Proteomic analysis of two populations of Schistosoma mansoni-derived extracellular vesicles: 15k pellet and 120k pellet vesicles

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Proteomic analysis of two populations of *Schistosoma mansoni*-derived extracellular vesicles: 15k pellet and 120k pellet vesicles

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Abstract

Helminth parasites secrete extracellular vesicles (EVs) into their environment that have potential roles in host-parasite communication, and thus represent potentially useful targets for novel control strategies. Here, we carried out a comprehensive proteomic analysis of two different populations of EVs – 15k pellet and 120k pellet EVs – from *Schistosoma mansoni* adult worms. We characterised the proteins present in the membranes of the EVs (including external trypsin-liberated peptides, integral membrane proteins (IMPs) and peripheral membrane proteins (PMPs)), as well as cargo proteins, using LC-MS/MS. A total of 286 and 716 proteins were identified in 15k and 120k pellets, respectively. Some of the most abundant proteins identified from both 15k and 120k pellets include known vaccine candidates such as *Sm*-TSP-2, saponin B domain-containing proteins, calpain glutathione-S-transferase, Sm29 and cathepsin domain-containing proteins. Other abundant proteins that have not been tested as vaccines include DM9 domain-containing protein, 13kDa tegumental antigen and histone H4-like protein. Sm23, a member of the tetraspanin family with known vaccine efficacy, was identified in the cargo and IMP compartments of only 15k pellet vesicles. Moreover, a collection of proteins with known or potential relevance in host-parasite communication including proteases, antioxidants and EV biogenesis/trafficking of both vesicle types were identified. Our results provide the first report of a comprehensive compartmental proteomic analysis of adult *S. mansoni*-derived EVs. Future research should investigate recombinant forms of these proteins as vaccine and serodiagnostic antigens as well as the roles of EV proteins in host-parasite communication.

Keywords: *Schistosoma mansoni*, extracellular vesicles, 15k pellet extracellular vesicles, 120k pellet extracellular vesicles, proteomics

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Abbreviations

EVs: extracellular vesicles

GST: glutathione-S-transferase

HSP: heat shock protein

IMPs: integral membrane proteins

MVBs: multivesicular bodies

PMPs: peripheral membrane proteins

TLPs: trypsin liberated proteins

TSPs: tetraspanins

TRPS: tunable resistive pulse sensing

1. Introduction

Schistosomiasis is one of the most important parasitic helminth infections, affecting nearly 240 million people worldwide, mainly in the tropics (1). Greater than 90% of the infections occur in Africa and, of these, the majority are due to either *Schistosoma mansoni* or *Schistosoma haematobium*, or both (2). Despite the use of mass drug administration with praziquantel to treat schistosomiasis over the last two decades, the infection still causes a loss of 1.53 million disability-adjusted life years. Indeed, in sub-Saharan Africa alone, up to 280,000 deaths every year are attributable to schistosomiasis (3).

Adult flukes possess a unique tegumental double bilayer, endowed with a population of stem cells that undergoes frequent regeneration and turnover, which is instrumental in immune escape pathways by making *S. mansoni* surface proteins inaccessible to host immune cells (4). Currently, there is no vaccine available for schistosomiasis and moreover, its treatment relies solely on a single drug, praziquantel, which warrants concern about emerging drug resistance (5) and emphasises the need for rigorous research into new drug targets and vaccine antigen discovery.

Schistosomiasis vaccine and diagnostic antigen discovery has focused on several developmental stages of the parasite with an emphasis on the tegument and excretory/secretory (ES) proteomes (6-13). Despite advances in the characterization of the secretome, substantially less is known about the molecular mechanisms involved in the release of these molecules. One such pathway is the release of extracellular vesicles (EVs) which have been recently described in *S. mansoni* (14, 15) and other *Schistosoma* spp. (16-18). EVs are membrane-enclosed vesicles that are continually secreted by different types of cells in disease and normal states (19). EVs usually include apoptotic bodies (originating from dying cells), microvesicles (MVs) (originating from plasma

membrane budding), and exosomes (released from multivesicular bodies). MVs and exosomes are the best characterized types of EVs (19) in terms of morphology, size and molecular composition (20). Several studies have demonstrated that parasite-derived EVs play a key role during helminth infections. For example, EVs are capable of modulating host innate immune responses (16, 21). In addition, the administration of exosomes to mice primes the immune response and reduces the severity of clinical signs in *Echinostoma caproni* infection (22). Moreover, immune reactivity towards *Schistosoma japonicum* EVs was identified when using sera from *S. japonicum*-infected rabbits (17). A recent study (23) demonstrated that, in a hamster vaccination-challenge model, secreted EVs from *Opisthorchis viverrini* and recombinant versions of EV surface tetraspanins (TSPs) induced antibody responses that resulted in signficantly reduced adult worm burdens after challenge infection. These findings serve as a baseline for the identification of potential target molecules for the development of schistosomiasis biomarkers and vaccines derived from EVs.

While the protein composition of schistosome EVs has been characterized at a crude, whole-vesicle level, the sub-vesicular distribution of those proteins has not been described. Herein, to our knowledge for the first time, we characterise the compartmental proteome content of *S. mansoni*-derived small EVs (120k pellet), and describe for the first time the presence of a relatively large schistosome EV (15k pellet) population and its proteome.

2. Material and methods

2.1 Ethics statement

The study obtained ethical approval (A2004, A2391 and A2395) from the Animal Ethics Committee at James Cook University. All studies involving mice were performed at the animal facilities of the Australian Institute of Tropical Health and Medicine in accordance with guidelines and protocols approved by the Animal Ethics Committee.

2.2 Mice and parasite material

Male 6-8 week old BALB/c mice were used for the study. All animals were maintained under standard conditions with food and water *ad libitum*. *S. mansoni* (Puerto Rican strain)-infected *Biomphalaria glabrata* snails were provided by the National Institute of Allergy and Infectious Diseases Schistosomiasis Resource Centre for distribution through the Biodefense and Emerging Infections Research Resources Repository, NIAID, National Institutes of Health, USA: *S. mansoni*, Strain NMRI; exposed *B. glabrata*, NR-21962.

2.3 Parasite culture and collection of excretory/secretory products

To harvest ES products, *S. mansoni* adult worms were obtained from infected mice as described previously (24). Briefly, mice were euthanized using an intraperitoneal injection of 0.2 ml pentobarbital/heparin solution followed by CO_2 gas administration and worms were perfused using perfusion solution (0.15 M sodium chloride with 0.03 M sodium citrate dehydrate in water) and collected in a container (24). Worms were washed three times using serum-free Basch media (0.5 µM hypoxanthine (Sigma), 1 µM serotonin (Sigma), 1 µM hydrocortisone (Sigma), 0.2 µM triiodothyronine (Calbiochem), 8 mg insulin (Lifetech), 0.5× MEM vitamin (Invitrogen), 2×

antibiotic/antimicotic (Invitrogen), 50 ml Schneider Drosophila medium (Invitrogen), 0.01 M HEPES (Invitrogen), and Eagle's basal medium (Invitrogen) to make 1 L final volume) (25) and cultured for 3 h at 37°C with 5% CO_2 in serum-free Basch media with a density of approximately 50 worm pairs in 5 ml of media. The culture media was discarded after 3 h, and replaced with fresh culture media. Worms were cultured for a further 10 days and ES products were collected every 24 h, centrifuged at 4°C (500 *g*, 2,000 *g* and 4,000 *g* for 30 min each) to pellet and discard parasite material such as tegumental debris and eggs, and kept at -80°C until further analysis. Worms were observed by microscopy daily to ensure that all were motile during *in vitro* culture. Immotile worms were removed as soon as they were detected.

2.4 EV isolation

EVs were isolated and purified following established methods previously described for *S. mansoni* (14). After differential centrifugation, samples were concentrated using a 10 kDa spin concentrator (Merck Millipore), and centrifuged at 15,000 *g* for 1 h at 4°C to pellet 15k vesicles, which were washed with PBS three more times and kept at -80°C until use. To collect 120k vesicles, supernatant was ultracentrifuged using an MLS-50 rotor (Beckman Coulter) at 120,000 *g* for 3 h at 4°C, and the resultant pellet was resuspended in 200 µl of PBS and subjected to Optiprep® discontinuous gradient separation. One ml of 40%, 20%, 10% and 5% iodixanol solutions prepared in 10 mM Tris-HCl, 0.25 M sucrose, pH 7.2, was layered in decreasing density using an ultracentrifuge tube, and the 200 µl containing the resuspended EVs was added to the top layer and ultracentrifuged at 120,000 *g* for 18 h at 4°C. A control tube was prepared in a similar way, replacing EVs with PBS to calculate the density of fractions. A total of

12 sample fractions were collected from the Optiprep® discontinuous gradient, and the excess Optiprep® solution was buffer exchanged using PBS containing 1× EDTA-free protease inhibitor cocktail (Santa Cruz, Dallas, TX, USA) using a 10 kDa spin concentrator. The absorbance was measured at 340 nm for each fraction obtained from the control tubes, and their densities were calculated. Finally, protein concentration of all sample fractions was measured using a Bradford Protein Assay Kit (ThermoFisher).

2.5 Tunable Resistive Pulse Sensing analysis of EVs

The particle concentration and size distribution of each fraction was analysed for both *S. mansoni*-derived 120k and 15k vesicle pellets by tunable resistive pulse sensing (TRPS) using a qNano instrument (Izon) following the manufacturer's instructions. 120k pellet vesicles from four different cultures were analysed using TRPS, and all fractions containing 120k vesicles (based on specific gravity/density analysis) from eight batches were pooled together. Finally, TRPS analysis was carried out again on the pooled sample before proteomic analysis was conducted. TRPS, concentration and purity analysis, was performed on the pooled 15k pellet sample isolated from eight different batches of cultured worms. Nanopore NP150 and NP300 were used for 120k and 15k vesicle pellets, respectively. Pressure and voltage values were set to optimize the signal to ensure high sensitivity. Samples were diluted 1:5 in electrolyte (Izon) before applying to the nanopore. Calibration was performed using CP100 and CP400 carboxylated polystyrene calibration particles (Izon) for 120k and 15k vesicle pellets, respectively, at a 1:2,000 dilution. Size distribution and concentration of particles were analyzed using the software provided by Izon (version 3.2). Purity analysis of both

ELVs and MVs (number of particles/µg of protein) was performed following methods described by Webber and Clayton (26).

2.6 Trypsin shaving and sequential protein extraction of purified *S. mansoni* EVs

Peptides from surface-accessible proteins were released by trypsin hydrolysis of EVs using a protocol described previously (27). Briefly, sequencing-grade trypsin (1 µg/µl) was added to purified EVs for 10 min at 37°C. Treated EVs were then centrifuged at 120,000 *g* at 4°C for 1 h, and surface peptides were obtained from the supernatant. EVs collected as a pellet were further processed to investigate proteins associated with the EV membrane and within the EV lumen. Briefly, EVs were resuspended in water and sonicated (3x) which resulted in the release of EV cargo content which was recovered from the supernatant by centrifugation at 120,000 *g* for 1 h at 4°C. Subsequently, the pellet was incubated with 0.1 M Na_2CO_3 (pH 11) on ice for 30 min and then centrifuged at 120,000 *g* at 4°C for 1 h to recover peripheral membrane proteins (PMPs) from the supernatant. Finally, integral membrane proteins (IMPs) were obtained by solubilizing the pellet in 1% Triton X-100/2% sodium dodecyl sulphate (SDS) at 37°C for 15 min.

2.7 In-gel digestion of proteins

For the proteomic analysis, each separated component of cargo, integral membrane and peripheral membrane proteins were electrophoresed on a 10% SDS-PAGE gel for 60 min at 140 V. Tryptic in-gel digestion was performed as described previously with minor modifications (28). In brief, the gel was stained with Coomassie blue (0.03%), destained overnight and each lane was cut into 7 slices (approximately 1 mm^2). Each band was destained by washing twice in 50% acetonitrile with 200 mM ammonium

bicarbonate at 37°C for 45 min, reduced with 20 mM dithiothreitol (Sigma) in 25 mM ammonium bicarbonate for 1 h at 65°C, alkylated with 50 mM iodoacetamide (IAM, Sigma) in 25 mM ammonium bicarbonate at 37°C for 15 min and digested with 100 ng/µl trypsin (Sigma) at 37°C for 18 h. Peptides were extracted in 50% acetonitrile with 0.1% trifluoroacetic acid. The last step was performed three times to maximize peptide recovery. All peptides were finally dried in a vacuum concentrator. Samples were then resuspended in 10 µl of 0.1% trifluoroacetic acid and tryptic peptides were desalted using a Zip-Tip® column (Merck Millipore) pipette tip according to manufacturer's protocol and dried in a vacuum concentrator before analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The experimental work-flow from sample collection to proteomic analysis is shown in Figure 1.

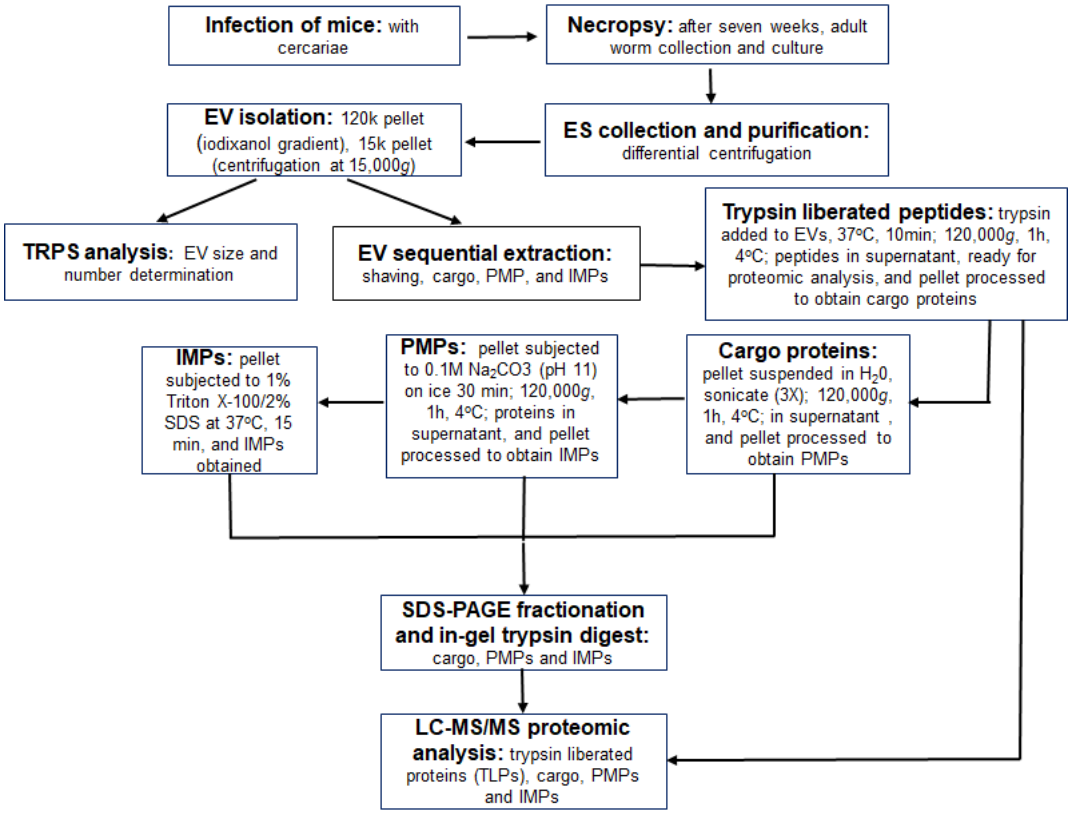


Figure 1. Overview of experimental workflow for proteomic analysis of *S. mansoni*-derived EVs. Seven weeks after infection of mice with *S. mansoni* cercariae, adult worms were perfused and cultured. Excretory/secretory (ES) products were collected and purified from cell-conditioned media using differential centrifugation (500 *g*, 2,000 *g* and 4,000 *g* at 4^{o}C, each for 30 min), followed by ultracentrifugation at 15,000 *g* at 4^{o}C for 1 h to pellet 15k pellet vesicles . The supernatant was then subjected to ultracentrifugation at 120,000 *g* at 4^{o}C for 3 h to pellet the 120k pellet vesicles. The purity and concentration of 120k vesicles were enhanced using an Optiprep® gradient. Size and concentration of EVs were determined by tunable resistive pulse sensing using a qNano instrument. For the proteomic analysis, EV surface-exposed proteins were obtained using trypsin shaving, then, sequential extraction resulted in purification of cargo proteins, PMPs and IMPs. Proteins were submitted to in-gel trypsin digestion and LC-MS/MS for proteomic analysis.

2.8 LC-MS/MS protein analysis

Tryptic fragments from each sample were resuspended in 8 µl of 0.1 % formic acid in LC-MS/MS-grade water and separated chromatographically by an Eksigent nanoLC 415 system using a 15 cm long Eksigent column (C18-CL particle size 3 µm, 120 Å, 75 µm ID) and a linear gradient of 3-40% solvent B (100 acetonitrile/0.1% formic acid [aq]) for 45 min followed by 40-80 % solvent B in 5 min. A pre-concentration step (10 min) was performed employing an Eksigent Trap-column (C18-CL, 3 µm, 120 Å, 350 µm x 0.5 mm) before commencement of the gradient. A flow rate of 300 nl/min was used for all experiments. The mobile phase consisted of solvent A (0.1% formic acid [aq]) and solvent B. Eluates from the RP-HPLC column were directly introduced into the PicoView ESI ionisation source of a TripleTOF 6600 MS/MS System (AB Sciex)

operated in positive ion electrospray mode. All analyses were performed using Information Dependant Acquisition. AnalystTF 1.7.1 (Applied Biosystems) was used for data analysis. Briefly, the acquisition protocol consisted of the use of an Enhanced Mass Spectrum scan with 15 sec exclusion time and 100 ppm mass tolerance. A cycle time of 1800 ms was used to acquire full scan TOF-MS data over the mass range 400–1250 m/z and product ion scans over the mass range of 100–1500 m/z for up to 30 of the most abundant ions with a relative intensity above 150 and a charge state of +2 − +5. Full product ion spectra for each of the selected precursors were then used for subsequent database searches.

2.9 Database search and protein sequence analysis

Data were searched against a database comprising the *S. mansoni* proteome obtained from WormBase ParaSite (http://parasite.wormbase.org/) appended to the common Repository of Adventitious Proteins (cRAP; http://www.thegpm.org/crap/) and their reversed decoy sequences using SearchGUI (v3.3.5). X!Tandem (vAlanin), Comet (v2018.01 rev.2), Tide (v3.2.x), MyriMatch (v2.1) and MS-GF + (v2016.01.02) were the search engines used, and the identification results were validated using PeptideShaker (v1.16.31). All searches were conducted employing the following search parameters: trypsin as digestion enzyme, maximum missed cleavages, 2; precursor ion mass tolerance ± 10 ppm, fragment ion tolerance ±0.1 Da; fixed modifications: carbamidomethylation of cysteine; variable modifications: deamidation of asparagine and glutamine, oxidation of methionine; Peptide Spectrum Matches (PSMs), peptides and proteins were validated at a 1.0% False Discovery Rate estimated using the decoy

hit distribution. Reported results correspond to those proteins showing ≥ 2 distinct unique peptides. An estimate of the relative abundance of the predicted proteins was assessed using the spectrum counting abundance index (29). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD015857 and 10.6019/PXD015857.

2.10 Bioinformatic analyses

Gene ontology (GO) annotations were obtained using the Blast2GO software (30), and GO terms were assigned as biological process or molecular function. Only children GO terms were used for subsequent analysis to avoid redundancy in GO terms. REViGO was used to summarize and plot GO terms (31). Protein family (Pfam) analysis was performed at the E-value <0.05 threshold using HMMER software (30). Signal peptides and transmembrane domains were predicted using SignalP v4.1 and TMHMM v2.0, respectively (32, 33).

3. Results

3.1 EV size, concentration and purity

S. mansoni adult worm-derived ELVs were purified using an iodixanol density gradient as described previously for other helminths (14, 34, 35). The density and protein concentration of all 12 fractions collected from the gradient were analysed. Fractions of 120k pellet vesicles from different batches with densities ranging from 1.09 to 1.22 g/ml (fractions containing *S. mansoni* small vesicles) as described previously (14) were combined and subjected to TRPS analysis using a qNano instrument to determine size

and concentration of 120k vesicles (Figure 2A). Likewise, 15k pellet vesicles collected from different batches of EVs were combined, protein concentration was measured and TRPS analysis was performed to obtain size and concentration (Figure 2B) of 15k vesicles-enriched sample. Accordingly, purity analysis of both 120k (4.1 X 10^8 particles/µg protein) and 15k pellet vesicles (1.6 X 10^7 particles/µg protein) (Figure 2C) was also performed as described previously (26).

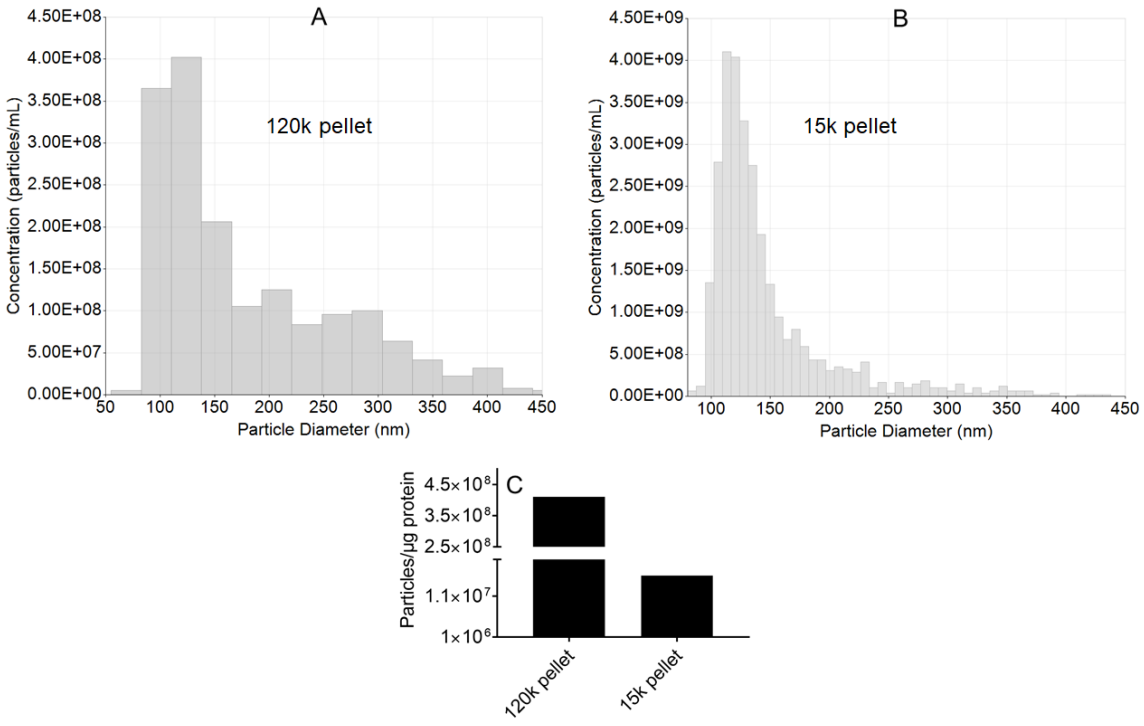
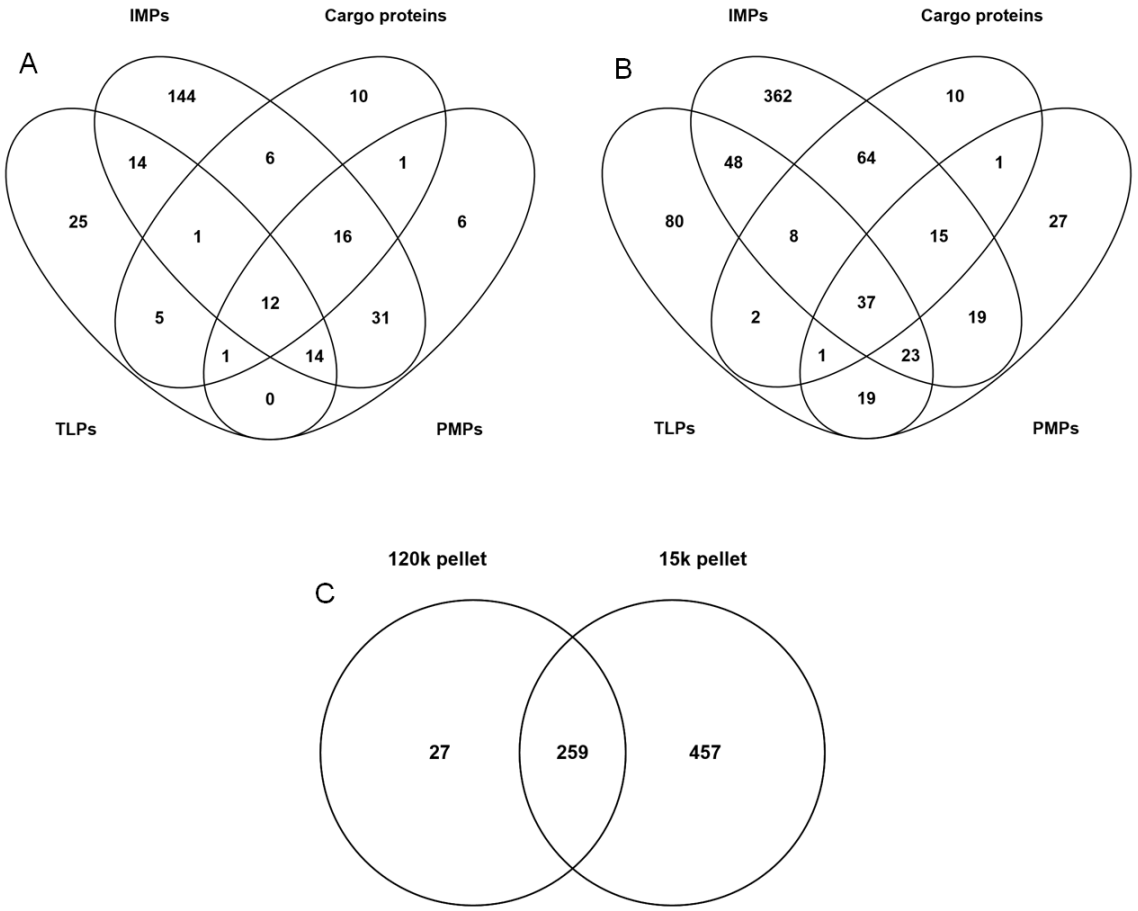


Figure 2. Tunable resistive pulse sensing (TRPS) analysis and purity on *S. mansoni*-drived extracellular vesicles. Particle size (x-axis) and number (y-axis) of 120k pellet (A) and 15k pellet vesicles (B) after purification were analysed by TRPS using a qNano instrument (iZon). The purity of both 120k and 15k pellet vesicles (C) was determined according to Webber and Clayton (26).

3.2 Proteomic analysis of *S. mansoni*-derived EVs

A proteomic analysis on each of the four components - TLPs, cargo proteins, IMPs and PMPs - of both 120k and 15k pellet vesicles was performed. In total, 286 and 716 different proteins were identified from *S. mansoni*-derived 120k (Figure 3A) and 15k pellet vesicles (Figure 3B), respectively. Of these, 27 were specific to 120k pellet and 457 specific to 15k pellet vesicles, while 259 proteins were identified in both types of EVs (Figure 3C). Antioxidants such as peroxiredoxin (Prx3) and proteins involved in exosome biogenesis or trafficking, such as vacuolar protein sorting-associated protein 28 homolog, were among the 27 120k vesicle-specific proteins identified (Table 1, Figure 4). 120k pellet vesicles contained 72 TLPs (Supplementary Table S1), 52 cargo proteins (Supplementary Table S2), 238 proteins in the IMP component (Supplementary Table S3) and 81 proteins in the PMP component (Supplementary Table S4). 15k pellet vesicles, on the other hand, contained 218 TLPs (Supplementary Table S5), 138 cargo proteins (Supplementary Table S6), 576 IMPs (Supplementary Table S7) and 142 PMPs (Supplementary Table S8). Some of the most abundant proteins identified, based on spectrum counting, in both *S. mansoni*-derived 120k (Supplementary Table S1-S4) and 15k pellet vesicles (Supplementary Table S5-S8), included the TSPs TSP-2 and TSP-4, glutathione-S-transferase (GST), saponin B domain-containing protein, DM9 domain-containing protein, cathepsin domain-containing proteins, 13 kDa tegumental antigen, and histone H4-like protein. However, the TSP Sm23 was only identified in 15k pellet vesicles.



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329 **Figure 3. Shared and specific adult *S. mansoni*-derived EVs proteins**. Venn diagram

330 representing the number of proteins identified from the four sub-vesicular components,

331 enumerating specific and shared proteins of 120k pellet (A) and 15k pellet vesicles (B)

332 and between the two vesicle types (C).

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341 **Table 1.** Functional annotation of proteins from adult *S. mansoni*-derived 120k pellet vesicles.

	Description	# Unique peptides			
Accession number	**Proteases/inhibitors**	**TLPs**	**Cargo**	**IMPs**	**PMPs**
Smp_032580.1	subfamily T1A non-peptidase homologue (T01 family)	2	4	0	0
Smp_070930.1	proteasome (prosome macropain) subunit alpha type 4	4	3	0	0
Smp_034490.1	Proteasome subunit beta type-6	0	2	0	0
Smp_187140.1	Cathepsin L-like proteinase precursor	0	3	4	5
Smp_301340.1	proteasome (prosome, macropain) subunit, beta type,1	0	2	0	0
Smp_214190.1	Calpain	4	4	11	3
Smp_340060.1	Mastin precursor	0	6	9	8
Smp_006390.1	cysteine protease inhibitor	0	0	3	0
Smp_030000.1	Putative aminopeptidase W07G4.4	10	0	7	0
Smp_103610.1	cathepsin B-like peptidase (C01 family)	0	0	4	2
Smp_343260.1	cathepsin L, a	3	0	7	3
Smp_083870.1	Putative aminopeptidase W07G4.4	4	0	0	0
Smp_029500.1	Thimet oligopeptidase	2	0	0	0
Smp_212920.1	20S proteasome subunit alpha 6	2	0	0	0
Smp_207080.1	Proteasome subunit alpha type-6	2	0	0	0
Smp_089670.1	Alpha-2-macroglobulin-like protein 1	5	35	0	2
Smp_090080.1	Estrogen-regulated protein EP45 precursor	0	5	0	0
Smp_075800.1	hemoglobinase (C13 family)	0	0	2	0
Smp_007550.1	Uncharacterized	2	0	0	0
	Biogenesis/vesicle trafficking				
Smp_035620.1	Multivesicular body subunit 12B	0	0	3	0
Smp_048940.1	Vacuolar protein sorting-associated protein 37B	0	0	3	0
Smp_055880.1	chmp1 (chromatin modifying protein) (charged multivesicular body protein), putative	0	0	8	0
Smp_067540.1	Vacuolar protein sorting-associated protein 28 homolog	0	0	2	0
Smp_034870.1	AP-2 complex subunit beta	0	0	4	0
Smp_055870.1	vesicle-associated membrane protein 7-like	0	2	11	2
Smp_068530.1	putative syntenin	0	2	8	3
Smp_074140.1	Annexin A13 (Annexin XIII)	3	4	13	9
Smp_013690.1	BRO1 domain-containing protein BROX	3	0	16	9
Smp_071630.1	Ras-related protein Rab-2A	0	0	6	0
Smp_077720.1	putative annexin	0	0	6	5
Smp_062300.1	Ras-related C3 botulinum toxin substrate 1	0	0	3	0
Smp_045550.1	putative annexin	0	0	11	0
Smp_067140.1	Ras-related protein Rab-7a	0	0	2	0
Smp_104670.1	ras-related protein Rab-8A isoform X1	0	0	3	0

Smp_139340.1	Ras-related protein Rab-27A	0	0	4	0
Smp_094820.1	Vacuolar protein sorting-associated protein VTA1-like protein	0	0	4	0
Smp_094420.1	Rab GDP dissociation inhibitor alpha	0	0	4	4
Smp_210430.1	Rab-protein 8	0	0	2	0
Smp_104310.1	rab11, putative	0	0	7	0
Smp_312770.1	Ras-related protein Ral-A	0	0	3	0
Smp_045710.1	Charged multivesicular body protein 4b	0	0	6	0
	Antioxidants				
Smp_309480.1	Uncharacterized	2	2	4	0
Smp_004470.1	Peroxiredoxin, Prx3	2	0	0	0
Smp_158110.1	Thioredoxin peroxidase	2	0	0	0
	Membrane structure				
Smp_040970.1	putative vacuolar proton atpases	0	2	23	5
Smp_041460.1	tetraspanin, putative (*Sm*-TSP-D76)	0	7	9	7
Smp_017730.1	200-kDa GPI-anchored surface glycoprotein	7	14	0	0
Smp_017430.1	CD63 antigen	0	5	9	5
Smp_055780.1	Multidrug resistance protein 1	4	4	50	24
Smp_140000.1	putative tetraspanin-CD63 receptor (*Sm*-TSP-4)	0	3	7	4
Smp_155310.1	tetraspanin, putative (*Sm*-TSP-1)	0	2	6	0
Smp_104270.1	Bis(5'-adenosyl)-triphosphatase enpp4	0	14	28	19
Smp_313560.1	alkaline phosphatase	2	4	19	4
Smp_344440.1	putative tetraspanin 18, isoform 1	0	4	11	6
Smp_015020.1	Sodium/potassium-transporting ATPase subunit alpha	0	0	11	0
Smp_004310.1	ATPase, H+ transporting, lysosomal, V0 subunit c	0	0	4	0
Smp_005720.1	Aquaporin-3 (AQP-3)	0	0	2	0
Smp_011560.1	putative tetraspanin	0	0	2	0
Smp_042910.1	osteopetrosis associated transmembrane protein	0	0	2	0
Smp_043990.1	basigin related	0	0	3	0
Smp_037540.1	putative alpha-amylase	0	0	6	0
Smp_032520.1	putative lysosome-associated membrane glycoprotein	0	0	3	0
Smp_031880.1	Immunoglobulin-like domain-containing protein	0	0	3	0
Smp_162510.1	SJCHGC07463 protein	0	0	6	0
Smp_128110.1	transporter, major intrinsic protein family protein	0	0	2	0
Smp_201730.1	SJCHGC09800 protein	0	0	2	0
Smp_091650.1	putative phospholipid-transporting ATPase IIB	0	0	18	0
Smp_153390.1	putative ectonucleotide pyrophosphatase/phosphodiesterase	0	0	2	0
Smp_123280.1	Major facilitator superfamily domain-containing protein 1	0	0	10	4
Smp_244680.1	protein VAC14 homolog	0	0	2	0
Smp_174490.1	expressed conserved protein	0	0	7	0

Smp_169610.1	putative zinc finger protein	0	0	2	0
Smp_159070.1	hypothetical protein MS3_09367	0	0	2	0
Smp_128940.1	putative metabotropic glutamate receptor	0	0	5	0
Smp_131840.1	25 kDa integral membrane protein	0	0	2	0
Smp_169870.1	putative cystinosin	0	0	3	0
Smp_095630.1	CD9-like protein Sm-TSP-1	0	0	2	0
Smp_130300.1	Sodium/potassium-transporting ATPase subunit alpha	0	0	4	0
Smp_123670.1	Lysosomal acid phosphatase	0	0	4	2
Smp_091240.1	Voltage-dependent anion-selective channel protein 2	0	0	4	2
Smp_165170.1	putative transient receptor potential cation channel,subfamily m, member	0	0	2	0
Smp_150500.1	Protein lifeguard 3	0	0	2	0
Smp_167240.1	SJCHGC05348 protein	0	0	5	0
Smp_210250.1	CDC50 family protein, LEM3 family	0	0	6	0
Smp_089700.1	integrin beta 2	0	0	4	0
Smp_129820.1	multi drug resistance-associated protein	0	0	4	0
Smp_166340.1	SJCHGC03061 protein	0	0	2	0
Smp_147070.1	Putative sodium-coupled neutral amino acid transporter 9	0	0	4	0
Smp_160160.1	putative sialin (solute carrier family 17 member 5) (sodium/sialic acid cotransporter) (ast) (membrane glycoprotein hp59)	0	0	5	0
Smp_162770.1	lysosome-associated membrane glycoprotein	0	0	3	0
Smp_099150.1	Pleckstrin homology domain-containing family B member 2	0	0	5	0
Smp_194920.1	T-cell immunomodulatory protein	0	0	10	7
Smp_164210.1	Synaptosomal-associated protein 25	0	0	16	0
Smp_246270.1	ATP-binding cassette sub-family B member 6, mitochondrial	0	0	16	7
Smp_344430.1	SID1 transmembrane family member 1	0	0	4	0
Smp_302090.1	putative ctl2	0	0	4	3
Smp_267000.1	ATPase, H+ transporting, lysosomal accessory protein 1	0	0	7	3
Smp_324850.1	SJCHGC01860 protein	0	0	3	0
Smp_300190.1	Aquaporin-9 (AQP-9) (Small solute channel 1)	0	0	2	0
Smp_340540.1	lysosome membrane protein 2	0	0	5	2
Smp_245370.1	Equilibrative nucleoside transporter 3	0	0	3	0
Smp_141010.1	Dysferlin	0	0	0	6
Smp_335630.1	tetraspanin 2 (*Sm*-TSP-2)	0	5	6	6
Smp_040080.1	SJCHGC08990 protein	0	0	7	5
Smp_074140.1	Annexin 13	0	0	4	0
Smp_069120.1	Synaptotagmin-2	0	0	2	0
Smp_127820.1	SJCHGC05417 protein	0	0	2	0

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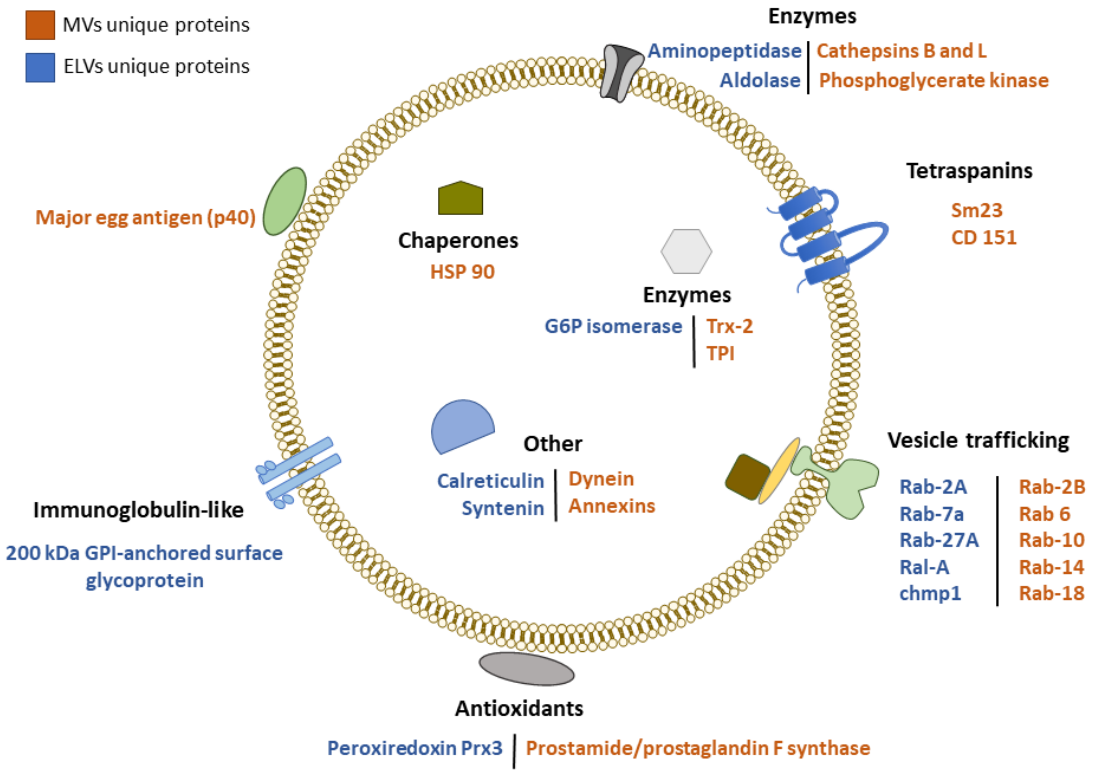

Figure 4. Schematic representation of most representative 120k and 15k pellet vesicle-unique proteins. Several unique membrane and cargo specific proteins were found in the different populations of extracellular vesicles isolated from the excretory/secretory products of *Schistosoma mansoni*.

A GO and protein family analysis of the identified proteins from both *S. mansoni*-derived 120k and 15k pellets was performed. The most abundant GO terms within the "biological process" ontology of 120k pellet vesicles (Figure 5A) were "primary metabolic process" followed by "cation transport", "carbohydrate metabolic process", "phosphorylation" and "ATP metabolic process"; whereas the most abundant biological process GO terms for 15k vesicles (Figure 5B) were "regulation of cellular process"

358 followed by “positive regulation of biological process”, “primary metabolic process”,
359 “cellular response to stimuli”, and “positive regulation of cellular process”.

360 The most prominent GO terms within the “molecular function” ontology of 120k
361 vesicles (Figure 6A) were “protein binding” followed by “transmembrane transporter
362 activity”, “transferase activity”, and “oxidoreductase activity”; whereas the most
363 prominent molecular function GO terms for 15k pellet vesicles MVs (Figure 6B) were
364 “heterocyclic compound binding” followed by “anion binding”, “carbohydrate
365 derivative binding” and “cytoskeletal protein binding”.

366

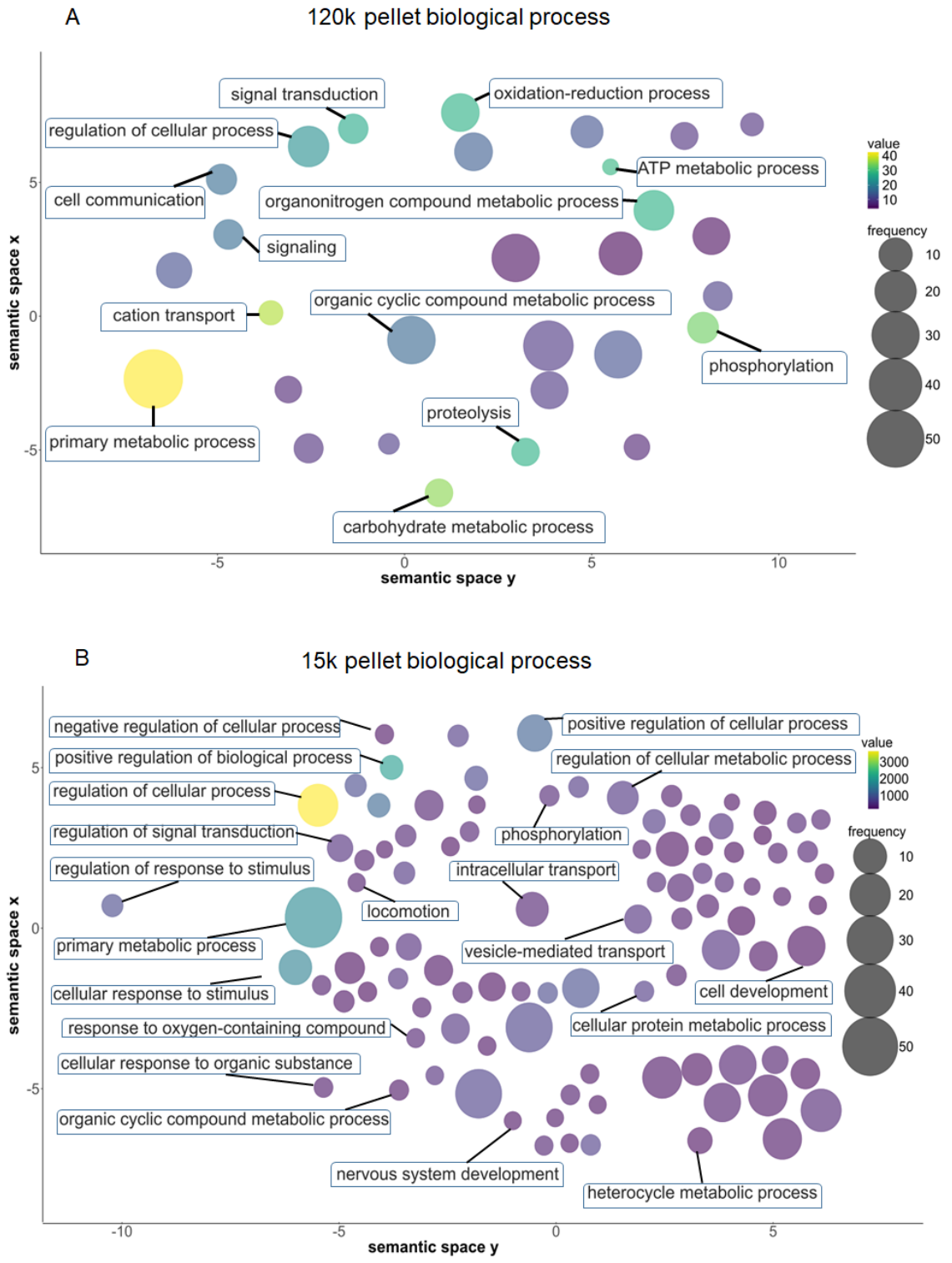

Figure 5. Bioinformatic analysis of gene ontology biological process terms in adult *S. mansoni*-derived 120k (A) and 15k vesicles (B). REViGO plot showing the most abundantly represented GO terms ranked by nodescore (Blast2GO). Increasing heatmap score signifies increasing nodescore from Blast2GO, while circle size denotes the frequency of the GO term from the underlying database.

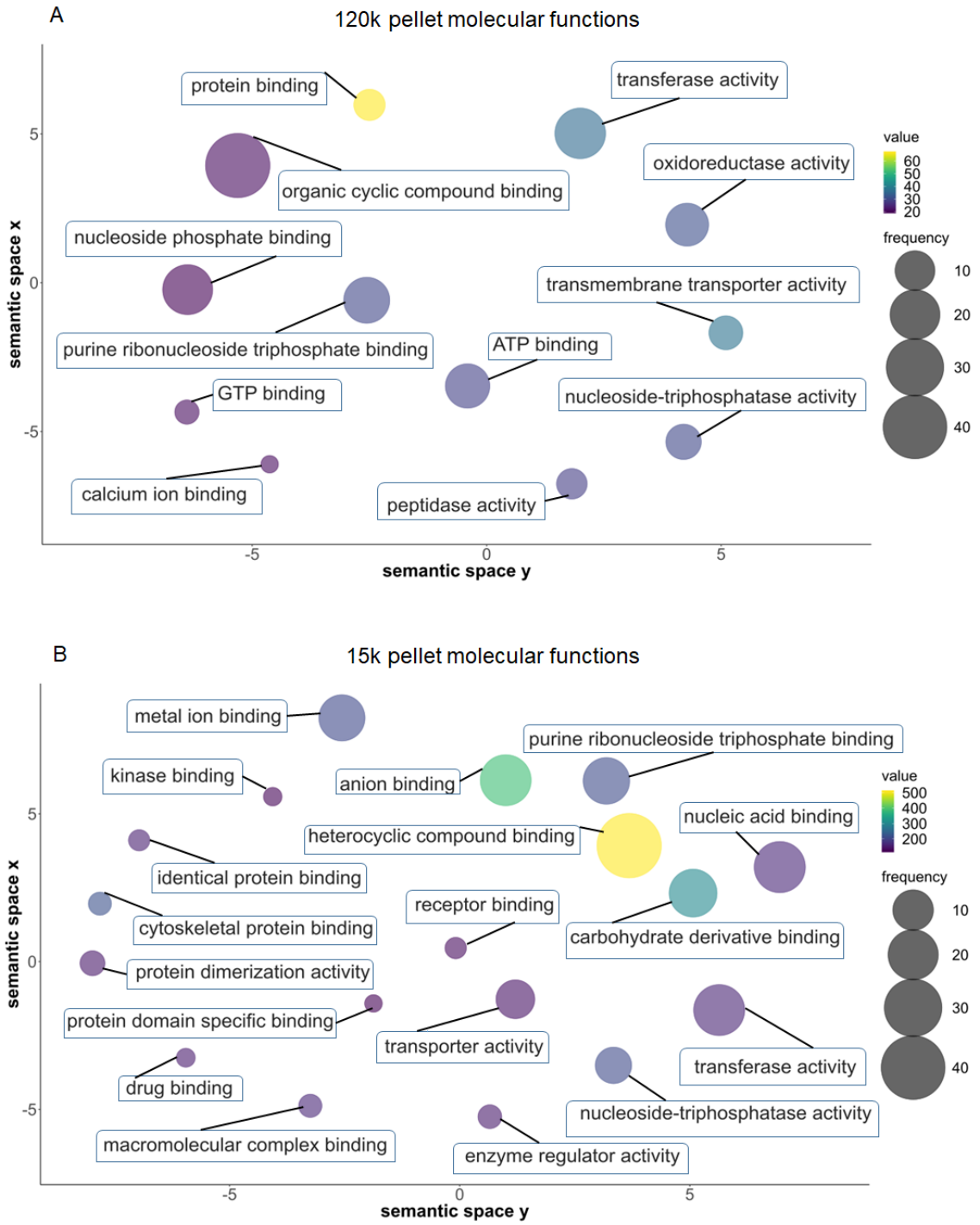

Figure 6. Bioinformatic analysis of gene ontology molecular function terms in adult *S. mansoni*-derived 120k (A) and 15k vesicles (B). REViGO plot showing the most abundantly represented GO terms ranked by nodescore (Blast2GO). Increasing heatmap score signifies increasing nodescore from Blast2GO, while circle size denotes the frequency of the GO term from the underlying database.

We have also reported the top 10 represented protein families in each vesicle type analysed (Figure 7). The eight most highly represented protein family domains (by amount of protein containing a particular domain) were common to both 120k and 15k pellet vesicles but presented different levels of abundance, including “EF-hand domain pair”, “ADP-ribosylation factor family”, “50s ribosome-binding GTPase” and “Ras” family proteins. In addition, proteins from the TSP family were abundant in the 120k vesicles but less well represented in the 15k vesicles. Protein families identified in both vesicle types analysed are presented in Supplementary Table S9 and Supplementary Table S10, respectively.

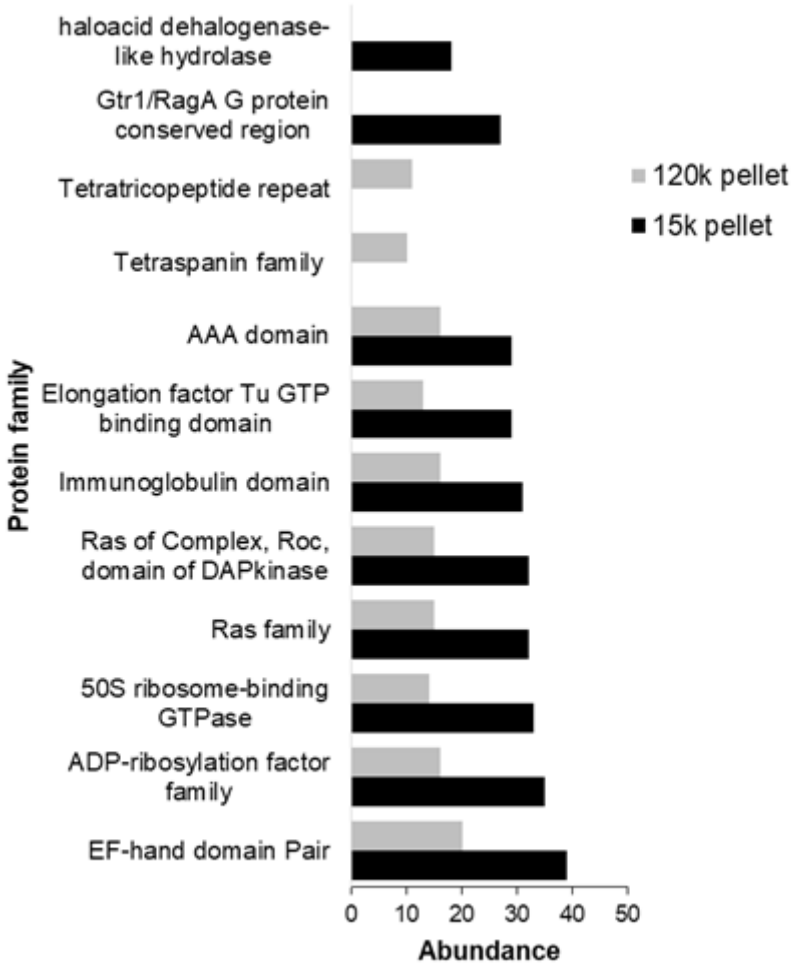

Figure 7. Pfam analysis of the proteins secreted by *S. mansoni*-derived EVs. Bar graph showing the top 10 most represented protein families of 120k and 15k pellet vesicles of adult *S. mansoni*.

From the total of 286 proteins identified in 120k pellet vesicles and 716 proteins identified in 15k pellet vesicles, 39 (13.6%) and 64 (8.9 %) contained a signal peptide, respectively (Supplementary Table S11 and 12), whereas 69 (24.1%) and 120 (16.8%) of the proteins identified in 120k pellet (Supplementary Table S13) and 15k pellet vesicles (Supplementary Table S14), respectively, contained transmembrane domains.

Functional annotation revealed proteins of potential relevance for host-parasite communication in both types of EVs analysed, including proteases, protease inhibitors and antioxidants in all compartments of EVs and proteins associated with membrane structures from 120k pellet (Table 1) and 15k pellet (Table 2). Moreover, previously described vaccine candidates, including, TSP-2, GST, Sm29, and calpain were identified in both vesicle types, while the TSP Sm23 was identified only in 15k Pellet. Moreover, proteases corresponding to cathepsin family proteins such as cathepsin B- and cathepsin L-like proteases were identified in both EV types analysed. Other proteases, including putative aminopeptidase W07G4.4, were released following trypsin shaving of both EV types. In addition, proteins from the annexin group, including annexin A3, annexin A7, annexin A8 and annexin A8-like protein 1, were identified only in MVs. Protease inhibitors such as serpin B9 and cystatin-B were also identified only in 15k pellet, while cysteine protease inhibitors were identified only in 120k pellet. Further, proteins with antioxidant or defence roles such as thioredoxin peroxidase were identified in both EV types analysed, whereas, peroxiredoxin Prx3 was only found in 120k pellet, and prostamide/prostaglandin F, thioredoxin peroxidase-2 and thioredoxin-2 were anti-oxidant proteins found only in 15k pellet (Table 1 and 2).

We identified proteins involved in vesicle biogenesis and trafficking exclusively in 120k pellet vesicles including chmp1 (chromatin modified protein), charged MVB proteins, vacuolar protein sorting-associated protein as well as Rab related proteins such as Rab-2A, Rab-7a, Rab-27A and Ral A. Moreover, other vesicle biogenesis proteins such as Ras related proteins (Rab-2B, Rab 6, Rab-10, Rab-14 and Rab-18) were identified only in 15k pellet. In addition, proteins with similar roles such as charged MVB protein 4b (SNF7 homolog associated with Alix 1), syntenin-1, annexin, Rab-8A and Rab-11A were identified from both vesicles types. Further, common exosome markers, such as heat shock protein (Hsp)-70, members of the TSP family (*Sm*TSP-1, *Sm*-TSP-2, *Sm*-TSP-4 and putative TSP-18), Rab protein families and enolase were identified in both EV types, and hsp-90 was identified only in 15k vesicles (Supplementary Tables S1-8).

Membrane transporters, channels, and other molecules involved in membrane structure were identified from different compartments of both EV types analysed including aquaporin, Na^+/K^+-transporting ATPase, H^+-transporting ATPase and phospholipid-transporting ATPase IIB. Moreover, other proteins with potential roles in membrane structure and signalling including, the TSPs noted above, as well as Sm13 and the 25 kDa integral membrane protein, were identified in both vesicle types, whereas other membrane proteins were unique to just one vesicle population, including the 200 kDa GPI-anchored surface glycoprotein, found in 120k pellet only, and Sm23, tegument antigen (I(H)A) and CD 151 homologue, found in 15k pellet vesicles only (Tables 1 and 2 and Supplementary Tables S1-8).

439

440 **Table 2.** Functional annotations of proteins from adult *Schistosoma mansoni*-derived 15k
441 pellet vesicles.

	Description	# unique peptides			
Accession number	**Proteases/inhibitors**	**TLPs**	**Cargo**	**IMPs**	**PMPs**
Smp_089670.1	Alpha-2-macroglobulin-like protein 1	2	11	3	2
Smp_089180.1	Ubiquitin carboxyl-terminal hydrolase 7	2	0	3	0
Smp_100090.1	High mobility group protein DSP1	2	0	0	0
Smp_103610.1	Cathepsin B-like peptidase (C01 family)	2	0	6	2
Smp_187140.1	Cathepsin L-like proteinase precursor	4	2	5	3
Smp_207080.1	Proteasome subunit alpha type-6	5	2	0	0
Smp_214190.1	Calpain	6	12	41	4
Smp_153960.1	Presenilin homolog	3	0	0	0
Smp_155720.1	Glycogen synthase kinase-3 alpha	3	0	0	0
Smp_141610.1	Cathepsin B-like cysteine proteinase	3	2	3	0
Smp_212880.1	Proteasome subunit beta type-5	5	0	0	0
Smp_172590.1	family S10 unassigned peptidase (S10 family)	4	4	10	3
Smp_247170.1	Major egg antigen	6	0	3	0
Smp_340060.1	Mastin precursor	13	13	17	9
Smp_343260.1	cathepsin L, a	23	0	12	12
Smp_346350.1	26S proteasome non-ATPase regulatory subunit 1	38	0	2	0
Smp_246110.1	Kyphoscoliosis peptidase	6	0	7	4
Smp_307450.1	U-actitoxin-Avd3s	8	0	0	0
Smp_303330.1	Hemoglobinase	7	0	0	0
Smp_032580.1	Proteasome subunit alpha type-5	0	3	2	0
Smp_013040.1	Lysosomal aspartic protease	0	2	6	0
Smp_074500.1	Proteasome subunit beta type-2	0	2	0	0
Smp_070930.1	Proteasome subunit alpha type-4	0	3	3	0
Smp_071610.1	Dipeptidyl peptidase 2	0	2	4	0
Smp_034490.1	Proteasome subunit beta type-6	0	5	4	0
Smp_082030.1	Protein dj-1beta	0	3	3	0
Smp_092280.1	20S proteasome subunit alpha 7	0	3	2	2
Smp_076230.1	Proteasome subunit alpha-type 7-like	0	2	0	0
Smp_212920.1	Proteasome subunit alpha type-1	0	4	0	0
Smp_301340.1	Proteasome subunit beta type-1-B	0	4	0	0
Smp_006390.1	Cystatin-B	0	0	6	0
Smp_008545.1	60 kDa heat shock protein, mitochondrial	0	0	3	0
Smp_027610.1	40S ribosomal protein S3	0	0	3	0
Smp_022400.1	Glucose-6-phosphate isomerase	0	0	11	0
Smp_030000.1	Putative aminopeptidase W07G4.4	0	0	21	0
Smp_028500.1	Caspase-3	0	0	2	0
Smp_018240.1	Transitional endoplasmic reticulum ATPase	0	0	19	0
Smp_019010.1	Dipeptidyl peptidase 3	0	0	9	0

Smp_029500.1	Thimet oligopeptidase	0	0	4	0
Smp_061920.1	UV excision repair protein RAD23 homolog B	0	0	5	0
Smp_056970.2	Glyceraldehyde-3-phosphate dehydrogenase	0	0	27	0
Smp_031730.1	Signal peptidase complex catalytic subunit SEC11C	0	0	2	0
Smp_067490.1	Sorcin	0	0	6	0
Smp_067480.1	Sorcin	0	0	6	0
Smp_033710.1	ATP-dependent RNA helicase DDX3Y	0	0	3	0
Smp_067060.1	Cathepsin B-like cysteine proteinase	0	0	5	0
Smp_089460.1	Calpain-B	0	0	13	0
Smp_083870.1	Putative aminopeptidase W07G4.4	0	0	5	0
Smp_099030.1	Casein kinase II subunit alpha	0	0	5	0
Smp_136640.1	Sorcin	0	0	5	0
Smp_085640.1	COP9 signalosome complex subunit 4	0	0	2	0
Smp_079770.1	Probable protein disulfide-isomerase ER-60	0	0	2	0
Smp_090800.1	Xaa-Pro dipeptidase	0	0	3	0
Smp_102040.1	Guanine nucleotide-binding protein subunit beta-2-like 1	0	0	3	0
Smp_072140.1	Rho-related GTP-binding protein RhoC	0	0	5	0
Smp_091470.1	Puromycin-sensitive aminopeptidase	0	0	4	0
Smp_104110.1	Ras-like GTP-binding protein RHO	0	0	4	0
Smp_089100.1	Glutathione hydrolase 7	0	0	12	0
Smp_075800.1	Hemoglobinase	0	0	7	0
Smp_247080.1	Aminopeptidase N	0	0	3	4
Smp_165490.1	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	0	0	3	0
Smp_155560.1	Serpin B9	0	0	2	0
Smp_213550.1	26S proteasome non-ATPase regulatory subunit 14	0	0	2	0
Smp_213240.1	Prolyl endopeptidase	0	0	7	0
Smp_243200.1	Calpain-7	0	0	2	0
	Biogenesis/vesicle trafficking				
Smp_099310.1	Protein transport protein Sec23A	2	0	3	0
Smp_118830.1	F-actin-capping protein subunit alpha-2	2	0	3	0
Smp_131870.2	Syntaxin-binding protein 1	3	0	0	0
Smp_157410.1	Cytoplasmic dynein 1 heavy chain 1	3	0	0	0
Smp_154420.1	clathrin heavy chain	3	0	14	3
Smp_332400.1	Coatomer subunit delta	12	0	3	0
Smp_333910.1	F-actin-capping protein subunit beta	12	0	5	0
Smp_008660.2	Severin	0	0	7	0
Smp_071630.1	Ras-related protein Rab-2B	0	0	11	0
Smp_077720.2	Annexin A5	0	0	4	0
Smp_004910.1	Ras-related protein Rab-14	0	0	4	0
Smp_005670.1	Ras-related protein Rab-11A	0	0	2	0
Smp_014020.1	Cell cycle control protein 50A	0	0	3	0
Smp_010090.1	Charged multivesicular body protein 5	0	0	3	0
Smp_008230.1	Ras-related protein Rab-18	0	0	4	0

Smp_014660.1	Ras-related protein Rab-10	0	0	4	0
Smp_048940.1	Vacuolar protein sorting-associated protein 37B	0	0	2	0
Smp_045710.1	Charged multivesicular body protein 4b	0	0	6	0
Smp_055880.1	Charged multivesicular body protein 1a	0	0	8	0
Smp_032150.1	Charged multivesicular body protein 3	0	0	3	0
Smp_047450.1	Synaptobrevin homolog YKT6	0	0	3	0
Smp_057320.1	Vesicle-fusing ATPase 2	0	0	4	0
Smp_035620.1	Multivesicular body subunit 12B	0	0	2	0
Smp_045500.1	Annexin A7	0	0	4	0
Smp_034870.1	AP-2 complex subunit beta	0	0	5	0
Smp_055870.1	Vesicle-associated membrane protein 8	0	0	7	0
Smp_104670.1	Ras-related protein Rab-8A	0	0	4	0
Smp_146400.1	Syntaxin-binding protein 1	0	0	3	0
Smp_074020.1	AP-2 complex subunit alpha	0	0	6	0
Smp_093230.1	Actin-related protein 10	0	0	2	0
Smp_068530.1	Syntenin-1	0	0	10	0
Smp_092770.1	Coatomer subunit gamma-2	0	0	2	0
Smp_079000.1	Charged multivesicular body protein 1b	0	0	3	0
Smp_104310.1	Ras-related protein Rab-11A	0	0	5	0
Smp_174580.1	Vesicular integral-membrane protein VIP36	0	0	2	0
Smp_169460.1	GTP-binding protein YPTC1	0	0	7	0
Smp_210250.1	Cell cycle control protein 50A	0	0	8	0
Smp_340230.1	Charged multivesicular body protein 2a	0	0	4	0
Smp_245450.1	Coatomer subunit alpha	0	0	3	0
Smp_337410.1	Tumor susceptibility gene 101 protein	0	0	3	0
Smp_163580.1	Ras-related protein Rab6	0	0	2	0
	Antioxidants				
Smp_194940.1	Prostamide/prostaglandin F synthase	4	0	7	0
Smp_158110.1	Thioredoxin peroxidase	3	3	7	3
Smp_309480.1	Thioredoxin peroxidase 2	8	6	9	5
Smp_008070.1	Thioredoxin-2	0	3	2	0
	Membrane structure				
Smp_121160.2	Mitochondrial dicarboxylate carrier	2	0	0	0
Smp_103200.1	V-type proton ATPase subunit D	2	0	8	0
Smp_099890.1	Receptor expression-enhancing protein 5	2	2	5	2
Smp_104270.1	Bis(5'-adenosyl)-triphosphatase enpp4	2	0	20	2
Smp_127820.1	SJCHGC05417 protein	2	0	0	2
Smp_130300.1	Sodium/potassium-transporting ATPase subunit alpha	3	0	12	0
Smp_130280.1	cell polarity protein	3	0	0	2
Smp_141010.1	Dysferlin	3	0	0	2
Smp_340630.1	putative endoplasmin	15	0	0	10
Smp_313560.1	alkaline phosphatase	9	4	17	6
Smp_315900.1	Plasma membrane calcium-transporting ATPase 3	10	0	2	0
Smp_005720.1	Aquaporin-3	0	2	5	0

Smp_020370.1	Reticulon-4	0	2	7	0
Smp_017430.1	23 kDa integral membrane protein	0	4	16	0
Smp_012440.1	Solute carrier family 2, facilitated glucose transporter member 1	0	7	13	0
Smp_041460.1	tetraspanin D76	0	2	8	0
Smp_153390.1	putative ectonucleotide pyrophosphatase/phosphodiesterase	0	7	5	3
Smp_155310.1	tetraspanin, putative (*Sm*-TSP-1)	0	2	6	0
Smp_140000.1	putative tetraspanin-CD63 receptor (*Sm*-TSP-4)	0	5	8	2
Smp_162770.1	lysosome-associated membrane glycoprotein	0	2	0	3
Smp_335630.1	tetraspanin 2 (*Sm*-TSP-2)	0	6	7	8
Smp_300190.1	Aquaporin-9 (AQP-9) (Small solute channel 1)	0	3	6	4
Smp_267000.1	ATPase, H+ transporting, lysosomal accessory protein 1	0	2	7	4
Smp_007260.1	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	0	0	3	0
Smp_011560.1	CD151 antigen	0	0	5	0
Smp_029390.1	V-type proton ATPase subunit B	0	0	14	0
Smp_015020.1	Sodium/potassium-transporting ATPase subunit alpha	0	0	22	0
Smp_024820.1	CD9 antigen	0	0	3	0
Smp_048230.1	High affinity copper uptake protein 1	0	0	4	0
Smp_039130.1	NPC intracellular cholesterol transporter 1	0	0	16	0
Smp_055780.1	Multidrug resistance protein 1	0	0	54	0
Smp_040080.1	Glycosylated lysosomal membrane protein B	0	0	7	0
Smp_059530.1	25 kDa integral membrane protein	0	0	3	0
Smp_091650.1	putative phospholipid-transporting ATPase IIB	0	0	20	2
Smp_136710.1	putative calcium-transporting atpase sarcoplasmic/endoplasmic reticulum type (calcium pump)	0	0	7	2
Smp_105680.1	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	0	0	2	0
Smp_105410.1	Solute carrier family 2, facilitated glucose transporter member 3	0	0	4	0
Smp_129820.1	Canalicular multispecific organic anion transporter 2	0	0	9	0
Smp_131890.1	Sodium- and chloride-dependent glycine transporter 1	0	0	2	0
Smp_127940.1	Sodium-driven chloride bicarbonate exchanger	0	0	2	0
Smp_123280.1	Major facilitator superfamily domain-containing protein 1	0	0	15	0
Smp_130230.1	Ras-related protein Rac1	0	0	2	0
Smp_128940.1	Metabotropic glutamate receptor 7	0	0	3	0
Smp_136240.1	Vesicle-associated membrane protein/synaptobrevin-binding protein	0	0	3	0
Smp_141680.1	Fasciclin-1	0	0	2	0

Smp_079220.1	ADP,ATP carrier protein	0	0	3	0
Smp_082810.1	Cell division control protein 42 homolog	0	0	7	0
Smp_069120.1	Synaptotagmin-2	0	0	11	0
Smp_075210.1	Prohibitin-2	0	0	3	0
Smp_127650.1	Secretory carrier-associated membrane protein 1	0	0	2	0
Smp_099150.1	Pleckstrin homology domain-containing family B member 2	0	0	4	0
Smp_162510.1	Solute carrier family 46 member 3	0	0	5	0
Smp_246400.1	Glycosyltransferase 1 domain-containing protein 1	0	0	6	4
Smp_173150.1	CD63 antigen	0	0	2	0
Smp_167240.1	Nicastrin	0	0	3	0
Smp_332300.1	Glucose-6-phosphate exchanger SLC37A2	0	0	2	0
Smp_194920.1	T-cell immunomodulatory protein	0	0	12	3
Smp_246270.1	ATP-binding cassette sub-family B member 6, mitochondrial	0	0	16	4
Smp_196110.1	Ferric-chelate reductase 1	0	0	6	0
Smp_176940.1	High affinity cationic amino acid transporter 1	0	0	4	0
Smp_333250.1	Putative phospholipid-transporting ATPase C4F10.16c	0	0	3	0
Smp_171870.1	Synaptic vesicle 2-related protein; Short=SV2-related protein	0	0	3	0
Smp_244710.1	ATP-binding cassette sub-family A member 3	0	0	2	0
Smp_344440.1	putative tetraspanin 18, isoform 1	0	0	6	14
Smp_343120.1	Plasma membrane calcium-transporting ATPase 3	0	0	13	0
Smp_160160.1	Sialin	0	0	2	0
Smp_340540.1	lysosome membrane protein 2	0	0	6	9
Smp_245370.1	Equilibrative nucleoside transporter 3	0	0	3	0
Smp_190770.1	LanC-like protein 2	0	0	0	3
Smp_248070.1	putative dock	0	0	0	4
Smp_315840.1	Kinase D-interacting substrate of 220 kDa	0	0	0	6
Smp_336620.1	Gamma-aminobutyric acid receptor subunit gamma-3	0	0	0	9
Smp_323680.1	SJCHGC06792 protein	0	0	0	8
Smp_095630.1	CD81 antigen	0	0	3	0

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443

444 ## 4. Discussion

445 Cells release different populations of EVs, which may either derive from the endosomal

446 pathway, formed by inward budding of the MVB membrane and allowing capture of

cytoplasmic cargo (exosomes, 30-150 nm in diameter), or bud directly from their plasma membrane (MVs, 100-1000 nm in diameter) (36). In this work, a proteomic analysis of two subpopulations of EVs secreted from adult *S. mansoni* was performed to gain more comprehensive coverage of these EV proteomes. The 120k pellet vesicles that we isolated fit the characteristics and flotation properties on a iodixanol-density gradient for classification as exosomes (37). Moreover, the size distribution and concentration of both *S. mansoni*-derived 120k and 15k pellet vesicles were analysed by TRPS, allowing us to confirm the presence of these EV types. Importantly, well-standardized techniques (38) were used to isolate these two EV types, giving us confidence in the purity of our vesicle samples.

The 120k pellet sample purified in the present study had a higher purity (number of particles/µg of protein) than the 15k pellet sample. The purity ratio was primarily described for exosomes (26), but, in general, since MVs are physically larger than exosomes, it is expected that they contain more proteins and hence the purity ratio should be lower. However, our results do not show significant differences in size between both types of samples, and we might speculate that the differences in protein composition might be due to the different release pathways followed by both EV types. Despite our results agreeing with previous studies (14, 26), it is worth noting that purity should be estimated for each organism or type of sample analysed. The particle diameter range for the two vesicle types appeared to be similar and overlapped (39). Accordingly, the size range of 120k vesicles had a wider range than that of 15k vesicles, however the mean size obtained corresponded to that of exosomes (19). 15k vesicles, however, had a relatively higher concentration (particles/ml) than 120k vesicles for any given size (Figure 2A and 2B). Although apoptotic bodies differ clearly from exosomes by their larger size, other vesicle

types are more difficult to separate since MVs with a similar size to exosomes can also bud at the plasma membrane (40). Moreover, the size overlap observed between the two vesicles analysed can be explained at least in part by the nanopore size used to measure the size distribution of these vesicles – NP150 was used to analyse 120k pellet (60 to 480 nm) whereas NP300 was used for 15k pellet vesicles (115 to 1150 nm). Several studies have reported helminth EVs that correspond to exosomes in terms of vesicle size, including *S. mansoni* (14, 15, 41), other trematodes (17, 42-45), nematodes (21, 34, 35, 46-51), and cestodes (52-54). To our knowledge, only one study was done prior to ours involved helminth-derived MVs which reported a 15k pelleted fraction from *F. hepatica* (44) with size range of 50-200nm and, furthermore, 15k pellet vesicles analysed in this study were within the expected size range for mammalian MVs (19). Using sequential extraction to attribute proteins to sub-vesicular compartments of EVs coupled with highly accurate tandem mass spectrometry, we identified more than twice as many 120k vesicles proteins than reported earlier (14).

To determine the location of each protein within their respective vesicles, we performed a sequential extraction as describe previously for *F. hepatica*- (44) and *Mycoplasma hyopneumoniae*-derived exosomes (27). Proteins identified with high abundance following trypsin shaving of both 120k and 15k pellet vesicles include a fatty acid binding protein with a role in biosynthesis of fatty acids and cholesterols in schistosomes (55), the antioxidants thioredoxin peroxidase (56) and GST (57, 58), dynein light chain (59) and membrane structure proteins, for example the Sm13 tegument antigen (60). Proteases and peptidases including calpain, cathepsins B, cathepsin L, cathepsin A, legumain, and a Pro-Xaa carboxypeptidase were also identified following trypsin shaving of both vesicle types analysed but with relatively less abundance. Similar peptidases were released following

trypsin shaving of the *F. hepatica*-derived EV surface (44). Whether these proteases and peptidases, many of which do not contain transmembrane or other membrane-anchoring motifs, are found on the surface of EVs *in vivo* remains to be determined. Proteases and other enzymes have been reported on the surface of mammalian exosomes following invagination of the cell membrane whereby molecules on the cell surface become localized to the luminal side of the endosomal membrane. Some of these proteases then make their way to the exosome surface through small invaginations of the endosomal membrane where they remain once the multi-vesicular body forms and eventually fuses with the cell membrane to release the newly formed exosome into the extracellular space (61). Another possibility is that soluble proteases bind to the exosome surface within either the endosomal compartment or following secretion of the exosomes into the extracellular space. Once localized to the exosome surface, proteases are free to encounter substrates either at the cell surface or within the extracellular matrix, including ectodomain shedding (62). Interestingly, most of the proteases associated with the mammalian cell surface are metalloproteases, particularly matrix metalloproteases (62), whereas there was only a single MMP (nardilysin or ADAM17) identified on the 15k vesicles surface and none were detected on 120k vesicles despite the presence of a family of leishmanolysin-like clan M metallopeptidases in the *S. mansoni* genome (63). Instead, cysteine proteases were abundantly represented on the surface of the schistosome vesicles, reflecting their genome-wide over-representation in many blood-feeding helminths (64), and suggestive of a role for EVs in feeding.

HSP-70 (65, 66) and TSPs (38, 67) are widely considered as biomarkers of EVs from mammalian cells; for example, HSP-70 has been reported from MVs (19, 68, 69) and the TSPs CD9, CD63 and CD81 have been found in exosomes, MVs and apoptotic bodies (40, 70, 71). Similarly, our data support these findings in that HSP-70 was identified in both

120k pellet (identified in cargo and PMP compartments) and 15k pellet (TLP and PMP compartments). Likewise, members of the TSP family including *Sm*-TSP-1, TSP-2, and TSP-4 were identified in cargo, PMP and IMP compartments of both vesicle analysed. Our data presented herein revealed that 15k and 120k vesicles share many proteins, notably the surface membrane TSPs, so distinguishing EV types by their proteomic content requires caution. While unique proteins were identified in both 120k and 15k pellet vesicles, confirmation of the specificity of these markers to each vesicle population, by western blotting or immunogold transmission electron microscopy, will be an important focus of future studies. A recent study has identified annexin-1 and CD63 (a protein from the tetraspanin family) as specific markers in MVs and exosomes, respectively, from human cells (69). We have identified several annexins that are present only in the 15k pellet vesicles; all tetraspanins identified were present in both populations. This highlights the difficulty in obtaining specific markers for non-human and non-mammalian EVs and the need to advance the field in this direction. Now that a catalogue of shared and unique EV sub-type proteins has been produced, antibodies to select candidate proteins can be generated to aid in classifying the various cellular/tissue origins of helminth EVs and to monitor their trafficking and release.

Previously described schistosomiasis vaccine candidates were identified in both 120k and 15k vesicles, including TSP-2 (72), GST (73, 74), Sm29 (75) and calpain (76-78), whereas Sm23 (73, 79) was identified from cargo and IMP components of 15k vesicles only. Indeed, TSP-2, GST, and Sm29 were among the most abundant proteins identified in both EV types, further supporting the notion of EVs as a major reservoir of vaccine candidates against schistosomiasis and other helminth infections. Our data also showed clear abundant of schistosome surface antigens such as TSP-1,TSP-2, TSP-4, as well as TSP-18 (relatively with less abundance) and a variety of other tegumental antigens in both EV

types analysed, some of which have been implicated in schistosome evasion of the host immune response (80, 81). Although TSP-1 has been reported to have vaccine efficacy in a mouse model (72), TSP-4 and TSP-18 remain to be tested. EVs secreted by other related trematodes were also found to be abundant in TSPs (44, 82). Further, antibodies generated against an EV surface TSP were able to block the internalization of *O. viverrini* EVs by human cholangiocytes (45), and EVs and recombinant TSPs from *O. viverrini* and other helminth parasites have been shown to be efficacious as vaccines in animal models (22, 23, 51).

One of the proteins identified from the integral membrane component of both vesicle types was annexin, a protein involved in a wide range of cellular processes, and, notably has an anti-inflammatory role (83). Moreover, annexins function as plasminogen receptors and enhance fibrinolysis-prolonging blood clot formation (84), roles that are likely to be important for an intra-vascular parasite. Together with the identification of other proteins with known and proposed roles in red cell lysis (saponin-like proteins) (85), haem storage (ferritin isoforms) and blood feeding (endoproteases and aminopeptidases), a role is emerging for EVs in the nutrient acquisition process in haematophagous trematode parasites. Moreover, enolase, found in both EV types in this study, has been identified as a tissue plasminogen activator which subsequently results in the generation of the potent fibrinolytic agent plasmin which could degrade blood clots forming around *S. mansoni* parasites *in vivo* (86).

Proteins involved in vesicle biogenesis and trafficking were particularly abundant in the IMP compartment of both vesicle types. Among these were members of the endosomal sorting complex required for transport-I and -III pathways with known essential roles in vesicle biogenesis and secretion in mammalian cells (87). Charged MVB proteins such as

chromatin modified protein (chmp1) were identified only in 120k pellet. It was unexpected to identify proteins involved in exososme biogenesis also in 15k vesicles. While every effort was made to minimise the occurrence of contamination of 15k sample by 120k pellet, we could not discount the possibility of isolation of 120k vesicles (if at all) in 15k pellet sample preparation. Moreover, while maximum effort was dedicated to keeping our ES, and hence EV samples, free of any egg-secreted molecules, the possibility of minor contamination with egg secretions cannot be wholly discounted. Even though the generation of MVs and exosomes occurs at distinct sites within the cell, common intracellular mechanisms and sorting machineries are involved in the biogenesis of both entities (reviewed in (19)). In many cases, these shared mechanisms hinder the possibility of distinguishing between the different vesicle sub-populations (20). Further, Rab GTPases/Ras family proteins (identified in IMP and PMPs) such as Rab-2A, Rab-7a, Rab-27A and Ral-A identified only in 120k pellet and Rab-2B, Rab 6, Rab-10, Rab-14 and Rab-18 in 15k pellet were identified, while Rab-8A as well as Rab-11A were identified from both vesicle types analysed. Ral-A is a small GTPase required for MVB biogenesis and EV release in *Caenorhabditis elegans* (88) and *F. hepatica* (89). In contrary to our findings, Rab10 has been detected in *S. japonicum*- (17) and *F. hepatica*-derived exosomes (89). It was unexpected to identify Rab proteins, usually involved in 120k vesicle biogenesis and trafficking (90), also in 15k samples, but this might be explained by the reasons mentioned earlier. Rab proteins are one of the main regulators of intracellular EV trafficking between subcellular compartments through processes including vesicle budding, mobility along the cytoskeleton, and membrane fusion (91).

5. Conclusions

Herein, we have isolated and characterised the proteomic composition of two sub-populations of *S. mansoni*-secreted EVs (120k and 15k pellet vesicles) using sequential extraction of proteins from the different sub-vesicular compartments to provide a comprehensive molecular snapshot. Future research should investigate whether *S. mansoni* EVs interact with defined host cell types as it is unclear whether they are internalised by host cells. A more comprehensive understanding of schistosome EV biology will facilitate development of methods to interrupt vesicle-mediated communication between host and parasite, as well as inter-parasite interactions. Schistosome EVs are clearly a rich source of target antigens for the development of novel approaches for preventing, treating and diagnosing infections, and we hope that further research continues to add to the schistosomiasis control toolbox.

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Appendix. SUPPLEMENTARY DATA

Supplementary Table 1. Surface-exposed proteins released by trypsin treatment of *Schistosoma mansoni*-derived 120k pellet vesicles.

Supplementary Table 2. Intra-vesicular cargo proteins identified after lysis of *Schistosoma mansoni*-derived 120k pellet vesicles.

Supplementary Table 3. Integral membrane proteins from *Schistosoma mansoni*-derived 120k pellet vesicles.

Supplementary Table 4. Peripheral membrane proteins proteins from *Schistosoma mansoni*-derived 120k pellet vesicles.

Supplementary Table 5. Surface-exposed proteins released by trypsin treatment of *Schistosoma mansoni*-derived 15k pellet vesicles.

Supplementary Table 6. Intra-vesicular cargo proteins identified after lysis of *Schistosoma mansoni*-derived 15k pellet vesicles.

Supplementary Table 7. Integral membrane proteins from *Schistosoma mansoni*-derived 15k pellet vesicles.

Supplementary Table 8. Peripheral membrane proteins proteins from *Schistosoma mansoni*-derived 15k pellet vesicles.

Supplementary Table 9. Protein families identified from *Schistosoma mansoni*-derived 120k pellet vesicles.

Supplementary Table 10. Protein families identified from *Schistosoma mansoni*-derived 15k pellet vesicles.

Supplementary Table 11. Signal peptide predictions for proteins identified from *Schistosoma mansoni*-derived 120k pellet vesicles.

Supplementary Table 12. Signal peptide predictions for proteins identified from *Schistosoma mansoni*-derived 15k pellet vesicles.

Supplementary Table 13. Transmembrane domain predictions for proteins identified from *Schistosoma mansoni*-derived 120k pellet vesicles.

Supplementary Table 14. Transmembrane domain predictions for proteins identified from *Schistosoma mansoni*-derived 15k pellet vesicles.