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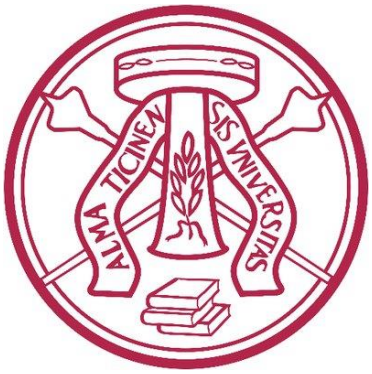
ADVANCING THE CLINICAL MANAGEMENT OF CYSTIC ECHINOCOCCOSIS PATIENTS: THREE YEARS OF CLINICAL AND EPIDEMIOLOGICAL RESEARCH



Tesi di Dottorato in Medicina Sperimentale

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Ai miei compagni di viaggio e ai pazienti, senza cui nulla sarebbe stato possibile.

Goodbye, and thanks for all the fish

A Hitchhiker's guide to the Galaxy – Douglas Adams

All of old. Nothing else ever. Ever tried. Ever failed. No matter. Try again. Fail again. Fail better

TABLE OF CONTENTS

ABSTRACT

The thesis illustrates the results of three years of clinical research in the field of Cystic Echinococcosis (CE). During these three years my work as a PhD student has mainly focused on three different aspects of CE-related research: CE epidemiology, clinical management, and studies on CE diagnosis with particular regard to serology and new molecular tools for the diagnosis of CE.

The first presented publication analyzed the state of the art on CE-related research in the Mediterranean area and showed that much was left to be done about the three topics I focused on during my PhD. The studies conducted in these three years have shown that CE is often misdiagnosed, especially in its rarer forms such as bone CE. Our group headed a multicenter study on the clinical management of this severely debilitating form of the disease to start building a consensus on the treatment and management of these patients. We also showed the safety of the "watch and wait" approach for the management of inactive cysts of the liver by updating previously published data. Another publication addresses our experience with the clinical management of cysts close to the Inferior Vena Cava (IVC), showing that the rate of IVC-related complications in our center was zero if patients were adequately treated, and that a stage-specific approach is also valid for this particular localization.

Regarding the epidemiology of CE, I have worked with Kazakh colleagues to publish data on the disease from the National Kazakh registry of CE, a publication that hopefully will serve to orient public health efforts in the country in the future. Another publication deals with the public health implications of Albendazole shortages in the treatment of CE – a problem which is not only restricted to Italy.

On serology, two publications are presented: one on the field use of Rapid Diagnostic Tests as confirmatory tests for the diagnosis of CE – a study that was never before carried out. The other on the different performance of diagnostic tests used for the serological diagnosis of CE.

Finally, a preliminary study on the alterations of miRNA expression levels in patients with CE is also included.

PART I – CYSTIC ECHINOCOCCOSIS, A TROPICAL NEGLECTED DISEASE

The first part of the thesis will focus on Cystic Echinococcosis (CE) and will try to give the reader a full account of the disease natural history, epidemiology and clinical management. All the information given here will serve as an introduction to the research results illustrated in the second part of the manuscript.

1) ECHINOCOCCOSIS – GENERAL INFORMATION

Cystic echinococcosis (CE) and Alveolar Echinococcosis (AE) are two zoonotic diseases caused by tapeworms belonging to the *Echinococcus* genus. CE is a cosmopolitan zoonosis present in countries across all continents excluding Antarctica, whereas AE is limited to the northern hemisphere [1]. Human CE affects an estimated 1.2 million people worldwide, with 1 to 3 million disability-adjusted life years (DALYs) lost globally every year, although these figures are likely to be underestimated [1–3]. *E. granulosus sensu lato* (sl), the causal agent of CE, develops it cycle between dogs and other canids (definitive hosts) and livestock, especially sheep (intermediate hosts), with humans as an accidental intermediate host. In humans, the parasite develops in its metacestode stage, forming cysts in organs and tissues, mainly in the liver. CE is mostly endemic in rural areas where sheep (and other livestock) breeding is practiced, such as central Asia and China, South America and Mediterranean countries[1]. In 2012 a joint FAO/World Health Organization (WHO) expert group classified *E. granulosus* second among the top eight ranked food-borne parasites of global public health importance[4]. However, even though the disease has a significant impact on health systems, it is still scarcely studied in endemic areas and research is mainly carried out in referral centers[5]. The aim of my work as a graduate student was to conduct clinical studies to provide information on the management of CE and its epidemiology. The results from three years of clinical research will be summarized in this thesis. During my three years as a graduate student I also had other research collaborations outside the topic of Echinococcosis. Results from these collaborations will be also presented, in a separate appendix.

2) CE – Life cycle and natural history

As previously stated, CE is a zoonotic disease caused by *E. granulosus sl.* This parasite belongs to the order of cestodes, flat, segmented worms that also include Tenia spp. The worm is a hermaphrodite and is as such self-sufficient for its own reproduction. The life cycle of *E. granulosus* involves definitive and intermediate hosts. The definitive host housing the adult worm is found in the intestine of dogs, other canids as well as felines, while the intermediate hosts are usually ungulates. Sheep are the main intermediate host, but several other species (cows, camels, horses, donkeys, pigs and goats) can be infected[6]. The life cycle of the parasite is presented in **Figure 1**

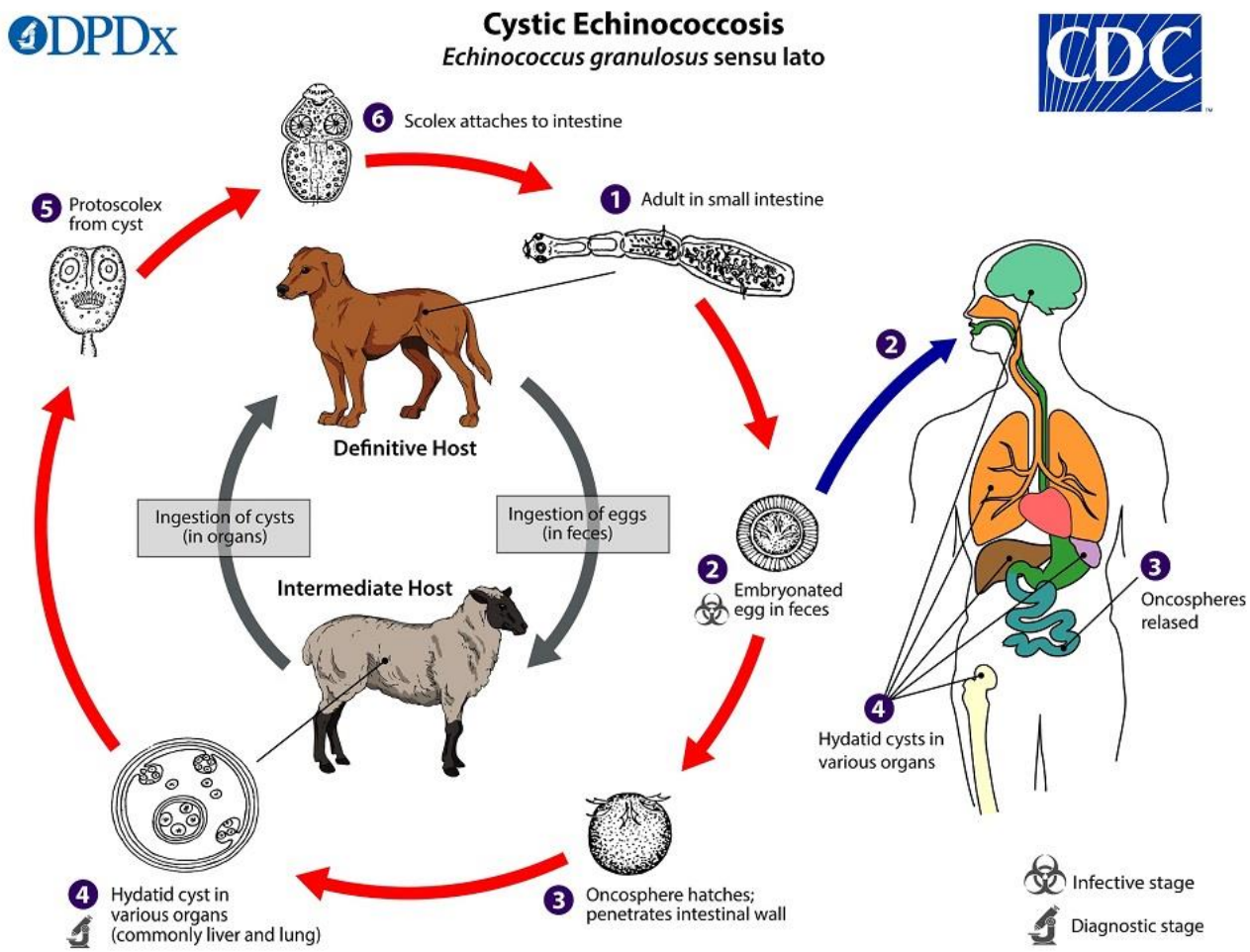

Figure 1 – Parasite life cycle - from[7]

The cycle starts when eggs are expelled with fecal material of infected dogs or other definitive hosts. These animals can be infected with a high load of worms and produce hundreds of eggs per day. Eggs are sensitive to exsiccation and to exposition to direct sunlight, but they can resist high temperature provided that humidity is high enough, as well as low temperatures[8,9].

Embrionated eggs are then ingested by intermediate hosts and hatch into oncospheres before penetrating the intestinal wall. From there, they are carried to the rest of the body via initial venous dissemination[9,10]. Once they reach their target site, the oncospheres encyst, giving rise to the formation of the "classical" metacestode or hydatid cyst. In fact, in all organs save for the bone, the cysts develop with the formation of three layers. The endocyst, which is the metabolically active part of the parasite, charged with producing brood capsules, protoscoleces and daughter vesicles. The parasite also produces a laminated layer (LL), an acellular, proteic membrane synthetized by the hexacanth larva as it develops and before synthesis for the

germinal layer starts. This “protein wall” plays a major role in immune evasion. The adventitial part of the cyst (pericyst) is host-derived and is composed of fibrous connective tissue. Its presence is useful during surgical procedures as it provides a clear surgical reference for the operating team. While developing, the cyst becomes fluid filled and brood capsules containing protoscoleces (PSC) are released in the cyst fluid. PSCs are thought to develop within ten months of infection, even though the exact timing has never been demonstrated and these data derive from animal studies[10].

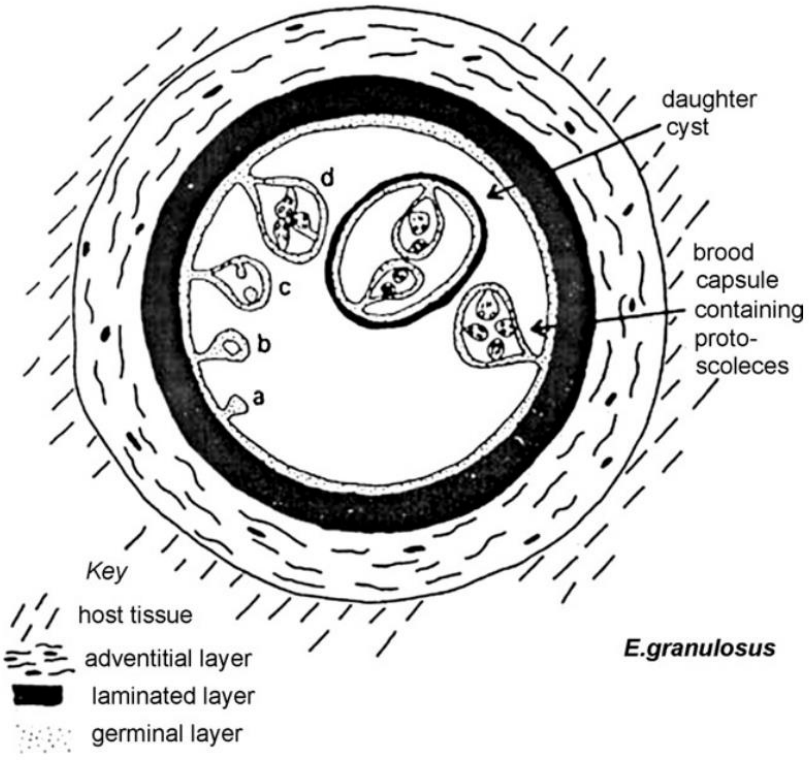

Figure 2 – Schematic representation of a CE cyst – from[11]

The cycle goes on when a definitive host has access to the viscera of an infected intermediate host. Once eaten, PSC attach to the intestinal walls of the hosts and develop into adult worms. The cycle then starts again. PSCs cannot pass the intestinal walls of mammals. For this reason, the consumption of infected organs by intermediate hosts is not infective[8–10].

Humans are “accidental” or “dead-end” intermediate hosts, as they very rarely perpetuate the parasite life cycle. Humans get infected with the ingestion of eggs present on food or contaminated, unwashed hands.

After infection, most of the times the cysts develop in the liver or lungs (50-70 and 10-20% of cases respectively), due to the initial hematogenous spreading via the portal veins and inferior vena cava. The spleen is the third anatomical location by frequency (10-15%) while other organs such as the bone, heart, brain are more rarely involved[8,9,12].

Cysts in intermediate hosts develop over the course of years in a process which is not clearly understood. Difficulties arise from several factors, including the fact that the aspect of the cysts on imaging does not always clearly match their degree of biological activity, and that once cysts are diagnosed they often must be treated[10]. Moreover, monitoring treatment requires years-long follow-up. The WHO-Informal Working Group for Echinococcosis (WHO-IWGE) has produced an ultrasound (US) classification that built on the one previously introduced by Gharbi and colleagues and that distinguishes between active, transitional and inactive stages[11,13]. This classification will be covered more extensively below. The growth rate of the cysts is also debated. Studies carried in humans and animals have found that cysts grow at a pace of around 1 cm a year, but this has also been found to be variable[10]. Moreover, a possible correlation between the growth rate and different genotypes (see below) still must be investigated.

2.1) The immune response in intermediate hosts

CE infection generates a complex immune response, as the parasite passes through several stages while in the body of the intermediate host. Briefly, studies on the immune response to infective oncospheres have shown that infection by onchospheres elicits a strong immune response, which is able to kill most parasites within days[10]. Oncospheres elicit a strong antibody response, acting together with complement to mediate protection against oncospheres, with the help of neutrophils. This strong immune response has been harnessed to develop the EG95 vaccine, that has proved highly efficacious in trials in several geographic regions[10]. The protection elicited by the vaccine can also be transmitted to the offspring of vaccinated animals via colostrum. The vaccine has been developed for the parasite G1 genotype and its efficacy against other genotypes is still unknown. This same initial immune response may be the basis of the high seroprevalence rates found in several studies, but no experimental data so far were produced to substantiate this hypohesis[10,14].

The immune response has been frequently investigated by using a mouse model where PSCs are injected in the peritoneum, mimicking secondary echinococcosis deriving from spillage of hydatid from ruptured cysts. The initial immune response has been shown to be of a Th1/Th2 phenotype, with an increased expression of IL-4, IL-5, IL-10 and IFN-gamma[15–17]. Studies have shown that the parasite is then able to downregulate both arms of this mixed immune response, which allows for parasite survival. Inflammation around the cysts (i.e. in host derived tissues) has been shown to be less active in live cysts than in degenerating cysts[10]. This may suggest that with the parasite proceeding towards inactivation, the mechanisms that allow for parasite tolerance are also less efficacious allowing for a renewed immune response[15,16]. However, the relationship of causality between cyst inactivation and inflammation in the host tissue still must be clarified, as cyst inactivation may well be the consequence of the renewed inflammation rather than its cause[10]. Parasite survival in the host does not rely solely on the isolation of the parasite from the host through the barrier effect of the LL. Actually, the LL has been shown to allow the exchange of molecules between the metacestode and the host [10]. This is proven by the high level of anti-hydatid cyst fluid (HCF) antibodies produced in intermediate hosts, and more recently by the discovery of exosomes (vescicules that contain a variable parasite-derived and host-derived cargo and that may represent a future target for diagnostic or therapeutic approaches) in HCF and plasma of hosts (Fratini et al, submitted)[18,19]. Furthermore, host immunoglobulins are found in HCF[10] While the LL does allow for the exchange of molecules between host and parasite, it also has immunomodulatory properties as shown by its ability to inhibit activation of antigen presenting cells and inhibit complement activation [10,20]

HCF contains parasite molecules with immuno-active properties, involved in the complex immune-mediated interplay between the parasite and the host[21]. Antibody, and more recently cytokine, responses against components of the HCF are explored and used for the immunodiagnosis of CE[22].

2.2) The immune response and applications in the diagnosis of CE

Diagnostic assays based on the detection of immune responses to parasitic antigens have shown considerable variability in results; this surely partially due to heterogeneity of antigenic preparations and lack of standardization in preparation of single antigens [23]. Furthermore, the influence of different genotypes on the diagnostic efficacy has been scarcely explored. Native or purified versions of HCF and of the two main antigens of HCF, AgB and Ag5, have been investigated and used from the serodiagnosis of CE [21]. Both molecules are expressed in all parasitic life stages have been shown to have immunomodulatory properties, but their biological role still has to be completely explored[10]. Other antigens such as EgTeg, EgTPx have been isolated but their role in *E. granulosus* immunity is not clear and they are not used in current serological assays[10].

Recombinant antigens have been proposed to overcome the lack of antigen standardization. Some of these antigens (2B2t, rAgB/2)[24–27] have been tested the diagnosis of CE (see below) with promising results [24–27]. However, most studies assessing the diagnostic performance of new serological tests have been carried on small cohorts of patients and without including a key piece of information, cyst stage, which is one of the key variables influencing serology responses, together with cysts number, localization, size, and previous treatment[27–30]. Seroassays have been shown to be falsely negative in a high proportion of both active (particularly CE1) and inactive cysts. Therefore, failure to describe the cyst stage distribution of the tested cohort nullifies a comprehensive and clinically-meaningful interpretation of any Se and Sp results.

Besides antibodies, other host-derived molecules are involved in the immune response to *E. granulosus* infection. These include interleukins (IL). As mentioned before, *E. granulosus* seems to be able to cause a switch in the immunophenotype from a Th1 or mixed Th1/Th2 phenotype to a down-modulated environment[16]. This observation has been used to study changes in the expression of different IL levels during infection[15,16]. In particular, IL-4 produced by whole blood stimulated with purified AgB has been proposed both as a diagnostic marker and as a marker of cyst activity[15].

Results from the first prospective study investigating the usefulness of this IL-4-based whole blood assays for the assessment of cyst viability and follow-up have been presented at congresses and are expected to be published shortly (Vola, A and Tamarozzi, F pers. comm.). At the same time, larger studies on immunoassays based on larger panels of cytochines are underway (Tamarozzi F, pers. comm.).

As previously mentioned, exosomes from *E. granulosus* have been recently isolated by the group of Siles-Lucas[19]; furthermore, host exosomes carrying parasite molecules have been recently isolated by Fratini and colleagues in plasma of subjects with CE (Fratini et al, submitted). These lipid vesicles [19], also found in other parasitic infections[18], seem to be able to pass the LL of the hydatid cyst and could play a role in host-parasite interplay by transporting excretory/secretory products of the parasite outside or host-derived molecules inside. They could also be potential future diagnostic targets or a way to vehicle therapeutics inside the cyst, once their trafficking between parasite and hosts will be elucidated.

Another recent advancement in the understanding of parasite biology, with potential diagnostic exploitation is represented by microRNAs (miRNAs), another class of molecules that have been intensively studied recently in both infectious and non-infectious diseases, as alterations in the composition of a host or parasite miRNome or in miRNA expression levels of these small molecules may give information on the status of such processes[31,32]. For instance, they have been studied in infections by *Schistosoma. japonicum*, *Schistosoma mansoni* and *Fasciola hepatica*[33–36]. In the case of *E. granulosus*, miRNAs from infected sheep have been shown to be differentially expressed in strains that had MHC profiles resistant or susceptible to *E. granulosus* infection[37,38]. A first explorative study by Dr Mara Mariconti, a post-doc from Prof. Brunetti's research unit. reported that some miRNAs previously shown to be associated with *E. granulosus* infection in mice models were also altered in humans with active cysts[39]. Further studies are needed to assess the potential of miRNAs for the diagnosis CE in general and specifically for active infections.

3) CE – classical and molecular epidemiology

As previously stated, CE is particularly present in areas where animal husbandry is practiced and is a cosmopolitan zoonosis, present in all continents except Antarctica[1,40]. The only territories that have been declared free from CE are small island nations or regions, namely Finland, Iceland and New Zealand (**Figure 3**).

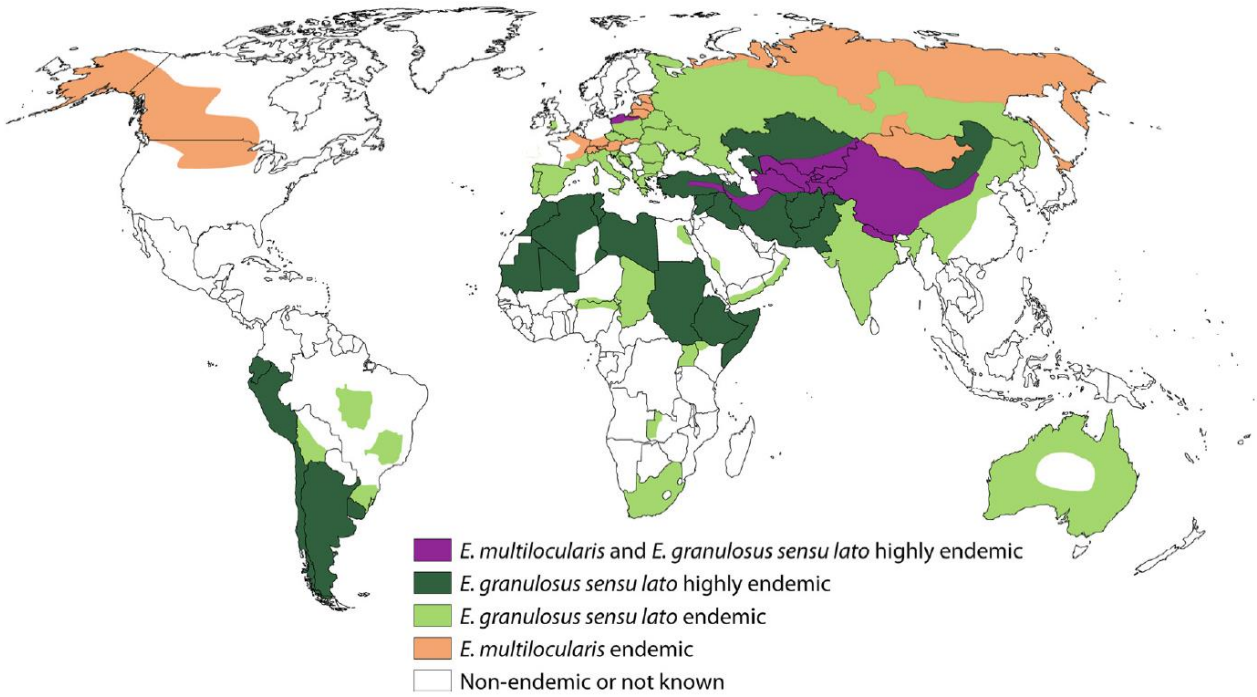

Figure 3 – Map showing the distribution of *E. granulosus* in the world, from[40]

The epidemiology of CE has not been well investigated in several countries that are thought to be endemic on the basis of the presence of previously reported human cases and of animal surveys[1]. However, a complete evaluation of the epidemiology of CE would require the collaboration of several parties including public health authorities, medical and veterinary experts and a strong commitment from political actors[5]. This often proves impossible, hindering control efforts. For this reason, the exact epidemiology of CE in most of Africa is not clearly known, as well as in several countries in Central Asia and other in South America[1,41,42]. While efforts to clarify the animal epidemiology of CE rely on animal studies including surveys on slaughtered intermediate hosts and on dogs[43–47], a complete evaluation of the animal epidemiology of CE requires enormous commitment from public health authorities. In fact, in many

countries where CE is hyperendemic, animals are often slaughtered at home and the offals are then left on the street for stray dogs to feed upon, or given to house dogs[48,49]. In several countries this practice is partly due to religious reasons as the ritual killing of animals in the house is a practice diffused in prevalently Islamic countries[48]. However, the lack of abattoirs designated for the slaughtering and inspections of animal carcasses[46,50,51]. Studies have also shown that when abattoirs are present, inspections by veterinarians are often badly conducted due to the scarce commercial value of offals from sheep[48]. Protocols for the appropriate disposals of offals from abattoirs are often lacking as well[49].

The epidemiology of human CE has been assessed in research projects and by public health authorities[52–55]. China, one of the most endemic countries worldwide, currently manages the widest public health surveillance network in the world. However, in many instances human surveillance programs are often lacking or non-existent. For instance, in Italy CE is not a notifiable disease[56], and public health estimates have been conducted on the basis of Hospital Discharge Records, which are an imperfect tool as they leave out outpatients and asymptomatic cases[57]. Recently, results from ultrasound-based surveys carried in Bulgaria, Turkey and Romania showsthat a consistent proportion of patients with CE did not know to be carrying the infection, which was expected., Active cysts where found in all ages and both sexes. The presence of active cysts in young patients may indicate continuing transmission. Most cysts were inactive, which is an expected result considering that they tend to spontaneously progress towards inactivation[52]. However, the most relevant result of the work carried out by Tamarozzi and colleagues was showing that surveillance systems for CE are often lacking. In fact, the survey carried out in Romania looked at around 1% of the country's rural population and found approximately the same number of cases notified by hospital records in the whole country in that same year[52]. The work shows well that official notification systems often underestimate the number of cases, as in Romania the projections obtained by the researcher showed that around 151.000 people could be affected by the disease and be asymptomatic, and that 40% of the patients may harbor active cysts[52]. The result is even more impressive considering that estimates for the global burden of CE until now gave the number of patients infected at one million people[2].

The HERACLES consortium also implemented a European Registry of Cystic Echinococcosis (ERCE)[58] which extended the Italian Registry of Cystic Echinococcosis (Registro Itailiano Echinococcosis Cystica –

RIEC)[59]. The registry has then been extended to extra-European countries. It currently contains data from more than 3000 CE patients. The main ambition of the registry is to serve as an epidemiological tool, however since reporting of cases is left to the initiative of single centers, this objective is far from being within reach. However, ERCE differs from surveillance systems present at country level as it allows for the collection of information regarding the stage of CE cysts, as well as their treatment. Thus, using ERCE on a wider scale would obtain information on the continuing transmission of CE in a country. ERCE is also a dataset for clinical studies. This use will be discussed below.

Human studies include seroprevalence surveys[14,60–63]. These studies aim to estimate of the prevalence of CE based on the number of subjects with a positive serology for CE. however, the immunology of CE is complex and false positives in seroassays may be cross-reactions or reflect environmental exposure to parasitic antigens without the establishment of an infection[10]. Subjects with positive serology but negative imaging often revert to seronegative after a relatively short time, hence f seropositivity should not be a marker for early infection[64]. However, although McPherson and colleagues demonstrated the superiority of US over serology in the assessment of CE epidemiology over twenty years ago, serosurveys continue to be employed[65,66].

Initial distinctions among different strains of *E. granulosus* were made on a morphological basis by spotting differences in adult worms in the definitive hosts or metacestodes and protoscoleces in intermediate hosts. Currently, *E. granulosus* is considered a species complex comprising 9 genotypes[9,67]. Since the advent of molecular methods, morphological differences have been matched with genomic data and initially 10 genotypes were proposed[68]. However, the G9 genotype has recently been assimilated to the G7 strain. The genotypes are grouped in different species and show a certain specificity for some species of intermediate hosts. The most prevalent genotype is G1, which accounts for 85% of CE cases worldwide and mainly parasites sheep in its natural cycle[67]. Initial studies were conducted using molecular techniques ranging from RFLP-PCR to sequencing using two mitochondrial genes (nad1 and cox1)[69]. The molecular resolution from these two genes has proven to be insufficient for in-depth analysis of phylogenetical relations between genotypes. For this reason, the use of nuclear genes has been explored in the last years[67,70]. After new data were obtained from nuclear markers, the elimination of the G2 genotype has been proposed[70]. However, a

consensus still must be reached on this point.

All known genotypes but G4 have conclusively been shown to be able to infect humans[8]. To this date there is no clear-cut data on whether infection by a different genotype translates into different clinical behaviors. A genotyping study with an adequate sample size has never been performed until recently. As part of the HERACLES project, a large sample size was genotyped using the sequencing of both Nad1 and Cox1, and this is expected to add information to our understanding of CE molecular epidemiology.

4) CE – CLINICAL PRESENTATION

4.1) Definitions

Cysts change their aspect over time, with a tendency towards inactivation that can either be spontaneous or in response to treatment. For the purpose of this manuscript, a few definitions will now be introduced.

- The inactivation of cysts is defined as. the disappearance of all of daughter vesicles).

- A cyst reactivation is defined as the appearance of daughter vesicles in the context of a previously inactive CE cyst.

- A relapse is defined as the appearance of a new cyst in the same or a proximal anatomical localization of a previous cyst that was treated by surgery

- Disease dissemination is defined as the appearance of new cysts in a distant anatomical localization from a first CE site, with clinically deduced possible cause (e.g. the dispersion of scoleces in the lungs following IVC embolization, or the dissemination of scoleces in the peritoneum following a liver cyst rupture).

4.2) Primary infection

In most cases, CE infection in humans is asymptomatic, and is diagnosed while the patient is undergoing imaging for other reasons[8,11]. Some patients present with symptoms due to the presence of CE cysts, which vary depending on the location of the cyst. While CE can theoretically affect all human organs, most patients carry a CE cyst in the abdomen, either in the liver (50-70%) or the spleen (5-15%)[11]. In this instances symptoms are fairly aspecific and include abdominal pain, discomfort and the perception of a mass in the abdomen if the cyst is very large.. Spleen CE has practically the same symptoms as liver CE, while other locations in the abdomen are rarer in frequency.

If CE cysts are in the lungs, which are the second localization by frequency, patients can experience cough or thoracic pain in the case of uncomplicated cysts[71]. symptomatic

Brain and cardiac CE cysts tend to be symptomatic given as the limited space for the expansion of the cysts [72,73]. This also applies to bone CE, where cysts grow with a branching pattern and do not develop the classical cyst structure[74,75].

Complicated cysts in the abdomen or lungs are usually symptomatic A classic complication of lung CE is the elimination of cyst fragments via the airways after the formation of a cysto-bronchial fistula[11]. Mechanical complications can also interest the biliary and urinary tract, as well as blood vessels. For instance, cysts near the Inferior Vena Cava (IVC) occasionally cause the leaking of the cyst in this blood vessel, with the release of parasitic material causing disease dissemination at best, potentially including pulmonary embolism [76–78]. Cyst rupture occurs [79]. but no study has assessed risk factors for cyst rupture, though experts report cases of rupture of cysts located shortly below the capsule of the liver, while in the case of lung CE, large cysts can rupture after the initiation of ABZ administration[11].

Anaphylaxis is one of the most feared complications of CE, but is a rare phenomenon, [79]. In general, mechanical complications are also associated with IgE elevation and hypereosinophylia, due to the sudden release of parasitic antigens from the cyst[8]. Cysts can also become infected with the formation of an abscess

4.3) Secondary infection

Secondary CE is defined as the appearance of cysts in anatomical sites that are distant from the primary localization, following the dispersion of protoscoleces from the primary cyst. Due to the long natural history of CE and the fact that infections are diagnosed years after initial contact with the parasite, it can be hard to establish whether patients have two primary localizations or if there has been disease spreading[75]. This is particularly evident in the case of patients with bone localizations. Most of the cases of secondary CE are, however, due to the diffusion of scoleces in the abdomen following the rupture of cysts. Other localizations include cerebral, spleen, musculoskeletal CE following hematogenous spreading and lung CE due to embolization[75,80]. Patients with peritoneal CE are often asymptomatic.Musculoskeletal CE tends to be more symptomatic, because of a mass effect due to the compression of vascular or nervous structures [8,81].

5) CE – DIAGNOSIS AND FOLLOW-UP

5.1) Imaging

imaging is the main tool for CE diagnosis. In particular US has had a key role in the diagnosis of CE as a portable, non-invasive imaging method. A US-based diagnosis of CE requires the detection of pathognomonic signs of CE cysts. The first attempts to a US classification where made by Gharbi,. Later, Caremani and colleagues also proposed their own classification[13]. At the start of the 2000s, the newly formed WHO-IWGE produced a standardized classification [82]. This classification essentially made some modifications to the one proposed by Gharbi. The classification distinguishes six cyst stages and three groups of cysts **(Figure 5)**. CE1 and CE2 cysts are considered active, CE3a and CE3b are considered transitional and CE4 and CE5 are considered inactive. These distinctions have led the WHO-IWGE to endorse a stage-specific approach to the treatment of uncomplicated CE cysts, which will be reviewed in more detail below.

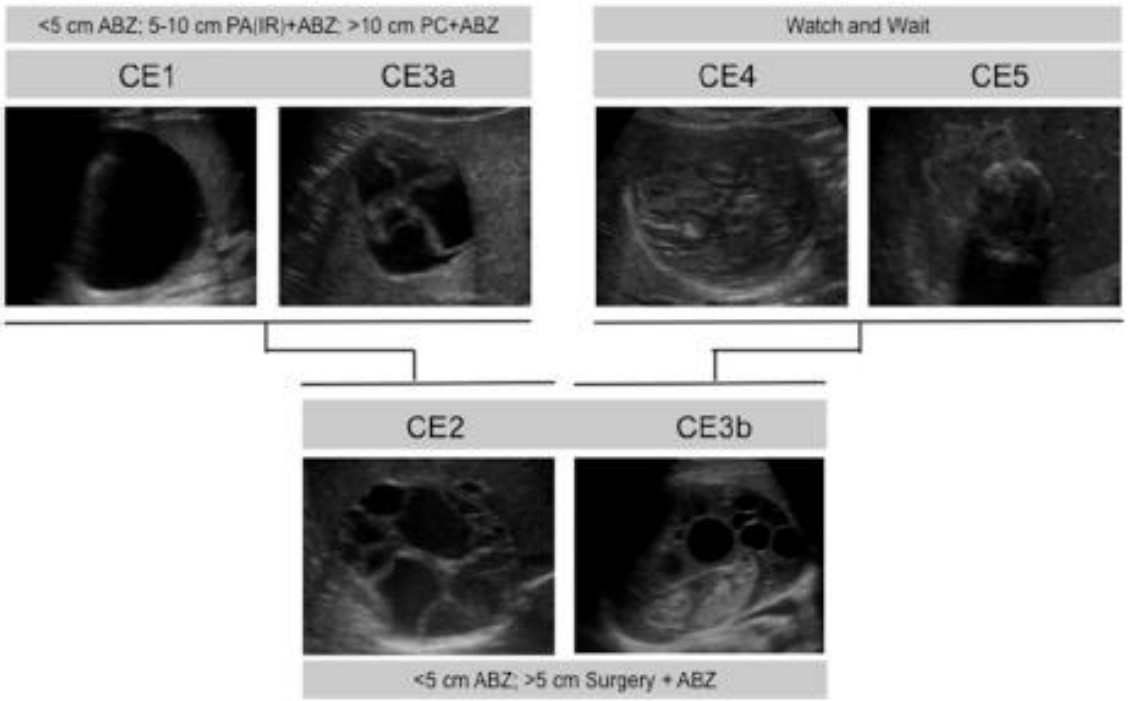

Figure 5 –Stage-specific clinical management of hepatic uncomplicated CE cysts (from[83]), in line with Expert Consensus recommendation[11]

Currently, members the WHO-IWGE are working on a technical manual for the management of CE, which will see the movement of CE3b cysts in the active group (Brunetti E and Tamarozzi F. pers. comm.).

the classification and the stage-specific approach proposed by the WHO-IWGE is are still a long way from being universally accepted although the situation has recently improved[84]. experience from the CE control program in the Rio Negro province of Argentina has shown that given enough time and tutorship, general physicians with no prior education on US or CE can learn to use the classification and the stage-specific approach endorsed by the WHO-IWGE[85]. Such programs are not widespread, however, as they require political and economical commitment from authorities, something which is rarely available for CE.

US allows for the diagnosis of CE cysts in the muscles and in the case of lung CE if the cyst is adjacent to the pleura, as well as cardiac CE[72,86]. Other anatomical locations that are not accessible to US exploration can be explored by either CT scan or MRI, and X-ray in the case of lung CE, although this should be followed by a second level exam if surgery is deemed necessary[87]. CE cysts explored via contrast-enhanced radiological examinations do not show contrastographic impregnation. This can be detected around the cyst if pericystic inflammation is present[72]. Stojkovic and colleagues showed that MRI is superior to CT for the diagnosis and staging of hepatic CE[87]. Currently, experts from the WHO-IWGE also recommend the use of MRI for extra-hepatic localizations (Tamarozzi F, pers. comm.). This is more complex for lung as lung MRI is often not available, even in centers in developed countries.

US and other imaging methods also allow for the non-invasive follow-up of CE patients. While many cysts tend to evolve towards inactivity during their natural history, in diagnosed humans with active cysts they should do so in response to treatment[10]. In fact, sometimes the administration of treatment can help clarify the nature of a suspect CE lesion, as the administration of ABZ for as little as one month can cause the detachment of the endocyst in CL lesions of parasitic nature. A course of ABZ has also been shown to cause temporary cyst solidification, in CE2 and CE3b cysts[88].

5.2) Serology

Serological assays are widely used for the diagnosis of CE. Several techniques (ELISA, Western Blot – WB, IHA, EIA and Rapid Diagnostic Tests - RDT) have been developed for the diagnosis of CE. The first

substrate used for the development of tests was native Hydatid Cystic Fluid (HCF), followed by native and recombinant antigens[22,24]. Problems concerning these different preparations have been illustrated in the Immunology section of this thesis. To date no study had rigorously assessed the role of serology in the differential diagnostic of CE. Our group recently published a study , whose results are presented and discussed below. The current consensus on the use of serology states that its role is complementary to imaging in the diagnosis of CE. Moreover, some studies have attempted to establish if these tests could be used in the follow-up of patients with CE, especially in surgically treated patients[89,90]. This as theoretically surgical treatment of a CE cyst should entail the elimination of all antigenic stimuli on the immune system, and patients should ideally revert to seronegativity if no relapse or new infection occurs. This hypothesis is hard to study and verify, as several surgical techniques are used in the surgical management of CE, and there is no absolute guarantee that all the parasitic tissue is eliminated during surgery[40]. Tests with native, purified and recombinant tests have been used and they have all shown that surgical patients cannot be followed-up by serology to detect relapses with the currently available tests[90].

RDTs have also been developed for the diagnosis of CE and tested in a few studies. Most of these assessed the performance of serological assays in a hospital setting, some did not include information on cyst stage[30,91,92]. Moreover, no study has ever assessed the potential of RDTs as confirmatory tests in resource limited settings, where operators with a specific experience on CE staging may be absent.

The first field study in resource limited settings on the use of a commercially available rapid test will be presented below.

The detection of antibodies belonging to the IgG4 subclass has been proposed to be more specific than other serological methods, as well as to correlate with the degree of activity of CE cysts.[10] However, the study that came to this conclusion used HCF from equine cysts with a possible bias due t differences in the infecting genotypes. Moreover, the study did not report the stages of the involved cysts[93].

5.3) Molecular and immuno-serological methods

Molecular methods are not widely used in the diagnosis of CE in humans.. PCR protocols have been developed for the diagnosis of CE in animals, using cyst samples from slaughtered animals[94], but parasitic DNA cannot be found in human serum, unless a cyst ruptures. In this case, DNA has also been found in the urine of patients. However, this is a rare instance and has little clinical utility in most patients[95].

Recently, studies using the ability of the parasite to condition the host immune system have been carried out. These studies have proposed the use of cytokines as diagnostic and prognostic markers for CE cysts. In particular, the detection of IL-4 by ELISA has shown promise as a new tool to diagnose CE, as well as to distinguish active cysts from inactive ones[15,16]. If these preliminary results are confirmed, this assay will be a welcome addition to the tests for the diagnosis of CE and its follow-up.

Another line of research that has been recently explored is the use of micro-RNAs (miRNAs) to diagnose CE[37,38], a. Our group published a paper exploring a possible diagnostic role of host-derived miRNAs in the diagnosis of CE, which will be presented in full below.

6) CE TREATMENT

Surgery has been traditionally considered the first line treatment for CE. Several techniques have been proposed, either radical or conservative. From the end of the eighties, a new class of drugs became available and showed activity against both CE and AE infections. Benzimidazoles are a class comprising several compounds, two of which are approved for human use and used in the treatment of CE: Mebendazole (MBZ) and ABZ. it soon then became clear that medical treatment alone could not treat all CE cysts. The need for other treatments led to the development of percutaneous procedures, the most widely used being the Puncture, Aspiration, Injection, Reasperation (PAIR) procedure. This technique was developed at the same time by a group of researchers in Pavia[96] and in Tunisia[97]. A second percutaneous procedure with percutaneous catheters was also developed for cysts larger than 10 cm[11]. Over time, it also became clear that inactive cysts very rarely reactivated. As such, a Watch and Wait (WW) approach was also proposed for uncomplicated inactive cysts. All these approaches were taken into account when formulating the Expert consensus for the diagnosis and treatment of Cystic and Alveolar Echinococcosis, whose recommendations are summarized in **Figure 5**[11].

6.1) Medical therapy

MBZ and ABZ exert their anti-parasitic activity by blocking the formation of b-tubulins polymers within the staminal cells of the parasite. They have also been shown to interfere with glucose uptake from the parasite, which proves damaging to *E. granulosus* due to the key role of glucose in this parasite's metabolism[98,99]. However, these drugs only have a parasitostatic activity in the treatment of CE, and it is thought that their effect is to impede the biological activity of the parasite while the immune system also fights the infection[99]. ABZ and MBZ are commonly used to treat other parasitic infections such as soil transmitted helminthiases or neurocysticercosis[100]. However, the duration of treatment for these infections is considerably shorter than the one required by CE and AE[100]. Currently, drugs are administrated over the course of months, as experts believe that this is required to exert an effect on cysts[11]. ABZ and MBZ were initially administered in courses of 28 days with one week of suspension, due to concern on the presence of side effects such as hepatotoxicity and medullary toxicity. This was based on animal studies, but subsequent studies have shown the safety profile of both drugs carries an acceptable risk/benefit ratio for patients undergoing prolonged administration[100,101]. However, most national pharmacovigilance authorities have not updated the recommendations for the use of ABZ and MBZ in the treatment of CE. Currently, France is the only country to accept the administration of ABZ in a continuous fashion[102]. ABZ has now become the drug of choice in the treatment of CE, with MBZ playing a secondary role. This is largely due to the better bioavailability of ABZ which translates in a lower dose needed to attain clinical efficacy[99].

Another problem with ABZ and less frequently MBZ is that the drugs can sometimes become unavailable, or not be available at all in some countries. Italy, for instance, has experience ABZ shortages that have impacted on the treatment of patients[102]. Two years ago the WHO-IWGE launched a survey to acquire data on ABZ availability from different countries in the world, and the second edition of the survey is currently underway.

5.5) Other candidate molecules evaluated for medical therapy

Several compounds have been tested in vitro or in vivo on mouse models to determine their possible anti-echinococcal activity. These include other benzimidazoles such as Oxfendazole, Flubendazole, Fenbendazole,

antimalarials such as mefloquine and artemisinin derivates, anti-cancer drugs and antibiotics or antivirals[99]. Most trials outside the benzimidazole class in vivo have been carried out to assess combination therapies with benzimidazoles, not single therapy protocols[99]. Another interesting point is the search for new formulations of existing benzimidazoles to increase the drug penetration into the cyst, something which affects ABZ efficacy. Liposomal formulations have been tested, as well as tablets containing nanopolimers[99]. Sodium salts of ricobendazole have also recently been used and have been tested in a mouse model. However, the largest obstacle to assessing new medical treatments is the expected long duration of treatment[99]. This involves recruiting patients for extended periods of time, increasing costs of clinical trials.

5.6) Surgical treatment

Radical and conservative techniques can be distinguished. In the former, CE cysts are completely removed from the liver parenchyma, with or without hepatic resection[40,103]. Different techniques can also be distinguished based on an open or closed cyst approach. One of the most diffused is the "total cystectomy" or pericystectomy approach, which uses the natural cleavage plan formed by the pericyst to remove the entire cyst without opening it. In conservative approaches, only the parasitic material is removed, while the resulting residual cavity is managed by capitonnage, omentoplasty or other techniques[103]. Recently, a standardized endocystectomy approach has been described by the group atHeidelberg University Hospital[104]. All open techniques should be executed by experienced teams and extreme caution should be used to avoid protoscoleces spillage from the cyst, as this is the most relevant risk factor for recurrence[104]. The use of surgical sponges soaked with 20% hypertonic saline has been recommended since the establishment of the WHO-IWGE expert consensus[11]. Since this solution is toxic for the peritoneum and the biliary tract and it is also able to cause hyperosmolar coma, the surgical field should also be covered by sponges soaked in 0.9% NaCl solution, [104]. Currently, studies on surgical patients suffer from the same limitations of those focusing on medical therapy, as it is impossible to effectively generate evidence on the best treatment of uncomplicated cysts without randomized controlled trials[103]. The same reasoning applies to the management of surgical complications, as one recent review found that questions are many and answers are lacking.

Cysts complicated by fistulization or rupture are generally treated by surgery, if the general conditions of the patient allow it. Infected cysts may be treated by surgery if percutaneous procedures are not available[11,103].

5.7) Percutaneous procedures

Percutaneous procedures were developed for the treatment of prevalently liquid CE cysts (CE1 and CE3a). The first technique to be developed was PAIR, and some other techniques have been proposed over time, all essentially variations of the PAIR protocol which sees a first step in the ultrasound-guided percutaneous puncture of the cyst, followed by aspiration of the cyst content, usually presenting itself as a clear, transparent fluid in young untreated cysts, whereas it becomes yellow and turbid in cysts that are spontaneously evolving towards inactivation or have been treated by ABZ[103,105,106]. After aspiration, a scolecidal agent (hypertonic saline or alcohol) is injected in the cavity, and is left there for twenty minutes to attain cyst sterilization. After this, the fluid is re-aspirated and the patient is kept in observation for twenty-four hours. The whole procedure is performed in the presence of an intensive care specialist, set to intervene in the case of collateral effects, mainly anaphylactic reactions. It should be noted that these are possible but quite rare instances[79,103].

Variations of the PAIR technique have been proposed, and recently a procedure without the use of scolecidal agents has been proposed by members of our group[83]. Further validations of this protocol will require prospective studies with a control group, an element lacking in the work of Firpo and colleagues.

PAIR recommended to treat cysts that are large more than 5 cm, while another kind of procedure using large bore catheters has gained ground as an alternative to surgical treatment in patients with large cysts (more than 10 cms)[11,107]. In this procedure, a catheter is inserted under US guidance in the cyst cavity, and is left in place draining the cyst content over days, while the patient is hospitalized. Scolecidal agents can be used in a similar fashion to what is done for the PAIR procedure[11].

Due to the use of scolecidal agents, patients undergoing percutaneous procedures should always be evaluated for the presence of a biliary fistula, especially if the cyst is larger than 7,5 cm. This has been shown to be a clear risk factor for the presence of fistulas[103]. The fact that this evaluation ideally requires access to or to cholangio-MRI has limited the use of percutaneous procedures in resource-limited settings.

All patients treated by a percutaneous procedure must undergo ABZ administration before the procedure and for the subsequent month, according to the WHO-IWGE[11]. This recommendation is also valid for patients undergoing surgery and is based on data from animal models showing that in case of peritoneal dissemination

of cyst content, one month of ABZ could prevent secondary CE. However, this recommendation is followed inconstantly in endemic countries[108].

The last recorded clinical trial to date has regarded the use of ABZ post-operatively in percutaneously treated patients, conducted by the group of Okan Akhan from the University of Ankara. The group concluded that 1 month of ABZ is a enough to reduce recurrence rates[109].

5.8) Watch and wait

Although not strictly a "treatment" strategy, watch and wait (WW) is recognized as a clinical management option for inactive cysts. Its rationale derives from the demonstration of the scarce reactivation rate of cysts that are found to be spontaneously inactive, or that have been treated according to the WHO-IWGE recommendations[110–113]. A limitation of currently published studies is relative to the fact that these are based on patients seen at referral centers for CE and the number of patients is not as high as one would want it to be to generate conclusive evidence. Generating data from hyperendemic countries is also hard, as in these settings surgeons tend to dominate the treatment of CE[108]. However, WW has been shown to avoid unnecessary treatment for patients, reducing risks and saving precious resources for health systems, as surgery for CE has been shown to be a costly procedure[114].

Hints for a watch and wait approach have been also pointed at by a work considering CE3b cysts. In this paper, the authors concluded that CE3b cysts had an indolent behavior after receiving medical treatment, with the development of a low number of complications. The authors then suggested that a WW approach for CE3b cyst could be implemented in selected cases in which surgery is not strictly indicated and that are compliant with medical therapy and a subsequent long-term follow-up. Data from other centers have not been generated on this approach.

PART II – Work carried out during the PhD program

. Most of my activity revolved around clinical management of CE patients including those with unusual locaations of CE such as bone CE. I also carried out some experimental studies on the possible role of miRNAs as diagnostic markers for CE

in collaboration with Dr Mara Mariconti, a member of our group who also won an European Society of Clinical Microbiology and Infectious Diseases research grant for young investigators. This has led to a field project on the use of RDTs as confirmatory tests in the diagnosis of CE, whose results will be illustrated here.

Other studies have used the data that our group has gathered over thirty years including experience with inactive cysts, with cysts in contact with the IVC and with splenic lesions suspect for CE.

Other two publications I authored deal with the consequences of shortages in Albendazole availability for CE patients in Italy and data from the Kazakh national registry of CE cases illustrating the incidence trends of CE in this hyperendemic country.

I have also written a review regarding research on CE in the Mediterranean region.

I also worked on projects unrelated with CE, whose results will be presented in the third section of this doctoral thesis.

1) Cystic Echinococcosis in the Mediterranean

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1.1) Abstract

Purpose of review: Cystic Echinococcosis (CE), a zoonotic disease caused by E. granulosus sensu lato is endemic in the Mediterranean, where pastoral activity is widespread, as the life cycle of this helminth involves sheep, as well as other livestock, as intermediate hosts. We review recent studies on CE from Mediterranean countries.

Recent findings: Reliable data on CE, from human and animal epidemiology to treatment, remain fragmented and insufficient to gauge the magnitude of the problem beyond local communitie. No major advances were seen the evidence base on which to base sound clinical decision-making. Summary: Despite a wealth of publications on the subject, CE remains a neglected disease also in the Mediterranean.

Conclusions: Hope is seen in the establishment of a European Registry for Cystic Echinococcosis, but implementation and maintenance of such an important tool will require hard work, political commitment and resources, monetary and otherwise.

Keywords: Cystic Echinococcosis, Epidemiology, Clinical Management, Mediterranean.

1.2) Introduction

Cystic echinococcosis (CE) and Alveolar Echinococcosis (AE) are two zoonotic diseases caused by tapeworms belonging to the Echinococcus genus. CE is a cosmopolitan zoonosis present in countries across

all continents excluding Antarctica, whereas AE is limited to the northern hemisphere [1]. Human CE affects an estimated 1.2 million people worldwide, with 1 to 3 million disability-adjusted life years (DALYs) lost globally every year, although these figures are likely to be underestimated [1–3]. E. granulosus sensu lato (sl), the causal agent of CE, develops it cycle between dogs and other canids (definitive hosts) and livestock, especially sheep (intermediate hosts), with humans as an accidental intermediate host. In humans, the parasite develops in its metacestode stage, forming cysts in organs and tissues, mainly in the liver. CE is mostly endemic in rural areas where sheep (and other livestock) raising is practiced, such as central Asia and China, South America and Mediterranean countries [1]. In 2012 a joint FAO/World Health Organization (WHO) expert group classified E. granulosus second among the top eight ranked food-borne parasites of global public health importance. Here we review studies on human and animal CE carried out in the Mediterranean countries in the last three years.

1.3) Materials and Methods

We performed a PubMed (MEDLINE) search for articles on CE published in English and French between 1st of January 2014 and the 30th of May 2017 reporting data from any of the following countries the Mediterranean region. The following search string was applied: (Cystic Echinococcosis OR E. granulosus) AND (Mediterranean OR Italy OR France OR Spain OR Algeria OR Morocco OR Bosnia Herzegovina OR Slovenia OR Egypt OR Syria OR Montenegro OR Albania OR Greece OR Turkey OR Cyprus OR Lebanon OR Israel OR Palestine OR Libya OR Malta OR Tunisia). We included original papers on CE epidemiology in humans, CE epidemiology in animals, molecular biology, clinical management. Review articles, case reports, case series considering less than 15 patients and studies carried out in countries outside the

Mediterranean region were excluded. (**Figure 1**). 90 records matched our inclusion criteria.

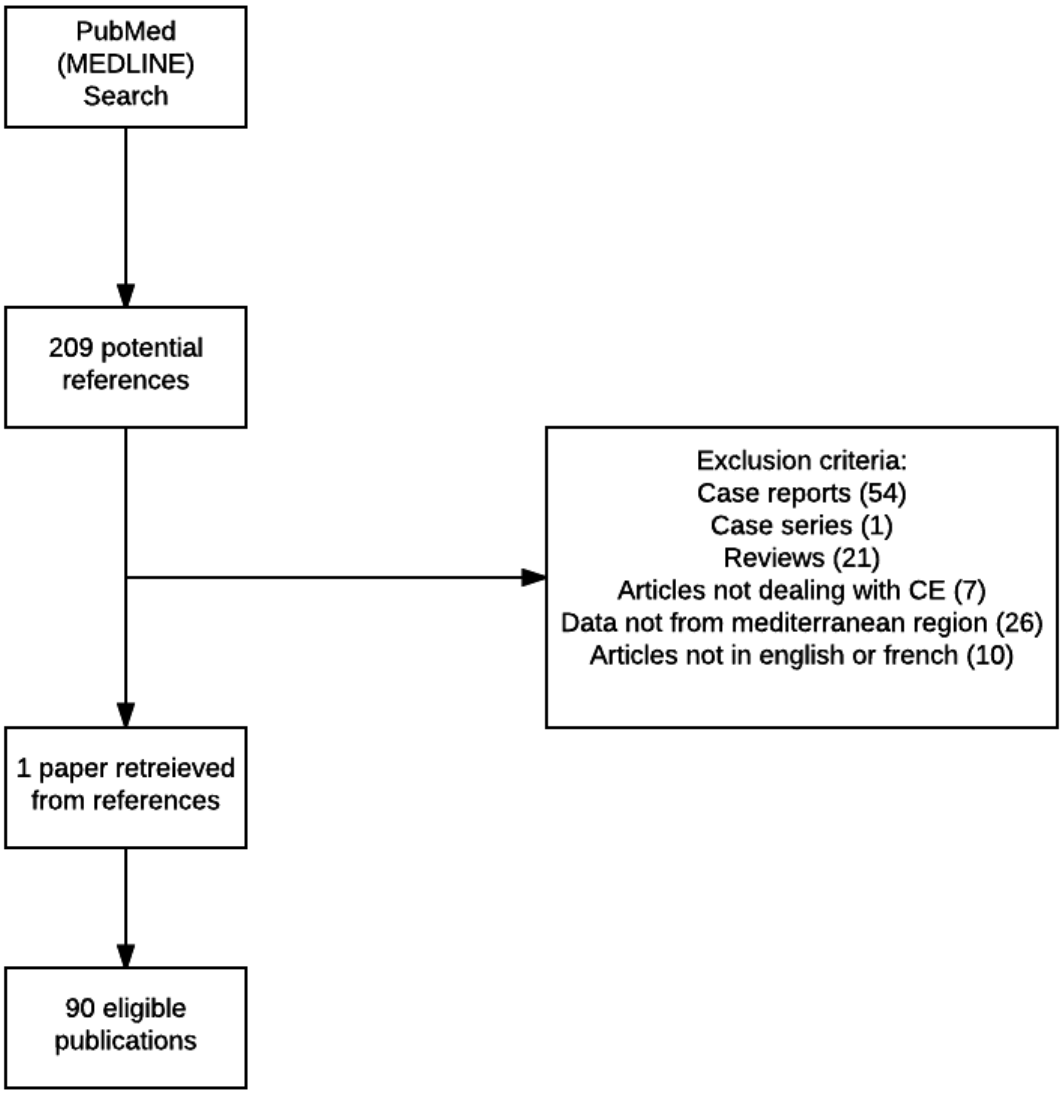

Figure 1 – Flow diagram detailing the publication inclusion process.

1.4) Risk factors for CE.

Among the articles who dealt with risk factors for infection with E. granulosus, two reported data from public health surveys in Morocco, [48,115] one assessed the level of knowledge about CE and the prevalence of risk factors in an endemic, rural area of the country and found that the local population had a scarce knowledge of the disease and of its risk factors, and that 67% of respondents fed dogs ruminant offal deemed

unfit for human consumption [48]. A similar lack of awareness on CE was found in the other study examining the social factors influencing the transmission of the disease including the role of markets and abattoirs [115]. The faulty management of abattoirs can favor transmission as state regulations, perceived as economically unfavorable, are not followed [115]. Dogs were seen gathering around slaughterhouses to feed on the remnants of the carcasses [115]. A similar situation emerged from a survey of abattoirs conducted in Algeria, where 42% of the slaughterhouses allow free access of dogs to fresh offal [49]. However, a study conducted in Tunisia on E. granulosus eggs present in soil found no difference between the contamination rates in sites close to slaughterhouses and far from them [116]. The authors suggested that behavior of dogs may be more important than the location of the sources of infection [116]. A study of an educational intervention on neglected zoonoses targeting the general population in Morocco [50] showed that although the intervention increased the awareness of the disease in target communities, target interventions for CE still need the surveillance on the pathogen in intermediate and definitive hosts to be effective [117]. A study from Turkey on the prevalence of risk factors among family members of breeders tried to correlate the presence of risk factors with serological results [61]. They found a positive serology for CE in 6.9% of the 1113 individuals screened, with a statistically significant association between the presence of CE and two age groups (30-39 and >60) and with the lack of routine veterinary supervision on animal health. Contact with dogs or ownership of cattle or sheep was not associated with a positive serology [61]. The significance of these findings should be considered with caution, given the limits of seroprevalence studies (see below). Moreover, these findings do not concur with those of another study carried out in Morocco, which found out that the ownership of dogs was significantly associated with the presence of CE cysts in patients screened by ultrasound. This study also found a borderline association with the ownership of cattle [54].

1.5) Burden of human CE.

We found thirty articles on the epidemiology or burden of human CE. Among seven articles from Spain, one compared a cohort of 16 non Spanish patients with 534 Spanish patients and found a higher incidence in the latter group [118]. The incidences were calculated taking the total Spanish and migrant population of Salamanca as references. The authors explain this difference with the fact that in Spain immigrants tend to seek medical advice less frequently than natives [118]. Another study found the mortality of CE to be of 3.1 cases per 100.000 inhabitants [119], while a paper on a cohort of 76 patients in a single center did not find any case of CE

related mortality [120]. Several studies in our review used Hospital Discharge Records (HDR) as a source of data to study CE epidemiology. HDRs provide information on the cause of the hospitalization defined according to the International Disease Classification. In Spain a study using the national HDR database and a study using HDRs in the Castilla – Leon region showed a reduction of the number of cases of CE in recent years, as did a single center study from Salamanca [121–123]. These studies noted that data from the official notification system showed fewer cases than those recorded in the studies themselves [121,122]. Conversely, three regions in Spain showed an increase in the number of reported cases over a 16 years' timeframe [121]. The seventh study from Spain considered the economic impact of CE on a single Province [124]. Estimates included in this study considered losses due to decreased production of meat and milk on the animal side, while the costs derived from health care expenses and the loss due to decreased productivity were considered for human estimates. Total estimated losses ranged from € 61,864 to € 360,466, with human associated costs accounting for 57% to 97% of the total expenses depending on the simulated scenario [124].

Among six papers published in Italy, one investigated the cost of surgery for abdominal CE in a single center and found the mean cost of an hospitalization for an abdominal surgical intervention to be 11,033 € [114]. Another article from Italy on Neglected Tropical Diseases in non-Italian patients showed that CE was more frequent in immigrants than in Italian patients [125]. A study of HDRs in Italy showed a mean number of 1379 hospitalizations per year, with most records being registered in southern and central Italy. Data from this study also showed that an official surveillance system is needed in Italy, as currently no notification based system is active [56]. This study also reported that a high number of hospitalizations to treat patients with CE were carried out as Day Hospital for medical treatment, which represents, to say the least, a waste in resources as medical therapy does not need supervision in the hospital [57]. In a study using HDRs in France, the overall number of cases were inferior to those of Italy and Spain [126]. While there seems to exist a reduction in the number of cases over time, the authors point out the persistence of autochthonous transmission, with possible high levels in Corsica [126]. Given the high number of immigrants coming to France from countries in the Maghreb where CE is endemic, the reported average 0.42/100.000 incidence rate is most likely underestimated. Moreover, HDR only account for CE patients admitted to hospitals to be treated for CE, with asymptomatic patients and those managed in an outpatient setting escaping this analysis [57,127]. A study analyzing hospital records from a single center was also published in Israel, with the majority

of cases occurring in nomads coming from the southern part of the country [128]. Other papers analyzed the epidemiological characteristics of patients seen in single centers in Turkey [129–133]. These studies showed the presence of a high number of patients, but they did all suffer the limitations of retrospective, single center studies. A systematic analysis of national data from Turkey would greatly help in clarifying the disease burden in the country.

Several studies in our review focused on the seroprevalence of CE [60,61,134,135].

Unfortunately, serology tells nothing about the presence of CE as serological tests present a high variability in results due to the interaction between test sensitivities and specificities and the fact that the performance of serological tests as tools for screening depends on the underlying prevalence of disease [136].

It is quite depressing that almost forty years from the publication of the seminal work by Macpherson and colleagues [66] this message has not come across the scientific community

Ultrasound is the only tool we have that can give us information about presence, location, stage size of cysts and be portable at the same time, and this is why over the years screening programs have been increasingly been carried out to assess CE prevalence in selected populations.o

A recent ultrasound study assessed the prevalence of CE in two endemic provinces in Morocco using portable ultrasound [54]. Such surveys can provide a more precise estimate of the infection prevalence compared to hospital based records and be the occasion to inform local health authorities and population on the existence of CE and its health implication. In this study most CE patients were asymptomatic and most cysts were inactive. However, the study also demonstrated an ongoing transmission of CE in the studied areas, as shown by a prevalence of 1.9% found by Chebli and colleagues, compared to 1.1 % reported in an earlier study [54,55].

Besides ultrasound studies, the many gaps in the official systems of data collection have prompted the set up of CE registries. The first such registry implemented was the Italian Registry of Cystic Echinococcosis (RIEC), an Italian national registry launched in 2012 that was then expanded into European Registry for Cystic Echinococcosis ERCE [58,59].

ERCE was launched in 2014 in the context of the HERACLES project (www.heracles.fp7.eu), an FP7 founded project that is still running at the time of this writing and that, among other things, addresses the epidemiology of CE on an international scale. Interestingly, data from ERCE published in 2016 showed that the number of cases present in the registry outnumbered cases reported through institutional channels already put in place by the European Centers for Disease control (ECDC)[58]. This confirms that the epidemiological figures of CE in Europe and in the Mediterranean region are vastly underestimated.

1.6) CE in animals.

Classical epidemiological studies on prevalence of CE in intermediate hosts are based on necropsy of slaughtered animals in abattoirs. One study carried out in Sardinia on animals tested after the drop of control measures saw a decrease in the number of cysts and an increase in cyst fertility [44]. The authors also proposed a morphological classification of cysts to recognize fertile cysts and monitor the level of parasite pressure in a control perspective [44]. Other studies have also assessed CE prevalence in other hosts and countries. Results from recent surveys are summarized in **Table 1** [44,137–139]. One article concerning the prevalence of infection by intestinal parasites in dogs examined in Crete found that shepherd dogs harbored taeniid eggs at a higher prevalence than household dogs [140]. However, no molecular methodology has been employed to identify the exact taeniid species of the eggs.

Sample origin	Host species (n)	Animal n	N infected (%)	Fertility rate	Article
Sardinia (1st Survey)	Sheep	1375	1029 (74.84%)	7.9%	Conchedda M. et al. [44]
Sardinia (2nd Survey)	Sheep	1414	916 (64.78%)	10%	
Corsica	Pig	2527	149 (5.9%)	30%	Uhmang G. et al. 2014 [137]
	Wild Boar	101	4 (4%)	25%	

	Cattle	2431	0 (0%)	0%	
	Sheep	5970	0 (0%)	0%	
Egypt	Water Buffaloes	120	5 (5.2%)	14.8%	Abbas I. 2016 [138]
Greece	Sheep	898	271 (30.2%)	64.5%	Chaligiannis I. et al. 2015 [139]
	Goats	483	38 (7.86%)	3.2 %	
	Buffaloes	38	16 (42%)	7.9%	
	Wild Boars	273	3 (1.1%)	0%	
	Deer	15	(0) 0%	0%	
Tunisia	Donkey	2010	173 (8.48%)	4.77%	Lahmar S. et al. 2014 [141]

Table 1 – Summary of data presented in articles concerning epidemiological surveys of cysts in intermediate hosts.

Ultrasound of cysts in intermediate hosts has also been proposed. Ultrasound in sheep had 83% sensitivity and 100% specificity in one study [142], and 88.7% sensitivity and 100% specificity in a second study [143]. Ultrasound was compared with necropsy examination and performed better than in previous reports. Dore and colleagues suggest that the integration of Ultrasound in programs trying to detect CE in sheep would allow for the culling of infected sheep before these reach the abattoirs, thus diminishing the possibility of disease transmission to canids [142]. However, this procedure appears to be difficult to implement in endemic areas. A Turkish team studied a small cohort of animals in which the total antioxidant capability (TAC) of lambs and sheep with CE was reduced compared to that of healthy subjects and suggested using ultrasound with the measurement of TAC for CE diagnosis. This seems difficult to reproduce on larger cohorts in endemic areas [144].

Another study looked at Rattus norvegicus as a potential intermediate host for CE [145], suggesting that rats can harbor fertile CE cysts and thus may contribute to the perpetration of the parasite life cycle.

Geospatial tools are another way to study prevalence in animals [43,146]. The authors of these paper use data from systems tracking the presence of zoonotic infections in cattle to spot foci of CE transmission. One survey using geospatial analysis tools assessed the prevalence of infected animals in dairy farms in north eastern Italy and found that, despite the fact that the region is considered hypoendemic, three clusters of transmission were present [146]. A similar approach was also used in Sardinia [43].

1.7) Genetic diversity

Molecular studies can supplement classical epidemiological data on CE epidemiology in the Mediterranean. Mitochondrial markers can differentiate E. granulosus into 9 genotypes: E. granulosus s.s. (G1-G3), E. equinus or "horse strain" (G4), E. ortleppi or "cattle strain" (G5) and E. canadiensis (G6-8 and G10), further divided into a "camel strain" (G6), a "pig strain" (G7) and two "cervid strains" (G8 and G10) [67]. We found 18 studies on the genetic diversity of E. granulosus using samples originating from countries in the Mediterranean region and coming from several hosts. Our findings are summarized in **Table 2**.

The G1 genotype is the most frequently isolated in the region and is responsible for 88% of the E. granulosus infections worldwide[67]. A study considering only G1 samples from different hosts [67] has shown that no host-specific substructure exists in the G1 genotype, as samples from different hosts were genetically close to each other. As the authors state, a high genetic diversity suggests a demographic expansion of the studied population and could constitute the background for the association of the G1 genotype with new species if the diffusion of the genotype in Europe widens, as well as pose the basis for the development of drug resistance [67].

Another study on phylogeny of E. granulosus considered both the classical mitochondrial markers and two nuclear markers[70]. The authors analyzed samples of E. granulosus sensu stricto (ss) originating from Mediterranean and other regions and concluded that when also nuclear markers are taken into account the G1 and G3 genotypes should be treated as different species. Their analysis of data from the sole mitochondrial markers also suggests that the G2 genotype should no longer be considered a distinct genotype. These findings need confirmation by further work [70].

Sample origin	Host species	Genotype	Article

Tunisia	Donkey	G4 and G1	Lahmar S. et al. 2014 [141]
Italy	Wild Boar	G3	Di Paolo A. et al. 2017 [147]
France	Pigs	G6-G7	Umhang G. et al. 2014 [137]
	Cattle	G6-G7	
	Wild Boars	G6-G7	
Egypt	Camels	G6	Yoshra AE. et al. 2015* [148]
	Pigs	G6, G7	
	Humans	G6, G7, G1	
Greece	Sheep	E. granulosus ss	Chaligiannis I. et al. 2015 [139]
	Goats	E. granulosus ss	
	Buffaloes	E. granulosus ss	
	Wild Boars	E. granulosus ss	
Albania	Sheep	G1	Kinkar L. et al 2016* [67]
Finland (patient from Algeria)	Humans	G1	
Greece	Humans	G1	
Italy	Cattle, Sheep	G1	
Spain	Sheep, Human, Wild Boar, Pig, Goat	G1	
Turkey	Cattle, Sheep	G1	
Turchia	Donkeys	G4	Simsek S. et al 2015 [149]
Italy	Wolf	E. granulosus ss	Gori F. et al. 2015

			[150]
Italy	Wolf	E. granulosus ss	Poglayen G. et al. 2017 [151]
Egypt	Buffaloes	G1	Abbas I. 2016 [138]
Greece	Sheep	E. granulosus ss	Roinioti E. et al. [152]
	Goats	E. granulosus ss	
	Cattle	E. granulosus ss	
Albania	Sheep	G1	Kinkar L. et al 2017* [70]
Finland (patient from Algeria)	Humans	G1	
Tunisia	Sheep	G1, G3	
France	Cattle, Sheep	G3	
Spain	Sheep	G1, G3	
Turkey	Cattle, Sheep	G1, G3, G4	
Libya	Dog	-	Boufana B. et al. 2015* [47]
Tunisia	Dog	-	
Palestine	Dog	-	
Tunisia	Wild Boars	E. granulosus ss	Boufana B. et al. 2014* [153]
	Camels	G1, G6	
	Cattle	G1	
	Goats	G1	
	Donkey	G1, G4	
	Sheep	G1	
	Dogs	-	
	Jackals	-	

Turkey	Human	E. granulosus ss	Bakal U. et al. 2015 [149]
Italy	Cattle	G1	Scala A. et al 2017 [43]
Algeria	Camels	G1, G6	Zait H. et al. 2016 [154]
	Cattle	G1	
	Goats	G1	
	Sheep	G1	
	Human	G1, G3, G6	

Table 2 – Summary of studies exploring the genetic diversity of E. granulosus in the Mediterranean area. Studies marked with a * include also samples not coming from the Mediterranean region.

1.8) Diagnostic serology

The diagnosis and management of CE relies on ultrasound with serology as a complementary tool. Its reliability is hampered by several factors, including the cyst size, stage and number, which influence serological results [155]. Research efforts have been trying to improve this situation by testing the role of new antigens in serologic tests for CE [135]. Current serological tests are based mainly on crude hydatid cyst fluid (HCF), or purified/enriched antigens obtained from cyst fluid. [22]. Overall, the diagnostic performances and reproducibility of results are not satisfactory due to the lack of standardized antigen preparations and to the poor sensitivity and specificity of the antigens [22]. Among the recent studies included in this review, a new assay using of Antigen 5 was described, with encouraging results [23,156]. Antibody tests based on P-29 and 2B2t antigens were proposed as useful for the serological follow – up of patients after surgery, however they became negative after surgery in most patients, but were not sensitive to detect recurrences [89,90]. Recently, the performances of Rapid Diagnostic Tests (RDTs) were also evaluated [91,157,158].

1.9) Imaging

A single study from Turkey used diffusion weighted MRI to differentiate CE1, CE2 and CE3 cysts from CE4 and CE5 cysts and found a of 75.9% - 87% and a specificity of 86.8% - 89.5% depending on the MRI

settings [159]. However, ultrasound is best at staging CE in the liver [87] and a far less expensive, repeatable more often than MRI [110,160].

1.10) Biological markers.

Research efforts have been aimed at discovering new markers of biological activity for CE cysts, as summarized in [161], as currently available serological markers unreliable for diagnosis and follow-up. After one study found that a Th2 immune response is dominant in patients with active cysts [67], an Italian group recently suggested that the production of IL-4 by white blood cells stimulated with an immunodominant antigen of E. granulosus could correlate with cyst activity, but the authors also admitted that a larger study was needed to provide evidence [15]. Another study. T regulatory cells also seem to play a predominant role in ovine CE [162]. A study considering a small group of mice infected with CE and breast cancer cells found out that a decrease in Th1 immunity led to an increase in the frequency of cancer metastasis, but this association has never been described in humans [163]. Serum sHLA-G levels were also found to correlate with CE activity, although this molecule would not be a suitable marker in individual patients [164]. Recently, the existence of parasitic exosomes of E. granulosus was also confirmed by a Spanish group [19]. The study of exosomal proteins could provide new markers for the detection of CE in humans and animals. A study by a team from Algeria also reported that proteome variations could be observed in cysts with different localizations [165], although only three patients were considered in this study.

1.11) Studies on clinical management

The clinical management of CE is complex [5]. Currently, treatment options include percutaneous treatments, surgery, the use of benzimidazoles and a watch and wait approach. The approach to clinical decision making should be guided by an ultrasound-based staging[11].

1.12) Drug Therapy

Benzimidazoles are currently the only available drug class to treat this disease, but they are only parasitostatic. This has led over the years to the search for more efficacious molecules or formulations [5,166]. Recently, a new albendazole salt has been patented [167,168]. The It has been suggested that the laminated layer of echinococcal cyst could trigger the induction of arginase and therefore NO production to modulate the

response from the host macrophages [169]; building on this finding, a group from Algeria published three papers on the anthelmintic effects of 6-gingerol, Punica gratum and Allium sativum extracts, to investigate their influence on NO production, but, even if confirmed, clinical applications are still very distant [170–172].

1.13) Percutaneous treatments

Studies on Percutaneous Treatments (PTs) have recently been published by Turkish teams. These studies have been testing catheter based techniques or variations of the Puncture, Aspiration, Injection, Re-aspiration (PAIR) technique and showed that the techniques are safe if carried out with the proper infrastructure and expertise [173,174]. Among the studies found in our review, one tested a variation of the PAIR technique in which the Re-aspiration step was not performed (PAI) on 25 patients [175]. This, in our opinion, is dangerous as permanence of scolecidal agent in the cavity increases the risk of chemical cholangitis, a dreaded complication of this technique. Further, the authors claim they treated also CE4 cysts, which are inactive and solid, and their follow-up was too short to conclusively evaluate the effectiveness of the procedure (12 months).

Another study explored PAIR in an outpatient setting and found that this technique can indeed be used without hospitalization. This is potentially interesting for low resource settings but given that only 33 cysts were treated in this way, larger studies are needed to confirm its safety [176]. A study proposed the Ormeci technique, an alternative to PAIR where the patient is treated in an outpatient setting with the scolecidal agent being left in situ with the addiction of polidocanol to prevent biliary fistulae, and does not receive benzimidazole derivatives to prevent relapses. This proposal appears debatable, for the same reasons outlined above and given that the author himself admits that treatment for recurrences would be needed on an "as needed" basis [106].

Interestingly, some of these studies used PTs on CE3b and CE2 cysts, which are currently not indicated for these two cyst types[109,175]. However they are retrospective and do not compare PTs with surgery. Prospective trials are needed to assess the efficacy of PTs in the treatment of CE3b [109] and CE2 cysts. A Watch and Wait

approach has been used for CE3b cysts in selected conditions [88], although once again the study was retrospective.

A study from a single center stated that in complicated cysts, surgery is superior to PTs [177], one paper tested ERCP in patients with biliary fistulae and found this method to be safe and effective [178]. These indications are well known [11].

A study from Egypt found that gamma radiation induced the apoptosis of E. granulosus metacestodes, but radiation therapy has provided unsatisfactory results in other studies [179,180].

Lastly, a team of surgeons from Morocco proposed a systematic approach for the management of cystobiliary fistulae in the treatment of CE cysts, which reduced the rate of complications and the duration of hospital stay, leading to a reduction in costs [181]. However, this strategy needs to be tested in larger, multicentric studies [181].

1.14) Conclusions

Many studies have been published on CE in the Mediterranean basin in the last 3 years. Unfortunately, the vast majority does not contribute any significant advancement in the areas surveyed by our review. Some hope can be found in initiatives aiming at providing in a systematic and prospective way the missing data on cyst evolution, either spontaneous or after different management approaches, in the countries surveyed, such as ERCE, but the obstacles to continuing data collection are daunting and coordinated efforts will be needed to keep the project going in the future. As long as clinical management remains hampered by the well-known lack of evidence base and short of costly randomized, prospective studies, the only advances in this area can come from incremental improvement obtained in referral centers with a large enough case load.

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2) Shortage of albendazole and its consequences for patients with cystic echinococcosis treated at a referral center in Italy

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2.1) Abstract

Albendazole (ABZ) is the best drug available to treat cystic echinococcosis (CE), a neglected tropical disease. CE patients often receive a continuous course of the drug for 6 to 12 months. In Italy, ABZ shortages occur almost on a yearly basis. We searched clinical records at the World Health Organization Collaborating Centre for the Clinical Management of CE (WHO CC) in Pavia, Italy to estimate the amount of ABZ prescribed to patients between January 2012 and February 2017. The cost of ABZ was estimated at 2.25 € per tablet based

on the current market price in Italy. Patients to whom ABZ had been prescribed were contacted to determine if they had experienced difficulties in purchasing the drug and to assess how such problems affected their treatment. Out of 348 identified CE patients, 127 (36.5%) were treated with ABZ for a total of 20,576 days. This led to an estimated cost of 92,592 €. Seventy-five patients were available for follow-up, 42 (56%) reported difficulties in obtaining ABZ. Of these patients, 4 (9.5%) had to search out of their region and 10 (23.8%) had to go out of the country. A total of 27 patients (64%) had to visit more than five pharmacies to locate the drug and 10 patients (23.8%) interrupted treatment due to ABZ non-availability. Shortages in ABZ distribution can disrupt CE treatment schedules and jeopardize patient health.

Keywords: Cystic Echinococcosis, Albendazole, Drug Shortage.

2.2) Introduction

Cystic echinococcosis (CE) is a zoonotic disease caused by the metacestode of a tapeworm from the E. granulosus sensu lato complex[6]. The parasite has a life cycle comprising a definitive host (dogs or other canids) and an intermediate host (sheep or other ungulates)[1]. Humans are accidental intermediate hosts, where the parasite larvae develop as cysts mainly in the liver and lungs[8]. CE has a high socioeconomic impact. Infection in humans causes estimated mean yearly losses ranging from 193,529,740 to 763,980,979 US dollars, and from 88,082 to 1,590,846 Disability Adjusted Life Years (DALYs), respectively, when underreporting is not assumed or is assumed[2,182]. Nevertheless, CE remains a neglected disease with little attention and funds devoted to research focusing on the clinical management of patients[5].

Human CE can currently be treated by surgery, percutaneous procedures, and medical therapy with benzimidazole derivatives. For inactive cysts, a watch-and-wait strategy can be adopted (**Figure 1**). However, the evidence base that should guide choice of treatment is poor due to lack of prospective, randomized studies[11,183]. Benzimidazole derivatives, especially albendazole (ABZ), are recommended for CE1 and CE3a cysts of the liver. However, unlike the vast majority of other helminthiases for which ABZ is used, long-term administration is needed for CE, ranging from 3 months to over 12 months in particular cases[5,11,166]. Here we

report on difficulties in the clinical management of CE patients treated at an Italian referral center due to the current shortage of ABZ.

2.3) Materials and methods

Patients

Patients recorded in the European Register of Cystic Echinococcosis (ERCE)[58] were included and seen at the World Health Organization Collaborating Center for the Clinical Management of CE (WHO CC) in Pavia, Italy from January 2012-February 2017 where they were prescribed ABZ. All CE patients were eligible for inclusion regardless of cyst location, cyst stage, or clinical severity. Demographic data and information on ABZ intake, including total duration of therapy and treatment scheme, were extracted from the ERCE.

Patient interview process

Eligible patients were contacted by phone or e-mail, or filled a questionnaire during follow – up visits to determine if they had experienced difficulties in acquiring ABZ. Interviews and e-mail exchanges with patients were conducted by two investigators (TM and AV). Each patient was administered a four-item questionnaire, inquiring: 1) whether purchase of the drug was perceived as difficult; 2) whether the patient had to visit more than five pharmacies to obtain the drug; 3) whether the patient had to search for the drug out of his region of residence or outside of Italy, and 4) if the patient had to interrupt treatment due to being unable to acquire the drug. A treatment interruption was defined as having to interrupt drug administration for at least three consecutive days.

Cost estimation

The official 2017 cost of ABZ in Italy obtained from the pharmacy of the San Matteo Hospital Foundation, Pavia is 2.25 € per 400 mg tablet. Costs deriving from ABZ use were estimated considering individual patient treatment duration recorded in the ERCE at a standard administration dose of 400 mg ABZ twice a day.

2.4) Ethical approval

This survey was conducted using patient data from the ERCE. At the time of registry enrollment, all patients provide written informed consent for their information to be used for research purposes. In addition, all patients are asked to provide consent to be contacted at a later date for research purposes. Prior to completing the

questionnaire, patients were again asked for consent to participate. Research-related use of the ERCE has been approved by the IRCCS San Matteo Hospital Foundation Ethical Committee.

2.5) Results

Of the 348 patients with CE registered by our center during the study period, 127 (36.5%) were prescribed ABZ for a total of 20,576 days. Ten patients (7.8%) were treated for extra-hepatic localizations including osteomuscular CE (n=4, 3.1%), CE of the lungs (n=5, 3.9%), the spleen (n=2, 1.6%). The median duration of treatment was 90 days (range 15 to 840 days) and the total estimated cost of medical treatment was 92,592 €. Eighty patients (63%) were treated solely with medical therapy for a median of 180 days (range 15 to 630 days). Thirty patients (23.6%) were treated surgically and they all received ABZ for a median of 30 days after surgery (range 28 to70 days). Fifteen surgical patients also received additional cycles of ABZ for a median of 180 days (range 30 to 570 days). Seventeen patients (13.4%) were prescribed ABZ after percutaneous procedures and took the drug for a median of 90 days (range 30 to 240 days). Of these patients, six took an additional course of ABZ for a median of 135 days (range 30 – 570). In addition, one patient (0.8%) was initially treated with a percutaneous procedure, but later required surgery and was also treated with ABZ for 570 days. We attempted to contact all 128 patients who had received ABZ as part of their management strategy. Out of 75 patients who were reachable by phone or e-mail and answered the questionnaire, 42 (56%) reported difficulties in obtaining ABZ. Of these patients, 4 (9.52%) had to look for ABZ out of their region and 10 (23.8%) had to go out of the country. A total of 27 patients (64%) had to visit more than five pharmacies to locate the drug and 10 patients (23.8%) interrupted treatment due to ABZ non-availability.

2.6) Discussion

Currently, four clinical management options are available for the management of CE, depending on the affected organ and cyst size: medical therapy with benzimidazoles, surgery, percutaneous techniques such as catheter-based procedures or the Puncture Aspiration Injection Reaspiration (PAIR) technique[96] and its derivates[83,106,175], and watch-and-wait[11,112]. Patients receiving surgery or percutaneous procedures for hepatic CE should receive ABZ from the day of the intervention to one month post-intervention to prevent secondary echinococcosis due to the dissemination of parasitic material[11].

Mebendazole (MBZ) became available for human use in the mid-1980s[101], with ABZ soon to follow. Since this time, no new drugs have been introduced to treat CE. Benzimidazoles appear to act on the parasite by

blocking beta tubulin formation[101,110], and are mainly parasitostatic[98,184], with ABZ showing better efficacy than MBZ[185]. Long-term outcome of medical therapy, however, depends on many variables, including the size and, most importantly, the stage of the cyst. For example, medical therapy is largely ineffective on CE2 or CE3b cysts, while better results are achieved with small CE1 and CE3a cysts [11,88,186] (**Figure 1**).

There are problems associated with the use of ABZ to treat CE. When first introduced for the treatment of CE, ABZ was administered with therapy interruptions, with cycles composed of 28 days of treatment followed by 14 days without treatment. This was because of limited long-term toxicity data available at the time, with early suspicions that long-term ABZ use was linked to tumor formation in animals[101]. However, it has subsequently been established that the drug can safely be used with continuous administration[101,185,187]. The optimal duration of therapy has not been formally assessed, but therapy duration generally varies between three and six months for abdominal CE [11,101,185,186,188], and in some cases lifelong administration has been used in the management of particular cases, such as bone infection[189,190]. Despite expert opinion that this treatment regimen is safe and carries a small risk of side effects [11,101,183] most countries, including Italy, still mandate a maximum of three treatment cycles of 28 days, separated by 14 days without treatment[101,183]. However, in many centers today, an adult patient is prescribed two 400 mg tablets of ABZ each day, meaning that they will need 60 tablets for one month of treatment. At present, this is still considered off-label use of the drug. In the last few years, the situation in Italy has been complicated further by the intermittent availability of ABZ.

ABZ has been on the official list of drugs with a shortage in Italy from the end of 2016 to February 2018 [191]. Shortages have also been anecdotally reported from several other countries [183], which has resulted in a WHO initiative to conduct an international survey investigating the drug's use and availability on an international scale[192]. The current study is the first to present data on the impact of ABZ shortages on CE patients. Although less effective, MBZ can also be used for the treatment of CE,[184,185,187,193] but it is often not available [183] and its price can be prohibitive[194]. In Italy, ABZ has been only intermittently available over the last 5 years and, since September 2017, availability has been extremely limited. The Italian National Drug Agency (Agenzia Italiana del Farmaco – AIFA) and the drug's producer (GlaxoSmithKlyne) have stated that the drug shortage has been caused by problems with drug production[191]. To make matters worse, while packages

containing sixty ABZ tablets are available in many other European countries, only three tablet packages are currently available in Italy. This is because ABZ production is being targeted at treatment of soil transmitted helminthiasis. This makes filling prescriptions and complying with the full course of therapy difficult for patients since pharmacies typically only stock a limited number of boxes of each drug[195].

The non-availability of drugs is a problem with which health systems throughout the world constantly struggle. This issue is not specific to tropical medicine, but can also impact other specialties, ranging from oncology to anesthesiology[194,196–199]. Ideally, health systems should work with the private sector to minimize the impact of drug shortages on patient care, but this is not always possible [197]. Moreover, the distribution of drugs between countries can be difficult, since permitted uses for each drug vary from one country to another. Due to the intermittent non-availability of ABZ in Italy during the last five years, patients followed in our center had substantial difficulties in receiving the correct treatment. In the current study, 56% of surveyed patients perceived the process of drug acquisition as being difficult. This difficulty was confirmed by the fact that a comparable proportion of patients had to visit more than five pharmacies to access a drug supply and that many patients had to locate the drug outside their home region or the country.

The lack of access to ABZ has been recognized as a key problem by the WHO-Informal Working Group on Echinococcosis[183]. While CE is typically a benign condition and most patients are asymptomatic at the time of diagnosis[11], medical treatment can be crucial, whether it is carried out as prophylaxis for secondary CE after invasive procedures or as a treatment[5,166]. Patients seen in our center and treated with medical therapy alone are prescribed ABZ for 3 to 6 months, with continuous administration, while patients who undergo surgery or percutaneous procedures receive a 1-month course as prophylaxis of secondary CE, which can be prolonged on the basis of ultrasound characteristics and percutaneous treatment type[83].

The total cost of medical treatment for patients followed in our center in the last five years was 92.592 €, using Italian market prices for ABZ. The Italian health system covers most of the costs of treatment, including medication costs, with younger, healthier, and wealthier individuals asked to cover part of their healthcare costs. While CE is undoubtedly a chronic pathology requiring follow-up, even in the case of inactive cysts [5,112], only the Piedmont region of Italy exempts patients from paying all CE-related expenses. Interestingly, Piedmont is not a highly endemic area for CE.

This study is part of a continuing effort to assess the burden of CE on health systems. We have previously reported on the costs of surgery for CE, showing that an intervention has a mean cost of 11,003 €, with some surgeries resulting in much higher costs[114]. ABZ can be used to treat patients who do not need surgery (**Figure 1**). However, overtreatment with surgery is a well-known problem for patients treated in non-referral centers[54]. ABZ is also used for prophylaxis of relapses and secondary CE after surgery or percutaneous procedures. The benefit of this practice has been shown in animal models[200]. As secondary CE can be severely disabling, [201] and relapses can require further surgeries, the cost of prophylactic ABZ can be justified by its long-term benefits.

The non-availability of ABZ in Italy can seriously compromise patients' treatment, as 23% of our cohort had to interrupt treatment for this reason. This difficulty is compounded by the fact that ABZ cannot be prescribed in Italy as a continuous treatment without being considered an off-label prescription. When ABZ is not available in Italy, importing the drug can be problematic if prescribed for off-label usage since the AIFA has ruled that imported drugs cannot be prescribed for off-label usage.

In the absence of ABZ, MBZ can be prescribed to patients suffering from CE. However, MBZ has a less favorable bioavailability than ABZ due to its lower solubility in fat and it requires higher doses to achieve the same effect as ABZ. This is known to hamper treatment compliance. Furthermore, MBZ is not officially recognized as an anthelmintic drug in several countries[183].

Unfortunately, no new drugs have been introduced to treat CE in the last three decades. Other drugs in the benzimidazole family have only been tested in animal models for their effect on E. granulosus[202,203]. Nitazoxanide has been employed in the treatment of CE, but only anecdotally and with unclear results[189,204]. Several other chemotherapeutic compounds, including extracts from plants have been tested in vitro or in vivo in animal models,[167,168,170] but even phase 1 clinical trials are not on the horizon. ABZ remains the main drug for the treatment of this disease, and lack of availability poses serious problems to patients and physicians.

2.7) Conclusions

Our study shows that the lack of access to ABZ disrupts treatment of CE. This is especially concerning for patients in need of perioperative prophylaxis and those for whom benzimidazole treatment is a bridge to surgery. As a consequence, we believe that policymakers should acknowledge this problem, which impacts

CE patients treated in both endemic and nonendemic areas [58,59]. Policymakers also need to officially recognize the continuous use of ABZ as a standard practice.

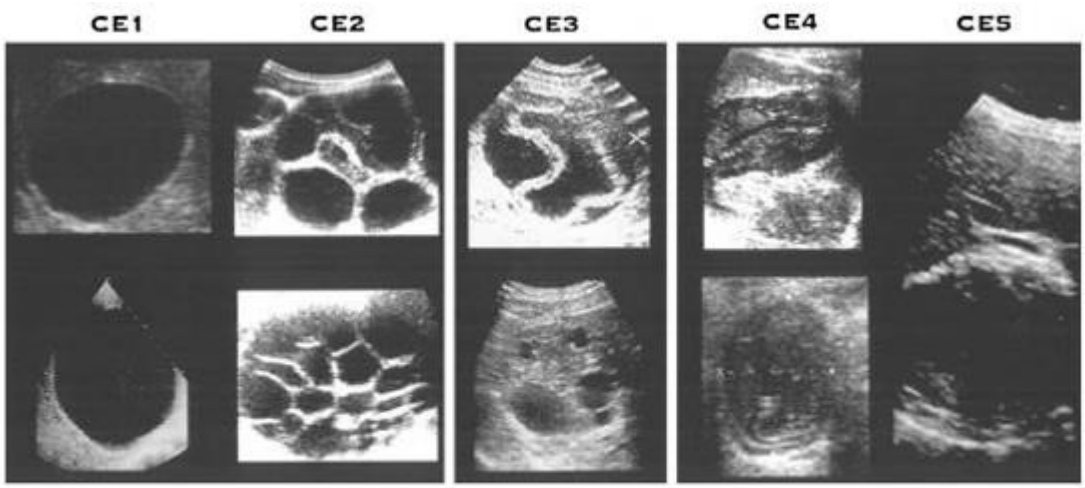

Figure 1 – Recommended treatment options for uncomplicated CE cysts of the liver. Modified from [11].

3) Incidence rates of surgically managed cystic echinococcosis in Kazakhstan, 2007-2016

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Keywords: Cystic Echinococcosis, Epidemiology, Central Asia, Kazakhstan, Disease Control

Running title: Incidence trends of surgically treated CE in Kazakhstan

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3.1) Abstract

Cystic echinococcosis (CE) is a zoonotic disease caused by the larval stage of the cestode *Echinococcus granulosus*. The parasite typically infects dogs and ungulates, with humans acting as dead-end hosts. Information on the epidemiology of CE is lacking from Central Asia, including from Kazakhstan where CE cases are reported centrally. This study presents data from the Kazakhstan Scientific and Practical Center for

Sanitary and Epidemiological Evaluation and Monitoring (SPCSEEM) on CE patients treated surgically, with a diagnosis confirmed by pathology. Evaluation of data from 2007 to 2016 indicated that the CE incidence rate decreased during this time period in most areas of Kazakhstan (country-level incidence rate of 5.6 vs 4.7 cases/100,000 population in 2007 and 2016, respectively). CE had a higher incidence in Southern Kazakhstan, with an incidence rate between 7.0 and 10.5 cases per 100,000 population, while northern regions had rates below 4.0 cases per 100,000 population. Moreover, despite the overall decrease, CE incidence continues to increase in the south. CE surveillance is needed, particularly in the south, to help inform policy makers and orient disease control efforts.

3.2) Introduction

Cystic echinococcosis (CE) is a disease caused by the larval stage of the cestode *Echinococcus granulosus.* Dogs act as definitive hosts, harboring the adult form of the tapeworm in their intestines, while ungulates act as intermediate hosts, developing metacestode cysts, which mainly affect the liver and lungs [1]. Humans act as "dead-end" or accidental intermediate hosts. According to the World Health Organization (WHO), over 1 million humans are affected worldwide with CE [2]. CE is more prevalent in sheep-raising areas, and often affects marginalized sections of society. The disease has a considerable economic impact due to both animal and human-related expenses[1,2,9,205]. While death is uncommon, morbidity can be significant if CE is not properly diagnosed and treated [2]. Currently, epidemiological data on the distribution of CE are scarce for some regions of the world [1]. This hinders efforts for CE control, as reliable data on the distribution of CE within a specific territory are crucial for evidence-based policy decisions and resource allocation. Central Asia is generally considered endemic for CE, but published data from many countries in this geographic area are scarce [206,207]. Previous publications from Kazakhstan have shown a high number of reported cases since 1994[51]. The annual mean country-level CE incidence rate rose from 0.9 cases per 100,000 population in 1974 to 5.9 cases per 100,000 population in 2000[208,209]. Studies published in 2003 and 2004 reported a 30% CE prevalence in sheep and a 50% prevalence in cattle, while *E. granulosus* infection prevalence in dogs ranged from 5% to 20% [8,9,10]. In this study, we provide an overview of the trends in human CE incidence rates from Kazakhstan for the years 2007-2016 using data on surgically treated cases from a national CE registry.

3.3) Materials and Methods

Data Source and Study Design

CE incidence rates were evaluated using data from the Kazakhstan Scientific and Practical Center for Sanitary and Epidemiological Evaluation and Monitoring (SPCSEEM) for the years 2007 to 2016. The SPCSEEM manages a disease surveillance system that collects data on the occurrence and spread of infectious diseases in Kazakhstan. CE is a notifiable disease in Kazakhstan and physicians working in hospitals throughout the country are mandated to report identified cases. Hospital administrators provide case information to regional public health centers in Kazakhstan. The SPCSEEM regional hubs then transmit monthly and annual reports to the main data center located in Almaty (**Figure 1**). As such, the dataset includes data from the entire country. However, the SPCSEEM is only notified of a CE case when a patient undergoes surgery and has their diagnosis confirmed via pathology. Collected data include demographics (name, date of birth, region, village, and district of origin), clinical and laboratory findings (cyst location and number, symptoms at presentation, serology, pre-operative diagnosis and pathology), and length of hospital stay. The dataset distinguishes new cases from disease relapses. If a patient is treated surgically within five years of the initial operation, the case is considered a relapse.

Statistical Analysis

Age- and sex-specific incidence rates (per 100,000 population) as well as age-standardized incidence rates were calculated for CE cases reported from each region (oblast) of Kazakhstan, as well as from the cities of Nur-Sultan and Almaty, from 2007 to 2016. The mean standardized incidence rate for the years 2007-2016 was also calculated. A direct standardization method was used to compare oblasts [11]. Regional comparisons were based on the WHO world standard population to adjust for age structure differences in each oblast over time[211]. Oblast- and city-level population numbers were obtained from the Annual Kazakhstan Population Datasheet from the Kazakhstan Agency of Statistics[12]. The least squares method was used to assess trends across time and the geometric mean was used to calculate average annual percent change in incidence rate. Two-way analyses of variance (ANOVA) was used to assess differences in sex and age groups. A p-value of <0.05 was deemed significant. Data analysis was carried out using SPSS 17.0 (IBM, USA).

3.4) Results

A total of 8,443 CE cases were reported from January 1, 2007 to December 31, 2016. Of these cases, 46.0% were male and the age of diagnosis ranged from 11 months to 80 years. Newly diagnosed patients accounted for 97.4% (n=8,224) of cases, while 2.6% (n=219) of cases were considered relapses. Cysts were found

primarily in the liver and lungs, with 6,106 cases (72.3%) with liver cysts, 1,836 cases (21.7%) with lung cysts, and 501 cases (5.9%) with cysts in other organs. **Figure 2** shows the total number of cases, as well as the number of cases by organ location, for the years 2007 to 2016. Data on cases with concurrent lung and liver involvement is not presented due to the low number of cases per year registered in the dataset (total 52, range 3-8, median 5).

The overall CE incidence rate decreased from 5.6 to 4.7 cases per 100,000 population from 2007 to 2016 (R^2=0.6686, p=0.004), which is indicative of a downward trend during the period under review as shown in **Figure 3**.

The 30-39 years age group had the highest overall incidence rate. There was a statistically significant difference among age classes **(p=0.001)**, with the lowest rate for children under 5 years of age (**Figure 4**). Women had a higher overall incidence rate than men (p=0.017) and this appears to be largely driven by the high incidence rate in women in the 30-39 years age group (**Figure 4**) **(p=0.017)**. Men had higher rates in the 15-19 and 20-29 years age groups, as well as in over 49 years age classes, although the difference was not significant except for the 70 years or older age group (p=0.189).

Figure 5 shows the geographic distribution of CE incidence rates in Kazakhstan. The mean nationwide incidence rate for the years 2007-2016 was 5.19 cases per 100,000 population (95% CI 4.91–5.47). Three oblasts in the southern part of the country (South Kazakhstan, Zhambyl, and Almaty) had the highest incidence rates, with 10.76, 8.73, and 7.43 cases per 100,000 population, respectively. The northern part of Kazakhstan, including the city of Nur-Sultan (formerly Astana City) and the oblasts of Kostanai, and Pavlodar, had the lowest incidence rates, with 1.32, 1.29, and 0.51 cases per 100,000 population, respectively (**Figure 5**). The percentage decrease in incidence rate between 2007 and 2016 ranged from 4.0% to 98.0% for all regions, except for Almaty, Aktobe, and East Kazakhstan (**Table 2**).

Discussion

To our knowledge, this is the first report on the trends in age-standardized CE incidence rates for the different regions in Kazakhstan. Our findings indicate that the overall CE incidence rate gradually declined in

Kazakhstan from 2007 to 2016. It should be noted that while the overall country-level incidence significantly decreased, the age-standardized CE incidence rate in Aktobe Oblast significantly increased in this time period, indicating that in some regions control measures may need to be reinforced. Moreover, a clear north-south gradient in disease distribution appears to exist[10,13]. The reported country-level CE incidence rate of 4.7 cases per 100,000 population for 2016 is higher than the rate of 1.4 cases per 100,000 population reported in 1990 [14]. Overall, the higher incidence rate may partially be explained by an improved diagnostic capability for CE, in addition to an increased awareness of the disease in the local population and by the medical community. This is also supported by the fact that the majority of cases in the dataset were new, while relapses accounted for only 2.6% of the total number.

Information on surgical procedures used is not available in the current version of the database. Therefore, some of the relapsed cases may represent the reactivation of a treated cyst (e.g. the appearance of daughter vesicles in a treated CE cyst that reached inactivation) instead of a true relapse (e.g. the appearance of a CE cyst in the same or proximal anatomical location from the original cyst). Moreover, cases of secondary CE (e.g. the appearance of new cysts due to the spillage of scoleces during a surgical procedure or consequent to cyst rupture) might be misclassified as relapses. Secondary CE most commonly presents in the peritoneum as scoleces are dispersed following the spontaneous, accidental or iatrogenic rupture of cysts, which are mainly located in the liver. Other rarer locations of secondary CE include other organs that can be reached by hematogenous spreading of scoleces[72,81,214].

In the current study, people in the 30-39 years age group had the highest CE incidence rate. This is expected as CE is most often diagnosed in this decade of adult life as cysts take a long time to develop and become symptomatic. Moreover, patients in this age range often undergo medical examinations for other reasons[8]. However, the incidence rate in children under 15 years of age was also considerable, suggesting ongoing local transmission[6,10,13]. Ongoing transmission in the younger age groups can be explained by the continuation of traditional livestock rearing practices that put young Kazakhs in contact with infected shepherd dogs [6,10,13]. A study published in 2003 found that rural dogs had an *E. granulosus* infection prevalence of between 5% and 10%, with some populations of shepherd dogs having a prevalence greater than 20% [13].

In the current study, women had an overall higher CE incidence rate than men, which concurs with the findings of previous studies. This finding may be explained by women having the predominate role in domestic

activities, including food preparation and caring for the family dog [15]. However, despite the fact that some surveys report a higher prevalence of dog contact among CE-affected individuals, these studies are often based on serosurveys and not on cases diagnosed by diagnostic imaging or other methods with greater accuracy [15]. Moreover, a recent analysis of risk factors from a prevalence survey in three endemic countries (Romania, Bulgaria, and Turkey) conducted by members of the HERACLES consortium, found no statistically significant difference between cases and controls in terms of contact with dogs[216]. Recently, the government of Kazakhstan has launched programs for the improvement of food quality, including actions targeting zoonotic diseases. Hopefully, these programs will contribute to decreasing disease transmission in the coming years[217]. This study shows that southern Kazakhstan has a higher CE incidence rate than the rest of the country, which is in line with previous reports[10]. Environmental differences may play a role, as southern Kazakhstan has a high humidity index, mild temperatures, and a long vegetative period, all of which are favorable conditions for maintaining *E. granulosus* eggs in the soil[217,218]. Secondly, socio-cultural practices in pastoral communities include routine contact with dogs that are allowed to consume raw offal from infected livestock [5,6,8,10,14]. Furthermore, southern Kazakhstan borders Uzbekistan and Kyrgyzstan, which are both considered to be hyperendemic for CE [1]. Commerce and movement of livestock have been increasing in Kazakhstan as a result of new plans to boost economic activities connected to animal husbandry. This could have increased the circulation of infected intermediate hosts, contributing to maintaining active disease transmission [217]. Individuals who reside along the border often travel between these countries and CE-infected migrants could have contributed to part of the disease burden detected in Kazakhstan.

A limitation of this study is that, in Kazakhstan, CE cases are reported to the SPCSEEM only after a patient undergoes surgery with the diagnosis confirmed by pathology. Since CE is asymptomatic in up to 70% of cases[18], many patients stay asymptomatic until an accidental diagnosis is made. In Kazakhstan, surgery has traditionally been the primary treatment for CE cases, which has led to the surgical treatment of inactive cysts. This practice is not restricted to Kazakhstan and has been reported from other countries[219,220]. However, some CE patients were likely treated medically or followed using a watch-and-wait approach, excluding them from inclusion in the centralized database. As our study is based on surgical data, our findings likely report an underestimated frequency of disease by not including medically-treated outpatients and asymptomatic cases [57]. Disease frequency could be more precisely reported by including these groups in future surveillance

programs. To this end, the recently launched European Registry for Cystic Echinococcosis (ERCE) could serve as a platform for centers in Kazakhstan, as it allows for the collection of clinical and epidemiological data. The registry has recently accepted its first member centers outside of the European Union[58]. The ERCE also allows for the collection of data from cases identified during ultrasound-based screenings. This information is needed to assess the prevalence of CE in a given geographical area, which is a crucial piece of information needed to inform policymakers on control measures[52]. Recently, an international collaboration between researchers from Kazakhstan and Italy implemented a standardized approach to the diagnosis of CE and a stage-specific approach to the treatment of these patients. The program started in 2016 in the Almaty Oblast and efforts are underway to extend the program to other parts of the country.

In conclusion, this is the first study to evaluate trends in the age-standardized CE incidence rates for the different regions in Kazakhstan. Overall, the CE incidence rate has declined over the past decade. However, the incidence rate continues to be high, especially in the southern part of the country, and new cases in children are troubling. Increasing incidence rates in Aktobe Oblast suggest that transmission is on the rise in some parts of the country. This trend calls for the early detection of disease using ultrasound screening and preventive actions among high-risk groups to lower the burden of CE in all regions of the country.

Acknowledgments

The authors wish to thank Dr. Alibek Mereke for helpful comments.

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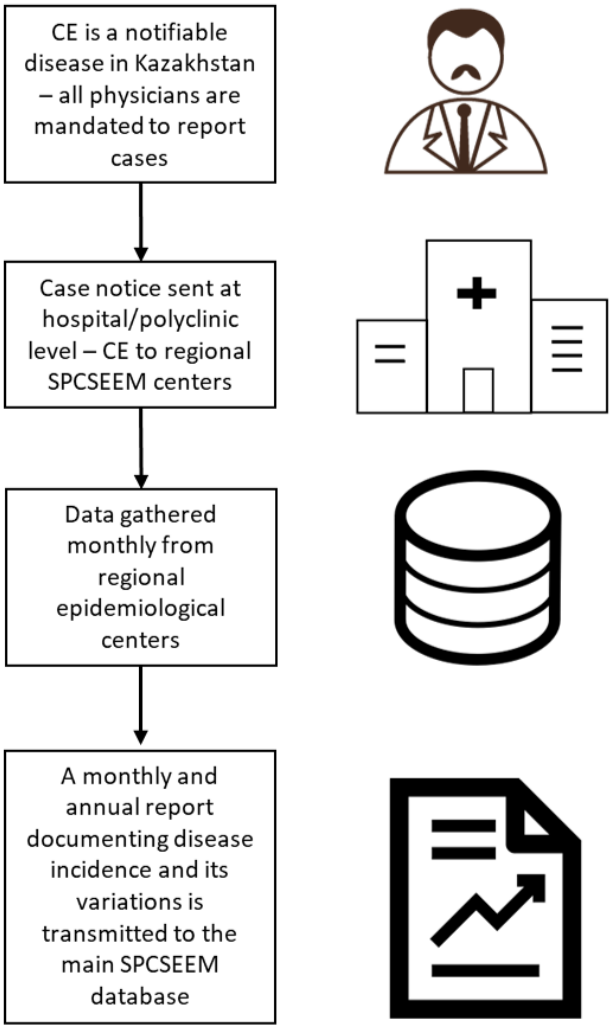

Figure 1 – Flowchart detailing disease notification and data gathering in the SPCSEEM database.

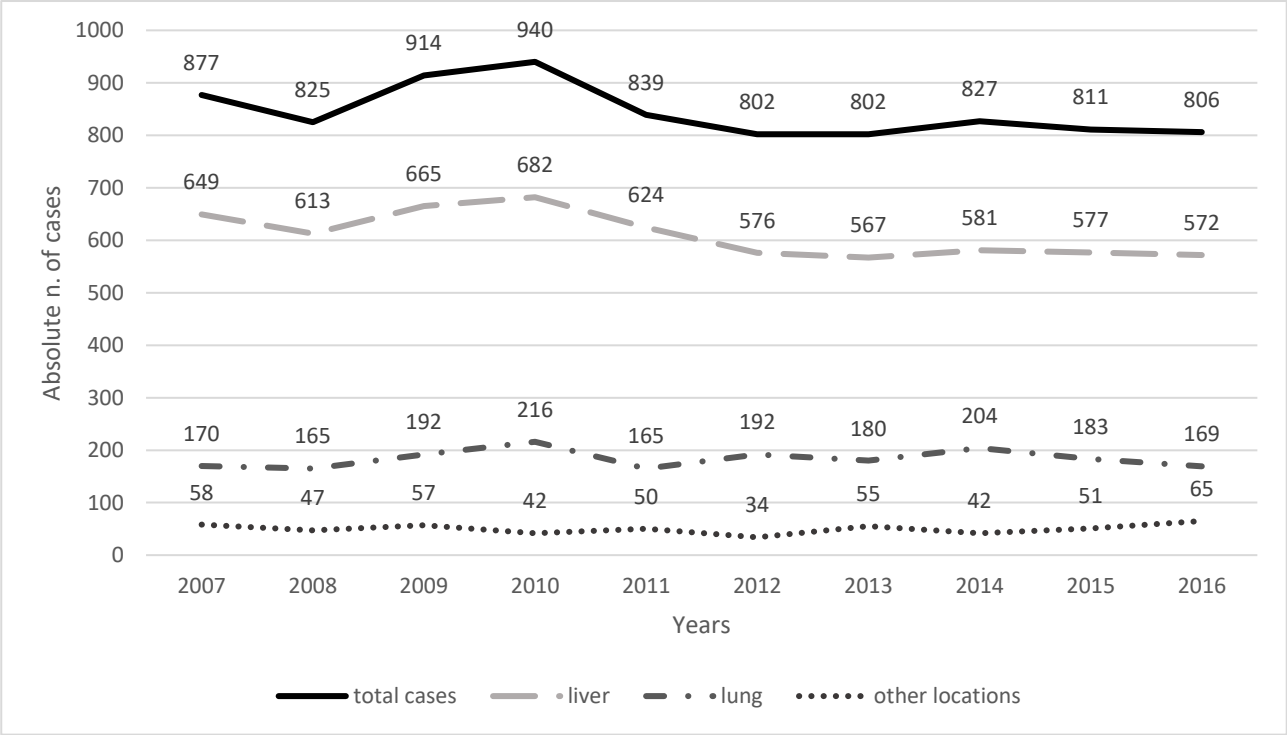

Figure 2 – Total number of reported surgically managed CE cases in Kazakhstan, as well as the number of cases by organ location, for the years 2007 to 2016. Data for cases with multiple organ localizations are not shown due to the reduced number of cases (52 overall, range 3-8, median five).

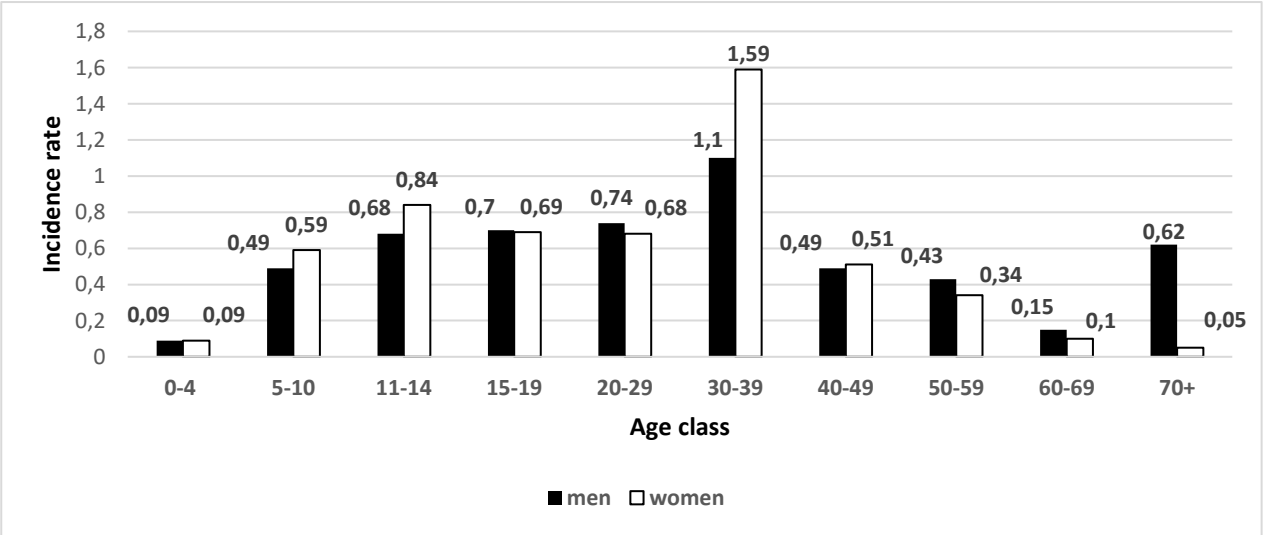

Figure 3 – Sex- and age-specific CE incidence rates (per 100,000 pop.) in Kazakhstan, 2007 - 2016

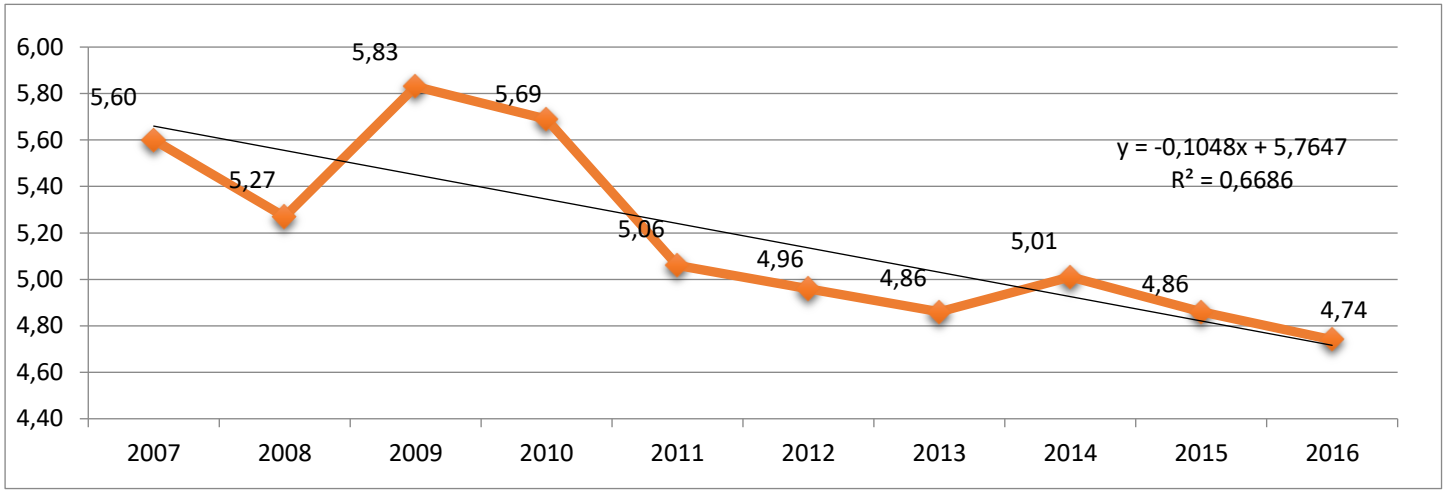

Figure 4 – Overall Mean annual standardized CE incidence rates (per 100,000 population) for Kazakhstan.

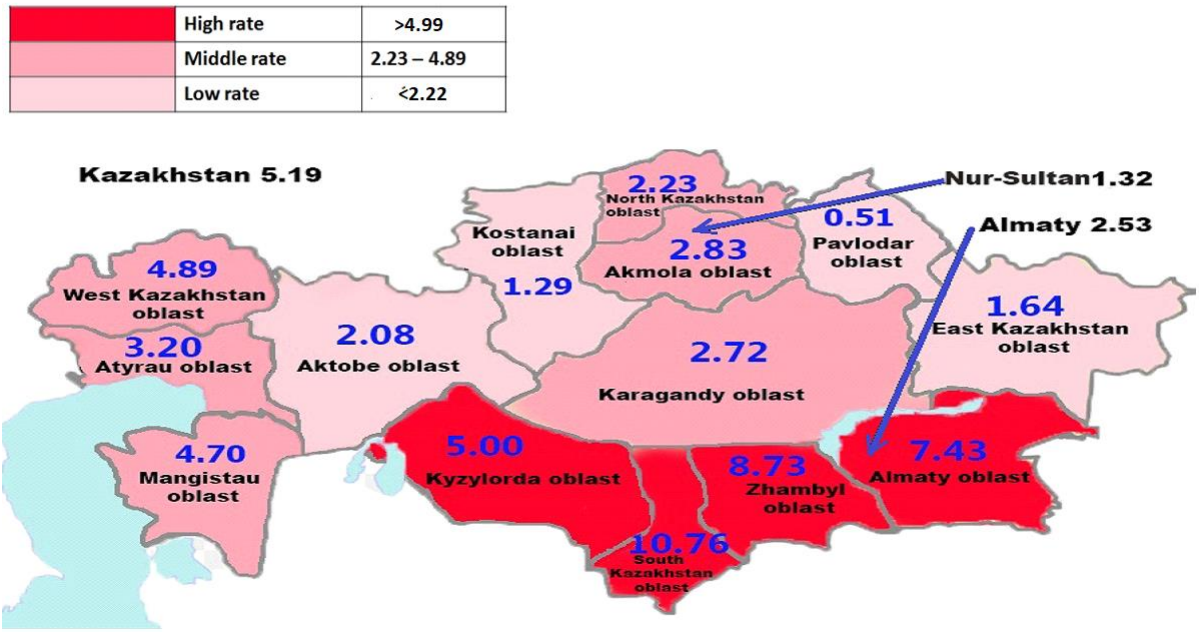

Figure 5 – Mean annual standardized CE incidence rates by region (per 100,000 pop.) in Kazakhstan, 2007-2016

4) Cystic Echinococcosis of the bone in Kazakhstan.

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Keywords: Echinococccosis, Bone Cystic Echinococcosis, Hydatid Disease, Clinical Studies.

4.1) Abstract

Cystic Echinococcosis (CE) is a parasitic zoonosis caused by E. granulosus primarily affecting the liver and lungs. CE of the bone is by far the most debilitating form of the disease and is very difficult to manage as it mimics malignant tumors. We reviewed bone CE cases admitted to a reference oncological hospital in Kazakhstan from January 2010 to February 2016. Among eight patients the mean age was 33,5 years, the male/female ratio was 1:3. Patients were examined by X-ray (8/8), CT (7/8), MRI (3/8). CE was in the spine (2 cases), pelvis (3 cases), and long bones (humerus, tibia and femur, one case for each). All patients were treated surgically. No perioperative albendazole was administered. No patient received albendazole afterwards. The mean hospital stay was 25 days. Interventions are urgently needed to assess the burden of CE in Kazakhstan and to inform clinicians of the existence of the disease.

4.2) Background

Cystic Echinococcosis (CE) is a parasitic disease caused by the cestode Echinococcosus granulosus. Its life cycle involves two hosts: dogs (but other carnivores as well) as the definitive host, and sheep (and possibly other herbivores) as intermediate hosts[8,75]. Humans are intermediate incidental hosts, or dead-end hosts[5,8]. Adult parasites are found in the intestine of definitive hosts. The eggs of the parasite are shed with the host's feces into the environment where the intermediate host, usually a sheep (or other herbivores), gets infected when grazing on contaminated ground. After ingestion of the egg, the embryo (oncosphere) hatches, penetrates the intestinal mucosa, enters into the host's circulatory system (via venous and lymphatic pathways), and develops into the characteristic vesicular metacestode when reaching a suitable anatomical site, assuming the intermediate host's immune system is unable to destroy the oncosphere[8]. This stage of the parasite is typically a unilocular, fluid-filled cystic lesion ('hydatid', 'hydatid cyst'), which grows (increasing in diameter from 1–5 cm per year) within the affected organ and harbors the protoscolices[11,75]. When the definitive host feeds on infected viscera, the cycle is complete[8]. In humans, the liver is the most frequent location (70% of cases), followed by the lungs (20% of cases), but any organ can be involved [8,11,75]. The nervous system is affected in 3% of cases, and the bone in 1 to 4% of cases[221]. The infection may remain asymptomatic for a very long time or manifest as a severe and debilitating condition[5,11]. To get a sense of the magnitude of the problem, over 250.000 Disability Adjusted Life Years are lost each year because of this zoonosis. CE also has a significant

economic impact, with at least $141,605,195 millions of dollars lost annually in animal production[2,5,205]. Despite all of the above, CE remains a neglected disease[2,5,205]. Osseous CE is one of the most severe forms of the disease [75,221]. Unlike in other organs, where a cyst with a clear cleavage plan is formed, bone CE spreads with an erosive/infiltrating pattern along medullary and trabecular channels[74]. The trabeculae are slowly reabsorbed due to pressure without any cortical extension[74]. The cysts then extend to surrounding soft tissues if the bone cortex is eroded[74].Vertebral echinococcosis is the most frequent form with 50% of osseous cases[221]. The hip and hip joint follow with 30% of cases, while the remaining 20% is seen in long bones[75]. The paucity of available data does not allow any reliable indications on clinical management [11,75,221]. The diagnosis of bone echinococcosis is primarily based on radiological and histopatological findings[74,221]. However, this diagnosis is most often made postoperatively, as the unspecific radiological aspects of the disease can simulate a variety of conditions, from inflammatory to neoplastic processes[190,221–224]. All this renders this disease extremely difficult to manage even for reference centers. In Kazakhstan, CE remains endemic. The country has seen a sharp, 5-fold rise in the incidence of the disease, according to a survey published in 2010, since 1995: from 1.2 to 6.7 per 100,000 people[213], with most cases being seen in rural settings.

4.3) Materials and Methods.

This retrospective analysis was carried out at the Kazakh Institute of Oncology and Radiology (KazIOR), Almaty, Kazakhstan. Records of patients diagnosed with CE of the bone between the 1st January 2010 and the 1st of February 2017 were included in the study. Patients with a final diagnosis different from CE were not considered for this study. The KazIOR is an Oncology reference center in Kazakhstan. The hospital has 430 beds and manages 8500 patients each year.

Study variables

For each patient we collected demographic data (name, surname, date of birth, region, village and district of origin), clinical data (symptoms at presentation, preoperative radiological, serological or pathological data, preoperative diagnosis), data on the presence or absence of other CE lesions, treatment data (Surgery Y/N, Albendazole Y/N, Secondary CE prophylaxis during surgery Y/N, use of prosthetics Y/N), treatment outcomes (recurrence Y/N, permanent or transient disabilities), data on the duration of the hospital stay.

4.4) Results

Of seventeen patients seen with a diagnosis of CE, eight patients matched our inclusion criteria. CE was present in the spine (four patients) pelvis (three patients), humerus (one patient), femur (one patient) and tibia (one patient). The median patient age was 33,5 years (range 19-55). Six patients were female, two were male.

Presenting symptoms included pain of the involved segment for all patients, edema (four patients) and difficulty walking (four patients). Seven patients were examined by X-ray, six patients underwent a CT scan, three patients underwent an MRI. Seven patients underwent an ultrasound examination of the affected segment. In all cases, a malignant tumor of the bone was initially suspected and all patients were treated with surgery. Despite all patients receiving pathological diagnosis of infection with E. granulosus, no patient received Albendazole as part of the clinical management. The median hospital stay was 22 days. Only one patient is currently undergoing a regular follow-up. Complications were primarily considered a consequence of surgical treatment, as one patient with CE in the spine developed paraplegia of the legs after the intervention and another patient sustained a permanent reduction in length of his right leg.

4.5) Discussion

Eight bone CE cases were seen in a single hospital in an endemic region. In our series the most frequent localization was the pelvis, followed by the spine and long bones. Only one patient presented with a CE cyst in organs other than the bone, consistent with the literature where for 40% of patients bone is the only location[75]. Such a high number of cases of bone CE in a single center confirms that CE is highly endemic in Kazakhstan. While the low number of patients with other localizations of CE could seem to contradict this conclusion, it should be noted that all correctly diagnosed CE cases in the Almaty region are treated at a dedicated center. However, knowledge about this disease is lacking as shown by the fact that CE was never included in the differential diagnosis in any of the patients in our cohort. Although CE of the bone is believed to be the consequence of a primary infection, some reported a coexistence of visceral and bone CE locations in 30-45% of cases [225]. In bone CE an early diagnosis is crucial to improve therapy outcomes and limit the damage caused by this chronic entity[221,225]. In our series, only one patient underwent a US scan of soft tissues surrounding the affected segment, and only three patients underwent an MRI. Even more worrisome is the fact that no patient received Albendazole as part of their clinical management and only one patient of our cohort is

currently undergoing a regular follow – up, a key element of clinical management given the high number of relapses. This is particularly important in bone CE where surgery is a complex, high risk procedure.

4.6) Conclusions

Our data confirm that bone CE is a highly debilitating disease and a serious clinical challenge as it is frequently misdiagnosed and treated inadequately. In our series, all cases were mistaken for malignant tumors after radiological examinations. Larger studies are needed to infer general conclusions about the pathology and natural history of the disease, as well as to build consensus for the management of this form of CE. Interventions are urgently needed to assess the burden of CE in the country and to inform clinicians of the existence of the disease.

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Research Capacity Strengthening and Knowledge Management to Improve Disease Control.

(Special Programme for Research and Training in Tropical Diseases) (to A.Duisenova)

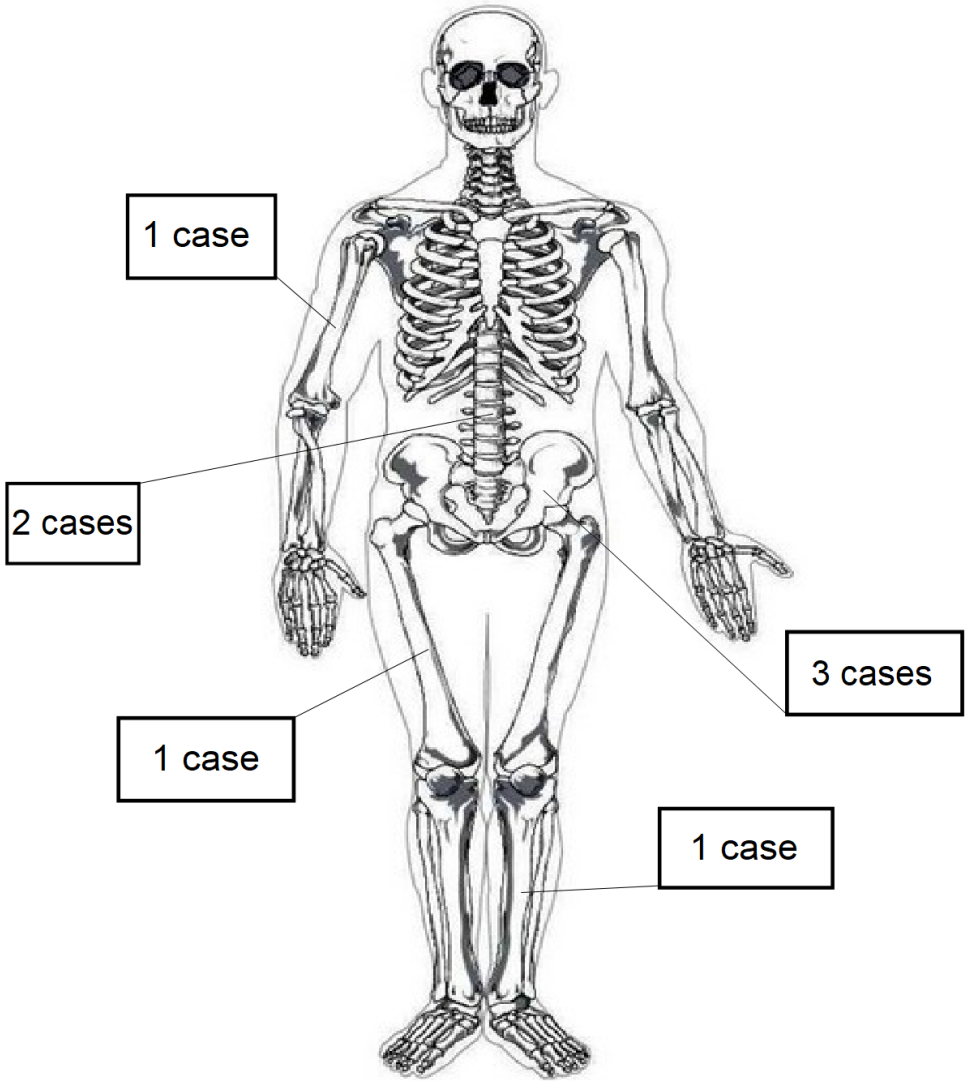

Figure 1 – Absolute numbers of the anatomical distribution of bone CE lesions in our study.

5) Cystic Echinococcosis of the bone; a European multicenter study.

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Running head: Bone cystic echinococcosis – a multicenter study

Keywords: Cystic echinococcosis, Bone echinococcosis, Clinical management, Multicenter study.

5.1) Abstract

Cystic Echinococcosis (CE) is a zoonosis caused by the larval stage of the tapeworm Echinococcus granulosus. In humans, the infection induces the formation of parasitic cysts mostly in the liver and lungs, but virtually any organ can be affected. CE of the bone is one of the rarest forms of the disease, yet it is also extremely debilitating for patients and hard to manage for clinicians. Unlike abdominal CE, there is currently no expert consensus on the management of bone CE. In this study we conducted a survey of the clinical records of seven European referral centers for the management of patients with CE and retrieved data on the clinical management of 32 patients with a diagnosis of bone CE. Our survey confirmed that patients endure chronic debilitating disease with a high rate of complications (84%). We also found that diagnostic approaches were highly heterogeneous. Surgery was extensively used to treat these patients, as well as albendazole, occasionally combined with praziquantel or nitaxozanide. Treatment was curative only for two patients, in one of whom after amputation of the involved bone. Our survey highlights the need to conduct systematic studies on bone CE, both retrospectively and prospectively.

5.2) Introduction

Cystic echinococcosis (CE), a zoonosis caused by the larval stage (metacestode) of the tapeworm Echinococcus granulosus sensu lato complex[6], is a chronic, complex and neglected parasitic disease, with a worldwide distribution. It is highly endemic in livestock raising areas[2,6]. The adults of E.granulosus reside in the small bowel of the definitive hosts (dogs and other canids) in whose faeces parasite eggs are released. After the ingestion by the intermediate hosts (ungulates such as sheep, goats or swines) the larvae released from the eggs penetrate the intestinal wall and migrate to various organs, especially liver and lungs, and develop into cysts[6]. When the definitive host ingests the infected viscera of the intermediate host, the cycle is completed. Humans may become dead-end intermediate hosts by ingesting parasite eggs[6]. Cysts can develop in any part of the body, although the liver and lungs are most frequently involved [72].

Osseus CE is very rare; it is generally reported in the literature that cysts develop in bones in <1% to 4% of cases reaching medical attention[75,190,223,226–228]. Although uncommon, osseous CE is highly disabling, with a

severe prognosis in terms of morbidity[75,221], comparable to that of locally malignant lesions of the bone due to its destructive growth [74,190,223,226,227,229,230]. CE of bone is less well known than abdominal CE, given its rarity. In an attempt to make the medical community aware of this problem, we have collected the clinical histories of a number of patients suffering from bone CE seen in several referral centers across Europe.

5.3) Materials and methods

Patients and centers

Physical or electronic archives of eight centers in four European countries (Table 1) were searched for clinical records of patients with bone CE from the start of each center activity to May 2015 for the majority of patients, while nine patients had data available until 2018. For each patient, demographic variables (sex, age at last follow–up visit, country of birth) and clinical variables were collected. These included duration of disease, calculated as the time span between the first diagnosis and the last follow–up visit, location of CE lesions, diagnostic modaility, pre- or post-operative etiological diagnosis, symptoms at diagnosis, disease- and treatment-related complications. The diagnosis was defined as certain if it was histologically confirmed; it was considered as probable if the diagnosis was based on the patient's history and imaging, laboratory and clinical features but no histological confirmation was obtained. The use of albendazole (ABZ) or other anthelmintic drugs, continuous or discontinuous administration of ABZ, medical or surgical treatment, number of surgical interventions, use of polymethylmethacrylate (PMMA) for reconstruction, and use of scolicidal agents to protect the operation field were also recorded. The total number of months of ABZ treatment received by each patient between the first diagnosis and the last follow–up visit was calculated. Finally, the treatment outcome at the time of the last follow–up visit was recorded and defined as "disease persistence" in the case of stable presence of parasite material as the result of an incomplete response of CE lesions to medical treatment or persistence of parasite material after surgical treatment; as "relapse" in the case of parasite reactivation with extension of CE lesions after initial surgical treatment – (either curative, or leaving behind some parasite material)- or after medical treatment; and as "freedom from disease" in the absence of CE lesions after treatment.

5.4) Results

Demographics

Data from thirty-two patients coming from 11 countries (one patient each for Afghanistan, Algeria, Bulgaria, United Kingdom, two patients each from Albania and Iraq, three patients from Turkey, seven patients from Romania and eight patients from Italy) are presented in **Table 2**.

Symptoms at diagnosis and diagnostic modalities

Presenting symptoms were pain (56%), neurological deficits (motor or sensory) (37%), and swelling of the involved bone segment (9%). Two (7%) patients were asymptomatic and lesions were found during exams performed for other reasons.

During the diagnostic process, 21 (66%) patients underwent an ultrasound (US) scan, 25 (78%) a CT scan, 26 (81%) an MRI scan, and 26 (81%) a plain X-ray examination; 15 (46%) patients underwent all four types of radiological examinations. In two (7%) patients scintigraphy was carried out. Serological tests were used in 27 (84%) patients, however the hereogeneity of the tests used in different centres and thoughout the investigated period within centres prevented any data analysis. In nine (28%) patients bioptical samples were also examined.

Twenty-six (81%) patients had a histopathological diagnosis of bone CE. For three patients (10%), the diagnosis was obtained by evaluation of bioptic samples obtained before surgery, while in the remaining patients from pathological analysis of surgical specimens. In eight patients (25%), CE was mentioned as the confirmed or possible etiology of the lesions before surgery. Twenty six patients (81%) had a definite diagnosis of CE of the bone, while in all other patients the diagnosis was classified as probable. The median disease duration was 17.5 years (range 0.5-57 years).

Bone CE topography in study patients.

Patients in our study had 46 CE lesions in bone. Most patients (n=22; 69%) had CE at only one bony site; six (19%) had two distinct lesions, and four (13%) had three. A total of nine (28%) patients had CE in the pelvic bones, seven (22%) in the ribcage, five (19%) in the femur and in two patients (6%) the humerus was involved. A total of 19 (59%) patients had lesions in the spine, which was the most common localization (**Figure 2**). Finally, 20 (63%) patients showed involvement of adjacent soft tissue.

A total of 20 (63%) patients had also visceral CE. In 12 patients (60%) the diagnosis of bone CE was made after the diagnosis of visceral CE, in seven (35%) patients the bone CE diagnosis was made before the patient was known to have visceral CE. For one (5%) patient, clinical records were not clear enough to clarify this aspect.

Disease complications, treatment and outcome.

Twenty-five (78%) patients developed complications. Neurological symptoms due to nerve compression occurred in 15 (47%) cases, either for the first time during follow-up or worsened if already present at diagnosis. In 10 (31%) cases, CE infiltration was complicated by bacterial superinfection. Ten (31%) patients developed pathological fractures. One (3%) patient had a bleeding episode, one (3%) had deep vein thrombosis, and one (3%) underwent amputation of her right arm. This last case was the only truly radical surgical procedure described for the entire cohort.

Twenty-nine (90%) patients underwent surgery. Of these, 25 (86%) patients did so more than once, with eight (27%) undergoing more than three surgical interventions. The total number of operations identified in the medical records of these patients was at least 66 (uncertainty arose from the records of three patients reporting “multiple surgery” without further details). Scolicidal agents were used in six surgical interventions (hypertonic saline in five cases, hydrogen peroxide in one case). PMMA was used in two (3.3%) surgical interventions, whilst prosthetic devices were used in three patients (10%), but had to be removed eventually due to superinfection or involvement of the prosthesis by the parasite. Eight (25%) patients also underwent percutaneous procedures, details of which could not be retrieved, for cysts located in the thigh, psoas or paraspinal muscles.

ABZ was used in the post-operative management of all patients, and was also administered to the two patients who did not undergo surgery. Four (13%) patients were treated with ABZ administered in cycles of various durations (1, 3 or 6 months) each year (on/off protocol) before moving to continuous administration. Fifteen (46%) patients were treated only with an on/off protocol and 12 (38%) patients were treated with lifelong administration from the start of medical treatment. Records were not clear in the case of one (3%) patient. Only one (3%) patient was treated with nitazoxanide in association with ABZ. Four (13%) patients were treated with praziquantel in combination with ABZ. Data on the duration of medical treatment was available for 27 patients (84%). For these patients, the median cumulative duration was 72 months (range 5 – 360).

Twenty-eight (88%) patients showed signs of disease persistence after treatment, including the three patients managed solely by medical therapy. Four (9%) surgical patients were initially declared disease free. Overall, 23 (75%) patients developed relapses, including the four patients initially considered to be free from disease. Information concerning relapses was lacking in the records of eight (27%) patients, which included both cases treated with ABZ only. Relapses occurred in nine patients treated with lifelong administration of ABZ (9/12, 75%) and in 13 patients treated with on/off protocols (13/16, 81%). Four (12%) patients died during the follow–up period, but from the available information it was not possible to determine whether the patients died due to CE-related complications or other, unrelated reasons. Of 32 patients, only two (6%) could be considered disease free after follow-up.

5.5) Discussion

Previous studies have reported a varied distribution of cysts within the skeleton: the spine (35%-50%), the pelvis (21%) and long bones, including the femur (16%) and tibia (10%)[74,75,229,231]. Involvement of the ribs, skull, scapula, humerus and fibula appears less common (2-6%)[74,75,231]. The disease is usually diagnosed between the fifth and the sixth decade[74,221] and is rarely encountered in childhood. Growth of the parasite in the bone tissue is a very slow process. The dynamics of bone involvement in CE are poorly understood: whether bone is a site of primary parasite implantation or is always secondary to infection in other organs is unclear. However, not all patients with bone CE also have cysts in other organs[75,230]. While visceral CE cysts expand at a slow pace and grow mostly concentrically, the rigid structure of the bone prevents this pattern to take place here as the cyst is not able to induce the development of an adventitial layer as is the case of other

organs, [74,221] and the metacestode development leads to the formation of exogenous vesicles containing protoscoleces along bone canals, conferring a branched, policystic appeareance to the lesion[74,221]. Bone destruction is likely the consequence of three factors: compression exerted by the growing parasite on surrounding tissues; ischaemic damage due to compression of blood vessels; and demineralization due to osteoclastic proliferation around the compressed bone tissue[74]. The localization of bone CE in our series is similar to that reported in previous publications[225,230]. In our cohort, the spine was the most frequent site followed by the pelvis and ribcage, with the humerus and femur being rarer locations. Most of our patients were symptomatic (94%). Symptoms and imaging features of bone CE are non-specific, making CE as a cause of bone disruption very hard to suspect in the first instance. This is also caused by a generally low level of clinical suspicion due to the rarity of CE in this location, against a background of the general neglect of CE[5]. Furthermore, due to the absence of pathognomonic radiological findings, the differential diagnosis includes neoplastic and inflammatory processes[74]. For this reason, patients commonly undergo multiple radiological examinations and a definitive diagnosis is often reached only post-operatively[74,223] as shown in our cohort. Even in case of clinical suspicion, serology may be negative, although data on this subject is scant[232–234]. Unfortunately the clinical records of our cohort regarding serology were also generally unclear or lacking altogether, so we can only provide information on the use of serological tests, but not a positivity rate. Furthermore, interpretation of such information would be hampered by the use of different tests across different centers and within centres over time, as one of the problems in CE serology is the lack of standardized tests and diagnostic algorithms [22,155].

Treatment options for bone CE have not been defined in a systematic way and currently include surgery which is used with or without administration of ABZ; in some cases, the use of other drugs such as praziquantel or nitazoxanide in combination with ABZ has also been reported in murine studies and in human case reports[204,226,235] but their efficacy remains unproven. Currently, no uniform protocol for the use of ABZ has ever been implemented[236].

Unfortunately, the retrospective nature of the study, the small number of cases, and the many variables which may influence such result, including different ABZ penetration in bone tissues depending on the extent and

location of the lesion, do not allow to draw conclusions on the difference in treatment outcome, if any, when ABZ was given continuously or in interrupted cycles.

Some authors are even sceptical about the actual role of medical treatment[190,229,230,237,238], supporting the opinion that only radical surgery is able to cure the disease[74,238]. In our cohort, all patients received prolonged courses of ABZ, which was not only longer than the current standard recognized by regulatory agencies (a maximum of three cycles each of 28 days, with a pause of fourteen days separating each cycle), but also longer than what is usually done for cystic echinococcosis in other organs, where three to six months of ABZ given continously are generally used [11]. Overall, prolonged administration was well tolerated and no patient had to stop the administration of ABZ. Our results suggest that the use of continous ABZ administrations should be implemented before and after surgery in all patients able to tolerate the treatment, as it is highly plausible that ABZ may halt the progression of the parasitic growth. This is supported by the fairly high survival rate of patients in our study over a long period of time.

With regards to surgery, radical treatment has been advocated as the sole treatment option able to cure the disease, but full removal of the parasitic tissue without harming the patient can be impossible[75,227,230]. Moreover, the persistence of parasitic material due to partial removal has a high potential for future reactivation of parasitic growth[190,226,227]. In our cohort, 97% of the patients were operated upon but a radical intervention was never possible with the exception of one case where the whole upper arm was amputated and patients experienced a high number of relapses. If not manged correctly, bone CE has a very poor prognosis in terms of long-term morbidity, compared to that of cancer[74,190,223,226,227,229,230]. In fact, vertebral CE was called "le cancer blanc" by Devé [74,221,226]. CE of the bone is a highly destructive process, capable of spreading from bone segments to the surrounding soft tissues and vice versa [75,226,227]: in our cohort, 27% of patients had more than one localization of bone CE and soft tissue involvement was extremely common.

Overall, our results suggest that while radical surgery should only be attempeted if there is a high degree of confidence in the possibility of removing all the parasitic material, palliative surgery should be attempted only in case a good degree of functionality can be restored. It should also be noted that the use of prosthetics for reconstructive surgery has been discouraged, as the parasite is able to stably attach to the materials used for their construction [221,227]. Bone allograft has also been considerd, but it is susceptible to parasite invasion if

relapses occur [221,226]. PMMA has been reported to be effective in the prevention of relapses [221,226,238] and the irrigation of the operational field with hypertonic saline or other scholecidal agents has been shown to reduce the rate of recurrence with a concentration and time dependent effect [75,226]. Traditional radiotherapy has proven to be completely ineffective[75,230,236], while other cutting-edge radioterapeutical approaches have not been applied so far.

5.6) Conclusions

Bone CE represents a challenge for clinicians and, given its relative rarity, available data are scarce. International collaboration can bring to light more cases than currently thought.

In our study we collected data from eight European centers to present a larger cohort of patients adding to current literature on bone CE, which mainly consists of single case reports or small case series, in an effort to bring this extremely serious and disabling manifestation of CE under the spotlight of the international medical community. We also show that patients diagnosed with bone CE present serious, sometimes life-threatening and often disabling complications, in accordance with previous analysis presented by other authors[226,227]. Whereas for liver CE a stage-specific approach has been at least partially agreed upon that allows a rational choice among different treatment options[11], no such thing exists for bone CE: as exemplified also in our study, the decision on the general management of patients with bone CE is largerly left in the hands of single physicians.

The only possibility of true advancement in the knowledge of this rare yet extremely disabling form of CE will necessary come only from broad, ideally prospective, multicentric studies, with common protocols for the management of the disease. Initiatives such the organization of a specific international database able to capture the very peculiar clinical features of osseous CE, complementing the existing ones such as the European Registry of Cystic Echinococcosis (ERCE)[58] are warranted given the higher level of complexity of this location compared to the hepatic one.

Acknowledgments

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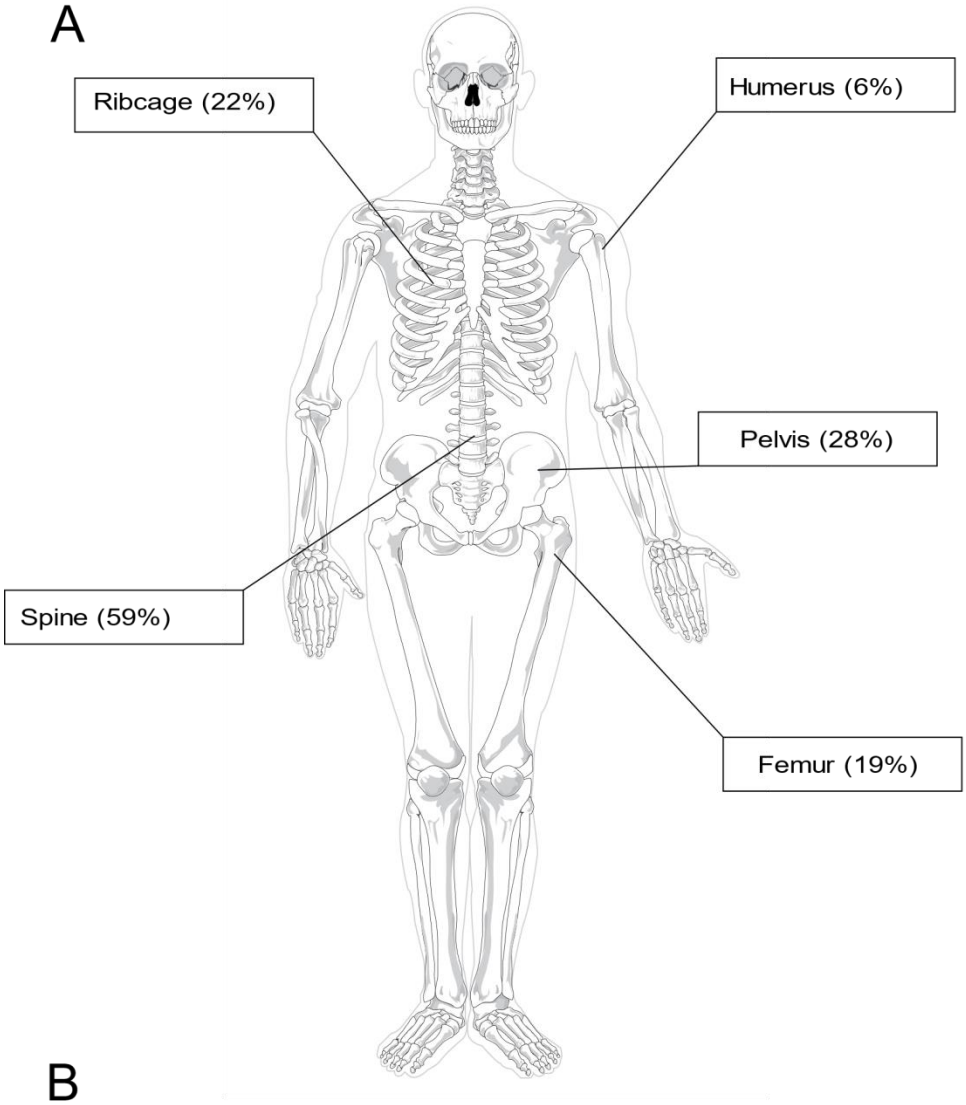
A



B

Percentage of bone CE locations over the total number of bone lesions (n=46)				
Osseus CE location				
Spine (23, 50%)	Pelvis (9, 19.6%)	Humerus (3, 6.5%)	Femur (5, 10.9%)	Ribcage (6, 13%)

Figure 1 – Distribution of bone CE lesions in our cohort of 32 patients. A) Percentage of patients with bone involvement of the indicated bone segments. B) Percentage of each bone segment involvement over the total number of bone lesions.

Country	Center	City	First year of center activity	Last Bone CE patient visit included in our cohort	Total number of patients with CE observed during center activity	N of patients with osseous CE
Italy	University of Pavia	Pavia	31 (1988 – 2018)	2018	888*	9
Romania	Carol Davila University	Bucarest	24 (1995 – 2018)	2016	1000*	6
Italy	San Bortolo Hospital	Vicenza	22 (1997 – 2018)	2018	50*	2
Italy	Hospital Sacro Cuore	Negrar (Verona)	31 (1988 – 2018)	2016	45*	1
Italy	Careggi University Hospital	Florence	19 (2000 – 2018)	2018	27*	2
Germany	University Hospital Heidelberg	Heidelberg	21 (1998 – 2018)	2016	800*	5
Germany	University Hospital	Düsseldorf	17 (1999 – 2016)	2016	106	3
United Kingdom	Hospital for Tropical Diseases	London	12 (2006 – 2018)	2016	155	4

Table 1 – Centers and number of patients per center involved in the study. *Estimated data.

n	32
Median Age at last follow - up (range)	52,5 (17 - 84)
Male (n%)	20 (63%)
Female n(%)	12 (37%)
Signs and symptoms	
Pain n (%)	18 (56%)
Neurological deficit n (%)	12 (37%)
Swelling of the involved segment n (%)	3 (9%)
Diagnostic tools	
Ultrasound n (%)	21 (66%)
CT n (%)	25 (78%)
MRI n (%)	26 (81%)
Scintigraphy n (%)	8 (25%)
X ray n (%)	26 (81%)
Serology n (%)	26 (81%)
Biopsy n (%)	12 (37%)
Pre-surgical Anatomopathological confirmation*	10 (31%)
Post-surgical Anatomopathological confirmation*	21 (66%)
No Anatomopathological confirmation*	6 (18%)
Clinical features	
Median age at diagnosis in years (range)	30,5 (10 - 63)
Median disease duration in years (range)	17,5 (6 - 62)

Patients with confirmed diagnosis	28 (88%)
Patients with disease persistence	28 (88%)
Patients with disease relapse	23 (71%)
Patients free from disease	2 (6%)

Table 2 – Demographic variables, signs and symptoms and diagnostic modality of bone CE. *The sum of histopathological investigations is higher than the number of patients having a definitive histopathological confirmation as patients received both a pre-surgical and post-surgical histopathological confirmation.

6) Clinical management of echinococcal cysts of the liver adjacent to the inferior vena cava – the experience of an Italian referral center

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Keywords: Cystic Echinococcosis, Inferior Vena Cava, Surgery, Treatment, Watch and Wait, Outcome.

6.2) Background

Cystic echinococcosis (CE) is a complex, chronic, and neglected disease with a worldwide distribution[1]. In humans, echinococcal cysts mainly form in the liver and lungs, and the clinical spectrum of infection ranges from asymptomatic to severe and rarely even fatal disease [2,8,11]. CE cysts are classified into six stages according to the World Health Organization Informal Working Group on Echinococcosis (WHO-IWGE) classification scheme[11]. Four management options are currentlyavailable for uncomplicated hepatic CE: surgery, percutaneous techniques, chemotherapy for active and transitional cysts, and a watch-and-wait approach for inactive cysts (**Figure 1**)[11,112,239] . Allocation of patients to these management options should be based on cyst stage, size, and location, presence of comorbidities, and available clinical expertise[11]. However, the limited

expertise on the disease outside referral centres, and the incomplete knowledge on the natural history of the infection, often result in overtreatment[240,241], and surgery is often still considered as the main, if not the only, approach irrespective of the above-mentioned variables[220,240]. Large studies on the surgical management of CE are flawed as in that they have failed to report the cyst stage as determined by ultrasound[181,242,243], details on follow-up are pèoor when available at all and CT was used as the main imaging method[220,244], despite being less reliable than ultrasound on cyst activity[87] ..

Cysts in the liver can be in contact with other structures such as the gallbladder, the biliary tree, the diaphragm, or the inferior vena cava (IVC),[11,245,246] leading to complications such as Budd-Chiari syndrome [76]. Contact with the IVC has been considered an indication for surgery in the fear of possible long-term complications deriving from compression or rupture, and reports detailing the removal of CE cysts after the development of IVC obstruction, thrombosis, and portal hypertension have been published[244,247–249]. However, information on the actual occurrence of complications in the presence of CE cysts in this location is scant. Currently, only one retrospective study reported the outcome of surgical approach to CE cysts in contact with the IVC, reporting post-surgical morbidity and case fatality rates of 35% and 5%, respectively in patients treated with radical surgery, and 17% and 0% respectively in those treated with conservative surgery [244]. Although patients were assigned to radical or conservative surgery based on a number of variables, and the two cohorts were not comparable, the authors concluded that radical surgery is less practicable than conservative surgery in patients with cysts in contact with the IVC and recommended the latter surgical approach. The authors considered only surgically treated patients with active, transitional, or complicated cysts; they did not provide information on cysts in inactive stages or treated with other methods.

The present study presents the almost 30 years' experience with CE cysts in contact with the IVC of a single referral center for the treatment of CE in northern Italy.

6.3) Methods

This is a retrospective descriptive study of patients with CE cysts in contact with the IVC. We reviewed the clinical records and imaging studies of all patients seen at the CE outpatient clinic at the Unit of Infectious and

Tropical Diseases at the San Matteo Hospital Foundation, Pavia, Italy, between January 1991 and July 2019. Patients with CE cysts in contact with the IVC were identified. Patients were included in the analysis if they had undergone a minimum of six months of follow-up. Patients with only one visit were excluded. One patient was excluded as he had interrupted follow-up at our center for eleven years after the first visit. Extracted data included: i) cyst stage at first visit in our centre and at the end of the follow – up; ii); clinical management, including information on therapy with albendazole (ABZ), surgery (SUR), percutaneous procedures (PER) or watch-and-wait (WW); iii) length of follow – up from the first visit in our centre expressed in months; iv) occurrence of IVC-related complications; v) reactivations, defined as the appearance of daughter cysts after a cyst had attained solidification. and vi) relapses, defined as the re-appearance of a CE cyst in the same location where the cyst was surgically removed. All operated patients were treated by the same surgical team and all patients received medical prophylaxis for relapses in case of invasive procedures.[11,183]

6.4) Results

Out of an estimated 900 patients seen at our center in the selected timeframe, 85 (8,5%) had liver cysts in contact with the IVC and matched our inclusion criteria. One patient had two cysts in contact with the IVC. We considered 86 cysts in our analysis.

Data on stage of the cyst in contact with the IVC at first visit in Pavia, prior treatments, and clinical management in our centre are presented in **Table 1.** The current stage-specific clinical management approach to uncomplicated hepatic CE applied in our centre is schematized in **Figure 1**. As shown, the majority of patients in the cohort were untreated at the time of first visit in Pavia (n=60, 69,8%). In particular, inactive cysts without a history of treatment were 17/24 or 71%.

Cyst characteristics at first visit in Pavia						
Size (mm)	CE1	CE2	CE3a	CE3b	CE4/CE5	Total
N	6	9	11	38	20	88
Median diameter	84.5	82	101	100.5	55	10

IQR	48	22	34	52	38	85
Clinical management before first visit in Pavia						
Treatment	CE1	CE2	CE3a	CE3b	CE4/CE5	Total
ABZ	0	3	0	5	2	10
ABZ+PER	0	0	0	1	1	2
ABZ+PER+SUR	0	0	0	1	0	1
ABZ+SUR	0	0	0	3	3	6
PER	0	0	1	0	0	1
PER+SUR	0	0	0	1	0	1
SUR	1	0	1	2	1	5
WW	5	6	9	25	17	62
Clinical management adopted in Pavia						
ABZ	2	7	6	8	0	23
ABZ+PER	2	0	3	3	0	8
ABZ+PER+SUR	1	0	0	1	0	2
ABZ+SUR	0	0	1	12	2*	15
PER	1	0	0	0	0	1
PER+SUR	0	0	0	1	0	1
SUR	0	2	1	8	0	11
WW	0	0	0	5	22	27
Total	6	9	11	38	20	88

Table 1 – Summary of treatments received by patients before and after Pavia took charge of their clinical management and cyst stages at first visit in Pavia * Albendazole and surgery were not applied to these inactive cysts for their specific treatment but as a joint management with that of other concomitantly present active cysts in other locations. IQR= interquartile range. ABZ=albendazole. PER=percutaneous treatment. SUR=surgery. WW=watch and wait.

Twenty-one patients in our cohort were treated by ABZ cycles alone, one patient with an inactive cyst receiving ABZ as part of the management of another cyst. Nine cysts reached inactivation, seven were unchanged (n=1 CE2, n=1 CE3a, n=5 CE3b) and three progressed to a CE3b stage from CE2. One patient underwent percutaneous treatment alone for a CE1 cyst, with the cyst reaching inactivation.

Eight patients were treated with ABZ plus percutaneous treatments, having no effect on a CE3a cyst, two CE3b cysts and allowing for the solidification of 5 cysts. One patient with an inactive cyst in contact with the IVC received ABZ as part of his clinical management after a CE3a cyst not in contact with the IVC was treated percutaneously.

Two patients did not respond to medical and percutaneous treatments on a CE1 and CE3b cyst and as such ultimately underwent surgical treatment due to the . One patient underwent percutaneous treatment and after a lack in response from a CE3b cyst, was operated. The percutaneous treatments on CE3b cysts were carried out before the publication of the WHO-IWGE expert consensus.

Sixteen patients were treated by ABZ plus surgery, one of these had an inactive cyst taken out after during surgery for an active cyst. Thirteen patients underwent surgery alone. One patient was operated outside of Pavia after the first visit notwithstanding having continued the follow-up in Pavia after the first visit, and detailed characteristics of the surgery were not available. Nineteen patients underwent conservative surgery, ten had radical operations. None of the surgical patients experience a relapse during the follow-up in Pavia.

Twenty-six patients were not treated and followed by watch and wait. Of these, 21 had inactive cysts at the first visit in Pavia, four had a CE3b cyst. Two patients were lost to follow-up, one patient has recently been re-evaluated and has been inserted in the waiting list for surgery, one has refused surgical intervention.

Among the inactive cysts at first visit in Pavia, no one, both in the untreated and treated subgroups (n=16 and 8 respectively) did reactivate.

Out of 62 patients presenting with active or transitional cysts at the first visit in Pavia, 44 achieved inactivation or were operated without relapses.

Thirteen patients with active or transitional cysts (5 CE2, 7 CE3b and 1 CE3a at the start of follow-up) had a total of 17 reactivations (median 1, range 1-3). Seven were treated using albendazole, five were ultimately treated by surgery after having received albendazole and/or albendazole+percutaneous treatment and one was treated using percutaneous procedures plus albendazole. Seven patients in this group ultimately presented a surgical cavity or an inactive cyst, the others still had active cysts at the end of follow-up.

Table 2 presents data on the follow-up time of the patients included in this cohort.

Stage at first visit in Pavia	n (%)	**Median total F-up (months)**	**Range**	**IQR**	**Median Treatment free F-up (months)**	**Range**	**IQR**
CE1	6	113	16-289	116	78	1-288	103
CE2	9	113	6-212	39	22	0-71	39
CE3a	10	43	7-106	58	18	0-67	58
CE3b	38	31	6-360	94	13	0-171	94
Previously treated CE4/CE5	8	43.5	6-153	103.5	38	6-144	37.5
Untreated CE4/CE5	17	50	13-180	116	44.5	1-154	40.5
Surgical cavity	-	-	-	-	-	-	-

Total	88	39.5	6-360	90.5	24	6-360	90.5
Stage at last f-up visit	**n (%)**	**Median total F-up (months)**	**Range**	**IQR**	**Median Treatment free F-up (months)**	**Range**	**IQR**
CE1	-	-	-	-	-	-	-
CE2	1	12	0	-	9	-	-
CE3a	2	7.5	7-8	1	0.5	0-1	1
CE3b	16	35	6-336	98.5	18	0-154	45
Treated CE4/CE5*	17	47.5	6-150	49	10	0-80	20
Untreated CE4/CE5	21	47.5	6-216	111.5	39.5	6-288	98
Surgical cavity	29	47.5	6-360	79	12	0-97	23
Total	88	39.5	6-360	90.5	24	0	50.5

Table 2 – Follow-up data for patients included in the cohort. Data about the total follow-up and treatment free follow-up is present, according to the stage of the cysts at initial presentation and at the last follow-up visit in Pavia. *Data for the two CE4 cysts treated by surgery is included in the Surgical cavity line.

None of our patients experienced IVC-related complications during follow-up. One of the patients presenting at the first visit in Pavia with a CE3b cyst had also a bilateral pulmonary localization and referred an episode of acute chest pain and shortness of breath in his youth. The history and clinical presentation could be compatible with an embolization from the cyst in contact with the IVC, however this is only speculative.

6.5) Discussion

The literature on natural history or treatment of CE cysts in contact with the IVC mostly consists of case reports. While these reports are useful, as each one increases clinicians' awareness of this condition, they do not provide knowledge that can be used to guide clinical management, Information on the follow-up of these patients is often not presented in case reports. While the largest descriptive study of a cohort of cysts in contact to the IVC to date did report on cyst staging an follow-up, it did not provide information on the use of treatments other than surgery and on the evolution of inactive cysts in contact with the IVC[244].

The CE cyst classification and stage-specific approach to treatment has not been adopted by most physicians [188,241,250] but of paramount importance, as hepatic surgery for CE, although showing a low mortality and post-surgical morbidity,[251,252] is not risk-free, bears considerable monetary cost, and should therefore be chosen only when needed.[114]. Moreover, contact with blood vessels makes the surgical intervention more challenging [244] and t riskier for the patient. In our experience, patients with CE cysts in contact with the IVC were managed with other approaches, either as the only approach or before resorting to surgery in selected cases. Patients with CE1 cysts did not have to be operated upon; only one patient with a CE3a cyst had to be operated, as the cyst was not solidified completely after medical and percutaneous treatments. Some patients with CE2 and CE3b cysts were also managed with non-surgical approaches, either because they could not be operated as a cost-benefit evaluation showed that a WW approach was more indicated, or because they refused the surgical intervention. In accordance with Ramia and colleagues[244], we found that conservative surgery was more frequently used in case of IVC contact. Unfortunately, it was not possible to extrapolate data on the reasons of their choice of either technique. Patients in our cohort had no relapse, although relapses tend to occur years after surgery, and patients in our surgical cohort will require a longer follow-up to provide definitive information on the incidence of relapses. The other main finding of our study concerns inactive cysts, of which none developed an IVC-related complication during their follow-up, irrespective of spontaneous or post treatment inactivation .. On the contrary, reactivations were seen in thirteen patients (14,6%) with cysts in active stages, consistently with previous findings[111,112].

To conclude, our experience suggests that the incidence of IVC-related complications in patients with CE cysts in contact with this vessel is probably rare and that these cysts may be managed according to a stage-specific approach if the clinical conditions of the patient allow it.

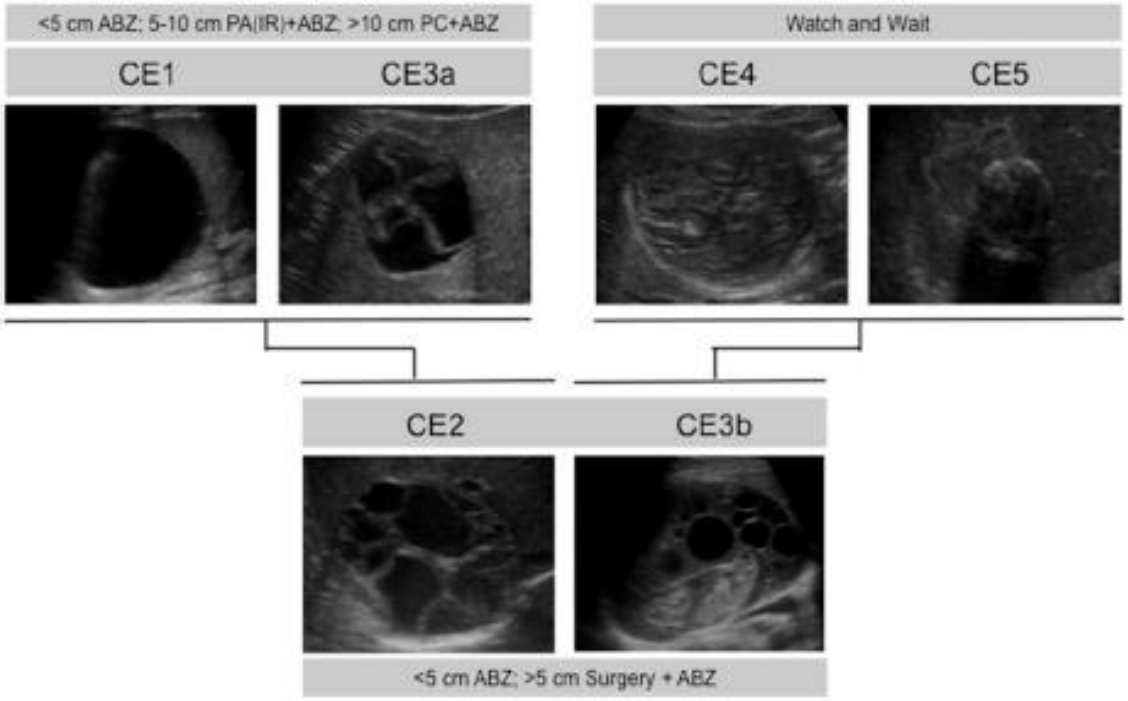

Figure 1 –Stage-specific clinical management of hepatic uncomplicated CE cysts applied in Pavia, (from[83]), in line with Expert Consensus recommendation[11]

7) Watch and Wait approach for Inactive Echinococcal Cyst of the Liver: an update

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Running title: Update of the Watch and Wait approach

7.1) Abstract

Human cystic echinococcosis (CE) is a chronic, complex and neglected infection causing severe disease in humans. Hepatic CE cysts are detected and classified mainly by ultrasound. Expert opinion and published data suggested that uncomplicated inactive liver cysts do not require treatment and only need to be monitored over time ("Watch and Wait").

Here we update our findings as published in 2014 on the "Watch and Wait" approach applied to inactive, asymptomatic cysts of the liver to keep the medical community informed.

Clinical data of patients who accessed the WHO Collaborating Center for CE at the University of Pavia-San Matteo Hospital Foundation from January 1991 to October 2017 were analyzed. Inclusion criteria were presence of one or more inactive uncomplicated cysts in the liver (CE4 or CE5), without any history of previous treatment, and an ultrasound-based follow-up of at least 24 months.

Fifty-three patients with 66 inactive cysts fulfilled the inclusion criteria. Of these, 11 patients are newly described here, 37 were part of our previously described cohort and the follow-up for 17 of them was further extended, and 5 were excluded from the previously published analysis as their follow-up was too short, but could be included now. 98.5% of cysts remained inactive over time without need for treatment and without development of complications. In only one patient (1.9% of patients), a reactivation of 1 cyst (1.5% of cysts) was observed.

Keywords: Cystic echinococcosis, "Watch and Wait" approach, *Echinococcus granulosus*, follow-up, inactive cyst.

7.2) Introduction

Cystic Echinococcosis (CE) is a chronic, complex and neglected parasitic zoonosis caused by the larval stage of *Echinococcus granulosus* sensu lato species complex that may cause severe disease in humans. The number of people affected by CE is estimated to be more than 1 million worldwide[253]. The life cycle of *E. granulosus* develops between a definitive host (dog or other canids), which harbors the adult tapeworms in the intestine and shed the parasite eggs with the feces, and an intermediate host (herbivores) where the larval stage develops as fluid-filled cysts in organs and tissues. Humans act as occasional intermediate hosts and acquire the infection through accidental ingestion of *E. granulosus* eggs. The larval cysts in humans are located in the liver in about 80% of cases but infection of almost any organ has been reported[72,205]

In 2010 the World Health Organization Informal Working Group on Echinococcosis (WHO-IWGE) published an expert consensus document on the diagnosis and clinical management of CE in humans[11] According to these recommendations, the decision on the best clinical management option, in case of hepatic localization of uncomplicated cysts, has to be guided by a "stage-specific approach", after assessment of the cyst stage on imaging (Figure 1), using ultrasound (US) as the reference technique, or magnetic resonance imaging.[5] In particular, uncomplicated inactive liver cysts (classified as CE4 and CE5 stages, Figure 1 and 2) do not require any treatment and need to be exclusively monitored over time using US[11]

An increasing number of published data, reporting that spontaneously inactivated cysts remain inactive in the majority of cases during the follow-up, is supporting the rationale of leaving uncomplicated inactive cysts untreated, which was based on observations that a good proportion of cysts become inactive spontaneously, and that cysts in general tend to remain stable over time[110–112,166] The reactivation rate of cysts that become inactive spontaneously appears to be lower (0%-6%) compared to that observed in cysts becoming inactive after therapy (25%-60%). Reactivation to stage CE3b, in any case, appears to occur in most cases within 2 years from first observation of inactivity[88,111,112,186]

In 2014, we published the first report on the long-term follow-up of spontaneously inactivated hepatic cysts on a clinical rather than epidemiological basis[112]. In that report, 28 patients (22% of the potentially eligible patients) could not be included in the cohort because they were visited for the first time in our center less than two years prior, thus not meeting the minimum follow-up length inclusion criterion. The "Watch and Wait" approach spares patients unnecessary treatments, including surgery, with attendant side effects, complications, and costs. As such, it is crucial to gather evidence on its safety in the appropriate cases. To this aim we update the results published in 2014 by our center, evaluating the results of "Watch and Wait" approach, in terms of safety and usefulness, in patients with spontaneously inactivated hepatic CE cysts visited from 1991 to 2017 in a single referral center in Italy.

7.3) Materials and Methods

Clinical data of patients who accessed the WHO Collaborating Center for CE at the University of Pavia-San Matteo Hospital Foundation from January 1991 to October 2017 were searched in our database and analyzed.

Data regarding demographic information and number, stage, size, and location of hepatic CE cysts were recorded.

Inclusion criteria were the presence of only one or more inactive uncomplicated cysts in the liver (classified as CE4 or CE5 using the WHO-IWGE classification, Figure 2) on US examination, a negative history of any previous treatment and an US-based follow-up of at least 24 months in our center. A minimum length of two years of follow-up was chosen because most reactivations happen within 24 months from first observation of inactivity[111,112,186]

Exclusion criteria were the presence of inactive CE but in extra-hepatic localization, the presence at the same time of cysts in active or transitional stage in any organ, and history of previous treatment. All patients who did not complete a minimum follow-up of 24 months were also excluded. Independently of inclusion or exclusion, patients who did not come to our clinic for more than 12 months as of October 2017 were considered lost to follow-up.

All patients were evaluated by an infectious disease clinician with more than 30 years of experience in US and in clinical management of CE (EB) using an US scanner with a 3.5-5 MHz convex probe.

The appearance of daughter vesicles within the cyst (passing from CE4-CE5 to CE3b stage) was considered a reactivation. Cyst super-infection, rupture, or appearance of any symptom related to the cyst were considered as complications.

Descriptive statistics were produced for demographic and clinical characteristics. Quantitative variables were expressed as mean and range. Interquartile ranges (IQR: 25th -75th percentile) were calculated for the median follow-up time. Qualitative variables were summarized as counts and percentages. The McNemar's test was performed to analyze the difference in cyst stage at the diagnosis and last follow-up visit for each patient.

7.4) Results

From January 1991 to October 2017, 179 patients with untreated CE4-CE5 echinococcal hepatic cysts were evaluated in our center. Of these, 53 (29.6%) fulfilled the inclusion criteria while 126 (70.4%) were excluded from the study for the following reasons: lost to follow-up after less than 2 years observation (n=102), previous

therapy (n=17), new diagnosis less than 2 years prior to the time of the visit (n=7). In the study period, 214 untreated hepatic CE4-CE5 cysts were observed, 66 (30.8%) of which in the patients eligible for inclusion in this study.

Included patients:

Of the 53 patients included in this study, 11 (20.8%) were seen for the first time after the date of data analysis of our previously published cohort, 37 (69.8%) were included in the previously published cohort and 5 (9.4%) patients were excluded from the previously published cohort because their follow-up was still too short, and now are included[112]. Of those included in the previous cohort, 17 were further followed in our center, therefore their follow-up was extended from the previous report. Twenty-two (41.5%) patients completed a follow-up of five years. The characteristics of the patients included in this work are summarized in table 1.

Among the monitored 66 cysts, 41 (62.1%) were classified as CE4 and 25 (37.9%) as CE5. Forty-five (68.2%) cysts were located in the right lobe, 10 (15.2%) in the left lobe, and 11 (16.7%) in the 4th segment. The mean diameter was 52 mm (range 14-94).

The median follow-up period of patients eligible for inclusion in the cohort was 52 months (IQR 36.6 - 90.2). During this time, cysts in 52 (98.1%) patients remained stable, while a reactivation (from CE4 to CE3b stage) was observed in one patient (1.9%) after a follow-up period of 24 months. This reactivation was the same described in 2014; no new reactivation events were observed[112]. Regarding the studied cysts, 65 (98.5%) remained inactive while 1 (1.5%) reactivated. No complications occurred during the follow-up period.

Excluded patients:

One hundred and two patients (81.0%) were excluded from the analysis because they were lost before 2 years of follow-up were completed, 7 (5.5%) were still in follow-up in our clinic but were not included because the follow-up period was less than 2 years on October 2017, and 17 (13.5%) were excluded due to previous therapy administered elsewhere despite being staged at diagnosis as CE4-5.

Forty-seven (46.1%) of the 102 patients excluded from the analysis due to loss to follow-up before the completion of 2 years, had already been excluded for the same reason also in our previous report.

In order to understand the reason of missing regular visits, we attempted to re-contact by telephone all patients excluded from the analysis due to loss to follow-up before the completion of at least 2 years observation. Thirty-nine (38.2%) patients could not be reached over the telephone number provided at the time of the visit (26 of them described in our previous report among excluded patients), 38 (37.3%) were currently followed in another hospital (11 of them described in our previous report among excluded patients) while 22 (21.6%) reported being in good clinical condition and decided by themselves to interrupt the follow-up (9 of them described in our previous report among excluded patients), although some expressed the intention to resume follow-up in the near future.

Two (2.0%) patients died for causes unrelated to CE and 1 (0.9%) patient reported to have suffered from complications, but it was not possible to clarify the nature and relation to the CE cyst by telephone (these 3 cases were already described in our previous report) (Figure 3).

7.5) Discussion

CE is a complex, chronic and neglected disease with a wide spectrum of clinical presentations and a chronic evolution requiring many years of follow-up. This is one of the reasons that makes prospective randomized clinical trials on this disease difficult, and clinical management recommendations still rely largely on expert opinion[205]

According to these recommendations, uncomplicated inactive cysts of the liver should be left untreated and simply monitored by US, the so-called ‘‘Watch and Wait’’ approach. This indication was originally based on publications reporting that a good proportion of cysts become spontaneously inactive and that cysts in general tend to remain stable over time[166] Additional fieldwork confirmed these observations, and results of two studies focusing on the long-term follow-up of spontaneously inactivated cysts on a clinical rather than epidemiological basis, provided support to this line of conduct.[54,85,110–112,] Unfortunately, this approach is still poorly followed[54] To add further data on the use of the “Watch and Wait” approach for uncomplicated spontaneously inactivated CE cysts of the liver, we present an update of a previously described cohort of patients followed-up in a single center by the same physician over more than 25 years[112]

In our study, more than 98% of the cysts observed for at least 2 years remained stable, without complication, strengthening the conviction that the "Watch and Wait" approach is a safe and useful method for the clinical management of inactive asymptomatic hepatic CE. This avoids overtreatment of patients with CE, with attendant and unjustified risks and costs. Loss to follow-up is unfortunately a common occurrence, and it may be that this group differs from those observed for at least 2 years as far as the incidence of complications is concerned. In an attempt to shed light on this issue, we contacted by phone those patients lost to follow-up before the two years observation was achieved, and apart from one case where an uncertain condition was reported that could have been a complication due to CE, none of those who could be contacted reported the development of any complication. Unfortunately, verifying the absence of reactivation, without the concomitant development of symptoms, is only possible if follow-up document area available, and this information could not be retrieved. It is also possible that complications may occur after an observation of 2 years, and therefore that the patients included in the analysis but lost to follow-up after an observation time >2 years and not re-contacted by phone would have experienced delayed complications. Although this hypothesis cannot be ruled out a priori with the data we retrieved, nearly half of the patients included in our analysis completed a longer follow-up period, reaching the goal of five years as recommended by the Expert Consensus document of the WHO[11], and no reactivation or complication occurred. In more than 25 years of cumulative observations, only one cyst reactivation was observed and this was the one described in 2014 in the first report of our cohor[112] This points out the need to follow-up these cysts in any case, as the biological mechanisms at the basis of progression to spontaneous inactivation or reactivation are still unknown and biomarkers of this dichotomous evolution are not yet available. We also need to implement a patient information and recall system to reduce the loss to follow-up.

In the absence of large, prospective, studies that can provide definitive recommendations for the clinical management of these patients, our data, still support the WHO-IWGE recommendations for the clinical management of inactive and asymptomatic echinococcal cysts of the liver.

8) Assessing the field performance of a Rapid Diagnostic Test for the serodiagnosis of abdominal cystic echinococcosis – field notes from the Peruvian Highlands and practical implications for public health

Manciulli T, Enríquez-Laurente R, Tamarozzi F, Lissandrin R, Elizalde M, Sedano C, Bardales K, Vola A, De Silvestri A, Tinelli C, Brunetti E, Santivanez S, Mariconti M.

8.2) Introduction

Cystic echinococcosis (CE) is a zoonotic parasitic disease caused by the larval stage (metacestode) of the dog tapeworm *Echinococcus granulosus* species complex. Dogs are the main definitive hosts of the parasite while ungulates, particularly sheep, are intermediate hosts [1]. Humans are accidental intermediate hosts: they acquire the infection by accidentally ingesting *E. granulosus* eggs shed in the environment through infected dog faeces [6]. In humans, the parasite larvae develop as fluid-filled cysts in different organs and tissues, liver and lungs being the main locations [8]. People can either remain asymptomatic or growing cysts may give rise to clinical manifestations, ranging from mild to severe, sometimes life-threatening conditions[11].

CE is distributed worldwide, especially in rural livestock-raising areas in the Mediterranean, Eastern Europe, North and East Africa, South America, Central Asia, China and Australia [1]. The global burden of CE has been estimated in over 1 million Disability-Adjusted Life Years, and over 2000 million USD lost yearly in animal productions [2]. Latin America is among the regions of the world with the highest prevalence of CE [254,255]. In Peru, in the areas of highest endemicity, prevalence in humans ranges between 5.5%-9.3% [53,255,256]. Despite these levels of infection, CE is not a notifiable disease in Peru.

CE diagnosis is based on imaging techniques, mainly ultrasound in the case of abdominal CE. The WHO Informal Working Group on Echinococcosis (WHO-IWGE) developed a standardized classification of CE cysts based on ultrasound morphology, which also indicates the biological activity of the cyst and guides the clinical management of the patients [11,257]. Serology plays a complementary role in the diagnosis of CE, but it is burdened by several limitations. In particular, currently available serological tests are not standardized, often require infrastructures and training to be performed, and their results are influenced by several factors relating to the patient (cyst stage, number, size, location, previous treatment), the test (antigenic preparation, test

format) and the underlying disease epidemiology [91,136,155,234]. These issues make the use of serology alone unreliable in field surveys aiming to estimate the prevalence of CE [136], although this approach is still being applied. In endemic, resource-limited settings, ultrasound expertise is also scant and the WHO-IWGE classification and management recommendations are not well known outside of referral centers. In such settings, a reliable, highly specific Rapid Diagnostic Test (RDT) to support the diagnosis of CE might be an appealing confirmatory test for cases raising suspicion of CE infection on imaging. Several reports have been published describing the performance of commercial and experimental RDTs for the diagnosis of CE [30,91,92,157,258]. The aim of the present work was to evaluate the performance of a commercial RDT as a potential point-of-care test in a filed setting, after having tested it in laboratory controlled conditions [30,91], for the diagnosis of abdominal CE cysts. We also aimed to test the reproducibility of the RDT, as well as its concordance with an ELISA test commercially available and used routinely in the laboratory setting the Italian center of this study, and the specificity of the test when used on individuals from a highly endemic area with no evidence of abdominal CE infection. The evaluation was carried out using sera collected during an ultrasound screening campaign in a highly endemic region of Peru.

8.3) Materials and Methods

Study area and community outreach

The study was approved by the Ethics Committee of the Universidad Peruana Cayetano Heredia, and consent to conduct the study was granted by the leaders of the communities involved.

The study was conducted in 3 rural communities in the Central Peruvian Highlands, in the villages of Ondores (Junin Region, District of Ondores), Corpacancha (Junin Region, District of Marcapomacocha) and Tomas (Yauyos Province, District of Tomas), situated at an elevation between 3,550 m and 4,200 m above sea level, (**Figure 1**), during 2 weeks in October 2017. Sheep are the main livestock bred in the area, but alpacas and llamas are also raised; animals are kept near the villages. Dogs are routinely kept as shepherds, but some are kept as pets.

The survey was carried out in Primary Care Centers (PCCs), which provide basic primary health care, and dental and obstetric assistance. Between August and September 2017, the local population was informed that the study would take place in the villages. General information about the infection and the project activities were provided through the local health center's outreach programs and on the radio in Quechua and Spanish languages. A census of the local population was carried out and this was an additional occasion to explain to the villagers the aim of the project and that all examinations would be carried out for free. All people older than five years were invited to participate. Volunteers attending the study sessions had the chance to sit down with a Spanish-speaking component of the team and pose questions about the study before signing the Informed Consent Form in Spanish and being assigned an alphanumeric code. The parents or legal guardians were asked to sign the Consent Forms in case of individuals <18 years of age. All study participants were asked information on previous CE infections and treatment, if any.

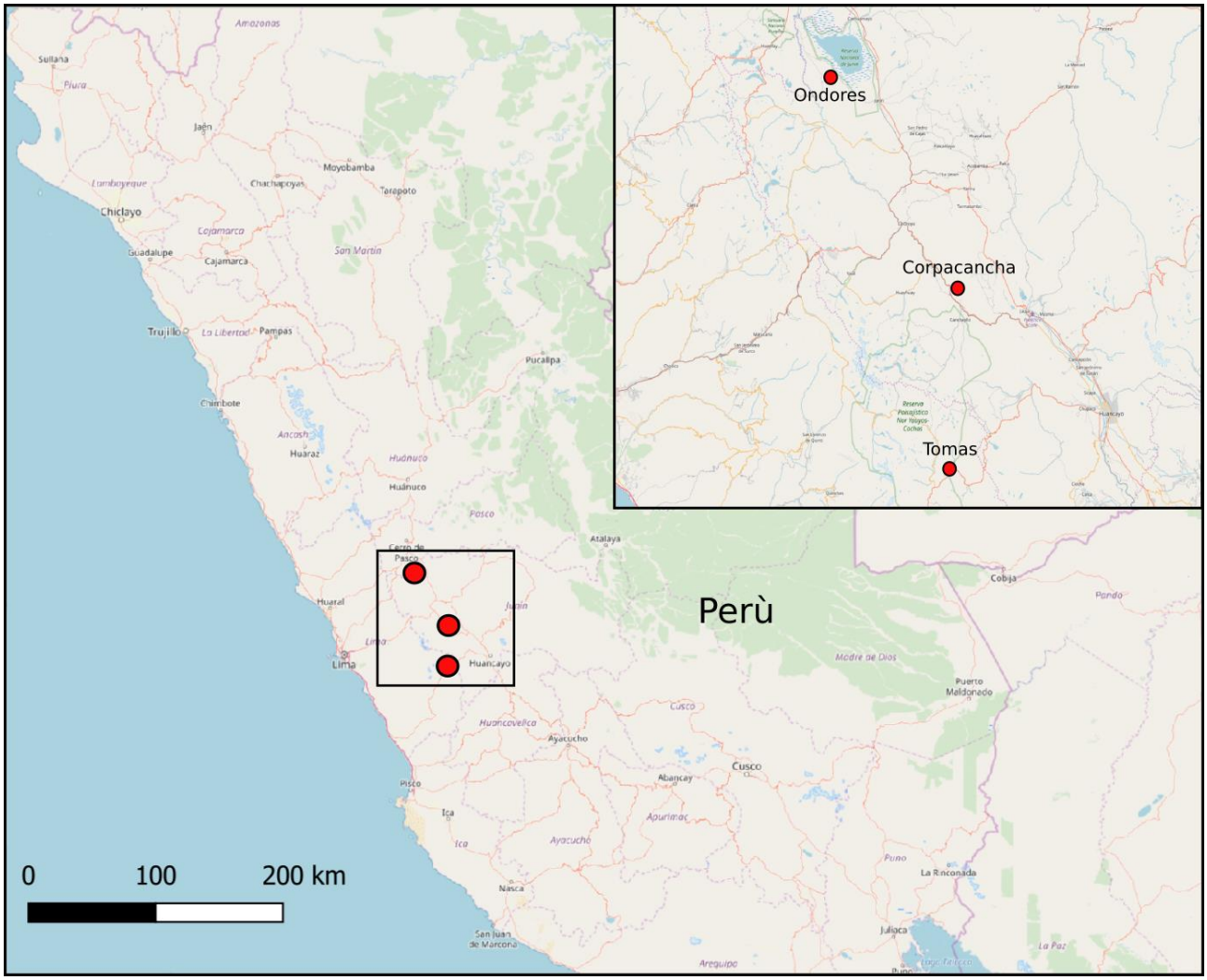

Figure 1 - Map showing the study sites included in the study

Ultrasonography

Abdominal CE was diagnosed by ultrasonography of the abdomen Ultrasonography was performed with 3 portable ultrasound machines equipped with convex probes: Mindray Z6 (Mindray, Shenzhen, China), Samsung UGEO PT60A (Yorba Linda, USA) and Sonosite 180 (FUJIFILM SonoSite, Bothell, Washington, USA). Three physicians trained in performing ultrasound scans and in the use of the WHO-IWGE classification for the diagnosis and staging of CE cysts performed the examinations. Cysts with pathognomonic signs of CE etiology were identified and staged according to the WHO-IWGE ultrasound classification, upon consent of the 3 ultrasound operators [11]. All individuals were informed about their ultrasound results and provided with a report in Spanish. This indicated whether further medical attention was required for CE-related or other conditions detected on ultrasound, and clinical management suggestion in case of abdominal CE, according to the WHO-IWGE Expert Consensus [11]. Individuals requiring medical attention were addressed to the attention of the PCC's in-charge officer for liaise with the national health system. Unfortunately, as discussed in the discussion, for practical constraints, it was not possible to perform chest X ray to all individuals participating to the study to rule out isolated lung CE.

Serology testing

Blood samples were collected from individuals with CE cysts on ultrasound and age- (+/- 5 years) and sex-matched uninfected volunteers in a ≈1:3 ratio.

Whole blood samples were centrifuged at 7000 g using a portable centrifuge, and the serum divided in two aliquots and frozen at -20 °C until used. Serum samples were divided in two aliquots, one to be used in Lima, one to be used in Pavia. All samples were labelled by the patient code and date of collection. Samples were stored in the parasitology lab of the Instituto Peruano de Parasitologia Clinica y Experimental (Lima, Peru). Here, sera were tested using the VIRapid® HYDATIDOSIS (Vircell, Granada, Spain) test, according to the manufacturer's instructions **(Figure 2)**.

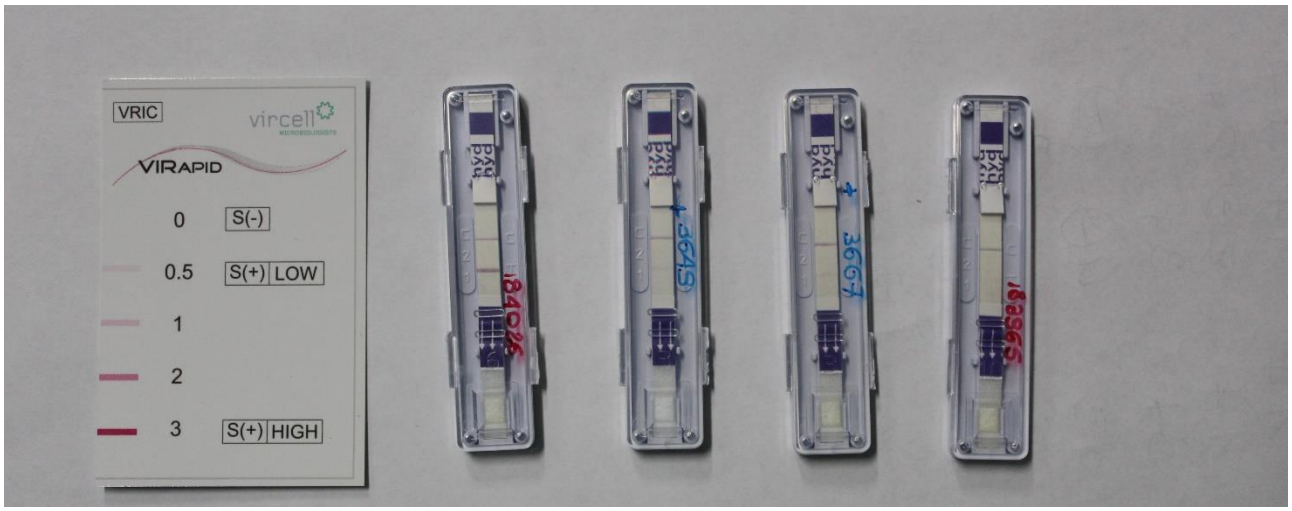

Figure 2 – Examples of positive (left) weak positive (two in the center) and negative (right) VIRAPID® Hydatidosis RDTs.

Serum aliquots to be tested in Pavia were shipped to the parasitology lab of San Matteo Hospital Foundation, in compliance with international and national legislation on transfer of biological material. Here, sera were tested with both the VIRapid® test and the commercial ELISA (Enzyme Linked Immune Sorbent Assay) test RIDASCREEN® Echinococcus IgG (R-Biopharm AG, Darmstadt, Germany), routinely used in the parasitology laboratory of the hospital. For the VIRapid® test, weak bands results were specifically noted for further analysis. For the ELISA test, Optical Density (OD) results were used to calculate and interpret a Sample Index (SI), as per manufacturer's instructions. ELISA results were considered positive for SI≥1.1, negative for SI <1.1. The RDT readers and the ELISA operator were blind to sample classification (CE/control; CE stage) and to results of other tests at the time of reading.

Statistical analysis

For the analysis, abdominal CE cysts were grouped into active (CE1, CE2 and CE3b), transitional (CE3a) and inactive (CE4 and CE5) [11]. When samples came from patients with more than one cyst, sera were classified according the stage of the cyst known to be more likely associated with a positive serology [155]. Sensitivity, Specificity, Negative Predictive Value (NPV), Positive Predictive Value (PPV), Positive Likelihood Ratio (LR+) and Negative Likelihood Ratio (LR-) for the **diagnosis of abdominal CE** were calculated for VIRapid® tests (Lima and Pavia setting with weak bands considered negative or positive in

two analysis scenarios) and the ELISA test. Individuals reporting a history of surgery for CE but no lesion on ultrasound referable to a CE cyst were either included in the analysis as controls or excluded from analysis in two analysis scenarios. Performance of ultrasound and serology tests combined serially were also calculated [155]. Sensitivity and specificity of different scenarios were compared using the McNemar's test; diagnostic accuracies were compared by comparing AUC (areas under the curve) using a non parametric approach for correlated receiver operating characteristic curves. Cohen's Kappa was used to evaluate the concordance between tests. Data were analyzed using STATA 15.0 (STATA Inc., College Station, Texas, USA).

8.4) Results

Ultrasound survey

Of the 1246 people registered during the census, 546 (43.8%) participated to the screening: 227 (35%) in Ondores, 213 (60%) in Corpacancha and 106 (30%) in Tomas. The median age of the participants was 35 years (range 5 - 87) and 347 (63.5%) were females. Of the 546 people who were examined by abdominal ultrasound, 33 (6.04%) had abdominal echinococcal cyst. The highest prevalence was observed in Corpacancha where the community had a prevalence of 13.6% (29/213). The total number of CE cysts found was 63. **Table 1** shows the distribution of cysts according to stages and abdominal organs affected.

	CE1	CE2	CE3a	CE3b	CE4	CE5	Total
Liver	13 (20.8%)	4 (6.4%)	8 (12.8%)	6 (9.6%)	18 (28.8%)	10 (16%)	59 (94.2%)
Spleen	0	0	0	1 (1.6%)	1 (1.6%)	0	2 (3.1%)
Kidney	1 (1.6%)	1 (1.6%)	0	0	0	0	2 (3.1%)
Total	14 (22.4%)	5 (8%)	8 (12.8%)	7 (11.2%)	19 (30.4%)	10 (16%)	63

Table 1 – Distribution of abdominal CE cysts by stage and organ.

One patient was found to have an abdominal cyst classified as CL. Albendazole *ex-juvantibus* treatment was recommended for this patient but he was excluded from this analysis as no information could be retrieved regarding following exams and final etiological diagnosis of the lesion.

Eighteen patients presented with focal liver lesions diagnosed as non-CE (12 simple cysts, two angiomas, one suspect fascioliasis and one complex liver cyst). None of these patients were unfortunately tested by serology

A history of surgical intervention for CE was reported by 6% (33/546) of the subjects. Interventions were most commonly for liver CE (63.7%, 21/33) followed by lung CE (30.3%, 10/33). Two subjects (6.06%, 2/33) reported a history of surgery for both liver and lung CE. Among individuals with a history of surgery for CE, five patients who had undergone lung surgery had abdominal CE cysts on ultrasound: four had liver CE cysts (n=3 CE3a, and n=1 CE4) and one had a splenic CE cyst (CE3b). No patient with a history of liver surgery still presented CE cysts on ultrasound.

Serology results

Serum was obtained from 114 volunteers, 33 cases with and 81 matched individuals without abdominal CE cysts visualized on ultrasound. VIRapid® test results in individuals with CE classified according to CE stage are summarized in Table 2.

Performance indexes of the tests, performed in the two country settings, are summarized in **Tables 2-4**. Overall, the analysis of the RDT performance shows that, both in Lima and Pavia, diagnostic performances were better when subjects with a history of surgery for CE were excluded, as expected due to the known persistence of detectable antibody titers for a long time even after successful surgery. The performance of the RDT was slightly better but not statistically different when applied in Lima, compared to Pavia if weak bands were considered negative (P for sensitivity was 0.08; p for specificity was 0.25 if post-surgical cases with no abdominal CE cysts were analyzed in the control group and 0.20 if post-surgical cases with no abdominal CE cysts were excluded from the analysis). Specificity was significantly better in Lima when weak bands in Pavia were considered positive ($p<0.001$ in both scenarios).

The ELISA test showed lower sensitivity and specificity compared to the RDT, in particular specificity was significantly higher compared to RDT performed in Pavia when weak bands were considered positive (p was

0.001 if post-surgical cases with no abdominal CE cysts were analyzed in the control group and 0.003 if post-surgical cases with no abdominal CE cysts were excluded from the analysis).

Diagnostic accuracy was not different if weak bands were considered negative (AUC Lima 0.79 AUC Pavia 0.71 AUC ELISA 0.71 p=0.13).

Table 2– VIRapid® test results (Lima setting) in individuals with CE classified according to CE stage.

	CE1	**CE2**	**CE3b**	**Total active**	**CE3a**	**CE4**	**CE5**	**Total inactive**
Positive	7 (77.8%)	3 (100%)	4 (100%)	14 (87.5%)	7 (100%)	2 (28.6%)	2 (66.7%)	4 (40.0%)
Negative	2 (22.2%)	0	0	2 (12.5%)	0	5 (71.4%)	1 (33.3%)	6 (60.0%)
Total	9	3	4	16	7	7	3	10

Table 3 – Test results and performance indexes of the VIRapid® test, Lima setting.

VIRapid Test, Lima setting							
Post-surgical cases with no abdominal CE cysts analyzed in the control group				Post-surgical cases with no abdominal CE cysts excluded from the analysis			
	CE pos	CE neg	Total		CE pos	CE neg	Total
Test pos	25	17	42	Test pos	25	8	33
Test neg	8	64	72	Test neg	8	48	56
Total	33	81	114	Total	33	56	89
Performance (95% CI)				Performance (95% CI)			
Sensitivity	0.76 (0.67-0.83)			Sensitivity	0.76 (0.65-0.84)		
Specificity	0.79 (0.69-0.85)			Specificity	0.86 (0.74-0.91)		
PPV	0.59 (0.48-0.67)			PPV	0.76 (0.63-0.82)		
NPV	0.89 (0.81-0.94)			NPV	0.86 (0.76-0.92)		
LR+	3.61 (2.72-4.27)			LR+	5.30 (3.77-5.90)		
LR-	0.31 (0.25-0.39)			LR-	0.28 (0.23-0.36)		

Table 4 – Test results and performance indexes of the VIRapid® test, Pavia setting. * One serum from one post-surgical case with no abdominal CE cyst at the time of screening was not available for analysis in Pavia.

VIRapid Test, Pavia setting							
WEAK BANDS CONSIDERED POSITIVE							
Post-surgical cases with no abdominal CE cysts analyzed in the control group*				Post-surgical cases with no abdominal CE cysts excluded from the analysis			
	CE pos	CE neg	Total		CE pos	CE neg	Total
Test pos	24	37	61	Test pos	24	23	47
Test neg	9	43	52	Test neg	9	33	42
Total	33	80	113	Total	33	56	89
Performance (95% CI)				Performance (95% CI)			

Sensitivity	0.73 (0.63-0.81)			Sensitivity	0.73 (0.62-0.81)		
Specificity	0.54 (0.43-0.62)			Specificity	0.60 (0.46-0.67)		
PPV	0.39 (0.30-0.48)			PPV	0.51 (0.39-0.61)		
NPV	0.83 (0.74-0.89)			NPV	0.79 (0.68-0.86)		
LR+	1.57 (0.14-2.06)			LR+	1.77 (1.25-2.31)		
LR-	0.51 (0.39-0.70)			LR-	0.46 (0.35-0.65)		
WEAK BANDS CONSIDERED NEGATIVE							
Post-surgical cases with no abdominal CE cysts analyzed in the control group*				Post-surgical cases with no abdominal CE cysts excluded from the analysis			
	CE pos	CE neg	Total		CE pos	CE neg	Total
Test pos	22	21	43	Test pos	22	12	34
Test neg	11	59	70	Test neg	11	44	55
Total	33	80	113	Total	33	56	89
Performance (95% CI)				Performance (95% CI)			
Sensitivity	0.67 (0.57-0.75)			Sensitivity	0.67 (0.56-0.76)		
Specificity	0.74 (0.63-0.80)			Specificity	0.79 (0.66-0.85)		
PPV	0.51 (0.41-0.60)			PPV	0.65 (0.52-0.73)		
NPV	0.84 (0.76-0.90)			NPV	0.80 (0.70-0.87)		
LR+	2.54 (1.84-3.20)			LR+	3.11 (2.17-3.80)		
LR-	0.46 (0.35-0.61)			LR-	0.42 (0.33-0.58)		

Table 5 – Test results and performance indexes of the RIDASCREEN® ELISA test. * Sera from 3 uninfected, 4 post-surgical, and 6 CE-infected individuals (3 CE1, 1 CE3a, 1 CE3b, 1 CE5) were not available for testing.

RIDASCREEN ELISA Test*							
Post-surgical cases with no abdominal CE cysts analyzed in the control group				Post-surgical cases with no abdominal CE cysts excluded from the analysis			
	CE pos	CE neg	Total		CE pos	CE neg	Total
Test pos	18	23	41	Test pos	18	13	31
Test neg	9	51	60	Test neg	9	40	49
Total	27	74	101	Total	27	53	80
Performance (95% CI)				Performance (95% CI)			
Sensitivity	0.67 (0.57-0.76)			Sensitivity	0.67 (0.55-0.77)		
Specificity	0.69 (0.57-0.76)			Specificity	0.76 (0.62-0.83)		
PPV	0.44 (0.33-0.53)			PPV	0.58 (0.45-0.67)		
NPV	0.85 (0.76-0.91)			NPV	0.82 (0.71-0.89)		
LR+	2.15 (1.51-2.80)			LR+	2.72 (1.85-3.45)		
LR-	0.48 (0.36-0.67)			LR-	0.44 (0.33-0.62)		

Cohen's Kappa of concordance between tests were from moderate to very good, as shown in Table 5. Interestingly, the worst concordance was obtained when comparing tests results from the two laboratories. Cohen's Kappa coefficients were overall increased by excluding post-surgical patients from the analysis.

Table 6 - Cohen's Kappa of concordance between tests.

Test 1	Test 2	Cohen's Kappa (95% CI) Post-surgical cases with no abdominal CE cysts analyzed in the control group	Cohen's Kappa (95% CI) Post-surgical cases with no abdominal CE cysts excluded from the analysis
VIRapid Lima	VIRapid Pavia (weak bands positive)	0.532 (0.386-0.677)	0.557 (0.394-0.720)
VIRapid Lima	VIRapid Pavia (weak bands negative)	0.717 (0.584-0.850)	0.689 (0.533-0.845)
ELISA	VIRapid Lima	0.602 (0.443-0.762)	0.598 (0.416-0.780)
ELISA	VIRapid Pavia (weak bands positive)	0.686 (0.549-0.823)	0.725 (0.578-0.872)
ELISA	VIRapid Pavia (weak bands negative)	0.853 (0.749-0.958)	0.920 (0.831-1.000)

Simulation of performance of ultrasound and serology tests for abdominal CE diagnosis

Field-applicable tools such as point-of-care ultrasonography and RDTs are suitable for prevalence studies. A further need in resource-poor endemic settings is the correct diagnosis of CE cases, and their subsequent clinical management. We therefore envisaged a hypothetical cross-sectional study of 5000 people in areas where the true prevalences of abdominal CE are 1% or 5%. Performance of ultrasonography was set at 0.98 sensitivity and 0.96 specificity [65]. VIRapid® test performance used for these calculations were those resulting from the Lima setting with individuals reporting surgery for CE but without abdominal CE cysts on ultrasound considered as non-infected (sensitivity 0.76, specificity 0.79). The expected results of a hypothetical screening study for estimating the prevalence of abdominal CE using ultrasonography alone, VIRapid® test alone, or the sequential application of ultrasonography followed by VIRapid® test only in individuals with evocative ultrasound imaging, are summarized in **Table 7**. As shown by the analysis, the use of serology alone would overestimate the disease prevalence, an estimate which would markedly improve if, instead, ultrasound alone was used in both prevalence settings. The introduction of the Rapid Test as a sequential test in individuals with possibly evocative lesions would slightly underestimate or overestimate the prevalence of CE, depending on the prevalence scenario.

Unfortunately it was not possible, with available data and due to the volunteer nature of blood donation in uninfected individuals, to perform a rigorous assessment of pre- and post-test probabilities of the RDT when applied in the differential diagnosis of hepatic lesions. However, when the RDT performance characteristics

were applied on a simulation of cases where the pre-test probability of a lesion being visualized on ultrasound was 50%, positive and negative post-test probabilities of VIRapid® test would be 78% and 22% respectively. If ELISA was applied as further confirmation on subjects with ultrasound lesions having a positive VIRapid® test, with a pre-test probability of 70%, positive and negative post-test probabilities of VIRapid® test would be 83% and 53%.

Table 7. The expected results of a hypothetical screening study for abdominal CE using ultrasonography alone, VIRapid® test alone, or the sequential application of ultrasonography followed by VIRapid® test only in individuals with evocative ultrasound imaging.

VIRapid test alone				**Ultrasonography alone**				**Ultrasonography followed by VIRapid test only individuals with evocative abdominal lesions**			
Sensitivity		0.76		Sensitivity		0.98		Net Sensitivity		0.74	
Specificity		0.79		Specificity		0.96		Net Specificity		0.99	
True prevalence 5%											
	RDT+	**RDT -**	Total		**US+**	**US-**	Total		**Test +**	**Test -**	Total
CE pos	190	60	250	**CE pos**	245	5	250	**CE pos**	186	64	2500
CE neg	998	3753	4750	**CE neg**	190	4560	4750	**CE neg**	40	4710	4750
Total	1188	3813	5000	Total	435	4565	5000	Total	226	4774	5000
PPV		0.16 (0.15-0.17)		PPV		0.56 (0.55-0.58)		PPV on US+		0.82 (0.78-0.86)	
NPV		0.98 (0.98-0.99)		NPV		0.99 (0.99-1.00)		NPV on US+		0.72 (0.67-0.76)	
Estimated prevalence		23.8%		Estimated prevalence		8.7%		Estimated prevalence		4.52%	
		True prevalence 1%									
	RDT+	**RDT -**	Total		**US+**	**US-**	Total		**Test +**	**Test -**	Total
CE pos	38	12	50	**CE pos**	49	1	50	**CE pos**	37	13	50
CE neg	1040	3911	4950	**CE neg**	198	4752	4950	**CE neg**	42	4908	4950
Total	1078	3923	5000	Total	247	4753	5000	Total	79	4921	5000

PPV	0.03 (0.03-0.04)	PPV	0.20 (0.19-0.21)	PPV on US+	0.47 (0.41-0.53)
NPV	1.00 (0.99-1.00)	NPV	1.00 (0.99-1.00)	NPV on US+	0.93 (0.89-0.96)
Estimated prevalence	21.6%	Estimated prevalence	4.94%	Estimated prevalence	1.58%

8.5) Discussion

In this study, we aimed to assess the performance of a commercially available RDT (VIRapid® HYDATIDOSIS) using field samples collected in an endemic area, during a screening campaign for CE. Prevalence of abdominal CE as assessed by abdominal ultrasound in the investigated area was similar to that found in other areas of the Andes in Peru [53,256,259]. In our hands, when tested using samples from selected patients in a controlled, laboratory setting using a selection of sera, the investigated RDT showed comparable sensitivity (74% vs 76%) and better specificity (96% vs 79%) compared to the results obtained in the field [91]. The comparable sensitivity obtained in the two settings (laboratory and field settings) may be due to a similar distribution of ultrasound detected CE cyst stages and localization in both cohorts, as it is known that these two factors influence substantially the sensitivity of serodiagnostic tests[155] The lower specificity observed in this field setting is also not surprising, considering the different cohorts, especially for what concerns the control samples. The positive serology results in the absence of CE cysts on ultrasound have been proposed to derive from exposure to infection without development of CE cysts[260], cross-reactions/non-specific reactions, inclusion among controls of people previously treated for CE still having detectable antibodies, and failure to detect CE infections in extra-abdominal organs (e.g. lungs). This latter scenario may be of particular relevance in our study setting, as a previous study from Peru evaluated in about 20% the relative proportion of lung CE on the total of CE infections in a screening site in the Central Peruvian Andes [256].Although one third of patients reporting previous surgery for CE had lung CE, this proportion cannot be used to reliably reflect the relative distribution of lung CE vs abdominal CE in our study population, as lung CE is more commonly symptomatic, therefore a comparatively larger proportion of patients with lung CE arrive to the medical attention than people, mostly asymptomatic, with liver CE [260]. Unfortunately, in our study we could not assess the presence of lung CE by chest X-ray, therefore we could not identify how many individuals classified as false-positive by

serology in our study were actually misclassified as they had lung CE. However, the sensitivity of serology in patients with lung CE is very low, as also evidenced by the aforementioned study by Gavidia and colleagues [256]. Therefore, it can be speculated that only small number of results classified as false positive would actually correspond to people with isolated lung CE, and could therefore not explain, alone, the CE prevalence overestimation deriving from the application of RDT alone in our screening simulations.

Our study highlights a limitation of serology tests, that is variable concordance of results between laboratories and between tests in the same laboratory, as evidenced also in other studies [261]. Concerning RDTs, this may be due in part to a subjective reading of fable bands, but also to batch-to-batch variability of the test itself. Indeed, when the Pavia operator read the photos of the RDTs performed in Lima that provided discordant results when tested on the same samples (n=29), only 60% (n=17) of tests were interpreted by the Pavia operator in the same manner as the original operator in Lima did, while in the remaining cases a fable band was recognized in the pictures but not read in the original tests in Lima (data not shown). The introduction of tests based on recombinant antigens, and the application of training and standardized methods for the performance and reading of assays may improve this aspect.

Point-of care tools are particularly suitable in resource-poor settings for both prevalence studies and in the clinical practice. Although the use of serology alone for the assessment of CE prevalence has been criticized [136], this approach is still unfortunately applied widely. When applying the test performances obtained in our field-based study to two hypothetical but plausible screening scenarios, it is evident that ultrasound alone or in combination, when possible, with a RDT with overall good performances applied to imaging-positive cases, could provide more reliable prevalence results than serology alone, which dramatically overestimate infection prevalence. This conclusion is also supported by past studies showing a significant difference between prevalences assessed by imaging and by serology[66,262,263]. Although removing individuals with previous surgery for CE from the analysis improves the performances of serology tests, we decided to carry out the simulation including these subjects category, as the real medical history and other factors influencing serology for CE such as type of surgery and time elapsed from intervention are not easy to determine reliably in a real-life, field setting. However, it seems reasonable to suggest not to perform ant RDT to discriminate post-surgical

CE relapses from residual post-surgical cavities, due to the known persistence of detectable antibody titers after surgery.

A further issue in resource-poor endemic settings is the correct diagnosis of CE cases, and their subsequent clinical management. It must be highlighted that ultrasonography is an operator-dependent tool, therefore both its sensitivity and, most importantly, its specificity, may vary considerably depending on the experience and specific expertise on CE of the operator. This factor as well as the prevalence of CE, the rate of CE cysts on the total of abdominal lesions found with ultrasound in a given population, influence the pre-test and post-test probabilities of a lesion being CE.

This study has several limitations, starting from the limited number of subjects included in the serology analysis and, as mentioned before, the impossibility of assessing the presence of lung CE by chest X-ray. Also, it must be stressed that most of the study participants (28/33 – 84%) came from a screening site with a high prevalence and as such the PPV of both ultrasound and rapid tests found in the study and applied in the simulations is particularly high. Finally, as mentioned previously, unfortunately we could not test specifically the value of the RDT for the classification of lesions that would have entered a differential diagnosis with the CE cohort.

Our results show that the sequential application of even one or more serology tests to cases with compatible/suggestive abdominal lesions on ultrasound may provide support to a less experienced clinician in clinical decision making when hepatic CE is suspected, with the recommendation to exclude from this approach people with a history of surgery for CE. However, prior knowledge of the local prevalence of CE and other pathologies/conditions which may enter in the differential diagnosis of CE cysts in the target population is imperative to define pre-test probabilities and therefore calculate post-test probabilities in different clinical settings. A further issue worth mentioning is the unfortunate scenario when a patient may have a non-CE lesion on ultrasound, testing positive on serology because of the presence of a lung CE indicing seropositivity. Again, the actual rate of occurrence of such scenario may be only ascertained by performing chest X ray to all individuals with any CE-evocating lesion upon ultrasound, which could not be performed here.

In conclusion, the commercial VIRapid® HYDATIDOSIS serological test applied in the field setting showed overall comparable sensitivity but lower specificity than those obtained in a controlled laboratory setting using a similar cohort of samples in terms of CE cyst stage distribution. The application of this RDT alone would not be reliable for the assessment of population prevalence of CE but could help clinical decision making in remote, resource-limited settings. Area-specific studies are needed to specify the role of serology in the confirmatory diagnosis of a CE-compatible lesion in different epidemiological and clinical contexts.

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9) Diagnostic performances of commercial elisa, iha, and wb in differentiation of hepatic echinococcal and non-echinococcal lesions: a retrospective analysis of data from a single referral centre

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9.1) Abstract

Objectives. The diagnosis of cystic echinococcosis (CE) is based on imaging. Serology supports imaging in doubtful cases, but no consensus exists on the algorithm to apply when imaging is inconclusive. We performed a retrospective analysis of serology results of patients with untreated hepatic CE and non-CE lesions, seen from 2005-2017, to evaluate their accuracy in the differential diagnosis of hepatic CE.

Methods. Serology results of three seroassays for echinococcosis (ELISA RIDASCREEN, IHA Cellognost, Western Blot LDBIO) and clinical characteristics of eligible patients were retrieved. Patients were grouped as having active or inactive CE and liquid or solid non-CE lesions. Sensitivity, specificity, and diagnostic accuracy were compared between scenarios encompassing different tests combinations.

Results. Eligible patients included 104 with CE and 257 with non-CE lesions. Sensitivity and diagnostic accuracy of WB were significantly higher than: i) IHA or ELISA alone; ii) IHA+ELISA interpreted as positive if both or either tests positive; iii) IHA+ELISA confirmed by WB if discordant. The best performances were obtained when WB was applied upon discordant or concordant negative IHA+ELISA. Analyses performed within "active CE (n=52) vs liquid non-CE (n=245)" and "inactive CE (n=52) vs solid non-CE (n=12)" groups showed overall similar results. Specificity was high for all tests (0.99-1.00) and did not differ between tests combinations scenarios

Conclusions. WB may the best test to apply in a one-test approach. Two first-level tests confirmed by WB seem to provide the best diagnostic accuracy. Further studies should be performed in different settings, especially where lower tests specificity is likely.

9.2) Introduction

Cystic echinococcosis (CE) is a neglected parasitic zoonosis caused by the larval stage (metacestode) of the tapeworm *Echinococcus granulosus sensu lato*. The parasite is transmitted between canids, definitive hosts, and ungulate intermediate hosts. CE is unevenly distributed over often vast and underserved rural areas where livestock-breeding communities live. Humans are accidental intermediate hosts, acquiring the infection through the ingestion of infective parasite eggs.

Echinococcal cysts may develop in humans in any organ or tissue, most commonly in the liver. Most infected people, especially those harbouring abdominal CE, are asymptomatic or pauci-symptomatic and often echinococcal cysts are diagnosed during imaging exams performed for other reasons. The diagnosis of CE is based on imaging, primarily ultrasound (US) for the abdominal locations[11]. CE cysts pass through different stages, as described in the WHO-IWGE (Informal Working Group on Echinococcosis) classification[11]. The differential diagnosis of CE cysts may be broad, ranging from harmless biliary cysts to malignancies. Pathognomonic features of CE, when present, can be visualized on US; however, US is an operator-dependent exam and good quality US machines and/or specific expertise on the recognition of CE characteristics are not widely available. Serology is used to support imaging in doubtful cases. However, currently available serology tests are not standardized, and their performances vary greatly, overall being quite unsatisfactory[21]. Several factors, including CE cyst location, stage, size, number, and previous treatments, influence the outcome of serology tests[155,264]. This induces a variable rate of false negative results. False positivity also occurs due to cross-reactivity with other parasites or other possible mechanisms such as exposure to the parasite in endemic areas and non-specific reactions[264]. Consequently, the correct interpretation of serology is challenging.

Although some diagnostic algorithms have been proposed[265], at present, there is no consensus on the application and interpretation of serology when imaging is inconclusive; most works investigating the performance of sero-assays were not designed to assess their usefulness for the differential diagnosis of detected lesions. We performed a retrospective analysis of data on three commercially available serological tests applied to patients with untreated hepatic CE cysts and non-CE lesions, seen at the outpatient clinic of the Unit of Infectious and Tropical Diseases, San Matteo Hospital Foundation, Pavia, Italy, a referral centre for CE located in a non-endemic area in northern Italy. The aim of this work was to evaluate the diagnostic accuracy of serology results in the differential diagnosis of hepatic CE.

9.3) Methods

The databases of the CE clinic were searched, and clinical and serological data of eligible patients diagnosed between 2005-2017 were retrieved, a period when the same three serology tests were consistently applied. Criteria for inclusion were: i) presence of only hepatic CE or suspect CE lesion(s) visualized on US; ii) having

been tested with the same three serology assays (see below); and iii) not having received previous treatment for CE.

Patients with CE were diagnosed and staged using US by experienced physicians. In some cases, the diagnosis of CE could be confirmed by observing a change in cyst morphology or seroconversion upon albendazole administration or after observing the presence of protoscoleces in the cyst fluid following percutaneous puncture. CE stages CE1, CE2, CE3a and CE3b (i.e. those cysts containing liquid components) were grouped as "active"; CE4 and CE5 cysts (i.e. with solid appearance) were grouped as "inactive". Patients with more than one cyst were classified as having active CE if at least one cyst was in stage CE1 to CE3b; patients were classified as having inactive CE if all cysts were in stage CE4-CE5. Non-CE lesions were classified as "liquid non-CE" if they had cystic appearance with liquid components, and as "solid non-CE" if no liquid component was observed on US. The diagnosis of these non-CE lesions was based on US, in some occasions flanked by other investigations including percutaneous aspiration, contrast-enhanced imaging, Doppler-US, and follow-up after *ex-juvantibus* albedazole intake showing no changes in cyst morphology.

The three commercial serology assays performed on all patients' samples included in the cohort were: ELISA RIDASCREEN Echinococcus IgG (R-biopharm, Darmstadt, Germany), IHA Cellognost Echinococcosis (Siemens, Erlangen, Germany), and Western Blot Echinococcus WB IgG (LDBIO Diagnostics, Lyon, France). All tests were carried out as routine diagnostic procedures and were performed and interpreted according to the manufacturers' instructions.

Results of serology tests were analysed anonymously and qualitatively (pos/neg). Sensitivity, specificity, positive and negative predicted values, positive and negative likelihood ratios, pre-test probability, and positive and negative post-test probabilities, with their 95% Confidence Intervals (CI), were calculated for each test and their combinations, on the whole cohort and on separated groups ("active CE vs liquid non-CE" and "inactive CE vs solid non-CE" lesions). Sensitivity and specificity of different scenarios were compared using the McNemar's test; diagnostic accuracies were compared by comparing AUC (area under the curve) using a non-parametric approach for correlated receiver operating characteristic curves [266]. Analyses were performed using MedCalc Bayesian Analysis Model (www.medcalc.com) and Stata 15.1 (StataCorp USA 2017).

9.4) Results

One hundred four patients with hepatic CE and 257 patients with non-CE lesions were included in the cohort. CE diagnosis in five patients with CE-suspect lesions was confirmed based on seroconversion (n=3), observation of hooks in the aspirated fluid (n=1), and progression from an active to inactive stage (n=1). In 27 patients, a non-CE diagnosis was confirmed by percutaneous aspiration (n=16), contrast-enhanced imaging or Doppler-US (n=7), and *ex-juvantibus* albedazole administration (n=4). Non-CE lesions included biliary cysts, hemangiomas, neoplasms, hematomas, abscesses, focal steatosis, and a textiloma.

Clinical and serological characteristics of the cohort are summarized in Table 1. In the non-CE group, only three patients had positive serology for CE, on IHA and/or ELISA. No patient with non-CE lesions was positive on WB.

CE COHORT				
GROUP STAGE	Number (%)	IHA pos (%)	ELISA pos (%)	WB pos (%)
ACTIVE	52 (50.0)	40 (76.9)	39 (75.0)	44 (84.6)
CE1	5 (4.8)	3 (60.0)	2 (40.0)	4 (80.0)
CE2	10 (9.6)	7 (70.0)	7 (70.0)	9 (90.0)
CE3a	13 (12.5)	11 (84.6)	12 (92.3)	10 (76.9)
CE3b	24 (23.1)	9 (79.2)	18 (75.0)	21 (87.5)
INACTIVE	52 (50.0)	10 (19.2)	10 (19.2)	16 (30.8)
CE4	28 (26.9)	6 (21.4)	9 (32.1)	11 (39.3)
CE5	24 (23.1)	4 (16.7)	1 (4.2)	5 (20.8)
TOTAL	104 (100)	50 (48.1)	49 (47.1)	60 (57.7)
NON-CE COHORT				
GROUP	Number (%)	IHA pos (%)	ELISA pos (%)	WB pos (%)
LIQUID	245 (95.3)	2 (0.8)	2 (0.8)	0 (0.0)
SOLID	12 (4.7)	0 (0.0)	0 (0.0)	0 (0.0)
TOTAL	257 (100)	2 (0.8)	2 (0.8)	0 (0.0)

Table 1. Clinical and serological characteristics of the CE and non-CE cohorts

We first analysed the whole cohort, and calculated the diagnostic performances of the three tests applied separately (scenario 1: IHA, 2: ELISA, and 3: WB) (Table 2, Table 3, web-only Supplementary Table 1). The specificity of WB was higher than that of IHA and ELISA, but the differences were not statistically significant. However, sensitivity and diagnostic accuracy of WB were significantly higher than those of the other single tests (p=0.012 sensitivity WB vs IHA; p=0.034 sensitivity WB vs ELISA; p=0.015 diagnostic accuracy WB vs IHA vs ELISA).

SCENARIO	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	AUC (95% CI)
Whole cohort					
1) IHA alone	0.48 (0.41-0.56)	0.99 (0.97-1.00)	0.96 (0.93-0.98)	0.82 (0.78-0.86)	0.736 (0.688-0.785)
2) ELISA alone	0.47 (0.42-0.52)	0.99 (0.97-1.00)	0.96 (0.93-0.98)	0.82 (0.78-0.86)	0.732 (0.683-0.780)
3) WB alone	0.58 (0.52-0.63)	1.00 (0.99-1.00)	1.00 (0.99-1.00)	0.85 (0.81-0.89)	0.788 (0.741-0.836)
4) IHA+ELISA	0.38 (0.33-0.44)	0.99 (0.98-1.00)	0.98 (0.95-0.99)	0.80 (0.75-0.84)	0.690 (0.643-0.737)
5) IHA and/or ELISA	0.57 (0.51-0.62)	0.99 (0.97-0.99)	0.95 (0.92-0.97)	0.85 (0.81-0.88)	0.788 (0.729-0.826)
6) IHA+ELISA; + WB if discordant	0.50 (0.45-0.55)	0.99 (0.98-1.00)	0.98 (0.96-0.99)	0.83 (0.79-0.87)	0.748 (0.700-0.796)
7) IHA+ELISA; + WB if discordant or neg	0.59 (0.54-0.65)	0.99 (0.98-1.00)	0.98 (0.96-0.99)	0.86 (0.82-0.89)	0.796 (0.749-0.844)
Active CE vs liquid non-CE					
1) IHA alone	0.77 (0.72-0.81)	0.99 (0.97-1.00)	0.95 (0.92-0.97)	0.95 (0.92-0.97)	0.880 (0.822-0.939)
2) ELISA alone	0.75 (0.69-0.80)	0.99 (0.97-1.00)	0.95 (0.92-0.97)	0.95 (0.92-0.97)	0.871 (0.811-0.931)
3) WB alone	0.85 (0.80-0.88)	1.00 (0.98-1.00)	1.00 (0.98-1.00)	0.97 (0.94-0.98)	0.923 (0.873-0.972)
4) IHA+ELISA	0.71 (0.66-0.76)	0.99 (0.98-1.00)	0.97 (0.95-0.99)	0.94 (0.91-0.96)	0.854 (0.791-0.916)
5) IHA and/or ELISA	0.81 (0.76-0.85)	0.99 (0.96-0.99)	0.93 (0.90-0.96)	0.96 (0.93-0.98)	0.898 (0.843-0.952)
6) IHA+ELISA; + WB if discordant	0.81 (0.76-0.85)	0.99 (0.98-1.00)	0.98 (0.95-0.99)	0.96 (0.93-0.98)	0.902 (0.847-0.956)
7) IHA+ELISA; + WB if discordant or neg	0.88 (0.84-0.92)	0.99 (0.98-1.00)	0.98 (0.95-0.99)	0.98 (0.95-0.99)	0.940 (0.896-0.984)
Inactive CE vs solid non CE					
1) IHA alone	0.19 (0.11-0.31)	1.00 (0.93-1.00)	1.00 (0.93-1.00)	0.22 (0.13-0.35)	0.596 (0.542-0.650)
2) ELISA alone	0.19 (0.11-0.31)	1.00 (0.93-1.00)	1.00 (0.93-1.00)	0.22 (0.13-0.35)	0.596 (0.542-0.650)
3) WB alone	0.31 (0.20-0.44)	1.00 (0.93-1.00)	1.00 (0.93-1.00)	0.25 (0.15-0.38)	0.654 (0.590-0.717)

4) IHA+ELISA	0.06 (0.02-0.15)	1.00 (0.93-1.00)	1.00 (0.93-1.00)	0.20 (0.11-0.32)	0.529 (0.497-0.561)
5) IHA and/or ELISA	0.33 (0.22-0.46)	1.00 (0.93-1.00)	1.00 (0.93-1.00)	0.25 (0.16-0.38)	0.663 (0.599-0.728)
6) IHA+ELISA; + WB if discordant	0.19 (0.11-0.31)	1.00 (0.93-1.00)	1.00 (0.93-1.00)	0.22 (0.13-0.35)	0.596 (0.542-0.650)
7) IHA+ELISA; + WB if discordant or neg	0.31 (0.20-0.44)	1.00 (0.93-1.00)	1.00 (0.93-1.00)	0.25 (0.15-0.38)	0.654 (0.590-0.717)

Table 2. Diagnostic performances of the three tests applied separately or in combination, on the whole cohort and divided by groups. The complete set of diagnostic performance parameters, including positive and negative likelihood ratios, and pre- and positive/negative post-test probabilities, is presented in the web-only Supplementary Table S1, Supplementary Table S2, and Supplementary Table S3. CI=Confidence Interval; PPV=positive predictive value; NPV=negative predictive value; AUC=area under the curve.

COMPARED SCENARIOS	**COMPARISON OF AUC p-value (superior scenario)**		
	Whole cohort	**Active CE vs liquid non-CE**	**Inactive CE vs solid non-CE**
1) IHA vs 2) ELISA vs 3) WB	**0.015 (3)**	0.171	0.081
1) IHA vs 4) IHA+ELISA	**0.002 (1)**	0.103	**0.005 (1)**
2) ELISA vs 4) IHA+ELISA	**0.003 (2)**	0.207	**0.005 (2)**
3) WB vs 4) IHA+ELISA	**<0.001 (3)**	**0.025 (3)**	**<0.001 (3)**
1) IHA vs 5) IHA and/or ELISA	**0.003 (5)**	0.207	**0.005 (5)**
2) ELISA vs 5) IHA and/or ELISA	**0.002 (5)**	0.103	**0.005 (5)**
3) WB vs 5) IHA and/or ELISA	0.618	0.289	0.783
4) IHA+ELISA vs 5) IHA and/or ELISA	**<0.001 (5)**	**0.035 (5)**	**<0.001 (5)**
3) WB vs 6) IHA+ELISA; + WB if disc	**0.014 (3)**	0.370	**0.010 (3)**
4) IHA+ELISA vs 6) IHA+ELISA; + WB if disc	**<0.001 (6)**	**0.020 (6)**	**0.005 (6)**
3) WB vs 7) IHA+ELISA; + WB if disc or neg	0.276	0.207	1.000
5) IHA and/or ELISA vs 7) IHA+ELISA; + WB if disc or neg	0.361	**0.024 (7)**	0.783
6) IHA+ELISA; + WB if disc vs 7) IHA+ELISA; + WB if disc or neg	**<0.001 (7)**	**0.039 (7)**	**0.010 (7)**

Table 3. Statistical significance of the comparisons of diagnostic accuracies (AUC, Area Under the ROC Curve) of different scenarios of the three tests applied separately or in combination, on the whole cohort and divided by groups. Statistically significant differences are indicated in bold. The complete set of comparisons including comparisons between sensitivities and specificities is presented in the web-only Supplementary Table S1, Supplementary Table S2, and Supplementary Table S3. Disc=discordant; neg=negative.

WB might be the test of choice for the differential diagnosis of hepatic CE when a single-test approach is used. However, WB is expensive, requires specifically trained personnel for its interpretation, and is often used only as a confirmatory second-level test. We therefore calculated the diagnostic performances of different scenarios where either WB is not available or is used only as a confirmatory test (Table 2, Table 3, web-only Supplementary Table S2 and S3).

When we simulated the instance where IHA and ELISA were applied in parallel and interpreted as positive if concordant positive (scenario 4), sensitivities and diagnostic accuracies were significantly lower than those of either IHA or ELISA as single tests (p=0.002 and p=0.003 respectively). WB still had, however, significantly better sensitivity and diagnostic accuracy than IHA+ELISA (p<0.001) (Table 2, Table 3, web-only Supplementary Table S2). On the contrary, in the instance when IHA and ELISA were applied in parallel and interpreted as positive if either test was positive (scenario 5), the tests combination had significantly higher sensitivity and diagnostic accuracy than the single tests (p=0.003 and p=0.002 respectively), while there was no significant difference with WB alone. Sensitivity and diagnostic accuracy were significantly higher for the scenario when IHA and ELISA were interpreted as positive if either test was positive (scenario 5) compared to the scenario when IHA and ELISA interpreted as positive if concordant positive (scenario 4) (p<0.001; Table 2, Table 3, web-only Supplementary Table S2).

When applied in case of IHA+ELISA discordant results, the addition of WB as a confirmatory test (scenario 6 vs scenario 4) had statistically higher sensitivity and diagnostic accuracy (p<0.001). However, WB alone was still significantly more sensitive and accurate than this combination test scenario (Table 2, Table 3, web-only Supplementary Table S3). Further, sensitivity and diagnostic accuracy were significantly higher for the scenario when WB was applied in case of IHA+ELISA discordant and concordant negative results (scenario 7), compared to the scenario when WB was applied in case of IHA+ELISA discordant results (scenario 6) (p=0.002 for sensitivity and p<0.001 for accuracy), while there was no significant difference with WB alone (scenario 3) (Table 2, Table 3, web-only Supplementary Table S3).

When we then applied the same analyses to the “active CE vs liquid non-CE” and “inactive CE vs solid non-CE” groups, overall the same results as the whole cohort applied to the “inactive CE vs solid non-CE”

comparison, while only a subset of comparisons reached statistical significance in the "active CE vs liquid non-CE" group. Results are detailed in Table 2, Table 3, and web-only Supplementary Tables S1, S2, and S3.

For all these analyses, performed on the whole cohort or the "active CE vs liquid non-CE" and "inactive CE vs solid non-CE" groups separately, specificity did not differ significantly between scenarios.

9.6) Discussion

The differential diagnosis of CE may be challenging. When imaging is inconclusive, serology plays a practical role in the diagnostic path; however, no consensus algorithm is available to guide the application and interpretation of serology. To our knowledge, this is the first study assessing the diagnostic accuracy of commercial seroassays for the differential diagnosis of hepatic CE, using a cohort of CE cases well characterized in terms of cyst stages and matching non-CE lesions, which were in differential diagnosis with CE. One previous study investigated the diagnostic performances of variable combinations of three in-house tests for the diagnosis of liver CE[28]. However, they used a heterogeneous cohort of CE cases without staging, and patients with cholelithiasis, a condition not entering differential diagnosis for hepatic CE, as controls. Although not comparable, the authors concluded that the best diagnostic accuracy was obtained using one or two first-line tests, followed by a highly specific third test, similarly to what can be derived from our data.

Our retrospective data show a surprisingly high specificity of all serology tests applied, with overall 1% false positive test rate in patients with non-CE lesions. An explanation may be that we assessed tests specificity on a particular cohort of patients, defined by the presence of lesions that might enter differential diagnosis with hepatic CE. Using this type of control population is sensible because serology is applied as a complementary test after the visualization on imaging of a lesion suggestive of CE. Similar to our results here, when we used the same type of control cohort to investigate the diagnostic accuracy of commercial rapid diagnostic tests, specificities were generally higher than those obtained in studies assessing the accuracy of seroassays using other control groups[91]. It must be noted, however, that our control cohort did not include any patient with alveolar echinococcosis (AE), due to the absence of patients with this serious condition cared for in our hospital. This is a limitation of our study, as AE is among the most important differential diagnoses of CE in co-endemic areas, and even the most species-specific serological tests show a high rate of cross-reactivity between the two species[267].

Our results indicate that WB may be the best single test to apply for the differential diagnosis of hepatic CE, when a one-test approach is chosen. WB alone was still generally superior to the application of two different tests. When we explored the scenario where WB was not available and two first-level tests were used in parallel, we found the rather counter-intuitive result that overall diagnostic accuracy was significantly higher when only one of the first-level tests was applied or when the two tests were used together but a true positive result was considered if just one out of two tests resulted positive. These findings derive from the high specificity of all tests observed in our study and should be interpreted with caution as this high specificity would not necessarily apply in areas where potentially cross-reacting conditions may occur more frequently. Indeed, although not statistically significant, scenario 4 (IHA and ELISA applied in parallel and interpreted as positive if concordant positive), intuitively, showed higher specificity than scenarios 1 (IHA alone), 2 (ELISA alone), and 5 (IHA and ELISA applied in parallel and interpreted as positive if at least one test was positive). Especially in settings where cross-reactivity may be frequent, the choice of favouring sensitivity over specificity should be evaluated carefully for its repercussions on clinical management. When feasible, the application of two first-level tests, confirmed by WB in case of discordancy or concordant negative results seems to provide the best diagnostic accuracy.

When examining the "active CE vs liquid non-CE" and "inactive CE vs solid non-CE" groups separately, overall results generally overlapped those of the whole cohort. The best diagnostic accuracy of the investigated serology tests (and their combinations) was found when serology was applied in the presence of active CE or non-CE lesions with "liquid content". This reflects the well-known higher sensitivity of serology tests in the presence of active and transitional compared to inactive cysts[155,264]. Taken as a whole, our results support the view that positive serology may be used to confirm a diagnosis of CE in the presence of reasonably high pre-test probability, while a negative test, even with WB, cannot exclude CE. The very low negative post-test probability observed in the "active CE vs liquid non-CE" group might suggest the possibility to interpret a negative serology as a CE-exclusion criterion in this group. However, this approach should be taken with caution and in light of cost-benefit considerations. That are whether it would be preferable to erroneously exclude a diagnosis of CE when serology is negative or to perform further diagnostic tests for CE notwithstanding a low probability of the lesion being CE. Besides the already mentioned absence of AE among the control conditions, this study has several limitations. The first is the retrospective design, which is burdened

by intrinsic selection bias. It was difficult in some cases, on the basis of clinical records, to disentangle whether serology results were taken into account or not in the final definition of CE vs non-CE case. Whenever possible, additional evidence confirming the diagnosis was sought. Also, no information was available on chest X ray of patients with non-CE hepatic lesions and serology-positive results, to exclude that seropositivity derived from an extra-abdominal CE. Finally, the generalizability of our results is also limited: we carried out the analysis on data from patients with only hepatic lesions, who had never been treated for CE, and seen in a reference centre located in a non-endemic area and with outstanding experience in the diagnosis of CE.

To conclude, our results suggest that: i) WB may the best single test to apply when a one-test approach is chosen; ii) the application of two first-level tests confirmed by WB in case of discordancy and concordant negative results seems providing the best diagnostic accuracy; and iii) serology should not be used to exclude CE in the presence of a compatible lesion. Further similar studies are needed to evaluate the performance of tests in different settings, with different expertise, available test, type and ratio of CE vs non-CE cases, and using a prospective, diagnostic benefit design.

TRANSPARENCY DECLARATION

Conflict of interest. The authors have nothing to disclose-

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10) Role of microRNAs in host defense against *Echinococcus granulosus* infection: a preliminary assessment

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10.1) Abstract

Cystic echinococcosis (CE) is a neglected helminthic zoonosis caused by the larval stage of the tapeworm *Echinococcus granulosus s.l.* MicroRNAs (miRNAs) are regulators of gene expression that have been linked with the pathogenesis of several human diseases, but little exists in the available literature about miRNAs in CE. Here we investigate the expression profiles of 84 microRNAs relevant to the function of lymphocytes and other immune cells during CE infection in the peripheral blood of patients with cysts in active and inactive stages. We applied the microRNA PCR Array technology to blood samples from 20 patients with a single hepatic CE cyst in either the active (CE3b) or inactive (CE4-CE5) stage. Our results show a 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) in patients with active cysts compared to those with inactive cysts.The high expression of these miRNAs in patients with active cysts suggests their role in a specific host immune response against the infection. Further work in this direction may help to shed light on the pathogenesis of human CE.

Keywords: MicroRNA, Cystic Echinococcosis, *Echinococcus granulosus*, hydatidosis, zoonosis, neglected disease

10.2) Introduction

Cystic echinococcosis (CE) is a chronic helminthic disease caused by the larval stage of the tapeworm *Echinococcus granulosus* sensu lato species complex. The life cycle of this parasite involves two hosts: dogs as definitive hosts and livestock (particularly sheep) as intermediate hosts, respectively. Humans act as accidental intermediate hosts. *E. granulosus* is distributed worldwide, with an estimated 1.2 million human cases [2] but CE is still a neglected disease [45]. In humans, the larval stage of the parasite develops as fluid-filled cysts that enlarge concentrically, up to a diameter of 20 cm or more and may survive for years or decades. These cysts are mainly localized in the liver (70%) and the lungs (20%) but may develop in any organ or tissue. The diagnosis and follow-up of CE is based on imaging. The WHO Informal Working Group on Echinococcosis (WHO-IWGE) developed a standardized classification of CE cysts based on ultrasound morphology[11], which also indicates the activity of the cyst and guides the clinical management of the patient [257]. Currently, the other diagnostic tool available for the diagnosis of CE is serology. However, serological tests suffer from many limitations, including false positives, false negatives and the scarce utility in the follow-up of patients due to their inability to distinguish between cysts that are definitely inactive and cysts with a potential for reactivation. Serology results are also influenced by several variables, including the cyst size, stage and number [155]. As such, new diagnostic and prognostic tools are much needed in the management of CE.

The mechanisms underlying the cohabitation of host and parasite 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 [268]. MiRNAs are small (19–24 nucleotide), non-coding RNAs that regulate gene expression post-transcriptionally by inhibiting protein translation or destabilizing target transcripts [269]. 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 [32,33,270]. MiRNAs are also involved in the pathogenesis of several human diseases including malignancies and diseases related to the immune response [32,33].

Recent studies showed that circulating miRNAs of both parasite and host origin can be detected in blood or fluids of humans and animals with helminth infections [271]. For this reason they are explored as potentially

diagnostic biomarkers for the early detection of parasite infection or related diseases. For example, human miR-192 has a potential utility as a noninvasive prognostic indicator for liver fluke-associated cholangiocarcinoma [35]. 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 [37,38].

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.

10.3) Materials and Methods

Sample collection

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-IWGE classification [11] 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. cysts without pathognomonic signs of CE on US were etiologically confirmed based on positive results on at least one serology test confirmed by presence of specific bands on Western Blot (ECHINOCOCCUS® Western blot IgG LDBIO Diagnostics, Lyon, France).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

Ethics statement

All patients signed an informed consent form for storage and scientific use of the leftover serum at the moment of blood sampling for routine serology. This retrospective study was performed according to the guidelines of Institutional Review Board of San Matteo Hospital Foundation, Pavia, Italy, on the use of biological specimens for scientific purposes in keeping with Italian Law (art.13 D.L gs 196/2003). All procedures performed in

studies involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

RNA extraction and reverse transcription

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).

For each sample, 250 ng of total RNA were retrotranscribed to cDNA using miScript II RT Kit (Qiagen, Hilden, Germany), as per manufacturer's protocol. Briefly, mature miRNAs were polyadenylated by poly(A) polymerase and converted into cDNA by reverse transcriptase with oligo-dT priming. The cDNA was then used for real-time PCR quantification of mature miRNA expression using the miScript miRNA PCR Assay (Qiagen, Hilden, Germania).

MicroRNA PCR Array

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.

Statistical analysis

The relative expressions of miRNAs were calculated using the 2-ΔCt method. The differences of miRNA levels between patients with inactive and active cysts were evaluated using a Mann-Whitney non-parametric test. All analyses were performed using Stata v 15.2 (StataCorp, USA) software. A $p<0.01$ was considered as significant.

Results

Sample RNA quality control

RNA concentration and purity were determined by NanoDrop ND-1000 spectrophotometer. The absorbance 260/280 ratio was ≥ 2 in all the samples. In addition, the integrity of RNA of several samples was determined by 1% agarose gel electrophoresis. The concentration of all the RNA samples was higher than 25 ng/ul, and the purity and integrity were suitable for microarray experiments in the present study.

miRNA profiling by miRNA PCR array analysis

To screen the expression of miRNAs in patients with different cyst stages, miRNA profiling by miRNA PCR array was performed on serum samples from 20 patients with CE with a single hepatic cyst. 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. In more detail, let-7g-5p, let-7a-5p, miR- 26a-5p and miR- 26b-5p showed an up-regulation of 15.3, 9.7, 11.2 and 8.4 folds respectively (all $p<0.01$; Fig 1.A); miR 195-5p and miR 16-5p, two members of the miR-15 family, showed an up-regulation of 5.7 and 12.3 folds respectively (all $p<0.01$; Fig. 1.B) and miR 30c-5p and miR 223-3p showed an up-regulation of 6.3 and 6.2 folds respectively (all $p<0.01$; Fig 1.C).

10.5) Discussion

CE is a chronic, complex and neglected disease with a worldwide distribution, especially in livestock-raising areas [1]. The spectrum of clinical manifestations ranges from asymptomatic to even fatal. The pathogenesis of CE is still unclear. The natural history of the cysts is not completely known; they appear to pass through different stages, from active to inactive forms [272], as classified by the WHO-IWGE [11], and to date the interplay between host and parasite remains largely unknown.

In the last years many research groups focused their attention on the relationships between miRNAs and immunity in both human and animals and on the changes occurring in the global miRNA expression patterns upon infection [36,273]. However, extremely little is known in this field regarding miRNAs in parasitic infections in the human host [271]

In this study we analyzed the expression profiles of 84 miRNAs involved in the regulation of the immune response in whole blood samples from 20 patients with CE in active and inactive stages using a miRNA PCR Array. Our results show a 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) in patients with active cysts compared to those with inactive cysts (fig. 1).

These miRNAs were already known to be involved in human diseases and widely studied in animal models. 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 [274]. 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, however results are still contrasting [34], 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 [275]. 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, and viruses, and also tumor cells [276] while miR-26b modulates the NF-jB pathway in alveolar macrophages by regulating PTEN [277]. 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 [278–280]. MiR-30 and miR-223 have a key role in the regulation of the innate immunity and in the type I interferon signaling and other cytokines, although their relative up-and down-regulation is not consistent among studies [281,282]. Both those miRNAs are also known to function as a tumor suppressor involved in many types of cancers [283,284].

Despite the small sample size analyzed, our 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 suggesting involvement of host miRNAs in the human-parasite interplay with the *E. granulosus* metacestode. These results add to the data obtained by Guo et al and Jiang et al, 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 gut during peroral *E. granulosus* infection, respectively [285,286]. The immune response to established hydatid cysts is poorly known; as summarized in a recent review[10], it is evident that immune modulatory mechanisms are in place, allowing the persistence of the established metacestode in the intermediate host for many years. In the intermediate host, cysts pass through different stages, in some occasions resulting in the spontaneous inactivation of the cyst. Although a different cell infiltrate can be observed surrounding intact and regressive cysts, it is unclear whether this is the cause of the effect of cyst structural changes. As a whole, however, current data deriving from mouse models point toward a possible damaging effect of a mixed Th1/Th2 immune response. Given the discrepancies in the literature on the correlation between different miRNAs and immune effector mechanisms,

it is not possible, at this stage, to attempt speculations on the up-regulation of miRNA found in our study in relation to the cyst activity and a defined pro- or anti-inflammatory environment. These preliminary data, however, open the way to further studies to investigate whether these miRNAs are related to *E. granulosus* infection and, in this case, the role of miRNAs in the pathogenesis of this complex disease.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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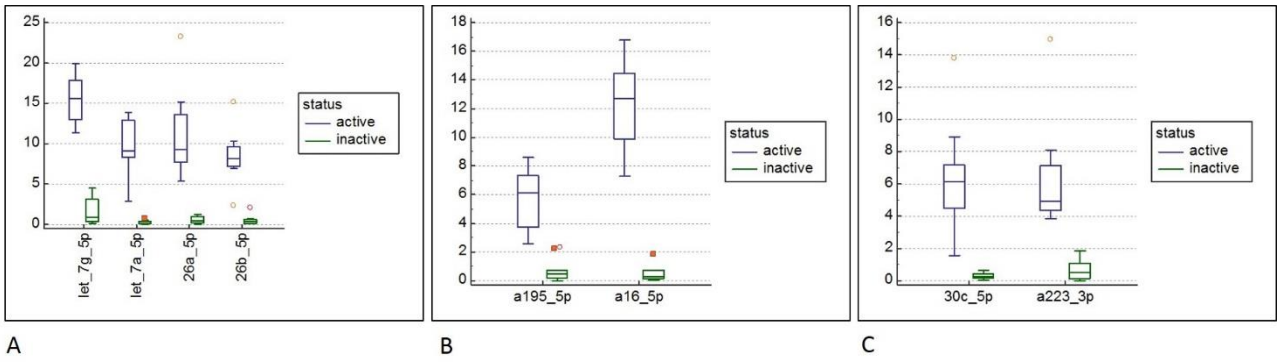

Figure 1. A, B and C: Expression levels of circulating miRNA in patients with active (blue) and inactive (green) cyst associated with the regulation of genes involved in the control of molecules connected with the immune system and signaling and transport of proteins.

11) GENERAL DISCUSSION AND CONCLUSIONS

This manuscript is the product of three years of research focused on diverse areas of CE research. As reviewed in the first presented article, much is left to be done for people suffering from CE. Despite the fact that this disease has a considerable impact on human and animal health[2], research on the topic continues to be underfunded and little attention is given to the problem, even in endemic settings[5,166]. A study published by our group and presented in section 2-2 of the thesis shows that patients willing to be treated in Italy had problems retrieving the ABZ, the main drug used to treat CE. This situation has been reported in other countries[287].

Several questions remain on the epidemiology of CE in different regions of the world, particularly in Asia, where countries that were considered less endemic during the existence of the soviet union have seen a return of CE cases[1,207,210] and Africa, where the epidemiology of the disease il largely unexplored[1,41,288]. In this thesis two papers on CE in Asia were presented (sections 2-2 and 2-3). One shows how knowledge of the disease is scarce even in endemic settings, a finding consistent with those of other studies[48,289,290] even though progresses seem to have been made in the last few years as evidenced by the decline in disease incidence shown by the analysis of the surgical incidence rates of CE in the country in the last ten years (section 2-3). The exact factors determining a decrease in CE, as discussed above, are not clear. In any case, the decrease in disease incidence has been shown to be not uniform in the country, with increases over time in some regions, as well as the presence of cases in children indicating active transmission[52,240].The study shows, however, that the surveillance system currently active in Kazakhstan does not provide information on the degree of activity of cysts. The fact that this valuable piece of information is omitted has implications for the clinical management of patients[11,84,250], especially when inactive cysts are treated by surgery, which may be an unnecessary intervention generating risks for the patient and costs for health systems[114]. Our group has previously shown in a publication that inactive cysts can be managed by a watch and wait approach, in accordance with expert recommendations[11,112], a conclusion supported by other studies[110,111,257]. Further data on the topic are presented in section (2-8) and reaffirm this important staple of CE clinical management. Data presented in section 2-9 of this manuscript confirm that this approach is also a viable option for inactive CE cysts adjacent to the IVC.

A further increase in data on CE epidemiology and clinical management is needed, however, and this can be achieved only with the collaboration of multiple centers sharing data on CE patients. To this end, the introduction of the ERCE has been greatly appreciated by a part of the CE community, even though critic points in the management of the registry remain to be addressed[58,59]. The value of inter-center collaboration is shown by the data published on bone CE by our group and scientists from eight different centers managing CE cases. While similar results have been published in a monocentric study[291], our study showed using data from multiple centers can be the start of building a consensus between experts.

Such efforts should be extended to the diagnosis of CE and to the use of serological tools, as shown by the fact that several published studies have failed to consistently analyze the role of different serological assays in the diagnosis of CE. The data presented in section 2-8 of the manuscript provides novel insights on this important topic, even though our study had several limitations connected to the retrospective nature of the study and due to the fact that serum samples did not come from a highly endemic area for CE for most of the considered patient cohort.

Data on the evaluation of a serological rapid test in an endemic, resource limited setting was also carried out by deploying a team in Perù. The study shows that a commercially available rapid test could act as a confirmatory test for the diagnosis of CE in situations where expert advise is lacking. The results also show that such tests are far from being used as screening tools, and further validation in different settings is needed, possibly using recombinant antigens who have shown promise in the diagnosis of CE[25,26].

Lastly, data on a possible new class of miRNAs has also been produced. These molecules have been shown to change their expression levels during other infectious diseases, and miRNAs from the hosts of parasites have in particular been studied during other helmintic infections as well as CE[32,37,38,271,292]. Our results provide the basis for the evaluation of a new potential diagnostic target for the diagnosis of CE, with potential applications in the follow-up of patients (section 2-9).

PART III – RESULTS FROM OTHER RESEARCH COLLABORATIONS

this part of the thesis contains results from other research collaborations I have contributed to during these three years.

1) Evaluation of the 'Active Melioidosis Detect' test as a Point of Care tool for the early diagnosis of melioidosis: a comparison with culture in Laos

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1.1) Abstract

Background. Melioidosis is difficult to diagnose clinically and culture of *Burkholderia pseudomallei* is the current, imperfect, gold standard. However, a reliable point of care test (POCT) could enable earlier treatment and improve outcomes.

Methodology/Principal findings. We evaluated the sensitivity and specificity of the "Active Melioidosis Detect" (AMD) Rapid Test (InBios, Seattle, USA) as a POCT, and determined how much it reduced the time to diagnosis compared with culture. We tested 106 whole blood, plasma and buffy coat samples, 96 urine, 28 sputum and 20 pus samples from 112 patients, of whom 26 (23.2%) were culture-positive for *B. pseudomallei*. AMD sensitivity and specificity were 65.4% and 87.2% respectively, the latter related to 10 weak positive reactions on urine samples, considered likely false positives. PPV was 60.7%, NPV was 89.3% and concordance rate between operators reading the test was 95.7%; time to diagnosis decreased by a median of 23 hours.

Conclusions. Our findings confirm that a strongly positive AMD result can reduce the time to diagnosis of melioidosis. However, the AMD currently has a disappointing overall sensitivity, especially with blood fractions, and specificity problems when testing urine samples.

Keywords

Melioidosis, *Burkholderia pseudomallei*, immunoassay, point of care technology, Laos.

1.2) Introduction

Melioidosis is an infectious disease caused by *Burkholderia pseudomallei*, a Gram negative, oxidase positive, saprophytic environmental bacillus.[293] The infection is highly endemic in South-East Asia and northern Australia,[294] but also widely under-reported.[295] The incidence is highest during the rainy season (May-October in SE Asia), especially following severe weather events.[296–298] The mortality rate ranges from 20 to 50%, rendering melioidosis a common cause of death in some areas.[299] In low resource settings, the wide differential diagnosis can prove problematic for physicians, which has been nicknamed 'the great mimicker'.[300]

Currently the 'gold standard' for the diagnosis is culture, using selective media such as Ashdown agar or selective broths for sites with a normal flora. However, the bacterium can be easily misidentified.[301] Moreover, the culture methods currently used have a sensitivity that may be as low as 60.2%.[302] Furthermore, culture takes several days before the diagnosis is confirmed,[303] leading to potentially fatal delays before the patient receives appropriate treatment.[304] New tools are therefore needed to diagnose the disease rapidly and accurately. Recently a qualitative, membrane-based lateral flow immunoassay Point-of-Care test (POCT) that detects the capsular polysaccharide of *B. pseudomallei*, the 'Active Melioidosis Detect' (AMD), has been developed by InBios (Seattle, USA).[305] The AMD has undergone a small number of evaluations for the direct detection of *B. pseudomallei* in a range of clinical samples [304–309] as well as for the detection of the organism in blood culture broths.[307,310] Such a test, which is easy to use and relatively cheap, could prove a very valuable tool in resource-limited settings.

In this study we aimed to evaluate the AMD as POCT for of suspected melioidosis, testing all available samples in real time as soon as possible after the admission of the patient, and to compare its sensitivity, specificity and time to presumptive diagnosis with that of culture.

1.3) Methods

Study site and population

The study was carried out in Mahosot Hospital, Vientiane, Lao PDR. The hospital serves patients from Vientiane Capital and is also a main national referral hospital for patients from other provinces. The number of patients with melioidosis diagnosed in the hospital has been steadily increasing over recent years.[311] Since

melioidosis is highly protean in its manifestations, study entry was simply based on a clinical suspicion of melioidosis by the responsible local physicians. Patients were actively recruited by one of the investigators (MCR) visiting the Adult and Paediatric Infectious Diseases, Ear-Nose-Throat, General Medicine, Adult Intensive Care Unit, Pulmonary, and Gastroenterology/Haematology wards at Mahosot Hospital at least once a day. The study was conducted throughout the rainy season (from June to October) in 2017.

Patient enrollment and sampling

All patients with clinically suspected melioidosis were considered for inclusion. The patient or a legal representative was asked to provide written informed consent before being enrolled. A standard set of samples was obtained as soon as possible after melioidosis was suspected by the local physicians. This included blood cultures, EDTA blood, throat swab, urine, sputum, pus, and body fluids (e.g. pleural or joint effusion, etc.) when clinically indicated and feasible. All patients from whom *B. pseudomallei* was isolated were started on the standard regimen[312] for the treatment of melioidosis as soon as possible after the laboratory informed the responsible clinician of the suspected diagnosis. When an AMD was positive, the result was comunicated by a member of the laboratory clinical team to the physician caring for the patient, explaining that the test was under evaluation, and a decision was made about the need for treatment based on the test result in the context of the clinical and epidemiological features. Treatment and outcome of each case were recorded on a standard proforma.

Laboratory procedures

All samples obtained were tested as soon as possible after their receipt in the laboratory. The AMDs were performed according to the manufacturer's instructions (see Supplementary Data) for all samples except blood. For these, an aliquot of the EDTA blood sample was taken and the remaining EDTA blood was separated into plasma and buffy coat fractions by centrifugation (2060rpm for 8 minutes). Thirty-five µl of each fraction was added to 1 drop of lysis buffer and then 35 µl of the mixture was added to three drops of chase buffer, mixed with a pipette and tested.

The results were read independently after 15 minutes by two different operators, one of whom was blind to clinical details. Line intensity was defined as "strong" if the line was clearly visible to the naked eye and in

a photograph, and "weak" if the line was hard to see with the naked eye and/or in a photograph. When discordant results were obtained, the AMD strips were reviewed by a third person who decided the line intensity. "Weak" lines were considered positive only if both investigators agreed that they were positive.

Blood cultures were processed as described:[313] the bottles were incubated in air at 37°C for 7 days, examined daily and, if turbid were subcultured onto Blood agar, plus Chocolate and MacConkey agar if Gram negative rods were seen on the Gram stain of the broth. Pus and sputum samples were cultured directly on non-selective media, Ashdown agar and in enrichment broths as described.[303] Centrifuged deposits of urine were cultured on Ashdown agar. Any Gram negative, oxidase positive rods, and all blood culture broths containing Gram negative rods, were tested with a latex agglutination reagent specific for the extracellular polysaccharide of *B. pseudomallei*.[314] Confirmation of identity of latex-positive colonies was made by testing with API20NE (bioMérieux, Basingstoke, UK). The time between the receipt of samples until the first positive AMD result and the first positive latex agglutination test were recorded.

Statistical analysis

We predicted the recruitment of 40 patients based on data from previous rainy seasons. Assuming a sensitivity of the AMD of 75%, the precision of the sensitivity with this sample size was estimated as being between 59% and 87% (95% Confidence Intervals), which would be similar to or higher than the reported sensitivity of culture (60.2%).[302] Data analysis was performed with the STATA statistical package (release 14.0, 2015, Stata Corporation, College Station, Texas, USA) and MedCalc (online version: https://www.medcalc.org/calc/diagnostic_test.php, Ostend, Belgium). We performed a Shapiro-Wilk analysis to test the normal distribution of quantitative variables. When quantitative variables were normally distributed, the results were expressed as the mean with 95% confidence intervals, otherwise median and interquartile ranges (IQR; 25th -75th percentile) were reported. Qualitative variables were summarized as counts and percentages to calculate sensitivities, specificities, PPV and NPV and the estimated 95% confidence intervals. Results were initally analysed by patient according to whether or not they had any positive culture for *B. pseudomallei*. Concordance between the AMD and culture were evaluated with Cohen's kappa; the kappa-statistic measure of agreement varies from 0 (no match) to 100 (perfect match). Analysis was also done by specimen type: AMD results on blood fractions were compared with concurrently

taken blood cultures, and otherwise AMD was compared with culture results on the same specimen. The Landis and Koch criteria[315] were used to categorise agreement between the two operators as follows: 0: poor; 0 –20: slight; 21 – 40: fair; 41 – 60: moderate; 61 – 80: substantial; 81 – 100: almost perfect. To assess statistical significance of the difference between time to diagnosis with AMD and culture, we used a Wilcoxon signed-rank test. A p-value of <0.05 was considered significant.

1.3) Results

Patients and samples

We enrolled 112 patients with suspected melioidosis during the study period of whom 59 were female (52.7%). Twenty-six (23%), including 12 females (46.2%), proved to have culture-positive melioidosis. From the 112 patients we collected 106 whole blood plasma and buffy coat samples, all of which had concurrent blood cultures; 96 urine samples (including 86 mid-stream urines and 10 catheter urines); 28 sputum samples and 20 pus samples. In addition, 107 throat swabs from these patients were cultured for *B. pseudomallei*, which is part of the normal local diagnostic workup of patients with suspected melioidosis.[316] Throat swabs and blood cultures were not available for five and six patients respectively, as these samples were not requested by the responsible physicians. Details of the patients and their ward locations are shown in Table 1.

Of the 26 patients with culture-positive melioidosis, 22 (84.2%) received specific treatment for melioidosis during hospital admission. Among the latter, eighteen patients survived (81.8%) and four died (18.2%). Three of the 26 culture-positive cases (12%) did not receive specific treatment for melioidosis (one refused treatment and two died before they could be treated), and one (3.8%) who had originally been admitted to Mahosot Hospital was subsequently transferred and treated elsewhere and information about their management and outcome were not available.

Analysis by patient

In total, 26 patients had melioidosis confirmed by culture. Of these, 17 (65.4%) had positive AMD results from at least one sample. Of the 86 patients with negative culture, 75 (87.2%) were negative by AMD on all available samples. The AMD thus showed a sensitivity of 65.4% (95%CI 44.3-82.8) and a specificity of 87.2% (95%CI 78.3-93.4), a PPV of 60.7% (95%CI 45.4-74.2) and a NPV of 89.3% (95%CI 83-93.4) for the

detection of culture-positive melioidosis in the 'by patient' analysis. The concordance rate between AMD and culture was 54.3% (95%CI 36.2-72.3).

Analysis by sample type

The agreement between the two operators reading the AMD results was 95.7% (95%CI 90.8-100), corresponding to an almost perfect match following Landis and Koch criteria.[315] The results were analysed by sample type, using culture as the gold standard, as shown in Table 2. Sensitivities ranged from 0% (95%CI 0-26.5) for buffy coat to 85.7% (95%CI 42.1-99.6) for pus. Specificities ranged from 79.6% (95%CI 70-87.2) for urine to 100% for whole blood, buffy coat, sputum and pus. Positive predictive values (PPV) ranged from 9.5% (95%CI 4.1-20.5) for urine to 100% for whole blood, sputum and pus, and negative predictive values (NPV) ranged from 88.7% (95%CI 88.7-88.9) for buffy coat to 98.7% (95%CI 93.7-99.7) for urine.

Positive urine results

Three patients had melioidosis confirmed by urine culture. Of these, two (66.7%) had a positive urine AMD result. Of the 93 patients with a negative urine culture, 19 (20%) had a positive urine AMD result. However, 9 (42.9%) of these 19 'false positives', came from patients who grew *B. pseudomallei* from other samples (including throat swabs) and were thus possibly detecting genuine antigenuria, whereas 10 (47.6%) were culture-negative on all samples obtained, resulting in an overall sensitivity for a positive urine AMD in detecting culture-positive melioidosis of 66.7% (95%CI 9.4-99.2) and a specificity of 79.6% (95%CI 70-87.2). Of the 9 positive AMDs from patients with positive cultures at other sites, eight were read as positive by both operators (three as strong lines and five as weak lines), while one was read as positive only by the operator totally blind to clinical details but not confirmed by the third operator. The latter patient had a deep pus sample that was positive by both culture and AMD. Of the other 8 patients, 7 had positive cultures and/or AMD from more than one site, whereas one was culture positive only from a throat swab.[316]

All 10 patients whose urine AMDs were positive but had no evidence of culture-positive melioidosis gave only weak lines in the AMD test. Eight of these were considered likely to be genuinely false-positive reactions, as the clinical presentations and courses of the patients were not consistent with melioidosis since

they were discharged in good condition despite having been treated with antibiotics that are not effective in melioidosis. The interpretation in the other two patients is unclear: one started IV ceftazidime but was discharged for treatment elsewhere and one was lost to follow up, so their outcomes are unknown.

These results are summarized in Table 3.

Time to diagnosis

In 16 (61.5%) culture-positive patients a positive AMD result was available earlier than culture, but only in five did this actually reduce the time until the patient received appropriate treatment, as in six cases clinicians decided to wait for culture results, in three cases treatment for melioidosis had already been given on the basis of clinical suspicion, and in two cases no melioidosis-specific treatment was given because one patient died too early and the other declined treatment. The time from sample collection to the first positive AMD result or presumptive positive culture result for those patients positive in both tests was reduced by a median of 23.1 hrs by the AMD, (IQR 6 hours) (p=0.003).

1.4) Discussion

Infection with *B. pseudomallei* is frequently severe, with septic shock occurring in up to 21% of patients.[317] The early administration of specific antibiotic treatment is key for securing a favorable outcome. A simple, sensitive and specific rapid diagnostic test which can be performed directly on clinical samples would be highly valuable, particularly since melioidosis is common in areas where laboratory facilities and training are often scarce. Culture, PCR and immunofluoresence assays (IFA)[318,319] often cannot be routinely used in melioidosis-endemic areas as they require dedicated facilities and highly trained staff. Tests for antibodies to *B. pseudomallei* using techniques such as indirect haemagglutination, whilst widely used, are beset with problems of both sensitivity and specificity,[320] although assays using better characterised antigens are being developed and show more promise as diagnostic tools.[321] Latex agglutination is useful for rapid testing of blood cultures growing Gram negative bacilli, but requires an initial incubation step, which inevitably delays the time to positive results.[322] The clinical management of melioidosis could thus be improved by a PoC test. This study built on our previous evaluation of the AMD, which was conducted at the same hospital,[307] but instead of using specimens passively received in the laboratory, we actively encouraged the submission of all relevant specimens (blood and urine, and sputum and pus whenever available) from suspected melioidosis patients as

soon as possible after hospital admission. Using this approach we found that for patients with a positive culture, the time to presumptive diagnosis was reduced by a median of 23 hours (p=0.003). In five cases, this actually led to a reduction in the time between admission and the start of treatment, and this might have happened in more patients had the test been fully evaluated and licensed. However, two of these five patients died, while three survived. A much larger study would be needed to determine whether this test actually has the potential to reduce mortality in patients with melioidosis.

In practical terms, the AMD is user-friendly as it requires little training and can give results in only 15 minutes. Moreover, in our hands the inter-operator concordance was good. Although the test is not yet available commercially, if marketed at relatively low cost it could be suitable for resource-limited melioidosis-endemic settings, where culture facilities are frequently not available.

Nonetheless, there is clearly room for improvement, as our 'by patient' performance analysis of the AMD showed an overall disappointing sensitivity (65.4%) and specifity (87.2%) for detecting culture-positive melioidosis. These are lower than the 85.7% sensitivity and 93.6% specificity by sample reported by Shaw and colleagues.[306] The low AMD specificity in our study was almost entirely due to weak positive reactions obtained on urine samples and read as positive at least by one operator strictly following the manufaturers' instructions. On clinical grounds we believe the majority of these to be false positives, the cause of which requires further investigation. Similar problems with urine samples were also reported by Shaw et al.[306]

In our 'by sample' analysis, the AMD performed directly on the various blood fractions had a very low sensitivity compared with blood cultures (0% for buffy coat, 16.7% for whole blood and 25% for plasma), as previously reported by Robertson et al.[304] but so far not analysed in other studies. This presumably relates to low levels of circulating antigen, even in patients with bacteraemia, and the comparatively large volumes of blood that are cultured. Sputum and pus samples showed good sensitivities in our study (80 and 85.7%, respectively) which were higher than the results obtained by Woods et al. (sputum sensitivity 33.3%, pus 47.1%)[307] and closer to the sensitivity for pus samples found by Shaw et al. (93.1%).[306] However, these two sample types were only available in 28 (25%) and 20 (22.4%) of the patients recruited, and seven (25%) and 5 (25%) of the 26 culture-positive patients respectively. Thus, our results suggest that the collection of these samples in patients suspected of having melioidosis is of great importance wherever possible.

One possible explanation for false negative cultures is prior antibiotic treatment, which is common in patients admitted to hospital in Laos.[323] Patients are routinely questioned about this by medical staff although they are often unaware of the agents they have taken or even whether these were antibiotics. However, we feel that this is unlikely to have had a significant impact in our study as the oral agents readily available in Laos, such as ampicillin and amoxicillin, have little or no activity against *B. pseudomallei*, and even in hospitals the agent most commonly used for empirical treatment of sepsis, ceftriaxone, has low clinical efficacy in melioidosis.[324]

The polysaccharide antigen detected by the AMD has been shown to be largely eliminated by renal filtration in a murine model, forming a single-walled nanotube structure that may allow its filtration through the glomerulus despite its high molecular weight of 300 kDa.[325] Thus, even when viable *B. pseudomallei* are not present in urine, antigenuria might result in a positive AMD. Urinary antigen detection appeared promising as a diagnostic test for melioidosis in our previous evaluation of the AMD [307] with a sensitivity of 86.7% compared with culture.[307] Urine AMD had a lower sensitivity in the current evaluation (66.7%), although we again found evidence of probable antigenuria in 9 patients who had a negative urine culture but a positive culture from other sites. However, in this evaluation we found problems with specificity of urine antigen detection. One possible explanation for this is that the methodology for testing urine in this study, as specified in the latest manufacturers' instructions, differed from that in our previous study, in which neat urine was tested. This requires further evaluation. We believe that the majority of these weak bands were likely to have been false positives as discussed above, but in future the use of an automated reader to quantify line intensity or urine concentration may help to distinguish between genuine antigenuria and false positive reactions.

1.5) Conclusions

Our study has confirmed the potential for a PoC test to enable earlier, potentially life-saving treatment in patients with melioidosis. Sensitivity and specificity are good with pus and sputum if these can be obtained, and a strong positive band on the AMD, as currently formulated, is sufficient evidence to start a patient on treatment for melioidosis. Urine antigen detection is promising, but further developments such as the modification of the test characteristics, implementation of an automated reader or the use of urine concentrators may help to distinguish between true-and false-positives. However, evaluations of the AMD

undertaken to date do not suggest that the AMD is likely to be useful for testing blood samples. It should also be noted that, as with all diagnostic tests, the sensitivity and specificity will never be 100% and so some patients will need to be managed on the basis of a strong clinical suspicion of melioidosis.

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Competing interests

None declaired.

Ethical approval

Ethical approval was granted by the Oxford Tropical Research Ethics Committee (protocol 507-17) and the National Ethics Committee for Health Research, Lao PDR (protocol 036).

2) Is there a role for bedside ultrasound in malaria? A review of the literature

ABSTRACT

Purpose

Point-of-care ultrasound (POCUS) has proven utility in the evaluation and treatment of many tropical diseases. Its role in malaria has been studied but the value for the clinician at the bedside is unclear. Our review aimed at summarizing existing studies to assess the usefulness, if any, of POCUS in malaria.

Methods

We used Boolean operators using keywords “malaria”, “acoustic”, “ultrasound”, “echography” and “ultrasonography” to search Pubmed, Scopus and Science Direct in three languages (Italian, French and English).

Results

We found twenty-two eligible references. Organs explored included the liver, spleen, heart, optic nerve sheath diameter (ONSD), kidney, lungs and cerebral vasculature. Multiple pathologic findings by ultrasound are reported but few demonstrate clinical utility. Current studies involve small number of patients with few trends emerging when comparing studies. Combining study results is limited due to the significant heterogeneity that exists between studies in regard to both methods of evaluation, as well as reporting of organ pathology and malaria severity.

Conclusions and assessment

A review of the current literature indicates that the use of ultrasound by clinicians adds little to the diagnostic evaluation of patients with malaria. Our review did find that measurements of the spleen, lungs, optic nerve sheath diameter and cerebral blood flow have potential utility in specific patient populations. Larger studies are needed to evaluate this utility persists when studied in a larger sample size.

Keywords: Malaria; Ultrasound; Diagnosis; Cerebral Malaria.

2.2) Introduction

Malaria is a disease caused by parasites belonging to the *Plasmodium* genus. In 2015, *P. falciparum* malaria was responsible for 214 million cases and 438.000 deaths, 90% of which occurred in Africa. African children under the age of 4 bear 90% of the burden of mortality from malaria, with estimates of around 306.000 deaths/year [326]. Rapid Diagnostic Tests (RDTs) and microscopy are usually employed to diagnose malaria. Microscopy is currently the gold standard for malaria diagnosis. However, its use can be difficult in some settings due to the lack of trained personnel [326]. Criteria for the diagnosis of severe malaria have been established by the WHO and include clinical parameters, parasitological findings and laboratory results [326].

The clinical presentation of malaria can vary widely based on both environmental factors as well as patient factors. Clinical findings in malaria include the presence of myalgia, arthralgia, fever, hypotension, chills, respiratory distress, GI tract symptoms such as diarrhea or vomiting and in the case of cerebral malaria, seizures, alteration of consciousness which can lead to coma. In the case of severe renal failure oliguria may also be present and urine might become dark (the so-called blackwater fever) [327]. Laboratory findings include a normocytic normochromic anemia, hypoglycemia, decreased platelet count. White blood cells are often normal, though they can be markedly elevated. Procalcitonin, CRP and fibrinogen levels are usually elevated, as well as fibrin levels [327]. Signs of a coagulopathy may also be present. Increases in serum lactate, creatinine, bilirubin, urate and muscle enzymes can be found. Acidosis may also be present [327]. Splenomegaly due to RBC sequestration is a common finding [328].

Patients who have lived their entire lives in a hyperendemic malaria zone may only present with fever and body aches while malaria can cause life threatening end organ damage in young children or travelers who have never been exposed previously. Particularly problematic for the clinician is that many other diseases that exist in tropical settings can appear with similar symptoms of fever, headache, confusion, and circulatory collapse. The diagnosis of severe malaria in endemic areas can be complicated: signs of severe malaria are nonspecific [329–331] and can be the consequence of other illnesses. Advanced laboratory testing is often not available where the burden of malaria is the highest. The presence of parasites does not necessarily translate into pathology:

in areas of high transmission, the prevalence of asymptomatic parasitemia in the population can be as high as 70% [332,333]. While rapid diagnostic tests and blood smears may help identify if a patient has a malaria infection this does not prove that the malaria is the cause of their presenting symptoms. Co-infection with other viral or bacterial pathogens may make it more difficult to tell if malaria is incidental in the diagnostic work up or the primary culprit of disease in a malaria endemic zone [331].

Imaging or testing available in low-resource settings that would allow the clinician to more accurately identify and risk stratify patients with severe malaria infection would be immensely helpful. Because malaria causes splenic sequestration and can lead to end organ damage in the liver, kidney, lungs and brain an imaging modality that allows a clinician to look pathology in these organs might have the potential to help in the diagnosis and treatment of patients infected with malaria. Given this known organ involvement, in undertaking this review our research question was: Does the current body of literature on ultrasound and malaria support the use of POCUS by clinicians as a diagnostic test in patients with suspected or confirmed malaria? Ultrasound is a well-suited tool for this purpose as it has been shown to have value in other tropical diseases that affect the abdominal and thoracic organs such as Tuberculosis, Schistosomiasis, Cystic Echinococcosis, and it has shown promise in the evaluation of Dengue. In the case of these conditions US has shown to be a precious instrument for the clinical management of patients with tropical diseases as it allows either the diagnosis of patients or their follow – up [334–336]. Current ultrasound technology has advanced to the point where ultrasound machines are extremely portable with excellent image quality. The nature of ultrasound allows for advanced imaging that is minimally invasive and highly repeatable [86,334,335].

In the case of malaria finding evidence of liver damage causing change in liver echotexture would be possible, as well as looking for evidence of RBC sequestration in the spleen. Thoracic ultrasound has advanced considerably in the past 15 years and finding pulmonary edema or pulmonary infiltrates in malaria patients might also be possible[337]. Ultrasound has also proven to be valuable in screening for elevated intracranial pressure by looking at optic nerve sheath diameter which is a known problem in cerebral malaria that is particularly devastating in pediatric patients [338]. We hoped to survey the literature to see if any of these ultrasound exams would be useful in the clinical care of malaria patients particularly in regard to risk stratification.

2.3) Materials and Methods

The main data sources used to carry out the literature search included three bibliographic databases: PubMed, Scopus and Science Direct. The computer search was conducted in 3 languages (English, French and Italian), combining the topic-related keywords "malaria", "acoustic", "ultrasound", "echography" and "ultrasonography", and using Boolean operators. We excluded papers on malaria in pregnancy in our review. The utility of ultrasound to evaluate the effects of malaria infections on fetal growth throughout pregnancy are well documented [339–341]. Fetal biometry that is used to defect fetal growth restriction is performed and interpreted by a physician with advanced fetal ultrasound credentialing and training. This is due to the fact that small errors in measurement can change fetal growth calculations substantially. We felt that fetal ultrasound in pregnancy has clearly proven benefits in expert hands. However, fetal biometry is beyond the scope of the average clinician in malaria endemic areas performing bedside ultrasound and so fell outside of our research question.

The last online search was performed on the 06th January 2019. Our initial search yielded 1822 potentially relevant references. The first screening based on title and/or abstract eliminated 1656 publications. During the review process, papers dealing with malaria in pregnancy were excluded for the reasons outlined above. In addition papers were excluded when the study population was less than 10 individuals. Review papers were excluded as they were summaries of primary literature sources that were already being included. No meta-analysis paper was found. After the review process (**Figure 1**), 22 references were obtained.

2.4) Patient cohorts

Out of 22 included papers, 10 (45%) included only adults [13,18–26], 11 (49,5%) only children [14,27–36] and 1 (4.5%) both children and adults [37]. Only 5 (22,7%) papers included both severe and non-severe malaria cases [337,342–345], while 5 (22,7%) did not stratify their populations [346–350]; 6 (27,2%) focused only on severe patients [23,24,26,29,32,36] and 3 (13,5%) distinguished between severe and cerebral malaria (CM) [28,33,35]. Among the papers that evaluated cases of severe malaria, the inclusion criteria differed: out of the 14 papers considering severe malaria patients, 11 (78,6%) based their inclusion criteria on WHO guidelines [342,344,345,351–355,357], while one paper divided patients into severe and non-severe on the sole basis of hemoglobin levels [343]. Two other papers used a Blantyre Coma Scale ≤2 to help further stratify patients [358,359]. A control group was included only in 5 (22,5%) of the papers [14,18,27,28,31]. The diagnosis of malaria was based on

microscopy in 20 papers (90,9%) [13,14,18–26,29–37], while the methodology was not specified for one paper (4.5%) [356]. In one case the diagnosis of malaria was based solely on RDT positivity [357]. Out of the considered studies, 2 (9%) were carried out in Europe [19,21], 3 (13,5%) in Oceania [30,34,37], 8 (36.4%) in the Indian subcontinent [13,20,22–26,32] and 9 (40,9%) in Africa [14,18,27–29, 31,33,35,36] (Table 1).

2.5) Organ Systems

The eligible papers examined seven different organ systems. The spleen was assessed in 10 (45%) articles [342,343,345–347,349,350,355,357,360], the liver in 7 (32%) [342,346,347,349,353,355,361], the optic nerve sheath diameter (ONSD) in 3 (14%) [342,345,358], the kidney in 1 (4.5%) [344], the heart in 6 (27%) [345,348,351,352,354,359], the cerebral vessels in 3 (14%)[345,356] and the lungs in 1 (4.5%) [337]. Some of the papers did not indicate the timing of assessment [342,347,349,350,352–355,361] while others employed multiple assessments in a matter of days [337,338,343] or hours [358]. Some papers compared US findings at the time of diagnosis and after recovery, in a period ranging from 21 days to 9 months [343,346,348,351]. Probe frequencies ranged from 1 MHz to 14 MHz The probes used in the study were not specified in 9 papers (41%) [338,343,349,350,353–355,359,361]. Table 2 summarizes the ultrasound methodology described in the reviewed studies. Heterogeneity between probe type (when listed), probe frequencies, and timing of assessment is significant (**Table 2**).

2.6) Examined organs and measurements

The **spleen** was assessed in ten papers (45%) [342,343,345–347,349,350,355,357,360]. Each paper used slightly different formulas to document spleen size. One study considered the maximum longitudinal dimension of the organ [342]. Another considered the spleen volume resulting by the formula 0.524 x length (cm) x width (cm) x depth (cm) [343]. Two studies used the geometrical formula for an ellipsoid [346,357]. Another study calculated the dimension of the spleen using Dittrich's formula, while also measuring length, width and depth [350]. Another study simply reported the presence of an enlarged spleen detected by ultrasound [360]. All studies but one found a correlation between the grade of malaria severity and the presence of splenomegaly, as in this paper malaria severity was not assessed [361]. Interestingly, all the studies are concordant in finding that children with severe malaria have more pronounced splenomegaly than those with uncomplicated malaria or healthy controls. However, one study found that children with CM and a pronounced splenomegaly had a higher chance of recovering from coma [357]. One paper found no correlation between spleen size and hemoglobin levels [343]. After treatment for malaria spleen

size was found to decrease in non-African patients with splenomegaly, but that was not the case in African patients [346].

The **heart** was assessed in 6 (27%) articles [345,348,351,352,354,359]. One article assessed the septal flattening, the ratio of right ventricular to left ventricular diameter, peak tricuspid regurgitation jet velocity and qualitative right and left ventricular contractility [345]. In two articles, the left ventricular end diastolic diameter (LVEDD) and left ventricular end systolic dimensions (LVESD) were measured. One paper also included the left ventricular myocardial performance index (LMPI), representing the "global" cardiac function along with a measurement of the inferior vena cava (IVC) collapsibility index (IVCCI) [359]. In one paper, the interventricular septal thickness (end diastolic DIVST), left ventricular posterior wall thickness (end diastolic DLVPWT), left ventricular ejection fraction (EF%), peak velocity of early filling phase (Ei), peak velocity of atrial filling phase (Ai) and Ei/Ai ratio were assessed [352]. One paper only reported a decrease in EF% and the presence or absence of diastolic disfunction, pericardial effusion, cardiac thrombi or valvular dysfunctions [354]. One study found that the left ventricular function was conserved in all assessed patients [345], while in another 86.3% of patients showed no pathological finding at echocardiography [348]. In another study no patient showed evidence of pericardial effusion [352], while in another 3.7% of patients did [354]. However, it should be noted that in the examined studies malaria patients were subject to cardiovascular problems in the presence of a normal US examination: 17% of the patients with normal ultrasound exams had cardiovascular complications and 11% had circulatory failure [352]. Lastly, one paper showed that an increase in the cardiac index (CI) and stroke index (SI) and a reduction of ejection fraction (EF) and fraction shortening (FS) can be present during the course of the disease [351].

The **cerebral circulation** was assessed in three (14%) studies using Transcranial Doppler (TCD) [338,345,356]. All studies assessed cerebral circulation in children. Murphy et al. compared cerebral circulation among children with cerebral, severe and uncomplicated malaria [345], while Newton et al just compared children with cerebral malaria with case controls [356]. Brien et al. compared children with signs of retinopathy and cerebral malaria with case controls [338]. Murphy et al. required a minimum of three recordings of the middle cerebral artery (MCA) on each side, calculating peak systolic flow velocity, mean flow velocity (time average peak), end-diastolic flow velocity and Pulsatility Index (PI). Results were averaged for each side, and the combined average was calculated [345]. The same measurements were calculated by Brien et al., who further divided findings into the

following diagnostic categories: microvascular obstruction, hyperemia, cerebral vasospasm, low flow, isolated posterior hyperemia, terminal intracranial hypertension (ICH) [338]. Newton et al. scanned the basilar artery (BA) and calculated mean peak flow velocities (Vm) and Gosling's pulsatility index (PI). The left to right ratios of the cerebral blood flow velocity (CBFV) and PI were calculated [14]. Murphy et al. reported that 19.4% patients had mean blood flow velocity >2 SD below mean value for age and 9.7% had a value >2 SD above the mean value for their age. No regional variation in cerebral blood flow was found [345]. Newton et al. support these results reporting irrelevant differences in the CBFV and PI of the right and left MCA and BA compared with those parameters in conscious children [356]. The same study, when comparing survivals and deaths, found a maximum MCA PI significantly higher in children who died, but no significant difference in other TCD parameters [356]. However, Brien et al. study found that 98% if children who are clinically diagnosed with cerebral malaria could be categorized into one of several phenotypes compared to the comparison group. Children with cerebral malaria and vasospasm phenotype have the highest incidence of neurologic sequelae (45%), while low flow phenotype is the most likely associated with death (32% compared to mortality of 22% for microvascular obstruction, 28% for hyperemia and 18% for vasospasm) [338].

One paper (4.5%) assessed the **kidney** volume in malaria by measuring the, length, width, anteroposterior diameter and cortical thickness. The volume was estimated using the ellipsoid formula [344]. No correlation was found between parasite counts and kidney sizes. No difference in renal volume was found between children who died and healthy controls [344].

One paper (4.5%) assessed the **lungs** in patients with malaria and sepsis by examining lung regions and defining aeration patterns from normal aeration to severe loss of lung aeration and lung consolidation [337]. However, only a total of 31 malaria patients were included in this study and less than half (13) had severe malaria. No correlation lung ultrasound findings and mortality was found in the subset of patients with malaria [337]. Interestingly patients with uncomplicated malaria were more likely to demonstrate lung pathology by ultrasound compared to those with severe malaria (51% vs. 39%). No definitive conclusions can be drawn given the small numbers of this study but lung findings in patients with malaria did not help with risk stratification in this study.

The **optic nerve sheath diameter (ONSD)** was assessed in three studies (13%) [342,345,358]. In Zha et al. measurements were obtained 3 mm posterior to the retina and a ROC curve was generated [342]. A similar approach

was also implemented by Murphy et al. and results were averaged for each eye [345]. Both studies adopted previously published criteria to determine if the measures were normal or altered [362,363]. Assessment of the ONSD compared patients with uncomplicated and severe malaria, plus a group of healthy controls [342]. The authors could not find any correlation between ONSD measurements and malarial severity. However, a study considering CM patients showed that 100% of CM patients had increased ONSD measurements [345]. In another study of CM 49% of patients had a ONSD of 4.3 mm or more [358]. An increased ONSD did not correlate with an increase in mortality, although in the group with increased ONSD there was a greater prevalence of neurological sequelae [358].

The **liver** was assessed in 7 articles (32%) [342,346,347,349,353,355,361]. Zha et al. measured its size along the right mid-clavicular line in the sagittal plane. Using the ROC curve test the threshold of >15.1cm was found to define hepatosplenomegaly [342]. Richter et al. defined hepatomegaly as an index of liver size exceeding the upper 95% percentile of a height-adjusted value in a healthy reference population [346]. In the Kochar et al. study, size and echo-texture of the liver, gall bladder, intrahepatic or extrahepatic bile duct dilatation and signs of portal hypertension were assessed in all the patients having serum bilirubin >10mg% [347]. Results of liver measurements were contradictory. Zha et al. reported a sensitivity of 58.3% and a specificity of 75.0% for patients with severe malaria [342]. A high prevalence of hepatomegaly was reported in two other studies, with prevalence of 24/29 (82.7%) [347] and of 25/29 (86.2%) [349]. However, a fourth study reported only 2.6% prevalence of hepatomegaly [346]. This variance may be explained by the fact that studies conducted by Kochat et al. and Kachawaha et al. measured liver only in patients with bilirubin >10 mg% [347,349], while in the study with a low percentage of hepatomegaly, more than half of the population was European and not living in a tropical area [346]. A further study considered the liver diameter alongthe midclavicular line and found that 58.5% of patients with malaria had hepatomegaly. The echogenicity of the liver was also evaluated, and the authors found that in patients with hepatomegaly and alterations in liver echogenicity there was a positive correlation with the elevation of liver enzymes [353]. Another study also examined the liver considering *P. vivax* malaria cases. The authors tried to correlate the presence of a *P. vivax* relapse with an increase in liver diameter measured on the mid-clavicular line and observed a positive correlation. However, the number of patients enrolled in this study was small and no information is given about the presence or absence of other conditions which could cause liver enlargement [361].

Parameters assessed for each organ investigated are summarized in **Table 3.**

2.7) Discussion

This study summarizes a heterogeneous collection of articles that attempted to identify ultrasound findings that would help risk stratify patients with malaria. Included papers assessed the use of ultrasound in multiple organs, with different methods and standard reference values [342,346,351]. Some studies gave no information on the type of probe used, while other studies evaluated the same organ using different probes. Most of the included studies had a small number of patients [343,345], some were done only in adults [342,353,354,361] others only in children [343–345]. Two studies did not define age groups at all [346,358]. The largest studies did not stratify patients by severity [346,358]. When malarial severity was defined, no common criteria were used [342,343]. Only five studies included a healthy control group [338,342,344,356,357]. Small sample size likely contributed to inconsistent results between studies. It is also possible that malaria's effect on organ systems varies between patient populations. As a result, drawing definitive conclusions about the utility of ultrasound in the care of patients with malaria is difficult.

Evaluation of the **spleen** did show a good specificity for severe malaria in one study (90.9%) [342] but with low sensitivity, and striking differences in prevalence across studies, ranging from 24% to 82.7% [345–347]. There may be differences in immune and non-immune populations with a reduction in spleen size documented after treatment in non-African patients but no change in the African cohort [346]. Kotlyar et al. suggested that an increase in spleen volume during an episode of severe malaria may have a protective effect for CM and mortality in children. The authors also reported that the absence of acute splenic enlargement in acute *P. falciparum* malaria may be associated with increased mortality [357]. This could be explained by the fact that the sequestration of parasites in the spleen could diminish the number of parasites invading the brain. However, these results need to be tested in larger studies, especially considering that African patients often manifest splenomegaly as a consequence of the chronic exposure to a multitude of tropical diseases [364,365]. Results of the assessment of the **heart** demonstrate a poor role of cardiac ultrasound in the diagnosis of malaria. Abnormalities found by these studies seem nonspecific and changes found are explained by the pathophysiologic mechanisms set in motion by the presence of an infection. Evaluation of the lung did not demonstrate any ability of pulmonary involvement in malaria patients to help with risk stratification. In both severe and uncomplicated malaria lung findings were present by ultrasound that were not seen on CXR. Larger studies are needed in this population to see if there is any clinically utility in these findings especially given the higher percentage of lung findings in the

uncomplicated malaria patients. Studies evaluating the **cerebral circulation** have inconclusive results, with low sensitivity for the detection of severe malaria in two studies [345,356]. One study did report a significant difference in cerebral circulation findings between children with known cerebral malaria and a comparison group of those without cerebral malaria. However, in this study both case and control populations were carefully selected. Cerebral malaria patients who were included had to have signs of retinopathy which is well-established as marker of increased severity in cerebral malaria. In this carefully selected sub-set of cerebral malaria patients there was a suggestion of prognostic value in specific patterns seen on transcranial doppler (TCD). This study differed from other studies on cerebral circulation both in patient selection and more specifically defined diagnostic categories seen on TCD.

Evaluation of **optic nerve sheath** has the most promise in the evaluation of malaria. However, these measurements are highly operator dependent and available studies have a small number of patients. Studies evaluating the **liver** have yielded contrasting results, the evaluation of either the dimensions or the echogenicity of the liver can show alterations in malaria patients, but these are nonspecific and have little value as a prognostic or diagnostic tool.

2.8) Conclusions

Our review found that the current published literature on the use of bedside ultrasound in the care of patients with malaria is heterogeneous. This is due to differences in study populations and study methodology.

Based on current evidence there is minimal utility of bedside ultrasound in the hands of the clinician to risk stratify the patient with malaria. As ultrasound becomes increasingly utilized in low-resource settings more research is needed, as there is suggestion of utility in certain patient populations. Measurement of cerebral perfusion and retinal perfusion could be a potentially useful tool but are far from having proven utility. Measurements of the spleen in children suffering from coma from cerebral malaria could serve as a prognostic tool and that measurement of the optic nerve sheath diameter in the context of cerebral malaria cases may hold promise as an adjunct to the clinical evaluation of such patients.

Ethical statements

1. **Conflict of interest:**

The authors declare that they have no conflict of interest

2. **Ethical approval**

This article does not contain any studies with human participants or animals performed by any of the authors.

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