# Novel markers for early detection and treatment follow-up of echinococcosis: analysis of microRNA and protein secretion mechanisms

**Final Technical Report**

## Introduction

Echinococcosis or hydatid disease is a chronic parasitic zoonosis that affects humans as well as domestic and wild mammals and is caused by cestode parasites of the genus *Echinococcus*. Cystic echinococcosis (CE) caused by *Echinococcus granulosus sensu lato* (s. l.) is worldwide distributed. CE is endemic in South America, especially in Argentina, southern Brazil, Uruguay, Chile, Peru and Bolivia (Cucher *et al.*, 2016). The life cycle of these parasites alternates between two hosts: wild and domestic canids as definitive hosts where the sexually mature adult stage develops, and wild and domestic ungulates as intermediate hosts where the metacestode larval stage grows and multiplies asexually, usually in the liver or lungs. Humans act as accidental intermediate hosts. Associated with poverty and poor hygiene practices, particularly in livestock-raising communities (Yang *et al.*, 2012), this disease constitutes essentially a preventable affection and is considered neglected by the World Health Organization. It has been estimated that 1-3.6 million DALYs are lost worldwide because of human CE (Craig *et al.*, 2007) and that up to $2 billion are lost annually in the livestock industry (Budke *et al.*, 2006). Regarding the pathogenesis of this zoonosis, CE remains asymptomatic during the initial stage of the infection and even for years during metacestode development if it is not growing in vital organs. The general morphology of the metacestode can be described as a fluid-filled cyst composed of a thin cellular layer surrounded by an acellular layer, where the fluid is called hydatid fluid and the cellular and acellular layers are called germinal and laminated layers, respectively.

Human echinococcosis diagnosis is based on image diagnosis together with the analysis of clinical and epidemiological data. However, this only allows to detect the parasite once it has already established, grew to an extent that triggers clinical signs and can be detected by imaging tests. Immunodiagnosis is only complementary, because its main drawbacks are cross-reactivity among different species of the genus *Echinococcus* and with other helminthiases, and low sensitivity since a relatively high percentage of patients (20-50%) do not show detectable levels of specific antibodies (Lorenzo *et al.*, 2005; Gottstein *et al.*, 2014), especially in the early stages of the disease (Pawlowski *et al.*, 2001). The level of circulating antibodies also depends on the localization and number of cysts: around 20% of patients with liver cysts and around 40% of patients with lung cysts do not produce detectable antibody levels yielding false negative results (Pawlowski *et al.*, 2001; Zhang *et al.*, 2012). In the case of brain, bone or calcified cysts there are usually no detectable antibody levels and if there are, they cannot be detected by the techniques currently used (Rigano *et al.*, 2002; Ortona *et al.*, 2003; Lawn *et al.*, 2004).

Recently, the use of circulating microRNAs (miRNAs) as disease biomarkers has emerged as a possible new diagnostic tool in several pathologies (Weiland *et al.*, 2012) such a cancer and diabetes. miRNAs are 22-nucleotide long non-coding RNAs that can be found both intra and extracellularly. Their use as diagnostic markers is based on the fact that they have been detected in different body fluids such as serum (Weber *et al.*, 2010). Furthermore, the level of several circulating miRNAs detected in plasma or serum varies in certain pathological states with respect to healthy patients. miRNAs circulate in the bloodstream in a stable form within extracellular vesicles (EVs) or in soluble protein complexes, remain stable even after several freeze-thaw cycles, are not sensible to nucleases and are tissue-specific or disease stage specific (Bernardo *et al.*, 2012; Fernandez-Mercado *et al.*, 2015).

In the case of infectious diseases, the use of miRNAs as biomarkers has as an additional advantage the specificity, since pathogens have unique or very divergent miRNAs with respect to their host miRNA homologues. In this respect, circulating miRNAs from viruses (reviewed in (Laganà *et al.*, 2013)), trematodes and nematodes have been detected in sera from infected hosts (Cheng *et al.*,

2013; Hoy *et al.*, 2014; Tritten *et al.*, 2014a; b; Quintana *et al.*, 2015). Recently, circulating miRNAs from *E. multilocularis* were shown to be detected in sera from experimental hosts (Guo and Zheng, 2017).

Due to the fact that neither image nor serological tests can be confidently used in the early stages of the disease, and that serological diagnosis of echinococcosis based on antibodies detection has low sensitivity in spite of the use of different antigenic sources (Lorenzo *et al.*, 2005), it is imperative to find new alternatives for epidemiological studies as well as for the individual diagnosis. In this way, the detection of *E. granulosus* s. l. miRNAs in body fluids may constitute a new diagnostic tool independent of the antibodies response and of greater specificity and sensitivity than current methodologies with special emphasis on early detection and the search of viability markers indispensable for post-treatment follow-up.

## Results and Discussion

*Aim 1) Identification of the extracellular miRNA repertoire secreted by* E. granulosus *s. l.*

To study miRNA secretion in *E. granulosus* s.l., we determined the complete repertoire of miRNAs secreted by the larval stages (metacestode and protoscolex) of *E. granulosus* s.l. towards the extra-parasite milieu (the culture medium) and in the case of the metacestode, to the inner parasite milieu (i. e., hydatid fluid) as well. To obtain metacestodes, we employed the secondary echinococcosis model that is based on the inoculation of freshly obtained protoscoleces from natural infections into the peritoneal cavity of mice. After at least 8 months of infection, metacestodes were recovered and incubated *in vitro*. Parasites were then incubated in the absence of foetal bovine serum in order to avoid cross-contamination with serum EVs and each type of sample was processed by a differential centrifugation protocol to fraction the excretion/secretion (E/S) products into the EV-enriched (P100) and EV-depleted (S100) extracellular compartments. Finally, the S100 samples were concentrated using a 3-kDa centrifugal device (Ancarola *et al.*, 2017).

In this sense, the presence of EVs was confirmed by transmission electron microscopy in the culture medium and hydatid fluid of the metacestodes (Fig. 1) and also, in the culture medium of protoscoleces.

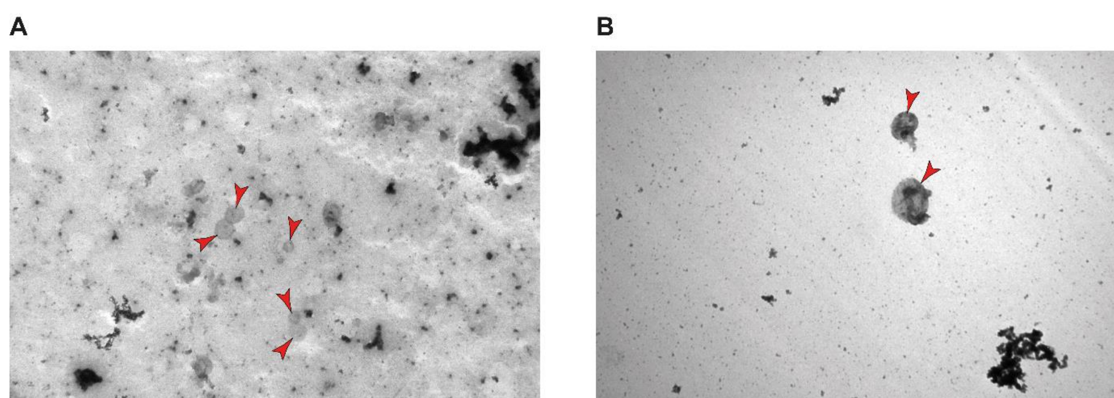


**Fig. 1:** Transmission electron microscopy images of extracellular vesicles (red arrowheads) from culture medium (A) and hydatid fluid (B) of *E. granulosus* s. l. metacestodes. Scale bars = 400 nm.

However, EVs were scarce in both types of metacestode samples. These results are in line with our previous observations in the related parasite, *Echinococcus multilocularis*, were EVs are mainly retained by the laminated layer (Ancarola *et al.*, 2017), the acellular layer produced by the metacestodes of the genus *Echinococcus* (Díaz *et al.*, 2011). This implies that EV content cannot freely reach the extra-parasite milieu and hence, would not represent the main means of transport to secrete molecules that could be in contact with the host, such as miRNAs and proteins. Consequently, we

proceeded to characterize whether the parasite secreted miRNAs by a non-vesicular pathway, i.e. not associated to EVs. For this, the presence of miRNAs in the different extracellular compartments (P100 and S100) was assessed by small RNA sequencing. As a result, parasite miRNAs in metacestode samples were predominantly found in the hydatid fluid (P100 and S100 fractions) while a low proportion was detected in the S100 fraction of the culture medium. Interestingly, in protoscoleces E/S products, parasite miRNAs were abundant in the EV-enriched fraction (P100) (Fig. 2). This suggests that the different life cycle stages of the parasite employ alternative ways to secrete miRNAs *in vitro* which are highly dependent on the morphology of the stage under study.

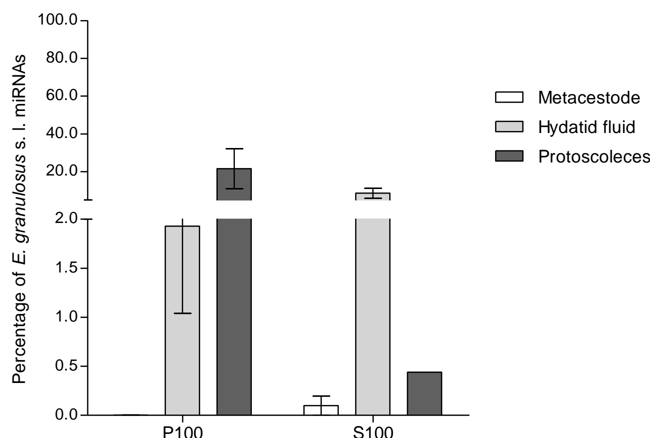


**Fig. 2:** Detection of *E. granulosus* s. l. microRNAs in the EV-enriched (P100) or EV-depleted (S100) fractions of the *in vitro* obtained excretion/secretion products. Percentage of read counts corresponding to parasite microRNAs.

With respect to the most abundantly secreted miRNAs, egr-miR-71-5p, egr-let-7-5p and egr-miR-4989-3p were found in all the samples, both in the P100 and S100 fractions. These miRNAs are either not expressed by the mammalian host (miR-71 and miR-4989) or show a highly divergent sequence with respect to the mammalian orthologue (let-7). Since these miRNAs were also abundantly detected in the E/S of *E. multilocularis* metacestodes *in vitro* (Ancarola *et al.*, 2020), they were chosen as candidate biomarkers of active infection.

In order to determine if those miRNAs that were most abundantly secreted to the extra-parasite milieu (i.e., P100 and S100 from culture media) could be detected by RT-qPCR, we assessed culture medium samples from *E. multilocularis* metacestodes. As a result, miR-71-5p could be readily detected in all the samples, while miR-4989-3p and let-7-5p were detected in two or one, respectively (Fig.3). In all cases, expression levels in S100 were higher. These results show that it is possible to detect extracellular miRNAs from *Echinococcus* spp. isolated from very diluted samples.

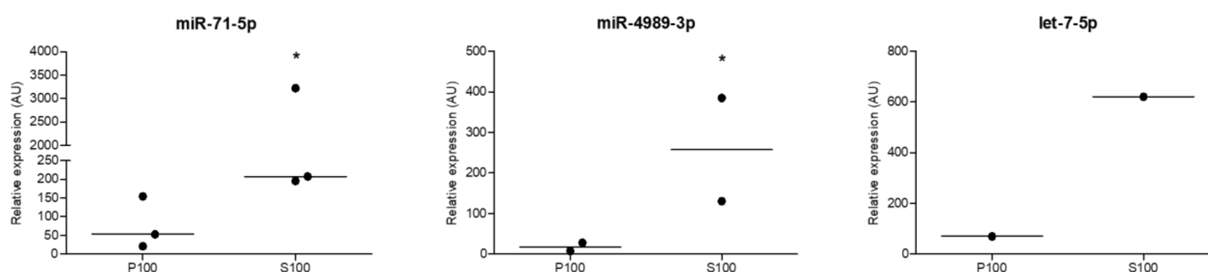


**Fig. 3:** RT-qPCR detection of selected ex-RNAs *in vitro*. Relative expression from selected miRNAs in culture medium from *E. multilocularis* metacestodes is depicted in arbitrary units (AU). Values were normalised to input culture medium. N = 3. Lines indicate the median values. * P<0.05.

*Aim 2) Identification of the protein secretome of* E. granulosus s. l.

As demonstrated in Aim 1, the metacestode stage of *E. granulosus* s. l. seems not to employ EVs to actively interact with the host. Hence, the molecules carried in EVs may not represent good candidate biomarkers of active infection, contrarily to molecules secreted through a non-vesicular pathway. In order to identify proteins specifically secreted by *E. granulosus* s. l. by the vesicular (EV) or non-vesicular (soluble form) pathways, we proceeded to characterize by proteomics (i.e., LC-MS/MS) the P100 and S100 fractions obtained from the E/S products of metacestodes and protoscoleces. Since each experimental infection yielded a limited amount of cysts, which were cultivated in batch in order to increase the amount of EV and soluble proteins obtained, samples were processed in order to isolate both RNA for Aim 1 and proteins for Aim 2 using Trizol reagent.

As a result, we observed that a very low number of proteins could be detected in metacestode P100 samples in contrast to results obtained from protoscoleces (Table 1). This could be related to the fact that i) metacestodes hardly secreted EV (to the extra- and intra-parasite milieus) *in vitro*; ii) metacestodes secrete very few soluble proteins *in vitro* to the culture medium in the absence of host stimuli and/or iii) proteins were isolated from the organic phase of Trizol preparations which may represent a very inefficient procedure for samples with low protein concentration.

**Table 1:** Number of proteins identified in the E/S products of *E. granulosus* s. l. N = 2

| Sample | P100 (EV-enriched) | S100 (EV-depleted) |
|---|---|---|
| Metacestode culture medium | 3 – 9 | 6 – 9 |
| Metacestode hydatid fluid | 1 - 6 | 44 - 144 |
| Protoscolex culture medium | 114 – 438 | 29 - 71 |

Due to the limitation of our approach, we proceeded to pool the results obtained from the different types of samples. Among the differentially secreted proteins between the P100 and S100 fractions, cestode- and platyhelminth- specific proteins of unknown function were detected (Table 2). These proteins are usually called "hypothetical proteins" since they lack conserved domains with other better characterised organisms and account for approximately 40 % of cestode genomes (Palevich *et al.*, 2018; Kamenetzky *et al.*, 2022). In the case of *Echinococcus*, hypothetical proteins specific of this genus can be sorted into 455 orthology groups (Fig. 4). These proteins are rarely studied even though they represent novelties in the evolution of these parasites that may fulfil relevant roles in their physiology and in the interaction with the host. Furthermore, since these proteins are not expressed by the mammalian host, they represent candidate biomarkers for active infection in the case of soluble proteins, or for treatment follow-up, in the case of EV-associated proteins when release of metacestode content occurs.

In this sense, in the EV-enriched samples, a hypothetical protein that contains transmembrane domains (i.e., reinforcing its secretion through the vesicular pathway) was detected. While in the EV-depleted samples, none of the proteins contained transmembrane domains and two displayed a signal peptide, confirming their secretory nature. Regarding RNA-binding proteins that may be acting as carriers of the miRNAs detected in the S100 samples, none of the proteins reported in mammals has been detected. This could imply that *E. granulosus* s. l. uses an alternate protein to secrete miRNAs or that a higher concentration of protein should be used to analyse these samples by LC-MS/MS.

**Table 2:** Hypothetical proteins detected exclusively in the EV-enriched (P100) or EV-depleted (S100) fractions of the E/S products of *E. granulosus* s. l. *in vitro*.

| | Hypothetical protein number | Conservation | Signal peptide[a] | Transmembrane domain[b] |
|---|---|---|---|---|
| **P100 (EV-enriched)** | 1 | Platyhelminths (Cestode+Trematode) | No | No |
| | 2 | Cestode | No | No |
| | 3 | Cestode (Taeniidae) | No | No |
| | 4 | Cestode (Taeniidae) | No | Yes |
| **S100 (EV-depleted)** | 5 | Platyhelminths (free-living + parasites) | No | No |
| | 6 | Cestode (Taeniidae) | Yes | No |
| | 7 | Platyhelminths (Cestode+Trematode) | Yes | No |

[a]: Predicted using SignalP version 5.0
[b]: Predicted using TMHMM version 2.0

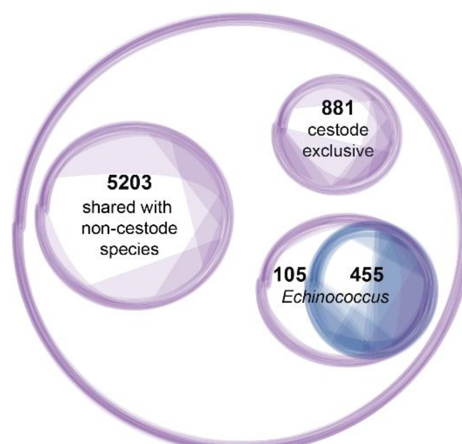


**Fig. 4:** Cestode genomes contain a high proportion of proteins with unknown function ("hypothetical proteins"). In this diagram, orthologous groups (OG) present in Cyclophyllidean cestodes (*Taenia*, *Hymenolepis* and *Echinococcus* genera) are depicted. The blue circle represents the number of OG corresponding to hypothetical proteins found exclusively in *Echinococcus*.

*Aim 3) Assessment of the diagnostic performance of the selected biomarkers in sera from experimental hosts*

Taking into consideration that the detection of circulating parasite miRNAs may indicate the presence of active infection, the diagnostic potential of *E. granulosus* s. l. miRNAs was assessed. Initially, in this project it was proposed to perform this Aim using samples from experimentally infected animals. Due to the pandemics, these experiments could not be performed, but we had access to plasma samples from patients. Patients were tested for routine diagnostic purposes using ELISA (RIDASCREEN Echinococcus IgG, R-Biopharm, Darmstadt, Germany) and cysts were classified

according to the WHO-IWGE classification into: active (CE1-2), transitional (CE3b), inactive (CE4, CE5) and control group (negative patients with no parasitic cysts). An equal number of samples from each group were analysed. All the CE patients had one single cyst localized in the liver.

Following the results obtained in Aim 1, we proceeded to assess the RT-qPCR detection of egr-miR-71-5p and egr-miR-4989-3p. As a result, only one patient showed a very low amplification signal (Ct = 39.3) for miR-71-5p and no signal was observed for miR-4989-3p (Fig 5). In all cases, the control endogenous miRNA (hsa-miR-423-5p) was successfully detected (Ancarola *et al.*, 2020). These results suggest that these two miRNAs may not represent good biomarkers of echinococcosis.

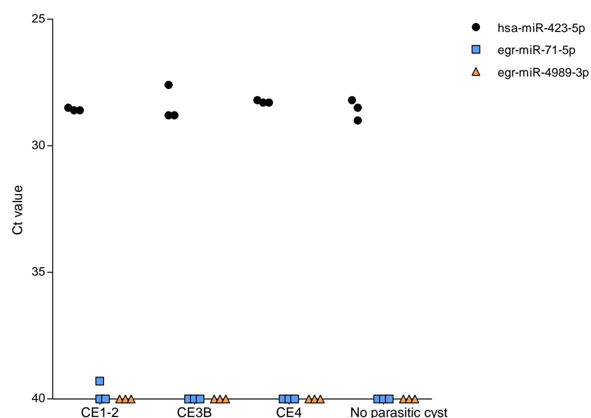


**Fig. 5:** RT-qPCR detection of selected microRNAs *in vivo*. Assessment of detection of Echinococcus spp. microRNAs in serum/plasma samples from cystic echinococcosis patients. Raw Ct values are shown. Ct = 40 means no amplification. N=3.

We proceeded then to perform a systematic review of the use of miRNAs as biomarkers of infectious diseases caused by helminth parasites. In the case of cestode parasites, current data focuses on infections caused by *Echinococcus* spp. Regarding patient samples, inconsistent results between different groups have been reported (Alizadeh et al., 2020; Ancarola et al., 2020), thus it is still a matter of debate whether *Echinococcus* spp miRNAs have biomarker potential. On the other hand, positive results were obtained in experimental infections with *E. multilocularis* (Guo and Zheng, 2017). However, in the case of nematodes and trematode infections, published data point to a trend of consistent parasite ex-RNA detection in serum or plasma for those species with life cycle stages circulating in peripheral blood or dwelling in vessels, e.g. filarial nematodes and schistosomes (Fig. 6).

## Conclusions

Since the discovery that miRNAs are present in human biofluids, such as serum and urine, the scientific community has invested huge efforts on studying their use as biomarkers of many diseases (Ghai and Wang, 2016). In the case of infectious diseases, the use of pathogen miRNAs as biomarkers promises the advent of highly specific diagnostic tools, since the different species of pathogens express sets of unique or divergent miRNAs with respect to the corresponding host homologues. Regarding human infections caused by helminths, diagnostic alternatives for both epidemiological studies and individual diagnosis are urgently needed since current techniques lack of enough sensitivity and specificity to accurately determine early infection, parasite viability for posttreatment follow-up, parasite burden and/or infection status (McCarthy et al., 2012). In this sense, it is highly relevant to study how parasites secrete such small RNAs in order to understand the meaning of parasite miRNA detection in the bloodstream of the host, i. e. active secretion versus passive release due to tegument disruption produced by chemotherapy.

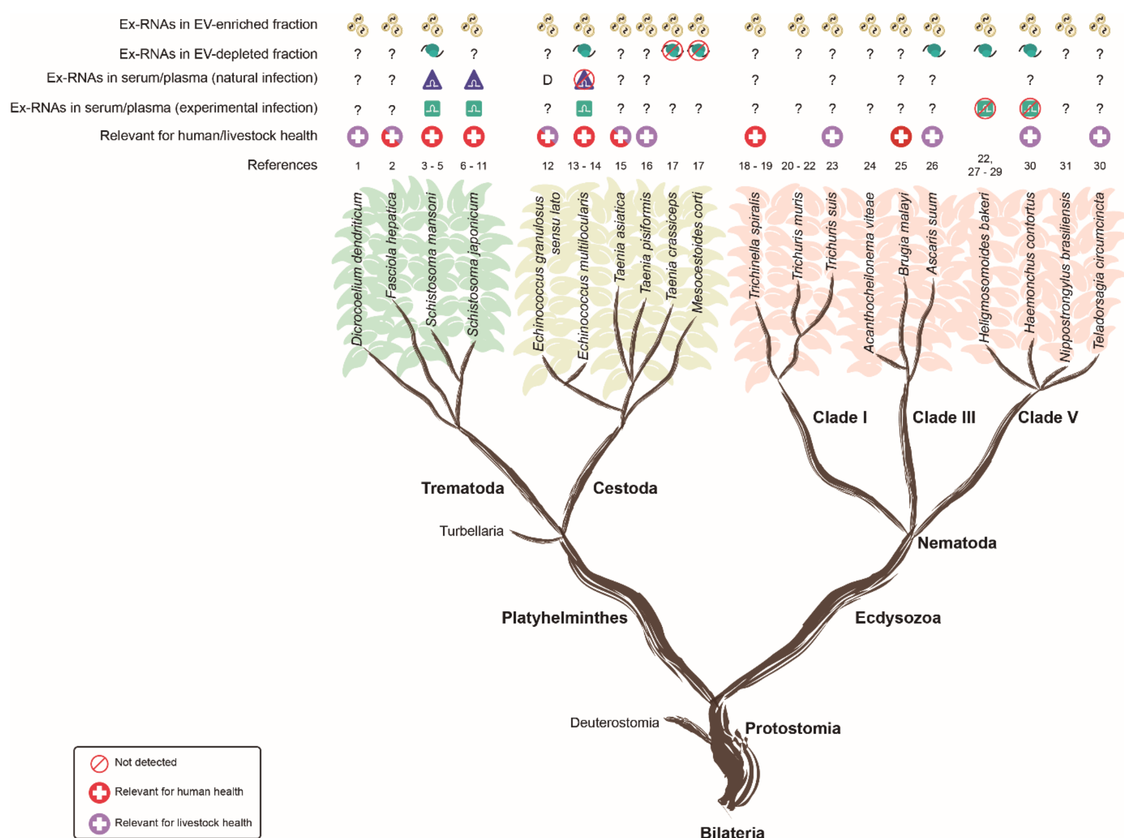


**Fig. 6:** Vesicular and non-vesicular ex-RNA detection in helminth parasites. "?": Missing data by November 2020. "D": discrepant results between reports. References correspond to the characterization of EV-enriched and/or EV-depleted samples obtained by the in vitro cultivation of parasites (Cucher *et al.*, 2021).

Despite the pandemics, here we could explore this subject thanks to the synergistic collaboration among the laboratories that composed the working group. We could deeply characterize the small RNA secretome of *E. granulosus* s. l. in both vesicular and non-vesicular fractions of the E/S products of larval stages. Also, we observed that the morphology of the metacestode stage restricts EV release, and hence EV content, to the extra-parasite milieu, while EVs are freely secreted in the protoscolex stage. Regarding extracellular RNA carriers, a non-vesicular origin of the miRNAs secreted by metacestodes has been proposed. Unfortunately, the selected parasite miRNAs were not detected in patient samples. However, a deeper characterization of the small RNA secretome *in vivo* (i.e. during *E. granulosus* infection) will provide a more realistic scenario. Finally, parasite-specific proteins with no orthologues in vertebrates (hosts) were detected in the E/S products obtained *in vitro*. In future studies, these proteins deserve a thorough characterization and evaluation as candidate biomarkers since they are highly divergent with respect to host proteins.

## Publications

**Ancarola, M. E., Lichtenstein, G., Herbig, J., Holroyd, N., Mariconti, M., Brunetti, E., Berriman, M., Albrecht, K., Marcilla, A., Rosenzvit, M. C., Kamenetzky, L., Brehm, K. and Cucher, M.** (2020). Extracellular non-coding RNA signatures of the metacestode stage of Echinococcus multilocularis. *PLoS Neglected Tropical Diseases* e0008890. doi: 10.1371/journal.pntd.0008890.

**Sotillo J, Robinson MW, Kimber MJ, Cucher M, Ancarola ME, Nejsum P, Marcilla A,**

**Eichenberger RM, Tritten L.** (2020). The protein and microRNA cargo of extracellular vesicles from parasitic helminths - current status and research priorities. Int J Parasitol. 50(9):635-645. doi: 10.1016/j.ijpara.2020.04.010.

**Cucher, M. A., Ancarola, M. E. M. and Kamenetzky, L.** (2021). The challenging world of extracellular RNAs of helminth parasites. *Molecular Immunology* **134**, 150–160. doi: 10.1016/j.molimm.2021.03.011.

**Kamenetzky, L., Maldonado, L. L. and Cucher, M. A.** (2022). Cestodes in the genomic era. *Parasitology Research* **121**, 1077–1089. doi: 10.1007/s00436-021-07346-x.

**García LCA, Pérez MG, Ancarola ME, Rosenzvit MC, Cucher MA.** (2022). *In vitro* system for the growth and asexual multiplication of *Taenia crassiceps* cysticerci. Parasitology. 149(13):1775-1780. doi: 10.1017/S0031182022001354.

**Congress presentations**

**Ancarola, M. E., Herbig, J., Maldonado L., Horvat S., Marcilla, A., Albrecht, K., Kamenetzky, L., Brehm, K. and Cucher, M.** Stage-dependent secretion of extracellular vesicles to the extra-parasite environment in the zoonotic cestode Echinococcus multilocularis. International Society for Extracellular Vesicles 2020 Workshop, EVs in Immunology. March 5-6, 2020, National Academy of Medicine, Buenos Aires, Argentina. Oral presentation, Abstracts Book p. 15.

**Ancarola, M. E., Lichtenstein, G., Herbig, J., Holroyd, N., E., Berriman, M., Albrecht, K., Marcilla, A., Rosenzvit, M. C., Kamenetzky, L., Brehm, K. and Cucher, M.** Extracellular RNA profile of Echinococcus multilocularis metacestodes with different tegument integrity. XXXII Reunión Anual de la Sociedad Argentina de Protozoología. November 18 - 20, 2020. Abstracts Book, p60. Virtual Meeting.

**García LCA, Pérez MG, Ancarola ME, Rosenzvit MC, Cucher MA.** Optimization of the in vitro culture of Taenia crassiceps: cysticerci density and culture media composition effects on parasite growth and survival. XXXII Reunión Anual de la Sociedad Argentina de Protozoología. November 18 - 20, 2020. Abstracts Book, p61. Virtual Meeting.

**Ancarola, M. E., Lichtenstein, G., Herbig, J., Holroyd, N., Mariconti, M., Brunetti, E., Berriman, M., Albrecht, K., Marcilla, A., Rosenzvit, M. C., Kamenetzky, L., Brehm, K. and Cucher, M.** Cestode parasites release extracellular vesicles containing small RNAs in a stage-dependent manner. Molecular Helminthology 2021. Virtual Meeting.

**García LCA, Pérez MG, Ancarola ME, Rosenzvit MC, Cucher MA.** Effects of parasite and host secreted factors on growth, asexual multiplication and survival of Taenia crassiceps cysticerci. Host-Pathogen Interaction Meeting 2021. November 8 – 12, 2021. Virtual Meeting.

**García LCA, Pérez MG, Ancarola ME, Rosenzvit MC, Cucher MA.** Effect of parasite and host factors on Taenia crassiceps asexual multiplication, growth and survival. XI Congreso de la Sociedad Argentina de Protozoología. Mendoza, Argentina. March 2022. Abstract Book, p137.

**Ancarola, M. E., Maldonado L., García L., Miles S., Mourglia-Ettlin G., Franchini G., Kamenetzky, L., Cucher, M.** Análisis comparativo por grupos de ortología de la composición proteica de vesículas extracelulares de parásitos platelmintos. XI Congreso de la Sociedad Argentina de Protozoología. Mendoza, Argentina. March 2022. Abstract Book, p140.

**Ancarola, M. E., Marcilla, A., Lichtenstein, G., Maldonado L., Holroyd, N., Mariconti, M., Brunetti, E., Berriman, M., Albrecht, K., Rosenzvit, M. C., Kamenetzky, L., Brehm, K., Cucher, M.** Parasite morphology affects extracellular vesicle secretion in cestodes. 15th International Congress of Parasitology. Symposium "Extracellular vesicles in host-helminth interaction". Copenhagen, Denmark. August 21 – 26, 2022.

**Missions**

Despite the COVID-19 pandemic reached Latin America 5 months after the beginning of this project, four missions could be carried out successfully at the collaborating laboratories in Uruguay and Brazil. These missions involved two research stays of the Principal Investigator of this project and a PhD student at the University of the Republic, Uruguay and one of the same student at Vale Institute, Brazil. During these missions, the PhD student was trained in animal models used to study cystic echinococcosis and small RNA sequencing, and also performed part of the experiments involved in this project. Also, part of the results and conclusions obtained have been published in three prestigious journals (PMID: 33773158, PMID: 33253209, PMID: 32652128) and were presented in international congresses. Despite the sanitary situation, we could start with this seed project thanks to the synergistic collaboration established among the different groups and the PGTF grant that allowed to immediately carry out the missions to each country.

## References

**Ancarola, M. E., Marcilla, A., Herz, M., Macchiaroli, N., Pérez, M., Asurmendi, S., Brehm, K., Poncini, C., Rosenzvit, M. and Cucher, M.** (2017). Cestode parasites release extracellular vesicles with microRNAs and immunodiagnostic proteins cargo. *International Journal for Parasitology* **47**, 675–686.

**Ancarola, M. E., Lichtenstein, G., Herbig, J., Holroyd, N., Mariconti, M., Brunetti, E., Berriman, M., Albrecht, K., Marcilla, A., Rosenzvit, M. C., Kamenetzky, L., Brehm, K. and Cucher, M.** (2020). Extracellular non-coding RNA signatures of the metacestode stage of Echinococcus multilocularis. *PLoS Neglected Tropical Diseases* e0008890. doi: 10.1371/journal.pntd.0008890.

**Bernardo, B. C., Charchar, F. J., Lin, R. C. Y. and Mcmullen, J. R.** (2012). A MicroRNA Guide for Clinicians and Basic Scientists : Background and Experimental Techniques. *Heart, Lung and Circulation* **21**, 131–142. doi: 10.1016/j.hlc.2011.11.002.

**Budke, C. M. C. M., Deplazes, P. and Torgerson, P. R. P. R.** (2006). Global socioeconomic impact of cystic echinococcosis. *Emerg Infect Dis* **12**, 296–303. doi: 10.3201/eid1202.050499.

**Cheng, G., Luo, R., Hu, C., Cao, J. and Jin, Y.** (2013). Deep sequencing-based identification of pathogen-specific microRNAs in the plasma of rabbits infected with Schistosoma japonicum. *Parasitology* **140**, 1751–1761. doi: 10.1017/S0031182013000917.

**Craig, P. S., Budke, C. M., Schantz, P. M., Li, T., Qiu, J., Yang, Y., Zeyhle, E., Rogan, M. T. and Ito, A.** (2007). Human Echinococcosis: A Neglected Disease? *Tropical Medicine and Health* **35**, 283–292. doi: 10.2149/tmh.35.283.

**Cucher, M. A., Macchiaroli, N., Baldi, G., Camicia, F., Prada, L., Maldonado, L., Avila, H. G., Fox, A., Guti??rrez, A., Negro, P., López, R., Jensen, O., Rosenzvit, M. and Kamenetzky, L.** (2016). Cystic echinococcosis in South America: Systematic review of species and genotypes of Echinococcus granulosus sensu lato in humans and natural domestic hosts. *Tropical Medicine and International Health* **21**, 166–175.

**Cucher, M. A., Ancarola, M. E. M. and Kamenetzky, L.** (2021). The challenging world of extracellular RNAs of helminth parasites. *Molecular Immunology* **134**, 150–160. doi: 10.1016/j.molimm.2021.03.011.

**Díaz, A., Casaravilla, C., Irigoín, F., Lin, G., Previato, J. and Ferreira, F.** (2011). Understanding thelaminatedlayerof larval Echinococcus I: structure. *Trends in Parasitology* **27**, 204–213.

**Fernandez-Mercado, M., Manterola, L., Larrea, E., Goicoechea, I., Otaegui, D. and Lawrie, C. H.** (2015). The circulating transcriptome as a source of non-invasive cancer biomarkers : concepts and controversies of non-coding and coding RNA in body fluids Circulating miRNAs as cancer biomarkers. *Journal of Cellular and Molecular Medicine* **19**, 2307–2323. doi: 10.1111/jcmm.12625.

**Gottstein, B., Wang, J., Blagosklonov, O., Grenouillet, F., Millon, L., Vuitton, D. A. and Müller, N.** (2014). Echinococcus metacestode: in search of viability markers. *Parasite (Paris, France)* **21**, 63. doi: 10.1051/parasite/2014063.

**Guo, X. and Zheng, Y.** (2017). Expression profiling of circulating miRNAs in mouse serum in response to Echinococcus multilocularis infection. *Parasitology* **144**, 1079–1087. doi: 10.1017/S0031182017000300.

**Hoy, A. M., Lundie, R. J., Ivens, A., Quintana, J. F., Nausch, N., Forster, T., Jones, F., Kabatereine, N. B., Dunne, D. W., Mutapi, F., Macdonald, A. S. and Buck, A. H.** (2014). Parasite-derived microRNAs in host serum as novel biomarkers of helminth infection. *PLoS neglected tropical diseases* **8**, e2701. doi: 10.1371/journal.pntd.0002701.

**Kamenetzky, L., Maldonado, L. L. and Cucher, M. A.** (2022). Cestodes in the genomic era. *Parasitology Research* **121**, 1077–1089. doi: 10.1007/s00436-021-07346-x.

**Laganà, A., Russo, F., Veneziano, D., Bella, S. Di, Giugno, R., Pulvirenti, A., Croce, C. M. and Ferro, A.** (2013). Extracellular circulating viral microRNAs: current knowledge and perspectives. *Frontiers in genetics* **4**, 120. doi: 10.3389/fgene.2013.00120.

**Lawn, S. D., Bligh, J., Craig, P. S. and Chiodini, P. L.** (2004). Human cystic echinococcosis: Evaluation of post-treatment serologic follow-up by IgG subclass antibody detection. *American Journal of Tropical Medicine and Hygiene* **70**, 329–335.

**Lorenzo, C., Ferreira, H. B., Monteiro, K. M., Rosenzvit, M., Kamenetzky, L., Garcı, H. H., Vasquez, Y., Naquira, C., Sa, E., Lorca, M., Last, J. A. and Gonza, G. G.** (2005). Comparative Analysis of the Diagnostic Performance of Six Major Echinococcus granulosus Antigens Assessed in a Double-Blind , Randomized Multicenter Study. *Journal of clinical microbiology* **43**, 2764–2770. doi: 10.1128/JCM.43.6.2764.

**Maldonado, L. L., Assis, J., Araújo, F. M. G., Salim, A. C. M., Macchiaroli, N., Cucher, M., Camicia, F., Fox, A., Rosenzvit, M., Oliveira, G. and Kamenetzky, L.** (2017). The Echinococcus canadensis (G7) genome: a key knowledge of parasitic platyhelminth human diseases. *BMC Genomics* **18**, 204. doi: 10.1186/s12864-017-3574-0.

**Ortona, E., Riganò, R., Buttari, B., Delunardo, F., Ioppolo, S., Margutti, P., Profumo, E., Teggi, A., Vaccari, S. and Siracusano, A.** (2003). An update on immunodiagnosis of cystic echinococcosis. In *Acta Tropica*, pp. 165–171. doi: 10.1016/S0001-706X(02)00225-5.

**Palevich, N., Britton, C., Kamenetzky, L., Mitreva, M., de Moraes Mourão, M., Bennuru, S., Quack, T., Scholte, L. L. S., Tyagi, R. and Slatko, B. E.** (2018). Tackling Hypotheticals in Helminth Genomes. *Trends in Parasitology* **34**, 179–183. doi: 10.1016/j.pt.2017.11.007.

**Pawlowski, Z. S., Eckert, J., Vuitton, D. A., Amman, R. W., Kern, P., Craig, P. S., Dar, K. F., De Rosa, F., Filice, C., Gottstein, B., Grimm, F., Macpherson, C. N. L., Sato, N., Todorov, T., Uchino, J., von Sinner, W. and Wen, H.** (2001). Echinococcosis in humans: clinical aspects, diagnosis and treatment. In *WHO/OIE Manual on Echinococcosis in humans and animals: a public health problem of global concern.* (ed. Eckert, J., Gemmel, M. A., Meslin, F. X., and Pawlowski, Z. S.), p. Paris.

**Quintana, J. F., Makepeace, B. L., Babayan, S. a, Ivens, A., Pfarr, K. M., Blaxter, M., Debrah, A., Wanji, S., Ngangyung, H. F., Bah, G. S., Tanya, V. N., Taylor, D. W., Hoerauf, A. and Buck, A. H.** (2015). Extracellular Onchocerca-derived small RNAs in host nodules and blood. *Parasites {&} vectors* **8**, 58. doi: 10.1186/s13071-015-0656-1.

**Rigano, R., Ioppolo, S., Ortona, E., Margutti, P., Profumo, E., Ali, M. D., Di Vico, B., Teggi, A. and Siracusano, A.** (2002). Long-term serological evaluation of patients with cystic echinococcosis treated with benzimidazole carbamates. *Clinical and Experimental Immunology* **129**, 485–492. doi: 10.1046/j.1365-2249.2002.01925.x.

**Tritten, L., Burkman, E., Moorhead, A., Satti, M., Geary, J., Mackenzie, C. and Geary, T.** (2014a). Detection of circulating parasite-derived microRNAs in filarial infections. *PLoS neglected tropical diseases* **8**, e2971. doi: 10.1371/journal.pntd.0002971.

**Tritten, L., O'Neill, M., Nutting, C., Wanji, S., Njouendoui, A., Fombad, F., Kengne-Ouaffo, J., Mackenzie, C. and Geary, T.** (2014b). Loa loa and Onchocerca ochengi miRNAs detected in host circulation. *Molecular and biochemical parasitology* **198**, 14–17. doi: 10.1016/j.molbiopara.2014.11.001.

**Weber, J. a, Baxter, D. H., Zhang, S., Huang, D. Y., Huang, K. H., Lee, M. J., Galas, D. J. and Wang, K.** (2010). The microRNA spectrum in 12 body fluids. *Clinical chemistry* **56**, 1733–1741. doi: 10.1373/clinchem.2010.147405.

**Weiland, M., Gao, X., Zhou, L. and Mi, Q.** (2012). Circulating microRNAs as biomarkers for human diseases Small RNAs have a large impact. *RNA biology* **9**, 850–859.

**Yang, Y., Clements, A. C. A., Gray, D. J., Atkinson, J.-A. M., Williams, G. M., Barnes, T. S. and McManus, D. P.** (2012). Impact of anthropogenic and natural environmental changes on Echinococcus transmission in Ningxia Hui Autonomous Region, the People's Republic of China. *Parasites {&} Vectors* **5**, 146. doi: 10.1186/1756-3305-5-146.

**Zhang, W., Wen, H., Li, J., Lin, R. and McManus, D. P.** (2012). Immunology and immunodiagnosis of cystic echinococcosis: An update. *Clinical and Developmental Immunology* **2012**, 165–171. doi: 10.1155/2012/101895.