Veterinary Parasitology 236 (2017) 22–33

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

# Isolation and characterization of exosomes derived from fertile sheep hydatid cysts

Mar Siles-Lucas[a,*], Carlos Sánchez-Ovejero[a], María González-Sánchez[a], Esperanza González[b], Juan Manuel Falcón-Pérez[b,c], Belgees Boufana[d,e], Federica Fratini[d], Adriano Casulli[d,e], Raúl Manzano-Román[a]

[a] Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), Cordel de Merinas 40-52, 37008 Salamanca, Spain
[b] Laboratory of Exosomes, Metabolomics Unit. CIC bioGUNE, CIBERehd, Derio, Spain
[c] IKERBASQUE, Basque Foundation for Science, Bilbao, Spain
[d] Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
[e] WHO Collaborating Centre for the Epidemiology, Detection and Control of Cystic and Alveolar Echinococcosis (in Animals and Humans), Italy

ARTICLE INFO

*Article history:*
Received 19 September 2016
Received in revised form 16 January 2017
Accepted 21 January 2017

*Keywords:*
*Echinococcus granulosus*
Cystic echinococcosis
Extracellular vesicles
Exosomes
Proteomics
Virulence factors

ABSTRACT

Cystic echinococcosis (CE) is a chronic and complex zoonotic disease. Information on the mechanisms involved in parasite establishment, growth and persistence remain limited. These may be modulated by a crosstalk between extracellular vesicles (EVs). EVs including exosomes and microvesicles are able to carry developmental signaling proteins which coordinate growth and establishment of several parasites. Here, an exosome enriched EV fraction was isolated from hydatid fluid (HF) of fertile sheep cysts. A proteomic analysis of this fraction identified a number of parasite-derived vesicle-membrane associated proteins as well as cytosolic proteins. Additionally, the exosomal enriched fraction contained proteins of host origin. Specific proteins –antigen B2 and TSPAN14- in the exosomal fraction were further assayed by immunoblot and transmission electron microscopy. To the best of our knowledge, this is the first report on the presence of parasite exosomes in fertile hydatid cyst fluid. Further characterization of the exosome cargo will allow the discovery of new markers for the detection of CE in humans and animals, and the treatment of CE patients, and provide new insights regarding the role of these EVs in the establishment and persistence of hydatid cysts.



## 1. Introduction

Cystic echinococcosis (CE) is a chronic zoonotic disease caused by the metacestode stage of *Echinococcus granulosus* sensu lato (s.l.) species complex and is typically transmitted in a pastoral life cycle involving sheep and dogs. CE has a worldwide social and economic impact on veterinary and public health (Cardona and Carmena (2013), being the cause of million USD annual economic burdens in many countries (Budke et al., 2006; Benner et al., 2010; Venegas et al., 2014). In Europe, high human CE incidence rates are found in pastoral areas e.g., in the Mediterranean basin (Rojo-Vazquez et al., 2011; Brundu et al., 2015; Tamarozzi et al., 2015; Rossi et al., 2016; Van Cauteren et al., 2016; Herrador et al., 2016).

The metacestode stage, or hydatid cyst consists of a fluid-filled bladder-like structure with an inner germinative layer (GL) surrounded by the acellular, parasite-secreted laminated layer (LL), and an adventitial layer generated as a consequence of the host inflammatory response. The LL is a microfibrillar matrix composed mainly of high-molecular-weight carbohydrates and represents the first line of defense for survival of the cyst both mechanically and immunologically (Díaz et al., 2011a, 2011b). The GL reproduces asexually into the cyst lumen, resulting in the formation of brood capsules containing protoscoleces. The GL actively secretes the LL components to the outer surface of the cyst and, together with the protoscoleces, produces different molecules found in the hydatid fluid (HF) contained within the cyst. HF also contains host plasma proteins (e.g., albumin and immunoglobulins), components that cross the adventitial and LL barriers by unknown mechanisms (Silva-Álvarez et al., 2016). Some of the HF components, including antigens B and antigen 5, reach the host and elicit a strong specific antibody response, but the mechanism involved in their transport across the cyst membranes remains unknown.

Understanding parasite biology is needed in order to elucidate and interfere with the mechanisms by which the parasite pro-

* Corresponding author.
*E-mail address:* mmar.siles@irnasa.csic.es (M. Siles-Lucas).

http://dx.doi.org/10.1016/j.vetpar.2017.01.022
0304-4017/

gresses to the chronic stage of the disease. In this sense, potential virulence factors are crucial for parasite survival and could provide new markers with potential application in the treatment, diagnosis and prognosis of CE patients. Novel specific and specialized mechanisms based on cell-to-cell communication through secreted extracellular vesicles (EVs) have recently been proposed to be highly relevant in this context (Coakley et al., 2015). Additionally, EVs from some parasites are known to transport parasite molecules to different host cells where their effect is exerted, e.g. for immune evasion (Schorey et al., 2015). These EVs include proteins, carbohydrates, lipids, microRNAs and other small RNAs, and are mainly found in specific vesicles known as exosomes.

Exosomes have been identified in a number of parasite species, including several nematodes, trematodes and protozoa, and their influence on the host cells of some of these groups has recently been reported (Schorey et al., 2015). To the best of our knowledge, no studies to date have been conducted on EVs derived from *E. granulosus* hydatid cysts. This is the first report on the isolation of EVs and the purification of exosomes from *E. granulosus* sheep fertile hydatid cysts and their proteomic characterization.

## 2. Material and methods

### 2.1. Parasite material

Hydatid cyst fluid (HF) was aseptically obtained from fertile sheep hydatid cysts at the Coreses slaughterhouse (Zamora, Spain) using sterile syringes. The HF from individual cysts was placed in 50 mL falcon tubes and kept on ice during transportation. HF was analyzed under a microscope to determine the presence and viability of protoscoleces by checking flame cell activity. Protoscoleces and other solid material were removed from the HF by centrifugation at 2000 × *g* for 3 min. Protoscoleces-free HF (200 mL) from 15 different fertile cysts exhibiting at least 90% of viable protoscoleces was pooled and stored at −80 °C.

### 2.2. Extracellular vesicles isolation, exosome purification and characterization

EVs were isolated from the pooled HF as follows: 200 mL of clear HF was centrifuged for 15 min at 5000 × *g*. The supernatant was filtered using low-protein binding 0.22 μm pore filters (Whatman Klari-Flex). The filtered HF was subjected to ultracentrifugation at 110,000 × *g* for 2 h at 4 °C using a Beckman Coulter Optima-XL ultracentrifuge (Beckman Coulter, Spain) with a 70Ti low-angle small volume fixed-angle rotor. The pelleted EVs were then washed with PBS and further ultracentrifuged at 110,000 × *g* for 2 h at 4 °C. The obtained EVs were resuspended in 0.5 mL PBS and stored at −80 °C.

For exosome purification, a discontinuous iodixanol gradient was used as described by Greening et al. (2015) with minor changes. Briefly, solutions of 5, 10, 20 and 40% iodixanol were prepared by mixing appropriate amounts of homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCL, pH 7.4) with an iodixanol working solution (0.25 M sucrose, 6 mM EDTA, 60 mM Tris-HCl, pH 7.4) and a stock solution of OptiPrepTM (60% w/v) aqueous iodixanol solution (Axis-Shield). The gradient was obtained by layering 4 mL of 40%, 4 mL of 20%, 4 mL of 10% and 3.5 mL of 5% solutions on top of each other in a 26.3 mL polyallomer centrifuge tubes (Beckman Coulter). Subsequently, 0.5 mL of PBS containing EVs was overlaid on top of the gradient which was then centrifuged for 18 h at 110,000 × *g* at 4 °C.

Gradient fractions of 1 mL were collected from the top of the gradient and their density directly measured by weighing. Each fraction was then diluted with 10 mL PBS and centrifuged for 3 h at 110,000 × *g* at 4 °C. The resulting pellets were resuspended in 200 μL nuclease-free water and stored at −80 °C for exosome visualization or lysis with RIPA buffer for protein extraction.

Size distribution within exosome-enriched EVs preparations were analyzed by measuring the rate of Brownian motion using a NanoSight LM10 system equipped with a fast video capture and particle-tracking software (NanoSight, Amesbury, U.K.). This nanoparticle tracking analysis (NTA) enables the determination of individual particle size distribution using samples in liquid suspension (Gercel-Taylor et al., 2012). The analysis settings were as follows: detection threshold, 10 Multi; frames processed, 767 of 768; frames per second, 25.62; calibration, 166 nm/pixel; blur, auto; min track length, auto; min expected size: 80 nm; temperature: 24 °C; viscosity: 0.90 cP. The size information of the Optiprep enriched exosomes, including the mean, mode, median and distribution was obtained after analyzing a recorded video.

Cryo-electron microscopy was performed on the same preparations applying a 4 μL droplet of the exosome enriched suspension to a 200 mesh R 2/2 Quantifoil® holey-carbon grid (Quantifoil, Germany). The excess solution was removed using Whatman filter paper and the grid was rapidly plunged into liquid ethane and transferred under liquid nitrogen into the microscope using a side entry nitrogen-cooled Gatan 914 cryoholder (Gatan, USA). Sample analysis was carried out using a JEOL JEM 2200F (Cs = 1.4 mm) transmission cryoelectron microscope (JEOL, USA), with an acceleration voltage of 200 kV and defocus ranging from −1.2 to −2.5 μm, accurately determined by using enhanced power spectra. Images were recorded under low dose conditions (10 electrons per A2) with a 2k × 2k Gatan Utrascan™ 1000 CCD camera (Gatan, USA).

### 2.3. Proteomic analysis

For the quantification and visualization of proteins in the obtained exosomal fraction, pelleted exosomes were lysed with 200 μL RIPA buffer. The proteins were then reduced and concentrated in a 7.5% polyacrylamide gel. The gel was stained with SYPRO Ruby (ThermoFisher, Spain) following the manufacturerís instructions, for the accurate relative quantification of the protein bands intensity. This was done by image analysis with a ChemiDoc MP Imaging system (Bio-Rad, Spain) in comparison with standards of known concentration. After that, a 20 μg sample was loaded and resolved onto 12% stacking polyacrylamide gels following staining with silver nitrate (mass spectrometry compatible). The stained gel was sliced into twelve portions including all the proteins at different molecular weights. Each slice was digested with trypsin (Kreimer et al., 2015), and the supernatant was purified with C18 stop-and-go two-dimensional chromatography (Rappsilber et al., 2003). The resulting peptides were analyzed in three technical replicates (three injections) by firstly loading in 1% formic acid on a C18 RP-pre-column Symmetry (5 μm particle size, 20 mm × 180 mm; Waters, USA), then on a BEH RP-C18 column (1.7 μm, 7.5 cm × 25 cm; Waters, USA) and ran in LC–MS/MS CID Top20 mode using a 120 min linear gradient to 40% ACN. Mascot software v2.3.2 (Matrix Science, UK) was used to generate peak lists from raw files and to perform database searches against *Echinococcus granulosus* and *Ovis aries* entries in protein databases (Swiss-Prot and TrEMBL). Only proteins with at least one unique peptide with 1% FDR confidence and mascot ion score of 20 were selected for further analyses. Each gel portion was treated as described above using three replicates. The “in silico” studies of the subcellular location and Gene Ontology (GO) assignments for the identified proteins were performed using the UniProt software.

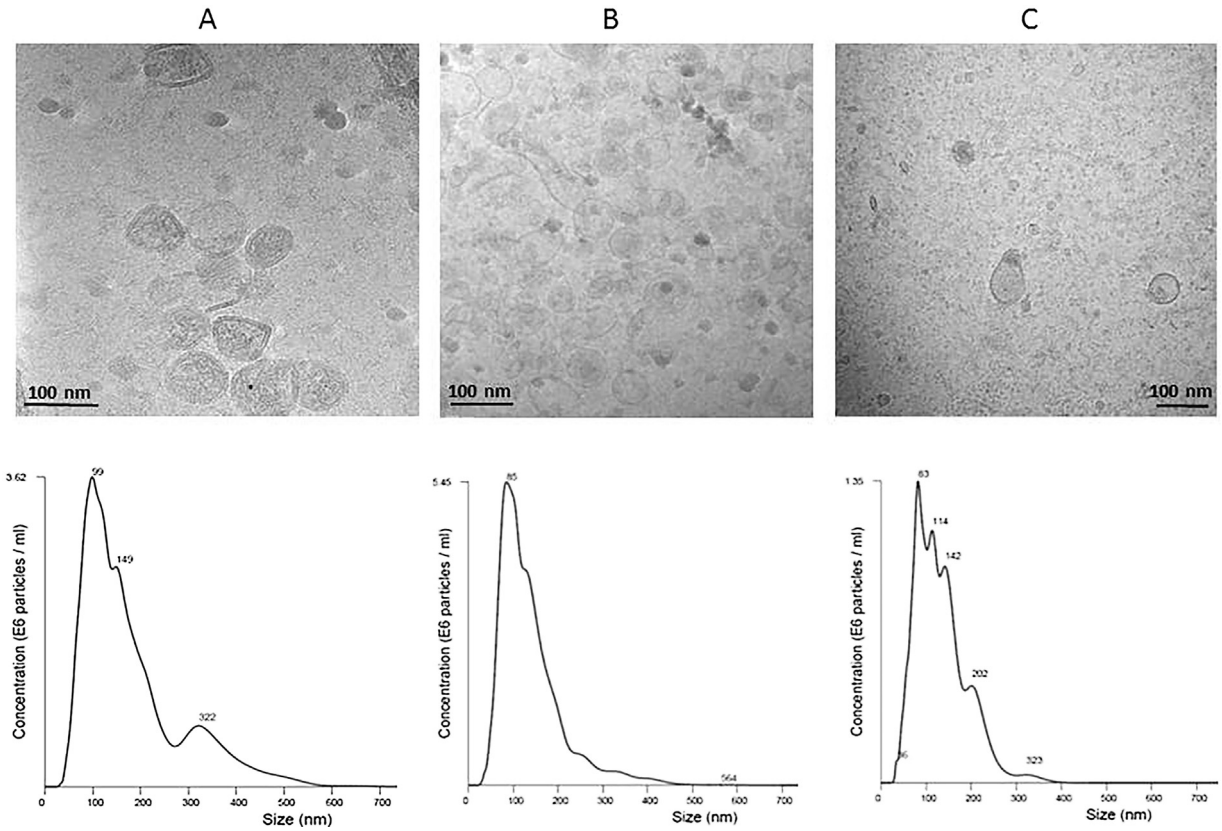


**Fig. 1.** Nanosight analysis of the OptiPrep density gradient fractions 6 (A), 7 (B) and 8 (C) containing hydatid fluid exosome-like vesicles. Isolated exosomes derived from fertile sheep hydatid cyst fluid were visualized by cryo-electron microscopy (upper images) and the size of particles in each fraction analyzed (lower graphs). Bars in the upper images represent 100 nm. Graphs indicate the concentration of particles per mL (Y axis) and the size of the particles in nm (X axis) found in each fraction.

### 2.4. Sequence comparison

Protein sequences identified in our exosomal preparation were compared with published *E. granulosus* protein sequences from proteomic studies of HF (Monteiro et al., 2010; Aziz et al., 2011), protoscoleces (somatic extract) (Monteiro et al., 2010; Hidalgo et al., 2016), GL (somatic extract) (Monteiro et al., 2010) and protoscoleces (secretory components) (Virginio et al., 2012). In addition, sequences identified in proteomic studies of *Fasciola hepatica* (Cwiklinski et al., 2015; Marcilla et al., 2012) and *Echinostoma caproni* exosomes (Marcilla et al., 2012) were also included for comparison.

UniProt IDs from the sequences identified in our exosomal preparation were transformed in GenBank IDs using UniProt. Sequences from each of the abovementioned publications were retrieved and compared with those in GenBank under non-redundant protein sequences (nr) restricted to *E. granulosus* (taxid:6210) using BlastP. Proteins were considered similar when their identity and coverage were ≥90% for *E. granulosus* sequences described by others and when their identity was ≥50% and their coverage was ≥75% for *F. hepatica* and *E. caproni* sequences. Genbank IDs of *E. granulosus* (taxid:6210) sequences fulfilling those cutoff values were compared with the GenBank IDs of the sequences identified in our exosomal preparation and coincident IDs were selected.

### 2.5. Immunoblot

Total HF and the protein extract derived from the exosomal preparation (15 μg each) were electrophoresed using a 15% acrylamide gel under reducing conditions and transferred onto a nitrocellulose membrane. The membrane was treated with blocking buffer (PBS plus 0.5% Tween 20 and 5% skimmed milk) for 1 h at 37 °C. A rabbit anti-B2 hyper-immune serum was obtained by immunization using 100 μg of the recombinant B2t antigen (Hernández-González et al., 2008) plus 1 mg saponin in PBS, in three subcutaneous weekly injections. The transferred proteins were incubated with this rabbit anti-B2 hyper-immune serum, the commercial rabbit anti-TSPAN14 antibody (Bioss Antibodies) and with a pre-immune rabbit serum, at 1:100 or 1:50 dilution in blocking buffer for 1 h at 37 °C. After washing with PBS plus 0.5% Tween 20 (three washes of 5 min each), strips containing the electrophoresed proteins were incubated with a peroxidase-labelled anti-rabbit total IgG (Sigma) at 1:2000 dilution in blocking buffer for 1 h at 37 °C. The reaction was developed with 5 mg of 4-Cl-naphtol and 20 μL $H_2O_2$ in 10 mL methanol plus 40 mL PBS. After developing, the reaction was stopped with distilled water.

### 2.6. Immuno-electron microscopy

Immuno-transmission electron microscopy with specific antibodies was performed to validate the presence of the antigen B2 identified by proteomics in the exosomal enriched preparation. For this analysis, the exosomes were diluted 1:10 in PBS and deposited on Formvar carbon-coated grids (Agar Scientific, UK) by glow discharge. These were first blocked for 10 min with 10% FBS and later separately immune-labeled with a rabbit anti-B2 hyperimmune serum, a rabbit anti-TSPAN14 antibody and with the corresponding pre-immune serum at 1:100 dilution in PBS plus 5% FBS for 30 min at room temperature, followed by incubation with protein A conjugated to 10-nm gold particles (Cell Microscopy Center, Utrecht University, The Netherlands). Samples were stained with uranyl

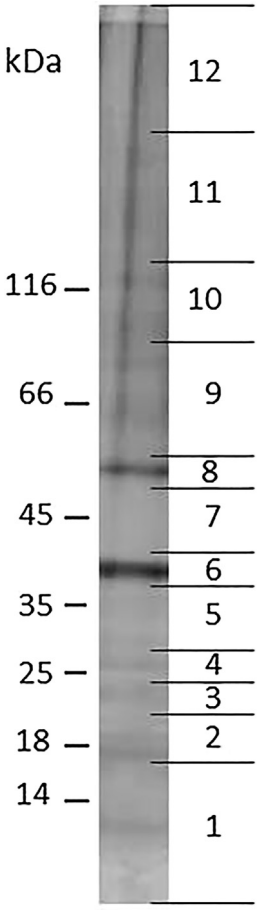


**Fig. 2.** SDS-PAGE (12%) profile of hydatid fluid exosomal proteins stained using silver nitrate. Fractions used for further MS/MS analysis and their relative molecular weight in kDa are indicated.

acetate and visualized using a JEOL JEM 1010 electron microscope (Tokyo, Japan) at 80 kV. Images were recorded with a 4k × 4k CMOS F416 camera from TVIPS (Gauting, Germany).

## 3. Results

### 3.1. Hydatid fluid exosome like vesicles: size, concentration and appearance

Fractions of density 1.07, 1.09 and 1.12 g/mL obtained after purification and fractionation of the EVs present in HF were analyzed by NTA. This analysis showed the presence of vesicles mainly in the first two fractions (Fig. 1). The NTA also showed the vesicles main peak sizes to range between 80 and 100 nm, which was compatible with the size of exosomes (Fig. 1). The concentration of exosomes obtained from 200 mL of HF was 21,128 particles/mL.

### 3.2. The proteome of E. granulosus exosome-like vesicles: identified proteins

After RIPA lysis of exosomes, the protein concentration was determined by the BCA method and 20 μg of the sample was electrophoresed on a 12% SDS-PAGE gel and stained using silver nitrate (Fig. 2). Protein bands were distributed with a wide molecular weight range (14–116 kDa), with two major bands of 40 and 50 kDa. Due to the complexity of the protein sample and the presence of major bands that could interfere with the identification of the total extract by MS/MS, twelve different gel slices were cut and used for further analysis to identify the exosome-like vesicles proteome (Fig. 2).

**Table 1**
Ten most common proteins identified in the proteome of exosome-like vesicles from *Echincococus granulosus* fertile sheep hydatid fluid.

| ID number | Protein name | Mascot score | EXOCARTA |
|---|---|---|---|
| I1WXU1 | Antigen5 | 23097 | – |
| A0A068WT80 | Alpha-mannosidase | 3331 | YES |
| A0A068X1L3 | Malate dehydrogenase | 3289 | YES |
| W6UUI4 | Severin/Gelsolin/Villin | 3124 | YES |
| A0A068WWM7 | Lipid transport protein | 2801 | – |
| Q6UZD9 | Antigen B subunit 4 | 2240 | – |
| A0A068WPN7 | Expressed protein | 1903 | – |
| U6J7F4 | Syndecan binding protein syntenin | 1897 | YES |
| Q24796 | EG10 (ezrin/moesin/radixin) | 1896 | YES |
| D1MH02 | Antigen B 4/1 | 1877 | – |

The MS/MS proteomic analysis of the sliced bands revealed that proteins of parasite and host origin were both present in the preparation. A total of 663 and 87 unique proteins were identified for *E. granulosus* and *O. aries* respectively (Supplementary Tables 1 and 2). Table 1 shows the 10 most abundant parasite-derived proteins. Among these, were some of the most relevant diagnostic antigens, including antigen 5, antigen B and EG10 which are identical to *E. granulosus* moesin/ezrin/radixin. Additional specific parasite antigens that were found to be abundantly represented in HF exosomes were the expressed protein A0A068WPN7 that includes a transmembrane domain, and the lipid transporting protein similar to *E. granulosus* apolipophorin A0A068WWM7. Molecules commonly found in exosomes from other organisms (Keerthikumar et al., 2016) were also among the most abundant proteins in our preparation, including alpha-mannosidase, malate dehydrogenase, severin, ezrin and syntenin.

Signatures of exosomes identified in HF exosome-like vesicles included tetraspanins, transporters and channels (ATPase and $Cl^-$ channels, lysosome-associated membrane proteins 1 and 2 and multidrug resistance associated protein), as well as molecules related to vesicle trafficking and multivesicular body biogenesis (annexins, Rabs, Rap 1B, RHO protein GDP dissociation inhibitor, actin-related protein 1/2, syntaxin, WD repeat containing proteins, ALIX, clathrin, tumor necrosis factor (TNF)-stimulated gene 101 and ubiquitin) (Fig. 3). Moreover, proteins often identified in exosome proteomic analysis were also identified here, including several membrane related and luminal exosomal proteins (Fig. 3) (Théry et al., 2009; Frühbeis et al., 2012; Natasha et al., 2014). These include several cytoskeletal proteins (actin, ezrin, fascin, fibronectin, gelsolin, moesin, myosin, perlecan, profilin and tubulins), enzymes (ATPases, dihydropyrimidinase related proteins, enolase, gapdh, peroxiredoxin, phosphoglycerate kinase 1, pyruvate kinase and aspartate amino transferase), molecules related to signal transduction (14-3-3, ADP-ribosylation factor 1, CDC42, epidermal grow factor receptor, G proteins, phosphoinositide-3-kinase, Ras homolog member family A and syntenin), chaperones (HSP70, HSP90 and cyclophilin A) and other molecules commonly found within EVs (ADP ribosylation factor, eukaryotic elongation factors 1A1 and 1A2, ferritin and histones).

A total of 87 host proteins were also identified in our EV enriched fraction (Supplementary Table 2). Comparable, host proteins (n = 73), including immunoglobulins and complement components, were found by Aziz et al. (2011) in HF from fertile sheep cysts. Surprisingly, only 8 of those proteins (carbonic anhydrase, fructose-bisphosphate aldolase, hemoglobin alpha, hemoglobin beta, peroxiredoxin, pyruvate kinase, serum albumin and triose phosphate isomerase) were also among the host proteins identified in our exosome sample. No complement components belonging to *O. aries*, and only three molecules rep-

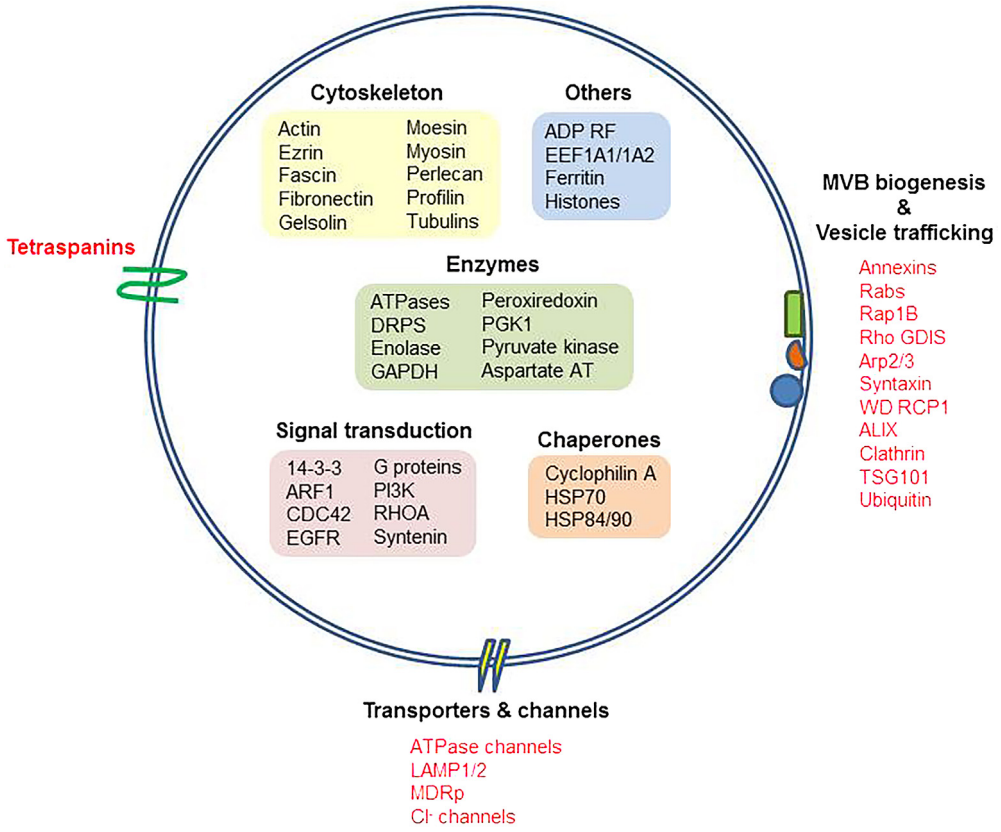


**Fig. 3.** Exosome signatures and exosomal proteins. Exosome signatures (in red) and proteins often identified in exosome proteomic analyses found in the proteome of the exosomes isolated from *Echinococcus granulosus* hydatid fluid of fertile sheep cysts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resenting the immunoglobulin superfamily (Ig lambda chain C region W5PSQ7, beta-2-microglobulin Q6QAT4 and the uncharacterized protein W5PSQ7) were found in our exosomal preparation. Interestingly, at least 25 of the identified *O. aries* proteins are often described as exosome signatures, including 14-3-3 proteins, actin, annexins, Cl- channels, cofilin, elongation factor 1-alpha, ferritin, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, heat shock protein alpha, histones, integrin beta, keratin type I, peroxiredoxin, phosphoglycerate kinase, pyruvate kinase, tetraspanin 14 and vimentin (Supplementary Table 2). Most of the 87 *O. aries* identified proteins (85.2%) have been previously reported in exosomes derived from other organisms (Keerthikumar et al., 2016) (Supplementary Table 2).

### 3.3. Sequence comparison

Sequences of proteins previously described in proteomic studies of exosome-enriched fractions from *F. hepatica* and *E. caproni* were retrieved (Cwiklinski et al., 2015; Marcilla et al., 2012). These studies identified proteins in total extract of vesicle-enriched fractions and included 87 and 73 protein sequences of parasite origin for *E. caproni* and *F. hepatica*, respectively (Marcilla et al., 2012). In addition, others identified on the exosome-surface and luminal proteins encompassed 180 parasite protein sequences in exosome-like vesicles of *F. hepatica* (Cwiklinski et al., 2015). Thirty six of 180 (20%) and 22 of 73 protein sequences (30.1%) in *F. hepatica* EV, and 33 of 87 (37.9%) protein sequences identified in *E. caproni* EV were found to be similar to proteins identified in our HF exosomes (Cwiklinski et al., 2015; Marcilla et al., 2012) (Table 2). From these, the highest proportion may represent exosomal signatures or is related to exosome biogenesis and release. The second most abundant group of proteins commonly identified in platyhelminth exosomes are those with catalytic activity, including nine proteins with peptidase activity (Table 2).

Our sequences were compared to sequences identified in proteomic studies for *E. granulosus* protoscoleces (Monteiro et al., 2010; Hidalgo et al., 2016), GL and HF (Monteiro et al., 2010; Aziz et al., 2011) as well as in the secretome of protoscoleces (Virginio et al., 2012). Proteomic studies of HF identified 64 *E. granulosus* protein sequences corresponding to 56 different proteins (Monteiro et al., 2010; Aziz et al., 2011), of which 38 sequences (59.4%) corresponding to 32 different proteins (57%) were similar to protein sequences identified in our exosomal proteome (Table 3). A total of 18 proteins (19 sequences) out of the 28 (45 sequences) reported for the excreted/secreted proteome of protoscoleces (Virginio et al., 2012) were also found to occur in the HF exosomes of this study, thus sharing 64.3% of proteins (Table 3). The proteome of the GL reported 19 different proteins (23 sequences; Monteiro et al., 2010), 12 of which (63.2%) were found in exosomes described in this study (Table 3). The most divergent proteome among those compared with the HF exosomal proteome was that corresponding to the somatic extract of protoscoleces, for which 106 sequences (80 proteins) were reported (Monteiro et al., 2010; Hidalgo et al., 2016). Only 35 proteins –43.7%- (46 sequences –43.4%-) were similar to sequences found in our exosomal preparation (Table 3).

### 3.4. Gene ontology assignments

Parasite proteins identified in the enriched EVs preparation were assigned to specific molecular functions (Fig. 4). The most common functions were “catalytic activity” and “binding”. Within the first category, “hydrolase activity” was the most common

**Table 2**
Proteins found in exosome-like vesicles of *Echinococcus granulosus*, *Fasciola hepatica* and *Echinostoma caproni*.

| Uniprot ID | GenBank ID | Protein name[3] | *Fasciola hepatica*[1,2] | *Echinostoma caproni*[2] |
|---|---|---|---|---|
| EXOSOME-RELATED[#] | | | | |
| Q56J98 | AAX73175.1 | 14-3-3 protein | BN1106_s3904B000042<br>AAW69422.1 | NP_989173.1 |
| U6JGI4 | EUB54777.1 | 14-3-3 protein beta:alpha | BN1106_s3904B000042<br>AAW69422.1 | NP_989173.1 |
| U6JEE0 | EUB63343.1 | 14-3-3 protein epsilon | BN1106_s3904B000042<br>AAW69422.1 | NP_989173.1 |
| U6J5Z8 | EUB61917.1 | 14-3-3 protein zeta | BN1106_s3904B000042<br>AAW69422.1 | NP_989173.1 |
| U6JLF5 | EUB56079.1 | Actin cytoplasmic A3 | BN1106_s2907B000133<br>XP_002578518.1 | CAX72862.1 |
| A0A068WV33 | CDS21485.1 | Actin cytoplasmic type 5 | BN1106_s2907B000133<br>XP_002578518.1 | CAX72862.1 |
| A0A068WDB3 | CDS16401.1 | Actin modulator protein | BN1106_s2349B000188 | XP_002572341.1 |
| W6UA11 | EUB57870.1 | Beta-enolase | BN1106_s3227B000227<br>Q27655.1 | CAK47551.3 |
| A0A068WIF6 | CDS19854.1 | Beta tubulin | BN1106_s55B000372<br>NP_954525.1 | ABO28786.1<br>XP_002578968.1 |
| U6IVD5 | EUB64783.1 | CDC42 | BN1106_s1908B000177 | |
| U6JME4 | EUB57191.1 | Charged multivesicular body protein | BN1106_s2316B000077 | |
| A0A068WA65 | CDS16566.1 | Charged multivesicular body protein 2a | | ABU40277.1 |
| W6UB32 | EUB55662.1 | Dynein light chain 2, cytoplasmic | | CAX76219.1 |
| W6UDQ8 | EUB58946.1 | Dynein light chain LC6, flagellar outer arm | | CAX76219.1 |
| E4WKZ9 | CBH41146.1 | Elongation factor 1-alpha | BAA25731.1 | AAC47901.1<br>AAS94011.1 |
| A0A068WF73 | CDS16232.1 | Elongation factor 2 | | CAA33804.1<br>CAX73469.1 |
| D0VLV3 | ACY30465.1 | Enolase | BN1106_s3227B000227<br>Q27655.1 | CAK47551.3 |
| U6JNP4 | EUB56065.1 | Ferritin | BN1106_s3950B000041 | |
| A0A096ZQK3 | AIS24256.1 | Glyceraldehyde-3-phosphate dehydrogenase | AAG23287.1 | AAB52408.1<br>AAG23287.1 |
| U6JIC8 | EUB56318.1 | Heat shock 70 kDa protein 4 | BN1106_s309B000234<br>CAA72216.1<br>AAS45710<br>XP_001198336.1 | CAM97394.1 |
| W6UM50 | EUB54574.1 | Heat shock protein HSP 90-alpha | O02192.1<br>AAW27659.1<br>NP_999808.1<br>AAY89890.1 | |
| W6U0E5 | EUB54575.1 | Heat shock cognate protein | BN1106_s309B000234<br>CAA72216.1<br>AAS45710.1<br>XP_001198336.1 | CAM97394.1 |
| U6JCI6 | EUB63925.1 | Histone H2B | | XP_002571395.1 |
| U6JN05 | EUB54192.1 | Histone H4 | BN1106_s10667B000018 | XP_002576634.1 |
| U6J226 | EUB54600.1 | Histone H3 | | AAT85893.1 |
| W6UHV2 | EUB57677.1 | Phosphoenolpyruvate carboxykinase [GTP] | CAB65311.1 | XP_001647935.1 |
| A0A068WNK0 | CDS21325.1 | Phospholipid-transporting ATPase | BN1106_s435B000242 | |
| U6JDZ7 | EUB54636.1 | Rab | BN1106_s844B000259 | |
| U6JRT3 | EUB61729.1 | Rab3 | BN1106_s258B000276 | |
| W6USR3 | EUB64338.1 | Ras-related protein Rab-8B | BN1106_s258B000276 | |
| A0A068WX24 | CDS22256.1 | Ras protein Rab 27A | BN1106_s1172B000096 | |
| U6JF50 | EUB59844.1 | Ras-related protein RABD2A | BN1106_s258B000276 | |
| W6UKC0 | EUB61616.1 | Ras-related protein Rab-10 | BN1106_s258B000276 | |
| W6UUI4 | EUB57054.1 | Severin | BN1106_s2349B000188 | XP_002572341.1 |
| W6UUP9 | EUB57119.1 | Sodium/potassium-transporting ATPase subunit alpha | BN1106_s521B000167 | |
| W6ULS3 | EUB59107.1 | Transforming protein RhoA | BN1106_s1908B000177 | |
| U6J8Q0 | EUB56891.1 | Transgelin | | XP_002572281.1<br>XP_002569800.1 |
| A0A068WVJ1 | CDS23799.1 | Tubulin alpha 1C chain | | CAP72045.1 |
| W6US08 | EUB61142.1 | Tubulin beta-1 chain | BN1106_s55B000372<br>NP_954525.1 | ABO28786.1<br>XP_002578968.1 |
| A0A068WML9 | CDS18873.1 | Tubulin beta 2C chain | BN1106_s55B000372<br>NP_954525.1 | ABO28786.1<br>XP_002578968.1 |
| A0A068WIF4 | CDS19545.1 | Vacuolar protein sorting associated protein 25 | BN1106_s1437B000141 | |

Table 2 (*Continued*)

| Uniprot ID | GenBank ID | Protein name[3] | *Fasciola hepatica*[1,2] | *Echinostoma caproni*[2] |
|---|---|---|---|---|
| CATALYTIC ACTIVITY | | | | |
| A0A068WD13 | CDS15503.1 | Cathepsin B* | BN1106_s793B000177<br>CAA80451.1 | |
| U6IZB4 | EUB62567.1 | Cathepsin L1* | BN1106_s8098B000020<br>AAR99519.1 | |
| A0A068WG50 | CDS18714.1 | Cysteine protease* | BN1106_s8098B000020<br>AAR99519.1 | |
| U6IXV3 | EUB64508.1 | Fructose-bisphosphate aldolase | BN1106_s4469B000065 | AAP06485.1<br>AAA57567.1 |
| U6J231 | EUB57800.1 | Glutamate dehydrogenase | BN1106_s5767B000030 | |
| A0A068WQB1 | CDS19835.1 | Guanine nucleotide binding protein subunit | BN1106_s1568B000271 | |
| W6UBY3 | EUB58066.1 | Guanine nucleotide-binding protein G(O) subunit alpha | BN1106_s378B000162 | |
| U6J0N2 | EUB62177.1 | Leucyl aminopeptidase* | BN1106_s617B000566<br>EUB62177.1 | |
| A0A068WNN8 | CDS19266.1 | Lysosomal Pro X carboxypeptidase* | BN1106_s1620B000120 | |
| A0A068 × 1L3 | CDS23843.1 | Malate dehydrogenase | BN1106_s1459B000183<br>AAT46071.1 | NP_956263.1 |
| W6UE99 | EUB59398.1 | Phosphoglycerate kinase | | AAZ17561.2 |
| A0A068WYA6 | CDS22665.1 | Proteasome subunit alpha type* | BN1106_s9050B000016 | |
| U6IX51 | EUB58437.1 | Proteasome subunit alpha type 6* | BN1106_s1259B000205 | |
| A0A068WI53 | CDS18151.1 | Proteasome subunit beta type* | BN1106_s6770B000051 | |
| A0A068WKP1 | CDS20308.1 | Proteasome prosome macropain subunit beta* | BN1106_s5855B000168 | |
| W6V3D8 | EUB60549.1 | Thioredoxin | BN1106_s4026B000080<br>AAF14217.1 | |
| Q8T6C4 | AAD02002.1 | Thioredoxin peroxidase | BN1106_s1614B000280<br>P91883.1 | ACV91889.1 |
| Q869D6 | AAN63052.1 | Thioredoxin glutathione reductase | CAM96615.1 | |
| A0A068 × 2Y0 | CDS24295.1 | Thioredoxin dependent peroxide reductase | BN1106_s1614B000280<br>P91883.1 | ACV91889.1 |
| A0A068WI25 | CDS19413.1 | Transitional endoplasmic reticulum ATPase | | AAP06321.1 |
| U6JN02 | EUB56511.1 | Triosephosphate isomerase | | AAF79172.1 |
| OTHERS | | | | |
| U6JHY6 | EUB54990.1 | Anoctamin | BN1106_s1581B000120 | |
| A0A068WR99 | CDS20191.1 | AP complex subunit beta | BN1106_s29B000536 | |
| W6UYG6 | EUB58609.1 | GTP-binding protein YPT1 | BN1106_s258B000276 | |
| A0A068W7S5 | CDS15844.1 | Myoferlin | BN1106_s3585B000136 | |
| A0A068WM60 | CDS20819.1 | Polyubiquitin | BN1106_s6576B000103<br>AAG22093.1 | AAG22093.1<br>CAX79713.1 |
| W6VCB0 | EUB64489.1 | Putative phosphoglycerate mutase | | AAX29976.1 |
| U6JJ69 | EUB57999.1 | Ras C3 botulinum toxin substrate 2 | BN1106_s1908B000177 | |
| A0A068W8V8 | CDS16453.1 | Ribosomal protein L40 | BN1106_s6576B000103<br>AAG22093.1 | AAG22093.1 |
| W6V5H0 | EUB61534.1 | T-complex protein 1 subunit beta | | AAX27387.2 |
| U6J7M1 | EUB63307.1 | von Willebrand factor A domain-containing protein 5A | BN1106_s4862B000066 | |

First and second rows of the tables show the UniProt and the GenBank (NCBI) IDs of the proteins identified in the exosomal-enriched fraction analyzed here. [1]Cwiklinski et al., 2015 (showing the IDs of the *F. hepatica* gene model sequences in WormBase ParaSite, http://parasite.wormbase.org/); [2]Marcilla et al., 2012 (showing the IDs of the GenBank database). [3]As annotated in the database. #Involved in exosome biogenesis/release or in exosome composition; *with peptidase activity.

(n = 103), including 35 "peptidase" and "serine type peptidase" proteins (Supplementary Table 1). This category also included 32 proteins in the "oxidoreductase activity" group (Supplementary Table 1). Proteins in the "binding" category included the following 13 "lipid binding" proteins: annexin, clathrin coat assembly protein, four different fatty acid binding proteins, glycolipid transfer protein, huntingtin interacting protein 1, PCTP protein, phosphatidylinositol-binding clathrin assembly protein LAP, phospholipase D, Phox and XK-related protein (Supplementary Table 1).

Gene ontology assignments under "cellular component" showed that the vast majority of the *E. granulosus* assigned proteins are located in the membrane (n = 153), containing a transmembrane helix (n = 128) (Fig. 4). The most common biological process in which the identified and assigned parasite molecules participate is "metabolic process" (n = 199), including 43 proteins related to carbohydrate metabolism and the tricarboxylic acid cycle.

With regards to proteins identified as being of host origin, the top three categories in the three gene ontology groups (molecular function, cellular component and biological process) coincided with those found in the identified parasite proteins (data not shown).

**Table 3**
Comparison of the protein sequences identified in exosomes from hydatid fluid with those described in hydatid fluid and protoscoleces.

| Uniprot ID | GenBank ID | Protein name[5] | HF[1,2] | GL[2] | PPS[2,3] | PPS ES[4] |
|---|---|---|---|---|---|---|
| Q56J98 | AAX73175.1 | 14-3-3 protein | 39015 | | | AAX73175.1 |
| U6JGI4 | EUB54777.1 | 14-3-3 protein beta:alpha | | EGC02757 | EGC02757<br>Q8MM75.1 | |
| U6JEE0 | EUB63343.1 | 14-3-3 protein epsilon | | | EGC02229<br>EGC02546 | EGC02546 |
| Q8MUA4 | AAM94863.1 | 14-3-3 protein homolog 2 | | | | AAM94863.1 |
| U6JLF5 | EUB56079.1 | Actin cytoplasmic A3 | 14260 | | | AAC37175.1 |
| A0A068WV33 | CDS21485.1 | Actin cytoplasmic type 5 | 14260 | | | AAC37175.1 |
| A0A068WDB3 | CDS16401.1 | Actin modulator protein | | | AAK00052.1 | |
| U6J0N8 | EUB62172.1 | Aminotransferase class III | 15805 | | CDJ18265.1 | |
| A0A068WJF9 | CDS20230.1 | Annexin | | | EGC00272 | EGC00272 |
| D1MH02 | ACZ51452.1 | Antigen B 4/1 | 36286 | | | |
| Q6EJE1 | AAP83172.1 | Antigen B subunit 2 | 40047 | | | |
| Q3YFP1 | AAZ76576.1 | Antigen B subunit 3 | 23354 | | | |
| Q6UZD9 | AAQ74963.1 | Antigen B subunit 4 | 36286 | | | |
| I1WXU1 | AFI71096.1 | Antigen 5 | 40093<br>16063<br>EGC03443 | | | |
| W6UXV8 | EUB58399.1 | Antigen B | 23354 | | | |
| A0A068WP33 | CDS19437.1 | Aromatic amino acid decarboxylase | | AAK39122.1 | AAK39122.1 | AAK39122.1 |
| A0A068WSK6 | CDS20657.1 | Aspartate aminotransferase | | | EL744715.1 | |
| A0A068WIF6 | CDS19854.1 | Beta tubulin | | | EGC04051 | |
| U6J063 | EUB64462.1 | Calpain A | | | | EL748263.1<br>EL749164.1 |
| A5YTY7 | ABQ96270.1 | Calreticulin | 81583 | | ABQ96270.1 | |
| A0A068WD13 | CDS15503.1 | Cathepsin B | 12681 | | | |
| U6J0K5 | EUB62203.1 | Citrate synthase | EGC00287 | EGC00287 | EGC00287<br>CDJ18230.1 | |
| W6UB32 | EUB55662.1 | Dynein light chain 2 | | | | EGC00967 |
| W6UDQ8 | EUB58946.1 | Dynein light chain LC6 | | | | EGC00967 |
| E4WKZ9 | CBH41146.1 | Elongation factor 1-alpha | | | EGC00741<br>CDJ17799.1 | BAF63675.1 |
| D0VLV3 | ACY30465.1 | Enolase | 24254 | | EGC03002<br>EGC04828<br>ACY30465.1 | ACY30465.1 |
| Q02970 | AAK51437.1 | Fatty acid-binding protein 1 | 21192 | | EGC00017<br>AAK51437.1 | AAK12096.1 |
| U6JNP4 | EUB56065.1 | Ferritin | 21730<br>82367 | EGC00020 | EGC00020 | |
| U6IXV3 | EUB64508.1 | Fructose-bisphosphate aldolase | 21731<br>EGC00369 | EGC00369 | CDJ17337.1 | EGC00369 |
| Q56JA3 | AAX73170.1 | Glucose-6-phosphate isomerase | 38910<br>Q56JA3 | | Q56JA3 | |
| A0T1W1 | ABK60175.1 | Glutathione S-transferase | 37276 | EGC00080 | EGC00080<br>ABK60175.1 | |
| A0A096ZQK3 | AIS24256.1 | GAPDH | 20892 | | EGC00305 | AAA61574.1 |
| U6JIC8 | EUB56318.1 | Heat shock 70 kDa protein 4 | 38737 | | EGC00004 | |
| W6U0E5 | EUB54575.1 | Heat shock cognate protein | | | EGC00004 | |
| U6JCI6 | EUB63925.1 | Histone H2B | EGC00502 | | | EGC00502<br>EGC03373 |
| U6JN05 | EUB54192.1 | Histone H4 | 11212 | | | |
| A0A068WJX2 | BP187957 | Isocitrate dehydrogenase | | BP187957 | | |
| U6JBW8 | EUB62057.1 | Major egg antigen p40 | 38533<br>EGC00939 | | EGC00939 | |
| A0A068 × 1L3 | CDS23843.1 | Malate dehydrogenase | 21740 | | EGC00028 | AAC28239.1 |
| Q24799 | CAA64731.1 | Myophilin | | | Q24799 | |
| W6UKD6 | EUB61528.1 | Perlecan | 16410 8053<br>EL757670 | | | |
| W6UHV2 | EUB57677.1 | Phosphoenolpyruvate carboxykinase | 38622 | EGC04068 | EGC04068 | |
| W6UE99 | EUB59398.1 | Phosphoglycerate kinase | | | EL740229.1 | EL740229.1<br>GT284890.1 |
| A0A068WM60 | CDS20819.1 | Polyubiquitin | 14646<br>EGC00481 | | | |
| A0A068WGU0 | CDS19295.1 | Prostaglandin H2 D isomerase | | | EGC03317<br>EGC04109 | |
| U6JIQ5 | EUB57723.1 | Protein DJ-1 | 17692 | | EGC00903 | |
| A0A068WL99 | CDS18444.1 | Purine nucleoside phosphorylase | | | EGC02514 | |
| U6J243 | EUB57787.1 | Pyruvate dehydrogenase E1 alpha | | EGC05022 | | |
| A0A068W8V8 | CDS16453.1 | Ribosomal protein L40 | 14646<br>EGC00481 | | | |
| U6JI56 | EUB60311.1 | Sarcoplasmic calcium binding protein | | | EGC04217 | |

Table 3 (*Continued*)

| Uniprot ID | GenBank ID | Protein name[5] | HF[1,2] | GL[2] | PPS[2,3] | PPS ES[4] |
|---|---|---|---|---|---|---|
| W6UUI4 | EUB57054.1 | Severin | | | AAK00052.1 | |
| U6IX85 | EUB57056.1 | Severin | | | CDJ17101 | |
| W6U3E3 | EUB55638.1 | Spondin-1 | 16815 | | | |
| A0A068WLT4 | CDS18591.1 | Superoxide dismutase [Cu-Zn] | | | BU493162.1 | |
| W6U646 | EUB56683.1 | Tegumental protein | | EGC03147 | | |
| Q8T6C4 | AAD02002.1 | Thioredoxin peroxidase | 82975<br>EGC00084 | EGC00084 | EGC00084<br>Q8T6C4 | |
| W6US08 | EUB61142.1 | Tubulin beta-1 chain | | | EGC04051 | |
| A0A068WFA0 | CDS17122.1 | UDP glucose 4 epimerase | | EGC00902 | | |
| A0A068WEG4 | CDS16022.1 | USP6 N terminal protein | | | EL748674.1 | |

First and second rows of the tables show the UniProt and the GenBank (NCBI) IDs of the proteins identified in the exosomal-enriched fraction analyzed here. HF, hydatid fluid; ES, excretory/secretory; PPS, protoscoleces; GL, germinative layer. Sequence ID numbers given by [1]Aziz et al., 2011 (showing the IDs of a custom-built protein database with Platyhelminthes proteins and EST sequences in GenBank); [2]Monteiro et al., 2010 (showing the IDs of *E. granulosus* ESTs in the LophDB public database); [3]Hidalgo et al., 2016 (showing the IDs of GenBank) and [4]Virginio et al., 2012 (showing the IDs of GenBank and the Platyhelminthes LophDB ESTpublic databases) are shown. Sequences with ID "EGC..." correspond to contig 1, with the exception of those marked with * which correspond to contig 2. [5]As annotated in the database.

### 3.5. Antigen B2 and the TSPAN14

To confirm the presence of antigen B2 and the host-derived TSPAN14 protein in the exosomal enriched sample, both immunoblot and immuno-gold staining experiments were performed. Immunoblots containing the total extract of exosomes and HF were assayed with a rabbit hyperimmune serum against antigen B2. Reactivity of this antibody resulted in the typical antigen B ladder pattern in both protein extracts (Fig. 5). The preimmune serum showed no reactivity with the HF and only a weak reaction for the exosomal extract was observed, displaying one of the major bands of this extract (between 45 and 66 kDa). The anti-TSPAN14 antibody showed a similar unspecific reaction to the preimmune serum with the exosomal extract, while no reaction was found with the same antibody against the HF. Additionally, exosomes were incubated with the anti-B2 serum and the anti-TSPAN14 antibody and the reaction was studied by immunoelectron microscopy. As shown in Fig. 5, reactivity against B2 and TSPAN14 was evident only in exosome-like vesicles, while the preimmune serum showed no label for the same preparation.

## 4. Discussion

Cellular signaling is a dynamic process that underlies all aspects of an organism development and function. In parasites, cell communication is also of paramount importance for the regulation of infection and parasite persistence. *E. granulosus* hydatid cysts are fluid-filled space-occupying lesions growing in internal organs of several vertebrate hosts. They possess a complex structure, including an external acellular laminated layer that functions as a barrier against host attack. Extracellular vesicles are cell-derived carriers of several molecules such as exosomes that have been shown to play an important role in intercellular communication. Exosomes are small vesicles containing proteins and nucleic acids that have been described from many organisms, including parasites (Silva-Álvarez et al., 2016; Marcilla et al., 2014). In the current study, we have shown that fertile hydatid cysts produce and contain exosomes that carry specific exosomal signatures as well as the major antigens commonly found in hydatid fluid.

Proteins identified in the exosomes isolated from HF show that *E. granulosus* could use the ESCRT-dependent biogenesis pathway to generate exosomes. A number of identified proteins show that EVs in HF originate through an intracellular endosomal/trans-Golgi transportation system (multivesicular bodies) and are released as intraluminal vesicles Piper and Katzmann. (2007). In this sense, the presence of tetraspanins and members of the endosomal sorting complexes required for transport (ESCRTs) e.g. the programmed cell death 6 interacting protein (ALIX), tumor susceptibility gene 101 protein (TSG101) and syndecan, among others, provide insights into the mechanisms used by *E. granulosus* cysts for exosome generation (Abels and Breakefield, 2016). The exosome biogenesis and release in *E. granulosus* could also be related to several additional proteins identified in our exosomal proteome, including the charged multivesicular body proteins 2 and 5, the Ral protein, Rab3, protein Wnt, vesicle associated membrane protein and acetylcholinesterase, SNARE proteins (Synaptotagmin-2 and 14), ceramide related proteins and myoferlin (Raposo and Stoorvogel, 2013). Additionally, most of the exosome signature proteins described in other organisms were found in our HF exosomal preparation, the most relevant depicted in Fig. 3 (e.g., annexins, tetraspanins and Rab proteins).

In addition to components commonly found in exosomes generated by most cell types, a series of parasite-specific molecules were found to be among the most abundant in the exosomal extract. These include antigen 5 and antigen B, the two immunodominant antigens found in HF. *In vitro* evidence suggests that antigen B may modulate host defenses by down-regulating neutrophils and dendritic cell-mediated innate responses as well as T-cell dependent mechanisms, which globally influence the intensity and quality of the adaptive immune responses (rev. by Virginio et al., 2007). Intriguingly, antigen B is absent in the LL and in the apical side of the tegumental syncytium of the GL which is in close contact with the LL (Sánchez et al., 1991, 1993). It is therefore unclear how antigen B and other HF antigens are transported outside of the cyst. Our results provide evidence that antigen B is associated with parasite-derived exosomes, but whether these exosomes could reach the host cells has still to be demonstrated. In this sense, it is well known that exosomes have antigen-presentation capacity and may act as immune-modulators due to their cargo molecules (Andreu and Yáñez-Mó, 2014).

Among the most abundant proteins identified in HF exosomes was the A0A068WWM7 lipid transport protein which is similar to apolipophorin. Cestodes are unable to synthesize *de novo* most of their own membrane lipids, including fatty acids, phospholipids and cholesterol (Bernthaler et al., 2009). Parasite lipid-binding proteins, including apolipophorin and antigen B, play a key role in cestode metabolism, as they are likely to be involved in the uptake of lipids or their precursors from the host.

Our exosomal proteome was compared with those available for other closely related parasites. *Fasciola hepatica* and *E. caproni* have been investigated in regard to their exosomal protein cargo (Marcilla et al., 2012; Cwiklinski et al., 2015). The low number of identified proteins in these publications, as compared to results seen in this study, could be attributed to a better coverage of proteins through the use of protein fractionation prior to the MS/MS analysis used here. Similarities ranged from 20% to 37.9%, with the majority of proteins common to the three parasites being those related to exosome biogenesis and release. This suggests

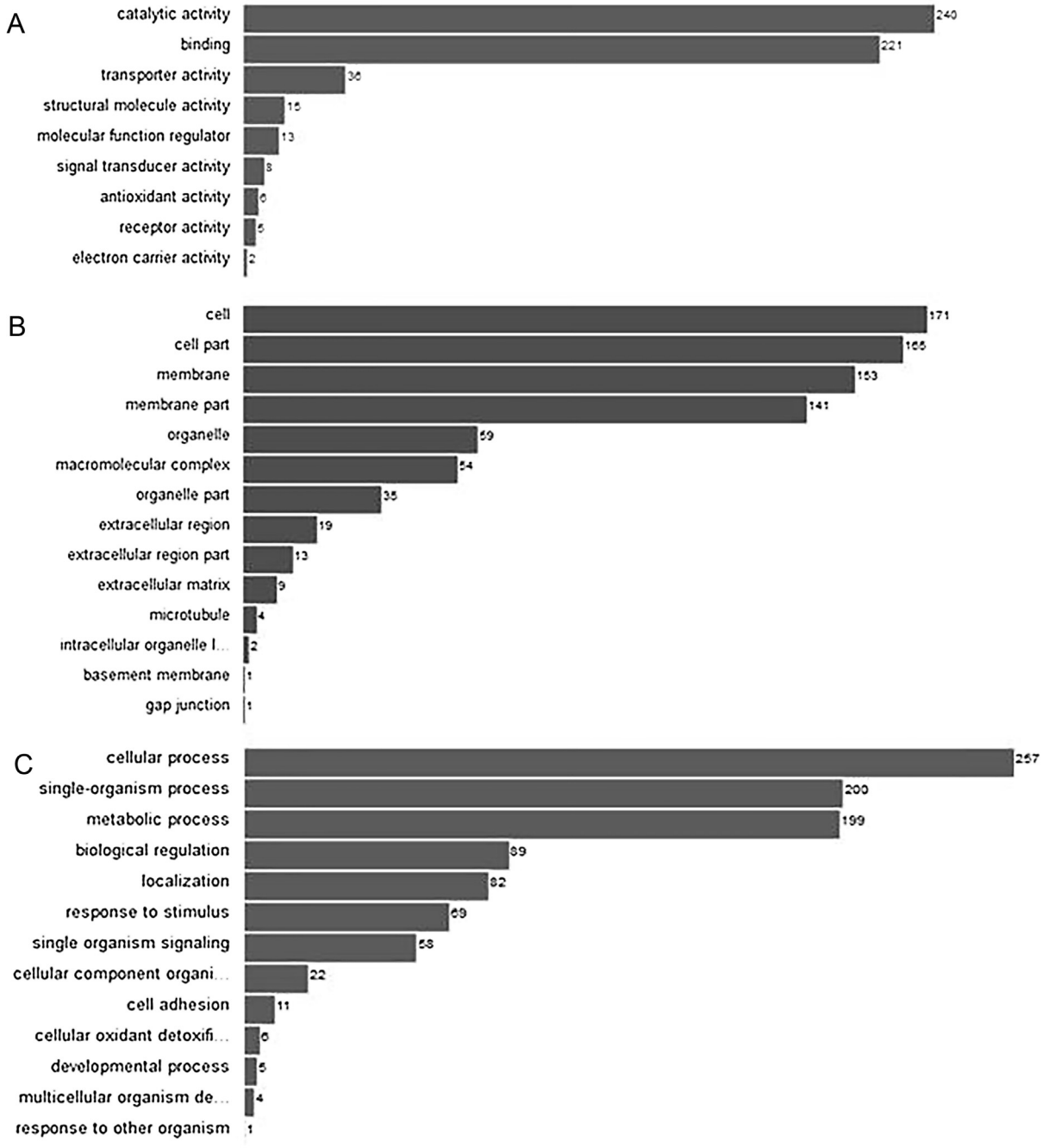


**Fig. 4.** Gene Ontology (GO). GO assignments of the *Echinococcus granulosus* identified proteins in the exosomal fraction. (A) Molecular Function, (B) Cellular Component, (C) Biological Process. Number of proteins in each category is indicated in the bars.

that plathyhelminths may utilize similar mechanisms for exosome production and discharge. A second group of proteins commonly abundant in the three parasites correspond to molecules with catalytic activity, including proteases.

Our HF exosome identified proteins were also compared with the available sequences of *E. granulosus* proteomes of cyst compartments that could contribute to exosomal release or contain exosomal components, including somatic and secretory protoscoleces extracts, somatic GL extracts and HF (Aziz et al., 2011; Monteiro et al., 2010; Virginio et al., 2012; Hidalgo et al., 2016). We speculate that both the GL and the protoscoleces contribute to the exosome-like population found inside fertile hydatid cysts, and that a proportion of the proteins present in HF are also part of the exosomal cargo. The high protein overlap between the proteins in the HF and the exosomes suggest that these vesicles are the primary mechanism of protein transport in the hydatid cyst, which is similar to reports on *F. hepatica* (Marcilla et al., 2012). However, the presence of some HF contaminant proteins in our exosomal preparation that could have co-purified, cannot be ruled out.

As mentioned, host proteins were also identified in our exosomal preparation. However, their number was much lower than those corresponding to parasite proteins (87 vs. 633). Host proteins found in our proteome were compared with proteins of host origin identified by Aziz et al. (2011) in the HF proteome using an approach similar to the one used here were a fractionation step was included prior to MS/MS protein analysis. Although the total

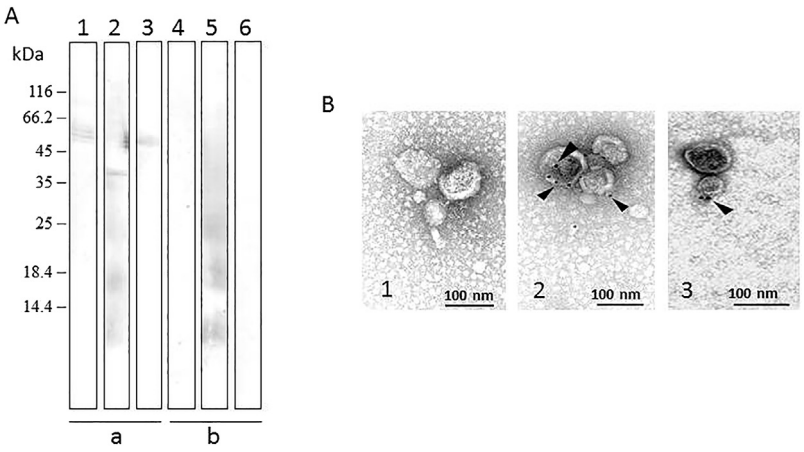


**Fig. 5.** Immunoblot and immunolocalization of antigen B and TSPAN14 in HF exosomes. Immunoblot (A) and immune-electron microscopy (B) assays of exosomes (1, 2, 3) and hydatid fluid (4, 5, 6) with a rabbit per-immune serum (1, 4), a rabbit anti-B2 serum (2, 5) and a rabbit anti-TSPAN14 antibody (3, 6). Relative molecular weight in kDa is indicated in (A). Arrowheads show the specific staining in (B). Bars in (B) indicate 100 nm.

number of host proteins found by those authors (n = 73) was similar to that found here, only 11% of the identifications coincided between the two proteomes. When the host proteins found in HF exosomes were analyzed, a high number of exosome-related proteins were found. Additionally, immunolocalization of a specific host molecule (TSPAN14) identified in the exosomal proteome, was found associated with exosomes isolated from hydatid fluid. The lack of reactivity of this same antibody against a total exosomal extract in immunoblot could be due to the low relative concentration of the TSPAN14 protein in the total extract, as indicated by its Mascot score (position 77 of 87; see Supplementary Table 2). Although our GO analysis showed that identified host proteins fell within the top categories assigned by GO to exosomal parasite proteins, whether exosomes of host origin could enter the hydatid cyst has still to be demonstrated. Our results most likely suggest that parasite exosomes could incorporate host proteins present inside the cyst during their formation. Nevertheless, we cannot rule out that host proteins present in the hydatid fluid are carried over during the exosomal enrichment procedure.

Gene ontology analysis of parasite proteins in HF exosomes showed that the main two categories of "molecular function" were catalytic and binding proteins, in which abundant peptidases, oxidoreductases and lipid binding proteins were found. Thus, exosomes could be considered as one of the main transporters of virulence factors in parasites.

## 5. Conclusions

We have demonstrated that *E. granulosus* cysts (in particular protoscoleces and GL) produce and release exosomes into the HF. The identified parasite exosomes carry virulence factors associated with cyst survival, including highly immunogenic and tolerogenic antigens and peptidases. Further studies are needed to investigate whether exosomes could mediate host-parasite interactions in CE.

## Acknowledgments

This study was funded by the European Community's Seventh Framework Programme under the Grant agreement FP7-602051 (Project HERACLES: Human cystic Echinococcosis ReseArch in CentraL and Eastern Societies; http://www.Heracles-fp7.eu/). We thank Dr. Germán Andrés and Milagros Guerra from the Electron Microscopy Unit at Centro de Biología Molecular Severo Ochoa and Dr. David Gil of the Electron Microscopy Facility from CIC bioGUNE for skillful technical assistance. Thanks are also given to Drs. M. Balsera and A. Peix (IRNASA-CSIC) for fruitful discussions.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2017.01.022.

## References

Abels, E.R., Breakefield, X.O., 2016. Introduction to extracellular vesicles: biogenesis, RNA cargo selection, content, release, and uptake. Cell. Mol. Neurobiol. 36, 301–312.
Andreu, Z., Yáñez-Mó, M., 2014. Tetraspanins in extracellular vesicle formation and function. Front. Immunol. 5, 442.
Aziz, A., Zhang, W., Li, J., Loukas, A., McManus, D.P., Mulvenna, J., 2011. Proteomic characterisation of *Echinococcus granulosus* hydatid cyst fluid from sheep, cattle and humans. J. Proteom. 74, 1560–1572.
Benner, C., Carabin, H., Sánchez-Serrano, L.P., Budke, C.M., Carmena, D., 2010. Analysis of the economic impact of cystic echinococcosis in Spain. Bull. World Health Organ. 88, 49–57.
Bernthaler, P., Epping, K., Schmitz, G., Deplazes, P., Brehm, K., 2009. Molecular characterization of EmABP, an apolipoprotein A-I binding protein secreted by the *Echinococcus multilocularis* metacestode. Infect. Immun. 77, 5564–5571.
Brundu, D., Piseddu, T., Stegel, G., Masu, G., Ledda, S., Masala, G., 2015. Response to comment on: retrospective study of human cystic echinococcosis in Italy based on the analysis of hospital discharge records between 2001 and 2012. Acta Trop. 144, 52.
Budke, C.M., Deplazes, P., Torgerson, P.R., 2006. Global socioeconomic impact of cystic echinococcosis. Emerg. Inf. Dis. 12, 296–303.
Cardona, G.A., Carmena, D., 2013. A review of the global prevalence, molecular epidemiology and economics of cystic echinococcosis in production animals. Vet. Parasitol. 192, 10–32.
Coakley, G., Maizels, R.M., Buck, A.H., 2015. Exosomes and other extracellular vesicles: the new communicators in parasite infections. Trends Parasitol. 31, 477–489.
Cwiklinski, K., de la Torre-Escudero, E., Trelis, M., Bernal, D., Dufresne, P.J., Brennan, G.P., O'Neill, S., Tort, J., Paterson, S., Marcilla, A., Dalton, J.P., Robinson, M.W., 2015. The extracellular vesicles of the helminth pathogen, *Fasciola hepatica*: biogenesis pathways and cargo molecules involved in parasite pathogenesis. Mol. Cell. Proteom. 14, 3258–3273.
Díaz, A., Casaravilla, C., Allen, J.E., Sim, R.B., Ferreira, A.M., 2011a. Understanding the laminated layer of larval *Echinococcus* II: immunology. Trends Parasitol. 27, 264–273.
Díaz, A., Casaravilla, C., Irigoín, F., Lin, G., Previato, J.O., Ferreira, F., 2011b. Understanding the laminated layer of larval *Echinococcus* I: structure. Trends Parasitol. 27, 204–213.
Frühbeis, C., Fröhlich, D., Krämer-Albers, E.M., 2012. Emerging roles of exosomes in neuron-glia communication. Front. Physiol. 3, 119.
Gercel-Taylor, C., Atay, S., Tullis, R.H., Kesimer, M., Taylor, D.D., 2012. Nanoparticle analysis of circulating cell-derived vesicles in ovarian cancer patients. Analyt. Biochem. 428, 44–53.
Greening, D.W., Xu, R., Ji, H., Tauro, B.J., Simpson, R.J., 2015. A protocol for exosome isolation and characterization: evaluation of ultracentrifugation

density-gradient separation, and immunoaffinity capture methods. Methods Mol. Biol. 1295, 179–209.
Hernández-González, A., Muro, A., Barrera, I., Ramos, G., Orduña, A., Siles-Lucas, M., 2008. Usefulness of four different *Echinococcus granulosus* recombinant antigens for serodiagnosis of unilocular hydatid disease (UHD) and postsurgical follow-up of patients treated for UHD. Clin. Vaccine Immunol. 15, 147–153.
Herrador, Z., Siles-Lucas, M., Aparicio, P., Lopez-Velez, R., Gherasim, A., Garate, T., Benito, A., 2016. Cystic echinococcosis epidemiology in Spain based on hospitalization records, 1997–2012. PLoS Negl. Trop. Dis. 10, e0004942.
Hidalgo, C., García, M.P., Stoore, C., Ramírez, J.P., Monteiro, K.M., Hellman, U., Zaha, A., Ferreira, H.B., Galanti, N., Landerer, E., Paredes, R., 2016. Proteomics analysis of *Echinococcus granulosus* protoscolex stage. Vet. Parasitol. 218, 43–45.
Keerthikumar, S., Chisanga, D., Ariyaratne, D., Al Saffar, H., Anand, S., Zhao, K., Samuel, M., Pathan, M., Jois, M., Chilamkurti, N., Gangoda, L., Mathivanan, S., 2016. ExoCarta: a web-based compendium of exosomal cargo. J. Mol. Biol. 428, 688–692.
Kreimer, S., Belov, A.M., Ghiran, I., Murthy, S.K., Frank, D.A., Ivanov, A.R., 2015. Mass-spectrometry-based molecular characterization of extracellular vesicles: lipidomics and proteomics. J. Proteome Res. 14, 2367–2384.
Marcilla, A., Trelis, M., Cortés, A., Sotillo, J., Cantalapiedra, F., Minguez, M.T., Valero, M.L., Sánchez del Pino, M.M., Muñoz-Antoli, C., Toledo, R., Bernal, D., 2012. Extracellular vesicles from parasitic helminths contain specific excretory/secretory proteins and are internalized in intestinal host cells. PLoS One 7, e45974.
Marcilla, A., Martin-Jaular, L., Trelis, M., de Menezes-Neto, A., Osuna, A., Bernal, D., Fernandez-Becerra, C., Almeida, I.C., Del Portillo, H.A., 2014. Extracellular vesicles in parasitic diseases. J. Extracell. Vesicles 3, 25040.
Monteiro, K.M., de Carvalho, M.O., Zaha, A., Ferreira, H.B., 2010. Proteomic analysis of the *Echinococcus granulosus* metacestode during infection of its intermediate host. Proteomics 10, 1985–1999.
Natasha, G., Gundogan, B., Tan, A., Farhatnia, Y., Wu, W., Rajadas, J., Seifalian, A.M., 2014. Exosomes as immunotheranostic nanoparticles. Clin. Ther. 36, 820–829.
Piper, R.C., Katzmann, D.J., 2007. Biogenesis and function of multivesicular bodies. Annu. Rev. Cell Dev. Biol. 23, 519–547.
Raposo, G., Stoorvogel, W., 2013. J. Cell Biol. 200, 373–383.
Rappsilber, J., Ishihama, Y., Mann, M., 2003. Stop and go extraction tips for matrix-assisted laser desorption/ionization nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal. Chem. 75, 663–670.
Rojo-Vazquez, F.A., Pardo-Lledias, J., Francos-Von Hunefeld, M., Cordero-Sanchez, M., Alamo-Sanz, R., Hernandez-Gonzalez, A., Brunetti, E., Siles-Lucas, M., 2011. Cystic echinococcosis in Spain: current situation and relevance for other endemic areas in Europe. PLoS Negl. Trop. Dis. 5, e893.
Rossi, P., Tamarozzi, F., Galati, F., Pozio, E., Akhan, O., Cretu, C.M., Vutova, K., Siles-Lucas, M., Brunetti, E., Casulli, A., HERACLES extended network, 2016. The first meeting of the European Register of Cystic Echinococcosis (ERCE). Parasit. Vectors 9, 243.
Sánchez, F., March, F., Mercader, M., Coll, P., Muñoz, C., Prats, G., 1991. Immunochemical localization of major hydatid fluid antigens in protoscoleces and cysts of *Echinococcus granulosus* from human origin. Parasite Immunol. 13, 583–592.
Sánchez, F., Garcia, J., March, F., Cardeñosa, N., Coll, P., Muñoz, C., Auladell, C., Prats, G., 1993. Ultrastructural localization of major hydatid fluid antigens in brood capsules and protoscoleces of *Echinococcus granulosus* of human origin. Parasite Immunol. 15, 441–447.
Schorey, J.S., Cheng, Y., Singh, P.P., Smith, V.L., 2015. Exosomes and other extracellular vesicles in host-pathogen interactions. EMBO Rep. 16, 24–43.
Silva-Álvarez, V., Folle, A.M., Ramos, A.L., Kitano, E.S., Iwai, L.K., Corraliza, I., Córsico, B., Ferreira, A.M., 2016. Echinococcus granulosus Antigen B binds to monocytes and macrophages modulating cell response to inflammation. Parasit. Vectors 9, 69.
Tamarozzi, F., Rossi, P., Galati, F., Mariconti, M., Nicoletti, G.J., Rinaldi, F., Casulli, A., Pozio, E., Brunett, E., 2015. The Italian registry of cystic echinococcosis (RIEC): the first prospective registry with a European future. Euro Surveill. 20, 21115.
Théry, C., Ostrowski, M., Segura, E., 2009. Membrane vesicles as conveyors of immune responses. Nat. Rev. Immunol. 9, 581–593.
Van Cauteren, D., Millon, L., de Valk, H., Grenouillet, F., 2016. Retrospective study of human cystic echinococcosis over the past decade in France, using a nationwide hospital medical information database. Parasitol. Res. 115, 4261–4265.
Venegas, J., Espinoza, S., Sánchez, G., 2014. Estimation of costs caused by cystic echinococcosis. Rev. Med. Chile 142, 1023–1033.
Virginio, V.G., Taroco, L., Ramos, A.L., Ferreira, A.M., Zaha, A., Ferreira, H.B., Hernández, A., 2007. Effects of protoscoleces and AgB from *Echinococcus granulosus* on human neutrophils: possible implications on the parasite's immune evasion mechanisms. Parasitol. Res. 100, 935–942.
Virginio, V.G., Monteiro, K.M., Drumond, F., de Carvalho, M.O., Vargas, D.M., Zaha, A., Ferreira, H.B., 2012. Excretory/secretory products from *in vitro*-cultured *Echinococcus granulosus* protoscoleces. Mol. Biochem. Parasitol. 183, 15–22.