HERACLES

(GA 602051)

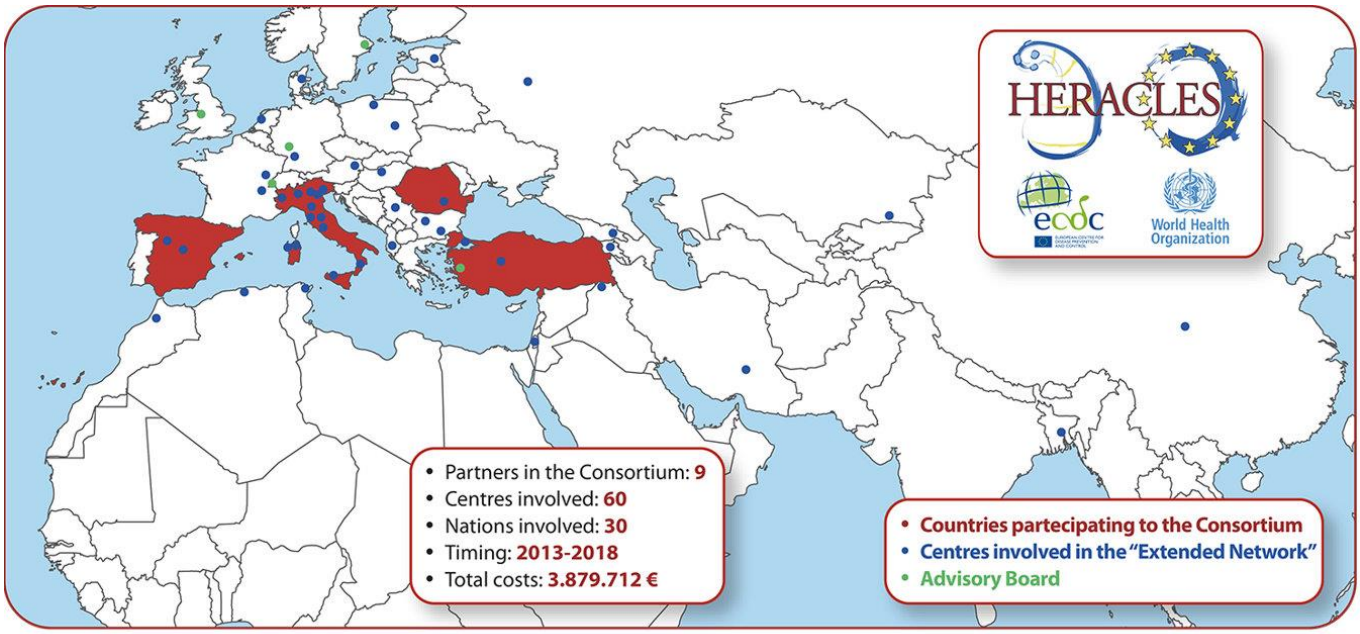
FINAL REPORT

PROJECT OBJECTIVES WITH MAIN ACHIEVEMENTS

The main objectives of **HERACLES** project:

- The assessment of CE prevalence in endemic rural areas of Romania, Bulgaria and Turkey (WP1);
- The enrolment of new patients in the European Register od CE (ERCE) (WP1);
- The Echino-Biobank and the validation of recombinant antigens (WP2);
- The host-parasite interplay studies on genotyping of human parasitic samples (WP3);
- The proteomic studies on biomarker discovery in exosomes from human plasma (WP3);
- The synthesis of new formulations of BMZ drugs and their efficacy in animal model (WP4);
- The dissemination of HERACLES achievements, raising awareness in the scientific community and the policy makers and improve the knowledge of the disease in the rural areas (WP5).

Figure 1.1. Synthesis of the core achievement of HERACLES project. http://www.heracles-fp7.eu/



CORE ACHIEVEMENTS

Funded by:

- Cross-sectional ultrasound study: **First "*original research*" ever published by *The Lancet Infectious Diseases* on CE**
- Biggest cross-sectional ultrasound-based population study: **conducted on 24,693 volunteers in Bulgaria, Romania, and Turkey**
- Estimated number of individuals who may be infected: **≈ 7,872 in Bulgaria, 37,229 in Romania, 106,237 in Turkey**
- Patients enrolled in the European Register (ERCE): **≈ 2,000 from 30 centres**
- International patent on anti-parasitic soluble drugs: **"Salts of benzimidazole compounds"**
- First proteomic description of **parasite exosomes in fertile hydatid cyst fluid**
- Identification of **potential biomarker candidates in human plasma samples by quantitative proteomic analysis**
- Samples in the Echino-BioBank: **≈4,500**
- Biggest study on genotyping of *E. granulosus* strains in humans: **742 samples from 26 countries**
- Scientific papers currently accepted by peer reviewed journals: **56**

Figure 1.2. HERACLES extended network generated from the inception of the project counting on 60 centres from Europe and Asia. http://www.heracles-fp7.eu/interactive_map.html

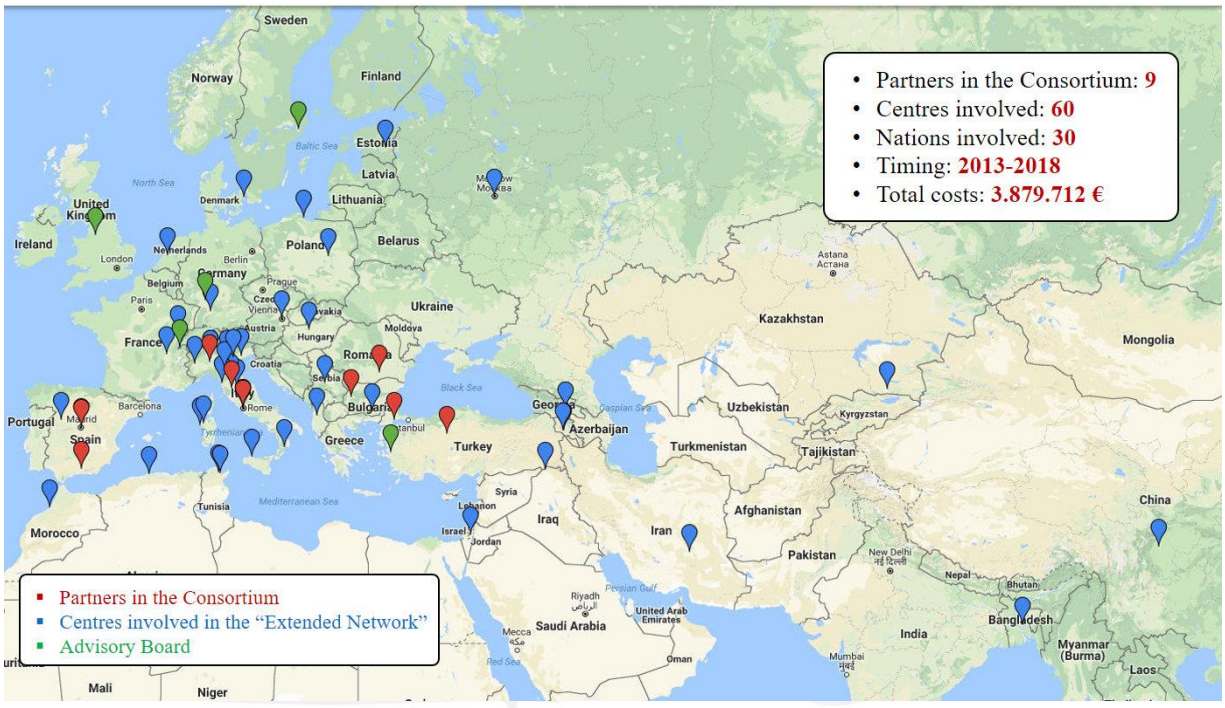
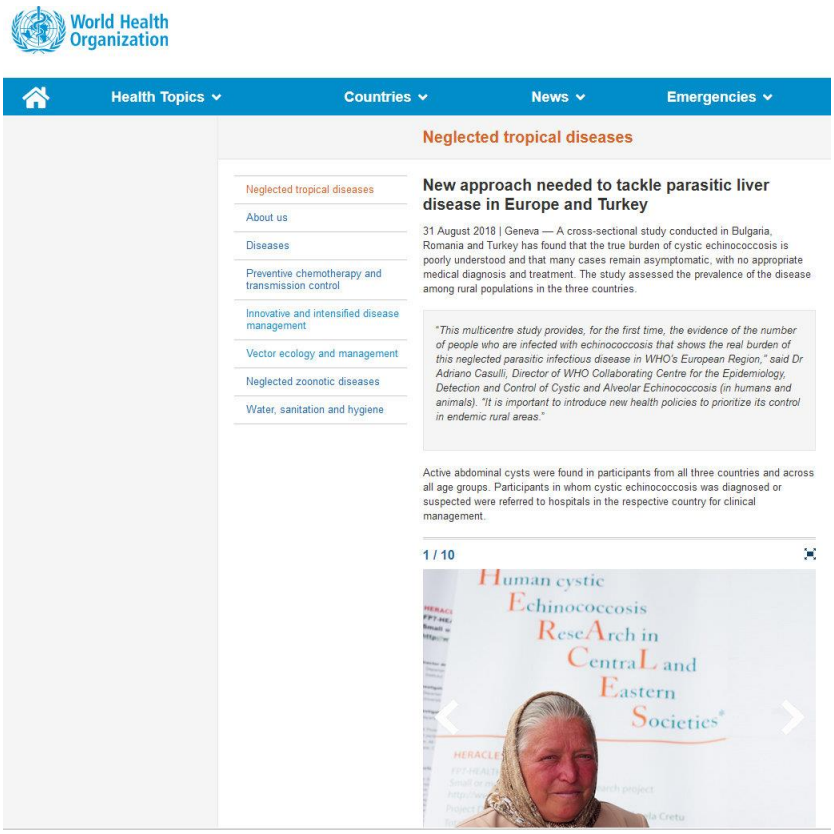


Figure 1.3. HERACLES project results promoted by the World Health Organization. http://www.who.int/neglected_diseases/news/new-approach-needeed-to-tackle-echinococcosis-europe/en/



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WP1: Ultrasound screening of Eastern European population and ERCE register

Objectives

The general objectives of WP1 were to:

1. Assess the prevalence and the burden of CE in rural underserved areas where sheep raising is practiced, by means of ultrasound screenings in Bulgaria, Romania and Turkey.
2. Build CE European Register including Bulgaria, Romania and Turkey based on the previous point.

Task 1.2: Ultrasound surveys (the burden of disease)

Background. Cystic echinococcosis is a neglected zoonotic infection that is distributed worldwide and prioritised by WHO for control efforts. The burden of human cystic echinococcosis is poorly understood in most endemic regions, including Eastern Europe. We aimed to estimate the prevalence of abdominal cystic echinococcosis in rural areas of Bulgaria, Romania, and Turkey.

Methods. We did a cross-sectional ultrasound-based survey that recruited volunteers from **50 villages in rural areas of Bulgaria, Romania, and Turkey**. These villages were in provinces with annual hospital incidence of cystic echinococcosis within the mid-range for the respective countries. All people who attended a session were allowed to participate if they agreed to be screened. Abdominal ultrasound screening sessions were hosted in public community structures such as community halls, primary health-care centres, schools, and mosques. Lesions were classified using an adapted WHO classification. We reported the prevalence of abdominal cystic echinococcosis adjusted by sex and age through direct standardisation, using the country's rural population as a reference.

Findings. From July 1, 2014, to Aug 3, 2015, **24,693 individuals** presented to screening sessions and 24,687 underwent ultrasound screening. We excluded a further six individuals due to missing data, leaving 24 681 people in our analysis.

Abdominal cystic echinococcosis was detected in 31 of 8,602 people screened in Bulgaria, 35 of 7,461 screened in Romania, and 53 of 8,618 screened in Turkey. The age and sex **adjusted prevalence of abdominal cystic echinococcosis was 0·41% (95% CI 0·29–0·58) in Bulgaria, 0·41% (0·26–0·65) in Romania, and 0·59% (0·19–1·85) in Turkey**. Active cysts were found in people of all ages, including children, and in all investigated provinces.

The estimated number of individuals who may be presently infected with CE in rural areas is around 151,000 (7,872 in Bulgaria, 37,229 in Romania, 106,237 in Turkey).

Interpretation. Our results provide population-based estimates of the prevalence of abdominal cystic echinococcosis. These findings should be useful to support the planning of cost-effective interventions, supporting the WHO roadmap for cystic echinococcosis control.

Figure 1.2.1. Study are of the abdominal US screenings on 24,693 people in a total of 50 villages and 15 districts/provinces of Bulgaria, Romania, and Turkey.

The absence of CE from top-level peer review journals contributes to its neglect status. According to its Editorial Board, this is the **first** "**original research**" (no review, no case report) **ever published on echinococcosis by The Lancet Infectious Diseases**:

Figure 1.2.2. HERACLES manuscript selected as cover for *The Lancet Infectious diseases.*

Task 1.5: CEE Registries

The European Register of Cystic Echinococcosis, ERCE (http://www.heracles-fp7.eu/erce.html) is an online prospective, observational, multicentre register of patients with probable or confirmed CE. ERCE was originally created as a tool for the surveillance of human CE, allowing national and international authorities to acknowledge the magnitude of the problem by reporting cases otherwise not captured by official systems, built to take into account the peculiar features of the infection, and to establish a prospective case retrieval to evaluate the evolution of individual cysts. ERCE has been individuated by the WHO-Informal Working Group on Echinoccocosis (WHO-IWGE) as the starting platform for the further development of an online prospective case retrieval tool to overcome the lack of prospective randomized trials on the comparative outcome of clinical management options (http://www.who.int/echinococcosis/resources/WHO_HTM_NTD_NZD_2017.01/en/). To this,

ERCE should be amended in order to fulfil requirements to drive such valid estimates from observational data according to available methodological literature. In November 2017, two preliminary meetings were held, one with professor Marcel Zwahlen (University of Bern, member of the IWGE steering group) expert in such methodologies, and one with delegates of the ERCE network most active centre, in order to set the ground for this future development.
As of July 31st 2018, **36 centres in 14 countries** (Albania, Austria, Bangladesh, Bulgaria, France, Georgia, Hungary, Iran, Italy, Kazakhstan, Poland, Romania, Spain, Turkey) **were adhering to ERCE** (Figure 1.5.1). Of these, 31 (86%) registered patients and, of these in turn, 18 (58%) recorded at least one visit occurring within the past 18 months. **A total of 1,967 patients were enrolled in ERCE** (Figure 1.5.2). Females accounted for 53% of registered patients; the age and sex distribution of patients registered in ERCE is shown in Figure 1.5.3. Most registered patients (62.3%) have 1 cyst; 25.5% have more than one cyst.

Figure 1.5.1. Map of the 14 countries with 36 ERCE affiliated centres, which have enrolled around 2,000 patients till now.

Data completeness and accuracy, in particular data on follow-up and cyst characteristics, are pivotal to the scope of the register and should be the aim of future ERCE development. As of July 31st 2018, 15 centres (48% of centres having recorded patients in ERCE) recorded also follow-up visits after those corresponding to the first patient's recording in ERCE. In these centres, a median of 21% patients also had follow-up visits recorded in ERCE. The cysts characteristics (location and stage) were recorded at least for some patients by 23 (74%) centres.

In December 2016, the **World Health Organization - Informal Working Group on Echinococcosis** individuated **ERCE as a starting platform to collect clinical data to derive evidence on treatment recommendations from case series, in the absence of randomized clinical trials**.

Figure 1.5.2. Number of patients recorded in ERCE by affiliated country.

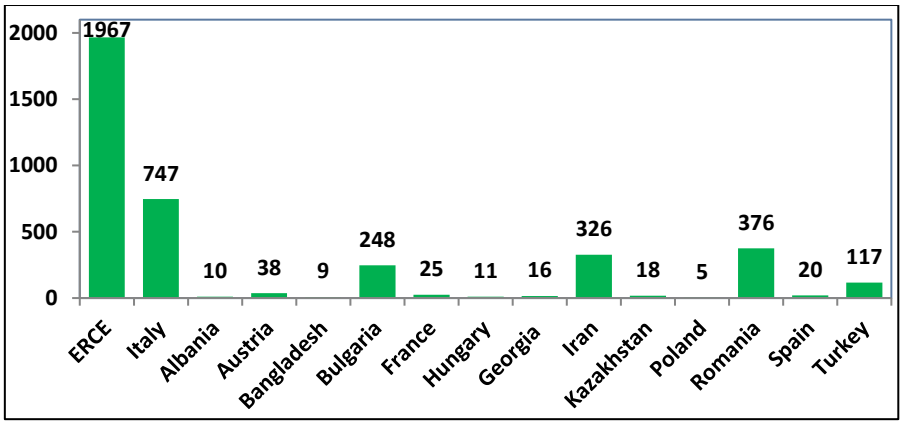

Figure 1.5.3. Age and sex of patients registered in ERCE.

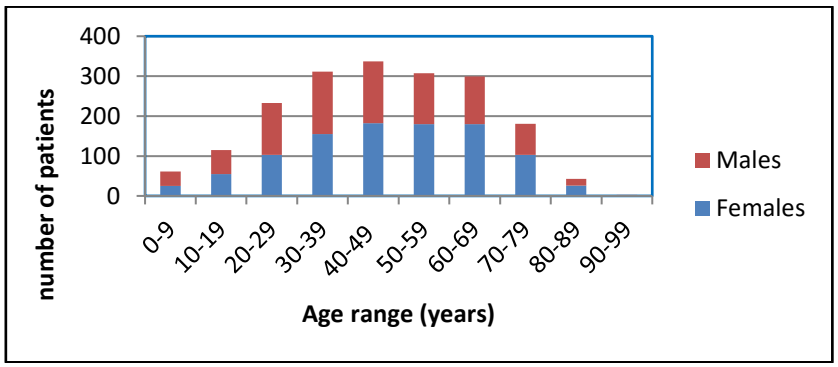

Figure 1.5.4. Commitment of the WHO-IWGE on ERCE as platform to collect clinical data to derive evidence on CE treatment recommendations.

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WP2: New molecular-based tools for detection, diagnosis and follow-up of CE

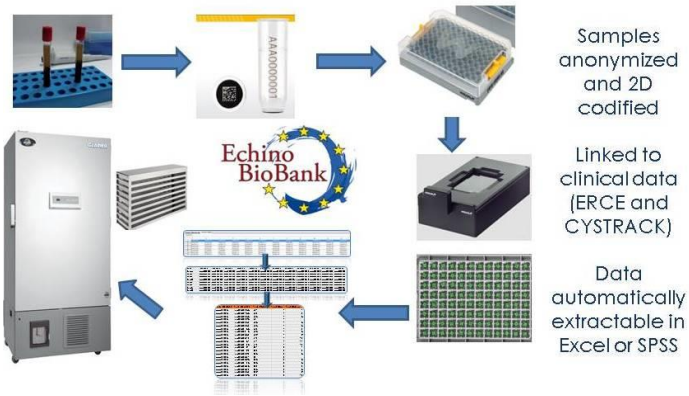
Objectives

The overall objective of the WP2 was to improve and standardize the serological detection of CE by diagnostic/follow-up tests in both humans and animals. To this end, serodiagnosis and serological follow-up of CE were addressed in a systematic manner, building up a sample repository linked with databases that include every clinical and epidemiological data relevant for proper validation of recently described recombinant antigens and respective antibodies. WP2 used these molecules in commercial kits based on inexpensive chromatographic and microfluidic devices that were developed and manufactured by the biotech company VIRCELL (Partner 9).

Task 2.1/2.2: Sample repository/Database development

Protocols for the collection, storage and treatment of samples have been described in an internal document and delivered to the partners. Clinical protocols for the definition of cohorts and samples have been elaborated. Additionally, a bio-bank facility has been built up and hosted by Partner 3. This **bio-bank collection has been registered and follows the legal rules stated by the EU**.
https://biobancos.isciii.es/ListadoColecciones.aspx? id=C.0003432

Figure 2.1.1. HERACLES Echino-Biobank organization.



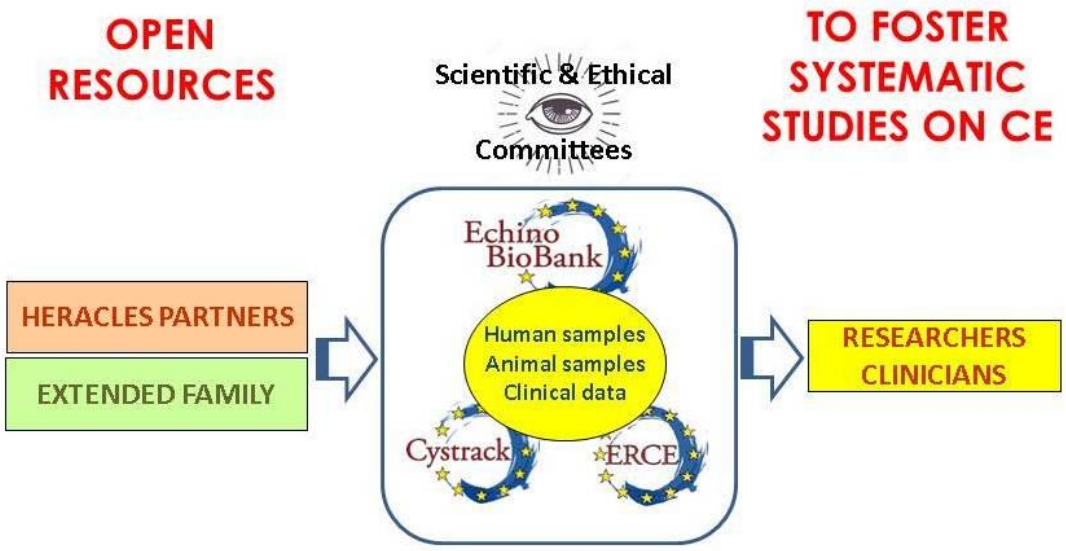
The Echino-Biobank has received samples from infected animals (cows, sheep and pigs) from Turkey and Spain, human samples including serum, plasma buffy coat and parasite material, generated during the screening performed in HERACLES, and also provided by partners and by other collaborators from patients visited in their hospitals and clinics, including prospective and retrospective patients. The Echino-Biobank also hosts a unique collection of native and recombinant antigens, as follows.

ANTIGEN	TYPE	QUANTITY
HYDATID FLUID	NATIVE	500 mg
Protoscolex	NATIVE	VARIABLE
Ag5 (semipurified)	NATIVE	50 µg
AgB1t	RECOMBINANT (GST)	10 mg plus clone
Ag2B2t	RECOMBINANT (GST)	10 mg plus clone
	RECOMBINANT	10 mg plus clone
Ag5t	RECOMBINANT (GST)	5 mg plus clone
AgMDH	RECOMBINANT (GST)	2 mg plus clone

AgAFFP	RECOMBINANT (GST)	2 mg plus clone
AgCaBP	RECOMBINANT (GST)	2 mg plus clone
AgDiPol (AgB1+AgB2+Ag5)	RECOMBINANT (GST)	10 mg plus clone
AgECP (exosomes)	RECOMBINANT (GST)	10 mg plus clone
AgADP (exosomes)	RECOMBINANT (GST)	10 mg plus clone

In summary, the **Echino-Biobank** is currently hosting the largest collection of CE samples organized in a biobank (**over 5,000 samples received**). The Echino-Biobank has also provided samples to HERACLES partners for their activities and to additional researchers working in the field of CE (**over 1,500 samples provided**).

Figure 2.1.2. HERACLES Echino-Biobank characteristics.



Task 2.3: Production and testing of recombinant antigens

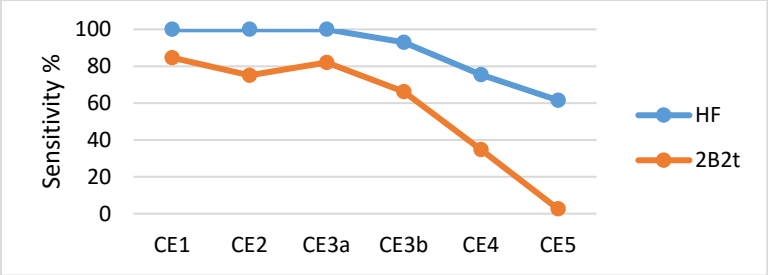
The recombinant **B1t, 2B2t, Ag5.2t, CaBP, AFFP and MDH** have been tested in ELISA by partner 3 and in VirClia (fluorescent ELISA) by partner 9. Tested serum samples represented retrospective CE patients with different clinical conditions (cyst classification, cyst number, etc.), healthy donors and patients with alveolar echinococcosis (AE). Their performance has been compared with hydatid fluid (HF) both for the diagnosis and follow-up of CE patients. In ELISA, none of the recombinant antigens showed higher sensitivity than the hydatid fluid, although they showed low cross-reactivity with AE patients and better specificity with healthy donors compared with HF (see table below). All antigens, including HF, showed a percentage of false negative patients. Results were similar in VirClia, a chemiluminiscent ELISA technique performed with the same antigens at Vircell SL (Partner 9).

ANTIGEN	SENSITIVITY	SPECIFICITY (HEALTHY DONORS)	SPECIFICITY (Alveolar Echinococcosis)
Hydatid fluid	97.8%	77.8%	47.6%
RecB1t	86.3%	90.1%	96.1%
Rec2B2t	80.3%	92.6%	97.6%
RecAg5t	78.6%	82.7%	78.6%
RecCaBP	33.9%	95.1%	90.5%
RecAFFP	28.6%	82.5%	59.5%
RecMDH	55.1%	80.1%	92.8%

The best recombinant candidates (B1, B2 and Ag5) were used to produce a multiclone containing specific immunogenic regions of the three antigens, called DiPOl. This chimera antigen did not result in better performance in ELISA, compared with the parental recombinant antigens or with the HF (see table below).

ANTIGEN	SENSITIVITY
Hydatid fluid	97.8%
RecB1t+Ag5t+2B2t (SUMMATORY)	96.1%
DiPOL (B1+B2+Ag5) (CHIMERA)	87.2%

The influence of the clinical variables (cyst number, stage, size and location, and the administration of albendazole to the patient before serum collection) was evaluated, in order to define those that were responsible of false negative results for each antigen. This evaluation shows that patients with inactive (CE4 and CE5) cysts are much less frequently positive than patients with active and transitional cysts, especially against the recombinant antigens, showing the potential of these antigens for the differentiation of inactive from active/transitional cysts. An example of this phenomenon is shown for the Rec2B2t compared with HF below.



In an attempt to find better recombinant candidates (enhancing sensitivity), Partners 3 and 9 decided to seek for alternatives based in the use of eukaryotic systems to produce the recombinant proteins. This should improve the similarities of the recombinant protein with the native antigen, compared with the recombinant protein produced in the procariote *Echerichia coli*. Specifically, codon usage and post-translational modifications (presence of carbohydrate moieties) were facilitated by cloning and producing the recombinant Ag5t in *Pichia pastoris* by Partner 9. Preliminary results of this new antigen in VirClia showed that the Ag5t obtained in *P. pastoris* had better performance than the Ag5t expressed in *E. coli* (see task 2.4). For the follow-up of patients (after surgery, aspiration, albendazole or in watch and wait), none of the tested antigens (including HF) showed promising results, due to persistence of antibodies in cured patients and to the presence of a high percentage of negative patients at the beginning of the follow-up period (specially for patients treated by surgery or aspiration) against the recombinant antigens. As an example, the results obtained with the RecB2t compared with HF are shown below.

We conclude that the detection of antibodies in serum is not a good tool for the follow-up of treated CE patients, and that alternative tools (e.g., antigen detection, definition of new circulating markers) should be investigated. In this sense, the study of new markers present in exosomes and the obtainment of monoclonal antibodies against these markers and against defined recombinant proteins, for the detection of the corresponding recombinant antigens, has been initiated.

Additionally, the usefulness of the detection of total IgG against the recombinant antigens and the HF have been preliminary tested in ELISA in screened patients, showing some potential for the recombinant antigens, specifically the 2B2t-GST, in the detection of screened people with a positive CE ultrasound image. In this group of patients, further testing is needed to get conclusions.

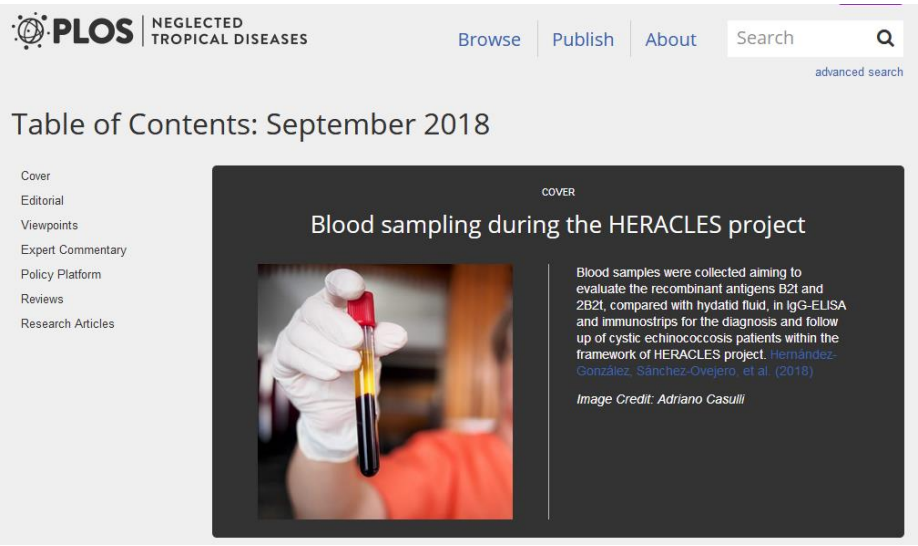
Progression to negativity by treatment outcome (cured *vs.* non-cured patients) among patients who underwent surgical intervention or percutaneous treatment

TEST	no. negative	Person-months follow-up (PM)	Neg. rate *100 PM (95% CI)	P-value[1]
Hydatid fluid (ELISA)				0.302
Cured (N=13)	3	296	1.01 (0.33-3.14)	
Not cured (N=3)	0	138	0.00	
B2t (ELISA)				**0.042**
Cured (N=4)	3	23	13.04 (4.21-40.44)	
Not cured (N=3)	0	138	0.00	

Progression to negativity by treatment response (good *vs.* poor) among patients who underwent drug treatment

TEST	no. negative	Person-months follow-up (PM)	Neg. rate *100 PM (95% CI)	P-value[1]
Hydatid fluid (ELISA)				NC
Good (N=18)	0	625	0.00	
Poor (N=45)	0	1417	0.00	
B2t (ELISA)				0.389
Good (N=14)	6	391	1.53 (0.69-3.42)	
Poor (N=35)	9	966	0.93 (0.48-1.79)	

Figure 2.1.3. Noteworthy, this publication has been elected to be the cover of PLoS Neglected Tropical Diseases in its September 2018 issue:



Task 2.4: Development of POC-LOC devices

Recombinant antigens will be selected, tested and produced to fit in POC/LOC devices (points 1 and 2). First trials in immunochromatography have been already undertaken for the antigen 2B2t by partners 3 and 9, resulting in a prototype preliminarily validated.

Some chosen recombinant antigens (Ag5, B1t, 2B2t, MDH) and also a chimera containing specific immunodominant regions of Ag5-B1t-2B2t have been expressed in another *E. coli* strain (Rosseta (DE3), designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. This strain supply tRNAs for the codons AUA, AGG, AGA, CUA, CCC, GGA on a compatible chloramphenicol resistant plasmid. Purification procedures were optimized, and a refolding method for rapid production of GST-tagged proteins has been developed for this recombinant antigens. This strategy has allowed enhanced antigen conditions to adapt these recombinant proteins to the different diagnostic platforms evaluated and testing the suitability of them for their inclusion as reactive in a rapid test. ELISA and a chemiluminescent diagnosis protocols have

been standardized as a method of evaluation prior to the optimization of a rapid test (partner 9). In our hands, Ag5 and the multiclone antigen are the best candidates to continue the antigenicity studies and their use in the rapid test design. Taking into account both the *in silico* analysis of these recombinant antigens as well as the results of this preliminary evaluation, Ag5 has been chosen to continue the studies of expression and antigenicity studies in a yeast model system. Protein expression in the microbial eukaryotic host *Pichia pastoris* offers the possibility to generate high amounts of recombinant protein in a fast and easy to use expression system. As a single-celled microorganism *P. pastoris* is easy to manipulate and grows rapidly on inexpensive media at high cell densities. Being a eukaryote, *P. pastoris* is able to perform many of the post-translational modifications performed by higher eukaryotic cells and the obtained recombinant proteins undergo protein folding, proteolytic processing, disulfide bond formation and glycosylation. Two expression strategies have been followed to assess both the intracellular expression of the protein (cloning in pPICZB vector) and its secretion outside the yeast (cloning in pHIL-S1). Both vectors used here contains the AOX1 promoter for tightly regulated, methanol-induced expression of the gene of interest. pPICZB vector has a C-terminal peptide containing the c-myc epitope and a polyhistidine (6xHis) tag for detection and purification of a recombinant protein and in the case of the secretion vector (pHIL-S1) the main feature is the alpha-factor secretion signal for secretion of the recombinant protein. An important advantage of this last strategy is the secretion of the recombinant protein into the growth medium, using a signal sequence to target the foreign protein to the secretory pathway of *P. pastoris*. With only low levels of endogenous protein secreted to the media by the yeast itself and no added proteins to the media, a heterologous protein builds the majority of the total protein in the medium and facilitates following protein purification steps. Purification procedures were optimized in both approach and the suitability of them was testing by a chemiluminescent diagnosis method. The recombinant proteins obtained in this expression system improve the levels of antigenicity comparing with the same version obtained in *E. coli* but are not similar in sensitivity to those obtained with the native antigens. In the last part of the project, purification and expression of Ag5 in *Pichia pastoris* was improved. A method to enhance multiple copies in *Pichia* transformed strains of copies of this antigen was tested. Studies have shown that increasing copy number of the expression vector is often directly proportional to the increase in protein production. Better expression was obtained and the purification of this antigen was optimized by FPLC. Deep evaluation is necessary with a high number of sera. Some of these results have been recently published, noteworthy the development and validation of an immunostrip containing the 2B2t recombinant antigen, in the following paper: *PLoS Negl Trop Dis*. 2018 Sep 6;12(9):e0006741.

A summary of the results found with the strips containing the 2B2t antigen is shown below. **The results did not guarantee the use of this recombinant in a commercial kit.**

In an attempt to discover new diagnostic markers, Partner 3, together with Partner 1, has approached the identification and proteomic characterization of exosomes inside hydatid cysts. The study has resulted in the finding of exosomes in hydatid fluid from sheep cysts, and the proteomic characterization has shown that both known (e.g., AgB, Ag5) and potentially new antigenic markers are transported inside the parasite exosomes. Details of this work are published in the paper: *Vet Parasitol.* 2017 Mar 15;236:22-33. Within **WP2**, Partner 2 carried out two activities aiming at evaluating the characteristics of commercially available serological assays for the diagnosis of CE. The first activity evaluated the factors influencing the serological response of patients with hepatic CE using commercially available ELISA and IHA tests routinely used in Partner 2 parasitology laboratory. It was found that serological responses as assessed by commercial tests depend on CE cyst activity, size and number, and time from treatment. Details of this work are published in the paper: *Am J Trop Med Hyg.* 2016; 94(1):166-71.

The second activity evaluated the diagnostic accuracy of three commercially available rapid diagnostic tests (RDTs) for the diagnosis of hepatic CE. RDTs are extremely appealing for use during screening campaigns and in rural health centres. Sera from patients with single hepatic CE cysts in

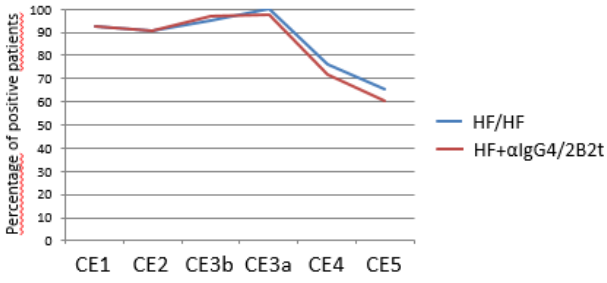
well-defined ultrasound stages (gold standard) and patients with non-parasitic cysts were analyzed by RDTs VIRapid HYDATIDOSIS (Vircell, Spain), Echinococcus DIGFA (Unibiotest, China), ADAMU-CE (ICST, Japan), and by RIDASCREEN Echinococcus IgG ELISA (R-Biopharm, Germany) as the test comparator. It was found that VIRapid test appears to perform best among the examined kits, with results comparable with the comparator test, but all tests are poorly sensitive in the presence of inactive cysts. Details of this work are published in the paper: *PLoS Negl Trop Dis.* 2016; 10(2):e0004444.

PRELIMINARY TRIALS IN IMMUNOSTRIPS (POINT OF CARE)

SPECIFICITY	HF/HF STRIPS	HF+anti-IgG4/2B2t STRIPS
Healthy donors (n=36)	2 (94,4%)	0 (100%)
Alveolar echinococcosis (n=50)	33 (34%)	16 (68%)

SENSITIVITY

	HF/HF STRIPS	HF+anti-IgG4/2B2t STRIPS
CE1 (n=13)	12 (92.3%)	12 (92.3%)
CE2 (n=21)	19 (90.5%)	19 (90.5%)
CE3b (n=108)	103 (95.4%)	105 (97.2%)
CE3a (n=47)	47 (100%)	46 (97.9%)
CE4 (n=102)	78 (76.5%)	73 (71.6%)
CE5 (n=38)	25 (65.8%)	23 (60.5%)
TOTAL	86.3%	84.5%



Main reference.

- Evaluation of the recombinant antigens B2t and 2B2t, compared with hydatid fluid, in IgG-ELISA and immunostrips for the diagnosis and follow up of CE patients. *PLoS Negl Trop Dis*. 2018 Sep 6;12(9):e0006741.

Other references.

- Preliminary Assessment of the Diagnostic Performances of a New Rapid Diagnostic Test for the Serodiagnosis of Human Cystic Echinococcosis. *Diagn Microbiol Infect Dis.* 2018. Apr 14. S0732-8893(18)30120-2.
- Structural and immunodiagnostic characterization of synthetic Antigen B subunits from *Echinococcus granulosus* and their evaluation as target antigens for cyst viability assessment. *Clin Infect Dis.* 2017 Nov 15.
- Rapid Diagnostic Tests for the Serodiagnosis of Human Cystic Echinococcosis. *Soc. Pathol. Exot.* 2017;110:20-30.
- Laboratory Diagnosis of *Echinococcus* spp. in Human Patients and Infected Animals. *Advances in Parasitology*, Academic Press, ISSN 0065-308X. 2017;96:159-257. Book chapter.
- Factors Influencing the Serological Response in Hepatic Echinococcus granulosus Infection. *Am J Trop Med Hyg.* 2016; 94(1):166-71.
- Comparison of the Diagnostic Accuracy of Three Rapid Tests for the Serodiagnosis of Hepatic Cystic Echinococcosis in Humans. *PLoS Negl Trop Dis.* 2016; 10(2):e0004444.
- Immuno-histochemical study of ovine cystic echinococcosis (*Echinococcus granulosus*) shows predominant T cell infiltration in established cysts. *Vet Parasitol.* 2015;209(3-4):285-8.

Serological Diagnosis and Follow-Up of Human Cystic Echinococcosis: A New Hope for the Future? *Biomed Res Int.* 2015; 2015:428205.

WP3: Host - parasite interplay

Objectives

The main objectives of the WP3 were to elucidate the molecular bases related to the pathogenicity of this disease, identify prognostic/diagnostic biomarkers for the clinical management of this disease and evaluate whether the clinical heterogeneity of CE patients is underpinned by a genetic heterogeneity.

Task 3.1: Eg haplotypes/genotypes/species characterization

This report is primarily an update on the progress of work relating to Task 3.1 which deals with the genetic diversity of CE metacestodes removed from surgically confirmed human patients. Progress has mainly been achieved in the following areas:

- Acquisition of CE samples for a total of 731 echinococcal cysts in the panel (Tab 3.1; Figure 3.1.1).
- **ND1 gene**: New primers were designed and successfully used to amplify other *Echinococcus* species namely, *E. canadensis* and *E. ortleppi*. We have successfully amplified and sequenced a total of 546 CE samples for the ND1 gene.
- **COX1 gene**: New primers were designed and successfully used to amplify and sequenced 457 CE samples for the COX1 gene which has resulted in the production of 1,828 contigs that now require analysis.
- Results of haplotype networks using the ND1 and COX1 nucleotide sequences are presented below (Figures 3.1.2 and 3.1.3).

Results from initial analysis of sequenced material has shown the dominance of the main haplotype with most human CE infections being caused by *E. granulosus* G1 genotype across all the included regions. The main common haplotype is shared worldwide with mostly single mutations seen here, so although haplotype diversity is high, nucleotide differences remain limited even within the *E. granulosus* G3 genotype. Using BLAST search, we compared our generated sequences with those deposited onto the NCBI GenBank and this showed most CE infection to be caused by *E. granulosus* G1 and G3 genotype and *E. canadensis*. There were also a few due to *E. ortleppi*.

Table 3.1. Numbers and sequencing of human metacestodes of *E. granulosus s. s.* in this study.

Country/Region	N	COX1	ND1
ALGERIA	44	43	44
ARGENTINA	5	5	4
ARMENIA	44	36	40
AUSTRIA	28	16	18
BOSNIA	1	1	1
BULGARIA	38	34	34
CHINA	40	19	25
DENMARK	3	3	3
ERITERA	1	1	1
GERMANY	1	1	1
HUNGARY	2	0	0
IRAQ	4	4	4
IRAN	21	5	18
ITALY	43	24	34
KAZAKHSTAN	1	1	1
KENYA/TURKANA	43	32	43
IRAQI KURDISTAN	66	41	54
LEBANON	1	1	1
MACEDONIA	1	1	1
MOROCCO	14	7	9
NETHERLANDS	5	5	5
PALESTINE	19	1	3

POLAND	27	1	6
ROMANIA	138	61	70
RUSSIA	12	7	10
SERBIA	3	2	3
SERBIA&MONTENEGRO	4	2	2
SPAIN	1	1	1
TUNISIA	44	38	42
TURKEY	77	64	68
TOTAL	**731**	**457**	**546**

Figure 3.1.3. Worldwide distribution of echinococcal cysts collected from humans in this study

Figure 3.1.2. *Echinococcus granulosus sensu stricto* haplotype network based on ND1 mitochondrial sequences.

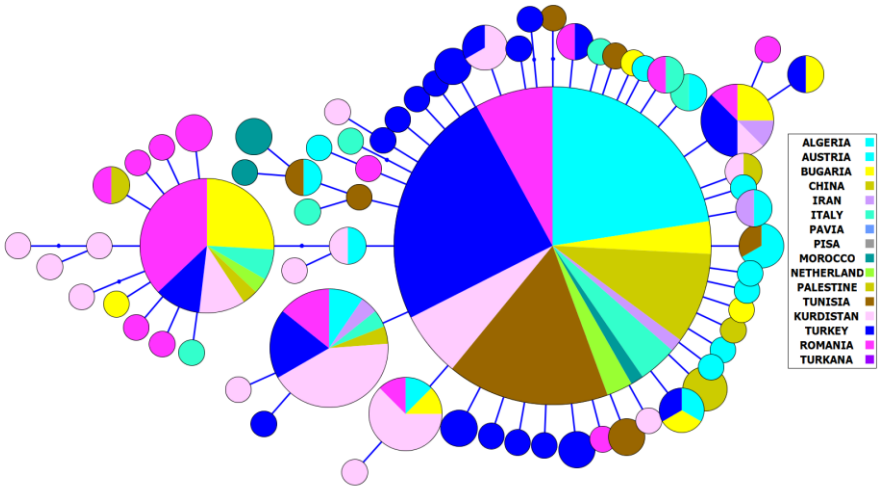
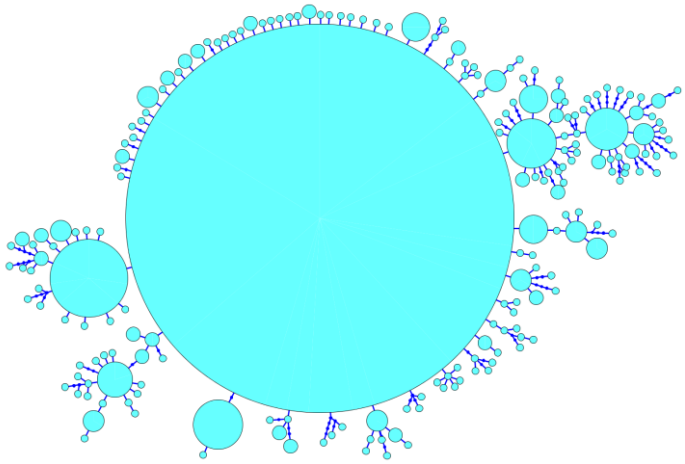


Figure 3.1.3. *Echinococcus granulosus sensu stricto* haplotype network based on COX1 mitochondrial sequences.



Main reference in preparation.

- Worldwide genetic diversity of *Echinococcus granulosus sensu stricto* from human patients (HERACLES project).

Other references.

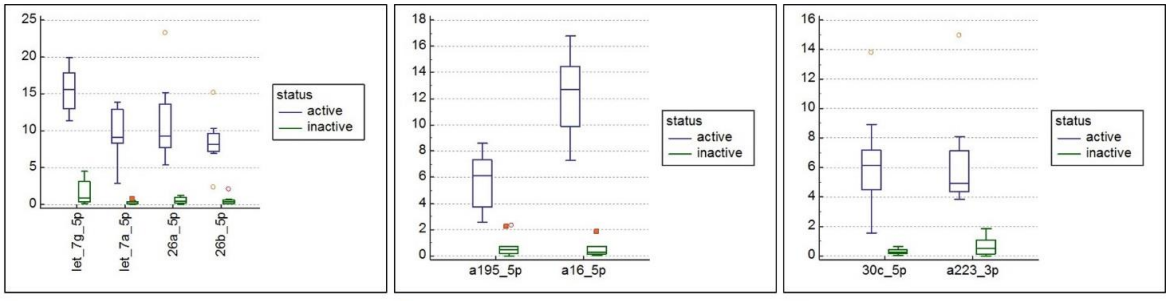
- Distinguishing *Echinococcus granulosus sensu stricto* genotypes G1 and G3 with confidence: A practical guide. *Infect Genet Evol*. 2018 Jun 21. 64:178-184.
- Global phylogeography and genetic diversity of the zoonotic tapeworm *Echinococcus granulosus sensu stricto* genotype G1. *Int J Parasitol*. 2018 May 19. S0020-7519(18)30099-7.
- Genetic diversity and phylogeography of the elusive, but epidemiologically important *Echinococcus granulosus sensu s*tricto genotype G3. *Parasitology*. 2018 Apr 17:1-10.
- The benefits of analysing complete mitochondrial genomes: Deep insights into the phylogeny and population structure of *Echinococcus granulosus sensu lato* genotypes G6 and G7. *Infect Genet Evol.* 2018 Jun 12. 64:85-94.
- Molecular phylogeny based on six nuclear genes suggests that *Echinococcus granulosus sensu lato* genotypes G6/G7 and G8/G10 can be regarded as two distinct species. *Parasitology* 2018. May 21:1-9.
- Human cystic echinococcosis in Turkey: a preliminary study on DNA polymorphisms of cysts removed from surgically confirmed patients. *Parasitology research*. 2018,117(4):1257-1263.
- Two haplotype clusters of *Echinococcus granulosus* (G1 genotype) in Iraqi Kurdistan support the hypothesis of a parasite cradle in the Middle East. *Acta Trop* 2017, 172:201-07.
- Genetic differentiation of the G6/7 cluster of *Echinococcus canadensis* based on mitochondrial marker genes. *Int J Parasitol*, 2017, S0020-7519(17)30208-4.
- High-resolution phylogeography of zoonotic tapeworm *Echinococcus granulosus sensu stricto* genotype G1 with an emphasis on its distribution in Turkey, Italy and Spain. *Parasitology*. 2016. Nov;143(13):1790-1801.
- First insights into the genetic diversity of *Echinococcus granulosus sensu stricto* (s.s.) in Serbia. *Vet Parasitol*. 2016; 15(223):57-62.
- A morphologically unusual *Echinococcus granulosus* (G1 genotype) cyst in a cow from Iraqi Kurdistan. *Epidemiology: Open Access*. 2015; 5:005.

Task 3.2: MicroRNA Array studies

The mechanisms that underlie the cohabitation of host and parasite in cystic echinococcosis are still unclear. Experimental studies in the past 30 years have tried to explain the strategies that parasites enact to survive within their hosts. Recent studies have shown that microRNAs (miRNAs) have an active role in the host-pathogen interaction and host immune responses to microorganisms. MiRNAs are small (19–24 nucleotide), non-coding RNAs that regulate gene expression post-transcriptionally by inhibiting protein translation or destabilizing target transcripts. MiRNAs play an important role in hematopoiesis, immune response and inflammation, and have emerged in recent years as important regulators of both innate and adaptive immune responses in a variety of murine model systems. MiRNAs are also involved in the pathogenesis of several human diseases including malignancies, inflammatory diseases, immune response related diseases and tumor occurrence. Recent studies showed that circulating miRNAs can be stably detected in blood or fluids of humans and animals with helminth infection. For this reason, they are being explored as potential diagnostic biomarkers for the early detection of parasite infection: for example, human miR-192 has a potential utility as a noninvasive prognostic indicator for *Opistorchis/Clonorchis* -associated cholangiocarcinoma. With respect to *Echinococcus* species miRNAs, they have recently been described providing a possibility of understanding their roles in host–parasite interaction, and their future potential use as diagnostic targets. Microarray technology is a valuable tool for the identification and characterization of gene expression profiles, due to its ability to analyze the differentially expressed genes of a whole genome in a single experiment. The aim of this study was to determine if human miRNAs related to immunity, are differentially expressed during *E. granulosus* infection.

For this purpose, we analyzed the expression profiles of 84 miRNAs in whole blood samples of 20 patients with CE in active or inactive stage using microarray technology. Twenty venous blood samples from 20 patients with a single hepatic CE cyst were included in the study. All patients were diagnosed by ultrasound (US). Cysts were classified according to the WHO standardized classification and were tested for routine diagnostic purposes in our diagnostic parasitology lab at the San Matteo Hospital Foundation, Pavia, Italy, using ELISA (RIDASCREEN® Echinococcus IgG, R-Biopharm, Darmstadt, Germany), and IHA (Cellognost® Echinococcosis IHA, Siemens Healthcare Diagnostics, Marburg, Germany) tests, as per manufacturer instructions. Samples were collected at our clinic, stored at −80°C until used and divided into two groups. Group 1 included 10 blood samples from patients with active cysts (CE3b) and group 2 included 10 blood samples from patients with inactive cysts (CE4 and CE5) that reached inactivation spontaneously. Total RNA was extracted from whole blood samples using TRIzol LS Reagent (Ambion; Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions, and quantified using a ND-100 Spectrophotometer (NanoDropTM Technologies, Wilmington, DE, USA).

Figure 3.2. A, B and C: Expression levels of circulating miRNA in patients with active (blue) and inactive (green) cyst associated with the regulation of genes involved in the control of molecules connected with the immune system and signaling and transport of proteins.



Quantification of miRNAs in blood was carried out using Human Immunopathology miScript miRNA PCR Array (Qiagen, Hilden, Germany). The Human Immunopathology miScript miRNA PCR Array profiles the expression of 84 miRNAs differentially expressed during immune responses. All reactions were carried out according to the manufacturer's protocols and recommendations using the IQ 5 Real time PCR (BioRad, Hercules, California, USA) detection system. The microarray analysis indicated an up-regulation in the expression of eight miRNAs (let-7g-5p, miR- 26a-5p, miR-195-5p, let-7a-5p, miR- 16-5p, miR- 26b-5p, miR- 30c-5p, and miR- 223-3p) in patients with active cysts compared with patients with inactive cysts. These miRNAs were already known to be crucially involved in immunity to diseases in humans. Let-7 family and miR-26 are known to have a direct role in immune responses such as proliferation and activation of macrophages, inflammation, apoptosis and/or oxidative damage. In particular, the let-7 family members have been shown to target interleukin (IL)-13, IL-10 and IL-6 in *in vivo* and *in vitro* models, while miR-26a5p and miR-26b-5p are frequently downregulated in various types of cancer, suggesting that these miRNAs function as tumor suppressors by targeting multiple oncogenes. MiR-26a also increases the expression of type I interferon, a signaling protein released by host cells in response to the presence of several pathogens, such as bacteria, parasites, viruses and also tumor cells while miR-26b modulates the NF-jB pathway in alveolar macrophages by regulating PTEN. MiR 195-5p and miR 16-5p belong to the miR-15 family, they are implicated in promoting apoptosis in a variety of cell types including immune cells, epithelial cells, and other tissue cells, that has been found in both animal and human studies. MiR-30 and miR-223 have a key role in the development and homeostasis of the immune system and in the regulation of type I interferon signaling and subsequent antiviral innate immunity. Both those miRNAs are also known to function as a tumor suppressor involved in many types of cancers.
To conclude, despite the small sample size analyzed, the study demonstrates a statistically significant up-regulation of eight miRNAs (let-7g-5p, let-7a-5p, miR- 26a-5p, miR- 26b-5p, miR- 195-5p, miR-16-5p, miR- 30c-5p, and miR- 223-3p) associated with the presence of active cysts and involvement of host miRNAs in the regulation of immune response against the parasite *E. granulosus* and/or in the hydatid cyst evolution. These results support the data obtained by Guo et al. (2017) and Jiang et al. (2016), who observed a dysregulation of the expression of several host miRNAs in *E. multilocularis*-infected mouse sera and in infected CE-resistant and -nonresistant sheep during *E. granulosus* infection, respectively.

Main reference.

- Role of microRNAs in host defense against *Echinococcus granulosus* infection: a preliminary assessment. *Immunologic Research*. 2018 (accepted)

Task 3.3: HLA-G polymorphism

The mechanisms underpinning the long-lasting co-habitation of the parasite and its host and the different outcomes after therapy are still unclear, but several immune evasion and immune modulation mechanisms have been suggested and a predominant Th2 may correlate with active infection or relapsing/unresponsive infection after treatment while skewing toward the Th1 arm would prevail in inactive infection and/or in patients who responded to therapy.
The immunogenetic component of CE infection has been largely investigated with conflicting results. A meticulous overview of the literature on this topic has been reviewed by Yang Y and colleagues in 2012. High-throughput genome wide studies in this disease confirm the importance of cytokines, immunoglobulins, HLA-class I and II classic dimers as central keys of the immune response.
However, the non-classical HLA molecules, such as HLA-G, have been neglected so far in the CE context in spite of their undiscussed anti-inflammatory and immunomodulatory properties in infection susceptibility/resistance and evolution.
The HLA-G gene maps on the telomeric end of the HLA region and codes for membrane-bound (G1, G2, G3, G4) and soluble (sG1, G5, G6, G7) proteins. HLA-G is constitutively expressed at the

maternal-fetal interface on extravillous cytotrophoblasts and in some tissues such as erythroid and endo, mesenchymal stem cells adult thymic medulla, cornea, nail matrix and pancreatic islets.
As no data are available on HLA-G and helminth infection, we looked at sHLA-G as a possible molecular mediator of the CE infection and investigated possible correlations between sHLA-G transcription level and CE natural history. To this aim, we analyzed serial serum samples from CE patients, collected at different time points during the infection, treated and non-treated at the first sampling. Temporal variations of sHLA-G blood levels were observed that significantly correlated to cysts stages and therapy responsiveness.
Partner 2 assessed the presence and levels of soluble HLA-G (sHLA-G) in sera from patients with CE using a commercial ELISA kit to determine whether host's HLA-G may have a role in the course of human CE.
It was found that sHLA-G blood levels fluctuate during patients follow up with a twofold increase of serum levels during the active or transitional phases of cysts, suggesting that cyst activity (e.g. the appearance of new daughter cysts) may trigger the over-shedding of soluble HLA-G molecules in the blood. It could be speculated that this may constitute a parasite immune evasion strategy and/or a host an immunomodulatory mechanism downregulating the inflammation caused by cyst activity. Also, although results may suggest sHLA-G levels could be statistically related to cyst viability, results from single patients are difficult to interpret and the use of sHLA-G as a diagnostic marker of cyst viability seems limited. Details of this work are published in the paper: *Parasite Immunol.* 2016; 38(7):414-8.

Main reference.

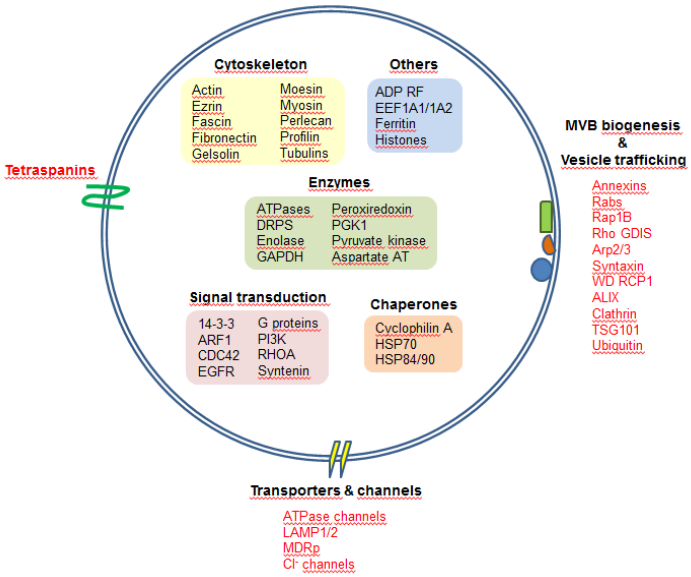
- Correlation of serum sHLA-G levels with cyst stage in patients with cystic echinococcosis: is it an immune-evasion strategy? *Parasite Immunol.* 2016; 38(7):414-8.

Task 3.4: Proteomic analysis

Isolation and characterization of *Echinococcus granulosus* exosomes in hydatid fluid: One of the goals of the HERACLES Project is the study of the host-parasite relationship in cystic echinococcosis, to define new disease markers. Cross-talk between cells could be mediated by extracellular vesicles, eg. exosomes, in several parasites. Exosomes are "Trojan horses" able to transport and deliver their cargo from cell to cell. The aim of this study is to isolate and characterize exosomes in the hydatid fluid of echinococcocal cysts.
Hydatid fluid collected from fertile sheep hydatid cysts, available at the EchinoBiobank, was used to isolate exosomes by ultracentrifugation and the OptiPrep method. Isolated exosomes were subjected to proteomic analysis, dividing the protein extract in 12 different fractions. Annotation and gene ontology analysis of the identified proteins was performed. Identified proteins were also compared with those identified by proteomics in exosomes from other parasites, and in different cyst compartments. The presence of two of the identified proteins in the isolated exosomes (antigen B2 and tetraspanin 14) was checked by immunoblot and immunoelectron microscopy. An exosome-enriched fraction was obtained from hydatid fluid (HF) of fertile sheep cysts. The proteomic analysis of this fraction identified a number of parasite-derived vesicle-membrane associated proteins as well as cytosolic proteins. A proportion of those proteins were found to be also present in other parasite exosomes, and proteins produced by protoscoleces and the germinal layer were found. Additionally, the exosomal enriched fraction contained proteins of host origin. Specific proteins -antigen B2 and TSPAN14- identified by proteomics were found in the extract and in intact exosomes as showed by specific antibody reaction.
We have demonstrated <u>for the first time</u> that *Echinococcus granulosus* cysts (in particular protoscoleces and GL) produce and release exosomes into the HF. The identified parasite exosomes carry virulence factors associated with cyst survival, including highly immunogenic and

tolerogenic antigens and peptidases. Further studies are needed to investigate whether exosomes could mediate host-parasite interactions in CE.

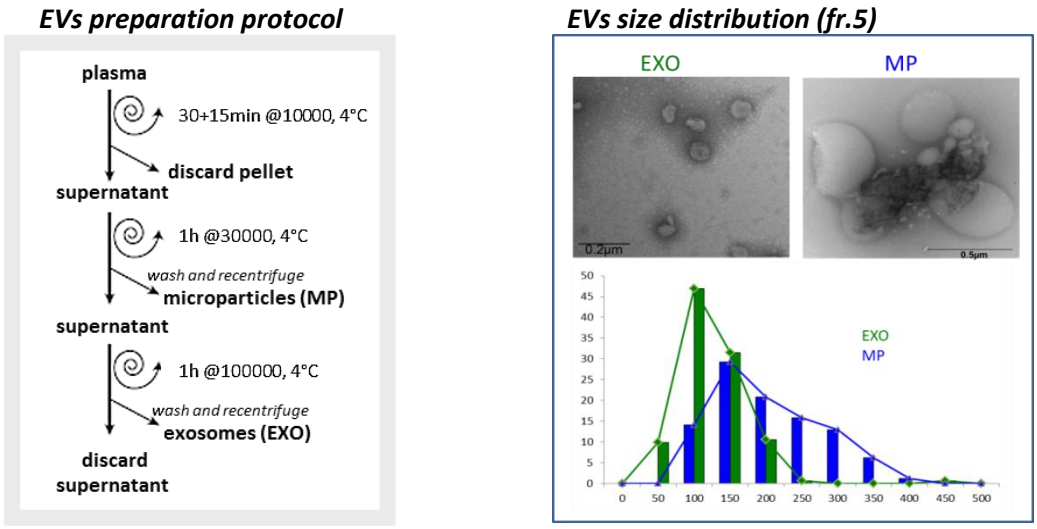
Figure 3.4.1. Exosome signatures and exosomal proteins. Exosome signatures (in red) and proteins often identified in exosome proteomic analyses found in the proteome of the exosomes isolated from Echinococcus granulosus hydatid fluid of fertile sheep cysts.



Echinococcosis Biomarkers Discovery: Proteomic investigation of exosomes from CE patient plasma: Recently there has been a growing interest in the study of extracellular vesicles (EVs). Proposed to mediate cell/cell communication in patho/physiological conditions, nowadays EVs represent sensible biomedical research targets, deeply investigated for biomarker identification or as drug delivery vehicles. We have set up a suitable method, based on differential centrifugations and density gradients, to efficiently separate from human plasma microparticles (MP) of various size (100–1000 nm) budding from the plasma membrane, and exosomes (EXO; 30–150 nm) generated within multivesicular endosomal compartments (MVB) and secreted when these compartments fuse with the plasma membrane. Gradient fractions 3-7 were characterized by transmission electron microscopy (TEM) and label-free quantitative proteomic analysis to generate a Protein Abundance Profile (PAP), which allow distinguishing between proteins shared by several types of EVs and proteins specifically enriched in EXOs or MPs.
Due to individual variability, vesicles from a total plasma volume of 100ml were collected for proteomic analysis. **A total of 517 EXO and 420 MP proteins, 354 common, were identified** with at least 2 peptides and FDR<1% at peptide level. Protein relative abundance was assessed by label-free quantitative analysis based on Top3 method and PAP profiles were submitted to hierarchical average linkage clustering (Gene Cluster 3.0) using Pearson's correlation as measure of profile similarity. Resulting dendrogram was manually analysed and clusters of correlated profiles ($R > 0.6$) were considered. For each cluster, a profile abundance was calculated as the median of PAPs. The **354 common proteins were grouped into 7 clusters** ($0.6 \leq R \leq 0.9$). 70% of proteins belong to cluster 3 (R=0.73) and Biological Process fold enrichment shows a 20-fold enrichment of proteins involved in glycolysis, while proteins involved in complex assembly, cellular structure morphogenesis and neurotransmitter secretion were enriched 5-7 fold. Among these we count proteins like Annexins; Integrins; RAB proteins; Intercellular Cell Adhesion Molecules (ICAM); protein involved in detergent resistant membrane microdomains (RAFT proteins); Actin related proteins (Arp2/3 complex, Calcium-Calmodulin complex, WAVE complex). The **163 exosome-specific proteins** were grouped into 7 clusters ($0.67 \leq R \leq 1$). 86% of proteins belong to clusters 4-7, which group proteins identified in fraction 5-6. Known markers of endocytosis and receptor

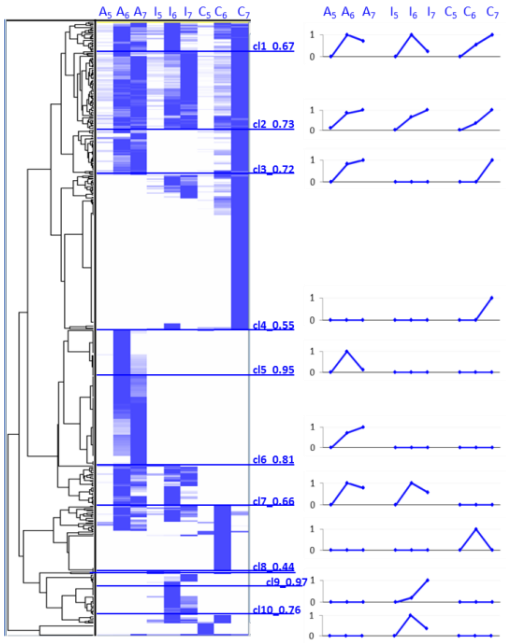
internalization were identified. The **66 microparticle-specific proteins** were grouped into 7 clusters ($0.82 \leq R \leq 1$). Markers of immune response and protein stabilization were identified.

Figure 3.4.2. Protocols for exosome isolation and enrichment.



These preliminary results on differentially enriched EVs from plasma of healthy people provide as first ascertainment that the **method is very sensitive to individual variability**, so that to get a "common" proteome a pool of at least 25 healthy subjects is necessary. Second, we **did not find any difference in the analysis of frozen plasma** (in our conditions, such as after centrifugation for 30min @ 10000 x g and addition of DMSO to a final concentration of 5%). Third, our **method allows distinguishing between proteins shared by several types of EVs and proteins specifically enriched in EXO or MP**. Among the common proteins are proteins known to be involved in vesicles trafficking and organization of membrane structures, but we also found proteins until now considered as exosomes markers (data not shown). We need to further validate this result to evaluate if those proteins are effectively shared or if represent a percentage of exosome contamination. Nonetheless, we found several proteins specifically enriched in exosome proteome and particularly in cluster 4, which suggest that our method can be suitable for disease biomarker research. Now, our encouraging preliminary results on plasma from healthy people suggest that the proposed procedure is suitable for EXO and MP investigation and adequately sensitive for biomarker identification.

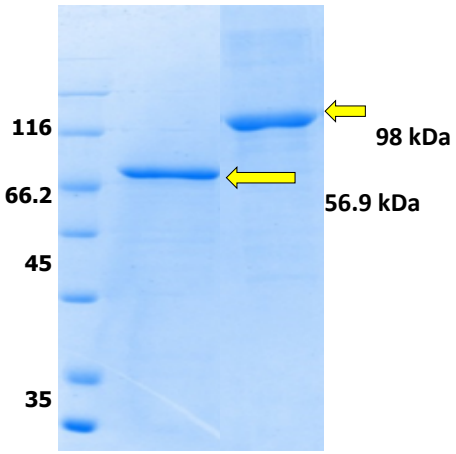
Figure 3.4.4. Cluster of protein abundance profile.



From **samples collected during 2014-2015 HERACLES survey**, we could organize **three pools of inactive cysts** (about 25 samples and 25ml each) and **two pools of active cysts** (about 25 samples and 25ml each). Two similar pools of controls were also analysed. We analysed just exosome fractions. A data overview of HUMAN proteins belonging from active, inactive and control experiments *vs* the list of proteins reported as the first 100 genes of **ExoCarta** (proteins mostly identified by exosome proteomics), showed: 158 active specific, 18 in ExoCarta; 82 inactive specific, 13 in ExoCarta; 88 common active-inactive, 25 in ExoCarta; 99 common active-inactive-control, 18 in ExoCarta; for a total of 74 ExoCarta Top100 proteins identified.

We generate a PAPs cluster by using just those proteins whose PAPs show a correlation ≥ 0.7 in the two biological replicates. Our results are rather thrilling, because there is a quite perfect clustering of common proteins identified in all experiments, contaminants, active specific, Echino-samples specific, control specific – indicating that we used good controls for echinococcosis but not healthy people, and inactive specific. Looking to pathways enrichment of human proteins we detected active metabolism, neurotransmitter and growth factor signaling for active cysts; an active metabolism and inhomogeneous disease activity, different from echinococcosis, in controls, which is a suggestion of specificity for our potential marker candidate lists. Considerably, we identified echino-proteins properly in clusters respectively of active and inactive cyst that represent potential diagnostic marker candidates. Finally, we have a list of potential diagnostic/prognostic candidates and many information available for further understanding the ECHGR biology.

Scanning this list, we focus on **two potential new markers**. The first is the **ECHGR Conserved Protein (A0A068WQT6)** very specific to the parasite and identified only in active-cyst samples, belonging to **cluster 6**. The second was suggested by the identification of several proteins in **clusters 5** and **6** (active cysts only) related to a probable immune evasion mechanism linked to the activation of a **Tyrosine protein-kinases (LYN, SRC)** pathway. Then we decide to investigate the ECHGR protein W6V0R9 homolog to ES62 in *Acanthocheilonema viteae*. These two protein have been cloned fused to GST and expressed/purified, mas recombinant proteins for antibody production to test plasma samples.



Main reference in preparation.

- Cystic Echinococcosis biomarker discovery in exosomes from human plasma (HERACLES project). Main reference.
- Isolation and proteomic characterization of exosomes in fertile hydatid cysts of sheep origin. *Veterinary Parasitology.* 2016; 236:22-23.
  Other references.
- Sensing parasites: Proteomic and advanced bio-detection alternatives. *J Proteomics.* 2016; S1874-3919(16)30005-7.

WP4: Increasing bioavailability of ABZ and new enantiomeric drug synthesis

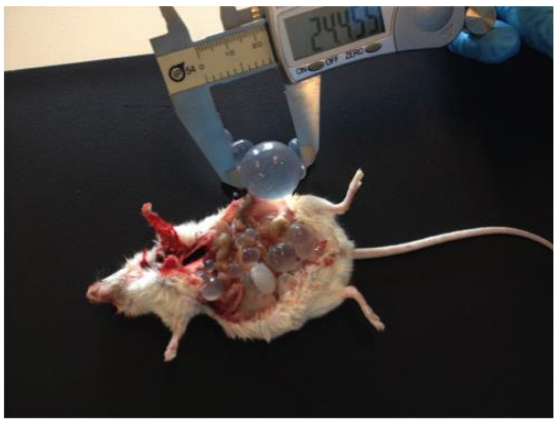
Objectives

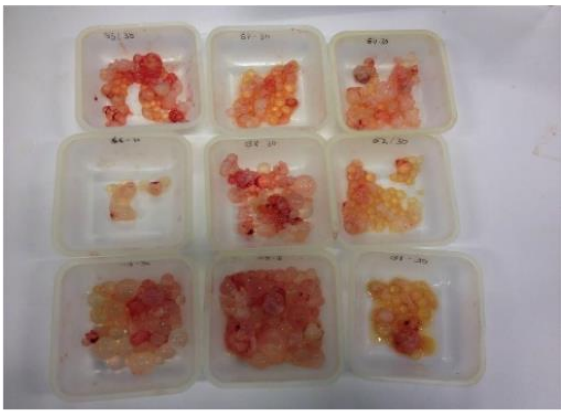
The objectives of WP4 were to synthetize new racemic and enantiomeric pure drug based on ABZ, determine, in an *in vivo* model and the efficacy of these new ABZ based drugs against *E. granulosus*.

Tasks 4.3/4.4/4.5: Experimental design in the animal model / Necropsy and sample collection / Pharmacokinetics

Among the management options for human CE, drug treatment and peri-interventional prophylaxis are currently based on poorly soluble, parasitostatic, benzimidazoles (BMZ), especially albendazole (ABZ). One drawback of ABZ, and of its active metabolite ricobendazole (RBZ, ABZ-SO), is the low intestinal absorption. As a consequence, high doses and repeated, long-term treatments are needed, exposing patients to side effects and decreased of quality of life. Cirilli and colleagues reported on novel, panted (https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2017021992 PCT/IT2016/000191), salt formulations of ABZ (ABZ-Na) and RBZ (RBZ-Na) and its enantiomers [(R)-(+)-RBZ-Na, (S)-(-)-RBZ-Na], which would allow improved intestinal absorption (Cirilli et al., 2017). We assessed the pharmacokinetic and efficacy of these new salt formulations in a murine model of CE. Infection was induced by intraperiotoneal infection with 1,500 viable (>95%) protoscoleces of *E. granulosus* sensu stricto (thereafter *E. granulosus*), obtained from naturally infected sheep, in 10-week old female BALB/c mice. Treatments were started after 9 months of infection, in 12 mice per group, with the compounds: ABZ and ABZ-Na (dissolved in dimethyl sulfoxide, DMSO); RBZ-Na, (R)-(+)-RBZ-Na, and (S)-(-)-RBZ-Na (dissolved in PBS); and DMSO (control group). Drugs were administered daily at 10 mg/kg body weight in 100 µl by oral gavage for 30 days. Drugs tested were synthesized according to the procedure previously described by Cirilli et al., 2017 and prepared fresh every day. On day 30 of treatment, mice were sacrificed and samples collected prior to dosing (Time 0) and after dosing at 15, 30, 180, and 420 minutes. Three mice per time point per group were sampled. Mice were deeply anesthetized and blood was drawn in EDTA by cardiac puncture. Plasma and cyst fluid samples were collected for pharmacokinetic analyses.

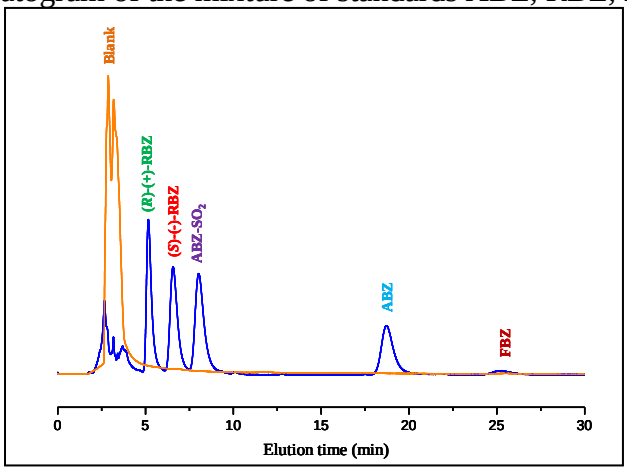
Figure 4.3.1. Secondary CE infection with *E. granulosus* in the mouse model.





Plasma was obtained from blood pools of each sampling time point within groups and stored at -20°C until use. After death by exsanguination, mice peritoneal cavity was opened, and the hydatid cysts were removed, separated from host tissue, and the whole cystic mass weighed (Figure 1). Cyst fluid samples were collected from five randomly selected cysts of each mouse per time point per group and stored at −20°C until use. One large and one medium-sized cysts from 6 animals of each group were randomly selected and processed for histochemical examination by trichrome, Periodic Acid-Schiff (PAS), and PAS-Alcian blue (PAS/AB) staining, and for scanning electron microscopy (SEM). The structural integrity of the cyst germinal layer (GL) and laminated layer (LL) was assessed by optical microscopy, while 5 areas of the GL were randomly sampled for SEM analysis. Plasma concentrations of ABZ, (R)-(+)-RBZ, (S)-(-)-RBZ and ABZ-SO_2 (ABZ sufone) were measured by HPLC after construction of calibration curves (Figure 2) pooled using blank plasma samples spiked with known amounts of ABZ, RBZ and ABZ-SO_2 containing a fixed amount of fenbendazole (FBZ) (internal standard); detection was done by UV absorption at 291 nm.

Figure 4.3.2. HPLC chromatogram of the mixture of standards ABZ, RBZ, ABZ-SO_2 and FBZ.



The mean weight of the whole cysts mass was compared between mice of each treated group and those of the control group and by treatment separately for the compounds dissolved in DMSO and in PBS by ANOVA tests. Treatment efficacy was calculated with the formula $(\mu_C - \mu_T) / \mu_C \times 100$, where μ_C is the mean weight of the whole cysts mass in the control group and μ_T in each treated group.

Experimental treatment of *E. granulosus* infected mice with BMZ salts significantly reduced the parasitic cyst mass in the murine model of CE (Figure 4.3.3, Table 4.3). Mean weight of the whole cysts mass in all treatment groups was significantly different from the control group, with the exception of (S)-(-)-RBZ-Na. ABZ-Na, resulted in significantly lower mean weight of the whole cysts mass when compared to ABZ treatment (p=0.037). Significant differences were detected among

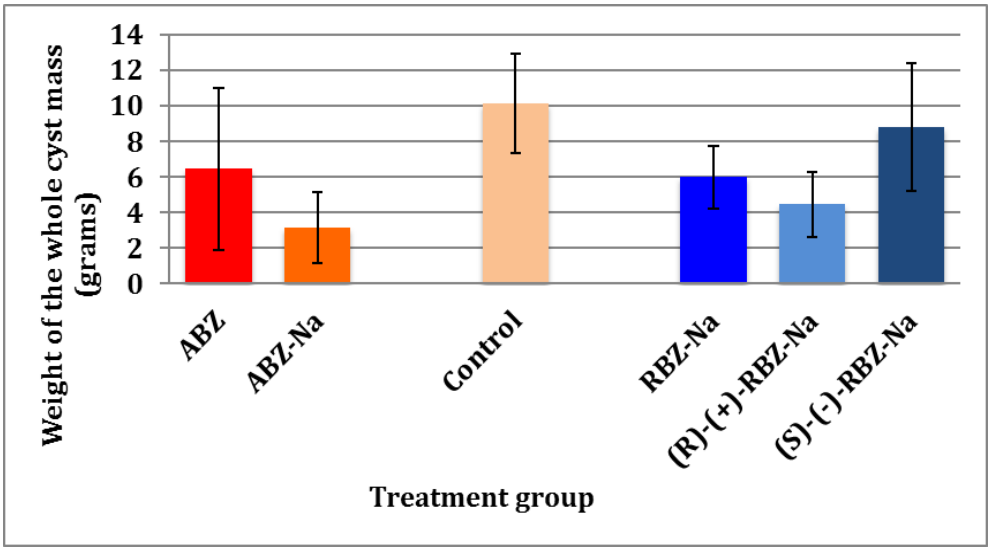
groups treated with the hydro-soluble drugs (p=0.001): mean weight of the whole cysts mass was significantly lower in the group treated with RBZ-Na compared to (S)-(-)-RBZ-Na (p=0.039), and with (R)-(+)-RBZ-Na compared to (S)-(-)-RBZ-Na (p=0.001). The highest efficacy was obtained by treatment with ABZ-Na (69.0%), followed by (R)-(+)-RBZ-Na (56.2%), RBZ-Na (41.0%), and ABZ (36.4%) and the lowest efficacy was identified upon treatment with (S)-(-)-RBZ-Na (13.3%).

Table 4.3. Cyst numbers, cyst weights and efficacies found in control and in treated mice.

Benzimidazole compounds	No. of mice°	Mean total weight of cysts (SD) in grams	Efficacy (%)
ABZ in DMSO	12	6.46 (4.55)	36.4*
ABZ-Na in DMSO	11	3.15 (1.98)	69,0*
RBZ-Na in PBS	11	5.99 (1.76)	41,0*
(R)-(+)-RBZ-Na in PBS	12	4.46 (1.81)	56,2*
(S)-(-)-RBZ-Na in PBS	11	8.81 (3.58)	13,3
Controls (DMSO)	12	10.16 (2.78)	/

SD, standard deviation; ° One mouse in each group ABZ-Na, RBZ-Na, and S-(+)-RBZ-Na died after infection, before treatment start; * Statistically significant (P<0.05 based on ANOVA).

Figure 4.3.3. Cystic mass weight (mean and SD) in the treatment groups.



Histopathology and scanning electron microscopy (SEM) confirmed that BMZ and their salts induce alterations in the germinal layer and laminated layer of *E. granulosus* metacestodes (Figures 4 and 5). In most cysts from drug-treated mice, with the exception of those treated with the poorly effective compound (S)-(-)-RBZ-Na, the GL was substantially reduced to a thin layer, with only a few discernible nuclei, while the GL of cysts in control animals consisted of 3-4 sequential cellular layers and clearly visible nuclei (Figure 4A and 4B). Other changes were desquamation, disruption, vacuolization, degeneration and also complete absence of germinal cells. The LL of cysts from drug-treated mice was distorted and irregular, with uneven PAS and PAS/AB staining compared to cysts from control mice, where the LL was evenly stained and showed its typical lamellated, layered appearance (Figure 4C and 4D). On SEM, the GL of cysts from control mice showed an amorphous, smooth surface, covered with numerous round cells, most likely being undifferentiated

cells, while in cysts from drug treated mice, the GL exhibited a much more fibrous surface and only occasionally rounded cells and developing brood capsules could be detected (Figure 5).

Figure 4.3.4. GL of control (A) and treated (B) mice stained by trichrome; LL of control (C) and treated (D) mice stained with PAS/AB.

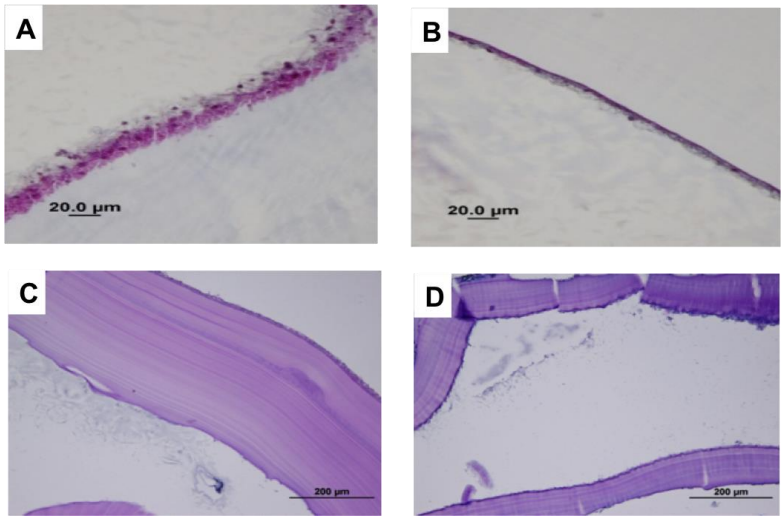
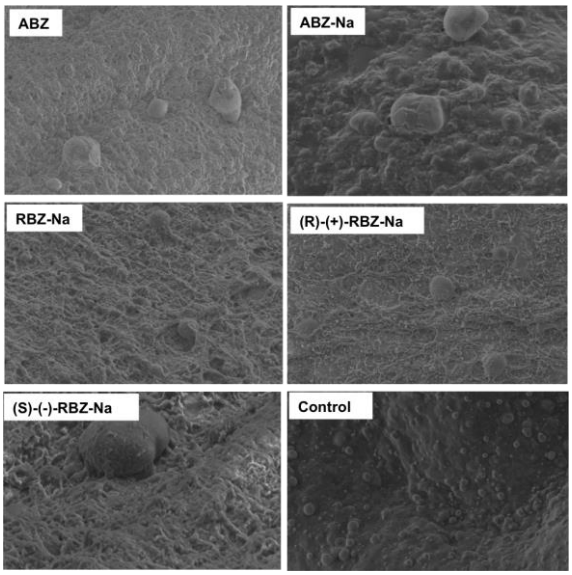


Figure 4.3.5. SEM analysis of the GL in cysts from control and treated groups.



Pharmacokinetics of BMZ and their salts in CE-infected mice as analyzed by HPLC (Figures 6 and 7). For all compounds, the most abundant metabolite detected in plasma at T_{max} (15 min) was the (S)-(-)-RBZ, with the exception of compound (R)-(+)-RBZ-Na where the latter metabolite was not detected. After administration of all compounds only the (S)-(-)-RBZ was detectable inside the cysts, with the exception of the administration of (R)-(+)-RBZ-Na, where only the only same molecule was detected both in the plasma and in the cyst. (Figure 7). Maximum levels of metabolites were delayed to 30 min in the fluid of cysts, compared with the corresponding levels in plasma.

Figure 4.3.6. Kinetics of the enantiomers of RBZ and the ABZ-SO_2 in plasma of mice treated with the different drugs.

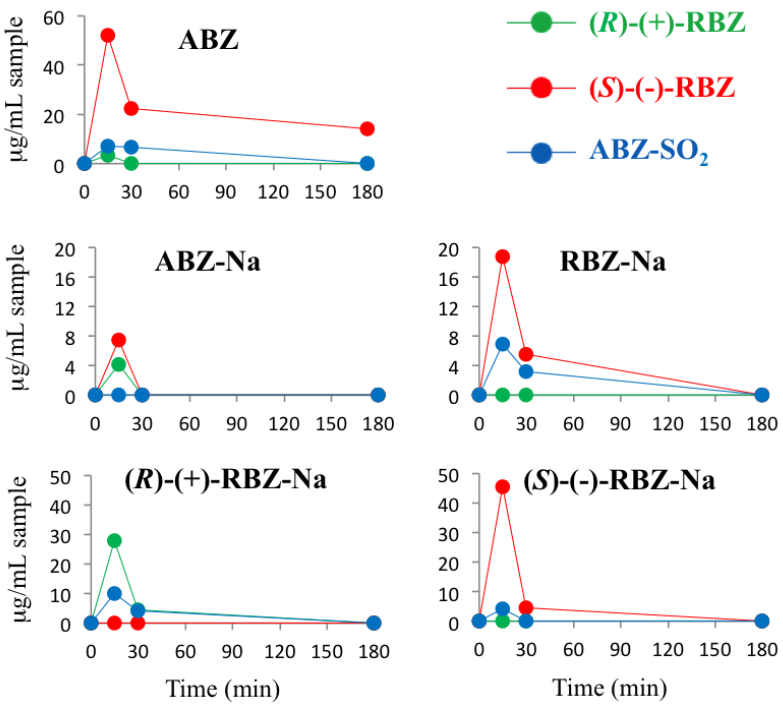
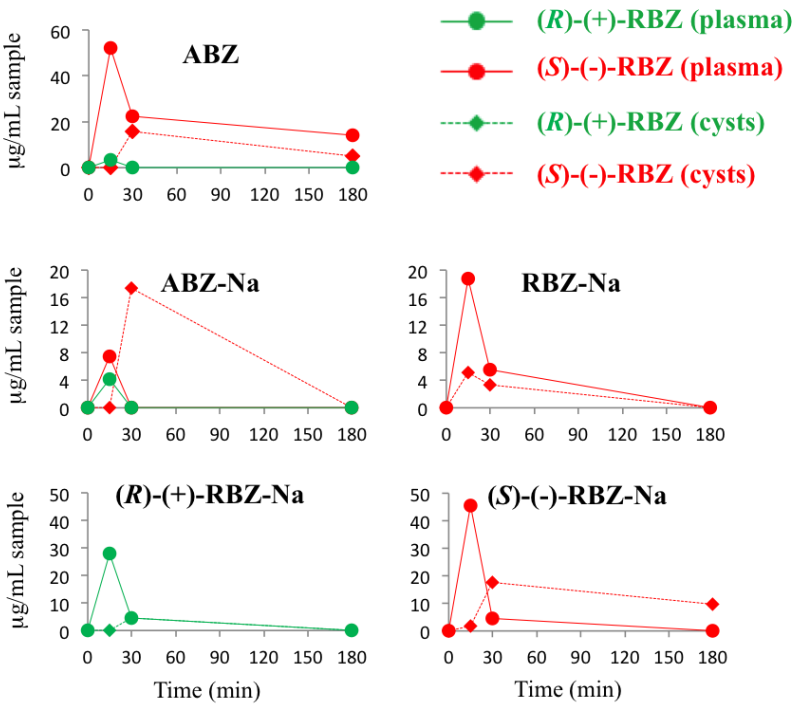


Figure 4.3.7. Kinetics of the enantiomers of RBZ in plasma and hydatid cyst fluid of mice treated with the different drugs.

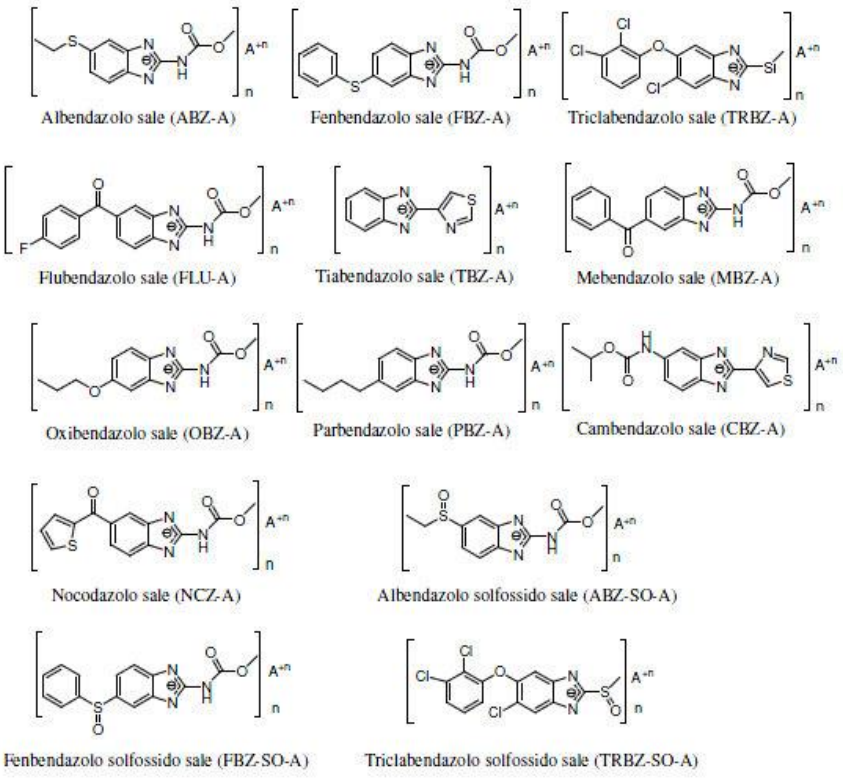


Our results show that sodium salt formulations of ABZ, RBZ, and RBZ S- and R-enantiomers show efficacy in a murine model of secondary CE. In particular, ABZ-Na and (R)-(+)-RBZ-Na showed the highest efficacy. Intra-cystic levels of (R)-(+)-RBZ were not measurable in any group, with the exception of mice treated with the parent drug (R)-(+)-RBZ-Na, indicating that intra-cystic level of (R)-(+)-RBZ could be below the detection limit of our technique even at the first assessed time point (15 min post-dosage). These data, together with the shape of the pharmacokinetic curves in plasma, are compatible with a faster kinetic of the measured metabolites, which would not allow the capture

of the actual Tmax in our experiments. However, when focusing on plasma levels of metabolites, it can be noted that the best efficacies were obtained after administration of the two compounds, ABZ-Na and (R)-(+)-RBZ-Na, also showing the highest circulating levels of (R)-(+)-RBZ. As it is known that the R-enantiomer formation is preponderant after treatment with a parent racemic drug in humans compared to mice, our results prove very promising and open the way for the formulation and testing of salt formulations of benzimidazoles with better efficacy for the treatment of human CE.

Task 4.6: New drug synthesis

International (Europe, USA) patent: **"Salts of compounds having a benzimidazolic structure, uses and process for the preparation thereof".** Application reference: PCT/IT2016/000191. ***"****The present invention relates to salts of anthelmintic compounds with a benzimidazolic structure, such as albendazole (ABZ), fenbendazole (FBZ), triclabendazole (TCBZ), or sulphoxides thereof, flubendazole (FLZ), mebendazole (MBZ), oxibendazole (OBZ), thiabendazole (TBZ), cambendazole (CBZ), parbendazole (PBZ), nocodazole (NCZ), the use and process for preparation thereof".* https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2017021992



According to the present invention, therefore, new salts of the above-mentioned compounds were prepared, said salts being **more water soluble** than the non-salified compounds. This chemical transformation enables a parenteral, as well as oral, administration of the drug, **greater bioavailability** and the **attainment of optimal therapeutic levels at lower doses** than those envisaged for the non-salified forms. It is therefore possible, by varying the stereochemistry of the sulphoxides, to **modulate the biological response and minimize side effects**, an extremely advantageous strategy for compounds intended for human use.

Recently, a simple synthetic strategy based on the presence of the acidic NH group in the benzimidazole nucleus, has been developed within the framework of HERACLES to obtain new water-soluble **salts of BMZ** (**S-BMZ**). Among these S-BMZ, research focused on **ricobendazole** [**RBZ**, also known as albendazole sulphoxide (**ABZSO**), the active chiral sulfoxide metabolite of albendazole, (ABZ)] and **triclabendazole sulfoxide** [**TCBZSO**]. Accordingly, the nitrogen atom of

the imidazole nucleus has been successfully deprotonated and salified through the addition of freshly ground NaOH at room temperature (**affordable process**). The transformation of racemic or enantiopure sulfoxides, which can be obtained on a semipreparative scale by enantioselective high-performance liquid chromatography (HPLC) on polysaccharide-based chiral stationary phases, into their Na salts [**RBZ-Na** and **TCBZSO-Na**] also offers the opportunity to **optimize the physicochemical parameters** of the ionizable drug molecules **without changing their pharmacologically active moiety**. Dissolution experiments via spectrophotometric measurements indicate that **RBZ-Na is highly soluble**: 22.83 mg mL^{-1} in water vs 0.06 mg mL^{-1} of the unsalified form. **TCBZSO-Na** has been also tested for solubility. Unlike **TCBZSO** that **is practically insoluble** (<0.005mg mL^{-1}), **TCBZSO-Na exhibits the notable solubility** of 19.3 mg mL^{-1}.
Moreover, the sulfur atom of the sulfoxide group is a stereogenic centre and, consequently, RBZ, and TCBZSO consist of an equimolar mixture of two **enantiomers** that **can be salified**. In vivo studies have demonstrated that the plasma concentration of the dextrorotatory form of RBZ [(*R*)-RBZ] is predominant over the levorotatory form [(*S*)-RBZ] in patients treated with ABZ. Moreover, an accumulation of (*R*)-RBZ has been observed in the cerebrospinal fluid of patients with neurocysticercosis. The pharmacokinetic studies available for RBZ have also demonstrated that the relative plasma concentrations of the dextro- and levorotatory enantiomers of RBZ vary according to species, age and gender of the host animal and species of parasite. The (*S*)-RBZ enantiomer has shown to be predominant in rats and mice, whereas the (*R*)-RBZ enantiomer is the prevalent species in sheep, goats, dogs, cattle and humans. These data point to a stereoselectivity in drug/organism interaction and suggest a **therapeutic application of enantiopure forms of sulfoxides**, thus the use of one **enantiomer alone** leads to **increased efficacy and/or less toxicity** compared to ABZ. To this end, another object of the present invention is to provide **new more soluble forms of chiral compounds** of RBZ and TCBZ-SO, usable as individual enantiomers, which can be obtained using processes that are **cost-effective and easy to implement** on a preparative, semi-industrial or industrial scale. In fact, the single enantiomers of sulfoxides can be easily produced by **HPLC resolution** on polysaccharide-based chiral stationary or by **asymmetric oxidation** of sulfur precursor with an inexpensive and readily available porphyrin-inspired chiral manganese complex using H_2O_2 as a terminal oxidant. The good yields (90-95%) and the **excellent enantioselectivities** (99% ee) **obtained by both methods suggests the scale-up of the production of the enantiomers from laboratory to industrial scale**.

PROTOCOL for PHASE I CLINICAL TRIAL IN HUMANS: This protocol, generated in **HERACLES** project, has been submitted in 2018 as part of **PROMETHEUS** proposal in the call topic: *New anti-infective agents for prevention and/or treatment of neglected infectious diseases (NID),* Topic identifier: SC1-BHC-15-2018.

Clinical study no. 1: The aim of this protocol is to implement a first in human **open label clinical trial** to evaluate the safety, tolerability and pharmacokinetics of escalating **Single Ascending Dose (SAD) of RBZ-Na and its enantiomers** in healthy volunteers. The results of this study will be used to select one of the two **enantiomers** to be tested along with the **RBZ-Na** in the study no. 2.
Clinical study no. 2: The aim of this protocol is is to implement a first in human **randomized, double-blind, ABZ-controlled** evaluation of the safety, tolerability, pharmacokinetics and early evidence on effectiveness of escalating **multiple ascending oral doses (MAD) of RBZ-Na** and one of its **enantiomers** in patients with cystic and alveolar echinococcosis using 2 different dosage regimes.

CLINICAL STUDY NO. 1: First in human, PHASE Ia: Single Ascending Dose (SAD) of Ricobendazole and its enantiomers
Study design: The proposed **Phase Ia** study is a randomised, twelve arms, open label clinical trial to evaluate the safety, tolerability and pharmacokinetics of escalating Single Ascending Dose (SAD) of

Investigational Medicinal Products (IMP) in healthy volunteers. The IMPs will be: RBZ-Na and its enantiomers [(*R*)-RBZ-Na and (*S*)-RBZ-Na]. This study will include the recruitment of **36 healthy volunteers**, the administration of the IMP and the monitoring of safety and tolerability (clinical and laboratory parameters). Moreover, the activities will include obtainment of plasma and urine samples from the volunteers for the pharmacokinetics of the three IMP. The single ascending doses will be 4, based on the results of the preclinical studies. Each dose for each of the three IMPs will be tested on 3 volunteers, for a total of 12 arms and of 36 volunteers (fig. 1). Each new arm will be administered the IMP only after 2 weeks monitoring for the assessment of safety of the preceding dose/group. The Phase Ia clinical trial will be conducted according to a "3+3" design (Lee et al., J Natl Cancer Inst 2009).

Primary Outcome Measures: Serious adverse events (SAE): Proportion of patients who present with SAE related to RBZ-Na, (*R*)-RBZ-Na or (*S*)-RBZ-Na, assessed both at clinical and laboratory (liver enzymes and full blood cells count) levels.

Secondary Outcome Measures: Adverse events (AE). Proportion of subjects who present with AE related to RBZ-Na, (*R*)-RBZ-Na or (*S*)-RBZ-Na, and the pharmacokinetic profiles.

Both SAE and AE will be clearly defined in the study protocol as well as in the investigator brochure, according to the European Directive 2001/20/EC and following updates, in particular the Guideline on good pharmacovigilance practices (GVP), EMA/876333/2011 Rev 4.

Pharmacokinetic Profiles evaluated: Based on the maximum plasma concentrations and the elimination half-life of the drug, the plasma samples for pharmacokinetics will be obtained at 13 time points (pre-dose or time 0, then at 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 48, and 72 hours after drug intake) on the day of drug administration. In addition, urine samples will be collected in four sequential periods during pharmacokinetics. The following PK parameters will be analyzed: Maximum plasma concentration (Cmax), Time to Cmax (Tmax), Elimination rate constant (Iz), Elimination half-life (T½), Area under the curve to the final sample (AUC0-t), Area under the curve to infinity (AUC∞), Oral clearance (CL/F), Oral volume of distribution (Vz/F).

Healthy volunteers will be recruited based on the following inclusion criteria: males and females, age range 18-55 years, non-smokers, body mass index range 18-30 kg/m^2, clinical laboratory tests (hepatic and renal profile, full blood count) within the normal range of the laboratory of the study site, negative results of HBsAg, anti-HCV antibodies, anti-HIV antibodies, QTc within normal ranges at ECG.

Main exclusion criteria: medical history of clinically significant concomitant disease, medical history of drug or alcohol abuse, concurrent use of any drugs, co-enrolment in other interventional trials, pregnant or lactating women, history of allergy to benzimidazole derivatives, creatinine>2xULN, AST or ALT >2xULN, Na, K, Chl beyond normal range, QTc>450ms; all tested within 2 months of beginning of the trial.

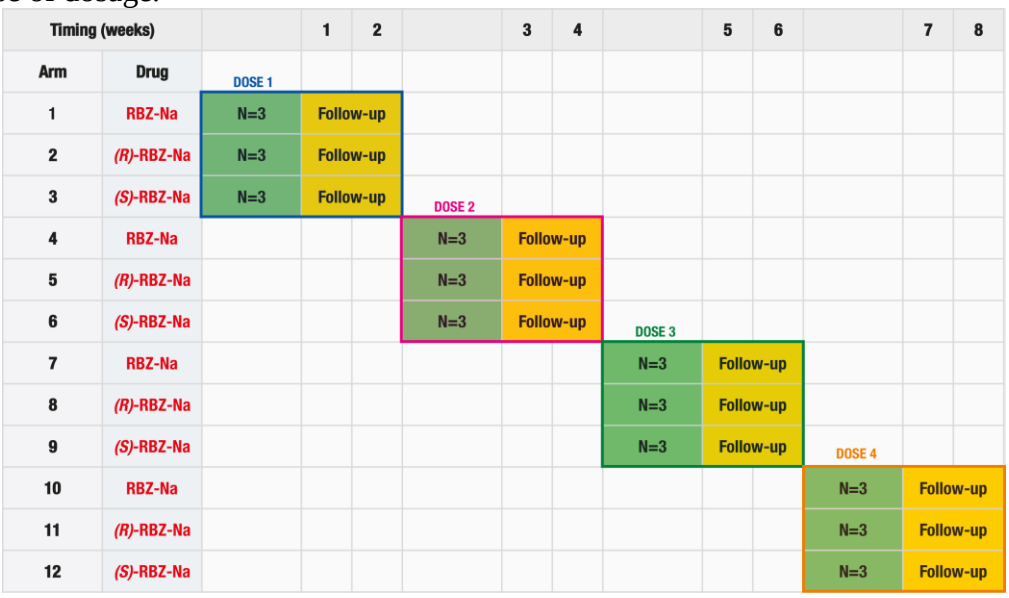
Cumulative efficacy information: **Benzimidazole (BMZ)** drugs **licensed for human use** (albendazole, triclabendazole, flubendazole, mebendazole, thiabendazole) are leading wide-spectrum anthelmintic drugs, and their therapeutic use is notably hampered by poor water solubility, resulting in low or erratic systemic bioavailability. The need of high dose and repeated and/or long-term treatments for some H-NIDs expose patients to side effects and decrease quality of life, favouring the emergence of anthelminthic resistance. This clinical trial is based on the use of **new water-soluble formulations of leading BMZ** with improved bioavailability. Recently, a simple synthetic strategy based on the presence of the acidic NH group in the benzimidazole nucleus, has been developed to obtain new water-soluble **salts of BMZ** (**S-BMZ**). Among these S-BMZ, this clinical trial focused on **ricobendazole** [**RBZ**, also known as albendazole sulphoxide (ABZSO), the active chiral sulfoxide metabolite of albendazole, (ABZ)]. The transformation of racemic or enantiopure sulfoxides into their Na salts [**RBZ-Na**; Patent: PCT/IT2016/000191] offers the opportunity to **optimize the physicochemical parameters** of the ionizable drug molecules **without changing their pharmacologically active moiety**. Dissolution experiments via spectrophotometric measurements

indicate that **RBZ-Na is highly soluble**: 22.83 mg mL^{-1} in water vs 0.06 mg mL^{-1} of the unsalified form (*J Pharm Biom Anal*. 2017;39:1-7).

Cumulative efficacy information: The **efficacy of RBZ-Na** and **its enantiomers** [(*R*)-RBZ-Na; (*S*)-RBZ-Na] has been recently assessed comparatively to conventional ABZ therapy in an experimental mouse model of cystic echinococcosis, and demonstrated that mice treated with ABZ-Na, (*R*)-RBZ-Na and (*S*)-RBZ-Na exhibited statistically significant lower cyst weights than mice treated with conventional ABZ therapy. Histology showed that treatments with RBZ-NA enantiomers and ABZ-Na resulted in degenerated germinal layer morphology and decreased thickness of the laminated layer of the parasite, thus confirming the improved efficacy of the ABZ and RBZ salt formulations compared to conventionally applied BMZ (see tasks 4.3-5). The selection of drug in this study, the starting dose and dose escalation will be based on pre-clinical study data and on the Single Ascending Dose study of Phase Ia.

Description and assignment of intervention: Each volunteer will be randomly assigned to one of the study arms: three IMP [RBZ-Na, (*R*)-RBZ-Na, and (*S*)-RBZ-Na] testing 4 ascending doses. The dose escalation will continue until at least two patients among an arm of three will experience a dose-limiting toxicities (i.e. ≥33% of patients with a dose-limiting toxicity at that dose level). The recommended dose for the Phase Ib study will defined as the dose level just below the toxic dose level of the Phase Ia study.

Figure 1. Scheme of the **Phase Ia** clinical trial aiming to establish the safety and tolerability of 3 drugs under 4 single ascending dose conditions and to determine pharmacokinetic exposure. Healthy volunteers will be exposed to a defined starting dose and undergo several rounds of safety assessment and increase of dosage.



CLINICAL STUDY NO. 2: First in human, PHASE Ib: Multiple Ascending Dose (MAD) of Ricobendazole and one of its enantiomers

Study design: The proposed **Phase Ib** study is a randomized, five arms, double-blind, ABZ-controlled evaluation of the safety, tolerability and pharmacokinetics of escalating Multiple Ascending Oral Doses (MAD) of **Investigational Medicinal Products (IMP)** in patients with cystic/alveolar echinococcosis, using 2 different dosage regimes. The IMPs will be: RBZ-Na and one of its enantiomers [(*R*)-RBZ-Na or (*S*)-RBZ-Na]. The IMPs will be administered at the highest safe dose and at doses scaled from highest safe dose. This study will include the recruitment of a total of **48 patients** with alveolar (AE) and cystic (CE) echinococcosis that will we randomized in 5 arms: 8 patients will be administered RBZ-Na scaled highest safe dose, 8 patients will be administered RBZ-

Na highest safe dose, 8 patients will be administered (*R*)-RBZ-Na or (*S*)-RBZ-Na scaled highest safe dose, 8 patients will be administered (*R*)-RBZ-Na or (*S*)-RBZ-Na highest safe dose; 16 patients will be administered standard ABZ therapy, as control arm (see fig. 2). The intervention phase on the patients will last for **28 days**. Further to administration of the IMP, subjects will be daily monitored with the collection of plasma and urine samples for the pharmacokinetic and metabolism analysis of the respective IMP. The selection of enantiomer drug, the starting dose and dose escalation will be based on pre-clinical study data and on the previous Single Ascending Dose (SAD) of Phase Ia.

Primary Outcome Measures: Serious adverse events (SAE): Proportion of patients who present with SAE related to RBZ-Na and (*R*)-RBZ-Na or (*S*)-RBZ-Na, assessed both at clinical and laboratory (liver enzymes and full blood cells count) levels.

Secondary Outcome Measures: Adverse events (AE). Proportion of subjects who present with AE related to RBZ-Na and (*R*)-RBZ-Na or (*S*)-RBZ-Na, and the pharmacokinetic profiles.

Both SAE and AE will be clearly defined in the study protocol as well as in the investigator brochure, according to the European Directive 2001/20/EC and following updates, in particular the Guideline on good pharmacovigilance practices (GVP), EMA/876333/2011 Rev 4.

Pharmacokinetic Profiles: *Time Frame*: blood samples are drawn at 15 time points up to 2 weeks and urine is collected at 5 intervals up to 3 days after dosing. The following PK parameters will be analyzed: Maximum plasma concentration (Cmax), Time to Cmax (Tmax), Elimination rate constant (Iz), Elimination half-life (T½), Area under the curve to the final sample (AUC0-t), Area under the curve to infinity (AUC∞), Oral clearance (CL/F), Oral volume of distribution (Vz/F).

Confirmed cystic/alveolar echinococcosis is defined as: pathognomonic radiological imaging and epidemiological risk factor, OR suggestive radiological imaging and positive serology and epidemiological risk factor, OR previous confirmed diagnosis by histology or PCR.

Patients will be recruited based on the following inclusion criteria: males and females, age range 18-55 years, indication for albendazole treatment according to WHO expert consensus, non-smokers, body mass index range 18-30 kg/m^2, clinical laboratory tests (hepatic and renal profile, full blood count) within the normal range of the laboratory of the study site, negative results of HBsAg, anti-HCV antibodies, anti-HIV antibodies, QTc within normal ranges at ECG.

Main exclusion criteria: medical history of clinically significant concomitant disease, medical history of drug or alcohol abuse, concurrent use of any drugs, co-enrolment in other interventional trials, pregnant or lactating women, history of allergy to benzimidazole derivatives, creatinine>2xULN, AST or ALT>2xULN, Na, K, Chl beyond normal range, QTc>450ms; all tested within 2 months of beginning of the trial.

Cumulative safety information: **Benzimidazole (BMZ)** drugs **licensed for human use** (albendazole, triclabendazole, flubendazole, mebendazole, thiabendazole) are leading wide-spectrum anthelmintic drugs, and their therapeutic use is notably hampered by poor water solubility, resulting in low or erratic systemic bioavailability. The need of high dose and repeated and/or long-term treatments for some H-NIDs expose patients to side effects and decrease quality of life, favouring the emergence of anthelminthic resistance. This clinical trial is based on the use of **new water-soluble formulations of leading BMZ** with improved bioavailability. Recently, a simple synthetic strategy based on the presence of the acidic NH group in the benzimidazole nucleus, has been developed to obtain new water-soluble **salts of BMZ** (**S-BMZ**). Among these S-BMZ, this clinical trial focused on **ricobendazole** [**RBZ**, also known as albendazole sulphoxide (ABZSO), the active chiral sulfoxide metabolite of albendazole, (ABZ)]. The transformation of racemic or enantiopure sulfoxides into their Na salts [**RBZ-Na**; Patent: PCT/IT2016/000191] offers the opportunity to **optimize the physicochemical parameters** of the ionizable drug molecules **without changing their pharmacologically active moiety**. Dissolution experiments via spectrophotometric measurements indicate that **RBZ-Na is highly soluble**: 22.83 mg mL^{-1} in water vs 0.06 mg mL^{-1} of the unsalified form (*J Pharm Biom Anal*. 2017;39:1-7).

Cumulative Efficacy information: The **efficacy of RBZ-Na** and **its enantiomers** [(*R*)-RBZ-Na; (*S*)-RBZ-Na] has been recently assessed comparatively to conventional ABZ therapy in an experimental mouse model of cystic echinococcosis, and demonstrated that mice treated with ABZ-Na, (*R*)-RBZ-Na and (*S*)-RBZ-Na exhibited statistically significant lower cyst weights than mice treated with conventional ABZ therapy. Histology showed that treatments with RBZ-NA enantiomers and ABZ-Na resulted in degenerated germinal layer morphology and decreased thickness of the laminated layer of the parasite, thus confirming the improved efficacy of the ABZ and RBZ salt formulations compared to conventionally applied BMZ (Vural et al., manuscript in final preparation for submission). The selection of drug in this study, the starting dose and dose escalation will be based on pre-clinical study data and on the Single Ascending Dose study of Phase Ia.

Description and assignment of intervention: Each patient will be randomly assigned to one out of the five study arms: the two IMP: RBZ-Na or one of the two enantiomers [(*R*)-RBZ-Na or (*S*)-RBZ-Na] with 2 doses each and the ABZ-control one. Each cohort will be composed by 8 patients testing one of the 2 API and 4 ABZ-controls. Patients and Investigators will be blinded to the drug assignment. The 2 different IMPs will be administered to human patients for 28 days.

Figure 2. Scheme of the **Phase Ib** clinical trial with a daily MAD over 28 days with 2 safe dosage regimes aims to determine their safety and tolerability, and to gain early evidence on effectiveness under multiple dose conditions accounting for accumulation and steady state pharmacokinetic exposure.

Main references.

- PATENT: Salts of compounds having a benzimidazolic structure, uses and process for the preparation thereof (https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2017021992).
- The sodium salt of the enantiomers of ricobendazole: preparation, solubility and chiroptical properties. *J Pharm Biom Anal*. 2017;39:1-7.
- Efficacy of new albendazole formulations against secondary cystic echinococcosis in experimentally infected mice (HERACLES project). Manuscript to be submitted.
- Progress in the Pharmacological Treatment of Human Cystic and Alveolar Echinococcosis: Compounds and Therapeutic Targets. *Plos NTD*. 2018 Apr 20;12(4):e0006422.

Additional publications.

- Shortage of Albendazole and Its Consequences for Patients with Cystic Echinococcosis Treated at a Referral Center in Italy. *Am J Trop Med Hyg*. 2018 Jul 23.

- A chromatographic study on the retention behavior of the amylose tris(3-chloro-5-methylphenylcarbamate) chiral stationary phase under aqueous conditions. *Journal of Separation Science* 20182018; 41(21):4014-4021.
- Unusual retention behavior of omeprazole and its chiral impurities B and E on the amylose tris (3-chloro-5-methylphenylcarbamate) chiral stationary phase. *J of Pharm Anal*. 2018. Aug;8(4):234-239.
- Analytical and semipreparative high performance liquid chromatography enantioseparation of bicalutamide and its chiral impurities on an immobilized polysaccharide-based chiral stationary phase. *J Chromatogr A.* 2016; 1445:166-71.
- Green high-performance liquid chromatography enantioseparation of lansoprazole using a cellulose-based chiral stationary phase under ethanol/water mode. *J Sep Sci.* 2016; 39:1418-24.
- Enantiomers of triclabendazole sulfoxide: analytical and semipreparative HPLC separation, absolute configuration, transformation into sodium salt. *J Pharm Biom Anal*. 2017;140:38-44.

WP5: Training and Dissemination

Objectives

- Establish the most effective methods for disseminating project results to a wide public, and in particular, to the rural populations where CE is a major health problem.
- Dissemination of the project results to inform the rural population at risk and the experts in the different fields (e.g., public health experts, clinicians, veterinarians, biologists).
- Training general practitioners working in rural areas at risk of CE in FASE and PAIR. Training local scientists in molecular identification of parasite and other advanced methods for the diagnosis and follow-up of patients affected by CE. This WP also focused on training of young women and scientists from Eastern Europe, to encourage their participation in research projects and improve their professional expertise.

Task 5.1-5.2-5.3: Dissemination of results, training and exploitation of results

All the groups, coordinated and encouraged by ALTA and ISS were very active in the dissemination of the project topic and results to the rural populations, as well as to the scientific community. These activities were aiming to disseminate HERACLES results, make advocacy for this neglected infectious disease and to improve the expertise of medical doctors living in endemic countries.

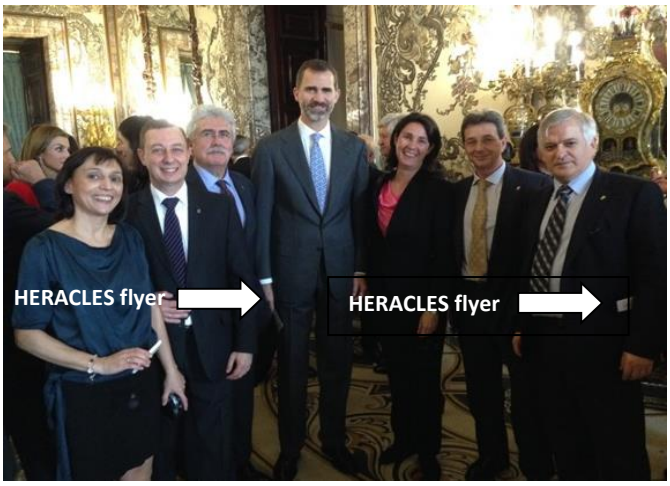
PUBLIC HEALTH EDUCATION CAMPAIGNS to 25,000 people and TRAINING to general practitioners and specialist physicians were provided during the US survey sessions in Romania, Bulgaria and Turkey.

Meeting of the European Register, ERCE. The first meeting of the European Register of Cystic Echinococcosis (ERCE) was held in Rome on November 12-13, 2015, with the aim of gathering together both ERCE active participants and those experts who were willing to join the Register, sharing and discussing experiences, issues and future developments. Fifty-two participants from 17 countries joined the meeting, including 5 no-EU countries (Albania, Iran, Georgia, Palestine and Serbia) interested in being actively involved in ERCE. During the meeting, ERCE activities carried out were presented, and training on the use of the Register was given.

Participants from 17 countries to the training course on the European Register for CE:

BROCHURES

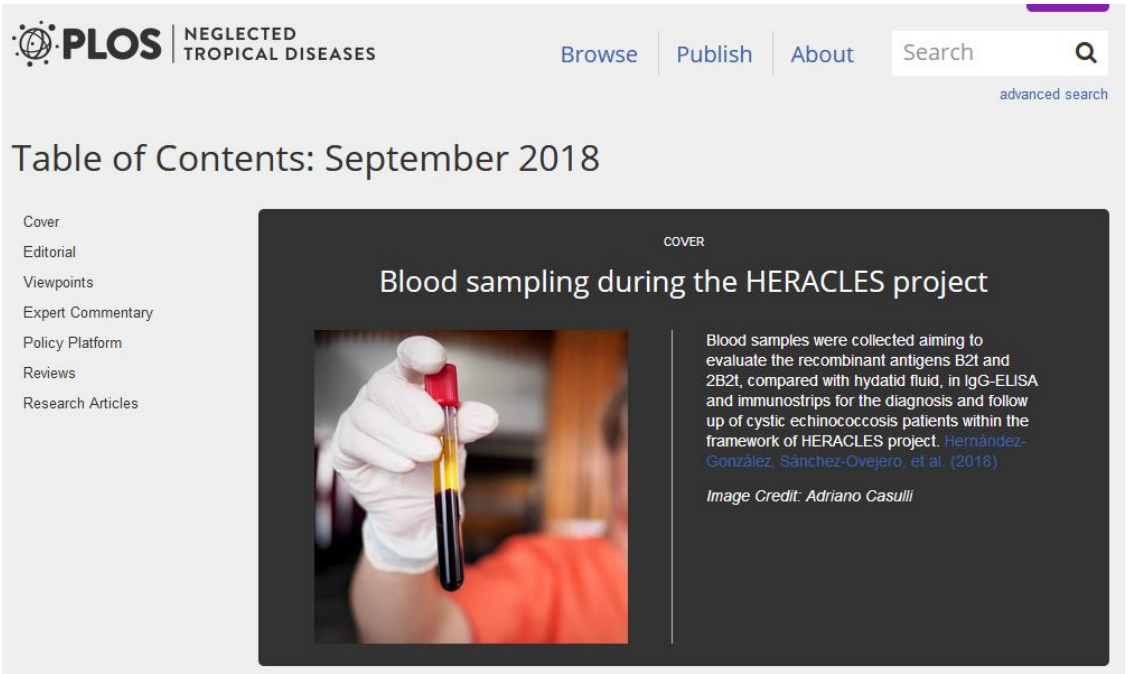
The HERACLES brochure showing core achievements has been delivered to around 1.000 people at different stands during last 5 year, including several authorities (King of Spain, President and Vice-President of CSIC in Spain, Spanish Ministry of Economy).



COVERS

- First original research ever published by *The Lancet Infectious Diseases* selected as cover for this prominent journal:

- Cover of the *PLoS Neglected Tropical Diseases* number of September 2018.



Main WEB dissemination of HERACLES activities

In English:

- WHO website: http://www.who.int/neglected_diseases/news/new-approach-needeed-to-tackle-echinococcosis-europe/en/
- CORDIS: https://cordis.europa.eu/news/rcn/129445_en.html
- http://www.heracles-fp7.eu/heracles_survey.html
- http://www.altaweb.eu/
- http://www.x-mol.com/paper/677375

In Italian:

- https://www.soipa.it/2018/05/22/impatto-dellechinococcosi-cistica-est-europa-progetto-heracles-lancet-infectious-diseases/
- http://www.sivempveneto.it/echinococcosi-cistica-condotto-dalliss-e-pubblicato-su-the-lancet-infectious-diseases-il-piu-grande-studio-epidemiologico-al-mondo/
- https://www.federfarma.it/Edicola/Filodiretto/VediNotizia.aspx?id=17157
- https://moh-it.pure.elsevier.com/en/publications/prevalence-of-abdominal-cystic-echinococcosis-in-rural-bulgaria-r

In Spanish:

- http://www.csic.es/web/guest/buscar?p_p_state=maximized&p_p_lifecycle=1&_contentviewerservice_WAR_alfresco_packportlet_struts_action=%2Fcontentviewer%2Fview&p_p_id=contentviewerservice_WAR_alfresco_packportlet&_contentviewerservice_WAR_alfresco_packportlet_nodeRef=workspace%3A%2F%2FSpacesStore%2Fc4160b7b-fa38-4941-af55-549e4d4e2830&p_p_mode=view&contentType=article
- https://www.agenciasinc.es/Noticias/La-hidatidosis-esta-mas-presente-entre-la-poblacion-de-lo-que-se-pensaba
- http://www.dicyt.com/noticias/la-hidatidosis-esta-mas-presente-entre-la-poblacion-de-lo-que-se-pensaba
- https://twitter.com/search?q=hidatidosis%20csic&src=typd&lang=es

In Turkish:

- http://www.ato.org.tr/news/show/380
- https://www.medimagazin.com.tr/guncel/genel/tr-kist-hidatik-prevalansini-saptamak-icin-8-binden-fazla-gonullu-tarandi-iste-sonuc-11-681-77338.html

In Bulgarian:

- Movie: https://www.youtube.com/watch?v=C7gUOByflNw&feature=youtu.be
- http://medicalnews.bg/blog/2018/05/25/%d0%91%d1%8a%d0%bb%d0%b3%d0%b0%d1%80%d1%81%d0%ba%d0%b8-%d0%bb%d0%b5%d0%ba%d0%b0%d1%80%d0%b8-%d0%b4%d0%be%d0%bf%d1%80%d0%b8%d0%bd%d0%b5%d1%81%d0%be%d1%85%d0%b0-%d0%b7%d0%bd%d0%b0%d1%87%d0%b8%d1%82/
- https://nauka.bg/project-proychvane-na-ehinokokozata/
- https://www.lexmedicanews.com/index.php/2018/05/24/bulgarski-lekari-v-prestizhno-mezhdunarodno-prouchvane/
- http://bnr.bg/kardzhali/post/100975710/lekari-obobshtiha-izsledvania-za-kucheshkata-tenia
- http://medicalnews.bg/blog/2018/05/25/Български-лекари-допринесоха-значит/
- http://rodopi24.blogspot.com/2018/05/7_27.html
- http://bnr.bg/kardzhali/post/100982365/prof-kamenna-vutova-higienata-moje-da-ni-spasi-ot-kucheshkata-tenia

In Romanian:

- https://www.medichub.ro/reviste/infectio-ro/voluntariat-in-cadrul-proiectului-european-heracles-experienta-privita-prin-ochii-unor-studenti-la-medicina-id-84-cmsid-67
- http://www.viata-medicala.ro/*articleID_14718-dArt.html

WP6: Project management

Consortium management tasks and achievements

The objectives stated in the Annex I are:

- Scientific and administrative coordination of the project.
- Monitor and guarantee the successful progress of the project within the timeframe, costs and quality limits as defined by the project contract signed with the EU and the Consortium Agreement signed among the beneficiaries.
- Establish effective communication among the consortium partners.
- Identify, protect and manage the Intellectual Property Rights (IPR) generated by the project and the patent applications.
- Correct management of ethical issues.

The main activities of scientific management accomplished by the project coordinator were:

Scientific coordination:

- Tasks allocation.
- Risk assessment and reallocation of the resources.
- Daily management of scientific activities, check the progress and set up of corrective actions.
- Involvement of the member of the governing bodies of the project in the strategic tasks of HERACLES
- Organization of the Steering Committee Meetings and conference calls.
- Following the scientific updates of the project website.

Ethics management:

- Evaluation, harmonization and improvements of the protocols for the surveys.
- Evaluation of the advises by the PIs from Pavia and the project coordinator, leaders of the WP1.

The administrative management is performed by ISS with the support from ALTA, and it deals with:

- Payment issues connected with the distribution of the EC contribution to the first report.
- Coordination of the administrative work and the administrative bodies of the different participants' institutions.
- Logistic organization of the meetings.
- Coordination of the activities related to the EC reporting.

HERACLES website: http://www.heracles-fp7.eu/

HERACLES final meeting

Held by beneficiary 1-ISS on September 17th-18th September 2018 at the Istituto Superiore di Sanità (ISS), Rome, Italy. The final meeting was combined with a workshop to highlight the impact of the HERACLES project at international level. Several relevant experts in the field of echinococcosis and of neglected infectious diseases were invited to give a lecture. The agenda of the meeting is available in the project website.

HERACLES: RESEARCH BASED, HUMAN FOCUSED