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Cystic echinococcosis in cattle: histological and proteomic features of inflammation

Tutor:

Prof. Stefano Rocca

Dottorando

Dott.ssa Ylenia Pilicchi

Co-Tutor:

Dott.ssa Tiziana Cubeddu

Prof.ssa Maria Filippa Addis

Prof. Antonio Scala

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Abstract

Cystic echinococcosis (CE) is caused by the larval stage of *Echinococcus granulosus* and is the most widespread zoonotic disease in both developed and developing countries. The World Health Organization (WHO) included CE in the list of Neglected Tropical Diseases in order to eliminate the disease and support the affected countries. *E. granulosus* has a multistage life cycle that involves two mammalian hosts, dogs as definitive hosts and herbivores as intermediate hosts. The adult form of the tapeworm measures approximately 7 mm, is hermaphrodite, equipped with six segments and able to make contact with the intestine of the definitive hosts in which it develops, producing fully mature eggs. These, released in the external environment through the faeces, show resistance to a wide range of conditions. Once ingested by the intermediate host, the larval stage of the parasite, called metacestode, develops in the internal organs in which it produces cystic lesions.

The aim of the study was to compare fertile and infertile cysts from naturally infected cattle by means of histological and proteomic analysis in order to elucidate the molecular cross-talk between host and parasite and obtain further information on the bovine host immune response against *E. granulosus*.

This survey is organised into two main parts based on histologic and proteomic investigations, respectively.

In this study, a total of 70 hydatids, 55 from lungs and 15 from livers, were collected from 22 naturally infected cattle, slaughtered between 2015-2018. Each cyst was measured during macroscopic examination and processed by routine histology. Fertility was assessed by microscopic examination of protoscoleces (PSCs) presence in the hydatid fluid (HCF). The adventitia layer (AL) (collagen capsule and inflammatory infiltrate) of each cyst was measured, and the number and distribution of giant cells (GCs) were scored in four categories.

Germinal layer (GL) categories were determined and also scored in three different groups. The evaluation of the immune response was carried out by indirect immunohistochemistry (IHC) in order to identify T and B lymphocytes, macrophages and T-reg cells, while molecular identification of *E. granulosus* was carried out by polymerase chain reaction (PCR) and the sequencing of two target genes.

As a result, lungs and livers revealed multiple cysts of varying sizes from 0.5 to 5 cm. Microscopically HCF observation, revealed the presence of 2 pulmonary fertile cysts and 68 infertile ones. The cysts were classified into four categories of inflammation (Absent, Mild, Moderate, Severe) based on a score index assigned to the following parameters: GCs, GL, Cd3 and Cd79a, grading them with a score. Two pulmonary cysts were assigned to absent inflammation, 18 to mild, 33 to moderate and 17 to severe. Indirect immuno-histochemical analysis showed a significant prevalence of T vs B lymphocytes. In addition, statistical analysis showed that the number of Cd3 positive cells is inversely proportional to the thickness of the collagen capsule (p-value < 0.05). Conversely, the number of MAC387 and FoxP3 positive cells was completely negligible and not scored. Molecular screening demonstrated that all isolates belonged to the G1 genotype, *i.e. E. granulosus sensu stricto* (also called *sheep strain*).

In the second part of the study, a total of 27 pulmonary tissue and 10 hydatid fluid samples were subjected to proteomic analysis. Specifically, 20 lung tissues surrounding cysts and 10 hydatid fluid samples were chosen based on the previous histologic classification. In addition, 7 samples from healthy bovine lungs were used as negative controls. Samples were prepared for analysis with filter aided sample preparation (FASP) and analysed with a Tandem Mass Tag (TMT)-based quantitative approach.

As a result of this second part of the study, a total of 5047 proteins were identified in lung tissues surrounding the cysts, of which 137 with significantly differential abundances in the comparison of lung tissue with different inflammation degrees.

In the hydatid fluid samples, a total of 2772 proteins were identified. HCF was analysed according to two different schemes: fertile *vs* infertile cysts, that showed the presence of 94 parasite and host proteins with significantly differential abundances, and sterile with different inflammation degrees in which 6 proteins were differential. In order to understand the mechanisms employed by *E. granulosus* to ensure its survival and by the host to defend itself from the parasite, we identified a total of 12 interesting proteins based on their abundance in hydatid fluid. Moreover, the comparative analysis of lung tissues and hydatid fluids showed a total of 19 statistically significant proteins shared between the groups based on the inflammation degrees. The functional association at a systemic level of proteins, performed through the web resource STRINGdb v11.0, showed the differential proteins map in Biological Process, Reactome Pathways and UniProt Keywords. The STRING analysis showed the association of 6 proteins to immune processes.

Discussing our results we can say that from a histopatological point of view, *E. granulosus* induces in the intermediate host a granulomatous tissue reaction, whose hallmarks are epithelioid and multinucleated giant cells directed to eliminate the foreign body. The fertility rate detected in our study (2.86%) is higher compared to the 0.76% detected in other studies on CE in Sardinia. Nevertheless, in other geographical areas fertility rates in cattle were less than 30%, consistently with our results that show low fertility rates. Lymphocytes characterisation carried out through immuno-histochemical analysis showed a high number of Cd3 positive cells compared to Cd79a positive cells, therefore a prevalence of T lymphocytes vs B, in both livers and lungs. Lungs results are consistent with data reported for sheep. In part, we could compare our degrees of inflammation to the suggested development phases that divide the established phases of *E. granulosus*, into 4 subclasses. The first one, ‘established maturing phase’ in which the metacestode starts to grow and is still susceptible to immune killing by the host could be compared to absent inflammation.

The second, ‘established stable phase’ in which the parasite could induce a response that is permissive in its regards and protective to the host, could be compared to mild inflammation. The third ‘establishment unstable stage’, in which the parasite develops into death process or produces daughter cysts, could be compared to moderate inflammation. Finally, the fourth, ‘established degenerative phase’ in which immunological features may involve a large infiltrate of leucocytes combined with high antibody levels, could be compared to severe inflammation.

Moreover, an important evidence provided by our study is the identification of *Echinococcus granulosus sensu stricto* (G1, sheep strain) in all examined samples, consistently with results reported by other surveys in Italy.

Based on the constantly growing search for biomarkers and the need of new effective vaccines, proteomic analysis is considered a very promising study area. Our study on lung tissue surrounding hydatid cysts provided numerous indications. The comparison of the different inflammation groups (Healthy controls *vs* Absent + Mild inflammation; Healthy controls *vs* Moderate + Severe; Absent + Mild *vs* Moderate + Severe) showed the absence of unique proteins attributable to one combination. This may be due to the tissue of origin (lung), the same for all analysed groups. Furthermore, the highest number of differential proteins was related to the Moderate + Severe vs Healthy Controls comparison. According to Principal Component Analysis, tissue samples clustered into three groups: Healthy controls, Absent + Mild and Moderate + Severe. This confirmed the histological findings. Obviously, the differences between healthy tissue and affected tissues were evident. In contrast, the histological appearance of cysts with absent and mild inflammation was very similar. Similarly, the differences between cysts with moderate and severe inflammation were very slight. In agreement with previous studies, our results confirm that serum proteins, such as serotransferrin and immunoglobulin, are very abundant in hydatid fluid as well as immunogenic proteins produced by the parasite as antigen 5 and antigen B.

The similarity between the host proteins of both plasma and HCF has been demonstrated by several studies, suggesting that *E. granulosus* might be capable to adsorb host proteins through the GL. In our study some differential proteins were overlapped in tissues and HCF. Two proteins, in particular, deserve attention: beta-2-microglobulin and peroxiredoxin. The first one is involved in chronic inflammation, while the second one is involved in protecting cells from oxidative stress. In conclusion, the results of this thesis highlight the complex interaction between the bovine host and *E. granulosus*. Therefore, the combination of histological and proteomic approaches applied in this work provided a better understanding of the host-parasite interplay and of the bovine host immune response against *E. granulosus*.

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Chapter I

Chapter I

Introduction and literature review

1.1 Global significance of Cystic Echinococcosis

The term cystic echinococcosis (CE) describes a widespread zoonotic infection caused by the larval stage of the cestode belonging to the genus *Echinococcus* (McManus D. P., 2012). *Echinococcus granulosus* is a Platyhelminth with a multistage life cycle that causes a cyst-forming disease in a wide range of mammalian hosts, mostly sheep and cattle. Humans are considered aberrant hosts, incidentally affected by the ingestion of parasite eggs shed by the dog (definitive host). The impact of E. *granulosus* in livestock is around $ 125.000 billion (US dollars) per year (Budke, 2006). These are probably underestimated data due to the lack of annual global reports both in humans and in animals.

The World Health Organization (WHO) included CE in the list of Neglected Tropical Diseases (NZDs) asking the various stakeholders to cooperate in order to eliminate the disease and support the affected countries (WHO, 2018).

NZDs are frequent in poor countries whose economy is dependent on livestock and wildlife, increasing the risk of transmission to humans (Mableson H.E., 2014).

Echinococcus granulosus causes significant health and economic problems in intermediate and aberrant hosts (Torgerson P.R., 2003) and has been recognized as part of foodborne diseases (FBDs) (Togerson P.R., 2015).

The FBDs are pathologies derived from the consumption of unsafe food. FBDs include diseases due to microbial pathogens, parasites, chemical contaminant and biotoxins (WHO, 2007). The issue of food security has become increasingly important due to the high mortality and morbidity caused by FBDs in the world population.

The Foodborne Disease Burden Epidemiology Reference Group (FERG), established by the World Health Organization (WHO) in 2006, includes six task forces:

- Enteric Diseases;
- Parasitic Diseases;
- Chemical and Toxin Task Forces;
- The Source Attribution Taskforce;
- Country Studies Task Force;
- Computational Task Force.

The former three study the FDBs from a qualitative point of view in order to establish the burden in terms of prevalence or incidence.

The Source Attribution Taskforce estimates the contribution to the burden of food and non-food origin, indicating which of the food can represent a health problem. Developing local investigation tools to analyse the weight of FBDs country by country, is a function of the Country Studies Task Force and finally, the Computational Task Force deals with data analysis.

CE has been included into the FERG in 2007 (Kuchenmüller T., 2009) together with other pathologies caused by Protozoa (*Giardia, Entamoeba, Cryptosporidium spp., Toxoplasma gondii*)*;* Platyhelminthes, Trematoda *(Fasciola spp., Opisthorchis spp., Clonorchis spp.)* and Cestoda *(Taenia solium, Echinococcus spp.);* Nematoda *(Anisakis simplex, Ascaris lumbricoides*).

CE has been analysed by the Parasitic Diseases Task Force (PDTF) constituted by experienced FERG members in the field of parasitology in order to estimate the number of infections, sequelae, deaths and Disability Adjusted Life Years (DALYs) (Togerson P.R., 2015).

FBDs are important causes of morbidity and mortality but the quantification of consequences attributable to these diseases is still impossible, due to the lack of data concerning developing countries. Thirty-one FBDs have been estimated by the FERG with subsequent over 600 million illnesses and 420,000 deaths in the world in 2010 (Havelaar A.H., 2015) (Hoffmann S., 2017).

The impact on human health caused by echinococcosis has been estimated through the DALYs system. DALY is an internationally applied metric established by WHO, calculated by adding the number of years of life lost to mortality (YLL) and the number of years lived with disability due to morbidity (YLD), DALY = YLL + YDL.

DALYs can be calculated using two approaches, using the prevalence to estimates the current burden in a population considering the previous cases or using, the latter used more frequently, the incidence in which both current and future health cases are included. (WHO, 2015). Every year, 188.000 cases of CE, resulting in 184.000 DALYs (0.98 DALYs per case) are estimated (Togerson P.R., 2015).

Table 1 Median number of CE foodborne illnesses, deaths, and Disability Adjusted Life Years (DALYs) with 95% uncertainty intervals, 2010

Pathogen	Illnesses (95% UI)	Deaths (95% UI)	DALYs (95% UI)	Proportion of foodborne-illness (95% UI)	Proportion of foodborne-DALYs (95% UI)	Foodborne llness (95% UI)	Foodborne deaths (95% UI)	Foodborne DALYs (95% UI)
Cestodes	596,838 (482,828–2,169,206)	48,269 (36,956– 70,978)	3,721,581 (2,942,173–5,416,945)	0.72 (0.35–0.79)	0.85 (0.65–0.93)	430,864 (334,389–774,703)	36,500 (25,652–50,063)	3,158,826 (2,411,585–4,122,032)
E. granulosus	188,079 (156,848–1,770,405)	2,225 (749–19,627)	183,573 (88,082–1,590,846	0.21 (0.15–0.30)	0.21 (0.15–0.29)	43,076 (25,881–371,177)	482 (150–3,974)	39,950 (16,996–322,953)
E. multilocularis	18,451 (11,384–29,619)	17,118 (10,184–27,346)	687,823 (409,190–1,106,320)	0.47 (0.04–0.75)	0.48 (0.01–0.76)	8,375 (656–17,005)	7,771 (243–15,896)	312,461 (9,083–640,716)

Modified table from (Togerson P.R., 2015) Illnesses are defined as the numbers of new cases in 2010.

Combining data of Cystic Echinococcosis and Alveolar Echinococcosis, the total burden is approximately of 871,000 DALYs of which:

- CE 184,000, 95% [UI 88,100–1.59 million] DALYs;
- AE 688,000, 95% [UI 409,000–1.1 million] DALYs.

Global costs for CE treatment has been estimated in 4.1 billion dollars per year, of which 46% destinated to human therapy and 54% associated with animal treatment (WHO, 2016) (WHO, 2017).

1.2 General morphology of the genus *Echinococcus*

1.2.1 Adult

The adult tapeworm measures approximately 7 mm (rarely exceeds this lenght), constituted by no more than six segments. The anterior end is characterized by a specialised attachment structure, called scolex, able to make contact with the intestine of the definitive host, by the presence of four muscular suckers on the rostrellum and two rows of hooks.

The body of the tapeworm is called strobila, and reproductive units, named proglottids, (2-6 segments), give it a peculiar segmented appearance.

The adult is hermaphrodite, it shows reproductive ducts in the lateral genital pore. The cirrus sac is prominent, horizontal or tilted anteriorly, while the vitellarium is globular.

The last proglottid could be almost completely filled by the uterus that dilates after fertilisation when eggs are fully developed.

1.2.2 Eggs

The eggs, produced in the terminal proglottid (definitive host), measure 30 to 40 μm in diameter. Eggs are composed by the oncosphere, or hexacanth embryo, that is the first larval stage surrounded by different envelops. The keratinised layer is responsible of the dark striated appearance and gives high resistance to a wide range of environmental temperatures

and physiochemical conditions, while the other layers disappear once released from the definitive hosts.

1.2.3 Metacestode

The metacestode is the larval stage of the parasite and develops in the intermediate hosts. It consists of a bladder surrounded by two layers, the acellular and outer one, called laminated layer, and the nucleated inner one, called germinal layer (GL). The latter is responsible for the asexual budding to brood capsules and protoscoleces develop from the inner wall of the brood capsules. The last component of cysts is the hydatid fluid (HF), formed by a complex of parasite-derived proteins, whose production is mostly due to GL. Some of its components are highly immunogenic (Manzano-Román R., 2015). Rupture of the cysts may result in the spillage of hydatid material and could cause an immediate anaphylactic reaction or a development of secondary cysts in contaminated sites (Katz A.M., 1958).

1.3 Life-cycle and transmission of *Echinococcus granulosus*

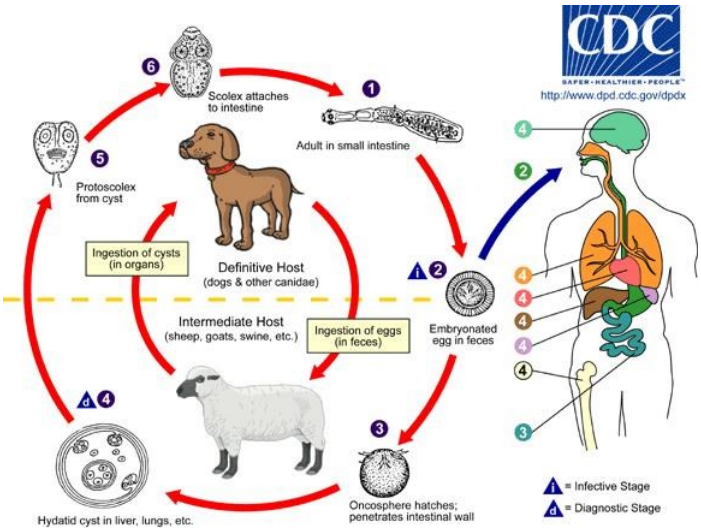

Figure 1 Life cycle of *E. granulosus* Image from the Centers for Disease Control and Prevention Image Library

Echinococcus needs two mammalian hosts for completion of its life cycle, a carnivore definitive host, in which the adult develops in the intestine, and an herbivore intermediate host in which metacestode grows-up in an internal organ.

Intermediate hosts become infected through the ingestion of eggs, released from the definitive hosts with the feces and spread on the grass and water.

In suitable environmental conditions, the eggs acquire an extreme resistance, and remain infective for several months in a wide range of temperatures, from 4°C to 15°C. However, they are susceptible to high temperature, UV exposition and desiccation (Gemmell M.A., 1968). Nevertheless, eggs are extremely resistant to low temperatures depending on the timing of exposure.

1.3.1 Definitive hosts

The definitive host becomes infected by the ingestion of viable protoscoleces. If the latter are contained in the cyst, the excystment is favourite by the masticatory process and furthermore by the pepsin action in the stomach. Protoscoleces undergo a change in their apical part constituted by suckers, rostrellum and hooks, invaginating within the mucopolysaccharide-coated basal region of the protoscolex tegument.

Evagination is first stage in the development of the adult form of the parasite in the definitive host . Protoscoleces evaginate in response to several stimuli as temperature, osmotic pressure and agitation. Evagination time is changeable, but generally starts after 6 hours and takes approximately three days (Thompson R.C.A., 1977). Aerobiosis is the essential condition for the evagination, whereas some enzimes and bile, even if not essential, can favour theprocess (Smyth J.D., 1967).

In the first phases after evagination, protoscoleces use their energy to quickly locate and attach to the mucosal surface of Lieberkühn's crypts. Some protoscoleces reach the crypts in six hours after infections, using glycogen as a reserve of energy (Smyth J.D., 1967).

Once in the small intestine, protoscoleces start the differentiation and divide into four phases: proglottisation, maturation, growth, and segmentation. Proglottisation and maturation lead to the germinal differentiation, while the others two to somatic differentiation (Thompson R.C.A., 1995). The proglottisation of protoscoleces is characterized by a high number of calcareus corpuscles, that disappeared at day 11 to 14. In this second stage, lateral excretory canals become evident and the first proglottid starts to be visible, due to a constriction that marks the area of the first segment. From the 14th to 17th days the genital rudiment divides into two parts, with the full development of a first segment. The formation of the second proglottid is anticipated by the formation of rudimentary tests in the first one. Male genitalia is completed at 20-28 days, when female's one is still developing. Starting from day 28 both male and female genitalia are fully mature, penultimate proglottid has developed, and a band or the third segment appears.

Finally, from day 33 to 37, ovulation and fertilisation of the last proglottid take place concurrently, the uterus is dilated and contains zygotes in division, and both genitalia degenerate in the terminal proglottid. In the penultimate genitalia are mature and developin the ante-penultimate one. The body is divided into three or four segments.

From 37 to 58 days, terminal proglottid appears gravid with several eggs, the penultimate contains zygotes and the strobila is divided in three to five segments.

The adult parasite starts the senescence after 6-20 months, even if it is reported that worms are capable to survive for 2 years or longer in the definitive hosts (Schantz P.M., 1988).

The eggs, from spherical to ellipsoid in shape, are constituted by several layers and measure 30 to 40 µm in diameter.

The first envelope called embryophore gives protection to the embryo (oncosphere). Thickness and impermeability of eggs are ensured by protection, due to some properties of embryophore, as keratin-like layers held together by a glue substance (Sakamoto T., 1981).

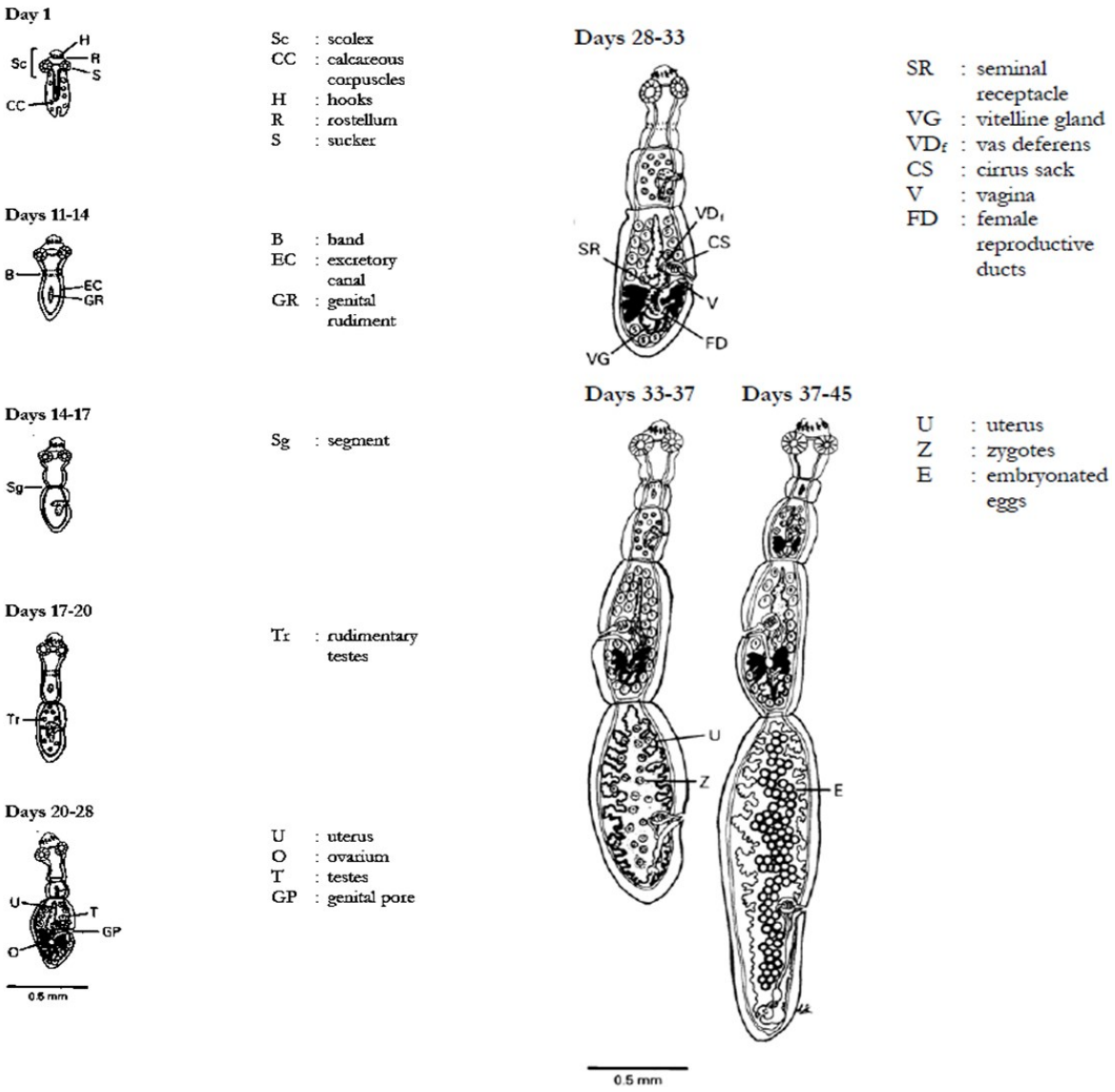

Figure 2 Stages of development to the adult form of *E. granulosus* - *Modiefied from (Eckert J., 2001)*

1.3.2 Intermediate hosts

Once ingested by the intermediate host, eggs undergo to the hatching enabled by the passive disaggregation of embryophoric blocks in the gastric and in the intestinal envinroment, exerted by proteolytic enzymes, such as pepsin and prancreatin (Thompson R.C.A. and Lymbery A.J., 1995).

The proper activation of the oncosphere begins after it's liberation from the envelope (Lethbridge R., 1980), by the modification in the membrane permeability exerted by the bile salts (Smyth J. D., 1969). The oncosphere in now enabled to penetrate the small intestine through hooks movements in the apical portion of the villi and reaches the lamina propria in 3 to 120 minutes after the hatching (Lethbridge R., 1980). Finally, oncosphere is passively carried through the vessels to the liver and other organs such as kidneys, spleen, muscles, brain and many others (Thompson R.C.A. and Lymbery A.J., 1995). Once arrived in the final location of the intermediate hosts, the oncosphere develops in metacestode larval stage, able to continue the parasite life cycle.

The full development in metacestode (post-oncospheral process) occurs in two weeks. During this time the oncosphere reorganizes itself in terms of cellular proliferation, hooks degeneration, muscular atrophy, vesciculization, and central cavity formation and finally development of both germinal and laminated layers (Heath D. D. and Lawrence S. B., 1976). The formation of protoscoleces begins within the hatching capsules, through an asexual asynchronous reproduction process, which leads to the simultaneous presence of different stages of protoscolic maturation. (Thompson R.C.A., 1995).

Germinal layer (GL) plays a role in the secretion of molecules probably involved in the evasion mechanism from host immune response, such as myo-inositol hexakiphosphate IP6, able to inhibit the complement activation, allowing cyst's establishment and survival (Breijo M., 2008).

GL is surrounded by the laminated layer (LL), a carbohydrate-protein complex of highly glycosylated mucin and glycoprotein (Kilejian A. and Schwabe C. W., 1971) which gives intracystic tension and works as a physiochemical barrier.

LL is a network of microfibrils with aggregates of electron-dense material. It has been demonstrated that LL is entirely of parasiteic orgine, produced by the GL and plays a role in the defence of metacestode favouring the protection from immunological attack. Immunoglobulin can pass through LL, an inert barrier, whereas the GL can regulate the penetration of molecules inside the cyst cavity. It has been recently demonstrated that LL protects the metacestode by preventing the nitric oxide production by the host , through the induction of an increase in the arginase activity of macrophages (Amri M. and Touil-Boukoffa C., 2015).

After the post-oncospheral development, the host produces the adventitial layer (AL), a fibrous capsule constituted by several layers of inflammatory cells. Depending on the strenght of the host immune response two possibilities can take place. A strong inflammatory reaction causes the degeneration and death of the parasite, whereas, as a second option, a favourable envinroment for the oncosphere can lead to the production of a fibrous capsule.

Several months may be necessary before protoscoleces production (fertile stage of metacestode). Although *E. granulosus* usually produces unilocular cavity cysts, the formation of secondary chambers, starting from the cyst wall and in communication with the central cavity, is not a rare event. Central cavity and chambers can be in communication by incomplete septa, or alternatively cysts may adhere to each other and produce clusters of small cysts of different size.

Each cyst may contain thousands of protoscoleces, each of which able to develops into an adult worm in the definitive host, even if some cysts are unable to produce protoscoleces (sterile metacestode).

Once produced, protocoleces are potentially ready for the ingestion from the definitive hosts, reach the upper duodenum where, following several signals as pH change, bile production and temperature increasing can evaginate e progress the cycle.evaginate . From four to six weeks are required to allow the protoscoleces to develop in adult and sexually mature worm.

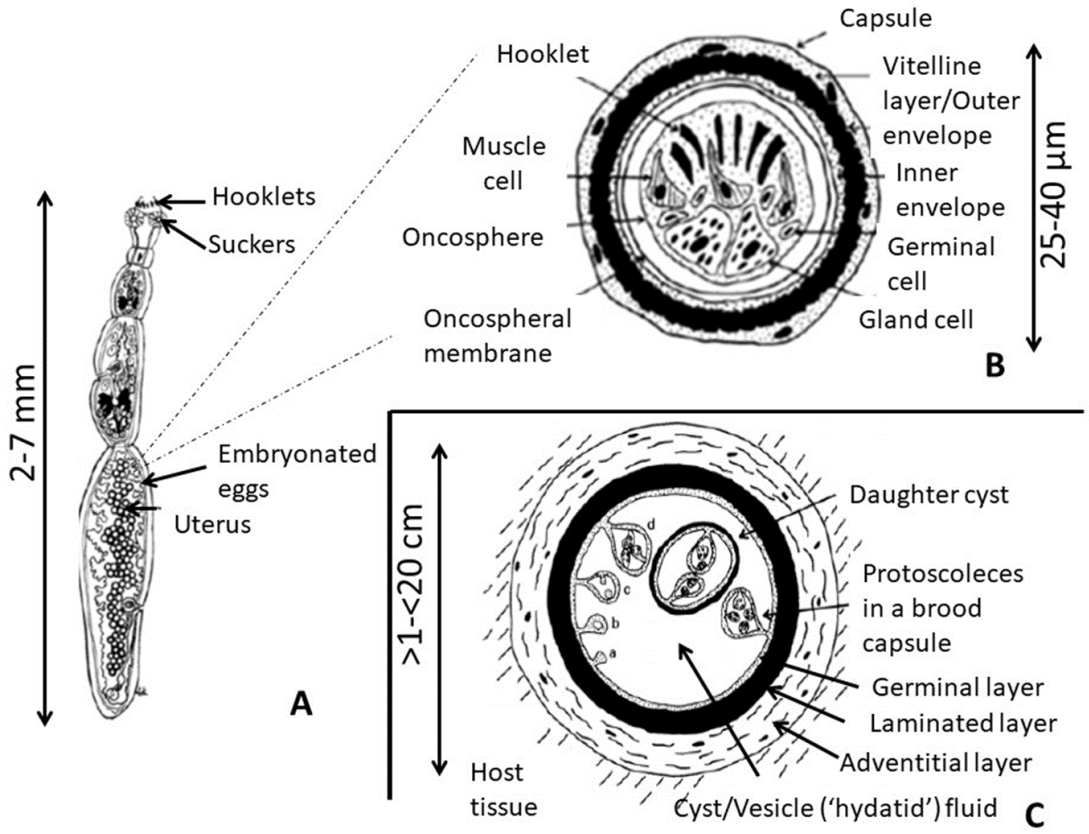

Figure 3 Stages of development of *E. granulosus* A: worm B egg C metacestode (Vuitton D., 2017)

1.4 Taxonomy

Humans and livestock echinococcosis are parasitic zoonotic diseases long known, with several names, since the ancient age, due to the larval or adult stage of the cestode belonging to the species *Echinococcus spp.*

Until the end of the 19th century different names, based on the morphology of the parasite or the host origin, have been used (Abuladze K. I., 1970). The first valid name was *Hydatigena granulosa*, given by Batsch in 1786 and based on a fertile cyst of sheep observed in Germany. In 1801, Rudolphi assigned the name *Echinococcus*, based on the aspect of protoscoleces. Hence from the union of these two names '*granulosa*' and '*Echinococcus*' originated the term *Echinococcus granulosus* still in use today (Romig T., 2015). From the end of 19th century, although there was still confusion about the names, the term was used to identify both the larval and adult stage of the parasite. *Echinococcus multilocularis*, causative agent of alveolar echinococcosis (AE) has been described by Leuckart in 1863.

1.4.1 Species of the genus *Echinococcus*

Based on the morphology of the adult worm, several species of *Echinococcus* have been described, *E. multilocularis* by Leuckart (1863) and *E. oligarthra* (Diesing K.M., 1863), *E. vogeli* in 1972 (Rausch R.L., 1972) and finally *E. shiquicus* in 2005 (Xiao N., 2005).

1.4.1.1 Subspecies

In order to clarify the relationships between species and subspecies of *E. granulosus*, an informal system of intraspecific strains has been proposed, that included eleven strains called: sheep, Tasmanian sheep, buffalo, horse, cattle, camel, pig, variant pig (or humas-pig), American cervid, Fennoscandian cervid, and lion strain.

Although the strains characterization was conducted on the basis of host-specificity, geographical distribution, morphology and stages of development, starting from 1990s molecular biology techniques start to become more useful for the strains identification and definition. Identification of *Echinoccocus* and *Taenia* species, subspecies and strains were conducted through the use of several molecular methods by specific nuclear or mitochondrial genomes. The publication of the partial sequences of two mitochondrial genes, cox1 and nad1, for seven strains of the species *E. granulosus* and for the other three species *E. multilocularis*, *E. vogeli* and *E. oligarthra*, was a decisive turning point in the determination of relationships and taxonomy of *Echinococcus* species and strains (Bowles J., 1992) (Bowles J. a. M., 1993). The nomenclature system was upgraded throughout the introduction of the term genotype instead of strains, even if after the terms were used as synonymous.

To the seven strains/genotypes of *E. granulosus*, three new of them were added: the American cervid strain (G8) (Bowles J. B. D., 1994), the variant-pig or human-pig strain (G9) (Scott J.C., 1997) and the Fennoscandian cervid strain (G10) (Lavikainen A., 2003).

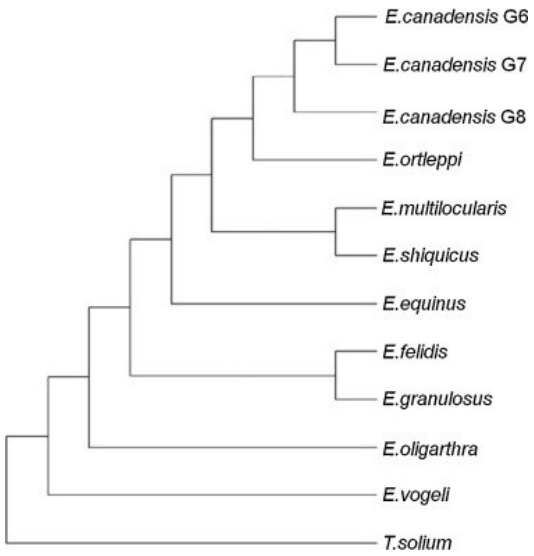

Figure 4 Cladogram on phylogenetic relationships, based on four mitochondrial genes (cox1, nad1, cob, rrn) (Romig T., 2015)

Currently, several species within the genus *Echinococcus* are recognised, showing differences in definitive and intermediate/aberrant hosts and in the geographical distribution. In humans, different species of the parasite are recognised as the causative agents of well characterised pathologic entities such as *Echinococcus granulosus* in the cystic echinococcosis (CE), *Echinococcus multilocularis* in the alveolar echinococcosis (AE) and *E. oligarthra* and *E. vogeli* in the polycystic echinococcosis (PE). The genus *Echinococcus* presents a high variability in terms of hosts specificity, morphology, antigenicity, development rate and transmission cycle.

Table 2 Taxonomy of the genus *Echinococcus*

Species	Subspecies	Strains	Genotypes
Echinococcus granulosus	*E. granulosus s.s.*	Sheep	G1
(Batsch 1796)		Tasmanian sheep	G2
		Buffalo	G3
	E. equinus	Horse	G4
	E. ortleppi	Cattle	G5
	E. canadensis	Camel	G6
		Pig	G7
		Intermedius (camel/pig)	G6/7
		American Cervid	G8
		Fennoscandian	G10
		Pig-human or pig variant	G9
	E. felidis	Lion	
	E. shiquicus		
E. multilocularis			
E. oligarthra			
E. vogeli			

1.4.2 *Echinococcus granulosus* complex

Molecular studies, based on mitochondrial DNA analysis, demonstrated the genetic variability of the complex *Echinococcus granulosus*. Ten distinct genotypes have been identified within this complex, including two sheep strains (G1 and G2), two bovid strains (G3 and G5), one horse strain (G4), one camel strain (G6), two pig strain (G7 and G9) and two cervid strains (G8 and G10).

For the parasite *E. granulosus* complex both wild-life and domestic cycle have been reported. The first one is characterised in the ancestral form by a wild cycle involving wolves and cervids (moose and reindeer), maintained by a predator-prey relationship, while the domestic cycle seems to have evolved from the cervid cycle adapting in ungulates due to the human intervention in the breeding process (Eckert J., 2001).

***1.4.2.1 Echinococcus granulosus sensu stricto*:** G1, G2 and G3 strains have been considered part of the same taxonomic group due to the phylogenetic relationship based on analyses on mitochondrial genome (Nakao M., 2006). *E. granulosus s.s* is cosmopolitan (worldwide distributed) and its definitive hosts are represented by domestic dog, wolf, dingo, jackal and other canids. The intermediates hosts involved in its cycle are sheep, goat, cattle, pig, camel, buffalo, horse, wild ungulates, marsupials, etc. Human cases have been reported.

1.4.2.2 Echinococcus equinus (G4) is present in Eurasia and Africa, involves dog as definitive host and horse, cervids and other equids as intermediate . Nohuman cases have been reported reported.

1.4.2.3 Echinococcus ortleppi (G5) is present in Eurasia and Africa, involving the domestic dog as definitive host and cattle as intermediates. Human diseases has been reported.

1.4.2.4 Echinococcus canadensis (G6, G7, G8, G10), distributed in Eurasia, Africa, North and South America, involves domestic dog and wolf as definitive hosts and pig, camel and cervids as intermediates, with some cases in humans . Based on an evolutionary species concept, some Authors suggested that *E. canadensis* should be split into three species: *Echinococcus intermedius for G6/G7* (as mentioned below in 1.5 Geographical distribution of *Echinococcus spp*. and molecular epidemiology CE in livestock and humans as reported by Deplazes et al., (2017)), *Echinococcus borealis* for G8, and *E. canadensis* for G10.

1.4.2.5 Echinococcus felidis, typical of Africa, involves lion as the definitive host and several intermediate hosts as hyena, warthog, zebra, wildebeest, bush pig, buffalo, antelopes, giraffe, hippopotamous. Human cases are not reported.

1.4.2.6 Echinococcus shiquiqus*,* distributed in the Tibetan Plateau, involves Tibetan fox as intermediate hosts and *Ochotona curzioniae* (named Tibetan plateau pika) as intermediates host. No human cases have been reported.

1.4.3 Echinococcus multilocularis

E. multilocularis is characterised by a sylvatic cycle involving foxes and rodents. Predation of wild animals and capture of rodents can infect domestic cat and dog as intermediate hosts. The cycle is reported in Europe, Japan and other regions. Another way of transmission could be perpetuated by domestic cats and rodents in some areas, although this chance is considered less important. *E. multilocularis* has been reported in Eurasia and North America affecting all foxes species wolf, racoon, dog, domestic dog and cat as definitive hosts, and arvicoline and microtine rodents and small herbivorous mammals including lagomorphs (e.g. pika); pigs, boars, horses, cattle, nutrias, primates and dog as intermediate hosts, even if the last seven species are considered as accidental hosts. Infection in humans is occasionally reported.

1.4.4 Echinococcus oligarthra

E. oligarthra, present in Central and South America affects wild felids (cougar, jaguar, ocelot, jaguarondi and Geoffroyi's cat) as definitive hosts, while agouti and opossum are the intermediates. Human cases have been reported.

1.4.5 Echinococcus vogeli

E. vogeli is distributed in Central and South America (Sousa O.E. and Thatcher V.E., 1969), withbush dog (*Speothos venaticus*) and domestic dog as definitive hosts and paca (*Cuniculus*) as intermediate. It involves also humans. The transmission of this strain is mostly guaranteed by a sylvatic predator/prey cycle, whereas the domestic cycle that involves the dog of some rural area of South America, seems to be responsible of the human's infection.

1.5 Geographical distribution of *Echinococcus spp.* and molecular epidemiology CE in livestock and humans

Echinococcus granulosus is widely distributed throughout the world, with the exception of few areas such as Iceland, Ireland and Greenland that are considered human CE free (Budke, 2006).

1.5.1 America

1.5.1.1 North America and US

In North America, livestock CE is mostly associated with *Echinococcus granlosus sensu lato* that involves sheep, swine and cattle as intermediate hosts.

Canada, Alaska and the northern part of US are characterised by the presence of *E. Canadensis* (G8-G10) that involves wolves (including *C. lupus*) and cervids (moose, *Alces alces*; caribou, *Rangifer tarandus*; and elk, or wapiti, *Cervus canadensis*).

A non-genotyped strain of *E. granulosus* is involved in the dog-sheep cycle in the western part of the US. G7 strain (*Echinococcus intermedius*) is responsible for the dog-swine cycle in Mexico, where *E. granulosus* G1 and *E. ortleppi* G5 are also present (Deplazes P., Global Distribution of Alveolar and Cystic Echinococcosis, 2017).

A formal surveillance for CE in wildlife species (canids and ungulates) doesn't exist , while examination at the slaughterhouse is a common practice for domestic livestock in US, Canada and México.

Human CE in North America is primarily due to *E. Canadensis* (G8), that has cervids as intermediates hosts. Data on CE in human in the North America territory lack due to absence of national register, however two studies demonstrated the presence of G8 strain in Alaska. G5 strain has been reported in Mexico (Maravilla P., 2004) (McManus D.P., 2002).

1.5.1.2 Central America

Cases of human CE have been occasionally reported in different countries of Central America as Costa Rica (Brenes Madrigal R.R., 1977), Guatemala, El Salvador, Honduras, Cuba, Panama (Sánchez G.A., 1992) (Sousa O.E. and Lombardo Ayala J.D., 1965) but information on molecular data and local transmission have not been described. Nevertheless, Cuba is the only country able to shows updates data on human CE (Deplazes P., Global Distribution of Alveolar and Cystic Echinococcosis, 2017).

1.5.1.3 South America

In South America, CE has an important socio-economic impact due to a high prevalence in livestock. It shows higher prevalence rates in Argentina (in particular Patagonia, Pampas, Coast), southwest of Bolivia, south of Brazil, South of Chile, southern and central Peru, and Uruguay. All species of *Echinococcus* responsible of CE in South America involve dogs as definitive host, and several mammalian species as intermediate hosts. The intermediate hosts of *E granulosus s.s* (G1-G3) are sheep, cattle, goats, alpaca and swine, for *E. ortleppi* (G5) is the cattle, *E. intermedius* G6 involves goats (Soriano S.V., 2010) and the swine is the intermediate host for G7 (Deplazes P., Global Distribution of Alveolar and Cystic Echinococcosis, 2017). The Pan American Health Organization (PAHO) applies a control for the CE in South America through post mortem examination, providing good information about the situation in livestock. On the other hand, since there is no systematic surveillance of infection in dogs, the real prevalence of the disease could be underestimated due to the limited data available.Similarly, due to several notification systems belonging to different countries of South America, the situation regarding human CE is usually underreported (PAHO, 2015), even if studies conducted between 2009 and 2015 reported 29,556 cases, most of which in Peru, 20,785.

Table 3 Genotypes and related geographical distribution of *Echinococcus granulosus* in America (Deplazes P., 2017)

Country	Human	Canids	Dog	Cervids	Sheep	Cattle	Goat	Alpaca	Pig
Canada		G8 G10		G8 G10					
México	G5	G7							G1 G7
United States	G8			G8 G10					Eradicated
Argentina	G1 G2 G5 G6		G1 G6		G1 G2 G3	G1	G1 G6	G1 G6	G1
Bolivia	G1								
Brazil	G1 G3 G5		G1 G3 G5		G1	G1 G5			G1 G7
Chile	G1 G6					G1 G3			
Peru	G1 G6				G1		G6	G6	G1 G7
Uruguay						G1 G5			

1.5.2 Asia and Eastern Europe

Echinoccocus species are widely distributed in the northern part of Asia (Russian Federation), involving humans, dogs, farmed animals and wildlife.

Several genotypes are involved in the infection as *E. granulosus* (G1-G3), *E. Canadensis* (G8, G10) and *E. intermedius* (G6/G7).

In Russia, the Federal Centre of Hygiene and Epidemiology reports 573 cases of human CE *per annum* (2006 – 2010). However, the data crossing between individual Russian district, regional centre and local scientific reports showed a higher number of cases per year, equal to 950 (Deplazes P., 2017), confirming CE as major health issue in Russia.

1.5.2.1 Middle East: Iran, Iraq; Israel, Jordan Kuwait, Lebanon, Oman, Palestine, Qatar, Saudi Arabia, Turkey and Yemen

The Middle East is considered one of most affected area by CE in the world (Cardona G. A. and Carmena D., 2013) (Dakkak A., 2010). Several factors are involved in local CE endemicity, such as a limited knowledge by the population regarding the transmission of the disease, a high number of stray dogs involved in the transmission, poor hygienic conditions in slaughterhouses in addition to home slaughtering; the rural/nomadic lifestyle accompanied by the use of sheep dogs on farms and the consumption of raw vegetables.

However, some other factors works as opposing fources acing against the CE transmission, such as the arid / semi-arid climatic conditions that prevent the survival of Echinococcus eggs, religious traditions that do not allow pig breeding and the habit of avoiding dogs in Muslim communities. (Dakkak A., 2010) (Harandi .M.F., 2011) (Rokni M. B., 2009).

E. granlosus s.s (G1-3) is the main responsible for both human and animal CE in the Middle East (Harandi .M.F., 2011) (Sharbatkhori M., 2009) (Utuk A. E., 2008), followed by *E. intermedius* (G6) with an increasing prevalence of the infection in humans (Rostami, 2015) (Al Kitani F. A., 2015). *E. intermedius* (G7) have been reported in Turkey and Iran (Fadakar B., 2015) (Eryıldız C. and Şakru N., 2012).

Even if sheep and goats are the most affected intermediate hosts, G1-G3 involves also cattle, buffalo, one- and two-humped camels, horse, donkey, pig, wild sheep (*Ovis orientalis*), goitered gazelle (*Gazalla subgutturosa*) and free-ranging Baboon (*Papio hamadryas*).

Human CE is often considered a disease of rural areas, but reports showed an increase in the number of the cases also in urban areas (Ok U.Z., 2007). Data available on human CE in the Middle East come from hospital reports, because some region as Iran, Palestine and Turkey recently took part to the European Register of CE (Rossi P., 2016).

1.5.2.2. South Asia: Afghanistan, Pakistan, India, Bhutan, Nepal, Bangladesh, Sri Lanka, Maldives

E. granulosus. genotypes (G1 and G3) are the most represented in the South of Asia, affecting mostly sheep and buffaloes. G1-G3 is considered the genotype with highest zoonotic potential, even if also G5 and G6 can infect humans. Prevalence based studies on intermediate hosts reported that CE is endemic in the majority of South Asia.

1.5.2.3 East Asia: China, Mongolia, Korea, Japan

Several genotypes have been reported in East Asia. *E. granulosus s.s* (G1-3) has been commonly isolated from humans, dogs, sheep, cattle and yaks. *E. intermedius* (G6) in humans, dogs, cattle and camels. G7, G10, G4 and G5 have been occasionally described.

1.5.2.4 South East Asia: Indonesia, Vietnam, the Philippines, Malaysia, Thailand and the Lao People's Democratic Republic

CE cycle in South East Asia is not maintained (McManus D.P., 2010), and the existence of CE is questioned, with the hypothesis thet infection could be restricted to wild animals. Infection in animals has been reported in 1974 in Indonesia in a dog (Carney W.P., 1974), bu further studies on 63 dogs in dogs did not demonstrate the presence of *E. granulosus.* Data are not available in livestock.

Table 4 Genotypes and related distribution of *Echinococcus granulosus* in Asia and European part of Russia (Deplazes P., 2017)

Country	Human	Wolf	Dog	Wild canids	Sheep	Camels	Goat	Equine	Cervid	Pig	Yak	Cattle/ Buffaloes	Cat
Armenia	G1-3				G1-3					G7		C: G1-3	
Kazakhstan			G1 G6/7										
Kyrgyzstan			G1 G4 G6/7										
Russian Federation	G1-3 G6 G10	G6 G10			G1-3		G1-3		G6 G8 G10				G1
Iran	G1 G2 G3		G1 G2 G3 G6	G1	G1 G3	G1 G3 G6	G1 G6 G7	G1 G6 G4				G1 G3 G6	
Iraq	G1											G1	
Jordan			G1 G4		G1								
Oman					G1	G1	G1					G1	
Palestine					G1 G2 G3								
Turkey	G1 G3 G6 G7		G1		G1 G3 G7	G1	G1	G1 G4				G1 G3	
Yemen	G1												
Afghanistan	G6												

Country	Human	Wolf	Dog	Wild canids	Sheep	Camels	Goat	Equine	Cervid	Pig	Yak	Cattle/ Buffaloes	Cat
Bhutan	G1-G3			G1-3							G1-3	G1-3 G5	
India	G1 G3 G5 G6			G1-G3	G1-G3		G1-G3			G3 G5		G1-G3 G5	
Nepal	G1-3			G1	G1 G5		G1 G5			G1-3		G1 G5	
Pakistan	G1			G1	G1 G3	G1	G1 G3					G1 G3	
China	G1 G3 G6 G7 G10		G1 G1-4 G6		G1	G1 G6	G6				G1	C: G1 C: G6	
Japan	G1											C: G1-3	
Mongolia	G1 G6/7 G10	G6/7 G10	G1										
Tibet					G1						G1	C: G1-3	

1.5.3 Australia and New Zealand

E. granulosus was introduced in both countries during the European settlement mostly through sheep livestock (Gemmell M.A., 1990), parasite's cycle was perpetuated between sheep and dog, or cattle and dog, causing a high rate of infection in humans. Furthermore, the maintenance of the parasite cycle is allowed by wild species suche as dingoes and macropods.

CE in animal decreased during the last 30 years and rarely affected sheep that came into contact with wild animals. The parasite is also infrequent in dog, and generally limited to rural areas where the infection is still present with a low prevalence, in the dog-cattle cycle. Dingoes and macropods marsupial in the eastern and southwest of Australia are susceptible to the infection (Jenkins D.J., 2014).

New Zealand and Australia have implemented successful control programs and the Ministry of Agriculture and Forestry declared New Zealand provisionally CE free in 2002 (Anonymous, 2012).

Human CE cases on the mainland of Australia are rarely reported. Some new cases have been identified, but most of these are attributable to immigrants infected before joining Australia (Thompson R.C.A and Jenkins D.J., 2014). In Tasmania no new cases have been reported (O'Hern J. A. and Cooley L., 2013).

1.5.4 Africa

1.5.4.1 North Africa: Morocco, Algeria, Tunisia, Libya, Egypt

CE is considered one of the most important diseases in North Africa, causing economic losses and human health issues.

The maintenance of the parasite cycle is mainly due to rural domestic transmission, involving dogs as definitive hosts and several intermediate hosts such as sheep, cattle, goats, camels, dromedaries, and donkeys. CE in wild animals involves golden wolves as definitive hosts and wild boars and antelope as intermediate hosts.

The main causes of CE persistence are epidemiological factors such as contacts between human population and livestock, scarce knowledge of the CE disease, high dog to human ratio, poor hygienic conditions in slaughterhouses or the practice of home slaughtering (El Berbri I., 2015). Moreover, the cycle is maintained due to the habit of feeding dogs with offal discarded (Kouidri M., 2012). Incidence rates in human are around 5-10 cases per 100.000 inhabitants (Torgerson P.R. and Macpherson C.N., 2011). CE endemicity is high in all North Africa's countries (Dakkak A., 2010).

1.5.4.2 Sub-Saharan Africa

CE is widespread in sub-Saharan Africa except in two arid zones in the northern part of the region (Sahara and Sahel). As well as North Africa, in sub-Saharan Africa, CE is highly endemic (Romig T. O. R., 2011) (Magambo J., 2006).

Several genotypes of *Echinococcus* are present in this area and, among these, *E. felidis*, seems to be widespread in Eastern and Southern Africa in wild animals with a cycle that involve lion, hyenas and warthogs. There are noreports in domestic animals or human species. Similarly to the previous one, *E. equinus* is widespread in South Africa, in a wildlife cycle that involve lions, wild dogs and zebras, with no reports in humans.

From Sudan to South Africa, *E. ortleppi* is widespread in the livestock cattle.

E.intermedius is typical of the arid region on the North where camels are the key points for the transmission of the parasite. G6 is the predominant genotype of the region, perpetuated by goats and wildlife animals. The number of human cases is lower if compared to other regions, leading to suppose that this genotype could be less infective for humans.

1.5.4.3. East Africa: South Sudan, Ethiopia, Eritrea, Somalia, Uganda, Kenya

Eastern part of Africa is mostly affected by *E. granulosus s.s.* caused by the sheep-dog cycle. Ethiopia is the area of the region with the highest prevalence in sheep, probably because this genotype is responsible for the all fertile cysts (Hailemariam Z., 2012) (Maillard S., 2007). In Kenya the prevalence is moderate even if G1-3 is the major responsible of the infection and cause of all fertile cysts in Maasailand (Addy F., 2012) (Romig T., 2011).

Both countries show a low prevalence in goats and all the fertile cysts are due to *E. intermedius* and *E. ortleppi*.

Cattle, affected by G1-3, are characterised mostly by infertile cysts, whereas infection due to *E. ortleppi* and G6 strain are less frequent but resulting in a higher rate of fertility (Mbaya H., 2014). South Sudan and Somalia show low rates of infection in sheep due to *E. intermedius* G6/7 (Omer R.A., 2010).

Ethiopia, Somalia and North-East of Kenia are characterised by low prevalence in camel, in contrast to North-West part that reported an extremely high prevalence in this species. Most of them are infected by G6/7 and in case of fertility by G1-3 (Dinkel A., 2004).

Wild animals are infected by *E. felidis* and G1-3 strains affect lions, hyenas and warthog. Cases of human CE has been mostly found in eastern South Sudan, southwest of Ethiopia and northwest of Kenya (Romig T., 2011) , but also in Uganda, Rwanda and Somalia (Babady N.E., 2009).

1.5.4.4. West and Central Africa: Nigeria, Burkina Faso, Cameroon, the Central African Republic, Democratic Republic of Congo

Poor information are available for these countries, however high rates of infection of camels are reported in the northern Nigeria while there is a low prevalence in sheep, goats and cattle. Scarce or old data are described in Burkina Faso, Cameroon, Central African Republic and the Democratic Republic of Congo, suggesting that these countries are poorly affected by CE.

1.5.4.5 Southern Africa: Angola, Zambia, Mozambique, Zimbabwe, Namibia and South Africa

Surveys conducted in livestock in 60' years demonstrated that the parasite is widespread in South Africa. These information were confirmed by molecular surveys that showed the presence of the genotypes G1-3, G4, G5, G6 and *E. felidis*. However, accurate data about the impact of the parasite in livestock, wild animals and humans still lack. Human CE is rarely reported due to the absence of surveys, but some case reports exist and indicate that CE is widespread in West and Central Africa.

Table 5 Genotypes and related geographical distribution of *Echinococcus granulosus* in Africa (Deplazes P., 2017)

Country	Human	Dog	Wild canids	Wild carnivores	Sheep	Camel	Cattle/ Buffalo	Pig	Warthog	Equine/ Wild Equine	Goat	Wild ruminants	Antelope	Swine	Wild boar
Algeria	G1 G2				G1 G2	G1 G2	G1								
Egypt	G1 G6 G7				G1 G6	G1 G5 G6	G6			G4				G6 G7	
Lybia	G1	G1			G1	G1	G1								
Morocco	G1				G1	G1	G1			G1	G1				
Tunisia	G1 G3 G6	G1	G1		G1	G1 G6	G1			G1 G4	G1		G1		G1
Ethiopia					G1-3	G1-3 G6/7	C: G1-3 C: G5 C: G6/7	G1 G5			G1 G6/7				
Ghana	G6														
Kenya	G1-3 G6	G1		G1-3 *E. felidis*	G1-3 G5	G1-3 G6/7	C: G1-3 C: G5 C: G6/7	G1 G5 G6			G1 G5 G6/7	G1-3			
Mali		G6													

Country	Human	Dog	Wild canids	Wild carnivores	Sheep	Camel	Cattle/ Buffalo	Pig	Warthog	Equine/ Wild Equine	Goat	Wild ruminants	Antelope	Swine	Wild boar
Mauritania	G6					G6	C: G6								
Namibia		G6/7		G1-3 G4			C: G5		*E. felidis*	We: G4 We: G5		G6/7 G4 G5			
Somalia						G6									
South Africa	G1-3 G5 G6/7			*E. felidis*											
South Sudan	G1-3				G6		C: G5 C: G6/7				G6/7				
Sudan	G6				G6	G5 G6	C: G5 C: G6				G6				
Uganda									G1 *E. felidis*						
Zambia							C: G5								

1.5.5 Europe

CE in Europe is caused mainly by *E. granulosus s.s.* G1-G3. *E. canadensis* G8 and G10 are associated to a low pathogenicity in northern Europe (Oksanen A., 2015). Human CE in Baltic region is mostly caused by *Echinococcus intermedius* G7 (Marcinkuté A., 2015).

As previously reported for North America, data on prevalence of human and livestock CE are fragmentary, but since 2016 FP7 HERACLES (Human cystic Echinoccocosis Research in Central and Eastern Societies) project intends to deepen the knowledge of the epidemiology of human CE (Rossi P., 2016).

In 2013, HERACLES was established (http://www.Heracles-fp7.eu/). The aim of HERACLES project are many, including assessing the prevalence of human abdominal CE through ultra-sound examination in the endemic rural area (Bulgary, Romany, Turkey), promoting educational activity and molecular diagnostic tools and implementing an international register of human CE. Moreover, the Echino-Biobank was founded to collect parasite and host patients sample for genetic and serological studies (Rossi P., 2016). ERCE (European Register of Cystic Echinococcosis) is the first CE register, while EurEchinoReg, European Echinococcosis Registry is dedicated to AE (Kern P., 2003).

The first meeting of ERCE has been held in 2015, in Rome (Italian National Health Institute, ISS) with 44 echinococcosis experts from 16 countries (Albania, Austria, Bulgaria, France, Georgia, Greece, Hungary, Iran, Italy, Palestine, Poland, Romania, Serbia, Spain, The Netherlands and Turkey). Among the aims of ERCE, there is the surveillance of human CE, establishing a multi-stage approach to treatment, data collection and the promotion of public health policies. The turning point is represented by the opportunities to conduct real-time studies and not only retrospective studies (Rossi P., 2016).

ERCE is a single multicentre database including patient data, demographic information, CE-related clinical data, biological samples as serum, plasma and cyst-derived material.

The deposited samples are registered in an official biobank collection and managed according to the European standards for biobanking (Law 1716/201) (Rossi P., 2016). ERCE and HERACLES project represent the opportunity to estimates the true number of CE cases in Europe, currently unknown data due to the absence of a mandatory notification for CE. The ERCE could be helpful tools for governments, the European Commission and European Agencies as ECDC (European Centre for Disease Prevention and Control), engaged in several activities as surveillance, epidemic intelligence, public health training and communication, in data collection and CE reporting and monitoring.

1.5.5.1 Western and Northern Europe: Iceland, Ireland, Great Britain, Norway, Sweden, Finland and Denmark

Iceland became free of CE infection after an effective control program (Schantz P.M., 1995) (Sigurdarson S., 2010).

United Kingdom is considered endemic for CE, particularly caused by *E granulosus* G1-3 found in dogs, sheep, cattle and *E. equinus* G4 found in horses, dog and two captive mammals as zebra and lemur (Boufana B., 2015).

Ireland is endemic only for *E. equinus* found in horses and dogs (Hatch C., 1970) (Kumaratilake L.M., 1986).

In Scandinavia, *E. canadensis* G8-G10 are responsible for CE, that involves wolves and cervids and in human cause a primarily lungs disease. Starting from 1960, only one case of CE has been reported in Finland (Oksanen A., 2015).

Human CE is historically present in UK,with a higher incidence value in Western Isles. has been generally decreased in the UK.

1.5.5.2 Central Europe: Belgium, The Netherlands, Luxembourg, Germany, Switzerland, Austria and Czech Republic

Austria, Germany, Switzerland are characterised by the endemicity of *E. ortleppi* G5 (cattle strain), although the absence of systematic data, some slaughterhouse reports, shows hardly absence of CE.

A single case of cyst in a pig due to G7 was found in Austria, but there are no current data from Germany, except sporadic cases of *E. granulosus s.s.* in sheep.

Cases are seldom reported in Switzerland and are generally referred to imported dog or sporadic cases in ruminant. *E. equinus* has been occasionally signaled in Switzerland, Germany, and Belgium. Between 2005 and 2007, CE was signaled in 6 cows, 267 pigs and 33 in sheep, in the Czech Republic. The disease, anyway, is considered infrequent if compared to the total number of slaughtered animals (Svobodová, 2014).

In central Europe the majority of human CE cases are described as imported cases. In the Netherlands hardly 30 cases per year are reported (Herremans T., 2010). In Switzerland around 50 cases per year are documented and described as imported cases. In Germany, the calculated incidence between 2001 and 2013 reported that on a total of 552 cases only 111 were in the autochthonous population (Torgerson P., 2017).

Retrospective studies conducted in Austria showed that inhabitants have been infected by G7 and immigrants by G1 or G6. Similarly, in Czech Republic 10 cases have been reported by hospitals since 2015, and only 1 of these affected a autochthonous (Stejskal F., 2014).

1.5.5.3 Eastern Central Europe: Poland and Baltic countries, Belarus, Ukraine, Moldova, Slovakia, Hungary

In Poland G1 and G7 occur in animals and humans (Dybicz, 2015). In Baltic countries (Lithuania, Latvia, Estonia), G7 infects human through the dog-pig cycle, pig, dog and cattle in Lithuania (Bruzinskaite R., 2009). In Estonia and Latvia, G8 and G10 are transmitted by wolves to cervid species (Marcinkuté A., 2015). In Estonia CE is historically referred to pigs, sheep and wild cervids, but new studies reported CE also in wild animals like moose and roe deer, infected by the grey wolf.

In Ukraine, CE is regularly reported in wolves and dog, but also wild boars and red deer, sheep and cattle, with different rates of prevalence based on the involved region.

The Republic of Moldova is endemic for CE, as estimated through a survey conducted on slaughterhouse in 2012 on the prevalence of CE in cattle, pig and sheep. The prevalence was higher in cattle and sheep, both affected by G1 and G3, whereas no pigs where found positive.

Slovakia and Hungary are historically affected by CE, due to familiar pig slaughtering (Seimenis A., 2003). Recently changes in the economic situation caused the decrease of family farms and the practice of veterinary meet inspection significantly contributed to decrease of human CE and the decline of CE in slaughterhouse animals.

In Slovakia, a decrease of the prevalence for CE in pigs has been reported after 2005.

In Moldova, Ukraine, and Belarus, the presence of Echinococcus species causing CE as well as *E. multilocularis* has been known for several decades. The Republic of Moldova is recognized as a CE endemic area, and human disease is considered a high-priority public health problem. Also in Ukraine, human CE cases are reported regularly, while a few cases have been registered in Belarus (Deplazes P., 2017).

1.5.5.4 Southeastern Central Europe: Romania, Bulgaria, Serbia, Croatia, Slovenia, Bosnia and Herzegovina, Kosovo, FYROM and Albania

Romania is considered highly affected by CE, and *E. granulosus s.s* is considered the prevalent strain. Both domestic and wild animals are involved as intermediate hosts in the cycles of *E. granulosus* G1 and *E. intermedius* G7 (Onac D., 2013).

In Bulgaria, the role of predominant strain is up to *E. granulosus* G1 able to infect jackals and wolves as definitive hosts and cattle, sheep and pig as intermediates (Breyer I., 2004).

In contrast, Slovenia showed a different situation with the low number of cases of domestic CE. Cystic echinococcosis is endemic in Serbia, mostly in sheep, although a decrease of infection rates has been reported in livestock in the last two decades (Bobic B., 2012).

Poor data have been reported for Croatia, in which CE was common in livestock between 1950s and 1970s. In 2009, a survey conducted in livestock reported a prevalence rate lower than 0.1% in Slovenia (EFSA, 2011). Human CE cases are sporadically reported and often without differentiation between AE and CE (Deplazes P., 2017).

Recent studies conducted in livestock (sheep and cattle) confirmed a high prevalence of CE in Bosnia and Erzegovina. Kosovo is characterised by a high rate of CE in cattle and Albania showed variable prevalence rates, mostly in sheep and cattle if compared to goats and swine. Unpublished data referred CE endemicity in intermediate hosts in FYROM.

Albania showed variable prevalence rates mostly in sheep and cattle if compared to goats and swine. Unpublished data referred CE endemicity in intermediate hosts in FYROM.

E. granulosus has been described since long time in Albania, but recently an increasing rate infections in dogs and human has been reported in the area surrounding Tirana, probably due to an invasive urbanization, incorrect disposal of urban waste and incomplete health care management and, most important, the presence of both dogs and sheep in the city and landfills (Pilaca A., 2014).

1.5.5.5 Southern Europe: Portugal, Spain, France, Italy, Greece

CE is considered the most important zoonosis in Southern Europe causing high morbidity rates both in humans and livestock (Seimenis A. , 2003). The transmission is mainly due to dog as definive hosts and small ruminant as intermediates, while the wild cycle is still unknown (Seimenis A., 2003). Nevertheless, a wild cycle, involving wild carnivores as definitive hosts and wild boars as intermediates, has been documented and reported in Spain (Martin-Hernando M.P., 2008), Italy (Busi M., 2007) and Corsica (Umhang G., 2014).

Four species of *Echinococcus* are present in Southern Europe, with *E. granulosus* (G1-G2-G3) as the most frequent in humans and animals (Beato S., 2013) and able to perpetuate both with sylvatic and domestic cycle (Busi M. S., 2007) (Varcasia A. C. S., 2007).

Echinococcus equinus G4 has been reported in horses from Spain and Italy (Gonzalez L.M., 2002) (Varcasia A., 2008a) while *E. ortleppi* (G5) was documented in cattle from France (Grenouillet F., 2014) and Italy (Casulli A., 2008).

The last genotype, *E. intermedius* G6/7, is present in all Southern Europe countries affecting goats, pigs and wild boars (Deplazes P., 2017).

Human CE is still considered a serious health problem in Southern Europe.

Table 6 Genotypes and related distribution of *Echinococcus granulosus* in Europe (Deplazes P., 2017)

Country	Human	Dog	Wolf	Wild canids	Cattle	Buffaloes	Equine	Pig	Wild boar	Swine	Sheep	Goat	Cervid	Red deer
Estonia		G1		G8 G10									G8 G10	
Latvia				G10										
Lithuania	G7	G7			G7			G7						
Moldova					G1 G3						G1 G3			
Poland	G7 G1							G7						
Slovakia	G7 G1-3							G7						
Ukraine								G7	G7					
Albania		G1												
Bulgaria				G1	G1					G1	G1			
Kosovo		G1-3			G1									
Romania	G1-3 G7				G1-3				G7		G1-3			G7
Serbia	G1 G7				G1 G7					G1 G7	G1			

Country	Human	Dog	Wolf	Wild canids	Cattle	Buffaloes	Equine	Pig	Wild boar	Swine	Sheep	Goat	Cervid	Red deer
France	G5	G6/7	G6/7		G1-3 G5			G6/7	G6/7		G1-3			
Greece						G1-3			G1-3		G1-3 G7	G1-3		
Italy	G1-3	G1-3	G1-3		G1-3 G5	G1-3	G4	G1 G7	G1		G1-3	G3		
Portugal	G1-3		G6/7		G1-3 G7						G1-3	G1-3		
Spain	G1		G1		G1		G4	G1 G7	G1 G7		G1	G1 G7		

1.6 Special focus on Italian epidemiology

Mediterranean region has been affected by CE for centuries due to the importance of livestock as source of income. In Italy, CE is widely distributed even if some differences have been found among the regions (Garippa G., 2006).

In the Northern regions, CE is considered sporadic with the lowest prevalence in Italy, less than 1% (Manfredi M.T., 2011).

In Central Italy, the prevalence in sheep was 22% in Abruzzo and 47% in Tuscany (Garippa G., 2006). In Basilicata prevalence in sheep was 67.7% (Cringoli G., 2007). In Campania, CE prevalence in sheep was between 33.3% and 75% (Cringoli G., 2007), 10.4 % in cattle (Rinaldi L., 2008) and 10.5% in buffaloes (Capuano F., 2006). In this region, where the sheepdogs cycle plays a critical role in the transmission to cattle and water buffaloes, an interesting situation has been reported. It appeared that the home slaughtering is almost absent and the presence of dogs in the legal slaughterhouse is forbidden. However, a geospatial survey supposed that free-ranging dogs come infected by ingestion of parasitized organs from sheep carcasses, and can play a role in cattle and water buffaloes infection (Cringoli G., 2007).

In Sicily, the prevalence in sheep is 57.6% with a fertility rate of 9,2% (Giannetto S., 2004), in cattle 67.1% with a fertility rate of 4% (Garippa G., 2006).

In Italy, the prevalence in pigs is around 9.4-11.1% (Varcasia A., 2006) (Garippa G., 2004) and 3.7% in wild boars (Varcasia A., 2008b), whereas, in horses is less than 1% (Varcasia A., 2008a). The prevalence reported in dogs is less than 6% (Garippa G., 2006) (Maurelli M.P., 2015) (Varcasia A., 2011). Finally, the prevalence in wolves is around 5.9-15% in the north of Italy (Guberti V., 2004) (Gori F., 2015).

Human CE in Italy is still considered a high health issue. The highest average incidence rates were recorded in the islands (Sardinia and Sicily) with a rate of 4.95/100.000 inhabitants.

In particular, 7.2 in Sardinia, and 4.2 in Sicily. In the South the reported incidence is around 2.1/100.000 inhabitants (except in Basilicata 5.6), in Central Italy is 1.2/100.000 inhabitants, whereas in the North is between 0.49 and 0.36/100.000 inhabitants from west to east respectively (Brundu D., 2014).

Underestimation of the burden of CE is due to the difficulty to report CE cases. To start tackling this problem, the Italian registry of echinococcosis (RIEC) has been set up by the WHO Collaborating Centre for the Clinical Management of Cystic Echinococcosis, (University of Pavia, San Matteo Hospital Foundation, in Pavia) and the Italian National Health Institute, ISS, in Rome, in 2012. Its development started in 2012 at the Sardinian Experimental Zooprophylactic Institute. Within HERACLES, the RIEC has been the template for the creation of the ERCE. At first, it included data from Bulgaria, Italy, Romania and Turkey, but later health centres from the other European countries expressed an interest in joining.

The main advantage of the national register is the merging of regional data, making them accessible at the same time to clinicians, epidemiologists and health authorities. This, combined to the compulsory notification, may represent a turning point in surveillance of disease in human (Tamarozzi F., 2015).

1.6.1 Special focus on Sardinia Island

Sardinia, the second large island of the Mediterranean area, could be considered an interesting epidemiological model for the study of CE (Scala A., 2006) The region has been subjected to three eradication program over the past half-century (1960, 1978, 1987) but prevalence is still high. Livestock is one of the main income source in Sardinia and is represented by around 3 million of sheep, 267.281 cattle and 164.647 swines (Istituto Zooprofilattico della Sardegna, 2016). CE in sheep has a prevalence of 76% with a fertility rate of 6.9% (Varcasia A., 2011). In pigs CE prevalence has been reported of 9.4% with a fertility rate around 6.5% (Garippa G., 2006). In 2010, an average annual incidence of 6.7% in humans has been reported, with hgher values in the rural areas (14.0%) (Conchedda M., 2010). In cattle, prevalence rates ranged from 19.6 and 41.5%, whereas fertility rates were between 0.76% and 2.6% (Scala A., 2004) (Varcasia A., 2006). Several genotypes of *Echinococcus* have been found in Sardinia except for *E. ortleppi* G5 (Varcasia A., 2006) (Busi M., 2007). In a recent survey conducted in the municipality of Arborea (Sardinia, Italy, 39°46′21.94″N; 8°34′52.64″E), in 160 intensive cattle farms, located away from sheep farms and fed with verified silage, have been analysed. Thirtyfive of 160 intensive farms were positive to CE (21.9%). In positive cattle, cysts were found in 71.2%, 55.8%, 1.9% in liver, lungs, and kidneys respectively. In 26.9% of cases cysts where present at the same moment in liver and lungs, whereas no fertile cysts were found (100% sterile). Biomolecular analysis on cystic samples through PCR screening and sequencing of mitochondrial genes, ND1 and cox1, confirmed that all isolates belonged to *E. granulosus s.s.* G1 (sheep strain) (Scala A., 2017).

Hence, being unlikely a domestic dog-bovine cycle, employment of new prophylactic actions as recombinant vaccines (Lightowlers M.W., 2003), management of stray-dogs, political plans and new health education programs are required to reverse this trend.

1.7 Aetiology of echinococcosis in humans

Currently four of the six acknowledged species of *Echinococcus* are considered a human health problem. In details *E. granulosus* is the causative agent of Cystic Echinococcosis (CE), *E. multilocularis* causes alveolar echinococcosis (AE), *E. vogeli* and *E. oligarthrus* are related to polycystic echinococcosis (PE), whereas the zoonotic potential of *E. shicuicus and E. felidis* has not yet been demonstrated (Mandal S. and Deb Mandal M., 2012) **.**

Human beings contract primary CE through oral uptake of *E. granulosus* eggs shed by infected dogs, direct hand-to-mouth transfer, due to close contact with infected definitive hosts, or ingesting contaminated uncooked food. The secondary contamination of food surface via atmospheric agents and insects, or by drinking contaminated water may be a further potential risk of infection (Eckert J. and Deplazes P., 2004).

Human AE is considered one of the most lethal helminthic infection. *E. multilocularis* in intermediates and aberrant hosts shows an alveolar structure in which several small vesicles proliferate in an exogenous tumour-like structure. In the early stage of infection, lesions may measure few millimetres, reaching the diameters of 15-20 mm/cm in human hosts. It has been reported that in patients with single-organ involvement, metacestode develops in around 99% of cases in liver and, later in the infection, metastases can spread from the first organ to others such as lungs, brain, bones etc. Transmission of *E. multilocularis* involved mainly foxes (sometimes wild canids, raccoon dogs, wolves or wild felids) as definitive hosts and small mammals (mostly rodents) as intermediate hosts. *E. granulosus* and *E. multilocularis* exploit the same infection mechanism in humans. PE due to *E. vogeli* or *E. oligarthrus* is only present in Central and South America. The wild cycle of *E. vogeli* involves bush dog as definitive host and pacas and agouti as intermediates.

Domestic dogs may infect humans acting as definitive hosts, after becoming infected by the ingestion of paca viscera (Rausch R.L. and D'Alessandro A., 2002).

Definitive hosts involved in the cycle of E. oligarthrus are wild felids, while intermediates one are agoutis, spiny rats, and pacas. How the infection is transmitted to humans is still unknown. Even if PE includes both *E. vogeli* and *E. oligarthrus* infections, the pathologies should be considered as different. *E. vogeli* share similar clinical symptoms with AE due to *E. multilocularis.* At the moment only three cases of PE caused by *E. oligarthrus* have been demonstrate, two of which showed an orbital location and the third a cardiac one (Eckert J. and Deplazes P., 2004).

1.7.1 Diagnosis of cystic echinococcosis

Imaging-based approach

Diagnosis of CE in intermediate and aberrant hosts is very difficult due to the absence of a long asymptomatic clinical phase. Pressure due to enlarging cysts and/or tissue fibrosis and necrosis in the affected organs trigger symptoms onset.

Imaging-based approach, particularly ultrasound (US), is considered the election technique for detecting lesions due to parasitic infections since 1970s. US is used not only to detect CE lesions, but also to monitor the development of cysts over the time (Frider B., 1999). Portable US is quiet inexpensive and used as a diagnostic tool of liver CE, while X-ray is used for lungs CE. Both are implied in population screening and follow-up.

In US, other imaging-based techniques are used such as computed axial tomography (CT) scans, magnetic resonance imaging (MRI), X-ray, angiography (AG) cholangiography (CAG), endoscopic retrograde cholangiography (ERC), percutaneous transhepatic cholangiography (PTC) and MRI-cholangiography (MRIC) (Pawlowski, 2001) (McManus D.P. Z. W., 2003) (Craig P. S., 2003).

As shown below, two are the main methods for cysts classification, Gharbi's and WHO IWGE's.They share similar contents, but some modifications are added in the second one:

- In Gharby classification, type II correspondes to CE3a and viceversa;
- Types are now grouped according their biological activity;
- Inclusion of solid cyst with daughter cysts (missing in Gharbi's);

Table 7 Comparison between Gharbi Classification and ultrasound classification of CE

Gharbi 1981	WHO IWGE 2001	Description	Stage
Type I	CE1	Unilocular unechoic cystic lesion with double line sign	Active
Type III	CE2	Multiseptated, "rosette-like" "honeycomb" cyst	
Type II	CE3a	Cyst with detached membranes (water-lily-sign)	Transitional
Type III	CE3b	Cyst with daughter cysts in solid matrix	
Type IV	CE4	Cyst with heterogenous hypoechoic/hyperechoic contents. No daughter cysts	Inactive
Type V	CE5	Solid plus calcified wall	

Modified table (Junghanss T., 2008)

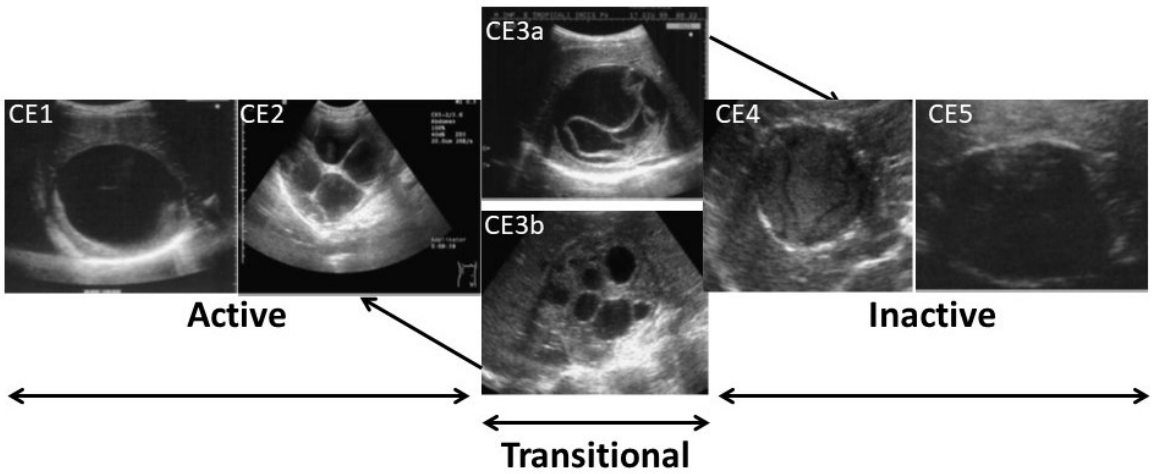

Figure 5 Ultrasound appearance of cystic lesions based on WHO-IGWE Classification (WHO Informal Group, 2003)

The World Health Organization Informal Working Group on Echinococcosis (WHO-IWGE) classified the liver cysts, into five types based on US imaging. CE1 to CE5.

CE1 and CE2 -> biologically active CE;

CE3a and CE3b -> biologically transitional CE;

CE4 and CE5 -> biologically inactive CE.

The challenge for US is detect small cyst <2 cm in diameter.

Serological detection

Detection of circulating antigens by the use of serological techniques is considered a useful tool in post-surgical follow-up, growth monitoring and cysts activity (Craig P. M., 1986). Serologic tests used for CE diagnosis are based on the detection of specific IgG antibodies, but several disadvantages as low sensitivity/specificity (Se/Sp) and low prognostic value for follow-up have been reported (Barnes T.S., 2012).

Hydatid fluid is the major antigenic source for echinococcosis immunodiagnosis, lipoproteins as antigen B (AgB) and antigen 5 (Ag5) are used in serological test for CE.

Casoni's skin test, is an immediate hypersensivity skin test used in the diagnosis of CE. Through an intradermal injection of sterilised hydatid fluid and an equal volume of saline solution, it induces a wheal response in case of positivity within 20 minutes. Nonetheless the antigens used are not standardised being crude extracts, able to induce immune anaphylactic reaction.

Different studies have been conducted on synthetic peptides or recombinant proteins derived from antigen B (AgB) or antigen 5 (Ag5), detected through ELISA or Immnublotting (Lorenzo C., 2005), although different rates of Se and Sp have been reported for the same recombinant antigen (Benabid M., 2013).

Recombinant antigen may be used to detect antibodies of pre-surgical cystic echinococcosis cases i.e. EpC1, cloned from a protoscoleces cDNA library, reliable in 92.2% of cases (Craig P.S., 2007) (Li J., 2004). Among the novel antigens: EgTeg tegumental protein, EgAP alkaline phosphatase, are considered high sensitive and specific.

Although the antigens have been used only in a limited sample group and moreover none of this is appropriate to screening-analysis or early diagnosis (Zhang W., 2012). Until now, the role of serology remains to confirm the diagnosis based on images in correlation with the involved species, target organ or antigen used (Kern P. d. S., 2017).

1.7.2 Treatments

For many years, surgery played a key role in CE treatment. Two different techniques were used, conservative and radical.

Conservative surgery consists of puncture of the cyst and evacuation of cyst content (membrane comprises), partial aspiration, introduction of the scolicidal agent and subsequent total aspiration. Associated risks are anaphylactic shock, chemical cholangitis or alveolare bronchial damage, secondary hydatidosis induction, due to hydatid fluid spillage, secondary bacterial infection and abscess formation, due to residual cavity left by the cyst removing.

Relapse rates after hydatidectomy in the liver are up to 20% (Atmatzidis K.S., 2005) while up to 11.3 in lungs (Ramos G., 2001).

Radical surgery is an alternative technique that provides the complete removal of the cyst, associated or not with liver or lung resection. Lung cysts can be removed sacrificing lung parenchima, and in both organs, segmentectomy or lobectomy may be used. Total cystectomy is the election method to decrease relapse rates. In radical surgery, intraoperative risks are higher than conservative one, but post-operative complications and relapse are lower (Atmatzidis K.S., 2005) (Demirleau J.C., 1974).

1.7.2.1 Additional treatment

The assumption of albendazole since one week before the surgery until three months after it and the use of soaked pads with anti-scolicidal agents in protection of surgical field, are useful methods in prevention of secondary CE infection and relapse.

In order to prevent cholangitis, is necessary avoid injection of scolicidal solution into cyst that communicates with biliary or bronchial tree, a careful inspection of the cystobiliary fistulae, bile content, determination of bilirubin in the aspirated fluid, and perform an anterograde cholangiography. A further hot point in post-surgery is the management of residual cavity. The ultimate choice would be the complete cyst removal to avoid the residual cavity formation, but in case of hydatidectomy, some precautions are necessary. In liver is required the drainage with suction and omentoplasty (Aktan A.O., 1993) whereas in lungs, obliteration of the residual cavity by capitonnage using multiple pursestring sutures is recommended (Ramos G., 2001) (Isitmangil T., 2002).

1.7.2.2 Percutaneous treatment

Treatment introduced in the mid-1980s were directed to the destruction of germinal layer with scolicidal agents or the removal of entire endocyst, through the technique of PAIR (i.e. **p**uncture the cyst, **a**spirate cyst fluid, **i**nject scolicidal agents and **r**e-aspirate the cyst content) (Men S., 1999) Several failures were reported on PAIR in multivesciculated cysts (CE2 and CE3b), thus confining its use for unilocular, with or without detached endocyst.

PAIR technique presents the same risks of the surgical treatments as anaphylactic shock, secondary echinococcosis due to spillage of cystic fluid and chemical cholangitis due to contact of scolicidal agent with the biliary tree.

The anatomical site plays a key role in safety and efficiency of PAIR technique, that is largely used in liver and extrahepatic abdominal cyst.

A further percutaneous technique is the radiofrequency thermal ablation (RF) through the uses of needle electrodes, but is not recommended due to the high relapse rate after few months (Brunetti E. and Felice C., 2004).

Three more percutaneous techniques have been reported and used when PAIR is not suitable, as in multivesciculated cysts or cyst with daughters cysts or solid material within.

-PEVAC: percutaneous evacuation;

-MoCaT: modified catheterization technique;

-DMFT: dilatable multi-function trocar.

Removal of the endocyst, cyst content and daughter cysts through aspiration, irrigation of the cavity with 10-20% saline solution and if necessary curettage. Catheterization of the cavity for 2-3 days after the treatment. The probability of anaphylactic shock and recurrence is high.

1.7.2.3 Chemotherapy

Studies reported that treatments with benzimidazole-carbamate compounds (BMZ), albendazole and mebendazole, induced very similar results while there are few differences in the administration method. Mebendazole is mostly absorbed in the small intestine and it is converted into poorly active hydroxylated products. Thereby, high doses of this drug are needed to invert metabolic conversion to have high levels of metabolites in the systemic circulation. Albendazole, in contrast, is metabolized to albendazole sulphoxide, an active antihelminthic molecule, with a higher efficalcy at lower doses.

The effect of albendanzole in patients with CE has been demonstrated in a study by Keshmiri and others, where around 80% of patients showed changes in the treated group while 13% in the untreated group.

Benzimidazole carbamates are safe if used for a short period in intestinal worm treatment (Horton J., 2000), whereas there are opposite evidences of side effect in the long term administration, such as alopecia and gastrointestinal symptoms. A further side effect is hepatotoxicity that causes an increase of liver enzymes. However, these symptoms are limited, not progressive and disappear on the treatment end. Side effect on bone marrow may cause aplastic anemia, but also, in this case, regular monitoring is an adequate precaution to eliminate most of side effects. Last but not least, teratogenic risks on fetus during the first pregnancy trimester reccomend to avoid the use not not only in pregnant women but also in women in childbearing age.

1.7.2.4 Watch and wait

Watch and wait is the last method, used when cysts are going to became calcified or consolidate without any therapy. Once reached this status there is no more danger for the patient. This method needs to be used concurrently with US for follow-up (Frider B., 1999).

1.7.2.5 Follow-up, prevention, and control

At least five years of follow-up are reccomended to patients after surgery, PAIR and/or drug treatments due to the high rates of relapse. Moreover, to prevent adverse effect due to albendazole, blood test and serum transaminases analysis are necessary for the first six months after treatment as well as ABZ-SO or mebendazole dose adjustment.

The control is warranted bysurveillance in safe slaughtering and prevention by the praziquantel administration in dogs. Sheep vaccination with EG95 vaccine, associated to slaughter control and therapy in dogs is strategy tried out in China and South America (Wen H., 2019).

1.8 Echinococcosis in animals

1.8.1 Diagnosis and treatment in definitive hosts

In definitive hosts, the adult parasites penetrate into the crypts of Lieberkühn between the villi, anchoring themselves to the epithelium with suckers and hooks (Thompson R.C.A., 1995). Despite this cruent method of attachment, parasite does not cause any relevant pathologies, except mild mucosa infiltration, increase of mucus production and flattening of epithelial cells (Eckert J., 2001).

The diagnosis is difficult in dog in which eggs of *Taenia* species and *Echinoccosus* are morphologically identical. Detections could occur in two ways: purgation using arecoline hydrobromide or arecoline acetarsol and necropsy (Eckert J. G. M., 1984).

Arecoline hydrobromide is a parasympathomimetic drug that acts on smooth muscle of small intestine, paralysing the adult worm and causing their spillage through the faeces. The advantage of this technique is the detection of a current infection. Purgation techniques have been used since 1958 in several control programs but now has been replaced by the introduction of novel anthelmintic drugs as praziquantel.

The necroscopic eamination allows a direct examination of the intestine, by the division it into several sections, opened by scissors and immersed them in physiological saline solution at 37°C, counting directly worms adhering to the mucosa with a stereoscopic microscope or a hand lens. In cases in which an accurate count is needed, it is better to left sections in physiological saline solution at 37°C for 30 minutes, in order to cause the shedding of the worms in the liquid, and collect the sediment for further studies.

A second strategy is the immunodiagnosis, that detects coproantigens released by *E. granulosus* in the faeces through ELISA tests, by the use of rabbit polyclonal antibodies produced against crude somatic worm extracts or preparation of excretory/secretory (E/S) product from immature intestinal worm stages (Deplazes P., 1992).

High specificity and sensitivity were reported (Deplazes P., 1994). ELISA can be used to detect serum antibodies.,but disadvantages are represented by the persistence of antibodies after worm's elimination, low sensitivity and unclear specificity (Lightowlers M. W. and Gottstein B., 1995).

1.8.2 Diagnosis in intermediate hosts

Hydatid cysts in intermediate hosts grow slowly and need years to become quite large to cause symptoms in animals. Hence, the diagnosis is mostly carried out during the slaughtering or necropsy. The lack of reliable exams in in-vivo diagnosis of CE is still a serious problem making necropsy the method of choice (Kumar A., 2016).

However, ultrasound (US) technique could be used in the examination of small animal like sheep and goats and also horses.

Necropsy is considered basic in control program involving intermediate hosts; the main target organs (liver and lungs) had to be sliced at 2 mm thickness and submitted to conventional histological examination, while a method as IFAT (Immunofluorescence Antibody Test), involving monoclonal antibodies, or PCR are helpful in the differential diagnosis of larval cestodes.

The most used antibodies detection methods are indirect haemagglutination test, counter immunoelectrophoresis (Raman M. and Chellappa D.J., 1998), ELISA (Kittelberger R., 2002), latex agglutination test (Martinez Gómez F., 1980) and enzyme linked immunoelectrotransfer blot and enzyme linked immunoelectrotransfer blot (EITB) (Dueger, 2003) (Jeyathilakan N., 2011). Currently, serum detection of antibodies against *E. granulosus* in intermediate hosts is possible, although necropsy is still the only method to confirm parasite presence. The sensitivity in experimentally infected sheep is high, in naturally infected sheep, is high variable while specificity is low because cross-reactivity may happen (Eckert J. G. M.-X., 2001).

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Aim of the thesis

Cystic echinococcosis (CE) represents a widely public health problem in particular in rural societies or developing countries. The World Health Organization included echinococcosis in its 2008–2015 strategic plans for the control of neglected tropical diseases.

Echinococcus spp. is a multi-stage life-cycle parasite, responsible of CE. It involves two mammalian hosts, a definitive one, mainly represented by carnivores (dogs and wild canids) and an intermediate host (sheep, cattle, goats, pig, and human as aberrant host) to complete its life-cycle. Its complex life-cycle is reflected in the host-parasite relationship, based on a balance between the host immune system and the evasion strategies of the parasite.

In intermediate hosts, *in-vivo* diagnosis is difficult due to the absence of clinical signs, hence necropsy is still considered the gold standard for a definitive diagnosis.

The first aim of our work was to conduct a comparative study of some features of fertile and sterile cysts in lungs and livers of naturally infected cattle by *E. granulosus*.

The following parameters were evaluated as cyst fertility based on the presence of viable protoscoleces in the hydatid fluid, the thickness of the collagen capsule, appearance of the germinal layer, distribution of giant cells in the adventitial layer, ratio of T / B lymphocytes in the host tissue and genotype of *Echinococcus granulosus* involved.

The morphological features have been described and compared in order to:

- understand the cause of the high infertility rate of cysts in cattle, identifying the features of both host and parasite tissues;
- characterize the immune response around fertile and infertile cysts through T and B lymphocyte scores;
- establish inflammations degrees based on the score of different parameters to evaluate their contribution to the progression of inflammatory response. (Chapter II).

The second aim was to characterize the protein patterns of both the hydatid fluid and the lung tissue surrounding the cysts in cattle echinococcosis, in order to identify the molecular cross-talk between host and parasite. To achieve this goal, protein extraction from hydatid fluid and tissue, sample preparation, labelling using Tandem Mass Tags (TMT), Liquid Cromathography Mass Spectrometry (LC-MS/MS) and statistical analysis were performed in order to identify proteins with significantly different abundandace (Chapter III).

Most of the existing survey are focused on the research of antigens in the serum of infected patients, whereas the knowledge about the local immune response is quite scarce. Another interesting aspect that should be elucidate is how the bovine, in contrast to other secondary hosts, manages to inactivate the cysts in the various organs. Moreover, data combining from cystic liquid and cystic tissue could represent a turning point in the knowledge of molecular cross-talk between host and *E. granulosus.*

Chapter II

2.1 Introduction

Cystic echinococcosis (CE), caused by the helminth *Echinococcus granulosus*, is a chronic zoonotic disease with a worldwide distribution, responsible for considerable public health problems and economic loss in animal production (Poglayen G., 2017). The life cycle of this parasite involves dogs and other canids as definitive hosts and herbivores as intermediate hosts. The last ones become infected after an oral uptake of eggs usually shed by dogs. Infective eggs contaminate grass and water and, once ingested, they develope into oncospheres. At this stage the parasite is able, through the portal vein, to reach liver, lungs and rarely other organs. Here, the oncosphere undergoes several reorganization events as degeneration, cell proliferation and differentiation resulting in the formation of metacestode (Negash K., 2013) (Khuroo M.S., 2002). The hydatid cyst is usually surrounded by a host-derived collagen capsule (adventitial layer, AL), and two parasite-derived layers, an inner nucleated germinal layer (GL) and an outer acellular laminated layer (LL) (Zhang W., 2003). The cyst cavity is filled with hydatid cyst fluid (HCF), considered the main responsible for the antigenic stimulation. The HCF, named hydatid sand, has a clear appareance and contain secretions of the parasite and host (Siracusano A., 2012).

Due to its genetic variability, *Echinococcus granulosus* can infect a high number of intermediate hosts including sheep, goats, cattle, camels, buffaloes, pigs, horses, donkeys and also humans, which are considered “dead-end” hosts (Thompson R.C. and McManus D.P., 2002). The aim of this work was the evaluation of immune response acted by naturally infected cattle against the G1 strain of *E. granulosus* focusing on a comparative study of the features of fertile and sterile cysts in lungs and livers.

2.2 Immunology of CE

The immune interplay strategy between intermediate hosts and *E. granulosus* is a matter of concern. Although the intermediate hosts exert a considerable inflammatory reaction against the parasite, this last developed several mechanisms to escape the host immune response, such as antigenic depletion, antigenic variation, immunologic indifference, diversion and subversion (Zhang W., 2012) (Zhang W., 2003). Intermediate hosts react against CE hydatid fluid and oncospheral antigens by the production of immunoglobulin (Ig) G (Zhang W., 2012), playing a critical role in parasite killing and protection against *E. granulosus* (Dempster R.P., 1992). In the early stages of infections, mechanisms adopted by the immune system to attack the parasite may involve antibody-dependent cell-mediated cytotoxic response (Rogan M.T., 1992), despite the low anti-oncospheral antibody levels (Zhang W., 2003). The activation of cell-mediated immunity involves infiltration of eosinophils, neutrophils, macrophages, and fibrocytes (Magambo J.K., 1995), which, however, cause only a mild inflammatory reaction, rather resulting in the formation of the fibrous layer able to separate the LL from the host tissue. With the progress of the infection (chronic phase), an increase of antibody levels may occur, particularly IgG, with a predominance of IgG1 and IgG4 subclasses (Daeki A.O., 2000), followed by IgM and IgE (Khabiri A. R., 2006). A hot point in the understanding of immune interplay between host and parasite in CE, is the high levels of both Th1 and Th2 cytokines (Riganò R., 1995) that usually downregulates each other (Pearce E.J. and MacDonald A.S., 2002). Analysing T cell profile of CE patients, an increase of Th2 profile has been found in the majority of cases, however, if compared with healthy controls, Th1 levels are also elevated. Perhaps, the role of macrophages in CE could help in the understanding Th1-Th2 controversy (Gottstein B., 2017). As reported by Mattila JT et al. in tuberculosis granulomas, Th1 and Th2 profile coexist, due to the concomitant expression of nitric oxide and arginase respectively (Mattila J.T., 2013.). Long

since, the immune response in hydatid disease is divided into two phases: pre-encystment and post-encystment (Rickard M.D. and Williams J.F., 1982).

In the first phase, called establishement, early post-oncospheral changes cause the formation of hydatid cyst. Once ingested by intermediate hosts, eggs hatch and release the oncosphere that moves to reach the target organ. Once arrived in its definitive location, the oncosphere loses its hooks and muscles and developed a central cavity to become a hydatid cysts, starting the deploying of LL (Díaz Á., 2017). In this stage, in naturally infected hosts, the parasite is susceptible to antibody-mediated IgG complement-dependent killing, but also exposed to a cellular inflammatory reaction due to the presence of neutrophils, macrophages, eosinophils and lymphocytes (Rickard M.D. and Williams J.F., 1982). In this early stage, oncospheres survive to the immune attack developing in hydatids, able to resist to antibodis and cell mediated inflammatory response in target organs, especially the liver and lungs (Rogan M.T., 2015). In the second phase (established phase), the parasite is lodged in a suitable organ, the hydatid is complete of GL and LL and starts growth. The presence of degenerate or dead cysts, after the first immunological attack, are the evidence that the parasite can suffer a subsequent killing during the establishment phase. The establishment phase cannnot be considered as a single period of development, due to the several changes that the individual cyst has to pass trhough. Indeed, the several possible combination of the strain of *E. granulosus*, the host and the organ involved, interfere with the developmental changes, such as time of growth, fertility, formation of daughter cysts, calcification (Brunetti E., 2011) (Bortoletti G., 2004). The laminated layer plays a role in the defence of the cysts against the nitric oxide released by activated macrophages (Steers N.J., 2001) and other inflammatory cells. It protects also the parasite acting as a barrier keeping away the inflammatory cells to the GL (Gottstein B. D. W., 2002). LL is able to contrast the complement cascade thanks to the presence of calcium inositol hexakisphosphate (Ins P6). Several studies reported different growth rates in many host species. Moreover, data on sheep demonstrated an

increase in the cyst volume after approximately 3 years post-infections (Díaz Á., 2017) (Torgerson P.R., 2009). Growth rates seem to be a strategy for the parasites survival. In cattle, has been demonstrated that small cysts (< 1 cm) are often calcified in both livers and lungs while rates of calcification decrease in cysts > 3 cm (Guo Z.H., 2011). A comparative study, conducted in experimentally infected macropods and sheep, demonstrated a bigger size, higher fertility rates, a thicker LL and a less well-developed AL in wallaby than in sheep, suggesting a less efficient host immune response in the former species, in particular for Th1 expression profile (Barnes T.S., 2011) (Barnes T.S., 2007). Factors involved in cyst fertility are still unknown, although is clear that age and size of the cysts could be relevant parameters (Lahmar S., 2013), as well as parasite strains, host species, target organ, considered able to influence fertility (Balbinotti H., 2012). Some studies reported the role of macrophages that, under Th1 cytokine control, can induce apoptosis and sterility (Cabrera G., 2008) (Spotin A., 2012). Paredes R. et al., (2007) demonstrated that in cattle, Ig were increased in infertile cysts if compared to fertile ones, suggesting that antibodies can contact the GL surface inducing apoptosis of embryonic cell and preventing the formation of protoscoleces. Other studies, reported the influences of Th1 profile on activated macrophages, that can induce apoptosis and sterility in the cyst (Spotin A., 2012) (Cabrera G., 2008). To a better understanding of the host immune response against the parasite, could be interesting to analyse the local response of individual cysts in addition to the systemic response in the intermediate hosts (Rogan M. T. et al., 2015).

2.3 Materials and Methods

2.3.1 Animals and cysts

Livers and lungs of 22 cattle with noticeable CE and healthy organs from 4 healthy cattle (livers and lungs used as control samples), slaughtered in Sardinia, were obtained between 2015-2018. Viscera were visually inspected for the detection and the collection of hydatid cysts. A total of seventy cysts were collected, 55 from lungs and 15 from livers, and each cyst was tested as an individual isolate. Hydatid cysts were removed from the parasitized organs and ranked as fertile (with viable PSCs) or infertile (without PSCs or with dead PSCs). Fertility was assessed by microscopic examination of PSCs presence, therefore HCF was microscopically examined through an unstained wet preparation (40X).

2.3.2 Histology

Excised cysts from target organs, livers and lungs, were collected ascertaining to include surrounding host tissue, for subsequent histopathological analysis. Cysts were sectioned, located in labelled cassettes, and fixed in 10% neutral formalin for 48h. Dehydration, clarification and paraffin embedding of tissues were carried out in an automatic tissue processor (HISTO-PRO 200), following standard procedures. The paraffin solidified blocks were cut with the Leica RM 2245 microtome, obtaining 3 μm sections and stained in an automatic multistaineir (ST5020, Leica Biosystems) with Haematoxylin and Eosin (HE). Sections were also stained with Masson Trichrome to highlight the collagen capsule. All slides were evaluated at light microscopy (Nikon Eclipse 80i), equipped with a Nikon Digital Sight Camera. Cysts from each cattle were identified with a progressive number and entered within an Excel database including related data as identification number (ID), species, organ and fertility.

2.3.3 Collagen capsule thickness measurement

Following the acquisition of photo collage of Masson's trichrome stain, AL (collagen capsule and inflammatory infiltrate) of each cyst was measured in a micrometer scale using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018). Three parameters were evaluated: Inflammatory Infiltrate Thickness (I. I. T.), Collagen Thickness (C. T.) and Σ Adventitial Layer Thickness (I. I. T.+ C. T.). Finally, a collagen grade has been obtained. Three measures were taken for each parameter: maximum, medium and minimum thickness, thus the final values were obtained by mean calculation for each sample and scored from 1 to 4 (thin to thick): CT1: 0-611 µm; CT2: 612-933 µm; CT3: 934-1369 µm; CT4: > 1370 µm (Box 1 A).

2.3.4 Evaluation of giant cells (GCs) and germinal layer (GL)

The number and distribution of giant cells (GCs) were scored in four categories considering simultaneously the presence of necrotic material in the AL.

GC1 = few GCs distributed in a regular layer in the AL; GC2 = high number of GCs distributed in a regular layer in the AL; GC3 = high number of GCs distributed in a regular layer mixed with necrotic material; GC4 = high number of GCs mixed with necrotic material (Box 1 B).

Germinal layer categories were determined and scored in three different groups through the examination of the following parameters: adhesion at LL, integrity of GL and production of PSCs. GL1 = attached to the LL, linear, intact and able to produce viable PSCs; GL2 = almost completely attached to the LL, linear and intact, not able to produce PSCs; GL3 = degenerated and not able to produce PSCs (Box 1 C).

2.3.5 Immunohistochemistry

Immunohistochemistry was carried out using the following antibodies: Cd3, clone F7.2.38, DAKO, (1:50) and Cd79a clone HM57, Abnova, (1:1000), MAC387 clone mac387 and FoxP3 clone 150D/E4, in order to identify T, B, macrophages and T-reg cells. Antigen retrieval was carried out in sodium citrate solution pH 6.0 ± 0.2 at 98°C for 20 minutes. Slides were incubated with primary antibodies overnight at 4°C. Secondary antibody binding reactions were developed with ImmPress kit peroxidase for 30 minutes RT. All slides were incubated with diaminobenzidine (ImmPACT DAB PEROXIDASE) to stain the reaction and counterstained in haematoxylin. Stained tissue sections were analyzed at 200X magnification. Cd3 and Cd79a positive cells were scored in 5 random fields of the AL of the cyst. Mean±SD were calculated for each sample group obtaining a lymphocyte grade for both T and B lymphocytes.

Cd3 grade 1 = 1-655; Cd3 grade 2 = 656-1262; Cd3 grade 3 = 1263-1729; Cd3 grade 4 >1730. Cd79a grade 1 = 0-61; Cd79a grade 2 = 62-158; Cd79a grade 3 = 159-364; Cd79a grade 4 >365 (Box 1 D).

2.3.6 Biomolecular analysis

HCF and GL from cysts were stored at -80°C in glass tubes. Molecular identification was carried out by polymerase chain reaction (PCR), amplifying fragments of the 2 mitochondrial genes NADH dehydrogenase 1 (ND1) and cytochrome C oxidase subunit 1 (cox1), using DNA extracted from the cysts (Scala, 2017). The nucleotide sequences were compared to those available in GenBank® using the basic local alignment search tool software (BLAST®) (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD, USA) and Mega 6.0 (Tamura K, 2013) (Scala A., 2017).

2.3.7 Statistical analysis

Statistical analyses were performed using Stata 11.2 Software (StataCorp LP). After performing descriptive statistics, categorical and ordinal variables were evaluated by Pearson (r) or Spearman rho (ρ) correlation coefficient, with Bonferroni adjustment and chi square (χ^2 test) or Fisher's exact test. Spearman correlation was used in order to assess the usefulness of GL grading in the histological evaluation of cystic lesions. A value of ρ approximately equal to 1 indicates a good correlation, a value near 0, indicates a poor correlation and a negative value indicates an inverse correlation. In addition, after checking the normality with Shapiro–Wilk test, a paired Student T-test was applied to evaluate the mean differences between T and B lymphocytes. A p-value < 0.05 was considered significant.

2.4 Results

In the present study, 70 cysts, 55 from lungs and 15 from livers of 22 naturally infected *E. granulosus* cattle, slaughtered in Sardinia between the years 2015-2018, have been analysed. Grossly, lungs and liver srevealed multiple cysts of varying sizes from 0.5 to 5 cm. In the lungs, cysts were either fully or partially embedded in the lung parenchyma (Fig. 6 A, B, C). Usually, the cysts were filled with clear cystic fluid and its aspiration caused the cyst collapse. In the livers, single to multiple cysts were observed, some of which (5//70) were calcified, gritty and hard to cut (Fig. 6 D, E, F).

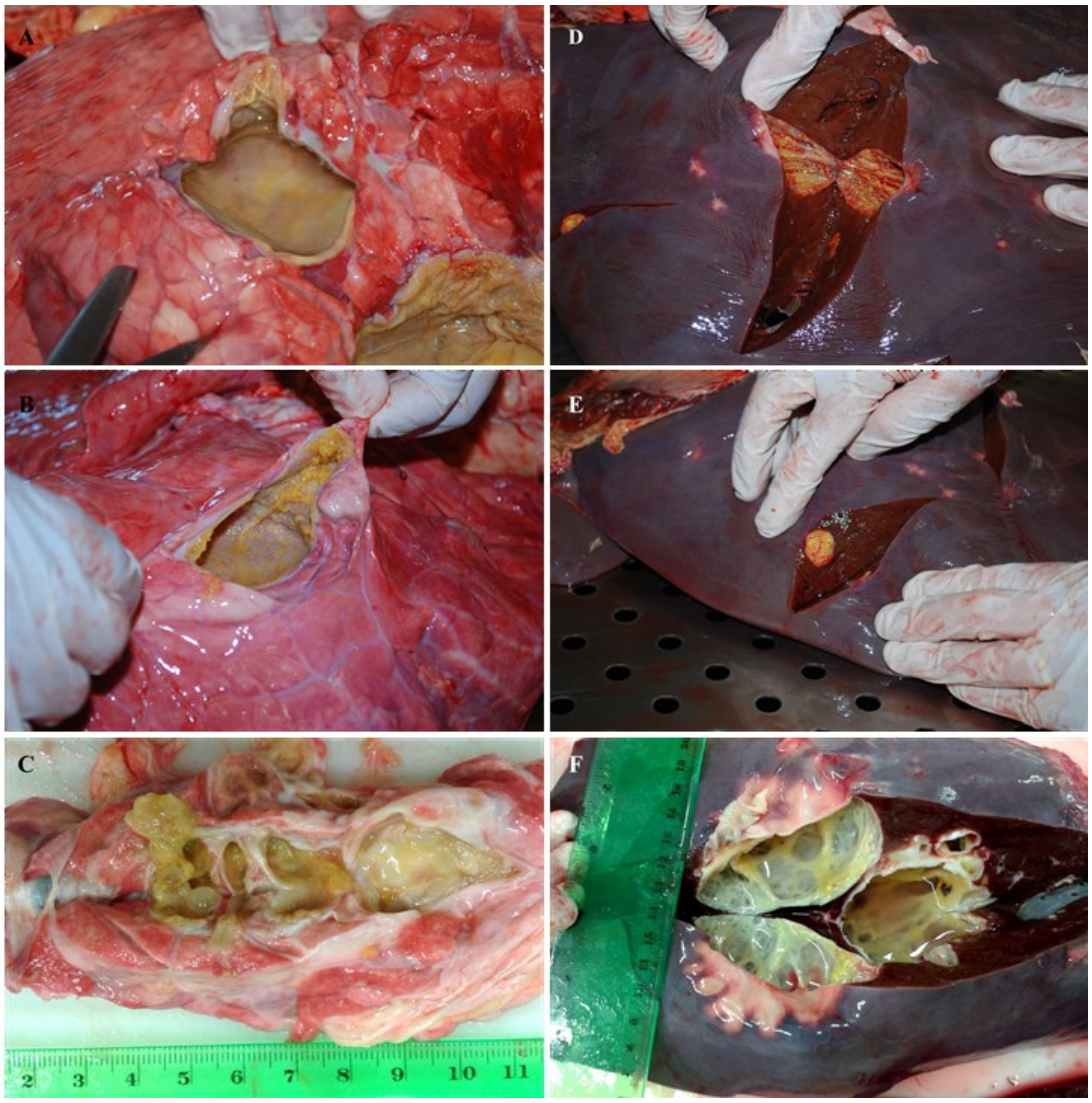


Figure 6 Hydatid cysts in cattle. A, B: unilocular hydatid cyst in lungs; C: multilocular hydatid cyst in lungs; D, E: unilocular hydatid cyst in liver; F: macroscopical features of a multilocular hydatid cyst in liver.

Microscopically observation of unstained wet preparation of HCF analysed considering morphology, showed the presence of viable PSCs (Fig. 7) in 2 pulmonary cysts classified as fertile (fertility rate of 2,86%), while the remaining 68, were classified as infertile for the absence of viable PSCs.

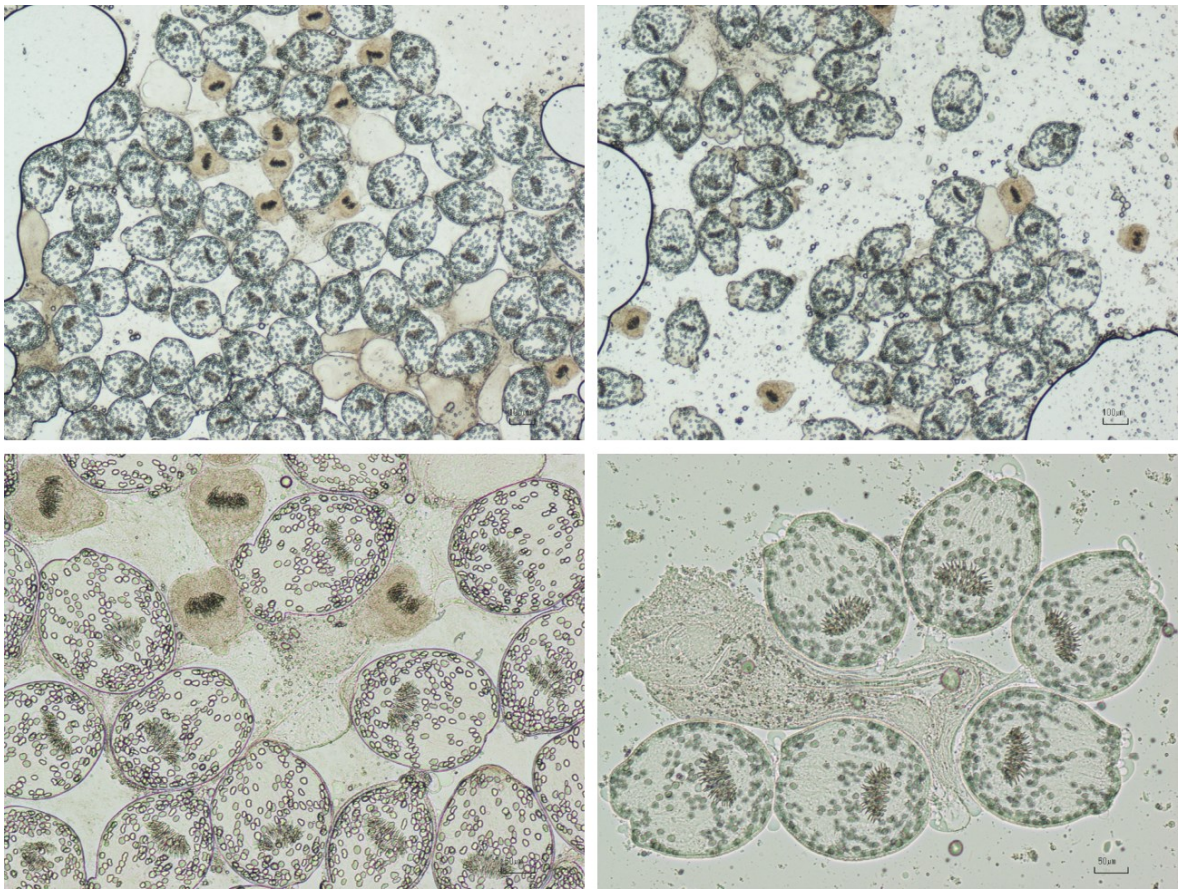


Figure 7 Wet preparation of hydatid fluid of fertile cysts from cattle showed vital and non vital (brown and small) PSCs.

2.4.1 Histology

Hydatid cysts were characterised by a thin germinal layer (GL) followed by an outer laminated layer (LL) and an outermost adventitial layer (AL). Parasitic membranes may appear continuous and intact, able to produce protoscoleces (PSCs) or disrupted/fragmented, containing degenerated PSCs. In particular, fertile cysts consisted in a thin, whole GL attached to an eosinophilic LL, from which buds brood capsules, containing PSCs toward the cyst lumen (Fig 8 A). While, infertile cysts, range from a thin and whole GL attached to

the LL to a degenerate and necrotic one, due to the surrounding cellular reaction. The AL showed variable features based on severity of inflammatory reaction. The inflammatory infiltrate comprised eosinophils, macrophages lymphocytes, epithelioid and multinucleated giant cells (Fig. 8).

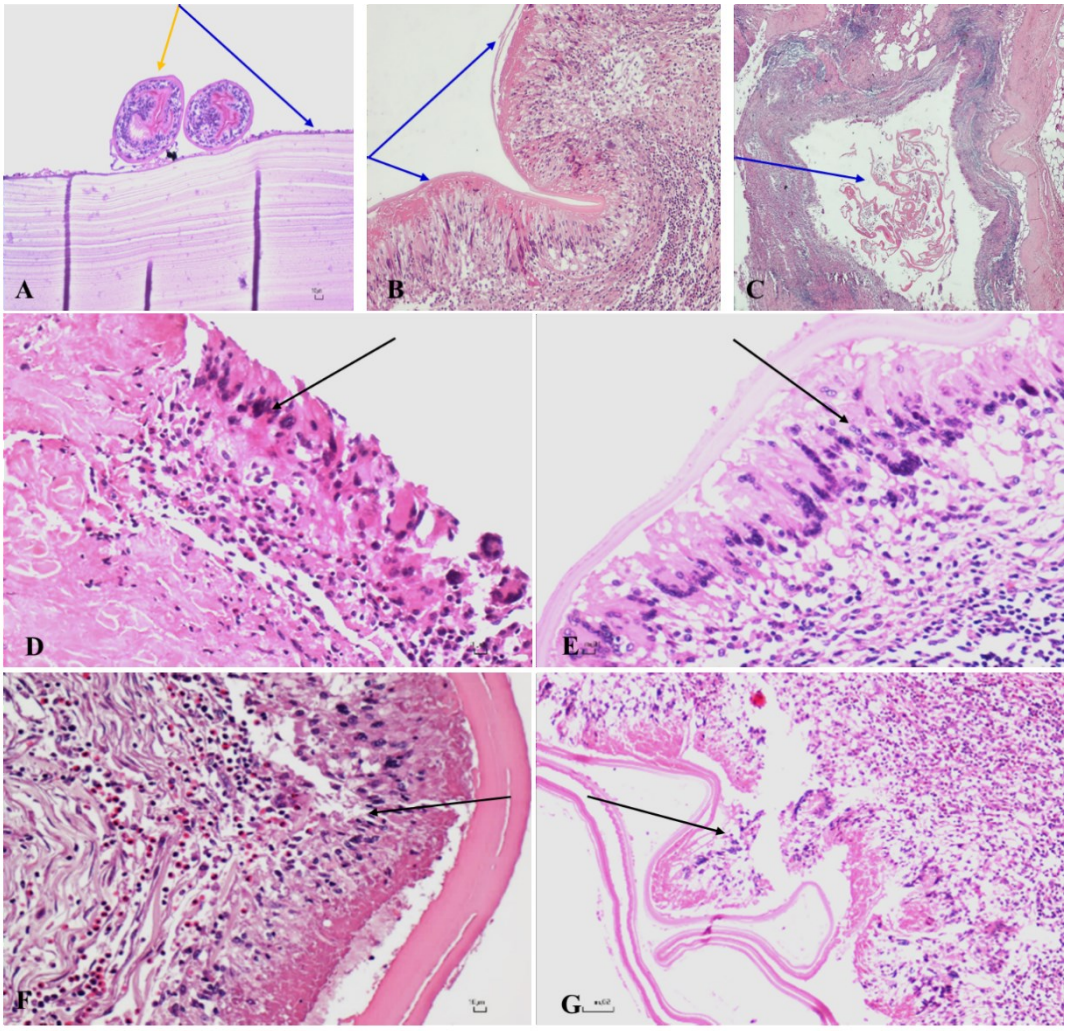


Figure 8 Histological features of GL and GCs. A: GL1 (blue arrow) able to produce vital PSCs (yellow arrow); B: GL2 completely attached to LL and detached in some areas (blue arrows); C: GL3 completely degenerated GL and LL (blue arrow). D: lung fertile cyst, absent inflammation, GCs classified as GC1: low number of GCs in the AL (black arrow); E: lungs infertile cyst, mild degree of inflammation, GCs classified as GC2, numerous GCs distributed in a uniform layer in the AL (black arrow); F:lungs infertile cysts, GCs classified as GC3, numerous GCs distributed in a uniform layer in the AL mixed with necrotic material (black arrow); G:lungs infertile cysts, GCs classified as GC4, high number of GCs presence of necrotic material (black arrow).

The cysts were classified into four categories based on a score index assigned to the following parameters: GCs, GL, Cd3 and Cd79a, grading them with a score from 1 to 15 according to the following criteria : Absent (1-4); Mild (5-8); Moderate (9-11); Severe (12-15) (Table 8).

Table 8: Inflammation's degrees summary table

N° of Cysts	Organ	GL	GCs	Cd3_ score	Cd79a_ score	Total score	Histological Grading
2	Lung	1	1	1	1	4	Absent
11	Lung	2	2-3	1-2	1-2	7-8	Mild
7	Liver	2	2-3	1-2-3	1-2	6-7-8	Mild
27	Lung	2-3	2-3-4	1-2-3-4	1-2-3-4	9-10-11	Moderate
6	Liver	2-3	2-3-4	1-2-3-4	1-2-3	9-10-11	Moderate
15	Lung	2-3	3-4	2-3-4	3-4	12-13-14-15	Severe
2	Liver	3	3-4	3-4	1-4	12-13	Severe

Inflammation degrees:

1. Absent inflammation (Fig.9A), includes 2 pulmonary fertile cysts characterized by a thick (>1370 μm) and compact AL with rare foci of inflammatory infiltrates in its outer side. The foci of inflammatory infiltrate were composed of few macrophages, T and B lymphocytes. Epithelioid cells were rarely observed and no sign of degeneration and necrosis were visible in the lung tissue. Giant cells were few and distributed in a regular layer in the AL (GC1 type) (Fig, 8 D) (Box 1 B). The GL was thin, whole and attached to an eosinophilic LL, and able to produce viable PSCs (GL1-grade) (Fig. 8A) (Box 1 C).
2. Mild inflammation (Fig.9B), comprised 18 infertile cysts (11 from lungs and 7 from liver). The lung parenchyma adjacent to the cysts was atelectatic in some areas and emphysematous in others. The areas surrounding the cysts were characterized by the presence of proliferative chronic pneumonia. The AL was thin with multi-focal inflammatory infiltrates extended into the surrounding tissue.

The AL shows fibrocytic cells on the outer side and collagen was replaced by inflammatory infiltrates in the inner part. A low number of eosinophils was observed. The inflammatory infiltrate was organized in a granulomatous reaction characterized by T and B lymphocytes, contiguous with palisading of derived epithelioid cells and multinucleated giant cells. The GCs were present in a high number and distributed in a regular layer classified as GC2 in 8 lung cysts and 7 in liver (Fig 8E) and as GC3 in 3 lung cysts and 2 in liver (Fig. 8F) (Box 1 B). The GL was thin and mostly whole attached to the LL. In some areas the GL appears degenerated and necrotic, not able to produce PSCs, and classified as GL2 in 11 lung cysts and 7 in liver (Fig. 8B) (Box 1 C).

3. Moderate inflammation (Fig.9C), included 33 infertile cysts (27 from lungs and 6 from liver). The areas of the lung parenchyma surrounding the cysts were characterized by chronic pneumonia. Lymphocytes infiltrate the parenchyma adjacent to the cysts causing atelectasis in some area and emphysema in other one. The inflammatory infiltrate was organized in a granulomatous reaction characterized by T and B lymphocytes contiguous with epithelioid cells and several multinucleated giant cells at the GCs were classified as GC2 (Fig 8E) in 12 lungs cysts and 2 liver cyst, GC3 (Fig 8F) in 14 lung cysts and 3 liver cysts and GC4 (Fig 8G) in 1 lungs cyst and 1 liver cyst (Box 1 B). A low number of eosinophils were detected. The cysts showed a very thin AL with diffuse inflammatory infiltrates extended into the surrounding tissue. AL interspersed with GL, LL, necrotic material and inflammatory infiltrate. The GL was classified as GL2 in 20 lung cysts and 2 liver cysts (Fig 8B), GL 3 in 7 lung cysts and in 4 liver cysts (Fig 8C). The GL was partially degenerated and detached from LL, between the two layers there was an area of necrotic material mixed with inflammatory infiltrate. (Box 1 C).

4. Severe inflammation (Fig.9D), included 17 infertile cysts (15 form lungs and 2 from liver). Lung parenchyma was affected by severe inflammation with a typical foreign body-reaction. Presence of severe granulomatous pneumonia in the areas surrounding the cysts, atelectasis in some areas and emphysema in others were observed. The cysts showed a very thin AL with diffuse inflammatory infiltrates extended into the surrounding tissue. The AL was almost completely interspersed with GL, LL, necrotic material and inflammatory infiltrate. The number of eosinophils was low. The inflammatory reaction was characterized by fibrocytic cells, T and B lymphocytes contiguous with epitheliod cells and several multinucleated giant cells, loses the features of common granulomatous reaction. GCs were classified as GC3 (Fig 8F) in 13 lungs cysts and 1 in liver, and finally as GC4 (Fig 8G) in 2pulmonary cysts and in 1 liver cyst (Box 1 B). The GL was degenerated, detached from LL, and between the two layers an interspersed of necrotic material and mixed with inflammatory infiltrate layer is present. GL was classified as GL2 (Fig 8B) in 11 pulmonary cyst, as GL3 (Fig 8C) in 4 pulmonary cysts, and in 2 liver ones (Box 1 C).

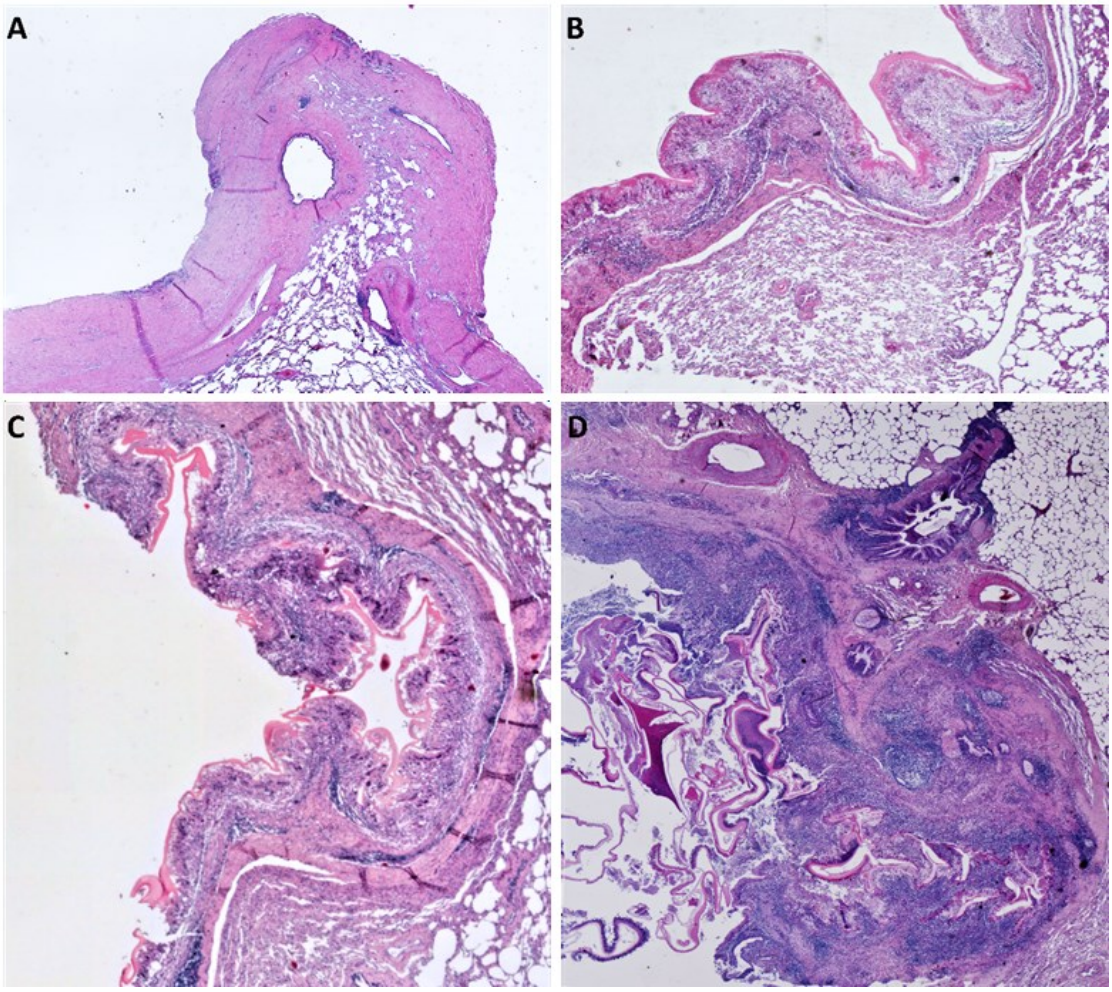


Figure 9 Inflammation degrees. A: Absent, thick and compact collagen capsule, GL able to produce PSCs, low number of GCs, T and B lymphocytes. B: Mild: thick and compact collagen capsule, GL unable to produce PSCs, GCs distributed in a regular layer, T and B lymphocytes. C: Moderate, thin collagen capsule, GL unable to produce PSCs, high nymber of GCs distributed in a regular layer, high number of T and B lymphocytes. D: Severe: thin collagen capsule interspersed with degenerated GL and LL, high number of T and B lymphocytes, GCs and necrotic material.

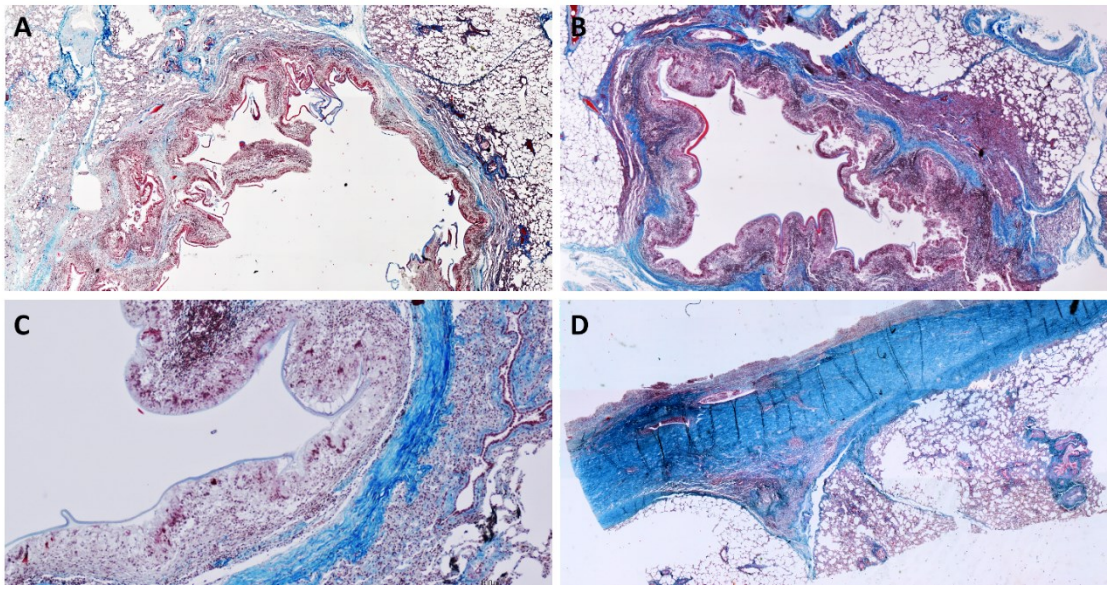


Figure 10 Collagen Thickness highleted by Masson trichrome stain A. CT1: 0-611 µm; B: CT2: 612-933 µm; C: CT3: 934-1369 µm; 4:CT4: > 1370 µm.

Table 9 Summary table: number of cysts for each category in lung and liver

	Lung				Liver		
	Absent	Mild	Moderate	Severe	Mild	Moderate	Severe
No. of cysts	2	11	27	15	7	6	2
CT1		3	9	3	1	1	
CT2		3	7	3	3		2
CT3		3	6	6	1	1	
CT4	2	2	5	3	2	4	
GL1	2						
GL2		11	20	11	7	2	
GL3			7	4		4	2
GC1	2						
GC2		8	12		5	2	
GC3		3	14	13	2	3	1
GC4			1	2		1	1
Cd3 grade 1	2	5	1		3	1	
Cd3 grade 2		6	11	1	3	1	
Cd3 grade 3			11	4	1	1	1
Cd3 grade 4			4	10		3	1
Cd79a grade 1	2	5	2		5	3	1
Cd79a grade 2		5	9		2	3	
Cd79a grade 3			12	2		1	
Cd79a grade 4			4	13			1

Box 1. Processing the inflammation degrees: a detailed description of involved features

Collagen Thickness degrees in lung and liver

1 = 0-611 µm, CT1 was found in 15 lungs cysts and 2 in liver;

2 = 612-933 µm, CT2 was found in 13 lungs cysts and 5 in liver;

3 = 934-1369 µm, CT3 was found in 15 lungs cysts and 2 in liver;

4 > 1370 µm, CT4 found in 12 lungs cysts and 6 in liver.

GCs categories in lung and liver

GC1 = few GCs distributed in a regular layer in the AL, 2 lungs cysts (Fig. 4D);

GC2 = high number of GCs distributed in a regular layer in the AL; 20 cysts in lungs and 7 in liver (Fig. 4E);

GC3 = high number of GCs distributed in a regular layer mixed with necrotic material; 30 cysts in lungs and 6 in liver (Fig. 4F);

GC4 = high number of GCs mixed with necrotic material; 3cysts in lungs and 2 in liver (Fig. 4G);

GL categories in lung and liver

GL1 = attached to the LL, linear, intact and able to produce viable PSCs, 2 lungs cysts; (Fig. 4A);

GL2 = almost completely attached to the LL, linear and intact, not able to produce PSCs, 42 cysts in lungs and 9 in liver (Fig. 4B);

GL3 = degenerated and not able to produce PSCs, 11 cysts in lungs and 6 in liver (Fig. 4C).

Cd3/Cd79a grade in lung and liver

Cd3 grade 1 = 1-655, was found in 8 lungs cysts and 4 in liver;

Cd3 grade 2 = 656-1262, was found in 18 lungs cysts and 4 in liver;

Cd3 grade 3 = 1263-1729,was found in 15 lungs cysts and 3 in liver;

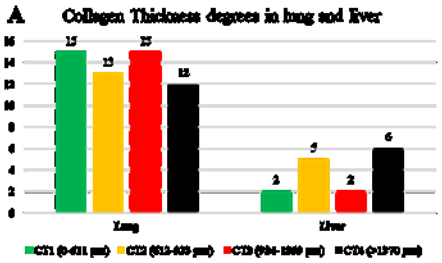
Cd3 grade 4 >1730, was found in 14 lungs cysts and 4 liver;

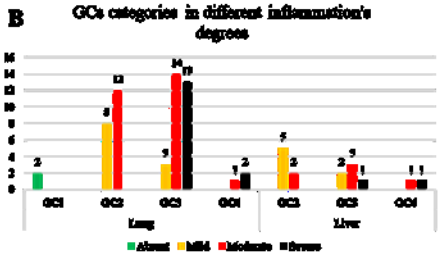
Cd79a grade 1 = 0-61, was found in 9 lungs cysts and 9 in liver;

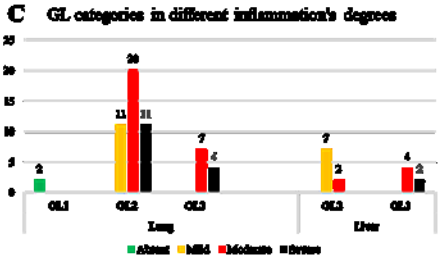
Cd79a grade 2 = 62-158, was found in 14 lungs cysts and 4 in liver;

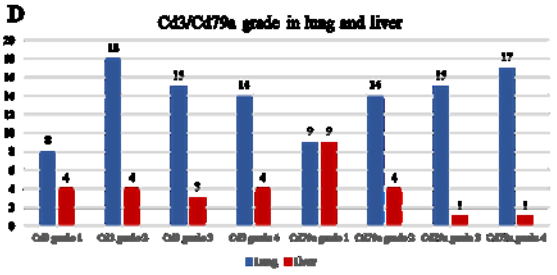
Cd79a grade 3 = 159-364,was found in 15 lungs cysts and 1 in liver;

Cd79a grade 4 >365 CT4 found in 17 lungs cysts and 1 liver.









2.4.2 Immunohistochemistry and lymphocytes score

Cd3 and Cd79a positive cells were scored in 5 random fields in the AL of the cyst (Fig. 11).

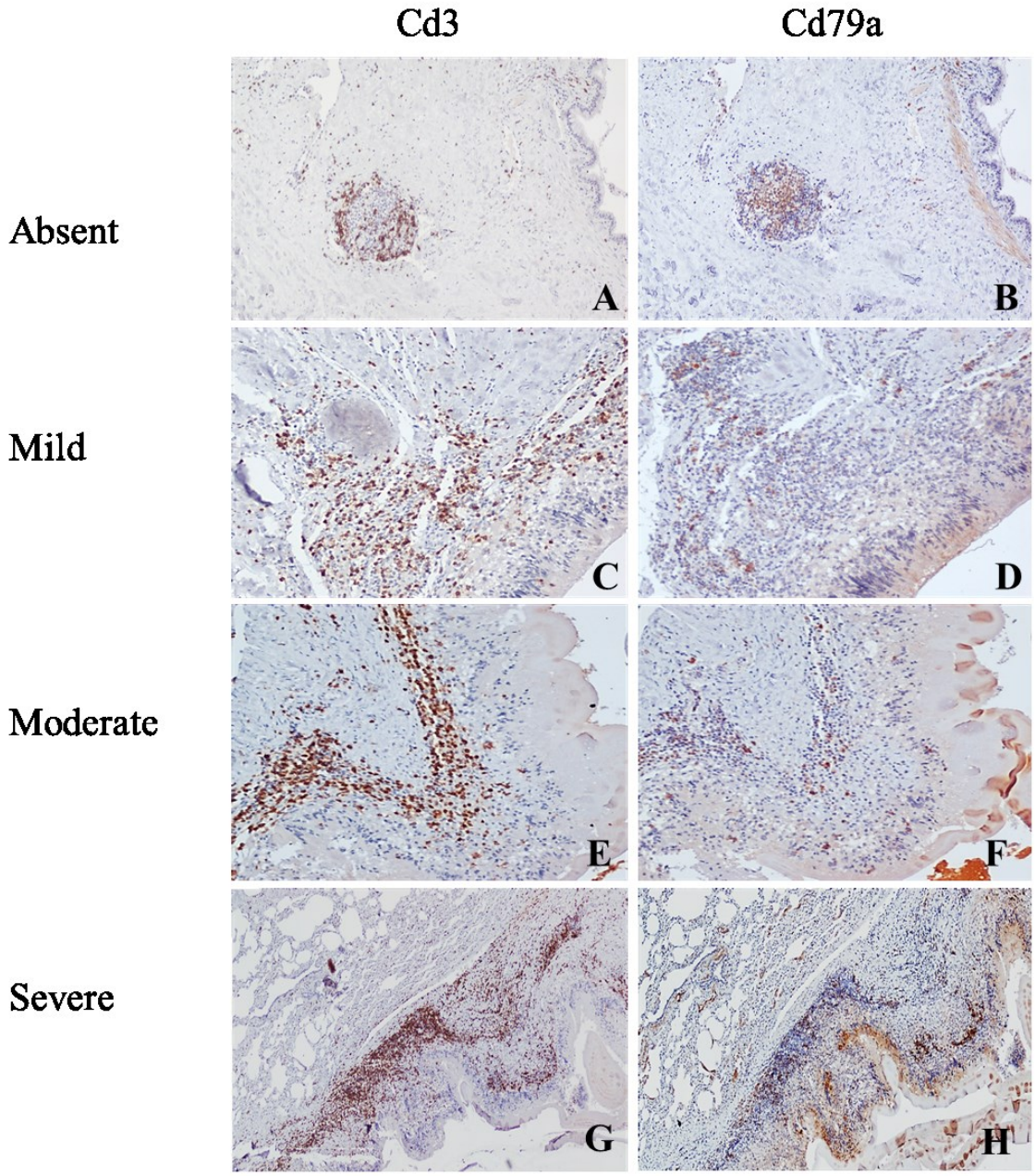


Figure 11 Immunohistochemistry stain in different inflammation degrees. A, B: Absent inflammation; C, D: Mild inflammation; E, F: Moderate inflammation; G, H: Severe inflammation.. A, C, E, G: T lymphocytes labelled by Cd3; B, D, F, H: Cd79a B lymphocytes labelled by Cd79a.

Indirect immunohistochemical analysis showed a significant prevalence of T vs B lymphocytes (mean± SD of T lymphocytes =1305.5 ± 657.81; mean± SD of B lymphocytes = 245.9 ± 271.9; T test = 15.8144 p-value <0.001) in the 70 samples analysed (raw data showed in 6.2 Supplementary table 1). In addition, statistical analysis showed that the number of Cd3 positive cells is inversely proportional to the thickness of the collagen capsule (p-value < 0.05). Cd3/Cd79 ratio was of 1.3 in the absent inflammation, 4.4 in mild/moderate and 7.6 in severe lungs inflammation. In liver, the ratio was of 11.9 in mild/moderate inflammation and 12.5 in severe inflammation, as showed in Fig. 12. Conversely, the number of MAC387 and FoxP3 positive cells was completely negligible and not scored.

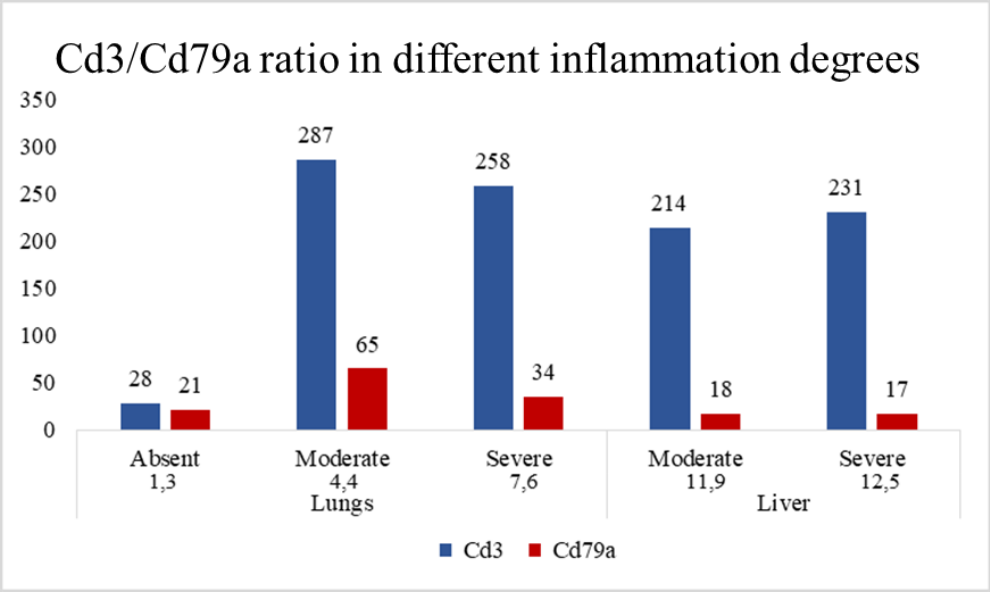


Figure 12 Cd3/Cd79a ratio in different inflammation degrees

2.4.3 Biomolecular analysis

Rapid PCR screening demonstrated that all isolates belonged to the G1 genotype, *i.e. E. granulosus sensu stricto* (also called *sheep strain*). This diagnosis was confirmed by sequencing the mitochondrial ND1 and cox1 genes; the sequences obtained were analysed and compared to those reported in GenBank® (Fig 13).

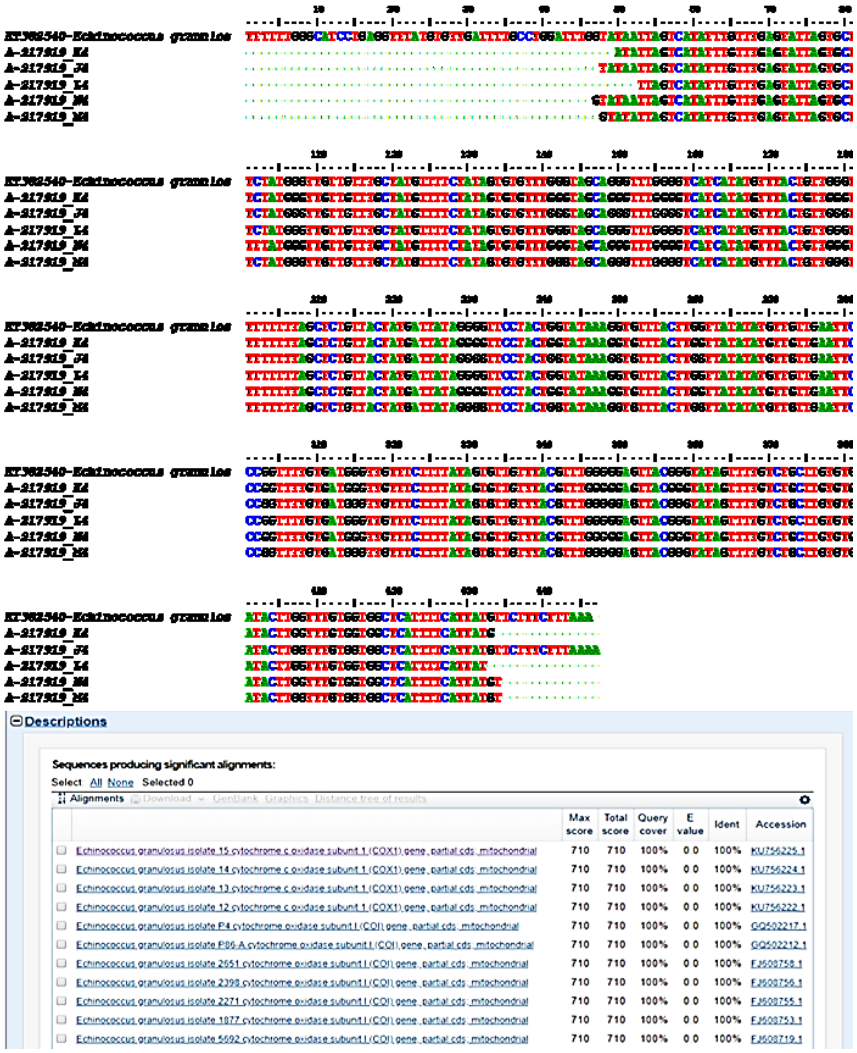


Figure 13 Cox1 sequences alignment

2.5 Discussion

In the current study 70 established cysts (GL, LL, AL) were found in lungs and liver of 22 naturally infected cattle and were analysed in order to identify the histopathological and immunohistochemical features and the inflammation degrees in the tissue surrounding the hydatid cysts and the genotypes of *E. granulosus* responsible for the infections.

E. granulosus induces in intermediate hosts a granulomatous tissue reaction, whose hallmarks are epithelioid and multinucleated giant cells directed to eliminate the foreign body (Hidalgo C., 2019) (Díaz A., 2000).

The fertility rate detected in our study (2,86%) is higher compared to the 0,76% detected in other studies on CE in Sardinia. (Scala A., 2004a) (Scala A., 2004b). Anyway, in other geographical areas, fertility rates in cattle were less than 30% (Muñoz J.P. and Sievers G., 2005), data consistent with our results that show a low fertility rates (2/70 fertile cysts founded), the slight increase of fertility may be due to the low number of samples analysed.

Immune interplay strategy between intermediate hosts and *E. granulosus* has always been a hot point in CE. *E. granulosus* induces a granulomatous tissue response characterised by cells of monocytic origin, particularly activated macrophages, called epithelioid cells, and multinucleated giant cells (Díaz Á., 2017), able to block and eliminate the foreign body (Hidalgo C., 2019). Lymphocytes characterisation carried out through immunohistochemical analysis showed a high number of Cd3 positive cells, T lymphocytes, if compared with Cd79a, B lymphocytes in both liver and lungs. Lungs results are consistent with data reported for sheep by Vismarra et al. (2015). However, in the previously mentioned study, has been reported a FoxP3 positive rates around 35% in the examined cysts, suggesting their key role in T cells response. However, in our study the clone used for Foxp3+ cell identification was not able to reveal positive cells in the examined cysts. T-reg activity played by FoxP3 positive cells need to be further investigated in order to confirm if

they are involved in hypo-responsiveness to *E. granulosus* infection, as reported for other helminth infections (Maizels R.M., 2004) (D'Elia, 2009). Likely, the role of macrophages in CE could help in understanding immune interplay between host and parasite in CE. Once again, the number MAC387 positive cells in our experiment was insignificant probably due to macrophage different subtypes.

The further characterisation of the localized responses of individual cysts in addition to systemic response in the intermediate hosts could be interesting for a better understanding of the immunological activity against the parasite (Rogan M.T., 2015).

In our study the Cd3 / Cd79a ratio was always in favor of the T lymphocytes (Cd3 +) for each degree of inflammation, with a trend that tended to increase in parallel with the increase in the degree of inflammation. A decrease in the number of T lymphocytes was observed only in the moderate inflammation group, which from the histological point of view corresponds to a situation of intermediate gravity and which for this reason could represent a phase of equilibrium between the two classes of lymphocytes.

In part, we could trace this trend back to the development phases suggested by Rogan et al. (2015) that suggest to divide the established phases of *E. granulosus*, into 4 subclasses.

The first one is the establishedt maturing phase in which the metacestode starts to grow and is still susceptible to immune killing by the host (Mourglia-Ettlin G., 2011). In this phase, the importance of macrophages (Rogan M.T., 1998) or eosinophils (Sakamoto T. and Cabrera P.A., 2003) role depends on the T-helper responses acted by the host. A hallmark of this phase is the LL, ables to protect the parasite against the inflammatory granulomatous reaction. In our study, cysts without an associated inflammatory reaction could be associated to this phase.

As the degree of inflammation progresses the cysts can be categorised in the second phase suggested by Rogan et al., (2015), the established stable phase.

In this stage the parasite could induce a response that is permissive in its regards and protective to the host (Siracusano A., 2012) (Vuitton D.A., 2003), but simultaneously, the growth capability of the parasite to can stop, due to several factors. Likely, the composition of collagen fibres of AL, may compress the cysts, as well as cell populations present in the AL that may inhibit the cyst growth (Rogan M.T., 2015). For all these reasons, a mild inflammation degree could be associated with the established stable phase, in which the host response and the immune modulation performed by the parasite appear to be in balance. Furthermore, the moderate inflammation degree could be compred to the third phase suggested by Rogan et al., (2015), called establishment unstable stage, in which the cyst develops into death process or revitalize. This instability is due to events that lead to the reorganization within the cyst, such as the collapse of cyst wall and GL, (Rogan M.T., 2006), the formation of daughter cysts and the infiltration of leucocytes. The initial degeneration stages of both GL and LL, in association with an increasing number of GCs mixed with necrotic material, in addition to the presence of eosinophils, witness the cyst instability at this stage. Finally, the severe inflammation degree could be compared with the last subclass proposed by Rogan et al., (2015), the established degenerative phase, in which immunological features may involve a large infiltrate of leucocytes combined with high antibody levels, but alsoa decreased levels of antibody in cysts in advance degree of inactivation (Rogan M. T. and Craig. P.S., 2002) (Tamarozzi F., 2013). A key role in development and degeneration of cysts is played by the LL, that protect the parasite from the immune attack (complement and inflammatory reaction), whose thickness can determine the cysts survival (Guo Z.H., 2011).

Moreover, data analysis showed that the thickness of the collagen capsule is inversely proportional to the number of positive Cd3 lymphocytes.

The type of GL is statistically associated with type of GCs and histologically, the GL type 3 (degeneration of both GL and LL) is associated with heavy infiltration of inflammatory cells in the inner side of the fibrous connective capsule, as confirmed by other study (Beigh A. B., 2017). In our survey, both fertile and infertile hydatid cysts from liver and lungs of 22 naturally infected cattle were analysed. Fertile or infertile cysts, classified as mild inflammation degrees, were characterized by a thick and compact collagen capsule, a normal organization of the LL and GL and an almost absent inflammatory reaction in the AL, according to (Barnes T.S., 2011), suggesting that their collection has occurred in the early post-infection period. The infertile cysts, were characterized by a connective capsule ranging from thick to thin, based on the inflammation degree, interspersed with an inflammatory infiltrate constituted by palisading foamy macrophages, multinucleated giant cells, lymphocytes, and the conservation status of host tissues (GL and LL), from disorganized to completely degenerated. The cysts classified in the mild inflammation degree in the present study, can be compared with the description of fertile hydatid cysts with low viability PSCs given by (Hidalgo C., 2019). This observation comes from the fact that we have workedwith naturally infected cattle with an unknown date of infection.

Besides, our study shows that the extent of the inflammatory response in the AL, from moderate to severe, affects the thickness of the connective capsule, causing its progressive thinning, due to degeneration. Our suggestions are confirmed by other studies, (Díaz Á., 2015) which propose that LL seems to be involved in the local inflammatory reaction. Moreover, another study (Hidalgo C., 2019) hints that in infertile cysts, LL is infiltrate and disorganized by host immune cells able not only to disrupt LL but also to infiltrate the lumen and probably induce infertility by destroying the GL.

GL absence due to its disruption is one of the common features of cysts degeneration combined with a LL collapsed and embedded in intensely eosinophilic material. Furthermore, in our study, infertile cysts classified from moderate to severe inflammation degrees showed necrotic material surrounded by laters of palisanding macrophages in particular multinucleated giant cells, infiltrate by lymphocytes consistently with what reported by Barnes T. S. et al., (2011) in sheep.

Last but not least, an important evidence provided by our study is the identification of *Echinococcus granulosus sensu stricto* (G1, sheep strain) in all examined samples, consistently with results reported by other surveys in Italy. G1 genotypes infection in cattle is characterised by low fertility values, suggesting that cattle couldn't be a suitable host *E. granulosus* (Scala A., 2004b). G1 genotype is responsible for the formation of sterile cysts in a large number of cattle, especially in the liver whereas a relative high fertility was found in lungs (Poglayen G., 2017). However, G5, bovine strain was found only once in Italy, in cattle imported in Northern Italy from Switzerland (Scala A., 2004b) (Rinaldi L., 2008) (Casulli A., 2008). These data are in contrast with what reported in cattle in Africa and in Asia, where infestations are sustained by G1-G3 genotypes and generate fertile cysts.

In conclusion, we can say that aspects that need to be investigated during CE infection are several and connected to each other in a complex way. Through our work we have tried to contribute to the identification of some histological features, such as GL and GCs type, as well as the thickness of collagen capsule and the inflammatory infiltrate, in order to allow to differentiate not only between fertile and infertile cysts, but also to characterize different inflammation degrees in the host tissue surrounding the hydatids.

Certainly, a multidisciplinary approach that includes not only molecular biology, histology and immunohistochemistry but also proteomics, could provide a more accurate view of the immunological mechanisms implemented by the intermediate host towards the parasite and vice versa.

2.6 Supplementary tables

Supplementary table 1

No. of cysts	Organ	GL	Fertility	GCs	Cd3_1	Cd3_2	Cd3_3	Cd3_4	Cd3_5	Σ Cd3	Cd3_ grade	Cd79a_1	Cd79a_2	Cd79a_3	Cd79a_4	Cd79a_5	Σ Cd 79a	Cd79a_ grade
1	1	2	0	2	318	209	208	162	136	1033	3	252	50	47	86	129	564	4
3	1	2	0	3	381	698	508	472	667	2726	4	33	35	30	23	14	135	2
4	1	2	0	3	429	338	154	194	453	1568	3	131	114	108	24	19	396	4
7	1	2	0	3	277	394	260	305	325	1561	3	51	67	52	77	22	269	3
8	1	2	0	2	101	226	236	128	96	787	2	97	42	18	26	26	209	3
9	1	2	0	3	199	363	269	382	385	1598	3	81	132	201	127	66	607	4
10	1	2	0	2	218	57	105	109	144	633	1	47	33	43	38	33	194	3
11	1	3	0	3	183	346	178	172	137	1016	2	62	29	46	175	132	444	4
12	2	2	0	2	64	49	98	115	109	435	1	26	29	1	16	29	101	2
13	2	2	0	2	14	68	71	60	34	247	1	24	6	40	10	24	104	2
14	2	2	0	2	46	23	48	30	21	168	1	9	25	5	0	0	39	1
17	1	2	0	3	362	224	134	184	319	1223	2	26	51	21	13	18	129	2
18	1	2	0	3	404	824	698	231	317	2474	4	185	100	27	53	15	380	4
19	1	2	0	2	147	153	121	118	147	686	1	41	28	30	29	22	150	2
50	1	1	1	1	0	82	124	0	0	206	1	83	0	0	0	102	185	1
51	1	1	1	1	0	24	0	0	47	71	1	0	20	0	0	4	24	1
52	1	2	0	3	536	428	672	537	617	2790	4	53	55	133	57	77	375	4
53	1	2	0	2	98	305	155	172	213	943	2	3	10	8	26	12	59	1
54	1	3	0	4	525	526	547	476	425	2499	4	52	74	260	40	48	474	4
55	1	2	0	2	130	216	426	379	208	1359	3	2	12	49	36	37	136	2
56	2	2	0	3	91	174	175	151	239	830	2	0	0	0	0	0	0	1
59	2	3	0	3	465	468	411	229	224	1797	4	13	12	2	12	9	48	1
60	2	2	0	2	217	305	267	349	307	1445	3	4	10	7	0	1	22	1
62	1	2	0	3	608	349	441	427	450	2275	4	56	72	71	455	444	1098	4
63	1	2	0	3	236	228	445	577	551	2037	4	702	12	13	502	131	1360	4
64	1	2	0	3	653	503	531	438	920	3045	4	410	205	352	10	55	1032	4
65	2	2	0	2	462	375	274	318	394	1823	4	37	37	32	23	10	139	2
66	2	3	0	3	344	246	278	244	314	1426	3	250	71	56	29	41	447	4
67	2	3	0	3	193	132	265	372	322	1284	3	2	3	11	5	7	28	1
68	2	2	0	2	610	438	387	386	271	2092	4	46	190	9	8	3	256	3
69	2	2	0	3	198	155	153	312	332	1150	2	3	4	5	5	5	22	1
70	1	2	0	2	174	138	430	457	227	1426	3	234	58	25	27	73	417	4
72	1	2	0	3	259	454	606	379	276	1974	4	34	133	130	53	21	371	4
73	2	3	0	4	83	31	74	48	30	266	1	0	0	0	0	0	0	1

No. of cysts	Organ	GL	Fertility	GCs	Cd3_1	Cd3_2	Cd3_3	Cd3_4	Cd3_5	Σ Cd3	Cd3_ grade	Cd79a_1	Cd79a_2	Cd79a_3	Cd79a_4	Cd79a_5	Σ Cd 79a	Cd79a_ grade
81	1	2	0	2	239	415	334	219	310	1517	3	49	107	74	160	58	448	4
82	1	2	0	2	426	311	153	321	246	1457	3	56	24	12	37	25	154	2
83	1	2	0	3	285	385	474	381	227	1752	4	15	28	26	19	14	102	2
84	1	2	0	2	426	221	257	350	257	1511	3	42	72	165	46	65	390	4
85	1	2	0	2	229	336	369	308	272	1514	3	83	75	87	41	20	306	3
89	1	2	0	3	481	290	368	402	364	1905	4	38	385	187	103	390	1103	4
91	1	2	0	3	367	436	403	140	227	1573	3	59	96	87	83	40	365	4
92	1	2	0	2	484	656	466	362	411	2379	4	24	74	20	34	13	165	3
93	1	3	0	3	426	400	357	473	356	2012	4	58	23	40	61	129	311	3
98	1	2	0	2	113	98	332	172	174	889	2	0	0	0	5	1	6	1
107	1	3	0	3	165	72	162	191	266	856	2	23	12	24	11	39	109	2
108	1	2	0	3	173	307	338	199	316	1333	3	12	10	36	111	50	219	3
109	1	3	0	3	280	186	134	227	278	1105	2	71	100	22	5	8	206	3
110	1	2	0	2	152	174	143	129	436	1034	2	17	28	11	13	22	91	2
111	1	3	0	3	108	250	174	219	285	1036	2	34	18	32	22	12	118	2
112	1	2	0	3	70	136	53	128	82	469	1	4	3	11	3	12	33	1
113	1	2	0	2	111	177	338	107	79	812	2	11	8	7	34	7	67	2
114	1	2	0	2	100	90	94	108	234	626	1	12	21	10	19	0	62	2
115	1	2	0	3	288	252	335	312	476	1663	3	42	349	11	3	42	447	4
116	2	3	0	3	134	208	125	187	109	763	2	18	8	13	11	15	65	2
117	2	3	0	4	491	323	529	122	265	1730	4	1	2	0	4	0	7	1
118	2	2	0	2	203	233	257	237	263	1193	2	8	2	2	2	3	17	1
119	1	3	0	4	91	150	19	36	48	344	1	1	4	6	2	1	14	1
120	1	2	0	3	391	195	215	170	217	1188	2	5	5	3	9	8	30	1
121	1	2	0	3	179	116	95	104	143	637	1	6	11	14	6	21	58	1
125	1	2	0	3	138	292	165	133	156	884	2	65	49	47	90	60	311	3
126	1	2	0	2	188	223	207	140	225	983	2	74	57	60	55	65	311	3
127	1	3	0	3	292	153	181	331	286	1243	2	55	42	35	58	18	208	3
128	1	2	0	3	259	236	408	230	244	1377	3	25	31	40	44	50	190	3
129	1	3	0	3	225	149	163	214	172	923	2	16	3	5	13	2	39	1
130	1	3	0	3	199	215	220	107	161	902	2	6	31	27	21	24	109	2
131	1	2	0	2	302	303	278	217	317	1417	3	80	74	29	14	28	225	3
132	1	2	0	2	197	185	148	317	254	1101	2	31	35	20	29	29	144	2
133	1	2	0	2	232	336	547	360	388	1863	4	32	32	22	29	58	173	3
1123	1	2	0	3	150	137	153	120	196	756	2	3	28	39	35	17	122	2
1288	1	3	0	4	642	354	126	279	343	1744	4	38	49	50	61	71	269	3

Supplementary table 1: No. of cyst: progressive number assigned to each sample; Organ: 1=lung, 2=liver; Inflammation's degrees: established based on histological evaluation; GL: classified in 3 types based on features mentioned in M&M; Fertility: 0=infertility, 1=fertility; GCs: classified in 4 degrees based on features mentioned in M&M; Cd3_1,2,3,4,5: score of Cd3 positive cells in 5 random field; Σ Cd3: sum of 5 field; Cd3 grade: 1= 1-655; 2= 656-1262; 3=1263-1729; 4 >1730; Cd79a_1,2,3,4,5: score of Cd79a positive cells in 5 random field; Σ Cd79: sum of 5 field; Cd79a grade: 1= 0-61; 2= 62-158; 3=159-364; 4 >365

Supplementary table 2

No. Of cysts	Organ	Collagen Thickness Max	Collagen Thickness Med	Collagen Thickness Min	Collagen mean	Collagen_grade
1	1	1853	1114	536	1167,67	3
3	1	851	511	476	612,67	2
4	1	806	466	268	513,33	1
7	1	670	851	401	640,67	2
8	1	4472	199	740	1803,67	4
9	1	1499	848	363,5	903,50	2
10	1	1655	686	489,5	943,50	3
11	1	640	504	559	567,67	1
12	2	3092	2315	162	1856,33	4
13	2	1051	928	332	770,33	2
14	2	1378	918	298	864,67	2
17	1	2173	1016	392,5	1193,83	3
18	1	1013	1512	144	889,67	2
19	1	1229	905	388	840,67	2
50	1	5601	3770	3044	4138,33	4
51	1	2621	1787	844	1750,67	4
52	1	2810	912	389	1370,33	4
53	1	892	931	599	807,33	2
54	1	2671	1690	1099	1820,00	4
55	1	710	439	279	476,00	1
56	2	4330	3352	1077	2919,67	4
59	2	1990	1054	373	1139,00	3
60	2	1499	1293	614	1135,33	3
62	1	3401	1722	930	2017,67	4
63	1	1238	535	172	648,33	2
64	1	1906	1084	602	1197,33	3
65	2	4026	1990	546	2187,33	4
66	2	573	1262	934	923,00	2
67	2	6055	1998	307	2786,67	4
68	2	336	91	90	172,33	1
69	2	943	358	114	471,67	1
70	1	2160	1957	1210	1775,67	4
72	1	2246	349	269	954,67	3
73	2	2853	2194	2122	2389,67	4

No. Of cysts	Organ	Collagen Thickness Max	Collagen Thickness Med	Collagen Thickness Min	Collagen mean	Collagen_grade
81	1	1283	570	380	744,33	2
82	1	311	271	138	240,00	1
83	1	1734	451	574	919,67	2
84	1	908	612	145	555,00	1
85	1	333	346	245	308,00	1
89	1	1902	1142	109	1051,00	3
91	1	1471	746	838	1018,33	3
92	1	1192	943	331	822,00	2
93	1	1979	1418	146	1181,00	3
98	1	2169	1738	610	1505,67	4
107	1	923	555	413	630,33	2
108	1	776	566	223	521,67	1
109	1	1612	836	599	1015,67	3
110	1	656	473	283	470,67	1
111	1	2954	1035	1449	1812,67	4
112	1	1071	840	864	925,00	2
113	1	412	841	254	502,33	1
114	1	1250	1410	432	1030,67	3
115	1	1437	1487	389	1104,33	3
116	2	3912	1777	1102	2263,67	4
117	2	864	810	389	687,67	2
118	2	589	800	454	614,33	2
119	1	133	130	47	103,33	1
120	1	1156	1777	308	1080,33	3
121	1	2014	2053	1259	1775,33	4
125	1	5661	2751	444	2952,00	4
126	1	426	352	743	507,00	1
127	1	2633	190	213	1012,00	3
128	1	1016	484	256	585,33	1
129	1	1865	1812	325	1334,00	3
130	1	1959	929	422	1103,33	3
131	1	500	496	355	450,33	1
132	1	586	486	456	509,33	1
133	1	964	602	389	651,67	2
1123	1	3626	929	570	1708,33	4
1288	1	409	333	186	309,33	1

Supplementary table 2: No. of cyst: progressive number assigned to each sample; Organ: 1=lung, 2=liver; Collagen Thickness Max (maximum), Med (medium) and Min (minimum) expressed in µm; Collagen mean: mean of the three parameters; Collagen_grade: 1=0-611; 2=612-933; 3=934-1369; 4 > 1370.

Supplementary table 3

N° cysts	Organ	I. I. T. Max	I. I. T. Med	I. I. T. Min	I. I. T. Mean	AL Thickness Max	AL Thickness Med	AL Thickness Min	AL Thickness Mean
1	1	4325	2598	1251	2724,67	6178	3712	1787	3892,33
3	1	1986	1192	1111	1429,67	2837	1704	1587	2042,67
4	1	1879	1086	689	1218,00	2685	1552	957	1731,33
7	1	2679	1276	267	1407,33	3349	2127	668	2048,00
8	1	2981	1794	317	1697,33	7453	1993	1057	3501,00
9	1	999	363	363,5	575,17	2428	1211	727	1455,33
10	1	1104	1602	489,5	1065,17	2759	2288	1309	2118,67
11	1	7011	2853	842	3568,67	7651	3357	1401	4136,33
12	2	458	326	680	488,00	3550	2641	842	2344,33
13	2	117	232	299	216,00	1168	1160	33	787,00
14	2	919	0	0	306,33	2297	918	298	1171,00
17	1	3260	2371	392,5	2007,83	5433	3387	785	3201,67
18	1	4053	2268	577	2299,33	5066	3780	721	3189,00
19	1	1502	741	167	803,33	2731	1646	555	1644,00
50	1	0	0	0	0,00	5601	3770	3044	4138,33
51	1	0	0	0	0,00	2621	1787	844	1750,67
52	1	703	228	97	342,67	3513	1140	486	1713,00
53	1	2081	621	400	1034,00	2973	1552	999	1841,33
54	1	15138	9580	6229	10315,67	17809	11270	7328	12135,67
55	1	2842	1025	651	1506,00	3552	1464	930	1982,00
56	2	2886	2234	718	1946,00	7216	5586	1795	4865,67
59	2	1996	1613	669	1426,00	3986	2667	1042	2565,00
60	2	0	0	0	0,00	1499	1293	614	1135,33
62	1	2783	2582	2170	2511,67	6184	4304	3100	4529,33
63	1	2890	2142	690	1907,33	4128	2677	862	2555,67
64	1	4447	2528	1403	2792,67	6353	3612	2005	3990,00
65	2	6039	1629	447	2705,00	10065	3619	993	4892,33
66	2	2866	1729	958	1851,00	3439	2991	1892	2774,00
67	2	673	856	132	553,67	6728	2854	439	3340,33
68	2	517	459	182	386,00	853	550	272	558,33
69	2	852	526	180	519,33	1795	884	294	991,00
70	1	2639	491	134	1088,00	4799	2457	1344	2866,67
72	1	3370	1395	220	1661,67	5616	1744	489	2616,33
73	2	2604	1926	1262	1930,67	5457	4120	3384	4320,33

N° cysts	Organ	I. I. T. Max	I. I. T. Med	I. I. T. Min	I. I. T. Mean	AL Thickness Max	AL Thickness Med	AL Thickness Min	AL Thickness Mean
81	1	1924	989	570	1161,00	3207	1649	950	1935,33
82	1	94	244	378	238,67	405	515	516	478,67
83	1	193	1053	144	463,33	1927	1504	718	1383,00
84	1	694	236	265	398,33	1602	848	510	986,67
85	1	1334	808	573	905,00	1667	1154	818	1213,00
89	1	871	711	595	725,67	2773	1853	704	1776,67
91	1	3432	2984	359	2258,33	4903	3730	1197	3276,67
92	1	1108	665	396	723,00	2300	1608	727	1545,00
93	1	495	638	341	491,33	2474	780	487	1247,00
98	1	929	434	261	541,33	3098	2172	871	2047,00
107	1	1129	678	338	715,00	2052	1233	751	1345,33
108	1	949	691	273	637,67	1725	1258	496	1159,67
109	1	2418	1951	282	1550,33	4030	2786	881	2565,67
110	1	1285	906	312	834,33	1941	1399	595	1311,67
111	1	1969	2414	165	1516,00	4923	3449	1654	3342,00
112	1	4282	3362	1056	2900,00	5353	4202	1920	3825,00
113	1	2332	361	382	1025,00	2744	1202	636	1527,33
114	1	2917	604	109	1210,00	4167	2014	541	2240,67
115	1	3353	2231	1558	2380,67	4790	3718	1947	3485,00
116	2	4781	2171	1346	2766,00	8693	3948	2448	5029,67
117	2	744	272	189	401,67	1608	1082	578	1089,33
118	2	884	534	194	537,33	1473	1334	648	1151,67
119	1	1197	1174	419	930,00	1330	1304	466	1033,33
120	1	2697	1184	1232	1704,33	3853	2961	1540	2784,67
121	1	3021	1369	1030	1806,67	5035	3422	2289	3582,00
125	1	1415	688	1776	1293,00	7076	3439	2220	4245,00
126	1	4159	3167	1735	3020,33	4261	3519	2478	3419,33
127	1	1128	1710	854	1230,67	3761	1900	1067	2242,67
128	1	4064	1937	1024	2341,67	5080	2421	1280	2927,00
129	1	2798	776	1300	1624,67	4663	2588	1625	2958,67
130	1	2939	1393	2531	2287,67	4898	2322	2109	3109,67
131	1	1999	1156	829	1328,00	2499	1562	1184	1748,33
132	1	2343	1135	114	1197,33	2929	1621	570	1706,67
133	1	1445	904	584	977,67	2409	1506	973	1629,33
1123	1	1554	3715	2281	2516,67	5180	4644	2851	4225,00
1288	1	3678	2992	744	2471,33	4087	3325	930	2780,67

Supplementary table 3: No. of cyst: progressive number assigned to each sample; Organ: 1=lung, 2=liver; I. I. T.: Inflammatory infiltrate thickness maximum, medium, minimum; I. I. T. mean: mean of the three measurement of inflammatory infiltrate thickness; A. L. thickness max, med, min: adventitia layer thickness maximum, medium and minmum, A. L. Mean: mean of the three measurement of inflammatory A. L. Thickness.

Supplementary table 4

No. of cysts	Organ	GL	GCs	CD3_ grade	CD79_ grade	Score	Grading
50	Lung	1	1	1	1	4	Absent
51	Lung	1	1	1	1	4	Absent
10	Lung	2	2	1	3	8	Mild
19	Lung	2	2	1	2	7	Mild
53	Lung	2	2	2	1	7	Mild
98	Lung	2	2	2	1	7	Mild
110	Lung	2	2	2	2	8	Mild
112	Lung	2	3	1	1	7	Mild
113	Lung	2	2	2	2	8	Mild
114	Lung	2	2	1	2	7	Mild
120	Lung	2	3	2	1	8	Mild
121	Lung	2	3	1	1	7	Mild
132	Lung	2	2	2	2	8	Mild
12	Liver	2	2	1	2	7	Mild
13	Liver	2	2	1	2	7	Mild
14	Liver	2	2	1	1	6	Mild
56	Liver	2	3	2	1	8	Mild
60	Liver	2	2	3	1	8	Mild
69	Liver	2	3	2	1	8	Mild
118	Liver	2	2	2	1	7	Mild
1	Lung	2	2	3	4	11	Moderate
3	Lung	2	3	4	2	11	Moderate
7	Lung	2	3	3	3	11	Moderate
8	Lung	2	2	2	3	9	Moderate
17	Lung	2	3	2	2	9	Moderate
55	Lung	2	2	3	2	9	Moderate
70	Lung	2	2	3	4	11	Moderate
81	Lung	2	2	3	4	11	Moderate
82	Lung	2	2	3	2	9	Moderate
83	Lung	2	3	4	2	11	Moderate
84	Lung	2	2	3	4	11	Moderate
85	Lung	2	2	3	3	10	Moderate
92	Lung	2	2	4	3	11	Moderate
107	Lung	3	3	2	2	10	Moderate

No. of cysts	Organ	GL	GCs	CD3_ grade	CD79_ grade	Score	Grading
108	Lung	2	3	3	3	11	Moderate
109	Lung	3	3	2	3	11	Moderate
111	Lung	3	3	2	2	10	Moderate
119	Lung	3	4	1	1	9	Moderate
125	Lung	2	3	2	3	10	Moderate
126	Lung	2	2	2	3	9	Moderate
127	Lung	3	3	2	3	11	Moderate
128	Lung	2	3	3	3	11	Moderate
129	Lung	3	3	2	1	9	Moderate
130	Lung	3	3	2	2	10	Moderate
131	Lung	2	2	3	3	10	Moderate
133	Lung	2	2	4	3	11	Moderate
1123	Lung	2	3	2	2	9	Moderate
59	Liver	3	3	4	1	11	Moderate
65	Liver	2	2	4	2	10	Moderate
67	Liver	3	3	3	1	10	Moderate
68	Liver	2	2	4	3	11	Moderate
73	Liver	3	4	1	1	9	Moderate
116	Liver	3	3	2	2	10	Moderate
4	Lung	2	3	3	4	12	Severe
9	Lung	2	3	3	4	12	Severe
11	Lung	3	3	2	4	12	Severe
18	Lung	2	3	4	4	13	Severe
52	Lung	2	3	4	4	13	Severe
54	Lung	3	4	4	4	15	Severe
62	Lung	2	3	4	4	13	Severe
63	Lung	2	3	4	4	13	Severe
64	Lung	2	3	4	4	13	Severe
72	Lung	2	3	4	4	13	Severe
89	Lung	2	3	4	4	13	Severe
91	Lung	2	3	3	4	12	Severe
93	Lung	3	3	4	3	13	Severe
115	Lung	2	3	3	4	12	Severe
1288	Lung	3	4	4	3	14	Severe
66	Liver	3	3	3	4	13	Severe
117	Liver	3	4	4	1	12	Severe

Supplementary table 4: No. of cysts: progressive number assigned to each sample; Organ; GL: germinal layer categories: 1, 2, 3; GCs: giant cells categories: 1, 2, 3, 4; Cd3 grade:1, 2, 3, 4 obtainded scoring Cd3 positive cells in 5 random field; Cd79 a grade:1, 2, 3, 4 obtainded scoring Cd79a positive cells in 5 random field; Score: sum of GL, GCs,Cd3 and Cd79 grade; Grading: Absent (1-4); Mild (5-8) Moderate (9-11); Severe (12-15).

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Chapter III

3.1 Introduction

Proteomics is defined as a branch of biotechnology concerned with applying the techniques of molecular biology, biochemistry, and genetics to analysing the structure, function, and interactions of the proteins produced by the genes of a particular cell, tissue, or organism, with organizing the information in databases, and with applications of the data (Merriam-Webster since 1828, s.d.). The proteome was defined by Mark Wilkins in 1996 as the "PROTein complement expressed by a genOME, or cells or tissues", pointing out that proteome, if compared with genome, can change under different conditions (Wilkins, 1996), due to post-translational modification in different sites by different ways (Bilal Aslam, 2017). Over the last two decades, proteomics assumed a key role in the understanding of biological systems in living organisms. Summarizing the advantages identified by Bilić et al., (2018), animal proteomics is a useful field to study biology and pathology of domestic species, compare healthy and diseased proteomes between species, use multiple sampling in time series studies, collect samples through non-invasive or minimally invasive methods, and use suitable species as models for human diseases. Moreover, the interaction of proteomic and genetic information may be supported by the knowledge of the population genetics of species of recorded breeding over the years.

Nowadays, veterinary proteomic research may contribute not only to animal health but also to diagnosis, treatments and prognosis of human diseases. In this respect, the identification and characterization of proteins from hydatid fluid and lung tissue surrounding *E. granulosus* cysts might help to discover new biomarker candidates for immunodiagnostics and vaccine development (Chemale G., 2003). Moreover, the knowledge on the parasite-host protein interplay is limited. It is important to investigate this field in order to compare fertile and infertile cysts, as well as infertile cysts in different stages of inflammation, to understand the interactions involved in survival and/or death of the parasite.

In this survey we describe the analysis and comparison of hydatid fluid samples and tissue surrounding hydatids of *E. granulosus* in fertile and infertile cysts from cattle lungs. The comparison of the identified proteins uncovered quantitative and qualitative differences whose biological significance is discussed in order to understand the dynamic protein pathways involved in host and pathogen responses during disease (Ceciliani F., 2014).

3.2 Materials and Methods

Hydatid fluid (HCF) and tissues collection

A total of 27 pulmonary tissue and 10 hydatid fluid samples were used for proteomic analysis. Particularly, 20 lung tissues surrounding cysts and 10 HCF samples were chosen based on the histologic classification (Chapter II). In addition, 7 samples from healthy bovine lungs were used as negative controls.

The proteomic analysis was divided into two trials in order to define proteome patterns of:

1. Cattle lung tissues surrounding fertile and infertile cysts at different inflammation degrees;
2. Hydatid fluid from fertile and infertile cysts.

Tissue samples chosen for proteomic analysis were divided into four categories as follows (Table 10):

- Group A: 2 samples from the "absent inflammation" category, and 5 samples from the "mild inflammation" category;
- Group B: 7 samples from the "moderate inflammation" category;
- Group C: 6 samples from the "severe inflammation" category;
- Group K (control group), including 7 samples of lung tissue from 4 healthy cattle.

In the same way, HCF of 10 hydatid cysts was grouped as follow (Table 11):

- Group A: 2 samples from the "inflammation absent" category;

- Group B: 2 samples from the “mild inflammation” category;
- Group C: 3 samples from the “moderate inflammation” category;
- Group D: 3 samples from the “severe inflammation” category.

Tissues and HCF samples were derived from the same hydatid cyst as shown in the table below. The cysts that had been labeled with sequential numbers were renamed using the letter of the reference group, followed by a progressive number. In summary, a total of 27 samples were used for the lung proteomic analysis from the following groups, respectively: 7 from A (Absent + Mild), 7 from B (Moderate), 6 from C (Severe), 7 from K (Healthy controls).

Table 10 Lungs tissue samples grouping based on the degree of inflammation

Group A (Absent + Mild)	Group B (Moderate)	Group C (Severe)	Group K (Healthy)
A1	B1	C1	K-12
A2	B2	C2	K-21
A3	B3	C4	K-22
A4	B4	C5	K-31
A5	B5	C6	K-31
A6	B6	C7	K-41
A7	B7		K-42

Table 11 Hydatid fluid samples grouping based on the degree of inflammation

Group A (Absent + Mild)	Group B (Moderate)	Group C (Severe)
A1	B1	C1
A2	B3	C3
A4	B4	C4
A5		

3.2.1 Tissue and hydatid fluid treatment

Tissue and hydatid fluid samples were immediately frozen after collection and stored at -80°C. Cold cut frozen lung and thawed HCF samples were homogenized in 500 mL lysis buffer (100 mM Triethyl Ammonium Bicarbonate, TEAB, 2% Sodium Dodecil Sulfate, SDS) using a Omni TH220 homogenizer (Omni International, Kennesaw, USA), followed by 2 cycles of sonication for 10", at maximum amplitude (Qsonica, Newtown, USA) on ice. After centrifugation at 16 000 g at 4 ºC for 20 minutes, clarified supernatant was transferred to a new tube.

3.2.2 Protein identification and quantification using Tandem Mass Tags (TMT) approach

After protein extraction from lung tissue and HCF, samples from each group were processed using a filter aided sample preparation (FASP) protocol and Tandem Mass Tag (TMT)-based quantitative approach. In brief, total protein concentration was determined using BCA assay (Thermo Scientific, Rockford, USA). For each sample and internal standard (pool of all samples), an amount of 35 µg of total proteins was diluted to a volume of 200 µL using urea buffer (8 M urea in 0.1 M Tris–HCl pH 8.5) and subjected to FASP protocol with some modifications (Wiśniewski J. R., 2009). Samples were transferred to the 10-kDa membrane filter units (Microcon YM-10, Merck Millipore), centrifuged (13 000 x g, 20 min, 20 °C) and washed subsequently with 200 µL of FASP-urea buffer. Proteins were alkylated (50 mM IAA, 20 min at room temperature in the dark), washed twice with urea buffer and then twice with TEAB (100 mM pH 8.5) (Thermo Scientific, Rockford, USA) followed by centrifugation. Proteins were digested by trypsin gold (Promega) (enzyme-to-protein ratio 1:35, v/v) at 37 °C overnight. Peptides were collected from filter by centrifugation, washed with 50 µL of TEAB/ACN (1:1, v/v), centrifuged and vacuum dried.

Before labelling, samples were resuspended in 50 µL of TEAB. TMT sixplex reagents (Thermo Scientific, Rockford, IL, USA) for lung tissues and TMT tenplex reagents (Thermo Scientific, Rockford, IL, USA) for hydatid fluid, were prepared according to manufacturer's procedure, as described previously (Horvatić A., 2019). An amount of 19 µL of specific TMT label was added to each sample for labelling (60 min, room temperature). The reaction was quenched using 5% hydroxylamine (Sigma-Aldrich, St. Louis, MO, USA). Five (nine in case of tenplexes) TMT-modified samples were randomly combined with the internal standard (labelled with TMT m/z 126), aliquoted, dried and stored at −20 °C for further analysis. In total, 6 sixplexes and 2 tenplexes were prepared.

High resolution LC-MS/MS analysis of TMT-labelled peptides was carried out using an Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were dissolved in loading solvent (1% ACN, 0.1% formic acid) and loaded onto the trap column (C18 PepMap100, 5 µm, 100A, 300 µm×5 mm), desalted for 12 min at the flow rate of 15 µL/min and separated on the analytical column (PepMap™ RSLC C18, 50 cm×75 µm) using a linear gradient of 5–45% mobile phase B (0.1% formic acid in 80% ACN) over 120 min, 45% to 90% for 2 min, held at 80% for 2 min and re-equilibrated at 5% B for 20 min at the flow rate of 300 nL/min. Mobile phase A consisted of 0.1% formic acid in water. Ionisation was achieved using nanospray Flex ion source (Thermo Fisher Scientific, Bremen, Germany) containing a 10 µm-inner diameter SilicaTip emitter (New Objective, USA). The MS operated in positive ion mode using DDA Top8 method. Full scan MS spectra were acquired in range from m/z 350.0 to m/z 1800.0 with a resolution of 70,000, 110 ms injection time, AGC target 1×106, a ± 2.0 Da isolation window and the dynamic exclusion 30 s. HCD fragmentation was performed at step collision energy (29% and 35% NCE) with a resolution of 17,500 and AGC target of 2×105.

Precursor ions with unassigned charge state, as well as charge states of +1 and more than +7 were excluded from fragmentation.

Acquired MS/MS spectra were analysed for protein identification and quantification using the SEQUEST algorithm implemented into Proteome Discoverer (version 2.0., ThermoFisher Scientific). Database search against *Bos taurus and Echinococcus granulosus* FASTA files (downloaded from SwissProt database on 11/5/2016, 6365 sequences) was performed according to the following parameters: two trypsin missed cleavage sites, precursor and fragment mass tolerances of 10 ppm and 0.02 Da, respectively; carbamidomethyl (C) fixed peptide modification, oxidation (M), deamidation (N,Q) and TMT sixplex (K, peptide N-terminus) dynamic modifications. The false discovery rate (FDR) for peptide identification was calculated using the Percolator algorithm in the Proteome Discoverer workflow based on the search results against a decoy database and was set at 1% FDR. At least two unique peptides and 5% FDR were required for reporting confidently identified proteins. Protein quantification was accomplished by correlating the relative intensities of reporter ions extracted from tandem mass spectra to that of the peptides selected for MS/MS fragmentation. The internal standard was used to compare relative quantification results for each protein between the experiments (6plexes for lung tissues and 10plex for HCF).

3.2.3 Data analysis

All statistics were performed using R v3.2.2 (Team RC., 2013). First, proteins with less than two unique peptides and the ones with 100% missing data were removed from the analysis. Sample outliers were detected per each group for each of the protein using the Dixon's test from R package *outliers* v0.14 (Komsta L., 2011). If any sample outlier was significant ($P < 0.05$) it was removed from further analysis. As most of the analysed proteins did not follow normal distribution according to the Shapiro-Wilk test, the difference in protein abundance

between groups was accessed by Kruskal-Wallis test in situations when there were three or more groups to compare, otherwise Wilcoxon-Mann-Whitney test was performed. Due to multiple comparisons problem, P-values were adjusted using the false discovery rate (FDR) q-value from R package *qvalue* v2.2.2 (Storey J.D., 2015). If any protein showed to be significant (FDR < 0.05), Conover *post-hoc* test was performed for pairwise multiple comparisons using the R package *PMCMR* v4.3 (Pohlert T., 2014). In situations when none of the proteins were significant, but there was some that showed suggestive significance (FDR > 0.05, but P-value < 0.05), Conover *post-hoc* test was also performed but these results should only be considered as suggestive. Only pairwise multiple comparisons with significant adjusted P-value (FDR < 0.05) after Conover *post-hoc* test were considered for further analysis. Fold change between two groups was calculated as mean (Group2)/mean(Group1) and expressed on log2 scale.

Principal Component Analysis (PCA) and volcano plots were designed using R package *ggplot2* v3.1.1 (Wickham H., 2009), heat map was designed using R package *ggplot2* v3.1.1 (Wickham H., 2009) and *ggdendro* 0.1-20 (de Vries A. and Ripley B.D., 2016), and Venn diagrams were designed using web tool InteractiVenn (http://www.interactivenn.net/) (Heberle H., 2015). Proteins GI accession numbers were converted into official gene symbol either by DAVID conversion tool (https://david.ncifcrf.gov/conversion.jsp), UniProtKB ID mapping (https://www.uniprot.org/uploadlists/) or from Mascot search engine implemented into Proteome Discoverer. Enrichment and pathway analysis was performed using STRINGdb v11.0 (https://string-db.org/), with the selection of appropriate organism and default settings with the exception of no more than 5 interactors to show in 1st shell. Pathway (KEGG Pathways, REACTOME Pathways) and annotation terms (GO Biological process, GO Molecular function, GO Cellular component) were extracted, and redundant Gene Ontology (GO) terms were removed using REVIGO (http://revigo.irb.hr/). Network of relationship between desired pathway (KEGG Pathways, REACTOME Pathways) or

annotation terms (GO Biological process, GO Molecular function, GO Cellular component) and proteins with significantly differential abundances between groups were designed using Cytoscape v3.7.1 (Shannon P., 2003).

3.3 Results

3.3.1 Lung tissues

A total of 5047 proteins were identified in lung tissues surrounding the cysts, according to set criteria (2 unique peptides, p-value and FDR < 0.05). The number of excluded proteins due to less than two unique peptides was equal to 2857; 31 proteins were excluded due to all the samples being not available (NA). The number of total proteins to be analysed was therefore 2159. A Conover *post-hoc* test identified 396 proteins. Following isoform removal, 142 proteins were analysed. One hundred and thirty-seven proteins were assigned to *Bos taurus* and 5 to *Echinococcus granulosus* (Table 12).

A Venn diagram resulting from the list of identified proteins in order to attribute these proteins to the various combination of the AC (Healthy controls *vs* Absent + Mild inflammation) AB (Healthy controls *vs* Moderate + Severe inflammation) and BC (Absent + Mild *vs* Moderate + Severe) samples is shown below (Fig. 14).

A total of 114 proteins were identified for the combination AB, 124 for AC, and finally 88 for the BC. No unique proteins were specifically attributed to one combination. Three proteins were identified as common between AB and BC, probably being characteristics of group B. Thirteen proteins were in common between AC and BC, probably being characteristic of group C. Thirty-nine proteins were common to AB and AC, probably being characteristic of group A. The proteins in common among all the combinations (AB, AC and BC) were 72.

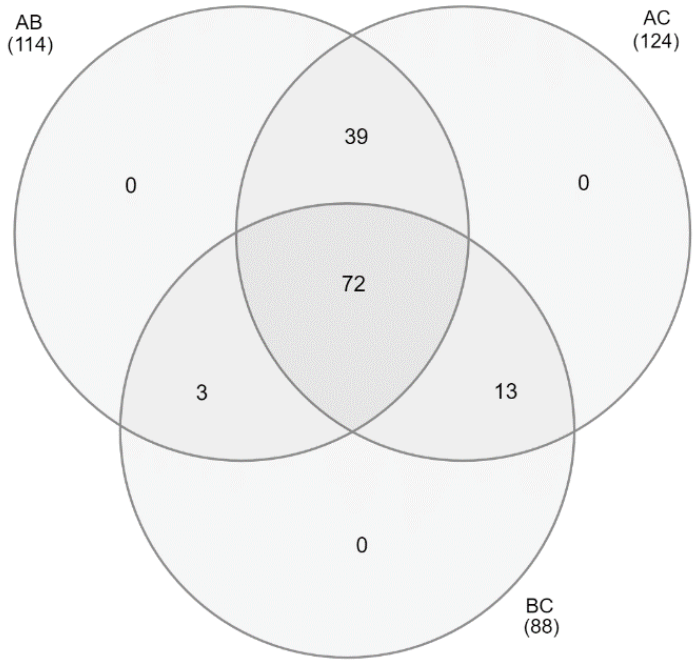

Figure 14 Venn diagram showing the distribution of proteins identified in the different groups of lung tissue surrounding cysts. AB: Healthy controls *vs* Absent + Mild inflammation; AC: Healthy controls *vs* Moderate + Severe; BC: Absent + Mild *vs* Moderate + Severe.

The statistically significant proteins for each group are illustrated in a volcano plot. For the Absent + Mild *vs* Negative controls comparison, 42 proteins were increased and 32 decreased (Fig. 15A). The comparison between Moderate + Severe *vs* Negative controls showed that 72 were increased and 51 decreased (Fig. 15B). The comparison between Moderate + Severe *vs* Absent + Mild showed that 7 were increased and 15 decreased (Fig. 15C).

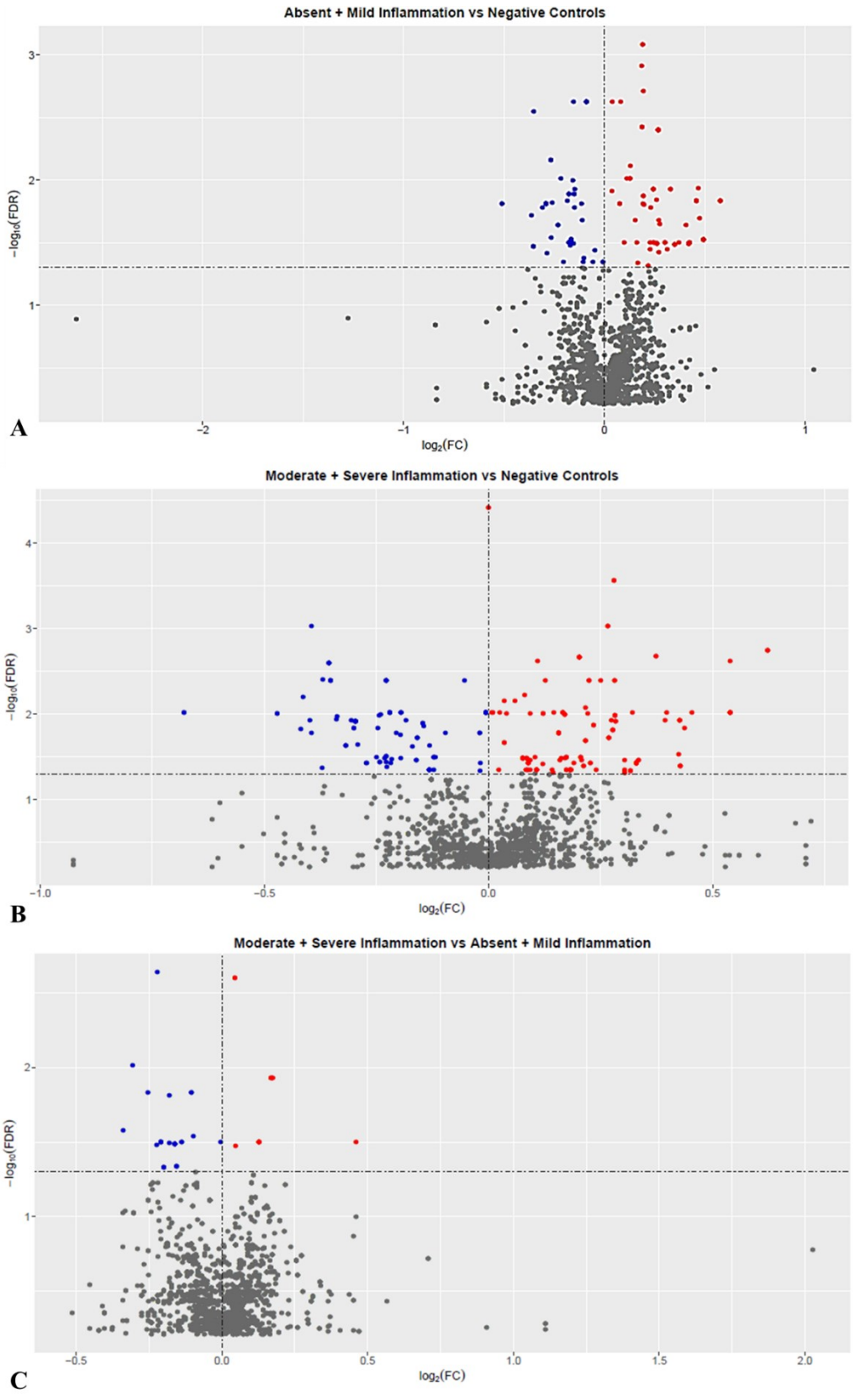

Figure 6 Volcano plot for lung tissues with different inflammation degrees. Volcano plot log_2Fold change (x-axis) and their associated log10 transformed p-values (y-axis) for peptides analysed by LC-MS/MS. Peptide significantly different between the groups are in red (increased), and in blue (decreased), non-significant are in grey.

PCA (Fig.16) and hierarchical clustering (Fig. 17) based on protein abundance values generated three main clusters for lung tissues, one including all K- samples, one including Absent + Mild inflammation group and one including Moderate + Severe inflammation. Absent + Mild was separated from Negative Controls and Moderate + Severe by the first component (17,42%) while Moderate + Severe was separated from Negative Controls by the second component (12.67%).

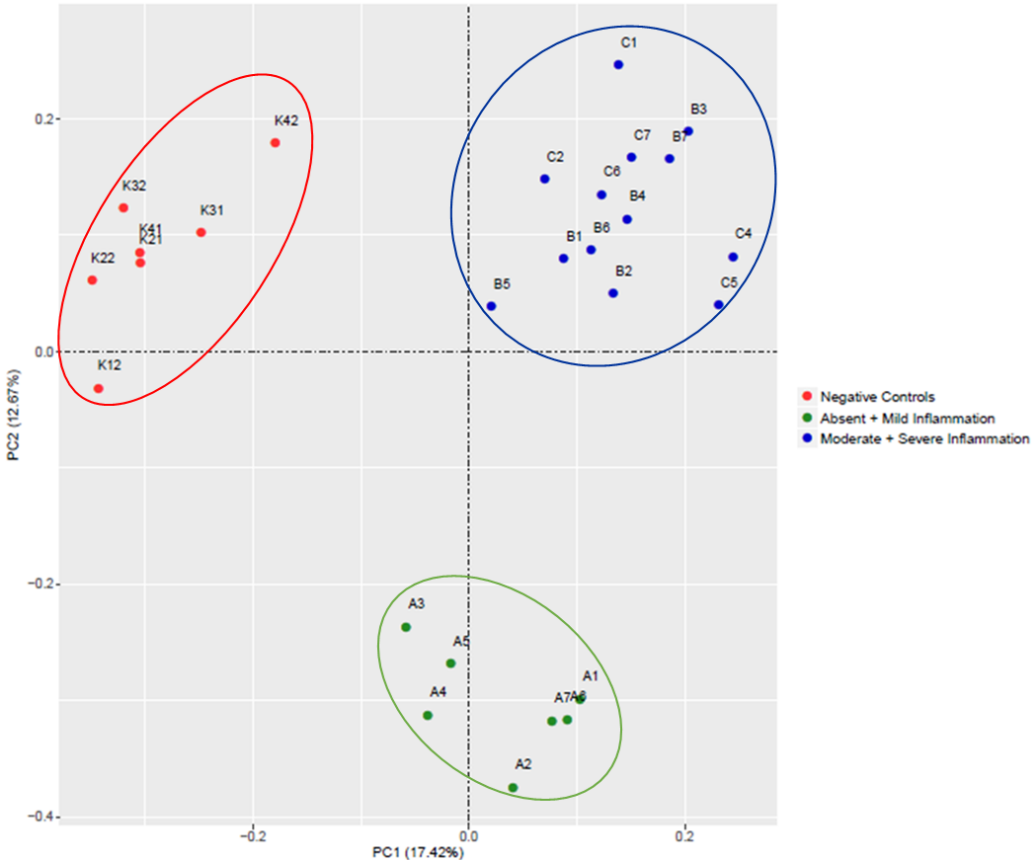

Figure 16 Clustering of samples according to protein abundance values. A: The panel shows the PCA clustering based on shotgun proteomics. K indicates control group; A: Absent + Mild inflammation group and B + C: Moderate + Severe group.

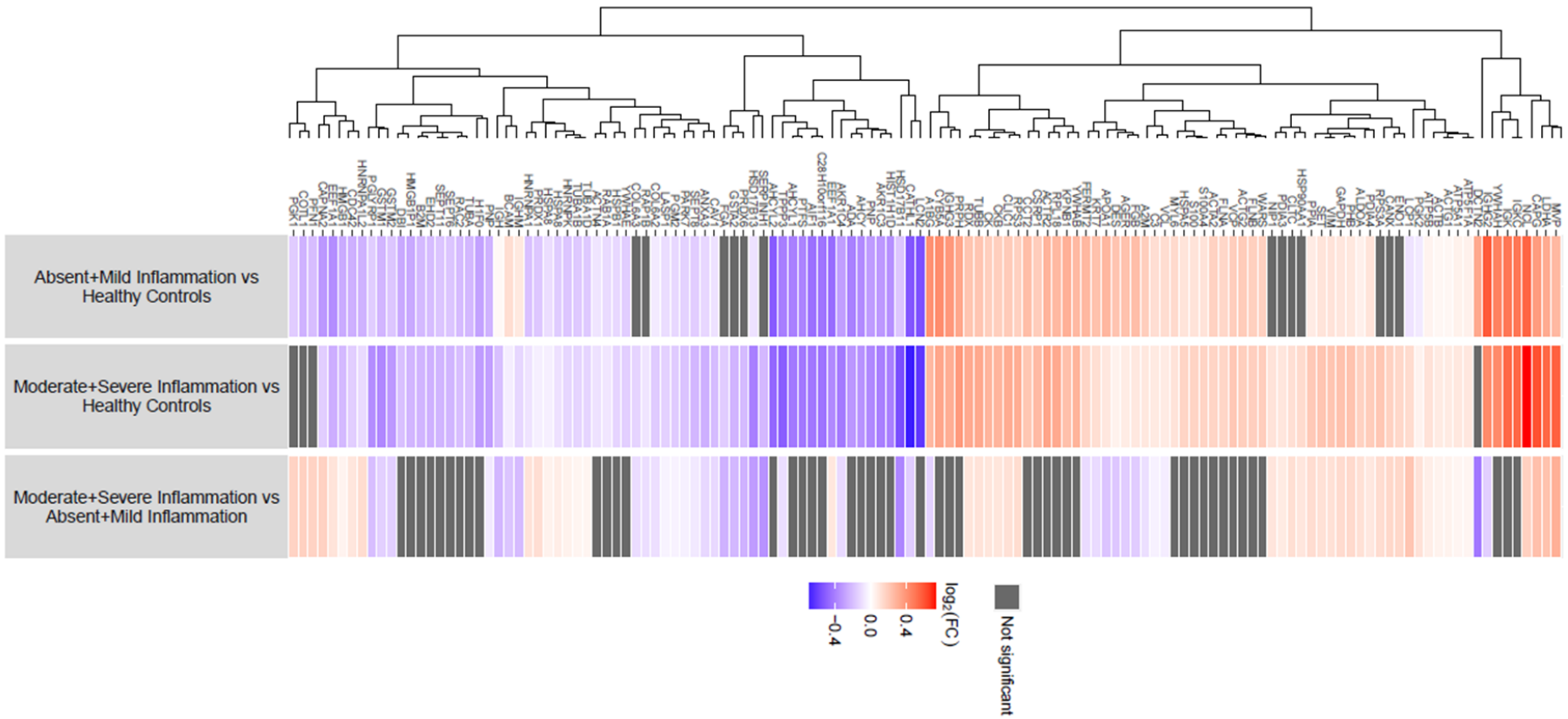

Figure 17 Clustering of samples according to protein abundance values. The Heat Map shows hierarchical clustering of proteins from lung tissue during Cystic echinococcosis infection. All of the proteins with differential abundance were classified into three groups: increased abundance in red, decreased in blue, and non-significant in grey.

Table 12 Parasite and host proteins with significantly differential abundances in lung tissue with different inflammation degrees. AB: Healthy controls vs Absent + Mild inflammation; AC: Healthy controls vs Moderate + Severe; BC: Absent + Mild vs Moderate + Severe

Gene Symbol	Species	Description	P	FDR	log2(Fold Change) AB	log2(Fold Change) AC	log2(Fold Change) BC
0	[Bos taurus]	Unnamed protein product	0.020665	0.038685	0.173918	0.184032	NS
0	[Bos taurus]	Unnamed protein product	0.018215	0.037387	0.420351	0.330068	NS
ACTA2	[Bos taurus]	ACTA 2 protein	0.019347	0.037822	0.169749	0.159882	NS
ACTG2	[Bos taurus]	Actin Gamma 2	0.015166	0.035777	0.193447	0.182176	NS
ACTN4	[Bos taurus]	Actinin-alpha-4	0.028147	0.046098	-0.0783	-0.08538	NS
ACTR2	[Bos taurus]	Actin-like Protein 2 Chain B	0.030826	0.047153	0.281413	0.335184	NS
ADA	[Bos taurus]	Adenosine deaminase	0.032803	0.047848	-0.39619	-0.32627	NS
AHCY	[Bos taurus]	Adenosylhomocysteinase	0.002756	0.01993	-0.36376	-0.33953	NS
AHCYL1	[Bos taurus]	AHCYL1 protein	0.00683	0.024585	-0.39395	-0.41807	NS
AHCYL2	[Bos taurus]	Adenosylhomocysteinase 3 isoform X1	0.00228	0.018457	-0.51076	-0.47154	NS
AKR1C3	[Bos taurus]	aldo-keto reductase family 1. member C3	0.004459	0.021262	-0.31877	-0.39829	NS
B2M	[Bos taurus]	Beta-2-microglobulin Chain B	0.013387	0.033305	-0.21573	-0.23043	NS
C28H10orf116	[Bos taurus]	Chromosome 10 open reading frame 116 ortholog	0.001688	0.016228	-0.43021	-0.43312	NS
CCT2	[Bos taurus]	T-complex protein 1 subunit beta	0.011126	0.030819	0.244685	0.212591	NS
CSRP1	[Bos taurus]	Cysteine and glycine-rich protein 1	0.031629	0.047336	0.187338	0.258507	NS
CYB5A	[Bos taurus]	Cytochrome B5	0.018215	0.037387	0.420351	0.330068	NS
DBI	[Bos taurus]	Acyl-CoA-binding protein	0.001246	0.015566	-0.26697	-0.19471	NS
EHD2	[Bos taurus]	EH-domain containing 2	0.003506	0.021259	-0.20395	-0.24044	NS
FLNA	[Bos taurus]	Filamin-1[	0.009654	0.029356	0.165276	0.162408	NS
FLNB	[Bos taurus]	Filamin B	0.031569	0.047336	0.185017	0.186463	NS
H1F0	[Bos taurus]	Histone H1.0	0.003799	0.021262	-0.28726	-0.30625	NS
HIST1H1D	[Bos taurus]	Histone H1.3	0.006363	0.023442	-0.34534	-0.39478	NS
HMGB1P1	[Bos taurus]	High-mobility group box 1-like	0.007666	0.025917	-0.26571	-0.22685	NS
HSPA5	[Bos taurus]	Endoplasmic reticulum chaperone BiP	0.025662	0.044156	0.101506	0.160032	NS
HSPB1	[Bos taurus]	Heat shock protein beta-1	0.010058	0.029562	-0.11853	-0.13137	NS
IGK	[Bos taurus]	IGK protein	0.003625	0.021259	0.492235	0.539015	NS
IGKC	[Bos taurus]	Immunoglobulin kappa light chain constant region	0.003625	0.021259	0.492235	0.539015	NS
KPNB1	[Bos taurus]	Importin subunit beta-1	0.010731	0.030819	0.2942	0.236337	NS
LCN2	[Bos taurus]	Neutrophil gelatinase-associated lipocalin	0.01173	0.030819	-0.54314	-0.61674	NS
MYL6	[Bos taurus]	Myosin light polypeptide	0.0217	0.038729	0.13819	0.106268	NS
PRPH	[Bos taurus]	Peripherin	0.000551	0.015566	0.328343	0.374287	NS
PTFS	[Bos taurus]	Prostaglandin F synthetase	0.005417	0.021262	-0.38251	-0.41911	NS
RAB1A	[Bos taurus]	Ras-related protein Rab-1A	0.020293	0.038363	-0.11058	-0.07474	NS
RAC2	[Bos taurus]	Ras-related C3 botulinum toxin substrate 2	0.02857	0.046098	-0.2087	-0.19992	NS
RPL18	[Bos taurus]	60S ribosomal protein L18	0.031178	0.047153	0.261852	0.316957	NS
S100	[Bos taurus]	S-100-related calcium-binding protein	0.027191	0.045559	0.104157	0.163223	NS
S100A4	[Bos taurus]	Protein S100-A4	0.027191	0.045559	0.104157	0.163223	NS
SEPT11	[Bos taurus]	Septin-11	0.001779	0.016228	-0.17766	-0.22798	NS
SEPT6	[Bos taurus]	Septin 6	0.001779	0.016228	-0.17766	-0.22798	NS
TUBA	[Bos taurus]	Tubulin Alpha	0.001418	0.015979	-0.21736	-0.19553	NS
VCL	[Bos taurus]	Vinculin	0.004054	0.021262	0.369884	0.453922	NS
VCP	[Bos taurus]	Transitional endoplasmic reticulum ATPase	0.011421	0.030819	0.183694	0.205454	NS
VCP	[Echinococcus granulosus]	Transitional endoplasmic reticulum ATPase	0.004382	0.021262	0.217591	0.283853	NS
VCP	[Bos taurus]	Valosin-containing protein	0.011421	0.030819	0.183694	0.205454	NS
WARS	[Bos taurus]	Tryptophan-tRNA ligase	0.020665	0.038685	0.173918	0.184032	NS
YWHAB	[Bos taurus]	14-3-3 protein beta	0.003221	0.021033	0.260709	0.282361	NS
YWHAE	[Bos taurus]	14-3-3 protein epsilon	0.002533	0.019408	-0.15113	-0.14679	NS
YWHAH	[Bos taurus]	14-3-3 protein eta isoform	0.004979	0.021262	0.473001	0.437568	NS
MVP	[Bos taurus]	Major vault protein	0.022022	0.039062	0.263034	0.559677	0.296643
LDHA	[Bos taurus]	Lactate dehydrogenase-A	0.000521	0.015566	0.349689	0.62293	0.273242
CAPG	[Bos taurus]	Macrophage-capping protein	0.002855	0.01993	0.367277	0.602369	0.235092
LCP1	[Bos taurus]	Lymphocyte cytosolic protein 1 (L-plastin)	0.028243	0.046098	-0.08391	0.133583	0.217494
GAPDH	[Bos taurus]	Glyceraldehyde-3-phosphate dehydrogenase	0.003377	0.021259	0.217314	0.397964	0.18065
COTL1	[Bos taurus]	Coactosin-like protein isoform X1	0.030342	0.046875	-0.23723	NS	0.172735
NCL	[Bos taurus]	Nucleolin	0.004712	0.021262	0.547033	0.719584	0.17255
CAPNS2	[Bos taurus]	Calpain small subunit 2	0.011582	0.030819	-0.31999	-0.15114	0.168849
PPIA	[Bos taurus]	Peptidyl-prolyl cis-trans isomerase A	0.000151	0.015566	0.11211	0.280203	0.168093

Gene Symbol	Species	Description	P	FDR	log2(Fold Change) AB	log2(Fold Change) AC	log2(Fold Change) BC
PGK1	[Echinococcus granulosus]	Phosphoglycerate kinase 1	0.011052	0.030819	-0.15489	NS	0.164929
PFN1	[Bos taurus]	Profilin-1	0.028712	0.046098	-0.19575	NS	0.161445
CANX	[Bos taurus]	Calnexin	0.007368	0.025662	NS	0.170786	0.147727
ENO1	[Bos taurus]	Alpha-enolase	0.016364	0.036608	NS	0.148287	0.146742
0	[Bos taurus]	Unnamed protein product	0.027119	0.045559	NS	0.155721	0.144903
PHB	[Bos taurus]	Prohibitin	0.020249	0.038363	0.087738	0.226175	0.138437
RPS3	[Bos taurus]	40S ribosomal protein S3	0.021515	0.038729	0.174029	0.303848	0.129818
HNRNPA1L2	[Bos taurus]	Heterogeneous nuclear ribonucleoprotein A1-like	0.017865	0.037387	-0.23892	-0.10941	0.129511
RPS3A	[Bos taurus]	Similar to ribosomal protein S3A	0.027663	0.045948	NS	0.211429	0.12749
PRDX1	[Bos taurus]	Peroxiredoxin-1	0.017057	0.036713	-0.17149	-0.04819	0.123305
CLTC	[Bos taurus]	Clathrin heavy chain 1	0.02154	0.038729	NS	0.122397	0.122397
ALDOA	[Bos taurus]	ALDOA protein	0.002918	0.01993	0.055025	0.170271	0.115246
SET	[Bos taurus]	protein SET	0.008124	0.026986	0.103209	0.216889	0.11368
CLIC1	[Bos taurus]	Chloride intracellular channel protein 1	0.016727	0.036608	0.195673	0.309055	0.113382
PGK2	[Bos taurus]	Phosphoglycerate kinase 2	0.028454	0.046098	-0.064	0.044438	0.108436
TUBB	[Bos taurus]	Tubulin beta-7 chain	0.002812	0.01993	0.217137	0.321578	0.104442
HNRNPA1	[Bos taurus]	heterogeneous nuclear ribonucleoprotein A1-like	0.005212	0.021262	-0.17694	-0.07466	0.102275
0	[Bos taurus]	Uncharacterized protein LOC518961	0.029358	0.046098	-0.04866	0.052675	0.101334
0	[Bos taurus]	Uncharacterized protein LOC527388	0.029358	0.046098	-0.04866	0.052675	0.101334
0	[Bos taurus]	Uncharacterized protein LOC528329	0.029358	0.046098	-0.04866	0.052675	0.101334
0	[Bos taurus]	Uncharacterized protein LOC617905	0.029358	0.046098	-0.04866	0.052675	0.101334
AIF1	[Bos taurus]	Allograft inflammatory factor 1 isoform X1	0.014541	0.034966	-0.35391	-0.25438	0.099536
RDX	[Bos taurus]	Radixin	0.02918	0.046098	0.218508	0.317168	0.09866
NIP1	[Echinococcus granulosus]	Eukaryotic translation initiation factor 3 subunit C	0.014385	0.034966	NS	0.104232	0.098239
CK	[Bos taurus]	Crystal Structure Of Bovine Retinal Creatine Kinase	0.007003	0.024843	0.181252	0.276997	0.095746
CKB	[Bos taurus]	Creatine Kinase B-type	0.007003	0.024843	0.181252	0.276997	0.095746
0	[Bos taurus]	Histone cluster 1. H4j-like	0.02632	0.044489	NS	0.08843	0.094422
CDC42	[Bos taurus]	Cell division control protein 42 homolog precursor	0.008729	0.028182	-0.24502	-0.15143	0.093589
HSP90AA1	[Bos taurus]	HSPCA protein	0.018773	0.037534	NS	0.135901	0.093304
PDIA4	[Bos taurus]	Protein disulfide isomerase-associated 4	0.01331	0.033305	0.115731	0.20865	0.092919
EEF1A1	[Echinococcus granulosus]	Elongation factor 1 alpha	0.001029	0.015566	-0.45626	-0.36606	0.090198
VIM	[Bos taurus]	Vimentin	0.003585	0.021259	0.135738	0.221468	0.08573
0	[Bos taurus]	Unnamed protein product	0.015853	0.035868	NS	0.093109	0.076413
PDIA3	[Bos taurus]	PDIA3 protein	0.015853	0.035868	NS	0.093109	0.076413
PGK1	[Bos taurus]	Phosphoglycerate kinase 1	0.032132	0.047829	NS	0.092928	0.066984
HSPA8	[Bos taurus]	HSPA8 protein	0.003823	0.021262	-0.15743	-0.0906	0.066832
ATP5B	[Bos taurus]	Mitochondrial ATP synthase. H+ transporting F1 complex	0.014251	0.034966	0.10758	0.173712	0.066132
HNRNPK	[Bos taurus]	Heterogeneous nuclear ribonucleoprotein K isoform X1	0.011744	0.030819	-0.17386	-0.12303	0.05083
ACTG1	[Bos taurus]	Chain A. Actin. Cytoplasmic 1	0.013105	0.033305	0.029294	0.076366	0.047072
0	[Bos taurus]	Unnamed protein product	0.008646	0.028071	-0.11165	-0.06565	0.045992
ACTB	[Bos taurus]	Actin Beta	0.000893	0.015566	0.063866	0.109624	0.045758
HMGB1	[Bos taurus]	High mobility group protein B1	0.004663	0.021262	-0.26082	-0.21585	0.044975
TUBA1B	[Bos taurus]	Tubulin alpha-1B	0.009391	0.028862	-0.16582	-0.12793	0.037891
TUBA1D	[Bos taurus]	Tubulin alpha-1D	0.009391	0.028862	-0.16582	-0.12793	0.037891
ATP5A1	[Bos taurus]	ATP synthase	0.018838	0.037534	0.052632	0.089084	0.036452
ATP5F1A	[Bos taurus]	Chain A Atp Synthase Alpha Chain Heart Isoform Mitochondrial Precu	0.018838	0.037534	0.052632	0.089084	0.036452

GI Accession	Gene Symbol	Species	Description	P	FDR	log2(Fold Change) AB	log2(Fold Change) AC	log2(Fold Change) BC
1.4E+09	EEF1A1	[Echinococcus granulosus]	Elongation factor 1 alpha	0.001029	0.015566	-0.45626	-0.36606	0.090198
289450	VIM	[Bos taurus]	Vimentin	0.003585	0.021259	0.135738	0.221468	0.08573
66952432	0	[Bos taurus]	Unnamed protein product	0.015853	0.035868	NS	0.093109	0.076413
1.46E+08	PDIA3	[Bos taurus]	PDIA3 protein	0.015853	0.035868	NS	0.093109	0.076413
77735551	PGK1	[Bos taurus]	Phosphoglycerate kinase 1	0.032132	0.047829	NS	0.092928	0.066984
1.58E+08	HSPA8	[Bos taurus]	HSPA8 protein	0.003823	0.021262	-0.15743	-0.0906	0.066832
89574045	ATP5B	[Bos taurus]	Mitochondrial ATP synthase.	0.014251	0.034966	0.10758	0.173712	0.066132
5.29E+08	HNRNPK	[Bos taurus]	Heterogeneous nuclear ribonucleoprotein K isoform X1	0.011744	0.030819	-0.17386	-0.12303	0.05083
3.85E+08	ACTG1	[Bos taurus]	Chain A. Actin. Cytoplasmic 1	0.013105	0.033305	0.029294	0.076366	0.047072
428	0	[Bos taurus]	Unnamed protein product	0.008646	0.028071	-0.11165	-0.06565	0.045992
22655316	ACTB	[Bos taurus]	Actin Beta	0.000893	0.015566	0.063866	0.109624	0.045758
123367	HMGB1	[Bos taurus]	High mobility group protein B1	0.004663	0.021262	-0.26082	-0.21585	0.044975
1.05E+09	TUBA1B	[Bos taurus]	Tubulin alpha-1B	0.009391	0.028862	-0.16582	-0.12793	0.037891
3.13E+08	TUBA1D	[Bos taurus]	Tubulin alpha-1D	0.009391	0.028862	-0.16582	-0.12793	0.037891
94574274	ATP5A1	[Bos taurus]	ATP synthase	0.018838	0.037534	0.052632	0.089084	0.036452
51247976	ATP5F1A	[Bos taurus]	Chain A Atp Synthase Alpha Chain	0.018838	0.037534	0.052632	0.089084	0.036452
2.96E+08	EEF1A1	[Bos taurus]	Eukaryotic translation elongation factor 1 alpha 1-like	0.0295	0.046196	-0.10254	-0.07514	0.027398
77567679	LASP1	[Bos taurus]	LIM and SH3 protein 1	0.023211	0.040546	-0.10382	-0.14168	-0.03787
1.52E+08	PGM2	[Bos taurus]	PGM2 protein	0.008419	0.027491	-0.12053	-0.15904	-0.03851
1575493	IGHG3	[Bos taurus]	IgG3 heavy chain constant region	0.004399	0.021262	0.466031	0.424324	-0.04171
5.29E+08	PARK7	[Bos taurus]	Protein/nucleic acid deglycase DJ-1 isoform X1	0.004863	0.021262	-0.138	-0.18425	-0.04625
8.1E+08	C3	[Bos taurus]	Complement component 3	0.009442	0.028862	0.152471	0.105353	-0.04712
1.11E+08	SEPT8	[Bos taurus]	Septin 8	0.00538	0.021262	-0.15215	-0.22048	-0.06833
82407788	PNP	[Bos taurus]	Purine Nucleoside Phosphorylase Chain A	0.00493	0.021262	-0.22635	-0.29659	-0.07024
2.96E+08	COL6A2	[Bos taurus]	Collagen alpha 2 type VI	0.015314	0.035777	-0.06359	-0.16085	-0.09727
5.77E+08	RAP1B	[Echinococcus granulosus]	Ras-related protein Rap-1b	0.030044	0.046561	NS	-0.08021	-0.09994
2.96E+08	RAP1B	[Bos taurus]	RAP1B. member of RAS oncogene family-like	0.030044	0.046561	NS	-0.08021	-0.09994
1.22E+08	TPPP3	[Bos taurus]	Tubulin polymerization-promoting protein family member 3	0.004331	0.021262	-0.4009	-0.50213	-0.10123
1.39E+09	COL6A3	[Bos taurus]	collagen alpha-3(VI) chain isoform X1	0.016944	0.036608	NS	-0.13232	-0.10394
2.96E+08	KRT7	[Bos taurus]	Keratin type II cytoskeletal 7	0.005442	0.021262	0.23085	0.121513	-0.10934
1.98E+08	FERMT2	[Bos taurus]	Fermitin family homolog 2	0.001331	0.015566	0.267127	0.15675	-0.11038
5.29E+08	AKR1C4	[Bos taurus]	Cytosolic dihydrodiol dehydrogenase 3	0.002073	0.018325	-0.30051	-0.41392	-0.11341
4.09E+08	A2M	[Bos taurus]	Alpha-2-macroglobulin	0.005268	0.021262	0.193851	0.070619	-0.12323
86823979	A1BG	[Bos taurus]	Alpha-1-B glycoprotein	0.00934	0.028862	0.406126	0.266879	-0.13925
5.29E+08	GSTM2	[Bos taurus]	Glutathione S-transferase	0.009954	0.029562	-0.17397	-0.31864	-0.14467
7582395	GSTM1	[Bos taurus]	Glutathione S-transferase	0.021269	0.038729	-0.22655	-0.3715	-0.14495
1.15E+09	IGH	[Bos taurus]	Immunoglobulin gamma heavy chain	0.004853	0.021262	0.57571	0.427827	-0.14788
89611	IGHG2	[Bos taurus]	Ig gamma-2 chain C region	0.004853	0.021262	0.57571	0.427827	-0.14788
2.96E+08	CATHL1	[Bos taurus]	Cathelicidin-1 precursor	0.002907	0.01993	-0.52581	-0.67938	-0.15357
3.12E+08	AGER	[Bos taurus]	Advanced glycosylation end product-specific receptor. splice	0.030057	0.046561	0.227928	0.07268	-0.15525
357	FGB	[Bos taurus]	Fibrinogen beta chain	0.004181	0.021262	0.242945	0.087092	-0.15585
33413902	IGHM	[Bos taurus]	Immunoglobulin mu heavy chain constant region	0.015832	0.035868	0.101235	NS	-0.16054
5.29E+08	CAV1	[Bos taurus]	Caveolin-1 isoform X1	0.002948	0.01993	-0.07598	-0.2442	-0.16822
2959456	DES	[Bos taurus]	Desmin	0.023509	0.04094	0.220852	0.049936	-0.17092
27807439	BCAM	[Bos taurus]	Basal cell adhesion molecule precursor	0.006845	0.024585	0.125159	-0.0545	-0.17966
162678	APOA1	[Bos taurus]	Apolipoprotein A-I precursor	0.009045	0.028862	0.270286	0.089267	-0.18102
3.71E+08	PGLYRP1	[Bos taurus]	Peptidoglycal recognition protein 1	0.00116	0.015566	-0.15302	-0.35233	-0.19931
2.96E+08	GSTA2	[Bos taurus]	Glutathione S-transferase A2	0.019006	0.037534	NS	-0.23002	-0.21296
3.95E+08	ANXA3	[Bos taurus]	Annexin A3	0.012217	0.031913	0.075844	-0.14806	-0.22391
1.49E+08	FGA	[Bos taurus]	Fibrinogen alpha chain	0.026051	0.044164	NS	-0.15653	-0.23714
3703050	PRDX6	[Bos taurus]	Ciliary body glutathione peroxidase	0.019349	0.037822	NS	-0.2103	-0.23836
88954109	HSD17B13	[Bos taurus]	Hydroxysteroid (17-beta) dehydrogenase 13	0.000241	0.015566	-0.08875	-0.39497	-0.30622
1.16E+08	SERPINH1	[Bos taurus]	Serpin H1	0.015504	0.035868	NS	-0.21823	-0.33805
86438493	HSD17B11	[Bos taurus]	Hydroxysteroid (17-beta) dehydrogenase 11	6.55E-05	0.015566	-0.17388	-0.55036	-0.37647
5.29E+08	DCTN2	[Bos taurus]	Dynactin subunit 2 isoform X1	0.014484	0.034966	0.32252	NS	-0.42216

3.3.2 Hydatid fluid

A total of 2772 proteins were identified in hydatid fluid according to set criteria (2 unique peptides and P value < 0.05). From the initial list, 1601 were excluded due to less than 2 unique peptides and 361 were considered after NA removals according to selection threshold.

The hydatid fluid samples were analysed according to two different schemes:

1) fertile *vs* infertile cysts in order to identify differences in the proteomic pattern of the two conditions;
2) sterile with different inflammation degrees, in order to characterize the proteome of each inflammation degree and evaluate the possible role of some proteins in the progression of inflammation.

The first analysis (fertile *vs* sterile) identified a total of 247 proteins. Following removal of the isoforms, the number of proteins was 94. Sixty-one proteins were assigned to *Bos taurus* while 33 were assigned to *Echinococcus granulosus* (Table 13).

PCA (Fig. 18 A) based on protein abundance values generated two main clusters for hydatid fluid, one including fertile cysts and the second one including infertile cysts. Fertile group was separated from infertile subgroup 1 (B4, C4, C3) by the first component (48.22%) while fertile group was separated from infertile subgroup 2 (A4, A5, B1, B3, C1) by the second component (24.51%).

The statistically significant proteins for each group were subjected to volcano plot analysis revealing that 36 proteins were increased and 35 were decreased for the comparison of Fertile *vs* Infertile groups (Fig. 18 B).

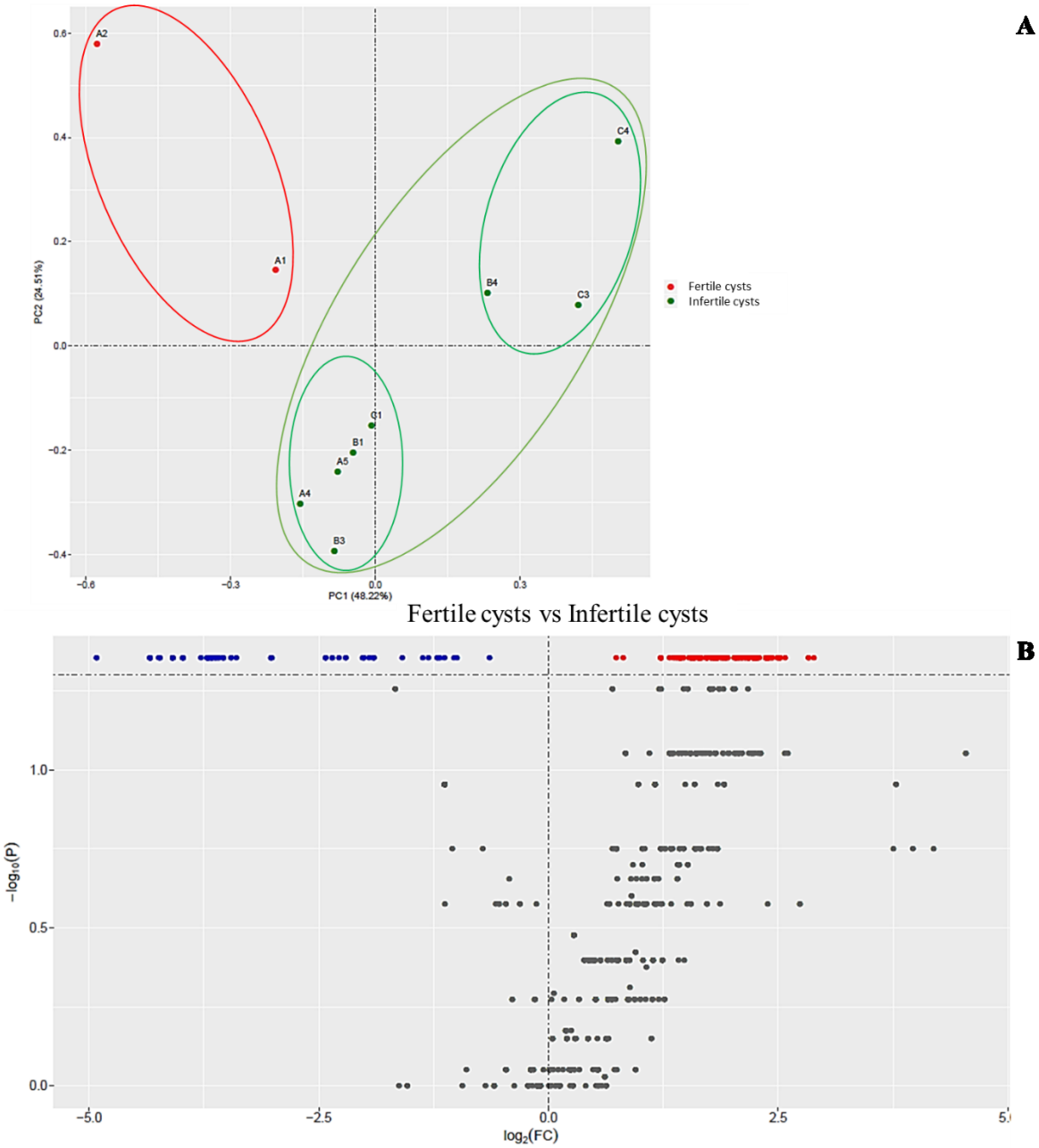

Figure 18 Clustering of samples according to protein abundance values. A: The panel shows the PCA clustering based on shotgun proteomics. Red dots indicate fertile cysts; green dots indicate infertile cysts. B: Volcano plot for lung tissues with different inflammation degrees. Volcano plot log_2Fold change (x-axis) and their associated log10 transformed p-values (y-axis) for peptides analysed by LC-MS/MS. Peptides significantly different between the groups are in red (increased), and in blue (decreased), non-significant are in grey.

Table 13 Parasite and host proteins with significantly differential abundances in hydatid fluid of fertile and infertile cysts

Species	Description	FC	Pvalue
[Bos taurus]	Serotransferrin precursor	7,40566	0,04444
[Bos taurus]	Aldolase C, fructose-bisphosphate	7,11364	0,04444
[Bos taurus]	Immunoglobulin gamma heavy chain	5,9589	0,04444
[Bos taurus]	Superoxide Dismutase Chain A	5,7364	0,04444
[Bos taurus]	Malate dehydrogenase peroxisomal isoform	5,66512	0,04444
[Bos taurus]	Antichymotrypsin alpha 1	5,6478	0,04444
[Bos taurus]	Transaldolase	5,6185	0,04444
[Bos taurus]	Thioredoxin	5,3956	0,04444
[Bos taurus]	Heparan sulfate proteoglycan	5,23305	0,04444
[Bos taurus]	Transgelin-2	5,18403	0,04444
[Bos taurus]	Carbonic Anhydrase	5,15764	0,04444
[Bos taurus]	Coactosin-like protein isoform X1	4,90625	0,04444
[Bos taurus]	Serpin A 3-7	4,81461	0,04444
[Bos taurus]	Hypothetical protein BOS_2215	4,79638	0,04444
[Bos taurus]	Prothymosin alpha	4,79638	0,04444
[Echinococcus granulosus]	Calmodulin	4,77656	0,04444
[Bos taurus]	Modulation Of Calmodulin Plasticity	4,77656	0,04444
[Bos taurus]	Unnamed protein product	4,68439	0,04444
[Bos taurus]	Pigment epithelium-derived factor precursor	4,67727	0,04444
[Bos taurus]	Acyl-CoA-binding protein Chain A	4,51389	0,04444
[Bos taurus]	Unnamed protein prodct	4,50719	0,04444
[Bos taurus]	GSTP1 protein	4,45946	0,04444
[Bos taurus]	Cyclophilin I	4,40881	0,04444
[Bos taurus]	Glucosephosphate isomerase	4,39831	0,04444
[Bos taurus]	Osteopontin, early T-lymphocyte activation	4,31349	0,04444
[Bos taurus]	Fructose-1,6-bisphosphatase	4,2803	0,04444
[Echinococcus granulosus]	Phosphoglycerate kinase	4,23567	0,04444
[Bos taurus]	Serpin A 3-1	4,19417	0,04444
[Bos taurus]	Transketolase	4,12598	0,04444
[Bos taurus]	NME1-NME2 protein-like	4,1055	0,04444
[Bos taurus]	Nucleoside diphosphate kinase B	4,1055	0,04444
[Bos taurus]	Lumican	4,05822	0,04444
[Bos taurus]	Purine nucleoside phosphorylase-like	3,80935	0,04444
[Bos taurus]	RBP4 protein	3,74436	0,04444
[Bos taurus]	Phosphoglycerate mutase 2	3,67692	0,04444
[Bos taurus]	Heat-responsive protein 12-like	3,55172	0,04444
[Bos taurus]	Enolase 1, (alpha)	3,53333	0,04444
[Bos taurus]	Serum albumin	3,50588	0,04444
[Bos taurus]	Unnamed protein product	3,49035	0,04444
[Bos taurus]	Endopin 2B	3,45455	0,04444
[Bos taurus]	Matrix metalloproteinase-9;	3,41207	0,04444
[Bos taurus]	Rho GDP-dissociation inhibitor 2	3,31897	0,04444
[Bos taurus]	ALDO A protein	3,29592	0,04444
[Bos taurus]	Fatty acid-binding protein	3,15741	0,04444
[Bos taurus]	Leukocyte elastase inhibitor isoform X1	3,12934	0,04444

Species	Description	FC	Pvalue
[Bos taurus]	Serine (or cysteine) proteinase inhibitor	3,12934	0,04444
[Bos taurus]	Serpin A 3-8	3,11377	0,04444
[Bos taurus]	Guanine deaminase	3,06983	0,04444
[Bos taurus]	14-3-3 protein epsilon	2,99673	0,04444
[Bos taurus]	L-lactate dehydrogenase A chain	2,99482	0,04444
[Bos taurus]	Plasminogen precursor	2,95536	0,04444
[Bos taurus]	Alpha-2-antiplasmin	2,93231	0,04444
[Bos taurus]	Albumin	2,91912	0,04444
[Bos taurus]	Carbonmonoxy Liganded Bovine Hemoglobin Ph 8.5 Chain	2,7123	0,04444
[Bos taurus]	Fibronectin 1	2,69655	0,04444
[Bos taurus]	Tyrosine 3/tryptophan 5 -monooxygenase activation protein	2,68278	0,04444
[Bos taurus]	Lymphocyte cytosolic protein 1 (L-plastin)	2,64423	0,04444
[Bos taurus]	Actin	2,5625	0,04444
[Bos taurus]	Antithrombin Chain B,	2,49046	0,04444
[Bos taurus]	Calmodulin	2,33275	0,04444
[Bos taurus]	Ribonuclease UK114	1,66471	0,04444
[Bos taurus]	Immunoglobulin light chain variable region	0,6402	0,04444
[Echinococcus granulosus]	Murinoglobulin-2	0,50099	0,04444
[Echinococcus granulosus]	Lysosomal alpha mannosidase	0,49044	0,04444
[Bos taurus]	Leucine-rich alpha-2-glycoprotein 1	0,45626	0,04444
[Echinococcus granulosus]	Basement membrane specific heparan sulfate	0,43205	0,04444
[Echinococcus granulosus]	Cathepsin B	0,40457	0,04444
[Echinococcus granulosus]	Hypothetical protein EGR_06427	0,33225	0,04444
[Echinococcus granulosus]	Fibulin-2	0,26792	0,04444
[Echinococcus granulosus]	Collagen alpha-1(IV) chain	0,26605	0,04444
[Echinococcus granulosus]	Papilin	0,2586	0,04444
[Echinococcus granulosus]	Hemicentin-1	0,24787	0,04444
[Echinococcus granulosus]	Hypothetical protein EGR_01530	0,24553	0,04444
[Echinococcus granulosus]	Expressed protein	0,21666	0,04444
[Echinococcus granulosus]	Hypothetical protein EGR_08643	0,21666	0,04444
[Echinococcus granulosus]	Egf domain protein	0,20567	0,04444
[Echinococcus granulosus]	Cysteine-rich secretory protein LCCL domain-containing	0,18595	0,04444
[Echinococcus granulosus]	Expressed conserved protein	0,18589	0,04444
[Echinococcus granulosus]	Hypothetical protein EGR_06216	0,18589	0,04444
[Echinococcus granulosus]	Expressed protein	0,12303	0,04444
[Echinococcus granulosus]	Antigen 5 38 kDa	0,09488	0,04444
[Echinococcus granulosus]	Kunitz protein 3 precursor	0,09107	0,04444
[Echinococcus granulosus]	WAP, kazal, immunoglobulin, kunitz and NTR domain-cont	0,09107	0,04444
[Echinococcus granulosus]	Antigen B4	0,08607	0,04444
[Echinococcus granulosus]	Tryptase	0,08322	0,04444
[Echinococcus granulosus]	Antigen 5 precursor	0,08172	0,04444
[Echinococcus granulosus]	Collagen alpha-1(XXVII) chain	0,08101	0,04444
[Echinococcus granulosus]	Fibrillar collagen chain FAp1 alpha	0,08101	0,04444
[Echinococcus granulosus]	Antigen B	0,07609	0,04444
[Echinococcus granulosus]	Antigen B1	0,06347	0,04444
[Echinococcus granulosus]	Antigen B 12kDa subunit	0,04948	0,04444
[Echinococcus granulosus]	Antigen B8/1	0,04948	0,04444
[Echinococcus granulosus]	Expressed protein	0,03309	0,04444
[Echinococcus granulosus]	Hypothetical protein EGR_08712	0,03309	0,04444

In the second analysis (sterile cysts with different inflammation degrees) a total of 166 proteins were differential. The number of proteins decreased to 6 after isoform removal, of which one assigned to *Echinococcus granulosus* and 5 to *Bos taurus* (Table 14).

PCA (Fig.20 A) based on protein abundance values generated three main clusters for hydatid fluid of infertile cysts, Severe group (C1, C4) and Moderate group (B3, B1) were separated by Mild group (A5, A5) from the first component (58,54%), while B4 and C3 samples did not cluster with the groups they belonged to. Volcano plots showed increased and decreased proteins, respectively in red and blue, in the three combinations of inflammation degrees. In Moderate *vs* Mild (Fig. 20 B), 14 proteins were increased and one decreased, in Severe *vs* Mild the number of increased proteins was 13 (Fig. 20 C) while in Severe *vs* Moderate (Fig. 20 D) 15 proteins were increased and 3 were decreased. The higher number of proteins shown in volcano plots is due to isoforms, subsequently removed. Finally, fold changes of the 6 statistically significant proteins are reported for the AB, AC, and BC combinations (Fig. 19). The levels of three proteins increase proportionally to the degree of inflammation compared to healthy tissue in AB and AC. The highest levels of L-lactate deidrogenase A, citrate synthase and immunoglobulin M are shown in the comparison between Moderate + Severe inflammation compared to negative controls.

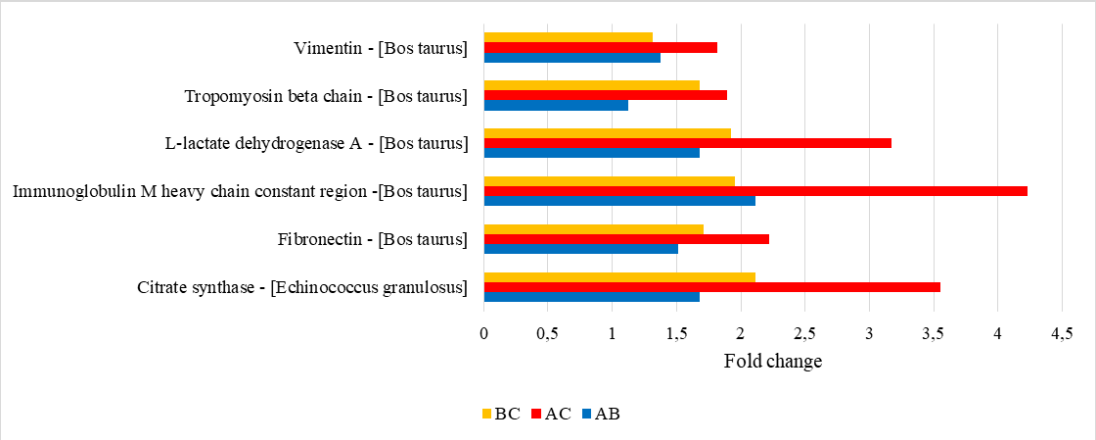

Figure 19 *Bos Taurus* and *Echinococcus granulosus* proteins significantly different in hydatid fluid of infertile cysts in different inflammation degrees. BC: Moderate vs Severe, AC: Mild vs Severe; AB:Mild vs Moderate

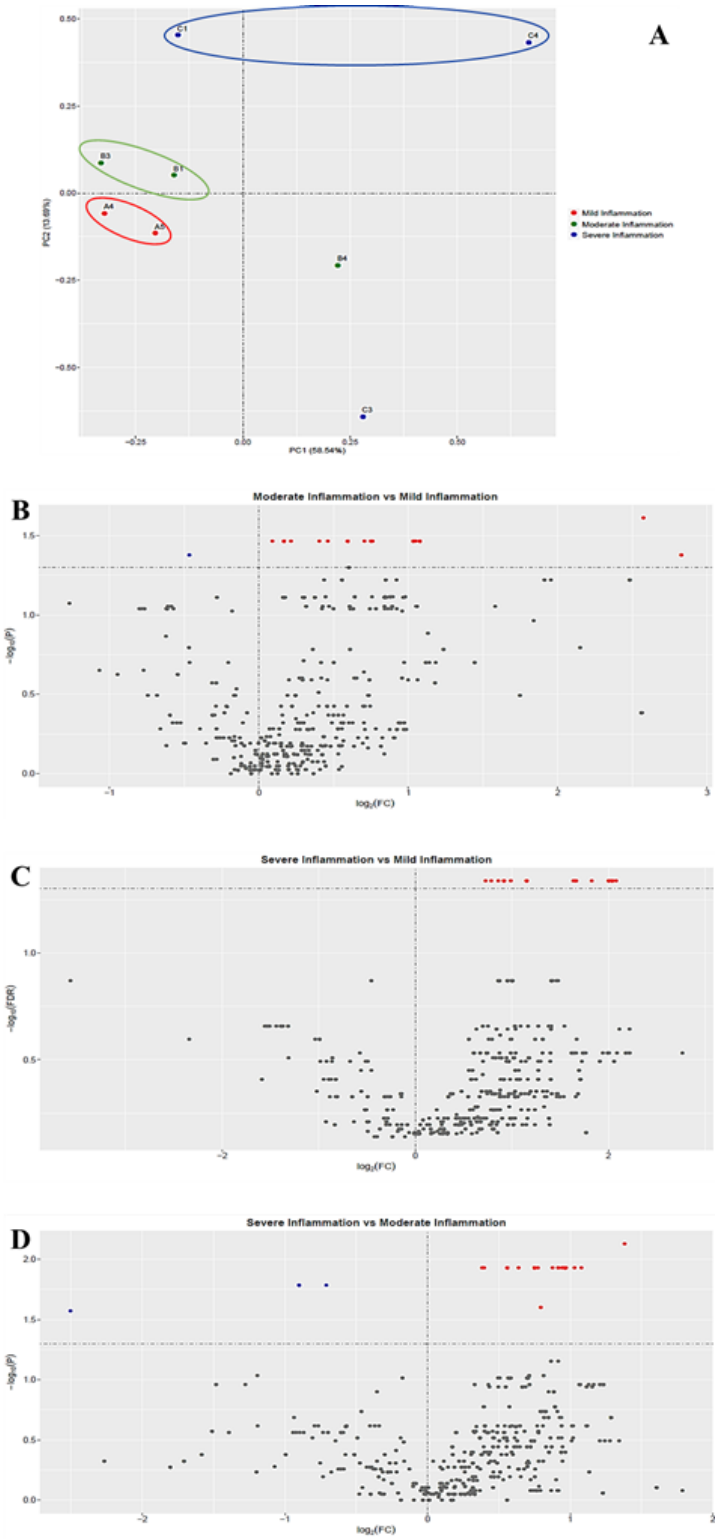

Figure 20 Clustering of samples according to protein abundance values. A: The panel shows the PCA clustering based on shotgun proteomics. Red dots indicate mild inflammation; green dots indicate moderate inflammation; blue dots indicate severe inflammation. B,C, D: Volcano plot for lung tissues with different inflammations degrees. Volcano plot log_2Fold change (x-axis) and their associated log10 transformed p-values (y-axis) for peptides analysed by LC-MS/MS. B: Moderate vs Mild inflammation; C: Severe vs Mild inflammation; D: Severe vs Moderate inflammation. Peptides significantly different between the groups are in red (increased), and in blue (decreased); non-significant are in grey

Table 14 Parasite and host proteins with significantly differential abundances in hydatid fluid of infertile cysts

Species	Description	Pvalue AB	FC AB	Pvalue AC	FC AC	Pvalue BC	FC BC
[Echinococcus granulosus]	Citrate synthase	0,034317	1,682266	0,001429	3,551724	0,011725	2,111274
[Bos taurus]	Fibronectin	0,034317	1,509142	0,001429	2,223629	0,011725	1,710718
[Bos taurus]	Immunoglobulin M heavy chain constant region	0,034317	2,110778	0,001429	4,227545	0,011725	1,957602
[Bos taurus]	L-lactate dehydrogenase A	0,034317	1,682796	0,001429	3,175627	0,011725	1,922819
[Bos taurus]	Tropomyosin beta chain	0,034317	1,123937	0,001429	1,890644	0,011725	1,682162
[Bos taurus]	Vimentin	0,034317	1,377778	0,001429	1,814286	0,011725	1,31682

Columns 3 and 4 show respectively P value and Fold Change for (A) Mild vs (B) Moderate; Columns 5 and 6 show respectively P value and Fold Change for (A) Mild vs (C) Severe; Columns 7 and 8 show respectively P value and Fold Change for (B) Moderate vs (C) Severe.

3.3.3 Proteins involved in the immune interplay between host and parasite

In order to understand the mechanisms acted by *E. granulosus* to ensure its survival and by the host to defend itself from the parasite, we identified a total of 12 interesting proteins based on their abundance in hydatid fluid (Fig. 21). Of the 12 proteins, only Cathepsin B was assigned to *Echinococcus granulosus* while the remaining to *Bos taurus*. Their role in parasite killing or survival will be described later.

Moreover, the comparative analysis of lung tissues and hydatid fluids showed a total of 19 statistically significant proteins shared between the groups (Fig. 22 A). The functional association at a systemic level of proteins, performed through the web resource STRINGdb v11.0, showed the differential proteins map in Biological Process, Reactome Pathways and UniProt Keywords. The STRING analysis showed the association of 6 proteins to immune processes (Fig. 22 B).

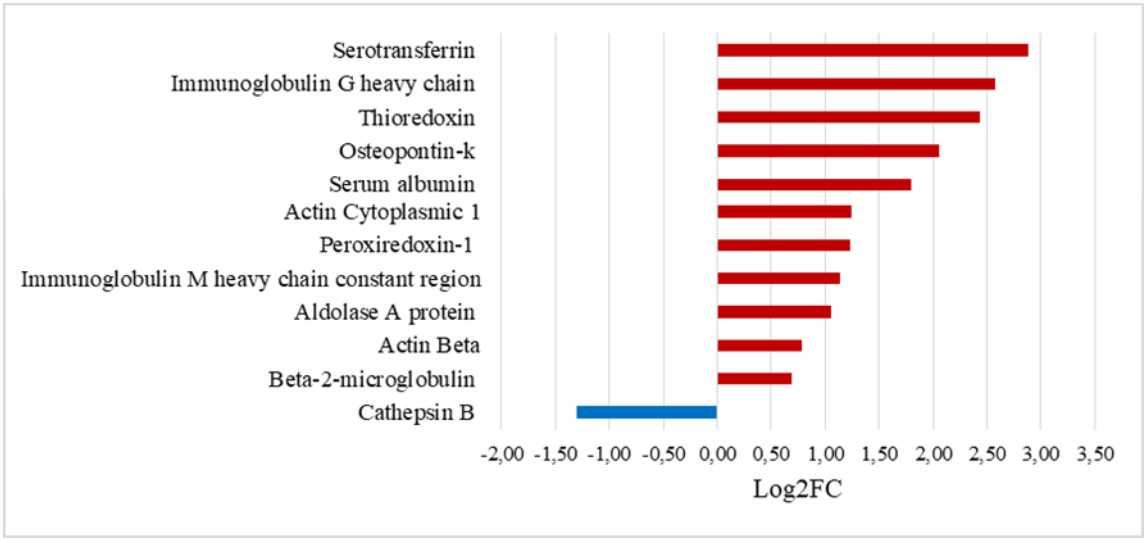

Figure 21 Proteins involved in immune interplay significantly different in fertile vs infertile cyst

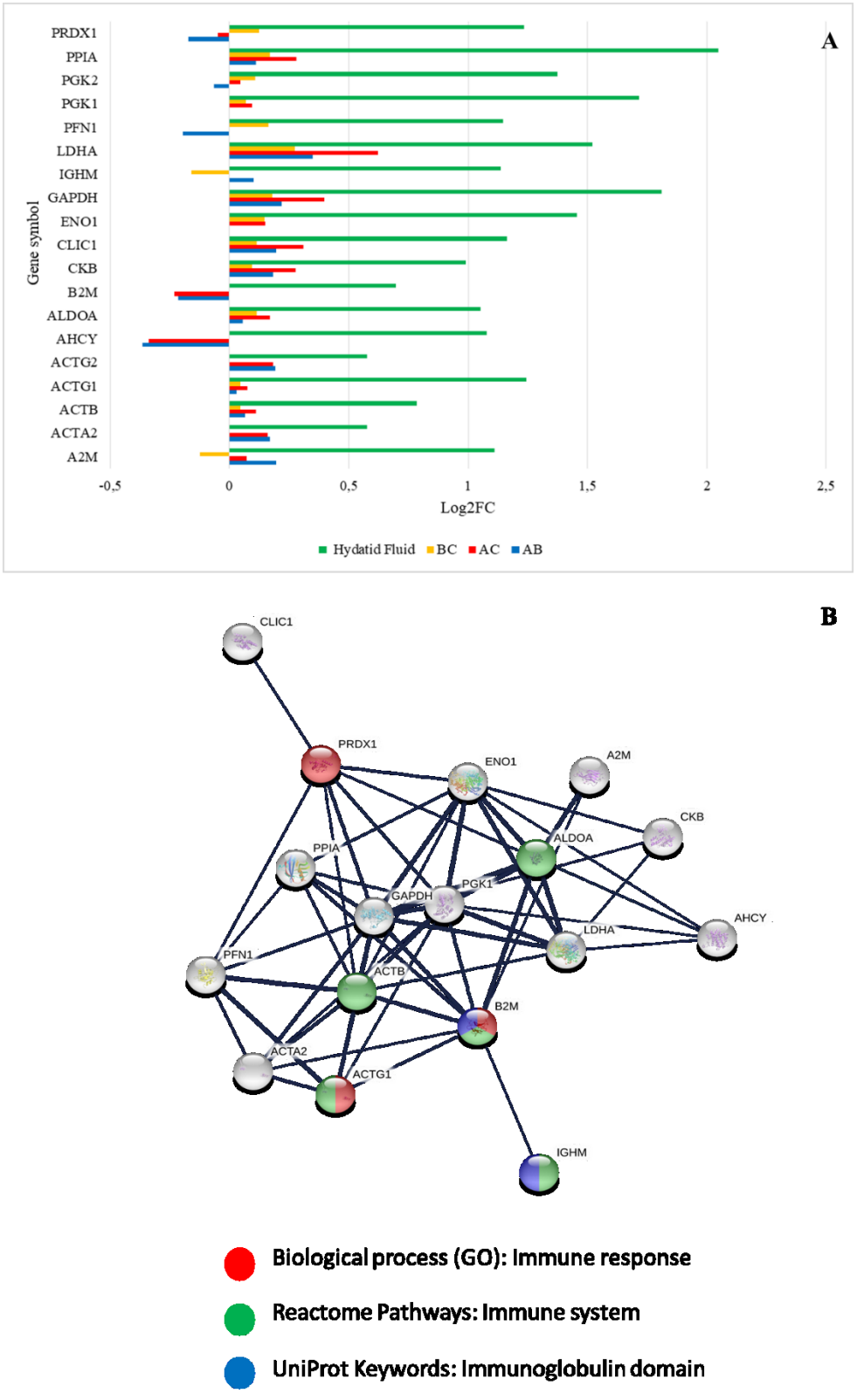

Figure 22 A: *Bos Taurus* proteins overlapping in lung tissues (different inflammation degrees) and in hydatid fluid. B: Protein network according to STRING. Proteins associated to Biological Process (Immune response), Reactome Pathways (Immune system), Uniprot Keywords (Immunoglobulin domains). Grey lines linking nodes represent the types of evidence used in predicting association. Meaning of network edges: confidence, line thickness indicates the strength of data support.

3.3.4 Gene Ontology (GO)

In order to obtain a protein-by-protein characterization of lung tissue and hydatid fluid proteomes, an extensive Gene Ontology analysis was carried out. In lung tissue, 100% of decreased proteins were associated to Biological process while among increased proteins 46% were associated to Biological process, 25% to Cellular component, 19% to Molecular function, 7% to Reactome and finally 3% to KEGG. In hydatid fluid, 44% of proteins were associated to Biological process, 19% to Cellular component, 18% to Molecular function, 10% to Reactome and finally 9% to KEGG.

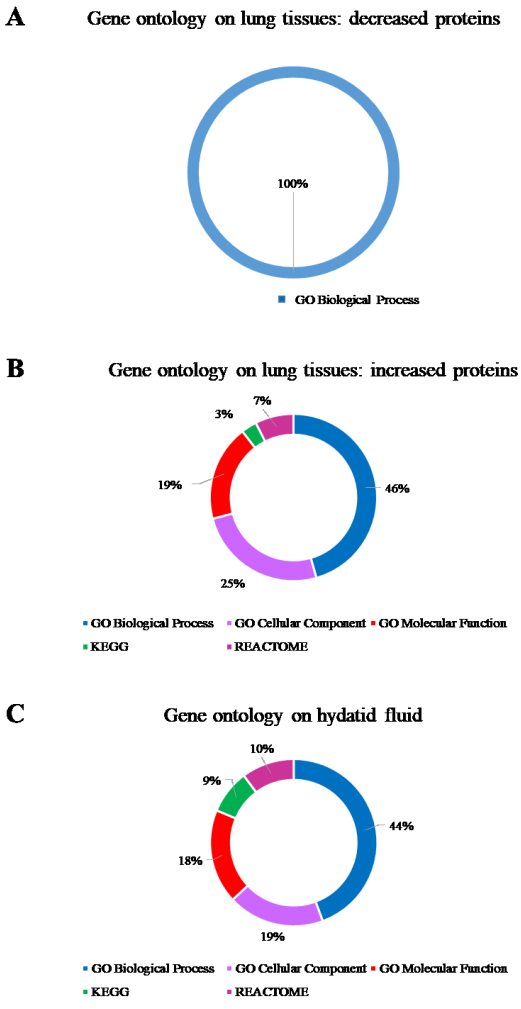

Figure 23 Gene ontology study of lung tissue and hydatid fluid proteome. GO analysis is shown for Biological Process, Cellular Function, Molecular Function, KEGG, Reactome.

3.4 Discussion

The search for biomarkers useful in the early diagnosis of cystic echinococcosis as well as the search for markers of cyst viability (Pagnozzi D. T. F., 2018) and the need of new effective vaccines (Miles S., 2019) are constantly growing. Several studies have focused on *E. granulosus* biomarkers in hydatid fluid (Zeghir-Bouteldja R., 2017) or serum (Pagnozzi D., 2016). However, information regarding reactions in the tissues surrounding cysts is still scarce (Longuespée R. C. R., 2017). Moreover, the analysis of HF proteome is considered a big challenge due to its content that is a mixture of E / S products from PSCs, from GL and host serum proteins (Chemale G., 2003). In the present study we performed a differential shotgun proteomic analysis in order to identify the differences in pathological and physiological conditions by analysing both lung tissues surrounding the cysts and fluid from fertile and infertile hydatids.

Our study on lung tissue surrounding hydatid cysts provided numerous indications. The Venn diagram resulting from the comparison of the different inflammation groups showed the absence of unique proteins attributable to one combination. This may be due to the tissue of origin (lung), the same for all analysed groups. Furthermore, the volcano plot showed that the highest number of differential proteins was related to the Moderate + Severe *vs* Negative Controls comparison. The second group with the greatest differences in terms of number of differential proteins was Absent + Mild *vs* Negative Controls. Finally, the lower number of differential proteins was related to the Moderate + Severe *vs* Absent + Mild comparison. This data is coherent with the progression of inflammation.

According to Principal Component Analysis, tissue samples clustered into three groups: Negative controls, Absent + Mild and Moderate + Severe. This confirmed what was described in the histological analysis (Chapter II).

Obviously, the differences between healthy tissue and affected tissues were evident. In contrast, the histological appearance of cysts with absent and mild inflammation was very similar. Similarly, the differences between cysts with moderate and severe inflammation were very slight. This explains why cysts were grouped in this way even after proteomic analysis.

Likewise, the heat map analysis showed a very similar trend in terms of increased and decreased proteins in the comparisons between combinations of inflammation and healthy tissue. In contrast, minor differences were identified in the comparison between cysts with Moderate + Severe inflammation and Absent + Mild. Also in this case, the difficulty in identifying substantial differences between the different degrees of inflammation is evident. In order to identify proteins involved in immune interplay between host and parasite, some proteins were taken into consideration. Particularly, Immunoglobulins, Cathepsin B, Thioredoxin, Osteopontin, beta-2-microglobulin and peroxiredoxin.

Longuespée R. et al. (2017) performed a proteomic study of human cystic echinococcosis in liver using FFPE (Formalin Fixed and Paraffin embedded) tissues in order to identify proteins involved in the immune response against the parasite. They reported a strong immunoglobulin response within the cysts and the presence of proteins involved in antigen presentation in the cyst wall where only one type of immunoglobulin (Ig kappa light chain V-III) was found (Longuespée R. C. R., 2017). In our study on tissue lung surrounding the cysts, IgG and IgK showed higher abundances in Negative controls *vs* Absent + Mild (AB) and Negative controls *vs* Moderate + Severe (AC) combinations, suggesting an increase of Ig levels in the comparison between healthy and disease. Interestingly, their abundance decreased in the correlation of Absent + Mild *vs* Moderate + Severe (BC).

In the survey mentioned above, some proteins assigned to *Echinococcus granulosus* were found in the liver and neither within the cyst or in the cyst wall; moreover, proteins assigned to human species were specifically present in the cyst and/or the cyst wall but not in the

liver. These data are consistent with our survey, in which of 5 proteins assigned to *E. granulosus* found in lung tissue, only one (Phosphoglycerate kinase) was found both in tissues and hydatid fluids. It is also interesting that the remaining 4 (Elongation factor 1 alpha, Eukaryotic translation initiation factor 3 subunit C, Ras protein Rap 1b, Transitional endoplasmic reticulum ATPase) were differential only in tissue and not in fluid.

In our study, the comparison of the hydatid fluid of fertile and sterile cysts led to the identification of 97 proteins of which 61 were assigned to *Bos taurus* and 36 to *Echinococcus granulosus*. According to PCA, the samples formed two main clusters. Fertile cysts appeared as a well-defined group while sterile ones were divided into two subgroups. In particular, the samples belonging to moderate and severe degrees of inflammation tended to overlap. This data continues to confirm the profound similarities between the two degrees of inflammation both from the histological and proteomic point of view. The number of increased and decreased proteins shown by the volcano plot were 36 and 35 respectively. In terms of abundance of statistically significant proteins, increased or decreased, in the comparison between only sterile cysts, the number is smaller compared to the fertile *vs* sterile comparison. This is due, once again, to the very blurred boundaries between different degrees of inflammation. Curiously, however, the PCA showed clustering in three different groups, leaving only two cysts outside the groupings.

According to Ammar Aziz (2011), our study confirms that the presence of serum proteins, such as serotransferrin and immunoglobulin, is very abundant in hydatid fluid as well as the immunogenic proteins produced by the parasite as antigen 5 and antigen B. The similarity between the host proteins of both plasma and hydatid fluid has been demonstrated by several studies (Monteiro K.M., 2010) (Chemale G., 2003) (Aziz A., 2011), suggesting that *E. granulosus* might be capable to adsorb host proteins through the germinal layer. The absorption mechanism could be due to an endocytic process stimulated by proteins such as albumin and IgG, as reported for other parasites (Ambrosio J., 1994) (Dunn J. and

Threadgold L.T., 1984). This action could have a dual purpose, *i.e.* use the proteins as nutrients or as inhibiting factors for the host's immune response (Schroeder H., 2009) (Gobert G.N. and McManus D.P., 2005).

The proteomic analysis of HF reveals the presence of the most important immunological antigens produced by the parasite: Ag5 (22 and 38 kDa) and AgB (isoforms 1, 4, 8), several glycolytic and pentose phosphate pathways components involved in the glucose metabolism, host serum proteins (albumin, immunoglobulin). According to the same study, a high amount of plasma proteins is present in the hydatid fluid, including immunoglobulins. Our findings show an increase in gamma immunoglobulins in infertile cysts fluid if compared to fertile ones. However, their presence in the fluid, would be due to an endocytic absorption and not to a specific response towards the parasite.

Furthermore, a study comparing HF from cattle, sheep and humans, suggests that the oxidative damage to DNA at GL level can induce infertility through apoptosis, suggesting the role of Cathepsin B in caspase activation (Vancompernolle K., 1998). Cathepsin B seems to play a role in activation (Mihalik R., 2004) (Bien S., 2010) or inhibition of apoptotic processes (Malla R., 2010). In our study, Cathepsin B showed an increase in HF from infertile if compared to fertile cysts, in line with its role in the oxidative damage in the germinal layer causing apoptosis and infertility.

Monteiro (2010) reported another protein that seems to assume a key role in the immune response*:* thioredoxin. The latter is able to engage and modulate several immune cell types and induce the proinflammatory cytokine production (Bertini R., 1999) pivotal for granuloma formation (Co D.O., 2004) In our study, thioredoxin produced by *Bos taurus* was increased in the infertile cysts if compared to fertile ones, suggesting its probable involvement in the host response against the parasite.

Bearing in mind the search for proteins involved in the immune response, osteopontin (OPN) seems to play an important role. The latter is synthetized by immune cells as T cells,

macrophages (including macrophage-derived cells), and NK cells (O'Regan A. and Berman J.S., 2000) and it is up-regulated when monocytes are stimulated to differentiate in macrophages (Atkins K., 1998). Osteopontin is a candidate in the granulomatous response against *E. granulosus* infection acting as proinflammatory molecule, supporting adhesion, inducing migration and modulating the function of a large number of immune cells (Monteiro K.M., 2010). Osteopontin exists in two distinct form, as cytokine in body fluids and as immobilized molecule in mineralized tissues. When present in tissues surrounding cysts, it may act in the inhibition of tissue calcification (Peng X., 2006). In our study osteopontin was increased in infertile cysts, suggesting its role as proinflammatory molecule, acting in the parasite killing.

Finally, in our study some differential proteins were overlapped in tissues and hydatid fluids. Of these 19 proteins, 6 were involved in immune response. Two proteins in particular deserve attention: beta-2-microglobulin and peroxiredoxin. The involvement of beta-2-microglobulin (B2M) in chronic inflammation is known (Topçiu-Shufta V., 2016). B2M is synthesized by lymphocytes, whose surface together with that of monocytes is rich in this protein. Furthermore, B2M is regulated by interferons and proinflammatory cytokines (Wilson A.M., 2007). Peroxiredoxins are involved in protecting cells from oxidative stress. PRDX possess thioredoxin or glutathione peroxidase and chaperone-like activities. PRDX1 is a proinflammatory factor present in plasma and body fluids, able to induce the activation of NFkB to foster inflammatory responses (Ishii T., 2012).

To the best of our knowledge, this is the first proteomic study of both lung tissues and HF from cattle affected by CE. Our approach shows for the first time that specific differential proteins are simultaneously present both in the tissue and in the fluid. Moreover, proteomic patterns were associated to different inflammation degrees as confirmed by the histopathological analysis. In line with Longuespée (2017), our study demonstrated the presence of proteins involved in larval development of *E. granulosus* in the tissue

surrounding the cysts. The parasite evidence in lung or liver tissue may be a turning point in molecular diagnosis, which could be obtained throughout a single biopsy.

In conclusion, the multidisciplinary approach combining the study of the tissue surrounding cysts and hydatid fluid proteome provided indications that can be exploited to provide a valid and reliable support to molecular pathology (Longuespée R., 2016).

3.5 Conclusion

To the best of our knowledge, this is the first work combining the histological and proteomic investigation of cystic echinococcosis (CE) in cattle aimed at evaluating the immune modulation during infection by *Echinococcus granulosus*.

It is common knowledge that the ability of the parasite to modulate the host immune response makes its survival possible. As a result, the study of mediators released by alive parasites and the understanding of the mechanisms involved in the helminth's ability to escape and redirect the host's immune system is a matter of concern (Hewitson J.P., 2009). Moreover, cattle ability to counteract infection by the sheep genotype of *Echinococcus granulosus* is a trending topic.

The results of this thesis, identifying some differential parasitic proteins in host tissues as well as differential hosts proteins increased in hydatid fluid of infertile cysts, highlight the complex interaction between the bovine host and *Echinococcus granulosus*. Particularly, a key role in immune response is suggested for Elongation factor 1 alpha, Eukaryotic translation initiation factor 3 subunit C, Ras protein Rap 1b, Transitional endoplasmic reticulum ATPase in lung tissues, and immunoglobulins, thioredoxin, peroxiredoxin, osteopontin and beta-2-microglobulin in hydatid fluid. In addition, only one protein produced by *Echinococcus granulosus*, cathepsin B, was found to be differential when comparing fertile and infertile cysts, suggesting its role in cyst infertility through the disruption of the germinal layer (GL). On this matter, GL would take a leading role as interface between host and parasite due to the high permeability of laminated layer (LL) to macromolecules (*e.g* thioredoxin and osteopontin). These data combined with the histologic features of GL and LL suggest their key role in the fertility or infertility of hydatid cysts. Indeed, if on one side *Echinococcus granulosus* is able to adsorb host proteins through the GL in order to use them as nutrients or to modulate the immune response by the host, on the

other hand bovines could use this mechanism to induce proinflammatory cytokines and consolidate the granulomatous reactions.

Moreover, the presence of parasite proteins in the host tissue may be exploited to facilitate diagnosis, for example through single biopsy as suggested by Longuespée (2017).

Although these indications will need to be confirmed by further studies, the combination of histological and proteomic approaches could represent a turning point in the understanding of pathological mechanisms and of the immune interplay between host and parasite.

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Appendix 1

List of publications

Addis M.F., Cubeddu T., Pilicchi Y., Rocca S., Piccinini R., 2019. Chronic intramammary infection by *Listeria monocytogenes* in a clinically healthy goat - A case report. BMC Veterinary Research;15:229.

Pisanu S., Cubeddu T., Cacciotto C., Pilicchi Y., Pagnozzi D., Uzzau S., Rocca S., Addis M.F., 2018. Characterization of paucibacillary ileal lesions in sheep with subclinical active infection by *Mycobacterium avium subsp. paratuberculosis*.Vet Res;49:117.

Poster "1st International Conference on xenobiotics and endogens in biological matrices":
Sanna M.A., Burrai G.P., Rocca S., Antuofermo E., Pirino S., Pilicchi Y., Alberti A., Addis M.F., Cubeddu T., 2019. Immunohistochemistry and nucleic acid hybridization in Veterinary Pathology.

Abstract 72° SISVET, 2018:
Pilicchi Y., Cubeddu T., Scala A., Varcasia A., Antuofermo E., Pirino S., Sanna M.A., Burrai G.P., Addis M.F., Rocca S., 2018. Preliminary histopathological and immunophenotipic characterization of tissues from sardinian cattle infected by *Echinococcus granulosus s.s*.

Abstract and Poster 72° SISVET, 2018:
Cubeddu T., Addis M.F., Pilicchi Y., Rocca S., Villa M., Piccinini R., 2018. Chronic mastitis by *Listeria monocytogenes* in a clinically healthy goat.

Abstract 72° SISVET, 2018:
Cubeddu T., Pisanu S., Cacciotto C., Pagnozzi D., Pilicchi Y., Vitiello V., Rocca S., Uzzau S., Addis M. F., 2018. Histopathological and molecular characterization of sheep ileal tissues with paucibacillary infection by *Mycobacterium avium subsp. paratuberculosis.*

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"If you find a path with no obstacles,
it probably doesn't lead anywhere"
Frank A. Clark

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