A Comprehensive Gender-related Secretome of *Plasmodium berghei* Sexual Stages

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**In Brief**
By a differential quantitative proteomic approach, we identified proteins released by *Plasmodium* gametocytes from a transgenic line defective in male gametogenesis and its parental line, when activated to form gametes. Gender specificity of gametocyte egress-secretome was defined by a comparative analysis of proteins quantified in the two lines. Candidate proteins selected for validation confirmed the predicted gender-specificity and showed that these secreted proteins localize to different types of vesicles, suggesting that emergence from the host cell involves a tightly coordinated secretory apparatus.

**Highlights**
- Quantitative analysis of *Plasmodium* sexual stage egress secretome.
- Activated gametocytes release gender-related proteins.
- Gametocyte egress process involves different types of vesicles.

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A Comprehensive Gender-related Secretome of Plasmodium berghei Sexual Stages

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Plasmodium, the malaria parasite, undergoes a complex life cycle alternating between a vertebrate host and a mosquito vector of the genus Anopheles. In red blood cells of the vertebrate host, Plasmodium multiplies asexually or differentiates into gamete precursors, the male and female gametocytes, responsible for parasite transmission. Sexual stage maturation occurs in the midgut of the mosquito vector, where male and female gametes egress from the host erythrocytes to fuse and form a zygote. Gamete egress entails the successive rupture of two membranes surrounding the parasite, the parasitophorous vacuole membrane and the erythrocyte plasma membrane. In this study, we used the rodent model parasite Plasmodium berghei to design a label-free quantitative proteomic approach aimed at identifying gender-related proteins differentially released/secreted by purified mature gametocytes when activated to form gametes. We compared the abundance of molecules secreted by wild type gametocytes of both genders with that of a transgenic line defective in male gamete maturation and egress. This enabled us to provide a comprehensive data set of egress-related molecules and their gender specificity. Using specific antibodies, we validated eleven candidate molecules, predicted as either gender-specific or common to both male and female gametocytes. All of them localize to punctuate, vesicle-like structures that relocate to cell periphery upon activation, but only three of them localize to the gametocyte-specific secretory vesicles named osmiophilic bodies. Our results confirm that the egress process involves a tightly coordinated secretory apparatus that includes different types of vesicles and may put the basis for functional studies aimed at designing novel transmission-blocking molecules.

Malaria currently remains one of the most deadly infectious diseases, despite a significant reduction in global morbidity and mortality because of improved control strategies. In 2018, WHO World Malaria Report estimated 228 million cases and 405,000 deaths. Children under five accounted for 67% of all malaria deaths. Most of the lethal events are caused by Plasmodium falciparum in sub-Saharan Africa.

Plasmodium parasite undergoes a complex, multi-stage life cycle involving a vertebrate host and a mosquito vector. Inside a red blood cell (RBC) of the vertebrate host, Plasmodium either multiplies asexually or differentiates into gamete precursors, the male and female gametocytes, responsible for the transmission of the disease.

An effective control toward the final goal of malaria eradication, must face the major challenge of the highly efficient spread of the disease. Because sexual stages are rarely detected by microscopy, it was long assumed that only a small fraction of malaria infected individuals were capable of infecting mosquitoes. It has now been demonstrated that gametocytes are instead highly prevalent in infected individuals (1). Moreover, mosquitoes can become infected also by biting asymptomatic individuals with low gametocyte densities. Transmission blocking interventions are then an essential aspect in malaria control (2). They may either target gametocytes in the human host or gametes and the subsequent vector stages in the mosquito.

Following uptake of circulating gametocytes, environmental cues in the mosquito midgut, such as a drop in temperature and the presence of xanthurenic acid, trigger gametogenesis (3). Important molecular and cellular changes quickly occur in activated gametocytes (4). Each female gametocyte forms a single macrogamete, whereas male gametocytes undergo three nuclear divisions, producing eight motile microgametocytes. For mating to occur, gametes must egress from the host RBC. This process takes place by successive inside-out rupture of two membranes surrounding blood stage parasites, the parasitophorous vacuole membrane (PVM) and RBC plasma membrane (4–6).

A few minutes after gametocyte activation, female-specific secretory organelles, the osmiophilic bodies (OBs), migrate...
to the parasite periphery and release their content in the parasitophorous vacuole (PV) lumen with concomitant rupture of the PVM. Male-specific OBs, also referred to as MOBs, have been characterized in *P. berghei* as small, club-shaped vesicles, morphologically distinct from female OBs. Upon activation, these vesicles cluster together to form larger structures that release their content in a few focal points (7).

Several OB-resident molecules have been functionally characterized and several were shown to be involved in gamete egress from the host cell (8). Gametogenesis in general and egress from the host erythrocyte (from now on defined as egress secretome) are tightly coordinated processes, central for transmission to the mosquito vector (9, 10). Molecules/pathways involved in these crucial steps of the parasite life cycle may represent novel promising targets for effective transmission-blocking interventions (11).

Here, we applied a quantitative proteomic approach to compare proteins differentially released or secreted by activated gametocytes of *Plasmodium berghei* wild type (wt) and a mutant deleted for the *actin II* gene, *actII(-)*, defective in male gametogenesis and egress. Gametocytes were activated *in vitro* by a drop in temperature and the addition of xanthurenic acid (XA). Proteins released by either male or female gametocytes were identified by comparative label-free quantitative proteomics. 

### EXPERIMENTAL PROCEDURES

**Experimental Design and Statistical Rationale**—Aim of this study is to identify gender-related gametocyte proteins released or actively secreted by *P. berghei* gametes during the egress from the host erythrocyte (from now on defined as egress secretome). By a combined approach of label-free quantitative proteomics and bioinformatics, we compared the abundance values of wt egress secretome with those of an *actinII* null mutant, blocked in male gamete maturation and egress but not in female gametogenesis, referred to as *actII(-)* (12) (Fig. 1).

As a control, we also compared the egress secretome of a second mutant, knocked out for the female-specific, OB-resident protein G377. Mutant parasites, *g377(-)*, are delayed in female gamete egress (7), whereas male gametogenesis is not affected. Being the egress of *g377(-)* females delayed but not abrogated, we expect only slight differences between this mutant and the wt. Nevertheless, the *g377(-)* line represents a valuable control, as it was produced with the same methodology of the *actII(-)* and both transgenic parasites were subjected to drug selection for comparable amounts of time.

Because of the use of an *in vivo* model and the complex procedure to recover highly enriched gametocytes, competent to undergo gametogenesis, proteomic experiments were performed only in two independent biological replicates (supplemental Tables S1 and S2). Nevertheless, we generated robust datasets for quantitative analysis, by applying stringent filtering conditions on MS data and specifically designed statistical analysis, described below.

**Construction of Proteomic Datasets**—Only parasite proteins identified with a minimum of two unique peptides in both replicates were included for further analyses. Protein abundance values were determined by label-free Top3 method (13) and the overall reproducibility between replicates ($R > 0.85; p < 0.05$) was evaluated using the Pearson’s correlation coefficient.
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Statistical Analysis—To further assess robustness of our quantitative datasets, we defined the variation range of abundance values between biological replicates (r1 and r2) of wt, actII(-) and g377(-) by calculating the ratio of the Top3 values of each identified protein in each biological replicate (r1/r2 and r2/r1). We then verified that the three datasets followed a similar distribution (Kolmogorov-Smirnov test, in XLSTAT2020.1.1) (Fig. 2A and 2B).

In order to assess variability of abundance values between replicates and measure the dispersion of data, we constructed two reference sets by combining either wt (r1/r2-r2/r1) with actII(-)(r1/r2-r2/r1), named reference A or wt (r1/r2-r2/r1) with g377(-) (r1/r2-r2/r1), named reference B (supplemental Table S3—Comparative analysis of Top3 ratios). Grubbs’ test was then used to filter them and exclude outlier values from successive analyses (p < 0.05 in XLSTAT2020.1.1). These reference distributions enabled us to define a threshold value when the distribution of the wt/actII(-) or wt/g377(-) mean Top3 values are compared (Fig. 2C).

As the actII(-) secretome is mainly composed of proteins released by activated female gametocytes, we then expect the wt/actII(-) abundance ratio value distribution to significantly differ from that of the reference data set A, mainly in the highest extreme ratio values that are predicted as proteins preferentially or specifically released by activated male gametocytes. We then considered as male-related candidates those proteins falling outside the third quartile (Q3) plus 1.5*IQR (interquartile range) of the wt/actII(-) distribution. At difference, when the wt/g377(-) distribution is considered, we expect that the abundance ratio values of these selected candidates fall within the distribution range (Fig. 2C).

This proteomic approach was further validated by analyzing expression profile and subcellular localization of 11 proteins, components of the egress secretome using specific antibodies raised under this study.

Experimental Animals—All animal experiments under this study were performed using female C57BL/6 mice (Charles River Wilmington, Massachusetts), according to D.Lgs 26/2014, which has transposed in Italy the European Directive 2010/63/EU on the protection of animals used for scientific purposes, the animal research protocols have been reviewed and approved by the Animal Welfare Body of the Istituto Superiore di Sanità (Italian National Institute of Health) and authorized by the Ministry of Health (authorization number 150/2016-PR of 19th February 2016).

Plasmodium berghei Synchronous Infections and Gamocyte Purification—Synchronous infections of the wt, the actII(-) and g377(-) parasitic lines were established in mice by intravenous injection of infective schizonts (14). Parasitemia, gametocytemia and sex ratio were carefully evaluated by observation at the optical microscope of Giemsa-stained blood smears. When gametocytes of the second synchronous infection cycle were fully mature, around 30 h post invasion (hpi), blood was collected by heart-puncture under anaesthesia and immediately diluted in G-buffer (1 × PBS, 20 mM glucose, 25 mM Heps pH 7.3, 5 mM sodium bicarbonate) that reversibly arrests P. berghei gametogenesis. Leukocytes were removed by using Plasmodipur filters (EuroProxima Arnhem, The Netherlands).

Schizont- or gametocyte-infected erythrocytes were separated from uninfected cells through Nycodenz density gradient centrifugation (14). In brief, infected blood was diluted 1:50 and loaded onto a 52% Nycodenz cushion in G-buffer and centrifuged 20 min at 300 × g. Gametocyte- or schizont-infected RBCs float at the interface, whereas uninfected or ring-infected RBCs are recovered in the pellet. Interface was sampled and gametocyte purity was evaluated by microscopic inspection (>95% in proteomic experiments). Nycodenz-purified gametocytes from mutants or wt were activated in vitro to form gametes in RPMI pH 8.0 supplemented with 50-μM xanthurenic acid and kept at 20 °C for 20 min, as previously described (15). Each proteomic experiment was performed on 2 × 10^7 wt and actII(-) and 10^7 g377(-) gametocytes. Female to male ratios, determined for wt, actII(-) and g377(-) were (1:1, 2:1 and 1:1 respectively).

Antibodies—Nonactivated and activated male gametocytes were immunolocalized by using, respectively, rabbit antibodies against the nuclear protein SET (16), diluted 1:100 and the monoclonal mouse anti-Tubulin antibody (Sigma-Aldrich St. Louis, Missouri) diluted 1:400. Nonactivated and activated females were identified by using rabbit antibodies against the OB-resident PbG377 (17), diluted 1:100. Anti-PbSU1, kindly provided by Mike Blackman, was used at a 1:100 dilution.

16 proteins identified in this study as released upon gametocyte activation were selected to raise specific mouse antibodies. Selection of candidates was based on their abundance ratio in wt/actII(-) data set. Relevant coding fragments were cloned in BamHI-NotI sites of PGEX6P-1 and expressed in E. coli as GST fusions. Amplified DNA regions and primers used for amplification, are detailed in supplemental Methods. Immune sera were obtained in BALB/c mice via intraperitoneal injections of purified recombinant polypeptides expressed in E.coli (50 μg in Freund’s complete adjuvant) followed by two additional injections (25 μg in Freund’s incomplete adjuvant) at 2-week intervals. Mice were bled 1 week after the third immunization.

Western Blot Analysis—Western blot analysis was performed at constant voltage (100 V) for 1 h, in transfer buffer (20% methanol, Tris 25 mM, Glycine 192 mM) using MINI TRANS-BLOT® Biorad Hercules, California apparatus. Primary (1:1000 dilution) and horseradish peroxidase-conjugated secondary (1:10000 dilution) antibodies were incubated in PBS-Tween (0.05%) +1% nonfat milk for 1 h. Membrane was developed using the ECL system (SuperSignalWest Pico, Thermo Fisher Waltham, Massachusetts) according to manufacturer’s instructions.

Indirect Immunofluorescence Assay (IFA)—Blood smears were fixed for 1 h in 4% paraformaldehyde at room temperature (RT). After washing in PBS, fixed erythrocytes were permeabilized with 0.1% Triton X-100 in PBS (10 min at RT), washed again and then blocked in PBS/3% BSA for 1 h. Slides were incubated 1h in primary antibody, washed in PBS and incubated 30 min in fluorescein- or rodamin-conjugated α-mouse or α-rabbit secondary antibodies (1:400 dilution). In the case of α-MDV1 mouse immune serum, the IgG were purified (protein G-agarose, Roche Italia) and directly conjugated with FITC according with manufacturer’s protocols (Zenon labeling kit, Thermo Fisher Scientific). Cell nuclei were stained with 4’,6-diamidino-2-phenilindolo (DAPI). Immune sera specificity was verified using pre-immune sera. Fluorescence pattern of 50-100 gametocyte-infected RBCs were inspected microscopically. Representative images were presented in relevant Figures.

Sample Preparation for Proteomic Analysis—2 × 10^7 Gametocytes were diluted in RPMI medium pH 8.0 supplemented with 50 μM Xanthurenic acid (XA) and kept 20 min at 20 °C to activate gametogenesis. Culture supernatant was acetone-precipitated at −80 °C overnight and subsequently recovered by centrifugation. Protein bands were separated by SDS-PAGE on 4–12% precast minigels (NuPAGE Bis-Tris; Invitrogen Carlsbad, California) and stained with NuPAGE Colloidal Coomassie (Invitrogen, Thermo Fisher Scientific). Gel lanes were cut into 24 slices and in-gel tryptic digestion was performed as already described (18). Briefly, gel slices were destained by washings in acetonitrile (ACN)/50 mM NH4CO3 (1:1), treated with 10 mM DTT (40 min at 56 °C) and 55 mM iodoacetamide (30 min in the dark at RT) to reduce and alkylate cysteines, shrunk with ACN and rehydrated for 40 min on ice with a solution of 12.5 ng/μl trypsin (Promega) in 50 mM NH4CO3. Trypsin solution was, then, replaced by 25 mM NH4CO3 and protein digestion carried out overnight at 37 °C. Supernatants were directly used for LC-MS/MS analysis.

Liquid Chromatography Tandem Mass Spectrometry—Nano-RPLC was performed using a nano-HPLC 3000 Ultimate (Thermo
Abundance ratio distributions of wt/actII(-) and wt/g377(-).

A. Top3 ratios of wt (light blue), actII(-) (pink) and g377(-) (green) follow the same log-normal distribution according with the Kolmogorov-Smirnov test (p > 0.05). The common median equal to 1 is indicated as a dotted line.

B. Cumulative distribution of Top3 ratio values. Dotted lines indicate the percentage of values falling within one standard deviation from the median (95.8% for wt; 91.2% for actII(-) and 85.3% for g377(-)).

C. Box plot representation of Top3 ratio value distributions of wt/actII(-) and wt/g377(-) and reference sets A (wt and actII(-) Top3 ratios) and B (wt and g377(-) Top3 ratios). Median value of wt/actII(-) is twice that of reference set A, being male proteome under-represented in actII(-) egress secretome. Red arrowhead indicates the maximum value of 6.1, at the boundary of the highest quartile of wt/actII(-) distribution, whereas red dots represent abundance ratio values external to the whisker identifying male-related candidates. Abundance ratio values of these selected candidates fall within the distribution range when wt/g377(-) or the reference B are considered.
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Fisher Dionex) connected in line to LTQ-XL linear ion trap (Thermo Fisher). Peptides derived from trypsin digestion were firstly loaded on a C18 RP-precolumn (300 μm i.d. × 5 mm; 5 μm particle size; LC Packings-Dionex, Thermo Fisher Scientific), washed by the loading pump at 20 μL/min with buffer A (5% ACN, 0.1% FA) for 5 min and then loaded on a homemade 12 cm × 75 μm-i.d. Silica Picotip (8 ± 1 μm) column (Picotip Emitter, New Objectives Littleton, MA) packed with Magic C18AQ (5 μm particle size; 200 Å pore size, Michrom Bioresources Auburn, California) for chromatographic separations. Peptides were eluted at 0.3 μL/min along a 60 min linear gradient from 15% to 60% of buffer B (95% ACN, 0.1% FA) and electrosprayed directly into the mass spectrometer with a spray voltage of 1.60–1.65 kV and a capillary temperature of 180°C.

The five most intense ions were sequentially selected and fragmented in CID mode: maximum injection time of 100 ms; m/z 50-2000 mass range; minimum signal threshold of 200 counts; isolation width of 2; normalized collision energy of 35. Wideband and multistage activation were enabled and dynamic exclusion allowed a repeat count of 2 within 30 s and exclusion time of 60 s.

Mass Spectrometry Data Processing and Analysis—Spectra files (available at ftp://massive.ucsd.edu/MSV000085650) were analyzed by Sequest HT search engine with Proteome Discoverer 1.4 (Thermo Fisher) running against a local database constructed merging proteomes of Mus musculus from Uniprot reviewed (Swiss-Prot release January 2020) and Pbergheria/ANKA from PlasmoDB, (release 46, November 2019) resulting in a total number of 21955 sequences and also against the decoy database. The Carboamidomethylation of cysteines was specified as fixed modification and the oxidation of methionine was set as variable modification; mass tolerance was set to 1 Da for precursor ion and 0.4 Da for fragment ions and a maximum of two missed cleavages was allowed. The Percolator tool was used for peptide validation based on the q-value and high confidence was chosen, corresponding to a false discovery rate (FDR) <1% on peptide-level. Proteins were identified with a minimum of 1 unique peptide and at least 2 first ranked peptides. Protein and peptide identification data are reported in supplemental Tables S1 and S2. Top3 abundance values were determined considering the three (or two) most abundant unique peptides for each protein. Top3 values of biological replicates were normalized on the replicate with the highest abundance (calculated as the sum of Top 3 values assigned to each identified protein). Top3 mean values of mutant lines were also normalized on wt taking into account the number of gametocytes used in each proteomic experiment (in the case of g377(-)) or the sex ratio (in the case of actII(-)).

RESULTS

Proteomic Analysis of Gender-Related Proteins Released during P. berghei Gamete Egress from the Host RBC—To define gender-related factors released during gametogenesis, we relied on a transgenic parasite line defective in male gamete maturation and egress, actII(-). As a control, we used the g377(-) mutant line, delayed in female egress (see Experimental Design and Statistical Rationale).

Synchronous infections of the wt and mutant parasites were obtained in mice by intravenous injection of purified fully mature infective schizonts. The conversion rate to gametocytes and the sex ratio were evaluated by visual inspection of Giemsa-stained blood smears.

Highly enriched (>95%) mature gametocytes were recovered from peripheral blood of mice infected with synchronous P. berghei parasites, 30 hpi, when young asexual stages (rings) and mature gametocytes are the major parasite populations circulating. Isolation of gametocytes was performed exploiting their distinct ability to float in Nycodenz gradient centrifugation, compared with uninfected and ring-infected RBCs.

Purified mature gametocytes from the wt and mutant parasites were induced to undergo gametogenesis in vitro. Proteins released in the same volume of gamete induction medium were precipitated and identified by MS in two independent biological replicates (supplemental Tables S1 and S2). Proteomic datasets included parasite proteins detected with a minimum of two unique peptides in both replicates (263 for the wt, 266 for the actII(-) and 129 for the g377(-) (see supplemental Fig. S1A). To assess consistency of quantitative data between each biological replicate, we calculated the ratio between each protein Top3 value in each biological replicate (r1/r2) and the reciprocal ratio (r2/r1). We then analyzed the distribution of these ratios in the three parasite lines. As shown in Fig. 2A, the three datasets follow the same distribution (Log-normal, Kolmogorov-Smirnov test, p < 0.05), with median equal to 1 and with more than 85% of the protein abundance values falling within 1 standard deviation of the median (Fig. 2B). This result indicates that most of the proteins have reproducible abundance values. In order to define a “threshold of significance” of experimental data, we constructed two reference distributions: the first one, reference A, contains wt and actII(-) Top3 ratios, whereas the second one, reference B, wt and g377(-). Proteins with outlier ratio values were excluded from successive analyses (see Experimental Design and Statistical Rationale). The final filtered datasets consisted of 254 wt, 255 actII(-) and 128 g377(-) proteins (supplemental Fig. S1B). 86.7% of proteins identified in the g377(-) secretome overlaps the wt data set. This is consistent with the fact that the egress from the host cell of g377(-) female gametocyte is only delayed but not abrogated.

Analysis of wt and actII(-) Egress Secretomes—187 proteins are in common between the wt and the actII(-), accounting for 73% of the two secretomes (supplemental Fig. S1B), but their relative abundance largely differ in the two parasite lines. They include molecules involved in various cellular processes, such as protein folding and transport, signaling, proteolytic activity and sexual stage development (supplemental Fig. S2A). We also detected known molecules implicated in the egress from the host erythrocyte such as the female-specific, OB-resident protein G377, MDV1 and GEST, expressed in both genders and localized to OBs (supplemental Table S3). Released proteomes also include proteins specifically detected in the wt or in the actII(-) parasites, 67 and 68 respectively, (supplemental Table S3). Analysis of the gene ontology (GO) assigned functions of the identified proteins revealed that 40% of the actII(-) and 15% of the wt unique proteins are involved in protein translation. (supplemental Fig.
S2B). This may be explained by mechanical rupture of a small fraction of the cells during manipulations. The higher percentage observed in the actII(-) sample is likely because of an increased membrane fragility of this mutant. For this reason, we expect that abundant, male-specific proteins may be also detected at a low level in mutant parasites.

As expected, the male-specific Actin II was detected only in the wt sample. In addition, 18 proteins, uniquely detected in the wt secretome, had been previously identified as more abundant in male gametocytes in the proteome of separate male and female gametocytes of *P. berghei* (19). These include proteins involved in the egress such as the merozoite TRAP-like protein (PBANKA_0512800) (20, 21) as well as proteins implicated in DNA replication, a process activated during male gametogenesis. These findings strongly support robustness of our proteomic approach, aimed to enrich for gender-specific molecules.

**Released Gametocyte Proteome Includes Gender-Specific Proteins**—In order to characterize proteins differentially released by activated male and female gametocytes, we decided to focus further analyses on the protein subset common to wt and actII-null gametocytes. To compare relative abundance of these two protein populations, we ranked them based on the wt/actII(-) Top3 ratio values (supplemental Table S3). As described above, the highest ratio values are expected for proteins specifically or preferentially secreted by male gametocytes. Based on the adopted experimental approach, proteins with abundance ratio values higher than 6.1 i.e. outside the third quartile (Q3) plus 1.5*IQR, (Fig. 2C) were considered as factors mainly secreted by male gametocytes. This value is well beyond the highest Top3 ratio value observed in the reference distribution A (see also Experimental Design and Statistical Rationale).

Consistently, the highest ranked proteins were two proteases, the *P. berghei* subtilisin-like protease 1 (SUB1) (22) and the *P. yoelii* aspartyl protease named microgamete surface protein (PyMiGS) (23), both shown to be involved in the egress of male gametocytes and localized to MOBs. A ratio value slightly below the threshold was assigned to Perforin II (PPLP2), implicated in the egress of male gametes (24). However, we cannot exclude a low level of expression in female gametocytes of *P. berghei*, where the specific transcript has been identified by RNA-Seq (PlasmoDB database). Further, in *P. falciparum* PPLP2 is expressed in gametocytes of both genders.

As a control, we compared mean Top3 values of the wt with that of a second parasite null mutant for the female-specific, OB-resident protein G377 (7). The lack of G377 causes a delay in the egress of female gametes, whereas male gametogenesis is not affected (7). As expected, the abundance ratio values of male-related candidates, previously selected, fall within the wt/g377(-) and the reference B distribution range (Fig. 2C).

To further validate the proposed egress secretome, we expressed peptides derived from 16 candidate proteins selected from the common data set (supplemental Table S4—Expressed Rel proteins) and predicted to be either differentially or equally released by male and female gametocytes. The peptides were selected based on the high similarity level to the *P. falciparum* orthologous proteins. Fifteen proteins, successfully expressed, were used to immunize Balb/C mice. Immune sera were first tested by Western blot on gametocyte preparations to verify their specificity. Eleven out of 15 immune sera recognized protein bands of the expected size (supplemental Fig. S3) and were then selected for further investigation.

Gender-related expression of the selected molecules was analyzed by specific antibodies in double IFA on fixed wt gametocytes. Gender specificity was determined using an immune serum against the nuclear protein SET, highly abundant in male gametocytes. A, proteins specifically expressed in male gametocytes; B, proteins specifically expressed in female gametocytes; C, proteins expressed in both genders. Nuclei are stained with DAPI. BF, bright field. Scale bar indicates 5 μM.

**FIG. 3.** Gender-specific expression of candidate proteins selected from gametocyte secretome. Expression profile of selected molecules was analyzed by specific antibodies in double IFA on fixed wt gametocytes. Gender specificity was determined using an immune serum against the nuclear protein SET, highly abundant in male gametocytes. A, proteins specifically expressed in male gametocytes; B, proteins specifically expressed in female gametocytes; C, proteins expressed in both genders. Nuclei are stained with DAPI. BF, bright field. Scale bar indicates 5 μM.
values around 1.5 were detected only in female gametocytes (Fig. 3B). The secreted ookinete proteins (Rel 2 and Rel 16), the subtilisin-like protease 2 (SUB2) (Rel 13), the MA3 domain-containing protein (Rel 5) and a conserved protein of unknown function (Rel 14) with ratios comprises between 5.5 and 1.65 were detected both in male and female gametocytes (Fig. 3C). This large range of Top3 ratio values may be explained by the fact that the secretion level of proteins common to both female and male gametocytes may largely differ between sexes. It is noteworthy that the SUB1 substrate SERA3, was also one of the highest ranked proteins (ratio value of 6.9). Even though expressed in both genders, this protein was previously reported to have a peripheral localization in males and a dotted pattern in the parasite cytoplasm in females (19). Here we show that the highest wt/actII(-) ratio of SERA3 may be explained by a different behavior of the protein upon gametocyte activation (see below). Overall, immune-localization results of the selected proteins are well in agreement with the prediction based on wt/actII(-) Top3 ratio and further support the robustness of the methodology applied.

P. berghei Gametocytes Release Different Classes of Vesicles—Most of the candidate proteins analyzed by IFA exhibit a punctuate vesicle-like pattern. This is not surprising considering that the released proteome is expected to be enriched for secreted proteins. To evaluate subcellular localization of the selected proteins, we performed double IFA with immune sera raised against two OB markers: G377, expressed only in female gametocytes, and MDV1 protein, found expressed in both genders.

Surprisingly, only three out of eleven proteins analyzed in this study localize to OBs (Fig. 4). As in the case of MDV1, the conserved protein of unknown function (PBANKA_0810800, Rel 14 immune serum) and the secreted ookinete protein (PBANKA_0619200, Rel 16 immune serum) were detected in both genders, whereas P. berghei microgamete surface protein (PbMiGS) (PBANKA_1449000, Rel 1 immune serum) was specifically localized to MOBs, as previously shown in P. yoelii (23).

The MiGS and PbSUB1, among the highest ranked proteins secreted upon gametocyte activation, are the only proteins, characterized so far, exclusively localized to MOBs. We
Previously demonstrated that PbSUB1 co-localizes with MDV1 in male gametocytes of *P. berghei* (22). By double-label immune localization, here we show that the two MOB-specific markers co-localize, both in nonactivated and activated male gametocytes (Fig. 5), suggesting a homogeneous composition of MOB proteome.

Interestingly, in this study, we show that the gametocyte egress-related secretome include proteins localized to vesicle-like structures, distinct from the OBs and MOBs. The extent of heterogeneity of vesicle population could not be measured as all the immune sera generated were raised in mice and direct labeling of the IgG gave faint fluorescence signals not suited for double IFA.

Rel Proteins Respond to Gametogenesis Stimuli—Early after gametocyte induction, OBs and MOBs relocate to the cell periphery to discharge their protein content into the PV with the concomitant disruption of the PVM. We asked whether these uncharacterized vesicles respond to stimuli that induce gametogenesis. To answer this question, we explored subcellular localization of candidate proteins in gametocytes activated for 5–8 min. IFA were performed using G377 as a marker of female gametocytes and tubulin as a marker of male gametocytes.

As shown in Fig. 6, upon gametocyte activation, candidate proteins relocalize to cell periphery, thus suggesting that they may participate to functions related to early stage of gamete differentiation and/or to the exit from the host RBC. An exception to this behavior is represented by the Sub1 substrate SERA3. This protein is secreted by activated male gametocytes, while remaining inside the cytoplasm of activated female gametocytes, consistent with Top3 ratio values described above.

In conclusion, double-label colocalization experiments validate the egress-related secretome and indicate that it contains gender-specific proteins and proteins common to both male and in female gametocytes. Several of them reside in vesicle-like structures distinct from the OBs and MOBs either common to male and female gametocytes or gender-specific. Despite their distinct subcellular localization, all of them respond to activation stimuli suggesting that they likely take part in the egress process.

DISCUSSION

It is now established that malaria long-term elimination cannot be achieved without controlling parasite transmission to mosquitoes. The discovery of new strategies targeting malaria transmission is viewed as particularly important also to reduce the spread of drug-resistant parasite strains (25).

When ingested by mosquitoes, gametocytes differentiate into gametes and egress from the host cell to fertilize. In about 24 h, the formed zygote transforms into a motile ookinete that escapes from the mosquito midgut. During this whole process, parasites must survive outside the host cell facing strong selective constraints. This exposure represents a tremendous bottleneck for parasite development and a large window to target the parasite through immunological or pharmacological measures. The midgut stages are thus viewed as prime targets for transmission-blocking interventions.

Using a comparative proteomic approach, we identified gender-related proteins secreted/released by *P. berghei* gametocytes upon activation to form gametes and egress from the host RBC. This data set of 263 proteins includes molecules predicted as male- or female-specific as well as proteins common to the two genders. It almost completely includes a gametocyte secretome of *P. berghei* consisting of 86 proteins, previously defined (21).

Our proteomic approach is based on comparative analysis of abundance ratio values of proteins released by the activated gametocytes of the wt and a transgenic line, actII(-), defective in male gamete maturation and egress, (12). Wt/actII(-) abundance ratio values enabled us to compile a ranking list of gender-related proteins, where the highest values of the distribution were assigned to proteins released specifically or preferentially by male gametocytes. Consistently, male-specific proteins already characterized, such as PyMiGS (23), SUB1 (22) or identified in this study, such as the Plasmepsin X and a putative lactate dehydrogenase, rank at the highest positions. To further confirm robustness of our approach we compared the egress secretome of the wt with that of a second mutant g377(-), delayed in female gametocyte egress. As expected, in this case the abundance ratio values of male-related candidates fall within the distribution range.
By means of specific antibodies, we validated our gender-related secretome defining sex specificity and subcellular localization of 11 candidate proteins, conserved between rodent and human malaria parasites. Three of them are specifically expressed in male gametocytes, two in females, whereas the remaining six proteins were detected in both genders. All of them localize to vesicle-like structures trafficked to parasite periphery upon gametocyte activation. A putative secreted ookinete protein (PBANKA_0619200) and a conserved protein of unknown function (PBANKA_0810800) localize to both OBs and MOBs, confirming that these gender-specific secretory organelles share in part their proteomes. Previous studies localized, in fact, the \( P. berghei \) gametocyte-specific proteins MDV1 (26) and GEST (26) to OBs of both genders. Interestingly, lack of MDV1 and GEST affects gamete egress preventing PVM disruption (26, 27).

However, in other described cases, OB-resident proteins have no clear role in gamete egress. For instance, the protein G377 is specifically expressed and highly abundant in female OBs of \( P. falciparum \) (28). When g377 is disrupted, OB formation is compromised (29), whereas egress from the host cell is not affected (30). As previously mentioned, in \( P. berghei \), lack of G377 causes morphological alteration of OBs and only a slight delay in female gamete egress (7). Further, new protein constituents of \( P. falciparum \) OBs, OBC13 and the Dipeptidyl aminopeptidase 2 (PfDPAP2) have only a marginal role in female gamete egress (30).

In rodent models, two proteases specifically expressed in male gametocytes, also identified in this study, are among MOB constituents, the \( P. berghei \) SUB1 (22) and the \( P. yoelii \) aspartyl protease MiGS (23, 31). Lack of PbSUB1 causes a significant delay in male gamete maturation and egress (22), whereas PyMiGS is dispensable for the egress process (23). Using specific antibodies, here we showed that MiGS is specifically expressed also in male gametocytes of \( P. berghei \) and demonstrated that MiGS and SUB1 coexist in the same MOBs.

Eight out of eleven proteins characterized in this study localize to vesicle-like structures distinct from OBs and MOBs, suggesting that these latter represent only a fraction of gametocyte vesicle population that respond to activation stimuli. This is consistent with several previous reports, in which proteins involved in gamete egress were shown to

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**Fig. 6.** Subcellular localization of candidate proteins in activated gametocytes. Rel antibodies against male- (A) and female (B)-specific proteins or against proteins detected in both genders (C) were used in IFA on fixed gametocytes activated for 5–8 min. Female gametocytes were specifically stained by anti-G377 antibodies, whereas male gametocytes by anti-Tubulin antibodies. Nuclei are stained with DAPI. BF, bright field. Scale bar indicates 5 \( \mu \)m.
localize to vesicles distinct from OBs. The protein MTRAP, for instance, belongs to the thrombospondin-related anonymous protein (TRAP) family expressed in the invasive blood stage merozoites (32) and in gametocytes of both P. berghei and P. falciparum (20, 31). This protein localizes to cytoplasmic vesicles but does not colocalize with MDV1 and is relocated to the cell periphery upon gametocyte activation. Disruption of the encoding gene blocks male and female gamete egress from the PVM (20, 31) in both Plasmodium species. Also the plasmodial perforin-like protein PPL2, shown to play a crucial role in the lysis of the erythrocyte membrane both in P. berghei (24) and P. falciparum (33) gametogenesis, localizes to intracellular vesicles other than OBs/MOBs and is secreted to the cell periphery upon gametocyte activation. In both Plasmodium species, pplp2 null mutants fail to permeabilize the host membrane but are still able to lyse the PVM. Finally, the patatin-like phospholipase (PfPATPL1) plays a key role in gametogenesis, as the conditional deletion of its encoding gene reduces the efficiency of gamete egress (34). PfPATPL1 does not co-localize neither with Pfg377 nor with PPLP2 and there is no change in its localization following gametocyte activation. Altogether, these observations suggest that different classes of cytoplasmic vesicles responding to activation stimuli contain proteins involved in gamete egress.

Interestingly, a single protein, the putative pantothenate transporter (PAT), has been characterized as a membrane component of both G377 and PPLP2-containing vesicles (31). In pat null mutants, membrane fusion events and the subsequent discharge of their content into the PV lumen is blocked. As a consequence, PVM is not disrupted and transmission to the mosquito is completely impaired (31).

In conclusion, overall these data indicate that secretory apparatus involved in gametocyte escape from the host cell is more complex than previously suspected and that the concerted action of molecules implicated in different steps of gamete maturation and egress may be accomplished by storing them in distinct vesicular structures.

In this context, our proteomic study provides a comprehensive data set of egress-related putative secreted molecules and their gender specificity. Our results may contribute to future functional studies aimed at identifying novel potential transmission-blocking candidates.

DATA AVAILABILITY

Raw files and MS search data have been deposited in MassIVE repository with the dataset identifier MSV000085650.

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Conflict of interest—Authors declare no competing interests.

Abbreviations—The abbreviations used are: DAPI, 4',6-diamidino-2-phenilindolo; hpi, hours post invasion; MOB, male osmiophilic body; OB, osmiophilic body; RBC, red blood cell; XA, Xanthurenic acid.

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