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RESEARCH ARTICLE

REVISED **Proteomic analysis of extracellular vesicles from a**
Plasmodium falciparum **Kenyan clinical isolate defines a core parasite secretome** [version 2; peer review: 2 approved, 1 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 that had been adapted to *in vitro* culture for a relatively shorter 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 (*Pf*EVs) 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 *Pf*EVs may play a role in parasite-host interactions. Comparison of this dataset with previously published datasets helps to define a core secretome present in *Pf*EVs.
Conclusions: *P. falciparum* extracellular vesicles contain virulence-associated parasite proteins. Analysis of *Pf*EVs 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 new targets for interventions or diagnostics.

Keywords

Malaria, Plasmodium falciparum, extracellular vesicles, exosomes, proteomics

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REVISED **Amendments from Version 1**

The reviewers comments focused both on the methodology we employed and how the data was analysed. This revised version of the paper has been adjusted to consider the reviewer's comments, largely through modifying the text to add clarity to both the implications and limits of this new data. Here are the major modifications:

-Figure 3 (Figure 3d) was modified to include comparison of our PfEV data with the proteome of 1) schizont postrupture vesicles and 2) plasma microparticles from individuals with acute *P. falciparum* infections.

-Additional text describing the result shown in Figure 3d and Table S2 was included (Result section, last paragraph)

-The Conclusion section was modified to include the limitation of the study

-Additional texts were also included in response to specific comments from the reviewers to add clarity to our text. These additional texts have been clarified in our responses to the reviewer's specific comments.

-Table S1 was modified to include the product description of the proteins identified. Two extra columns were added to Table S2 to show the details of the data shown in Figure 3d.

See referee reports

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[1]. The pathogenesis mechanisms of severe malaria are not completely understood, but relevant factors include parasite burden[2], induction of host inflammatory responses[3], 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[4]. 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[5,6]. However, results from other studies suggest that endothelial activation and inflammation during malaria parasite infections can be independent of cytoadhesion of IEs[7–9], and effectors secreted by the parasite may play an important role[7,10–13].

One way secreted effectors are released from cells is through extracellular vesicles (EVs) that can be classified into two major types; exosomes and microvesicles[14,15]. Exosomes are vesicles of endocytic origin with diameter of 30–150 nm. They are generated through inward invagination of the limiting membrane of late endosomes leading to formation of intraluminal vesicles (ILVs)[16]. During the process of inward invagination of the endosomal membrane, many cytosolic proteins, RNA, and lipids are sorted into the ILVs[16]. Late endosomes containing multiple ILVs are called multivesicular bodies (MVBs) which when they merge with plasma membrane of the cell, release the ILVs as exosomes into the extracellular space[17]. By contrast, microvesicles are larger, with a diameter of 100–1000 nm, and are generated through outward invagination of the plasma membrane[18]. EVs 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[14,15,19,20]. The physiological significance of these vesicles is becoming increasingly appreciated in many disease processes, including cancer[15,21–23] and infectious diseases[12,24–28].

Increasing evidence suggests that EVs play an important role in intercellular communications[12,14,21,24,28–33]. In the context of cancer and infectious diseases, EVs can be used to sabotage the host defence mechanism[28,34–38], prepare the host cell for invasion[21,22,29,39,40] and acquire nutrients from the environment[41–44]. 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 (*Pf*EVs) were recently identified in two studies that used long-term adapted laboratory isolates, and proposed that *Pf*EVs play a role in cell-cell communications and gametocytogenesis[12,24]. As EVs represent an extended phenotype of the cell[15], unravelling the bioactive molecules in *Pf*EVs will contribute to our understanding of the biology and virulence mechanisms of *P. falciparum*. Analogy with other pathogens would suggest that *Pf*EVs 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 *Pf*EVs or pack a less extensive set of effectors into them, whereas more recently culture adapted *P. falciparum* clinical isolates might release *Pf*EVs containing a greater variety and depth of effectors. Here we present analysis of the protein content of *Pf*EVs 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 *P. falciparum* EV proteome datasets to define a core *Pf*EV content.

Materials and methods
Parasite culture
The Kenyan isolate (referred to as isolate 9605)[45] was obtained from a child admitted to Kilifi County Hospital with cerebral malaria in 2009. The isolate was adapted to *in vitro* culture and at the time of conducting this study it had been grown *in vitro* for a maximum of 70 cycles, as opposed to many years that most laboratory isolates such as 3D7 have been cultured for. The genome of this isolate was sequenced using Illumina at the Wellcome Trust Sanger Institute, Hinxton, Cambridge and the full genome assembled, (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[46].

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 before addition to *P. falciparum* culture. 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 then 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 culture-conditioned media was centrifuged one more 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 was filtered at 0.2µm and the flow-through frozen at -80°C until use.

Purification of *Pf*EVs by density gradient ultracentrifugation

To purify *Pf*EVs, the frozen supernatant generated above was thawed on ice and loaded on quick-seal ultracentrifugation tubes (Bechman 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[47–49] 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 13.5ml and centrifuged at 150, 000g for 2 hours and the pellet re-suspended in 400µl 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 using silver stain (Bio-Rad).

Proteomic analysis by mass spectrometry

Fractions confirmed to contain *Pf*EV 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% CH_3CN 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% CH_3CN and dried in

a SpeedVac (Thermo Fisher Scientific). The peptides were resuspended in 20µl 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 × 20 mm, 5µm) at 10µL/min for 15 min, then peptides were separated on a 75 µm id × 25 cm PepMap column (3 µm) at a flow rate of 300 nl/min over a 90 min linear gradient of 4–32% CH_3CN/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 1×10^6 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 both the *Plasmodium falciparum* 3D7 and 9605 protein databases, human protein database (from UniprotKB, October 2014, www.uniprot.org), and a contaminate database supplied by MaxQuant. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[50] 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 0.50 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 *Pf*EV proteome, gene ontology analysis for enrichment of cellular components was carried out using PlasmoDB (http://plasmodb.org/plasmo/showApplication.do).
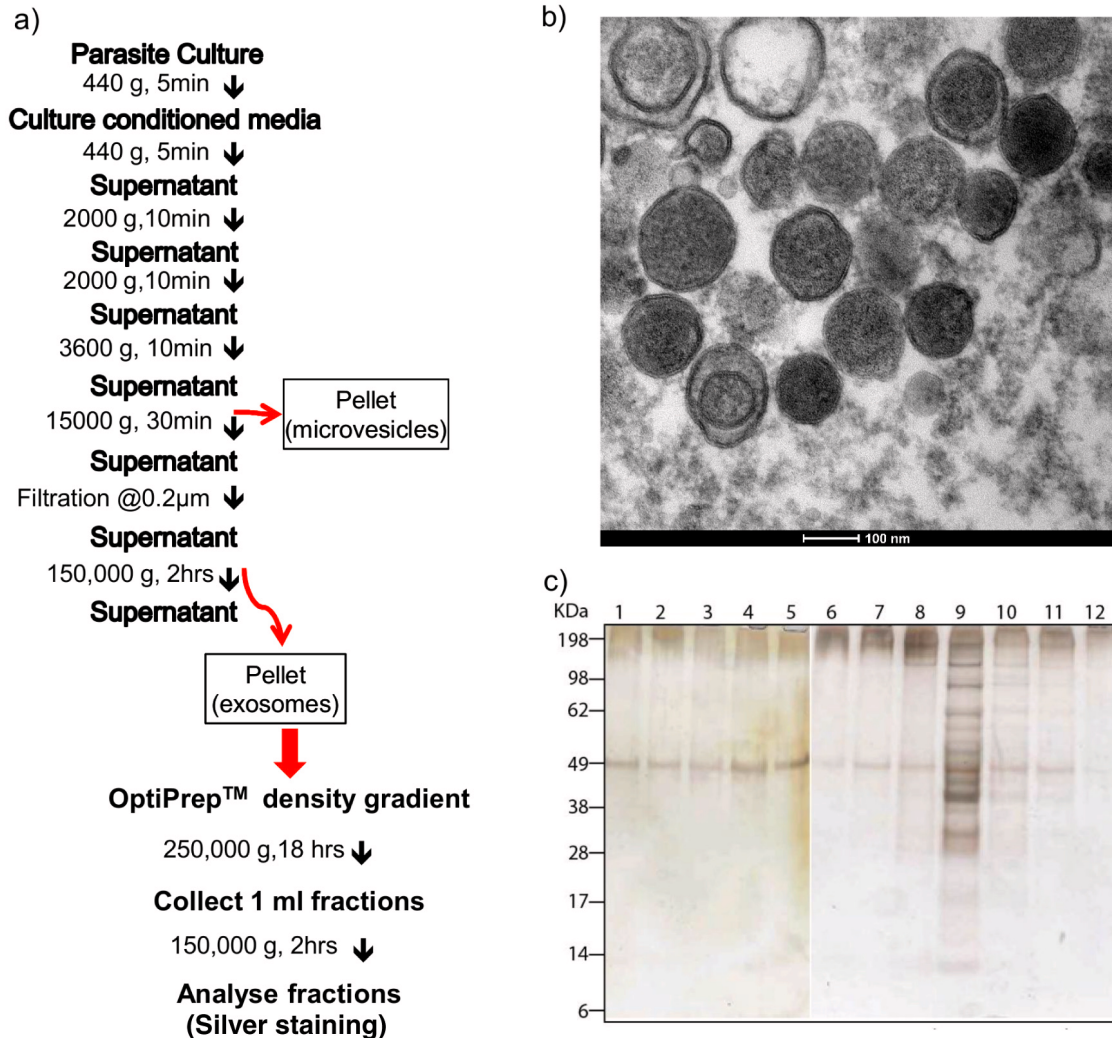
Figure 1. Isolation of PfEVs. A). Schematic showing the steps followed to purify *Pf*EVs. **B**). Transmission electron microscopy was used to confirm the presence of vesicles in the pellet. The sizes ranged between 27–411nm (median= 132 nm and mean±SD = 143nm≥66). 73% were below 150nm and 90% were below 200 nm. **C**). Protein extract from each of the density gradient fraction was run on SDS-PAGE gel and stained with silver. The common band is albumin and lanes 9, 10 and 11 corresponding to fractions 10,11 and 12 seem to contain *Pf*EV proteins. This gel only shows analysis of *Pf*EVs from TR time point. The original uncropped image for both RT and TR time points is available at https://osf.io/wdg96/[81].

Results

Extracellular vesicles were purified from culture conditioned media of a recently adapted *P. falciparum* isolate

We isolated *Pf*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. Examining the pellet by transmission electron microscopy revealed vesicles (Figure 1b) with median size of 132nm and mean±SD of 143nm±66. This primarily overlaps with the size range of exosomes (30–150nm), but also overlaps with the size range of microvesicles (100–1000nm) and it is likely that both are present. 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^3, consistent with the range described for exosomes (1.08–1.22 g/cm^3)[51].

Purified extracellular vesicles contained both host and parasite proteins

To identify the protein content in the isolated *Pf*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. 1194 protein groups were initially identified. Of these, 50 protein groups were potential contaminants (keratin, trypsin, etc), 25 were "Reverse Database" entry (False hits) and 557 were "Only identified by site". After exclusion of all these groups, 594 protein groups were left for analysis. Of the remaining 594 proteins, the majority of non-*P. falciparum* proteins were of serum/albumax origin such as complement proteins. Only few erythrocyte proteins such as haemoglobin (alpha and beta chains), band 3 anion transport protein, erythrocyte band 7 integral membrane protein, spectrin, glycophorin A and C were identified. 153 *P. falciparum* proteins were also identified (Table S1) which will be the subject of the subsequent analysis.

Virulence associated parasite proteins were significantly enriched in the *Pf*EV proteome

We identified 61 and 149 *P. falciparum* proteins in *Pf*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). The observed high overlap between the RT and TR *Pf*EV proteome may be partly due to the temporal overlap between the two samples at the ring and trophozoite stages, which include the most metabolically active stage where most protein export occurs.
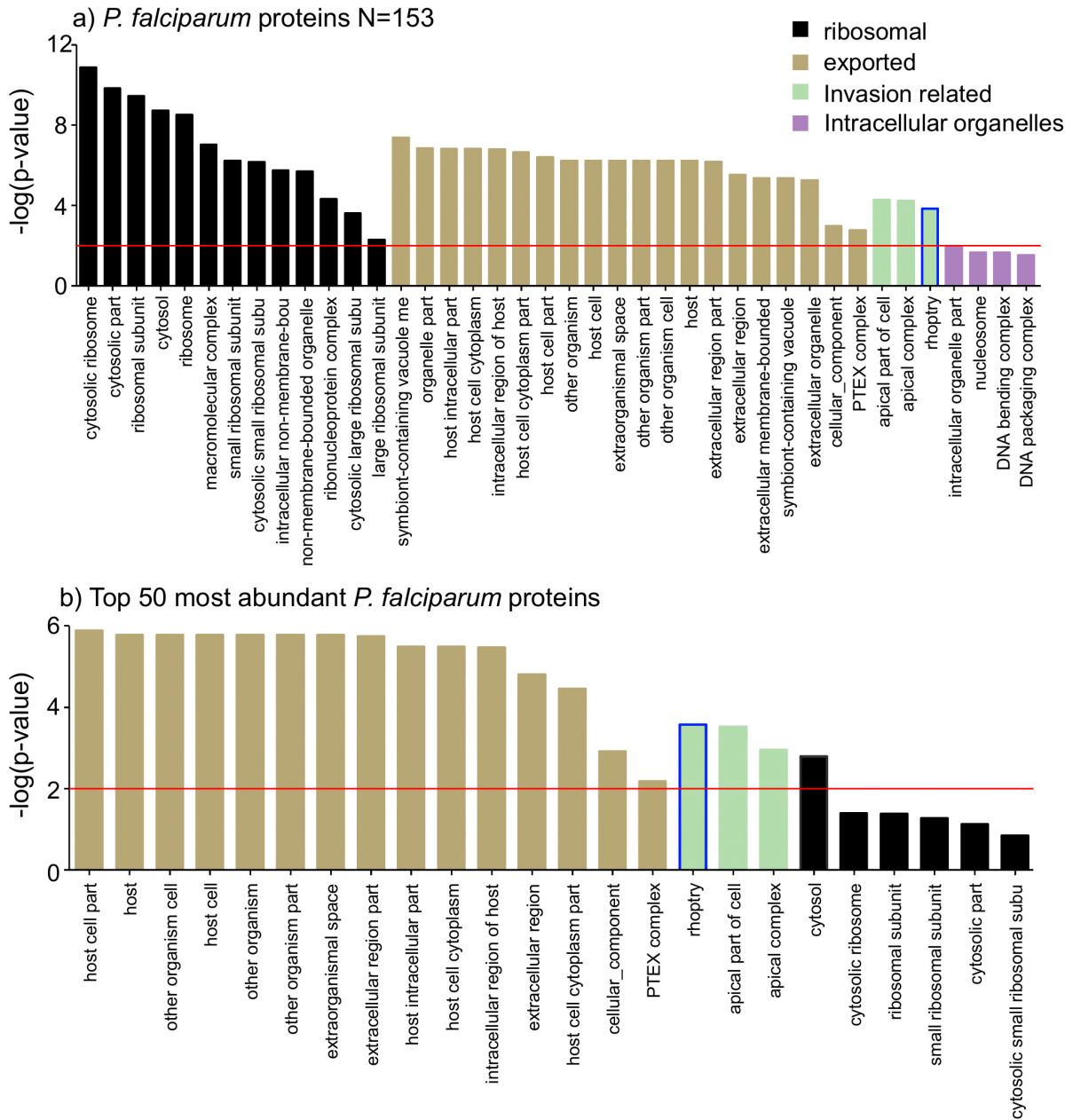
GO term enrichment analysis for cellular components revealed that terms related to proteins commonly found in EVs from other systems such as ribosomes[52,53] were also significantly enriched in *Pf*EVs (Figure 2a). In addition, terms related to virulence associated parasite specific proteins were significantly enriched (Figure 2a). Here we use the term "virulence associated proteins" to refer to 1) proteins involved in remodelling of the IE such as those exported to cytosol/surface of the IE and 2) proteins involved in invasion of the erythrocytes. These 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). By contrast, GO-terms associated with proteins of intracellular organelles such as nucleus, ER, and mitochondria were not significantly enriched in the *Pf*EV proteome (Figure 2a). Among the apically associated proteins, rhoptry associated proteins were particularly enriched (Figure 2a). *Pf*EVs from the RT sample were enriched for proteins found in exomembrane compartments of the IEs (Table S1), but proteins associated with invasion were absent, as expected given the early time point of this sample.

Parasite proteins exported to the infected erythrocytes and those secreted by the merozoite were the most abundant *Pf*EV proteins

When the list of *Pf*EV proteins was stratified by the number of unique peptides, the top 50 most abundant (≥4 unique peptides) were enriched for 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 MC-2TM), J-dots (HSP40 and HSP70-x), IE membrane (Glag3.1, RhopH3, RESA, KAHRP, *Pf*EMP3) and parasitophorous membrane (PTEX complex, ETRAMP family) were identified (Table S1). Interestingly while REX1/2, two proteins localised in the MCs[54] were detected in the *Pf*EV proteome, REX3, which localises in the IE cytosol[54] as a soluble protein, was not detected. Further, *Pf*HRP2 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 *Pf*EMP1, Rifins, Stevor, PHIST and FIKK are exported to or via these exomembrane compartments. Among these multigene protein families, PHIST and Rifin families were represented in *Pf*EVs (Table S1 and Table S2) but no *Pf*EMP1 and Stevor proteins were detected, consistent with previous finding by Mantel *et al.*[12]. As noted in the Methods, peptide data was searched against both the 3D7 and 9605 proteomes, so the absence of PfEMP1 peptides is likely not due to sequence variation in this highly polymorphic antigen. It should be noted that other proteins that localise at the knobs beneath the IE surface membrane, such as KAHRP and *Pf*EMP3, and proteins linked to transport of *Pf*EMP1 to the surface of the IE (PTP1, PTP6) were found[55]. These observations suggest that *Pf*EVs are selectively loaded, and do not simply contain a cross section of all exported proteins.

The second most abundant class of parasite proteins enriched in *Pf*EVs was merozoite antigens discharged from the secretory endomembranous compartments of the apical end (Figure 2b and Table S1). 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 region 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 (*Pf*Rh) 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[12]. Several dense granule proteins released during merozoite invasion were also among the most abundant *Pf*EV 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).

Figure 2. Virulence-associated parasite proteins are significantly enriched in *Pf* EV 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 proteins (apical complex). Note the rhoptry proteins (green bar, highlighted with blue line), especially those of the rhoptry bulb were significantly enriched. 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 significantly enriched.

Comparing PfEVs datasets to define a core PfEV proteome

The proteomic data of the Kenyan clinical isolate had substantial overlap with a previously published PfEV proteome[12] (Figure 3a). Between these two studies 184 PfEV proteins have now been identified (Figure 3a and Table S2). 53/84 proteins detected by Mantel et al.[12] were also found in our Kenyan isolate, while 100 proteins (54% of the 184) were unique to the Kenyan isolate (Figure 3a). GO term enrichment analysis based on cellular components revealed that virulence associated proteins, as defined above, and ribosomal proteins were both significantly enriched in the

PfEV proteins shared between the two studies, suggesting that these parasite proteins form the core PfEV proteome (Figure 3b). However, for the PfEV proteins identified only in the Kenyan isolate, ribosomal and virulence associated exomembrane proteins were significantly enriched, but invasion-related proteins were not (Figure 3c). Variation between isolates therefore seems to primarily occur in the ribosomal and exported proteins, although it is important to emphasize that technical variation between methodologies of the two studies could also contribute to these differences.
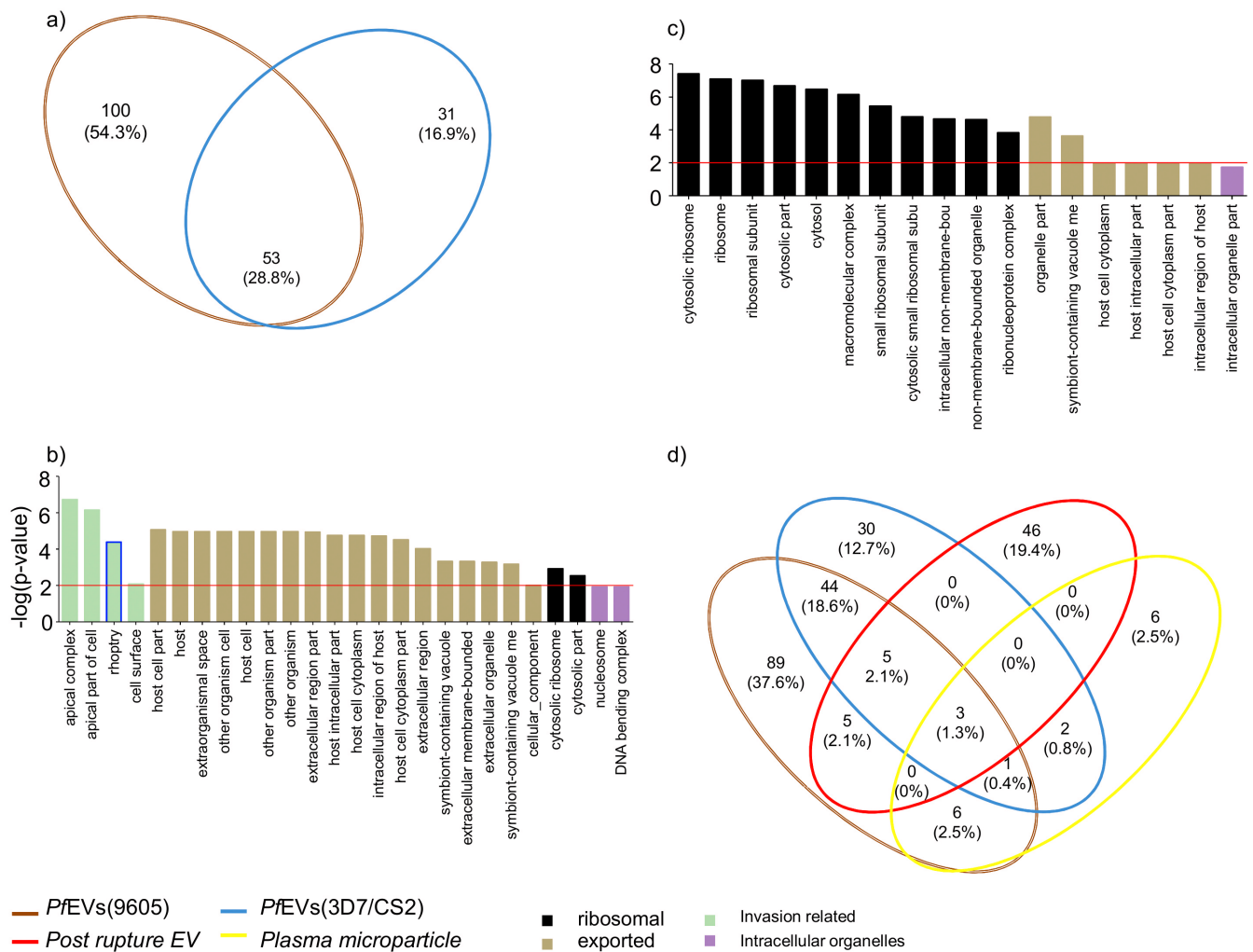
Figure 3. Exported and invasion related *P. falciparum* proteins form the core proteome of *Pf*EVs. A) Venn diagram showing that the proteome of *Pf*EVs from the Kenya isolate had extensive overlap with a previously published *Pf*EV proteome (Mantel *et al.*[12]). **B**) *Pf*EV core proteins: GO-terms enrichment analysis for cellular components of the *Pf*EV proteins common in both the Kenyan and the long-term laboratory isolates used in Mantel *et al.*[12]. Plotted is the –log p-value (Bonferroni adjusted for 53 comparisons) against the GO terms. **C**) 9605 specific *Pf*EVs proteins: GO-term enrichment analysis showing that the *Pf*EV proteins specific to the Kenyan isolate. Plotted is the –log p-value (Bonferroni adjusted for 46 comparisons) against the GO terms. **D**) Venn diagram showing the overlap between the *Pf*EV proteome identified in 1) this study (dark orange), 2) the previous *Pf*EV proteome (Mantel *et al.* Cell Host Microbe 2013) (blue), 3) Schizont post rupture vesicles (Millholland *et al.* MCP 2011) (red), 4) plasma microparticles from patients with acute *P. falciparum* infection (Antwi-Baffour *et al.* proteome Sci 2017) (yellow).

We further compared the *Pf*EV proteome of the Kenyan isolate with two other published proteomes from 1) EVs released during rupture *of P. falciparum* schizonts[56] and 2) plasma microparticles isolated from individuals with acute *P. falciparum* infection[57]. For the post rupture vesicles, we downloaded two files annotated to contain data for sample type "ruptured"[56]. In these two files, we identified 60 *P. falciparum* proteins (Table S2), of which 13 were also present in our *Pf*EV proteome (Figure 3d and Table S2). 8 of the 13 were also present in the *Pf*EV proteome described in Mantel *et al.*[12] (Figure 3d and Table S2). The shared proteins between our study and that of Millholland *et al.*[57] were not enriched in invasion-related antigens and only one shared protein, MSP1, was an invasion related antigen (Table S2). The microparticle proteome from *P. falciparum* infected individuals[57] included only 18 *P. falciparum* proteins, of which 10 were present in our *Pf*EV data (Figure 3d and Table S2). The shared proteins largely consisted of proteins commonly identified in EVs such as heat shock proteins, while the rhoptry proteins enriched in the *Pf*EV proteome of the Kenyan isolate were absent (Table S2). Therefore, while our data could potentially contain both microparticles or *P. falciparum* schizont post rupture vesicles, the majority of proteins identified in this study have not previously been identified in these two sample types.

Discussion

In this study, we characterised the protein content of EVs released by a Kenyan *P. falciparum* clinical isolate, 9605. The *Pf*EV purification protocol we used included a centrifugation step at 15000g for 30 min followed by a filtration at 0.2μm (Figure 1a) to exclude vesicles larger than 200 nm. This step likely led to exclusion of most of the vesicles that fall within what is described as microvesicles that originate from the plasma membrane, and may explain why known host erythrocyte surface antigens were not abundant in our proteomic analysis. However, while the median size of the *Pf*EVs falls within that described for exosomes, it does also overlap microvesicle size ranges, so the data could formally contain both vesicle types.

Proteomics identified 153 *P. falciparum* proteins, of which the most abundant were virulence associated proteins, specifically those involved in erythrocyte invasion and host cell remodelling. Previous proteomic analysis of *Pf*EVs from a long-term laboratory isolate identified 84 *P. falciparum* proteins[12], of which 53 were identified again in this study, showing a high degree of overlap. The finding of an additional 100 proteins in this study could be due to technical differences in sample acquisition, vesicle purification or mass spectrometry between the two studies. However, it is also well known that long-term laboratory *P. falciparum* isolates can down-regulate genes involved in pathogenesis *in vivo* 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[58] 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[59]. Therefore, it is possible that due to the relatively shorter time the Kenyan isolate

spent in *in vitro* culture conditions, 9605- *Pf*EVs proteome may represent a closer reflection of that released by the parasite under *in vivo* conditions. Caution is required due to the technical differences noted above, and the fact that while 9605 isolate is undoubtedly closer to a true clinical isolate than 3D7, it has still been cultured for up to 70 cycles. Analysis of further long and short-term laboratory-adapted isolates processed in exactly the same way will be required to formally test this hypothesis.

The biogenesis of *Pf*EVs is not known, but the proteomic analysis described here revealed several parasite proteins with potential role in biogenesis and transport of endosomal vesicles. These include Rab GTPases (*Pf*Rab7, *Pf*Rab6), and *P. falciparum* pyruvate kinase (PfPK) (Table S2), which in other systems are required for transport[60] as well as release of exosomes[61]. The apicomplexan Rab11A has been speculated to be involved in the transport of vesicles derived from endosome-like compartments[62]. *Pf*EVs also contain several cytosolic proteins commonly found in EVs such as LDH, GAPDH, ENO1, and pyruvate kinase (PK) which are thought to be sorted into ILVs during the formation of MVBs[16] suggesting similar mechanism may be involved in the biogenesis of *Pf*EVs.

One of the protein groups most significantly enriched in *Pf*EVs were virulence associated proteins exported to the exomembrane compartments of the IEs[63] (Figure 2), suggesting a proportion of the *Pf*EVs may have an origin in these vesicular compartments. Previous immunoprecipitation of an MC resident protein SEMP1[64], which is also among the most abundant *Pf*EV proteins (Table S1), co-precipitated 14 proteins of which 13 were identified here as being present in *Pf*EVs (Table S1/ S2), further reinforcing the link between *Pf*EVs and the exomembrane compartments within the IEs[63]. Functional analysis of the *P. falciparum* genes described here may therefore shed more light on the process of *Pf*EV biogenesis and release.

Another vesicular compartment closely linked to the contents of the *Pf*EVs is the secretory endomembrane compartments of the merozoite apical end, such as the dense granules and the rhoptry. Comparison of our data with vesicles released post rupture of the schizont showed only a small degree of overlap, so the abundance of these protein classes was unlikely due to the presence of post rupture vesicles among our *Pf*EVs. In particular, proteins from the rhoptry, specifically those from the rhoptry bulb region, were among the most dominant identified (Table S2), while these were absent from the previously published proteome of schizont post rupture vesicles. The rhoptry bulb has a honeycomb appearance by transmission microscopy, which has been postulated to be formed by internal membranes and/or vesicles[65], and it has been further hypothesised that the rhoptry might be the equivalent of multivesicular body (MVB) in higher eukaryotes[66]. 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[67], which could well be *Pf*EVs. Further, antibody targeting PTEX150 released from dense granules[68] precipitated

82 parasite proteins[69], of which 42 (51%) are present in *Pf*EVs (Table S2). The possibility that key components required for early establishment of the exomembrane compartments such as PTEX[70–72], RAP[73], and RhopH[67,74,75] complexes are in part secreted in vesicles will be important to confirm using immunoelectron microscopy.

Screens of large panel of *P. falciparum* proteins using plasma from malaria exposed individuals have identified multiple potential vaccine candidates[76–78]. The *Pf*EV proteome contains several of these antigens (Table S2). *Pf*EVs contain not only parasite proteins, but also TLR agonist such as parasite nucleic acids (Abdi *et al.* unpublished data) that might potentiate immune response[79]. In this context, immunisation of mice with *P. yoelii* EVs elicited immune response that provided protection against lethal infection[26,80] Further study of *Pf*EVs could therefore be of interest for vaccine development.

Conclusions
In summary, we have purified *Pf*EVs from a relatively short-term adapted Kenyan isolate. The physical characteristics of the *Pf*EVs primarily overlaps what is known for exosomes, but the sample may also contain microvesicles. Proteomic analysis broadened the previous list of *Pf*EV components, and suggests that a proportion of *Pf*EVs are closely linked to exomembrane and endomembrane compartments in the IEs and merozoites. A major limitation of the study is that a single *P. falciparum* isolate was used and the proteomic data was generated from a single experiment. As such while we can use the data presented in this study to generate hypotheses we cannot make strong conclusions. Therefore, an expanded study of *Pf*EV protein, RNA, lipid and metabolite content from a range of isolates, and functional validation of *Pf*EV components, biogenesis mechanisms, and their role in parasite-parasite and parasite-host interaction is clearly required.

Data availability
The raw mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[50] 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/[81].

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.

Supplementary material
Table S1: List of *P. falciparum* proteins identified in *Pf*EVs.
'*Pf*EVs_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 *Pf*EV proteins.
'Combined list of *Pf*EVs antigens' are the total number of *Pf*EVs antigens identified in both the Kenyan isolate and the previously published study by Mantel *et al.*[12] and some of their characteristics. 'GOterms_cellcomp_*Pf*EVs_core' is GO-terms enrichment analysis for cellular components of the *Pf*EVs proteins common in both the Kenyan and the long-term laboratory isolates used in Mantel *et al.*[12] Part of this analysis is shown graphically in Figure 3b. 'GOterms_cellcomp_9605specific' is the GO-term enrichment analysis showing that the *Pf*EV proteins specific to the Kenyan isolate. Part of this analysis is graphically shown in Figure 3c. Postrupture vesicles (Millholland *et al.* MCP 2011) are the *P. falciparum* proteins identified in the supplementary files named rbc_z01_to_z34.csv and rbc_01_to_10.csv annotated to contain data for the sample type "ruptured", and plasma microparticles represent proteome of plasma microparticles identified in the study by Antwi-Baffour *et al.* proteome Sci 2017.

Click here to access the data.

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Version 2

Reviewer Report 05 January 2018

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We accept the manuscript with reservation as authors have replied positively to most of our queries. However, since proteome analysis has been performed on limited samples, we think that some of the experiment(s) that we suggested are doable as author(s) could have raised some antibodies for published exosome markers and performed the co-localization studies/western studies.

Competing Interests: No competing interests were disclosed.

We confirm that we have read this submission and 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.

Reviewer Report 08 December 2017

https://doi.org/10.21956/wellcomeopenres.14296.r28264

✓ Matthias Marti (iD)
Wellcome Trust Center for Molecular Parasitology, University of Glasgow, Glasgow, UK

The revised version of this MS has been significantly improved and addressed most concerns. In particular additional comparison with previously published studies have been added. My only remaining comment is that there is still no clear statement as to what the different vesicle populations might be.
Given the sampling regime and their different composition, I would assume that they are a mixture of iRBC-derived microvesicles and materials released during egress invasion ("exosomes"). It would be helpful if this could be clarified in the discussion.

Competing Interests: No competing interests were disclosed.

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

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Version 1

Reviewer Report 01 September 2017

✔   **Alister G. Craig** (iD)

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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

1. Mantel PY, Hoang AN, Goldowitz I, Potashnikova D, et al.: Malaria-infected erythrocyte-derived microvesicles mediate cellular communication within the parasite population and with the host immune system.*Cell Host Microbe*. 2013; **13** (5): 521-34 PubMed Abstract | Publisher Full Text

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

Reader Comment 08 Nov 2017

Abdirahman Abdi, Kemri-Wellcome Trust Research programme, Kenya

This paper extends the work of Mantel et al 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 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 lines.

Response: We hypothesize that more recently adapted *P. falciparum* isolates might contain a broader repertoire of EV components than long-term adapted isolates such as 3D7. However, we completely agree that the data in this paper does not prove or disprove this hypothesis - while 3D7, from which previous data has been obtained, has been in culture in different labs around the world for decades, the Kenyan isolate we used has been cultured for about 70 cycles, so is still some distance from a truly clinical isolate. We have deleted the term "clinically relevant" from the abstract as suggested. We have also modified the Discussion to make it clear the limits of interpretation of this data and the need for further work with a broader range of *P. falciparum* strains. The reviewer's suggestion to track changes in quantity or content of *Pf*EVs released by an isolate over multiple cycles in *in vitro* culture is an excellent one, and one that we plan to pursue in future studies.
New text added:
Caution is required due to the technical differences between studies noted above, and the fact that while 9605 isolate is undoubtedly closer to a true clinical isolate than 3D7, it has still been cultured for up to 70 cycles. Analysis of further long and short-term laboratory-adapted isolates processed in exactly the same way will be required to formally test this hypothesis.

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)?

Response:The rationale for using fractionation was to increase the breadth of the proteins we could detect by mass spectrometry; this was also the approach used by previously published proteomic work on *P. falciparum* EVs (Mantel *et al* Cell Host & Microbe 2013, PMID: 23684304), making our data more directly comparable to this previous data. As this field expands we totally agree that additional approaches need to be tested including FASP which is an excellent suggestion.

3. What was the proportion of "contaminants" (false hits) that were excluded from the analysis?

Response: New text added:

1194 protein groups were initially identified. Of these, 50 protein groups were potential contaminants (keratin, trypsin, etc), 25 were "Reverse Database" entry (False hits) and 557 were "Only identified by site". After exclusion of all these groups, 594 protein groups were left for analysis. Of the remaining 594 proteins, the majority of non-*P. falciparum* proteins were of serum/albumax origin such as complement proteins. Only few erythrocyte proteins such as haemoglobin (alpha and beta chains), band 3 anion transport protein, erythrocyte band 7 integral membrane protein, spectrin, glycophorin A and C were identified. 153 *P. falciparum* proteins were also identified (Table S1) which will be the subject of the subsequent 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 model system used and one shows EV activation of monocytes and neutrophils but not endothelium.

Response: We certainly agree that these are very early days for the field, and functional studies are needed to investigate more directly the phenotypic impact of *Pf*EVs. We have added an additional recent reference that links *Pf*EVs with endothelial activation *in vitro (Mantel et al Natcomm 2016,* PMID:27721445*).*

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 .   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?

Response: There is some temporal overlap between the two different samples collected - one came from media added at the ring stage and harvested at the trophzoite stage (RT sample), while the second comes from fresh media added at the trophozoite stage and harvested in the following cycle at the ring stage (TR). The two samples (RT and TR) therefore both contain trophzoite stage parasites which are the most metabolically active stage in the *P. falciparum* life cycle, where a large amount of protein export occurs. It is therefore reasonable to expect that *Pf*EVs produced just before the RT time point would be quite similar to those produced at the beginning of the TR time point, resulting in considerable overlap. In fact, the only stage of the parasite life cycle that we can say the two samples do not overlap at all is during egress and merozoite invasion, and as this would predict, invasion-related proteins were detected only in the TR sample. In addition, while the reviewer is absolutely right that transcription in *P. falciparum* is tightly controlled, the proteins produced will have a very large range of half-lives. The overlap in samples is therefore not completely unexpected, but we completely agree a tighter time course analysis is necessary to define the proteins packaged in *Pf*EVs over the various stages of the asexual cycle.
New text added:
The observed high overlap between the RT and TR *Pf*EV proteome may be partly due to the temporal overlap between the two samples at the ring and trophozoite stages, which include the most metabolically active stage where most protein export occurs.

6. The lack of discovery of PfEMP1 peptides be related to their absence from the 9605genome 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.

Response: Assembled full genome sequence of isolate 9605 is available including the subtelomeric region. As noted in the Methods, and now also emphasised in the Results, we used both the 9605 and 3D7 repertoires in our peptide searches, so we don't think the lack of PfEMP1 in our proteome is due to this issue. Our findings are also consistent with that of Mantel et al 2013 which was done on the reference isolate 3D7 and also lacked PfEMP1

peptides. We agree with the reviewer that this is an interesting finding.
New text added:
Method section;
The genome of this isolate was sequenced using Illumina at the Wellcome Trust Sanger Institute, Hinxton, Cambridge and the full genome assembled.
Result section;
As noted in the Methods, peptide data was searched against both the 3D7 and 9605 proteomes, so the absence of PfEMP1 peptides is likely not due to sequence variation in this highly polymorphic antigen. It should be noted that other proteins that localise at the knobs beneath the IE surface membrane, such as KAHRP and *Pf*EMP3, and proteins linked to transport of *Pf*EMP1 to the surface of the IE (PTP1, PTP6) were found [51] . These observations suggest that *Pf*EVs are selectively loaded, and do not simply contain a cross section of all exported proteins.

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?*

Response: EVs, specifically exosomes, are generated through inward budding of endosomal membranes forming intraluminal vesicle (ILV) within multivesicular bodies (MVB) (reviewed in   Abels, ER 2016, PMID:27053351). During this process, cytosolic molecules such as RNA and proteins including glycolytic enzymes are sorted into the ILVs which are eventually released to the extracellular environment as exosomes when the MVB merges with the plasma membrane. Ribosomal proteins are commonly identified in EVs proteome, including that of *Plasmodium* (Mantel et al, PMID:23684304 Martin-Jauler, L 2016, PMID:27900319, Bosque, A et al PMID:27086912, Dozio, V et al PMID:28473883and this study). It is worth noting here that some of the *Plasmodium* ribosomal proteins identified in EVs such as PfP2 (Pf3D7_0309600) (Table S2) have been previously shown to be exported to the surface of the infected erythrocyte within a narrow time window during the schizont stage (Das, S et al PlosPath 2012, PMID:22912579).
However, while this suggests that there might be a biological basis for identification of ribosomal proteins in *Pf*EVs, we cannot rule out the possibility that they were co-purified with the EVs as contaminants during the ultracentrifugation process. Given that our *Pf*EV proteome has greater depth than the previously published version, an increase in ribosomal hits is not unexpected irrespective of the process in which the ribosomal proteins end up in EVs. We have now included text in the result section to clarify this.

New text added:
Introduction paragraph 2;

They are generated through inward invagination of the limiting membrane of late endosomes leading to formation of intraluminal vesicles (ILVs)[2]. During the process of inward invagination of the endosomal membrane, many cytosolic proteins, RNA, and lipids are sorted into the ILVs[2]. Late endosomes containing multiple ILVs are called multivesicular bodies (MVBs) which when they merge with plasma membrane of the cell, release exosomes into the extracellular space

Result section:
However, ribosomal and virulence associated exomembrane proteins were significantly enriched in the Kenyan *Pf*EV proteome, but invasion-related proteins were not (Figure 3c). Variation between isolates therefore seems to primarily occur in the ribosomal and exported proteins, although it is important to note that technical variation between methodologies of the two studies could also contribute to these differences.

Discussion section paragraph 4.
*Pf*EVs also contain several cytosolic proteins commonly found in EVs such as LDH, GAPDH, ENO1, and pyruvate kinase (PK) which are thought to be sorted into ILVs during the formation of MVBs [2] suggesting similar mechanism may be involved in the biogenesis of *Pf* EVs.

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".*

Response: Parasite exported to the surface of the infected erythrocytes that mediate cytoadhesion to the endothelial walls and those exported to the cytosol of the infected erythrocyte that have a role in modifying the physical properties of the infected are known to be critical for parasite virulence. We therefore defined parasite proteins predicted to be involved in remodelling of the infected erythrocytes as virulence associated. This includes members of the multigene families, rifins, stevors, PfEMP1 and PHIST, and those involved in export process itself such as the translocon machinery. In addition, we also considered proteins involved in erythrocyte invasion to be virulence-associated as they are essential for parasite multiplication rates. We have inserted a sentence clarifying these definitions into the Result section.
Text added:
Result section;
Here we use the term "virulence associated proteins" to refer to 1) proteins involved in remodeling of the IE such as those exported to cytosol/surface of the IE and 2) proteins involved in invasion of the erythrocytes.

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?*

Response: We completely agree with the reviewer that many parasite proteins are likely to be released during bursting of the infected erythrocytes ad could be potentially exposed to the immune system as a result. However, proteins packaged in EVs might elicit better immune response as in other systems EVs are known to contain not only proteins but also TLR agonists such as nucleic acids, which can potentiate immune response (Kasturi, S. P Nature 2011, PMID:21350488).
We have modified the paragraph on this accordingly. As suggested, we have also deleted

the sentence including the phrase "fine-tuning the host immune response, preparing the host cell for invasion and acquiring nutrients from the environment", which we agree are perhaps too speculative at this stage. This has been replaced with a sentence on the limitations of the study.

Text added:

Discussion section last paragraph

*Pf*EVs contain not only parasite proteins, but also TLR agonist such as parasite nucleic acids (Abdi et al unpublished data) that might potentiate the immune response (Kasturi, S. P Nature 2011, PMID:21350488).

Conclusions:

A major limitation of the study is that a single *P. falciparum* isolate was used and the proteomic data was generated from a single experiment. As such while we can use the data presented in this study to generate hypotheses we cannot make strong conclusions. Therefore, an expanded study of *Pf*EV protein, RNA, lipid and metabolite content from a range of isolates, and functional validation of *Pf*EV components, biogenesis mechanisms, and their role in parasite-parasite and parasite-host interaction is clearly required.

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".*

Response: This is an excellent suggestion, and we have now included the gene names as recommended.

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

Response: We agree that immunoblots or other assays would be useful validation tools, but unfortunately there are no antibodies available for the vast majority of the proteins identified in the *Pf*EVs, limiting the possibility for such study. We used the extensive overlap between the *Pf*EVs proteins identified in the previous study and our study as primary validation, and are in the process of raising antibodies to other targets to provide additional secondary validation.

12. *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 –) 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?*

Response: We completely agree that data from additional samples will be extremely useful, and are in the process of generating this data. However, those datasets are likely to take

> some time to acquire, and in keeping with the ethos of Wellcome Open, we thought it useful to publish this initial dataset as rapidly and openly as possible to maximize utility for other researchers.
> We believe the single most logical experiment that follow the data described in this manuscript is to validate the content of PfEVs using multiple isolates ranging from long term laboratory isolate, short term culture adapted clinical isolates and *ex vivo* clinical isolates which we are in the process of doing it.
>
> ***Competing Interests:*** These are authors response to reviewers comments.

Reviewer Report 17 August 2017

https://doi.org/10.21956/wellcomeopenres.12873.r24571

? **Pawan Malhotra**
Malaria Biology Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, Delhi , India
Inderjeet Kaur
Malaria Biology Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, Delhi, India

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 confirm that we have read this submission and 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.

Reader Comment 08 Nov 2017

Abdirahman Abdi, Kemri-Wellcome Trust Research programme, Kenya

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__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.

Response: Unfortunately, the typical exosomal markers such as tetraspanins are not conserved in *P. falciparum* and therefore commercial antibodies to exosome markers cannot be used to judge the purity of *P. falciparum* extracellular vesicles. We did identify in the PfEV proteome homologs of proteins such as GAPDH, ENO1, HSP90, PKM, LDH, PGK that are commonly found in EVs in other cell systems.

__2__. Like-wise authors would have used similar techniques to confirm the presence of additional proteins that they found in their analysis.

Response: We agree that this would have been the ideal, but as noted in response to reviewer1, antibodies were not available to do additional validation. We are in the process of generating those antibodies, and plan to use them in future studies.

__3__. It is not clear that how many biological replicates were used in the present study.

Response: Although we have carried out the isolation of PfEVs from more than 20 biological replicates and obtained consistent results in terms of their densities, the proteomic analysis was only performed on one sample for each of the two-time points (RT and TR). We appreciate this is a limitation and we have clarified it in the Conclusion section of the revised manuscript.

Added text:
A major limitation of the study is that a single *P. falciparum* isolate was used and the proteomic data was generated from a single experiment. As such while we can use the data presented in this study to generate hypotheses we cannot make strong conclusions. Therefore, an expanded study of *Pf*EV protein, RNA, lipid and metabolite content from a range of isolates, and functional validation of *Pf*EV components, biogenesis mechanisms, and their role in parasite-parasite and parasite-host interaction is clearly required.

4. *I also think a comparison between earlier studies and present study could have been represented by Venn diagram.*

Response: A Venn diagram was included as Figure 3a, and has been expanded in this revised version (Figure 3d).

5. *Authors could have used some markers to prove their hypothesis that EVs are not derived from infected erythrocyte membranes.*

Response: It was not our intention to suggest that EVs are not derived from infected erythrocyte surface membranes, only that some of the standard infected erythrocyte markers such as PfEMP1 are lacking. We have modified any sentence that suggested that "EVs are not derived from the infected erythrocyte surface membrane" to make this clearer.

Competing Interests: Comments shown here are authors reply to the reviewer's comments

Reviewer Report 28 July 2017

https://doi.org/10.21956/wellcomeopenres.12873.r24252

? **Matthias Marti** (iD)

Wellcome Trust Center for Molecular Parasitology, University of Glasgow, Glasgow, UK

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
1. Millholland MG, Chandramohanadas R, Pizzarro A, Wehr A, et al.: The malaria parasite progressively dismantles the host erythrocyte cytoskeleton for efficient egress.*Mol Cell Proteomics*. 2011; **10** (12): M111.010678 PubMed Abstract | Publisher Full Text
2. Antwi-Baffour S, Adjei JK, Agyemang-Yeboah F, Annani-Akollor M, et al.: Proteomic analysis of

microparticles isolated from malaria positive blood samples.*Proteome Sci*. 2016; **15**: 5 PubMed Abstract | Publisher Full Text

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Yes

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Yes

If applicable, is the statistical analysis and its interpretation appropriate?
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Are all the source data underlying the results available to ensure full reproducibility?
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Are the conclusions drawn adequately supported by the results?
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Reader Comment 08 Nov 2017

Abdirahman Abdi, Kemri-Wellcome Trust Research programme, Kenya

General comment: 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:

Response: It was certainly not our intention to suggest that the EVs analysed in this study can only be exosomes. We mentioned the term exosomes in our Results and Discussion sections only in reference to the sizes and densities of the vesicles we have isolated. In response, we have gone through the text to make this distinction much clearer, and now use PfEVs in place of exosomes in all appropriate places. For example, in the Background section last paragraph, we replaced the term *P. falciparum* exosomes with PfEVs.
Also in the Methods under subtitle, preparation of culture conditioned media, we deleted the statement "presumed to predominantly contain exosomes"
It was also not our intention to be definitive about the source of PfEVs. We simply noted our observations that proteins commonly thought to localize membranous compartments

within the infected erythrocytes such as Maurer's clefts and merozoite's apical organelles are enriched in our *Pf*EV proteome. This led us to suggest the possibility of some of the *Pf* EVs being derived from these compartments.

We have adjusted the text to make this clearer.

"Examining the pellet by transmission electron microscopy revealed vesicles (Figure 1b) with median size of 132nm and mean±SD of 143nm±66. This primarily overlaps with the size range of exosomes (30-150nm), but also overlaps with the size range of microvesicles (100-1000nm) and it is likely that both are present"

Specific comments
1. *There is no systematic comparison between RT and TR proteome.*

Response: We did compare the RT and TR proteomes and made comments to that effect in the text. We clarified this in paragraph 2 of the Result section and Table S1. "Of the 61 proteins identified in the RT samples, 57 were also present in the TR sample".

2. *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 and should be included in the comparative analysis.*

Response:We agree with the reviewer that the TR sample may contain material released during egress and invasion events.

We looked at the paper that the reviewer suggested (Millholland, MG et al MCP 2011) and we identified 60 *P. falciparum* proteins in two files in the supplementary (rbc_z01_to_z34.csv and rbc_01_to_10.csv) labelled to contain data for the sample type "ruptured". Of these, 13 were in our 9605 *Pf*EV proteome (Figure 3d and Table S2): four ribosomal, 2 histones, MSP1, MAHRP2, actin-1, ETRAMP5, Hsp70-1, EXP2, and fructose-biphosphate aldolase. 8 of these 13 were also in the proteome of the vesicles described in Mantel et al Cell Host Microbe 2013 (Figure 3d and Table S2). We now discuss this analysis in the Results section and have added new display items, Figure 3d and Table S2, to show the comparisons. Overall, the *P. falciparum* post-rupture vesicles were not enriched in invasion-related antigens. Of the 13 proteins that were in the overlap between our study and that of Millholland, MG et al MCP 2011, only one, MSP1, is an invasion related antigen. We added a paragraph describing this analysis to the Result section.

New text:

We further compared the *Pf*EV proteome of the Kenyan isolate with two other published proteomes from 1) EVs released during rupture *of P. falciparum* schizonts   and 2) plasma microparticles isolated from individuals with acute *P. falciparum* infection. For the post rupture vesicles, we downloaded two files annotated to contain data for sample type "ruptured". In these two files, we identified 60 *P. falciparum* proteins (Table S2), of which 13 were also present in our *Pf*EV proteome (Figure 3d and Table S2). 8 of the 13 were also present in the *Pf*EV proteome described in Mantel *et al*[12] (Figure 3d and Table S2). The shared proteins between our study and that of Millholland *et al* were not enriched in invasion-related antigens and only one shared protein, MSP1, was an invasion related

antigen (Table S2). The microparticle proteome from *P. falciparum* infected individuals included only 18 *P. falciparum* proteins, of which 10 were present in our *Pf*EV data (Figure 3d and Table S2). The shared proteins largely consisted of proteins commonly identified in EVs such as heat shock proteins, while the rhoptry proteins enriched in the *Pf*EV proteome of the Kenyan isolate were absent (Table S2). Therefore, while our data could potentially contain both microparticles or *P. falciparum* schizont post rupture vesicles, the majority of proteins identified in this study have not previously been identified in these two sample types.

Note that recently another EV proteome was published that should also be included in this analysis.

Response*:* We thank the reviewer for this reference. We don't believe that this is a direct comparator to our work, as it focussed on plasma microparticles (microvesicles) which was pelleted down by centrifuging at 19000g. In our analysis, the bulk of microvesicles would have been either pelleted down at an early step using a 15000g spin or excluded in the filtration step (as illustrated in Fig.1a). Nevertheless, as suggested we compared our list with this dataset and found 10 of the 18 *P. falciparum* genes identified and shown in Table 3 of Antwi-Baffour et al Proteome Sci 2017 paper. As with the comparison with egress material, we now show the result of this analysis in Figure 3b and Table S2 and discuss it in the last paragraph of the Result section. Most of the 10 shared antigens belonged to those commonly present in EVs such as heat shock proteins, actin, tubulin, enolase and elongation factor-1alpha (Table S2). The rhoptry antigens which were particularly enriched in the *Pf*EVs described in our study were absent.
New text describing this comparison is shown above.

3*. In the comparison with the proteome data from Mantel the majority of additional proteins are ribosomal (Figure 3c). These are unlikely vesicle-derived but rather contaminants. Is there an explanation for this?*

Response: Ribosomal proteins are commonly identified in EV datasets including that of Mantel *et al* Cell Host Microbe 2013 (Table S2). Given that our *Pf*EV proteome has greater depth, an increase in ribosomal hits is not surprising, although whether these are contaminants, or true components of EVs cannot be determined. However, as shown in Table S2, only 19 of the 100 PfEV proteins that we identified as specific to the Kenyan isolate are ribosomal proteins. So, while the proportion of ribosomal proteins in the additional *Pf*EV proteins identified in the Kenyan isolate is high compared to their proportion in the proteome of *P. falciparum,* the majority of additional proteins identified in this study were not ribosomal proteins.

added text
However, ribosomal and virulence associated exomembrane proteins were significantly enriched in the Kenyan *Pf*EV proteome, but invasion-related proteins were not (Figure 3c). Variation between isolates therefore seems to primarily occur in the ribosomal and exported proteins, although it is important to note that technical variation between methodologies of the two studies could also contribute to these differences.

4. *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.*

Response: It is certainly true that there were subtle but potentially important methodological differences between the two studies. In our study, we included an additional two steps in the processing of the culture conditioned media - a centrifugation step at 15000g and a filtration step at 0.2um (Figure 1a) before taking the culture media through ultracentrifugation and density gradient to purify the PfEVs. We agree with the reviewer that as a result strict comparisons are not possible. It was not our intention to overstate the implications of a deeper proteome being found in this study, which the reviewer is right could be due to these technical differences. We have therefore modified the text accordingly (Discussion section paragraph one and two). However, it is our understanding based on Fig.S1A of the Mantel *et al* manuscript that media from mixed culture was used to generate the PfEV proteome in that study, meaning that it is quite possible that it also included some material released during egress. Indeed, Fig 3C and Table S1 of that study clearly identify proteins found in the micronemes and rhoptries (EBA181, RhopH3, RhopH2 etc) that are among those that the reviewer suggests could be released during egress (comment #6 below). We therefore think that the methodological differences may not be that stark, but there is no question that a systematic comparison of multiple strains prepared in exactly the same way is needed before any definitive statements can be made. The text has been modified accordingly.
Added text:
The finding of an additional 100 proteins in this study could be due to technical differences in sample acquisition, vesicle purification or mass spectrometry between the two studies.

5. *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?*

Response: Host erythrocyte derived proteins were detected in the *Pf*EVs although not many, and are described in the text. It was certainly not our intention to imply that PfEVs are not derived from the IE surface, instead we simply wanted to note that some IE surface proteins such as PfEMP1 appear to be absent from them in both this study and Mantel et al. We have modified our interpretation of the absence of PfEMP1 in the proteome of PfEVs to read that "*Pf*EVs are selectively loaded, and do not simply contain a cross section of all exported parasite proteins".

6. *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.*

Response: As noted in our reply to comment #2 above, it is certainly true that egress vesicles could be present in our dataset, although there was not a substantial overlap between our PfEV proteome dataset and that of a previous publication of schizont post

rupture vesicles (Millholland et al MCP 2011). Specifically, the shared proteins did not include the well-known rhoptry and microneme proteins (Figure 3d and Table S2). The text has been modified accordingly (Result section, last paragraph).

7: 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 proteins might occur at the erythrocyte membrane and thus explain why only some RBC membrane localized parasite proteins are detected in EVs.

Response: It was certainly not our intention to suggest that the EVs we analysed can only be exosomes. We mentioned the term exosomes in our Result and Discussion sections only in reference to the sizes and densities of the vesicles we have isolated. As illustrated in Figure 1a, we aimed to purify vesicles with sizes less than 200 nm but we understand the method we used to isolate the vesicles, ultracentrifugation, is non-specific and the sizes of the exosomes and microvesicles overlap. That is why we used the term *P. falciparum* extracellular vesicles to describe the vesicles we purified to include both exosomes and microvesicles. We have gone through the text to make this distinction much clearer, and now use PfEVs in place of exosomes in all appropriate places. We also agree with the reviewer that selective packaging of host protein into PfEVs might occur, and now note this in the text "*Pf*EVs are selectively loaded, and do not simply contain a cross section of all exported proteins".

8. 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?

Response: We observed that several proteins commonly thought to localize to the Maurer's Clefts and other membranous compartments within the infected erythrocytes are enriched in our *Pf*EV proteome. This led us to suggest the possibility of some of the *Pf*EVs being derived from these compartments. Similar suggestions were made by Mantel et al in Cell Host Microbe 2013 (discussion section, paragraph 2 "interestingly, within the small fraction of parasite proteins identified in RMVs, proteins resident in Maurer's clefts are enriched. ………this suggest that RMVs are formed from Maurer's clefts structures and iRBCs subregions that excludes knobs". As to the question of whether Maurer's clefts are in fact MVBs, this is a fascinating suggestion, but not one that we can directly address with our data.

Competing Interests: These are authors reply to the reviewer's comments