Review Article

# Expression of cysteine proteinases and cystatins in parasites and use of cysteine proteinase inhibitors in parasitic diseases. Part I: Helminths

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## ABSTRACT

Cysteine proteinase (CP) is a new era as well as interesting topic in several publications during the last two decades. CPs enable several different biological activities in parasite biology and pathogenesis such as digestion of host proteins for nutrition, invasion through cellular and tissue barriers, processing secondary protein modifications for parasite survival as well as manipulation of the host immune system (immunomodulation). In this regard, CPs, like heat shock proteins, are suggested to be virulence factors and serodiagnostic markers. Therefore, CPs data are utilized as drug targets or vaccine candidates through use of their inhibitors as well as in diagnosis of several parasitic diseases. MEROPS is an on-line database for classification, characterization and structural properties of all identified proteinases and their inhibitors. Parasites express not only proteolytic enzymes for their survival and long persistence, but also inhibitors; cystatins, serpins and aspins to inhibit cysteine, serine and aspartic proteinases, respectively, both of the host and their own. On the other hand, CPs inhibitors (CPIs) are either general, inhibiting members of all classes of proteinases, or specific; inhibiting only one class of proteinases. However, a new classification was adopted in 2007, according to their structure; either with low molecular weight peptidomimetic inhibitors or those composed of one or more peptide chains. In spite of that, the majority of research studies used the old classification, general and specific CPIs. The main objective of the this review is to present updated data for all identified CPs and CYSs expressed in helminths and use of their CPIs as chemotherapeutic drug targets or as protective vaccine candidates. The secondary objective is to simplify, as possible, new approaches for drug and vaccine development as well as accurate diagnostic methods in parasitological researches utilizing the terrible evolution in bioinformatics and technology.

**Key Words:** Apoptosis, caspases, cathepsin, cystatins, cysteine proteinase, cysteine proteinase inhibitor, drug target, MEROPS, vaccine.

**Abbreviations: AEP:** Asparaginyl endopeptidase; **CP:** Cysteine proteinase; **CPI:** Cysteine proteinase inhibitor; **CYS:** Cystatin; **EM:** Electron microscopy; **E/S:** Excretory/secretory products; **IFN:** Interferon; **MW:** Molecular weight; **NEJ:** Newly excysted juvenile; **TNF:** Tumor necrosis factor; **WB:** Western blotting.

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## INTRODUCTION

Proteinases, known also as peptidases, proteases and peptide hydrolases, are enzymes for catabolizing proteins and polypeptides by cleavage of their peptide bonds. They are divided into two broad types according to their capability of formation of transient covalent bond. Metallo- and aspartyl-proteinases catalyze proteins without formation of covalent bond through use of the activated oxygen of a water molecule with the aid of a metal cation or without it, respectively. Proteinases which form a transient covalent bond, use the catalytic oxygen of serine (serine proteinases) or threonine (threonine proteinases), or the sulfur of an essential cysteine (cysteine proteinases, CPs). In CPs, the amino acid which provides the functional group, and makes an initial attack on the carbonyl carbon of a peptide bond to be cleaved (i.e. the nucleophile) is the sulfur of the -SH functional group of the active site cysteine. This sulfur molecule is particularly nucleophilic because of the indispensable presence of a chemical base (e.g. histidine)[1].

Through proteins catalytic function, CPs have several clinical applications. In human, it was found that any equilibrium disturbance between CPs and their natural inhibitors in ingested food leads to pathogenesis of several diseases such as cancer, rheumatoid arthritis, emphysema and osteoporosis. It is clear now that their proteolytic activity affect several specific pathological processes, e.g. calpains, specific-tissue CPs, showed several implications in cancer development such as cell transformation, invasion and apoptosis. Therefore, they are suggested by several studies as potential anti-cancer targets[2]. They also influence response of cancer cells to chemotherapeutic drugs through their activation[3]. Moreover, it was documented that both cathepsins S and L are involved in

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pathogenesis of rheumatoid arthritis through degradation of extracellular matrixes, suggesting use of their inhibitors as new drug targets[4]. Cathepsins also play specific roles in lung homeostasis and pathophysiological processes in several pulmonary diseases; emphysema, chronic bronchitis and silicosis[5]. Cathepsin K is now considered as novel therapeutic target in treatment of metabolic bone disease, e.g. osteoporosis[6].

In helminths, CPs play several essential roles in development of life cycle stages such as egg hatching, larva molting and migration. They were found to change or influence Th1/Th2 profile in their hosts to create a suitable environment for sustained parasite burden in the host, i.e. favoring for virulence and pathogenesis of the diseases they caused. Besides, they have role in host tissue degradation, evasion of host immune response(s) and nutrition balance. Therefore, they are considered important virulence factors, and their inhibitors are of scientific interest for parasitologists in the last two decades to discover new drug and vaccine targets[7-9]. Furthermore, it is known that trypsin family serine proteases are involved in protein digestion in vertebrates' intestine, whereas CPs of the papain family and aspartic proteases take the role in primitive invertebrates. In a review article, Turk *et al*.[10] discussed roles played by lysosomal cathepsins in cellular processing, however, protein degradation is the most important. In trematodes gut, cathepsins D and B are involved in hemoglobin digestion and albumin degradation, respectively, while asparaginyl endopeptidase (AEP) shared with both cathepsins B and L in protein digestion[11]. On the other hand, one of the most important immune responses against gastrointestinal nematodes is the release of IL-4 and IL-13 (Th2-type cytokines). Meanwhile, squamous cell carcinoma antigens are secreted from human gut epithelial cells through IL-4 and/or IL-13 cytokines. In their work, the investigators found that these antigens inhibited several parasite-derived CPs, indicating new mechanism of immune response against helminthic infections[12].

Cystatins (CYSs) are reversible, tight-binding CPs inhibitors which are divided into three major categories according to their molecular weights (MW) and presence of disulfide bridge; the 1st (type I) includes those with no disulfide bridge and with mean MW 11 kDa, type II includes those with two bridges and mean MW 14 kDa, while type III are glycoproteins with high MW (60 to 120 kDa)[13]. Filarial nematodes not only express CPs, but also express CYSs which proved to possess potent immunomodulatory activity. CYSs stimulate production of tumor necrosis factor-α (TNF-α) and IL-10 with interference of antigen processing and presentation of dendritic cells and macrophages[14]. In addition, the mechanism through which *Fasciola* spp. alter host immune response to favor their survival was illustrated. The investigators identified immunomodulatory proteins (helminth defense molecules) which bind with macrophage plasma membrane to be processed by lysosomal cathepsin L leading to interference with macrophage function (antigen processing)[15]. On the other hand, CYSs, similar to CPs, were suggested as therapeutic drug targets to relieve some inflammatory diseases. It was reported that *Av*17, a CYS expressed in *Acanthocheilonema viteae*, the rodent filaria, significantly decreased IL-4 production, reduced eosinophils recruitment with their effector molecules, and lowered mast cells sensitization, suggesting its role to relieve allergic manifestations occurred in other helminthic infections[16]. Later, German investigators experimentally demonstrated the mechanism through which *Av*17 could manipulate macrophage-mediated inflammatory reactions, reducing progression of inflammatory diseases, e.g. colitis[14], and allergic diseases[17]. Another example is CYS derived from *S. japonicum*, which was suggested as potential drug to reduce progression of inflammatory diseases[18,19].

Inhibitors of CPs (CPIs), reported in several publications in the last two decades were mainly confined to E64, phenyl vinyl sulfones (PVSs) and histone deacetylases (HDACs) inhibitors. E64 is the most common CPI used in several studies to recognize characters, properties and functions of a certain studied CP. It showed efficient inhibitory activities on the major CPs of clan CA identified in helminths; papain (family C1) and calpain, cathepsins B and L (family C2)[20,21]. Vinyl sulfones gained much interest in pharmaceutical research and offered the potential of new direction for chemotherapy of helminthic diseases. They are characterized by high selectivity for CPs over serine proteases, stable inactivation of the target CP and relative inertness in the absence of the protease target active site. These characters make them with desirable pharmacokinetic and safety profiles in experimental studies in rodents, dogs and primates[22]. Sequence analyses and substrate profiling validated PVS as drug target due to its significant reduction in parasite burden and pathology in *S. mansoni* murine model[23,24]. PVS proved to have *in vitro* lethal effect on *F. gigantica* adult worms as evident by electron microscopy (EM)[25]. On the other hand, HDACs form a conserved enzyme family that controls gene transcription and hence its expression *via* the removal of acetyl residues from histones. The latter is the major component of chromatin, and inhibition of deacetylation influences gene expression and regulates genomic functions, through different mechanisms[26]. For example, HADC inhibitors, through gene regulation, induce apoptosis *via* regulation of reactive oxygen species production[27], or receptors death[28], or mitochondrial pathway[29]. *S. mansoni* possesses three HDACs (class I)[30] and several HDACs (class II)[31]. In spite of presence of expressed sequence tags (ESTs) encoding multiple putative members of HDAC (class III) in *S. mansoni* genome, their presence was denied in all developmental stages[26]. In their review, Andrew *et al.*[26] assigned certain criteria to develop new anti-parasitic drug utilizing HDAC inhibitors; selection of compounds with high potency, high safety profile to be used during childhood and pregnancy;

low cost, suitable activity against drug-resistant strains, and could be used in combination with other already potential drugs. HDACs inhibitors (valproic acid; trichostatin A, and sodium-butyrate; BA) were evaluated in treatment of schistosomiasis in several studies[23,24, 31-33].

## Classification

**MEROPS**, a website assigned for Welcome trust foundation, is an updated information for proteinases database and it includes their classification as well as details for their homologs, inhibitors, substrates and specificity[34]. The classification is based on similarities at the tertiary and primary structural levels in the amino acids sequences. The classification is hierarchical, based on sequence comparisons of the domains known to be important for activity. A protein that has been sequenced and characterized biochemically is chosen as a representative (holotype), and all sequences that represent species variants of the holotype are grouped into a protein species. The sequences of statistically significant related protein species are grouped into a family. Families that are believed to have a common ancestor are grouped into a clan. In other words, a family contains related sequences, and a clan contains related structures.

During the period 2008-2014, a series of articles were published by Rawlings and his colleagues[35-39]. The focus of these publications is to through much light on the data provided by MEROPS, which are very useful for designing inhibitors. Therefore, these publications provided 1) methods to distinguish between each proteinase and its interactions with substrates and/or inhibitors, 2) new tools to compare proteinases and their inhibitors in different strains of the same organism or between organisms, 3) data related to preference for individual amino acids (e.g. arginine) near the cleavage site, however, cysteine, tryptophan, asparagine, histidine and methionine are rare amino acids around cleavage sites, 4) data for other proteolytic enzymes, with establishment of orthologues that could be detected in other species, 5) data for establishment of new indexes for gene names and peptidase substrates, 6) detailed description of the small-molecule peptidase inhibitors including naturally occurring compounds such as pepstatin, bestatin and amastatin, as well as synthetic inhibitors that could be generated in a laboratory, and finally 7) details of cleavage positions in substrates, natural and synthetic as well as physiological and biochemical. It is worthy to identify the cleavage sites in substrates as it determines proteinases physiological roles and its specificity, which can help in the design of better and more selective synthetic substrates and inhibitors[35-38]. In a later publication in 2014[39], it was observed that the sequence of family names is not consecutive because some families were removed from the database, either because a sequence relationship has been discovered to another family in the database, or if experimental studies showed that the activity is not that of a peptidase. The number of the identified proteinases exceeds 4000, and each was assigned an unique MEROPS identifier in spite of its removal from a certain family to another. However, comparisons of the intron–exon structure of eukaryote genes was recently developed which proved to be useful in understanding their evolution[39].

Furthermore, MEROPS website presents a high quality snapshot of the relative similarities of parasite proteinases which provides a useful context for new work on parasite proteinases. Atkinson *et al.*,[40] reported that sequence similarity networks (SSNs) allowed very large groups of homologous proteins to be viewed together and clustered by similarity. To create SSNs, the sequence similarity between each pair of protein sequences, classified within a MEROPS clan or family, is calculated using the sequence alignment program BLAST. Thus, SSNs allowed for proteins with correlated protein structural similarity to be marked and statistically correlated with a BLAST E-value. It was found that the shorter the edge connecting two proteins, the more similar the pair of proteins. Moreover, SSNs could be extracted to view sets of protein attributes tailored to specific interests using network visualization software termed Cytoscape[41].

MEROPS in its previous releases grouped certain proteinases showing great homology to a sequenced and biochemically characterized CP, termed "type example, TE". Accordingly, a review article was published in 2009, and CPs were classified into nine clans with TE, and one unclassified clan without TE; 1) CA (TE: Lysosomal cathepsins), 2) CD (TE: Caspase), 3) CE (TE: Adenain), 4) CF (TE: Pyroglutamyl peptidase), 5) CH (TE: Hedgehog protein), 6) CL (TE: Sortase B), 7) CM (TE: Hepatitis C virus peptidase), 8) CN (TE: Sindbis virus-type peptidase), and 9) CO (TE: Dipeptidyl peptidase VI)[1]. However, because some families were removed from clan to another, and discovery of new clans and family members, MEROPS (release 11.0) classified CPs into ten clans; CA, CD, CE, CF, CL, CM, CN, CO, CP and CQ and one unassigned that includes 9 CPs[34]. Almost all helminthic CPs are associated with deubiquitinating activity (DUB), which is deubiquitinating peptidase. It is a peptidase that can catalytically remove the ubiquitin covalently attached to specific lysine side chains, and this enzyme is also referred to as isopeptidase[42].

Clan CA includes the majority of helminthic cathepsins (80%) which belong to family C1. Among clan CD, two families include the rest of helminthic CPs; caspases (C14) and legumains (C13). Members of C14 are identified in all creatures, and they are either C14A and C14B. The first includes only caspases, while the second contains both metacaspases (C14B M) and paracaspases (C14B P). Caspase is an abbreviation of cysteine-dependent, aspartate-specific peptidase, as they are specific peptidases for protein substrates containing aspartate[43]. They are either inflammatory or apoptotic caspases, and the latter are

either initiator or effector[44]. Family C13 includes two CPs which play important roles in parasite-host interactions; GPI–protein transamidases and AEP/legumains. Several pathogens have surface-bound GPI-anchored proteins, so they have the ability to elicit immune response and they are also able to bind to host cell-surface receptors, allowing the pathogen entry into the cells[45].

**Cysteine proteinases:** In a review article, Dzik[46] discussed all molecules; proteinases and others, expressed and/or secreted by different helminths to establish their long existence in the host. The reviewer addressed and discussed all CPs functions on biochemical basis; suitable pH and temperature, specific type of proteolytic nutrient or tissue, CPs localization in different developmental stages, different sites in the host and vector or snail for CPs expression with their associated enzymes and hormones-like, and lastly type of host immune response, either humoral and/or cellular, with special emphasis on pro-inflammatory cytokines. He also discussed helminths which produce protein homology with interferon-γ (IFN-γ) such as *Taenia crassiceps* and *Trichuris muris* or homology with migration-inhibitory factor such as *T. spiralis* and filarial worms.

## Trematodes

• ***Schistosoma* spp.:** Several studies were conducted in the last two decades to identify different CPs in schistosomes and to elucidate their functions that enable *Schistosoma* spp. to penetrate host skin, i.e. overcome host dermal defense barrier, and to travel in host circulation, i.e. overcome host immune response either humoral or cellular. The most two important CPs are cathepsin B (clan CA, family C1) and AEP/legumain (clan CD, family C13). They were previously termed as *Sm*31 and *Sm*32, respectively, and were thought to be involved in hemoglobin degradation. Localization of both CPs was investigated, and high expression levels were observed in adult female extracts, while lower levels were immunolocalized in cercarial cecum and flam cells. The obtained results suggested that both CPs might play a role with flam cells excretory and osmoregulatory functions[47].

In 2005, RNA interference technology, a reverse-genetics tool for identification of gene functions, was used to silence expression of the gene encoding *S. mansoni* cathepsin B1 (*Sm*CatB1). Treated schistosomes showed significant growth retardation without reduction in hemoglobin degradation when compared to control schistosomes[48]. However, *Sm*CatL3 is a new cathepsin L member identified in *S. mansoni* expressed sequence taq (EST) and the investigators claimed its contribution in the proteases cascade catalyzing host nutrients[49]. One of the vaccine trials conducted by El-Ridi and her colleagues[50] showed that (*Sm*CatB1) produced 60% protection with significant increased levels of antigen-specific antibody responses as well as Th2 cytokines (IL-4, IL-5 and IL-13) 6 days post the challenge infection. The investigators also showed that combined immunization with *Sm*CatB1, *F. hepatica* cathepsin L, *Schistosoma* glyceraldehyde 3-phosphate dehydrogenase (SG3PDH) and peroxiredoxin (PRX-MAP) increased the protection to 83%. In addition, *Sm*CatB1 proved its capacity to act simultaneously as immunogen and adjuvant[50]. Another study used *Sm*CatB1 with a toll-like receptor 9 agonist as an adjuvant to Th1 response, and conferred ~ 55-60% reduction in worm and both intestinal and hepatic egg burdens[51]. One year later, the same investigators immunized mice with *Sm*CatB1 formulated in Montanide, as an adjuvant, against challenge infection. Similar results were obtained (55-60% reduction) in all parasitological parameters, but with significant high production of specific antibodies against *Sm*CatB1, and both types of cytokines including IFN-γ, IL-12, and TNF-α (Th1), and IL-5 and IL-4 (Th2). i.e. balanced mixed Th1/Th2 response[52]. Another vaccine trial was recently conducted by Egyptian investigators in mice and hamsters utilizing gut-derived CP (*Sm*CatL3), in combination with *Sm*CatB1 and SG3PDH. Results showed that combined tri-vaccine produced highest level of protection against challenge infection (70-76%) in both experimental animals. Accordingly, it was claimed that if the vaccine would show promising results in non-human primates, it will be progressed to phase 1 safety trials in human[53].

DNA vaccine construct of the gene encoding legumain (*Sm*32) was investigated in *S. mansoni*-infected mice, and it produced 37% reduction in egg burden[54]. In another study aiming to use legumain as vaccine candidate, the investigators found that immunization should be conducted with polymerizable peptides (pool of multiple epitopes), and was dependent on either strain of immunized mice (inbred, C57BL/6 or outbred, Swiss)[55]. Legumain was also investigated as serodiagnostic marker in schistosomiasis *mansoni*, when sera from immunized rabbits recognized the 32 kDa which is an excretory/secretory product (E/S) of adult *Schistosoma* vomitus. The investigators recommended use of polyclonal monospecific antibodies against *Sm*32 in utilizing ELISA or Western blot (WB) techniques for serodiagnosis[56].

Cloning and characterization of gene encoding calpain (clan CA, family C2) of *S. japonicum* (Philippine strain) was conducted, and the investigators compared its sequence with that of the Chinese strain. On the light of their results, and those obtained from another previous study for the same gene homologue of *S. mansoni*, the investigator suggested its potential use as vaccine candidate[57]. For calpain immunolocalization, mice were immunized with recombinant *S. japonicum* calpain, and its specific monoclonal antibodies were generated. It was shown that these antibodies localized calpain mainly in the cercaria and to some extent in adult tegument. Therefore, calpain was suggested to produce protective immunity and the investigators suggested its potentiality as vaccine

candidate[58]. The therapeutic efficacy of the vaccine formulation of *S. mansoni* calpain (*Sm*-p80) was evaluated, and showed reduction in worm and egg burden. The investigators claimed that their study was the first to report killing of adult schistosomes with a vaccine candidate[59].

On the other hand, caspases-3 and -8 were identified in *S. mansoni* schistosomula and were suggested to be involved in proteolytic apoptosis of skin T lymphocytes (mainly CD4 and CD8)[60]. Investigating caspase-3 and -7 activities in *S. japonicum* showed their expression in all the developmental stage, with highest expression levels in day 14 post infection. These results suggested the important role of caspases in schistosomes' survival and development within their host, hence the investigators recommended further studies to validate using caspase as potential drug target[61].

• ***Fasciola* spp.:** Cathepsin L expressed in E/S of *F. hepatica* (*Fh*CatL) proved to inhibit Th1 host immune response through suppression of IFN-γ production. The investigators showed that this suppression effect was not observed in mice with Il-4 deficient, suggesting independence on IL-4[62]. Six different cathepsin L fragments were detected in sequence analysis of its encoding gene in *F. gigantica* suggesting the presence of more than one gene controlling cathepsin L expression, as previously detected in *F. hepatica*[63]. *Fh*CatL also showed *in vitro* suppression of T cell proliferation, as its incubation with sheep or human T lymphocytes significantly reduced surface CD4 expression, which was inhibited by addition of E64[21]. One year later, Indian investigators reviewed the role of *Fh*CatL CPs in host' tissue penetration, nutrient digestion and immunoevasion. All throughout various developmental stages, 13 *Fasciola* cathepsin L were identified, and immunolocalized mainly in the gut epithelial cells of adult and juvenile stages. The reviewers also discussed the vaccine trials utilizing both cathepsins L (1 and 2) in sheep and cattle[64]. However, in another review article published in the same year, Australian investigators claimed 18 cathepsin L isolated from *Fasciola* spp., including a novel one identified by their *Fasciola* evolutionary studies[65]. A study conducted in Poland identified, cloned and characterized a new isoform of cathepsin L termed *Fh*CPW (*F. hepatica* CP-Warsaw). Bioinformatics analysis allowed the investigators to predict presence of potential HLA-II epitopes at the outer surface of *Fh*CPW1 suggesting its potential use as vaccine candidate[66]. Furthermore, one of the cathepsin L1 isoforms was cloned from cDNA library of *F. gigantica* and named *Fg*CatL1H. Its expression was highly observed in metacercaria and newly excysted juveniles (NEJs), which decreased gradually in next developmental stages. Immunolocalization showed it was expressed in the digestive tract epithelial cells. It showed *in vitro* proteolytic activity in collagen type I, laminin, and IgG, suggesting its role in host tissue migration and immunomodulation[67].

The diagnostic accuracy of recombinant *F. hepatica* cathepsin L was evaluated in ELISA using sera from sheep and calves experimentally or naturally infected with *F. hepatica* and other parasites. Interestingly, sensitivity and specificity changed according to the host type (sheep or cattle) that ranged from 96.5-100%. Specific antibodies against cathepsin L were first detected 5-7 weeks post infection (in experimentally infected hosts). The test was also evaluated using sera from naturally infected cattle from other endemic countries (Netherlands, Ecuador and Vietnam), and gave similar results[68]. Since then, several studies utilized cathepsin L1 in serodiagnosis of human fascioliasis in several countries; Iran[69]; Spain[70], India[71-73], Canada[74], and Thailand[75]. On the other hand, *Fh*CatL3 expressed from juvenile *F. hepatica* was found to be the most efficient cathepsin in cleavage of collagen types I and II, an essential step in tissue invasion for parasite establishment[76,77]. Two isoforms were also identified for *Fh*CatL3, and cloned, and the investigators observed their maximum expression levels from NEJ immediately after excystment[78].

The first report of cathepsin B expressed in *F. hepatica* juveniles, its cloning and identification of its immunogenicity was conducted in Australia. Recombinant *Fh*CatB proved its antigenicity only early after infection suggesting its temporal expression within few days of infection for parasite survival in the host[79]. Genes encoding cathepsin B from three different stages of *F. gigantica*; adult, NEJ and metacercaria were cloned and sequenced and 64-79% similarity was observed. Immunolocalization studies revealed enormous transcripts in metacercaria (cecal epithelial cells) and juvenile stages (epithelial cells of the proximal part of the digestive tract), more than in adult stage (tegument and tissues of the reproductive system). In addition, *Fg*CatB1 transcripts were detected in all stages, while those of *Fg*CatB2 and FgCatB3 were only expressed in metacercaria and NEJs. The investigators suggested involvement of *Fg*CatB1 in general digestive function, while the other cathepsin B might have a role in host tissue digestion for gut penetration and migration within host hepatic tissues[80]. Anti-recombinant *Fg*CatB2 antibodies showed no cross reaction with the other parasites' antigens using WB, suggesting its use as serodiagnostic marker in early fascioliasis[81]. On the other hand, monoclonal antibodies (McAbs) raised against recombinant *Fg*CatB3 showed their strong reactivity and specificity using indirect ELISA and WB, with no cross reaction with other sera obtained from patients with other parasites. Immunolocalization showed similar results in metacercaria and NEJs[82]. Based on its high reactivity and specificity, one of these McAbs was selected and used in sandwich ELISA to detect circulating *Fg*CatB3 in naturally infected cattle, and experimentally infected mice, as well health control animals. Both animal groups were also naturally or experimentally infected with other parasites. Results revealed that McAbs' accuracy was 98.5% and 98.9%, for

natural and experimental infections, respectively, and it could detect fascioliasis from day 1 to 35 post infection[83].

Several studies utilizing CP in protection against challenge infection without recognition of the used CP were conducted in endemic countries such as Cuba[84], Poland[85,86], and Egypt[87]. In 2008, Czech investigators with a group of Egyptian investigators succeeded in using a mimotope, which mimics the structure of *Fg*CatL epitope, in protection of sheep against challenge infection with *F. gigantica*. Results revealed significant IL-4 production which induced humoral antibodies to bound with NEJs and prevent occurrence of challenge infection[88]. Similar results were obtained for fascioliasis *hepatica* in sheep and goats in two studies conducted in Mexico[89,90]. In the later study, Quil A adjuvant was used and gave better results than using mimotope alone. Later, recombinant *Fg*CatL1 was used in experimentally infected mice to prevent challenge infection, and it didn't decrease the protection level too much compared to non-vaccinated and adjuvant vaccinated mice. However, it produced significant increase in Th1 and Th2 responses with marked decrease in hepatic pathological lesions[91]. Recently, tegument of the *F. hepatica* NEJs was analyzed using liquid chromatography and tandem mass spectrometry. Eighteen glycoproteins were detected, among them glycosylated fractions of *Fh*CatB3 and two isoforms of *Fh*CatL3. The investigators recommended further analysis and bioinformatics data to recognize the specific structures of these glycosylated fractions in cathepsin B3 and L3 for developing a new vaccine candidate and optimizing its efficacy[92]. Another study utilized *Fh*CPW encapsulated in transgenic plant-based system for oral delivery without its degradation in the host' gut. In addition, hepatitis B virus core protein (HBcAg) was used as a carrier to maximize host' immune response. The investigators vaccinated rats against challenge infection with *F. hepatica*. Although the vaccine gave substantial protection rate (65.4%), the investigators recommended vaccination of livestock animals with plant-based system with future identified antigens with high antigenic epitopes efficient enough to produce maximum immune response without adjuvants[93].

In addition to cathepsins, *Fasciola* spp. E/S showed the ability to induce early apoptosis of host eosinophils, which was found to be both time- and concentration-dependent. Caspase-3 expression from NEJs was suggested to immunomodulate host immune response during migration through hepatic tissues. Apoptosis of hepatic eosinophils was observed 21 days post infection in experimentally infected rate[94]. Similar results were obtained in a study conducted in Spain, and the investigators detected the highest percentage of caspase-3 eosinophils at the periphery of necrotic foci of hepatic tissues, mainly one and four weeks post infection, and in the infiltrate around adult flukes in the bile ducts at 17 weeks post infection[95]. Two members of clan CD, family C13; glycosylphosphatidylinositol: protein transamidase and AEP in *F. gigantica* juvenile and adult flukes were reported. Both CPs were immunolocalized also in intestinal epithelium of juvenile flukes and adult tegument. In addition, immunoblotting against sera of infected mice showed their expression four weeks after infection[96].

One of *Fasciola* spp. CPs (*Fas* antigens), categorized in MEROPS among the unassigned clan (family C1), was suggested as serodiagnostic marker for fascioliasis. The investigators used immunoelectrophoresis to isolate three antigens *Fas*1, *Fas*2 and crude E/S, then they utilized ELISA to survey IgG *F. hepatica* seroprevalence in alpacas (*Lama pacos*), in an endemic area in Peru. Their results suggested *Fas*1 use as serodiagnostic markers as it gave more or less similar results of crude E/S[97]. However, *Fas*2 also gave 92.4% sensitivity and 83.6% specificity when evaluated for serodiagnosis of fascioliasis in children living in the same endemic area in Peru[98]. Recently, results of a study conducted by the same Peruvian investigators which utilized *Fas*2 IgM-ELISA suggested its use as specific serodiagnostic test to detect acute human fascioliasis[99]. In the other hand, results of an Egyptian study utilizing *Fas*-1 showed 91.1% sensitivity and 89.1% specificity[100].

• ***Clonorchis sinensis*:** Sequence analysis of the cloned gene encoding CP from adult *C. sinensis* showed a 28.5 kDa protein, with proteolytic activity inhibited by addition of several general CPIs. It also showed significant specific reactivity with sera obtained from patients with clonorchiasis, and Korean investigators suggested its use as serodiagnostic marker[101]. Later, primers were selected from the gene encoding CP, which was obtained from GenBank termed *Cs*CP. The primers were evaluated in serodiagnosis of clonorchiasis using dot-ELISA and WB, and gave 91.7% sensitivity and 97.6% specificity[102]. One year later, *Cs*CP was mainly localized in adult' intestine as well as oral sucker, excretory bladder and tegument of cercariae and metacercaria. WB Results showed its binding with sera of rat anti *Cs*CP and rabbit anti-E/S as well as from a patient with clonorchiasis. In addition, ELISA showed that r*Cs*CP gave ~87% sensitivity and ~70% specificity, confirming its potential use in serodiagnosis[103]. Using immunogenetic ELISA, monoclonal IgG and egg yolk immunoglobulin (IgY) coated with magnetic beads were utilized against *Cs*CP to detect and capture circulating antigen in an attempt to diagnose clonorchiasis. In addition, intensity of infection was calculated as heavy, moderate and mild according to fecal egg number, and results showed positive correlation between ELISA optical density and egg counts. However, cross reactions were detected with schistosomiasis *japonicum* and paragonimiasis[104]. In an attempt to control clonorchiasis, Chinese investigators used *Bacillus subtilis* spores to deliver *Cs*CP to grass crab in culture, as a model of freshwater fish. *Cs*CP was coated in a protein and used as diet for grass crab, and the results showed significant increase of specific IgM levels in immunized group compared to the controls which fed on the same diet without *Cs*CP. The investigators

recommended use of feed-based recombinant spores to prevent metacercaria formation in freshwater fish[105].

Three cathepsins were reported in several publications for *C. sinensis*; L, F and B, while cathepsin C was reported only once in 2014. **Cathepsin L** with a MW 24 kDa shared 34% and 52% similarity with those detected in *F. hepatica* and *S. mansoni*, respectively[106]. Recombinant CP with 40-58% similarity to cathepsin L identified from other flukes; showed 96% sensitivity and 96.2% specificity versus 88% and 100% for crude E/S extract, respectively. In addition, it was localized in adult' intestinal epithelial cells and its intrauterine eggs[107]. Using SDS-PAGE, metacercarial E/S showed two bands (28 and 40 kDa) with proteolytic activities, as proved by addition of general CPI. Korean investigators showed that both expressed CPs were involved *in vitro* metacercarial excystation of *C. sinensis*[108]. In addition, immunolocalization of *Cs*CatL in adult' intestine and the tegument of all developmental stages suggested its involvement in nutrients digestion and host tissue invasion[109]. Similar immunolocalization results of the 41.5 kDa *Cs*CatL were obtained, and the investigators showed the serodiagnostic potentiality of its recombinant with 62.5% sensitivity and 81.7% specificity[110]. Moreover, the same investigators observed the ability of r*Cs*CatL to elicit production of specific IgG1 and IgG4, with significant predominance of the later. Although cross reaction occurred in sera from patients with *S. japonicum* and *P. westermanii*, the investigators suggested its use in seroprevalence surveys conducted in areas not endemic for both parasites[111].

The 28 kDa protein from E/S of adult *C. sinensis* showed down regulation using general CPI, and phylogenetic analysis suggested its nature as **cathepsin F**[112]. A novel gene encoding *C. sinensis* cathepsin F (*Cs*CatF-6) which showed its expression in different developmental stages, was identified and characterized. It showed proteolytic activities on human hemoglobin, collagen, fibronectin, IgG and albumin. It was localized in the intestine of metacercaria, juvenile and adult stages, suggesting its role in nutrients digestion[113]. A total of 12 genes were identified encoding *Cs*CatF, and three families were described; *Cs*CatF-4; -6 and -11. *Cs*CatFs were expressed in the intestine of all developmental stages with change of transcripts number according to the life cycle requirements. The Korean investigators suggested similar role; nutrient digestion[114].

**Cathepsin B** identified in *C. sinensis* showed 63% and 50% similarity with those identified in *S. japonicum*, and *F. hepatica*, respectively. It was mainly localized in the intestine of adults and metacercaria. Using ELISA and WB, r*Cs*CatB showed specific reactivity with sera from patients with clonorchiasis, suggesting its potential use in serodiagnosis[115]. Bioinformatics analysis showed four isoforms of *Cs*CatB (1-4) which their recombinants showed high IgG production. Type of IgG and time of its production peak differed according to *C. sinensis* developmental stage which mainly express its *Cs*CatB isoform. However, IgG1 and IgG2a showed high levels after combined immunization. In addition, significant reduction in both worm and egg burdens was observed in immunization with *Cs*CatB2 and *Cs*CatB3[116]. One year later, combined *Cs*CatB isoforms showed its involvement in digestion of various human proteins such as albumin, fibronectin, hemoglobin and IgG. It also showed its ability to promote human cell proliferation, i.e. carcinogenic[117].

Lastly, **cathepsin C** was identified and characterized in *C. sinensis* and it showed more than 50% similarity with those detected in other flukes. It was immunolocalized in metacercaria cyst wall, as well as adult worm' suckers and reproductive organs. However, further studies were recommended to utilize it as potential diagnostic antigen or drug target against clonorchiasis[118].

Apart from cathepsins, three other CPs were identified; caspase-3, legumain and AEP. Hepatocytes apoptotic findings in clonorchiasis 4-12 weeks post-infection were correlated with mRNA expression of *Fas*, *Fas*L, and caspase-3 using semi-quantitative reverse transcriptase PCR. These results suggested role of *Fas*/*Fas*L-mediated pathway in host hepatic cells apoptosis occurred due to caspase-3 expression to facilitate tissue invasion and establish clonorchiasis[119]. Recombinant legumain expressed in adult E/S proved its potential use in serodiagnosis[120]. Later, AEP was characterized in *C. sinensis* and it was immunolocalized in the intestine of all developmental stages. The investigators suggested its involvement in nutrients degradation[121].

• ***Opisthorchis viverrini*:** Three CPs; AEP[122], cathepsin F[123] and cathepsin B[124] were detected in somatic extracts as well as E/S of all *O. viverrini* developmental stages. Based on phylogenetic analysis, CPs showed high similarity with those detected in *C. sinensis*, and moderate one with those detected in other flukes. While *Ov*AEP1 was mainly immunolocalized in the adult' gut, suggesting its role in nutrients degradation, both cathepsins were detected in the adult' reproductive system beside its gut. Recombinants *Ov*AEP1, *Ov*CatB1 and *Ov*CatF1 showed 85%, 67% and 62% sensitivity and 100%, 81% and 84% specificity, respectively suggesting their serodiagnostic potentiality[122,125,126]. Moreover, r*Ov*CatB1 was found to elicit high IgG production[125], whereas use of egg yolk immunoglobulin (IgY) with rabbit antibody raised against *O. viverrini* somatic antigens in sandwich ELISA increased sensitivity and specificity levels to 93% and 78.57%, respectively[126]. Meanwhile, *Ov*CatF1 was highly expressed in bile ducts of infected mice several weeks post infection, suggesting its involvement in cholangiocarcinogenesis[123]. Capability of *O. viverrini* cathepsin B (*Ov*CatB1) to trans-activate *Ov*CatF1 at pH different from that required for auto-activation was reported. Combined cathepsins more efficiently degraded hemoglobin more than each

CP alone, suggesting regulatory role of *Ov*CatB1 on the latter, and involvement of both in development of cholangiocarcinoma[124].

• ***Paragonamus westermanii*:** In a series of publications[127-130], E/S from *P. westermanii* NEJs induced superoxide production from granulocytes stimulated by IgG degradation. This effect was related to CPs proteolytic activity expressed in E/S and it was concentration-dependent[127]. In addition, CPs possess proteolytic activity that regulates IL-8 production from human eosinophils, and it was also concentration-dependent[128]. CPs showed their role in regulating activation and degranulation of eosinophils when stimulated with IgG[129]. Incubation of human eosinophils with E/S produced high levels of eosinophil-derived neurotoxin *in vitro*, suggesting marked degranulation, which was inhibited by addition of E64 as CPI. The investigators attributed the pulmonary inflammatory lesions around the flukes to eosinophils degranulation[130]. However, other investigators attributed eosinophils apoptosis to active biological factors in E/S from NEJs which stimulate caspase-3 to mediate apoptosis[131].

It was found that the 27 and 28 kDa CPs expressed in E/S from NEJs were immunolocalized in the lining of metacercaria' excretory bladder[132]. Similar results were obtained, and the investigators added that these CPs might have a role in accelerating metacercarial excystment[133]. However, another study immunolocalized both CPs at the tegument, and their proteolytic activity was for fibrillary proteins, e.g. collagen, but neither for hemoglobin nor immunoglobulins. In addition, the investigators found two distinct isoforms for CP with MW 28 kDa (a and b), and the first was profoundly expressed during active invasion of host' tissue and migration of NEJs to reach their habitat[134]. Later, the 27 kDa CP was found to induce eosinophils degranulation[135], most probably it is the same CP reported by Shin's series publications (2000-2005)[127-130].

On the other hand, recombinant 28-kDa CP from adult *P. westermanii* E/S was investigated in serodiagnosis using immunoblotting. It showed 86.2% sensitivity and 98% specificity[136]. Also, recombinant gene encoding CP from the adult flukes was investigated for its potential use in serodiagnosis. Using WB, it gave 90% sensitivity and 100% specificity, while in ELISA, it gave 93% for both sensitivity and specificity[137]. Proteomic analysis of E/S from adult *P. westermanii* showed that at least 10% of the identified protein spots were CPs, with MW between 27 and 35 kDa. The majority of these CPs reacted strongly with sera from patients with paragonimiasis[138]. Another CP; hemoglobinase (legumain trematode type clan CA, family C13) was immunolocalized in the adult' intestinal epithelium. Phylogenetic analysis showed its similarity with those detected in some strains of *F. hepatica* and *Schistosoma* spp., to degrade human hemoglobin[139].

## Cestodes

• ***T. solium*:** A CP was detected in E/S of *T. solium* metacestodes *in vitro* cultures and the investigators detected higher activity of CP in phosphate buffer saline culture than in either PRMI or PRMI plus fetal calf serum. They also showed that the detected CP inhibited human T lymphocytes ($CD4^+$) *in vitro*, and peptidase activity was inhibited by addition of specific CP inhibitor, indicating that $CD4^+$ depletion was due to CP activity[20]. Four years later, signs of apoptosis of the cultured human $CD4^+$ were detected using EM. Therefore, the investigators postulated the nature of the detected CP in E/S to belong to clan CD, family C14 (caspases)[140]. Apoptosis of lymphocytes was also detected in the inflammatory reaction around *T. solium* metacestodes *in vitro* cultures. Mexican investigators showed their DNA fragmentation and confirmed expression of caspases from *T. solium* metacestodes[141]. Using immunohistochemical techniques, caspase-3 antibodies were detected between the inflammatory reactions around viable *T. solium* metacestodes. The results confirmed the role of caspase CP in immunoevasion through apoptosis[142].

In 2005, two purified CPs; 29 kDa[143] and 48 kDa[144], were identified in *T. solium* metacestodes, which showed peptidase degradation activity of human IgG. Both studies suggested that this peptidase activity might play a role in pathogenesis of cysticercosis, and recommended use of CP as vaccine candidate or CPI as drug target. One year later, immunization of mice with purified 48 kDa CP revealed 72% reduction of parasite burden in immunized mice[145].

Another CP was identified and characterized also in *T. solium* metacestodes; cathepsin L which showed degradation activity of human IgG and bovine serum albumin, but not collagen. Using WB analysis, Korean investigators showed its antigenicity against sera from patients with neurocysticercosis, however, cross reactions occurred with sera from patients with sparganosis and fascioliasis, but neither against paragonimiasis nor clonorchiasis[146]. Immuno-WB and ELISA were employed for diagnosis of cysticercosis utilizing cathepsin L purified from human cysts. The investigators found that specificity of immuno-WB was higher than ELISA (98% versus 92.7%), whereas ELISA showed sensitivity higher than immuno-WB for patients either with a single or multiple cysts[147]. Three years later, the same investigators accompanied with Cysticercosis Working Group in Peru identified the same CP in *T. solium* oncosphere and expressed by *Cysticerci*. They used it in a vaccination trial, and the results revealed high IgG levels, but without satisfactory protection[148]. Gene encoding cathepsin L was also used by Japanese investigators to differentiate between *Taenia* spp. utilizing loop-mediated isothermal amplification (LAMP) technique. In their study, LAMP proved to be rapid, simple, specific as well as sensitive tool for species identification, as the gene primers used in their study could differentiate

between *Taenia* spp. in DNA extracted from stool samples with five eggs/gm stool[149].

In addition to caspases and cathepsins identified in the previous studies,Yan and his colleagues[150] identified other CPs in *T. solium* genome such as several members of sapiens (clan CA) including calpain, ubiquitinyl hydrolase-L1 and ubiquitin-specific peptidase 14 representing families C2, C12 and C19, respectively. The investigators discussed their findings and predicted the characters and roles played by CPs expression using phylogenetic analysis and with comparison with similar CPs detected in other helminths such as *E. multilocularis*, *S. mansoni*, and *Caenorhabditis elegans* as models for of different helminth' classes.

• ***Echinococcus*** **spp.:** Japanese investigators conducted proteinase inhibition studies with *E. multilocularis* antigens extracted from protoscoleces, and found that *Em*18, *E. multilocularis* protein, is a fragment product of *Em*10 degradation. They used recombinant *Em*18 in serodiagnosis of alveolar echinococcosis utilizing ELISA and immunoblotting. Both assays showed satisfactory sensitivity (87.1% versus 90.3%, respectively). When they tested against sera from patients with hydatid cyst (no. 33) and neurocysticercosis (no. 10) as well as healthy controls (no. 15), only one serum sample of hydatid cyst case showed positive reaction by both assays[151]. Later, the same investigators identified, localized and characterized two isoforms of cathepsin L in E/S of *E. multilocularis* metacestodes, named as *Em*CLP1 and *Em*CLP2. Germinal layer, brood capsule and protoscolex were the sites of both CPs localization. Recombinant CPs showed IgG, albumin, collagens (type I and IV) and fibronectin degradation. Accordingly, they suggested that both isoforms have their proteolytic activity for parasite nutrition and survival as interaction against the host immune response[152,153]. In addition, *E. multilocularis*, similar to *T. solium*, showed expression of caspase 3 as observed in the immunohistochemical studies conducted in mice experimentally infected with *E. multilocularis*. Six months post infection, lymph node metastases with significant monocytes apoptosis were observed in infected mice in comparison to the controls. This was associated with proportional increased levels of caspase 3 expression and and TNF-α, and the investigators attributed pathological metastasis to inhibition of host immune response as a result of monocytes apoptosis due to caspase 3 proteolytic activity[154].

In two studies conducted in Chile[155], and China[156], the investigators postulated caspase expression in infertile hydatid cysts, which showed DNA fragmentation and higher expression levels of caspase 3. The Chinese study recommended further studies to employ induction of apoptosis as alternative non-surgical strategy in treatment of hydatid cysts[156]. One year later, a study conducted in Iran, and the investigators claimed that caspase 3 expressed from infertile cysts is a suppression mechanism in cystic echinococcosis due to apoptosis of the germinal layer, while that expressed from fertile cysts is survival mechanism due to host lymphocyte apoptosis[157].

• ***Spirometra*** **spp.:** It was in 2000 when the first report was published by Korean investigators who detected cathepsin L (27 kDa) in *Spirometra erinacei*, the causative agent of human sparganosis. CP proteolytic activity was highly expressed in plerocercoid followed by coracidium, with neither activity in the immature ova nor adult stages. These findings suggested CPs involvement in only the invasive stages of sparganosis[158]. Utilizing ELISA, Chinese investigators succeeded to use recombinant gene encoding cathepsin L of *S. erinaceieuropaei* in serodiagnosis of human sparganosis, with no cross reaction with sera from patients with cysticercosis[159]. Since then, several studies were published to investigate several cathepsins with different MWs; 37.87 kDa[160], 23.4 kDa[161], 36 kDa[162,163], and to analyze sequencing of their encoding genes. The objective of their studies was to select the most antigenic epitope to manufacture best primers used for serodiagnosis with better accuracy. Recently, results of a study conducted in Japan revealed the important role played by the 23 kDa cathepsin expressed from plerocercoids in host invasion and disease progression[164].

## Nematodes

• ***T. spiralis*****:** Several serine, cysteine and metalloproteinases, 25-55 kDa, were identified in E/S from *in vitro* cultures of *T. spiralis* muscle larvae, however, serine proteinases constituted the majority of the detected proteinases[165]. Apoptotic proteolytic activity due to caspase 9 was detected using immunohistochemical analysis by finding its expression in the infected muscle, and EM as shown by swollen mitochondria[166]. In addition, another two caspases members (3 and 8) were detected in *T. spiralis*-infected muscles and the investigators found decreased expression levels after cyst formation around the larva. They also observed absence of their expression either in the inflammatory reactions around the cyst or in normal muscles. Analyses of all factors involved in TNF-α and its receptor signaling pathway to mediate apoptosis showed correlation association with CPs expression. In addition, real time PCR analysis of the infected muscles showed upregulation of the genes encoding both CPs, with high expression until cyst formation[167]. Later, up- and downregulation of factors involved in apoptosis of the infected muscles were postulated as adaptive mechanism for survival of *T. spiralis* and chronicity of trichinosis[168]. Furthermore, apoptotic proteolytic activity in small intestine of *T. spiralis*-infected mice due to caspase 3 was reported. The investigators detected markers of endoplasmic reticulum stress in mice' intestine 3-7 days after infection[169]. Finally, cathepsin F was identified in adult *T. spiralis* and muscle larvae. The investigators

purified it and obtained serum from rabbits immunized by the purified protein. Sera antibodies recognized a band with 46 kDa when added to extracts from adult, muscle larvae as well as E/S of muscle larvae. In addition, they found its localization on the cuticle. The investigators hypothesized the role of cathepsin F in the developmental stages to the extent that it might be used as a potential vaccine candidate and drug target[170].

• **Filarial worms:** Using RNA interference technology, two genes encoding expression of cathepsins L and Z were validated for CPs expression from third-stage larvae (L3) of *O. volvulus*. American investigators utilized immuno-EM, using antibodies against each CP, and found that both CPs were essential factors for ecdysis, in the last molting step to L4. The investigators suggested use of these CPs as vaccine candidates and/or drug targets[171]. Similar results were obtained for only cathepsin L[172] and both CPs in *B. malayi*[173]. Also in *B. malayi*, cathepsin B was found to have essential roles in various developmental functions such as nutrients digestion, growth and microfilariae molting. Indian investigators showed that parasite exposure to E64 resulted in extensive decrease in their motility and viability leading to death after 8 hours. They attributed their results to high levels of reactive oxygen species (ROSs) due to increased activity of both glutathione enzymes (reductase and s-transferase). Increased ROSs might lead to alteration of mitochondrial membrane permeability due to oxidation of fatty acids and protein induced by ROSs. In addition, they found that E64 activated ced-3, a homolog of mammalian caspase 3, which contributed in apoptosis[174].

• ***Angiostrongylus cantonensis*:** Angiostrongyliasis is the most common cause of eosinophilic meningitis in Southeast Asia where rats are the definitive host, while humans are incidentally infected through drinking or ingestion of contaminated water and vegetables. Larvae are found in the central nervous system, causing permanent brain and nerve damage. Apoptotic CPs; caspase-3, -8, and -9 were identified within the leukocyte infiltrates in the brain parenchyma and subarachnoid space in *A. cantonensis*–infected mice[175]. In 2011, Chinese investigators identified cathepsin B in *A. cantonensis* 4$^{th}$ stage larvae which was cloned and characterized. It showed ~50% homologue similarity with other nematodes cathepsin B, and activated the expression of CD40, MHC II, and CD80 in host dendritic cells. The investigators suggested the potential role played by these CPs in invading central nervous cells through immunomodulatory mechanism[176]. One year later, sequence analysis of the gene encoding the identified cathepsin showed that it contains a specific hemoglobinase motif to hydrolyze several host proteins including hemoglobin and human IgG. Using WB, cathepsin B was found in E/S from 4$^{th}$ stage larvae, and the investigators suggested its role in parasite nutrition[177]. In the same year, Brazilian investigators immunoblot *A. cantonensis* E/S against pooled sera from patients with angiostrongyliasis and healthy controls. They identified 17 antigenic proteins including cathepsin B. The investigators recommended molecular cloning of the identified antigenic spots for development of accurate serodiagnostic marker[178]. Two members of cathepsin B were immunolocalized in *A. cantonensis*; *Ac*CathB-1, with a native structure was identified in the adult stages as well as in 1$^{st}$ and 3$^{rd}$ larval stages, while the *Ac*CathB-2 is mainly localized in its digestive tract[179]. Functions of both cathepsin B were investigated, and found that both enabled larvae penetrating host small intestine to reach central nervous system[180,181].

• **Hook worms:** Proteolytic activity was observed against cathepsins L and B-specific substrates in E/S from adult and infective larvae of *A. caninum*, and were termed as *Ac*CP-1 and -2[182]. The same Australian investigators observed that purified *Ac*68 antigen, which was used for serodiagnosis of cutaneous larva migrans, shared ~50% similarity with *Ac*CP-1[183]. In the same year, Poland investigators designed primers to catch CP conserved coding regions in *A. ceylanicum*. They used rapid amplification of cDNA ends (RACE-PCR), followed by sequencing of its products. They found that it showed homology with sequences of genes encoding *Ac*CP-1 of *A. caninum*, but they termed it *Acey*-1. The investigators suggested its exclusive production from the gland cells of adult stage to be involved in blood digestion[184]. When the investigators immunized hamsters with DNA construct of *Acey*-1, high production of IgG was obtained 7 days after immunization[185]. In 2004, American investigators conducted two studies and found that hemoglobin digestion by *A. caninum* was by enzyme synergistic cascade of aspartic proteases and *Ac*CP-2[186]. They utilized *Ac*CP-2 in dogs immunization, and the obtained results revealed high specific IgG antibodies, significant decrease in both intestinal worm burden, and fecal egg counts. The investigators also observed *Ac*CP-2 expression in the intestinal brush-border membrane[187]. In 2009, proteomic analysis of *A. caninum* E/S showed three cathepsins B[188]. Two years later, *Ac*CP-2 expression was described in both egg and larval stages, and the investigators suggested its role in early development of *A. caninum*[189]. In *N. americanus*, hemoglobin degradation was also proved to be multi-enzyme cascade by proteolytic activity of aspartic protease (*Na* APR-1), *Na*CP-3 and metalloprotease (*Na*MEP-1). The investigators immunolocalized the native proteases in the intestine of adult *N. americanus*, and the results showed inability of recombinant *Na*CP-3 to cleave hemoglobin, but instead cleaved globin fragments[190].

• ***Anisakis simplex*:** Apoptosis of mammalian cells was demonstrated in *A. simplex* and Japanese investigators showed its *in vitro* occurrence by two mechanisms; rapid which is mediated by $H_2O_2$, and delayed mediated by expressed caspase-9[191]. Papain (clan CA family C1), was also detected in *A. simplex* 3$^{rd}$ stage larvae, and the investigators postulated its role in protein digestion in primitive invertebrates[192].

**Cystatins (CYSs):** Long persistence of the majority of helminths in their host was partially attributed to parasite' possession of molecules with immunomodulatory functions, CYS is one of them. Therefore, CYSs are suggested as virulence factors for their regulatory role because they are reversible, tightly binding CPIs. From another point of view, helminths CYSs possess cellular function to protect them against unwanted proteolysis through regulation of intracellular and extracellular protein breakdown. When a parasite CYS is described to inhibit parasite CP, it means that it regulates its expression to avoid unwanted CP proteolytic activity.

**In nematodes,** onchocystatin (*Ov*7) was the first discovered helminth CYS, with an essential role in regulating *O. volvulus* larval molts[193]. Then, several addition roles were reported, e.g. regulating expression of several cathepsins. In filarial nematodes, they proved to induce protein degradation associated with cleavage of the MHC-II, through regulation of cathepsins L and S[194], or cathepsin F[195]. In GIT nematodes, nippocystatin, derived from *Nippostrongylus brasiliensis* E/S proved to induce protein degradation within host antigen-presenting cells through regulation of cathepsins B and L[196]. In addition, CYS-2 identified in free-living nematode *Caenorhabditis elegans* showed its expression in germ cells and embryos, but not in mature oocysts, suggesting its role during oogenesis and fertilization. The investigators also showed that parasites with mutant gene encoding *Ce*CYS-2 caused sterility[197]. Later, the binding affinity of *A. lumbricoides* CYS (*Al*CYS) to four cathepsins showed high regulatory activity on cathepsins C, L and S and lower to some extent on cathepsin B. The investigators analyzed *Al*CYS structure and discussed the mechanism(s) behind variations in its regulatory activity to different cathepsin[198].

**In filarial nematodes,** CYSs regulated legumain expression involved in MHC-II processing pathway in host B cells, using its proteolytic activity[199]. Beside their role in regulation of parasitic CPs expression, CYSs immunomodulatory functions were reported. In *O. volvulus*, recombinant *Ov*7 inhibited host cathepsins L and S leading to increased TNF-α production and IL-12 downregulation, followed by profound increased levels of IL-10. The investigators considered it as virulence factor contributed in cellular hypo-responsiveness and parasite persistence within its host[200]. Moreover, upregulation of nitric oxide production by IFN-γ-activated macrophages was reported through proteolytic activities of *Av*17 and *Ov*7 in *A. viteae* and *O. volvulus*, respectively[201]. In a review article, Hartmann and Lucius[202] summarized the mechanism(s) through which CYSs from parasitic nematodes elicit multiple, specific activities to immunomodulate host immune response using different strategies. The reviewers suggested development of immunotherapeutic drugs utilizing this CYSs potentiality. Gene encoding *Av*17 was sequenced, and the investigators recognized several binding sites for the transcription factors, e.g. antigen-presentation and necrosis factor. The obtained results suggested *Av*17 potentiality as vaccine candidate for filarial diseases[203]. For *B. malayi*, a study was conducted to analyze expression of three CYSs (*Bm*CYS-1, -2 and -3), and compared their results to those obtained for *C. elegans*. The investigators detected differences in the parasitic filaria, the most important of which is CYS-2 possession an additional AEP regulatory function. They also observed that CYS-2 was expressed in all developmental stages to block host CPs, whereas the other CYSs didn't possess the additional regulatory role and their expression was restricted in mosquitos for other CYSs functions[204]. American investigators evaluated the efficacy of recombinant *Bm*CYS-1 and *Bm*CYS-2 as a protective vaccine against *B. malayi* challenge infection in gerbils. Results showed that the vaccine with either CYS did not reduce worm burden, but significantly changed parasite' habitat, i.e. heart and lung instead of lymphatics. As never previously reported in the literature that a vaccine induced selection of parasite' habitat, the investigators recommended further studies to explain this unique rare phenomena[205].

**In GIT nematodes,** detection of IgG captured by commercial *T. spiralis* CYS dot ELISA versus conventional ELISA using crude E/S antigen was evaluated in serodiagnosis of trichinosis in experimentally infected mice. Egyptian investigators correlated their results with light and heavy infection as well as before and after treatment. Results showed that dot ELISA gave 86.6% and 100% positivity one week after infection for light and heavy infection, respectively versus negative results by conventional ELISA. After chemotherapy, it gave positive results only from sera of mice with infected muscles, while conventional ELISA gave positive results in all infected mice, i.e. with or without infected muscle[206]. Later, it was found that E/S from *T. spiralis* included a non-inhibitory CYS, termed multi-CYS-like domain protein (MCD-1), as it failed to inhibit papain *in vitro*. Its expression was confined during the early intestinal phase in trichinosis[207]. Also, when *Ts*MCD-1 was used in plasmid DNA vaccine, it gave unsatisfactory results, 38% reduction in worm burden[208]. However, a 45.9 kDa CYS-like protein (*Ts*CLP) was identified and characterized from *T. spiralis* intestinal larvae 6 hours post infection. Recombinant *Ts*CLP showed its potentiality to be used either as serodiagnostic marker for early trichinosis (2 weeks post infection), or as vaccine candidate as it produced 61,2% reduction in number of muscle larvae[209]. For *A. cantonensis*, a novel CYS was detected and characterized in the fourth larval stage. The investigators detected its regulatory function on parasite cathepsin B to upregulate nitric oxide production from IFN-γ activated by host macrophages[210].

**In trematodes,** Egyptian investigators conducted three studies using commercial CYS-capture ELISA. Serodiagnosis of human fascioliasis before and after triclabendazole treatment was assessed to detect IgG and

IgM antibodies captured by CYS. In comparison with IHAT, both IgG and IgM CYSs showed 100% sensitivity and specificity for early diagnosis of acute fascioliasis and gave negative results in 95% of cured patients after 4 months[211]. For serodiagnosis of early patent schistosomiasis *mansoni*, capture-ELISA was used to detect IgM antibodies against *S. mansoni* CYS in comparison to those against soluble gg antigen (SEA). For all cases, stool analysis was frequently examined for early recognition of patent schistosomiasis. Results showed that IgM antibodies captured by CYS diagnosed early schistosomiasis better than those captured by SEA. The investigators found IgM antibodies against SEA only in 5% of all seropositive cases detected by IgM against CYS[212]. In the third study, IgG CYS capture-ELISA gave both 100% sensitivity and specificity in comparison to soluble worm antigen preparation (SWAP) in conventional ELISA (85% sensitivity)[213]. In clonorchiasis, CYS-capture ELISA showed no cross reaction with sera obtained from patients with other parasites, while conventional ELISA using adult E/S crude extracts showed cross reactivity with sera from patients with opisthorchiasis, cysticercosis, and sparganosis[214].

A multi-domain molecule was cloned from *F. hepatica* protein with high MW and multiple peptides (type III), and it showed CYS proteolytic regulatory function on *F. hepatica* cathepsin L1. The investigators suggested its use as potential vaccine candidate or drug target[215]. In addition, three CYSs with low MW were described in *F. gigantica* E/S and termed *Fg*Stefin-1, -2 and -3. *Fg*Stefin-1 (type I, without disulfide bridge), was immunolocalized in adult' tegument and intestinal epithelium, and showed its regulatory function on the major *Fasciola* cathepsin L. Its recombinant showed both intra- and extra-cellular protective functions, i.e. regulating parasite' CPs expression and inhibiting host CPs expressed on tegmental surface[216]. On the other hand, *Fg*Stefin-2 (type II, with single peptide) showed its expression in the intestinal epithelium of metacercaria, NEJs and adult stages, as well as reproductive organs. It regulated proteolytic activity of cathepsin B to protect the parasite from intra-cellular auto-proteolysis, and the investigators recommended further studies to elucidate role of *Fg*Stefin-2 expressed in the reproductive organs[217]. Recently, *Fg*Stefin-3 (type II) was identified and its recombinant showed potent regulatory function of cathepsin L, similar to the other CYSs[218]. Similarly, stefin-1 was expressed from all *C. sinensis* developmental stages, and it was immunolocalized where *Cs*CatF synthesis. Its recombinant showed regulatory activity on *Cs*CatF as well as proteolytic inhibitory effects on human cathepsins B and L[219]. Similar results regarding site of expression and inhibitory proteolytic activities were reported for *Cs*Stefin-2[220].

In *S. japonicum*, Chinese investigators characterized a molecule in *S. japonicum* which induced apoptosis and showed its transcripts mainly in all developmental stages in the host. They termed it inhibitor apoptosis protein (*Sj*IAP)[221]. Then, the same institute reported a new molecule termed cytokine-induced apoptosis inhibitor (*Sj*CIAP), which showed its transcription in all developmental stages, even the eggs. They experimentally demonstrated *Sj*CIAP capability to inhibit caspase activity in both human cell lines or schistosome lysates, i.e. regulating parasite CPs, similar to CYS function. Therefore, they suggested development of drug targeting *Sj*CIAP for treatment of schistosomiasis *japonicum*[222]. In 2014, two studies were conducted by the Chinese institute; the first investigated use of *Sj*IAP combined with adenovirus vectors, for vaccine delivery, to protect mice from challenge infection. Although it showed moderate protection, there was significant increased levels of IgG (IgG1 IgG2a and IgG2b), cytokines (IFN-γ and IL-2), and lymphocyte proliferation. Therefore,the investigators suggested its use as vaccine candidate[223]. The second study characterized a cDNA fragment synthesized from *S. japonicum* RNA which contains baculovirus IAP repeat (BIR) domain and termed it *Sj*BIRP. Its increased transcripts were detected in schistosomula, with high expression in females than males. Investigating its function, *Sj*BIRP showed *in vitro* inhibitory activity, i.e. regulatory function on parasite' caspases 3 and 7, suggesting its use as potential drug target for schistosomiasis *japonicum*[224]. Finally, recombinant *S. japonicum* CYS produced significant results regarding inhibition of lysosomal CPs of murine dendritic cells, reduction in antigen presenting cells as well as increased cytokines levels (Il-4 and transforming growth factor-β). The investigators claimed considering *Sj*-CYS as parasite-derived immunosuppressive factor[225].

**In cestodes,** a 11 kDa CYS was identified in *Spirometra erinacei*, and the investigators characterized its proteolytic activity to regulate endogenous papain and the 27 kDa CP[226]. Using extensive genomic data, a Chinese investigator reported that cestodes CYSs differ from those of trematodes, in possession of two tandem repeated domains, without a conserved disulfide bond[227].

## Cysteine proteinase inhibitors

**In trematodes,** a vinyl sulfone compound, K11777, was evaluated in murine schistosomiasis *mansoni*, in a dose of 25 mg/kg twice daily, administered for the first two weeks post-infection. It produced significant reduction in both worm and egg burdens as well as hepatic granulomas[23]. French investigators showed the efficacy of two HDACs inhibitors (trichostatin A and valproic acid) against *S. mansoni* larval and adult worms *in vitro*. Apoptotic was rapid in trichostatin A, with increased caspase 3/7 in both inhibitors[31]. Aza-peptide Michael acceptors belong to a new class of irreversible inhibitors showed potent efficacy to AEP/legumains expressed in *S. mansoni* (SmAE), and the investigators suggested its use as potential drug target[228]. In 2013, two studies were conducted in

Egypt evaluating CPIs in treatment of schistosomiasis in comparison or combined with praziquantel (PZQ). The first study evaluated phenyl vinyl sulfone (PVS) and valproic acid (VA) in in *S. mansoni*-experimentally infected mice. Results showed that PZQ was more efficient in reducing worm and egg burdens, whereas PVS showed the best results in reducing hepatic granuloma, and combined therapy was recommended[24]. In the second study, the investigators evaluated PVS, fluromethylketone (FMK), and sodium nitro prussid (SNP). Results showed that FMK (50 mg/kg) combined with PZQ gave the best reduction in intestinal and hepatic egg burdens as well as granuloma size and numbers. However, PVS alone or combined with PZQ showed satisfactory efficacy in reducing hepatic granuloma size[229]. In an attempt to develop drug target efficient to inhibit 3 CPs; cruzain, rhodesain and cathepsin B from *T. cruzi*, *T. brucei*, and *S. mansoni*, respectively, a group of investigators from Brazil and USA synthesized 24 compounds known as thiosemicarbazones and thiazole derivatives. Using their screening approach, the investigators observed five compounds with high potent inhibitory activity against *Sm*CatB, with significant efficacy against schistosomula *in vitro* culture. However, only one compound showed its efficacy against adult schistosomes[230].

For treatment of fascioliasis, a selective cathepsin inhibitor (Ep-475) was evaluated in mice. The parasitological measures included serum levels of liver enzymes, worm burden and fecundity as well as egg viability. Results showed that Ep-475 reduced liver damage, and significantly reduced fluke size and egg viability, but without significant reduction in total worm burden[231]. Using recent technology in virtual screening and MYBRIDGE-HitFinder database for *Fh*CatL3 and human cathepsin L to identify their binding sites for a substrate, the investigators predicted five compounds with specific *Fh*CatL3 inhibitory activities. The study was a proposal for drug design approach for fascioliasis, and the investigators recommended further studies utilizing this approach to discover new specific inhibitors for parasitic CPs[232]. One year later, another computational approach was utilized to develop new specific inhibitor against *Fh*CatL3. One of chalcones compounds (C34) was identified with high potency to induce *in vitro* both motility and viability loss of NEJs. So, NEJs should not be able to penetrate host gut wall[233].

In **nematodes**, efficacy of two vinyl sulfone compounds (K11002 and K11777) were evaluated in treatment of hook worms. Both compounds were incubated with various life cycle of *A. ceylanicum*, and orally administered to infected hamsters in a single dose. In comparison with albendazole treatment, results showed that K11002 *in vitro* killed adult worms and eggs with no larvicidal efficacy, while K11777 gave similar results comparable with albendazole. Combined CPI with albendazole increased hook worms mortality compared to single chemotherapy. The investigators recommended further studies to search for other CPIs to obtain an efficient chemotherapeutic drug for hook worms[234].

## CONCLUDING REMARKS

1. **CPs** are expressed enzymes essential for protein breakdown with formation of transient covalent bond using cysteine sulfur. In helminths, they have several functions including nutrients digestion, host tissue degradation and evasion of host immune response.

2. Helminthic **CYSs** are molecules responsible for their long persistence in their host. They are expressed to inhibit and/or regulate parasitic CPs functions as well as to inhibit host CPs. Therefore, both CPs and CYs are considered virulence factors.

3. **CPIs are** of scientific interest in Parasitology research for development of new drug and vaccine targets. The most common CPIs include E64, phenyl vinyl sulfones (PVSs) and histone deacetylases (HDACs) inhibitors.

4. **MEROPS**, a website for all proteinases database, provides updated information including their homologs, inhibitors, substrates and specificity. The majority of helminthic CPs belong to clan CA, while the rest belongs to clan CD.

5. In **trematodes**, all CPs are expressed in adult flukes E/S beside schistosomulae in schistosomes and metacercaria and NEJs in the other flukes. Cathepsins B and L are the main CPs as they are involved in the proteases cascade catalyzing host proteins or tissues to provide parasite nutrients for survival and growth, and to facilitate larval penetration and migration within its host. Caspases are expressed in *Schistosoma* and *Fasciola* spp. and were reported as potential drug targets. While legumain was reported in *Schistosoma* spp., *Fas* was reported in *Fasciola* spp., and both were utilized as serodiagnostic markers. Several isoforms of cathepsins B and L are expressed in *Schistosoma* and *Fasciola* spp., and were utilized for protection and vaccine trials. Cathepsin B is involved in development of cholangiocarcinoma in clonorchiasis and opisthorchiasis.

6 . In **Cestodes**, all CPs are reported in tissue larval invasion, namely cysticercosis, hydatid disease and sparganosis caused by *T. solium*, *Echinococcus* and *Spirometra* spp., respectively. Caspases are the main CPs expressed in cysticercosis and hydatid disease. Caspase 3 is expressed mainly in infertile hydatid cyst, and was suggested as an alternative non-surgical strategy in its treatment. Cathepsin L is the only CP utilized as serodiagnostic marker in neurocysticercosis and sparganosis. Gene encoding cathepsin L was used to differentiate between *Taenia* spp. in DNA extracted from stool samples with five eggs/gm stool. In addition, two CPs with MW 29 kDa and 48 kDa

are utilized as potential drug targets or vaccine candidate in neurocysticercosis.

7. In **Nematodes**, several CPs are reported. Caspase is involved for apoptotic proteolysis in *T. spiralis* and *A. cantonensis*. Cathepsins B and L are expressed in *O. volvulus*, and *B. malayi* for nutrients digestion, growth and microfilariae molting. Cathepsin B is also expressed in *A. cantonensis* to facilitate larval penetration to reach central nervous system. In hook worms, CPs are expressed for blood digestion.

8. **CYSs** are mainly expressed in **tissue nematodes** as they have immunomodulatory functions through regulation of several cathepsins expression. In *T. spiralis*, CYS was utilized either as serodiagnostic marker for early trichinosis, or as vaccine candidate. In **trematodes**, CYSs were used for serodiagnosis of early patent schistosomiasis *mansoni*, and clonorchiasis or as potential vaccine candidate or drug target in fascioliasis.

9. **CPIs**, general and specific,were mainly investigated in treatment of schistosomiasis and fascioliasis.

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