Executive Summary

Cystic echinococcosis (CE), a chronic helminthic disease caused by the infection with the larval stage (metacestode) of the tapeworm *Echinococcus granulosus sensu lato*, is one of the most widespread zoonotic diseases. In the EU, two forms of echinococcosis are clinically recognized in humans: CE, caused by *E. granulosus* complex and alveolar echinococcosis caused by *Echinococcus multilocularis*. However, human CE is by far the most prevalent and probably accounts for more than 95% of the estimated 2-3 million global cases with 200,000 new cases diagnosed annually. In many parts of the world, however, CE causes severe healthcare and economic losses due to treatment costs, lost wages, and livestock-associated production losses. In the European Union (EU) and adjacent countries (AC), CE is endemic in southern and eastern countries such as Spain, Italy, Greece, Macedonia, Bulgaria, Moldova, Romania, Serbia and Turkey. In Eastern Europe, the number of CE human cases has increased in recent years, with high endemicity in pastoral communities. Thus, within EU, some southern countries (such as Spain and Italy) and eastern countries (such as Bulgaria, Romania and Turkey) are those where human CE is still a major health and economic problem.

Summary description of project context and objectives

There is an urgent need to raise the awareness of CE and bring the magnitude of the problem to the attention of policymakers and general public in these endemic countries. For these reasons, **HERACLES collaborative project** is designed to provide new insights into parasite/host relationship associated with the epidemiology, clinical manifestation, parasite infectivity, host immunity, improvement of therapeutic treatment and new tools for the detection, diagnosis and follow-up of CE.

The main objectives of **HERACLES** project are:

- Create the European Register for the surveillance and clinical management of CE to provide baseline data for risk calculations and to establish a prospective case retrieval (WP1);
- Establish and register the Echino-Biobank as a representative collection of genetic *E. granulosus sensu lato* isolates and blood/serum/plasma samples from animal and human CE patients and related databases to standardize clinical and epidemiological research in this field (WP2);
- Set-up and validate new molecular-based PoC-LoC kits for immunological surveillance, rapid diagnosis and follow-up in humans by testing recombinant antigens (WP2);
- Identify cyst stage-specific biomarkers for CE through proteomic studies of exosomes from CE patient plasma (WP3);
- Evaluate whether the clinical heterogeneity of CE patients is underpinned by a genetic diversity through genotyping of human parasitic samples (WP3);
- Synthesis of new racemic and enantiopure formulations of BMZ drugs with a higher bioavailability and efficacy against the parasite in an *in vivo* model (WP4);
- Train experts working in Eastern Europe and Turkey, as they are crucial to fight this disease (WP5);
- Disseminate HERACLES achievements, raising awareness in the scientific community and the policy makers and improve the knowledge of the disease in the rural endemic areas (WP5).

Description of main S&T results/foregrounds

WP1 (Ultrasound surveys). We did a cross-sectional ultrasound-based survey that recruited volunteers from 50 villages in rural areas of Bulgaria, Romania, and Turkey. Lesions were classified using an adapted WHO classification. We reported the prevalence of abdominal CE adjusted by sex and age through direct standardisation, using the country's rural population as a reference. 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 CE 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). Our results provide population-based estimates of the prevalence of CE. These findings should be useful to support the planning of cost-effective interventions, supporting the WHO roadmap for CE control.
WP1 (ERCE). The **European Register of Cystic Echinococcosis** was created as an online prospective, observational, multilingual and multicentre register of patients with CE (ERCE; http://www.heracles-fp7.eu/erce.html). ERCE is focusing on the clinical management and public awareness of CE. **Thirty-six centres in 14 countries** (Albania, Austria, Bangladesh, Bulgaria, France, Georgia, Hungary, Iran, Italy, Kazakhstan, Poland, Romania, Spain, Turkey) **were adhering to ERCE**. **Around 2,000 patients were enrolled in ERCE by informed consent**.
WP2 (Biobank and recombinant antigens antigens). This tool was aiming to generate a repository of samples to standardize research in the bio-medical field of CE. The registered **Echino-Biobank** was fulfilling the EU law for biobanking and hosted at the IRNASA-CSIC. Samples were linked with their respective clinical data through the two databases developed during HERACLES (ERCE and CYSTRACK). Additionally; **eight recombinant antigens** (named B1t, B2t, 2B2t, Ag5t, MDH, CaBP, AFFP and DiPol) were produced at the IRNASA-CSIC. The Echino-Biobank **received around 4,500 samples** and has provided to eight institutions working in CE for a total of 1,500 samples. The best individual antigens (B1t; 2B2t and Ag5t) were used for the design and production of a multiantigenic recombinant protein called DiPol (a combination of B1, B2 and Ag5 antigens).
WP3 (Exosomes in Hydatid Fluid). Exosomes were isolated from cysts and subjected to proteomic analysis. The proteomic analysis of this fraction identified a number of parasite-derived vesicle-membrane associated proteins as well as cytosolic proteins. We have **demonstrated for the first time that *E. granulosus* cysts produce and release exosomes into the hydatid fluid**.
WP3 (Proteomics). We set up an efficient methodology, based on differential centrifugations and density gradients, to separate **exosomes** from plasma, further characterized by transmission electron microscopy and quantitative proteomics. Then, we applied our approach for the discovery of CE biomarkers for diagnosis and viability definition. Our first ascertainment is that the method is very sensitive to individual variability. Second, we did not find any difference in the analysis of frozen plasma in our conditions. Third, our method allows distinguishing between proteins shared by several types of extracellular vesicles and proteins specifically enriched in exosomes. Finally, the application of our method to CE patient plasma pools allowed to the identification of potential **biomarker candidates**.
WP3 (Genotyping). This task was aiming to confirm species/haplotypes infecting humans and investigate the intraspecific genetic diversity within/between populations. A total of **742 hydatid cysts removed from surgically confirmed cases** were included in this study. To date we have successfully amplified the whole ND1 and COX1 mitochondrial genes. 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*.
WP4 (CE infection in animal model). Animal model has been successfully established via intraperitoneal injection of protoscoleces belonging to *E. granulosus sensu stricto*. After 10 months developed hydatid cysts has been detected. A **patent on salts of benzimidazoles (Sodium salt of Ricbendazole, RBZ-Na)** has been obtained and drugs were synthetized and tested. This study was aiming to determine the parasitostatic effects of different formulations of BMZ-Na against echinococcal cysts on immunocompetent BALB/c mice. Experimental treatment of *E. granulosus* infected mice with **BMZ-Na significantly reduced the parasitic cyst mass** in the murine model of CE. Moreover, the 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.
WP4 (New drug synthesis). The international patent "Salts of compounds having a benzimidazolic structure, uses and process for the preparation thereof" was generated. *"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". 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.

WP5 (Training and dissemination). Intense and very focused disseminating and training activities have been implemented by all the partners targeting the population screened during the ultrasound surveys (n=**24,696**). The main tools were flyers, posters, seminars and other graphical material. The project web site was updated with a lot of material and information on HERACLES.

Potential impact and main dissemination activities and exploitation of results

Potential Impact

Human CE remains highly endemic in pastoral communities, particularly in regions of the Mediterranean littoral and Eastern Europe. In Eastern Europe, the number of cases has increased in recent years.

CE urgently needs attention. Diagnosis is complex and clinical management is difficult because of the poor evidence base due to lack of prospective, randomized studies that require serious funding, which is missing because CE is under the radar. **HERACLES offered a chance to break this vicious circle and to put CE on the radar. The consequences of this "simple" act can be most relevant for populations suffering from a condition that remains invisible despite its high burden.** Complex and chronic diseases with a low case fatality rate clustering in poor rural areas are the ideal research topic to be funded with the public money as less attractive for private investments which depend on quick results with a consequent high revenue rate. Public health services and funding agencies should fill this gap.

The overall objective of HERACLES was to provide evidence of the real burden of the disease, improve our understanding of the biological mechanisms underpinning pathogenesis of CE, and in translating this understanding into easy-to-use **molecular based kits for the diagnosis, clinical management and follow-up.**

The results from HERACLES will support governments, the European Commission and related European agencies such as ECDC and EFSA to harmonize data collection, monitoring and reporting of CE.

Dissemination activities

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.

The main dissemination approaches from the project start included:

- **Public health education campaigns** directed to 25,000 people living rural endemic areas and **trainings** to general practitioners on ultrasonography and to specialist physicians on percutaneous treatments of CE (PAIR, CAT, Mo-CAT), were provided during the ultrasound 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.

- **Development of a Project website**
  The project web site (http://www.heracles-fp7.eu/) has been developed by ALTA and is on line by the beginning of the project. The web site has an original design of the layout, based on the colours of the project logo. In the HOME page the aim and scope of the project, the main objectives and the logos of the beneficiaries.
  In the web site there is a very impressive HERACLES interactive map with all the centres involved, in some way, in the project (members of the consortium, Advisory Board and "extended family").
  In the several sections, there is a description of the background, the project, the workplan and the consortium. It is important to underline that in the main page there is a link to ERCE (European register of Cystic Echinococcosis) section, patronised by HERACLES, where the aims of ERCE are well described, as well as the centres involved, the contacts and the general information. The ERCE embedded site and the map were constantly updated an improved. The box with new and event reports the main achievements and HERACLES dates. Under Project Details the administrative data of the project are reported. Under communications, there are HERACLES documents that can be downloaded (a leaflet, an overview document, a summary of HERACLES numbers and a link to the agencia iberoamericana DiCYT that has published an article related to HERACLES). The reserved area, accessible via the project web site, is password-protected and contains several important documents related to the project GA, agendas, slides and material of the meetings (presentations). It is also intended as archive for example the surveys protocols, SOPs, contacts, etc. The reserved area of the web site is constantly updated.

- **Brochure**
  The HERACLES brochure showing core achievements has been delivered to around 1.000 people at different stands during last 5 year, including several authorities.

- **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

- **COVERS**

  First original research ever published by *The Lancet Infectious Diseases* selected as cover for this prominent journal (**Prevalence of abdominal cystic echinococcosis in rural Bulgaria, Romania, and Turkey: a cross-sectional, ultrasound-based, population study from the HERACLES project.** Tamarozzi F, Akhan O, Cretu CM, Vutova K, Akinci D, Chipeva R, Ciftci T, Constantin CM, Fabiani M, Golemanov B, Janta D, Mihailescu P, Muhtarov M, Orsten S, Petrutescu M, Pezzotti P, Popa AC, Popa LG, Popa MI, Velev V, Siles-Lucas M, Brunetti E, Casulli A. Lancet Infect Dis. 2018 Jul;18(7):769-778).

  Cover of the *PLoS Neglected Tropical Diseases* number of September 2018 (**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**. Hernández-González A, Sánchez-Ovejero C, Manzano-Román R, González Sánchez M, Delgado JM, Pardo-García T, Soriano-Gálvez F, Akhan O, Cretu CM, Vutova K, Tamarozzi F, Mariconti M, Brunetti E, Vola A, Fabiani M, Casulli A, Siles-Lucas M. PLoS Negl Trop Dis. 2018 Sep 6;12(9):e0006741).

- **Publications/chapter of books/reviews**

  See A1 for publications.

- **Participation at scientific events to present HERACLES results**

  See list in A2.

  Exploitation activities

  The exploitation plans of HERACLES have considered return on investment of EU funding as one of its main goals. This plan has included these two main sections:

- **Results** to be exploited **commercially or industrially**. The main developments resulting from the project is the **International (Europe, USA) patent**: **"Salts of compounds having a benzimidazolic structure, uses and process for the preparation thereof".** 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.
- **Results** to be **disseminated to stakeholders other than industry**, including health authorities, health workers and patients, contributing to standards and policy developments in CE. These have included new epidemiological data and registries (i.e. the European Register of Cystic Echinococcosis - ERCE), training of health workers, and risk awareness for general population. In particular the **World Health Organization** supported the dissemination of the project results advocating new approaches to tackle parasitic liver disease in Europe an Turkey (https://www.who.int/neglected_diseases/news/new-approach-needeed-to-tackle-echinococcosis-europe/en/)

- The results with potential for industrial or commercial application (even if it requires further research and development, or private investment) have been protected in conformity with the current legislation and with the legitimate interests of all participants. Specifically, **1 patent** (already granted) has been produced within HERACLES (see Section B of this report).
  Scientific publications, technical notes and advertising with data generated during the grant period have increased the visibility of the exploitable results arising from HERACLES for the scientific community. Results have been presented at appropriate meetings and on the consortium web page, in product brochures, internet marketing campaigns and emailing campaigns.

Project website and relevant contact details

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

Contractors involved:

- Istituto Superiore di Sanità (ITALY)
- Università degli Studi di Pavia (ITALY)
- Agencia Estatal Consejo Superior de Investigaciones Cientificas (SPAIN)
- Spitalul Clinic Colentina Bucuresti (ROMANIA)
- Hacettepe Universitesi (TURKEY)
- Hospital for Active Treatment of Infectious and Parasitic Diseases "Prof. I. Kirov" (BULGARIA)
- Namik Kemal Universitesi (TURKEY)
- Vircell S.L. (SPAIN)
- ALTA Ricerca e Sviluppo in Biotecnologie Srlu (ITALY)

Coordinator contact details

Dr. Adriano Casulli, Dep of Infectious Diseases, ISTITUTO SUPERIORE di SANITÀ (NATIONAL INSTITUTE of HEALTH) Viale Regina Elena, 299, 00161 Rome, ITALY
adriano.casulli@iss.it