immune responses, to acquire nutrients, and/or to prepare host cells for invasion. One of the ways that effector molecules are secreted is through extracellular vesicles (EVs) such as exosomes. Recently, the malaria parasite *P. falciparum* has been shown to produce EVs that can mediate transfer of genetic material between parasites and induce sexual commitment. Characterizing the content of these vesicles may improve our understanding of *P. falciparum* pathogenesis and virulence.

Methods: Previous studies of *P. falciparum* EVs have been limited to long-term adapted laboratory isolates. In this study, we isolated EVs from a Kenyan *P. falciparum* clinical isolate adapted to *in vitro* culture for a short period and characterized their protein content by mass spectrometry (data are available via ProteomeXchange, with identifier PXD006925).

Results: We show that *P. falciparum* extracellular vesicles (PEVs) are enriched in proteins found within the exomembrane compartments of infected erythrocytes such as Maurer’s clefts (MCs), as well as the secretory endomembrane compartments in the apical end of the merozoites, suggesting that these proteins play a role in parasite-host interactions. Comparison of this novel clinically relevant dataset with previously published datasets helps to define a core secretome present in *Plasmodium* EVs.

Conclusions: *P. falciparum* extracellular vesicles contain virulence-associated parasite proteins. Therefore, analysis of PEVs contents from a range of clinical isolates, and their functional validation may improve our understanding of the virulence mechanisms of the parasite, and potentially identify targets for interventions or diagnostics.

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**Introduction**

*Plasmodium falciparum* malaria remains a major public health problem, with 212 million cases of malaria and half a million deaths due to severe malaria reported worldwide in 2015. The pathogenesis mechanism of severe malaria is not completely understood, but relevant factors include parasite burden, induction of host inflammatory responses, and obstruction of movement of blood in the microvasculature of important organs such as the brain due to adhesion of parasite infected erythrocytes (IEs) to vascular endothelial cells. Binding of IEs to vascular endothelial cells through endothelial protein C receptor (EPCR) has been hypothesized to cause endothelial activation and inflammation, contributing to pathogenesis. However, results from other studies suggest that endothelial activation and inflammation during malaria parasite infections can be independent of cytoadhesion of IEs, and effectors secreted by the parasite have been suggested to play a more important role.

One way secreted effectors are released from cells is through extracellular vesicles (EVs) that can be classified into two major types: exosomes and microvesicles. Exosomes are vesicles of endocytic origin with diameter of 30–150 nm released to the extracellular space when the endosomal multivesicular body (MVB) merges with plasma membrane of the cell. By contrast, microvesicles are larger, with a diameter of 100–1000 nm, and are generated through outward invagination of the plasma membrane. These vesicles can transfer biologically active effector molecules such as lipids, nucleic acids, metabolites and proteins from one cell to another thereby modifying the properties of the recipient cells. The physiological significance of these vesicles is becoming increasingly appreciated in many disease processes, including cancer and infectious diseases.

Increasing evidence suggests that secreted extracellular vesicles (EVs) play an important role in intercellular communications. In the context of cancer and infectious diseases, EVs can be used to sabotage the host defence mechanism, prepare the host cell for invasion, and acquire nutrients from the environment. Identifying the content of EVs released by pathogenic organisms will help to understand their basic biology, and potentially identify targets for intervention or diagnostics.

In malaria, *P. falciparum* EVs (PfEVs) have only been recently identified in two studies that used long-term laboratory isolates, and it has been proposed that PfEVs play a role in cell-cell communications and gametocytogenesis. As EVs represent an extended phenotype of the cell, unravelling the bioactive molecules in PfEVs will contribute to our understanding of the biology and virulence mechanisms of *P. falciparum*. Analogy with other pathogens would suggest that *P. falciparum* exosomes may play a role in immunomodulation, nutrient acquisition and invasion in vivo. Given that long-term *P. falciparum* laboratory strains, such as 3D7, have not been exposed to the human host environment for decades, it is possible that they may have adapted to release fewer PfEVs or pack a less extensive set of effectors into them, whereas more recently culture adapted *P. falciparum* clinical isolates might release PfEVs containing a greater variety and depth of effectors.

Here we present analysis of the protein content of PfEVs isolated from a Kenyan clinical isolate that has been grown *in vitro* for a shorter period than established laboratory strains, and compare it with published *Plasmodium* EV proteome datasets to define a core *Plasmodium* EV content.

**Materials and methods**

**Parasite culture**

The Kenyan isolate (referred to as isolate 9605) was obtained from a child admitted to Kilifi County Hospital with cerebral malaria in 2009. The parasite was adapted to *in vitro* culture and at the time of conducting this study the parasite had been grown *in vitro* for a maximum of 70 cycles, as opposed to many years in the case of most laboratory isolates such as 3D7. The genome of this isolate was sequenced using both Illumina and PacBio platforms at the Wellcome Trust Sanger Institute, Hinxton, Cambridge, (manuscript in preparation). With the publication of the genome, the isolate will be made available to the community through the European malaria reagents repository (http://www.malariaresearch.eu/reagents), University of Edinburgh. Parasite culture was carried out under standard conditions.

**Preparation of *P. falciparum* culture-conditioned media**

*P. falciparum* 9605 culture was tightly synchronised by repeated sorbitol treatment and expanded to 6 flasks, each containing 500 µl packed cells at 5–10% parasitemia. RPMI culture media supplemented with AlbumaxII (Gibco) was used to grow the parasite. AlbumaxII was depleted of exosomes by centrifuging at 150,000g for 2 hours. 50ml of the media was added to each flask when parasites were at early ring stages and harvested after 24 hours when the parasite grew to mature trophozoites (referred to as the ring-to-trophozoite, or RT sample). The culture was diluted with fresh blood (supplied by NHS, Cambridgeshire, UK) to maintain parasitemia within 5–10% in the following cycle. Fresh media was added and then harvested again 24 hours later, when the parasites had returned to early ring stage (referred to as the trophozoite-to-ring, or TR sample). Culture-conditioned media harvested at each step was processed as outlined in Figure 1. The culture was transferred to 50ml Falcon tubes and centrifuged at 440g for 5 minutes to pellet the erythrocytes. The supernatant was centrifuged more one time at 440g for 5 minutes, then twice at 2000g for 10 minutes, once at 3600g for 10 minutes, and finally at 15000g for 30 minutes, each time using new 50ml Falcon tube. The pellet obtained after the 15000g spin was stored at -80°C for future analysis, as it potentially contains microvesicles. The supernatant, presumed to predominantly contain exosomes, was filtered at 0.2µm and the flow-through frozen at -80°C until use.

**Purification of PfEVs by density gradient ultracentrifugation**

To purify PfEVs, the frozen supernatant generated above was thawed on ice and loaded on quick-seal ultracentrifugation tubes (Beckman Coulter cat# 343322), then centrifuged at 150, 000g for 2hrs using an Optima XE90 ultracentrifuge and 70.1TI rotor (Beckman Coulter). The pellet was washed twice by re-suspending in cold PBS and centrifuging at 150,000g for 2 hours after each wash. The final pellet was loaded onto OptiPrep™ density gradient
medium prepared as described\(^{45-47}\) and centrifuged at 250,000g for 18 hours. 1ml fractions were collected from the top of the gradient into 1.5ml Eppendorf tubes. To estimate the density of the purified vesicles, the weights of the tubes containing the fractions were measured using weighing machine (Sartorius, PB221S). Each fraction was diluted in PBS to 13.5 ml and centrifuged at 150,000g for 2 hours and the pellet re-suspended in 400 µl of 8M urea, 2.5% SDS in 50 mM phosphate buffer, pH 8.0 to extract proteins, before concentrating using a 3KDa MWCO concentrator (Pierce™). A fifth of each fraction was analysed for the presence of proteins by running on SDS-PAGE and staining with silver stain (Bio-Rad).

Proteomic analysis by mass spectrometry
Fractions confirmed to contain PfEV proteins by silver staining were first reduced by adding DTT to final concentration of 5mM for 10 minutes at 70 °C, then alkylated by adding iodoacetamide (IAA) to final concentration of 10 mM and incubated for 30 minutes at room temperature in the dark. The samples were then separated by SDS-PAGE (NuPAGE 4–12% Bis-Tris Gel, Life Technology). The gel was fixed in 40% methanol/2% acetic acid for 30 minutes, stained with colloidal Coomassie (Sigma) overnight, and finally destained with 30% methanol until the background was cleared. Each lane was excised to four pieces, and the gel pieces were destained in 50 mM ammonium bicarbonate/50% CH₃CN until the gel pieces were completely destained, then they were digested by trypsin (Pierce MS Grade, Thermo Fisher Scientific) overnight at 37°C. Peptides were extracted with 0.5% formic acid (FA)/50% CH₃CN and dried in a SpeedVac (Thermo Fisher Scientific). The peptides were resuspended in 20 µl of 0.5% FA just before LC-MS/MS analysis on a LTQ Orbitrap Velos (Thermo Fisher) hybrid mass spectrometer equipped with a nanospray source, coupled with an Ultimate 3000 RSLCnano System. Samples were first loaded and desalted on a PepMap C18 trap (0.1 mm id x 20 mm, 5µm) at 10 µL/min for 15 min, then peptides were separated on a 75 µm id x 25 cm PepMap column (3 µm) at a flow rate of 300 nl/min over a 90 min linear gradient of 4–32% CH₃CN/0.1% FA, 130 min/cycle. All instrument and columns were from Thermo Fisher Scientific. The LTQ Orbitrap Velos was operated in the “Top 15” data-dependant acquisition mode. The 15 most abundant and multiply-charged precursor ions in the MS survey scan in the Orbitrap (m/z 380 – 1600, with the lock mass at 445.120025) were dynamically selected for CID fragmentation (MS/MS) in the LTQ Velos ion trap. The ions must have a minimal signal above 3000 counts. The preview mode of FT master scan was disabled. The Orbitrap resolution was set at 30,000 at m/z 400 with one microscans. The isolation width for the precursor ion was set at 2 Th. The normalized collision energy was set at 35% with activation Q at 0.250 and activation time for 10 msec. The dynamic exclusion mass width was set at ±10 ppm and exclusion duration for 60 seconds. To achieve high mass accuracy, the AGC (Automatic Gain Control) were set at 1×10⁶ for the full MS survey in the Orbitrap with a maximum injection time at 150 msec, and 5000 for the MS/MS in the LTQ Velos with a maximum injection time at 100 msec.
The raw files were processed in MaxQuant (Version 1.5.3.30, www.MaxQuant.org), and searched against the *Plasmodium falciparum* 3D7 and 9605 protein databases, human protein database (from UniprotKB, October 2014, www.uniprot.org), and a contaminant database supplied by MaxQuant. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE<sup>46</sup> partner repository, with the dataset identifier PXD006925.

Parameters used were mainly in default values, with some modifications: trypsin with maximum 2 missed cleavages sites; peptide mass tolerance at first search was set at 20 ppm and main search was at 4.5 ppm; MS/MS fragment mass tolerance at 0.5 Da, and top 8 MS/MS peaks per 100 Da and a minimum peptide length of 7 amino acids were required. Fixed modification for Carbamidomethyl and variable modification for Acetyl (Protein N-term), Deamidated (NQ) and Oxidation (M) were used. False discovery rates (FDR) were estimated based on matches to reversed sequences in the concatenated target-decoy database. The maximum FDR at 1% was allowed for both proteins and PSMs. Peptides were assigned to protein groups, a cluster of a leading protein(s) plus additional proteins matching to a subset of the same peptides.

Gene ontology (GO) enrichment analysis
To assess whether certain classes of proteins are enriched in P/EVs proteome, gene ontology analysis for enrichment of cellular components was carried out using PlasmoDB (http://plasmodb.org/plasmo/showApplication.do).

Results
Vesicles with features characteristics of exosomes can be purified from culture conditioned media of a recently adapted *P. falciparum* isolate
We isolated P/EVs from culture-conditioned media of a Kenyan *P. falciparum* clinical isolate adapted to *in vitro* culture. Culture-conditioned media was harvested as shown in Figure 1a, and vesicles pelleted using ultracentrifugation. Examination of the pellet by transmission electron microscopy revealed vesicles (Figure 1b) with median size of 132nm, mean±SD of 143nm±66. To further purify the pelleted vesicles, the pellet was re-suspended in PBS and subjected to OptiPrep density gradient ultracentrifugation as described in the Methods. 1ml fractions drawn from the top of the gradient were collected, and as shown in Figure 1c, lane 9, 10, and 11 (corresponding to fractions 10, 11, and 12) contained several protein bands, whereas the rest of the fractions contained a single band representing albumin, a common contaminant co-pelleted with EVs. The density of the fractions 10, 11, and 12 ranged between 1.06–1.17g/cm<sup>3</sup>, consistent with the range described for exosomes (1.08–1.22 g/cm<sup>3</sup>)<sup>46</sup>.

The purified vesicles contained both host and parasite proteins
To identify the protein content in the isolated P/EVs, fractions identified as containing proteins through silver-staining (Figure 1c) were run on an SDS-PAGE gel and stained by colloidal Coomassie. Each lane was cut into four pieces and processed for mass spectrometry analysis as described in the Methods. 153 *P. falciparum* proteins (Table S1) and few erythrocyte proteins such as haemoglobin (alpha and beta chains), band 3 anion transport protein, erythrocyte band 7 integral membrane protein, and spectrin were identified. Serum/albumax and other common contaminants were excluded from the analysis.

Virulence associated parasite proteins were among the protein class significantly enriched in the P/EVs proteome
We identified 61 and 149 *P. falciparum* proteins in the P/EVs purified from the culture conditioned media of ring-to-trophozoite (RT) and trophozoite-to-rings (TR) time points, respectively (Table S1). 57 of the 61 parasite proteins in the RT time point were also present in the TR, showing reproducibility of enrichment methods and giving a total of 153 *P. falciparum* proteins (Table S1). GO term enrichment analysis for cellular components revealed that terms related to proteins commonly found in EVs such as ribosomes were significantly enriched in P/EVs (Figure 2a). In addition, terms related to virulence associated parasite specific proteins were also significantly enriched (Figure 2a). The virulence associated proteins included proteins residents in membrane bound organelles that form in the IE cytosol such as Maurer’s clefts (MCs), and merozoite secretory organelles such as the rhoptry, the microneme and the dense granules (Figure 2a). In contrast, GO-terms associated with proteins of intracellular organelles such as nucleus, ER, and mitochondria were not significantly enriched in the P/EVs proteome (Figure 2a). Among the apically associated proteins, rhoptry associated proteins were the most dominant (Figure 2a). P/EVs from the RT sample were enriched for proteins found in exomembrane compartments of the IEs (Table S1).

Parasite proteins found in membrane-bound organelles from IE cytosol and the merozoite were the most abundant P/EV proteins
When the list of P/EV proteins was stratified by the number of unique peptides, the top 50 most abundant (>2 unique peptides) were parasite proteins that are exported to exomembrane compartments within the infected erythrocytes beyond the parasite plasma membrane (Figure 2b and Table S1). Proteins found across this exomembrane network, including MCs (SBP1, REX1/2, MAHRP1/2 and PMC-2TM), J-dots (PHISP40 and PHISP70-x), IE membrane (Glag3.1, RhopH3, RESA, KAHRP, PiEMP3) and parasitophorous membrane (PTEX complex, ETRAMP family) were identified (Table S1). Interestingly while REX1/2, two proteins localised in the MCs<sup>46</sup> were detected in the P/EVs proteome, REX3, which localises in the IE cytosol<sup>10</sup> as a soluble protein, was not detected. Further, *P. falciparum* REXHRP2 which is secreted into the extracellular space as a soluble protein was also not detected, suggesting most parasite proteins identified in the proteomic analysis are associated with membranes.

Several multigene family proteins such as PiEMP1, Rifins, Stevor, PHIST and FK1K are exported to or via these exomembrane compartments. Among these multigene protein families, PHIST and Rifin families were represented in P/EVs (Table S1 and Table S2) but no PiEMP1 and Stevor proteins were detected, consistent with previous finding by Mantel et al.<sup>12</sup>. This is despite the presence of other proteins that localise at the knobs beneath the IE surface membrane, such as KAHRP and PiEMP3, and...
Figure 2. Virulence-associated parasite proteins are significantly enriched in PIEVs proteome. A). Gene Ontology enrichment analysis for cellular components (N=153). Plotted is the –log p-value (Bonferroni adjusted for 56 comparisons) against the GO terms. The horizontal red line indicates the cut-off for significance (p<0.01). The most significant GO terms were associated with ribosomal, exported and invasion genes (apical complex). Note the invasion related genes are denominated by rhoptry genes (green bar, highlighted with blue line), especially the rhoptry bulb. The rhoptry has been hypothesized to be the equivalent of the endosomal multivesicular body containing vesicles to be secreted to the extracellular environment as exosomes. Enrichment for genes related to intracellular organelles (purple bars) such as mitochondria, ER and nuclear was not significant. B). GO-terms associated with the top 50 most abundant proteins (#unique peptide≥4). Plotted is the –log p-value (Bonferroni adjusted for 38 comparisons) against the GO terms. Genes exported to the vesicular compartments within the cytosol of the parasitized red blood cells and those secreted from the endomembrane compartments of the merozoites such as the rhoptry and dense granules were the most abundant genes.
proteins linked to transport of PfEMP1 to the surface of the IE (PTP1, PTP6). These observations suggest that PfEVs are not derived from the IE plasma membrane.

The second most abundant class of parasite proteins enriched in PfEVs was merozoite antigens discharged from the secretory endomembranous compartments of the apical end (Figure 2b and Table S1). The rhoptry proteins were the most enriched (Figure 2 and Table S1). These include the Rhoph complex (RhopH1 (Clag3.1) Rhoph2 and Rhoph3), the RAP complex (RAP2 and RAP3), RALP1 and RON3. Notably, these proteins are found in the bulb of the rhoptry organelle. Except for RON2, proteins from the rhoptry neck region, such as other RONs or members of the reticulocyte binding protein homologue (PRH) family except RH4, were not detected. Some microneme resident proteins such as EBA-175 and EBA-181 were also present (Table S1) but notably absent was AMA1, consistent with a previous report\(^\text{12}\). Several dense granule proteins released during merozoite invasion were also among the most abundant PfEVs proteins. These included those that contribute to establishment of a translocon at the PVM for protein export (PTEX members; HSP101, PTEX150, and EXP2) and proteins exported into the invaded erythrocyte early after invasion (SBP1, RESA, MAHRP1).

Comparing PfEVs datasets to define a core PfEV proteome

The proteomic data of the Kenyan clinical isolate had substantial overlap with the only previously published PfEVs proteome\(^\text{12}\) but was significantly deeper. Between these two studies there are now 184 PfEV proteins identified. 53/84 proteins detected by Mantel et al.\(^\text{12}\) were common to our Kenyan isolate and 100 proteins (54% of the 184) were unique to our clinical isolate (Figure 3a). GO term enrichment analysis based on cellular components revealed that the PfEVs proteins shared between the Kenyan and the long-term laboratory isolates were dominated by virulence associated proteins found in secretory membranous compartment of the merozoite or the cytosol of the IE suggesting that these class of parasite proteins form the core PfEV proteome (Figure 3b). The PfEV proteins detected only in the Kenyan isolate were enriched for ribosomal and virulence associated exomembrane proteins (Figure 3c).

**Figure 3. Exported and invasion related *P. falciparum* proteins form the core proteome of PfEVs. A) Venn diagram showing that the proteome of PfEVs from the Kenya isolate had good overlap with the only previously published PfEVs proteome (Mantel et al.)\(^\text{12}\), but was also significantly deeper, covering more proteins. B) GO-terms enrichment analysis for cellular components of the PfEVs proteins common in both the Kenyan and the long-term laboratory isolates used in Mantel et al.\(^\text{12}\). Plotted is the \(-\log p\text{-value} \) (Bonferroni adjusted for 53 comparisons) against the GO terms. GO terms associated with exported and invasion related proteins were significantly enriched. C) GO-term enrichment analysis showing that the PfEV proteins specific to the Kenyan isolate. Plotted is the \(-\log p\text{-value} \) (Bonferroni adjusted for 46 comparisons) against the GO terms.
Discussion
In this study, we characterised the protein content of EVs released by a Kenyan *P. falciparum* clinical isolate, 9605. We show that these *PfEVs* have physical characteristics close to exosomes, and proteomics identified 153 *P. falciparum* proteins, of which the most abundant were virulence associated proteins.

Previous proteomic analysis of *PfEVs* from long-term laboratory isolates identified 84 *P. falciparum* proteins. The expanded list identified here suggests that the protein content of *PfEVs* from clinical isolates might be broader than that of long-term laboratory isolates. While analysis of further long and short-term laboratory-adapted isolates will be required to formally test this hypothesis, it is well known that long-term laboratory *P. falciparum* isolates can down-regulate genes involved in pathogenesis in *in vitro* but are not required for *in vitro* culture, such as genes associated with cytoadherence. For example, the common laboratory isolate 3D7 predominantly expresses only a limited number of *var* genes which encode for the variant surface antigen required for sequestration. Further, genes encoding for exported and sexual stage proteins have been shown to be upregulated in short-term culture adapted clinical isolates (48–86 in *vitro* cycles) as compared to long-term laboratory isolates. Therefore, due to the relatively shorter time the Kenyan isolate spent in *in vitro* culture conditions, 9605- *PfEVs* proteome may represent a closer reflection of that released by the parasite under *in vivo* condition. Caution is required of course, and technical differences in *EV* purification or proteomic detection between studies clearly cannot be ruled out as the cause of the differences observed. More studies of *PfEVs* from clinical isolates is clearly required.

The biogenesis of *PfEVs* is not known, but this proteomic analysis revealed several parasite proteins with potential role in biogenesis and transport of endosomal vesicles. These include Rab GTPases (*PfRab7*, *PfRab6*), and *P. falciparum* pyruvate kinase (*PfPK*), which in other systems are required for transport as well as release of exosomes. The apicomplexan Rab11A has been speculated to be involved in the transport of vesicles derived from endosome-like compartments. In addition, the most abundant proteins were from exomembrane compartments of the IEs (Table S2), suggesting an origin in these vesicular compartments. Previous immunoprecipitation of an MCs resident protein *SEMP1*, which is also among the most abundant *PfEVs* proteins (Table S1), co-precipitated 14 proteins of which 13 were identified here as being present in *PfEVs* (Table S1/S2), further reinforcing the link between *PfEVs* and the exomembrane compartments within the IEs. Functional analysis of the *P. falciparum* genes described here may therefore shed more light on the process of *PfEVs* biogenesis and release.

Another vesicular compartment closely linked to *PfEVs* is the secretory endomembrane compartments of the merozoite apical end, such as the dense granules and the rhoptry. Proteins from the rhoptry, specifically those from the rhoptry bulb region, were among the most dominant identified (Table S2). The rhoptry bulb has a honeycomb appearance by transmission microscopy, which has been postulated to be formed by internal membranes and/or vesicles, and it has been further hypothesised that the rhoptry might be the equivalent of multivesicular body (MVB) in higher eukaryotes. It is therefore possible that the content of the merozoite endomembrane compartments particularly those of the rhoptry and the dense granules are secreted as EVs. This possibility is supported by a recent study that showed 50% of RhopH3 is released into the culture media during merozoite invasion as insoluble membrane-associated protein that can only be pelleted down from the culture media by ultracentrifugation, which could well be *PfEVs*. Further, antibody targeting PTEX150 released from dense granules precipitated 82 parasite proteins, of which 42 (51%) are present in *PfEVs* (Table S2). The possibility that key components required for early establishment of the exomembrane compartments such as PTEX, RAP, and RhopH have complexes are in part secreted in vesicles will be important to confirm using immunoelectron microscopy.

Previously, typical intracellular *Plasmodium* antigens have been proposed as potential subunit vaccine candidates including some identified in screens of large panel of *P. falciparum* proteins using plasma from malaria exposed individuals. *PfEVs* contain several of these antigens (Table S2) which might explain how they become accessible to the host immune system. Further, immunisation of mice with *P. yoelii* EVs provided protection against lethal infection and data in PlasmoDB shows a high proportion of the genes encoding parasite proteins in *PfEVs* have a non-synonymous to synonymous mutations ratio (dN/dS) of above 1, suggesting they might be under host immune pressure.

Conclusions
In summary, we have purified *PfEVs* with physical characteristics of exosomes from a short-term adapted Kenyan clinical isolate. Proteomic analysis broadened the list of *PfEVs* components, and suggests that *PfEVs* are closely linked to exomembrane and endomembrane compartments in the IEs and merozoites. We hypothesise that, in addition to the parasite-parasite communication role described before, *PfEVs* might have a role in fine-tuning the host immune response, preparing the host cell for invasion and acquiring nutrients from the environment. Further functional validation of *PfEVs* components, and expanded study of *PfEVs* protein, RNA, lipid and metabolite content from a range of isolates, is clearly required.

Data availability
The raw mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository, with the dataset identifier PXD006925. Project name: ‘Proteomic analysis of extracellular vesicles from a *Plasmodium falciparum* Kenyan clinical isolate defines a core parasite secretome’. The original gel image for Figure 1c is available at [https://osf.io/wdg96](https://osf.io/wdg96).
Ethical statement
Ethical approval was obtained from Kenya Medical Research Institute Scientific and Ethical Review Unit (KEMRI/SERU/CGMRC/022/3149), and written informed consent was obtained from the guardian of the child whose parasite sample was used in this study. The study methods were carried out in accordance with the approved guidelines.

Competing interests
No competing interest were disclosed

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Supplementary material
Table S1: List of P. falciparum proteins identified in PfEVs.
‘PRIEVs_9605_parasite_proteins’ contains the list of the proteins ordered in terms of abundance. RT stands for Ring to Trophozoite sample while TR stands for Trophozoite to rings sample. ‘GOterms_cellcomp_all’ contains GO term analysis based on cellular component. The –log p-value was generated from the Bonferroni adjusted p-value. Part of this analysis is shown graphically in Figure 2a. ‘Top50.GOterms_cellcomp’ is the GO terms enrichment analysis based on cellular components for the top 50 most abundant proteins. Part of this analysis is shown graphically in Figure 2b.
Click here to access the data.

Table S2: The combined PfEV proteins.
‘Combined list of PfEVs antigens’ are the total number of PfEVs antigens identified in both the Kenyan isolate and the previously published study by Mantel et al13 and some of their characteristics. ‘GOterms_cellcomp_PfEVs_core’ is GO-term enrichment analysis for cellular components of the PfEVs proteins common in both the Kenyan and the long-term laboratory isolates used in Mantel et al13. Part of this analysis is shown graphically in Figure 3b. ‘GOterms_cellcomp_9605specific’ is the GO-term enrichment analysis showing that the PfEV proteins specific to the Kenyan isolate. Part of this analysis is graphically shown in Figure 3c.
Click here to access the data.

References


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This paper extends the work of Mantel et al. 1 using an isolate of Plasmodium falciparum more recently derived from patient material. Microvesicles, or extracellular vesicles (EV) have previously been shown to activate immune cells and trigger gametocytogenesis, and a deeper understanding of the proteins displayed by them could support further hypotheses about their role in vivo. The data presented here increases the list of proteins identified and suggests that EV are derived from internal vesicular structures rather than the infected erythrocyte membrane.

Comments:

1. The term “clinically relevant” in the abstract is not supported by any clinical data about the infection the parasite line 9605 was derived from. Additionally, after 70 cycles of in vitro culture, while this isolate is closer to its patient origins than 3D7 it is not known how closely it still represents the original sample. This is acknowledged by the authors later in the paper and will require further work to define the changes that occur during culturing. For example, it may be possible that parasites reduce the release of EV or alter the spectrum of proteins released, but no data are provided to support this other than the single 9605 line.
2. What was the rationale to use “fractionated” in-gel digestion for (membrane-rich) EVs? Did the authors try alternative protocols that facilitate the identification of membrane-bound proteins such as FASP (filter-aided sample preparation)?
3. What was the proportion of “contaminants” (false hits) that were excluded from the analysis?
4. The potential role of EV in malaria infections is an exciting field but the data supporting their direct effect on endothelial activation, independent of cytoadherence, are limited. Of the references cited (#7, 10-12) two are from mouse studies, one does not identify whether EV were involved in the in vitro model system used and one shows EV activation of monocytes and neutrophils but not endothelium.
5. The finding of 57 out of 61 parasite proteins from the RT sample being in the TR sample might show some reproducibility but could also be considered surprising given the temporal regulation of expression seen in the erythrocytic cycle of P. falciparum. Does this suggest that proteins exported to the erythrocytic cytoplasm and vesicles within this compartment are retained for the whole cycle despite relatively stringent control of mRNA production?
6. Could the lack of discovery of PfEMP1 peptides be related to their absence from the 9605 genome sequence available for proteomic analysis? How well did the subtelomeric regions assemble for this isolate? The pool of this protein within the cell is thought to be significant and it has been associated with the Maurer’s Clefts, so its lack is interesting.

7. Some comment on the dominance of ribosomal proteins in the list of hits specifically identified in this study, compared to Mantel et al, would be useful. Is this a technical issue? How might this be interpreted in terms of the origin of EV?

8. The presence of virulence proteins in exosomes appears to be one of the major findings in this manuscript. Perhaps, the authors should detail the criteria used to include proteins under “virulence-associated parasite proteins”.

9. The point being made in the final paragraph of the discussion is not entirely clear. The display of internal parasite antigens by EV could explain exposure of these to the immune system, but this is also covered by the rupture of infected erythrocytes. What is the specific role of EV in this context? Some clarification would be useful. Along similar lines, what is meant in the conclusions about “fine-tuning the host immune response, preparing the host cell for invasion and acquiring nutrients from the environment”. Which parts of the dataset contribute to these suggestions?

10. In the supplementary material, it would be useful to have a column with the common names of proteins in Table S1, as shown in Table S2 under “product description”.

11. Have any of the proteins identified in the proteomic analysis been validated using an independent method, like Western blot or enzyme immunoassays?

Perhaps the major limitation of the study is that the proteomic description of EVs is limited to a single isolate and a single “experiment” / culture. It would have been desirable to see a similar analysis for few clinical isolates or, at least, in more than one culture from the same isolate, although this is partially mitigated through the comparison with previously published data. This would have been helpful to appreciate the experimental and biological variability. It is not uncommon for proteomics (or any –omics) studies to generate a number of interesting hits (proteins of interest) followed by hypotheses or plausible explanations about the biological phenomenon under study. The risks associated with this approach (sharpshooter fallacy) can be partially circumvented by proposing hypotheses that can be tested experimentally or by validating the mass spectrometry findings using alternative methods. What would be the single most important experiment or that would follow logically from the description provided in the manuscript?

References

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes
Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 17 August 2017

doi:10.21956/wellcomeopenres.12873.r24571

**Pawan Malhotra, Inderjeet Kaur**
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The study by Abdi et al illustrates the proteome of extracellular vesicles from the clinical isolates that have not passed through many passages. The study is important and provides additional information than previously published couple of studies describing the exosomes proteomes. I recommend indexing after major modifications. Some specific comments are as follows:

1. Authors should have used some known exosome markers to show the purity of vesicles. They could have carried-out western blot or immunofluorescence analysis using the specific exosome protein antibodies to prove this point.
2. Like-wise authors would have used similar techniques to confirm the presence of additional proteins that they found in their analysis.
3. It is not clear that how many biological replicates were used in the present study.
4. I also think a comparison between earlier studies and present study could have been represented by Venn diagram.
5. Authors could have used some markers to prove their hypothesis that EVs are not derived from infected erythrocyte membranes.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

**Are the conclusions drawn adequately supported by the results?**
Partly

*Competing Interests:* No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Referee Report 28 July 2017

doi:10.21956/wellcomeopenres.12873.r24252

**Matthias Marti**
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The study by Abdi et al. describes proteomic analysis of *P. falciparum*-derived extracellular vesicles from a clinical isolate. The presented data expand our knowledge of the protein content of these vesicles and provide a basis for diagnostic and functional studies.

The manuscript is well written, however the data analysis and presentation is lacking some detail that would be helpful for interpretation. Most importantly the authors conclude they have analyzed exosomes originating from apical organelles rather than EVs derived from the IE surface. This may be true, see also comments below, but then the title and description of data should be altered.

Some specific comments below:

- There is no systematic comparison between RT and TR proteome. It is assumed that the TR-specific proteome is enriched in egress material but that remains to be confirmed (see below).
- The TR sample includes egress and invasion events. Therefore materials released during this phase will be present in the sample. An egress proteome has been published\(^1\) and should be included in the comparative analysis.
- Note that recently another EV proteome was published that should also be included in this analysis\(^2\).
- In the comparison with the proteome data from Mantel et al the majority of additional proteins is ribosomal (Figure 3c). These are unlikely vesicle-derived but rather contaminants. Is there an explanation for this?
- The conclusion that the Kenyan strain contains a larger variety of EV proteins in comparison to 3D7/CS2 cannot be drawn as different populations of EVs (with this study including egress vesicles) were analysed.
- PHIST and Rifin proteins are detected in EVs but it is concluded EVs are not derived from the IE surface. This would be surprising since the previous analysis also included RBC proteins and detected a number of surface antigens including glycophorins. Was host proteome also investigated here?
- It is mentioned that many merozoite antigens are detected, mostly from rhoptries but also micronemes. This would again suggest egress vesicles (or what the authors call exosomes) rather than vesicles released during between invasion and egress.
The authors conclude that they may have analyzed exosomes rather than EVs, and that exosomes may form from apical organelles. The argument that EVs can only be exosomes is not conclusive. The authors argue that EVs cannot be derived from the erythrocyte despite identifying components of the erythrocyte membrane in their proteome (e.g. band 3, top right paragraph on page 5). Selective cargo loading of *P. falciparum* proteins might occur at the erythrocyte membrane and thus explain why only some RBC membrane localized parasite proteins are detected in EVs.

The authors conclude that EVs may derive from Maurer’s clefts as several Maurer’s clefts proteins were identified. In combination with their conclusion that PfEVs are only comprised of exosomes, do the authors suggest that Maurer’s clefts are multivesicular body like compartments? Are there any reports about vesicles inside Maurer’s clefts?

References

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
No

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.