The role of the fibrinolytic system in the migration of *Fasciola hepatica* juveniles across the host intestine

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Summary

Summary

Neglected tropical diseases (NTDs) comprise a group of twenty infectious conditions that collectively affect over 1.5 billion people worldwide, primarily in low-income countries. Although NTDs are not associated with high mortality rates, they cause substantial morbidity that undermines economic productivity, thereby perpetuating the cycle of poverty (1). A significant proportion of NTDs are caused by helminth parasites, with recent estimates indicating that more than one billion people living in sub-Saharan Africa, Asia, and Central and South America are infected with at least one of these organisms (2). Helminth parasites also affect a large proportion of domestic animals that are used in livestock practices, thereby representing an important threat to animal welfare and global food security. This is particularly relevant in low-income regions where NTDs are most prevalent, as these areas are projected to experience the highest population growth in the coming decades, underscoring the urgent need for more sustainable and efficient food production systems to comply with global nourishing standards that guarantee human health and well-being. In this regard, ruminants are key to food security in arid and semi-arid regions of the globe where land is not suitable for crop production, as they process grasses and forages—inedible to humans—into high-quality, protein-rich foods (3).

One of the most prevalent parasitic infections of ruminants is fasciolosis, a parasitic disease caused by helminth trematodes of the genus *Fasciola*, including *F. hepatica* and *F. gigantica*. Owing to its remarkable capacity to adapt to new hosts and environments, *F. hepatica* shows a cosmopolitan distribution, with cases of infection reported on every continent except Antarctica (4). Since these parasites harbour a significant zoonotic potential and cases of human infection are particularly concentrated in rural and low-income areas of the world, human fasciolosis is classified by the World Health Organization as a food-borne NTD requiring targeted interventions for its effective elimination (5,6). The definitive hosts of *F. hepatica*, including humans and non-human animals, become infected by the ingestion of metacercariae, which are commonly attached to edible water plants, such as wild watercress. Upon ingestion, metacercariae excyst in the duodenum releasing the so-called newly excysted juveniles (FhNEJ), which cross the intestinal wall and migrate through the peritoneum and liver parenchyma before reaching the major hepatic bile ducts, where adult flukes mature and produce eggs that are shed with faeces (7).

The standard treatment for fasciolosis is triclabendazole (TCBZ), one of the few commercially available anthelmintics effective against both juvenile and adult stages of the

parasite. However, the ongoing selection of TCBZ-resistant isolates underscores the urgent need for novel therapeutic and control strategies against this parasite (8). In this context, vaccination represents a promising alternative to pharmacological treatment, offering the potential for cost-effective, sustainable, and long-term control of fasciolosis. To date, numerous efforts have been undertaken to develop a vaccine against *F. hepatica*, yet none have achieved the levels of protection required for commercialisation. Most of these studies have attempted to target the adult stages of this parasite, which are highly proficient in immune evasion and reside inside a niche that is largely inaccessible to host immune effectors (9). In light of this, vaccine strategies targeting *F. hepatica* earlier in development may represent a more successful approach for the effective control of fasciolosis. To this end, a deeper understanding of the biology of *F. hepatica* juveniles, including the detailed characterisation of host-parasite relationships that sustain parasite growth, migration, and survival within the mammalian organism, is crucial for identifying novel targets for intervention (3,10).

Tissue migration is a hallmark of the life cycles of many helminth parasites that has been conserved throughout evolution. This process is facilitated by parasite motility and the action of parasite-secreted proteases that enable the degradation of host tissue components that would otherwise physically block parasite migration. Furthermore, endogenous mechanisms supporting tissue migration are complemented by the ability of parasites to hijack the mammalian host's proteolytic machinery. A paradigmatic example of this is the interaction of tissue and/or blood-borne parasites with the host fibrinolytic system through the expression of proteins that recruit the zymogen plasminogen to stimulate its conversion into plasmin, the main fibrinolytic protease (11,12). Whether the pre-hepatic juvenile stages of *F. hepatica* utilise a similar strategy to facilitate tissue migration prior to their definitive establishment within the bile ducts remains to be addressed.

Based on these considerations, the main objective of this thesis is to expand our knowledge of host-parasite relationships during the early stages of fasciolosis, with a particular focus on the potential interplay between FhNEJ and the host's fibrinolytic system and the role of this interaction in the migration of these parasites through the host intestine. This knowledge may inform the identification of parasitic molecules that can be targeted for therapeutic or immunological interventions, ultimately leading to the development of novel strategies that prevent parasite maturation and block disease transmission within affected populations.

References

1. Hotez PJ, Molyneux DH, Fenwick A, Kumaresan J, Sachs SE, Sachs JD, *et al*. Control of neglected tropical diseases. N Engl J Med. 2007 Sep 6;357(10):1018–27.
2. Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. Helminth infections: the great neglected tropical diseases. J Clin Invest. 2008 Apr 1;118(4):1311–21.
3. Fitzpatrick JL. Global food security: the impact of veterinary parasites and parasitologists. Vet Parasitol. 2013 Aug 1;195(3–4):233–48.
4. Nyindo M, Lukambagire AH. Fascioliasis: an ongoing zoonotic trematode infection. Biomed Res Int. 2015;2015:786195.
5. World Health Organization. Ending the neglect to attain the Sustainable Development Goals: a road map for Neglected Tropical Diseases 2021-2030. Geneva: World Health Organization; 2020.
6. Pan American Health Organization (PAHO). Operational guidelines for the elimination of human fascioliasis as a public health problem in the Americas. Pan American Health Organization; 2024.
7. Siles-Lucas M, Becerro-Recio D, Serrat J, González-Miguel J. Fascioliasis and fasciolopsiasis: current knowledge and future trends. Res Vet Sci. 2021 Jan;134:27–35.
8. Kelley JM, Elliott TP, Beddoe T, Anderson G, Skuce P, Spithill TW. Current threat of triclabendazole resistance in *Fasciola hepatica*. Trends Parasitol. 2016 Jun;32(6):458–69.
9. Deslyper G, Doherty DG, Carolan JC, Holland CV. The role of the liver in the migration of parasites of global significance. Parasites Vectors. 2019 Dec;12(1):531.
10. González-Miguel J, Becerro-Recio D, Siles-Lucas M. Insights into *Fasciola hepatica* juveniles: crossing the fasciolosis rubicon. Trends Parasitol. 2021 Jan;37(1):35–47.
11. González-Miguel J, Siles-Lucas M, Kartashev V, Morchón R, Simón F. Plasmin in parasitic chronic infections: friend or foe? Trends Parasitol. 2016 Apr;32(4):325–35.
12. Diosdado A, Simón F, Serrat J, González-Miguel J. Interaction of helminth parasites with the haemostatic system of their vertebrate hosts: a scoping review. Parasite. 2022;29:35.

Bibliographic review

1. *Fasciola hepatica* biology and fasciolosis

Fasciola hepatica, commonly known as the liver fluke, is a helminth parasite belonging to the genus *Fasciola*, which is classified within the family Fasciolidae, subclass Digenea, class Trematoda, and phylum Platyhelminthes (1). The genus *Fasciola* includes two species, *F. hepatica* and *F. gigantica*, which infect a wide range of animals, including humans, causing a chronic and debilitating disease known as fasciolosis. These parasites have distinct geographic distributions: while *F. hepatica* is distributed globally, representing the helminth parasite with the widest latitudinal, longitudinal, and altitudinal distribution (2), *F. gigantica* is confined to tropical Africa, the Middle East, eastern Europe, and southern and eastern Asia (3,4). This section provides a comprehensive overview of the biology and life cycle of *F. hepatica*, along with a detailed review of the disease associated with infection by this parasite.

1.1. *Fasciola hepatica* life cycle

F. hepatica has an indirect life cycle that includes freshwater snails as intermediate hosts, where the asexual phases of the parasites develop, and a mammalian definitive host, where adult worms mature. Definitive hosts, typically ruminants and humans, become infected by ingestion of water plants contaminated with infective metacercariae, which excyst upon arrival to the duodenum, thereby releasing the newly excysted juveniles (FhNEJ). FhNEJ cross the intestinal wall, after which juvenile flukes migrate through the peritoneum and liver parenchyma until they reach the major hepatic bile ducts, where adult worms develop and produce fertilised eggs that are shed with faeces (4). In total, the life cycle of *F. hepatica* takes 14–23 weeks to complete (2). A graphic summary of the life cycle of *F. hepatica* is found in **Figure 1**.

1.1.1. Development of *Fasciola hepatica* within the intermediate host

F. hepatica eggs are yellowish-brown in colour, oval in shape, 130–150 μm long by 63–90 μm wide, and have a distinctive operculum (1,5). They harbour a fertilised ovum and a number of yolk granules. They are produced by adult flukes in the major bile ducts, passed from the common bile duct into the duodenum, and subsequently excreted with faeces. Eggs can remain viable in the environment up to several months (1). If climatic conditions are suitable, the fertilised ovum embryonates and develops into a miracidium, which is released to the environment upon egg eclosion. Miracidia are ciliated and highly mobile, with a short lifespan of only 24 hours. Glycogen stores support the active locomotion of

miracidia, facilitating their rapid location of, and subsequent penetration into, an intermediate snail host (1).

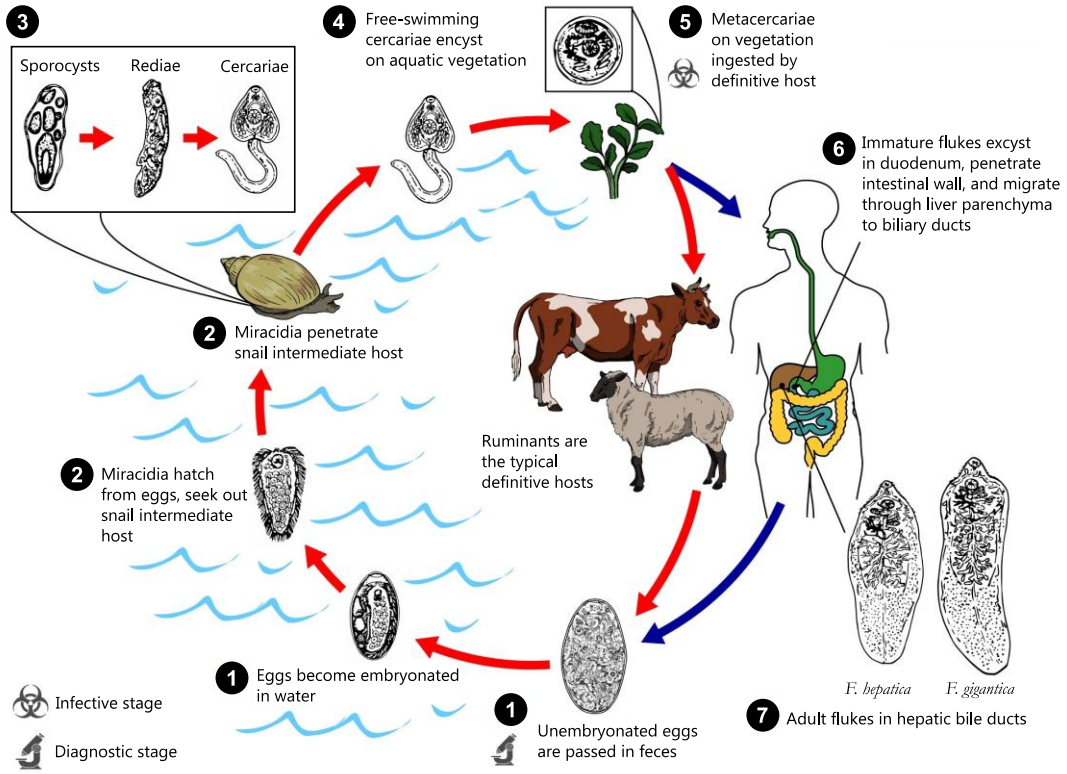

Figure 1. The life cycle of *Fasciola hepatica*. The life cycle of *F. hepatica* is divided in five phases. [1] Egg shedding by the definitive host followed by egg embryonation (development of the miracidia) in the environment; [2] hatching of miracidia and penetration into the intermediate snail host; [3] asexual development of the parasite within the snail; [4] emergence of cercariae from the snails and encystment on surfaces (typically water plants); [5] ingestion of metacercariae by definitive hosts; [6] metacercariae excystment in the duodenum and migration of juvenile flukes towards the hepatic bile ducts; [7] development of adult flukes and onset of oviposition (1). Source: Centers for Disease Control and Prevention (CDC), https://www.cdc.gov/dpdx/fascioliasis/index.html.

Intermediate hosts are freshwater snails of the Lymnaeidae family. About thirty different lymnaeid species can serve as intermediate hosts for *F. hepatica*, with *Galba truncatula* being the most common in Europe. Miracidia are attracted to water snails, which reside in water bodies and close to the surface, by light and chemical cues, and penetration is achieved by mechanical forces and the secretion of proteolytic enzymes. Upon snail penetration, the miracidium develops into the sporocyst, which migrates through snail blood or lymph vessels towards the digestive gland of the snail. There, the sporocyst germinal cells divide and give rise to a morula from which the next larval stage, the redia, develops (1). Up to four redial generations may develop inside the same snail host, depending on environmental

conditions (6). Upon sporocyst rupture, the rediae are released and their germinal cells multiply to eventually develop into the cercaria, which has a long tail and two suckers (oral and ventral). Four to seven weeks after miracidium infection, cercariae emerge from the snail and swim through the water to eventually attach to a solid surface—usually leaves of freshwater plants—by means of their ventral sucker. This process occurs within the first two hours after cercarial shedding. Upon settlement, the cercarial tail is lost and encystment begins by the production of proteins and mucopolysaccharides that form the cyst wall. Encysted cercariae, known as metacercariae, are infective to the definitive host and can survive for more than a year in the environment (1,7). Intermediate snail hosts play an important role in the life cycle of *F. hepatica* by supporting the clonal expansion of the parasite, with up to 3,200–4,000 metacercariae produced from a single miracidium (1,8).

1.1.2. Development of *Fasciola hepatica* within the definitive host

The definitive hosts of *F. hepatica* are most commonly herbivorous domestic animals, particularly ruminants involved in livestock production, including sheep, goat, and cattle, although a variety of other domestic and wild animals can also support *F. hepatica* development. These include horses, donkeys, mules, camelids, buffaloes, deer, wild boars, various marsupials, rabbits, hares, and nutria (2). Humans are also susceptible to *F. hepatica* infection, but human fasciolosis endemic areas are primarily concentrated in rural areas where basic sanitation standards are not always met (4). The infection route for definitive hosts is the ingestion of metacercariae, which are commonly found in food products such as grazing pasture for domestic animals, or freshwater plants, particularly wild watercress, for humans (9).

Upon ingestion, excystment of metacercariae occurs in two phases: the activation phase and the emergence phase. *In vitro*, metacercariae activation is stimulated by carbon dioxide (CO_2), reducing conditions, and temperature, while bile is required for emergence (1,10). Although the stimuli required for excystment *in vivo* are more difficult to determine, the physicochemical factors influencing excystment *in vitro* are sequentially encountered along the digestive system of *F. hepatica* mammalian hosts. For instance, the rumen of sheep, goat, and cattle performs a fermentative digestion process that generates reducing conditions and the production of considerable amounts of CO_2 (11,12), while bile is present in the small intestine, further down the digestive tract. Additionally, stomach or abomasum enzymes including pepsin and pancreatin increase the excystment rate of metacercariae *in vitro* (10), highlighting the positive role that the stomach environment plays in metacercariae activation *in vivo*. Excystment of *F. hepatica* metacercariae is an active process, relying on

both the host's physicochemical conditions and factors expressed by the encysted parasite, including B- and L-like cysteine peptidases (13).

The ventral plug of metacercariae opens in the small intestine, right below the *ductus choledocum* (common bile duct) in the second portion of the duodenum, resulting in the release of FhNEJ. These parasites exhibit dorsoventral flattening and their size varies between 100–170 μm in length due to continuous elongation-contraction cycles during active locomotion (14). Bile facilitates FhNEJ emergence by stimulating muscle activity and by activating FhNEJ-derived enzymes that are required to digest components of the cyst wall (1,15). However, certain bile components, such as deoxycholic acid, are toxic to FhNEJ (15,16), prompting their rapid migration across the intestinal wall within the first two to three hours after ingestion of metacercarie (1,2).

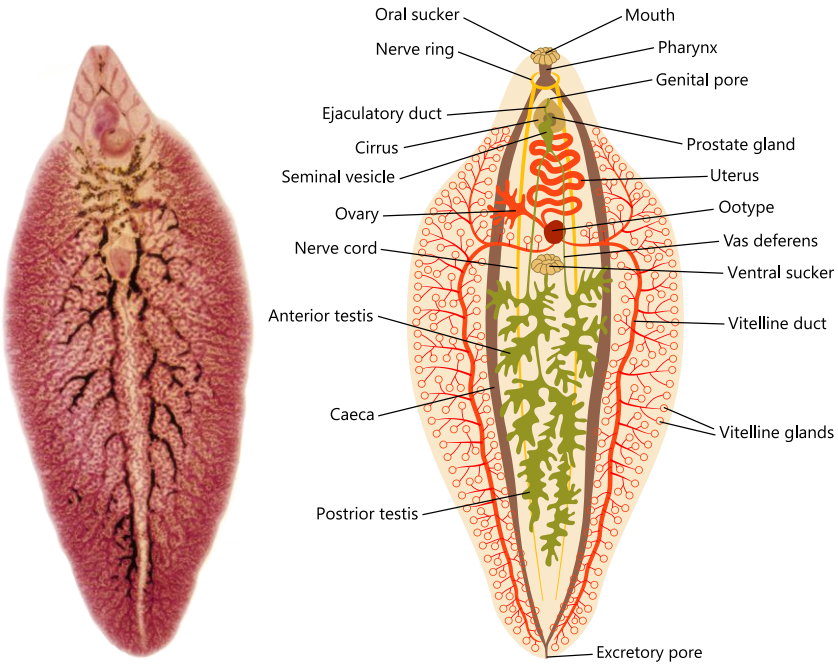

Figure 2. Adult *F. hepatica* morphology and major anatomic structures. The main structures of the female reproductive system of *F. hepatica* are the vitelline glands, the vitelline duct, the ovary, the ootype, and the uterus. Eggs produced in the ootype contain an unfertilised oocyte and approximately 30 vitelline cells, which synthesise shell proteins and support nutrition during embryonation. Vitelline cells are produced in the vitelline glands, and oocytes and eggs are produced in the ovary and ootype, respectively. The uterus, where fertilization between an oocyte and a spermatozoon occurs, leads to the genital pore, through which fertilised eggs are released to the exterior. The main structures of the male reproductive system of *F. hepatica* are the anterior and posterior testis, the vas deferens, the seminal vesicle, the cirrus, the prostate gland, and the ejaculatory duct. Spermatozoa are produced in the testis and stored in the seminal vesicle (17). The digestive tract of *F. hepatica* is divided in two portions: the foregut, which includes the mouth, the pharynx, and the oesophagus; and the caeca (intestines). Most food is ingested through the oral sucker, which leads to the caeca, while the ventral sucker is mainly used to maintain position while feeding (18). Waste material is excreted through the excretory or protonephridial pore, which helps maintain a homeostatic fluid composition and

represents a site for extracellular vesicle release (19). The nervous system of *F. hepatica* innervates the musculature, reproductory organs, and the suckers. It is divided in central and peripheral nervous systems (CNS and PNS, respectively). The CNS is formed by two ganglia located around the pharynx from which three major nerve cords or trunks (ventral, dorsal, and lateral) originate. The PNS comprises minor nerve cords and subtegumental nerve plexuses (20,21). Source: National Center for Veterinary Parasitology, USA, https://www.ncvetp.org/trematodes (left) and modified from Adobe Stock, https://stock.adobe.com (right).

After crossing the intestinal wall, juvenile flukes crawl through the peritoneal mesothelium using their ventral sucker until they access the left lobe of the liver through the Glisson capsule, which occurs approximately six days after ingestion. Juvenile parasites migrate through the liver parenchyma for 5–6 weeks while feeding on blood and liver tissue, resulting in considerable growth of the parasites (22). Juvenile migration across the liver parenchyma is facilitated by the immunotolerant environment that predominates in this organ (23). The migratory behaviour of juvenile parasites across the intestinal wall, peritoneum, and liver parenchyma is supported by parasite movement, glycogen stores, and the secretion of proteolytic enzymes (24). Liver migration by juvenile parasites causes severe tissue damage that characterises the acute pathology associated with *F. hepatica* infection.

Around seven weeks after infection, migrating juveniles reach the bile ducts, where they mature into adult parasites. Adult parasites are dorsoventrally flattened and typically measure 4.88–14.07 mm wide by 11.64–29.00 mm long (**Figure 2**) (25). These parasites are obligate blood-feeders that occasionally feed on hyperplastic bile duct epithelial cells through their digestive system. The digestive tract of *F. hepatica* is blind-ended, and food is ingested through the oral sucker and digested through the activities of proteolytic enzymes expressed by epithelial cells that outline the gut (gastrodermal cells). These cells are also involved in nutrient absorption from digested food, although small nutrients including glucose, amino acids, and simple lipids can also be absorbed via the tegument. Undigested material is regurgitated to the exterior through the oral sucker (22). Blood digestion involves different parasite-derived enzymes. These include saposins, which lyse erythrocytes, leading to the release of haemoglobin; cathepsin L1 (FhCL1), which digests haemoglobin into smaller peptides; and leucine aminopeptidase (FhLAP), which further degrades haemoglobin peptides into single amino acids that are used for biosynthetic purposes (26,27). Members of the family Fasciolidae are hermaphroditic and preferentially reproduce via cross-fertilization, although self-fertilization can also occur (1). Eggs appear in faeces 9–16 weeks after infection, depending on the definitive host species (2,5), and adult parasites can produce up to 20,000 eggs per day. The lifespan of adult parasites extends to

11 years in sheep, 9–12 months in cattle, and up to 13 years in humans, causing a chronic pathology in the definitive host (1,2).

1.2. Fasciolosis

F. hepatica is one of the etiologic agents of fasciolosis, a chronic and debilitating disease that represents an economically important affection of domestic livestock and a human zoonosis of growing public health concern. By affecting millions of ruminants worldwide, fasciolosis represents a major threat to animal welfare and global food security (28). Cases of human infection are predominantly concentrated in tropical areas of the globe; thus, the Word Health Organization (WHO) categorises human fasciolosis as a food-borne Neglected Tropical Disease (NTD) (29).

1.2.1. Epidemiology

F. hepatica has a cosmopolitan distribution, with cases of infection reported in all continents except for Antarctica (30,31) (**Figure 3**). The global prevalence of cattle and ovine fasciolosis is 17% and 13%, respectively, although it varies significantly between and within continents (31). In goat and other non-human animals, the prevalence is generally lower (32). In Spain, the overall prevalence of fasciolosis estimated from regional studies is 57.56% in cattle and 3.13% in small ruminants (31). Animal fasciolosis poses a significant threat to animal welfare and causes substantial economic losses in the global livestock industry, estimated at 3,200 million United States Dollars per year. This is caused by liver condemnation, poor-quality carcasses, decreased growth and conception rates, sudden mortality—although less frequent—, and a reduction in the production yield of animal-derived subproducts, including milk and wool (32,33). For instance, in an endemic area located in Galicia (northwest Spain), it has been recently estimated that cows presenting high loads of *F. hepatica* produce 4.5% less milk per day than their uninfected counterparts. This translates to 1.387 kg of milk lost per cow per day (34), underscoring the significant burden that fasciolosis poses on livestock productivity and its serious implications for global food security (28).

It is widely acknowledged that the current prevalence of human fasciolosis ranges from 2.4 to 17 million people (2,35). However, a recent meta-analysis reviewing the available literature assessing the prevalence of fasciolosis in the general population concluded that approximately 50 million people are infected worldwide (36). The highest prevalence was found in South America (9–9.09%), followed by Africa (2.66–4.8%), Asia (2–3.02%), and

North America (1.40%) (31,36). Additionally, it is estimated that 180 million people living in endemic areas are at risk of infection (37). Because human infection is highly prevalent in rural areas and associated with poverty (35), the WHO has classified human fasciolosis as a zoonotic NTD of concern that requires targeted public health interventions for its elimination (38). The disease inflicts significant morbidity in humans, with an estimated global loss of 90,000 years of full health due to infection-related disabilities (39). In this regard, children are especially vulnerable to the long-term sequelae associated with fasciolosis, caused by delayed growth and impaired neurocognitive development (36). It should be noted that the figures on the prevalence of human fasciolosis provided here are derived from recent meta-analyses that included only publications studying the prevalence of fasciolosis in the general population (31,36). Consequently, the figures do not encompass all countries and the actual global prevalence of fasciolosis is certainly higher. For example, studies assessing the prevalence of human fasciolosis in the general population were not available within Europe, but case reports describing individual cases of human fasciolosis have been published in virtually every European country (40). As an example, 3,933 cases of human infection were reported in Europe in 2005 (41).

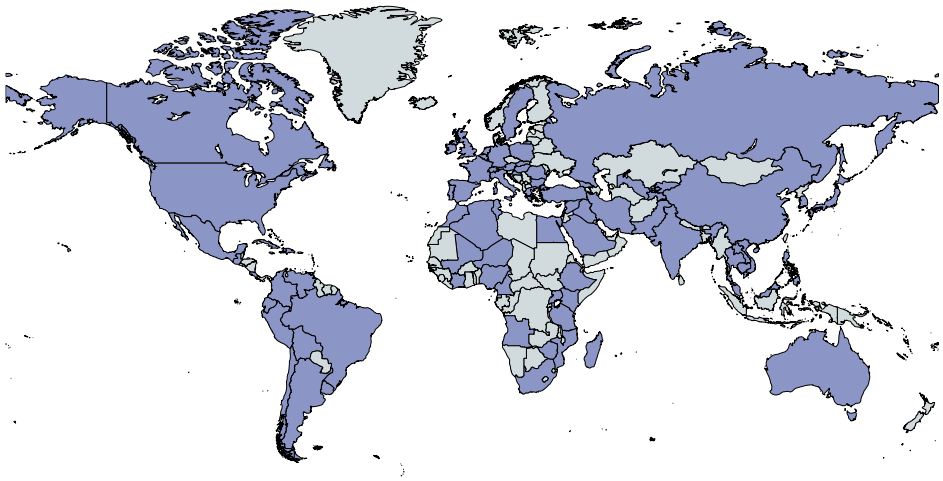

Figure 3. Global distribution of human fasciolosis. Countries with reported cases of human fasciolosis are highlighted in purple based on combined data from WHO, 2013 (40), Hostos-Infantes *et al.*, 2023 (36) and Lan *et al.*, 2024 (31). Source: Created with https://www.mapchart.net/.

The widespread geographic success of *F. hepatica* is mediated by biological, environmental, and social factors. *F. hepatica* adapts to a wide range of organisms and environments, with about thirty species of snails and fifty species of mammals recognised as suitable hosts for *F. hepatica* (42). This might be partly explained by an enrichment of transposable elements in *F. hepatica* genome, which favours phenotypic adaptability by promoting mutagenesis and

the rapid generation of genetic variants better adapted to changing physiological and environmental conditions (43). Moreover, the ability of this parasite to cause mild, non-fatal infections, along with its longevity and remarkable immune evasion mechanisms, are additional biological factors maximising parasite survival and transmission (42).

Environmental factors promoting the spread of *F. hepatica* include climate change and the anthropogenic modification of ecosystems. Increasing average temperatures accelerate different phases of *F. hepatica* life cycle, including egg embryonation and larval development within the intermediate snail host (7,44). In addition, drier conditions resulting from global warming cause the need to implement expansive irrigation and water storage systems that represent favourable niches for lymnaeid colonization (44). In general, non-human animal infections are influenced by man-guided livestock movements and inadequate herd management, including a misuse of anthelmintics that fosters the selection of drug-resistant isolates (32,42). Regarding human fasciolosis, it is more prevalent in low-income countries due to limited access to anthelmintics and to disease detection services. In these settings, consumption of uncontrolled wild edible plants and water contaminated with metacercariae also facilitates infection (42). Interestingly, a high prevalence of fasciolosis in livestock does not always correlate with higher infection rates in human communities living in close proximity, pinpointing the role that socio-cultural factors play on the transmission of *F. hepatica* among humans (42,45).

1.2.2. Pathology and clinical signs

The pathology of fasciolosis is multifactorial, depending on host-related factors such as species, age, and health status, as well as parasite-dependent factors, including strain and infective dose (parasitic load). Fasciolosis is most commonly mild and subclinical, but heavy infections in highly susceptible hosts, including cattle, sheep, and goat, can result in sudden death (18). In general, fasciolosis is divided in four clinical phases: the incubation phase (2–3 months), spanning from the ingestion of metacercariae to the appearance of the first symptoms; the invasive or acute phase (2–4 months), encompassing juvenile migration through the liver parenchyma towards the bile ducts; the latent phase (several months), including juvenile maturation into adult worms and the onset of oviposition; and the biliary or chronic phase (months–years), when clinical signs related to occupation of the biliary ducts by adult parasites start to appear (2,18). The pre-patent period refers to the time from metacercariae ingestion to the excretion of eggs in faeces (25). The most clinically relevant phases of fasciolosis are the invasive (acute) and the biliary (chronic) phases (2,18), which will be further elaborated upon below.

Crossing of FhNEJ through the duodenum is generally asymptomatic and does not cause any histopathological signs other than an occasional infiltrate of eosinophils in the lamina propria and submucosa of the duodenum (18). Therefore, the pathology associated with the acute phase derives from the migration of juvenile flukes through the liver parenchyma, leading to tissue damage via multiple mechanisms. These include the erosion of tissue by the parasite's spines and suckers, the ingestion of hepatocytes by juvenile flukes, the secretion of tissue-degrading enzymes, and the activation of an immune response aimed at targeting the parasite that inadvertently damages host tissues (1,18,19,46). During this phase, and starting one week post-infection, migratory tracts can be observed in the livers of infected sheep with a mild to severe infiltrate of eosinophils and macrophages (**Figure 4A**). At the earliest phases, the acute phase is generally asymptomatic in sheep (18), but in humans it causes toxic and allergic reactions that result in fever, urticaria, abdominal pain, and gastrointestinal (GI) disturbances, such as nausea and diarrhoea (2,35). Later on, tissue damage by migrating juveniles accumulates, resulting in haemorrhagic spots, fibrosis, granulomas, and liver congestion, which are observed from 6 weeks post-infection and can cause sudden death in heavily infected sheep. At 12 weeks post-infection, most juveniles have entered the bile ducts, causing bile duct hyperplasia and inflammation.

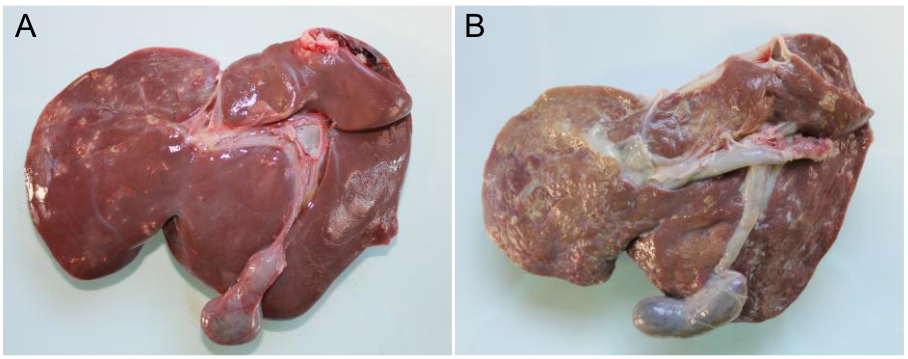

Figure 4. Liver pathology associated with *F. hepatica* infection. A) Gross liver pathology in sheep at 17 days post-infection (acute or migratory phase), showing pale-yellowish foci across the liver corresponding to the migratory tracts of juvenile parasites. B) Gross liver pathology in sheep at 16 weeks post-infection (chronic phase), showing a rough hepatic surface with white-yellowish scars corresponding to the healing of necrotic migratory tracts, along with dilation of the major bile ducts. Source: Courtesy of Prof. José Pérez Arévalo (Faculty of Veterinary Medicine, University of Córdoba, Spain).

After the latent phase, which is commonly asymptomatic, the chronic or biliary phase of fasciolosis begins. This phase is characterised by a pathology of the biliary tree associated with the obstruction of bile ducts by adult parasites and the mechanical degradation of the bile duct wall, resulting from focal ruptures caused by parasites during blood feeding. The

occasional ingestion of hyperplastic bile duct epithelial cells by adult parasites, along with a chronic eosinophilic and granulomatous inflammatory response in the liver, play major roles in the pathology associated with chronic fasciolosis (19,47,48). Altogether, adult feeding habits and a sustained inflammatory response cause an enlargement of the hepatic lymph nodes, the gallbladder, and the liver (hepatomegaly), diffuse cirrhosis, hyperplasia and inflammation of the bile ducts (hyperplastic cholangitis), thickening and necrosis of the bile duct walls, and local haemorrhages (**Figure 4B**). Ultimately, these responses cause weight loss and anaemia in domestic animals, including sheep, cattle, and goat (18). In humans, the clinical signs associated with chronic fasciolosis are similar, the most pathognomonic being cholangitis (inflammation of the bile ducts), cholecystitis (inflammation of the gallbladder), and cholelithiasis (formation of gallstones) (2,35).

Interestingly, aberrant juvenile migration can result in ectopic human fasciolosis. While the intestines are the most commonly reported ectopic site, other locations have also been documented, including the skin, the subcutaneous tissue and muscle, the eye, the pancreas, various lymph nodes, the lungs, the peritoneum, and the epidural space of the dorsal spine. These cases are rare and typically present with non-pathognomonic symptoms and an absence of eggs in faeces, making diagnosis more challenging (49).

1.2.3. Immune response to *Fasciola hepatica* infection

Early fasciolosis is characterised by a mixed Type 1/Type 2 T-helper cell immune response (Th_1/Th_2) with secretion of interleukin-4 (IL-4), interferon-γ (IFN-γ), IL-10, and transforming growth factor β (TGF-β). The concomitant release of Th_2 and Th_1 mediators in response to *F. hepatica* infection suggests that the host attempts to mount a robust protective Th_1 immune response to eliminate the parasite that is effectively inhibited by it (48,50). Eosinophils and macrophages are also recruited to the intestinal mucosa to kill FhNEJ via antibody-dependent cellular cytotoxicity (ADCC) (48,50). It has been experimentally shown that FhNEJ are susceptible to ADCC *in vitro* mediated by rat, sheep, and cattle antibodies (51), but these parasites have mechanisms to evade such responses, which are discussed in **Section 2.2.3**.

During early fasciolosis, an induction of B cells is also observed and characterised by predominant production of immunoglobulin G subclass 1 (IgG1) antibodies, which are associated with a non-protective Th_2 response. As the infection progresses, the Th_2 response is amplified and characterised by an increased production of IL-5 and eosinophilia, along with the suppression of Th_1- and Th_{17}-mediated responses. The Th_2-

skewed immune context induced by *F. hepatica* secreted proteins does not trigger the standard 'weep-and-sweep' response that leads to parasite expulsion during infections with GI nematodes. Instead, this Th_2-dominated response is primarily focused on repairing the tissue damage induced by migrating juveniles. The effective suppression of Th_1 responses by the parasite during early infection explains why primary infections fail to elicit protective immunity, which facilitates subsequent reinfections of the same host (50).

During the chronic phase of fasciolosis, production of anti-inflammatory (IL-4, IL-5, and IL-13) and regulatory (IL-10 and TGF-β) cytokines prevails along with an induction of alternatively-activated M2 macrophages and regulatory T cells, which constitute a tolerogenic/anergenic immune state that is a hallmark in many chronic helminth infections. Although M2-activated macrophages are crucial for tissue repair, they have a reduced ability to kill migrating juveniles compared to their M1 counterparts. Overall, the immune response during chronic fasciolosis is associated with tissue repair and wound healing mechanisms rather than cytotoxic responses. While this response effectively protects the host from excessive tissue damage derived from parasite occupation, it paradoxically promotes their successful establishment and development within the mammalian organism (48,50).

The ability of *F. hepatica* to suppress Th_1 and Th_{17}-driven inflammation can modify the outcome of concurrent co-infections and the efficacy of vaccines against other pathogens. For instance, *F. hepatica* increases the susceptibility of infected hosts to different microbial infections that require Th_1 responses for pathogen clearance (50), and reduces the antibody titters stimulated by vaccination against foot and mouth disease, a highly contagious infection of cattle (52). That said, the modulatory effects exerted by *F. hepatica* on vaccines against other pathogens are complex and influenced by multiple factors, including the timing of vaccination and infection with *F. hepatica* as well as the amount of parasites used for infection (52,53). Conversely, the immunomodulatory potential of *F. hepatica*-derived molecules has been proven to be beneficial in animal models of hyper-inflammatory and autoimmune disorders (54–56). In this regard, it has recently been suggested that *F. hepatica* may improve the outcome of viral diseases causing inflammation, not only by mitigating the harmful immune pathology associated with some of these infections (57) but also by reducing viral entry into host cells (58).

1.2.4. Diagnosis of fasciolosis

Diagnosis of *F. hepatica* is performed through direct and indirect detection methods. The detection of eggs in faeces by faecal egg count (FEC) is the gold-standard direct method for the diagnosis of fasciolosis. This method has historically relied on sedimentation techniques; however, in recent years, optimised filtration- and sedimentation-based commercial kits for FEC have been developed, which demonstrate improved sensitivity and accuracy compared to standard sedimentation procedures. The main limitation of FEC is that it only detects chronic infections, which is not optimal considering that most of the pathology comes from juvenile migration across tissues during acute infection, i.e., before eggs can be detected in faeces. Additionally, the diagnostic accuracy of FEC is influenced by various factors, including host age, parasite burden, intermittent egg shedding (59), and the level of expertise of the personnel performing the analysis.

Enzyme-linked immunosorbent assay (ELISA) is currently the benchmark technique for indirect diagnosis of fasciolosis. The primary advantage of immunological methods lies in their ability to provide a diagnosis during the pre-patent period, this is, when eggs are not yet present in faeces. However, these methods are costlier than FEC and may result in cross-reactivity with other parasite species, potentially leading to false-positive diagnoses. ELISA-based methods for the diagnosis of fasciolosis can either be direct (sandwich ELISA) or indirect, depending on whether they detect *Fasciola* antigens or host-derived antibodies, respectively. Consequently, direct ELISA detects only active infections, whereas indirect ELISA has the limitation that antibodies can be detected after infection resolution (59). Both approaches are suitable for detecting infections in serum and milk samples, and direct ELISA methods are also available for detecting coproantigens in stool samples (59,60). While indirect ELISA typically detects positive cases 2–3 weeks post-infection, certain direct ELISA methods can detect them as early as one week post-infection (61). Regarding direct ELISA, commercial kits based on the monoclonal antibody MM3, which detects *F. hepatica* excreted/secreted products (ESPs) in faeces, are amongst the most commonly used for the direct immunological detection of fasciolosis (59,62). For indirect ELISA, different antigens have been used for plate coating and antibody detection, yielding different but acceptable sensitivities, including ESPs, cathepsin proteases, kunitz-type proteins, glutathione S-transferase (GST), fatty acid binding proteins (FABPs), and tegumental antigens (59).

The aforementioned techniques are constrained by their inability to differentiate between *Fasciola* species, which is particularly relevant in regions where *F. hepatica* and *F. gigantica* co-

exist. Species-specific detection relies on molecular methods that amplify specific regions of the parasite genome. These include standard polymerase chain reaction (PCR) and different versions of it: real-time (RT)-PCR, which can be used for quantification purposes, thereby providing information about infection intensity; PCR followed by digestion of the amplified fragments with restriction enzymes (restriction fragment length polymorphism, RFLP), which can detect species-specific polymorphisms that are differentially enriched for restriction enzyme target sites; and multiplexed PCR, which allows for the detection of multiple DNA fragments in the same reaction. The main limitation of PCR-based detection methods is that the source of DNA is generally parasite eggs, which are only present in the chronic phase of infection. Additionally, their dependence on sophisticated DNA extraction protocols and specialised laboratory equipment, including thermal cyclers, make them unsuitable to become the gold-standard technique in field settings (59). In this scenario, loop-mediated isothermal amplification (LAMP) techniques have recently emerged as a viable alternative for the detection of *Fasciola* species, since they yield similar results as conventional PCR and the DNA amplification reaction is performed at a constant temperature, facilitating its application in low-resource areas (63,64).

1.2.5. Treatment of fasciolosis

Different anthelmintic drugs are commercially available to treat fasciolosis, including albendazole, ricobendazole, triclabendazole (TCBZ), nitroxinil, closantel, oxyclozanide, rafoxanide, and clorsulon. Given its effectiveness towards both adult and juvenile stages of *F. hepatica*, TCBZ is currently the most widely used drug to treat fasciolosis. Closantel is also active against juvenile parasites; however, its efficacy begins at five weeks post-infection, whereas TCBZ is effective against juveniles as early as two days after infection (59). TCBZ is administered orally in sheep, cattle, and goat, and there is currently a commercially available formulation for humans. Upon administration, TCBZ is metabolised into various derivatives, with TCBZ sulfoxide exhibiting the highest activity. These metabolites enter adult flukes through oral ingestion, although passive diffusion through the tegument surface has also been observed *in vitro*. TCBZ targets the microtubules, disrupting their assembly and subsequent spindle formation, which inhibits cell division and ultimately induces apoptosis in the testis, vitelline glands, and the ovary. As a result, parasites die within 48–96 hours post-treatment (65). Additionally, inhibition of adenylate cyclase activity has been proposed as a potential alternative or complementary mechanism of action of TCBZ (65,66).

Under-dosing of TCBZ, the repeated use of this drug, and mass-treatment of flocks in endemic areas have prompted the selecion of TCBZ-resistant (TCBZ-R) *F. hepatica* isolates (59). This is an alarming and ongoing phenomenon with an expanding number of countries reporting TCBZ resistance, not only in farms (67,68) but also in humans (69,70). One plausible mechanism behind TCBZ resistance is altered target binding; however, since no differences in β-tubulin protein sequence or expression levels have been detected between TCBZ-R and -sensitive (TCBZ-S) flukes, the interest has shifted to other possible explanations. These include altered drug uptake or increased efflux, which is supported by an improvement of TCBZ efficacy on resistant flukes when co-administered with inhibitors of P-glycoprotein (Pgp)-linked drug efflux pumps. Additionally, TCBZ-R flukes show increased drug metabolism, suggesting that an alteration in the activities of drug-metabolising enzymes, including cytochrome P450 and GST, might be distorted in these isolates (71). Recently, a genome-wide analysis of TCBZ resistance conducted by Beesley *et al.* (72) provided important insights into the genetic basis of TCBZ resistance. Through experimental gene crossing and linkage mapping, the authors identified a haplotype within the *F. hepatica* genome containing different single-nucleotide polymorphisms enriched in TCBZ-R flukes. Among the genes located within this genomic region, ABCB1 (which encodes the Pgp-linked drug efflux pump), FABPV (a member of the FABP protein family), and two Ras-related proteins were identified as putative mediators of TCBZ resistance, at least within the studied isolates. Importantly, the identified locus exhibited Mendelian dominant inheritance and was also subject to drug selection under natural field conditions (72). Strategies to overcome TCBZ resistance include improved livestock management practices and drug-related approaches. The latter encompass the use of drug combinations, reducing drug metabolism to increase drug availability, re-purposing of existing drugs, and the use of alternative treatments, including natural compounds such artemisinin derivatives (59,71). Nonetheless, the increasing prevalence of TCBZ resistance in *F. hepatica* creates a pressing need to explore alternative control strategies, with vaccines emerging as a particularly promising solution (59,73).

1.2.6. Vaccine-based approaches to fasciolosis control

Vaccines represent one of the most promising alternatives to anthelmintics for achieving cost-effective, sustainable, and long-term control of fasciolosis. Since vaccine-derived chemical residues are neither retained in the animals nor passed onto the pasture, vaccines are also considered safe for both consumers and the environment (59,74). The first attempt at developing a vaccine against fasciolosis was carried out in 1967 using γ-ray-attenuated

metacercariae (75). Since then, multiple strategies have been explored in both laboratory models (mouse, rat, rabbit) and livestock (cattle, sheep, goat) (**Supp. File 1**), yielding significant variability in protection rates and exhibiting poor reproducibility between different trials. This highlights that developing a vaccine against *F. hepatica* remains a challenge, partly due to the sophisticated mechanisms that this parasite has evolved to evade host immune responses, including those elicited with vaccination (24,47) (**Section 2.2.3**).

Given the suboptimal performance of vaccination trials conducted to date, innovative approaches are proposed for improving the efficacy of *F. hepatica* vaccines. These include an evaluation of tegument proteins on the surface of juvenile flukes as potential vaccine candidates and exploring the potential of fluke glycans and exosome function to interfere with host-parasite interactions. Moreover, it is widely accepted that an effective vaccine will require a combination of antigens, but the optimal candidate combination has yet to be identified (51). Interestingly, as shown in **Supplementary File 1**, 73.5% of the vaccination trials using native antigens yielded significant protection rates to varying extents, whereas only 42.3% of trials employing recombinant proteins resulted in significant vaccine efficacies. This suggests that protein conformation or post-translational modifications (PTMs), such as phosphorylation and glycosylation, may be critical for epitope recognition and the induction of an effective immune response in the host. Nonetheless, vaccination trials conducted under controlled conditions have demonstrated poor reproducibility, highlighting significant animal-to-animal variation in response to infection and potential differences between different *F. hepatica* isolates in terms of pathogenicity and batch-to-batch fitness. This lack of reproducibility underscores the need for improved trial design including the careful calculation of the number of animals needed to obtain statistically robust results, which can be accurately estimated through power analysis (76).

That said, the methods by which vaccine efficacy is measured are currently being revisited. Most trials have focused on maximizing the reduction of fluke burden, with percent reductions of over 70% generally accepted as the goal for commercialisation. However, it is increasingly acknowledged that post-challenge evaluation of vaccine efficacy could also be assessed based on the ability of vaccines to reduce the clinical pathology to a level sufficient to prevent the significant economic losses associated with it. In cattle, the threshold for substantial economic losses is estimated at 30–40 flukes per animal (51), which translates to different percent efficacies depending on the intensity of infection and may be far below 70% in low intensity infections. In addition, egg viability and embryonation rates might also be regarded as indicators of vaccine efficacy provided that

a reduction in these parameters could substantially reduce parasite transmission within herds. In some trials, a reduction in egg embryonation in vaccinated vs. non-vaccinated animals has been observed even in the absence of a reduction in fluke burden or faecal egg counts, which is compelling (76). Additionally, recent estimates suggest that low percent efficacies can be weighed out by increasing vaccination coverage, with analyses revealing that a vaccine with 43% efficacy could benefit cattle production if 90% of the herd were vaccinated. Such vaccines are more likely to be successful than others with higher efficacies but lower coverage (51).

Another important aspect to consider when understanding the underperformance of vaccine trials is that they have mainly employed antigens derived from the adult stage of *F. hepatica* in their formulations (24,79,84). The antigenic complexity of adult parasites, characterised by redundant protein isoforms that facilitate immune evasion, together with their location within the bile ducts, which represent an immunologically 'safe' environment, contribute to the low efficacy observed in vaccination trials (84). In light of this, the juvenile stages of *F. hepatica* have recently gained attention in vaccine development (24,51,84), as these parasites are not fully developed and potentially more susceptible to host defences, their anatomic location is more accessible to immune effectors, and they cause the majority of tissue damage—and subsequent economic losses—associated with *F. hepatica* infection (**Section 1.2.2**). Targeting juvenile flukes would not only reduce the clinical pathology associated with fasciolosis and the resulting economic burden in the livestock industry, but also prevent the development of adult worms and subsequent egg shedding, thereby blocking disease transmission within affected populations. However, vaccination trials utilizing antigens from juvenile flukes are still scarce, particularly those conducted in the parasite's natural hosts (**Table 1**).

Table 1. Vaccination trials using antigens from the pre-hepatic stages of *F. hepatica*.

Antigen	Form	Host	Protection (%)	Ref.
Crude extract	Native	Rat	92.60%	van Milligen *et al.* (2000) (77)
FhpCL3	Recombinant	Rat	52.00%	Reszka *et al.* (2005) (78)
FhCB	Recombinant	Rat	60.00%	Jayaraj *et al.* (2009) (79)
FhCB + FhCL5 (a)	Recombinant	Rat	82.86%	
FhCB + FhCL1g (a)	Recombinant	Rat	65.71%	
FhCB + FhCL5 + FhCL1g (a)	Recombinant	Rat	62.86%	
FhCL3-1 + FhCL3-2 + FhCB3	Recombinant	Rat (male)	47.00%	Wesolowska *et al.* (2018a) (80)
FhCL3-1	Recombinant	Rat (male)	40.00%	
FhCL3-2	Recombinant	Rat (male)	58.00%	

Antigen	Form	Host	Protection (%)	Ref.
FhCL3-1 + FhCL3-2 + FhCB3	Recombinant	Rat (female)	n.s.	Wesolowska *et al.* (2018b) (81)
FhCL3-1	Recombinant	Rat (female)	n.s.	
FhCL3-2	Recombinant	Rat (female)	n.s.	
FhCL5 + FhCB2 (a)	Recombinant	Sheep	n.s.	Norbury *et al.* (2018) (82)
FhCL5 + FhCB2 (a)	Recombinant	Sheep	40.5%	
FhTeg1 + FhTeg5	Recombinant	Rat	48.2%	McCusker *et al.* (2020) (83)
FhTeg1 + FhTeg5	Recombinant	Cattle	n.s.	

Protection is assessed based on a reduction in fluke burden. Differences in protection outcomes observed under apparently identical conditions are explained by variations in factors not specified in this table, such as adjuvants, vaccination protocols, antigen dosages, or administration routes. Trials using attenuated metacercariae are not included in this table but can be found in Spithill *et al.*, 1999 (75). (a) FhLC1g and FhCL5 are specific to metacercariae and adult parasites, respectively, but are included in this table when used in combination with juvenile-specific antigens. CB, cathepsin B; CL1g, cathepsin L1g; CL3, cathepsin L3; CL5, cathepsin L5; n.s., not significant; pCL3, immature cathepsin L3; Teg1, tegument glycoprotein 1; Teg5, tegument glycoprotein 5.

2. Host-parasite relationships during early-stage fasciolosis

As highlighted in the previous section, focusing on the pre-hepatic stages of *F. hepatica* may offer a promising path to improve the efficacy of vaccines against fasciolosis, as juvenile parasites theoretically 'hold the key' to disrupting the infection cycle and overcoming the limitations of adult-based control strategies. Achieving this goal requires a deeper understanding of the biology of *F. hepatica* juveniles, including their mechanisms of interaction with host tissues. This section provides a thorough review of FhNEJ biology and their mechanisms of trans-intestinal invasion, migration, and evasion of host immune defences. However, to fully understand the complex interactions between *F. hepatica* and the mammalian host during the early phases of infection, it is essential to first examine the structure and histology of the intestinal epithelium, as this constitutes the initial site of host-parasite engagement.

2.1. The small intestine: structure and function

In addition to fulfilling important physiologic functions related to digestion and nutrient absorption, the gut represents the first and most extensive line of defence against pathogens that enter the organism through the intestinal tract (85). This organ is anatomically divided into two main sections: the small intestine and the colon. The small intestine is further segmented into three regions, arranged from proximal to distal: the duodenum, the jejunum, and the ileum (86). Histologically, the small intestine is divided in four layers: the mucosa, the submucosa, the *muscularis externa*, and the serosa (87) (**Figure 5A**). The mucosa itself is also divided into different layers and it organises into villi and crypts of Lieberkühn. Villi are finger-like protrusions of tissue aimed at increasing the total surface area of the intestinal epithelium to maximise nutrient absorption. In contrast, crypts are invaginations of the epithelium that extend into the underlying extracellular matrix (ECM) (86,87) (**Figure 5B**).

2.1.1. The epithelium of the intestinal mucosa

The intestinal epithelium is the apical-most layer of the intestinal mucosa. The intestinal mucosa is composed of three distinct layers, arranged from luminal to basal: the intestinal epithelium, consisting of a monolayer of epithelial cells with functional specialisations; the lamina propria, a layer of loose connective tissue that provides mechanical support to the epithelium and contains blood capillaries for nutrient absorption; and the *muscularis mucosae*, a thin layer of smooth muscle cells that facilitates lymphatic drainage through rhythmic

contractions. The intestinal epithelium is separated from the lamina propria by the basement membrane (BM) (85), a specialized form of ECM that is found right beneath epithelial cells and surrounding myocytes, adipocytes, and Swann cells (88). Beyond physically blocking pathogen entry, the intestinal mucosa is equipped with robust immune defences to prevent pathogen infiltration and dissemination.

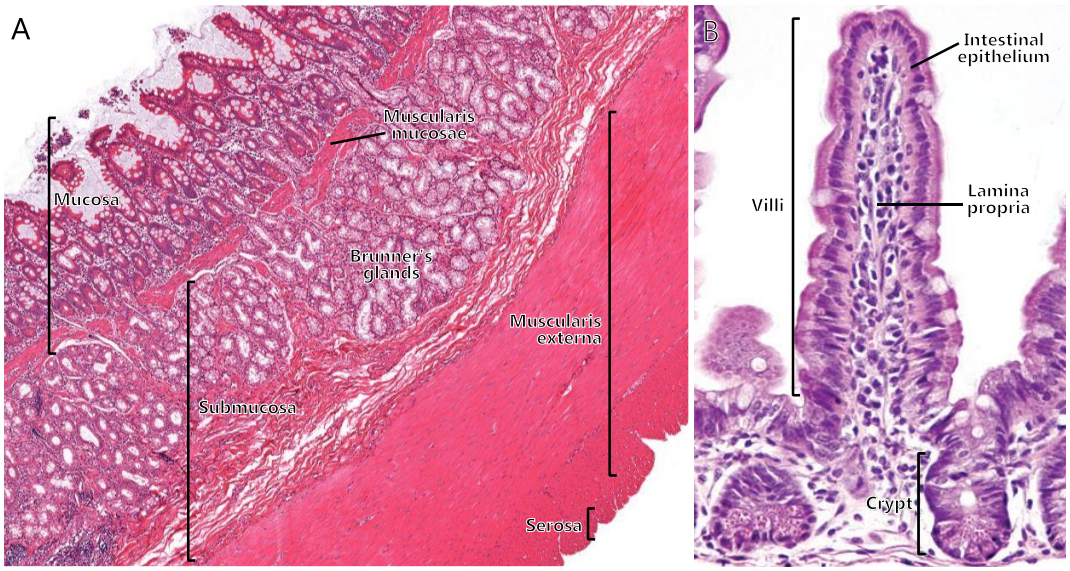

Figure 5. Structure of the small intestine. A) Haematoxylin and eosin (H&E) staining of the human duodenum. The small intestine is divided in the mucosa, the submucosa, the *muscularis externa*, and the serosa. The mucosa contains a columnar epithelium supported by the BM, the lamina propria, and the surrounding smooth muscle (*muscularis mucosae*). The submucosa contains the major blood and lymph nodes and harbours mucus-secreting Brunner's glands, which are only found in the duodenum portion of the small intestine. The *muscularis externa* is formed by an inner layer of circular smooth muscle and an outer layer of longitudinal smooth muscle. The serosa is composed by a thick layer of fibrous tissue outlined by mesothelial cells that separates the intestine from the peritoneal cavity (87,89,90). B) H&E staining of the mouse duodenum. The intestinal epithelium and the underlying BM and lamina propria organise into invaginations known as crypts of Lieberkühn and finger-like projections called villi. Smooth muscle fibres from the *muscularis mucosae* extend along the vertical axis of each villus, typically surrounding a lymphatic capillary that terminates at the villus tip. Sources: https://histologyguide.com/ (A); adapted from Van der Flier and Clevers, 2009 (86) (B).

2.1.1.1. Cellular components

The intestinal epithelium represents the tissue with highest turn-over rates in adult mammals, a function that is maintained by a pool of self-renewing Leucine-rich repeat-containing G-protein coupled receptor 5-positive (LGR5+) adult stem cells, the so-called intestinal stem cells (ISCs). ISCs are found throughout the entire intestinal tract and reside at the bottom of the crypt. These pluripotent cells divide into transit-amplifying cells, also known as progenitor cells, which self-renew for 4–5 times before giving rise to the five

major different epithelial subsets that form the intestinal epithelium: enterocytes, goblet cells, Paneth cells, enteroendocrine cells, and tuft cells (86,91,92). However, recent advances in single-cell sequencing technologies combined with spatial mapping techniques have provided unprecedented insights into the complexity of the intestinal cellular landscape, with 16 to 24 epithelial cell types with distinct transcriptional identities identified within the gut, depending on the study (93,94). While ISCs generate new daughter progenitor cells, differentiated cells move up the crypt–villus axis to reach the top of the villi a few days after terminal differentiation, when they undergo anoikis (programmed cell death induced by detachment from the underlying ECM) and are shed into the gut lumen (86). This process occurs within 3–5 days in mice and one week in the human intestine (95). The only exception to this are Paneth cells, which migrate downwards back to the crypt base where they live for 1–2 months before being cleared by infiltrating macrophages (86,96). Strikingly, recent studies have shown that tuft cells can function as damage-induced, ISC-like regenerative cells in the human intestine, capable of giving rise to all the epithelial lineages found in this tissue in the event of an intestinal injury (97).

Enterocytes are the most abundant cell type of the intestinal epithelium and contain distinct microvilli at their apical surface, in contact with the gut lumen. They are responsible for nutrient absorption and secrete enzymes that aid in food digestion (92). Goblet cells are specialised in the secretion of the mucus layer that covers the epithelium, which protects it from the erosive effects of gut fluids involved in food digestion (98). Paneth cells play important roles in antibacterial defence by producing bactericidal molecules and participate in the maintenance of the stem cell population by secreting Wingless-related integration site signal proteins (86,92). Enteroendocrine cells regulate the digestive activities of the gut by secreting serotonin and other peptide hormones that regulate the behaviour of different cell types, including intestinal neurons (92). Tuft cells, also known as 'brush' cells owing to their well-developed microvilli apparatus, are the least-abundant cell type under steady-state conditions, comprising less than 1% of the total cells in the mouse intestinal epithelium. These cells function as sentinels with important chemosensory functions and they play a central role in initiating type 2 immune responses following infection with GI nematodes (99).

2.1.1.2. Cellular junctions

Epithelial cells are interconnected by intercellular junctions located at the apical and basolateral domains, and attached to the underlying ECM via cell-matrix junctions located at their basal surface. Different types of intercellular junctions connect the cytoskeleton of

neighbouring cells to promote cell-to-cell attachment: tight junctions, adherens junctions, and desmosomes (arranged apical to basal). Gap junctions are located below desmosomes and they are not strictly considered intercellular junctions, as they function as channel-forming units that facilitate the transfer of water-soluble molecules between adjacent cells rather than mediating cell-to-cell attachment (88). Except for desmosomes, intercellular junctions are also involved in controlling the paracellular transport of small molecules and solutes (100). Cell-matrix junctions are found in the basal membrane of epithelial cells and they are classified as actin-linked cell-matrix junctions (e.g., focal adhesions) and hemidesmosomes depending on the cytoskeleton protein they bind to: either the actin cytoskeleton or intermediate filaments, respectively (**Figure 6**). Altogether, these intercellular and cell-matrix junctions are found throughout the entire digestive system, including the stomach, the intestines, the gallbladder, and the bile ducts (100,101).

Table 2. Epithelial intercellular and cell-matrix junctions.

Junction	Transmembrane adhesion protein	Extracellular ligand	Intracellular adaptor protein	Cytoskeletal ligand
Intercellular junctions				
Tight junctions	Claudins, occludin, tricellulin, JAM, others (a)	Equivalent protein on neighbouring cell	ZO proteins	Actin filaments, microtubules (b)
Adherens junctions	Classical cadherins	Equivalent protein on neighbouring cell	α/β catenin, p120-catenin, vinculin	Actin filaments
Desmosomes	Non-classical cadherins (desmoglein, desmocollin)	Equivalent protein on neighbouring cell	Plakoglobin, plakophilin, desmoplakin	Intermediate filaments
Cell-matrix junctions				
Actin-linked cell matrix junction	Integrins	ECM proteins	Talin, kindlin, vinculin, paxillin, FAK, etc.	Actin filaments
Hemidesmosomes	Integrins	ECM proteins	Plectin, BP230	Intermediate filaments

Information extracted from Welcome *et al.*, 2018 (100), Alberts *et al.*, 2022 (88), and Kollmann *et al.*, 2025 (102). This table represents a general model of intercellular and cell-matrix junctions, although there are certain exceptions to this. For instance, some integrins can also participate in intercellular junctions; and additional adhesion molecules not listed here are involved in transient intercellular attachment in special circumstances. (a) Claudins represent the major transmembrane proteins in tight junctions, and they are essential for their formation and regulation. Occludin is not essential for tight junction assembly but is involved in limiting junctional permeability. Tricellulin is an integral component of tight junctions at points where three cells meet (88,100). (b) Tight junctions connect to microtubules when ZO adaptor proteins bind to either cingulin or paracingulin proteins at the cytoplasmic side. ECM, extracellular matrix; FAK, focal adhesion kinase; JAM, junctional adhesion molecule; ZO, zonula occludens.

Intercellular and cell-matrix junctions are formed by transmembrane adhesion proteins and intracellular adaptor proteins (**Table 2**). Transmembrane adhesion proteins such as claudins interact with structures at the extracellular space: either another transmembrane adhesion

protein expressed by the neighbouring cell (intercellular junctions) or a protein in the basal ECM (cell-matrix junctions). Adaptor proteins such as zonula occludens (ZO) proteins link the transmembrane adhesion protein with cytoskeleton proteins, typically actin or intermediate filaments, depending on the type of junction. In general, apical or basolateral localization of intercellular junction proteins relies on intracellular transport from the endoplasmic reticulum (ER) to the appropriate domain of the lateral plasma membrane, which depends on clathrin-mediated intracellular vesicle trafficking (103,104).

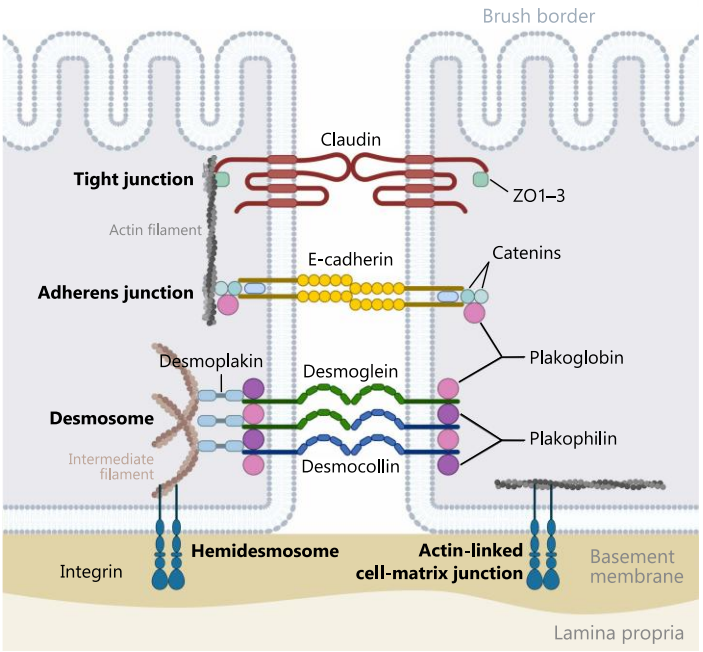

Figure 6. Schematic description of the epithelial junctions. Depicted is the intercellular gap between two intestinal epithelial cells and the underlying BM and lamina propria. From apical to basal, intercellular junctions include tight junctions, adherens junctions, and desmosomes. Cell-matrix junctions include actin-linked cell-matrix junctions (e.g., focal adhesions) and hemidesmosomes. Source: Adapted from Kollmann *et al.*, 2025 (102). Created with Biorender.com.

In addition to regulating cell-to-cell attachment, intercellular junctions also function as intracellular signalling platforms through the engagement of adaptor proteins that interact with signal transducers that regulate epithelial cell proliferation, differentiation, and cytoskeleton dynamics, among others (105–107). Intracellular signalling can ultimately lead to changes in gene transcription and protein expression, for example, through the engagement of ZO proteins and catenins (105,108). Another important functional aspect of intercellular junctions, particularly tight junctions, is their role as a fence that separates the apical and basolateral domains of the plasma membrane, establishing a polarised functional specialisation. In this regard, tight junctions confine transmembrane proteins,

such as glucose and other micronutrient transporters, to their appropriate membrane domains, preventing their unwanted diffusion to other regions (88). This apical–basal compartmentalisation specified by tight junctions is crucial for the proper functioning of the epithelium as a whole (100).

Finally, disruption of intercellular junctions can cause epithelial barrier dysfunction, which is a hallmark of miscellaneous pathological conditions of the intestine, including inflammatory bowel disease (IBD) and Crohn's disease (100,109). Infectious agents such as bacteria and viruses can also disrupt tight junctions, partly through the induction of pro-inflammatory cytokines and the subsequent activation of ECM-degrading enzymes (110–113). Although viruses access the intracellular space rather than traversing epithelial barriers through the paracellular route, some rely on tight junction disruption for cell entry when their receptors are located at the basolateral domain (i.e., below tight junctions) of the host epithelial cell membrane (111).

2.1.2. The extracellular matrix of the intestinal mucosa

The intestinal mucosa contains two types of ECM: the BM, which is a specialised form of ECM that is found directly beneath epithelial cells; and the interstitial matrix (IM), which is the ECM portion of the lamina propria (114). The IM also represents the ECM portion of other intestinal tissue layers, including the submucosa, the *muscularis externa*, and the serosa (85). The lamina propria is a thin layer of connective tissue located beneath the BM and mainly composed of ECM, which is produced by various cell types within the lamina propria, including fibroblasts and myofibroblasts. Cellular components of the lamina propria also include non-ECM producing cells, such as scattered immune cells and endothelial cells that surround blood and lymphatic capillaries found within this tissue layer (88,115).

In general, ECMs are dynamic structures constituted by a complex network of macromolecules that give mechanical support to tissues. In addition, these structures represent a physical barrier against pathogen entry and synchronise cell behaviour by modulating intracellular signalling pathways through the engagement of cell surface receptors (85,116). While all the ECMs share common macromolecules, their types and ratios vary depending on the tissue type. The different ECM components are classified into fibrous proteins (e.g., collagens), glycoproteins (e.g., laminin, nidogen, fibronectin), and gel-forming polysaccharides of the family of glycosaminoglycans (GAGs; e.g., hyaluronan). Except for hyaluronan, all the other GAGs found in the ECM are linked to a core protein,

forming the so-called proteoglycans (PGs; e.g., perlecan, decorin, and aggrecan). These macromolecules confer hydration and elasticity to the tissue while providing resilience to tensile and compressive forces (85,88). The components of the ECM of the intestinal mucosa are summarized in **Table 3**.

Table 3. The main components of the ECM of the intestinal mucosa.

Component	Molecule type	Secreting cells	Functions
Basement membrane			
Collagen type IV (a)	Fibrous protein (network-forming)	Mesenchymal, enteroendocrine and epithelial cells	BM stabilization; interaction with transmembrane receptors
Laminins	Glycoprotein	Epithelial and mesenchymal cells	BM assembly; modulation of epithelial homeostasis
Nidogen (entactin)	Glycoprotein	Epithelial and mesenchymal cells	BM stabilization; interaction with ECM components
Perlecan	Proteoglycan	Epithelial and mesenchymal cells	Cell differentiation, proliferation, adhesion and migration
Interstitial membrane			
Collagens I and III	Fibrous protein (fibril-forming)	Subepithelial fibroblasts and myofibroblasts	Regulate cell adhesion, tissue development and homeostasis
Fibronectin	Glycoprotein	Fibroblasts, epithelial cells	Regulates cell adhesion, migration, differentiation, growth and survival
Elastin/tropoelastin	Fibrous protein	Fibroblasts, smooth muscle cells	Modulates intestinal tissue stretching; confers tissue elasticity
Hyaluronan (b)	GAG	Fibroblasts, smooth muscle cells	Mediates intestinal ECM interactions
Decorin	Proteoglycan	Epithelial cells, fibroblasts, smooth muscle cells	ECM integrity

Information extracted from Pompili *et al.*, 2021 (85). (a) Collagens represent the major protein of ECMs. They are, in fact, the most abundant protein in mammals, constituting around 25% of their total protein mass (88). (b) Also known as hyaluronic acid or hyaluronate (88). BM, basement membrane; ECM, extracellular matrix; GAG, glycosaminoglycan.

The BM is a very thin layer of ECM—typically 40–120 nm thick—containing the network-forming proteins laminin and collagen type IV as the main components. Laminins, which represent the main organisers of the BM structure, are composed of three polypeptide chains (α, β, and γ) that are held together by disulphide bonds and arranged in a bouquet-like shape. Like the fibril-forming collagens type I and III, particularly enriched in the IM of the lamina propria, type IV collagen is also composed by a triple-stranded helix; however, the helical structure of type IV collagen is interrupted in some regions to allow bending, which facilitates its arrangement into networks rather than fibrils. Laminins and type IV collagen interact with additional BM proteins, including nidogen and perlecan, forming a complex network of proteins and PGs (88).

The ECMs are dynamic structures that undergo constant turnover, which is essential to guarantee tissue homeostasis. In this regard, an imbalance in the equilibrium between ECM

deposition and degradation in the intestine can lead to several pathologies, including IBD and related syndromes that are characterised by aberrant intestinal fibrosis (85,88). ECM remodelling is regulated by growth factors secreted by epithelial and mesenchymal cells, by mechanical forces, and by matrix-degrading enzymes and their inhibitors. In mammals, ECM degradation is mainly performed by zinc-dependent endopeptidases, including astacins, pappalysins, matrix metalloproteinases (MMPs), serralysins, a desintegrin and metalloproteinase proteases (ADAMs), and ADAM with thrombospondin proteases (ADAMTS), though certain serine proteases, such as plasmin, can also contribute to this process (85). Among these, MMPs have a significant contribution to ECM remodelling in the mammalian intestine (**Table 4**). Additionally, many pathogens, including bacteria, fungi, and parasites like *F. hepatica*, express proteases that degrade ECM components to break through tissue barriers and disseminate within the host organism (117,118).

Table 4. Substrate specificities of MMPs.

Name	**Synonym**	**ECM substrates**	**Non-ECM substrates** (a)
MMP-1	Collagenase-1	Collagen I, II, III, VII, VIII, X, gelatin, proteoglycan link protein, aggrecan, veriscan, tenascin, nidogen	MMP-2, MMP-9
MMP-2	Gelatinase-A	Collagen I, IV, V, VII, X, XI, XIV (b), gelatin, elastin, fibronectin, vitronectin, laminin-1, laminin-5, tenascin-C, galectin-3, aggrecan, decorin, hyaluronidase-treated versican, proteoglycan link protein, osteonectin (c)	MMP-1, MMP-9, MMP-13
MMP-3	Stromelysin-1	Collagen III, IV, V, IX, X, aggrecan, fibronectin, laminin, vitronectin, gelatin, elastin, tenascin-C, versican, perlecan, decorin, proteoglycan link protein, nidogen, osteonectin	MMP-1, MMP-2/TIMP-2 complex, MMP-7, MMP-8, MMP-9, MMP-13
MMP-7	Matrilysin	Collagen IV, X, gelatin, aggrecan, fibronectin, laminin, vitronectin, elastin, decorin, proteoglycan link protein, nidogen, tenascin-C, osteonectin, β4 integrin, elastin	MMP-1, MMP-2, MMP-9, MMP-9/TIMP-1 complex
MMP-8	Collagenase-2, neutrophil collagenase	Collagen I, II, III, V, VII, VIII, X, gelatin, aggrecan, fibronectin, proteoglycan link protein	
MMP-9	Gelatinase-B	Collagen IV, V, VII, X, XIV, gelatin, elastin, galectin, aggrecan, vitronectin, versican, proteoglycan link protein, fibronectin, nidogen, osteonectin	
MMP-10	Stromelysin-2	Collagen III, IV, V, gelatin, aggrecan, fibronectin, elastin, proteoglycan link protein	MMP-1, MMP-8
MMP-11	Stromelysin-3	Collagen IV, gelatin, laminin, fibronectin	
MMP-12	Metalloelastase, macrophage elastase	Collagen IV, gelatin, elastin, proteoglycan monomer, fibronectin, vitronectin, laminin, nidogen	
MMP-13	Collagenase-3	Collagen I, II, III, IV, IX, X, XIV, gelatin, aggrecan, perlecan, tenascin-C, fibronectin, osteonectin	MMP-9, PAI-2

Name	Synonym	ECM substrates	Non-ECM substrates (a)
MMP-14	MT1-MMP	Collagen I, II, III, gelatin, elastin, fibronectin, laminin-1, vitronectin, proteoglycans, dermatan sulphate, tenascin-C, nidogen	MMP-2, MMP-13
MMP-15	MT2-MMP	Fibronectin, laminin, perlecan, tenascin-C, nidogen, aggrecan	MMP-2
MMP-16	MT3-MMP	Collagen III, gelatin, fibronectin	MMP-2
MMP-17	MT4-MMP	Aggrecan (cartilage) (119)	
MMP-18	Collagenase-4	(d)	
MMP-19		Gelatin	
MMP-20	Enamelysin	Amelogenin (enamel component)	
MMP-21		(d)(e)	
MMP-22		(d)	
MMP-23		(d)(e)(f)	
MMP-24	MT5-MMP	(d)	MMP-2, MMP-13
MMP-25	MT6-MMP, leukolysin	Galectin-1 (g), osteonectin (120)	MMP-2
MMP-26		Collagen IV, denatured collagen, fibronectin, vitronectin, gelatin	MMP-9
MMP-28	Epilysin	(d)(e)	
MMP-29		(d)	
Meprin		Collagen I, III, IV, laminin-1, laminin-5, fibronectin, nidogen (121)	

The information has been extracted, compiled, and updated from Lijnen *et al.,* 2001 (122) and Engelse *et al.,* 2004 (123). (a) Only MMP precursors are listed. A full list of intracellular, surface-exposed/intercellular, and chemokine/cytokine non-ECM substrates is available at Cauwe *et al.,* 2010 (124), Cauwe *et al.,* 2007 (125), and Rodríguez *et al.,* 2010 (126), respectively. (b) Only degrades relaxed collagen (inactive towards intact helical collagen) (127). (c) Also known as secreted protein, acidic and rich in cysteine (SPARC). (d) At the time of writing this text, the ECM substrate for these MMPs remains unknown. (e) Although still unidentified, these MMPs likely cleave ECM proteins as they are involved in biological processes that require ECM degradation and remodelling, including foetal development [MMP-21 (128)], cancer progression and/or aggressiveness [MMP-21 (129) and MMP-23 (130)], ovarian follicular development [MMP-23 (131)], and wound healing [MMP-23 (132) and MMP-28 (133,134)]. (f) MMP-23 is membrane anchored but structurally different from MT-MMPs (131,135). (g) Galectin-1 is not an ECM protein itself but interacts with ECM proteins (136). ECM, extracellular matrix; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase ; PAI-2, plasminogen activator inhibitor 2 ; TIMP-1, tissue inhibitor of metalloproteinase 1.

The MMPs are zinc-dependent endopeptidases that cleave multiple substrates, including intracellular proteins (124), cytokines and chemokines (126), intercellular proteins (e.g., epithelial junction proteins) (125), cell surface proteins (e.g., mucin and defensins), latent growth factors, proteases and protease inhibitors, clotting factors, and virtually all structural ECM proteins (**Table 4**) (85,122). By doing so, these enzymes play a major role in cell migration and the maintenance of intestinal homeostasis (**Figure 7**). Despite being encoded by different genes, MMPs share significant homology and structural domain organisation, and they are secreted as inactive zymogens that are converted into their catalytically active forms by different proteases, including plasmin, trypsin, chymotrypsin, kallikrein, cathepsins, neutrophil elastase, urokinase, and other MMPs (122,137,138). MMP activity is

finely regulated by different inhibitors, with tissue inhibitors of metalloproteinases (TIMPs) representing the central regulatory factors of these proteases (85).

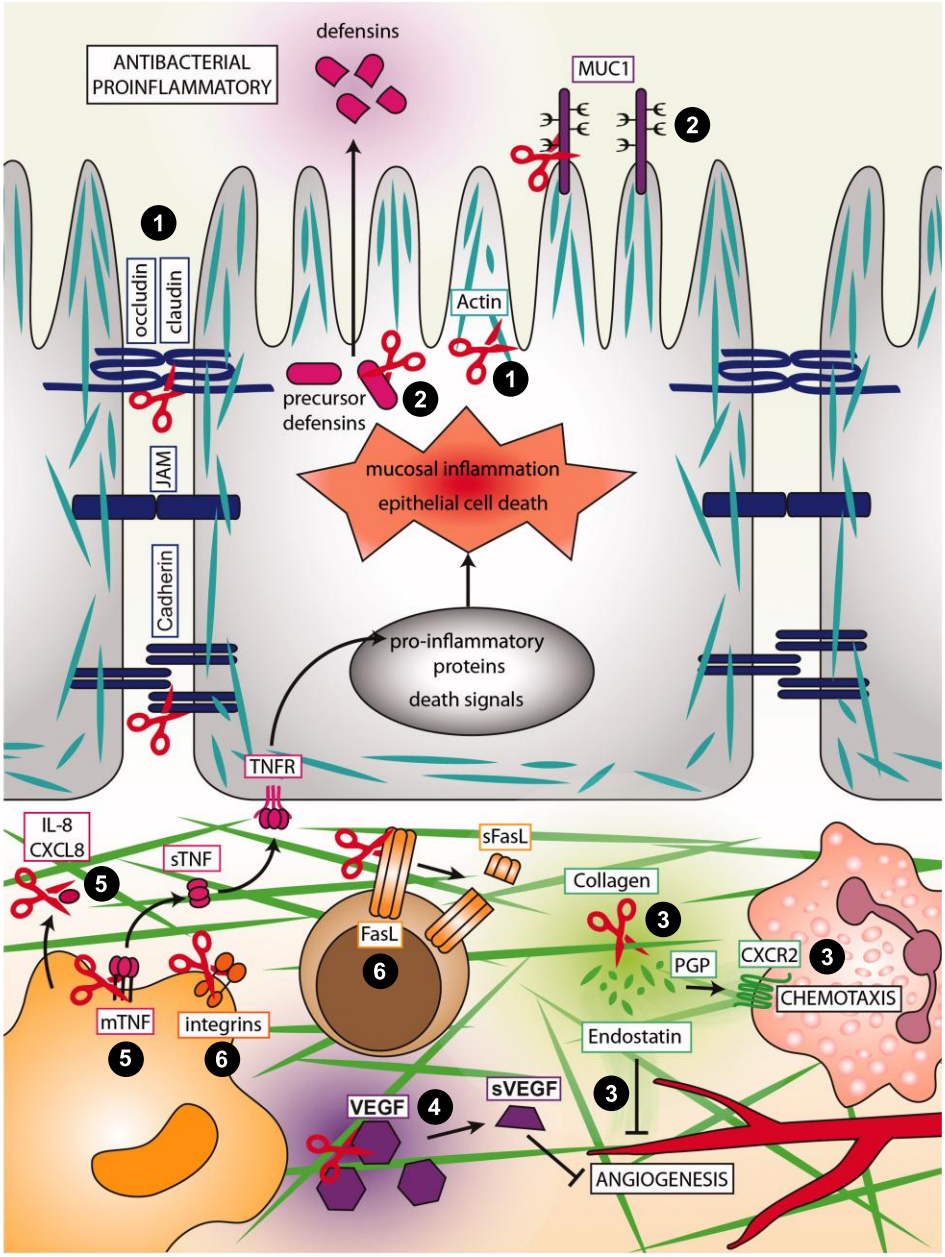

Figure 7. MMP activities that modulate intestinal barrier integrity and inflammation. MMPs have a vast range of extracellular, pericellular, and intracellular substrates. [1] Within the epithelial layer, MMPs can degrade intercellular junction molecules (e.g., cadherins, occludins, and claudins) and intracellular structural proteins (e.g., actins), leading to altered cell shapes and epithelial barrier disruption. [2] The protective antibacterial properties of the intestinal epithelium are partly regulated by MMP-mediated cleavage of antimicrobial defensins and MUC1, which directly kill bacteria and inhibit their adhesion to the cell membrane, respectively. [3] The degradation of collagen in the submucosa by MMPs

results in the release of collagen fragments, including PGP and endostatin. PGP levels are increased during intestinal inflammation and functions as a neutrophil chemoattractant via the CXCR2 receptor. Endostatin is a potent anti-angiogenic factor produced from collagen cleavage by MMP-9. [4] Angiogenesis in the intestinal epithelium is also regulated by MMP-mediated release of soluble VEGF from the ECM. [5] MMPs are important mediators of intestinal inflammation by cleaving cytokines and chemokines produced by different inflammatory cells, including macrophages (in orange), lymphocytes (in brown), and neutrophils (in pink), into more or less potent molecules. Cleavage of IL-8/CXCL8 by MMP-9 potentiates neutrophil recruitment to inflamed sites. Cleavage of membrane-bound TNF by MMP-13 releases soluble TNF, which exerts potent pro-inflammatory responses by signalling through the TNFR. Additionally, cleavage of the pro-inflammatory cytokine IL-1β by MMP-1, -3, and -9 inhibits its pro-inflammatory and angiogenic effects (not represented in this figure). [6] MMPs modulate leukocyte adhesion and lymphocyte apoptosis by cleaving adhesion molecules (e.g., integrins) and apoptotic mediators (e.g., FasL), respectively. CXCR2, C-X-C motif chemokine receptor 2; IL-8/CXCL8, interleukin-8, also known as C-X-C motif chemokine ligand 8 (CXCL8); FasL, Fas ligand; mTNF, membrane-bound tumour necrosis factor; MUC1, mucin-1; PGP, proline-glycine-proline; sTNF, soluble tumour necrosis factor; TNFR, tumour necrosis factor receptor; VEGF, vascular endothelial growth factor. Source: Adapted from de Bruyn *et al.*, 2016 (139).

Finally, along with its essential role in the maintenance of tissue architecture, the ECM is also an important regulator of intracellular signalling pathways that ultimately modulate cell adhesion, motility, and proliferation in response to ECM alterations (85,88,116). In this regard, ECM components have also been described to trigger changes at the protein expression level in parasitic organisms. For instance, the interaction between the protozoan parasite *Trypanosoma cruzi* and ECM proteins induces the reprogramming of the parasite's metabolism, which is considered a mechanism that facilitates parasite adaptation to the newly encountered host microenvironment (140).

2.1.3. Immune functions of the intestinal mucosa

In addition to functioning as a physical barrier against pathogen entry, the intestine represents the largest compartment of the immune system. The majority of immune cells are diffusely distributed throughout the lamina propria, although lymphocytes can occasionally be found in the intestinal epithelium, between adjacent epithelial cells. Additionally, a special form of organised lymphoid tissue, collectively known as gut-associated lymphoid tissue (GALT), is located in the submucosa and the lamina propria of the mucosa (85,89).

The GALT is covered by microfold (M) cells that are specialised in the uptake and transport of antigens (e.g., bacteria, viruses, and inert particles) from the gut lumen to underlying dendritic cells (DCs) that present them to B and T lymphocytes, leading to antibody production and the onset of cytotoxic responses (89). In the duodenum, activated B cells (plasma cells) are particularly primed to produce immunoglobulin A (IgA) antibodies, which decrease along the proximal–distal axis of the small intestine (141). Adaptive immune cells are located in macroscopic lymphoid GALT sub-structures known as Peyer's patches

and isolated lymphoid follicles. Of note, the size and density of Peyer's patches increases from the jejunum to the ileum, and they are rare in the duodenum. In addition to M-cell-mediated antigen presentation, DCs found within the lamina propria can send extensions between epithelial cells to directly uptake antigens in the gut lumen (89). Interestingly, DCs in the proximal small intestine present a tolerogenic phenotype compared to those found further down the digestive tract, which is beneficial to tolerate oral antigens. This implies that adaptive immune responses in the proximal small intestine are skewed towards immunoregulatory Type 2 adaptive responses, a feature that is hypothesised to result from co-evolution with helminths that reside within this niche (141).

Innate immune responses in the intestinal epithelium are mainly triggered by intestinal epithelial cells, which express an array of pathogen-recognition receptors (PRRs) that recognise pathogen-associated molecular patterns. PRRs in the intestinal epithelium of the small intestine include toll-like receptors (TLRs), which are expressed at the cell or endosomal membranes, and cytoplasmic PRRs. Cytoplasmic PRRs encompass those belonging to the RNA helicase family (e.g., retinoic acid inducible gene protein 1 and melanoma differentiation-associated gene 5) and nucleotide-binding and oligomerisation domain (NOD)-like receptors (NLRs). NLRs containing a pyrin domain (NLRPs) are important modulators of innate inflammatory responses in the intestine. Upon activation, these receptors assemble into multi-protein complexes called inflammasomes that activate inflammatory caspases, such as caspase-1, to form a proteolytic complex that culminates in the activation of IL-1β and IL-18 precursors into their active forms. PRRs are also expressed by non-epithelial immunocompetent cells located in the lamina propria and their activation ultimately leads to the recruitment of leukocytes and DCs (142).

Paneth and goblet cells also perform important defensive roles in the small intestine by secreting antimicrobial peptides (e.g., lysozyme, α-defensins, phospholipase A, and regenerating islet-derived protein IIIA) and mucus proteins, respectively, the latter representing an important physical barrier against bacterial infiltration (98,143). That said, Paneth cells also secrete inflammatory cytokines and express cell-surface receptors that prime and modulate both innate and adaptive immune responses in the intestine (143). In addition to secreting the protective mucus layer that covers the intestinal epithelium, goblet cells are also involved in pathogen sensing by delivering luminal antigens to immune cells in the underlying lamina propria through the formation of goblet cell-associated antigen passages. Additionally, a subset of goblet cells function as sentinels that increase mucus secretion upon sensing of antigens derived from pathogenic bacteria through TLR-

dependent signalling, which reinforces the mucus barrier and aids in pathogen clearance (98).

Finally, tuft cells warrant special attention for their central role in the immune defence against intestinal parasites. Upon infection with GI nematodes, the intestinal epithelium responds by triggering an alarmin-mediated signalling axis that ultimately stimulates goblet cell hyperplasia, increased mucus secretion, and smooth muscle hypercontractility, leading to the so-called 'weep-and-sweep' response that facilitates worm expulsion (144). In 2016, it was discovered that this response is orchestrated by tuft cells (145–147), which have been described in the mouse, rat, human, and ovine digestive tract (99) and constitutively secrete the alarmin IL-25 (IL-17E). In the presence of GI nematodes, secretion of IL-25 by tuft cells increases, triggering a positive feedback loop that results in tuft and goblet cell hyperplasia and increased mucus secretion, leading to efficient worm clearance (145–147).

2.2. *Fasciola hepatica* juvenile biology

The FhNEJ (**Figure 8**) are highly efficient parasites whose primary objective is to reach their definitive destination within the bile ducts. Accomplishing this represents a significant challenge, as migrating towards the distant biliary tree while evading host immune defences incurs a high energy cost. FhNEJ rely on potent metabolic pathways that enable efficient energy production, which is primarily invested in fuelling motility and circumvent immune responses mounted against them.

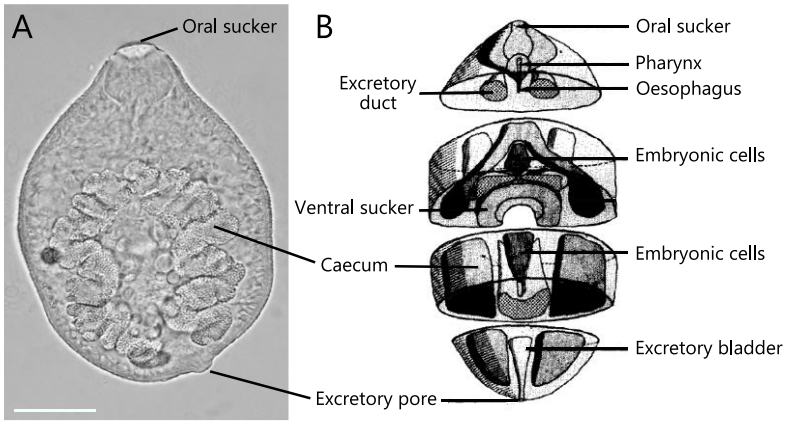

Figure 8. FhNEJ morphology and major anatomic structures. A) Brightfield image of a whole-mounted FhNEJ acquired using a spinning disk Dragonfly High Speed Confocal Microscope System (Andor, Oxford Instruments). Scale bar, 50 µm. B) Gross anatomy of FhNEJ highlighting the main organ systems. The digestive system of FhNEJ starts at the oral sucker, which is connected to the pharynx. The pharynx connects with the oeasophagus, which bifurcates into two lateral intestines or caeca (148). The excretory system of *F. hepatica* is protonephridial and plays a central role in osmoregulation and the maintenance of fluid homeostasis. It is composed by two lateral ascending ducts containing

flame cells that run forward and descend backwards at the level of the pharynx. These ducts merge into the main excretory duct, which drains into the excretory bladder. The excretory bladder empties its contents through the excretory pore (also known as protonephridial pore) (19,148). Sources: Photography by Judit Serrat Fernández (acquired at the Microscopy and Image Analysis Facility of the Institute of Functional Biology and Genomics, Salamanca, Spain) (A); Bennett and Threadgold, 1973 (148) (B).

The tegument of FhNEJ plays a central role in sensing and responding to host-derived signals, which is essential for their successful establishment within the mammalian organism. Different aspects related to FhNEJ metabolism, immune evasion, and migration are discussed in the following sections, as a deeper knowledge on these processes could inform the development of improved control strategies for fasciolosis.

2.2.1. The tegument of FhNEJ

The tegument of *F. hepatica*, including that of FhNEJ, is the outermost layer of the parasite, thereby representing the host-parasite interface that is continuously exposed to host tissues and immune effector mechanisms. As such, molecules expressed within the tegument surface are considered promising potential vaccine candidates against this parasite (149). The tegument is a syncytial structure composed of various cells with interconnected cytoplasms (84). This structure is rich in secretory bodies and is covered by a dense layer of highly glycosylated molecules, forming the so-called tegument glycocalyx (149,150). In addition to fulfilling important biological functions for parasite survival, including nutrient absorption, metabolism, osmoregulation, and protection against gut lumen compounds (19), the tegument plays a central role in the interaction between FhNEJ and host tissues through the expression of ECM-degrading proteases, detoxifying enzymes, and immune-modulatory molecules, among others (151). Moreover, the tegument represents a dynamic structure undergoing continuous turnover, enabling the rapid adaptation of the parasites to a constantly changing host microenvironment encountered during migration thtough the intestine, the peritoneum, and the hepatic parenchyma (84).

The tegument of FhNEJ closely resembles that of adult parasites (**Figure 9**), with the main difference being the presence of only a single type of secretory bodies in FhNEJ (148). Secretory bodies are produced in Golgi complexes of tegumental secretory cells and contribute to glycocalyx turnover (84). FhNEJ contain T0 secretory cells that produce T0 secretory bodies. Later in parasite development, T0 cells are replaced by T1 and T2 secretory cells, which produce their corresponding secretory bodies (19), reflecting the different glycocalyx coat compositions required for parasite adaptation to the different niches within the host organism across different developmental stages (84). In this regard, FhNEJ T0 bodies contribute to a glycocalyx composition that protects from gut lumen

hydrolytic enzymes, emulsification by bile components, and increasing surface tension (152).

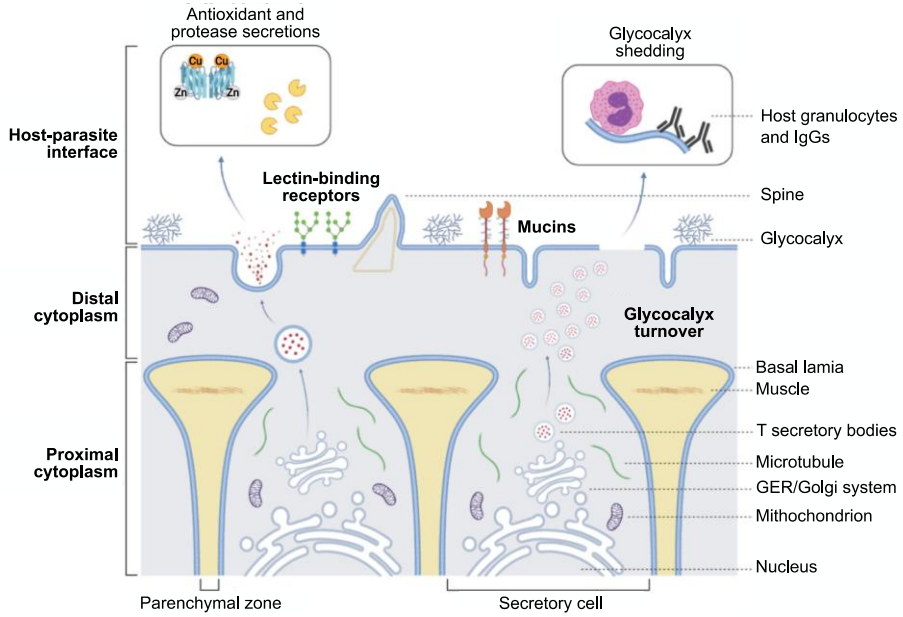

Figure 9. The tegument of FhNEJ. The tegument of *F. hepatica*, including that of FhNEJ, is a syncytial structure composed by multiple secretory cells whose cytoplasms are connected and organised into two functionally-distinctive zones (proximal and distal cytoplasm). Secretory cells produce different types of secretory T bodies depending on the developmental stage of the parasite, which contribute to the contents of the outermost glycocalyx layer that covers the tegument surface. The tegument also represents a site of secretion/excretion of different soluble products, including proteases and antioxidant molecules. GER, granular endoplasmic reticulum. Source: González-Miguel *et al.*, 2021 (84).

Underdeveloped spines are found across the entire FhNEJ tegument surface. These are protein-rich outward projections whose abundance increses by 8-fold during parasite development. In the adult, these structures are important to maintain fluke attachment to the bile duct wall and in damaging and rupturing the endothelium of blood vessels, which facilitates blood feeding (19). Although less numerous, the spines in FhNEJ and liver-migrating juveniles aid in their migration through the mammalian organism, causing the mechanical degradation of tissues and contributing to the liver damage that characterises the acute phase of infection (**Section 1.2.2**). Finally, sensory papillae are also found at specific regions of the tegument surface, especially surrounding the oral and ventral suckers. Although the functions of these structures is far from understood, they contain a central single cilium and presumably function as mechano- and/or chemoreceptors (19,148).

Beyond the tegument, an additional interaction interface between the parasite and host tissues is provided by molecules secreted by the parasite to the extra-parasitic environment,

either in the form of soluble ESPs or within the cargo of extracellular vesicles (EVs). It is generally accepted that ESPs are released from the gut and/or the tegument through exocytosis or non-classical secretory pathways (153). Strikingly, the repertoire of proteins in FhNEJ ESPs is much more diverse than that of adult parasites, probably reflecting that FhNEJ ESPs play essential roles in host tissue invasion and immune evasion. These functions are less critical in adult parasites, which might rather invest their energy reserves toward the mass production of eggs (154). FhNEJ-derived ESPs are mainly composed by proteases (including cysteine- and serine-type proteases, metalloproteinases, and leucine aminopeptidases), protease inhibitors, and anti-oxidant molecules, many of them having important immunomodulatory properties (154,155). *F. hepatica* EVs were first described in adult flukes and later on demonstrated to also be secreted by eggs and FhNEJ (156). These vesicles are classified in two major types based on their size and they are released from the gut, the protonephridial (excretory) system, and the tegument, to a lesser extent (153,157). With subtle differences, the content of these vesicles is similar across developmental stages and they contain different types of molecules, including proteins, lipids, and microRNAs (miRNAs), that manipulate host cell behaviour (153,158,159).

2.2.2. Metabolic adaptation

From excystment to liver penetration and subsequent migration through the liver parenchyma, FhNEJ experience growth at an unexpectedly high rate (1), a process that is supported by self-renewing and pluripotent neoblast-like stem cells located throughout the FhNEJ parenchyma (160). In fact, liver-migrating juveniles double in size approximately every two weeks while the parasite's digestive and reproductive systems fully develop (22). This rapid growth creates a need for significant metabolic re-adaptations in developing juveniles. Due to high oxygen availability, metacercariae and FhNEJ mainly depend on aerobic metabolic routes for energy production. However, as parasite size increases and accesses the bile ducts, which represents an essentially anaerobic environment, oxygen diffusion towards the internal organs is reduced. This forces a metabolic switch, particularly at deeper tissues, from aerobic to anaerobic energy metabolism (84,154).

F. hepatica is completely dependent on carbohydrate metabolism to produce adenosine triphosphate (ATP), since other biomolecules, such as amino acids and lipids, are used for biosynthetic purposes rather than energy production. FhNEJ and the free-living stages of *F. hepatica* metabolise glucose through oxygen-dependent reactions, including the tricarboxylic acid (TCA) cycle, which represents the most efficient pathway to obtain energy from carbohydrates, and aerobic acetate production. As oxygen availability decreases,

aerobic acetate production becomes the primary source of energy production, which becomes the dominant metabolic route in liver-migrating juveniles. Later on, in the bile ducts, oxygen is scarce, and parasites switch to a completely anaerobic metabolism for ATP production, mainly through malate dismutation and anaerobic glycolysis, to a lesser extent (22). These metabolic adaptations are reflected at the proteomic level, as metacercariae and axenically-maintained FhNEJ one and three hours post-excystment show a preferential expression of proteins belonging to aerobic metabolism pathways, including the TCA cycle, when compared to axenically-maintained FhNEJ twenty-four hours post-excystment (154). Interestingly, similar changes in protein expression are observed as early as 2.5 hours post-excystment in FhNEJ that have crossed the intestinal wall in an *ex vivo* model of trans-intestinal migration, suggesting that molecules found within the host intestine may function as molecular cues to prime FhNEJ metabolic adaptation (151).

2.2.3. Immune evasion

One of the most important immune mechanisms against FhNEJ is ADCC, which depends on antibody binding to the FhNEJ surface followed by parasite recognition by eosinophils and macrophages through their surface-exposed immunoglobulin Fc receptors. This mechanism has been shown to effectively kill FhNEJ *in vitro* (50); however, these parasites have evolved mechanisms to evade these responses. One such mechanism is glycocalyx turnover, which occurs every 2–3 hours and sloughs off antigen-antibody complexes attached on the surface of FhNEJ that would stimulate ADCC (48,84). Glycocalyx turnover is especially active at the FhNEJ stage, consistent with their increased exposure to host immune defences compared to adult parasites that inhabit the major hepatic bile ducts, an immunologically 'safe' microenvironment where bile components suppress both cell-mediated and antibody-mediated immune responses (19,161). Another mechanism by which FhNEJ evade ADCC involves the expression of a TGFβ-like molecule (FhTLM), a TGFβ-related protein that suppresses nitric oxide production by ADCC-activated macrophages, ultimately inhibiting cytotoxic responses towards the parasite (162,163). Finally, the rapid movement of FhNEJ through host tissues also facilitates immune evasion as it enables the parasite to outpace immune cells attempting to mount a response on their surface (48).

Immune evasion throughout *F. hepatica* development is also mediated by the expression of different molecules at their tegument surface or excreted/secreted, either as soluble ESPs or within the cargo of EVs (151,154,164). It should also be noted that molecules found within the secretome of *F. hepatica* may also be partly accounted by the vomitus, i.e., released

to the exterior together with undigested gut contents that are regurgitated during normal feeding (149). In addition to these, *F. hepatica*-derived miRNAs are increasingly recognised as important regulators of host immune responses by modulating the expression of immune response genes in host cells (159). The most abundant proteins in FhNEJ ESPs are cysteine-type peptidases, especially clade L and B cathepsins (154,155). Other than fulfilling important digestive functions at the intestinal level, these proteases can cleave different IgG subclasses, thereby blocking antibody-mediated responses against migrating parasites (165). In addition, adult-derived clade L cathepsin peptidases can cleave all IgG antibody subclasses and inhibit the development of pro-inflammatory Th_1 responses that would efficiently kill the parasite (166,167). Interestingly, FhNEJ-derived cathepsin L3 (FhCL3) has been recently shown to be internalised by host dendritic cells and stimulate inflammasome activation and subsequent production of IL-1β (168), a cytokine that is also stimulated by the nematode *Heligmosomoides polygyrus* to inhibit anti-parasitic 'weep-and-sweep' responses in the gut (169). The secretome of FhNEJ is also rich in detoxifying enzymes that counteract the activities of oxygen and nitrogen reactive species secreted by immune effector cells, including peroxiredoxin (FhPrx), thioredoxin (FhTrx), and superoxide dismutases (FhSOD) (154). Additional proteases identified within FhNEJ ESPs with potential immune evasion properties are clade B cathepsins and FhLAPs (48,155,167).

Non-enzymatic modulators expressed and secreted by FhNEJ include protease inhibitors, such as serpins, cystatins, kunitz-type molecules (FhKTMs), and steffins (154). Serpins inhibit serine proteases and their presence in the FhNEJ secretome is compelling provided that serine proteases within FhNEJ ESPs are scarce, indicating that serpins secreted by FhNEJ are essentially employed to regulate host-derived serine proteases (170). In fact, certain FhNEJ-derived serpins can inhibit the activities of digestive enzymes found in the host gut lumen, potentially protecting the parasite from the erosive effects of gut secretions. Additionally, FhNEJ-derived serpins can inhibit the activities of serine proteases involved in vascular permeability, inflammatory responses, and the complement system, facilitating parasite survival during infection (170,171). Different types of FhKTMs are present in the secretome of FhNEJ, and they function as inhibitors of host-derived cysteine proteases, including endosomal/lysosomal cathepsins. Since these enzymes are involved in antigen processing and presentation on the surface of antigen-presenting cells, internalisation of secreted FhKTM by host cells would impair the onset of adaptive immune responses (172).

A family of helminth molecules that mimic host antimicrobial peptides and inhibit innate inflammatory responses, collectively known as helminth defence molecules (FhHDMs), are also abundant in the secretome of FhNEJ and play an important role in immune evasion

at the earliest phases of infection (154,173). Finally, some of the abovementioned molecules, including FhTLM, FhPrx, and FhTrx, have been demonstrated to stimulate the differentiation of macrophages into an M2 phenotype, representing an efficient immune evasion mechanism as these cells are key to tissue repair but ineffective at eliminating helminths (47,48).

2.2.4. Trans-intestinal invasion and migration

Parasite migration through host tissues is considered an evolutionary advantage as it associates with faster growth rates and greater parasite size, which correlates with increased fecundity in certain parasite species (174). Tissue migration is also a hallmark in the life cycle of *F. hepatica*, as this parasite travels through different organs before permanently establishing in its definitive location. In general, FhNEJ migration is supported by active locomotion and the secretion of different molecules that aid in the migration process. The musculature of FhNEJ is mostly found beneath the tegument and comprises an outer circular muscle layer and an inner longitudinal muscle layer that are responsible for the rhythmic waves of contraction and relaxation that facilitate piston-like movement of the parasite. As explained in **Section 1.1.2**, bile is essential for metacercariae excystment but toxic to FhNEJ, thereby functioning as a negative chemotactic cue that stimulates the rapid trans-intestinal migration of FhNEJ (19). Bile further contributes to FhNEJ migration by stimulating parasite locomotion, as certain bile components have been shown to stimulate FhNEJ muscle contractility *in vitro* (15,19). Bile toxicity probably explains why *F. hepatica* juveniles access the liver through the abdominal cavity instead of the ampulla of Vater (hepatopancreatic ampulla), which directly connects the gut duodenum with the main bile duct and represents the preferred migratory route for the closely related trematode species *Clonorchis sinensis* and *Opistorchis viverrini*.

Although the precise mechanisms governing FhNEJ trans-intestinal migration are far from understood (84), cathepsins are considered the most important proteases contributing to this process. Cathepsins are papain-like cysteine-type proteases that show a temporal pattern of expression, reflecting the different proteolytic requirements that the parasite has during development and migration (**Figure 10**). These proteases are found within the pool of genes that are most highly transcribed across the different developmental stages of *F. hepatica* (175) and they are present in the FhNEJ tegument and secretome (151,154,155). Interestingly, cathepsin peptidases have been identified within the cargo of adult-derived EVs (164) but not in those secreted by FhNEJ, although this could be explained by their abundance being below detection levels, by them being secreted only as soluble ESPs, or

by the need of host-derived stimuli for their encapsulation within EVs (158). Cathepsins are produced by gastrodermal cells that line the gut and they are secreted as inactive zymogens containing a pro-peptide that inhibits the catalytic activity of the protease, which protects the parasite from unwanted proteolysis. These zymogens are activated by other proteases (e.g., legumains) or via auto-catalysis, which occurs under acidic conditions such as those found in the parasite's gut lumen (175,176).

In FhNEJ, cathepsins B1, B2, B3 (FhCB1–3), and FhCL3 represent around 20% of the total proteins secreted during the first 24 hours post-excystment (154). FhCL3 expression at the pre-hepatic stages of *F. hepatica* is important for trans-intestinal migration, as this protease is very efficient at cleaving collagen, a structural component of the intestinal ECM that physically blocks FhNEJ migration (118,177). Cathepsin L4 (FhCL4) has recently been identified in the tegument of FhNEJ but only upon gut passage, suggesting that host-derived stimuli may be important for the expression of molecules that may potentially be important for FhNEJ migration through host tissues (151). As the parasite develops and migrates through the liver parenchyma, the expression of FhCBs and FhCL3 diminishes and FhCL1 and cathepsin L2 (FhCL2) become more abundant in their secretomes. Just like FhCL3, FhCL2 aids in the migration through the liver parenchyma owing to its potent collagenolytic properties (173). Later on, as parasites enter the bile ducts and mature into adult worms, the expression of FhCBs and FhCL3 is completely shut down and the repertoire of cathepsins expressed at this stage is dominated by FhCL1, FhCL2, and cathepsin L5 (FhCL5), with FhCL1 playing an essential role in nutrition through the degradation of haemoglobin into smaller peptides (27,175).

Finally, recent studies have postulated that the interaction between parasites and host-derived enzymes, including plasmin, the main protease of the fibrinolytic system, may contribute to parasite migration across host tissues and reduce the energy cost associated with this process (178,179). In fact, various proteins identified within the ESPs of adult *F. hepatica* have been shown to interact with plasminogen (PLG), the zymogen of plasmin, and to stimulate its conversion into the catalytically active protease. This has been suggested to facilitate immune evasion and the long-term establishment of adult parasites within the mammalian organism, while providing a plausible mechanistic explanation to the neurological disorders that are occasionally associated with *F. hepatica* infection (180). However, whether similar mechanisms to leverage host fibrinolysis also operate in FhNEJ and whether these would contribute to their migration across the intestinal wall remains unexplored.

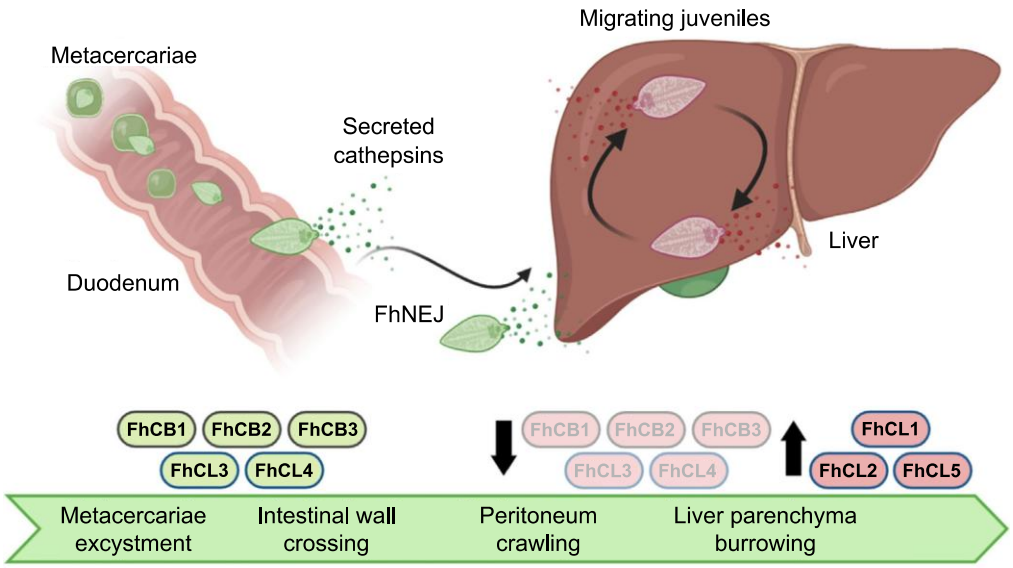

Figure 10. Stage-specific expression of cathepsin proteases by *F. hepatica*. At the FhNEJ and pre-hepatic stages of the parasite, the expression of FhCB1–3, FhCB9 (not represented in this figure), and FhCL3–4 prevails and aids in trans-intestinal migration. Later on, the expression of these cathepsins diminishes and they are virtually absent in adult parasites, which express FhCL1, FhCL2, and FhCL5, the former playing a central role in blood feeding. FhCB1–3, *F. hepatica* cathepsin B1–3; FhCL1–5, *F. hepatica* cathepsin L1–5; FhNEJ, *F. hepatica* newly excysted juvenile. Source: González-Miguel *et al.*, 2021 (84).

3. The fibrinolytic system of vertebrates

The fibrinolytic system plays a crucial role in maintaining blood homeostasis by removing fibrin clots from the vascular endothelium that would otherwise cause pathological conditions. The fibrinolytic system involves a series of enzymes, receptors, and inhibitors that work in concert to control the process of fibrinolysis. With a few exceptions, most functions of the fibrinolytic system depend on plasmin, a broad-spectrum serine protease that cleaves fibrin and multiple additional substrates. This section contains an in-depth review of the different functions of the fibrinolytic system and its components, with a particular focus on the roles of this system beyond fibrin breakdown and clot removal. In this regard, we present a comprehensive and exhaustive compilation of all fibrinolysis-unrelated plasmin substrates available from the literature, which have been summarised in **Supplementary File 2**.

3.1. The fibrinolytic system as part of the haemostatic system

The fibrinolytic system is a component of the haemostatic system of vertebrates, which functions to keep blood in a liquid state while resolving lesions that appear in the vascular endothelium to prevent haemorrhages (181). The haemostatic system is divided in three phases: platelet aggregation, coagulation, and fibrinolysis, also known as primary, secondary, and tertiary haemostasis, respectively (182).

Platelet aggregation starts with their interaction with subendothelial collagen and immobilised von Willebrand factor (VWF), which become accessible to circulating platelets only in the event of an injury. Upon interacting with collagen and immobilised VWF, platelets become activated, resulting in the engagement of their surface-exposed integrin receptors with multiple ligands, including fibrinogen, facilitating platelet-platelet aggregation (183). Propagation of the platelet plug is achieved via the secretion of a plethora of agonists by activated platelets, which stimulates the activation of nearby platelets and promotes their interaction via fibrinogen bridges (181,183). Alongside platelet aggregation, vascular injuries initiate the coagulation cascade through the exposure of subendothelial tissue factor, which culminates in the deposition of fibrin on the platelet plug, thereby stabilising it. This fibrin-stabilised platelet plug is now referred to as a blood clot (182,183). Once the vascular lesion is resolved, fibrinolysis—that is, activation of the fibrinolytic system—generates plasmin, a protease that degrades fibrin and stimulates blood clot dissolution and the restoration of normal blood flow (**Figure 11**).

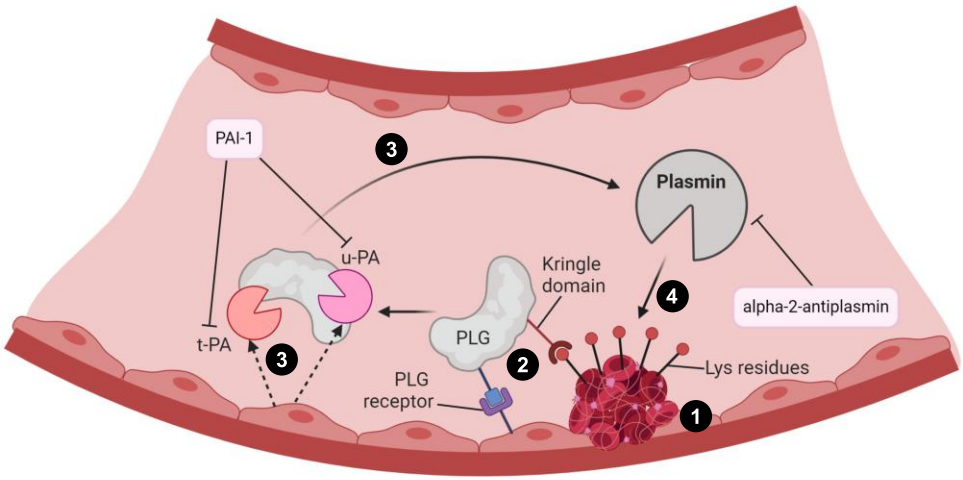

Figure 11. Overview of the fibrinolytic cascade. After vascular repair, dissolution of the blood clot starts by binding of the zymogen plasminogen (PLG) to lysine residues of the fibrin within the clot [1,2], which exposes the cleavage site for proteases that function as PLG activators: the tissue-type and urokinase-type plasminogen activators (t-PA and u-PA, respectively). [3,4] Upon cleavage, PLG is converted into its catalytically active form, the serine protease plasmin, which degrades fibrin. Fibrin degradation by plasmin stimulates blood clot dissolution and the restoration of normal blood flow. The activities of the fibrinolytic system are regulated by different inhibitors, including the PLG activator inhibitor 1 (PAI-1) and α2-antiplasmin, which inhibit t-PA/u-PA and plasmin, respectively (184). PLG, plasminogen; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor 1. Created with Biorender.com.

3.2. Plasminogen and plasmin

Plasminogen (PLG) is the zymogen of the primary fibrinolytic protease plasmin. The conversion of PLG into its catalytically-active form is crucial for the execution of the functions of the fibrinolytic system. PLG is a glycoprotein of 791 amino acids and 92 kilodaltons (kDa) that is present in plasma at a concentration of 1.5–2 μM (185). PLG is primarily produced in the liver by hepatocytes, although alternative organs have also been proposed as secondary sources of PLG, including the adrenal glands, the kidneys, the brain, the spleen, the thymus, and the gut (186). Circulating PLG contains a glutamic acid on its terminal site, thus being referred to as Glu-PLG. Glu-PLG has a closed and compact conformation and is converted into a more relaxed and open molecule upon hydrolysis of the Arg68–Met69, Lys77–Lys78, or Lys78–Val79 peptide bonds by plasmin (185,187). These PLG forms with alternative lysine amino-termini are collectively known as Lys-PLG (187).

The PLG molecule is divided in seven different structural domains (186,188,189) (**Figure 12**). The kringle domains (K1–K5) play a key role in fibrinolysis as they contain lysine-binding sites (LBS) that mediate the interaction between PLG and C-terminal lysine

residues found in PLG receptors or partner proteins. Only K1, K2, K4, and K5 contain a functional LBS. K1 and K5 LBS show the highest affinities for lysine residues; however, only the K1 LBS is available for ligand binding in the circulating or closed form of PLG (189). Evidence that PLG binding to its receptors is mediated by the LBS of kringle domains is demonstrated by the strong inhibition of this interaction in the presence of lysine analogues, such as aminocaproic acid (ε-ACA) and tranexamic acid (TXA), or by carboxypeptidase B (CpB), an enzyme that cleaves C-terminal lysine residues (190–192). PLG is converted into plasmin by proteolytic cleavage, a function that is executed by the proteases tissue-type and urokinase-type PLG activators (t-PA and u-PA, respectively). The t-PA and the u-PA cleave the peptide bond between amino acids Arg561–Val562, which constitute the activation loop (AL) (**Figure 12**). This results in the generation of plasmin, a trypsin-like serine protease composed of two polypeptide chains that are bound by two disulphide bridges (185,186,188). The mechanism of PLG activation is summarised in **Figure 13**.

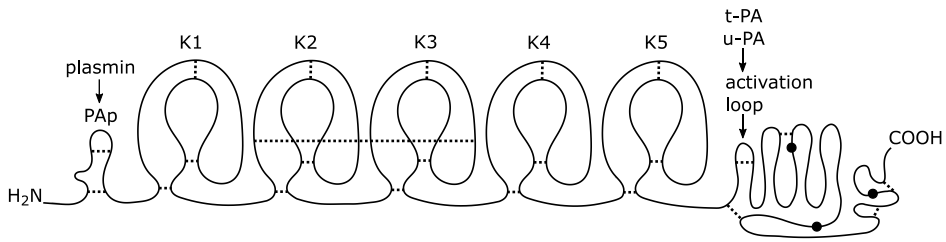

Figure 12. The structure of PLG. The N-terminal side of PLG contains the pre-activation or pan-apple domain (PAp), which is cleaved by plasmin to generate Lys-PLG. The PAp is followed by five kringle domains (K1–K5) and the activation loop, which contains the peptide bond that is cleaved by t-PA and u-PA. The C-terminal portion of PLG includes the catalytic triad of plasmin (His603, Asp646, and Ser741; marked with dots). Dashed lines indicate disulphide bridges. PAp, pan-apple domain; K1–K5, kringle domains 1–5; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

3.3. Plasminogen receptors

PLG receptors (PLG-Rs) are crucial in fibrinolysis by promoting a conformational change in the PLG molecule that exposes the cleavage site for t-PA and u-PA, thereby facilitating plasmin generation (188). Additionally, PLG-Rs protect plasmin from inactivation, since binding of this protease to PLG-Rs and plasmin inhibitors involves the same kringle domains (189). PLG-Rs are diverse and they are expressed by multiple cell types, including monocytes/macrophages, fibroblasts, platelets, endothelial cells, and carcinoma cells (193).

Cellular PLG receptors are divided based on their sensitivity to CpB and are summarised in **Table 5** (194). There are three types of CpB-sensitive PLG receptors, all of them including

proteins with C-terminal lysines: first, the plasminogen receptor KT (PLG-R_{KT}), a transmembrane protein; second, intracellular proteins lacking transmembrane domains, such as α-enolase, cytokeratin 8, S100A10 (in complex with membrane-bound annexin A2), histone 2B (H2B), and RuvB-like protein (195); and third, proteins that require proteolytic pre-processing of their C-terminal sites to expose basic or lysine-like residues, including actin.

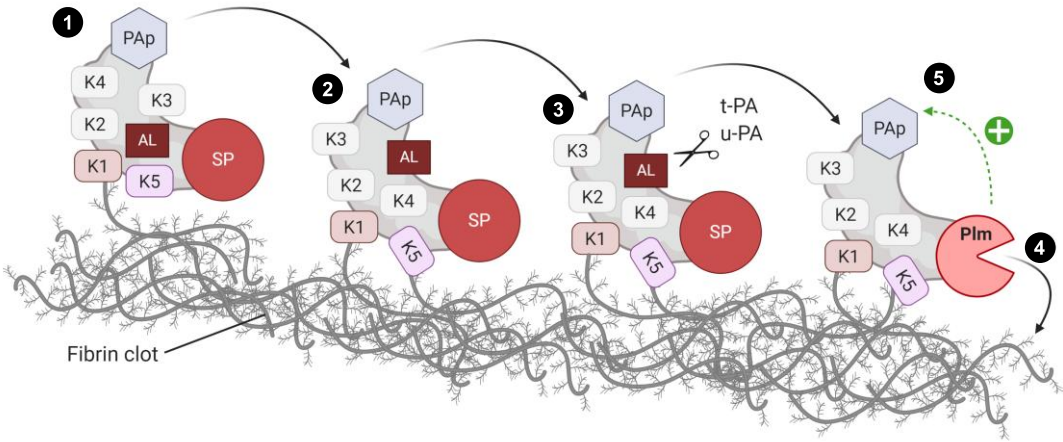

Figure 13. The mechanism of PLG activation. [1] Glu-PLG binds to its receptors or partner proteins (e.g., fibrin), via the K1. [2] The initial recruitment of Glu-PLG to its partner protein causes a mild conformational change in the Glu-PLG molecule that exposes the K5, which now interacts with lysine residues of fibrin. The interaction between Glu-PLG and fibrin through additional kringle domains triggers a major conformational change that further opens the PLG molecule and exposes the AL (188). [3] The t-PA and u-PA cleave Glu-PLG at the AL position, leading to plasmin generation. [4,5] The resulting plasmin cleaves its substrate (fibrin, in this figure) and additional Glu-PLG molecules that bind to the receptor. Plasmin-mediated cleavage of Glu-PLG represents a positive feedback mechanism that accelerates the fibrinolytic cascade by producing Lys-PLG, which has a conformation that is more easily activated by t-PA and u-PA (187,188). Note that the AL is only exposed in the Glu-PLG molecule in the presence of PLG receptors (fibrin, in this example) or after cleavage of its N-terminal domain by plasmin. PAp, pan-apple domain; K1–K5, kringle domains; AL, activation loop; SP, serine protease domain; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; Plm, plasmin. Created with Biorender.com.

In turn, CpB-insensitive PLG receptors are classified based on their ability to stimulate plasmin generation from bound PLG. CpB-insensitive receptors that do not stimulate plasmin generation include tissue factor and non-protein gangliosides. The functional role of these proteins remains unclear. CpB-insensitive receptors that do stimulate plasmin generation even in the absence of C-terminal lysines include integrins $\alpha_M\beta_2$ and $\alpha_5\beta_1$ and amphoterin. Whether these proteins undergo proteolysis to reveal C-terminal basic residues prior to plasmin activation is not known (194).

Finally, in addition to promoting the conformational change that exposes the AL in the PLG molecule and protecting plasmin from its inhibitors, PLG-Rs also function to co-

localise PLG with its activators, ensuring that plasmin generation is highly localised at the required sites (194,196). For instance, PLG-R_{KT} and S100A10 (p11) have been demonstrated to directly bind t-PA, potentially using the same C-terminal lysine as for PLG binding. These receptors stimulate plasmin generation by co-recruiting t-PA to PLG bound to an adjacent PLG-R_{KT} or S100A10 (p11) molecule (190,196). Moreover, both PLG-R_{KT} and S100A10 (p11) co-localise with the u-PA receptor (u-PAR) at the cell surface (196,197).

Table 5. PLG receptors in eukaryotic cells.

PLG-R	Cell type	C-terminal lysine	Major cellular location	Secretory pathways
Annexin-A2	Endothelial cells, cells of the monocytoid lineage	Absent (a)	Cytosol and/or nucleus	Translocation depends on p11 and phosphorylation and activity of L-type like Ca^{2+} channels and Ca^{2+} (b)
Actin	Endothelial cells, carcinoma and catecholaminergic cells, PC-3, HT1080	Absent (c)	Cytoskeleton	Unknown
Amphoterin	Neurons	Absent (d)	Cytoplasmic and extracellular	Unknown
Integrin α5β3	Endothelial cells	Absent	Integral membrane protein	Classical ER and Golgi pathway
Integrin αMβ2	Neutrophils, monocytes, macrophages	Absent	Integral membrane protein	Classical ER and Golgi pathway
Integrin α2bβ3	Platelets, rheumatoid arthritis synovial fibroblasts	Absent	Integral membrane protein	Classical ER and Golgi pathway
Cytokeratin 8	Hepatocellular, breast carcinoma cells	Present	Cytoskeleton	Unknown
α-enolase	Monocytes, neutrophils, carcinoma and lymphoid cells, myoblasts, neurons	Present	Cytosol	L-type-like Ca^{2+} channel and intracellular Ca^{2+} (e)
GAPDH (198)	Macrophages	Present	Cytosol	Translocation depends on PTMs and extracellular iron concentration (199)
Histone 2B	Neutrophils, monocytoid and endothelial cells	Present	Nucleus	L-type-like Ca^{2+} channel and intracellular Ca^{2+} (f)
S100A10 (p11)	Endothelial cells, HT1080 cells	Present	Cytosol and nucleus	L-type-like Ca^{2+} channel and intracellular Ca^{2+} (g)
PLG-R_{KT}	Monocytes, macrophages, neurons	Present	Integral membrane protein	Classical ER and Golgi pathway
TATA-binding protein-interacting protein	Monocytoid cells	Present	Nucleus	Unknown

Information extracted and adapted from Plow *et al.*, 2012 (200). (a) Extracellular localisation and PLG binding depend on heterotetramerization with S100A10 (p11) dimers, which contain C-terminal lysines. (b) Associates with plasma membrane

via phosphatidylserine. Unlike other PLG-Rs, extracellular localisation is associated with increased protein expression (189). (c) Requires proteolytic cleavage prior to PLG binding to expose C-terminal basic residues (201). (d) Amphoterin is highly rich in lysine residues, which might explain why it can still bind PLG even in the absence of a C-terminal lysine (202). (e) Externalisation also occurs via exosomes; and PTMs including acetylation and phosphorylation may regulate membrane association (203,204). (f) Associates with plasma membrane via phosphatidylserine and heparin sulphate. (g) Associates with annexin-A2 to form the heterotetrameric PLG-R annexin-A2/S100A10. PLG-R, plasminogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase, PLG-R_{KT}, plasminogen receptor KT; PTM, post-translational modification.

3.3.1. Plasminogen receptors as moonlighting proteins

As shown in **Table 5**, many PLG-Rs are intracellular proteins with canonical cytosolic functions that are shuffled to the extracellular space in response to relevant stimuli, such as fibrin or inflammatory mediators (189,191). Except for annexin A2, whose extracellular localisation correlates with an increase in mRNA expression (189), relocation of other PLG-Rs to the extracellular space occurs in the absence of increased protein expression. Although the mechanisms of translocation of intracellular PLG-Rs are not completely understood, experiments using inhibitors of Ca^{2+} channels suggest that Ca^{2+} signalling plays a major role in regulating this process (189,200). Using intracellular proteins as PLG-Rs instead of proteins constitutively expressed on the plasma membrane represents a safety mechanism to guarantee that the broad-spectrum proteolytic activity of the host fibrinolytic system is activated only when necessary. This is particularly important given that plasma contains PLG at remarkably high concentrations (185,190,200). For instance, in mice, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) relocates to the plasma membrane of migrating macrophages upon administration of thioglycolate, an inducer of acute inflammation in the peritoneal cavity (198), and treatment of human blood monocytes with lipopolysaccharide up-regulates the expression of α-enolase on the cell surface (205). This characteristic behaviour supports the classification of certain PLG-Rs as moonlighting proteins.

The concept of protein moonlighting was first introduced in 1999, following a growing number of reports that challenged the long-held belief in the one gene-one protein-one function paradigm (206). Moonlighting proteins include proteins that have multiple functions based on their expression levels, cellular location, and binding partners—these last two being closely interrelated, since relocating proteins to other cellular compartments allows for their interaction with additional partner proteins that were not available in the original location. This classification excludes proteins that are a result of gene fusions, splice variants, homologous but non-identical proteins, proteins that require specific PTMs to execute a specific function—although PTMs might be involved in cellular re-location—,

and proteins that have a single function but can operate in different cellular compartments (207). Moonlighting proteins are generally devoid of trafficking signals, hence their relocation to alternative cellular locations, including the extracellular space, depends on non-canonical shuffling mechanisms (208). Based on these characteristics, the majority of the best described PLG-Rs (**Table 5**) could be considered moonlighting proteins. Among these, α-enolase and GAPDH are well-recognised moonlighting proteins with housekeeping glycolytic activities that can be relocated to additional cellular subcompartments to perform non-glycolytic functions, including the activation of the host fibrinolytic system at the extracellular space (198,204,209).

Although moonlighting proteins have been primarily linked to pathogenic processes—especially in the context of bacterial, fungal, and, more recently, parasitic infections (207,208)—they also perform essential physiological functions in higher eukaryotes, including the regulation of the cell cycle, apoptosis, and inflammatory responses (198,204,207). It is theorised that protein moonlighting may have arisen to diversify the number of functions that can be executed with a relatively stable set of proteins, while representing an evolutionary adaptation that minimises the energy cost associated with performing multiple biological functions. This phenomenon also aids in the integration of different functions within the same biological process and establishes connections between seemingly unrelated biological pathways (207).

3.4. Plasminogen activators

PLG is converted to plasmin upon proteolytic cleavage by its physiologic activators, t-PA and u-PA (184). Both activators are trypsin-like serine proteases containing kringle domains that mediate lysine-dependent protein interactions, although the u-PA kringle is not involved in the interaction of this PA with partner proteins (184,210). Through a positive feedback loop that potentiates the fibrinolytic cascade, plasmin cleaves both t-PA and u-PA, converting these proteases into two-chain variants with enhanced proteolytic activities (184).

Studies using genetically modified mice have revealed interesting insights into the relative contributions of each PA to fibrinolysis. Defects in fibrin degradation are only observed in PLG knock-out (KO) or t-PA/u-PA double KO mice, but not in t-PA or u-PA single-mutants, suggesting that these activators can compensate for each other during fibrinolysis (211,212). The t-PA and u-PA are overexpressed upon disruption of tissue homeostasis, which guarantees a spatial and temporal restriction of plasmin generation that safeguards

the organism from unwanted plasmin-mediated proteolysis under physiologic conditions (190).

3.4.1. The tissue-type plasminogen activator

The t-PA is a 70 kDa serine protease with different structural domains, including a finger domain, an epidermal growth factor (EGF)-like domain, two kringle domains homologous to those found in PLG, and the catalytic triad, located in amino acids His322, Asp371, and Ser478. Binding of t-PA to fibrin involves the finger and the second kringle domains. The t-PA is produced as a single polypeptide chain of 527 amino acids that is converted to a disulphide-bound two-chain variant upon proteolytic cleavage of the Arg275–Ile276 peptide bond by plasmin. This variant shows enhanced proteolytic activity compared to the single-chain protease (213).

The t-PA is mainly produced and secreted by endothelial cells in response to different stimuli, including thrombin, histamine, bradykinin, adrenaline, acetylcholine, vasopressin, and shear stress (184). Therefore, the fibrinolytic functions of t-PA are primarily confined to the vascular space, specifically in relation to blood clot dissolution (184,213,214). The catalytic activity of t-PA is higher when bound to other proteins rather than in solution. These proteins include fibrin and PLG-Rs like annexin A2/S100A10 and PLG-R_{KT}. By concomitant binding of PLG and t-PA, annexin A2/S100A10 and PLG-R_{KT} enable the formation of tripartite complexes that co-localise the effector proteins required for plasmin generation while protecting t-PA from the activity of its inhibitor, the PA inhibitor 1 (PAI-1) (214).

3.4.2. The urokinase-type plasminogen activator

The u-PA, also known as urokinase, two-chain u-PA (tcu-PA), or high molecular weight (MW) u-PA, is a 54 kDa serine protease composed of two peptide chains linked by a disulphide bond. It contains three structural domains: an EGF-like domain, a single kringle domain, and the catalytic triad, which involves amino acids Asp255, His204, and Ser356 (213). The EGF-like and kringle domains are involved in binding of u-PA to its physiologic receptor, u-PAR (215). In contrast with PLG and t-PA, the kringle domain of u-PA does not contain an LBS (213) and does not mediate the interaction with partner proteins (184,215), as shown by the fact that a kringle-deficient mutant of u-PA still binds to u-PAR with only a slight reduction in binding stability (210). Moreover, binding of u-PA to partner proteins is dispensable for its catalytic activity, since receptor-bound and soluble u-PA are equally efficient in substrate cleavage (215,216). That said, the ability of u-PA to generate

plasmin from PLG increases in the presence of fibrin, but this is due to the conformational change induced in the PLG molecule upon binding to fibrin rather than a direct effect of fibrin in u-PA activity (184).

The u-PA is synthesised and secreted as a precursor zymogen of 411 amino acids that lacks catalytic activity, known as pro-u-PA or single-chain u-PA (scu-PA). The pro-u-PA is cleaved at the Lys158–Ile159 peptide bond by different proteases, including plasmin, kallikrein, trypsin, human cathepsins B and L, and u-PA itself (217–220), generating an active enzyme of two polypeptide chains linked by a disulphide bond. As shown in **Figure 14**, additional proteolytic events generate subsequent u-PA-derived molecules with different biological functions (221).

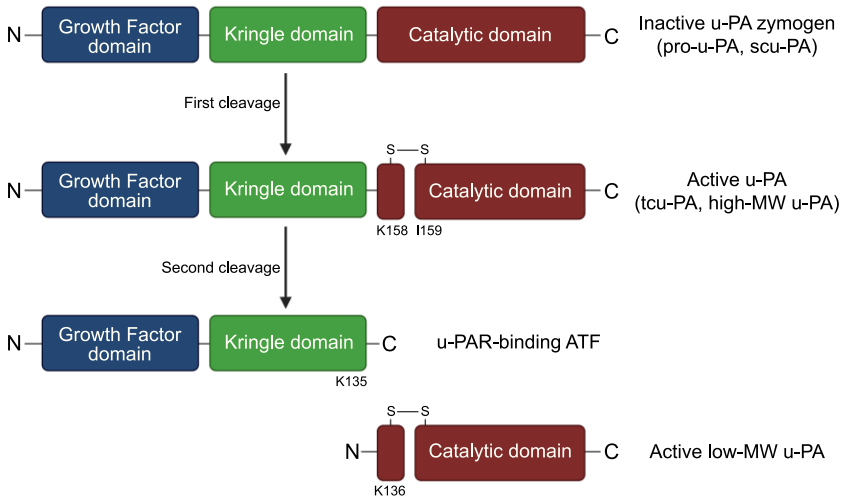

Figure 14. Structure and cleavage of the u-PA. The u-PA is synthesised as a single-chain inactive zymogen (pro-u-PA) of 411 amino acids containing an EGF-like domain, a kringle domain, and the catalytic triad. Upon proteolytic cleavage between amino acids Lys158 and Ile159, the pro-u-PA is converted into the active form of the protease, which is formed by two polypeptide chains linked by a disulphide bond. After activation, a second round of proteolytic cleavage at Lys135–Lys136 generates the ATF and active low-MW u-PA (213,221). Low-MW u-PA is generated in the presence of high concentrations of plasmin (213). u-PA, urokinase-type plasminogen activator; pro-u-PA, precursor of u-PA; scu-PA, single-chain u-PA; tcu-PA, two-chain u-PA; MW, molecular weight; u-PAR, u-PA receptor; ATF, amino-terminal fragment. Adapted from Hamada *et al.*, 2024 (221). Created with Biorender.com.

The u-PA is produced by many different cell types including endothelial cells, leukocytes, renal epithelial cells, and some tumour cells (184). This protease is also expressed by epithelial cells of the intestine, presumably to loosen intercellular junctions and facilitate epithelial cell turnover at the top of the villi (222). The ubiquitous expression of u-PA underpins its central role in mediating the fibrinolysis-unrelated functions of the fibrinolytic system, including pericellular proteolysis during tissue remodelling and repair, leukocyte

migration, and tumour invasion, among many others (213,223). In addition to PLG, the u-PA also cleaves certain growth factors and MMP precursors, thereby modulating their activities (214).

3.4.2.1. The receptor of the urokinase-type plasminogen activator

The u-PAR is a 50–60 kDa glycosylated protein that belongs to the lymphocyte antigen 6 (Ly6) superfamily of proteins, which are characterised by the presence of Ly6 and u-PAR (LU) domains. The u-PAR contains three LU domains, termed D1–D3 and a glycosyl-phosphatidylinositol (GPI) anchor that is added post-translationally and mediates cell surface localisation after trafficking to the extracellular space (221,224) (**Figure 15**). Both u-PA and pro-u-PA bind to u-PAR through a large surface that involves the three LU domains. The D1 domain, together with a portion of the D1–D2 linker region, participate in binding of u-PAR to the somatomedin B (SMB) domain of vitronectin, a major component of the ECM (224,225).

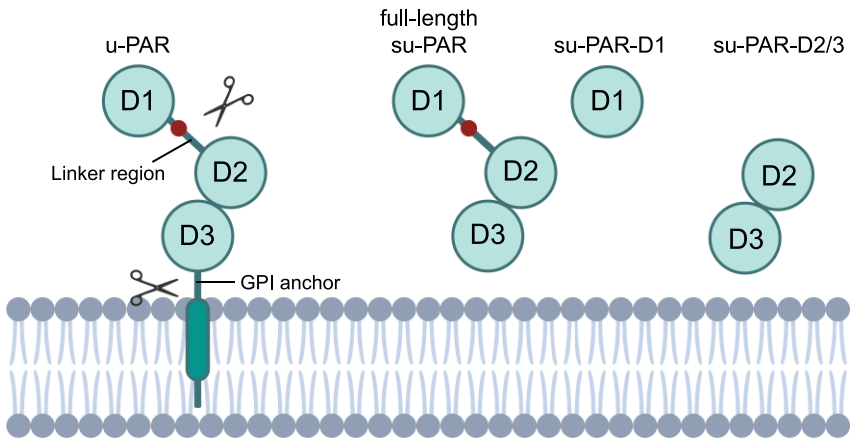

Figure 15. Schematic representation of the u-PAR and its soluble derivatives. Cleavage of the u-PAR at either the GPI anchor (by phospholipase C and D, cathepsin G, or plasmin) or the D1–D2 linker region (by chymotrypsin, elastase, MMPs, cathepsin G, plasmin, or u-PA) generates three different soluble forms of u-PAR (full length su-PAR, su-PAR-D1, and su-PAR-D2/D3). The su-PAR-D2/D3 can be shed or remain membrane-bound (224). The roles of these u-PAR derivatives are not fully understood (224,225), but given that cleaved u-PAR fails to interact with vitronectin and integrins, it is generally considered that u-PAR cleavage inhibits u-PAR-mediated cell adhesion and intracellular signalling. However, certain soluble u-PAR derivatives can also trigger novel signaling pathways involved in leukocyte chemotaxis, highlighting their role in the modulation of inflammatory responses (224,225). u-PAR, urokinase-type plasminogen activator receptor, GPI, glycosylphosphatidylinositol; su-PAR, soluble u-PAR. Adapted from Smith *et al.*, 2010 (224) and Rasmussen *et al.*, 2021 (226). Created with Biorender.com.

The u-PAR is expressed on the surface of endothelial and smooth muscle cells, neutrophils, monocytes/macrophages, T cells, fibroblasts, and different cancer cells, where it regulates intracellular signalling pathways that modulate cell behaviour (221,225). Since u-PAR is

GPI-anchored and does not contain transmembrane domains or cytosolic tails, it cannot directly relay signals to the intracellular space. To do so, u-PAR is connected with a series of signalling receptors, including tyrosine kinase receptors, integrins, and G protein-coupled receptors, that regulate signalling downstream of u-PAR (227).

3.5. Inhibitors of the fibrinolytic system

Inhibition of fibrinolysis may occur upon t-PA, u-PA, or directly at the level of plasmin. The main inhibitors of the fibrinolytic system are α2-antiplasmin, α2-macroglobulin, the thrombin activatable fibrinolysis inhibitor (TAFI), PAI-1–3, nexin-1, and neuroserpin. α2-antiplasmin is the primary plasmin inhibitor present in plasma, followed by α2-macroglobulin, which is about ten times less efficient than α2-antiplasmin in plasmin inhibition (184,185). The main inhibitor of t-PA and u-PA both at the vascular and extra-vascular space is PAI-1 (184,185,228), whereas PAI-2 is relevant during pregnancy (184,228) and PAI-3 activities are restricted to embryonic development (229). TAFI is a plasma carboxypeptidase that cleaves C-terminal lysine and arginine residues found in some PLG-Rs, thereby inhibiting their interaction with PLG (184,213). Nexin-1 and neuroserpin are involved in the regulation of fibrinolysis in the CNS (228).

PAI-1 is a 50 kDa protein produced by a variety of cells including endothelial, smooth muscle, and liver cells, among others (228). Upon binding of PAI-1 to t-PA or u-PA, a quaternary complex containing PAI-1, the PA, plasmin, and the PLG-R is formed. This complex is removed from the plasma membrane through low-density lipoprotein-mediated endocytosis and subsequent degradation via the lysosomal pathway (190,230,231). This is also the case during the inhibition of u-PA/u-PAR plasmin-independent functions. After PAI-1 binding to u-PA/u-PAR, internalisation of the ternary complex results in intracellular degradation of PAI-1 and u-PA and the recycling of free u-PAR back to the cell surface (232). PAI-1 spontaneously acquires a latent (inactive) conformation under physiological pH and temperature, with an active half-life of approximately two hours. However, the half-life of PAI-1 can be significantly enhanced artificially through the introduction of specific amino acid modifications, which extend the duration of its inhibitory effects by up to 72-fold (233).

Except for α2-macroglobulin and TAFI, the abovementioned inhibitors of the fibrinolytic system belong to the serine protease inhibitor (serpin) superfamily (71). Serpins are highly conserved protease inhibitors found across evolution, from viruses to vertebrates, that inhibit serine protease activity through a so-called 'suicide mechanism' that begins with

serpin binding to the catalytic domain of the serine protease via reversible bonds. This is followed by serpin cleavage by the serine protease, which results in the formation of a covalent bond between the serine protease catalytic triad and the reactive centre of the serpin, creating an irreversible serpin-enzyme complex that lacks catalytic activity (228,234).

3.6. Fibrinolysis-unrelated functions of the fibrinolytic system

Over thirty years ago it was recognised that PLG not only interacts with fibrin but also with cell surfaces, via a myriad of receptors that are broadly expressed by virtually all cell types except for red blood cells (194) (**Table 5**). Alongside the multiplicity of plasmin substrates described in the literature, which have been extensively reviewed in **Supplementary File 2**, the broad cellular expression of PLG-Rs implies that the fibrinolytic system is involved in very diverse physiologic and pathologic processes that are both unrelated and independent of fibrin degradation and clot removal. Notably, adding further complexity to the kaleidoscope of biological processes regulated by the fibrinolytic system, these functions are not solely mediated by the pleiotropic effects of generated plasmin. Rather, additional fibrinolytic factors, including t-PA, u-PA, u-PAR, and PAI-1, can also modulate fibrinolysis-unrelated processes independently of plasmin generation and even of their own proteolytic activities (225,235–239).

The fibrinolytic system is an important regulator of inflammatory and immune responses (**Figure 16**), a function that is mainly executed by u-PA/u-PAR and plasmin through their ability to trigger inflammation-related intracellular signalling pathways. For instance, fibrin degradation by plasmin generates multiple fibrin degradation products (FDPs), including fragments D and E, D-dimer, Bβ1–42, Bβ15–42, and α chain (alphastatin), that stimulate the production of both pro- and anti-inflammatory mediators by different immune cells (240). Regarding u-PAR, its contribution to intra-vascular fibrinolysis is minimal, as shown by an absence of thrombotic disorders or spontaneous fibrin deposition in u-PAR KO mice (241), which do present significant alterations in neutrophil recruitment and macrophage activation (242,243). Moreover, u-PA/u-PAR play crucial roles in the maintenance of epithelial barrier function and the regulation of intestinal inflammatory responses, as shown by increased expression levels of these factors during intestinal epithelial barrier breakdown induced by pro-inflammatory cytokines (244) (**Figure 16**).

An important aspect of plasmin-mediated regulation of inflammation is that involving the complement system, an interaction that is complex and somewhat controversial (245), with some studies reporting plasmin(ogen) as a complement activator and others, as a

complement inhibitor. However, given the intricate network of interconnected pathways that constitute the complement system, it is expected that plasmin(ogen) exerts both stimulatory and inhibitory effects depending on the specific arm of the complement cascade being regulated (246). In general, plasmin activates the complement pathway downstream of C3, for instance, by cleaving C5 and generating C5-derived fragments that are chemotactic to neutrophils. However, plasmin also has potent complement inhibitory effects by degrading C3b, which lies upstream in the complement cascade and plays a major role in amplifying complement-mediated responses (245,246). Along with this, the fibrinolytic system also regulates inflammation resolution and dendritic cell recruitment to infected sites, the latter representing a prerequisite for the onset of adaptive immune responses (247,248).

A key aspect of inflammatory and immune responses is cell migration. Therefore, in the context of inflammation and immunity, the contribution of the fibrinolytic system is also reflected in the ability of plasmin to facilitate cell migration. Plasmin stimulates cell migration through the direct degradation of ECM components, including laminin, fibronectin, and PGs, as well as by cleaving and activating MMP precursors (**Supp. File 2**). Plasmin activates the precursor zymogens of MMP-1, -2, -3, -9, -10, -12, and -13, either via direct cleavage or by activating other MMPs that, in turn, cleave and activate the MMP precursor (249–256). The MMP-1 zymogen is activated by plasmin through the generation of an intermediate form with minimal catalytic activity that is more readily activated by MMP-3 than the intact pro-MMP-1 molecule (249). Similarly, MMP-2 is activated in a two-step process that involves the generation of an inactive intermediate by membrane-type 1 (MT1)-MMP that is subsequently converted into fully active MMP-2 by plasmin cleavage (252). Regarding the activation of the MMP-9 zymogen (pro-MMP-9), plasmin does not directly cleave and activate pro-MMP-9 but rather, MMP-9 generation in the presence of plasmin depends on plasmin-mediated activation of MMP-3, which is a potent activator of the MMP-9 zymogen (254,255,257). Interestingly, the u-PA activates MMP-2 and MMP-9 irrespective of plasmin generation by direct cleavage of their precursor zymogens (137,138).

Figure 16 (next page). The pleiotropic effects of the fibrinolytic system in inflammation. [1] Plasmin cleaves different components of the complement system, resulting in either activation or inhibition of the cascade. [2] Fibrin is deposited at virtually any site of overt tissue damage, where it represents a scaffold for leukocyte recruitment. Therefore, fibrin removal by plasmin would inhibit the inflammatory response. [3] However, fibrin degradation by plasmin generates FDPs with chemotactic properties. Chemotaxis is also stimulated by plasmin cleavage of chemokines, generating truncated versions that stimulate leukocyte recruitment. [4] The involvement of the fibrinolytic system in cell adhesion is mediated by u-PAR binding to the SMB domain of vitronectin, a major component of the ECM, and by PAI-1, which competes with u-PAR for binding to the SMB (not represented in this figure) (258). [5] Plasmin promotes cell migration by directly cleaving ECM proteins and by activating MMP zymogens into their active forms. The u-PA is also capable of cleaving

and activating MMPs irrespective of plasmin generation. [6] Inflammation-related intracellular signalling is regulated by FDPs and the u-PAR, which signal through MAPK, PKC, and NF-κβ or through GPCRs and EGFRs, respectively (240,259). [7] The fibrinolytic system participates in inflammation resolution by stimulating the polarisation of macrophages towards an anti-inflammatory or pro-resolution M2 phenotype and by promoting apoptosis of leukocytes, fibrin clearance, and the stimulation of anti-inflammatory cytokine release. CCL21, C-C motif chemokine ligand 21; PLG, plasminogen; PLG-R, plasminogen receptor; MMP, matrix metalloproteinase; pro-MMP, precursor (zymogen) of MMP; FDP, fibrin degradation product; u-PA, urokinase-type plasminogen activator; u-PAR, receptor of the u-PA; ECM, extracellular matrix, EGFR, epidermal growth factor receptor; GPCR, G protein-coupled receptor; FPR, formyl peptide receptor; MAPK, mitogen-activated protein kinase; NF-κβ, nuclear factor κβ; PKC, protein kinase C. Created with Biorender.com.

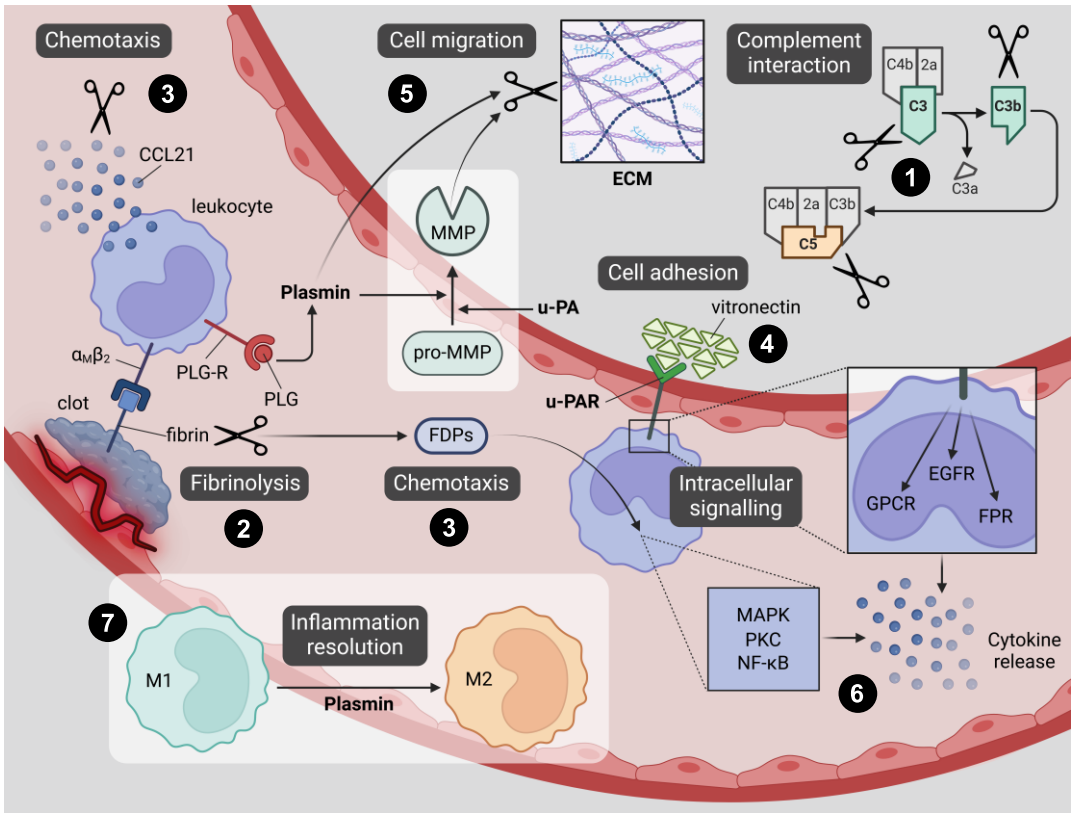

Figure 16 (legend on the previous page).

4. The interaction between clinically-relevant endoparasites and the host fibrinolytic system

Given the pleiotropic functions of the fibrinolytic system outlined in the preceding section, it is clear that this system may represent a crucial mechanism exploited by various pathogens to migrate across host tissues more efficiently and to circumvent host-derived immune defences. Accordingly, the interaction between pathogens and the fibrinolytic system has been described in very distant organisms, including bacteria, fungi, and parasites (178,179,260,261), thereby representing a paradigmatic example of evolutionary convergence wherein phylogenetically unrelated species develop analogous strategies to fulfil a specific biological purpose. This section reviews the current knowledge on the interaction between human and veterinary endoparasites of clinical relevance and the host fibrinolytic system, including insights into the mechanisms of these interactions and their potential functional significance during parasitic infections.

4.1. Parasitic proteins that interact with the fibrinolytic system

The interaction with the fibrinolytic system is a well-documented and nearly universal phenomenon in bacteria (extensively reviewed in Refs. 260 and 262), which stimulate plasmin generation from host-derived PLG either by expressing PLG-Rs on their surface, by secreting them, and/or by producing PLG activators that directly cleave and activate PLG. Plasmin generation at the bacterial surface or pericellular space allows for the cleavage of multiple substrates, including fibrin and various ECM components (**Supp. File 2**), thereby facilitating bacterial dissemination and survival inside the mammalian organism (260,262).

Although less-well studied, the interaction between clinically-relevant endoparasites and the fibrinolytic system of their hosts has also been experimentally confirmed and presumed to be important for infection success (178,179). A summary of parasitic proteins that interact with components of the fibrinolytic system is shown in **Table 6**. This table provides an updated compilation of data from Figuera *et al.*, 2013 (263), González-Miguel *et al.*, 2016 (178), and Diosdado *et al.*, 2022 (179), which reviewed parasitic fibrinolysis-interacting proteins employing distinct methodologies. In this table, only studies demonstrating the interaction between a specific parasitic protein and host fibrinolytic factors were included, either through the use of recombinant proteins (the majority of studies) or protein-protein interaction techniques, such as immunoprecipitation followed by mass spectrometry (e.g., Ref. 264). A complete list of helminth parasites that interact with fibrinolytic factors,

including those where the interaction has been experimentally confirmed using complex protein extracts, is available in Ref. 179. As shown in **Table 6**, parasitic proteins that interact with the fibrinolytic system are diverse and they are expressed in different antigenic compartments and evolutionary stages of parasites. This suggests that this interaction is important for the migration of juvenile parasites across host tissues prior to their long-term establishment inside the mammalian organism as well as for the survival of adult phases within their physiological definitive niche.

Table 6. Fibrinolysis-interacting proteins expressed by clinically-relevant endoparasites.

Protein	**Parasite**	**Fibrinolytic function**	**Location** (a)	**Stage** (a)	**Biological process** (b)	**PMID** (c)
Actin	*Dirofilaria immitis*	PLG-R, increase in t-PA/u-PA expression	Surface, ESP, somatic	Adult	Fibrinolysis (d), survival, pathogenesis	25875022
Annexin	*Clonorchis sinensis*	PLG-R	Surface, somatic	Adult, metacercaria	Immune modulation	24861011
	Schistosoma bovis	PLG-R	Surface, somatic	Adult, schis-tosomula	Survival	21889851
	Spirometra erinaceieuropaei	PLG-R	Surface, somatic, ESP	Adult, spargana	Immune evasion, tissue invasion	39601902
Enolase	*Babesia microti*	PLG-R	Surface, cytosol	Intraerythro-cytic	Migration, invasion	28443086
	Clonorchis sinensis	PLG-R	Surface, ESP	Egg, cercaria, metacercaria, adult	Migration, invasion, immune evasion, pathogenesis	21382423
	Cryptosporidium parvum	PLG-R	Surface	Oocyst	Migration, invasion	28569179
	Echinostoma caproni	PLG-R	ESP, somatic	Adult	Establishment and pathogenesis (tissue destruction)	17462631
	Fasciola hepatica	PLG-R	Surface	NEJ	Migration, adhesion, survival	39213442
	Giardia intes-tinalis (e)	PLG-R			Adhesion to host cells	28770921
	Leishmania mexicana	PLG-R	Surface, EVs	Promastigote	Migration, invasion	23178693, 17653767
	Onchocerca volvulus	PLG-R	Somatic	Larva L3, microfilaria, adult	Migration (ECM degradation)	12818429
	Plasmodium berghei/ falciparum	PLG-R	Surface	Ookynete	Development inside mosquito vector	21949403

Protein	**Parasite**	**Fibrinolytic function**	**Location** (a)	**Stage** (a)	**Biological process** (b)	**PMID** (c)
	Schistosoma bovis	PLG-R	Surface, somatic and ESP	Adult		20609522
	Schistosoma japonicum	PLG-R	Surface, somatic	Schistoso-mula, adult	Migration, development	20512506
	Schistosoma mansoni	PLG-R	Surface	Schistoso-mula, adult	Fibrinolysis, survival	26658895
	Spirometra mansoni	PLG-R	Surface, ESP	Spargana, adult	Tissue invasion	34031713
	Taenia multiceps	PLG-R		Protoscolex, adult		26412520
	Taenia pisiformis	PLG-R	Surface, ESP	Metacestode	Invasion, migration	25623259
	Taenia solium	PLG-R	Surface, somatic	Cysticercus, adult	Migration, invasion, survival	29466706, 29657009
	Trichinella spiralis	PLG-R	Surface	Larva, adult	Migration, invasion	31806006
	Trichomonas vaginalis	PLG-R	Surface		Adhesion, virulence	18070902
FBAL	*Clonorchis sinensis*	PLG-R	Somatic, ESP	Adult	Fibrinolysis, survival, nutrition	33612738
	Dirofilaria immitis	PLG-R, increase in t-PA/u-PA expression	Surface, ESP, somatic	Adult	Fibrinolysis, survival, pathogenesis	25875022
Galectin	*Dirofilaria immitis*	PLG-R, increase in u-PA expression	Surface, ESP, somatic	Adult	Fibrinolysis, survival, pathogenesis	25666684
GAPDH	*Babesia microti*	PLG-R		Intraerythro-cytic	Migration, invasion	31355216
	Clonorchis sinensis	PLG-R	Surface, somatic	Egg, metace-rcaria, adult	Migration, invasion	25300416
	Dirofilaria immitis	PLG-R, increase in u-PA expression	Surface, somatic	Adult	Fibrinolysis, survival, pathogenesis	25666684
	Onchocerca volvulus	PLG-R	Somatic	Larva L3, microfilaria, adult	Migration (ECM degradation)	15955451
	Schistosoma mansoni	PLG-R	Surface	Adult	Fibrinolysis, survival	32098782
	Trichomonas vaginalis	PLG-R	Surface		Invasion	19380472
HSP60 (f)	*Plasmodium falciparum*	PLG-R, PLG internalization	Cytosol	Intraerythro-cytic	Inhibit inflammatory responses (ALFs)	32847585
LACK	*Leishmania mexicana*	PLG-R	Surface, secreted	Promastigote	Migration, invasion	21272581
NAPc2	*Ancylostoma caninum*	decrease u-PA expression				25672417
PGM	*Schistosoma mansoni*	PLG-R	Tegument	Schistosom-ula, adult	Fibrinolysis, survival	36083036

Protein	**Parasite**	**Fibrinolytic function**	**Location** (a)	**Stage** (a)	**Biological process** (b)	**PMID** (c)
Serpin	*Echinococcus multilocularis*	Plasmin inhibition				17727977
SMP-1	*Leishmania mexicana*	PLG-R	EVs	Promastigote	Migration, invasion	23178693
SP2	*Schistosoma mansoni*	PA, enhances t-PA activity	Surface (only males), somatic, ESP	Adult	Migration, invasion	29677188
TGH-2	*Brugia malayi*	Increase in PAI-1 transcription	ESP	Microfilaria, adult		11035752
VAL18	*Schistosoma mansoni*	PLG-R	ESP	Cercaria, schistoso-mula	Migration, invasion	29477861

(a) A specific location or development stage does not necessarily indicate that the interaction is undetectable in others; rather, it may also reflect that non-specified fractions/stages were not analysed in the publication. (b) Biological process attributed to the interaction, whether experimentally demonstrated or not. (c) PubMed identification number. (d) Fibrinolysis refers to the ability of the parasite to degrade surrounding fibrin clots that would otherwise lead to immobilisation, which is particularly relevant for parasites residing inside the vascular system. (e) The interaction was studied *in silico* using different bioinformatics-based molecular modelling techniques. (f) PLG interaction was identified by immunoprecipitation followed by mass spectrometry, which revealed multiple interacting proteins; only HSP60 is included here for simplicity. Blank cells indicate information that was not reported in the publication. NAPc2, anticoagulant protein c2; FBAL, fructose-1,6-bisphosphate aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; HSP60, heat shock protein 60; LACK, *Leishmania* homolog of receptors for activated C-kinase; PGM, phosphoglycerate mutase; SMP-1, small myristoylated protein 1; SP2, serine protease 2; VAL18, venom allergen-like protein 18; TGH-2, transforming growth factor β homolog; PLG-R, plasminogen receptor; ESP, excreted/secreted product; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; NEJ, newly-excysted juvenile; ALF, angiostatin-like fragments.

4.1.1. Mechanisms of interaction between clinically-relevant endoparasites and the fibrinolytic system

The most common mechanism of interaction between clinically-relevant endoparasites and the fibrinolytic system described in the literature relies on the expression of proteins that interact with PLG at the host-parasite interface—either the parasite surface or as part of their secretomes—thereby functioning as parasitic PLG-Rs. Binding of PLG to these receptors is functionally relevant as it stimulates plasmin generation from bound PLG by host-derived PAs. Some parasites also express proteins at their surface that not only bind PLG but also t-PA and/or u-PA (265), facilitating the colocalisation of fibrinolytic factors that are needed for efficient plasmin generation at the parasitic surface or periparasitic space. By using lysine analogues, most studies summarised in **Table 6** also revealed that the interaction between parasitic PLG-Rs and host PLG is, to varying degrees, dependent on the presence of this amino acid in parasitic PLG-Rs, mirroring the mechanism of interaction between PLG and its receptors in the mammalian host (**Section 3.2**).

Interestingly, proteases that function as PAs are rare among parasites, with a serine protease from *Schistosoma mansoni* (SmSP2) being the only parasitic protease described as capable of performing this function. SmSP2 not only cleaves PLG to generate catalytically-active plasmin, but also converts single-chain t-PA into its double-chain form, which exhibits approximately ten times greater proteolytic activity than the single-chain variant (213,266). Proteases that cleave PLG have also been identified in *Plasmodium falciparum*. However, these enzymes are not classified as PAs since they do not produce plasmin but PLG-derived angiostatin-like fragments (ALFs) that modulate host inflammatory responses (267). An additional mechanism of interaction between parasites and the host fibrinolytic system involves parasite-induced alterations in the protein expression levels of certain fibrinolytic factors. For instance, ESPs of the nematode *Dirofilaria immitis* stimulate the expression of t-PA and u-PA and inhibit the expression of PAI-1 in a cell line representing the arterial endothelium, resulting in an overall state of increased fibrinolysis (268–270).

4.1.2. Anatomic location of parasitic fibrinolysis-interacting proteins

As shown in **Table 6**, parasitic proteins that interact with host fibrinolytic factors are mainly expressed on the parasite surface or excreted/secreted in the form of ESPs. Notably, most fibrinolysis-interacting proteins identified in these parasites are intracellular proteins involved in cellular architecture and/or metabolism. Despite lacking signal peptides and transmembrane domains, these proteins are relocated to the parasite surface to bind host fibrinolytic factors. The mechanisms responsible for surface relocation of these proteins are not completely understood but may involve lipid binding before translocation to the outer membrane, interaction with partner proteins containing signal sequences for extracellular export, or secretion through EVs. Some PLG-Rs have indeed been identified in the cargo of *Leishmania mexicana* EVs (271), and a recent proteomic analysis of FhNEJ EVs (158) revealed that these structures harbour enzymes identified in other parasite species (179) as proteins that interact with certain fibrinolytic components, supporting the notion that FhNEJ EVs could represent a pathway for the secretion of fibrinolysis-interacting proteins to the periparasitic space. Regardless of the specific secretion pathway, extracellular translocation is thought to require PTMs and is followed by the non-covalent association of the protein with the extracellular environment through as yet unidentified mechanisms (272).

Similar to PLG-Rs expressed by host cells, the observation that most parasitic fibrinolysis-interacting proteins have canonical functions unrelated to plasmin generation argues in favour of a moonlighting potential of these proteins (**Section 3.3.1**). Moonlighting proteins

utilised by these pathogens for invasion and dissemination purposes tend to be housekeeping proteins, including core metabolic enzymes and cytoskeleton or cell cycle proteins, with highly conserved protein sequences across species. This confers an evolutionary advantage by minimising the recognition of parasitic fibrinolysis-interacting proteins by the host immune system (207).

4.2. Functional relevance of the parasite-fibrinolysis interaction

Since plasmin enhances cell migration through direct cleavage of ECM proteins and activation of ECM-degrading MMPs, plasmin generated by the binding of host fibrinolytic factors to parasitic PLG/PA-Rs could facilitate parasite migration across host tissues prior to their establishment within the definitive niche. This function enhances the invasive potential of parasites and establishes parasitic fibrinolysis-interacting proteins as important virulence factors (179).

The interaction between clinically-relevant endoparasites and the host fibrinolytic system is also linked to immune evasion and modulation strategies, as suggested or experimentally shown in different parasites, including *C. sinensis* (273), *Trichinella spiralis* (274), *L. donovani* (275), and *P. falciparum* (267,276,277). Whether or not immune modulation in these cases depends on plasmin generation has not always been experimentally tested. However, given that plasmin cleaves immunoglobulins, complement molecules (**Supp. File 2**), and regulates intracellular signalling cascades that orchestrate inflammatory responses (**Section 3.6**), it is plausible that plasmin generation on the parasite surface and/or periparasitic space represents a key mechanism for modulating host immune responses, not only in the aforementioned organisms but universally across all parasites that interact with the host fibrinolytic system. In line with this, *P. falciparum* was shown to evade complement-mediated immune attacks through the stimulation of plasmin generation from host-derived PLG (276,277). Similarly, *F. hepatica* has also evolved mechanisms to evade complement-mediated immunity (171), but whether this is mediated by the interaction between *F. hepatica* and the host fibrinolytic system (180) has not been investigated.

Finally, owing to its potent fibrinolytic functions, leveraging the host fibrinolytic system may facilitate the degradation of fibrin clots that would otherwise cause parasite immobilisation, which is particularly relevant for the survival of parasites residing within the vascular system, such as *S. mansoni* and *D. immitis* (178). In addition, the ability of *D. immitis* to stimulate plasmin generation from host-derived PLG enhances wound healing, which is a hallmark of many helminth infections (**Section 1.2.3**) (48,50). A graphic summary

of the functional relevance of the interaction between clinically-relevant endoparasites and the host fibrinolytic system is available in **Figure 17**.

4.3. Parasitic fibrinolysis-interacting proteins as vaccine targets

Since parasitic proteins that interact with the host fibrinolytic system are located at the interface between both organisms, one of the most compelling applications of parasitic fibrinolysis-interacting proteins is their potential use in vaccine development. However, many of these proteins are metabolic enzymes with significantly conserved sequences across various species (278), which poses serious challenges to their use as vaccine targets. In addition, the interaction between clinically-relevant endoparasites and host fibrinolysis appears to be highly redundant as it involves multiple parasitic proteins. This redundancy implies that targeting a single molecule might not be sufficient to disrupt plasmin generation at the parasite surface or periparasitic space (279). Despite such limitations, the use of parasitic fibrinolysis-interacting proteins for vaccine development has been explored and still holds promise. As shown in **Table 7**, vaccines using fibrinolysis-interacting proteins have been tested in parasites belonging to different taxonomic ranks, including the trematodes *S. japonicum* (280), S. *mansoni* (281), and *C. sinensis* (273), the nematode *T. spiralis* (274), and the protozoan *Babesia microti* (282), with either variable or non-existent protection rates.

Table 7. Vaccination trials using parasitic fibrinolysis-interacting proteins (published between 2010 and 2024).

Protein	Function	Parasite	Protection (a)	Method	Model	N (b)	Ref.
Annexin	PLG-R	*Clonorchis sinensis* (c)		Cytokine profile	Rat		(273)
Enolase	PLG-R	*Babesia microti*	Around 40%	Count of infected erythrocytes and qRT-PCR	Mouse	4	(282)
	PLG-R	*Schistosoma japonicum*	24.28%	Egg count in liver	Mouse	10	(280)
			21.45%	Egg count in faeces			
FBAL	PLG-R	*Trichinella spiralis*	48.70%	Count of adult flukes in intestine	Mouse	25	(274)
			52.50%	Count of larvae per gram of muscle			
PGM	PLG-R	*Schistosoma mansoni*	n.s.	Count of adults in portal vein	Mouse	10	(281)
			n.s.	Egg count in liver			

Only parasitic proteins whose interaction with a component of the fibrinolytic system has been experimentally confirmed are shown. (a) Percentage reduction in parasitic load during challenge infections in immunised animals compared to non-immunised controls. (b) Number of animals employed per experimental group. (c) This study demonstrates that vaccination causes a potentially protective immune response, but it does not address whether this results in protection against challenge infections. Blank cells indicate information that was not provided or reported in the original publication.

FBAL, fructose-1,6-bisphosphate aldolase; PGM, phosphoglycerate mutase, PLG-R, plasminogen receptor; n.s., not significant.

4.4. Final remarks

An interesting aspect of the interaction between clinically-relevant endoparasites and the host fibrinolytic system is that, in addition to potentially serving as a mechanism by which these infectious agents invade their hosts, it may also underlie some of the pathogenesis linked to parasitic infections. This is particularly evident in cardiopulmonary dirofilariosis, a disease affecting primarily dogs and cats and caused by infection with the nematode *D. immitis*. Adult worms reside in the pulmonary arteries and right ventricle of the heart, where they can persist for years, eventually causing a chronic inflammatory vascular pathology (283). Plasmin generated as a result of the recruitment of PLG at the surface of *D. immitis* stimulates the proliferation and migration of canine endothelial and smooth muscle cells (268,270,284), leading to proliferative endarteritis (107), the hallmark of cardiopulmonary dirofilariosis. Similarly, cutaneous lesions associated with *L. mexicana* infection are smaller in mice with genetic depletion of PLG compared to their wild-type littermates (285); and the interaction between *F. hepatica* and host PLG has been proposed to underlie some of the neurological disorders occasionally associated with human fasciolosis (180). Therefore, parasites must precisely regulate the exploitation of the host fibrinolytic system to balance the benefits that this interaction has to their survival with the long-term pathogenic effects that excessive fibrinolysis may impose on the host.

As demonstrated in **Table 6**, the interaction between clinically-relevant endoparasites and the host fibrinolytic system appears to be nearly universal, suggesting that this phenomenon is essential for the success of parasitic infections. However, only a minority of studies reporting such interactions have conducted functional assays to experimentally assess whether the interaction between the parasite and the fibrinolytic system serves physiological roles. The aforementioned studies on *D. immitis* (268,270,284) and *L. mexicana* (285) exemplify this gap. While these studies clearly indicate that the host fibrinolytic system is involved in the pathogenesis associated with these infections, evidence is still lacking regarding its significance for the dissemination and the stable establishment of parasites within the mammalian organism. To the best of our knowledge, this link has thus far only been demonstrated in *P. falciparum*. Using *in vivo* models and a cutting-edge methodology, Alves e Silva *et al.* (265) convincingly demonstrated that the interaction between *P. falciparum* and the host fibrinolytic system is required for both the development of the parasite within its mosquito vector and the successful infection of the mammalian host (265). These results

were shown to have important practical applications, as blocking the interaction between *P. falciparum* and PLG in the mosquito vector efficiently hindered the development of the parasite within the mosquito and its subsequent transmission to mammalian hosts (286,287).

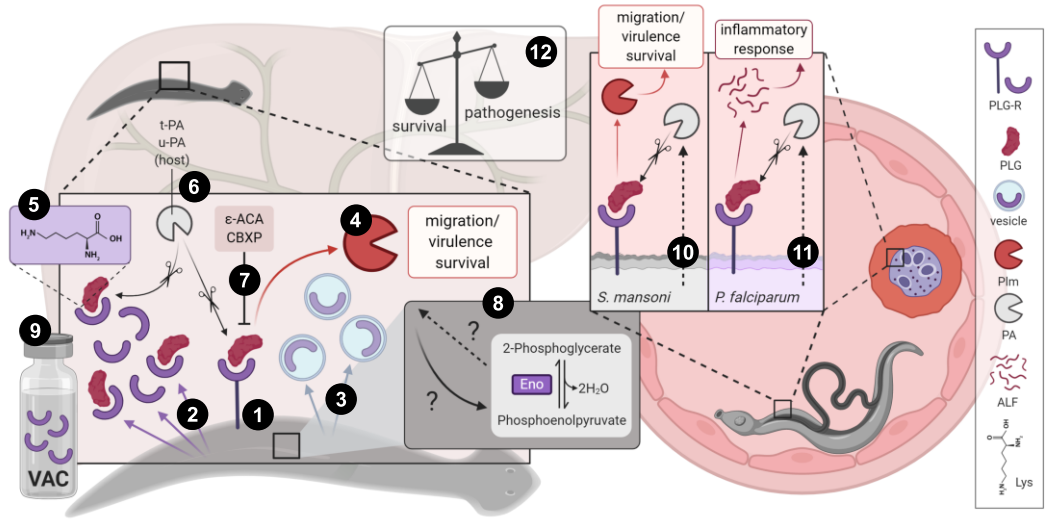

Figure 17. Relevant aspects of the interaction between clinically-relevant endoparasites and the fibrinolytic system of their mammalian hosts. The most common mechanism of parasite-fibrinolysis interaction is through the expression of PLG-Rs that allow for PLG recruitment at the parasite surface [1] and subsequent plasmin generation [4] in the presence of host-derived PAs [6]. Parasitic PLG-Rs are expressed on the parasite surface [1] or secreted, either freely [2] or within EVs [3]. [5,7] The interaction between PLG and parasitic receptors is generally lysine-dependent, as demonstrated by its inhibition in experiments including ε-ACA and/or carboxypeptidase during incubation with PLG. [8] Parasitic PLG-Rs are often intracellular enzymes (e.g., α-enolase) with moonlighting potential. The mechanism of secretion or surface re-location of these proteins is not completely understood. [9] Some parasitic PLG-Rs have been used in vaccination trials with varying efficacies (**Table 7**). [10,11] Enzymes capable of directly cleaving PLG have been identified in *S. mansoni* and *P. falciparum*. The protease in *P. falciparum* is not strictly considered a PA, as it does not generate plasmin but ALFs that modulate host inflammatory responses. [12] An overstimulation of the host fibrinolytic system by parasites can have pathogenic consequences; therefore, a balance must exist between the benefits gained by the parasite and the long-term negative effects that are generated in the host as a result of sustained increased fibrinolysis stimulated by the parasite. A *F. hepatica* juvenile is represented in the liver, and a male/female pair of *S. mansoni* parasites with granuloma-encapsulated eggs are represented inside a blood vessel. PLG, plasminogen; PLG-R, plasminogen receptor; Plm, plasmin; PA, plasminogen activator; VAC, vaccine; ALF, angiostatin-like fragment; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; ε-ACA, aminocaproic acid; CBXP, carboxypeptidase; Eno, α-enolase. Created with Biorender.com.

Even though the evidence coming from physiologically-relevant experimental models is scarce, the importance of fibrinolysis-interacting proteins in the virulence and survival of parasites is underscored by a proteomic study showing that, in sharp contrast to their attenuated counterparts, virulent strains of *Histomonas meleagridis*, a protozoan parasite of poultry, overexpress proteins identified as PLG-binding proteins in other parasite species,

including GAPDH, FBAL, actin, and heat-shock protein 60 (**Table 6**). Furthermore, the proteins overexpressed in the virulent strain were present in different truncated forms and containing different PTMs, supporting their sub-cellular relocation and the notion that their overexpression might be related to mechanisms independent of their canonical functions (288). Moreover, a comparative study by Tritten *et al.* (289) revealed intriguing differences in protein compositions of the secretomes of free-living and parasitic nematodes. Specifically, some of the canonical fibrinolysis-interacting proteins identified in certain parasites, including FBAL and enolase (**Table 6**), were present in ESPs of all studied parasitic nematodes but not in the secretome of the free-living nematode *Caenorhabditis elegans* (289). Altogether, this body of evidence supports the notion that the interaction between parasites and the fibrinolytic system of their hosts may be a critical determinant of infection success, not only by influencing parasite migration, evasion, and virulence, but also by potentially shaping the nature of parasitism.

References

1. Andrews SJ, Cwiklinski K, Dalton JP. Chapter 1. The discovery of *Fasciola hepatica* and its life cycle. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 1–23.
2. Mas-Coma S, Valero MA, Bargues MD. Fascioliasis. In: Toledo R, Fried B, editors. Digenetic Trematodes. Springer International Publishing; 2019. p. 71–103.
3. Knubben-Schweizer G, Rössler AS, Schade-Weskott E, Torgerson PR. Chapter 6. Epidemiology and control. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 180–211.
4. Siles-Lucas M, Becerro-Recio D, Serrat J, González-Miguel J. Fascioliasis and fasciolopsiasis: current knowledge and future trends. Res Vet Sci. 2021 Jan;134:27–35.
5. Ubeira FM, Martínez-Sernández V, González-Warleta M, Mezo M. Chapter 10. Diagnostics for animal and human fasciolosis. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 308–38.
6. Rondelaud D, Belfaiza M, Vignoles P, Moncef M, Dreyfuss G. Redial generations of *Fasciola hepatica*: a review. J Helminthol. 2009 Sep;83(3):245–54.
7. Dreyfuss G, Sindou P, Hourdin P, Vignoles P, Rondelaud D. Chapter 2. *Fasciola hepatica* larval development within the intermediate host. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 23–65.
8. Hodgkinson JE, Cwiklinski K, Beesley N, Hartley C, Allen K, Williams DJL. Clonal amplification of *Fasciola hepatica* in *Galba truncatula*: within and between isolate variation of triclabendazole-susceptible and -resistant clones. Parasites Vectors. 2018 Jun 26;11(1):363.
9. Mas-Coma S, Bargues MD, Valero MA. Human fascioliasis infection sources, their diversity, incidence factors, analytical methods and prevention measures. Parasitology. 2018 Nov;145(13):1665–99.
10. Dixon KE. The physiology of excystment of the metacercaria of *Fasciola hepatica* L. Parasitology. 1966 Aug;56(3):431–56.
11. Membrive CMB. Anatomy and physiology of the rumen. In: Millen DD, De Beni Arrigoni M, Lauritano Pacheco RD, editors. Rumenology. Springer International Publishing; 2016. p. 1–38.

12. Owens FN, Basalan M. Ruminal fermentation. In: Millen DD, De Beni Arrigoni M, Lauritano Pacheco RD, editors. Rumenology. Springer International Publishing; 2016. p. 63–102.

13. Robinson MW, Menon R, Donnelly SM, Dalton JP, Ranganathan S. An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*. Mol Cell Proteomics. 2009 Aug;8(8):1891–907.

14. Bennett CE. Surface Features, Sensory Structures, and Movement of the newly excysted juvenile *Fasciola hepatica* L. J Parasitol. 1975;61(5):886–91.

15. Sukhdeo MV, Keith S, Mettrick DF. The effects of bile on the locomotory cycle of *Fasciola hepatica*. J Parasitol. 1988 Jun;74(3):493–5.

16. Li S, Kim TI, Yoo WG, Cho PY, Kim TS, Hong SJ. Bile components and amino acids affect survival of the newly excysted juvenile *Clonorchis sinensis* in maintaining media. Parasitol Res. 2008 Oct;103(5):1019–24.

17. Hanna REB, Fairweather I, Robinson MW. Chapter 4. The reproductive system of *Fasciola hepatica*. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 112–45.

18. Sangster NC, Martínez-Moreno Á, Pérez J. Chapter 5. Pathology, pathophysiology and clinical aspects. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 145–80.

19. Robinson MW, Hanna REB, Fairweather I. Chapter 3. Development of *Fasciola hepatica* in the mammalian host. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 65–112.

20. Halton DW, Gustafsson MKS. Functional morphology of the platyhelminth nervous system. Parasitology. 2009/04/06 ed. 1996;113(S1):S47–72.

21. Magee RM, Fairweather I, Johnston CF, Halton DW, Shaw C. Immunocytochemical demonstration of neuropeptides in the nervous system of the liver fluke, *Fasciola hepatica* (Trematoda, Digenea). Parasitology. 2009/04/06 ed. 1989;98(2):227–38.

22. Tielens AGM, van Hellemond JJ. Chapter 8. Metabolism. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 256–78.

23. Deslyper G, Doherty DG, Carolan JC, Holland CV. The role of the liver in the migration of parasites of global significance. Parasites Vectors. 2019 Dec;12(1):531.

24. Cwiklinski K, Dalton JP. Omics tools enabling vaccine discovery against fasciolosis. Trends Parasitol. 2022 Dec;38(12):1068–79.

25. Calvani NED, Šlapeta J. Chapter 13. *Fasciola gigantica* and fasciola hybrids in Souteast Asia. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 423–61.

26. Acosta D, Cancela M, Piacenza L, Roche L, Carmona C, Tort JF. *Fasciola hepatica* leucine aminopeptidase, a promising candidate for vaccination against ruminant fasciolosis. Mol Biochem Parasitol. 2008 Mar;158(1):52–64.

27. Lalor R, Cwiklinski K, Calvani NED, Dorey A, Hamon S, Corrales JL, *et al.* Pathogenicity and virulence of the liver flukes *Fasciola hepatica* and *Fasciola gigantica* that cause the zoonosis Fasciolosis. Virulence. 2021 Dec 31;12(1):2839–67.

28. Fitzpatrick JL. Global food security: the impact of veterinary parasites and parasitologists. Vet Parasitol. 2013 Aug 1;195(3–4):233–48.

29. World Health Organization. Global report on Neglected Tropical Diseases 2023. 1st ed. Geneva: World Health Organization; 2023.

30. Nyindo M, Lukambagire AH. Fascioliasis: an ongoing zoonotic trematode infection. Biomed Res Int. 2015;2015:786195.

31. Lan Z, Zhang XH, Xing JL, Zhang AH, Wang HR, Zhang XC, *et al.* Global prevalence of liver disease in human and domestic animals caused by *Fasciola*: A systematic review and meta-analysis. J Glob Health. 2024 Sep 19;14:04223.

32. Mehmood K, Zhang H, Sabir AJ, Abbas RZ, Ijaz M, Durrani AZ, *et al.* A review on epidemiology, global prevalence and economical losses of fasciolosis in ruminants. Microb Pathogen. 2017 Aug;109:253–62.

33. Hayward AD, Skuce PJ, McNeilly TN. The influence of liver fluke infection on production in sheep and cattle: a meta-analysis. Int J Parasitol. 2021 Oct;51(11):913–24.

34. González-Warleta M, Castro-Hermida JA, Figueira M, López J, Conesa D, López-Quílez A, *et al.* Bayesian hierarchical modelling of the geospatial distribution of fasciolosis in dairy cattle and the impact on production: application to the main milk-producing region (Galicia) in Spain. Vet Parasitol. 2024 Jan;325:110091.

35. Gandhi P, Chandra R, Schmitt EK, Chen CW, Samantray S, Venishetty VK, *et al.* Chapter 14. Global impact of human fasciolosis. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 461–93.

36. Rosas-Hostos Infantes LR, Paredes Yataco GA, Ortiz-Martínez Y, Mayer T, Terashima A, Franco-Paredes C, *et al.* The global prevalence of human fascioliasis: a systematic review and meta-analysis. Ther Adv Infect Dis. 2023 Dec;10:20499361231185413.

37. Cwiklinski K, O'Neill SM, Donnelly S, Dalton JP. A prospective view of animal and human Fasciolosis. Parasite Immunol. 2016 Sep;38(9):558–68.

38. Pan American Health Organization (PAHO). Operational guidelines for the elimination of human fascioliasis as a public health problem in the Americas. Pan American Health Organization; 2024.

39. Torgerson PR, Devleesschauwer B, Praet N, Speybroeck N, Willingham AL, Kasuga F, *et al.* World Health Organization estimates of the global and regional disease burden of 11 foodborne parasitic diseases, 2010: a data synthesis. PLoS Med. 2015 Dec;12(12):e1001920.

40. World Health Organization. Sustaining the drive to overcome the global impact of neglected tropical diseases: second WHO report on neglected diseases. Geneva: World Health Organization; 2013.

41. Fürst T, Keiser J, Utzinger J. Global burden of human food-borne trematodiasis: a systematic review and meta-analysis. Lancet Infect Dis. 2012 Mar;12(3):210–21.

42. Alba A, Vazquez AA, Hurtrez-Boussès S. Towards the comprehension of fasciolosis (re-)emergence: an integrative overview. Parasitology. 2021 Apr;148(4):385–407.

43. Choi YJ, Fontenla S, Fischer PU, Le TH, Costábile A, Blair D, *et al.* Adaptive radiation of the flukes of the family Fasciolidae inferred from genome-wide comparisons of key species. Mol Biol Evol. 2020 Jan 1;37(1):84–99.

44. Cuervo PF, Bargues MD, Artigas P, Buchon P, Angles R, Mas-Coma S. Global warming induced spread of the highest human fascioliasis hyperendemic area. Parasites Vectors. 2024 Oct 21;17(1):434.

45. Mas-Coma S, Valero MA, Bargues MD. Chapter 2. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Adv Parasitol. 2009;69:41–146.

46. Cwiklinski K, Robinson MW, Donnelly S, Dalton JP. Complementary transcriptomic and proteomic analyses reveal the cellular and molecular processes that drive growth and development of *Fasciola hepatica* in the host liver. BMC Genomics. 2021 Dec;22(1):46.

47. Tanabe MB, Caravedo MA, Clinton White AJ, Cabada MM. An update on the pathogenesis of fascioliasis: what do we know? Res Rep Trop Med. 2024;15:13–24.

48. Flores-Velázquez LM, Ruiz-Campillo MT, Herrera-Torres G, Martínez-Moreno Á, Martínez-Moreno FJ, Zafra R, *et al.* Fasciolosis: pathogenesis, host-parasite interactions, and implication in vaccine development. Front Vet Sci. 2023 Dec;11(10):1270064.

49. Taghipour A, Zaki L, Rostami A, Foroutan M, Ghaffarifar F, Fathi A, *et al.* Highlights of human ectopic fascioliasis: a systematic review. Infect Dis (Lond). 2019 Dec;51(11–12):785–92.

50. Donnelly S, Flynn R, Mulcahy G, O'Neill S. Chapter 9. Immunological interaction between *Fasciola* and its host. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 278–308.

51. Spithill TW, Toet H, Rathinasamy V, Zerna G, Swan J, Cameron T, *et al.* Chapter 12. Vaccines for *Fasciola*: new thinking for an old problem. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 379–423.

52. Costa M, Mansilla F, Manuel Sala J, Saravia A, Ubios D, Lores P, *et al. Fasciola hepatica* infection modifies IgG1 specific immune response to foot-and-mouth disease virus induced by vaccination. Vaccine. 2024 Jan 25;42(3):541–7.

53. Krump L, Hamilton CM, Sekiya M, O'Neill R, Mulcahy G. The effect of *Fasciola hepatica* infection on respiratory vaccine responsiveness in calves. Vet Parasitol. 2014 Mar 17;201(1–2):31–9.

54. Dalton JP, Robinson MW, Mulcahy G, O'Neill SM, Donnelly S. Immunomodulatory molecules of *Fasciola hepatica*: candidates for both vaccine and immunotherapeutic development. Vet Parasitol. 2013 Aug;195(3–4):272–85.

55. Walsh KP, Brady MT, Finlay CM, Boon L, Mills KHG. Infection with a helminth parasite attenuates autoimmunity through TGF-β-mediated suppression of Th_{17} and Th_1 responses. J Immunol. 2009 Aug 1;183(3):1577–86.

56. Lalor R, Tanaka A, Shiels J, Dixit A, Hoadley S, Dufourd E, *et al.* An immunoregulatory amphipathic peptide derived from *Fasciola hepatica* helminth defense molecule (FhHDM-1.C2) exhibits potent biotherapeutic activity in a murine model of multiple sclerosis. FASEB J. 2025 Feb 28;39(4):e70380.

57. Siles-Lucas M, González-Miguel J, Geller R, Sanjuan R, Pérez-Arévalo J, Martínez-Moreno Á. Potential influence of helminth molecules on COVID-19 pathology. Trends Parasitol. 2021 Jan;37(1):11–4.

58. Serrat J, Francés-Gómez C, Becerro-Recio D, González-Miguel J, Geller R, Siles-Lucas M. Antigens from the helminth *Fasciola hepatica* exert antiviral effects against SARS-CoV-2 *in vitro*. Int J Mol Sci. 2023 Jul 18;24(14).

59. Rufino-Moya PJ, Zafra Leva R, Martínez-Moreno Á, Buffoni L, Valderas García E, Pérez Arévalo J, *et al.* Advancement in diagnosis, treatment, and vaccines against *Fasciola hepatica*: a comprehensive review. Pathogens. 2024 Aug 7;13(8).

60. Mezo M, González-Warleta M, Castro-Hermida JA, Muiño L, Ubeira FM. Field evaluation of the MM3-SERO ELISA for detection of anti-*Fasciola* IgG antibodies in milk samples from individual cows and bulk milk tanks. Parasitol Int. 2010;59(4):610–5.

61. Alvarez Rojas CA, Jex AR, Gasser RB, Scheerlinck JPY. Techniques for the diagnosis of *Fasciola* infections in animals: room for improvement. Adv Parasitol. 2014;85:65–107.

62. Mezo M, González-Warleta M, Carro C, Ubeira FM. An ultrasensitive capture ELISA for detection of *Fasciola hepatica* coproantigens in sheep and cattle using a new monoclonal antibody (MM3). J Parasitol. 2004 Aug;90(4):845–52.

63. Martínez-Valladares M, Rojo-Vázquez FA. Loop-mediated isothermal amplification (LAMP) assay for the diagnosis of fasciolosis in sheep and its application under field conditions. Parasites Vectors. 2016 Feb 5;9:73.

64. Amiri S, Shemshadi B, Shirali S, Kheirandish F, Fallahi S. Accurate and rapid detection of *Fasciola hepatica* copro-DNA in sheep using loop-mediated isothermal amplification (LAMP) technique. Vet Med Sci. 2021 Jul;7(4):1316–24.

65. Álvarez LI, Lanusse CE, Williams DJL, Fairweather I, Hodgkinson JE. Chapter 7. Flukicudal drugs: pharmaco-therapeutics and drug resistance. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 211–56.

66. Kelley JM, Elliott TP, Beddoe T, Anderson G, Skuce P, Spithill TW. Current threat of triclabendazole resistance in *Fasciola hepatica*. Trends Parasitol. 2016 Jun;32(6):458–69.

67. Kahl A, von Samson-Himmelstjerna G, Helm C, Hodgkinson J, Williams D, Weiher W, *et al.* Efficacy of flukicides against *Fasciola hepatica* and first report of triclabendazole resistance on German sheep farms. Int J Parasitol Drugs Drug Resist. 2023 Dec;23:94–105.

68. Larroza M, Aguilar M, Soler P, Mora J, Roa M, Cabrera R, *et al.* Triclabendazole resistance in *Fasciola hepatica*: first report in sheep from the Santa Cruz province, Argentinian Patagonia. Vet Parasitol Reg Stud Reports. 2023 Oct;45:100927.

69. Winkelhagen AJS, Mank T, de Vries PJ, Soetekouw R. Apparent triclabendazole-resistant human *Fasciola hepatica* infection, the Netherlands. Emerg Infect Dis. 2012 Jun;18(6):1028–9.

70. Morales ML, Tanabe MB, White ACJ, Lopez M, Bascope R, Cabada MM. Triclabendazole treatment failure for *Fasciola hepatica* infection among preschool and school-age children, Cusco, Peru. Emerg Infect Dis. 2021 Jul;27(7):1850–7.

71. Fairweather I, Brennan GP, Hanna REB, Robinson MW, Skuce PJ. Drug resistance in liver flukes. Int J Parasitol Drugs Drug Resist. 2020 Apr;12:39–59.

72. Beesley NJ, Cwiklinski K, Allen K, Hoyle RC, Spithill TW, La Course EJ, *et al.* A major locus confers triclabendazole resistance in *Fasciola hepatica* and shows dominant inheritance. PLoS Pathog. 2023 Jan;19(1):e1011081.

73. Geary TG, Sakanari JA, Caffrey CR. Anthelmintic drug discovery: into the future. J Parasitol. 2015 Apr;101(2):125–33.

74. Molina-Hernández V, Mulcahy G, Pérez J, Martínez-Moreno Á, Donnelly S, O'Neill SM, *et al. Fasciola hepatica* vaccine: we may not be there yet but we're on the right road. Vet Parasitol. 2015 Feb;208(1–2):101–11.

75. Spithill TW, Smooker PM, Sexton JL, Bozas E, Morrison CA, Creaney J, *et al.* Chapter 11. Development of vaccines against *Fasciola hepatica*. In: Fasciolosis. 1st ed. UK: CABI; 1999. p. 377–411.

76. Cwiklinski K, Drysdale O, López Corrales J, Corripio-Miyar Y, De Marco Verissimo C, Jewhurst H, *et al.* Targeting secreted protease/anti-protease balance as a vaccine strategy against the helminth *Fasciola hepatica*. Vaccines (Basel). 2022 Jan 20;10(2).

77. van Milligen FJ, Cornelissen JB, Bokhout BA. *Fasciola hepatica*: an antigen fraction derived from newly excysted juveniles, containing an immunoreactive 32-kDa protein, induces strong protective immunity in rats. Exp Parasitol. 2000 Mar;94(3):163–71.

78. Reszka N, Cornelissen JBWJ, Harmsen MM, Bieńkowska-Szewczyk K, de Bree J, Boersma WJ, *et al. Fasciola hepatica* procathepsin L3 protein expressed by a baculovirus recombinant can partly protect rats against fasciolosis. Vaccine. 2005 Apr 27;23(23):2987–93.

79. Jayaraj R, Piedrafita D, Dynon K, Grams R, Spithill TW, Smooker PM. Vaccination against fasciolosis by a multivalent vaccine of stage-specific antigens. Vet Parasitol. 2009 Mar 23;160(3–4):230–6.

80. Wesołowska A, Basałaj K, Norbury LJ, Sielicka A, Wędrychowicz H, Zawistowska-Deniziak A. Vaccination against *Fasciola hepatica* using cathepsin L3 and B3 proteases delivered alone or in combination. Vet Parasitol. 2018 Jan 30;250:15–21.

81. Wesołowska A, Basałaj K, Norbury LJ, Sielicka A, Wędrychowicz H, Zawistowska-Deniziak A. Sex and vaccination: insights from female rats vaccinated with juvenile-specific proteases from *Fasciola hepatica*. Vet Parasitol. 2018 May 15;255:91–6.

82. Norbury LJ, Basalaj K, Zawistowska-Deniziak A, Sielicka A, Wilkowski P, Wesolowska A, *et al.* Intranasal delivery of a formulation containing stage-specific recombinant proteins of *Fasciola hepatica* cathepsin L5 and cathepsin B2 triggers an anti-fecundity effect and an adjuvant-mediated reduction in fluke burden in sheep. Vet Parasitol. 2018 Jul 15;258:14–23.

83. McCusker P, Toet H, Rathinasamy V, Young N, Beddoe T, Anderson G, *et al.* Molecular characterisation and vaccine efficacy of two novel developmentally regulated surface tegument proteins of *Fasciola hepatica*. Vet Parasitol. 2020 Oct;286:109244.

84. González-Miguel J, Becerro-Recio D, Siles-Lucas M. Insights into *Fasciola hepatica* juveniles: crossing the fasciolosis rubicon. Trends Parasitol. 2021 Jan;37(1):35–47.

85. Pompili S, Latella G, Gaudio E, Sferra R, Vetuschi A. The charming world of the extracellular matrix: a dynamic and protective network of the intestinal wall. Front Med. 2021 Apr;8:610189.

86. van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol. 2009;71:241–60.

87. Llanos Fernández JL, Zilvetty Pardo ES, Llano Fernández D. Capítulo 19. Sistema digestivo: tubo digestivo. In: Atlas: Histología. Cochabamba: Universidad Privada del Valle; 2019. p. 205–9.

88. Alberts B, Heald R, Johnson A, Morgan D, Raff M, Roberts K, *et al.* Chapter 19. Cell junctions and the extracellular matrix. In: Molecular Biology of the Cell. 7th ed. New York: W. W. Norton & Company; 2022. p. 1105–46.

89. Mowat AM, Agace WW. Regional specialization within the intestinal immune system. Nat Rev Immunol. 2014 Oct;14(10):667–85.

90. Sorenson RL, Brelje C. Chapter 14. Gastrointestinal tract. In: Atlas of human histology: A guide to microscopic structure of cells, tissues and organs. 3rd ed. USA; 2014. p. 223–367.

91. Meran L, Baulies A, Li VSW. Intestinal Stem Cell Niche: The Extracellular Matrix and Cellular Components. Stem Cells Int. 2017;2017:7970385.

92. Alberts B, Heald R, Johnson A, Morgan D, Raff M, Roberts K, *et al.* Chapter 22. Stem cells in tissue homeostasis and regeneration. In: Molecular Biology of the Cell. 7th ed. New York: W. W. Norton & Company; 2022. p. 1279–313.

93. Hickey JW, Becker WR, Nevins SA, Horning A, Perez AE, Zhu C, *et al.* Organization of the human intestine at single-cell resolution. Nature. 2023 Jul 1;619(7970):572–84.

94. Elmentaite R, Kumasaka N, Roberts K, Fleming A, Dann E, King HW, *et al.* Cells of the human intestinal tract mapped across space and time. Nature. 2021 Sep;597(7875):250–5.

95. Oda M, Hatano Y, Sato T. Intestinal epithelial organoids: regeneration and maintenance of the intestinal epithelium. Curr Opin Genet Dev. 2022 Oct;76:101977.

96. Beumer J, Clevers H. Cell fate specification and differentiation in the adult mammalian intestine. Nat Rev Mol Cell Biol. 2021 Jan;22(1):39–53.

97. Huang L, Bernink JH, Giladi A, Krueger D, van Son GJF, Geurts MH, *et al.* Tuft cells act as regenerative stem cells in the human intestine. Nature. 2024 Oct;634(8035):929–35.

98. Gustafsson JK, Johansson MEV. The role of goblet cells and mucus in intestinal homeostasis. Nat Rev Gastroenterol Hepatol. 2022 Dec;19(12):785–803.

99. Bas J, Jay P, Gerbe F. Intestinal tuft cells: sentinels, what else? Semin Cell Dev Biol. 2023 Dec;150–151:35–42.

100. Welcome MO. Intercellular network of junctions of the gastrointestinal Tract. In: Welcome MO, editor. Gastrointestinal Physiology: Development, Principles and Mechanisms of Regulation. Springer International Publishing; 2018. p. 201–25.

101. Rao R, Samak G. Bile duct epithelial tight junctions and barrier function. Tissue Barriers. 2013 Oct 1;1(4):e25718.

102. Kollmann C, Niklas C, Ernestus K, Hiller GG, Hörner M, Burkard N, *et al.* The phenotype of necrotizing enterocolitis correlates with distinct changes of intestinal junctional proteins. Pathol Res Pract. 2025;265:155731.

103. Pan YY, Deng Y, Su S, Yin JH, Chen YH, Wang LC, *et al.* Structure composition and intracellular transport of clathrin-mediated intestinal transmembrane tight junction protein. Inflammation. 2023 Feb;46(1):18–34.

104. Ivanov AI, Naydenov NG. Chapter 2. Dynamics and regulation of epithelial adherens junctions: recent discoveries and controversies. In: Jeon KW, editor. International Review of Cell and Molecular Biology. Academic Press; 2013. p. 27–99.

105. Hartsock A, Nelson WJ. Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. Biochim Biophys Acta. 2008 Mar 1;1778(3):660–9.

106. Zihni C, Balda MS, Matter K. Signalling at tight junctions during epithelial differentiation and microbial pathogenesis. J Cell Sci. 2014 Aug 15;127(16):3401–13.

107. Müller L, Hatzfeld M, Keil R. Desmosomes as signaling hubs in the regulation of cell behavior. Front Cell Dev Biol. 2021;9:745670.

108. González-Mariscal L, Domínguez-Calderón A, Raya-Sandino A, Ortega-Olvera JM, Vargas-Sierra O, Martínez-Revollar G. Tight junctions and the regulation of gene expression. Semin Cell Dev Biol. 2014 Dec 1;36:213–23.

109. Horowitz A, Chanez-Paredes SD, Haest X, Turner JR. Paracellular permeability and tight junction regulation in gut health and disease. Nat Rev Gastroenterol Hepatol. 2023 Jul 1;20(7):417–32.

110. Ngendahayo Mukiza C, Dubreuil JD. *Escherichia coli* heat-stable toxin b impairs intestinal epithelial barrier function by altering tight junction proteins. Infect Immun. 2013 Aug;81(8):2819–27.

111. Vaswani CM, Varkouhi AK, Gupta S, Ektesabi AM, Tsoporis JN, Yousef S, *et al.* Preventing occludin tight-junction disruption via inhibition of microRNA-193b-5p attenuates viral load and influenza-induced lung injury. Mol Ther. 2023 Sep 6;31(9):2681–701.

112. Caron TJ, Scott KE, Fox JG, Hagen SJ. Tight junction disruption: *Helicobacter pylori* and dysregulation of the gastric mucosal barrier. World J Gastroenterol. 2015 Oct 28;21(40):11411–27.

113. Al-Sadi R, Abdulqadir R, Ma TY. Editorial: Role of matrix metalloproteinases and other inflammatory mediators in the disruption of the intestinal tight junction barrier. Front Immunol. 2023;14:1194827.

114. Bonnans C, Chou J, Werb Z. Remodelling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol. 2014 Dec;15(12):786–801.

115. Khan S, Hashmi GS, Ii J. Histology and functions of connective tissues: a review. University J Dent Scie. 2015;1(1):1–2.

116. Hastings JF, Skhinas JN, Fey D, Croucher DR, Cox TR. The extracellular matrix as a key regulator of intracellular signalling networks: ECM regulation of intracellular signalling networks. Br J Pharmacol. 2019 Jan;176(1):82–92.

117. Singh B, Fleury C, Jalalvand F, Riesbeck K. Human pathogens utilize host extracellular matrix proteins laminin and collagen for adhesion and invasion of the host. FEMS Microbiol Rev. 2012 Nov;36(6):1122–80.

118. Robinson MW, Corvo I, Jones PM, George AM, Padula MP, To J, *et al.* Collagenolytic activities of the major secreted cathepsin L peptidases involved in the virulence of the helminth pathogen, *Fasciola hepatica*. PLoS Negl Trop Dis. 2011 Apr 5;5(4):e1012.

119. Clements KM, Flannelly JK, Tart J, Brockbank SMV, Wardale J, Freeth J, *et al.* Matrix metalloproteinase 17 is necessary for cartilage aggrecan degradation in an inflammatory environment. Ann Rheum Dis. 2011 Apr;70(4):683–9.

120. Starr AE, Bellac CL, Dufour A, Goebeler V, Overall CM. Biochemical characterization and N-terminomics analysis of leukolysin, the membrane-type 6 matrix metalloprotease (MMP25): chemokine and vimentin cleavages enhance cell migration and macrophage phagocytic activities. J Biol Chem. 2012 Apr 13;287(16):13382–95.

121. Herzog C, Haun RS, Kaushal GP. Role of meprin metalloproteinases in cytokine processing and inflammation. Cytokine. 2019 Feb;114:18–25.

122. Lijnen HR. Plasmin and matrix metalloproteinases in vascular remodeling. Thromb Haemost. 2001 Jul;86(1):324–33.

123. Engelse MA, Hanemaaijer R, Koolwijk P, van Hinsbergh VW. The fibrinolytic system and matrix metalloproteinases in angiogenesis and tumor progression. Semin Thromb Hemost. 2004 Feb;30(1):71–82.

124. Cauwe B, Opdenakker G. Intracellular substrate cleavage: a novel dimension in the biochemistry, biology and pathology of matrix metalloproteinases. Crit Rev Biochem Mol Biol. 2010 Oct;45(5):351–423.

125. Cauwe B, Van den Steen PE, Opdenakker G. The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases. Crit Rev Biochem Mol Biol. 2007 Jun;42(3):113–85.

126. Rodríguez D, Morrison CJ, Overall CM. Matrix metalloproteinases: what do they not do? New substrates and biological roles identified by murine models and proteomics. Biochim Biophys Acta. 2010 Jan 1;1803(1):39–54.

127. Seltzer JL, Eisen AZ. Native type I collagen is not a substrate for MMP2 (gelatinase A). J Invest Dermatol. 1999 Jun;112(6):993–4.

128. Ahokas K, Lohi J, Illman SA, Llano E, Elomaa O, Impola U, *et al.* Matrix metalloproteinase-21 is expressed epithelially during development and in cancer and

is up-regulated by transforming growth factor-beta1 in keratinocytes. Lab Invest. 2003 Dec;83(12):1887–99.

129. Zhao Z, Yan L, Li S, Sun H, Zhou Y, Li X. Increased MMP-21 expression in esophageal squamous cell carcinoma is associated with progression and prognosis. Med Oncol. 2014 Aug;31(8):91.

130. Moogk D, da Silva IP, Ma MW, Friedman EB, de Miera EVS, Darvishian F, *et al.* Melanoma expression of matrix metalloproteinase-23 is associated with blunted tumor immunity and poor responses to immunotherapy. J Transl Med. 2014 Dec 10;12:342.

131. Ohnishi J, Ohnishi E, Jin M, Hirano W, Nakane D, Matsui H, *et al.* Cloning and characterization of a rat ortholog of MMP-23 (matrix metalloproteinase-23), a unique type of membrane-anchored matrix metalloproteinase and conditioned switching of its expression during the ovarian follicular development. Mol Endocrinol. 2001 May;15(5):747–64.

132. Xia H, Hu Q, Yang Y, Yuan H, Cai Y, Liu Z, *et al.* Effect of matrix metalloproteinase 23 accelerating wound healing induced by hydroxybutyl chitosan. ACS Appl Bio Mater. 2023 Apr 17;6(4):1460–70.

133. Saarialho-Kere U, Kerkelä E, Jahkola T, Suomela S, Keski-Oja J, Lohi J. Epilysin (MMP-28) expression is associated with cell proliferation during epithelial repair. J Invest Dermatol. 2002 Jul;119(1):14–21.

134. Lohi J, Wilson CL, Roby JD, Parks WC. Epilysin, a novel human matrix metalloproteinase (MMP-28) expressed in testis and keratinocytes and in response to injury. J Biol Chem. 2001 Mar 30;276(13):10134–44.

135. Velasco G, Pendás AM, Fueyo A, Knäuper V, Murphy G, López-Otín C. Cloning and characterization of human MMP-23, a new matrix metalloproteinase predominantly expressed in reproductive tissues and lacking conserved domains in other family members. J Biol Chem. 1999 Feb 19;274(8):4570–6.

136. Troncoso MF, Elola MT, Blidner AG, Sarrias L, Espelt MV, Rabinovich GA. The universe of galectin-binding partners and their functions in health and disease. J Biol Chem. 2023 Dec;299(12):105400.

137. Shin SM, Cho KS, Choi MS, Lee SH, Han SH, Kang YS, *et al.* Urokinase-type plasminogen activator induces BV-2 microglial cell migration through activation of matrix metalloproteinase-9. Neurochem Res. 2010 Jul;35(7):976–85.

138. Keski-Oja J, Lohi J, Tuuttila A, Tryggvason K, Vartio T. Proteolytic processing of the 72,000-Da type IV collagenase by urokinase plasminogen activator. Exp Cell Res. 1992 Oct;202(2):471–6.

139. de Bruyn M, Vandooren J, Ugarte-Berzal E, Arijs I, Vermeire S, Opdenakker G. The molecular biology of matrix metalloproteinases and tissue inhibitors of metalloproteinases in inflammatory bowel diseases. Crit Rev Biochem Mol Biol. 2016;51(5):295–358.

140. Mattos EC, Canuto G, Manchola NC, Magalhães RDM, Crozier TWM, Lamont DJ, *et al.* Reprogramming of *Trypanosoma cruzi* metabolism triggered by parasite interaction with the host cell extracellular matrix. PLoS Negl Trop Dis. 2019 Feb 6;13(2):e0007103.

141. Brown H, Esterházy D. Intestinal immune compartmentalization: implications of tissue specific determinants in health and disease. Mucosal Immunol. 2021 Nov 1;14(6):1259–70.

142. Santaolalla R, Abreu MT. Innate immunity in the small intestine. Curr Opin Gastroenterol. 2012 Mar;28(2):124–9.

143. Clevers HC, Bevins CL. Paneth cells: maestros of the small intestinal crypts. Annu Rev Physiol. 2013;75:289–311.

144. Bąska P, Norbury LJ. The role of the intestinal epithelium in the "weep and sweep" response during gastro-intestinal helminth infections. Animals (Basel). 2022 Jan 12;12(2).

145. Gerbe F, Sidot E, Smyth DJ, Ohmoto M, Matsumoto I, Dardalhon V, *et al.* Intestinal epithelial tuft cells initiate type 2 mucosal immunity to helminth parasites. Nature. 2016 Jan 14;529(7585):226–30.

146. von Moltke J, Ji M, Liang HE, Locksley RM. Tuft-cell-derived IL-25 regulates an intestinal ILC2-epithelial response circuit. Nature. 2016 Jan 14;529(7585):221–5.

147. Howitt MR, Lavoie S, Michaud M, Blum AM, Tran SV, Weinstock JV, *et al.* Tuft cells, taste-chemosensory cells, orchestrate parasite type 2 immunity in the gut. Science. 2016 Mar 18;351(6279):1329–33.

148. Bennett CE, Threadgold LT. Electron microscope studies of *Fasciola hepatica*. 13. Fine structure of newly excysted juvenile. Exp Parasitol. 1973 Aug;34(1):85–99.

149. Wilson RA, Wright JM, de Castro-Borges W, Parker-Manuel SJ, Dowle AA, Ashton PD, *et al.* Exploring the *Fasciola hepatica* tegument proteome. Int J Parasitol. 2011 Nov;41(13–14):1347–59.

150. Ravidà A, Cwiklinski K, Aldridge AM, Clarke P, Thompson R, Gerlach JQ, *et al.* *Fasciola hepatica* surface tegument: glycoproteins at the interface of parasite and host. Mol Cell Proteomics. 2016 Oct;15(10):3139–53.

151. Becerro-Recio D, Serrat J, López-García M, Molina-Hernández V, Pérez-Arévalo J, Martínez-Moreno Á, *et al.* Study of the migration of *Fasciola hepatica* juveniles across the intestinal barrier of the host by quantitative proteomics in an *ex vivo* model. PLoS Negl Trop Dis. 2022 Sep;16(9):e0010766.

152. Bennett CE, Threadgold LT. *Fasciola hepatica*: development of tegument during migration in mouse. Exp Parasitol. 1975 Aug;38(1):38–55.

153. Cwiklinski K, De Marco Verissimo C, McVeigh P, Donnelly S, Dalton JP, Cameron T, *et al.* Chapter 11. Applying 'omics' technologies to understand *Fasciola* spp. biology. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 338–79.

154. Cwiklinski K, Jewhurst H, McVeigh P, Barbour T, Maule AG, Tort J, *et al.* Infection by the helminth parasite *Fasciola hepatica* requires rapid regulation of metabolic, virulence, and invasive factors to adjust to its mammalian host. Mol Cell Proteomics. 2018;17:792–809.

155. Di Maggio LS, Tirloni L, Pinto AFM, Diedrich JK, Yates Iii JR, Benavides U, *et al.* Across intra-mammalian stages of the liver fluke *Fasciola hepatica*: a proteomic study. Sci Rep. 2016 Sep 7;6:32796.

156. Sánchez-López CM, Trelis M, Jara L, Cantalapiedra F, Marcilla A, Bernal D. Diversity of extracellular vesicles from different developmental stages of *Fasciola hepatica*. Int J Parasitol. 2020 Aug;50(9):663–9.

157. de la Torre-Escudero E, Gerlach JQ, Bennett APS, Cwiklinski K, Jewhurst HL, Huson KM, *et al.* Surface molecules of extracellular vesicles secreted by the helminth pathogen *Fasciola hepatica* direct their internalisation by host cells. PLoS Negl Trop Dis. 2019 Jan;13(1):e0007087.

158. Trelis M, Sánchez-López CM, Sánchez-Palencia LF, Ramírez-Toledo V, Marcilla A, Bernal D. Proteomic analysis of extracellular vesicles from *Fasciola hepatica* hatching eggs and juveniles in culture. Front Cell Infect Microbiol. 2022 Jun 3;12:903602.

159. Ricafrente A, Nguyen H, Tran N, Donnelly S. An evaluation of the *Fasciola hepatica* miRnome predicts a targeted regulation of mammalian innate immune responses. Front Immunol. 2020;11:608686.

160. McCusker P, Clarke NG, Gardiner E, Armstrong R, McCammick EM, McVeigh P, *et al.* Neoblast-like stem cells of *Fasciola hepatica*. PLoS Pathog. 2024 May;20(5):e1011903.

161. Correia L, Podevin P, Borderie D, Verthier N, Montet JC, Feldmann G, *et al.* Effects of bile acids on the humoral immune response: a mechanistic approach. Life Sciences. 2001 Oct 5;69(20):2337–48.

162. Japa O, Hodgkinson JE, Emes RD, Flynn RJ. TGF-β superfamily members from the helminth *Fasciola hepatica* show intrinsic effects on viability and development. Vet Res. 2015 Mar 11;46:29.

163. Sulaiman AA, Zolnierczyk K, Japa O, Owen JP, Maddison BC, Emes RD, *et al.* A trematode parasite derived growth factor binds and exerts influences on host immune functions via host cytokine receptor complexes. PLoS Pathog. 2016 Nov;12(11):e1005991.

164. Marcilla A, Trelis M, Cortés A, Sotillo J, Cantalapiedra F, Minguez MT, *et al.* Extracellular vesicles from parasitic helminths contain specific excretory/secretory proteins and are internalized in intestinal host cells. PLoS ONE. 2012;7(9):e45974.

165. Carmona C, Dowd AJ, Smith AM, Dalton JP. Cathepsin L proteinase secreted by *Fasciola hepatica in vitro* prevents antibody-mediated eosinophil attachment to newly excysted juveniles. Mol Biochem Parasitol. 1993 Nov;62(1):9–17.

166. Berasain P, Carmona C, Frangione B, Dalton JP, Goñi F. *Fasciola hepatica*: parasite-secreted proteinases degrade all human IgG subclasses: determination of the specific cleavage sites and identification of the immunoglobulin fragments produced. Exp Parasitol. 2000 Feb;94(2):99–110.

167. Donnelly S, O'Neill SM, Stack CM, Robinson MW, Turnbull L, Whitchurch C, *et al.* Helminth cysteine proteases inhibit TRIF-dependent activation of macrophages via degradation of TLR3. J Biol Chem. 2010 Jan 29;285(5):3383–92.

168. Celias DP, Corvo I, Silvane L, Tort JF, Chiapello LS, Fresno M, *et al.* Cathepsin L3 from *Fasciola hepatica* induces NLRP3 inflammasome alternative activation in murine dendritic cells. Front Immunol. 2019 Mar 22;10:552.

169. Zaiss MM, Maslowski KM, Mosconi I, Guenat N, Marsland BJ, Harris NL. IL-1β suppresses innate IL-25 and IL-33 production and maintains helminth chronicity. PLoS Pathog. 2013;9(8):e1003531.

170. De Marco Verissimo C, Jewhurst HL, Tikhonova IG, Urbanus RT, Maule AG, Dalton JP, *et al.* *Fasciola hepatica* serine protease inhibitor family (serpins): purposely crafted for regulating host proteases. PLoS Negl Trop Dis. 2020 Aug;14(8):e0008510.

171. De Marco Verissimo C, Jewhurst HL, Dobó J, Gál P, Dalton JP, Cwiklinski K. *Fasciola hepatica* is refractory to complement killing by preventing attachment of mannose binding lectin (MBL) and inhibiting MBL-associated serine proteases (MASPs) with serpins. PLoS Pathog. 2022 Jan 10;18(1):e1010226.

172. Smith D, Cwiklinski K, Jewhurst H, Tikhonova IG, Dalton JP. An atypical and functionally diverse family of Kunitz-type cysteine/serine proteinase inhibitors secreted by the helminth parasite *Fasciola hepatica*. Sci Rep. 2020 Nov 26;10(1):20657.

173. Robinson MW, Donnelly S, Hutchinson AT, To J, Taylor NL, Norton RS, *et al.* A family of helminth molecules that modulate innate cell responses via molecular mimicry of host antimicrobial peptides. PLoS Pathog. 2011 May;7(5):e1002042.

174. Read AF, Skorping A. The evolution of tissue migration by parasitic nematode larvae. Parasitology. 1995 Sep;111(Pt 3):359–71.

175. Cwiklinski K, Donnelly S, Drysdale O, Jewhurst H, Smith D, De Marco Verissimo C, *et al.* The cathepsin-like cysteine peptidases of trematodes of the genus *Fasciola*. In: Advances in Parasitology. Elsevier; 2019. p. 113–64.

176. Pritsch IC, Tikhonova IG, Jewhurst HL, Drysdale O, Cwiklinski K, Molento MB, *et al.* Regulation of the *Fasciola hepatica* newly excysted juvenile cathepsin L3 (FhCL3) by its propeptide: a proposed 'clamp-like' mechanism of binding and inhibition. BMC Mol Cell Biol. 2020 Dec 7;21(1):90.

177. Corvo I, Cancela M, Cappetta M, Pi-Denis N, Tort JF, Roche L. The major cathepsin L secreted by the invasive juvenile *Fasciola hepatica* prefers proline in the S2 subsite and can cleave collagen. Mol Biochem Parasitol. 2009 Sep;167(1):41–7.

178. González-Miguel J, Siles-Lucas M, Kartashev V, Morchón R, Simón F. Plasmin in parasitic chronic infections: friend or foe? Trends Parasitol. 2016 Apr;32(4):325–35.

179. Diosdado A, Simón F, Serrat J, González-Miguel J. Interaction of helminth parasites with the haemostatic system of their vertebrate hosts: a scoping review. Parasite. 2022;29:35.

180. González-Miguel J, Valero MA, Reguera-Gomez M, Mas-Bargues C, Bargues MD, Simón F, *et al.* Numerous *Fasciola* plasminogen-binding proteins may underlie blood-brain barrier leakage and explain neurological disorder complexity and heterogeneity

in the acute and chronic phases of human fascioliasis. Parasitology. 2019 Mar;146(3):284–98.

181. Versteeg HH, Heemskerk JWM, Levi M, Reitsma PH. New fundamentals in hemostasis. Physiol Rev. 2013 Jan;93(1):327–58.
182. Stassen JM, Arnout J, Deckmyn H. The hemostatic system. Curr Med Chem. 2004 Sep;11(17):2245–60.
183. Gale AJ. Continuing education course #2: current understanding of hemostasis. Toxicol Pathol. 2011 Jan;39(1):273–80.
184. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. Br J Haematol. 2005 May;129(3):307–21.
185. Lijnen HR. Elements of the fibrinolytic system. Ann N Y Acad Sci. 2001;936(1):226–36.
186. Castellino FJ, Ploplis VA. Human plasminogen: structure, activation, and function. In: Waisman DM, editor. Plasminogen: structure, activation, and regulation. Boston, MA: Springer US; 2003. p. 3–17.
187. Miles LA, Castellino FJ, Gong Y. Critical role for conversion of Glu-plasminogen to Lys-plasminogen for optimal stimulation of plasminogen activation on cell surfaces. Trends Cardiovasc Med. 2003 Jan;13(1):21–30.
188. Law RHP, Caradoc-Davies T, Cowieson N, Horvath AJ, Quek AJ, Encarnacao JA, *et al.* The X-ray crystal structure of full-length human plasminogen. Cell Rep. 2012 Mar 29;1(3):185–90.
189. Godier A, Hunt BJ. Plasminogen receptors and their role in the pathogenesis of inflammatory, autoimmune and malignant disease. J Thromb Haemost. 2013 Jan;11(1):26–34.
190. Flick MJ, Bugge TH. Plasminogen-receptor (KT): plasminogen activation and beyond. J Thromb Haemost. 2017 Jan;15(1):150–4.
191. Das R, Pluskota E, Plow EF. Plasminogen and its receptors as regulators of cardiovascular inflammatory responses. Trends Cardiovasc Med. 2010 May;20(4):120–4.
192. McCormack PL. Tranexamic Acid. Drugs. 2012 Mar 1;72(5):585–617.
193. Didiasova M, Wujak L, Wygrecka M, Zakrzewicz D. From plasminogen to plasmin: role of plasminogen receptors in human cancer. Int J Mol Sci. 2014;15(11):21229–52.

194. Miles LA, Ny L, Wilczynska M, Shen Y, Ny T, Parmer RJ. Plasminogen receptors and fibrinolysis. Int J Mol Sci. 2021 Feb 8;22(4).

195. Hawley SB, Tamura T aki, Miles LA. Purification, cloning, and characterization of a profibrinolytic plasminogen-binding protein, TIP49a. J Biol Chem. 2001 Jan 5;276(1):179–86.

196. Miles LA, Parmer RJ. Plasminogen receptors: the first quarter century. Semin Thromb Hemost. 2013 Jun;39(4):329–37.

197. Miles LA, Lighvani S, Baik N, Andronicos NM, Chen EI, Parmer CM, *et al.* The plasminogen receptor, Plg-R(KT), and macrophage function. J Biomed Biotechnol. 2012;2012:250464.

198. Chauhan AS, Kumar M, Chaudhary S, Patidar A, Dhiman A, Sheokand N, *et al.* Moonlighting glycolytic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH): an evolutionarily conserved plasminogen receptor on mammalian cells. FASEB J. 2017 Jun;31(6):2638–48.

199. Sirover MA. Subcellular dynamics of multifunctional protein regulation: mechanisms of GAPDH intracellular translocation. J Cell Biochem. 2012 Jul;113(7):2193–200.

200. Plow EF, Doeuvre L, Das R. So many plasminogen receptors: why? J Biomed Biotechnol. 2012;2012:141806.

201. Miles LA, Andronicos NM, Baik N, Parmer RJ. Cell-surface actin binds plasminogen and modulates neurotransmitter release from catecholaminergic cells. J Neurosci. 2006 Dec 13;26(50):13017–24.

202. Parkkinen J, Raulo E, Merenmies J, Nolo R, Kajander EO, Baumann M, *et al.* Amphoterin, the 30-kDa protein in a family of HMG1-type polypeptides. Enhanced expression in transformed cells, leading edge localization, and interactions with plasminogen activation. J Biol Chem. 1993 Sep 15;268(26):19726–38.

203. Didiasova M, Zakrzewicz D, Magdolen V, Nagaraj C, Bálint Z, Rohde M, *et al.* STIM1/ORAI1-mediated Ca^{2+} influx regulates enolase-1 exteriorization. J Biol Chem. 2015 May 8;290(19):11983–99.

204. Díaz-Ramos À, Roig-Borrellas A, García-Melero A, López-Alemany R. *a* -Enolase, a multifunctional protein: its role on pathophysiological situations. J Biomed Biotechnol. 2012;2012:1–12.

205. Wygrecka M, Marsh LM, Morty RE, Henneke I, Guenther A, Lohmeyer J, *et al.* Enolase-1 promotes plasminogen-mediated recruitment of monocytes to the acutely inflamed lung. Blood. 2009 May 28;113(22):5588–98.

206. Jeffery CJ. Moonlighting proteins. Trends Biochem Sci. 1999 Jan;24(1):8–11.

207. Singh N, Bhalla N. Moonlighting Proteins. Annu Rev Genet. 2020;54:265–85.

208. Karkowska-Kuleta J, Kozik A. Moonlighting proteins as virulence factors of pathogenic fungi, parasitic protozoa and multicellular parasites. Mol Oral Microbiol. 2014 Dec;29(6):270–83.

209. Didiasova M, Schaefer L, Wygrecka M. When place matters: shuttling of enolase-1 across cellular compartments. Front Cell Dev Biol. 2019;7:61.

210. Bdeir K, Kuo A, Sachais BS, Rux AH, Bdeir Y, Mazar A, *et al.* The kringle stabilizes urokinase binding to the urokinase receptor. Blood. 2003 Nov 15;102(10):3600–8.

211. Bugge TH, Flick MJ, Danton MJ, Daugherty CC, Romer J, Dano K, *et al.* Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator. Proc Natl Acad Sci U S A. 1996 Jun 11;93(12):5899–904.

212. Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, Bronson R, *et al.* Physiological consequences of loss of plasminogen activator gene function in mice. Nature. 1994 Mar 31;368(6470):419–24.

213. Rijken DC, Lijnen HR. New insights into the molecular mechanisms of the fibrinolytic system. J Thromb Haemost. 2009 Jan;7(1):4–13.

214. Nicholl S, Roztocil E, Davies M. Plasminogen activator system and vascular disease. CVP. 2006 Apr 1;4(2):101–16.

215. Crippa MP. Urokinase-type plasminogen activator. Int J Biochem Cell Biol. 2007 Jan 1;39(4):690–4.

216. Aisina RB, Mukhametova LI. Structure and function of plasminogen/plasmin system. Russ J Bioorg Chem. 2014 Nov;40(6):590–605.

217. Mahmood N, Mihalcioiu C, Rabbani SA. Multifaceted role of the urokinase-type plasminogen activator (uPA) and its receptor (uPAR): diagnostic, prognostic, and therapeutic applications. Front Oncol. 2018;8:24.

218. Goretzki L, Schmitt M, Mann K, Calvete J, Chucholowski N, Kramer M, *et al.* Effective activation of the proenzyme form of the urokinase-type plasminogen activator (pro-uPA) by the cysteine protease cathepsin L. FEBS Lett. 1992 Feb 3;297(1–2):112–8.

219. Kobayashi H, Schmitt M, Goretzki L, Chucholowski N, Calvete J, Kramer M, *et al.* Cathepsin B efficiently activates the soluble and the tumor cell receptor-bound form

of the proenzyme urokinase-type plasminogen activator (Pro-uPA). J Biol Chem. 1991 Mar 15;266(8):5147–52.

220. Torres-Paris C, Chen Y, Xiao L, Song HJ, Chen P, Komives EA. The autoactivation of human single-chain urokinase-type plasminogen activator (uPA). J Biol Chem. 2023 Oct 1;299(10):105179.

221. Hamada M, Varkoly KS, Riyadh O, Beladi R, Munuswamy-Ramanujam G, Rawls A, *et al.* Urokinase-type plasminogen activator receptor (uPAR) in inflammation and disease: a unique inflammatory pathway activator. Biomedicines. 2024 May 24;12(6).

222. Gibson PR, Birchall I, Rosella O, Albert V, Finch CF, Barkla DH, *et al.* Urokinase and the intestinal mucosa: evidence for a role in epithelial cell turnover. Gut. 1998 Nov 1;43(5):656–63.

223. Diaz A, Martin-Jimenez C, Xu Y, Merino P, Woo Y, Torre E, *et al.* Urokinase-type plasminogen activator-mediated crosstalk between N-cadherin and β-catenin promotes wound healing. J Cell Sci. 2021 Jun 1;134(11).

224. Smith HW, Marshall CJ. Regulation of cell signalling by uPAR. Nat Rev Mol Cell Biol. 2010 Jan;11(1):23–36.

225. Desmedt S, Desmedt V, Delanghe JR, Speeckaert R, Speeckaert MM. The intriguing role of soluble urokinase receptor in inflammatory diseases. Crit Rev Clin Lab Sci. 2017 Mar;54(2):117–33.

226. Rasmussen LJH, Petersen JEV, Eugen-Olsen J. Soluble urokinase plasminogen activator receptor (suPAR) as a biomarker of systemic chronic inflammation. Front Immunol. 2021;12:780641.

227. Ferraris GMS, Sidenius N. Urokinase plasminogen activator receptor: a functional integrator of extracellular proteolysis, cell adhesion, and signal transduction. Semin Thromb Hemost. 2013 Jun;39(4):347–55.

228. Schaller J, Gerber SS. The plasmin-antiplasmin system: structural and functional aspects. Cell Mol Life Sci. 2011 Mar;68(5):785–801.

229. Meijers JCM, Herwald H. Protein C inhibitor. Semin Thromb Hemost. 2011 Jun;37(4):349–54.

230. Strickland DK, Kounnas MZ, Argraves WS. LDL receptor-related protein: a multiligand receptor for lipoprotein and proteinase catabolism. FASEB J. 1995 Jul;9(10):890–8.

231. Herz J, Strickland DK. LRP: a multifunctional scavenger and signaling receptor. J Clin Invest. 2001 Sep;108(6):779–84.

232. Sillen M, Declerck PJ. A narrative review on plasminogen activator inhibitor-1 and its (patho)physiological role: to target or not to target? Int J Mol Sci. 2021 Mar 8;22(5).

233. Berkenpas MB, Lawrence DA, Ginsburg D. Molecular evolution of plasminogen activator inhibitor-1 functional stability. EMBO J. 1995 Jul 3;14(13):2969–77.

234. Khan MS, Singh P, Azhar A, Naseem A, Rashid Q, Kabir MA, *et al.* Serpin inhibition mechanism: a delicate balance between native metastable state and polymerization. J Amino Acids. 2011;2011:606797.

235. Plouët J, Moro F, Bertagnolli S, Coldeboeuf N, Mazarguil H, Clamens S, *et al.* Extracellular cleavage of the vascular endothelial growth factor 189-amino acid form by urokinase is required for its mitogenic effect. J Biol Chem. 1997 May 16;272(20):13390–6.

236. Fredriksson L, Li H, Fieber C, Li X, Eriksson U. Tissue plasminogen activator is a potent activator of PDGF-CC. EMBO J. 2004 Oct 1;23(19):3793–802.

237. Su EJ, Fredriksson L, Geyer M, Folestad E, Cale J, Andrae J, *et al.* Activation of PDGF-CC by tissue plasminogen activator impairs blood-brain barrier integrity during ischemic stroke. Nat Med. 2008 Jul;14(7):731–7.

238. Rogove AD, Siao CJ, Keyt B, Strickland S, Tsirka SE. Activation of microglia reveals a non-proteolytic cytokine function for tissue plasminogen activator in the central nervous system. J Cell Sci. 1999 Nov 15;112(22):4007–16.

239. Hu K, Yang J, Tanaka S, Gonias SL, Mars WM, Liu Y. Tissue-type plasminogen activator acts as a cytokine that triggers intracellular signal transduction and induces matrix metalloproteinase-9 gene expression. J Biol Chem. 2006 Jan 27;281(4):2120–7.

240. Jennewein C, Tran N, Paulus P, Ellinghaus P, Eble JA, Zacharowski K. Novel aspects of fibrin(ogen) fragments during inflammation. Mol Med. 2011 May 1;17(5):568–73.

241. Dewerchin M, Nuffelen AV, Wallays G, Bouché A, Moons L, Carmeliet P, *et al.* Generation and characterization of urokinase receptor-deficient mice. J Clin Invest. 1996 Feb 1;97(3):870–8.

242. Gyetko MR, Sud S, Kendall T, Fuller JA, Newstead MW, Standiford TJ. Urokinase receptor-deficient mice have impaired neutrophil recruitment in response to pulmonary *Pseudomonas aeruginosa* infection. J Immunol. 2000 Aug 1;165(3):1513–9.

243. Genua M, D'Alessio S, Cibella J, Gandelli A, Sala E, Correale C, *et al.* The urokinase plasminogen activator receptor (uPAR) controls macrophage phagocytosis in intestinal inflammation. Gut. 2015 Apr 1;64(4):589.

244. Cheng Y, Hall TR, Xu X, Yung I, Souza D, Zheng J, *et al.* Targeting uPA-uPAR interaction to improve intestinal epithelial barrier integrity in inflammatory bowel disease. eBioMedicine. 2022 Jan 1;75:103758.

245. Draxler DF, Sashindranath M, Medcalf RL. Plasmin: a modulator of immune function. Semin Thromb Hemost. 2017 Mar;43(2):143–53.

246. Foley JH. Plasmin(ogen) at the nexus of fibrinolysis, inflammation, and complement. Semin Thromb Hemost. 2017 Mar;43(2):135–42.

247. Perucci LO, Vago JP, Miles LA, Sousa LP. Crosstalk between the plasminogen/plasmin system and inflammation resolution. J Thromb Haemost. 2023 Oct;21(10):2666–78.

248. Loef EJ, Sheppard HM, Birch NP, Dunbar PR. Plasminogen and plasmin can bind to human T cells and generate truncated CCL21 that increases dendritic cell chemotactic responses. J Biol Chem. 2022 Jul;298(7):102112.

249. Suzuki K, Enghild JJ, Morodomi T, Salvesen G, Nagase H. Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin). Biochemistry. 1990 Nov 6;29(44):10261–70.

250. Sonoki A, Okano Y, Yoshitake Y. Dermal fibroblasts can activate matrix metalloproteinase-1 independent of keratinocytes via plasmin in a 3D collagen model. Exp Dermatol. 2018 May;27(5):520–5.

251. Saunders WB, Bayless KJ, Davis GE. MMP-1 activation by serine proteases and MMP-10 induces human capillary tubular network collapse and regression in 3D collagen matrices. J Cell Sci. 2005 May 15;118(Pt 10):2325–40.

252. Baramova EN, Bajou K, Remacle A, L'Hoir C, Krell HW, Weidle UH, *et al.* Involvement of PA/plasmin system in the processing of pro-MMP-9 and in the second step of pro-MMP-2 activation. FEBS Lett. 1997 Mar 24;405(2):157–62.

253. Carmeliet P, Moons L, Lijnen R, Baes M, Lemaître V, Tipping P, *et al.* Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. Nat Genet. 1997 Dec;17(4):439–44.

254. Hahn-Dantona E, Ramos-DeSimone N, Sipley J, Nagase H, French DL, Quigley JP. Activation of proMMP-9 by a plasmin/MMP-3 cascade in a tumor cell model.

Regulation by tissue inhibitors of metalloproteinases. Ann N Y Acad Sci. 1999 Jun 30;878:372–87.

255. Ramos-DeSimone N, Hahn-Dantona E, Sipley J, Nagase H, French DL, Quigley JP. Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. J Biol Chem. 1999 May 7;274(19):13066–76.

256. Orbe J, Rodríguez JA, Calvayrac O, Rodríguez-Calvo R, Rodríguez C, Roncal C, *et al.* Matrix metalloproteinase-10 is upregulated by thrombin in endothelial cells and increased in patients with enhanced thrombin generation. Arterioscler Thromb Vasc Biol. 2009 Dec;29(12):2109–16.

257. Okada Y, Gonoji Y, Naka K, Tomita K, Nakanishi I, Iwata K, *et al.* Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT 1080 human fibrosarcoma cells. Purification and activation of the precursor and enzymic properties. J Biol Chem. 1992 Oct 25;267(30):21712–9.

258. Deng G, Curriden SA, Wang S, Rosenberg S, Loskutoff DJ. Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release? J Cell Biol. 1996 Sep;134(6):1563–71.

259. Blasi F, Carmeliet P. uPAR: a versatile signalling orchestrator. Nat Rev Mol Cell Biol. 2002 Dec;3(12):932–43.

260. Bhattacharya S, Ploplis VA, Castellino FJ. Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. J Biomed Biotechnol. 2012;2012:482096.

261. Chen SM, Zou Z, Guo SY, Hou WT, Qiu XR, Zhang Y, *et al.* Preventing *Candida albicans* from subverting host plasminogen for invasive infection treatment. Emerg Microbes Infect. 2020 Dec;9(1):2417–32.

262. Lähteenmäki K, Edelman S, Korhonen TK. Bacterial metastasis: the host plasminogen system in bacterial invasion. Trends Microbiol. 2005 Feb;13(2):79–85.

263. Figuera L, Gómez-Arreaza A, Avilán L. Parasitism in optima forma: exploiting the host fibrinolytic system for invasion. Acta Tropica. 2013 Oct;128(1):116–23.

264. El Chamy Maluf S, Icimoto MY, Melo PMS, Budu A, Coimbra R, Gazarini ML, *et al.* Human plasma plasminogen internalization route in *Plasmodium falciparum*-infected erythrocytes. Malar J. 2020 Aug 26;19(1):302.

265. Alves e Silva TL, Radtke A, Balaban A, Pascini TV, Pala ZR, Roth A, *et al.* The fibrinolytic system enables the onset of *Plasmodium* infection in the mosquito vector and the mammalian host. Sci Adv. 2021 Feb;7(6).

266. Leontovyč A, Ulrychová L, O'Donoghue AJ, Vondrášek J, Marešová L, Hubálek M, *et al.* SmSP2: a serine protease secreted by the blood fluke pathogen *Schistosoma mansoni* with anti-hemostatic properties. PLoS Negl Trop Dis. 2018 Apr;12(4):e0006446.

267. Melo PMS, Bagnaresi P, Paschoalin T, Hirata IY, Gazarini ML, Carmona AK. *Plasmodium falciparum* proteases hydrolyze plasminogen, generating angiostatin-like fragments. Mol Biochem Parasitol. 2014 Jan;193(1):45–54.

268. González-Miguel J, Morchón R, Siles-Lucas M, Simón F. Fibrinolysis and proliferative endarteritis: two related processes in chronic infections? The model of the blood-borne pathogen *Dirofilaria immitis*. PLoS ONE. 2015;10(4):e0124445.

269. González-Miguel J, Morchón R, Mellado I, Carretón E, Montoya-Alonso JA, Simón F. Excretory/secretory antigens from *Dirofilaria immitis* adult worms interact with the host fibrinolytic system involving the vascular endothelium. Mol Biochem Parasitol. 2012 Feb;181(2):134–40.

270. González-Miguel J, Morchón R, Carretón E, Montoya-Alonso JA, Simón F. Can the activation of plasminogen/plasmin system of the host by metabolic products of *Dirofilaria immitis* participate in heartworm disease endarteritis? Parasites Vectors. 2015 Apr 1;8:194.

271. Figuera L, Acosta H, Gómez-Arreaza A, Dávila-Vera D, Balza-Quintero A, Quiñones W, *et al.* Plasminogen binding proteins in secreted membrane vesicles of *Leishmania mexicana*. Mol Biochem Parasitol. 2013 Jan;187(1):14–20.

272. Ceruti P, Principe M, Capello M, Cappello P, Novelli F. Three are better than one: plasminogen receptors as cancer theranostic targets. Exp Hematol Oncol. 2013 Dec;2(1):1–11.

273. He L, Ren M, Chen X, Wang X, Li S, Lin J, *et al.* Biochemical and immunological characterization of annexin B30 from *Clonorchis sinensis* excretory/secretory products. Parasitol Res. 2014 Jul;113(7):2743–55.

274. Yang Y, Bai X, Li C, Tong M, Zhang P, Cai W, *et al.* Molecular characterization of fructose-1,6-bisphosphate aldolase from *Trichinella spiralis* and its potential in inducing immune protection. Front Cell Infect Microbiol. 2019;9:122.

275. Swenerton RK, Zhang S, Sajid M, Medzihradszky KF, Craik CS, Kelly BL, *et al.* The oligopeptidase B of *Leishmania* regulates parasite enolase and immune evasion. J Biol Chem. 2011 Jan 7;286(1):429–40.

276. Ernest M, Rosa TFA, Pala ZR, Kudyba HM, Sweeney B, Reiss T, *et al. Plasmodium falciparum* gametes and sporozoites hijack plasmin and factor H to evade host complement killing. Microbiol Spectr. 2023 Jun 15;11(3):e0449322.

277. Reiss T, Theis HI, Gonzalez-Delgado A, Vega-Rodriguez J, Zipfel PF, Skerka C, *et al.* Acquisition of human plasminogen facilitates complement evasion by the malaria parasite *Plasmodium falciparum*. Eur J Immunol. 2021 Feb;51(2):490–3.

278. Ghosh AK, Jacobs-Lorena M. Surface-expressed enolases of *Plasmodium* and other pathogens. Mem Inst Oswaldo Cruz. 2011 Aug;106 Suppl 1(0 1):85–90.

279. Figueiredo BC, Da'dara AA, Oliveira SC, Skelly PJ. Schistosomes enhance plasminogen activation: the role of tegumental enolase. PLoS Pathog. 2015 Dec;11(12):e1005335.

280. Yang J, Qiu C, Xia Y, Yao L, Fu Z, Yuan C, *et al.* Molecular cloning and functional characterization of *Schistosoma japonicum* enolase which is highly expressed at the schistosomulum stage. Parasitol Res. 2010 Aug;107(3):667–77.

281. Pirovich DB, Da'dara AA, Skelly PJ. Glycolytic enzymes as vaccines against schistosomiasis: testing *Schistosoma mansoni* phosphoglycerate mutase in mice. J Parasitol. 2024 Mar 1;110(2):96–105.

282. Liu X, Zheng C, Gao X, Chen J, Zheng K. Complete molecular and immunoprotective characterization of *Babesia microti* enolase. Front Microbiol. 2017;8:622.

283. González-Miguel J, Morchón R, Siles-Lucas M, Oleaga A, Simón F. Surface-displayed glyceraldehyde 3-phosphate dehydrogenase and galectin from *Dirofilaria immitis* enhance the activation of the fibrinolytic system of the host. Acta Trop. 2015 May;145:8–16.

284. González-Miguel J, Larrazabal C, Loa-Mesón D, Siles-Lucas M, Simón F, Morchón R. Glyceraldehyde 3-phosphate dehydrogenase and galectin from *Dirofilaria immitis* participate in heartworm disease endarteritis via plasminogen/plasmin system. Vet Parasitol. 2016 Jun 15;223:96–101.

285. Maldonado J, Marina C, Puig J, Maizo Z, Avilan L. A study of cutaneous lesions caused by *Leishmania mexicana* in plasminogen-deficient mice. Exp Mol Pathol. 2006 Jun;80(3):289–94.

286. Wang S, Ghosh AK, Bongio N, Stebbings KA, Lampe DJ, Jacobs-Lorena M. Fighting malaria with engineered symbiotic bacteria from vector mosquitoes. Proc Natl Acad Sci U S A. 2012 Jul 31;109(31):12734–9.

287. Pascini TV, Jeong YJ, Huang W, Pala ZR, Sá JM, Wells MB, *et al.* Transgenic *Anopheles* mosquitoes expressing human PAI-1 impair malaria transmission. Nat Commun. 2022 May 26;13(1):2949.

288. Monoyios A, Hummel K, Nöbauer K, Patzl M, Schlosser S, Hess M, *et al.* An alliance of gel-based and gel-free proteomic techniques displays substantial insight into the proteome of a virulent and an attenuated *Histomonas meleagridis* strain. Front Cell Infect Microbiol. 2018;8:407.

289. Tritten L, Ballesteros C, Beech R, Geary TG, Moreno Y. Mining nematode protein secretomes to explain lifestyle and host specificity. PLoS Negl Trop Dis. 2021 Sep;15(9):e0009828.

Supplementary File 1

Supplementary Table 1 contains an updated compilation of the vaccine trials conducted in livestock against fasciolosis, originally summarised by Flores-Velázquez *et al.* (2023), Spithill *et al.* (2022), Cwiklinski and Dalton (2022), and Toet *et al.* (2014). The information presented in these reviews has been updated (as of December 2024) through a bibliographic search on PubMed database to identify additional vaccination trials against fasciolosis in livestock published from 2015 onward, using the search term "(fasciol*[Title]) AND (vaccin*[Title])", to ensure a comprehensive coverage of relevant studies (under the premise that trials published before 2015 are adequately covered in the aforementioned reviews).

Supplementary Table 1. Vaccination trials against fasciolosis in livestock.

Antigen	Form	Host	*Fasciola* species	Protection (%)	PMID (a)
SINGLE VACCINES					
Cathepsin L					
FhCL	Native	Sheep	*F. hepatica*	69.0%	8119370
FgCL	Native	Cattle	*F. gigantica*	n.s.	9421734
FgCL	Native	Sheep	*F. gigantica*	56.0%	22451733
FhCL1	Native	Cattle	*F. hepatica*	42.5%	8945548
FhCL1	Native	Sheep	*F. hepatica*	34.0%	10085042
FhCL1	Recombinant	Goat	*F. hepatica*	n.s.	28385539
FhCL1	Recombinant	Sheep	*F. hepatica*	n.s.	27661612
FhCL1	Recombinant	Goat	*F. hepatica*	n.s.	22001704
FhCL1	Recombinant	Goat	*F. hepatica*	n.s.	19665401
FhCL1/FhCL2	Mimitope	Sheep	*F. hepatica*	47.0%	18812010
FhpCL1	Recombinant	Sheep	*F. hepatica*	n.s.	29525280
FhCL1	Recombinant	Cattle	*F. hepatica*	48.0%	20600503
FhCL1	Recombinant	Goat	*F. hepatica*	n.s.	23083835
FhCL1	Mimitope	Sheep	*F. hepatica*	51.0%	22513301
FhCL1	Mimitope	Sheep	*F. hepatica*	57.5%	33872793
FhCL1	Mimitope	Sheep	*F. hepatica*	n.s.	33373968
FhCL1	Mimitope	Sheep	*F. hepatica*	67.17%	
FhCL1	Mimitope	Goat	*F. hepatica*	62.2%	39000332
FhCL1	Mimitope	Goat	*F. hepatica*	75.3%	
FhCL1	Mimitope	Goat	*F. hepatica*	55.4%	33845124
FhCL1	Mimitope	Goat	*F. hepatica*	70.4%	
FhCL1	Mimitope	Goat	*F. hepatica*	46.9-79.5%	24218177
FhCL2	Mimitope	Goat	*F. hepatica*	n.s.	33845124
FhCL2	Mimitope	Goat	*F. hepatica*	50.4%	39000332
FhCL2	Mimitope	Sheep	*F. hepatica*	n.s.	33872793
FhCL2	Native	Sheep	*F. hepatica*	n.s.	10085042
FasAc14p	Synthetic peptide	Sheep	*F. gigantica*	23.1%	17549696

Antigen	Form	Host	*Fasciola* species	Protection (%)	PMID (a)
Cathepsin CPFhW					
CPFhW	Recombinant	Sheep	*F. hepatica*	35.5%	30483259
CPFhW	Recombinant	Cattle	*F. hepatica*	56.2%	
CPFhW	Recombinant inclusion bodies	Cattle	*F. hepatica*	54.0%	17481823
CPFhW	Recombinant inclusion bodies	Sheep	*F. hepatica*	n.s.	
Excreted/secreted products					
	Mimitope	Sheep	*F. hepatica*	52.39%	33373968
Fatty acid-binding protein					
Fh15	Recombinant	Sheep	*F. hepatica*	43.0%	17275191
FgFABP	Recombinant	Buffalo	*F. gigantica*	35.0%	15986249
FgFABP	Recombinant	Buffalo	*F. gigantica*	n.s.	21750874
Sm14	Recombinant	Goat	*F. hepatica*	n.s.	20835620
Sm14	Recombinant	Goat	*F. hepatica*	n.s.	22001704
Sm14	Recombinant	Sheep	*F. hepatica*	98.5%	12911521
FhFABP	Native	Cattle	*F. hepatica*	55.0%	3661829
FgFABP	Native	Cattle	*F. gigantica*	31.0%	9421734
Fh12	Native	Sheep	*F. hepatica*	n.s.	11337125
Fh12	Native	Sheep	*F. hepatica*	24.0%	15567592
Fh12	Native	Sheep	*F. hepatica*	42.0%	18308471
Fh15	Recombinant	Sheep	*F. hepatica*	n.s.	11337125
Glutathione S-transferase					
FgGST	Recombinant	Buffalo	*F. gigantica*	n.s.	21750874
FhGST	Recombinant	Goat	*F. hepatica*	n.s.	23261152
FhGST	Native	Sheep	*F. hepatica*	57.0%	1978849
FhGST	Native	Cattle	*F. hepatica*	49-69%	9032888
FhGST	Native	Goat	*F. hepatica*	n.s.	20035898
FgGST	Native	Cattle	*F. gigantica*	n.s.	9421734
FgGST	Native	Sheep	*F. gigantica*	n.s.	11900929
SbGST	Recombinant	Cattle	*F. gigantica*	n.s.	12695038
Haemoglobin					
FhHb	Native	Cattle	*F. hepatica*	43.8%	8945548
FhHbF2	Recombinant	Cattle	*F. hepatica*	n.s.	18621914
Helminth defense molecule					
MF6p/FhHDM1	Synthetic peptide	Sheep	*F. hepatica*	6.0%	29525280
MF6p/FhHDM1	Native	Sheep	*F. hepatica*	15.0%	
Kunitz-type molecule					
FhKTM	Native	Sheep	*F. hepatica*	n.s.	Bozas *et al.* (pers. comm.) (b)
FhKTM	Native	Cattle	*F. hepatica*	n.s.	
Leucine amino-peptidase					
FhLAP	Recombinant	Sheep	*F. hepatica*	49-89%	21939713
FhLAP	Recombinant	Buffalo	*F. gigantica*	n.s.	21376699
FhLAP	Native	Sheep	*F. hepatica*	89.0%	10085042
Metacercariae (γ-ray attenuated)					
Compilated in Spithill *et al.* (1999)					

Antigen	Form	Host	*Fasciola* species	Protection (%)	PMID (a)
Soluble antigens (total)					
FhSAMS	Native	Cattle	*F. hepatica*	n.s.	39513742
FhSAMS	Native	Cattle	*F. hepatica*	n.s.	
Surface membrane and tegument proteins (triton-soluble)					
FgTSMTP 60 kDa (Enolase)	Native	Sheep	*F. gigantica*	41.3%	26970372
FgTSMTP 32 kDa	Native	Sheep	*F. gigantica*	14.1%	
FgTSMTP 28 kDa	Native	Sheep	*F. gigantica*	19.2%	
Paramyosin					
	Native	Sheep	*F. hepatica*	45%	Morrison *et al.* (pers. comm.) (b)
	Native	Sheep	*F. hepatica*	n.s.	
	Native	Cattle	*F. hepatica*	47.0%	
	Native	Cattle	*F. gigantica*	n.s.	9421734
Peroxiredoxin					
FhPrx	Recombinant	Goat	*F. hepatica*	n.s.	20153792
FhPrx	Recombinant	Goat	*F. hepatica*	n.s.	22001704
FgPrx	Recombinant	Buffalo	*F. gigantica*	n.s.	21376699
Phospho-glicerate kinase					
FhPGK/pCMV	cDNA	Sheep	*F. hepatica*	n.s.	31550657
FhPGK/pCMV	cDNA	Sheep	*F. hepatica*	n.s.	
FhPGK/pCMV	cDNA	Sheep	*F. hepatica*	5.8%	27078643
Tetraspanin					
FhTSP2	Recombinant, fused to *E. coli* LTB protein	Cattle	*F. hepatica*	n.s.	34835144
Thioredoxin glutathione reductase					
FhTGR	Recombinant	Cattle	*F. hepatica*	8.2%	27270384
FhTGR	Recombinant	Cattle	*F. hepatica*	3.8%	
FhTGR	Recombinant	Cattle	*F. hepatica*	23.0%	
14-3-3					
14-3-3z	Recombinant	Sheep	*F. hepatica*	n.s.	30078585
COMBINATION VACCINES					
FhCL1 + Hb	Native	Cattle	*F. hepatica*	51.9%	8945548
FhCL1 + FhCL3	Recombinant	Cattle	*F. hepatica*	37.6%	29373193
FhCL1 + FhCL3	Recombinant	Cattle	*F. hepatica*	n.s.	
FhCL1 + FhCL2	Native	Cattle	*F. hepatica*	55.0%	10425237
FhCL1 + FhCL2	Mimitope	Sheep	*F. hepatica*	n.s.	33872793
FhCL1 + FhCL2	Mimitope	Goat	*F. hepatica*	32.4%	33845124
FhCL1 + FhCL2	Native	Sheep	*F. hepatica*	n.s.	10085042
FhCL1 + FhLAP	Recombinant chimeric	Sheep	*F. hepatica*	25.5%	31036453
FhCL1 + FhLAP	Recombinant chimeric	Sheep	*F. hepatica*	30.7%	
FhCL1 + FhLAP	Recombinant chimeric	Sheep	*F. hepatica*	40.6%	
FhCL1 + FhCL2 + FhLAP	Native	Sheep	*F. hepatica*	79.0%	10085042

Antigen	Form	Host	*Fasciola* species	Protection (%)	PMID (a)
FhCL1 + FhHDM + FhLAP + FhPrx	Recombinant	Sheep	*F. hepatica*	37.2%	33509286
FhCL1 + FhHDM + FhLAP + FhPrx	Recombinant	Sheep	*F. hepatica*	n.s.	
FhCL1 + FhPrx + Sm14	Recombinant	Goat	*F. hepatica*	n.s.	22001704
FhCL2 + Hb	Native	Cattle	*F. hepatica*	72.4%	8945548
FhCL2 + Hb	Native	Cattle	*F. hepatica*	72.0%	9682340
FhCL2 + Hb	Native	Cattle	*F. hepatica*	11.0%	
FhCL2 + Hb	Native	Cattle	*F. hepatica*	29.0%	10425237
FhCL5 + FhCB2	Recombinant	Sheep	*F. hepatica*	20.9%	30105973
FhCL5 + FhCB2	Recombinant	Sheep	*F. hepatica*	40.5%	
FhFABP + FhGST	Recombinant	Buffalo	*F. gigantica*	35.0%	21750874
FhGST + FhTeg (total tegument antigen)	Native	Cattle	*F. hepatica*	33.0%	35202313
FhTeg1 + FhTeg5	Recombinant	Cattle	*F. hepatica*	n.s.	32971381
FhStf1 + FhStf2 + FhStf3 + FhKT1	Recombinant	Sheep	*F. hepatica*	17.4%	35214614
FhStf1 + FhStf2 + FhStf3 + FhKT1	Recombinant	Sheep	*F. hepatica*	n.s.	
FhPrx + FhTrx + FhTGR	Recombinant	Sheep	*F. hepatica*	n.s.	37826973
FhPrx + FhTrx + FhSOD1 + FhSOD3	Recombinant	Sheep	*F. hepatica*	n.s.	37826973
FhKT1 + FhSrp1 + FhStf1	Recombinant	Sheep	*F. hepatica*	n.s.	

Protection is assessed based on a reduction in fluke burden. Differences in protection outcomes observed under apparently identical conditions (based on antigen, host, and *Fasciola* species) are explained by variations in factors not specified in this table, such as adjuvants, vaccination protocols, antigen dosages, or administration routes. (a) PubMed identification number. (b) Cited as a personal communication in Spithill *et al.* (1999). CB2, cathepsin B2; CL1, cathepsin L1; CL2, cathepsin L2; CL5, cathepsin L5; FABP, fatty acid-binding protein; GST, glutathione S-transferase; HDM, helminth defence molecule; Hb, haemoglobin; KTM, kunitz-type molecule; KT1, kunitz-type inhibitor; LAP, leucine aminopeptidase; n.s., not significant; PGK, phosphoglycerate kinase; pCL1, immature cathepsin L1; SAMS, soluble *Fasciola hepatica* antigens associated with Montanide 763 AVG and saponin adjuvants; Stf1-3 stefin 1-3; SOD1,2, superoxide dismutase 1,2; Teg1, Teg5, tegument glycoprotein 1,5; TSMP, triton-soluble surface membrane and tegumental proteins; Prx, peroxireductase; Trx, thioreductase; TGR, thioredoxin glutathione reductase.

References

Cwiklinski K, Dalton JP. Omics tools enabling vaccine discovery against fasciolosis. Trends Parasitol. 2022 Dec;38(12):1068–79.

Flores-Velázquez LM, Ruiz-Campillo MT, Herrera-Torres G, Martínez-Moreno Á, Martínez-Moreno FJ, Zafra R, *et al.* Fasciolosis: pathogenesis, host-parasite interactions, and implication in vaccine development. Front Vet Sci. 2023 Dec;11(10):1270064.

Spithill TW, Smooker PM, Sexton JL, Bozas E, Morrison CA, Creaney J, *et al.* Chapter 11. Development of vaccines against *Fasciola hepatica*. In: Fasciolosis. 1st ed. UK: CABI; 1999. p. 377-411.

Spithill TW, Toet H, Rathinasamy V, Zerna G, Swan J, Cameron T, *et al.* Chapter 12. Vaccines for *Fasciola*: new thinking for an old problem. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 65–112.

Toet H, Piedrafita DM, Spithill TW. Liver fluke vaccines in ruminants: strategies, progress and future opportunities. Int J Parasitol. 2014 Oct;44(12):915–27.

Supplementary File 2

The database PubMed was explored to search for original articles describing plasmin substrates. To this end, the following search terms were applied: "(plasmin[Title]) AND (cleav*[Title])", "(plasmin[Title]) AND (degrad*[Title])" and "(plasmin[Title]) AND (hydrol*[Title])"; which yielded 90, 148, and 29 results, respectively (as of 10th October 2024). After a manual pre-selection of relevant articles based on their titles, and excluding those for which access was unavailable, a total of 186 articles were considered for further analysis. Some references not containing the specified search terms were incorporated based on prior knowledge of the topic and/or because they were identified as relevant during the elaboration of the main text. **Supplementary Table 2** provides a summary of all plasmin substrates described in these articles, along with the associated physiological relevance of this proteolytic event, whether experimentally demonstrated or not.

Supplementary Table 2. Fibrinolysis-unrelated plasmin substrates.

Substrate	**Physiological relevance**	**PMID** (a)
Aggregin	coagulation (platelet aggregation)	2148055
α-2-macroglobulin (α2M)	α2M clearance from plasma	2435319
Amyloid β-protein	amyloidosis (including Alzheimer's Disease)	32694536; 11588612; 10471309
	production of non-amyloidogenic fragments, protection against the development of Alzheimer's disease	12446968
Amyloid β-protein (aggregates)	prevents amyloid β-induced neurotoxicity	10818128
Amyloid β-protein (precursor)	amyloidosis (including Alzheimer's Disease)	11263499
Angiopoietin-like protein 4 and 8 (ANGPTL4/8)	triglyceride metabolism	36763533
Angiotensin II	blood pressure	4339394
Annexin A2	inflammation (cytokine production by macrophages)	17413040
	inflammation (intracellular signalling)	16373665
Antibodies (opsonizing)	immune evasion (*Francisella tularensis*)	19752236
β-2-glycoprotein (β2GPI)	angiogenesis (inhibition)	17872974
	thrombosis	9596664
β-glycan	signalling by type I and type II TGF-β	8068006
Brain-derived neurotrophic factor, precursor (pro-BDNF)	hypoccampal plasticity (long-term memory)	15486301
C1 inhibitor	pathology of inflammatory disorders (systemic lupus erythematosus, respiratory distress syndrome, rheumatoid arthiritis)	9234243

Substrate	Physiological relevance	PMID (a)
C3	leukocyte chemotaxis, increase in vascular permeability	4226271
	complement system inhibition (immune evasion by *Staphylococcus aureus*)	23071827
	chemotaxis of mast cells and neutrophils (by C3a and C5a)	20870944
	leukocyte chemotaxis (by C3a and C5a), activation of complement system (induction of membrane attack complex (MAC) assembly)	27077125
C3b	complement system inhibition (immune evasion by *S. aureus*)	23071827
	complement system inhibition (immune evasion by *Haemophilus influenzae*)	22124123
	complement system inhibition (immune evasion by *Leptospira interrogans*, invasion)	26822552
	complement system inhibition (immune evasion by *Moraxella catarrhalis*)	26099590
	complement system inhibition (immune evasion by pathogenic bacteria)	22451663
	complement system inhibition (immune evasion by *Streptococcus pyogenes*)	26945067
C3b proteolytic fragment (iC3b)	complement pathway regulation	25556624
	complement system inhibition (immune evasion by *S. pyogenes*)	26945067
C5	chemotaxis of mast cells and neutrophils (by C3a and C5a)	20870944
	leukocyte chemotaxis (by C3a and C5a), activation of complement system (induction of membrane attack complex (MAC) assembly)	27077125
	complement system inhibition (immune evasion strategy by *M. catarrhalis*)	26099590
	complement system inhibition (immune evasion by pathogenic bacteria)	22451663
	complement system inhibition (immune evasion by *L. interrogans*, invasion)	26822552
Cadherin-5 (VE-cadherin)	*S. pneumoniae* epithelial migration	18725422
Casein	digestion of milk components during lactation	6240266
		1369431; 13885559
C-C motif chemokine ligand 1 (CCL1/HCC-1)	inflammation, generation of a truncated variant that stimulates leukocyte chemotaxis	11544332
C-C motif chemokine ligand 2 (CCL2) (b)	enhances CCL2 chemotactic activities, excitotoxic neurodegeneration	17301181
	inflammation (central nervous system)	21486949
C-C motif chemokine ligand 21 (CCL21)	inflammation, generation of a truncated variant that stimulates dendritic cell chemotaxis	35690148
	lymphocyte migration, immune cell adhesion	27301418
Chromogranin A	cardiovascular disease (hypertension)	17991725
	cell adhesion (neuroendocrine tumours)	12297497
	stress-related catecholamine responses mediated by neuroendocrine cells	11342539
Collagen type IV	skeletal muscle degeneration	2947809
	tumour invasion (c)	2144209
Collagen type XIX	Release of fragment NC1(XIX), which has anti-tumour activities	25668817
ECM	capillary morphogenesis (vessel formation)	30784190
	human endometrial stromal cell viability, proliferation, and migration (development of endometriosis)	38735446
	ECM degradation, migration of *S. pneumoniae*	16113819
	pathological ECM accumulation, glomerulonephritis	16788698
	metastatic potential of thyroid tumour cell line	10524570
	glomerular ECM accumulation, renal disease	7540230

Substrate	**Physiological relevance**	**PMID** (a)
Epithelial sodium channel (ENaC)	renal disorders (proteinuria)	18981180
	general ENaC functions	22966015
	pulmonary blood-gas exchange (lung oedema)	32133621
Factor VIII	coagulation	19126539; 4274479; 17189254; 6444272
Factor Xa (FXa)	coagulation	8663221
Fibrin (d)(e)	angiogenesis	12197472
	angiogenesis (inhibition of tubule formation by endothelial cells) [Bβ43-63]	20068569
	angiogenesis (inhibits endothelial cell migration and tubule formation), [fragment E]	10987275
	angiogenesis (inhibits the migration and tubule formation of human dermal microvascular endothelial cells) [alphastatin]	14512300
	angiogenesis [fragment E]	1280677
	coagulation (increased tissue factor production), fibrinolysis (increases u-PA production), inflammation (increased IL-1 secretion), [D-dimer]	1845845
	diagnosis of coagulation and thrombolytic disorders	4265363
	ECM degradation, migration of *S. pneumoniae*	16113819
	endothelial barrier integrity (reduces vascular leakage during shock) [Bβ15-42]	19401765
	inflammation (inhibits leukocyte transmigration during myocardial ischemia-reperfusion injury) [Bβ15-42]	12663071
	inflammation (neutrophil and fibroblast chemotaxis) [Bβ1-42]	3359049
	inflammation (IL-6 production by macrophages) [fragment E]	10102564
	inflammation (production of IL-6 and IL-1β by monocytes) [D-dimer]	8199021
	mitigates pathology associated with ischemia/reperfusion (reduces leukocyte infiltration) [Bβ15-42]	20405575; 19114899
	pathology of anti-phospholipid syndrome	15100323
	smooth muscle cell migration and proliferation (restenosis and atherogenesis) [fragment E]	10713318
	tumour progression of oral squamous cell carcinoma [Bβ15-42]	15723073
	wound healing	28135202
	wound healing, cell adhesion, inflammation (increased IL-1β production) [fragment E]	11266115
	human endometrial stromal cell viability, proliferation, and migration (development of endometriosis)	38735446
	ovarian cancer progression	2139349
Fibrinogen (d)	antigenic properties of FDPs	17561232; 4258329
	coagulation	128845; 94836; 4262076; 236019
	complement system inhibition (immune evasion by *L. interrogans*, invasion)	26822552
	cytotoxicity on kidney cells	6857591
	progression of sarcoma (in mice)	6459937
	postoperative recovery from hepatectomy (relevance of FDPs)	2156347
	production of vasoactive peptides	7353036

Substrate	**Physiological relevance**	**PMID** (a)
	vasoconstriction/dilation by FDPs	155325
Fibroblast growth factor 2 (FGF-2)	fibroblast proliferation, tissue repair	8789459
Fibronectin	ECM degradation, migration of *S. pneumoniae*	16113819
	invasion, migration (*Borrelia burgdorferi*)	10417158
	phagocytosis	7252334
	vitroretinal surgery	20450255
	tumour metastasis	2947330
	skeletal muscle degeneration	2947809
Fragment E	clinical applications (distinguish between fibrinolysis and fibrinogenolysis)	6449096
Gammaglobulins	treatment of adult autoimmune thrombocytopenic purpura	6683424
Growth hormone	all growth hormone-related functions, including glucose uptake	10702910
		4234815
Hemagglutinin (of *Influenza* virus)	virus entry	6461136; 4795670
High density lipoprotein (HDL)	development of atherosclerosis	6460340
Histidine-rich glycoprotein (HRG)	cell adhesion, angiogenesis, coagulation, fibrinolysis and clearance of immune complexes, necrotic cells and pathogens	19712047; 38272220
Immunoglobulin G (IgG)	IgG metabolism (adaptive immune responses)	8037734
Insulin-like growth factor-binding protein-5 (IGFBP-5)	bone remodelling	9374687
Interferon (gamma)	interferon gamma-mediated immune responses	2529320
Interleukin 8 (IL-8)	neutrophil chemotaxis	1828038
Kallikrein	formation of active fragments with potential to regulate coagulation	2949543
Kininogen	acetaminophen (APAP)-induced liver injury	33827130
	coagulation (contact mediated clotting and activation of factor XII)	157559
Lactoglobulin (β)	food industry applications	10552596; 10552597
Laminin	ECM degradation, migration of *S. pneumoniae*	16113819
	epidermal BM assembly and differentiation	18460030
	invasion, migration (*B. burgdorferi*)	10417158
	long-term potentiation	10684901
	neuronal degeneration	9428515
	tissue remodelling, wound healing	6455777
	vitroretinal surgery	20450255
	tumour metastasis	2947330
	skeletal muscle degeneration	2947809
MMP (collagenase, not specified)	osteoid removal by osteoblasts	2554972
MMP-2	pathologies related to MMP-2 dysregulation	9679953
Myelin	demyelinating pathologies	6167674
	inflammatory demyelinating diseases	2936427; 2411869
Neural cell adhesion molecule L1	neural development and regeneration	10574721

Substrate	**Physiological relevance**	**PMID** (a)
NR2A subunit of N-methyl-D-aspartate receptors (NMDA)	disorders of the central nervous system, including ischemia and spinal cord injury	19240037
Osteopontin	integrin-mediated cell adhesion, bone mineralization, inhibition of ectopic calcification, wound healing, inflammation, regulation of immune cell functions, and tumour growth	20071328
Perlecan	release of bound basic FGF (angiogenesis)	8626565
Glycoproteins GPIIb-IIIa, GPIb, GPIa-Iia	platelet aggregation, acute myocardial infarction	2169924
Prolactin, placental lactogen	pregnancy-related endothelial cell dysfunctions (preeclampsia)	34601001
Pro-MMP-1	collagen degradation, wound healing	10733668
	ECM (collagen) degradation in physiological and pathological conditions (g)	2176865
	ECM degradation in the skin	29498767
	ECM degradation, vascular regression	15870107
pro-MMP-2 (h)	potentially all MMP-2 physiologic roles	9089282
pro-MMP-3	ECM degradation, aneurism formation	9398846
	ECM degradation, tumour cell invasion (MDA-MB-231 breast carcinoma cell line)	10224058
	tumour cell invasion (MDA-MB-231 breast carcinoma cell line)	10415742
Pro-MMP-9	degradation of pro-MMP-9 upon pre-treatment with divalent cation chelators, inactivation of its functions (inflammation, metastasis)	22677171
	inflammation (macrophage invasion) (f)	20424186
	ECM degradation, aneurism formation (f)	9398846
Pro-MMP-10	mediates thrombin-dependent vascular effects	19762781
Pro-MMP-12	ECM degradation, aneurism formation	9398846
Pro-MMP-13	ECM degradation, aneurism formation	9398846
Protease-activated receptors (PARs)	inflammation (monocyte recruitment)	25165151
	coagulation (platelet aggregation)	14973136; 10610687
Proteoglycan	degradation of arterial connective tissue	2969314
	cartilage destruction associated with rheumatoid arthritis	6235859
Pro-u-PA	tumour cell metastasis	1837419
SARS-CoV-2 Spike	SARS-CoV-2 cell entry	36193902
Syndecan-4	arteriosclerosis (development of arteriosclerotic plaques), recruitment of mononuclear blood cells to the plaque	16087677
Synuclein (α)	Parkinson's disease	22619171
Thrombin receptor	platelet activation	8760030
Trypsin inhibitor (inter-α)	inflammation, kidney injury	92450
Tumour necrosis factor α (TNF-α)	ovulation (sheep)	10208979
u-PA receptor (uPAR)	potentially all u-PA functions (inflammatory, tumour invasion, others)	15358545
Vascular endothelial growth factor (VEGF)	angiogenesis	12417332
Vitronectin	invasion, migration (*B. burgdorferi*)	10417158
von Willebrand factor	coagulation	3160727; 38271661;

Substrate	Physiological relevance	PMID (a)
		28279966; 24449821; 6444272

Only articles describing physiological plasmin substrates were included; studies describing engineered substrates and/or plasmin cleavage events with potential applications in the food industry were excluded. (a) PubMed identification number. (b) Also known as monocyte chemoattractant protein-1 (MCP-1). (c) Since tissue sections were used for some of the experiments, the authors suggest that collagen type IV degradation might also occur via plasmin-mediated activation of a latent collagenase, as demonstrated by Pins *et al.*, 2000 (PMID: 10733668). (d) Papers describing plasmin cleavage of fibrinogen or fibrin were included in the table only if this activity was related to non-fibrinolytic functions (i.e., generation of FDPs that regulate inflammatory responses). (e) The FDP that exerts the mentioned function is indicated in square brackets. (f) This study also concludes that plasmin can directly cleave and activate pro-MMP-9. However, this observation could be explained by the presence of pro-MMP-3 in the conditioned cell media that the authors employed in their MMP-9 activation assays. (g) This study shows that plasmin generates a pro-MMP-1 intermediate devoid of catalytic activity that is subsequently activated by MMP-3. (h) Plasmin cleaves and activates an intermediate form of MMP-2 that is generated by MT1-MMP cleavage of pro-MMP-2. Blank cells indicate information that was not reported in the publication. BM, basement membrane; ECM, extracellular matrix; FDP, fibrin degradation product, FGF, fibroblast growth factor; MMP, metalloproteinase; u-PA, urokinase-type plasminogen activator; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Hypothesis, objectives, and thesis outline

Hypothesis and objectives

The migration of *Fasciola hepatica* newly excysted juveniles (FhNEJ) across the intestinal wall is considered a crucial step for infection success. However, the molecular mechanisms driving this process are not completely understood, especially regarding the interaction between FhNEJ and host-derived molecules that could facilitate trans-intestinal migration and the successful establishment of these parasites within the mammalian organism. This knowledge is particularly relevant in light of the growing prevalence of *F. hepatica* infections that are resistant to the standard treatment, triclabendazole, coupled with the lack of an effective vaccine against this cosmopolitan parasite, which altogether highlight the need for the development of novel intervention strategies against fasciolosis. In this regard, juvenile-directed approaches would not only reduce disease transmission among affected populations by decreasing the amount of eggs that are shed into the environment but also mitigate the pathology associated with *F. hepatica* infection, which is primarily caused by the migration of juvenile parasites across host tissues prior to their definitive establishment within the hepatic bile ducts.

Given the well-described ability of helminth parasites, including adult *F. hepatica*, to interact with the fibrinolytic system of their mammalian hosts, we hypothesised that FhNEJ may similarly exploit host fibrinolysis to migrate more efficiently across the intestinal wall by leveraging the broad-spectrum catalytic activity of plasmin, the central fibrinolytic protease. To address this hypothesis, we proposed the following objectives:

1. To investigate whether FhNEJ express molecules at their tegument surface, which constitutes the host-parasite interface, that interact with different components of the host fibrinolytic system, and to analyse whether this interaction leads to plasmin generation within the microenvironment surrounding the parasite. This objective has been addressed in **Chapter 1** and **Chapter 3**.

2. To determine the identity of fibrinolysis-interacting proteins expressed at the tegument of FhNEJ, which could represent potential candidates for novel intervention strategies against early-stage fasciolosis. This objective has been addressed in **Chapter 1** and **Chapter 3**.

3. To assess whether FhNEJ-induced plasmin generation enhances the degradation of laminin, a major structural component of the intestinal extracellular matrix (ECM),

potentially facilitating the migration of these parasites across the intestinal wall. This objective has been addressed in **Chapter 2**.

4. To shed light into the functional relevance of the interaction between FhNEJ and the host fibrinolytic system using physiologically-relevant experimental models of early-stage fasciolosis. This objective has been addressed in **Chapter 4**.

Thesis outline

In **Chapter 1**, the capacity of a tegument-enriched protein extract from FhNEJ (FhNEJ-Teg) to bind plasminogen (PLG) is investigated, and the anatomic location of PLG-binding proteins is determined through immunofluorescent staining of whole-mounted FhNEJ. In this chapter, we also identify the potential PLG-binding proteins in FhNEJ-Teg and partially validate these results using recombinant versions of selected candidate proteins. Finally, we employ enzymatic assays to confirm that PLG binding by FhNEJ-Teg might have important functional consequences, as it leads to the generation of plasmin, the ultimate effector of the fibrinolytic system.

In **Chapter 2**, we demonstrate that FhNEJ contain proteins at their tegument surface that bind to laminin, one of the most abundant components of the intestinal basement membrane. Following a similar strategy as that employed in Chapter 2, we identify the potential laminin-binding proteins in FhNEJ-Teg and partially validate these results using recombinant versions of some of the identified proteins. In this chapter, we also describe the ability of the juvenile-specific cathepsin L3 to bind to and degrade laminin, and confirm that FhNEJ-induced plasmin generation—described in Chapter 1—increases the ability of these parasites to degrade this major ECM protein. Finally, we reveal that the interaction between FhNEJ and laminin triggers changes in the protein expression profile of these parasites that could facilitate their survival and adaptation to the newly encountered host microenvironment.

In **Chapter 3**, we investigate the interaction between FhNEJ and the urokinase-type PLG activator (u-PA), which is the major PLG activator expressed in the intestine. Our results show that FhNEJ-Teg contains proteins that bind to the precursor zymogen of u-PA (pro-u-PA). Following a similar strategy as that employed in previous chapters, we identify the proteins responsible for pro-u-PA binding and partially validate these findings using

recombinant versions of selected candidate proteins. In this chapter, we also demonstrate that FhNEJ-Teg express one or more proteases that cleave pro-u-PA and stimulate its conversion into the catalytically-active enzyme, u-PA. Finally, we demonstrate that FhNEJ-induced pro-u-PA activation positively feeds back into the capacity of these parasites to stimulate plasmin generation from host-derived PLG.

In **Chapter 4**, we assess the functional relevance of the interaction between FhNEJ and the fibrinolytic system described in previous chapters by using a co-culture system of mouse primary small intestinal epithelial cells (mPSIEC) and a mouse model of early-stage fasciolosis. Our experiments reveal that co-incubation of mPSIEC with both FhNEJ and PLG leads to plasmin generation in the pericellular space, which correlates with increased u-PA secretion and collagen degradation in mPSIEC culture supernatants. Additionally, by using multiplexed assays, we show that FhNEJ increase the secretion of different matrix metalloproteinases to the extracellular space in a PLG-independent manner. In this chapter, a proteomic analysis of mPSIEC whole-cell lysates reveals that co-stimulation with FhNEJ and PLG alters the abundance of proteins in host cells that could potentially facilitate trans-intestinal migration and contribute to immune evasion by FhNEJ. Finally, experimental infections were conducted in mice with impaired fibrinolysis to assess the physiologic relevance of the interaction between FhNEJ and the host fibrinolytic system in the migration of juvenile parasites within the mammalian organism.

Chapter 1

Fasciola hepatica juveniles interact with the host fibrinolytic system as a potential early-stage invasion mechanism

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The trematode *Fasciola hepatica* is the most widespread causative agent of fasciolosis, a parasitic disease that mainly affects humans and ruminants worldwide. During *F. hepatica* infection, newly excysted juveniles (FhNEJ) emerge in the duodenum of the mammalian host and migrate towards their definitive location, the intra-hepatic biliary ducts. Understanding how *F. hepatica* traverses the intestinal wall and migrates towards the liver is pivotal for the development of more successful strategies against fasciolosis. The central enzyme of the mammalian fibrinolytic system is plasmin, a serine protease whose functions are exploited by a number of parasite species owing to its broad spectrum of substrates, including components of tissue extracellular matrices. The aim of the present work is to understand whether FhNEJ co-opt the functions of the host fibrinolytic system as a mechanism to facilitate trans-intestinal migration. To that end, a tegument-enriched antigenic extract of FhNEJ (FhNEJ-Teg) was obtained *in vitro*, and its capability to bind the zymogen plasminogen (PLG) and enhance its conversion to the active protease, plasmin, were analysed by a combination of enzyme-linked immunosorbent, chromogenic, and immunofluorescent assays. Additionally, PLG-binding proteins in FhNEJ-Teg were identified by bidimensional electrophoresis coupled to mass spectrometry analysis, and the interactions were validated using FhNEJ recombinant proteins. Our results show that FhNEJ-Teg contains proteins that bind PLG and stimulate its activation to plasmin, which could facilitate the traversal of the intestinal wall by FhNEJ and contribute to the successful establishment of the parasite within its mammalian host. Altogether, our findings contribute to a better understanding of host-parasite relationships during early fasciolosis and may be exploited from a pharmacological and/or immunological perspective for the development of treatment and control strategies against this global disease.

Introduction

Fasciolosis caused by the trematode *Fasciola hepatica* is considered the widest-spread helminth disease, affecting wild and domestic animals and up to 17 million people worldwide (1). Definitive hosts (typically ruminants raised as livestock and humans) become infected by ingestion of metacercariae commonly attached to water plants that are used for consumption. Upon ingestion, metacercariae excyst in the duodenum and release the newly excysted juvenile flukes (FhNEJ), which cross the intestinal wall within the first two to three hours after infection and migrate throughout the peritoneum towards the liver. After burrowing through and feeding from hepatic tissue, migrating juveniles finally establish in the major biliary ducts, where they mature into adult worms and egg shedding starts (2). Given that human fasciolosis hyper-endemic areas are found in regions where people live in substandard socio-economic conditions (2–4), this parasitosis is also classified by the World Health Organization as a Neglected Tropical Disease (5). Nowadays, the emergence of resistant isolates against the drug of choice, triclabendazole, the lack of an effective vaccine against *F. hepatica*, and the geographic expansion of this parasite due to climate change turns fasciolosis into a disease of growing public health concern (6,7).

Classical vaccine strategies against *F. hepatica* have focused on targeting the adult phase of this parasite, which has certain drawbacks considering that i) migrating juveniles produce substantial tissue damage during the acute stage of the disease (8), which occasionally causes sudden death of the definitive host (9), and ii) adult flukes reside in the biliary tree, an anatomical niche that preferentially induces immunotolerance. In this scenario, interventions aimed at blocking the migration of juveniles and the development of adult flukes may be more effective in fasciolosis prevention and control, but the molecular mechanisms involved in FhNEJ survival and development inside the host are not yet fully understood. Given that crossing of the intestinal wall by FhNEJ can be regarded as a 'point of no return' in fasciolosis in terms of therapeutic control (8), it is of utmost importance to precisely understand the molecular events that regulate this process.

Crossing of the intestinal wall by FhNEJ is driven, among others, by cathepsin proteases, which are papain-like cysteine peptidases that are capable of degrading components of the intestinal extracellular matrix (ECM) (10,11). *F. hepatica* cathepsins are amongst the most highly expressed proteins in *F. hepatica*, which evidences the importance of these enzymes in *F. hepatica* biology and development, and they are divided in two families (B and L) that

show a temporal pattern of expression, being FhCL3 and FhCB1, FhCB2, and FhCB3 the most highly expressed isotypes in FhNEJ (12).

In addition to their endogenous repertoire of proteases, it is possible that FhNEJ co-opt proteolytic functions of the host in order to migrate more efficiently in terms of energy expenditure. A paradigmatic example of exploitation of host resources is the interaction between parasites and plasminogen (PLG), the central zymogen of the mammalian fibrinolytic system (13–15), which has been described in adult *F. hepatica* via the secretion of PLG-binding proteins and proposed to be a mechanism of survival inside the biliary ducts (16,17). PLG is converted into its active form, plasmin, by the serine proteases tissue-type or urokinase-type plasminogen activators (t-PA and u-PA, respectively). PLG binding to fibrin and other partner proteins occurs via specialised protein domains found in the PLG molecule called kringle, which have high affinity for lysine residues of partner proteins (18). Although t-PA expression is mostly restricted to the vascular endothelium, u-PA is expressed in different tissues and can therefore stimulate plasmin generation in the extravascular space (19). This, in conjunction with the wide array of plasmin substrates, including ECM components (19), matrix metalloproteinases (20), and the C3 and C5 complement molecules (21), explains why host-derived plasmin could be a highly valuable tool for parasites to migrate through the mammalian organism, modulate immune responses, and overall increase their chances of survival within their definitive hosts (13–15).

The aim of the present work is to investigate whether FhNEJ interact with the fibrinolytic system and promote plasmin generation at their surface as a potential mechanism to facilitate trans-intestinal migration. This study reveals the pro-fibrinolytic potential of FhNEJ and broadens our knowledge of host-parasite relationships during early infection by *F. hepatica*.

Materials and methods

In vitro excystment of *F. hepatica* metacercariae

FhNEJ were obtained by *in vitro* stimulating the excystment of 5000 *F. hepatica* metacercariae (Ridgeway Research Ltd) as previously described (22). Briefly, pure CO_2 (99.5% purity) was bubbled in 10 mL of ice-cold distilled water for 30 s, followed by supplementation with sodium dithionite to a final concentration of 0.02 M. This solution was added to metacercariae and incubated for 1 h at 37 ºC. Following incubation, the

metacercariae were washed twice with room-temperature distilled water and resuspended in 5 mL of Hank's balanced salt solution (Sigma) supplemented with 10% lamb bile (obtained from a local abattoir) and 30 mM HEPES (Sigma) pH 7.4. The parasites were incubated for 4 h at 37 ºC and FhNEJ were manually recovered under a magnifier using a 20 µL pipette and immediately subjected to protein extraction.

Protein extraction of the tegument fraction

After two washes in sterile phosphate-buffered saline (PBS), 3500 FhNEJ were resuspended in 300 µL of PBS containing 1% Nonidet P-40 substitute (NP40; Sigma cat. nr. 74385) and incubated at room temperature for 30 min with mild rotation. Next, parasites were centrifuged 5 min at 300 x*g* and the supernatant containing the FhNEJ tegument-enriched protein extract (FhNEJ-Teg) was frozen at -80 ºC until use (23). Protein concentration was determined using the Pierce BCA Protein Assay kit (Thermo Fisher).

Plasminogen binding assay

PLG binding was assessed by enzyme-linked immunosorbent assay (ELISA) as previously described (24). In brief, 96 well plates (Costar) were coated overnight at 4 ºC with 0.5 µg of FhNEJ-Teg or 1 µg of recombinant protein (25) diluted in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Wells coated in 1% bovine serum albumin (BSA) were used as negative controls. After washing three times with PBS containing 0.05% Tween-20 (PBST), wells were blocked with 1% BSA diluted in PBS for 30 min at 37 ºC and increasing amounts (0 µg to 3 µg) of PLG (Origene) diluted in blocking solution were added to the wells and incubated for 1 h at 37 ºC. In parallel, a competition assay was performed in which 50 mM of 6-aminocaproic acid (ε-ACA; Sigma) was added to the wells together with PLG. After three washes with PBST, the wells were incubated with an anti-PLG primary antibody raised in sheep (Acris Antibodies) followed by horseradish peroxidase (HRP)-conjugated anti-sheep secondary antibody (Sigma). Primary and secondary antibodies were diluted 1:2000 and 1:4000, respectively, in blocking solution, and incubated for 1 h at 37 ºC. Wells were washed three times with PBST between and after antibody incubations. Finally, PLG binding was revealed by adding 1.5 mM of the chromogenic substrate ortho-phenylene-diamine (Sigma) diluted in substrate buffer (25 mM citric acid, 45 mM Na_2HPO_4, 0.04% H_2O_2, pH 5). The samples were incubated at room temperature in the dark for 5–10 min, and the reaction was stopped by adding an equal volume of 3 N sulphuric acid. Optical densities (OD) were measured at 492 nm in a

Multiskan GO spectrophotometer (Thermo Fisher). The assays were performed in technical triplicate.

Detection of plasminogen binding on the surface of FhNEJ by immunofluorescence

F. hepatica metacercariae were excysted as described above and FhNEJ were recovered every hour after addition of excystment medium and cultured in RPMI-1640 culture media (Thermo Fisher) at 37 ºC in a 5% CO_2 atmosphere. 50 FhNEJ per condition were washed three times in PBS and incubated with blocking solution (0.1% BSA in PBS) supplemented with 100 μg/mL of human PLG (Origene) for 3 h at 37 ºC. In parallel, a competition assay was performed that included 50 mM of ε-ACA during PLG incubation. Two extra sets of FhNEJ incubated in blocking buffer alone served as negative controls for PLG staining and to control for unspecific background staining derived from the secondary antibody. After PLG incubation, FhNEJ were fixed in 4% paraformaldehyde (Santa Cruz) for 1 h at room temperature and probed with 13 μg/mL of anti-human PLG antibody raised in sheep (Innovative Research) followed by incubation with Alexa Fluor 568 donkey anti-sheep IgG (Thermo Fisher) diluted 1:500. FhNEJ were washed three times with blocking buffer between incubations and primary and secondary antibodies were diluted in blocking solution and incubated overnight at 4 ºC. Surface staining with concanavalin A (Sigma) was performed by including this compound at a final concentration of 5 μg/mL in the secondary antibody mix. Finally, FhNEJ were washed three times in blocking solution and whole-mounted in glass slides using a 9:1 glycerol solution containing 0.1 M propyl gallate. Specimens were viewed using a spinning disk Dragonfly High Speed Confocal Microscope System (Andor, Oxford Instruments) located at the Microscopy Facility of the Institute of Functional Biology and Genomics (IBFG, Salamanca, Spain) under the 40x/0.95 dry objective lens. Laser settings were maintained across experimental conditions and 12–15 FhNEJ were acquired per condition. Stacks of 30 slices (1 μm/slice) spanning the entire FhNEJ volume were acquired and images were processed in FIJI software (26) by getting a sum slices Z projection of each FhNEJ. For visualisation purposes, histogram widths were equally adjusted in all conditions, and images were converted to RGB colour and exported as .tiff files.

Plasminogen activation assay

This assay was performed in 96 well plates (Costar) in a final volume of 100 µL by measuring the amidolytic activity of generated plasmin on a plasmin-specific chromogenic substrate, as previously described (24). In every well, 2 µg of PLG (Origene) were diluted in PBS together with 3 µg of D-Val-Leu-Lys 4-nitroanilide dihydrochloride chromogenic substrate (S-2251, Sigma) and 1 µg of FhNEJ-Teg or recombinant protein in the presence or absence of 15 ng t-PA (Sigma) or 10 ng u-PA (Sigma). FhNEJ-Teg or recombinant proteins were replaced by equal amounts of BSA and incubated in the presence or absence of PLG activators to control for both the capability of t-PA or u-PA to activate unbound PLG and the spontaneous conversion of PLG into plasmin, respectively. Microplates were incubated for 3 h at 37 ºC and substrate cleavage was assessed by measuring absorbance at 405 nm every 30 min in a Multiskan GO spectrophotometer (Thermo Fisher). All the reactions were performed in technical triplicate.

Detection of plasminogen activation on the surface of live FhNEJ

This assay is an adaptation of the PLG activation assay described in the previous section including incubation with live FhNEJ. FhNEJ were obtained by stimulating the excystment of *F. hepatica* metacercariae as described (see section "*In vitro* excystment of *F. hepatica* metacercariae") followed by recovery in RPMI-1640 culture media (Thermo Fisher) for 3 h at 37 ºC in a 5% CO_2 atmosphere after excystment. In every well, 2 µg of PLG (Origene) were diluted in PBS together with 3 µg of S-2251 (Sigma) and 20 FhNEJs in the presence or absence of 15 ng t-PA (Sigma) plus 10 ng u-PA (Sigma). Some wells contained FhNEJ-Teg in place of live FhNEJ as a positive control for plasmin generation. Wells containing PBS only, FhNEJ in PBS, or PLG plus t-PA and u-PA (without FhNEJ) were used as negative controls. Microplates were incubated for 3 h at 37 ºC and substrate cleavage was assessed by measuring absorbance at 405 nm every 30 min in a Multiskan GO spectrophotometer (Thermo Fisher). All the reactions were performed in biological quadruplicate.

Bidimensional (2D) electrophoresis of FhNEJ-Teg

First, FhNEJ-Teg extract was purified using the ReadyPrep 2-D Cleanup Kit (BioRad) and the protein pellets resuspended in rehydration buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholami-dopropyl)dimethylammonio]-1-propanesulfonate). The extract was then divided in aliquots of 125 µL, supplemented with 0.05 M dithiothreitol (DTT; Sigma) and 0.2% ampholytes pH 3–10 (BioRad) and added to 7 cm ReadyStrip IPG Strips with a

linear pH range of 3–10 (BioRad) for passive rehydration overnight at 20 ºC in a Protean IEF Cell equipment (BioRad). Isoelectric focusing was performed the next day by using a constant amperage of 50 µA per strip in the Protean IEF Cell equipment (BioRad). Next, the proteins in the strips were reduced with DTT (0.02 g/mL) and alkylated with iodoacetamide (0.0025 g/mL) for 10 min and 15 min, respectively, at room temperature. Both DTT and iodoacetamide were diluted in equilibration buffer containing 6 M urea, 2% sodium dodecyl sulphate (SDS), 1.5 M Tris-HCl pH 8.8, 30% glycerol, and bromophenol blue, and the separation for the second dimension was performed using 12% SDS-polyacrylamide gel electrophoresis (PAGE). These gels were stained with silver using in-house prepared reagents following the standard protocol (excluding formaldehyde and glutaraldehyde from the formulations to ensure compatibility with subsequent analysis by mass spectrometry) or transferred to a nitrocellulose membrane for immunoblot detection of PLG binding. Silver-stained gels were imaged using a Chemidoc MP Imaging System (BioRad).

Immunoblot detection of plasminogen binding

The proteins in the 2D gels were transferred to nitrocellulose membranes using a constant amperage of 400 mA for 90 min at 4 ºC. Blots were blocked for 1 h at room temperature with 2% BSA diluted in PBST and incubated overnight at 4 ºC with 25 µg/mL of PLG (Origene) diluted in blocking solution. PLG binding was detected by adding an anti-PLG primary antibody raised in sheep (Acris Antibodies) followed by HRP-conjugated anti-sheep secondary antibody (Sigma). Primary and secondary antibodies were diluted 1:1000 and 1:2000, respectively, in blocking solution and incubated for 90 min at 37 ºC. Membranes were washed three times with PBST between incubations and protein spots with bound PLG were revealed with 4-chloro-naphtol following standard procedures. Blots were imaged in a Chemidoc MP Imaging System (BioRad). Spot matching between silver-stained gels and the blots and the assignment of molecular weights (MW) and isoelectric points (*pI*) of each protein were performed using the PDQuest Software v.8.0.1 (BioRad).

Spot analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

Protein spots in the silver stained gels were manually excised using 1000 µL sterile pipette tips and sent for proteomic analysis at the Proteomics Facility of the Central Support Service for Experimental Research (SCSIE, University of Valencia). In-gel protein

digestion of every spot was performed using sequencing-grade trypsin (Promega) as described elsewhere (27). In brief, 100 ng of trypsin were used for each sample, and digestion was performed overnight at 37 ºC. Trypsin digestion was stopped with 10% trifluoroacetic acid (TFA) and the supernatant was removed. Next, samples were subjected to double acetonitrile (ACN) extraction and the peptide mixtures were dried in a speed vacuum and resuspended in 9 µL of 2% ACN, 0.1% TFA.

LC-MS/MS was carried out as follows: 5 µL of digested peptide mixtures were loaded onto a trap column (3µ C18-CL, 350 µm × 0.5 mm; Eksigent) and desalted with 0.1% TFA at 5 µL/min during 5 min. The peptides were then loaded onto an analytical column (3µ C18-CL 120 Å, 0.075 × 150 mm; Eksigent) equilibrated in 5% ACN plus 0.1% formic acid (FA). Elution was carried out with a linear gradient of 7–40% B in A for 20 min (A: 0.1% FA; B: ACN plus 0.1% FA) at a flow rate of 300 nL/min. Peptides were analysed in a mass spectrometer nanoESI qQTOF (6600plus TripleTOF; ABSCIEX). Sample was ionised in a Source Type: Optiflow<1 µL Nano applying 3.0 kV to the spray emitter at 200 ºC. Analysis was carried out in a data-dependent mode. Survey MS1 scans were acquired from 350–1400 m/z for 250 ms. The quadrupole resolution was set to 'LOW' for MS2 experiments, which were acquired 100–1500 m/z for 25 ms in 'high sensitivity' mode. The following switch criteria were used: charge 2+ to 4+, minimum intensity, and 250 counts per second. Up to 100 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s. Finally, the rolling collision energies equations were set for all ions as for 2+ ions according to the following equations: $|CE| = (slope) \times (m/z) + (intercept)$.

Protein identification

ProteinPilot v5.0 search engine (ABSCIEX) was used for protein identification. ProteinPilot default parameters were used to generate a peak list directly from 6600plus TripleTOF .wiff files. The Paragon algorithm (28) of ProteinPilot v5.0 was used to search the Uniprot_trematoda database (200604, 362615). The following parameters were used: Trypsin specificity, IAM cys-alkylation, no taxonomy restriction, and the search effort set to rapid analysis. Protein grouping was done using the Pro Group algorithm (ABSCIEX). Only proteins belonging to *F. hepatica* and having an Unused value ≥ 2 were used for subsequent analyses, and protein isoforms were manually grouped to facilitate downstream data interpretation. Gene Ontology (GO) analysis in the Biological Function category of PLG-binding proteins identified via 2D-MS was performed using the software Blast2GO v5.2 (Biobam Bioinformatics).

Statistical analysis

Plots were created with Prism 9 software (GraphPad Software) and statistical analyses were performed with the R Commander package (29). Comparison between two groups used an unpaired Student's *t*-test. Comparison between three or more groups used an Analysis of Variance (ANOVA) test followed by a Tukey post-hoc analysis for pairwise comparisons. Unless otherwise stated, the differences were not significant.

Results

The tegument-enriched antigenic fraction of FhNEJ contains plasminogen-binding proteins

The ability of FhNEJ-Teg to bind PLG was analysed by ELISA. To this end, wells were coated with this extract, incubated with PLG, washed, and PLG binding was detected using standard downstream ELISA procedures. This experiment showed that FhNEJ-Teg contains proteins that are capable of binding PLG in a concentration-dependent manner as compared to the negative control, 1% BSA (**Figure 1**). In parallel to this, and aiming at underpinning the molecular mechanism of PLG interaction with FhNEJ-Teg proteins, a competition assay was performed in which the lysine analogue ε-ACA was added to the reaction. In this case, PLG binding was completely abrogated, suggesting that PLG binding to FhNEJ-Teg proteins is specific and mediated by its kringle domains.

In order to reveal the spatial distribution of PLG-binding proteins on the surface of FhNEJ, PLG binding was addressed via immunofluorescent staining by incubating FhNEJ in the presence or absence of PLG prior to fixation (**Figure 2** and **Supp. Figure 1**). Although we found some inter-individual variability in the staining pattern of FhNEJ incubated in the presence of PLG, 92.3% of all FhNEJ incubated with PLG showed specific staining at their posterior area, which, based on the respective brightfield images, coincides with the excretory (protonephridial) pore (**Figure 2B panel i**). In addition to PLG staining at the excretory pore, which was observed in all FhNEJ that were positive for PLG staining, 15.4% of all analysed specimens also showed specific PLG staining at the oral sucker (**Figure 2B panel ii**), and 7.7% of them revealed PLG binding all over the tegument surface (**Figure 2B panel iii**). FhNEJ incubated in the absence of PLG, in the presence of PLG together with 50 mM of ε-ACA, or with secondary antibody alone

(**Figure 2A**, **2C**, and **2D**, respectively) had some unspecific background staining that was less intense than that observed in FhNEJ incubated in the presence of PLG.

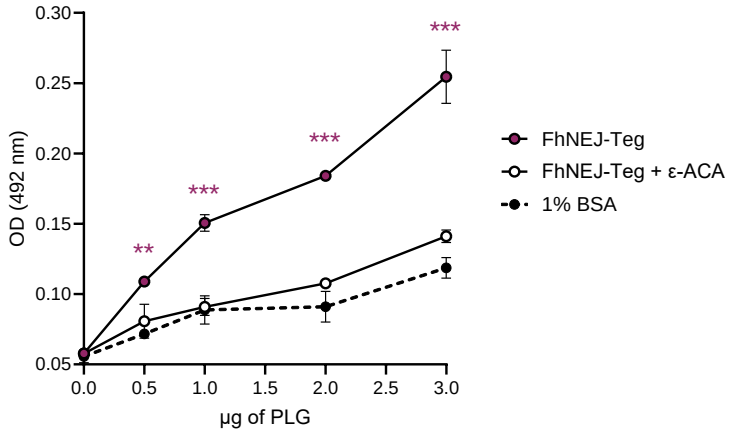

Figure 1. FhNEJ-Teg contains proteins that bind PLG in a concentration- and lysine-dependent manner. FhNEJ-Teg binding to PLG was detected via ELISA by coating wells with 0.5 µg FhNEJ-Teg and incubating with increasing amounts of human PLG. In parallel, a competition assay was performed by including 50 mM of ε-ACA during PLG incubation. FhNEJ-Teg-coated wells and incubated with 1% BSA served as negative controls for PLG binding. Data points indicate the mean of three technical replicates ± SD. Asterisks indicate significant differences between FhNEJ-Teg and the rest of the groups (**p≤0.01; ***p≤0.001; one-way ANOVA followed by Tukey contrasts for pairwise comparisons).

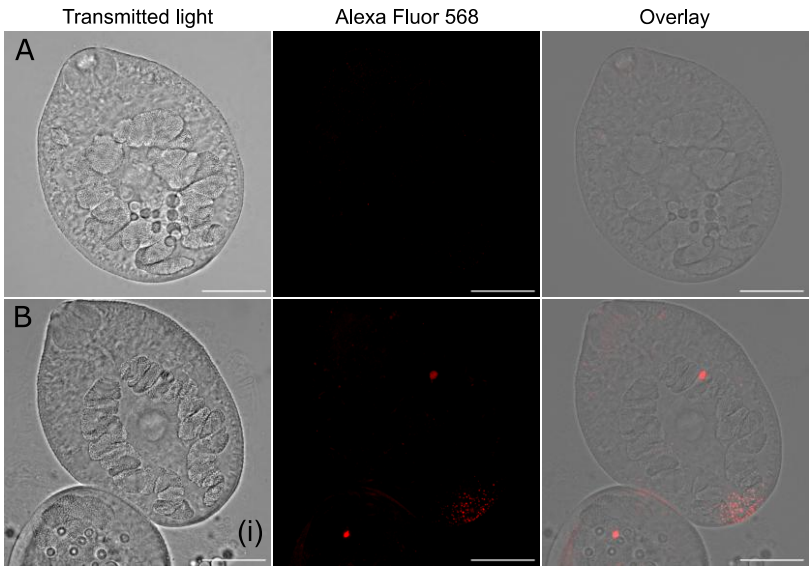

Figure 2 (continued on the next page). Detection of PLG binding on FhNEJ by immunofluorescence reveals distinct localisation patterns of PLG-binding proteins. FhNEJ were whole-mounted and PLG binding (Alexa Fluor 568) was detected by immunolocalisation using a confocal laser microscope. FhNEJs were incubated in the absence (A) or presence (B) of 100 µg/mL of PLG; in the presence of PLG plus 50 mM ε-ACA (C), or with secondary-antibody alone (D). PLG binding was only observed in FhNEJ incubated with PLG (B) and detected around the excretory

(protonephridial) pore (B, panel i), the oral sucker (B, panel ii), and/or all over the tegument surface (B, panel iii). Scale bars, 50 µm. Images represent the sum projection of 31 planes spanning the entire FhNEJ volume.

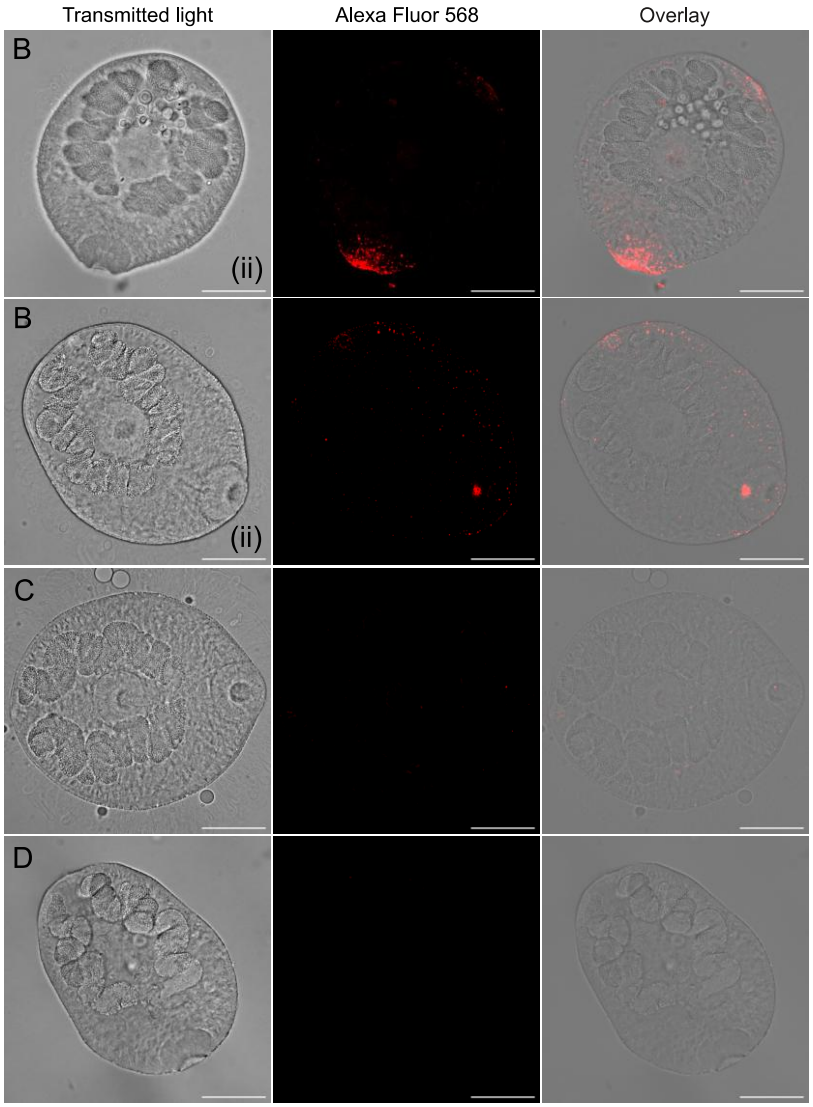

Figure 2 (legend on the previous page).

Binding of plasminogen by FhNEJ-Teg proteins enhances plasmin generation

In order to determine whether PLG binding by FhNEJ-Teg proteins facilitates the conversion of bound PLG to the catalytically active protease, plasmin, we performed an enzymatic assay by co-incubating FhNEJ-Teg with PLG and a plasmin-specific chromogenic substrate. This assay was performed in the presence or absence of the physiologic PLG activators, t-PA or u-PA, to investigate whether plasmin generation

from bound PLG is dependent on host-derived factors or FhNEJ-Teg contains proteases that are capable of cleaving and activating PLG themselves. As observed in **Figure 3**, the conversion of PLG to plasmin by both t-PA (**Figure 3A**) and u-PA (**Figure 3B**) is enhanced in the presence of FhNEJ tegument proteins.

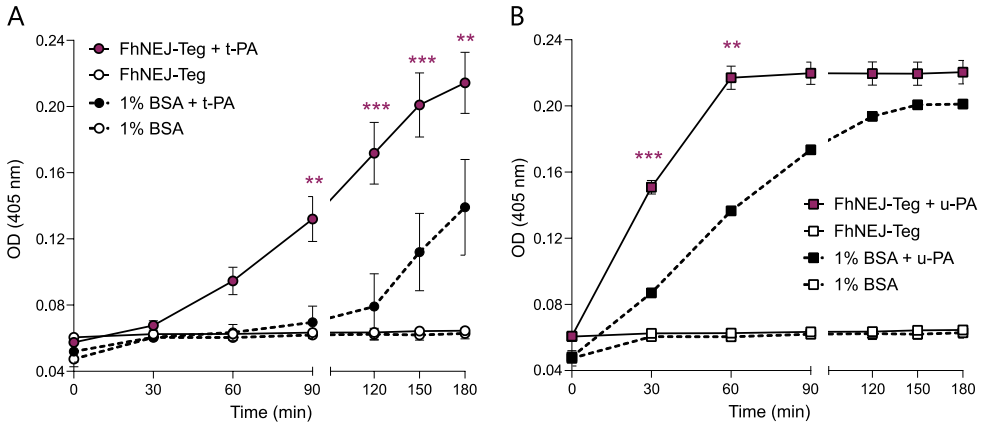

Figure 3. PLG binding by FhNEJ-Teg proteins facilitates its conversion to plasmin. 1 µg of FhNEJ-Teg was incubated with human PLG, a chromogenic substrate for plasmin (S-2251), and either t-PA (A) or u-PA (B), and plasmin generation was assessed by measuring substrate cleavage over time (absorbance at 405 nm). In some instances, FhNEJ-Teg was replaced by 1% BSA as a negative control. Data points indicate the mean of three technical replicates ± SD, and asterisks indicate significant differences between FhNEJ-Teg + t-PA (A) or u-PA (B) and its BSA + PA counterpart (**p≤0.01; ***p≤0.001; one-way ANOVA followed by Tukey contrasts for pairwise comparisons).

We next investigated whether plasmin generation also occurs in the presence of live FhNEJ, so we set up a similar enzymatic assay to test for plasmin activation using live parasites next to the FhNEJ-Teg extract (**Figure 4**). We confirmed that FhNEJ were alive throughout the entire experiment by observing under a stereoscope that they remained highly mobile. Twenty FhNEJ per well were used, since, based on our expertise, this amount of FhNEJ brings about approximately 1 µg of tegument-enriched proteins following our standard extraction procedures, which is the amount that we employed in the abovementioned enzymatic assay. Remarkably, we observed that live FhNEJ incubated with PLG are capable of enhancing plasmin generation in the presence of PLG activators, and that this effect is significantly higher than that observed in wells containing PLG and its activators without FhNEJ. Additionally, the potentiation of plasmin generation by 20 live FhNEJ almost identically replicates the effects obtained when 1 µg of FhNEJ-Teg is added to the reaction (**Figure 4**).

2D analysis of FhNEJ-Teg and identification of PLG-binding proteins by mass spectrometry

In order to determine the identity of the PLG-binding proteins in FhNEJ-Teg, the proteins of this extract were first separated using 2D electrophoresis spanning a MW range of 15–260 kDa and *pI* 3–10 (**Figure 5A**). Secondly, 2D gels were electro-transferred to nitrocellulose membranes in order to detect the presence of PLG-binding proteins by ligand blotting (**Figure 5B**). By matching the spots in the ligand-blot with those in the homologous silver-stained gel, we identified a total of 33 protein spots that were manually excised from the 2D gel and analysed by LC-MS/MS.

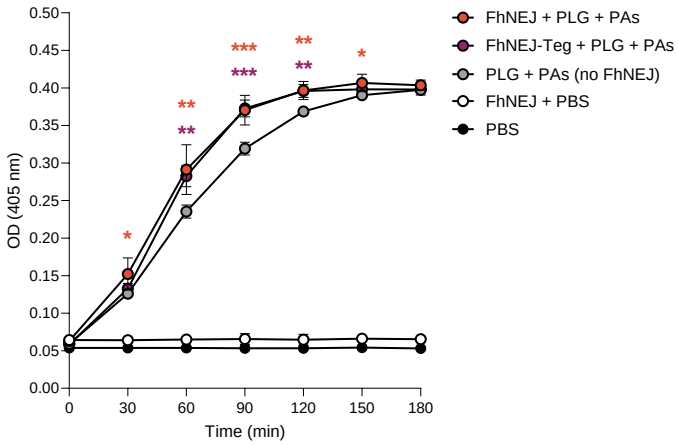

Figure 4. Detection of plasmin generation by live FhNEJ. 20 FhNEJ per well were incubated with PLG, PLG activators (PAs), and a chromogenic substrate for plasmin (S-2251), and the capability of live FhNEJ to stimulate plasmin generation was determined by measuring substrate cleavage (absorbance at 405 nm) every 30 minutes. Data points indicate the mean of four biological replicates ± SD. Wells containing PBS, FhNEJ in PBS, and PLG + PAs without FhNEJ serve as negative controls; and wells containing PLG + PAs + FhNEJ-Teg (1 µg per well) serve as a positive control for the stimulation of plasmin generation by FhNEJ-derived proteins. Upper asterisks indicate significant differences between the negative control (PLG + PAs without FhNEJ) and its counterpart wells containing live FhNEJ. Lower asterisks indicate significant differences between the negative control (PLG + PAs without FhNEJ) and its counterpart wells containing 1 µg of FhNEJ-Teg extract. The differences between the remaining conditions were not statistically significant (*p≤0.05; **p≤0.01; ***≤0.001; one-way ANOVA followed by Tukey contrasts for pairwise comparisons).

Based on similarity to *F. hepatica* sequences contained in the Uniprot_trematoda database, our proteomic analysis identified an average of 12 proteins per spot and revealed that the tegument-enriched fraction of FhNEJ contains 279 potential PLG binding protein isoforms, corresponding to 133 proteins. **Figure 6A** shows the proteins (including isoforms) that were most recurrently identified in the pool of potential PLG binding proteins, being the juvenile-specific cathepsin L3 (FhCL3) the most recurrently identified

PLG-binding protein in the pool of potential PLG-binding proteins detected by MS in FhNEJ-Teg. We also identified cathepsins belonging to additional juvenile-specific clades, such as FhCB2 and FhCB3, and PLG receptors identified in other parasite and non-parasite pathogen species, such as enolase, actin, heat-shock protein 70 (hsp70), annexin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and fructose-bisphosphate aldolase (FBAL) (**Figure 6A** and **Supp. Table 1**), as potential PLG-binding proteins (13–15). GO annotation of the identified potential PLG-binding proteins revealed that these are mostly involved in biological processes related to proteolysis, regulation of catalytic activity, and carbohydrate metabolism (32%, 14%, and 12%, respectively) (**Figure 6B**).

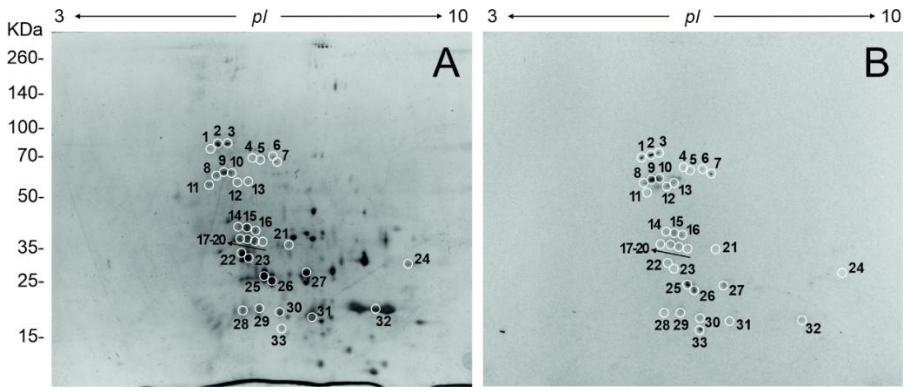

Figure 5. Bidimensional electrophoresis of FhNEJ-Teg and detection of PLG binding by immunoblotting. A) 40 µg of FhNEJ-Teg was separated in two dimensions using IPG strips with a pH range of 3–10 and 12% SDS-PAGE gels. B) Immunoblot detection of PLG binding to FhNEJ-Teg proteins. PLG-binding protein spots are circled and numbered.

Validation of 2D-MS results with recombinant juvenile-specific cathepsins

Our 2D-MS approach showed that juvenile-specific cathepsins B2, B3, and L3 are potentially involved in PLG binding, and we validated these results using recombinant versions of them. The recombinant cathepsins B2, B3, and L3 used for validation had 84.6%, 81.9% and 90.2% identity, respectively, to the sequences identified by MS (**Supp. Figure 2**) and were catalytically-inactive (25). Our ELISA-based PLG binding assay confirmed that FhCB2, FhCB3, and FhCL3 are capable of binding PLG in a concentration-dependent manner, being FhCB2 and FhCL3 the proteins with greatest affinity for PLG (**Figure 7**). PLG binding by these proteases was dramatically reduced in the presence of the lysine analogue ε-ACA, which indicates that PLG binding is specific and occurs via its kringle domains.

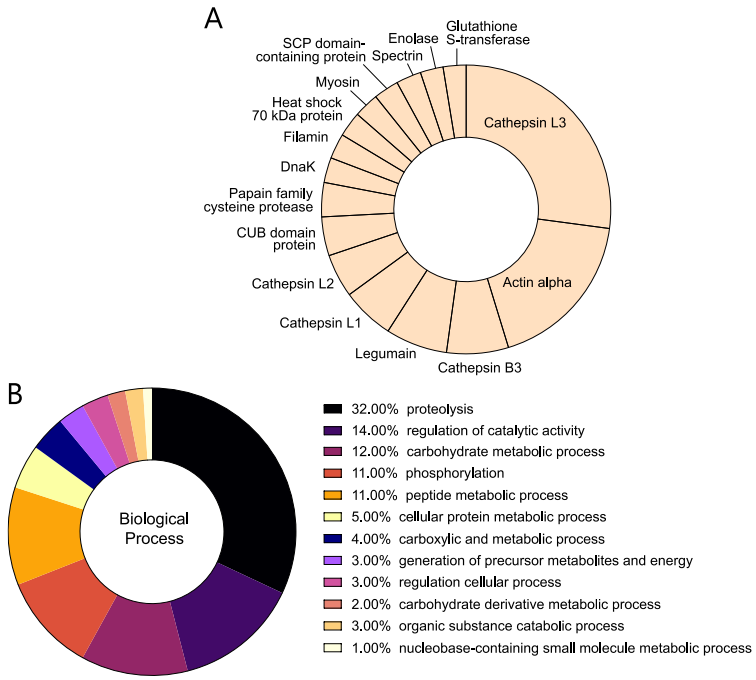

Figure 6. Identification of potential PLG-binding proteins in the tegument of FhNEJ-Teg by mass spectrometry. Protein spots detected in the ligand blot were manually excised and analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). A total of 33 protein spots were identified, corresponding to 133 different proteins. A) Abundance distribution of the 16 most recurrently identified proteins as potential PLG receptors in FhNEJ-Teg. B) Gene Ontology analysis of the potential PLG-binding proteins identified in the PLG-reactive protein spots.

Additionally, we performed plasmin activation enzymatic assays in the presence of recombinant FhCB2, FhCB3, and FhCL3 to address whether binding of PLG by these proteins is functionally relevant in terms of plasmin generation. These assays showed that binding of PLG by FhCB2 and FhCB3 enhances PLG cleavage and its conversion to plasmin (**Figure 8**), although to different extents and requirements in terms of PLG activators. Addition of FhCB2 to the reaction enhances PLG conversion to plasmin preferentially by u-PA over t-PA (**Figure 8A**), while the opposite holds true when FhCB3 is the protein involved in PLG binding (**Figure 8B**). Interestingly, and even though **Figure 7** shows that FhCL3 is capable of binding PLG, stimulation of bound PLG into plasmin by this protein is less potent than that driven by FhCB2 and FhCB3 and only statistically significant at later time points and when t-PA is added to the reaction (**Figure 8C**).

Discussion

The interaction between parasites and the fibrinolytic system of the host has been demonstrated in very diverse organisms, including bacteria, fungi, and parasites, and proposed to be beneficial for parasite survival, migration, and immune evasion inside the vertebrate host (13–15,30). Adult flukes of *F. hepatica* express and secrete proteins that are capable of interacting with PLG and stimulate its conversion to the effector protease, plasmin (16,17), and we hypothesised that this interaction may also occur in the juvenile stages of the parasite and represent a mechanism for FhNEJ to overcome the intestinal wall and initiate tissue migration throughout the mammalian host.

In order to test this hypothesis, we obtained a tegument-enriched antigenic fraction from freshly excysted FhNEJ (FhNEJ-Teg) and analysed PLG binding by ELISA. This experiment showed that FhNEJ-Teg contains proteins that are capable of binding PLG in a concentration- and lysine-dependent manner. Binding of PLG to its physiologic receptors depends on specific protein domains of the PLG molecule, termed kringle domains, which are capable of binding lysine residues present in partner proteins (18). Therefore, the fact that this mechanism is maintained in FhNEJ-Teg reinforces the idea that this binding is specific and biologically relevant in the context of tissue invasion and parasite establishment within the mammalian organism.

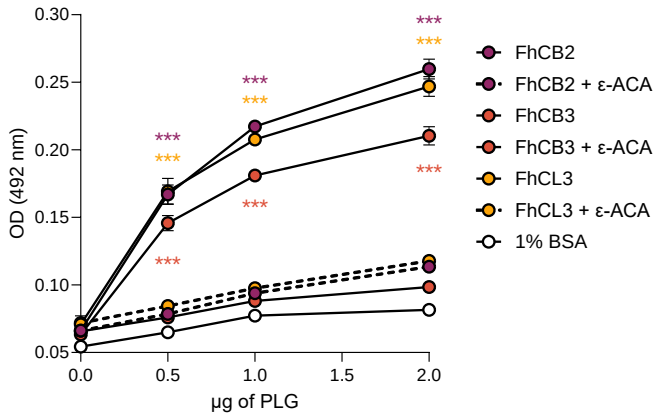

Figure 7. The juvenile-specific cathepsins FhCB2, FhCB3, and FhCL3 bind PLG in a concentration- and lysine-dependent manner. The capability of FhNEJ cathepsins B2, B3, and L3 to bind increasing amounts of PLG was assessed via ELISA using recombinant and catalytically-inactive versions of these proteins. In parallel, a competition assay was performed that included 50 mM of ε-ACA during PLG incubation; and incubation with 1% BSA served as a negative control for PLG binding. Data points indicate the mean of three technical replicates ± SD. Asterisks indicate significant differences between every protein and both its 50 mM ε-ACA counterpart condition and the negative control, 1% BSA (***≤0.001; one-way ANOVA followed by Tukey contrasts for pairwise comparisons).

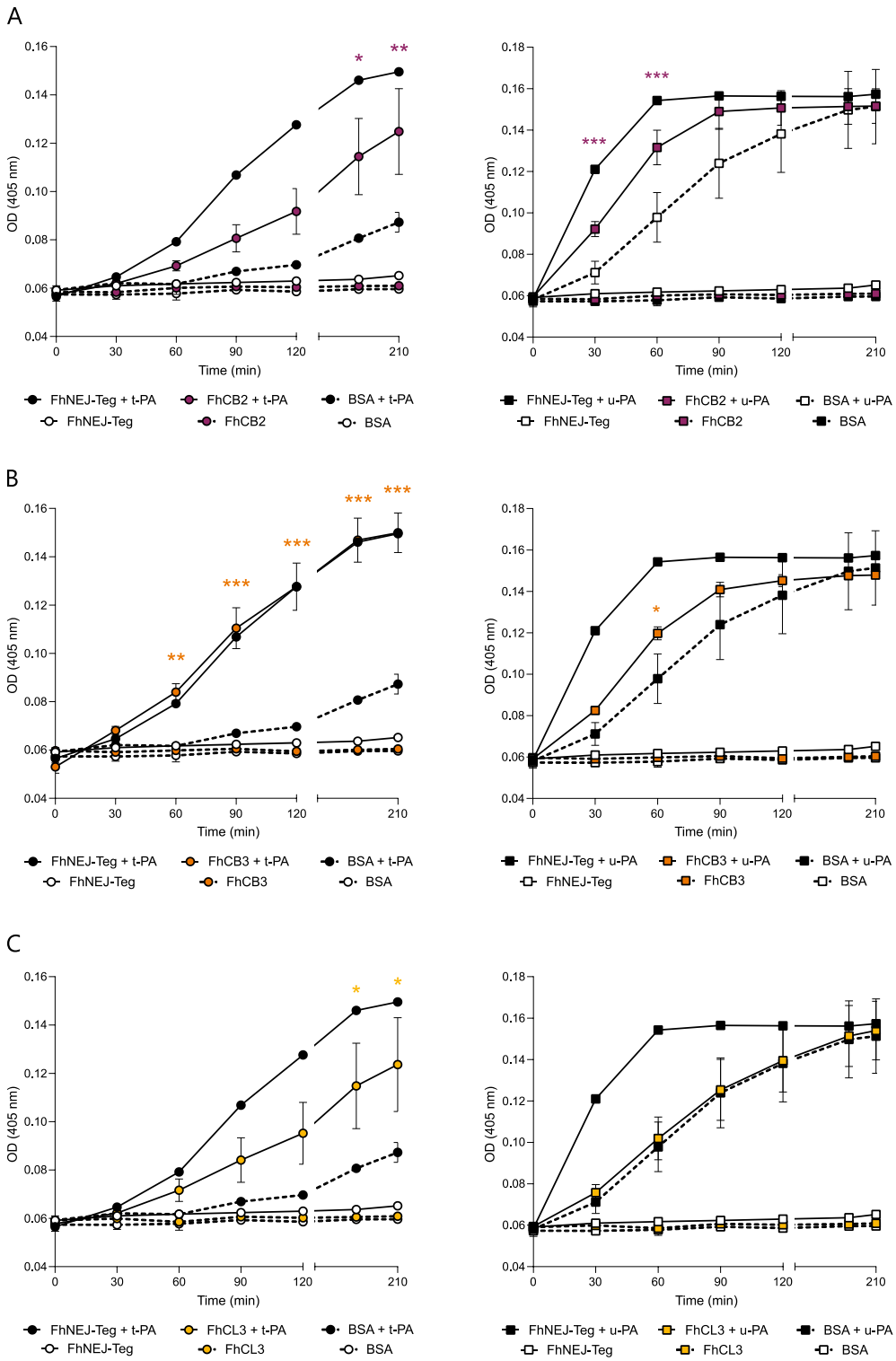

Figure 8 (legend on the next page).

Figure 8 (previous page). PLG binding by FhNEJ cathepsins enhances plasmin generation. 1 µg of recombinant FhCB2 (A), FhCB3 (B), or FhCL3 (C) were incubated with human PLG, a chromogenic substrate for plasmin (S-2251), and either t-PA (left panels) or u-PA (right panels), and plasmin generation was assessed by measuring substrate cleavage (absorbance at 405 nm). In some instances, recombinant cathepsins were replaced by 1% BSA as a negative control. Data points indicate the mean of three technical replicates ± SD, and asterisks indicate significant differences between conditions incubated in the presence of cathepsins + PLG activators and their 1% BSA + PLG activators counterpart (*p≤0.05; **p≤0.01; ***≤0.001; one-way ANOVA followed by Tukey contrasts for pairwise comparisons).

Complementary to our ELISA assays, we performed immunofluorescent staining of FhNEJ surface-bound PLG. Despite observing some interindividual variability, we detected a stable pattern of staining at the excretory (protonephridial) pore, which represents a major site of protein secretion together with the tegument and the gut (31). In addition, some FhNEJ also showed specific PLG labelling at the oral sucker, which is involved in FhNEJ tissue migration via alternate attachment and release together with the ventral sucker (8). This observation further advocates for a role of PLG binding on the surface of FhNEJ in the traversal of the intestinal wall and subsequent tissue migration. Finally, we found an additional pattern of PLG staining that was specific not only at the excretory pore and/or the oral sucker but widely spread all over the tegument, suggesting that the mechanisms of translocation of PLG-binding proteins to the surface of FhNEJ may be manifold.

Next, we sought to analyse whether PLG binding to FhNEJ-Teg proteins is functionally relevant by measuring whether it enhances conversion of bound PLG to its catalytically-active form, plasmin. Three hours was considered a biologically relevant time span to measure plasmin generation given that FhNEJ take around two to three hours to cross the intestinal wall after excystment (1), meaning that plasmin generation should occur within this timeframe if it were to be involved in FhNEJ migration through the intestinal epithelium. By incubating FhNEJ-Teg proteins with a chromogenic substrate for plasmin and the PLG activators t-PA or u-PA, we confirmed that binding of PLG by tegument proteins potentiates its conversion to plasmin by both activators. Unlike described in *Schistosoma bovis* (32), a closely-related trematode species, we were unable to detect any plasmin activity in the absence of PLG activators, indicating that FhNEJ-Teg does not contain proteases that are capable of directly activating PLG. We envision that the mechanism behind enhanced PLG activation by FhNEJ-Teg is most likely similar to that described for canonical PLG receptors, i.e., through the induction of a conformational change in bound PLG molecules that exposes the cleavage sites for t-PA and/or u-PA, thus facilitating the generation of plasmin (19,33). Remarkably, when FhNEJ-Teg extract was replaced by an equivalent amount of live FhNEJ, we confirmed that PLG binding by FhNEJ proteins enhances plasmin generation in the presence of PLG activators. Given

that these FhNEJ remained alive throughout the entire experiment, we cannot rule out the possibility that plasmin generation in non-tegument surfaces, including the gut, or by FhNEJ excreted/secreted products, as observed in adult *F. hepatica* (17), could also account for the observed enhancement of plasmin generation. In relation to this, a recent publication using laser microdissection to generate exclusive tissue fractions of the tegument and the gut of adult *F. hepatica* showed that detergent-based extraction of *F. hepatica* tegument antigens, as used in the present study, enriches this antigenic fraction with proteins derived from the parasite's gastrodermal cells (34). Therefore, it remains a possibility that FhNEJ-Teg PLG-binding proteins detected in this study may have arisen from gut secretions and adhered to the parasite's surface during incubation prior to fixation (12). Notwithstanding the origin of the tegument PLG-binding proteins, and based on the capacity of plasmin to degrade components of the ECM (35), these results support our original idea that PLG fixation on the tegument could represent a mechanism for FhNEJ to harness the functions of the host fibrinolytic system that provides an additional source of proteolytic activity to facilitate traversal of the intestinal wall and the successful establishment of the parasite within the vertebrate host. In the future, these findings could be further validated using *in vivo* or *ex vivo* models of *F. hepatica* infection (36,37).

The PLG activation/plasmin generation activities of t-PA and u-PA are related to variable physiological processes due to differential tissue expression of these proteases. Whereas t-PA expression is almost restricted to the vascular endothelium (18,19), u-PA is synthesised by a wider range of cell types, such as endothelial cells, macrophages, neutrophils, renal epithelial cells, some tumour cells (18,38), and epithelial cells of the small intestine (39). As a result, t-PA activity is mostly restricted to intra-vascular fibrinolysis (19), whereas u-PA is considered a master driver of cell migration (40) in different physiological settings (19,41). The observation that plasmin generation seems to be preferentially enhanced when u-PA—rather than t-PA—is added to the reaction is most likely explained by the fact that u-PA is capable of partially activating soluble (unbound) PLG, whereas t-PA is not (33). Despite this technical constraint, we presume that u-PA may be the activator involved in plasmin generation from FhNEJ-bound PLG in a real physiological setting given that this is the PLG activator that is most highly expressed in the small intestine (39).

Aiming at identifying the proteins that are responsible for PLG binding in FhNEJ-Teg, we performed a proteomic analysis using 2D electrophoresis and ligand blotting coupled to mass spectrometry that identified 133 proteins potentially capable of binding PLG

(summarised in **Supp. Table 1**). We partly validated these results by performing an ELISA-based PLG binding assay with recombinant versions of cathepsins B2, B3, and L3, whose presence at the FhNEJ tegument surface has been previously described (12). It is not surprising that a large number of proteins are used by FhNEJ for PLG recruitment provided that in pathogenic organisms, including parasites, PLG binding and plasmin activation is also highly redundant since multiple surface receptors normally coexist for this purpose (13–15). From an evolutionary perspective, redundancy is an efficient strategy to ensure the maintenance of essential biological functions since it provides for back-up proteins to take over in case of loss of function of other effector proteins due to genetic mutations (42). However, since utilising multiple proteins for one single and same (redundant) function would result in genetic drift and subsequent loss of redundancy, a successful strategy to preserve redundancy is to employ proteins that have both overlapping (redundant) and unique (non-redundant) functions so that selective pressure in favour of the proteins' independent roles guarantees the maintenance of their expression over time (43). This is precisely what we observe in this study: the use of proteins with highly divergent biological functions for PLG binding/plasmin activation purposes (see **Figure 6A** for a GO annotation of the potential PLG-binding proteins identified by 2D-MS), which reinforces the notion that the interaction between FhNEJ and the fibrinolytic system of the host ensures parasite fitness and thus represents an essential mechanism for parasite survival inside the mammalian organism. Unfortunately, the fact that FhNEJ interact with the host fibrinolytic system via such redundant mechanisms poses great difficulties to confront this interaction through classic control and therapeutic strategies (13).

Among others, we identify some of the best characterised PLG receptors in different bacterial, fungal, and parasite species as potential PLG-binding proteins in FhNEJ-Teg, such as enolase, GAPDH, and FBAL (14,15,30). These proteins are also present in the surface of certain tumour cells and their over-expression is linked to metastasis (44), further highlighting the role that the PLG/plasmin system plays in biological processes in which cell migration is required. Interestingly, these canonical PLG receptors are glycolytic enzymes devoid of transmembrane domains and hence their classical biological role is cytosolic. In fact, the third most represented biological function identified in the pool of PLG-binding proteins in FhNEJ-Teg is carbohydrate metabolism, which is not surprising considering that the use of glycolytic enzymes for PLG binding purposes has been well documented in many pathogenic and non-pathogenic organisms, including multicellular parasites (14). The mechanism by which FhNEJ enolase, GAPDH, and

FBAL may be transported to the tegument surface, the outermost layer of FhNEJ, still remains an open question. In other organisms, several mechanisms for cell surface expression of these proteins have been proposed, including relocation to the plasma membrane as part of a protein complex, binding to lipid molecules followed by translocation to the outer membrane, and non-covalent association to the cell membrane after active secretion to the extracellular space (14,44). Alternatively, these proteins could also be released to the extracellular space as part of the cargo of extracellular vesicles (EVs), which have recently been identified in FhNEJ (45) and proposed to represent the secretion route of *F. hepatica* molecules that lack consensus N-terminal signal sequences for their secretion via the endoplasmic reticulum/Golgi pathway (12). In line with this, a proteomic analysis of FhNEJ EVs revealed that these secretory structures harbour enzymes identified in the present study as potential PLG-binding proteins, namely GAPDH (Accession A0A2H1BWY6), heat-shock protein 70 (Accession B1NI98), heat-shock protein 90 (Accession A0A2H1BZF7), and CUB-domain protein (Accession A0A2H1C1S8) (46). Additionally, adult *F. hepatica* smallest EVs—the so-called 120K EVs—harbour GAPDH (47), and another report demonstrates that these 120K EVs are most likely released via the protonephridial (excretory) system (31). Although we do not know whether FhNEJ smallest or 120K-like EVs harbour GAPDH and/or other PLG-binding proteins identified in this study—because the proteome of FhNEJ EVs has so far been analysed in total FhNEJ EVs and not separate populations (46)—, these observations coincide with the staining pattern of PLG binding observed in our immunofluorescence images, which is most prominent in the protonephridial (excretory) pore. Altogether, these findings support the idea that some of the PLG-binding proteins that we detected on the surface of FhNEJ may be released as part of the cargo of FhNEJ EVs.

In addition to glycolytic enzymes, proteolysis is the most represented biological function of the potential PLG-binding proteins identified in our 2D-MS analysis. Remarkably, the most recurrently identified potential PLG-binding proteins belong to the superfamily of cathepsin proteases. More specifically, we identify FhCL3 and FhCB3 as potential PLG-binding proteins, and FhCL1, FhCL2, and FhCB2, to a lesser extent. This is consistent with the developmental stage-specific expression of these proteases, being FhCB1-3 and FhCL3 mostly expressed in the juvenile stages of the parasite that are found in the duodenum (36,48,49). However, one of the limitations of our 2D-MS approach is that it is performed under denaturing conditions, which may expose internal PLG-binding epitopes in some proteins that are not employed for PLG-binding in a real physiological

context. Therefore, we set out to validate our 2D-MS results by assessing PLG binding in an ELISA experiment, which is performed under native conditions, using recombinant and catalytically-inactive versions of the juvenile cathepsins B2, B3, and L3 (25). This experiment confirmed the capacity of these proteins to bind PLG in a concentration- and lysine-dependent manner, and our plasmin activation enzymatic assays confirmed that FhCB2 and FhCB3 also serve as enhancers of plasmin generation from bound PLG, although with different kinetics and involving different PLG activators. Whereas FhCB2 induces plasmin generation by u-PA and not t-PA, the opposite holds true for FhCB3, and even though we confirmed that FhCL3 is capable of binding PLG, this protein is apparently less effective in stimulating its conversion to plasmin.

Previous experiments performed in our lab following a similar 2D-MS strategy showed that cathepsins present in excretory/secretory products of *F. hepatica* adult flukes are capable of binding PLG (17). To the best of our knowledge, *F. hepatica* is the only parasite whose cathepsins have been identified as PLG-binding proteins (13), and given that we validated our results using catalytically-inactive recombinant versions of them, we can also conclude that this is the first time that a proteolysis-independent function is described for these proteases in relation to FhNEJ migration. Therefore, we propose that *F. hepatica* cathepsins shall be regarded as moonlighting proteins, which are defined by their capability to play two or more unrelated roles depending on their cellular or developmental context (50). Proteins that are constitutively expressed and have high levels of expression are more likely to develop moonlighting functions because their constitutive nature makes it more likely for them to be involved in advantageous interactions with protein and non-protein partners. Similarly, proteins that arise from gene duplication events are likely to moonlight given that gene duplication creates a permissive space in which new functions can be developed without compromising the canonical role of the protein (50). *F. hepatica* cathepsin peptidases are amongst the most abundantly expressed proteins in FhNEJ (51) and they are also products of gene duplication events (52), which makes them good candidates to acquire moonlighting roles including the PLG-binding function that we describe in this study.

From a clinical perspective, the interaction between FhNEJ and the fibrinolytic system described here may contribute to and/or exacerbate the focal haemorrhages that are caused by migratory juveniles during the acute phase of infection (1,53). Furthermore, our results could also be relevant in relation to the neurological manifestations associated with this disease—including paralysis, walking problems, speech disorders, disorientation, amnesia, delusional disorders, convulsions and coma, among others (54)—, which have

been postulated to be mediated by increased blood-brain barrier permeability caused by an aberrant fibrinolytic balance in infected patients (17).

In conclusion, we have demonstrated for the first time that FhNEJ interact with the fibrinolytic system of the mammalian host as a potential early stage invasion mechanism by binding PLG and enhancing its conversion into the active enzyme, plasmin. We further determined that PLG binding by FhNEJ is highly redundant and mainly driven by juvenile cathepsin peptidases, which advocates for a previously uncharacterised proteolytic-independent role of these enzymes in relation to FhNEJ trans-intestinal migration. Altogether, this work contributes to a deeper understanding of host-parasite relationships at the earliest phases of fasciolosis, and sheds light into a potential FhNEJ migration mechanism that may be worth exploiting from a pharmacological and immunological perspective for the development of more successful treatment and control strategies against this widespread parasitic disease.

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References

1. Mas-Coma S, Valero MA, Bargues MD. Fascioliasis. In: Digenetic Trematodes. Springer International Publishing; 2019. p. 71–103.
2. Siles-Lucas M, Becerro-Recio D, Serrat J, González-Miguel J. Fascioliasis and fasciolopsiasis: current knowledge and future trends. Res Vet Sci. 2021 Jan;134:27–35.
3. Webb CM, White AC, Gotuzzo E, Lopez M, Bascope R, Cabada MM, *et al.* Socioeconomic factors associated with *Fasciola hepatica* infection among children from 26 communities of the Cusco region of Peru. Am J Trop Med Hyg. 2018 Nov 7;99(5):1180–5.
4. Angles R, Buchon P, Valero MA, Bargues MD, Mas-Coma S. One health action against human fascioliasis in the Bolivian Altiplano: food, water, housing, behavioural

traditions, social aspects, and livestock management linked to disease transmission and infection sources. IJERPH. 2022 Jan 20;19(3):1120.

5. World Health Organization. Ending the neglect to attain the Sustainable Development Goals: a road map for Neglected Tropical Diseases 2021-2030. Geneva: World Health Organization; 2020.
6. Afshan K, Fortes-Lima CA, Artigas P, Valero MA, Qayyum M, Mas-Coma S. Impact of climate change and man-made irrigation systems on the transmission risk, long-term trend and seasonality of human and animal fascioliasis in Pakistan. Geospat Health. 2014 May 1;8(2):317.
7. Bargues MD, Artigas P, Angles R, Osca D, Duran P, Buchon P, *et al.* Genetic uniformity, geographical spread and anthropogenic habitat modifications of lymnaeid vectors found in a One Health initiative in the highest human fascioliasis hyperendemic of the Bolivian Altiplano. Parasites Vectors. 2020 Dec;13(1):171.
8. González-Miguel J, Becerro-Recio D, Siles-Lucas M. Insights into *Fasciola hepatica* juveniles: crossing the fasciolosis rubicon. Trends Parasitol. 2021 Jan;37(1):35–47.
9. Lalor R, Cwiklinski K, Calvani NED, Dorey A, Hamon S, Corrales JL, *et al.* Pathogenicity and virulence of the liver flukes *Fasciola hepatica* and *Fasciola gigantica* that cause the zoonosis fasciolosis. Virulence. 2021 Dec 31;12(1):2839–67.
10. Caffrey CR, Goupil L, Rebello KM, Dalton JP, Smith D. Cysteine proteases as digestive enzymes in parasitic helminths. PLoS Negl Trop Dis. 2018 Aug 23;12(8):e0005840.
11. McGonigle L, Mousley A, Marks NJ, Brennan GP, Dalton JP, Spithill TW, *et al.* The silencing of cysteine proteases in *Fasciola hepatica* newly excysted juveniles using RNA interference reduces gut penetration. Int J Parasitol. 2008;38(2):149–557.
12. Cwiklinski K, Donnelly S, Drysdale O, Jewhurst H, Smith D, De Marco Verissimo C, *et al.* The cathepsin-like cysteine peptidases of trematodes of the genus *Fasciola*. In: Advances in Parasitology. Elsevier; 2019. p. 113–64.
13. Diosdado A, Simón F, Serrat J, González-Miguel J. Interaction of helminth parasites with the haemostatic system of their vertebrate hosts: a scoping review. Parasite. 2022;29:35.
14. Figuera L, Gómez-Arreaza A, Avilán L. Parasitism in optima forma: exploiting the host fibrinolytic system for invasion. Acta Tropica. 2013 Oct;128(1):116–23.

15. González-Miguel J, Siles-Lucas M, Kartashev V, Morchón R, Simón F. Plasmin in parasitic chronic infections: friend or foe? Trends Parasitol. 2016 Apr;32(4):325–35.

16. Bernal D, de la Rubia JE, Carrasco-Abad AM, Toledo R, Mas-Coma S, Marcilla A. Identification of enolase as a plasminogen-binding protein in excretory-secretory products of *Fasciola hepatica*. FEBS Lett. 2004 Apr 9;563(1–3):203–6.

17. González-Miguel J, Valero MA, Reguera-Gomez M, Mas-Bargues C, Bargues MD, Simón F, *et al.* Numerous *Fasciola* plasminogen-binding proteins may underlie blood-brain barrier leakage and explain neurological disorder complexity and heterogeneity in the acute and chronic phases of human fascioliasis. Parasitology. 2019 Mar;146(3):284–98.

18. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. Br J Haematol. 2005 May;129(3):307–21.

19. Nicholl S, Roztocil E, Davies M. Plasminogen activator system and vascular disease. CVP. 2006 Apr 1;4(2):101–16.

20. Lijnen HR. Elements of the fibrinolytic system. Ann N Y Acad Sci. 2001;936(1):226–36.

21. Amara U, Flierl MA, Rittirsch D, Klos A, Chen H, Acker B, *et al.* Molecular intercommunication between the complement and coagulation systems. J Immunol. 2010 Nov 1;185(9):5628–36.

22. Hernández-González A, Valero ML, Pino MS del, Oleaga A, Siles-Lucas M. Proteomic analysis of *in vitro* newly excysted juveniles from *Fasciola hepatica*. Mol Biochem Parasitol. 2010 Aug;172(2):121–8.

23. García-Campos A, Ravidà A, Nguyen DL, Cwiklinski K, Dalton JP, Hokke CH, *et al.* Tegument glycoproteins and cathepsins of newly excysted juvenile *Fasciola hepatica* carry mannosidic and paucimannosidic N-glycans. PLoS Negl Trop Dis. 2016 May 3;10(5):1–26.

24. González-Miguel J, Morchón R, Mellado I, Carretón E, Montoya-Alonso JA, Simón F. Excretory/secretory antigens from *Dirofilaria immitis* adult worms interact with the host fibrinolytic system involving the vascular endothelium. Mol Biochem Parasitol. 2012 Feb;181(2):134–40.

25. Barbour T, Cwiklinski K, Lalor R, Dalton JP, De Marco Verissimo C. The zoonotic helminth parasite *Fasciola hepatica*: virulence-associated cathepsin B and cathepsin L

cysteine peptidases secreted by infective newly excysted juveniles (NEJ). Animals. 2021;11(12):3495.

26. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, *et al.* Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012 Jun 28;9(7):676–82.
27. Shevchenko A, Jensen ON, Podtelejnikov AV, Sagliocco F, Wilm M, Vorm O, *et al.* Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. Proc Natl Acad Sci USA. 1996 Dec 10;93(25):14440–5.
28. Shilov IV, Seymour SL, Patel AA, Loboda A, Tang WH, Keating SP, *et al.* The paragon algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol Cell Proteomics. 2007 Sep;6(9):1638–55.
29. Fox J, Bouchet-Valat M. Rcmdr: R Commander. 2021. Available from: https://socialsciences.mcmaster.ca/jfox/Misc/Rcmdr/
30. Ayón-Núñez DA, Fragoso G, Bobes RJ, Laclette JP. Plasminogen-binding proteins as an evasion mechanism of the host's innate immunity in infectious diseases. Biosci Rep. 2018 Oct 31;38(5):1–16.
31. de la Torre-Escudero E, Gerlach JQ, Bennett APS, Cwiklinski K, Jewhurst HL, Huson KM, *et al.* Surface molecules of extracellular vesicles secreted by the helminth pathogen *Fasciola hepatica* direct their internalisation by host cells. PLoS Negl Trop Dis. 2019 Jan;13(1):e0007087.
32. Ramajo-Hernández A, Pérez-Sánchez R, Ramajo-Martín V, Oleaga A. *Schistosoma bovis*: plasminogen binding in adults and the identification of plasminogen-binding proteins from the worm tegument. Exp Parasitol. 2007 Jan;115(1):83–91.
33. Aisina RB, Mukhametova LI. Structure and function of plasminogen/plasmin system. Russ J Bioorg Chem. 2014 Nov;40(6):590–605.
34. Bennett A, de la Torre-Escudero E, Dermott S, Threadgold L, Hanna R, Robinson M. *Fasciola hepatica* gastrodermal cells selectively release extracellular vesicles via a novel atypical secretory mechanism. IJMS. 2022 May 15;23(10):5525.
35. Syrovets T, Simmet Th. Novel aspects and new roles for the serine protease plasmin. Cell Mol Life Sci. 2004 Apr 1;61(7–8):873–85.

36. Becerro-Recio D, Serrat J, López-García M, Molina-Hernández V, Pérez-Arévalo J, Martínez-Moreno Á, *et al*. Study of the migration of *Fasciola hepatica* juveniles across the intestinal barrier of the host by quantitative proteomics in an *ex vivo* model. PLoS Negl Trop Dis. 2022 Sep;16(9):e0010766.

37. Robb E, McCammick EM, Wells D, McVeigh P, Gardiner E, Armstrong R, *et al*. Transcriptomic analysis supports a role for the nervous system in regulating growth and development of *Fasciola hepatica* juveniles. PLoS Negl Trop Dis. 2022 Nov 7;16(11):e0010854.

38. Mondino A, Blasi F. uPA and uPAR in fibrinolysis, immunity and pathology. Trends Immunol. 2004 Aug;25(8):450–5.

39. Gibson PR, Birchall I, Rosella O, Albert V, Finch CF, Barkla DH, *et al*. Urokinase and the intestinal mucosa: evidence for a role in epithelial cell turnover. Gut. 1998 Nov 1;43(5):656–63.

40. Vassalli JD. The urokinase receptor. Fibrinolysis. 1994;8(Suppl. 1):172–81.

41. Rijken DC, Lijnen HR. New insights into the molecular mechanisms of the fibrinolytic system. J Thromb Haemost. 2009 Jan;7(1):4–13.

42. Nowak MA, Boerlijst MC, Cooke J, Smith JM. Evolution of genetic redundancy. Nature. 1997 Jul;388(6638):167–71.

43. Ghosh S, O'Connor TJ. Beyond paralogs: the multiple layers of redundancy in bacterial pathogenesis. Front Cell Infect Microbiol. 2017 Nov 15;7:467.

44. Ceruti P, Principe M, Capello M, Cappello P, Novelli F. Three are better than one: plasminogen receptors as cancer theranostic targets. Exp Hematol Oncol. 2013 Dec;2(1):1–11.

45. Sánchez-López CM, Trelis M, Jara L, Cantalapiedra F, Marcilla A, Bernal D. Diversity of extracellular vesicles from different developmental stages of *Fasciola hepatica*. Int J Parasitol. 2020 Aug;50(9):663–9.

46. Trelis M, Sánchez-López CM, Sánchez-Palencia LF, Ramírez-Toledo V, Marcilla A, Bernal D. Proteomic analysis of extracellular vesicles from *Fasciola hepatica* hatching eggs and juveniles in culture. Front Cell Infect Microbiol. 2022 Jun 3;12:903602.

47. Cwiklinski K, de la Torre-Escudero E, Trelis M, Bernal D, Dufresne PJ, Brennan GP, *et al*. The extracellular vesicles of the helminth pathogen, *Fasciola hepatica*: biogenesis pathways and cargo molecules involved in parasite pathogenesis. Mol Cell Proteom. 2015 Dec;14(12):3258–73.

48. Robinson MW, Menon R, Donnelly SM, Dalton JP, Ranganathan S. An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*. Mol Cell Proteom. 2009 Aug;8(8):1891–907.

49. Becerro-Recio D, Serrat J, López-García M, Sotillo J, Simón F, González-Miguel J, *et al.* Proteomics coupled with *in vitro* model to study the early crosstalk occurring between newly excysted juveniles of *Fasciola hepatica* and host intestinal cells. PLoS Negl Trop Dis. 2022 Oct;16(10):e0010811.

50. Singh N, Bhalla N. Moonlighting Proteins. Annu Rev Genet. 2020;54:265–85.

51. Cwiklinski K, Jewhurst H, McVeigh P, Barbour T, Maule AG, Tort J, *et al.* Infection by the helminth parasite *Fasciola hepatica* requires rapid regulation of metabolic, virulence, and invasive factors to adjust to its mammalian host. Mol Cell Proteom. 2018;17:792–809.

52. Cwiklinski K, Dalton JP, Dufresne PJ, La Course J, Williams DJ, Hodgkinson J, *et al.* The *Fasciola hepatica* genome: gene duplication and polymorphism reveals adaptation to the host environment and the capacity for rapid evolution. Genome Biol. 2015 Dec;16(71):1–13.

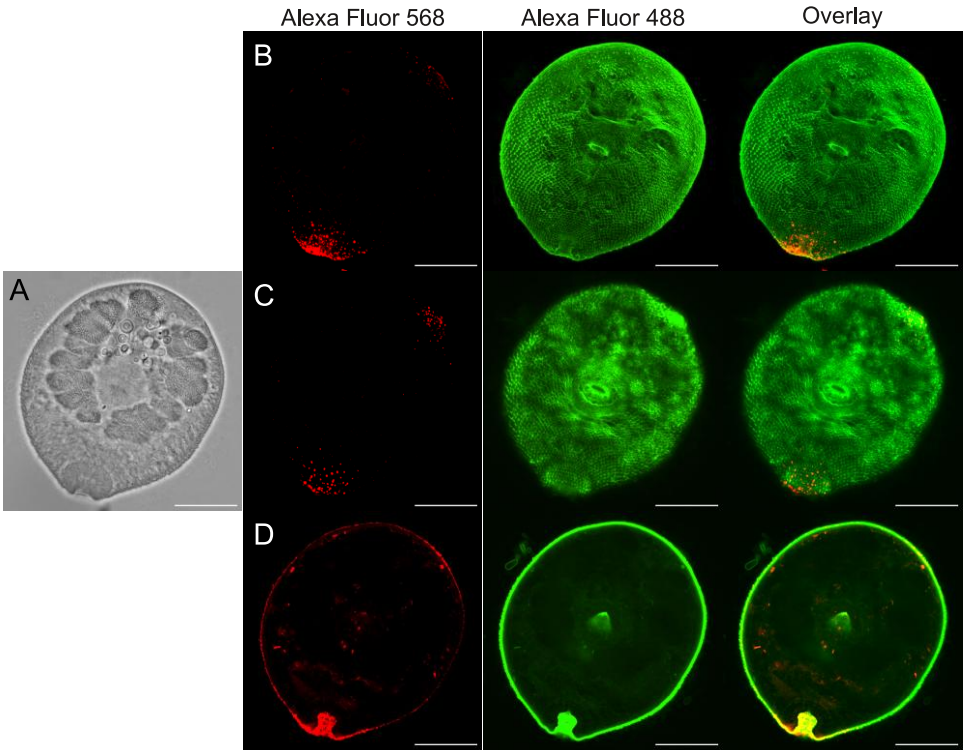
53. Dalton JP, editor. Fasciolosis. 2nd ed. UK: CABI; 2021.

54. Mas-Coma S, Agramunt VH, Valero MA. Neurological and ocular fascioliasis in humans. Adv Parasitol. 2014;84:27–149.

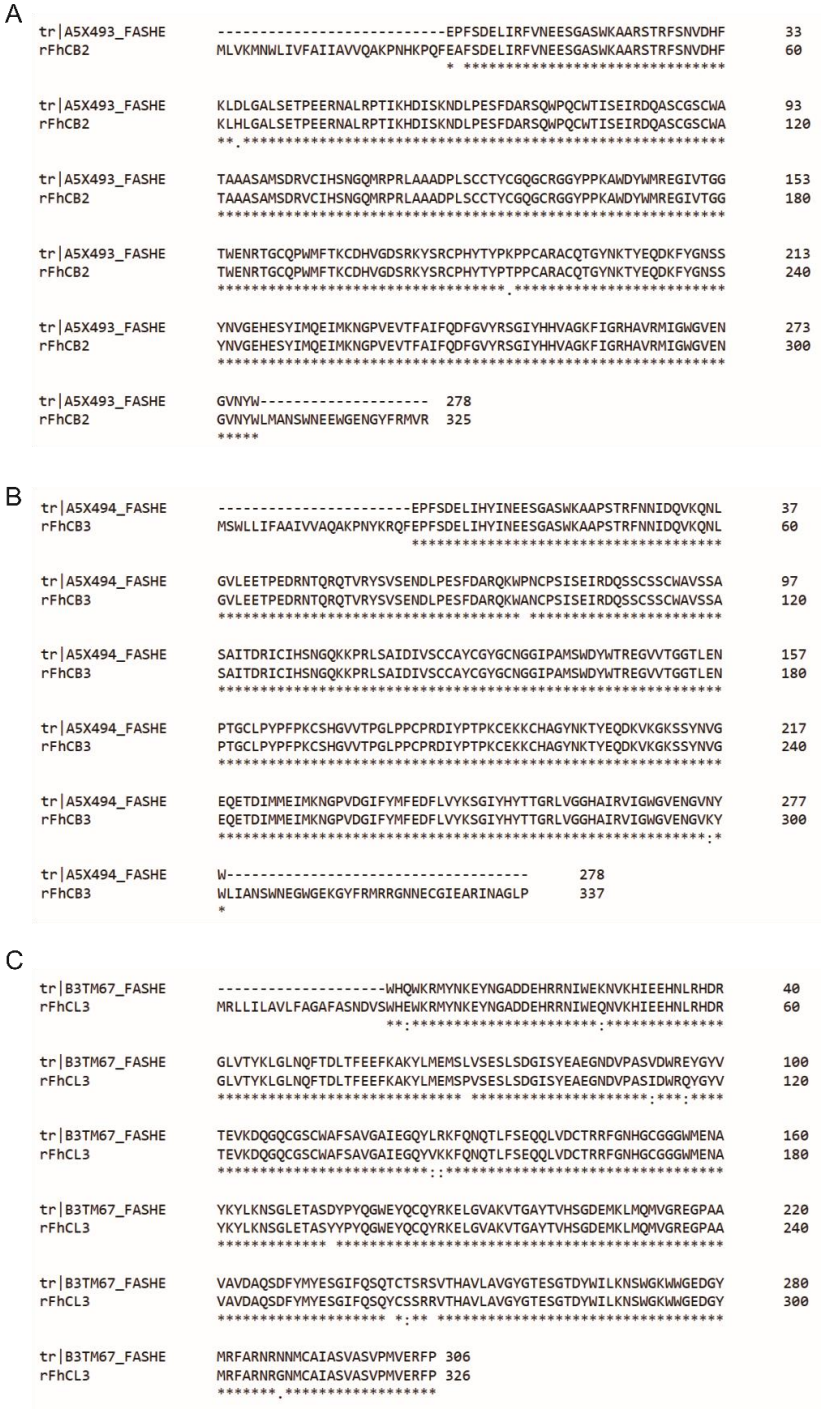
Supplementary information

Supplementary Table 1. Potential PLG-binding proteins in the tegument-enriched antigenic fraction of FhNEJ identified by 2D-LC-MS/MS. Available at: https://doi.org/10.1371/journal.pntd.0010936.s001

Supplementary Figure 1. Detection of PLG binding on whole-mounted FhNEJ by immunofluorescence. Representative image of an FhNEJ incubated in the presence of PLG (Alexa Fluor 568) and counterstained with concanavalin A (Alexa Fluor 488) to highlight the FhNEJ surface. Panels show the transmitted light image **(A)**, the sum of all acquired Z projection slices spanning the entire FhNEJ volume **(B)**, and the surface **(C)** or middle **(D)** plane of the projection to highlight specific PLG staining at the FhNEJ surface. Scale bars, 50 μm.



Supplementary Figure 2. Sequence alignment between recombinant FhCB2 **(A)**, FhCB3 **(B)**, or FhCL3 **(C)** used in our validation assays (top sequences) with those identified by 2D-MS (bottom sequences).



Chapter 2

Molecular characterisation of the interplay between *Fasciola hepatica* juveniles and laminin as a mechanism to adhere to and break through the host intestinal wall

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Fasciola hepatica is the main causative agent of fasciolosis, a zoonotic parasitic disease of growing public health concern. *F. hepatica* metacercariae are ingested by the host and excyst in the intestine, thereby releasing the newly excysted juveniles (FhNEJ), which traverse the gut wall and migrate towards the biliary ducts. Since blocking *F. hepatica* development is challenging after crossing of the intestinal wall, targeting this first step of migration might result in increased therapeutic success. The intestinal extracellular matrix (ECM) is constituted by a network of structural proteins, including laminin (LM) and fibronectin (FN), that provide mechanical support while acting as physical barrier against intestinal pathogens. Here, we employed enzyme-linked immunosorbent and immunofluorescent assays to test for the presence of LM- and FN-binding proteins in a tegument-enriched antigenic fraction of FhNEJ, and further determined their identity by bidimensional electrophoresis coupled to mass spectrometry. Additionally, we performed enzymatic assays that revealed for the first time the capability of the juvenile-specific cathepsin L3 to degrade LM, and that LM degradation by FhNEJ proteins is further potentiated in the presence of host plasminogen. Finally, a proteomic analysis showed that the interaction with LM triggers protein changes in FhNEJ that may be relevant for parasite growth and adaptation inside the mammalian host. Altogether, our study provides valuable insights into the molecular interplay between FhNEJ and the intestinal ECM, which may lead to the identification of targetable candidates for the development of more effective control strategies against fasciolosis.

Introduction

Fasciola hepatica is the most common causative agent of fasciolosis, and it has a complex life cycle that includes an intermediate snail host, where the asexual phases of the parasite multiply, and a definitive mammalian host, where the parasites develop into sexually-mature flukes. Definitive hosts, usually ruminants and humans, become infected upon ingestion of aquatic plants or water contaminated with metacercariae, which release the newly excysted juveniles (FhNEJ) upon arrival to the duodenum. Shortly after excystment, FhNEJ cross the intestinal wall and migrate through the peritoneum to eventually penetrate the liver. At this point, immature flukes initiate a migration period through the liver parenchyma until they finally gain access into the major hepatic biliary ducts, where adult parasites develop and egg shedding starts (1). By infecting millions of ruminants worldwide, *F. hepatica* infection causes major economic losses in the livestock industry (2) and represents a major threat to animal welfare and global food security (3). In addition, current estimations on the prevalence of human fasciolosis reveal that up to 17 million people may be infected worldwide (4). Since human infections are particularly concentrated in regions where people live in substandard economic conditions, human fasciolosis is considered a neglected foodborne zoonotic disease of growing public health concern (5). Increasing reports of human and veterinary cases of fasciolosis that are resistant to the standard drug, triclabendazole, together with the lack of an effective vaccine, raise awareness on the need to find alternative treatment and control strategies against *F. hepatica* (1). Given that crossing of the intestinal wall by FhNEJ is considered a 'point of no return' in fasciolosis in terms of therapeutic control (6), a detailed understanding on the processes that drive FhNEJ trans-intestinal migration may be useful in this regard.

The intestinal mucosa represents the first and most extensive line of defence against pathogens that gain access to the mammalian organism through the intestinal tract. Structurally, this tissue is formed by a monolayer of diversely specialised epithelial cells that sit on top of the intestinal extracellular matrix (ECM), which is externally outlined by a thin layer of smooth muscle cells (7). The intestinal ECM consists of an acellular mesh of structural proteins that give mechanical support to the tissue while acting as a physical barrier against invading pathogens (7–9). In addition to physically blocking pathogen entry, the intestinal mucosa is endowed with potent immune mechanisms to prevent pathogen invasion (10), and beyond its supportive and defensive roles, the ECM is also

regarded as a master regulator of cell activity by triggering intracellular signalling events that regulate cell differentiation and migration (11).

The intestinal ECM is divided in two sub-compartments. Sitting right beneath the epithelial layer we find the basement membrane, which is mainly formed by collagen type IV, laminin (LM), nidogens, and perlecan. Immediately below the basement membrane, there is the interstitial layer, whose major components are collagen I and III, fibronectin (FN), and elastin (7). In order to break through the intestinal epithelium and succeed at host invasion, intestinal pathogens have evolved a myriad of mechanisms to overcome the intestinal ECM and evade immune responses that are initiated in this tissue towards them (8,9). A paradigmatic example of such mechanisms is the expression of ECM-binding proteins—collectively termed adhesins—and proteases by intestinal pathogenic bacteria that bind to and degrade a variety of ECM components (8,12). The importance of ECM-binding and degrading proteins for successful tissue invasion is evidenced by studies showing that blocking these factors results in a dramatic reduction in the invasive capacity of pathogens (8). In addition to endogenous proteases, many intestinal pathogens hijack the functions of the host fibrinolytic enzyme plasmin, which is a broad-spectrum serine protease that degrades several components of the intestinal ECM (13), for colonisation purposes. The ability of degrading ECM components both by endogenous proteases and by an induction of plasmin generation from the host-circulating zymogen plasminogen (PLG) is a widespread phenomenon in bacterial, fungal, and protozoan pathogens (8), but remains largely unexplored in helminth parasites, including *F. hepatica*.

Here, we employed a combination of enzyme-linked immunosorbent (ELISA), immunofluorescent, and enzymatic assays to study the interaction between FhNEJ and LM, one of the major components of the intestinal basement membrane, and we further analysed the consequences of the FhNEJ-LM crosstalk at a proteomic level to better understand the biological relevance of this event in the establishment of infection. Collectively, these results broaden our knowledge on host-parasite relationships in early fasciolosis, which may pave the way for the identification of attractive targets for future anti-*Fasciola* therapeutics.

Materials and methods

In vitro excystment of *F. hepatica* metacercariae

An amount of 5000 *F. hepatica* metacercariae (Ridgeway Research Ltd.) were excysted *in vitro* as previously described (14). In brief, CO_2 was bubbled in 10 mL of ice-cold distilled water for 30 s, the solution was supplemented with 0.02 M of sodium dithionite, and added to metacercariae for 1 h at 37 ºC. Next, metacercariae were washed twice with distilled water, resuspended in 5 mL of Hank's balanced salt solution (Sigma) supplemented with 10% lamb bile (obtained from a local abattoir) and 30 mM HEPES (Sigma) pH 7.4, and incubated at 37 ºC. FhNEJ were manually recovered under a stereomicroscope using a 20 µL pipette and immediately subjected to protein extraction.

Protein extraction of the tegument- and somatic-enriched antigenic fractions of FhNEJ

Upon excystment, FhNEJ were processed as previously described (15,16). Briefly, FhNEJ were washed twice in sterile phosphate-buffered saline (PBS), resuspended in 200–300 µL of PBS containing 1% Nonidet P-40 (NP-40; Sigma), and incubated at room temperature for 30 min with mild rotation. Following incubation, FhNEJ were centrifuged 5 min at 300 x*g* and the supernatant containing the tegument-enriched protein fraction (FhNEJ-Teg) was frozen at -80 ºC until use. Protein pellets containing naked FhNEJ were resuspended in 200 µL of RIPA buffer (Sigma) and disrupted by ultrasound (5 cycles of 30 s). The samples were then centrifuged at 1000 x*g* for 5 min and the clarified supernatant containing the somatic-enriched protein fraction (FhNEJ-Som) was transferred to clean tubes and frozen at -80 ºC. Protein concentration was determined using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific).

LM and FN binding assays

LM and FN binding were assessed by ELISA as previously described (17), with minor modifications. Briefly, microtiter 96-well plates were coated overnight at 4 ºC with either 1 µg of FhNEJ-Teg, 0.5 µg of recombinant *F. hepatica* cathepsin CL3 (FhCL3) (18), or 0.5 µg of *F. hepatica* glutathione S-transferase (FhGST) (kind gifts from Prof. J.P. Dalton) diluted in 200 µL of 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6. Wells coated with 1% bovine serum albumin (BSA) served as negative controls for unspecific binding of LM and FN. We used recombinant *Saccharomyces cerevisae* enolase (ScENO; Sigma) as a positive control for LM binding as it shares over 50% sequence identity with enolase

from *Paracoccidioides brasiliensis*, which has been previously described to bind LM (19). Recombinant actin from the roundworm *Dirofilaria immitis* (DiACT) (20) was used as a positive control for FN binding (unpublished data from our lab). Wells coated with 0.5 μg of FN (Santa Cruz Biotechnology) or LM (Santa Cruz Biotechnology) of human and mouse origin, respectively, were used as primary antibody controls. Following two washes in PBS containing 0.05% Tween-20 (PBST), wells were blocked with 1% BSA for 1 h at 37 ºC and increasing amounts of LM (0 μg to 10 μg per well) or FN (0 μg to 10 μg per well) diluted in blocking solution were added and incubated for 2 h at 37 ºC. Wells were washed three times in PBST and incubated for 2 h at 37 ºC with the corresponding primary antibodies diluted in blocking solution: anti-LM raised in rabbit (1:2000; Abcam) or anti-FN raised in mouse (1:500; Santa Cruz Biotechnology). Following three washes in PBST, wells were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG (Sigma) diluted 1:3000 and 1:2000, respectively, in blocking solution and further incubated for 1 h at 37 ºC. Finally, wells were washed three times in PBST and LM/FN binding was revealed by adding 100 μL of 1.5 mM of the chromogenic substrate ortho-phenylene-diamine (Sigma) diluted in 25 mM citric acid, 45 mM Na_2HPO_4, 0.04% H_2O_2, pH 5. The reaction was stopped by adding 100 μL of sulphuric acid 2 N and optical densities (OD) were measured at 492 nm in a Multiskan GO spectrophotometer (Thermo Fisher Scientific). LM and FN binding assays were performed in triplicate.

Detection of LM binding on the surface of FhNEJ by immunofluorescence

F. hepatica metacercariae were excysted as described in the section "*In vitro* excystment of *F. hepatica* metacercariae" and FhNEJ were recovered every hour after addition of excystment medium and transferred to plates containing RPMI-1640 culture media (Thermo Fisher Scientific) supplemented with 30 mM HEPES (Sigma), 0.1% glucose (Sigma), and 50 μg/mL gentamycin (Sigma) at 37 ºC in a 5% CO_2 atmosphere (21). One hour after recovery, 80 FhNEJ per condition were washed three times in PBS and incubated with blocking solution (0.1% BSA in PBS) supplemented with 100 μg/mL of LM (Santa Cruz Biotechnology) for 2 h at 37 ºC. Two extra sets of FhNEJ incubated in blocking buffer alone served as negative controls for LM binding and to control for unspecific background staining derived from the secondary antibody. After LM incubation, FhNEJ were fixed in 4% paraformaldehyde (Santa Cruz Biotechnology) for 1 h at room temperature and probed with anti-LM antibody raised in rabbit (Abcam) diluted 1:100 followed by incubation with Alexa Fluor 488 donkey anti-rabbit IgG (Thermo Fisher Scientific) diluted 1:500. FhNEJ were washed three times with blocking

buffer between incubations, and primary and secondary antibodies were diluted in blocking solution and incubated overnight at 4 ºC. Finally, FhNEJ were washed once in blocking solution, twice in PBS, and whole-mounted in glass slides using a 9:1 glycerol solution containing 0.1 M propyl gallate. Specimens were viewed using a spinning disk Dragonfly High Speed Confocal Microscope System (Andor Oxford Instruments) located at the Microscopy Facility of the Institute of Functional Biology and Genomics (IBFG, Salamanca, Spain) under the 40×/0.95 dry objective lens. A minimum of 15 FhNEJ were acquired per condition. Stacks of 31 slices (1 μm/slice) spanning the entire FhNEJ volume were acquired, and images were processed in FIJI software v.1.53t (22) by getting a maximum Z projection of each FhNEJ. For visualisation purposes, histogram widths were equally adjusted in all conditions and the images were converted to RGB colour and exported as .tiff files.

Bidimensional (2D) electrophoresis of FhNEJ

Bidimensional electrophoresis of FhNEJ-Teg was performed as previously described (23). First, FhNEJ-Teg extract was purified using the ReadyPrep 2-D Cleanup Kit (BioRad) and protein pellets were resuspended in rehydration buffer (7 M urea, 2 M thiourea, and 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). The protein extract was then divided in aliquots of 125 μL, supplemented with 0.05 M dithiothreitol (DTT; Sigma), and 0.2% ampholytes pH 3–10 (BioRad), and added to 7 cm ReadyStrip IPG Strips with a linear pH range of 3–10 (BioRad) for passive rehydration overnight at 20 ºC in a Protean Isoelectric Focusing (IEF) Cell equipment (BioRad). IEF was performed the next day by using a constant amperage of 50 μA per strip in the Protean IEF Cell equipment (BioRad). Next, the proteins in the strips were reduced with DTT (0.02 g/mL) and alkylated with iodoacetamide (0.0025 g/mL) for 10 min and 15 min, respectively, at room temperature. Both DTT and iodoacetamide were diluted in equilibration buffer containing 6 M urea, 2% sodium dodecyl sulphate (SDS), 1.5 M Tris-HCl pH 8.8, 30% glycerol, and bromophenol blue. Separation in the second dimension was performed using 12% SDS-polyacrylamide gel electrophoresis (PAGE). These gels were stained with silver using in-house prepared reagents following the standard protocol (excluding formaldehyde and glutaraldehyde from the formulations to ensure compatibility with subsequent analysis by mass spectrometry) or transferred to a nitrocellulose membrane for immunoblot detection of LM binding. Silver-stained gels were imaged using a Chemidoc MP Imaging System (BioRad).

Immunoblot detection of LM-binding proteins

Immunoblot detection of LM-binding proteins was performed as previously described (17,23), with minor modifications. In brief, the proteins in the 2D gels were transferred to a nitrocellulose membrane using a constant amperage of 400 mA for 90 min at 4 ºC. The blot was blocked for 1 h at room temperature with 2% BSA diluted in PBST and incubated overnight at 4 ºC with 5 μg/mL of LM (Santa Cruz Biotechnology) diluted in blocking solution. LM binding was detected by adding an anti-LM primary antibody raised in rabbit (Abcam) followed by HRP-conjugated anti-rabbit IgG (Sigma). Primary and secondary antibodies were diluted 1:2000 and 1:4000, respectively, in blocking solution and incubated for 90 min at 37 ºC. Membranes were washed three times with PBST between antibody incubations and protein spots with bound LM were detected using enhanced chemiluminescence (Clarity Western ECL Substrate, BioRad) on a ChemiDoc MP Imaging System (BioRad). Spot matching between the silver-stained gel and the counterpart blot was performed using the PDQuest Software v.8.0.1 (BioRad).

Spot analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

Proteomic analysis of LM-binding spots was performed as previously described (23). Briefly, protein spots in the silver-stained gels were manually excised using 1000 μL sterile pipette tips and sent for proteomic analysis at the Proteomics Facility of the Central Support Service for Experimental Research (SCSIE, University of Valencia). In-gel protein digestion of every spot was performed using sequencing-grade trypsin (Promega) as described elsewhere (24). In brief, 100 ng of trypsin were used for each sample, and digestion was performed overnight at 37 ºC. Trypsin digestion was stopped with 10% trifluoroacetic acid (TFA) and the supernatant was removed. Next, samples were subjected to double acetonitrile (ACN) extraction and the peptide mixtures were dried in a speed vacuum and resuspended in 9 μL of 2% ACN, 0.1% TFA.

LC-MS/MS was carried out as follows: 5 μL of digested peptide mixtures were loaded onto a trap column (3μ C18-CL, 350 um × 0.5 mm; Eksigent) and desalted with 0.1% TFA at 5 μL/min during 5 min. The peptides were then loaded onto an analytical column (3μ C18-CL 120 Å, 0.075 × 150 mm; Eksigent) equilibrated in 5% ACN plus 0.1% formic acid (FA). Elution was carried out with a linear gradient of 7–40% B in A for 20 min (A: 0.1% FA; B: ACN plus 0.1% FA) at a flow rate of 300 nL/min. Peptides were analysed in a mass spectrometer nanoESI qQTOF (6600plus TripleTOF; ABSCIEX). Samples were

ionised in a Source Type: Optiflow < 1 μL Nano applying 3.0 kV to the spray emitter at 200 ºC. Analysis was carried out in a data-dependent mode. Survey MS1 scans were acquired from 350–1400 m/z for 250 ms. The quadrupole resolution was set to 'LOW' for MS2 experiments, which were acquired 100–1500 m/z for 25 ms in 'high sensitivity' mode. The following switch criteria were used: charge 2+ to 4+, minimum intensity, and 250 counts per second. Up to 100 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s. Finally, rolling collision energies equations were set for all ions as for 2+ ions according to the following equation: |CE|= (slope)×(m/z)+(intercept).

Protein identification within LM-binding spots

The identification of proteins contained within LM-binding spots was performed as previously described (23). Briefly, proteinPilot v5.0 search engine (ABSCIEX) was used for protein identification. ProteinPilot default parameters were used to generate a peak list directly from 6600plus TripleTOF .wiff files and the Paragon algorithm (25) of ProteinPilot v5.0 was used to search the Uniprot_trematoda database (200604, 362615). The following parameters were used: trypsin specificity, IAM cys-alkylation, no taxonomy restriction, and search effort set to rapid analysis. Protein grouping was completed using the Pro Group algorithm (ABSCIEX). Only proteins belonging to *F. hepatica* and having an Unused value ≥ 2 were used for subsequent analyses, and protein isoforms were manually grouped to facilitate downstream data interpretation. Clades of cathepsin L entries with unspecified descriptions were assigned based on similarity to canonical cathepsin L prosegment sequences via multiple sequence alignment with Clustal Omega, and those corresponding to cathepsin B entries were determined by phylogenetic comparison of the entire protein sequence. Gene Ontology (GO) analysis in the Biological Function category of LM-binding proteins identified by 2D-MS was performed using the software Blast2GO v5.2 (Biobam Bioinformatics).

LM degradation assays

LM degradation by FhCL3 and by FhNEJ-Teg-induced plasmin generation from bound PLG were analysed. In the first setting, recombinant FhCL3 zymogen was activated *in vitro* by incubation for 2 h at 37 ºC in an appropriate volume of C-P buffer (0.1 M citrate-phosphate, 100 mM NaCl, 2 mM DTT, 10 μg/mL dextran sulfate, pH 4.5) (18) so that the final concentration of mature enzyme in the mix was 1 μM. In the second setting, 1.5 μg of FhNEJ-Teg were incubated in the presence or absence of 3 μg of PLG (Origene)

and 15 ng of the urokinase-type PLG activator (u-PA, Sigma). In both instances, the reactions were performed in a total volume of 150 μL. A condition containing 1 μM of human plasmin (Origene) was used at as a positive control for LM degradation (26). The reaction mixtures were supplemented with 0.1 μg of LM (Santa Cruz Biotechnology) and incubated at 37 ºC for 3 h. For visualization of LM degradation, equal amounts of samples were loaded after incubation into either in-house prepared 6% polyacrylamide or pre-cast 4–20% Bis-Tris (GenScript) gels, and protein bands were revealed by silver stain or Sypro Ruby (Sigma) following standard procedures. Band intensity was quantified by densitometry using the FIJI software v.1.53t (22).

Analysis of LM-induced proteomic changes in FhNEJ by Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS)

In order to test whether LM interaction triggers proteomic changes in FhNEJ, we employed a methodology described by others (27–29), with minor modifications. First, three untreated 6 cm^2 plates were coated with 76.44 μg LM (Santa Cruz Biotechnology) diluted in 3 mL of coating medium (0.2 M $NaHCO_3$, pH 9.5) overnight at 4 ºC. Three uncoated plates were used as negative controls and incubated likewise in coating medium without LM. The next day, plates were washed three times with PBS and 400 FhNEJ per plate were added in Dulbecco's Modified Eagle Medium (Capricorn Scientific) supplemented with 2% foetal bovine serum (Capricorn Scientific) and incubated 3 h at 37 ºC in a 5% CO_2 atmosphere. After incubation, FhNEJ were harvested and subjected to protein extraction for the isolation of FhNEJ-Teg and FhNEJ-Som as described in the section "Protein extraction of the tegument- and somatic-enriched antigenic fractions of FhNEJ".

Quantitation of protein concentration in all samples was performed using a detergent-compatible kit (Protein Quantification Assay; Machery-Nagel) following the manufacturer's instructions. Prior to gel separation, 10 μg of each protein sample were diluted in an appropriate volume of 2×Laemmli Sample Buffer (BioRad) supplemented with β-mercaptoethanol followed by denaturation at 95 ºC for 5 min. Gel separation was performed using Any kD Mini-Protean TGX Precast Protein Gels (BioRad) at 200 V for 5 min, followed by fixation in 40% ethanol/10% acetic acid for 1 h and stained with colloidal Coomassie (BioRad) for 1 h. After destaining in milliQ water, in-gel digestion of protein samples was performed with sequencing grade trypsin (Promega), as described elsewhere (24). Specifically, gel bands were cut and digested using 500 ng of trypsin in 50 μL of 50 mM ammonium bicarbonate solution and digestion was stopped by addition of

TFA to a final concentration of 1%. Peptides were then recovered by performing a double extraction with ACN, dried in a rotatory evaporator, and resuspended in the appropriate volume of 2% ACN/0.1% TFA to a final concentration of 0.3 μg/μL.

For library construction, all *F. hepatica* samples were pooled together and loaded onto a trap column (3μ C18-CL 120 Å, 350 μm × 0.5 mm; Eksigent) and desalted with 0.1% TFA at 5 μL/min for 3 min. The peptides were then loaded onto an analytical column (3μ C18-CL 120 Å, 0.075 × 150 mm; Eksigent) equilibrated in 5% ACN/0.1% FA. Elution was performed over 60 min in a linear gradient of 7–40% solvent B in A (A: 0.1% FA; B: ACN, 0.1% FA) at a flow rate of 300 nL/min and analysed in a mass spectrometer nanoESI qQTOF (6600plus TripleTOF; ABSCIEX). Eluted peptides were ionised applying 2.8 kV to the spray emitter. Analysis was carried out in a data-dependent mode. Survey MS1 scans were acquired from 350–1250 m/z for 250 ms. The quadrupole resolution was set to 'UNIT' for MS2 experiments, which were acquired 100–1500 m/z for 150 ms in 'high sensitivity' mode. Protein identification for library construction was performed with ProteinPilot v5.0 (SCIEX) using default parameters to generate a peak list directly from 6600plus tripleTOF .wiff files. The Paragon algorithm (25) was used to search a database containing the predicted proteome of *F. hepatica* (PRJEB25283, https://parasite.wormbase.org/Fasciola_hepatica_prjeb25283/Info/Index (accessed on 10th March 2022) appended to the cRAP contaminant database (https://www.thegpm.org/crap/ (accessed on 10th March 2022) (15).

For SWATH-MS analysis, 5 μL of each peptide mixture were loaded onto a trap column (NanoLC Column, 3μ C18-CL, 75 μm × 15 cm; Eksigent) and desalted with 0.1% TFA at 2 μL/min during 10 min. The peptides were then loaded onto an analytical column (LC Column, 3μ C18-CL, 75 um × 12 cm; Nikkyo) equilibrated in 5% ACN/0.1% FA. Peptide elution was carried out with a linear gradient of 5 to 40% solvent B (A: 0.1% FA; B: ACN, 0.1% FA) for 90 min at a flow rate of 300 nL/min and analysed in a mass spectrometer nanoESI qQTOF (6600 TripleTOF, ABSCIEX). The tripleTOF was operated in SWATH (data-independent acquisition) mode, in which a 0.050 s TOF MS scan from 350–1250 m/z was performed, followed by 0.080 s product ion scans from 350–1250 m/z on the defined windows (3.05 s/cycle). We used 37 SWATH windows with 15 Da window width from 450 to 1000 Da. The .wiff files obtained from SWATH individual acquisitions were analysed using PeakView 2.1 (SCIEX). First, protein areas were calculated and normalised to the total sum of the areas of all the quantified proteins. Statistical treatment was performed using MarkerView 3.0 (SCIEX), and evaluation of the differences between stimulated and control samples was performed using Student's *t*-test

coupled to Welch post-hoc correction. Only proteins with a p-value<0.05 were considered as differentially expressed. Discriminant Analysis with Pareto Scaling was performed for the reduction in dimensionality and plotted in R software, and protein descriptions and Uniprot accession numbers were retrieved using the software Blast2GO v6.0.3 (Biobam Bioinformatics). Clades of cathepsin B entries were determined by phylogenetic comparison of the entire protein sequence.

Immunoblotting

Equal amounts of proteins from FhNEJ-Som samples used for SWATH-MS analysis were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. Total protein staining was performed with Sypro Ruby (Thermo Fisher Scientific) according to manufacturer's instructions followed by incubation for 1 h at room temperature in blocking buffer (PBST containing 2% BSA). *F. hepatica* cathepsin B1–3 anti-serum produced in rabbit (kind gift from Prof. J.P. Dalton) was diluted 1:500 in blocking buffer and added to the blot overnight at 4 ºC. After incubation with HRP-conjugated secondary antibody (Sigma) diluted 1:2000 in blocking buffer, protein bands were detected using enhanced chemiluminescence (Clarity Western ECL Substrate, BioRad) on a ChemiDoc MP Imaging System (BioRad).

Statistical analysis

Plots were created with Prism 9 software (GraphPad Software) and statistical analyses were performed with the R Commander package (30). Comparison between three or more groups was completed with Analysis of Variance (ANOVA) test followed by Tukey post-hoc correction for pairwise comparisons. Asterisks indicate significant differences between the corresponding experimental groups (**p≤0.01, ***p≤0.001). Unless otherwise stated, the differences were not significant.

Results

The FhNEJ tegument-enriched protein fraction contains proteins that bind to LM but not FN

First, after *in vitro* excystment of *F. hepatica* metacercariae, FhNEJ isolation, and extraction of their tegument-enriched protein fractions, we analysed whether FhNEJ-Teg contains proteins that are capable of interacting with two proteins of the intestinal ECM, such as

LM and FN. To this end, we set up an ELISA-based experiment in 96-well microtiter plates by coating wells with 1 µg of FhNEJ-Teg or 1% BSA as a control for unspecific binding. Upon incubation of the wells with increasing amounts of LM, we observed that binding of LM by FhNEJ-Teg proteins is concentration-dependent, statistically greater than that obtained in wells coated with 1% BSA, and similar to that mediated by our positive control, ScENO (**Figure 1A**). In parallel to this, a similar experiment was performed by adding human FN instead of LM to FhNEJ-Teg-coated wells to analyse FN binding by FhNEJ-Teg proteins. Interestingly, this experiment showed that FhNEJ-Teg does not contain proteins that bind to FN (**Figure 1B**). In parallel to this, LM binding on the surface of FhNEJ was also confirmed by immunofluorescent staining of whole-mounted parasites (**Figure 1C–E**), which revealed specific binding of this ECM protein all over the FhNEJ surface.

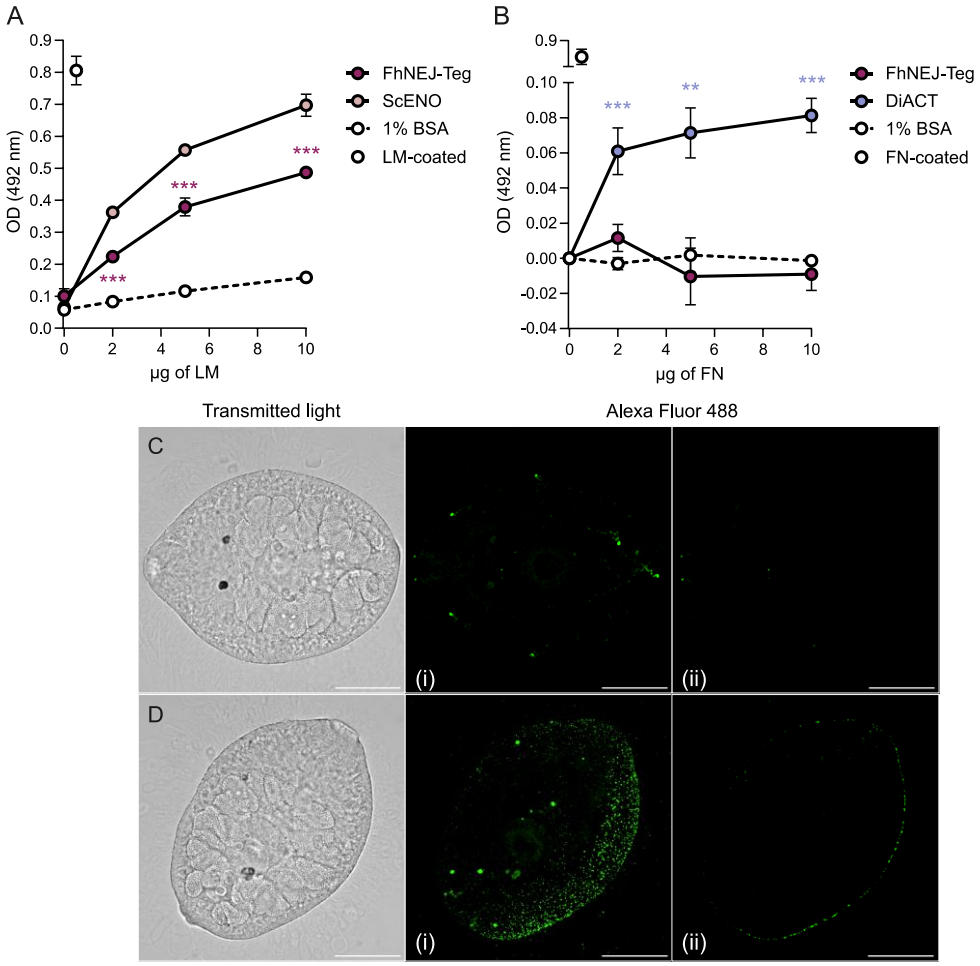

Figure 1 (continued on the next page).

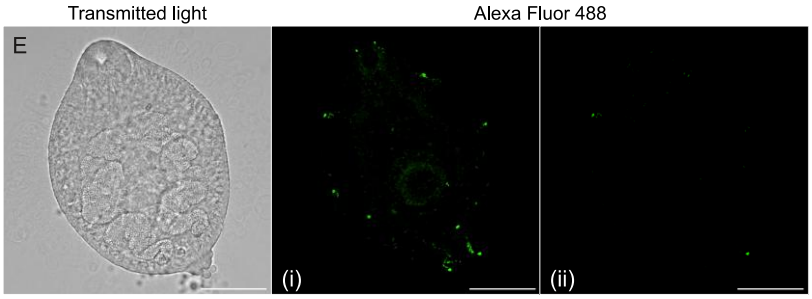

Figure 1. FhNEJ-Teg contains proteins that interact with LM, but not FN. Binding of FhNEJ-Teg proteins to either LM (A) or FN (B) was detected via ELISA by coating wells with FhNEJ-Teg followed by incubation with increasing amounts of LM or FN. ScENO and DiACT served as positive controls for LM and FN binding, respectively, and 1% BSA was used as a negative control for unspecific binding of both ECM proteins. Optical densities (OD) were measured at 492 nm. Dots indicate the mean of three technical replicates ± SD. Asterisks indicate significant differences between the corresponding group and the negative control (**p≤0.01; ***p≤0.001; one-way ANOVA). (C–E) FhNEJ were whole-mounted and LM binding on their surface (Alexa Fluor 488) was detected on a confocal laser microscope. Panels show representative images of FhNEJ incubated in the absence (C) or presence (D) of 100 µg/mL of LM, or with secondary antibody alone (E). A maximum projection of all acquired planes spanning the entire FhNEJ volume (i), and the middle plane of each projection (ii) are shown to highlight specific staining at the parasite surface. Scale bars, 50 µm.

Identification of potential LM-binding proteins in FhNEJ-Teg

Aiming at identifying the proteins contained within FhNEJ-Teg that are involved in LM binding, we first separated the proteins contained within this extract by 2D electrophoresis. Separation was performed in two gels so that the protein spots in one of the gels were revealed by silver staining (**Figure 2A**) and the proteins contained in the other gel were transferred to a nitrocellulose membrane to detect LM binding by standard western blot procedures (**Figure 2B**). After matching the LM-reactive spots that appeared in the blot with those in the homologous silver-stained gel, we detected a total of 14 protein spots that were sent for analysis by MS.

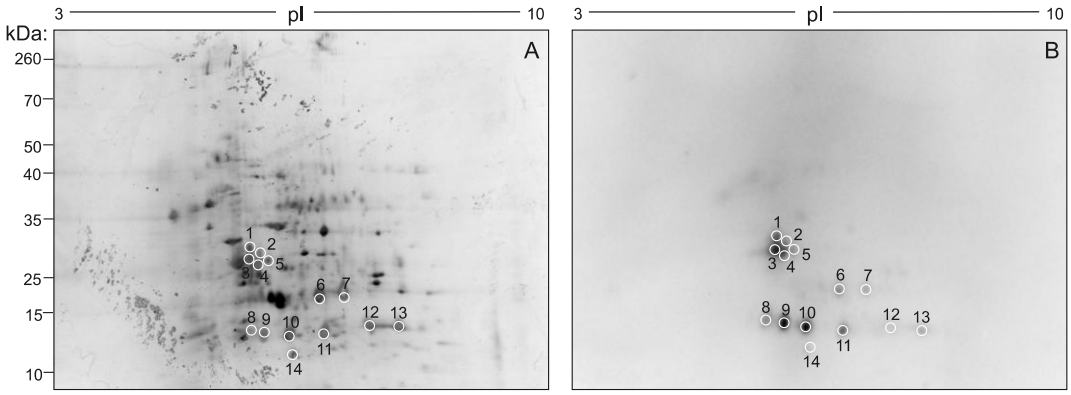

Figure 2. 2D electrophoresis of FhNEJ-Teg and detection of LM-binding proteins by immunoblotting. 40 µg of FhNEJ-Teg were separated in two dimensions using IPG strips with a pH range of 3–10 and 12% SDS-PAGE. This

procedure was performed in duplicate so that the proteins in one of the gels were revealed by silver staining (A) and the others were transferred to a nitrocellulose membrane for detection of LM binding by immunoblotting (B). LM-binding protein spots are circled and numbered.

Based on similarity to *F. hepatica* sequences contained in the Uniprot_trematoda database, we identified a total of 80 proteins in FhNEJ-Teg with potential to bind LM and an average of 16 proteins per spot (**Supp. Table 1**). **Figure 3A** shows the proteins that were most recurrently identified, and **Figure 3B** represents the GO annotation under the Biological Process category of all the potential LM-binding proteins identified by 2D-MS. FhCL3 stands out as the protein that is most recurrently identified within LM-reactive spots, and GO annotation reveals that the identified potential LM-binding proteins contained within FhNEJ-Teg are involved in proteolysis, mitotic cell cycle, microtubule cytoskeleton organisation, phosphorylation, regulation of catalytic activity, small molecule metabolic process, catabolic process, and cellular nitrogen compound metabolic process.

We next sought to validate our 2D-MS results via ELISA-based LM-binding assays using recombinant versions of FhCL3 and FhGST. To this end, we coated microtiter plate wells with the abovementioned recombinant proteins in a neutral buffer that preserves the proteins' native conformations and incubated them with increasing amounts of LM, which confirmed that FhCL3 and FhGST are capable of binding LM in a concentration-dependent manner and to a significantly greater extent than that showed by the negative control, 1% BSA (**Figure 4**).

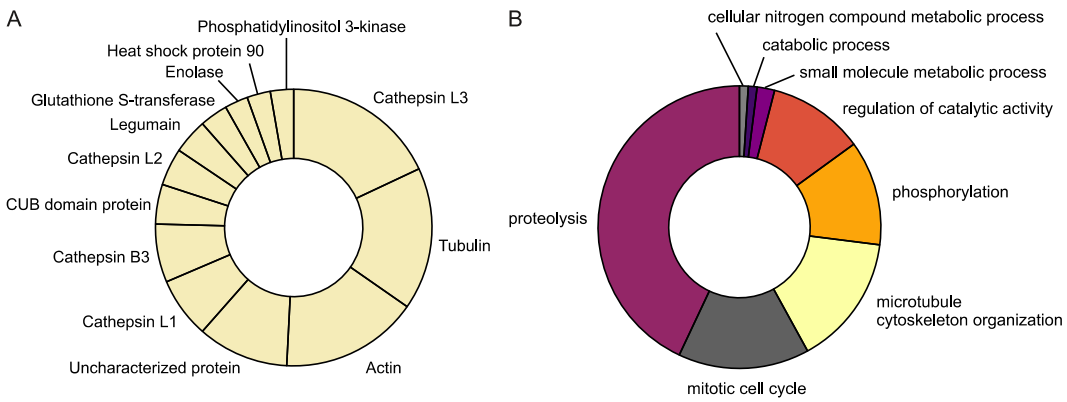

Figure 3. MS analysis of potential LM-binding proteins contained within LM-reactive spots. Protein spots detected in the silver-stained gel containing LM-binding proteins were manually excised and analysed by MS. A total of 14 protein spots were identified, containing 80 different proteins with an average of 16 proteins per spot. (A) Abundance of the most recurrently identified proteins in the pool of potential LM-binding proteins. (B) GO analysis under the Biological Process category of the potential LM-binding proteins identified by MS.

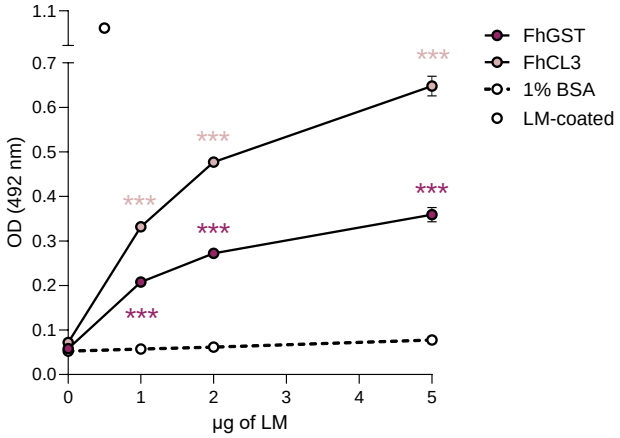

Figure 4. FhCL3 and FhGST bind to LM in a concentration-dependent manner. The capability of FhCL3 and FhGST to bind to LM was assessed via ELISA using recombinant versions of these proteins. Wells coated with 1% BSA served as negative controls for unspecific LM binding. Optical densities (OD) were measured at 492 nm. Dots indicate the mean of three technical replicates ± SD, and asterisks indicate significant differences between the indicated protein and the negative control, 1% BSA (***p≤0.001; one-way ANOVA).

LM degradation by FhCL3

Based on our observation that FhCL3 is capable of binding LM, we next tested whether this protein would also be proteolytically active towards this ECM component. To this end, we set up an enzymatic assay where we co-incubated mature FhCL3 with LM for three hours to assess LM degradation by this enzyme. Auto-catalytic activation of FhCL3 was performed and confirmed by SDS–PAGE on a 12% polyacrylamide gel prior to incubation with LM, which revealed a ~35 kDa band corresponding to the mature enzyme and a ~12 kDa band corresponding to the protease pro-peptide (**Supp. Figure 1**). Incubation of mature FhCL3 with LM resulted in the complete fading of protein bands corresponding to LM to a level comparable to that shown by our positive control, plasmin (**Figure 5**), indicating that FhCL3 is capable of degrading this ECM protein.

FhNEJ-Teg contains proteins that act as cofactors for plasmin-mediated degradation of LM

We have recently shown that FhNEJ-Teg contains proteins that interact with the host fibrinolytic system by binding PLG and promoting its conversion into the active protease, plasmin (23), which is a broad-spectrum serine protease that degrades multiple substrates, including LM (26,31). Based on this, we sought to determine whether FhNEJ-Teg-induced plasmin generation from bound PLG could potentiate LM degradation by mixing

FhNEJ-Teg with PLG, u-PA, and LM. In some instances, FhNEJ-Teg, PLG, and u-PA were omitted, and plasmin was added as a positive control for LM degradation. After incubation for three hours at 37 ºC, the samples were loaded into 6% polyacrylamide gels for visualization of LM degradation. This experiment showed that LM degradation is enhanced in the presence of FhNEJ-Teg proteins together with PLG and u-PA, confirming that PLG-binding proteins contained within the FhNEJ-Teg extract promote LM degradation by stimulating plasmin generation from bound PLG. Remarkably, FhNEJ-Teg in the absence of PLG and u-PA also gave a significant decrease in LM band intensity as compared to control lanes containing LM, PLG, and u-PA (**Figure 6**).

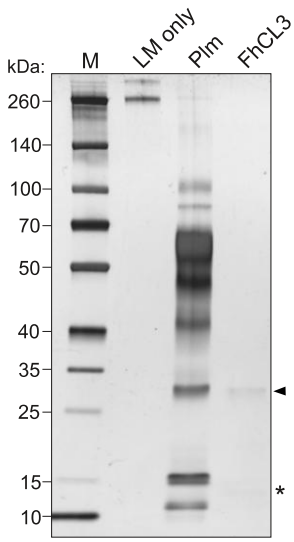

Figure 5. LM degradation by FhCL3. Mature FhCL3 (1 µM) was incubated with LM (0.1 µg) for three hours at 37 ºC and LM degradation was assessed by SDS-PAGE and silver staining. A condition containing an equivalent amount of plasmin (Plm) in place of FhCL3 was used as a positive control for LM degradation. Lane 1, MW marker; lane 2, LM only; lane 3, LM + Plm; lane 4, LM + mature FhCL3; 4–20% Bis-Tris polyacrylamide gel stained with silver. Arrowhead indicates mature FhCL3, asterisk indicates FhCL3 pro-peptide.

Proteomic changes induced in FhNEJ upon interaction with LM

In order to study whether the interaction with LM triggers proteomic changes in FhNEJ, we incubated live FhNEJ in plates coated with or without LM and isolated both FhNEJ-Teg and FhNEJ-Som to perform a quantitative analysis of differentially expressed proteins (DEPs) by SWATH-MS. This analysis identified a total of 495 and 302 proteins in FhNEJ-Teg and FhNEJ-Som, respectively. Discriminant Analysis showed that samples of each experimental condition clustered together (**Supp. Figure 2**), and the analysis revealed that incubation with LM resulted in overexpression of 5 and 14 proteins in FhNEJ-Teg and FhNEJ-Som, respectively, and downregulation of one protein in both

antigenic compartments (**Table 1**). Among others, we identified a member of the GST family of proteins to be overexpressed in FhNEJ-Teg upon incubation with LM; and we also detected an overexpression of the juvenile-specific cathepsin B2 (FhCB2) in FhNEJ-Som of LM-incubated FhNEJ compared to their control counterparts. Overexpression of FhCB2 in FhNEJ-Som upon culture in LM-coated plates was validated by western blot analysis (**Supp. Figure 3**).

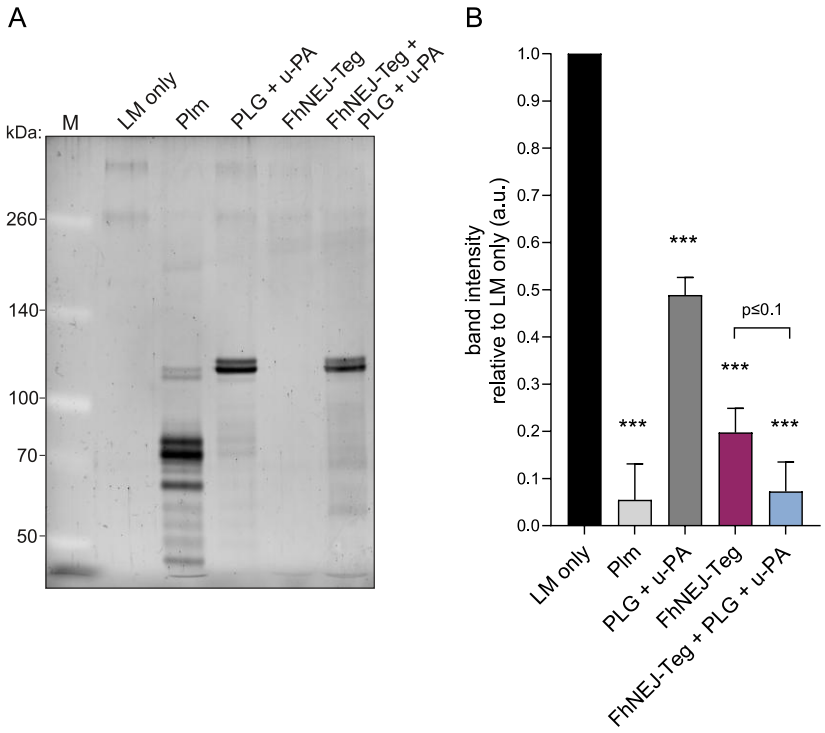

Figure 6. FhNEJ-Teg contains proteins that act as cofactors for plasmin-mediated LM degradation. LM was incubated either alone or with FhNEJ-Teg in the presence or absence of PLG and u-PA and incubated for three hours at 37 ºC. LM incubated with Plm served as a positive control for LM degradation; and LM incubated with PLG + u-PA (without FhNEJ-Teg) was used to control for background LM degradation derived from u-PA-induced Plm generation from soluble PLG. (A) Representative image of three independent experiments. Lane 1, MW marker; lane 2, LM only; lane 3, LM + Plm; lane 4, LM + PLG + u-PA, lane 5, LM + FhNEJ-Teg; lane 6, LM + FhNEJ-Teg + PLG + u-PA; 6% polyacrylamide gel stained with Sypro Ruby. (B) Quantification of LM degradation. Bars indicate the mean of three independent experiments ± SD, and asterisks indicate significant differences between each condition and lanes containing LM only (***p≤0.001; one-way ANOVA).

Discussion

In order to characterise the molecular mechanisms used by FhNEJ to cross the intestinal wall and initiate the migration process that will eventually drive them to the liver, we first sought to analyse whether this parasitic stage is capable of specifically interacting with

components of the intestinal ECM, namely LM and FN. Given that the tegument, the outermost layer of the parasite, is constantly exposed to host tissues, thereby representing the host-parasite interface (6), we tested for the presence of LM- and FN-binding proteins in a tegument-enriched protein fraction of FhNEJ (FhNEJ-Teg). This experiment showed that FhNEJ-Teg is capable of binding LM, but not FN, in a concentration-dependent manner. Since LM is a major component of the intestinal basement membrane, which is the tissue layer that sits immediately underneath the intestinal epithelium, whereas FN is found in deeper tissue layers, such as the interstitial matrix (7), these results suggest that FhNEJ are specifically adapted to interact with the tissue microenvironment that they encounter immediately after excystment.

Table 1. DEPs identified by SWATH-MS in FhNEJ incubated in LM-coated plates as compared to their control counterparts and separated by antigenic compartment.

FhNEJ-Teg DEPs	**Uniprot Accession**	**p-Value**	**Log2FC**
Alkylated DNA repair protein alkB 1	A0A4E0RF73	0.012	1.95
Calcium-binding mitochondrial carrier protein SCaMC-1	A0A4E0RTX3	0.037	1.76
Cargo transport protein erv29	A0A4E0RGI5	0.034	1.18
Golgi-associated plant pathogenesis protein 1	A0A2H1C4H4	0.044	1.01
Glutathione S-transferase omega class	A0A4E0RYR4	0.036	0.53
Propionyl-CoA carboxylase alpha chain	A0A4E0S3G7	0.012	−1.21
FhNEJ-Som DEPs	**Uniprot Accession**	**p-Value**	**Log2FC**
Nuclear protein localization protein 4 NADH	A0A4E0R4V7	0.037	2.15
NADH dehydrogenase	A0A4E0R2Z1	0.012	2.1
Glucose-6-phosphate isomerase	A0A4E0RGH6	0.049	1.85
40S ribosomal protein S2	A0A4E0RD65	0.032	1.8
Programmed cell death protein 6	A0A4E0S2T2	0.023	1.71
Alpha-aminoadipic semialdehyde synthase	A0A4E0RQ3	0.008	1.7
60S ribosomal protein L6	A0A2H1BY45	0.01	1.66
Superoxide dismutase	A0A2H1CLW2	0.032	1.62
Eukaryotic ribosomal protein L18	A0A2H1C1P4	0.027	1.46
Thioredoxin peroxidase	A0A4E0RVH8	0.038	1.44
Cathepsin B2	A0A4E0RNG2	0.027	1.41
Solute carrier family 2 facilitated glucose transporter member 3	A0A4E0RGV2	0.042	1.37
Spectrin beta chain	A0A4E0RR49	0.035	1.19
Heat shock protein 70	B1NI98	0.034	0.99
Oxoglutarate dehydrogenase	A0A4E0RQS7	0.044	−1.22

We next sought to determine the identity of the proteins contained within FhNEJ-Teg that are involved in LM binding by 2D electrophoresis and ligand blotting coupled to MS, which revealed the presence of 80 potential LM-binding proteins in this antigenic extract. The juvenile-specific FhCL3 stands out as the protein that is most recurrently identified in the pool of LM binding proteins, together with tubulin, actin, cathepsin L1, FhCB3,

CUB domain protein, cathepsin L2, legumain, FhGST, enolase, heat-shock protein 90, and phosphatidylinositol 3 kinase (PI3K). Since 2D-MS is performed under denaturing conditions, which could potentially expose internal LM-binding motifs in proteins that are not used for LM binding in a real physiological context, we validated some of these results by ELISA, which is performed under native conditions. We focused on FhCL3 and FhGST given their biological importance during the early immature stage of the parasite (15,32,33), and confirmed that these proteins are indeed capable of binding to LM specifically and in a concentration-dependent manner. To the best of our knowledge, this is the first time that an ECM-binding function is described for FhCL3 and FhGST, supporting a role for these proteins as adhesins (12).

Next, we performed a functional annotation analysis on the potential LM-binding proteins that we identified by 2D-MS and observed that these proteins are mainly involved in biological processes of proteolysis, mitotic cell cycle, microtubule cytoskeleton organisation, phosphorylation, regulation of catalytic activity, small molecule metabolic process, catabolic process, and cellular nitrogen compound metabolic process. The fact that some of the FhNEJ-Teg proteins that interact with LM are related to cytoskeleton organisation is compelling considering that ECM components, including LM, are important triggers of intracellular signalling events that culminate in remodelling of the cellular cytoskeleton, which is required for migration of cells across tissues (34–37). Furthermore, cell proliferation at the juvenile stage of the parasite is particularly important to sustain parasite growth and development, which is mainly supported by stem cell-like cells known as neoblasts (38) and the PI3K-protein kinase B (Akt) signalling pathway (39). Interestingly, LM has been shown to stimulate the PI3K-Akt pathway, which, in turn, enhances fibroblast proliferation (40). Altogether, these results suggest that the interaction between FhNEJ and LM may not simply serve as an anchoring/adhesion mechanism to the intestinal mucosa, but also operate as a spatial cue that triggers intracellular molecular events in FhNEJ related to parasite migration and development inside the mammalian organism, which most certainly deserves further attention.

FhCL3 is regarded as a master driver of FhNEJ migration through the intestinal wall owing to its capability to degrade collagen (41–43), a major component of this tissue layer together with LM (7). In view of this, and considering that this protein was most recurrently identified as a LM-binding protein in our 2D-MS analysis, we next tested whether FhCL3 would also have the ability to degrade bound LM, as described for cathepsin L isoforms expressed by adult flukes (44,45). Our results revealed that mature

FhCL3 is indeed capable of hydrolysing LM to a similar or event greater extent as our positive control, the main fibrinolytic protease plasmin. Since LM integrity is maintained under acidic conditions (46), we are confident that the observed LM degradation is not due to loss of LM structure under the specific assay conditions that we used, and that LM degradation by FhCL3 also occurs in a physiological context provided that this protease is most active at less acidic pH values, such as those found in the host duodenum (18).

We have recently demonstrated that FhNEJ interact with the host fibrinolytic system by expressing proteins that bind to PLG, which facilitates the conversion of this zymogen into its catalytically active form, plasmin (23). PLG activation occurs upon binding of PLG to receptors or partner proteins so that a conformational change is induced in the PLG molecule that exposes the cleavage sites for the tissue-type and urokinase-type PLG activators (t-PA and u-PA, respectively), which selectively cleave PLG and convert it into its catalytically active form, plasmin (13,47). Provided that plasmin is a broad-spectrum serine protease that degrades multiple ECM components, including LM (26,48), the stimulation of plasmin generation from bound PLG by FhNEJ-Teg proteins could potentially be used to degrade intestinal ECM components and support FhNEJ migration through the intestinal wall (49–51). Based on this, we set out to analyse whether PLG binding by FhNEJ-Teg would potentiate the endogenous, FhCL3-mediated, capacity of FhNEJ to degrade LM. To this end, we co-incubated FhNEJ-Teg with LM in the presence or absence of PLG and u-PA to analyse whether subsequent LM degradation would be further enhanced in the presence of these fibrinolytic factors. We included u-PA over t-PA in the reaction mixes provided that u-PA is the PLG activator that is selectively expressed by the intestinal epithelium (52). Although PLG is preferentially activated when bound to its physiologic receptors (47), u-PA is also capable of cleaving soluble (unbound) PLG (53), which resulted in certain background LM degradation due to plasmin generation from unbound PLG (**Figure 6**, compare lanes 4 and 2). Despite this technical constraint, we confirmed that FhNEJ-Teg contains proteases that degrade LM (**Figure 6**, compare lanes 5 and 4) and, most interestingly, that this proteolytic activity is further enhanced when the reaction is performed in the presence of PLG and u-PA (**Figure 6**, compare lanes 6 and 5), which validates our hypothesis that FhNEJ-Teg proteins act as cofactors for plasmin-induced LM degradation through PLG binding and the stimulation of plasmin generation from bound PLG. Collectively, our LM degradation assays reveal that FhNEJ are endowed with a potent and redundant proteolytic machinery that is purposely crafted for degrading the major structural components of the intestinal basement membrane, namely collagen (41–43) and LM (this study), which act as a

physical barrier that needs to be overcome by FhNEJ for the successful establishment of the parasite within its mammalian host.

Finally, and provided that ECM components are potent triggers of intracellular signalling events in eukaryotic cells (11) and parasites (27,54), we envisioned that binding to LM would not simply be used for adhesion purposes, but that the interaction with this ECM component may also trigger changes in the protein expression profile of FhNEJ. To test this, FhNEJ were added to LM-coated plates for three hours since this is the average time that the parasites take to cross the intestinal wall (4), meaning that proteomic changes induced by LM interaction should occur within this time frame if they were to be relevant in a real physiological setting. After incubation, FhNEJ were harvested and differences in protein expression between LM-incubated and control FhNEJ were quantified by MS. Among others, we found differential expression of several proteins related to glucose metabolism upon incubation of FhNEJ with LM. More specifically, we detected an overexpression of glucose 6-phosphate isomerase, the enzyme that catalyses the second step of glycolysis, and downregulation of oxoglutarate dehydrogenase, which is involved in the tricarboxylic acid (TCA) cycle, a metabolic pathway that fuels oxidative phosphorylation. This finding suggests that host LM could function as a molecular trigger of the gradual metabolic switch that is required during parasite growth and development, which limits oxygen diffusion towards deeper tissues and generates a strong dependence on anaerobic metabolism for energy production (6,55). This idea is apparently conflicting with the fact that we also detected overexpression of enzymes involved in aerobic energy metabolism (e.g., NADH dehydrogenase and alpha-aminoadipic semialdehyde synthase) in FhNEJ upon incubation with LM. However, a downregulation of oxoglutarate dehydrogenase expression would most likely shift FhNEJ metabolism to gradually depend on anaerobic pathways provided that, in humans, a congenital deficiency of this enzyme leads to severe impairment of the TCA cycle and oxidative respiration (56,57). Of note, increased expression of glycolytic enzymes and downregulation of enzymes of the TCA cycle has also been detected in FhNEJ after crossing the intestinal wall in an *ex vivo* model of trans-intestinal migration (33).

In addition to metabolic changes, we detected an overexpression of FhCB2 in FhNEJ-Som upon incubation with LM. Western blot analysis of FhNEJ-Som confirmed that FhCB2 is overexpressed in FhNEJ upon interaction with LM, which was also interpreted as a validation of our statistical and bioinformatic analyses to identify DEPs. Based on what is known for *Schistosoma mansoni* orthologs (58), FhCBs have been suggested to be involved in the immunomodulatory mechanisms that operate in FhNEJ to control innate

immune pathways that would otherwise stimulate adaptive pro-inflammatory responses towards them (59). Therefore, overexpression of FhCBs upon direct contact with LM, as shown by our results, appears to be particularly important during intestinal crossing given the highly proficient immune mechanisms that operate in this organ (10). This said, it remains a possibility that additional juvenile-specific cathepsin B clades, namely FhCB1 and FhCB3, are upregulated upon interaction with LM provided their high degree of homology, which hinders the precise annotation of these proteins.

Finally, we also detected overexpression of detoxifying enzymes, such as thioredoxin and FhGST, in FhNEJ upon incubation with LM, which adds an extra layer of biological relevance to FhGST expression during FhNEJ development besides its protective antioxidant functions (60). The fact that FhGST is specifically overexpressed in FhNEJ-Teg upon incubation with LM, and that our 2D-MS and ELISA-based experiments revealed that FhGST is an LM-binding protein located in this antigenic compartment, advocates for the existence of a positive feedback loop between FhNEJ and the host intestinal ECM aimed at improving FhNEJ adhesiveness to the intestinal mucosa, which might be necessary provided the mechanical force exerted by intestinal fluids that FhNEJ need to resist so as not to be swept down the gastrointestinal tract before gut passage.

In conclusion, we have shown that the tegument-enriched antigenic fraction of FhNEJ contains an array of proteins that are capable of interacting with LM, a major component of the intestinal basement membrane. Among these, FhCL3 stands out provided the ability of this protease to degrade this important intestinal ECM component, an enzymatic function that is described for the first time in this study. Additionally, our results also demonstrate that the endogenous capacity of FhNEJ to degrade LM is further enhanced by co-opting the proteolytic activity of host plasmin. Finally, our results suggest that the interaction between FhNEJ and LM triggers intracellular signalling pathways that might be relevant during FhNEJ migration, development, and adaptation to the newly encountered host tissue microenvironment. Since the intestinal ECM functions as a physical barrier that blocks pathogen invasion, proteins that bind to and degrade ECM components shall be regarded as important virulence factors in *F. hepatica* that may be worth targeting for fasciolosis treatment and control during early infection.

Acknowledgements

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References

1. Siles-Lucas M, Becerro-Recio D, Serrat J, González-Miguel J. Fascioliasis and fasciolopsiasis: current knowledge and future trends. Res Vet Sci. 2021 Jan;134:27–35.
2. Toet H, Piedrafita DM, Spithill TW. Liver fluke vaccines in ruminants: strategies, progress and future opportunities. Int J Parasitol. 2014 Oct;44(12):915–27.
3. Fitzpatrick JL. Global food security: the impact of veterinary parasites and parasitologists. Vet Parasitol. 2013 Aug 1;195(3–4):233–48.
4. Mas-Coma S, Valero MA, Bargues MD. Fascioliasis. In: Toledo R, Fried B, editors. Digenetic Trematodes. Springer International Publishing; 2019. p. 71–103.
5. Nyindo M, Lukambagire AH. Fascioliasis: an ongoing zoonotic trematode infection. Biomed Res Int. 2015;2015:786195.
6. González-Miguel J, Becerro-Recio D, Siles-Lucas M. Insights into *Fasciola hepatica* juveniles: crossing the fasciolosis rubicon. Trends Parasitol. 2021 Jan;37(1):35–47.
7. Pompili S, Latella G, Gaudio E, Sferra R, Vetuschi A. The charming world of the extracellular matrix: a dynamic and protective network of the intestinal wall. Front Med. 2021 Apr 16;8:610189.
8. Singh B, Fleury C, Jalalvand F, Riesbeck K. Human pathogens utilize host extracellular matrix proteins laminin and collagen for adhesion and invasion of the host. FEMS Microbiol Rev. 2012 Nov;36(6):1122–80.
9. Betanzos A, Bañuelos C, Orozco E. Host invasion by pathogenic amoebae: epithelial disruption by parasite proteins. Genes. 2019 Aug 14;10(8):618.
10. Mowat AM, Agace WW. Regional specialization within the intestinal immune system. Nat Rev Immunol. 2014 Oct;14(10):667–85.

11. Hastings JF, Skhinas JN, Fey D, Croucher DR, Cox TR. The extracellular matrix as a key regulator of intracellular signalling networks: ECM regulation of intracellular signalling networks. Br J Pharmacol. 2019 Jan;176(1):82–92.

12. Kline KA, Fälker S, Dahlberg S, Normark S, Henriques-Normark B. Bacterial adhesins in host-microbe interactions. Cell Host Microbe. 2009 Jun;5(6):580–92.

13. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. Br J Haematol. 2005 May;129(3):307–21.

14. Hernández-González A, Valero ML, Pino MS del, Oleaga A, Siles-Lucas M. Proteomic analysis of *in vitro* newly excysted juveniles from *Fasciola hepatica*. Mol Biochem Parasitol. 2010 Aug;172(2):121–8.

15. Becerro-Recio D, Serrat J, López-García M, Sotillo J, Simón F, González-Miguel J, *et al.* Proteomics coupled with *in vitro* model to study the early crosstalk occurring between newly excysted juveniles of *Fasciola hepatica* and host intestinal cells. PLoS Negl Trop Dis. 2022 Oct;16(10):e0010811.

16. García-Campos A, Ravidà A, Nguyen DL, Cwiklinski K, Dalton JP, Hokke CH, *et al.* Tegument glycoproteins and cathepsins of newly excysted juvenile *Fasciola hepatica* carry mannosidic and paucimannosidic N-glycans. PLoS Negl Trop Dis. 2016 May 3;10(5):1–26.

17. Li Q, Liu H, Du D, Yu Y, Ma C, Jiao F, *et al.* Identification of novel laminin- and fibronectin-binding proteins by far-western blot: capturing the adhesins of *Streptococcus suis* type 2. Front Cell Infect Microbiol. 2015 Nov 16;5:82.

18. Barbour T, Cwiklinski K, Lalor R, Dalton JP, De Marco Verissimo C. The zoonotic helminth parasite *Fasciola hepatica*: virulence-associated cathepsin B and cathepsin L cysteine peptidases secreted by infective newly excysted juveniles (NEJ). Animals. 2021;11(12):3495.

19. Marcos CM, Fátima da Silva J, Oliveira HC, Moraes da Silva RA, Mendes-Giannini MJS, Fusco-Almeida AM. Surface-expressed enolase contributes to the adhesion of *Paracoccidioides brasiliensis* to host cells. FEMS Yeast Res. 2012 Aug;12(5):557–70.

20. González-Miguel J, Morchón R, Siles-Lucas M, Simón F. Fibrinolysis and proliferative endarteritis: two related processes in chronic infections? The model of the blood-borne pathogen *Dirofilaria immitis*. PLoS ONE. 2015;10(4):e0124445.

21. De Marco Verissimo C, Jewhurst HL, Dobó J, Gál P, Dalton JP, Cwiklinski K. *Fasciola hepatica* is refractory to complement killing by preventing attachment of

mannose binding lectin (MBL) and inhibiting MBL-associated serine proteases (MASPs) with serpins. PLoS Pathog. 2022 Jan 10;18(1):e1010226.

22. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, *et al.* Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012 Jun 28;9(7):676–82.
23. Serrat J, Becerro-Recio D, Torres-Valle M, Simón F, Valero MA, Bargues MD, *et al.* *Fasciola hepatica* juveniles interact with the host fibrinolytic system as a potential early-stage invasion mechanism. PLoS Negl Trop Dis. 2023 Apr;17(4):e0010936.
24. Shevchenko A, Jensen ON, Podtelejnikov AV, Sagliocco F, Wilm M, Vorm O, *et al.* Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. Proc Natl Acad Sci U S A. 1996 Dec 10;93(25):14440–5.
25. Shilov IV, Seymour SL, Patel AA, Loboda A, Tang WH, Keating SP, *et al.* The Paragon algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol Cell Proteomics. 2007 Sep;6(9):1638–55.
26. Nakagami Y, Abe K, Nishiyama N, Matsuki N. Laminin degradation by plasmin regulates long-term potentiation. J Neurosci. 2000 Mar 1;20(5):2003–10.
27. Mattos EC, Canuto G, Manchola NC, Magalhães RDM, Crozier TWM, Lamont DJ, *et al.* Reprogramming of *Trypanosoma cruzi* metabolism triggered by parasite interaction with the host cell extracellular matrix. PLoS Negl Trop Dis. 2019 Feb 6;13(2):e0007103.
28. Mattos EC, Schumacher RI, Colli W, Alves MJM. Adhesion of *Trypanosoma cruzi* trypomastigotes to fibronectin or laminin modifies tubulin and paraflagellar rod protein phosphorylation. PLoS ONE. 2012 Oct 4;7(10):e46767.
29. Liskovykh M, Lee NC, Larionov V, Kouprina N. Moving toward a higher efficiency of microcell-mediated chromosome transfer. Mol Ther Methods Clin Dev. 2016;3:16043.
30. Fox J, Bouchet-Valat M. Rcmdr: R Commander. 2021. Available from: https://socialsciences.mcmaster.ca/jfox/Misc/Rcmdr/
31. Nicholl S, Roztocil E, Davies M. Plasminogen activator system and vascular disease. CVP. 2006 Apr 1;4(2):101–16.

32. Robinson MW, Menon R, Donnelly SM, Dalton JP, Ranganathan S. An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*. Mol Cell Proteomics. 2009 Aug;8(8):1891–907.

33. Becerro-Recio D, Serrat J, López-García M, Molina-Hernández V, Pérez-Arévalo J, Martínez-Moreno Á, *et al.* Study of the migration of *Fasciola hepatica* juveniles across the intestinal barrier of the host by quantitative proteomics in an *ex vivo* model. PLoS Negl Trop Dis. 2022 Sep;16(9):e0010766.

34. Seetharaman S, Etienne-Manneville S. Cytoskeletal crosstalk in cell migration. Trends Cell Biol. 2020 Sep;30(9):720–35.

35. Colognato H, Yurchenco PD. Form and function: the laminin family of heterotrimers. Dev Dyn. 2000 May 24;218(2):213–34.

36. Schéele S, Nyström A, Durbeej M, Talts JF, Ekblom M, Ekblom P. Laminin isoforms in development and disease. J Mol Med. 2007 Jul 16;85(8):825–36.

37. Gu YC, Kortesmaa J, Tryggvason K, Persson J, Ekblom P, Jacobsen SE, *et al.* Laminin isoform–specific promotion of adhesion and migration of human bone marrow progenitor cells. Blood. 2003 Feb 1;101(3):877–85.

38. McCusker P, McVeigh P, Rathinasamy V, Toet H, McCammick E, O'Connor A, *et al.* Stimulating neoblast-like cell proliferation in juvenile *Fasciola hepatica* supports growth and progression towards the adult phenotype *in vitro*. PLoS Negl Trop Dis. 2016 Sep 13;10(9):e0004994.

39. Cwiklinski K, Robinson MW, Donnelly S, Dalton JP. Complementary transcriptomic and proteomic analyses reveal the cellular and molecular processes that drive growth and development of *Fasciola hepatica* in the host liver. BMC Genomics. 2021 Dec;22(1):46.

40. Liu P, Chen H, Yan L, Sun Y. Laminin α5 modulates fibroblast proliferation in epidural fibrosis through the PI3K/AKT/mTOR signaling pathway. Mol Med Report. 2020 Jan 28;21(3):1941–1500.

41. Corvo I, O'Donoghue AJ, Pastro L, Pi-Denis N, Eroy-Reveles A, Roche L, *et al.* Dissecting the active site of the collagenolytic cathepsin L3 protease of the invasive stage of *Fasciola hepatica*. PLoS Negl Trop Dis. 2013 Jul 11;7(7):e2269.

42. Corvo I, Cancela M, Cappetta M, Pi-Denis N, Tort JF, Roche L. The major cathepsin L secreted by the invasive juvenile *Fasciola hepatica* prefers proline in the S2 subsite and can cleave collagen. Mol Biochem Parasitol. 2009 Sep;167(1):41–7.

43. Robinson MW, Corvo I, Jones PM, George AM, Padula MP, To J, *et al.* Collagenolytic activities of the major secreted cathepsin L peptidases involved in the virulence of the helminth pathogen, *Fasciola hepatica*. PLoS Negl Trop Dis. 2011 Apr 5;5(4):e1012.

44. Norbury LJ, Beckham S, Pike RN, Grams R, Spithill TW, Fecondo JV, *et al.* Adult and juvenile *Fasciola cathepsin* L proteases: different enzymes for different roles. Biochimie. 2011 Mar;93(3):604–11.

45. Berasain P, Goni F, McGonigle S, Dowd A, Dalton JP, Frangione B, *et al.* Proteinases secreted by *Fasciola hepatica* degrade extracelullar matrix and basement membrane components. J Parasitol. 1997 Feb;83(1):1.

46. Freire E, Coelho-Sampaio T. Self-assembly of laminin induced by acidic pH. J Biol Chem. 2000 Jan;275(2):817–22.

47. Miles LA, Parmer RJ. Plasminogen receptors: the first quarter century. Semin Thromb Hemost. 2013 Jun;39(4):329–37.

48. Faisal Khan KM, Laurie GW, McCaffrey TA, Falcone DJ. Exposure of cryptic domains in the alpha 1-chain of laminin-1 by elastase stimulates macrophages urokinase and matrix metalloproteinase-9 expression. J Biol Chem. 2002 Apr 19;277(16):13778–86.

49. Figuera L, Gómez-Arreaza A, Avilán L. Parasitism in optima forma: exploiting the host fibrinolytic system for invasion. Acta Tropica. 2013 Oct;128(1):116–23.

50. Diosdado A, Simón F, Serrat J, González-Miguel J. Interaction of helminth parasites with the haemostatic system of their vertebrate hosts: a scoping review. Parasite. 2022;29:35.

51. González-Miguel J, Siles-Lucas M, Kartashev V, Morchón R, Simón F. Plasmin in parasitic chronic infections: friend or foe? Trends Parasitol. 2016 Apr;32(4):325–35.

52. Gibson PR, Birchall I, Rosella O, Albert V, Finch CF, Barkla DH, *et al.* Urokinase and the intestinal mucosa: evidence for a role in epithelial cell turnover. Gut. 1998 Nov 1;43(5):656–63.

53. Aisina RB, Mukhametova LI. Structure and function of plasminogen/plasmin system. Russ J Bioorg Chem. 2014 Nov;40(6):590–605.

54. Guillén N. Role of signalling and cytoskeletal rearrangements in the pathogenesis of *Entamoeba histolytica*. Trends Microbiol. 1996 May;4(5):191–7.

55. Dalton JP, editor. Fasciolosis. 2nd ed. UK: CABI; 2021.

56. Yap ZY, Strucinska K, Matsuzaki S, Lee S, Si Y, Humphries K, *et al.* A biallelic pathogenic variant in the OGDH gene results in a neurological disorder with features of a mitochondrial disease. J Inherit Metab Dis. 2021 Mar;44(2):388–400.

57. Guffon N, Lopez-Mediavilla C, Dumoulin R, Mousson B, Godinot C, Carrier H, *et al.* 2-Ketoglutarate dehydrogenase deficiency, a rare cause of primary hyperlactataemia: report of a new case. J Inherit Metab Dis. 1993;16(5):821–30.

58. Donnelly S, O'Neill SM, Stack CM, Robinson MW, Turnbull L, Whitchurch C, *et al.* Helminth cysteine proteases inhibit TRIF-dependent activation of macrophages via degradation of TLR3. J Biol Chem. 2010 Jan 29;285(5):3383–92.

59. Cwiklinski K, Donnelly S, Drysdale O, Jewhurst H, Smith D, De Marco Verissimo C, *et al.* The cathepsin-like cysteine peptidases of trematodes of the genus *Fasciola*. In: Advances in Parasitology. Elsevier; 2019. p. 113–64.

60. Ryan S, Shiels J, Taggart CC, Dalton JP, Weldon S. *Fasciola hepatica*-derived molecules as regulators of the host immune response. Front Immunol. 2020 Sep 2;11:2182.

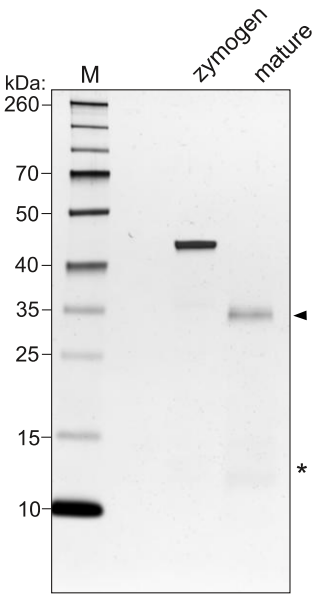
61. Coleman JL., Roemer Elizabeth J., Benach Jorge L. Plasmin-coated *Borrelia burgdorferi* degrades soluble and insoluble components of the mammalian extracellular matrix. Infect Immun. 1999 Aug 1;67(8):3929–36.

62. Goldfarb RH, Murano G, Brundage R, Siegal GP, Terranova V, Garbisa S, *et al.* Degradation of glycoprotein and collagenous components of the basement membrane: studies with urokinase-type plasminogen activator, alpha-thrombin, and plasmin. Semin Thromb Hemost. 1986 Oct;12(4):335–6.

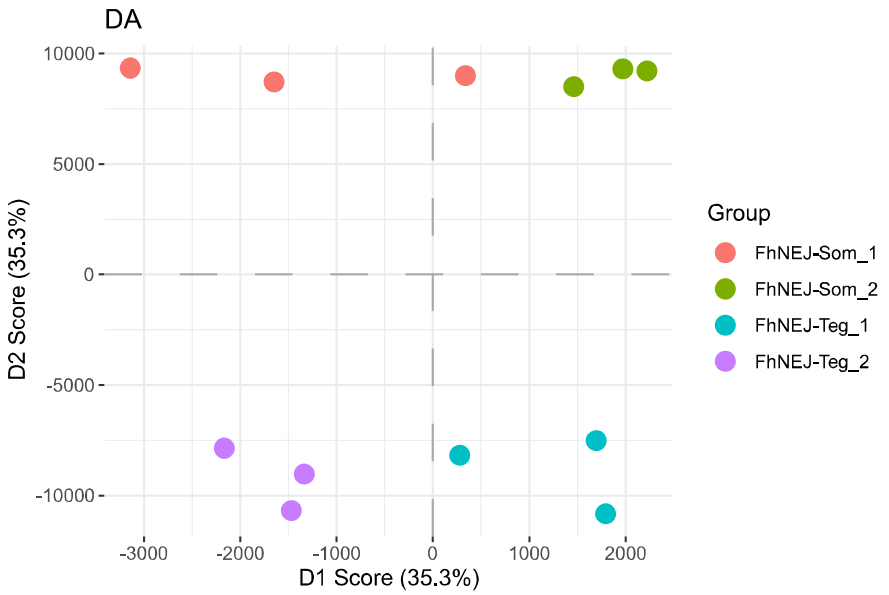
Supplementary information

Supplementary Table 1. Potential LM-binding proteins in the tegument-enriched antigenic fraction of FhNEJ identified by 2D-LC-MS/MS. Available at: https://www.mdpi.com/article/10.3390/ijms24098165/s1.

Supplementary Figure 1. Auto-catalytic activation of FhCL3 was confirmed by SDS–PAGE on a 12% polyacrylamide gel. Lane 1, MW marker; lane 2, empty lane; lane 3, FhCL3 zymogen; lane 4, activated FhCL3. Arrowhead indicates active FhCL3; asterisk indicates the FhCL3 pro-peptide.

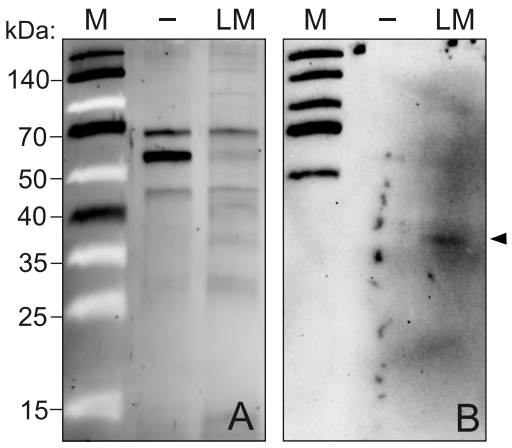


Supplementary Figure 2 (next page). Discriminant Analysis of tegument- and somatic-enriched antigenic fractions of FhNEJ incubated in control or LM-coated plates and analysed by SWATH-MS. FhNEJ-Teg_1, tegument-enriched antigenic fraction from control FhNEJ; FhNEJ-Teg_2, tegument-enriched antigenic fraction from FhNEJ incubated with LM; FhNEJ-Som_1, somatic-enriched antigenic fraction from control FhNEJ; FhNEJ-Som_2, somatic-enriched antigenic fraction from FhNEJ incubated with LM.



Supplementary Figure 2 (legend on the previous page).

Supplementary Figure 3. Western blot analysis of cathepsin B2 levels in somatic-enriched samples (FhNEJ-Som) from FhNEJ incubated in control and LM-coated plates. A small fraction of FhNEJ-Som samples used for SWATH-MS analysis was analysed by SDS-PAGE and transferred to a nitrocellulose membrane for the detection of FhCB2 by western blot. **(A)** Total protein staining by Sypro Ruby. **(B)** Immunoblot using FhCB1–3 anti-serum. Lane 1, MW marker; lane 2, FhNEJ-Som from control FhNEJ; lane 3, FhNEJ-Som from FhNEJ incubated in LM-coated plates. Arrowhead indicates FhCB2.



Addendum

With this addendum, the authors provide additional evidence supporting the conclusions drawn from Figure 6 of the original paper. Although we previously demonstrated that plasmin generation is significantly greater in samples containing FhNEJ-Teg, PLG, and u-PA, compared to those with PLG and u-PA alone (23), we conducted a plasmin activity assay on the samples used in Figure 6, which confirmed increased plasmin generation in samples containing FhNEJ-Teg + PLG + u-PA (Figure 6A, lane 6) relative to PLG + u-PA alone (Figure 6A, lane 4) (**Figure 7A**). This assay links the increased LM degradation observed in Figure 6A, lane 6 (FhNEJ-Teg + PLG + u-PA) to enhanced plasmin generation in these samples stimulated by PLG binding to FhNEJ-Teg proteins. Furthermore, the new data confirm that u-PA alone does not degrade LM (**Figure 7B,C**), consistent with previous reports (61,62).

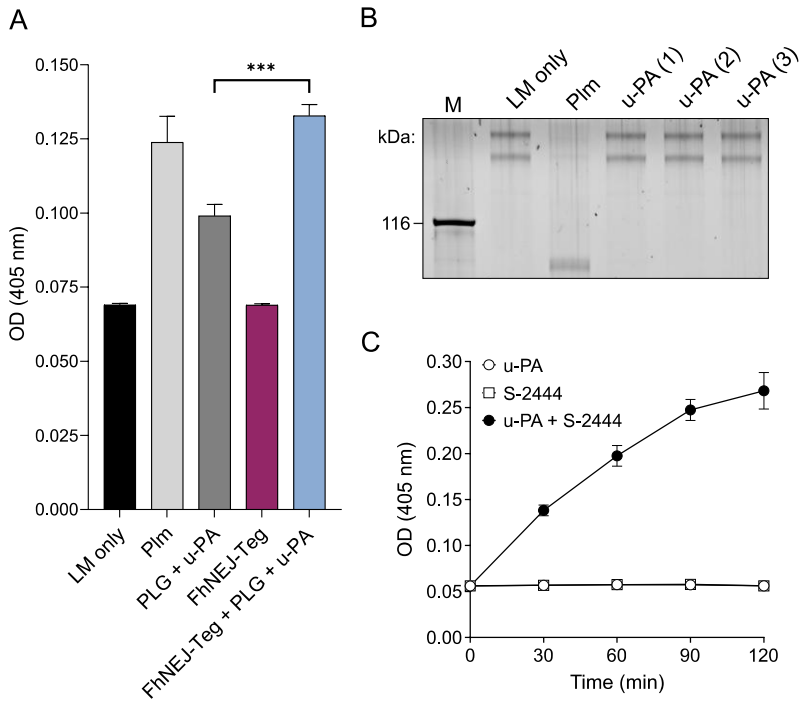


Figure 7. Detection of plasmin activity in samples used in Figure 6 of the original paper. A) Equal volumes of samples were incubated with a plasmin-specific chromogenic substrate and plasmin activity was quantified as described in Serrat *et al.*, 2023 (23). Bars indicate the mean of three independent experiments ± SD, and asterisks indicate significant differences between relevant groups (***p≤0.001; one-way ANOVA). B) u-PA was incubated with LM as described in Figure 6 and--- LM degradation was assessed by SDS-PAGE. C) Catalytic activity of the u-PA stock used in panel B was tested by mixing an aliquot of the reaction mixes with a u-PA-specific chromogenic substrate (S-2444; 5-Diagnostics), followed by quantification of u-PA activity as described for plasmin in panel A.

Chapter 3

Binding and cleavage of pro-urokinase by a tegument extract of *Fasciola hepatica* newly excysted juveniles activate the host fibrinolytic system

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Plasmin, the final product of fibrinolysis, is a broad-spectrum serine protease that degrades extracellular matrix (ECM) components, a function exploited by multiple pathogens for dissemination purposes. The trematode *Fasciola hepatica* is the leading cause of fasciolosis, a major disease of livestock and an emerging zoonosis in humans. Infection success depends on the ability of *F. hepatica* newly excysted juveniles (FhNEJ) to penetrate the host intestinal wall, a process that remains incompletely understood. We have previously shown that FhNEJ are capable of binding plasminogen (PLG), the zymogen of plasmin, on their tegument surface, which leads to plasmin generation in the presence of host-derived PLG activators and the subsequent degradation of laminin, a major component of the intestinal ECM. Here, we describe the interaction between a tegument extract of FhNEJ and the precursor of the urokinase-type PLG activator (pro-u-PA). We found that *F. hepatica* cathepsins B3, L3, enolase, and glutathione S-transferase mediate this interaction, suggesting a multifactorial or moonlighting role for these proteins. Additionally, our results revealed that the tegument of FhNEJ contains a protease that is capable of cleaving and activating pro-u-PA into its catalytically active form, which positively impacts the capacity of the parasites to generate plasmin from host PLG. Collectively, our findings indicate that FhNEJ interact with the host fibrinolytic system at multiple levels, reinforcing the potential of targeting this interaction as a strategy to prevent FhNEJ trans-intestinal migration and infection success.

Introduction

The co-option of the functions of the mammalian fibrinolytic system by infectious agents, including bacteria, fungi, and parasites, is increasingly recognised as a paradigmatic example of how these organisms exploit host resources to improve their fitness and secure their successful establishment within their mammalian hosts (1,2). The classical function of the fibrinolytic system is the degradation of fibrin clots that appear in the vascular endothelium upon injury. When these lesions have been repaired, the zymogen plasminogen (PLG) binds to lysine residues present on fibrin molecules that constitute the clot and undergoes a conformational change that exposes the target sites of its activators, the serine proteases tissue-type and urokinase-type PLG activators (t-PA and u-PA, respectively). Upon cleavage, PLG is converted into its catalytically active form, the serine protease plasmin, which degrades fibrin and contributes to the dissolution of the clot and the restoration of normal blood flow (3).

Plasmin is a broad-spectrum protease with multiple extravascular substrates, including components of the extracellular matrix (ECM), immunoglobulins, and molecules of the complement system (4–6), among others. Plasmin activity is directed to the extravascular space by u-PA, which, as opposed to its blood-specific counterpart, t-PA, is expressed by a wider array of cell types, including endothelial cells, macrophages, neutrophils, intestinal epithelial cells, and certain tumour cells (3,7,8). The u-PA, also known as urokinase, is synthesised and secreted as a precursor zymogen of 411 amino acids (pro-u-PA) that only exhibits catalytic activity after proteolytic cleavage into its active form, u-PA. Pro-u-PA cleavage and subsequent activation are mediated by different proteases, including plasmin, kallikrein, trypsin, and human cathepsins B and L (9–11), and cleaved u-PA is formed by two polypeptide chains that are linked via a disulphide bond. Overall, the broad spectrum of plasmin substrates, together with the expression of u-PA by various cell types, engages the fibrinolytic system in multiple extravascular processes that require cell migration, including inflammatory responses, embryogenesis, wound healing, and cancer cell dissemination (7,9,12). These extravascular functions of the fibrinolytic system are exploited by parasites for migration, feeding, immune evasion, and survival inside their mammalian hosts (1,2,13), and the helminth *Fasciola hepatica* is no exception (14–16).

F. hepatica, commonly known as the liver fluke, is a parasitic trematode and the most widespread causative agent of fasciolosis, a re-emerging food-borne neglected zoonotic disease that affects more than fifty million people worldwide and a large proportion of

wild and domestic animals (17,18). Globally, *F. hepatica* infection is most prevalent in cattle and sheep, with infection rates reaching up to 90%, thereby inflicting significant economic losses on the livestock industry—estimated at up to $3.2 billion per year. These losses are primarily due to liver condemnation, severe morbidity, mortality in cases of hyper-infection, and reduced yield and quality of carcasses and animal-derived products, such as milk and wool. By doing so, *F. hepatica* infection poses major challenges to global food security, particularly in low-income countries where most pressure on increasing food production is expected to occur provided that the population in these areas is predicted to double in the next few decades (19). Moreover, fasciolosis also impacts agricultural practices in certain areas of the world where livestock are still employed as a source of traction power and manure in crop production (18). Definitive hosts become infected upon ingestion of aquatic plants or water contaminated with metacercariae, which excyst and release the newly excysted juveniles (FhNEJ) upon arrival at the duodenum. FhNEJ cross the intestinal wall within the first 72 hours post-infection, reaching the peritoneal cavity and then the liver, where they migrate through until gaining access to the major hepatic biliary ducts, where adult flukes mature and produce eggs that are shed with faeces (20,21). While intestinal penetration of FhNEJ is not associated with clinical symptoms, the migration of juvenile flukes through the liver parenchyma and the chronic presence of adults in the biliary ducts cause allergic reactions, hepatic congestion and inflammation, bile duct hyperplasia, and cholangitis, among other conditions (22,23).

Our laboratory has recently shown that FhNEJ interact with the host fibrinolytic system by binding PLG and stimulating its conversion to plasmin, which, in turn, potentiates the capacity of these parasites to degrade laminin (LM), one of the major components of the intestinal ECM (16,24). This mechanism of co-opting host resources during the early stages of infection potentially facilitates FhNEJ penetration of the intestinal epithelium while minimising the energy expenditure typically associated with this process. Given that u-PA is expressed by epithelial cells of the intestine, presumably to loosen intercellular junctions and facilitate epithelial cell turnover at the top of the villi (8), this study investigated the ability of FhNEJ to exploit the functions of this fibrinolytic protease for invasion and migration.

Materials and methods

In vitro excystment of *F. hepatica* metacercariae and protein extraction of the tegument-enriched fraction

FhNEJ were obtained by *in vitro* stimulation of the excystment of *F. hepatica* metacercariae (Ridgeway Research Ltd.) as previously described (16). First, CO_2 (99.5% purity) was bubbled in 10 mL of ice-cold distilled water for 30 s and supplemented with sodium dithionite to a final concentration of 0.02 M. This solution was added to 5000 metacercariae and incubated for 1 h at 37 ºC. The metacercariae were then washed twice with distilled water, resuspended in 5 mL of Hank's balanced salt solution (Sigma) supplemented with 10% lamb bile (obtained from a local abattoir) and 30 mM HEPES (Sigma), pH 7.4, and incubated for 3–5 h at 37 ºC. FhNEJ were manually recovered under a stereoscope every hour after the addition of excystment media and incubated at 37 ºC and 5% CO_2 for 3 h in plates containing 4 mL of RPMI-1640 culture media (Thermo Fisher Scientific) supplemented with 30 mM HEPES (Sigma), 0.1% glucose (Sigma), and 50 μg/mL gentamycin (Sigma) to induce recovery (25). A total of 1839 FhNEJ were subjected to protein extraction of the tegument-enriched fraction (FhNEJ-Teg), which was performed in 300 μL of PBS containing 1% Nonidet P-40 substitute (NP-40; Sigma), as previously described (16,26), in the absence of protease and phosphatase inhibitors. The protein concentration was 0.42 mg/mL, as determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific).

u-PA and pro-u-PA binding assays

Binding of u-PA and pro-u-PA to FhNEJ-Teg or recombinant proteins was assessed by enzyme-linked immunosorbent assay (ELISA), as previously described (16). Briefly, microtiter 96-well plates (Thermo Fisher Scientific) were coated overnight at 4 ºC with either 0.5 μg of FhNEJ-Teg, 1 μg of recombinant *F. hepatica* cathepsin zymogens B3 (FhCB3) or L3 (FhCL3), glutathione S-transferase (FhGST), or enolase (FhENO) (kind gifts from Prof. J.P. Dalton) diluted in 200 μL of carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Wells coated with 1% bovine serum albumin (BSA) were used as negative controls. After three washes in PBS containing 0.05% Tween-20 (PBST), the wells were blocked with either 1% BSA or 1% milk in PBS for 30 min at 37 ºC, and increasing amounts (0 μg to 1 μg) of human pro-u-PA (Abcam) or human u-PA (Sigma) diluted in blocking solution were added to the wells and incubated for 1 h at 37 ºC. A competition assay was performed in parallel by adding 50 mM of the lysine analogue 6-

aminocaproic acid (ε-ACA; Sigma) to the wells during incubation with u-PA and pro-u-PA. After three washes with PBST, the wells were incubated for 1 h at 37 ºC with an anti-human-u-PA primary antibody raised in sheep (Innovative Research) diluted 1:500 in blocking solution, followed by incubation for 1 h at 37 ºC with a horseradish (HRP)-conjugated anti-sheep secondary antibody (Sigma) diluted 1:1000 in blocking solution. The wells were washed three times with PBST between and after antibody incubations. Finally, the binding of u-PA and pro-u-PA was revealed by adding 100 µL per well of substrate buffer (25 mM citric acid, 45 mM Na_2HPO_4, 0.04% H_2O_2, pH 5.0) containing 1.5 mM of the chromogenic substrate ortho-phenylene-diamine (Sigma). The wells were incubated at room temperature in the dark, and the reaction was stopped after 15 min by adding an equal volume of 3 N sulfuric acid. Optical density (OD) was measured at 492 nm with a Multiskan GO spectrophotometer (Thermo Fisher Scientific). The assay was performed twice in technical triplicates. The values of every condition assayed were blanked to the mean of the condition containing 0 µg of pro-u-PA to eliminate background signal derived from binding of the primary antibody to the proteins used for coating. Primary antibody specificity for both pro-u-PA and u-PA was tested under the abovementioned conditions in wells coated with 250 ng of pro-u-PA (Abcam) or u-PA (Sigma). The ability of ε-ACA to block lysine-dependent interactions was tested by coating the wells with 0.5 µg of FhNEJ-Teg followed by incubation with 2 µg of human PLG in the presence or absence of 50 mM ε-ACA. PLG binding was detected following standard ELISA procedures with an anti-PLG-specific primary antibody, as described elsewhere (16).

Bidimensional (2D) electrophoresis of FhNEJ-Teg

The FhNEJ-Teg extract (80 µg) was purified using the ReadyPrep 2-D Cleanup Kit (BioRad) to precipitate proteins, and protein pellets were resuspended in 250 µL of rehydration buffer (7 M urea, 2 M thiourea, and 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate). The extract was then divided into two aliquots of 125 µL, supplemented with 0.05 M dithiothreitol (DTT; Sigma) and 0.2% ampholytes pH 3–10 (BioRad), and added to two 7 cm ReadyStrip IPG strips (40 µg per strip) with a linear pH range of 3–10 (BioRad) for passive rehydration overnight at 20 ºC in a Protean IEF Cell equipment (BioRad). Isoelectric focusing (IEF) was performed with a Protean IEF Cell equipment (BioRad) at 20 ºC under the following conditions: 15 min at 250 V, 2 h at 4000 V, and 16000 V/h at 4000 V. A maximum amperage of 50 µA per strip was used. Next, the proteins in the strips were reduced with DTT (0.02 g/mL) and alkylated

with iodoacetamide (0.0025 g/mL) for 10 min and 15 min, respectively, at room temperature. Both DTT and iodoacetamide were diluted in equilibration buffer containing 6 M urea, 2% sodium dodecyl sulphate (SDS), 1.5 M Tris–HCl pH 8.8, 30% glycerol, and bromophenol blue. Separation in the second dimension was performed using 12% SDS-polyacrylamide gel electrophoresis (PAGE) following standard procedures. One of the gels was immediately stained with silver using in-house-prepared reagents and following standard procedures (excluding formaldehyde and glutaraldehyde from the formulations to ensure compatibility with subsequent analysis by mass spectrometry) and imaged using a Chemidoc MP Imaging System (BioRad). The proteins contained in the second gel were electro-transferred onto a nitrocellulose membrane for detection of pro-u-PA binding by ligand blotting (see the next section).

Detection of pro-u-PA-binding proteins by ligand blotting

The proteins in the 2D gel were electrotransferred onto nitrocellulose membranes using a constant amperage of 400 mA for 90 min at 4 ºC. The blots were blocked for 1 h at room temperature with 2% BSA diluted in PBST and incubated overnight at 4 ºC with 2 μg/mL pro-u-PA (Abcam) diluted in blocking solution. After washing in PBST, pro-u-PA binding was detected by adding an anti-u-PA primary antibody raised in mice (Santa Cruz Biotechnology) diluted 1:500, followed by incubation with an HRP-conjugated anti-mouse secondary antibody (Sigma) diluted 1:2000. The antibodies were diluted in blocking solution and incubated for 90 min at 37 ºC. Protein spots bound to pro-u-PA were revealed by enhanced chemiluminescence (Clarity Western ECL Substrate; BioRad) and imaged on a ChemiDoc MP Imaging system (BioRad). PDQuest Software v.8.0.1 (BioRad) was used to match spots between the silver-stained gel and the corresponding blot and to estimate the experimental molecular weight (MW) and isoelectric point (*pI*) values of every spot.

Spot analysis by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

Individual protein spots in the silver-stained gels were manually excised under sterile conditions and sent for proteomic analysis at the Proteomics Facility of the Central Support Service for Experimental Research (SCSIE, University of Valencia), as previously described (16). Protein identification was performed via the software ProteinPilot v5.0 (ABSCIEX) to search the UniProt-trematoda database (200604). Only proteins belonging to *F. hepatica* and having an Unused value ≥ 2 (meaning confidence of identification ≥

99%) were used for subsequent analyses, and protein isoforms were manually grouped to facilitate downstream data interpretation. Clades of cathepsin L entries with unspecified descriptions were assigned on the basis of similarity to canonical cathepsin L prosegment sequences via multiple sequence alignment with Clustal Omega (27), and those corresponding to cathepsin B entries were determined by phylogenetic comparison of the entire protein sequence (16). Protein sequences were retrieved via UniProt ID mapping, and Gene Ontology (GO) analysis was subsequently performed via OmicsBox v3.0.30 software (Biobam Bioinformatics).

In vitro activation of *F. hepatica* cathepsin zymogens

The activation of *F. hepatica* recombinant cathepsin L1, L2, L3, and B2 was performed by mixing zymogens in an appropriate volume of citrate-phosphate (C-P) activation buffer (0.1 M citrate phosphate, 100 mM NaCl, 2 mM DTT, 10 μg/mL dextran sulphate, pH 4.5) (28) such that the final concentration of active enzyme after incubation for 2 h at 37 ºC was 50 μM. Zymogen activation was verified by resolving an aliquot of the activated mixture on a pre-cast 4–20% Bis-Tris gel (GenScript), followed by silver staining via custom-prepared reagents and standard procedures. In parallel, zymogen activation was assayed in an enzymatic assay using a cathepsin-specific fluorogenic substrate (Z-Gly-Pro-Arg-AMC; Bachem), as previously described (29). Briefly, activated cathepsins (7.5 mM) were mixed in 100 μL of sodium acetate reaction buffer (100 mM sodium acetate, 1 mM EDTA, 1 mM DTT, 0.01% Brij-35, pH 7.0) containing 20 μM fluorogenic substrate, and their hydrolytic activity was monitored over time at 37 ºC as fluorescence relative units (RFU) in a FluoStar Omega fluorimeter (BMG LabTech) using excitation/emission wavelengths of 380/460 nm. The broad-spectrum protease inhibitor E-64d at 100 μM (Sigma) was used to control that the recorded fluorescent signal was specific to cathepsin proteolytic activity. All the reactions were performed in triplicate.

Pro-u-PA activation assays

This assay was performed in 96-well plates (Thermo Fisher Scientific) in a final volume of 100 μL by measuring the amidolytic activity of generated u-PA on a u-PA-specific chromogenic substrate. In each well, 0.01 μM pro-u-PA (Abcam) was mixed with 0.5 mM pyro-Glu-Gly-Arg-pNa chromogenic substrate (5-Diagnostics) together with increasing amounts of FhNEJ-Teg (1–3 μg) or 0.05 μM recombinant activated cathepsin L1, L2, L3, or B2. Wells containing pro-u-PA (0.01 μM), plasmin (0.05 μM), and chromogenic substrates (0.5 mM), and wells containing u-PA (0.01 μM) and chromogenic substrates

(0.5 mM) were used as controls for pro-u-PA cleavage and substrate specificity, respectively. The microplates were incubated for up to 24 h at 37 ºC, and substrate cleavage was assessed by measuring the absorbance at 405 nm at different time points in a Multiskan GO spectrophotometer (Thermo Fisher Scientific). All the reactions were performed in triplicate, and the experiment was performed twice.

Plasmin activity assay

This assay was performed in 96-well plates (Thermo Fisher Scientific) in a final volume of 100 μL of PBS by measuring the amidolytic activity of the generated plasmin on a plasmin-specific chromogenic substrate, as previously described (16). Briefly, 1 μg of PLG (Origene) was added to every well together with 0.01 μM pro-u-PA (Abcam) and 2 mM pyro-Glu-Phe-Lys-pNa chromogenic substrate (5-Diagnostics) in the presence or absence of 1 μg of FhNEJ-Teg. Wells containing different paired combinations of FhNEJ-Teg, PLG, and pro-u-PA, or individual compounds alone were used as controls for background signals. Wells containing 0.0051 μM u-PA (Sigma)—corresponding to the estimated amount of u-PA generated in wells containing FhNEJ-Teg + pro-u-PA at 24 hours after incubation, as shown in **Figure 3A**—were used to control for unspecific activity of the u-PA generated in the wells toward the plasmin-specific chromogenic substrate. The microplates were incubated for up to 24 h at 37 ºC, and substrate cleavage was assessed by measuring the absorbance at 405 nm at different time points in a Multiskan GO spectrophotometer (Thermo Fisher Scientific). All the reactions were performed in triplicate, and the experiment was performed twice.

Statistical analysis

Plots were created with Prism 10 software (GraphPad Software), and statistical analyses were performed with either Prism 10 software (GraphPad Software) or the R Commander package (30). Comparisons between more than two experimental groups were performed via analysis of variance (ANOVA) followed by Tukey post-hoc analysis for pairwise comparisons. Unless otherwise stated, the differences were not significant.

Results

The tegument-enriched fraction of FhNEJ contains proteins that bind pro-u-PA

First, the ability of FhNEJ-Teg to bind to u-PA and its precursor, pro-u-PA, was assessed using an ELISA-based binding assay. In this experiment, wells were coated with FhNEJ-Teg and incubated with increasing amounts of either u-PA or pro-u-PA. A condition including the lysine analogue ε-ACA in the wells with pro-u-PA/u-PA was included to study whether this amino acid plays a role in the interaction of these factors with FhNEJ-Teg proteins. Our results demonstrate that FhNEJ-Teg contains proteins that bind to both the zymogen pro-u-PA and the active protease, u-PA, in a concentration-dependent manner, with notably lower binding affinities observed for u-PA (**Figure 1**). This low binding of u-PA to FhNEJ-Teg could not be explained by differential antibody affinity towards this factor, as shown in **Supplementary Figure 1A**. Our studies also revealed that pro-u-PA/u-PA binding occurs in a lysine-independent manner, as shown by the inability of ε-ACA to abrogate it. The ability of ε-ACA to block lysine-dependent interactions was confirmed by the incubation of FhNEJ-Teg with PLG (**Supp. Figure 1B**), an interaction that is strongly inhibited by this lysine analogue (16).

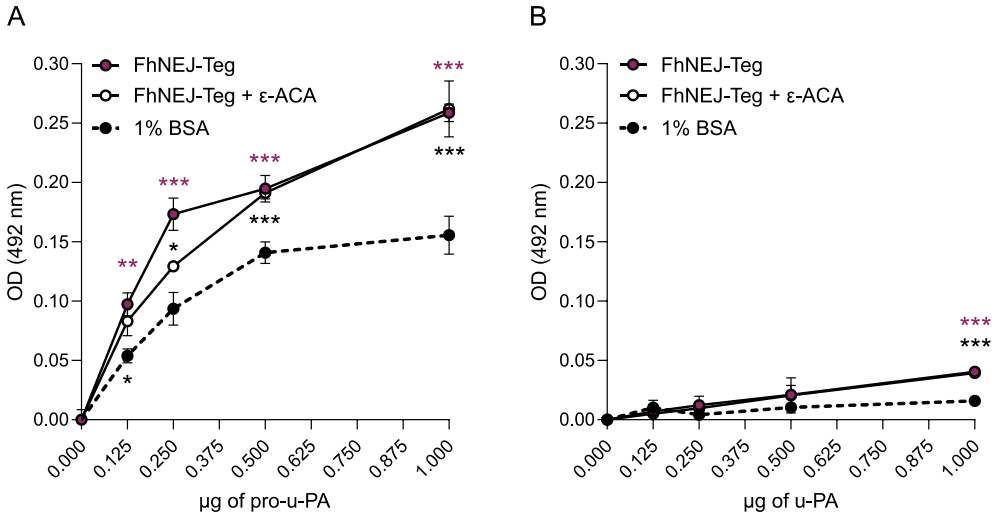

Figure 1. Binding of u-PA and its precursor by FhNEJ-Teg proteins. A,B) Binding of FhNEJ-Teg proteins to pro-u-PA (A) or u-PA (B) was detected via ELISA by coating wells with FhNEJ-Teg (0.5 µg) and incubating with increasing amounts of these factors. In parallel, a condition where ε-ACA was present during pro-u-PA/u-PA incubation was included to assess whether the binding of these fibrinolytic factors to FhNEJ-Teg proteins occurs via lysine residues. Wells coated with 1% BSA served as controls for nonspecific binding to pro-u-PA/u-PA. The graphs are representative of two independent experiments. The data points indicate the means of three technical replicates ± SD, purple asterisks indicate significant differences between pro-u-PA/u-PA and the negative control (1% BSA), and black asterisks

indicate significant differences between the negative control (1% BSA) and pro-u-PA/u-PA incubated in the presence of ε-ACA (**p≤0.1; ***p≤0.001; one-way ANOVA). Note that, in Panel B, the data points corresponding to FhNEJ-Teg and FhNEJ-Teg + ε-ACA overlap.

Identification of pro-u-PA-binding proteins in the tegument of FhNEJ

To identify the pro-u-PA-binding proteins contained within FhNEJ-Teg, we combined 2D electrophoresis, ligand blotting, and LC-MS/MS. Ligand blot analysis revealed 20 protein spots within the FhNEJ-Teg extract that appeared to bind pro-u-PA (**Figures 2A** and **2B**). Subsequent LC-MS/MS analysis revealed that these spots contained 116 different protein isoforms that corresponded to 52 different proteins with the potential to bind to pro-u-PA (**Supp. Table 1**). On average, each spot contained seven proteins, with the most common proteins identified being actin, cathepsins L1 and L2, FhCL3, FhCB3, FhGST, heat shock protein 70, annexin, and FhENO (**Figure 2C**). The sequences of 86 out of the 116 identified protein isoforms were retrieved and used for GO analysis to gain insights into their cellular locations (**Figure 2D**) and the molecular functions executed by these proteins (**Figure 2E**). This analysis revealed that putative pro-u-PA-binding proteins in FhNEJ-Teg are involved in proteolysis and cytoskeleton organisation, among others, and they are located mainly in the nucleus or associated with the extracellular space. Finally, a panel of recombinant *F. hepatica* proteins was used to validate the LC-MS/MS results in an ELISA-based binding assay, which revealed that FhCB3, FhCL3, FhGST, and FhENO bind to pro-u-PA in a concentration-dependent manner. While FhCL3, FhGST, and FhENO bound to pro-u-PA with similar affinities, FhCB3 exhibited lower efficiency in pro-u-PA binding (**Figure 2F**). The alignments of the proteins identified by 2D-MS and the corresponding recombinant versions used for validation assays are provided in **Supplementary Table 2**.

FhNEJ-Teg contains proteins that activate pro-u-PA into its catalytic form

Since pro-u-PA requires cleavage to become active, we next examined whether FhNEJ-Teg contains proteases capable of converting pro-u-PA into active u-PA. To test this hypothesis, a u-PA-specific activity assay was performed by co-incubating FhNEJ-Teg with pro-u-PA in the presence of a u-PA-specific chromogenic substrate and substrate cleavage was monitored over time. Wells containing plasmin instead of FhNEJ-Teg were included as positive controls for the generation of u-PA from its precursor (31), and wells containing mature u-PA served as controls for substrate specificity. Our results revealed that, compared with the absence of the extract, FhNEJ-Teg increased u-PA activity upon

incubation with pro-u-PA over time in a dose-dependent manner (**Figure 3A**). Importantly, such activity was not an artefact induced by FhNEJ-Teg buffer components, as verified in **Supplementary Figure 2A**.

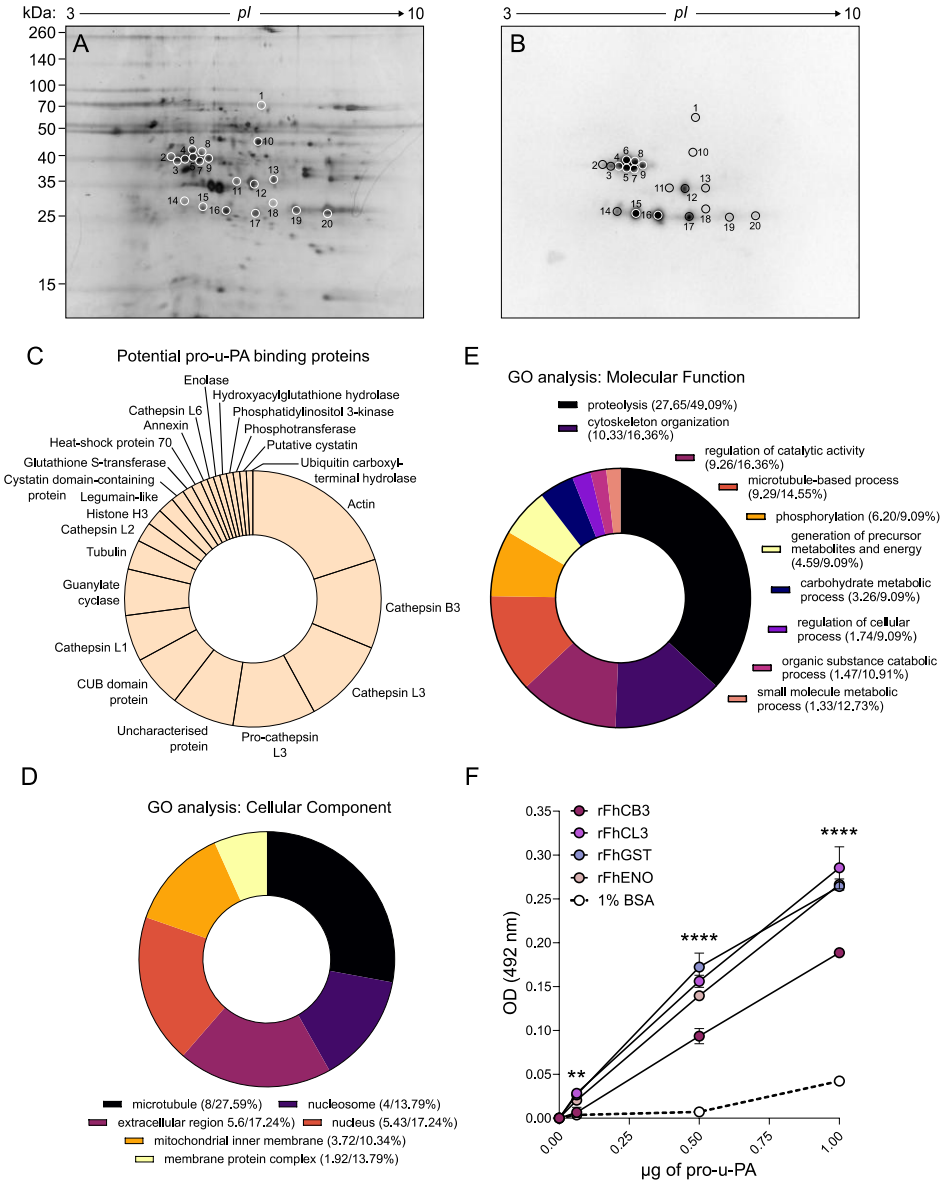

Figure 2. Identification of pro-u-PA-binding proteins in FhNEJ-Teg. A,B) FhNEJ-Teg proteins were resolved by 2D electrophoresis in duplicate so that proteins in one of the gels were visualised by silver stain (A), and the others were transferred onto a nitrocellulose membrane to detect pro-u-PA binding by standard ligand blot procedures (B). Protein spots are circled and numbered. C) Abundance distribution of the most recurrently identified proteins in the pool of potential pro-u-PA binding proteins (circled spots). D,E) GO analysis of the potential pro-u-PA-binding proteins identified by 2D-MS in the biological process (D) and cellular component (E) categories plotted according to

node score. The values in parentheses indicate the node score/percent of sequences annotated in each category. F) Binding of the recombinant proteins FhCB3, FhCL3, FhGST, and FhENO to pro-u-PA was detected via ELISA by coating the wells with the recombinant proteins (1 µg) followed by incubation with increasing amounts of pro-u-PA. The data points indicate the mean of three technical replicates ± SD, and the asterisks indicate significant differences between all the recombinant proteins and the negative control (1% BSA), with identical p values for each comparison (**p≤0.01; ****p≤0.0001; one-way ANOVA), except in the wells incubated with 0.0625 µg of pro-u-PA. In this condition, the difference between FhCB3 and 1% BSA was not significant. Note that some data points overlap: those representing FhCB3 and 1% BSA (0.0625 µg of pro-u-PA), those representing FhCL3 and FhGST (0.0625 µg of pro-u-PA), and those representing FhGST and FhENO proteins (1 µg of pro-u-PA).

We next tested the ability of recombinant *F. hepatica* cathepsins L1, L2, L3, and B2 to cleave and activate pro-u-PA. First, the activation of each cathepsin zymogen into its mature form was confirmed by SDS-PAGE and by an enzymatic assay using a cathepsin-specific fluorogenic substrate (**Supp. Figure 2B,C**) (28,29). The active enzymes were subsequently used to assess pro-u-PA activation in a u-PA enzymatic assay, as described above for FhNEJ-Teg. Our results revealed that none of the assayed *F. hepatica* cathepsins were capable of cleaving and activating pro-u-PA (**Figure 3B**) under the conditions used in this experiment. Notably, we used the same concentrations of mature cathepsins as plasmin, which serves as a positive control for u-PA generation from pro-u-PA (31), and we confirmed that u-PA activity is not inhibited by the buffer used for cathepsin activation (**Figure 3B**, u-PA + C-P buffer).

Activation of pro-u-PA by FhNEJ-Teg enhances plasmin generation from host PLG

Finally, to test whether pro-u-PA activation by FhNEJ-Teg proteins is sufficient to trigger the fibrinolytic route in the presence of host PLG, we set up an enzymatic assay to study plasmin generation in the presence of pro-u-PA, FhNEJ-Teg, and PLG. To that end, these compounds were incubated either alone or in different combinations in the presence of a plasmin-specific chromogenic substrate, and substrate cleavage over time was measured as a surrogate for plasmin generation in the wells. Although the condition containing pro-u-PA and PLG resulted in a progressive increase in substrate cleavage due to residual plasmin in the PLG stock used (**Supp. Figure 3A**), the results demonstrated that the u-PA generated from pro-u-PA by FhNEJ-Teg can cleave host PLG, leading to a significant increase in plasmin generation in the wells compared with those incubated without the extract (**Figure 4**). Notably, increased plasmin generation in wells containing FhNEJ-Teg, pro-u-PA, and PLG was not caused by FhNEJ-Teg buffer components (**Supp. Figure 3B**).

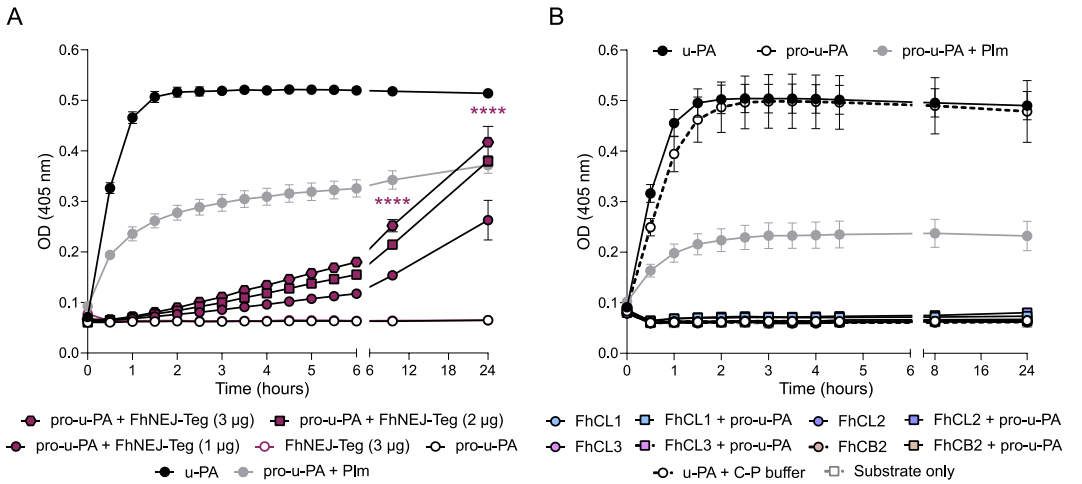

Figure 3. FhNEJ-Teg stimulates the catalytic activity of pro-u-PA. A) Increasing doses of FhNEJ-Teg (1 µg to 3 µg per well) were incubated with human pro-u-PA and u-PA-specific chromogenic substrate, and u-PA generation was assessed by measuring substrate cleavage over time (absorbance at 405 nm). The figure shows a representative result of two independent experiments where the data points indicate the means of three technical replicates ± SD. Differences between all FhNEJ-Teg doses + pro-u-PA and pro-u-PA alone were significant from three hours onwards, but significance is shown only at the latest timepoints for visualization purposes (****p≤0.0001; one-way ANOVA). B) Catalytically-active recombinant *F. hepatica* cathepsin peptidases L1, L2, L3, and B2 were incubated with human pro-u-PA and a u-PA-specific chromogenic substrate, and u-PA generation was assessed by measuring substrate cleavage over time (absorbance at 405 nm). Wells containing u-PA alone were used to control for substrate specificity, and wells containing pro-u-PA alone were used to confirm that this zymogen lacks catalytic activity. Wells containing u-PA in the presence of C-P activation buffer were used to ensure that the components of cathepsin activation (C-P) buffer did not inhibit u-PA catalytic activity. The data points indicate the means of three technical replicates ± SD. None of the differences between active cathepsins incubated alone or in the presence of pro-u-PA were significant (one-way ANOVA). In both panels, wells containing pro-u-PA with plasmin instead of FhNEJ-Teg serve as positive controls for u-PA generation from pro-u-PA (31).

Discussion

F. hepatica has evolved sophisticated mechanisms to migrate through the organism of its mammalian host, including the expression of an armoury of papain-like cysteine peptidases, known as cathepsins, that degrade tissue components and facilitate parasite invasion and establishment within the definitive host (32–35). In addition to these endogenous mechanisms, our laboratory has recently shown that FhNEJ are capable of stimulating plasmin generation from host-derived PLG, which further enhances the ability of these parasites to degrade intestinal ECM components *in vitro*, potentially facilitating parasite migration through the intestinal wall (16,24). Since FhNEJ do not produce proteases capable of directly cleaving and activating PLG but rely on host-derived PLG activators to convert this zymogen into plasmin (16), we hypothesised that they might also interact with and modulate the activity of the primary intestinal PLG

activator, u-PA, to further potentiate plasmin generation from circulating PLG. To test this hypothesis, we first sought to determine whether FhNEJ interact with u-PA and its precursor, pro-u-PA.

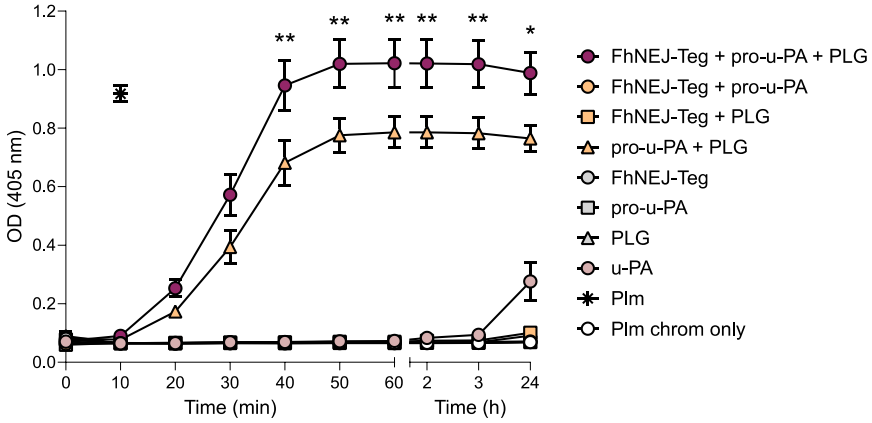

Figure 4. Stimulation of u-PA generation from pro-u-PA by FhNEJ-Teg proteins enhances plasmin generation from host PLG. Pro-u-PA was incubated in the presence or absence of FhNEJ-Teg and PLG, and plasmin generation was assessed by measuring the cleavage of a plasmin-specific chromogenic substrate (absorbance at 405 nm) over time. Wells containing u-PA and the plasmin-specific chromogen were used to control for unspecific activity of u-PA towards this substrate, and wells containing plasmin (Plm) alone were used to control for substrate specificity. The figure shows a representative result of two independent experiments, where the bars indicate the mean of three technical replicates ± SD. Asterisks indicate significant differences between pro-u-PA + PLG with and without FhNEJ-Teg (***p≤0.001; one-way ANOVA). Note that the data points corresponding to FhNEJ-Teg + pro-u-PA, FhNEJ-Teg + PLG, FhNEJ-Teg, pro-u-PA, PLG, u-PA, and Plm only overlap.

To this end, we co-incubated a tegument-enriched fraction of FhNEJ (FhNEJ-Teg) with increasing amounts of either pro-u-PA or u-PA and analysed their interaction by ELISA. We focused on FhNEJ-Teg proteins because the tegument represents the interface that is in direct contact with host tissues, and we have previously shown that this extract contains proteins that bind to host PLG and stimulate plasmin generation in the presence of the host-derived activators t-PA and/or u-PA (16,36). Our results showed that FhNEJ-Teg proteins interact with the precursor of u-PA in a concentration-dependent manner, an interaction that is more efficient than their binding to the active enzyme, u-PA. Moreover, the observation that the binding of u-PA to FhNEJ-Teg proteins is significant only at high concentrations (1 μg) suggests that this interaction may not occur under physiological conditions. Since protein interactions between FhNEJ-Teg and PLG are mediated by kringle domains present in the PLG molecule (16), which bind to lysine residues of partner proteins, we tested whether the kringle domain of pro-u-PA also mediates the interaction with this extract. Our results showed that the interaction between pro-u-PA and FhNEJ-Teg proteins is not mediated by the kringle domain, as shown by

the inability of the lysine analogue ε-ACA to abrogate it, which is not surprising considering that a kringle-deficient mutant of u-PA still binds to its physiologic receptor, the urokinase-type plasminogen activator receptor (u-PAR), although with lower binding stability (37).

To determine the identity of the FhNEJ-Teg proteins that bind to pro-u-PA, we performed a 2D-MS analysis of this extract, which revealed that many of them coincided with those identified as PLG-binding proteins in the past (16), such as actin, FhCL3, FhENO, FhGST, and heat shock protein 70. Since 2D-MS is performed under denaturing conditions, which might expose cryptic pro-u-PA binding sites that are not available for pro-u-PA binding under physiologic conditions, we partly validated the 2D-MS results by ELISA and using recombinant versions of some of the identified proteins, including the FhCB3 and FhCL3 zymogens, FhGST, and FhENO. These assays are conducted under native or physiological conditions, providing a more accurate reflection of the *in vivo* situation. We selected these proteins on the basis of our previous observations of their ability to bind either PLG, LM, or both (16,24,38). Our findings indicate that the same protein is capable of establishing interactions with different components of the host fibrinolytic system. Interestingly, the ability to establish interactions with multiple fibrinolytic factors is common to other helminth proteins that interact with the fibrinolytic route (13). This is also the case for the physiologic receptor of u-PA, the u-PAR, which interacts with u-PA via a central cavity that leaves the external receptor surface accessible for interaction with other proteins, such as vitronectin and integrins, thereby functioning as a hub that colocalises all the protein effectors that need to be engaged to execute the different biological functions that are mediated by u-PA/u-PAR (39). Notably, the pro-u-PA-binding proteins identified in the present study not only coincide with those that we recently identified as PLG-binding proteins but are also capable of binding LM *in vitro*, one of the major components of the intestinal ECM (16,24,40). By doing so, these pro-u-PA/PLG/LM-binding proteins expressed at the FhNEJ surface might function as multiprotein receptors that orchestrate the fine interplay between fibrinolytic effectors and ECM proteins that is needed to focalise fibrinolysis towards tissue components of the intestinal wall that would otherwise block FhNEJ migration.

Notably, our GO analyses revealed that some of the identified pro-u-PA binding proteins in FhNEJ-Teg, such as FhENO and FhGST, are intracellular enzymes with canonical cytosolic functions. The detection of intracellular and nuclear proteins in our 2D-MS analysis could be attributed to detergent-dependent exposure of these proteins during the

extraction of FhNEJ-Teg, which could accidentally enrich this extract with proteins that are not naturally present on the parasite's surface. However, studies by others have demonstrated that intracellular and nuclear proteins can be relocated to the cell surface to perform noncanonical functions, including PLG binding (41–48). In line with this, the relocation of intracellular proteins for PLG binding purposes has also been described in other parasitic helminths (13,38). On the basis of this knowledge, we propose that our findings reflect a moonlighting role for *F. hepatica* enolase, GST, and histones 3 and 2B rather than nonspecific protein binding or detergent-mediated accidental extraction to the tegument fraction. Protein moonlighting has supposedly evolved as a strategy to amplify the number of functions that can be executed with a stable set of proteins by simply shuffling them from one cellular location to another, which allows for their interaction with additional partner proteins (e.g., extracellular proteins) that were not available in their original location (49). From an evolutionary perspective, moonlighting of housekeeping and cytoskeletal proteins at the parasite surface is advantageous because these proteins are highly conserved across species and cannot be targeted by the host immune system without undesired self-recognition.

To the best of our knowledge, the mechanisms by which intracellular moonlighting proteins are translocated to the cell surface remain largely unknown. It has been proposed that this process may involve lipid binding prior to translocation to the outer membrane, interaction with partner proteins that contain signal sequences for extracellular export, or secretion as part of the cargo of extracellular vesicles. Regardless of the secretion pathway, extracellular translocation is believed to require post-translational modifications (PTMs) and is followed by the noncovalent association of the protein with the extracellular environment via an as yet unidentified mechanism (32,50). Interestingly, some of the potential pro-u-PA proteins identified in this study, such as heat-shock protein 70 (Accession B1NI98) and the CUB domain protein (Accession A0A2H1C1S8), are present in FhNEJ EVs (51). In addition, one of the FhCL3 protein isoforms (Accession Q9GRW4) identified in this work as a potential pro-u-PA binding protein appears in spots (14, 16, 17, and 20) with different isoelectric points, suggesting that this protein may acquire distinct PTMs prior to its translocation to the parasite surface.

The glycolytic enzyme enolase is a paradigmatic example of a moonlighting protein that contributes to the virulence potential of very different pathogenic organisms, including parasites (13), fungi (52), bacteria (53), and even viruses (54). Recently, *F. hepatica* enolase has been described as a moonlighting protein that is capable of interacting with PLG and the intestinal ECM protein LM (38), and we now show that this glycolytic enzyme is also

capable of interacting with pro-u-PA, which, together with PLG, represents a leading driver of extravascular plasmin-mediated proteolysis. In addition to FhENO, FhGST, which was identified and validated as a pro-u-PA binding protein in the present study, was also identified as a LM-binding protein in previous studies (24). In light of these findings, the acquisition of novel protein functions that avoid *de novo* transcription and translation (i.e., moonlighting) could be regarded as an efficient energy-saving strategy employed by FhNEJ to rapidly adapt to the constantly changing host microenvironment and rather expend their vital energy stores in growth and metabolic reprogramming, two processes that are crucial for infection success at the earliest phases of infection (55).

Next, we wanted to address whether FhNEJ-Teg contains proteases that are capable of directly cleaving pro-u-PA and converting it to its catalytically active form, u-PA. To this end, we co-incubated FhNEJ-Teg in the presence of pro-u-PA and a u-PA-specific chromogenic substrate and monitored substrate cleavage over time as a surrogate for u-PA generation. This experiment revealed that FhNEJ-Teg potentiates u-PA-specific substrate cleavage in a dose-dependent manner, which is presumably mediated by a protease that directly cleaves pro-u-PA and activates its catalytic activity. However, the binding of pro-u-PA to its physiologic receptor, u-PAR, enhances the catalytic activity of this zymogen even in the absence of proteolytic cleavage (56). Therefore, whether the increased pro-u-PA activation that we detected in the presence of FhNEJ-Teg proteins is due to pro-u-PA cleavage and subsequent u-PA generation, to binding-mediated enhancement of pro-u-PA enzymatic activity in the absence of cleavage, or both, remains to be determined. Nonetheless, the fact that FhCL3 was identified and validated as a pro-u-PA binding protein yet failed to stimulate its catalytic activity points towards the former. While the cleavage and activation of pro-u-PA for dissemination purposes is well documented in bacteria (57,58), to our knowledge, this is the first instance of such a function being described in a helminth parasite, with only one previous study reporting t-PA activation by the blood fluke *Schistosoma mansoni* (13,59).

Since cathepsin peptidases are highly expressed at the FhNEJ stage and some of their human orthologues can cleave and activate pro-u-PA (10,11,32), we tested whether the increased u-PA activity observed in our enzymatic assay was mediated by *F. hepatica* cathepsins. Owing to the technical challenges encountered during the activation of FhCB3, the ability of this protease to cleave and activate pro-u-PA could not be tested. Our studies revealed that recombinant *F. hepatica* cathepsins L1, L2, L3, and B2, in striking contrast to their human orthologues, are not involved in pro-u-PA cleavage and activation. Given that FhCLs and FhCB2 do not activate pro-u-PA and that proteases

that typically cleave and activate pro-u-PA under physiological conditions are predominantly serine proteases, including plasmin, trypsin, kallikrein, and tryptase (9), it is tempting to speculate that pro-u-PA activation is carried out by a serine protease expressed in the tegument of FhNEJ. Unlike their cysteine-type counterparts, serine proteases of *F. hepatica* are poorly described, and a dipeptidyl peptidase (DPP) that shares similarity with human DPP-2 and DPP-4 is one of the very few, if not the only, serine proteases characterised in this parasite (60,61). Considering that human DPP-4 is a PLG receptor on the surface of certain cell types (62,63) and that this protease was identified in previous studies by us as a potential PLG-binding protein in the tegument of FhNEJ (16), one could speculate that *F. hepatica* DPP might be the protease responsible for the pro-u-PA cleavage identified in the present study.

Our enzymatic assay revealed a significant but discrete and dose-dependent increase in pro-u-PA cleavage in the presence of FhNEJ-Teg, suggesting that the protease(s) in the FhNEJ-Teg extract responsible for this increase, if any, might be present at very low concentrations, or that this function is executed by a limited number of them. In this scenario, minimal u-PA activity at the parasite surface might still be sufficient to generate small but localised amounts of plasmin from tegument-bound PLG (16), which, in turn, would cleave and activate surface-bound pro-u-PA (31) in a positive feedback loop that would ultimately lead to potent plasmin generation in the periparasitic space. To test this hypothesis, we co-incubated pro-u-PA, PLG, and a plasmin-specific chromogenic substrate in the presence or absence of FhNEJ-Teg to assess whether the generation of u-PA from pro-u-PA by FhNEJ-Teg proteases would stimulate PLG activation into plasmin. Although the incubation of PLG with pro-u-PA resulted in some background plasmin generation, this effect was further enhanced in the presence of FhNEJ-Teg proteins, confirming that pro-u-PA cleavage and activation by FhNEJ-Teg proteases positively influence the ability of these parasites to generate plasmin from tegument-bound PLG.

The large number of potential pro-u-PA binding proteins identified in this work indicates that FhNEJ proteins interact with pro-u-PA via highly redundant mechanisms, as is the case for their interaction with PLG (16). Biochemical redundancy is a hallmark of essential biological functions, as it endows an organism with backup mechanisms to ensure the maintenance of that function even in the absence of a specific effector (64). Although biochemical redundancy emphasises the idea that the interaction between FhNEJ and the host fibrinolytic system might represent a key process for parasite survival, it also implies that targeting this parasite-host relationship for therapeutic or

control purposes is very challenging. However, the fact that the interaction between FhNEJ and pro-u-PA does not seem to be mediated by lysine residues suggests that the interaction mechanism in this case might be more specific than that between FhNEJ-Teg and PLG and thus more easily targetable from a pharmacological or immunological perspective. In relation to this, targeting the u-PA/u-PAR interaction using synthetic peptide antagonists and monoclonal antibodies has recently arisen in the oncology field as a valuable strategy to block cancer cell migration and tumour progression (65,66). It is tempting to speculate that similar strategies could be applied to infections by *F. hepatica* and potentially other parasites that interact with the host fibrinolytic system for migration and invasion inside their mammalian host. Theoretically, such an approach would not elicit undesirable side effects on host fibrinolysis since pronounced species specificity exists in the interaction between u-PA and its physiologic receptors (65,67).

In conclusion, this study elucidates the interaction between FhNEJ and host-derived pro-u-PA and reveals how FhNEJ-Teg proteins enhance pro-u-PA activation through direct cleavage by an as yet unidentified protease, which, in turn, significantly increases the ability of FhNEJ to activate the host fibrinolytic system. These findings complement previous work from our laboratory that revealed that FhNEJ interact with host PLG to stimulate plasmin generation and potentiate the endogenous capacity of these parasites to degrade LM, a major component of the intestinal ECM (16,24). Collectively, these data reveal the multilayered activation of the host fibrinolytic system on the surface of FhNEJ, which might be worth exploring for immunological or therapeutic interventions against early-stage fasciolosis.

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References

1. González-Miguel J, Siles-Lucas M, Kartashev V, Morchón R, Simón F. Plasmin in parasitic chronic infections: friend or foe? Trends Parasitol. 2016 Apr;32(4):325–35.
2. Figuera L, Gómez-Arreaza A, Avilán L. Parasitism in optima forma: exploiting the host fibrinolytic system for invasion. Acta Trop. 2013 Oct;128(1):116–23.

3. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. Br J Haematol. 2005 May;129(3):307–21.

4. Barthel D, Schindler S, Zipfel PF. Plasminogen is a complement inhibitor. J Biol Chem. 2012 May 25;287(22):18831–42.

5. Syrovets T, Simmet Th. Novel aspects and new roles for the serine protease plasmin. Cell Mol Life Sci. 2004 Apr 1;61(7–8):873–85.

6. Bhattacharya S, Ploplis VA, Castellino FJ. Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. J Biomed Biotechnol. 2012 Oct 14;482096.

7. Syrovets T, Lunov O, Simmet T. Plasmin as a proinflammatory cell activator. J Leukoc Biol. 2012 Sep;92(3):509–19.

8. Gibson PR, Birchall I, Rosella O, Albert V, Finch CF, Barkla DH, *et al.* Urokinase and the intestinal mucosa: evidence for a role in epithelial cell turnover. Gut. 1998 Nov 1;43(5):656–63.

9. Mahmood N, Mihalcioiu C, Rabbani SA. Multifaceted role of the urokinase-type plasminogen activator (uPA) and its receptor (uPAR): diagnostic, prognostic, and therapeutic applications. Front Oncol. 2018;8:24.

10. Goretzki L, Schmitt M, Mann K, Calvete J, Chucholowski N, Kramer M, *et al.* Effective activation of the proenzyme form of the urokinase-type plasminogen activator (pro-uPA) by the cysteine protease cathepsin L. FEBS Lett. 1992 Feb 3;297(1–2):112–8.

11. Kobayashi H, Schmitt M, Goretzki L, Chucholowski N, Calvete J, Kramer M, *et al.* Cathepsin B efficiently activates the soluble and the tumor cell receptor-bound form of the proenzyme urokinase-type plasminogen activator (Pro-uPA). J Biol Chem. 1991 Mar 15;266(8):5147–52.

12. Mondino A, Blasi F. uPA and uPAR in fibrinolysis, immunity and pathology. Trends Immunol. 2004 Aug;25(8):450–5.

13. Diosdado A, Simón F, Serrat J, González-Miguel J. Interaction of helminth parasites with the haemostatic system of their vertebrate hosts: a scoping review. Parasite. 2022;29:35.

14. Bernal D, de la Rubia JE, Carrasco-Abad AM, Toledo R, Mas-Coma S, Marcilla A. Identification of enolase as a plasminogen-binding protein in excretory-secretory products of *Fasciola hepatica*. FEBS Lett. 2004 Apr 9;563(1–3):203–6.

15. González-Miguel J, Valero MA, Reguera-Gomez M, Mas-Bargues C, Bargues MD, Simón F, *et al.* Numerous *Fasciola* plasminogen-binding proteins may underlie blood-brain barrier leakage and explain neurological disorder complexity and heterogeneity in the acute and chronic phases of human fascioliasis. Parasitology. 2019 Mar;146(3):284–98.

16. Serrat J, Becerro-Recio D, Torres-Valle M, Simón F, Valero MA, Bargues MD, *et al.* *Fasciola hepatica* juveniles interact with the host fibrinolytic system as a potential early-stage invasion mechanism. PLoS Negl Trop Dis. 2023 Apr;17(4):e0010936.

17. Rosas-Hostos Infantes LR, Paredes Yataco GA, Ortiz-Martínez Y, Mayer T, Terashima A, Franco-Paredes C, *et al.* The global prevalence of human fascioliasis: a systematic review and meta-analysis. Ther Adv Infect Dis. 2023 Dec;10: 20499361231185413.

18. Mehmood K, Zhang H, Sabir AJ, Abbas RZ, Ijaz M, Durrani AZ, *et al.* A review on epidemiology, global prevalence and economical losses of fasciolosis in ruminants. Microb Pathogen. 2017 Aug;109:253–62.

19. Fitzpatrick JL. Global food security: the impact of veterinary parasites and parasitologists. Vet Parasitol. 2013 Aug 1;195(3–4):233–48.

20. Mas-Coma S, Valero MA, Bargues MD. Fascioliasis. In: Digenetic Trematodes. Springer International Publishing; 2019. p. 71–103.

21. Andrews SJ, Cwiklinski K, Dalton JP. Chapter 1. The discovery of *Fasciola hepatica* and its life cycle. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 1–23.

22. Sangster NC, Martínez-Moreno Á, Pérez J. Chapter 5. Pathology, pathophysiology and clinical aspects. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 145–80.

23. Siles-Lucas M, Becerro-Recio D, Serrat J, González-Miguel J. Fascioliasis and fasciolopsiasis: current knowledge and future trends. Res Vet Sci. 2021 Jan;134:27–35.

24. Serrat J, Torres-Valle M, López-García M, Becerro-Recio D, Siles-Lucas M, González-Miguel J. Molecular characterization of the interplay between *Fasciola hepatica* juveniles and laminin as a mechanism to adhere to and break through the host intestinal wall. Int J Mol Sci. 2023 May 3;24(9).

25. De Marco Verissimo C, Jewhurst HL, Dobó J, Gál P, Dalton JP, Cwiklinski K. *Fasciola hepatica* is refractory to complement killing by preventing attachment of

mannose binding lectin (MBL) and inhibiting MBL-associated serine proteases (MASPs) with serpins. PLoS Pathog. 2022 Jan 10;18(1):e1010226.

26. García-Campos A, Ravidà A, Nguyen DL, Cwiklinski K, Dalton JP, Hokke CH, *et al.* Tegument glycoproteins and cathepsins of newly excysted juvenile *Fasciola hepatica* carry mannosidic and paucimannosidic N-glycans. PLoS Negl Trop Dis. 2016 May 3;10(5):1–26.
27. Sievers F, Higgins DG. The Clustal Omega multiple alignment package. Methods Mol Biol. 2021;2231:3–16.
28. Barbour T, Cwiklinski K, Lalor R, Dalton JP, De Marco Verissimo C. The zoonotic helminth parasite *Fasciola hepatica*: virulence-associated cathepsin B and cathepsin L cysteine peptidases secreted by infective newly excysted juveniles (NEJ). Animals. 2021;11(12):3495.
29. Pritsch IC, Tikhonova IG, Jewhurst HL, Drysdale O, Cwiklinski K, Molento MB, *et al.* Regulation of the *Fasciola hepatica* newly excysted juvenile cathepsin L3 (FhCL3) by its propeptide: a proposed 'clamp-like' mechanism of binding and inhibition. BMC Mol Cell Biol. 2020 Dec 7;21(1):90.
30. Fox J, Bouchet-Valat M. Rcmdr: R Commander. 2021. Available from: https://socialsciences.mcmaster.ca/jfox/Misc/Rcmdr/
31. Kasai S, Arimura H, Nishida M, Suyama T. Proteolytic cleavage of single-chain pro-urokinase induces conformational change which follows activation of the zymogen and reduction of its high affinity for fibrin. J Biol Chem. 1985 Oct 5;260(22):12377–81.
32. Cwiklinski K, Donnelly S, Drysdale O, Jewhurst H, Smith D, De Marco Verissimo C, *et al.* The cathepsin-like cysteine peptidases of trematodes of the genus *Fasciola*. In: Advances in Parasitology. Elsevier; 2019. p. 113–64.
33. Corvo I, O'Donoghue AJ, Pastro L, Pi-Denis N, Eroy-Reveles A, Roche L, *et al.* Dissecting the active site of the collagenolytic cathepsin L3 protease of the invasive stage of *Fasciola hepatica*. PLoS Negl Trop Dis. 2013 Jul 11;7(7):e2269.
34. Corvo I, Cancela M, Cappetta M, Pi-Denis N, Tort JF, Roche L. The major cathepsin L secreted by the invasive juvenile *Fasciola hepatica* prefers proline in the S2 subsite and can cleave collagen. Mol Biochem Parasitol. 2009 Sep;167(1):41–7.
35. Robinson MW, Corvo I, Jones PM, George AM, Padula MP, To J, *et al.* Collagenolytic activities of the major secreted cathepsin L peptidases involved in the

virulence of the helminth pathogen, *Fasciola hepatica*. PLoS Negl Trop Dis. 2011 Apr 5;5(4):e1012.

36. González-Miguel J, Becerro-Recio D, Siles-Lucas M. Insights into *Fasciola hepatica* juveniles: crossing the fasciolosis rubicon. Trends Parasitol. 2021 Jan;37(1):35–47.
37. Bdeir K, Kuo A, Sachais BS, Rux AH, Bdeir Y, Mazar A, *et al.* The kringle stabilizes urokinase binding to the urokinase receptor. Blood. 2003 Nov 15;102(10):3600–8.
38. O'Kelly E, Cwiklinski K, De Marco Verissimo C, Calvani NED, López Corrales J, Jewhurst H, *et al.* Moonlighting on the *Fasciola hepatica* tegument: enolase, a glycolytic enzyme, interacts with the extracellular matrix and fibrinolytic system of the host. PLoS Negl Trop Dis. 2024 Aug;18(8):e0012069.
39. Llinas P, Le Du MH, Gårdsvoll H, Danø K, Ploug M, Gilquin B, *et al.* Crystal structure of the human urokinase plasminogen activator receptor bound to an antagonist peptide. EMBO J. 2005 May 4;24(9):1655–63.
40. Pompili S, Latella G, Gaudio E, Sferra R, Vetuschi A. The charming world of the extracellular matrix: a dynamic and protective network of the intestinal wall. Front Med. 2021 Apr 16;8:610189.
41. Das R, Burke T, Plow EF. Histone H2B as a functionally important plasminogen receptor on macrophages. Blood. 2007 Nov 15;110(10):3763–72.
42. Herren T, Burke TA, Das R, Plow EF. Identification of histone H2B as a regulated plasminogen receptor. Biochemistry. 2006 Aug 8;45(31):9463–74.
43. Qiao G, Wu A, Chen X, Tian Y, Lin X. Enolase 1, a moonlighting protein, as a potential target for cancer treatment. Int J Biol Sci. 2021;17(14):3981–92.
44. Itakura M, Yamaguchi K, Kitazawa R, Lim SY, Anan Y, Yoshitake J, *et al.* Histone functions as a cell-surface receptor for AGEs. Nat Commun. 2022 May 27;13(1):2974.
45. Das M, Ithychanda SS, Plow EF. Histone 2B facilitates plasminogen-enhanced endothelial migration through protease-activated receptor 1 (PAR1) and protease-activated receptor 2 (PAR2). Biomolecules. 2022 Jan 26;12(2).
46. Luah YH, Chaal BK, Ong EZ, Bozdech Z. A moonlighting function of *Plasmodium falciparum* histone 3, mono-methylated at lysine 9? PLoS ONE. 2010 Apr 19;5(4):e10252.

47. Brix K, Summa W, Lottspeich F, Herzog V. Extracellularly occurring histone H1 mediates the binding of thyroglobulin to the cell surface of mouse macrophages. J Clin Invest. 1998 Jul 15;102(2):283–93.

48. Li X, Ye Y, Peng K, Zeng Z, Chen L, Zeng Y. Histones: the critical players in innate immunity. Front Immunol. 2022;13:1030610.

49. Singh N, Bhalla N. Moonlighting proteins. Annu Rev Genet. 2020;54:265–85.

50. Ceruti P, Principe M, Capello M, Cappello P, Novelli F. Three are better than one: plasminogen receptors as cancer theranostic targets. Exp Hematol Oncol. 2013 Dec;2(1):1–11.

51. Trelis M, Sánchez-López CM, Sánchez-Palencia LF, Ramírez-Toledo V, Marcilla A, Bernal D. Proteomic analysis of extracellular vesicles from *Fasciola hepatica* hatching eggs and juveniles in culture. Front Cell Infect Microbiol. 2022 Jun 3;12:903602.

52. Arvizu-Rubio VJ, García-Carnero LC, Mora-Montes HM. Moonlighting proteins in medically relevant fungi. PeerJ. 2022 Sep 13;10:e14001.

53. Henderson B, Martin A. Bacterial moonlighting proteins and bacterial virulence. In: Between pathogenicity and commensalism. Berlin, Heidelberg: Springer Berlin Heidelberg; 2011. p. 155–213.

54. Vadlamani S, Karmakar R, Kumar A, Rajala MS. Non-metabolic role of alpha-enolase in virus replication. Mol Biol Rep. 2023 Feb;50(2):1677–86.

55. Cwiklinski K, Jewhurst H, McVeigh P, Barbour T, Maule AG, Tort J, *et al.* Infection by the helminth parasite *Fasciola hepatica* requires rapid regulation of metabolic, virulence, and invasive factors to adjust to its mammalian host. Mol Cell Proteom. 2018;17:792–809.

56. Higazi AAR, Cohen RL, Henkin J, Kniss D, Schwartz BS, Cines DB. Enhancement of the enzymatic activity of single-chain urokinase plasminogen activator by soluble urokinase receptor. J Biol Chem. 1995 Jul 21;270(29):17375–80.

57. Järvinen HM, Laakkonen L, Haiko J, Johansson T, Juuti K, Suomalainen M, *et al.* Human single-chain urokinase is activated by the omptins PgtE of *Salmonella enterica* and Pla of *Yersinia pestis* despite mutations of active site residues. Mol Microbiol. 2013 Aug;89(3):507–17.

58. Sanderson-Smith ML, Zhang Y, Ly D, Donahue D, Hollands A, Nizet V, *et al.* A key role for the urokinase plasminogen activator (uPA) in invasive Group A streptococcal infection. PLoS Pathogens. 2013 Jul;9(7):1–9.

59. Leontovyč A, Ulrychová L, O'Donoghue AJ, Vondrášek J, Marešová L, Hubálek M, *et al.* SmSP2: a serine protease secreted by the blood fluke pathogen *Schistosoma mansoni* with anti-hemostatic properties. PLoS Negl Trop Dis. 2018 Apr;12(4):e0006446.

60. Carmona C, McGonigle S, Dowd AJ, Smith AM, Coughlan S, McGowran E, *et al.* A dipeptidylpeptidase secreted by *Fasciola hepatica*. Parasitology. 1994 Jul;109(Pt 1):113–8.

61. Kašný M, Mikeš L, Hampl V, Dvořák J, Caffrey CR, Dalton JP, *et al.* Chapter 4. Peptidases of trematodes. In: Advances in Parasitology. Elsevier; 2009. p. 205–97.

62. Gonzalez-Gronow M, Grenett HE, Weber MR, Gawdi G, Pizzo SV. Interaction of plasminogen with dipeptidyl peptidase IV initiates a signal transduction mechanism which regulates expression of matrix metalloproteinase-9 by prostate cancer cells. Biochem J. 2001 Apr 15;355(Pt 2):397–407.

63. Mario Gonzalez-Gronow, Steven Kaczowka, Govind Gawdi. Dipeptidyl peptidase IV (DPP IV/CD26) is a cell-surface plasminogen receptor. Front Biosci. 2008;13(5):1610–8.

64. Nowak MA, Boerlijst MC, Cooke J, Smith JM. Evolution of genetic redundancy. Nature. 1997 Jul;388(6638):167–71.

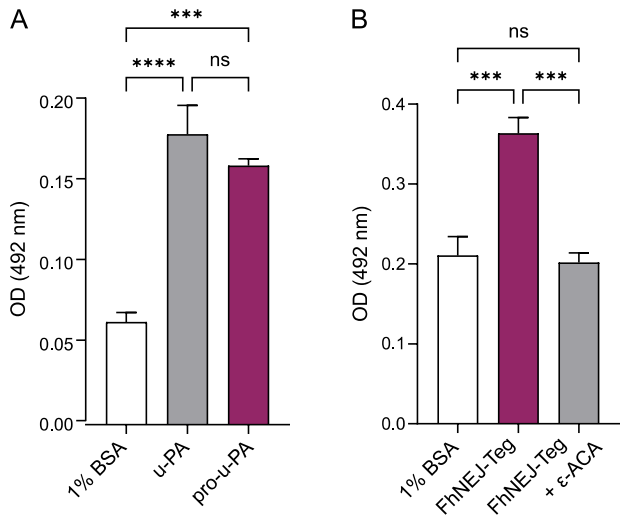
65. Metrangolo V, Ploug M, Engelholm LH. The urokinase receptor (uPAR) as a "Trojan horse" in targeted cancer therapy: challenges and opportunities. Cancers (Basel). 2021 Oct 27;13(21):5376.

66. Yuan C, Guo Z, Yu S, Jiang L, Huang M. Development of inhibitors for uPAR: blocking the interaction of uPAR with its partners. Drug Discov Today. 2021 Apr;26(4):1076–85.

67. Lin L, Gårdsvoll H, Huai Q, Huang M, Ploug M. Structure-based engineering of species selectivity in the interaction between urokinase and its receptor. J Biol Chem. 2010 Apr;285(14):10982–92.

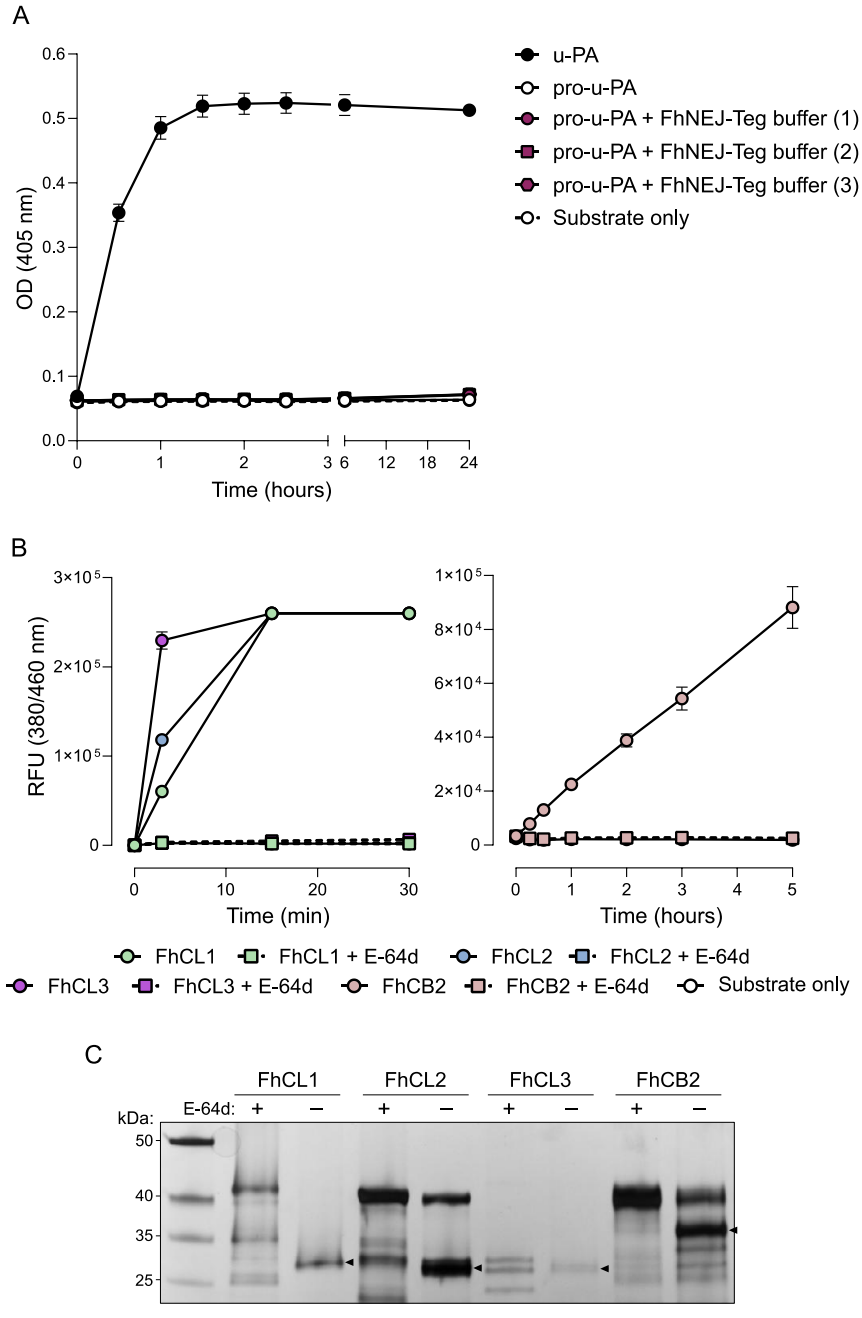
Supplementary information

Supplementary Figure 1. A) The specificity of the primary antibody used to detect pro-u-PA and u-PA binding was tested by ELISA. The wells were coated with either u-PA or pro-u-PA, followed by incubation with the u-PA-specific primary antibody used in the assay presented in **Figure 1**. **B)** Detection of PLG binding to wells coated with FhNEJ-Teg or 1% BSA in the presence or absence of ε-ACA (50 mM) was used to test the ability of this compound to inhibit lysine-dependent interactions (16). In both panels, the bars indicate the means of three technical replicates ± SD, and the asterisks indicate significant differences between the indicated groups (***p≤0.001, ****p≤0.0001; ns, not significant; one-way ANOVA).

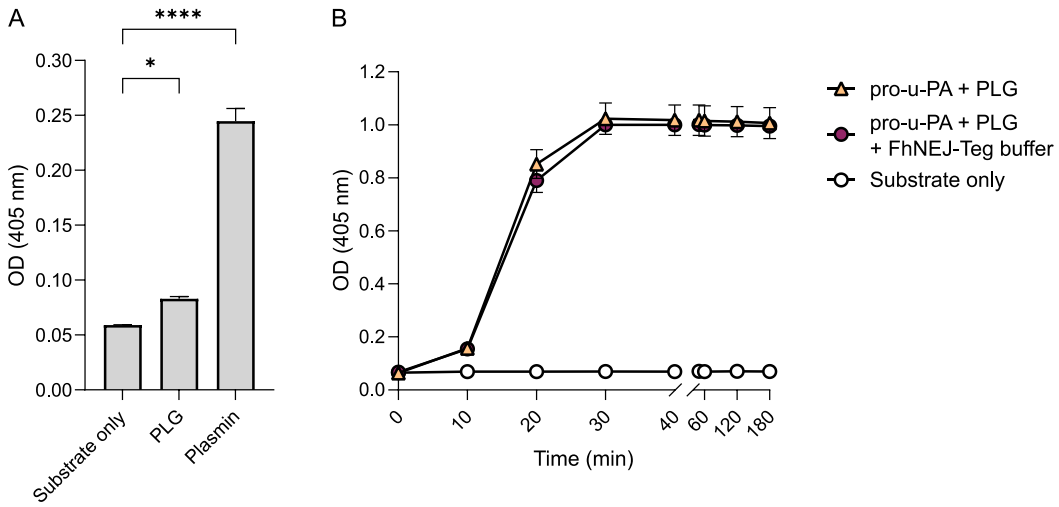


Supplementary Figure 2 (figure on the next page). A) Pro-u-PA was incubated with increasing volumes of FhNEJ-Teg buffer (equivalent to those used in **Figure 3A**) and a u-PA-specific chromogenic substrate to control that the observed pro-u-PA activation in **Figure 3A** was not caused by FhNEJ-Teg buffer components. Note that the data points corresponding to pro-u-PA, pro-u-PA + FhNEJ-Teg buffer (1), pro-u-PA + FhNEJ-Teg buffer (2), pro-u-PA + FhNEJ-Teg buffer (3), and the substrate only overlap. **B,C)** Activation of *F. hepatica* cathepsin zymogens was checked via an enzymatic assay employing an Gly-Pro-Arg-AMC fluorogenic substrate (B) and via SDS-PAGE followed by silver staining (C). In B, the data points corresponding to the substrate only, FhCL1 +

E-64d, FhCL2 + E-64d, FhCL3 + E-64d, and FhCB2 + E-64d overlap. In C, activated enzymes (arrowheads) in the absence of E-64d (−) were loaded next to their zymogen counterparts, which were incubated with C-P buffer in the presence of E-64d (+) to prevent autocatalytic activation. kDa, molecular weight in kilodaltons.



Supplementary Figure 3. A) The residual plasmin activity in the PLG stock used in the assay shown in **Figure 4** was tested by mixing PLG with a plasmin-specific chromogenic substrate. Wells containing plasmin instead of PLG served as positive controls for substrate cleavage. The bars indicate the means ± SD of three technical replicates, and the asterisks indicate significant differences (*p≤0.05; ****p≤0.0001; one-way ANOVA). **B)** Pro-u-PA + PLG were incubated with an equivalent volume of FhNEJ-Teg buffer as that used for FhNEJ-Teg in **Figure 4** and a plasmin-specific chromogenic substrate to confirm that the registered plasmin generation in **Figure 4** was not caused by FhNEJ-Teg buffer components.



Supplementary Table 1. Potential pro-u-PA-binding proteins in the tegument-enriched fraction of FhNEJ identified by 2D-MS. Only entries with Unused ≥ 2 and corresponding to *F. hepatica* are shown. Experimental molecular weight (MW, in kilodaltons) and isoelectric point (*pI*) values were estimated using the PDQuest Software v.8.0.1 (BioRad). Available at: https://veterinaryresearch.biomedcentral.com/articles/10.1186/s13567-025-01449-4#additional-information.

Supplementary Table 2. Alignments of protein sequences of the recombinant proteins used for validation experiments (**Figure 2F**) and those identified by 2D-MS analysis (**Supp. Table 1**). The table shows percent identity matrices created by Clustal Omega (27). Available at: https://veterinaryresearch.biomedcentral.com/articles/10.1186/s13567-025-01449-4#additional-information.

Chapter 4

Early host-parasite interaction models reveal a key role for fibrinolysis in *Fasciola hepatica* intestinal migration

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Fasciola hepatica is the most common etiologic agent of fasciolosis, a parasitic disease that affects millions of ruminants worldwide and a zoonotic human infection of public health concern. Upon ingestion of infective metacercariae, *F. hepatica* newly excysted juveniles (FhNEJ) emerge in the duodenum and cross the intestinal wall to initiate a migration route that culminates with their establishment within the hepatic bile ducts. The ability of FhNEJ to exploit the broad-spectrum activities of host plasmin, the central protease of the fibrinolytic system, has been proposed as a strategy employed by these parasites to migrate across the intestinal wall while minimising energy expenditure. In this study, mouse intestinal epithelial cells (mPSIEC) were stimulated with FhNEJ and plasminogen (PLG), the zymogen of plasmin, to understand whether FhNEJ-stimulated plasmin generation modulates processes relevant to parasite migration through the intestinal wall, including extracellular matrix (ECM) degradation and the secretion of ECM-degrading enzymes. Plasmin-mediated cellular responses were further examined by proteomic analysis of mPSIEC whole-cell lysates. In parallel, the contribution of the fibrinolytic system in FhNEJ migration was studied *in vivo* by infecting mice with *F. hepatica* metacercariae following pharmacological inhibition of fibrinolysis. Co-stimulation of mPSIEC with FhNEJ and PLG led to increased plasmin generation in the intestinal pericellular space, which was associated with enhanced collagen degradation and secretion of the urokinase-type plasminogen activator. In addition, using independent cell culture replicates and a stringent statistical pipeline, we identified a robust set of differentially expressed proteins in mPSIEC following stimulation with FhNEJ and PLG. These proteins were involved in cell adhesion, migration, ECM remodelling, immune evasion, and fibrinolysis. Despite inter-experimental variability, FhNEJ migration in mice was reduced upon pharmacological inhibition of fibrinolysis, supporting the contribution of host fibrinolysis to parasite invasion *in vivo*. Altogether, this work provides unprecedented insights into the role of the host fibrinolytic system in FhNEJ migration across mammalian tissues, thereby advancing our understanding of host-parasite relationships during early-stage fasciolosis and highlighting interesting directions for future research in this area.

Introduction

A paradigmatic example of host-parasite relationship is the ability of parasites to engage with the fibrinolytic system of their definitive hosts, a strategy that is considered a key aspect of parasite dissemination and survival within the mammalian organism (1–3). The most common mechanism of interaction between parasites and host fibrinolysis is through the expression of lysine-rich proteins at the host-parasite interface—either on the parasite surface or secreted into the tissue microenvironment surrounding the parasite—that interact with plasminogen (PLG), the main zymogen of the fibrinolytic route. Upon binding to its receptors (PLG-Rs), PLG undergoes a conformational change that exposes the target sites of its activators, the proteases tissue-type and urokinase-type PLG activators (t-PA and u-PA, respectively), which convert PLG into its catalytically active form, the serine protease plasmin (4).

The classical function of the fibrinolytic system is to degrade the fibrin network that stabilises blood clots in the vascular endothelium (4); however, due to the broad spectrum of plasmin substrates, this system also participates in multiple biological processes that are both unrelated and independent of fibrin degradation and blood clot removal, including cell migration (5,6). Plasmin facilitates cell migration by degrading different components of the extracellular matrix (ECM), including collagens, laminin, fibronectin, and proteoglycans (7–11), as well as by cleaving and activating the zymogens of different metalloproteinases (MMPs), which are considered master regulators of ECM remodelling during homeostasis, development, and disease (12). Additionally, fibrinolysis-unrelated substrates of plasmin also include complement molecules and immunoglobulins (13). Based on this, the stimulation of plasmin generation from host PLG within the tissue microenvironment surrounding parasites has been postulated as a mechanism facilitating immune evasion and their migration through host tissues (2,3,13).

Fasciola hepatica is one of many parasites whose interaction with the host fibrinolytic system has been experimentally shown (14–18). *F. hepatica* is a helminth trematode with an indirect life cycle that includes freshwater snails as intermediate hosts, where the asexual phases of the parasites develop, and a mammalian definitive host, where adult flukes mature. Definitive hosts, typically ruminants and humans, become infected by ingestion of water plants contaminated with infective metacercariae, which excyst in the duodenum and release the newly excysted juveniles (FhNEJ). Shortly after excystment, FhNEJ cross the intestinal wall, and the juvenile flukes subsequently migrate through the

peritoneum and liver parenchyma before entering the major hepatic bile ducts, where they mature into adults and produce fertilised eggs that are shed with the faeces (19).

F. hepatica is the most widespread etiologic agent of fasciolosis, a chronic and debilitating disease that affects millions of ruminants worldwide, thereby representing an economically important affection of domestic livestock that poses major threats to animal welfare and global food security (20,21). Since cases of human infection are predominantly concentrated in tropical areas of the globe, human fasciolosis is categorised by the Word Health Organization a food-borne neglected tropical disease of growing public health concern requiring targeted intervention for its elimination (22,23).

Using *in vitro* assays, we and others have previously shown that FhNEJ interact with the host fibrinolytic system by multiple mechanisms. These include the expression of proteins that bind to PLG, the precursor zymogen of u-PA (pro-u-PA), or both at the host-parasite interface (14–16,18), as well as the expression of proteases that can stimulate pro-u-PA conversion into its catalytically active form, which further potentiates plasmin generation from host PLG (15). Notably, FhNEJ-stimulated plasmin generation contributes to the endogenous capacity of these parasites to degrade laminin (24), one of the major components of the intestinal basement membrane (25), indicating that the interaction between FhNEJ and host fibrinolysis may facilitate their migration across the intestinal wall. Since FhNEJ trans-intestinal migration is considered a crucial step for infection success, a deeper understanding of the mechanisms governing this process, including the contribution of the host fibrinolytic system, may offer potential avenues for developing novel therapeutic and control strategies against this widespread parasite (26).

Although the interaction between parasites and host fibrinolysis system is well-established (1–3), only a minority of studies have performed functional assays using physiologically-relevant experimental models to assess whether it serves any physiological roles (1). The abovementioned interplay between *F. hepatica* and the host fibrinolytic system exemplifies this gap. In the present study, we addressed this limitation by exploring the functional relevance of the interaction between FhNEJ and host fibrinolysis using a co-culture system of epithelial cells derived from the mouse small intestine, which simulates the first contact between FhNEJ and host tissues (27,28), and a mouse model of early-stage fasciolosis (29).

Materials and Methods

Culture of mouse primary small intestinal epithelial cells

C57BL/6 mouse primary small intestinal epithelial cells (mPSIEC; Cell Biologics), were cultured as previously described (27,28) and following the manufacturer's instructions. Briefly, cells were plated in 6 cm^2 dishes (Corning) pre-coated with a gelatin-based coating solution (Cell Biologics) and grown in complete epithelial cell medium (Cell Biologics) in a humidified, 5% CO_2 atmosphere at 37 ºC. Cell medium was replaced every 48 h and cells were tripsinised and split at a 1:3 ratio when confluence was reached following standard cell culture procedures.

Excystment of *F. hepatica* metacercariae

F. hepatica metacercariae (Italian strain; Ridgeway Research Ltd) were excysted as previously described (14). Briefly, metacercariae were incubated for 1 h at 37 ºC in a solution containing CO_2 and 0.02 M sodium dithionite (Sigma), followed by three washes with distilled water and incubation in excystment medium [Hank's balanced salt solution (Sigma) supplemented with 10% lamb bile (obtained from a local abattoir) and 30 mM HEPES (Sigma), pH 7.4] at 37 ºC. FhNEJ were manually recovered under a stereomicroscope using a 20 µL pipette every hour after addition of excystment medium, immediately transferred to a clean plate containing complete epithelial cell medium, and incubated in a humidified, 5% CO_2 atmosphere at 37 ºC to induce recovery for 1 h prior to transfer to mPSIEC cultures.

In vitro interaction model

FhNEJ were co-cultured with mPSIEC as previously described (27,28), with some modifications. At passage 5, mPSIEC were cultured in 6-well plates until confluence was reached. At that point, 200 FhNEJ were added to the corresponding wells in 2 mL of complete epithelial cell medium in the presence or absence of 10 µg/mL of human PLG (Origene). A condition where cells were co-incubated with 200 FhNEJ, PLG (10 µg/mL), and 50 mM of the lysine analogue 6-aminocaproic acid (ε-ACA; Sigma) was included to assess whether the mechanism of FhNEJ-induced plasmin generation is lysine-dependent. Cells left unstimulated, cells stimulated only with 200 FhNEJ, and cells stimulated only with PLG (10 µg/mL) were used as controls. The parasites were separated from the cells 24 h after stimulation, and whole-cell lysates and cell culture supernatants were harvested

for downstream analyses. First, 1 mL of each culture supernatant was carefully aspirated and transferred to 1.5 mL tubes, followed by centrifugation for 5 min at 13000 x*g* and 4 ºC. The pellets containing cell debris were discarded and clean supernatants were frozen at -80 ºC until use. Whole-cell lysates were obtained by washing the cells in pre-warmed PBS followed by scraping in 200 µL of RIPA buffer (Sigma). Whole-cell lysates were transferred to 1.5 mL tubes, vigorously vortexed for 30 s and centrifuged for 5 min at 13000 x*g* and 4 ºC. Pellets containing the cell debris were discarded and supernatants were transferred to clean 1.5 mL tubes and stored at -80 ºC until use. Protein concentrations were determined using the Pierce BCA Protein Assay (Thermo Fisher) and ranged between 1.03–1.37 mg/mL, depending on the sample. Each experimental condition was conducted in triplicate.

Plasmin activity in cell culture supernatants

Plasmin activity in cell culture supernatants collected from mPSIEC in the *in vitro* interaction model was assayed by measuring the amidolytic activity of generated plasmin on a plasmin-specific chromogenic substrate, as previously described (14), with minor modifications. In every well of a transparent, flat-bottom, microtiter 96-well plate, equal volumes of cell culture supernatant were mixed with 1 mM of D-Val-Leu-Lys 4-nitroanilide dihydrochloride chromogenic substrate (S-2251, Sigma) in a total volume of 100 µL of PBS. The microplates were incubated for up to 24 h at 37 ºC and substrate cleavage was assessed by measuring absorbance at 405 nm every hour in a Multiskan GO spectrophotometer (Thermo Fisher). Wells containing 0.02 µM of plasmin (Origene) instead of cell culture supernatant were used to control for substrate specificity. All the reactions were performed in technical triplicate.

Analysis of u-PA levels in cell culture supernatants

The levels of u-PA in cell culture supernatants (100 µL) were measured by enzyme-linked immunosorbent assay (ELISA) using the Mouse PLAU/Upa (Urokinase-Type Plasminogen Activator) ELISA kit (FineTest) and following the manufacturer's instructions. The reaction was stopped 11 min after addition of the 3,3',5,5'-tetramethylbenzidine substrate, and the amount of u-PA in the samples was quantified by fitting a regression line to the linear portion of a standard curve with known u-PA concentrations. All the reactions were performed in technical duplicate.

Analysis of total collagen in cell culture supernatants

The levels of total collagen in cell culture supernatants (20 μL) were measured by enzymatic digestion of collagen into N-Gly terminal peptides that react with a dye reagent to form a fluorescent complex, using the Collagen Assay Kit (Assay Genie) and following the manufacturer's instructions. The amount of collagen in the samples was quantified by fitting a regression line to the linear portion of a standard curve with known collagen concentrations. All the reactions were performed in technical duplicate.

Zymography

The ability of plasmin to degrade the mPSIEC coating solution was measured by preparing 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels using standard protocols but replacing water with the mPSIEC coating solution (Cell Biologics). Next, different amounts of plasmin (Origene) were mixed with non-reducing sample buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 0.01% bromophenol blue) in a total volume of 18 μL and samples were loaded onto the gel in duplicate. Electrophoresis was performed at constant 15 mA for 20 min followed by 30 mA. After electrophoresis, the gel was soaked twice in wash buffer (5 mM $CaCl_2$, 2% Triton-X, pH 7.4) for 30 min at room temperature, followed by overnight incubation at 37 ºC in digestion buffer (5 mM $CaCl_2$, 0.1 M glycine, pH 8.4) (30). All the incubations were performed with mild shaking. After incubation, the gels were stained with Coomassie brilliant blue staining, following standard procedures, and imaged in a Chemidoc MP Imaging System (BioRad).

Analysis of MMP levels in cell culture supernatants

The levels of MMP-2, MMP-3, MMP-8, pro-MMP-9, and MMP-12 in mPSIEC culture supernatants (25 μL) were analysed on a Luminex xMAP platform using a 5-plex mouse MMP magnetic bead panel (Millipore) and following the manufacturer's instructions. Multiplex assays were analysed on a Liquichip Luminex 100 Liquid Array Multiplexing System XYP Lab (Luminex Corporation, Qiagen) with Luminex Xponent 3.1 software (Luminex Corporation), following the manufacturer's technical guidelines. Data analysis was performed with the Milliplex Analyst 5.1 Software (Merck). All the reactions were performed in technical duplicate. The experiment and analysis were conducted at the Flow Cytometry Facility of the National Centre for Biotechnology (Spanish National Research Council; CNB-CSIC, Madrid, Spain).

Proteomic analysis of mPSIEC whole-cell lysates

Sample preparation. Protein concentrations were determined using the Macherey-Nagel Protein Quantification Assay (Macherey-Nagel) and equal amounts of protein were reduced with 2 mM dithiothreitol at 60 ºC for 20 min, and alkylated with 5 mM iodoacetamide dilutes in 50 mM ammonium bicarbonate at room temperature for 30 min. Next, proteins were purified using the SP3 protocol (31,32) to eliminate detergents prior to in-solution protein digestion. Purified proteins were digested overnight at 37 ºC with 100 ng of sequencing-grade trypsin (Promega) diluted in 50 mM ammonium bicarbonate and the reaction was stopped by addition of 0.1% trifluoroacetic acid.

LC-MS/MS analysis. Liquid chromatography (LC) was performed by loading 200 ng of digested peptides (diluted in 20 µL of 0.1% formic acid) onto an Evotip Pure tip (EvoSep) following the manufacturer's instructions, followed by separation using the Evosep One system on an analytical column Performance 15 cm × 150 µm, 1.5 µm (Evosep). The eluted peptides were ionized in a captive Spray with 1700 V at 200 ºC and tandem mass spectrometry analysis (MS/MS) was performed in a TimsTOF fleX mass spectrometer (Bruker) in data-independent acquisition Parallel Accumulation-Serial Fragmentation (diaPASEF) mode.

Data processing and protein quantification. The PASER system (Bruker) was used to send the raw data for subsequent analysis and quantification with DIA-NN v1.8 software (https://github.com/vdemichev/DiaNN). First, an *in silico*-predicted spectral library was built from the Uniprot *Mus musculus* database using DIA-NN v1.8, using QuantUMS (high precision) as the quantification strategy. After raw data normalisation and quantification, proteins were filtered at 1% false discovery rate (FDR) to ensure confidence in protein identification. The proteomic analysis was carried out at the Proteomics Unit of the Central Support Service for Experimental Research (SCSIE) of the University of Valencia (Spain), a member of the Instituto de Salud Carlos III (ISCIII) ProteoRed Proteomics Platform.

Statistical analysis. Peak intensities of all the conditions were transformed to the logarithm with base e using the log1p function in R. Next, a stringent statistical pipeline employing three distinct methodologies was applied to identify differentially expressed proteins (DEPs) with high robustness, as previously described (33). First, the glmnet package in R was employed to fit an elastic-net regularised regression model to select those variables (proteins) that better explain each experimental condition (34) (**Supp.**

Figure 1). To refine the model, the nearZeroVar function from the caret package in R was applied to identify variables with near-zero variance, indicating very low variability across observations. These proteins were discarded from subsequent analysis. The values of the regularisation parameters (alpha and lambda) were optimised with the train function of the caret package through cross-validation re-sampling to obtain the regularised model that best fit our data. The log-transformed expression values of the selected proteins were standardised through Z-score normalisation to ensure direct comparability of protein expression levels across samples, followed by representation in heatmaps. Second, feature selection by elastic-net was validated through Partial Least Squares Discriminant Analysis (PLS-DA) using the mixOmics package in R (35) (**Supp. Figure 2**). This package contains the vip function, which estimates the importance of each variable in the projection. When vip>1.5, the influence of the variable on the response is very high (33). Third, differential expression analysis between selected pairs of experimental conditions was performed using the limma package in R to get fold-change (FC) differences in protein expression along with the corresponding p-values (36). Only proteins that were selected by both elastic-net and PLS-DA (vip>1.5) and exhibited a significant fold-change (p-value≤0.05), as calculated by limma, were considered differentially expressed. This analysis was performed by the Statistics and Omics Data Analysis Unit of the Central Support Service for Experimental Research (SCSIE) of the University of Valencia, Spain.

Analysis of interaction effects. To identify proteins whose differential expression was specifically driven by the interaction of FhNEJ and PLG, rather than by either factor alone, an interaction effect analysis was conducted on the list of DEPs identified using the abovementioned statistical pipeline in the Untreated vs. FhNEJ+PLG comparison. To ensure that only proteins influenced by the interaction between FhNEJ and PLG were retained, proteins that were also differentially expressed in either the Untreated vs. FhNEJ or Untreated vs. PLG comparisons were excluded unless their expression in the combined treatment deviated significantly from the expected additive effect. This approach is conceptually similar to established methodologies in gene interaction studies aimed at detecting synergistic genetic perturbations, where interaction effects are assessed by comparing the observed combined effects to the expected additive effects of individual perturbations (37,38). To this end, an expected additive FC was computed as the sum of the individual effects of FhNEJ and PLG, based on FC values obtained from the Untreated vs. FhNEJ and Untreated vs. PLG comparisons. An interaction score was then calculated as the difference between the observed FC in the Untreated vs. FhNEJ+PLG comparison and the expected additive FC. Proteins common to all three

comparisons were considered to be differentially expressed as a result of the interaction between FhNEJ and PLG only if their absolute interaction score exceeded a threshold. To avoid arbitrary thresholding, interaction scores were filtered using the 1.5 × IQR rule, where IQR is the interquartile range, a standard method for outlier detection based on Tukey's definition of extreme values (39). Therefore, the interaction threshold was defined as: median interaction score + (1.5 × IQR); where the median interaction score is the median of all the interaction scores calculated across proteins in the Untreated vs. FhNEJ+PLG condition. This analysis ensured that only proteins exhibiting substantial interaction effects—whether synergistic or antagonistic—were retained for further investigation. In addition to this analysis, a complementary approach was performed to identify proteins whose differential expression in the FhNEJ+PLG condition, when compared to FhNEJ alone, could not be explained solely by the effect of PLG. In this case, proteins were filtered based on their FC values in the Untreated vs. PLG comparison. A threshold was defined as the median absolute FC ± (1.5 × IQR) in this comparison, capturing the typical range of PLG-induced expression changes. Only proteins in the FhNEJ vs. FhNEJ+PLG comparison with an absolute FC exceeding this threshold were retained.

Animal ethics protocol

All animals received human care in accordance with the guidelines for the protection of animals used for scientific purposes (Directive 2010/63EU, Decision 2012/707/UE, and RD 53/2013). All procedures were approved by the Ethical Animal Experimentation Committee of the University of Córdoba and by the Junta de Andalucía (project nr. 2021P|/22).

In vivo model

20 wild-type, 49–55-day-old female C57BL/6 mice (Charles River Laboratories) were divided in two groups: one group (n=10) was injected intraperitoneally with mouse PLG activator inhibitor 1 (PAI-1) recombinant stable mutant (50 μg per mouse; Innovative Research) to inhibit the fibrinolytic route, as previously described (40); and the control group (n=10) was injected intraperitoneally with sterile phosphate-buffered saline (PBS). All mice were challenged with *F. hepatica* metacercariae 30 min after injection, following a protocol recently described by our lab (29). First, mice were orally infected with 125 *F. hepatica* metacercariae as follows: metacercariae were resuspended in a total volume of 50 μL of PBS and oral infection was performed using a sterile 20–200 μL pipette tip

trimmed 0.5 cm and pre-washed with PBS containing 0.01% Triton X-100 to prevent the metacercariae from sticking to the plastic tip. Second, 8 days after challenge with *F. hepatica* metacercariae, mice were euthanised after 24 h fasting through CO_2 overdose followed by cervical dislocation. Prior to dissection, 2 mL of sterile PBS were injected into the peritoneal cavity, taking care to avoid puncturing any organs. Peritoneal fluid was collected by making a small abdominal incision, followed by careful aspiration with a pipette tip. The liquid was transferred to 1.5 mL tubes and immediately frozen at -80 ºC. For the recovery of juvenile worms from the hepatic parenchyma, livers were carefully dissected with tweezers under a stereomicroscope and the number of juvenile parasites was manually counted by independent, trained, and experimental group-blinded researchers that were unfamiliar with the objective of the experiment. The experiment was conducted over two consecutive days, using 5 mice per group per day, with the same reagents and personnel. Animal handling was performed by qualified staff at the Experimental Animal Facility of the University of Córdoba (Spain). Mice were housed in groups of 4 per cage, with aspen wood bedding, and provided food and water *ad libitum* on a standard dry pellet diet for rodents. The experiment was conducted after 1 week of acclimatisation at 22 ± 3 ºC, 50-60% relative humidity, and a 12 h light/dark cycle.

PAI-1 and t-PA activity assays

The activities of PAI-1 and t-PA in peritoneal fluids of mice were measured using the commercially available Mouse active PAI1 ELISA Kit (Innovative Research) and Mouse Active tPA ELISA Kit (Innovative Research), following the manufacturer's instructions. The amounts of active PAI-1 and t-PA in the samples were quantified by fitting a regression line to the linear portion of a standard curve with known active PAI-1/t-PA concentrations. All the reactions were performed in technical duplicate.

Statistical analysis

Plots were created, and statistical analyses were performed using Prism 10 software (GraphPad Software). Unless otherwise stated, graph bars represent the mean of three cell culture replicates, each calculated as the average of two or three technical replicates. Error bars represent either the standard error of the mean (SEM) or the standard deviation (SD) to reflect the precision of the sample mean as an estimate of the population mean or the variability among individual technical measurements, respectively. The data from the *in vivo* experiment was analysed using a two-way analysis of variance (ANOVA), considering both treatment groups and the day on which the experiment was

conducted as factors. Based on the result of this analysis, the data was plotted separately for each day and presented as mean ± SEM. Since normality in data distribution cannot be confidently determined with small sample sizes (41), non-parametric methods were chosen to determine statistically significant differences between experimental conditions across biological replicates. Specifically, the Mann-Whitney U test was used to assess differences between two experimental conditions; and comparisons between more than two experimental conditions were performed via Kruskal-Wallis test followed by Dunn's post-hoc analysis of pairwise comparisons. Unless otherwise stated, the threshold for significance was set at $\alpha \leq 0.1$.

Results

FhNEJ stimulate plasmin generation in the pericellular space of mouse intestinal epithelial cells

In order to study whether FhNEJ stimulate plasmin generation from host PLG in an *in vitro* system of host-parasite interactions (27,28), mPSIEC were co-incubated for 24 hours with FhNEJ in the presence of host PLG and without the exogenous addition of PLG activators. A condition were cells were co-incubated with FhNEJ, PLG, and the lysine analogue ε-ACA was included to assess whether FhNEJ-induced plasmin generation is mediated by lysine residues present in FhNEJ-derived PLG-interacting proteins, as previously described (14). Plasmin generation in cell culture supernatants of mPSIEC was assessed using a plasmin-specific chromogenic substrate, which revealed that cells incubated with PLG alone showed a small degree of plasmin generation that was increased in the presence of FhNEJ. This effect was observed across three independent cell culture replicates and was completely abrogated in the presence of ε-ACA, indicating that the observed increased plasmin generation is mediated by lysine residues (**Figure 1**).

FhNEJ increase u-PA levels in mPSIEC culture supernatants and promote collagen degradation in a PLG-dependent manner

We next addressed how mPSIEC respond to the presence of FhNEJ in terms of u-PA release and collagen degradation, and whether these responses would be modulated by the interaction between FhNEJ and PLG. The levels of u-PA in cell culture supernatants of mPSIEC left unstimulated, stimulated with FhNEJ and PLG, or simulated with either component alone were assessed through ELISA, and total collagen in these samples was

quantified by measuring the amount of N-Gly terminal peptides generated upon treatment of the samples with a collagenolytic enzyme. These experiments revealed that, in contrast to cell culture supernatants from mPSIEC single-treated with either FhNEJ or PLG, cell culture supernatants from mPSIEC stimulated with both FhNEJ and PLG exhibited a statistically significant increase in u-PA levels (**Figure 2A**) and a decrease in collagen (**Figure 2B, Supp. Figure 3A**) compared to supernatants from untreated mPSIEC. In line with this, standard zymography performed with increasing amounts of recombinant plasmin revealed that this protease degrades the ECM coating solution used to culture mPSIEC (**Supp. Figure 3B**). Altogether, these results show that the stimulation of mPSIEC with both FhNEJ and PLG results in increased u-PA release and ECM degradation in this *in vitro* cell system.

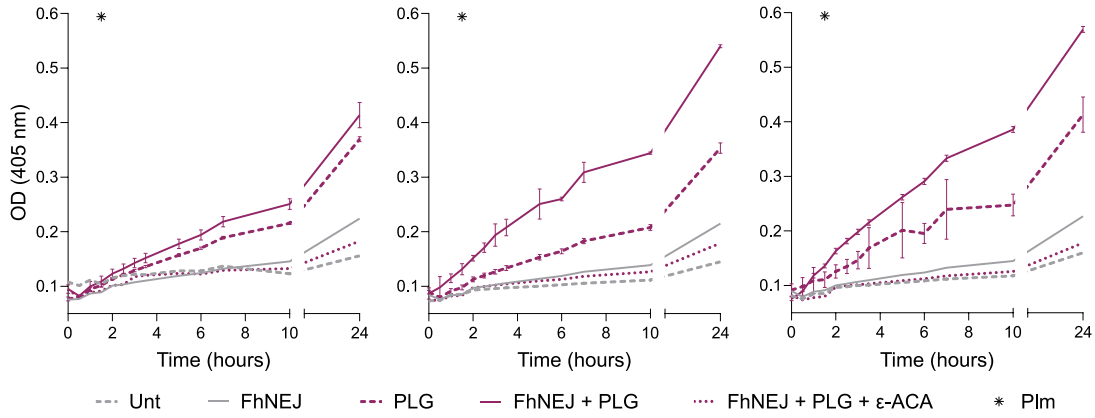

Figure 1. Mouse intestinal epithelial cells facilitate FhNEJ-induced plasmin generation from PLG. mPSIEC cultures were incubated with FhNEJ and PLG and plasmin generation on cell culture supernatants (OD at 405 nm) was assessed 24 hours after stimulation in an enzymatic assay by mixing equal amounts of cell culture supernatants with a plasmin-specific chromogenic substrate. Cells left untreated (Unt) or single-treated with either FhNEJ or PLG served as negative controls for FhNEJ-induced plasmin generation, and cells incubated with FhNEJ and PLG in the presence of ε-ACA were used to assess whether FhNEJ-induced plasmin generation is mediated by lysine residues. A condition was included where the plasmin-specific chromogenic substrate was incubated with recombinant plasmin (Plm) instead of cell culture supernatant to control for substrate specificity. Each graph represents one of three independent cell culture replicates. Lines indicate the mean of three technical replicates ± SD. Error is displayed only for the PLG-only and FhNEJ+PLG conditions for simplification purposes.

Regulation of MMP levels in mPSIEC culture supernatants by FhNEJ

We went on to evaluate whether FhNEJ influence the secretion of different ECM-degrading MMPs by mPSIEC, and whether this would be influenced by PLG. To that end, the levels of MMP-2, -3, -8, -12, and pro-MMP-9 were analysed through multiplexed assays in cell culture supernatants of mPSIEC left unstimulated, stimulated with FhNEJ and PLG, or stimulated with either component alone (**Figure 3**). This experiment revealed

that MMP-2 and pro-MMP-9 are the MMPs showing greater concentrations in the cell culture supernatants of mPSIEC. Specifically, the levels of MMP-2 were similar across the different experimental conditions, whereas those of pro-MMP-9 were only detectable in the conditions incubated with FhNEJ (FhNEJ-only and FhNEJ+PLG). Similar results were obtained for MMP-3, -8, and -12, despite their levels in mPSIEC culture supernatants being much lower than those observed for MMP-2 and pro-MMP-9. Overall, although some experimental conditions could not be quantified due to values falling below the detection limits of the technique, these results indicate that MMP levels in cell culture supernatants substantially increase upon incubation of mPSIEC with FhNEJ, regardless of PLG availability.

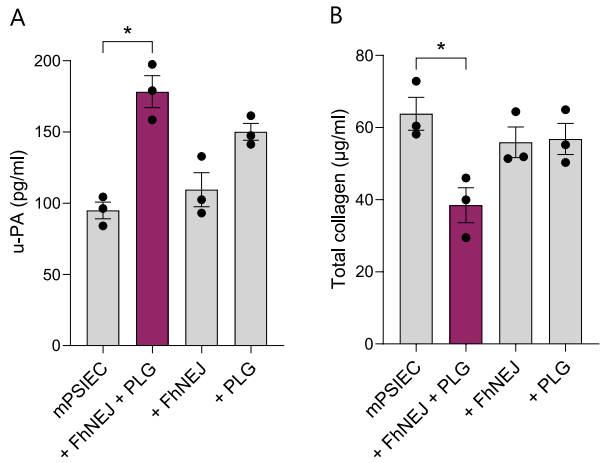

Figure 2. FhNEJ stimulate u-PA release and collagen degradation by mPSIEC in the presence of PLG. mPSIEC cultures were stimulated with FhNEJ and PLG and the levels of u-PA (A) and total collagen (B) were analysed in cell culture supernatants 24 hours after stimulation by ELISA (A) or by measuring the amount of N-Gly terminal peptides generated after enzymatic digestion with a collagenolytic enzyme (B). In both panels, cells left untreated or single-treated with either FhNEJ or PLG serve as negative controls. Data points represent the value obtained for each independent cell culture replicate, calculated as the average of two technical replicates, and bars represent the mean ± SEM. Only statistically significant differences are shown (*p≤0.1; Kruskal-Wallis test followed by Dunn's post-hoc test for pairwise comparisons).

Proteomic profiling reveals mPSIEC responses driven by the interaction between FhNEJ and host PLG

To characterise in greater detail how mPSIEC respond to FhNEJ and whether these responses are modulated by their interaction with host PLG, a proteomic analysis was conducted in whole-cell lysates from mPSIEC left unstimulated, stimulated with FhNEJ and PLG, or stimulated with either component alone. This analysis identified a total of 8136 proteins with FDR > 1% that were included in the differential expression analysis. We compared the protein expression profiles of untreated cells to cells stimulated with

both FhNEJ and PLG to identify proteins that are differentially regulated by the interaction between both factors, which results in increased plasmin activity in cell culture supernatants (**Figure 1**). In order to keep only proteins regulated by the combination of FhNEJ and PLG, and not by either component alone, DEPs identified in both the untreated vs. FhNEJ and the untreated vs. PLG comparisons that overlapped with DEPs identified in the untreated vs. FhNEJ+PLG comparison were not considered as differentially regulated by FhNEJ+PLG unless their FC values showed an interaction effect. As a result of this interaction analysis, we identified a total of 84 DEPs in mPSIEC treated with FhNEJ and PLG that are specifically attributable to the combined effect of both components (**Table 1**).

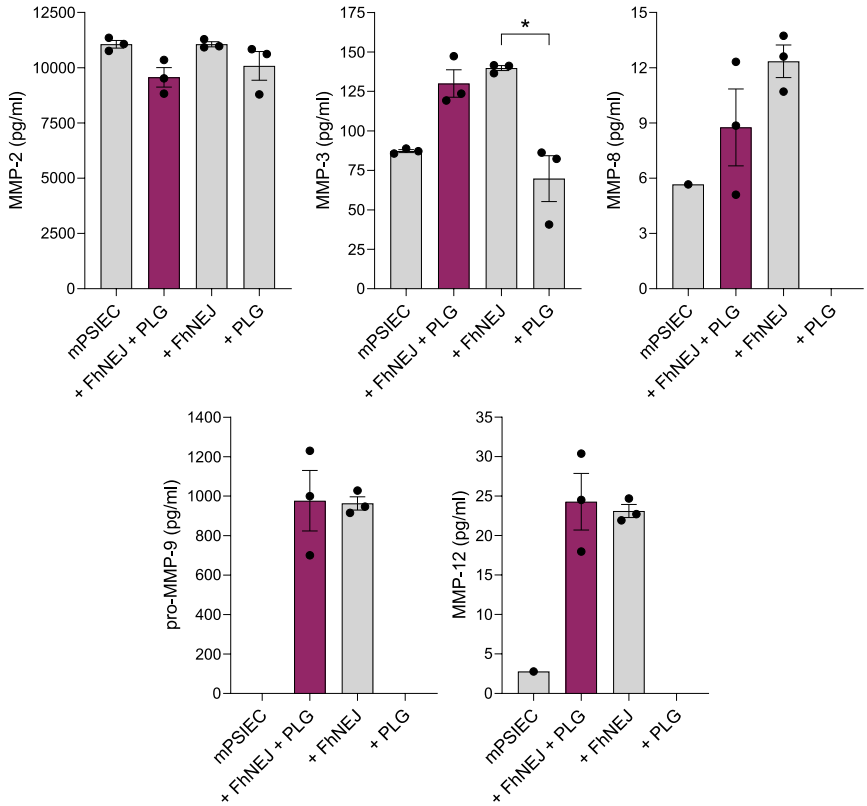

Figure 3. Increased secretion of MMP-3, MMP-8, MMP-12, and pro-MMP-9 by mPSIEC upon stimulation with FhNEJ. mPSIEC cultures were stimulated with FhNEJ and PLG, with either component alone, or left unstimulated, and the levels of MMP-2, -3, -8, -12, and pro-MMP-9 were simultaneously assayed using multiplexed assays. In all graphs, data points represent the value obtained for each independent cell culture replicate, calculated as the average of two technical replicates, and bars represent the mean ± SEM. Only statistically significant differences are shown (*p≤0.1; Kruskal-Wallis test followed by Dunn's post-hoc test for pairwise comparisons). Note that statistical analysis could not be performed for MMP-8, MMP-12, and pro-MMP-9 due to some MMP levels being below the detection limit of the technique (PLG-only in MMP-8 analysis; PLG-only in MMP-12 analysis; and Untreated and PLG-only in pro-MMP-9 analysis).

In addition to proteins shown in **Table 1**, we also considered as differentially regulated as a result of the interaction between FhNEJ and PLG those DEPs identified by comparing mPSIEC stimulated with FhNEJ+PLG to cells stimulated with only FhNEJ. Following a similar rationale as described above, we excluded proteins regulated by PLG alone by discarding those DEPs in the FhNEJ vs. FhNEJ+PLG comparison that were common to those identified in the untreated vs. PLG comparison unless their fold change under the FhNEJ+PLG condition exceeded the typical range of PLG-induced expression changes. By doing so, we identified a total of 15 proteins that were differentially regulated in mPSIEC as a result of the stimulation with both FhNEJ and PLG (**Table 2**). Notably, this analysis identified PLG (Plg) as significantly upregulated in FhNEJ+PLG-treated cells compared to cells treated with FhNEJ alone, highlighting the accuracy of the approach.

Table 1. DEPs in untreated vs. FhNEJ+PLG-treated mPSIEC. A negative fold-change indicates that proteins were overexpressed in FhNEJ+PLG-treated vs. untreated mPSIEC. The opposite is true when the fold-change is positive.

Genes	logFC	adjPVal	Genes	logFC	adjPVal
Cap1	-11.5641	1.16E-08	**Mrgbp**	0.315304	0.041192
Eif1ad11	-10.8284	0.044884	**Sgo2**	0.321909	0.044884
Zfp606	-9.98762	1.16E-08	**Tmem184b**	0.337123	0.042419
Serpinb5	-9.09	1.29E-05	**Cdca2**	0.337796	0.044267
A130006I12Rik	-8.8194	6.9E-06	**Aurka**	0.337847	0.037707
S1pr2	-8.79117	6.89E-05	**Glul**	0.353169	0.029492
Pptc7	-7.5837	3.26E-07	**Blm**	0.365228	0.032615
Tmem14c	-0.70435	0.044884	**Actn4**	0.36718	0.046035
Rab3b	-0.67455	0.031743	**Rab38**	0.367389	0.026216
Mmp11	-0.67003	0.046035	**Aurkb**	0.370126	0.047626
Dennd3	-0.4902	0.004343	**Sash1**	0.377697	0.044267
Desi2	-0.44875	0.044884	**Rpl35**	0.398069	0.012174
Dhx38	-0.44049	0.035042	**Acsl4**	0.398548	0.007476
Srsf2	-0.43185	0.041835	**Cox6a1**	0.405347	0.028322
Gorasp1	-0.38835	0.011505	**Esco2**	0.419492	0.018064
Ccn2	-0.37626	0.046035	**0610010K14Rik**	0.476796	0.044884
Atrx	-0.32326	0.04661	**Arhgap20**	0.48969	0.044267
Slc25a30	-0.31699	0.046035	**Cmip**	0.490543	0.046035
P4ha2	-0.26881	0.044267	**Aven**	0.490844	0.041192
Slc12a7	-0.2512	0.026216	**Gmnn**	0.495586	0.047626
Scel	-0.24909	0.044267	**Cdc34**	0.522914	0.040503
Gpam	-0.23204	0.046035	**Suco**	0.539981	0.018465
Sppl2a	0.191843	0.042419	**Cetn2**	0.553036	0.040283
Ska3	0.199186	0.0426	**Nifk**	0.619508	0.046035
Lama5	0.223652	0.040283	**Fnip1**	0.677284	0.006195
Slc9a3r2	0.224492	0.046404	**Lrig1**	0.745655	0.00283
Dvl2	0.239475	0.046035	**Sema3a**	0.831001	0.031743
Cdv3	0.244815	0.035681	**Serpind1**	0.858629	0.032615
Notch2	0.247477	0.022438	**Fn1**	0.888985	0.031603

Genes	logFC	adjPVal	Genes	logFC	adjPVal
Lamb1	0.250854	0.018889	**Zfhx4**	1.130508	0.010738
Prkd2	0.254831	0.048562	**Thbs1**	1.239887	0.035681
Diaph3	0.255139	0.018889	**Serpinb7**	1.278409	0.003469
Rnf4	0.25624	0.044267	**A2m**	1.423682	0.016865
Prr11	0.265523	0.04991	**Itih4**	1.425836	0.026216
Spdl1	0.271143	0.031743	**Tmc5**	1.469909	0.002405
Melk	0.27314	0.048562	**C7**	1.471351	0.003517
Calu	0.28254	0.022262	**Dnah14**	1.608509	0.026216
Fam50a	0.286497	0.011574	**Mettl25**	7.646163	4.28E-05
Mphosph10	0.292922	0.028712	**Pigr**	8.709757	4.38E-06
Msmo1	0.302965	0.031743	**Sema3d**	10.34484	3.39E-07
Ror1	0.311029	0.021179	**Lamtor3-ps**	12.05803	0.00283
Tns2	0.313294	0.017259	**Alb**	12.61229	2.51E-07

By combining both analyses, we identified 94 unique DEPs in double-treated mPSIEC, which are involved in cell adhesion and migration, ECM remodelling, mucosal immunity, and fibrinolysis. These included cyclase-associated protein 1 (Cap1), serpin b5, the Ras-related protein Rab3b, actinin-4 (Actn4), MMP-11 (Mmp11), the tissue inhibitor of metalloproteinase 3 (Timp3), fibronectin 1 (Fn1), quiescin sulfhydryl oxidase 1 (Qsox1), semaphorin 3D (Sema3d), the polymeric immunoglobulin receptor (Pigr), carbohydrate sulfotransferase 2 (Chst2), the complement molecule C7, the sphingosine 1-phosphate receptor 2 (S1pr2), alpha-2 macroglobulin (A2m), and carboxipeptidase 2 (Cpb2), also known as the thrombin activatable fibrinolysis inhibitor (TAFI).

Table 2. DEPs in FhNEJ-treated vs. FhNEJ+PLG-treated mPSIEC. A negative fold-change indicates that proteins were overexpressed in FhNEJ+PLG-treated vs. FhNEJ-treated mPSIEC. The opposite is true when the fold-change is positive.

Genes	logFC	adjPVal
Cap1	-11.5928	2.45E-08
Cpb2	-8.97008	9.76E-07
Ube2l6	-8.30553	9.76E-07
Qsox1	-9.00044	9.76E-07
Sema3d	10.18779	9.76E-07
Klhl30	10.2297	4.84E-06
Chst2	7.69985	5.86E-06
Timp3	-0.59491	0.004089
Rab3b	-0.50349	0.010792
Plg	-1.34697	0.014915
Dennd3	-0.42075	0.022473
Phip	-0.55656	0.025486
Ccn1	-0.45733	0.043756
Scel	-0.36049	0.043756
Slco2a1	-0.33878	0.044832

The contribution of the fibrinolytic system to FhNEJ migration in a mouse model of fasciolosis

Finally, in an effort to understand the contribution of the host fibrinolytic system in the migration of *F. hepatica* juveniles during the earliest stages of infection in a physiologically-relevant experimental system, we employed a mouse model of acute fasciolosis (29) to quantify the amount of juvenile flukes that successfully migrate to the livers of control mice and mice whose fibrinolytic route had been pharmacologically inhibited prior to challenge with *F. hepatica* metacercariae (**Figure 4A**). Due to technical constraints, the experiment was performed across two consecutive days, employing five mice per group per day. Statistical analysis using two-way ANOVA revealed that the day of the experiment gave statistically significant differences in FhNEJ counts (**Supp. Table 2**), as PAI-1-treated mice from day 1—but not control mice—exhibited significantly different amounts of liver-migrating flukes than their counterparts from day 2 (**Supp. Figure 4**). Therefore, PAI-1-treated mice could not be treated as absolute replicates and data was subsequently plotted and analysed separately for each day. This analysis revealed that PAI-1 treatment prior to *F. hepatica* infection resulted in a significant decrease in the number of juvenile flukes recovered from the livers of infected mice eight days after challenge with *F. hepatica* metacercariae, but only in the experiment conducted on day 1 (**Figure 4B**). To assess potential differences in PAI-1 treatment efficacy across different experimental days, we measured the levels of fibrinolysis in the peritoneal fluids of all mice, which were collected immediately after euthanasia. However, no statistically significant differences were observed in either PAI-1 or t-PA activities between control and PAI-1-treated mice on any experimental day (**Supp. Figure 5**).

Discussion

Aiming to understand the contribution of the fibrinolytic system in biological processes that might facilitate FhNEJ migration, commercially-available primary intestinal epithelial cells derived from the mouse small intestine (mPSIEC) were stimulated with FhNEJ (27,28) and PLG for 24 hours, after which cell culture supernatants and mPSIEC whole-cell lysates were harvested for downstream analyses. Additionally, mPSIEC left unstimulated or stimulated with either FhNEJ or PLG alone served as negative controls, and an additional subset of mPSIEC stimulated with FhNEJ, PLG, and the lysine analogue ε-ACA was used to assess whether plasmin generation depends on the presence

of lysine residues in FhNEJ-derived PLG-interacting proteins, as previously described (14). Although FhNEJ take only a few hours to cross the intestinal wall (19), we extended the stimulation period to up to 24 hours, as previously described (28), to account for potential delays in cellular responses due to the artificial and reductionist nature of the co-culture system.

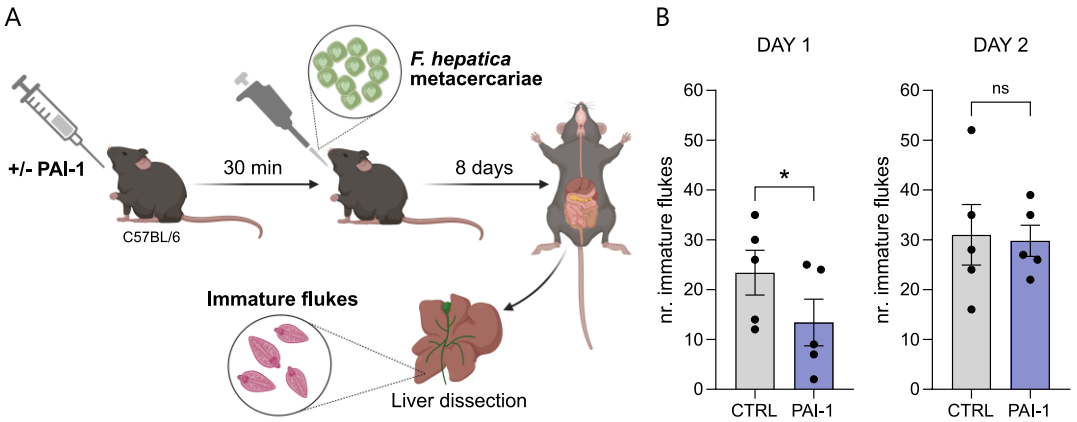

Figure 4. Study of the influence of the fibrinolytic system in FhNEJ migration in a mouse model of fasciolosis. A) Schematic representation of the experimental design. Thirty minutes prior to infection with 125 *F. hepatica* metacercariae, mice were injected with either PBS (control) or a recombinant, stable-mutant version of PAI-1 to inhibit the fibrinolytic route, as previously described (40). Eight days after challenge with *F. hepatica* metacercariae, mice were euthanised and the number of juvenile flukes in the livers were manually counted under a stereomicroscope. B) Counts of juvenile flukes recovered from the livers of PAI-1-treated and control mice eight days after challenge with *F. hepatica* metacercariae. Data points represent the number of juveniles recovered in the liver of each mouse, and bars indicate the mean ± SEM. Asterisks indicate significant differences between experimental conditions (ns, not significant; *p≤0.1; Mann-Whitney U test). Panel A created with Biorender.com.

First, our enzymatic assays revealed that cell culture supernatants from mPSIEC stimulated with both FhNEJ and PLG showed increased plasmin activity compared to those from control or single-treated cells, indicating that FhNEJ stimulate plasmin generation in the pericellular space of the mouse small intestine in the presence of host PLG. Since FhNEJ cannot directly cleave and activate PLG (14), and mPSIEC were stimulated in the absence of exogenous addition of either t-PA or u-PA, these results suggest that mPSIEC express the PLG activator(s) required to convert PLG into plasmin, which might have important physiological and pathological implications. Notably, FhNEJ-induced plasmin generation was completely abrogated in the presence of ε-ACA, indicating that this phenomenon depends on the interaction between lysine residues present in PLG-interacting proteins expressed by FhNEJ and the PLG kringle domains, replicating the mechanism of PLG activation utilised by physiologic PLG-Rs.

Since u-PA is expressed by epithelial cells of the small intestine, presumably to loosen intercellular junctions and facilitate epithelial cell turnover at the top of the villi (42), we studied whether the combination of FhNEJ and PLG would increase the availability of this PLG activator in the mPSIEC extracellular space. Results revealed that cell culture supernatants of mPSIEC stimulated with both FhNEJ and PLG contained significantly more u-PA than untreated or single-treated cells, suggesting that plasmin generation in the pericellular space may initiate a positive feedback loop that enhances u-PA release by double-treated cells. These findings indicate that plasmin may enhance fibrinolysis within the small intestinal micro-environment through two complementary mechanisms: the well-established ability of this protease to cleave and activate the zymogen form of u-PA (43,44) and the stimulation of u-PA secretion by intestinal cells, as demonstrated in the present study.

Plasmin is considered a master regulator of cell migration through the degradation of different ECM components (5,6), including collagen type IV and laminin (11,45), two major structural proteins of the basement membrane underlying the intestinal epithelium (25). To determine whether FhNEJ-mediated plasmin generation enhances the degradation of intestinal ECM components, we quantified the levels of total collagen in cell culture supernatants of mPSIEC incubated with FhNEJ, PLG, or both. Decreased collagen levels in cell culture supernatants was interpreted as increased degradation of this ECM protein, as previously described (46). Our results revealed that collagen levels slightly decrease in mPSIEC supernatants treated with either FhNEJ or PLG, but the reduction becomes significant only when both are combined, indicating that FhNEJ-induced plasmin generation promotes collagen degradation. This notion is supported by the observation that plasmin is capable of degrading the ECM coating solution employed for mPSIEC culture. Although the interaction between helminth parasites and the host fibrinolytic system is well-established and has long been proposed to facilitate tissue migration through enhanced ECM degradation (1–3), to our knowledge, this is the first experimental demonstration of increased ECM degradation resulting from parasite-induced plasmin generation in a trematode, as this phenomenon had only been shown in the nematode *Dirofilaria immitis* (46). Considering the potent collagenolytic properties of FhCL3 (47,48), which is highly expressed and secreted by FhNEJ (49), the residual, non-statistically significant collagen degradation that we detected in cell culture supernatants from mPSIEC only treated with FhNEJ is somewhat unexpected. However, this could be explained by the fact that the commercial kit that we used in our experiments fails to detect the specific collagen isoforms that are degraded by FhCL3, that such isoforms are

not present in the ECM coating employed here to culture mPSIEC, or that the extent of collagen degradation by FhNEJ under physiologic conditions is less pronounced than that observed in *in vitro* experiments performed in the absence of host cells (47,48).

In addition to directly degrading ECM components, plasmin activates the precursor zymogens of MMP-1, -2, -3, -9, -10, -12, and -13, either via direct cleavage or by activating other MMPs that, in turn, cleave and activate the MMP precursor (50–57). MMPs are highly proficient at degrading ECM components (12), and given their close relationship with the fibrinolytic system (5), we addressed whether FhNEJ-mediated plasmin generation would simulate MMP release by mPSIEC (58), which could facilitate FhNEJ trans-intestinal migration by expanding the arsenal of enzymes that are available in the extracellular space for ECM degradation. Our results showed that FhNEJ substantially stimulate the secretion of MMP-3, -8, -12, and pro-MMP-9 by mPSIEC, a response that was not further stimulated in the presence of PLG, indicating that FhNEJ-induced plasmin generation does not affect the secretion of these enzymes by host cells. Since MMP-like enzymes in the FhNEJ secretome constitute only 0.04% of the total protein content (59), we believe that their contribution to the measured MMP levels in cell culture supernatants is likely negligible. Regardless of MMP secretion, it remains to be addressed whether FhNEJ-induced plasmin generation in the intestinal epithelium facilitates the activation of MMP precursors that could contribute to the observed collagen degradation and ultimately facilitate the migration of these parasites across the host intestine.

Since plasmin influences biological processes not only by regulating the activity of proteolytic enzymes but also by modulating intracellular signalling pathways that culminate in changes in gene expression (60–63), we conducted a proteomic analysis on whole-cell lysates of mPSIEC treated with both FhNEJ and PLG to investigate how these cells respond to elevated plasmin levels in the pericellular space. To this end, we applied a stringent statistical pipeline (33) combined with an analysis of interaction effects to identify proteins with statistically different abundances in FhNEJ+PLG-treated mPSIEC compared to untreated cells that are specific to the combination of FhNEJ and PLG. This analysis included elastic-net penalised regression, which has emerged as a powerful and validated approach for robust feature selection, enabling the reliable identification of condition-specific gene and protein expression signatures in diverse biomedical fields (64–67). Additionally, differential expression was performed with the limma package, which applies a combination of statistical principles that outperform ordinary *t*-tests in proteomics data analysis when sample sizes are small (36,68). In parallel

to this, we also compared the proteomes of FhNEJ-treated and FhNEJ+PLG-treated mPSIEC to reduce the overall variability and enhance the detection of subtle but biologically meaningful proteomic changes specifically induced by the combined action of FhNEJ and PLG that may be overlooked in the broader untreated vs. FhNEJ+PLG-treated contrast. Supporting the accuracy of this approach, only the comparison between FhNEJ-treated and FhNEJ+PLG-treated cells captured PLG as a significantly abundant protein in whole-cell lysates of double-treated mPSIEC. Given that mPSIEC were thoroughly washed before harvesting, we assume that the amount of residual PLG in these samples was negligible, reinforcing that comparing the protein expression profiles of FhNEJ-treated and FhNEJ+PLG-treated cells effectively fine-tunes the analysis to detect subtle proteomic shifts that are undetectable in the untreated vs. FhNEJ+PLG differential expression analysis.

Some of the DEPs resulting from the interaction between FhNEJ and PLG are involved in biological processes that could be beneficial to FhNEJ trans-intestinal migration and survival within the mammalian organism, including cell adhesion and migration, cytoskeleton dynamics, ECM remodelling, mucosal immunity, and fibrinolysis. For instance, we identified Cap1, serpin b5, Rab3b, and Actn4 as differentially regulated upon mPSIEC stimulation with both FhNEJ and PLG. Cap1 is involved in cell adhesion and motility through the regulation of actin cytoskeleton dynamics (69), serpin b5 is a non-inhibitory serpin that regulates u-PA/u-PAR-mediated cell adhesion (70), and Rab3b is required for the distribution of the major tight junction (TJ) adaptor protein zonula occludens 1 to the apical membrane of epithelial cells (71,72). Similarly, depletion of Actn4 inhibits the recruitment of occludin to TJs, thereby disrupting the formation of functional intercellular junctions (73). Differential expression of these proteins in mPSIEC stimulated with both FhNEJ and PLG indicates that plasmin might regulate parasite adhesion to the intestinal epithelium and modulate epithelial junction dynamics, potentially compromising epithelial barrier integrity and facilitating FhNEJ migration across the host intestine.

Additionally, some of the DEPs identified in mPSIEC incubated with both FhNEJ and PLG play important roles in ECM remodelling. Among others, we found differential expression of Mmp11 and Timp3 in double-treated cells, suggesting that FhNEJ-induced plasmin generation alters the protease/anti-protease balance of the MMP system to ultimately regulate ECM degradation. Overexpression of Mmp-11 is particularly compelling as this metalloproteinase degrades collagen type IV (74), a major structural component of the intestinal basement membrane (25). In the context of ECM

remodelling, it is noteworthy that we also detected downregulation of the ECM protein fibronectin in whole-cell lysates of mPSIEC incubated with both FhNEJ and PLG. Decreased Fn1 expression in double-treated mPSIEC aligns with Mmp-11 overexpression in these cells, as MMP-11 was shown to supress fibronectin gene expression in an *in vivo* zebrafish model of ECM remodelling (75). Moreover, the overexpression of Qsox1, which is required for the incorporation of laminin into the ECM (76), provides further evidence that FhNEJ regulate tissue integrity through the modulation of ECM dynamics/deposition in a plasmin(ogen)-dependent manner.

Another notable functional aspect of the interaction between FhNEJ and PLG, highlighted by our proteomic analysis, is the identification of proteins linked to host immune responses and, potentially, to FhNEJ immune evasion mechanisms. Among others, we found downregulation of Sema3d, Pigr, Chst2, and the complement molecule C7 in mPSIEC treated with both FhNEJ and PLG. Sema3d has been recently shown to promote pancreatic cancer progression by stimulating macrophage polarisation into a protumourigenic M2 phenotype (77). Since M2 polarization is a hallmark of anti-*Fasciola* immune responses (78), this finding offers novel insights into how these responses might be regulated at the intestinal level during infection with FhNEJ. In the context of FhNEJ trans-intestinal migration, the downregulation of Pigr in mPSIEC upon stimulation with FhNEJ and PLG is particularly noteworthy, as this protein is a key regulator of mucosal immunity by facilitating the translocation of immunoglobulin A (IgA) to the apical compartment of the intestinal epithelium (79). In fact, mice lacking Pigr expression show increased bacterial infiltration into the liver due to impaired IgA-mediated antimicrobial defence in the gut (80). In line with this, the downregulation of Chst2 upon stimulation with FhNEJ and PLG may also represent a mechanism to evade mucosal immunity, as this protein is involved in lymphocyte homing to peripheral lymphoid organs and presumably to inflamed sites by modulating the synthesis of carbohydrate ligands that interact with L-selectin molecules expressed on the lymphocyte surface (81,82).

The complement molecule C7 is essential for the assembly of the lethal, pore-forming membrane attack complex, a key effector in antibacterial defence. Previous studies had shown that *F. hepatica* expresses molecules that inhibit complement proteases, thereby evading complement-mediated responses (83). To the best of our knowledge, the present study provides the first evidence that FhNEJ modulate the expression of C7 in mouse intestinal cells, an effect observed only upon co-stimulation with both FhNEJ and PLG. Intriguingly, we also observed a downregulation of the complement protein C3 in FhNEJ-treated mPSIEC compared to untreated controls (**Supp. Table 1**), suggesting that

FhNEJ modulate the expression of complement molecules through plasmin(ogen)-dependent and -independent mechanisms. The S1pr2 is another immune-related protein that we identified as differentially regulated in double-treated mPSIEC. This protein regulates the onset of type 2 immune responses in the lung by inhibiting the secretion of interleukin 33 (IL-33) by lung macrophages (84). IL-33 is also secreted by intestinal epithelial cells and has recently been shown to represent an indispensable trigger of intestinal type 2 immune responses that lead to successful gastrointestinal nematode expulsion (85). Based on this, the observed S1pr2 overexpression in mPSIEC upon stimulation with both FhNEJ and PLG may serve to reduce IL-33 release by intestinal epithelial cells and suppress the onset of anti-helminth immunity in the intestine, providing an intriguing direction for future investigation.

Moreover, our proteomic analysis indicated that plasmin itself might also be regulating fibrinolysis in the small intestine tissue microenvironment, as double-treated mPSIEC differentially regulate the expression of two major fibrinolysis inhibitors: A2m and TAFI, which were downregulated and overexpressed, respectively, in mPSIEC upon stimulation with FhNEJ and PLG. This finding suggests that fine-tuning fibrinolysis may benefit the parasite by balancing proteolytic activity such that it facilitates tissue penetration while preventing excessive ECM degradation, which could inadvertently lead to severe tissue damage and exacerbated inflammatory responses in the intestine.

Finally, we employed a mouse model of acute fasciolosis (29) to conclusively determine whether the fibrinolytic system is involved in the intra-mammalian migration of *F. hepatica* juveniles during early infection. To that end, mice were injected with either PBS or PAI-1 to inhibit the fibrinolytic route prior to infection with *F. hepatica* metacercariae. We utilised a recombinant and stable-mutant version of PAI-1, as previously described (40), because the wild-type inhibitor has a very short half-life of approximately two hours (86). Eight days post-infection was selected as the time point for quantifying infection success based on previous studies indicating that juvenile recovery from the livers of infected mice is highest at this time (29). Results showed that the number of liver-migrating flukes in PAI-1-treated mice was significantly lower than that observed in control mice, but only in the experiment conducted on day 1.

Since differences in juvenile counts across experimental days were observed only in PAI-1-treated mice, but not in their control counterparts, we suspected that variations in PAI-1 treatment efficacy between days might explain these results. To investigate this possibility, fibrinolysis was evaluated by measuring the levels of active PAI-1 and t-PA in

peritoneal fluids collected immediately after euthanasia. Although the differences in active PAI-1 and t-PA levels between PAI-1-treated and control mice on day 1 were more consistent with the expected inhibition of fibrinolysis than those observed in mice from day 2, these differences were small and did not reach statistical significance. This is likely due to the fact that peritoneal fluids were collected eight days after PAI-1 treatment, exceeding the maximum half-life of six days achieved for this factor through random mutagenesis (86). Altogether, these results highlight the complexity of working with genetically diverse organisms such as *F. hepatica*, which likely constitutes one of several factors contributing to the inconsistent and variable outcomes reported in vaccine trials over the past decades (86–88). This variability not only hinders data interpretation but also raises ethical concerns regarding the use of large animal cohorts to overcome inter-individual heterogeneity and meet significance thresholds that are inherently arbitrary. Notwithstanding these points, these findings provide unprecedented insights into the role of the fibrinolytic system in FhNEJ migration *in vivo*, supporting the long-standing hypothesis that FhNEJ-stimulated plasmin generation from host PLG contributes to the dissemination of *F. hepatica* juveniles through mammalian tissues.

In summary, this study demonstrates that plasmin is generated in the pericellular space of mouse intestinal epithelial cells in the presence of FhNEJ and host-derived PLG. This process enhances collagen degradation and u-PA release into the extracellular environment, potentially facilitating FhNEJ migration across the intestinal wall. Additionally, FhNEJ-induced plasmin generation triggers changes in the expression levels or abundance of proteins involved in biological processes that could be critical during the earliest stages of infection, including cell adhesion and migration, ECM remodelling, immune evasion, and fibrinolysis. Finally, despite the inherent challenges associated with *in vivo* models of *F. hepatica* infection, our findings provide the first functional evidence supporting the long-proposed role of the fibrinolytic system in facilitating the migration of *F. hepatica* juveniles through mammalian tissues. Collectively, these results underscore the contribution of the fibrinolytic system in parasite dissemination during early-stage fasciolosis, providing interesting insights into host-parasite relationships established in the initial stages of infection and highlighting potential avenues for future research in this area.

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References

1. Diosdado A, Simón F, Serrat J, González-Miguel J. Interaction of helminth parasites with the haemostatic system of their vertebrate hosts: a scoping review. Parasite. 2022;29:35.
2. González-Miguel J, Siles-Lucas M, Kartashev V, Morchón R, Simón F. Plasmin in parasitic chronic infections: friend or foe? Trends Parasitol. 2016 Apr;32(4):325–35.
3. Figuera L, Gómez-Arreaza A, Avilán L. Parasitism in optima forma: exploiting the host fibrinolytic system for invasion. Acta Trop. 2013 Oct;128(1):116–23.
4. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. Br J Haematol. 2005 May;129(3):307–21.
5. Lijnen HR. Plasmin and matrix metalloproteinases in vascular remodeling. Thromb Haemost. 2001 Jul;86(1):324–33.
6. Syrovets T, Lunov O, Simmet T. Plasmin as a proinflammatory cell activator. J Leukoc Biol. 2012 Sep;92(3):509–19.
7. Hantaï D, Festoff BW. Degradation of muscle basement membrane zone by locally generated plasmin. Exp Neurol. 1987 Jan;95(1):44–55.

8. Oudart JB, Brassart-Pasco S, Vautrin A, Sellier C, Machado C, Dupont-Deshorgue A, *et al.* Plasmin releases the anti-tumor peptide from the NC1 domain of collagen XIX. Oncotarget. 2015 Feb 28;6(6):3656–68.

9. Goldfarb RH, Murano G, Brundage R, Siegal GP, Terranova V, Garbisa S, *et al.* Degradation of glycoprotein and collagenous components of the basement membrane: studies with urokinase-type plasminogen activator, alpha-thrombin, and plasmin. Semin Thromb Hemost. 1986 Oct;12(4):335–6.

10. Richardson M, Hatton MW, Moore S. The plasma proteases, thrombin and plasmin, degrade the proteoglycan of rabbit aorta segments *in vitro*: an integrated ultrastructural and biochemical study. Clin Investig Med Med Clin Exp. 1988 Apr;11(2):139–50.

11. Nakagami Y, Abe K, Nishiyama N, Matsuki N. Laminin degradation by plasmin regulates long-term potentiation. J Neurosci. 2000 Mar 1;20(5):2003–10.

12. Lu P, Takai K, Weaver VM, Werb Z. Extracellular matrix degradation and remodeling in development and disease. Cold Spring Harb Perspect Biol. 2011 Dec 1;3(12).

13. Pala ZR, Ernest M, Sweeney B, Jeong YJ, Pascini TV, Alves E Silva TL, *et al.* Beyond cuts and scrapes: plasmin in malaria and other vector-borne diseases. Trends Parasitol. 2022 Feb 1;38(2):147–59.

14. Serrat J, Becerro-Recio D, Torres-Valle M, Simón F, Valero MA, Bargues MD, *et al.* *Fasciola hepatica* juveniles interact with the host fibrinolytic system as a potential early-stage invasion mechanism. PLoS Negl Trop Dis. 2023 Apr;17(4):e0010936.

15. Serrat J, Torres-Valle M, De Marco Verissimo C, Siles-Lucas M, González-Miguel J. Binding and cleavage of pro-urokinase by a tegument extract of *Fasciola hepatica* newly excysted juveniles activate the host fibrinolytic system. Vet Res. 2025 Jan 25;56(1):20.

16. O'Kelly E, Cwiklinski K, De Marco Verissimo C, Calvani NED, López Corrales J, Jewhurst H, *et al.* Moonlighting on the *Fasciola hepatica* tegument: enolase, a glycolytic enzyme, interacts with the extracellular matrix and fibrinolytic system of the host. PLoS Negl Trop Dis. 2024 Aug;18(8):e0012069.

17. Bernal D, de la Rubia JE, Carrasco-Abad AM, Toledo R, Mas-Coma S, Marcilla A. Identification of enolase as a plasminogen-binding protein in excretory-secretory products of *Fasciola hepatica*. FEBS Lett. 2004 Apr 9;563(1–3):203–6.

18. González-Miguel J, Valero MA, Reguera-Gomez M, Mas-Bargues C, Bargues MD, Simón F, *et al.* Numerous *Fasciola* plasminogen-binding proteins may underlie blood-

brain barrier leakage and explain neurological disorder complexity and heterogeneity in the acute and chronic phases of human fascioliasis. Parasitology. 2019 Mar;146(3):284–98.

19. Andrews SJ, Cwiklinski K, Dalton JP. Chapter 1. The discovery of *Fasciola hepatica* and its life cycle. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 1–23.
20. Mehmood K, Zhang H, Sabir AJ, Abbas RZ, Ijaz M, Durrani AZ, *et al.* A review on epidemiology, global prevalence and economical losses of fasciolosis in ruminants. Microb Pathog. 2017 Aug;109:253–62.
21. Fitzpatrick JL. Global food security: the impact of veterinary parasites and parasitologists. Vet Parasitol. 2013 Aug 1;195(3–4):233–48.
22. World Health Organization. Global report on Neglected Tropical Diseases 2023. 1st ed. Geneva: World Health Organization; 2023.
23. Pan American Health Organization (PAHO). Operational guidelines for the elimination of human fascioliasis as a public health problem in the Americas. Pan American Health Organization; 2024.
24. Serrat J, Torres-Valle M, López-García M, Becerro-Recio D, Siles-Lucas M, González-Miguel J. Molecular characterization of the interplay between *Fasciola hepatica* juveniles and laminin as a mechanism to adhere to and break through the host intestinal wall. Int J Mol Sci. 2023 May 3;24(9).
25. Pompili S, Latella G, Gaudio E, Sferra R, Vetuschi A. The charming world of the extracellular matrix: a dynamic and protective network of the intestinal wall. Front Med. 2021 Apr 16;8:610189.
26. González-Miguel J, Becerro-Recio D, Siles-Lucas M. Insights into *Fasciola hepatica* juveniles: crossing the fasciolosis rubicon. Trends Parasitol. 2021 Jan;37(1):35–47.
27. Becerro-Recio D, Serrat J, López-García M, Sotillo J, Simón F, González-Miguel J, *et al.* Proteomics coupled with *in vitro* model to study the early crosstalk occurring between newly excysted juveniles of *Fasciola hepatica* and host intestinal cells. PLoS Negl Trop Dis. 2022 Oct;16(10):e0010811.
28. González-Miguel J, Becerro-Recio D, Sotillo J, Simón F, Siles-Lucas M. Set up of an *in vitro* model to study early host-parasite interactions between newly excysted juveniles of *Fasciola hepatica* and host intestinal cells using a quantitative proteomics approach. Vet Parasitol. 2020 Feb;278:109028.

29. López-García M, Cwiklinski K, Becerro-Recio D, Ruiz-Campillo MT, Molina-Hernández V, Pérez-Arévalo J, et al. Deciphering the proteomic profile of the parasite Fasciola hepatica after initial vertebrate host infection reveals new insights into its early-stages. Mol Cell Proteomics. 2025;101005.

30. Reed KL, Fruin AB, Gower AC, Stucchi AF, Leeman SE, Becker JM. A neurokinin 1 receptor antagonist decreases postoperative peritoneal adhesion formation and increases peritoneal fibrinolytic activity. Proc Natl Acad Sci. 2004 Jun 15;101(24):9115–20.

31. Moggridge S, Sorensen PH, Morin GB, Hughes CS. Extending the compatibility of the SP3 paramagnetic bead processing approach for proteomics. J Proteome Res. 2018 Apr 6;17(4):1730–40.

32. Hughes CS, Moggridge S, Müller T, Sorensen PH, Morin GB, Krijgsveld J. Single-pot, solid-phase-enhanced sample preparation for proteomics experiments. Nat Protoc. 2019 Jan;14(1):68–85.

33. Pinheiro T, Lip KYF, García-Ríos E, Querol A, Teixeira J, van Gulik W, *et al.* Differential proteomic analysis by SWATH-MS unravels the most dominant mechanisms underlying yeast adaptation to non-optimal temperatures under anaerobic conditions. Sci Rep. 2020 Dec 18;10(1):22329.

34. Friedman J, Hastie T, Tibshirani R. Regularization paths for generalized linear models via coordinate descent. J Stat Softw. 2010;33(1):1–22.

35. Rohart F, Gautier B, Singh A, Lê Cao KA. mixOmics: an R package for 'omics feature selection and multiple data integration. PLoS Comput Biol. 2017 Nov;13(11):e1005752.

36. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015 Apr 20;43(7):e47.

37. Schrode N, Seah C, Deans PJM, Hoffman G, Brennand KJ. Analysis framework and experimental design for evaluating synergy-driving gene expression. Nat Protoc. 2021 Feb;16(2):812–40.

38. Schrode N, Ho SM, Yamamuro K, Dobbyn A, Huckins L, Matos MR, *et al.* Synergistic effects of common schizophrenia risk variants. Nat Genet. 2019 Oct;51(10):1475–85.

39. Mariella E, Grasso G, Miotto M, Buzo K, Reilly NM, Andrei P, *et al.* Transcriptome-wide gene expression outlier analysis pinpoints therapeutic vulnerabilities in colorectal cancer. Mol Oncol. 2024 Jun;18(6):1460–85.

40. Alves e Silva TL, Radtke A, Balaban A, Pascini TV, Pala ZR, Roth A, *et al.* The fibrinolytic system enables the onset of *Plasmodium* infection in the mosquito vector and the mammalian host. Sci Adv. 2021 Feb;7(6).

41. Ahad NA, Yin TS, Othman AR, Yaacob CR. Sensitivity of normality tests to non-normal data. Sains Malays Vol 40 Issue 6 June 2011 Pages 637-641. 2011;

42. Gibson PR, Birchall I, Rosella O, Albert V, Finch CF, Barkla DH, *et al.* Urokinase and the intestinal mucosa: evidence for a role in epithelial cell turnover. Gut. 1998 Nov 1;43(5):656–63.

43. Mahmood N, Mihalcioiu C, Rabbani SA. Multifaceted role of the urokinase-type plasminogen activator (uPA) and its receptor (uPAR): diagnostic, prognostic, and therapeutic applications. Front Oncol. 2018;8:24.

44. Wun TC, Ossowski L, Reich E. A proenzyme form of human urokinase. J Biol Chem. 1982 Jun 25;257(12):7262–8.

45. Mackay AR, Corbitt RH, Hartzler JL, Thorgeirsson UP. Basement membrane type IV collagen degradation: evidence for the involvement of a proteolytic cascade independent of metalloproteinases. Cancer Res. 1990 Sep 15;50(18):5997–6001.

46. González-Miguel J, Morchón R, Carretón E, Montoya-Alonso JA, Simón F. Can the activation of plasminogen/plasmin system of the host by metabolic products of *Dirofilaria immitis* participate in heartworm disease endarteritis? Parasites Vectors. 2015 Apr 1;8:194.

47. Robinson MW, Corvo I, Jones PM, George AM, Padula MP, To J, *et al.* Collagenolytic activities of the major secreted cathepsin L peptidases involved in the virulence of the helminth pathogen, *Fasciola hepatica*. PLoS Negl Trop Dis. 2011 Apr 5;5(4):e1012.

48. Corvo I, Cancela M, Cappetta M, Pi-Denis N, Tort JF, Roche L. The major cathepsin L secreted by the invasive juvenile *Fasciola hepatica* prefers proline in the S2 subsite and can cleave collagen. Mol Biochem Parasitol. 2009 Sep;167(1):41–7.

49. Cwiklinski K, Jewhurst H, McVeigh P, Barbour T, Maule AG, Tort J, *et al.* Infection by the helminth parasite *Fasciola hepatica* requires rapid regulation of metabolic,

virulence, and invasive factors to adjust to its mammalian host. Mol Cell Proteom. 2018;17:792–809.

50. Suzuki K, Enghild JJ, Morodomi T, Salvesen G, Nagase H. Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin). Biochemistry. 1990 Nov 6;29(44):10261–70.
51. Sonoki A, Okano Y, Yoshitake Y. Dermal fibroblasts can activate matrix metalloproteinase-1 independent of keratinocytes via plasmin in a 3D collagen model. Exp Dermatol. 2018 May;27(5):520–5.
52. Saunders WB, Bayless KJ, Davis GE. MMP-1 activation by serine proteases and MMP-10 induces human capillary tubular network collapse and regression in 3D collagen matrices. J Cell Sci. 2005 May 15;118(Pt 10):2325–40.
53. Baramova EN, Bajou K, Remacle A, L'Hoir C, Krell HW, Weidle UH, *et al.* Involvement of PA/plasmin system in the processing of pro-MMP-9 and in the second step of pro-MMP-2 activation. FEBS Lett. 1997 Mar 24;405(2):157–62.
54. Carmeliet P, Moons L, Lijnen R, Baes M, Lemaître V, Tipping P, *et al.* Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. Nat Genet. 1997 Dec;17(4):439–44.
55. Hahn-Dantona E, Ramos-DeSimone N, Sipley J, Nagase H, French DL, Quigley JP. Activation of proMMP-9 by a plasmin/MMP-3 cascade in a tumor cell model. Regulation by tissue inhibitors of metalloproteinases. Ann N Y Acad Sci. 1999 Jun 30;878:372–87.
56. Ramos-DeSimone N, Hahn-Dantona E, Sipley J, Nagase H, French DL, Quigley JP. Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/ stromelysin-1 cascade enhances tumor cell invasion. J Biol Chem. 1999 May 7;274(19):13066–76.
57. Orbe J, Rodríguez JA, Calvayrac O, Rodríguez-Calvo R, Rodríguez C, Roncal C, *et al.* Matrix metalloproteinase-10 is upregulated by thrombin in endothelial cells and increased in patients with enhanced thrombin generation. Arterioscler Thromb Vasc Biol. 2009 Dec;29(12):2109–16.
58. Raza SL, Nehring LC, Shapiro SD, Cornelius LA. Proteinase-activated receptor-1 regulation of macrophage elastase (MMP-12) secretion by serine proteinases. J Biol Chem. 2000 Dec 29;275(52):41243–50.

59. Di Maggio LS, Tirloni L, Pinto AFM, Diedrich JK, Yates Iii JR, Benavides U, *et al.* Across intra-mammalian stages of the liver fluke *Fasciola hepatica*: a proteomic study. Sci Rep. 2016 Sep 7;6:32796.

60. Burysek L, Syrovets T, Simmet T. The serine protease plasmin triggers expression of MCP-1 and CD40 in human primary monocytes via activation of p38 MAPK and janus kinase (JAK)/STAT signaling pathways. J Biol Chem. 2002 Sep 6;277(36):33509–17.

61. Cheng TL, Chen PK, Huang WK, Kuo CH, Cho CF, Wang KC, *et al.* Plasminogen/thrombomodulin signaling enhances VEGF expression to promote cutaneous wound healing. J Mol Med Berl Ger. 2018 Dec;96(12):1333–44.

62. Pendurthi UR, Ngyuen M, Andrade-Gordon P, Petersen LC, Rao LVM. Plasmin induces Cyr61 gene expression in fibroblasts via protease-activated receptor-1 and p44/42 mitogen-activated protein kinase-dependent signalling pathway. Arterioscler Thromb Vasc Biol. 2002 Sep 1;22(9):1421–6.

63. Tykhomyrov AA, Nedzvetsky VS, Aĝca CA, Guzyk MM, Korsa VV, Grinenko TV. Plasminogen/plasmin affects expression of glycolysis regulator TIGAR and induces autophagy in lung adenocarcinoma A549 cells. Exp Oncol. 2020 Dec;42(4):270–6.

64. Ebrahimi V, Sharifi M, Mousavi-Roknabadi RS, Sadegh R, Khademian MH, Moghadami M, *et al.* Predictive determinants of overall survival among re-infected COVID-19 patients using the elastic-net regularized Cox proportional hazards model: a machine-learning algorithm. BMC Public Health. 2022 Jan 5;22(1):10.

65. Janizek JD, Dincer AB, Celik S, Chen H, Chen W, Naxerova K, *et al.* Uncovering expression signatures of synergistic drug responses via ensembles of explainable machine-learning models. Nat Biomed Eng. 2023 Jun;7(6):811–29.

66. Torang A, Gupta P, Klinke DJ 2nd. An elastic-net logistic regression approach to generate classifiers and gene signatures for types of immune cells and T helper cell subsets. BMC Bioinformatics. 2019 Aug 22;20(1):433.

67. Zhang K, Ye B, Wu L, Ni S, Li Y, Wang Q, *et al.* Machine learning-based prediction of survival prognosis in esophageal squamous cell carcinoma. Sci Rep. 2023 Aug 19;13(1):13532.

68. Kammers K, Cole RN, Tiengwe C, Ruczinski I. Detecting significant changes in protein abundance. EuPA Open Proteom. 2015 Jun;7:11–9.

69. Zhang H, Ghai P, Wu H, Wang C, Field J, Zhou GL. Mammalian adenylyl cyclase-associated protein 1 (CAP1) regulates cofilin function, the actin cytoskeleton, and cell adhesion. J Biol Chem. 2013 Jul 19;288(29):20966–77.

70. Endsley MP, Hu Y, Deng Y, He X, Warejcka DJ, Twining SS, *et al.* Maspin, the molecular bridge between the plasminogen activator system and beta1 integrin that facilitates cell adhesion. J Biol Chem. 2011 Jul 15;286(28):24599–607.

71. Yamamoto Y, Nishimura N, Morimoto S, Kitamura H, Manabe S, Kanayama H omi, *et al.* Distinct roles of Rab3B and Rab13 in the polarized transport of apical, basolateral, and tight junctional membrane proteins to the plasma membrane. Biochem Biophys Res Commun. 2003 Aug 22;308(2):270–5.

72. Sunshine C, Francis S, Kirk KL. Rab3B regulates ZO-1 targeting and actin organization in PC12 neuroendocrine cells. Exp Cell Res. 2000 May 25;257(1):1–10.

73. Nakatsuji H, Nishimura N, Yamamura R, Kanayama HO, Sasaki T. Involvement of actinin-4 in the recruitment of JRAB/MICAL-L2 to cell-cell junctions and the formation of functional tight junctions. Mol Cell Biol. 2008 May;28(10):3324–35.

74. Motrescu ER, Blaise S, Etique N, Messaddeq N, Chenard MP, Stoll I, *et al.* Matrix metalloproteinase-11/stromelysin-3 exhibits collagenolytic function against collagen VI under normal and malignant conditions. Oncogene. 2008 Oct 23;27(49):6347–55.

75. Jenkins MH, Alrowaished SS, Goody MF, Crawford BD, Henry CA. Laminin and Matrix metalloproteinase 11 regulate Fibronectin levels in the zebrafish myotendinous junction. Skelet Muscle. 2016;6:18.

76. Ilani T, Alon A, Grossman I, Horowitz B, Kartvelishvily E, Cohen SR, *et al.* A secreted disulfide catalyst controls extracellular matrix composition and function. Science. 2013 Jul 5;341(6141):74–6.

77. Thielman NRJ, Funes V, Davuluri S, Ibanez HE, Sun WC, Fu J, *et al.* Semaphorin 3D promotes pancreatic ductal adenocarcinoma progression and metastasis through macrophage reprogramming. Sci Adv. 2024;10(42):eadp0684.

78. Donnelly S, Flynn R, Mulcahy G, O'Neill S. Chapter 9. Immunological interaction between *Fasciola* and its host. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 278–308.

79. Johansen FE, Kaetzel CS. Regulation of the polymeric immunoglobulin receptor and IgA transport: new advances in environmental factors that stimulate pIgR expression and its role in mucosal immunity. Mucosal Immunol. 2011 Nov;4(6):598–602.

80. Hendrikx T, Lang S, Rajcic D, Wang Y, McArdle S, Kim K, *et al.* Hepatic pIgR-mediated secretion of IgA limits bacterial translocation and prevents ethanol-induced liver disease in mice. Gut. 2023 Oct;72(10):1959–70.

81. Begolli G, Marković I, Knežević J, Debeljak Ž. Carbohydrate sulfotransferases: a review of emerging diagnostic and prognostic applications. Biochem Medica. 2023 Oct 15;33(3):030503.

82. Kawashima H, Petryniak B, Hiraoka N, Mitoma J, Huckaby V, Nakayama J, *et al.* N-acetylglucosamine-6-O-sulfotransferases 1 and 2 cooperatively control lymphocyte homing through L-selectin ligand biosynthesis in high endothelial venules. Nat Immunol. 2005 Nov;6(11):1096–104.

83. De Marco Verissimo C, Jewhurst HL, Dobó J, Gál P, Dalton JP, Cwiklinski K. *Fasciola hepatica* is refractory to complement killing by preventing attachment of mannose binding lectin (MBL) and inhibiting MBL-associated serine proteases (MASPs) with serpins. Skelly PJ, editor. PLOS Pathog. 2022 Jan 10;18(1):e1010226.

84. Gong C, Jin Y, Wang X, Mao J, Wang D, Yu X, *et al.* Lack of S1PR2 in macrophage ameliorates sepsis-associated lung injury through inducing IL-33-mediated type 2 immunity. Am J Respir Cell Mol Biol. 2024 Mar;70(3):215–25.

85. Zhao M, Ren K, Xiong X, Xin Y, Zou Y, Maynard JC, *et al.* Epithelial STAT6 O-GlcNAcylation drives a concerted anti-helminth alarmin response dependent on tuft cell hyperplasia and Gasdermin C. Immunity. 2022 Apr 12;55(4):623-638.e5.

86. Berkenpas MB, Lawrence DA, Ginsburg D. Molecular evolution of plasminogen activator inhibitor-1 functional stability. EMBO J. 1995 Jul 3;14(13):2969–77.

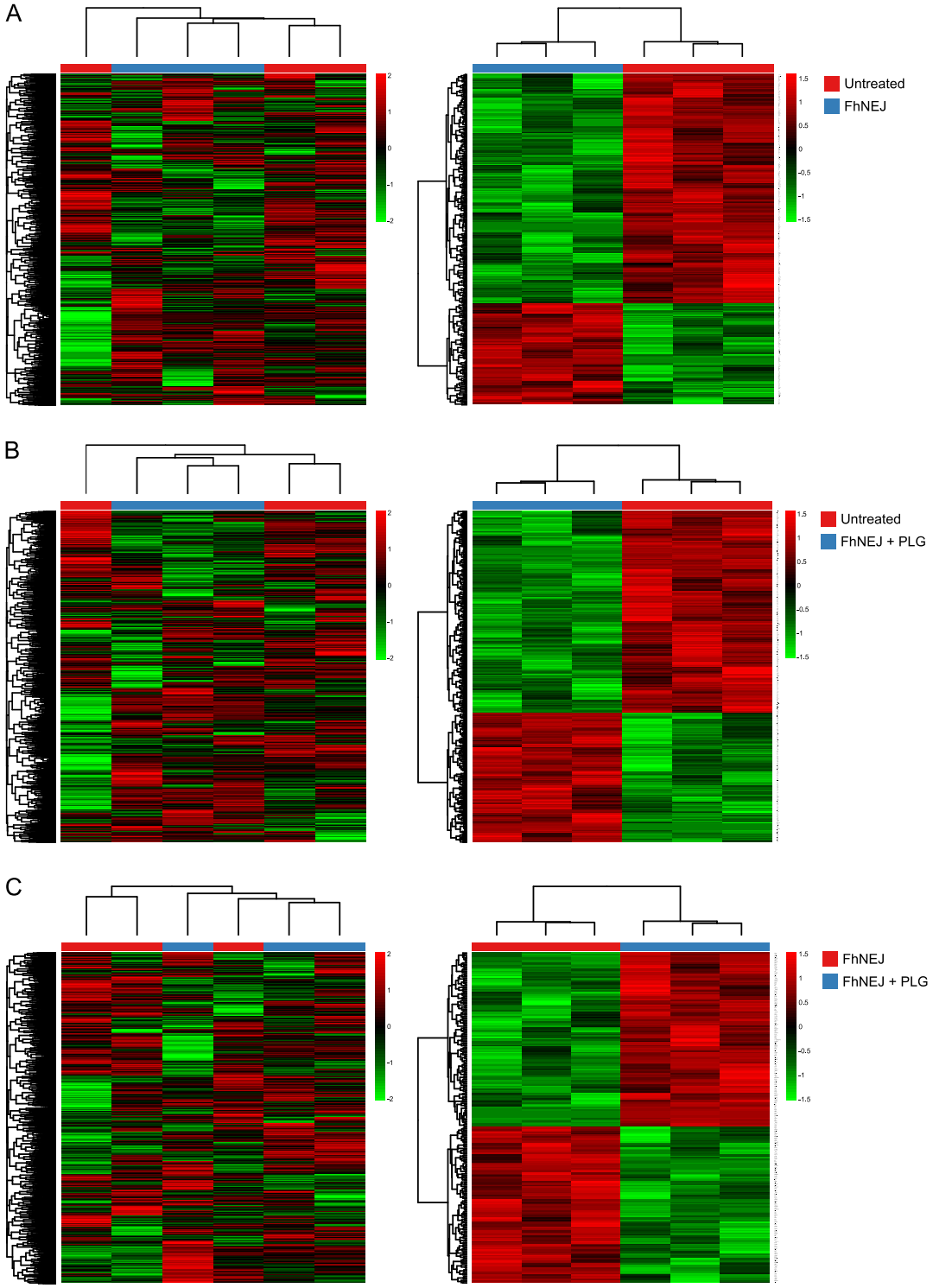
87. Spithill TW, Toet H, Rathinasamy V, Zerna G, Swan J, Cameron T, *et al.* Chapter 12. Vaccines for *Fasciola*: new thinking for an old problem. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 379–423.

88. Cwiklinski K, Drysdale O, López Corrales J, Corripio-Miyar Y, De Marco Verissimo C, Jewhurst H, *et al.* Targeting secreted protease/anti-protease balance as a vaccine strategy against the helminth *Fasciola hepatica*. Vaccines. 2022 Jan 20;10(2).

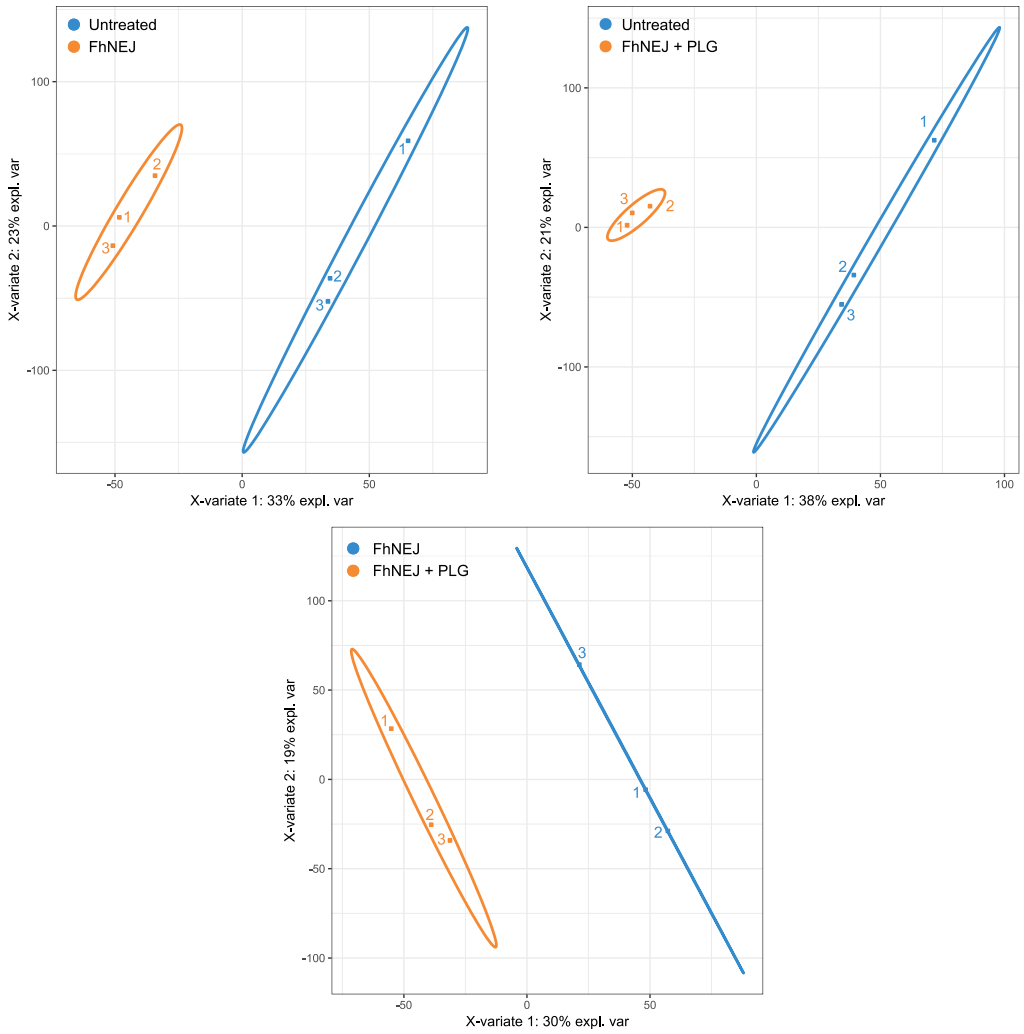
Supplementary information

Supplementary Figure 1 (Figure on page 254). Feature selection by elastic-net penalised regression improves sample clustering. An elastic-net penalised regression model was performed to select for proteins that are specifically expressed in each experimental condition. The figures show heatmap representations of protein expression levels (normalized to Z-score) of all the proteins identified (left panels) or proteins selected as specific to each experimental condition after feature selection through elastic-net regularised regression (right panels), for selected, biologically-relevant pairwise comparisons: untreated mPSIEC vs. mPSIEC treated with FhNEJ **(A)**; untreated mPSIEC vs. mPSIEC treated with both FhNEJ and PLG **(B)**; and FhNEJ-treated mPSIEC vs. mPSIEC treated with both FhNEJ and PLG **(C)**. Heatmaps created with R software.

Supplementary Figure 2 (Figure on page 255). PLS-DA analysis of selected pairwise comparisons was performed to identify the proteins most relevant for distinguishing between experimental conditions. Only those elastic-net pre-selected proteins that contributed substantially to the newly projected bidimensional space (as determined by vip>1.5) were considered as specific to each experimental condition. Plots are shown for selected, biologically-relevant pairwise comparisons: untreated mPSIEC vs. FhNEJ-treated mPSIEC (top left); untreated mPSIEC vs. mPSIEC treated with both FhNEJ and PLG (top right); and FhNEJ-treated mPSIEC vs. mPSIEC treated with both FhNEJ and PLG (bottom). Plots created with R software.

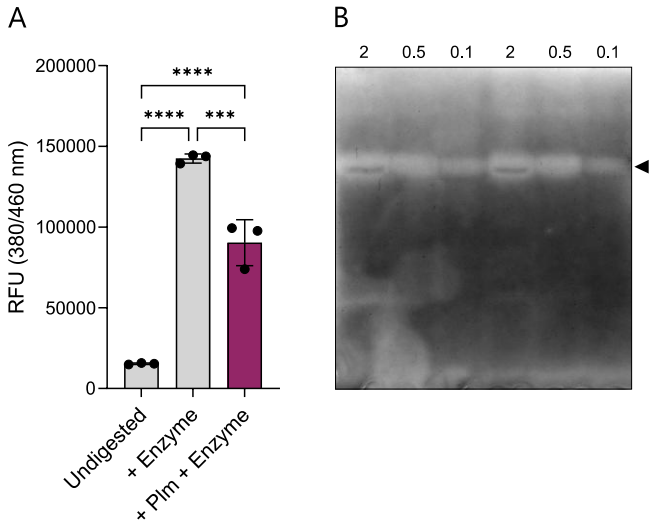


Supplementary Figure 1 (legend on page 253).

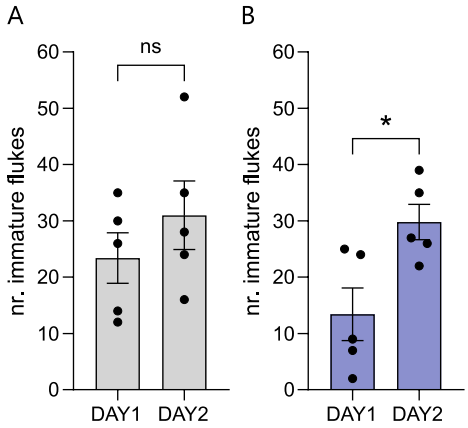


Supplementary Figure 2 (legend on page 253).

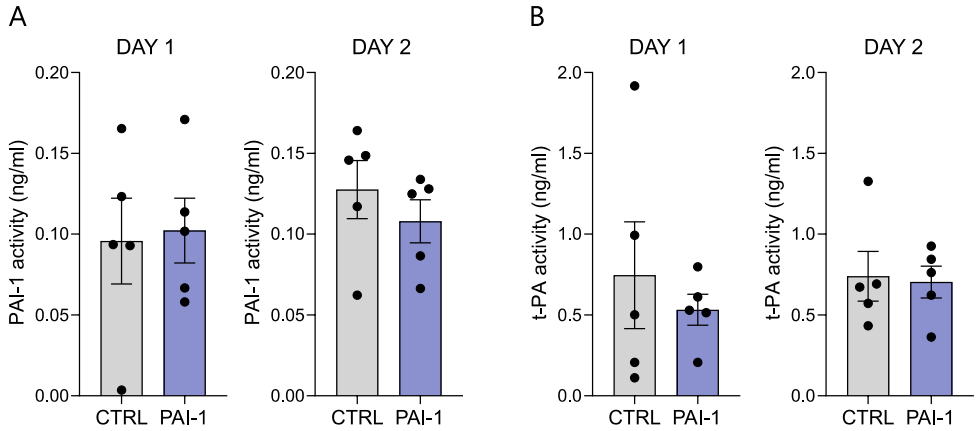
Supplementary Figure 3. A) The effect of plasmin on the detection of total collagen was determined using the same commercial kit as in Figure 2B by pre-incubating total collagen with 2 μM plasmin for 24 hours at 37 ºC followed by enzymatic digestion and detection of N-Gly terminal peptides, as specified by the manufacturer's instructions. Total collagen left undigested ("Undigested") or digested without plasmin pre-incubation ("+ Enzyme") served as controls. Bars indicate the mean of three technical replicates ± SD (***p≤0.001, ****p≤0.0001; one-way ANOVA followed by Tukey post-hoc analysis of pairwise comparisons). RFU, relative fluorescent units (excitation 380 nm, emission 460 nm). **B)** Zymography showing plasmin-mediated degradation of the ECM coating used for mPSIEC culture. Different amounts of plasmin (2 μM, 0.5 μM, and 0.1 μM) were loaded onto 10% SDS-PAGE gels prepared using mPSIEC coating solution instead of water. Light bands (arrowhead) corresponding to degradation of the mPSIEC coating solution by plasmin were revealed by Coomassie brilliant blue staining.



Supplementary Figure 4. Fluke recovery in control and PAI-1-treated mice across experimental days. The number of flukes recovered from control **(A)** and PAI-1-treated **(B)** mice was plotted separately to assess possible variation in intra-group responses across days. Data points represent the number of juveniles recovered in the liver of each mouse, and bars indicate the mean ± SEM. Asterisks indicate significant differences between experimental conditions (ns, not significant; **p≤0.1; Mann-Whitney U test).



Supplementary Figure 5. Analysis of PAI-1 and t-PA activities in peritoneal fluids of *F. hepatica*-infected mice. Eight days after challenge with *F. hepatica* metacercariae, mice were euthanised and the levels of active PAI-1 **(A)** and t-PA **(B)** in peritoneal fluids were quantified by ELISA using commercially-available kits. Data points represent the levels of active PAI-1 or t-PA in the peritoneal fluid of each mouse, and bars indicate the mean ± SEM. None of the differences were significant (Mann-Whitney U test).



Supplementary Table 1. DEPs in untreated vs. FhNEJ-treated mPSIEC. A negative fold-change indicates that proteins were overexpressed in FhNEJ-treated vs. untreated mPSIEC. The opposite is true when the fold-change is positive.

Protein	logFC	adjPVal
Rpl32	-11.656	1.73E-08
Cdc42	-11.6507	2.79E-08
Abpa12a	-8.81047	0.0000338
S1pr2	-8.67745	2.79E-08
Plin2	-1.07837	0.000559
Jagn1	-0.46458	0.049104
Ttyh3	0.308236	0.049048
Cdca8	0.424581	0.032809
Pzp	0.891437	0.008727
Bcl7b	1.024835	0.04689
Itih2	1.563109	0.023871
C3	1.606383	0.038488
Cpn1	7.886917	0.0000338
Map6	8.331462	0.00000468
Qsox1	8.616831	0.00000145
Itih3	8.676878	1.73E-08
Hp	9.253502	0.0000643
Hexim2	10.7249	0.000000717
Alb	13.12146	0.000149

Supplementary Table 2. Two-way ANOVA of the number of recovered *F. hepatica* juvenile flukes in the livers of control and PAI-1-treated mice eight days after challenge with *F. hepatica* metacercariae. SS, sum of squares; DF, degrees of freedom; MS, mean square; F, F statistic; DFn, degrees of freedom of the numerator; DFd, degrees of freedom of the denominator.

Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	3.522	0.3642	ns	No	
Day	26.19	0.0215	*	Yes	
Treatment	5.704	0.2519	ns	No	
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	96.8	1	96.8	F (1, 16) = 0.8725	P=0.3642
Day	720	1	720	F (1, 16) = 6.489	P=0.0215
Treatment	156.8	1	156.8	F (1, 16) = 1.413	P=0.2519
Residual	1775	16	111		

General discussion

General discussion

Parasites have evolved remarkable mechanisms to exploit host resources by subverting key physiological pathways to facilitate their survival and transmission. A paradigmatic example of this is the ability of parasites to interact with the fibrinolytic system of their definitive hosts, a strategy that theoretically facilitates parasite dissemination and survival within the mammalian organism (1,2). The classical function of the fibrinolytic system is to degrade fibrin clots that form on the vascular endothelium, a process that depends on the conversion of the plasminogen (PLG) zymogen into the serine protease plasmin by the tissue-type and urokinase-type PLG activators (t-PA and u-PA, respectively) (3). However, the multiplicity of plasmin substrates and the broad cellular expression of u-PA, the main extra-vascular PLG activator, engage the fibrinolytic system in a myriad of fibrinolysis-unrelated functions that participate in biological processes that are unrelated to blood clot removal, including cell adhesion, wound healing, angiogenesis, inflammation/immunity, and cell migration. Intriguingly, these processes are generally altered in mammalian hosts during parasitic infections, suggesting that manipulating these pathways may provide survival advantages to parasites.

The most common mechanism of interaction between parasites and host fibrinolysis is through the expression of PLG-binding lysine-rich proteins at the host-parasite interface, either the parasite surface or secreted into the tissue microenvironment surrounding the parasite (1). The interaction between PLG and fibrin—and non-fibrin PLG-interacting proteins, collectively known as PLG receptors or PLG-Rs—is essential for the execution of fibrinolytic functions, as it causes a conformational change in the PLG molecule that exposes the target sites of t-PA and u-PA, thereby facilitating its conversion into plasmin (3). *Fasciola hepatica* is one of many parasites whose interaction with the host fibrinolytic system has been experimentally shown. Specifically, *in vitro* studies demonstrated that adult stages of *F. hepatica* secrete proteins that interact with PLG (4,5), promoting its subsequent activation into plasmin (4). This interaction was presumed to facilitate the survival of adult flukes inside the bile ducts and was also proposed to underlie some of the pathology related to *F. hepatica* infection, particularly in relation to the neurological symptoms that occasionally appear in patients with acute or chronic fasciolosis (4). In this thesis, we aimed to analyse the broader significance of the interaction between *F. hepatica* and the host fibrinolytic system by investigating whether this capacity is conserved in the juvenile stages of the parasite, specifically in the newly excysted juveniles (FhNEJ) that emerge in the duodenum shortly after the ingestion of metacercariae. This interaction could have

important functional implications in the migration of these parasites across the intestinal wall, provided the involvement of plasmin in the degradation of extracellular matrix (ECM) components that act as a physical barrier against pathogen dissemination. Our results demonstrate that FhNEJ express PLG-Rs at their tegument surface that interact with two core components of the fibrinolytic system, namely PLG and the precursor of u-PA (pro-u-PA). These findings give rise to several noteworthy considerations regarding the nature and implications of this interaction.

First, we identified a large number of proteins that interact with PLG, pro-u-PA, or both (**Chapter 1**, **Chapter 3**), indicating that the interaction between FhNEJ and the fibrinolytic system occurs via highly redundant mechanisms. Since biochemical redundancy allows for the maintenance of essential biological functions by providing back-up mechanisms that can operate when a specific effector protein is lost (6), this finding highlights that hijacking host fibrinolysis is functionally relevant for the survival of FhNEJ within their definitive hosts. The functional relevance of this interaction is also underscored by the observation that it occurs in pathogens belonging to distant taxonomic groups, including bacteria, fungi, protozoans, and multicellular parasites (1,2,7,8), thereby representing a paradigmatic example of evolutionary convergence wherein unrelated organisms have evolved a similar strategy to fulfil essential biological functions.

Second, most of the proteins that we identified as PLG-Rs in the tegument surface of FhNEJ are intracellular or cytosolic proteins with canonical functions unrelated to PLG binding, which argues in favour of their moonlighting potential. Proteins such as enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutathione S-transferase (GST), and certain histones are well-recognised moonlighting proteins in other organisms (9–11) that we identified as PLG- and/or pro-u-PA-interacting proteins in the FhNEJ tegument (**Chapter 1**, **Chapter 3**). The identification of the juvenile-specific cathepsins B2, B3, and L3 (FhCB2–3, FhCL3) as proteins that interact with fibrinolytic factors is particularly noteworthy provided that this is the first time that a moonlighting function is described for members of this family of enzymes (9). Moreover, since our PLG-binding assays were conducted using the zymogen versions of these proteases, our results demonstrate that cathepsins contribute to FhNEJ migration through a mechanism that is independent of their catalytic activity. Notably, some of the PLG- and/or pro-u-PA-binding proteins (FhGST and FhCL3) were also identified and validated as capable of binding to laminin (**Chapter 2**), one of the major components of the intestinal ECM (12). Given the well-known ability of plasmin to degrade laminin (13), these results indicate that proteins that

concomitantly interact with PLG, pro-u-PA, and laminin act as interaction hubs that efficiently localise proteolytic activity towards intestinal ECM components that would otherwise block FhNEJ migration. Indeed, results showed that FhNEJ-induced plasmin generation from host PLG enhances the endogenous capacity of these parasites to degrade laminin (**Chapter 2**).

Third, the biochemical redundancy underlying the interaction between FhNEJ and host fibrinolysis limits the feasibility of targeting this interaction to block FhNEJ migration and prevent disease progression. This limitation is further exacerbated by the fact that many of the fibrinolysis-interacting proteins that we identified in the tegument of FhNEJ are housekeeping proteins (e.g., glycolytic enzymes such as enolase and GAPDH) with highly conserved sequences across organisms, including mammals. However, an interesting observation is that some of the PLG/pro-u-PA-interacting proteins in the tegument of FhNEJ, including FhCL3, were identified in protein spots of similar molecular weight but differing isoelectric points (**Supp. Table 1** in **Chapter 1**, **Supp. Table 1** in **Chapter 3**), indicating that these proteins contain post-translational modifications (PTMs) when exposed to the tegument surface of FhNEJ. The acquisition of PTMs has been proposed as one of the mechanisms by which moonlighting proteins are shuffled to alternative cellular locations to perform non-canonical functions (14). In the context of the potential applicability of our findings, the fact that fibrinolysis-interacting proteins at the FhNEJ surface harbour PTMs, potentially different to those expressed by host cells, opens up an interesting opportunity to selectively block FhNEJ migration while minimising undesired self-recognition. The feasibility of such an approach is supported by the recent identification of FhNEJ enolase as a PLG-interacting protein that is recognised by sera of experimentally-infected sheep (15), suggesting that this enzyme may indeed represent a potential vaccine candidate against early-stage fasciolosis. Moreover, our analyses revealed that the interaction with PLG involves enolase proteoforms with distinct isoelectric points (**Supp. Table 1** in **Chapter 1**), indicating that, just like FhCL3, this protein also contains PTMs when exposed on the FhNEJ surface that could potentially differ from those present in enolase expressed by host cells. The presence of PTMs in tumour-associated antigens (TAAs), which are absent in the equivalent proteins expressed by non-transformed cells, enhances their recognition as foreign antigens and increase their potential use as therapeutic anti-cancer vaccines (16). Remarkably, one such TAA is citrullinated enolase, which represents an effective target against different malignancies in mice by boosting anti-cancer immunity with minimal cross-reactivity to healthy tissue (17).

Another noteworthy aspect of the interaction between FhNEJ and the host fibrinolytic system revealed in this thesis is that these parasites express a protease or set of proteases that cleave pro-u-PA into its catalytically-active form (**Chapter 3**)—a function that is rarely observed in helminth parasites (1). The ability of FhNEJ to activate pro-u-PA stands in contrast to their inability to directly cleave and activate PLG (**Chapter 1**). Considering that u-PA is capable of cleaving up to 773 ± 391 molecules of a synthetic chromogenic substrate per minute (18), pro-u-PA activation by FhNEJ may represent a strategy to optimise plasmin generation in the microenvironment surrounding the parasites, as this indirect route is likely to result in greater plasmin production than direct cleavage and activation of PLG. Since all the *F. hepatica* cathepsin peptidases that we tested failed to activate pro-u-PA (**Chapter 3**), we argue that pro-u-PA activation might be carried out by dipeptidyl peptidase 4, which is among the very few serine proteases characterised in this parasite. Interestingly, a dipeptidyl peptidase is overexpressed in FhNEJ upon their interaction with mouse intestinal epithelial cells in an *in vitro* cell-based model of early host-parasite relationships in fasciolosis (19). Alternatively, the fact that metalloproteinase-like enzymes present in snake venoms cleave and activate pro-u-PA (20), and that these enzymes are detected in the secretome of FhNEJ (21), offer an intriguing clue regarding the potential identity of the proteases responsible for pro-u-PA cleavage and activation by FhNEJ. Identifying the protease(s) expressed by FhNEJ that are responsible for pro-u-PA activation is compelling, as this function is presumably less redundant than the promiscuous lysine-mediated interactions underlying PLG binding, and may therefore represent a more specific and tractable target for therapeutic intervention. In addition, the high species-specificity of the interaction between u-PA and its physiological receptor, u-PAR (22), a member of the Ly6 superfamiliy of proteins, further underscores the potential of the FhNEJ-pro-u-PA interaction as a selective target against early-stage fasciolosis. Interestingly, members of the u-PAR/Ly6 superfamily of proteins have been described in *F. hepatica* (23) and other trematodes, including *F. gigantica* (24), *Opistorchis viverrini* (25), and *Schistosoma mansoni* (26), and proposed as potential vaccine candidates against these parasites (26,27).

Additional key findings of this thesis emerge from the early host-parasite interaction models presented in **Chapter 4**. Notably, mouse small intestinal epithelial cells (mPSIEC) exposed to both FhNEJ and PLG, which enhances plasmin generation in the pericellular space, exhibited increased secretion of u-PA. The u-PA and its receptor play instrumental roles in cell migration and metastasis, with their elevated expression in human tumours being strongly associated with invasiveness and poor prognosis (28). In this context, our findings

suggest that FhNEJ may co-opt host proteolytic pathways to loosen epithelial barriers and compromise their integrity, which could facilitate parasite migration at the earliest phases of infection. This notion is strongly reinforced by the enhanced collagen degradation that we observed in mPSIEC incubated with both FhNEJ and PLG. Complementing these findings, the proteomic analysis of mPSIEC co-stimulated with FhNEJ and PLG offers novel insights into the broad spectrum of biological processes potentially modulated by FhNEJ-induced plasmin activity within the intestinal tissue microenvironment. While previous chapters emphasised the role of plasmin in facilitating tissue penetration by FhNEJ through enhanced proteolysis, this proteomic analysis reveals the potential influence of plasmin on the regulation of host immune responses, underscoring the multifaceted impact of manipulating host fibrinolysis during early infection. In particular, the differential expression of the polymeric immunoglobulin receptor and the sphingosine 1-phosphate receptor 2 in mPSIEC stimulated with both FhNEJ and PLG—but not either factor alone—warrants further attention given the central role of these proteins in mucosal immunity (29,30).

Finally, this thesis presents the first functional assessment of the role played by the interaction between *F. hepatica* and the host fibrinolytic system in facilitating the migration of juvenile flukes across the mammalian host *in vivo*. Notwithstanding the variability observed across experiments conducted on different days, a significant reduction in hepatic fluke recovery was recorded in mice from one experiment in which the fibrinolytic pathway had been pharmacologically inhibited prior to infection with *F. hepatica* metacercariae. This result constitutes the first conclusive evidence that the host fibrinolytic system actively contributes to the migration of pre-hepatic *F. hepatica* juveniles towards the liver (**Chapter 4**). Equally important, however, are the broader implications of these findings regarding the experimental modelling of *F. hepatica* infections. The high variability and the frequent lack of statistical significance in *in vivo* experiments—long recognised in vaccination studies—underscore the inherent challenges of working with a biologically complex and genetically diverse parasitic organism.

As a helminth parasite with sexual reproduction, *F. hepatica* undergoes meiotic recombination and random gamete fusion during self- or cross-fertilisation, generating considerable genetic diversity among the developing embryo. Consequently, each sub-batch of infective metacercariae used in experimental infections potentially harbours genetically distinct FhNEJ, resulting in marked inter-individual variability in phenotypic traits such as trans-intestinal migratory proficiency, anthelmintic resistance, or susceptibility

to vaccine-induced immune responses (31–33). Phenotypic diversity between FhNEJ is exemplified by the different distribution patterns of PLG-binding proteins that we observed in the surface of these parasites by immunofluorescence (**Chapter 1**). In addition to parasitic genetic diversity, other factors, such as phenotypic variability in the host and stochastic differences in parasite establishment (biological randomness), might contribute to the observed experimental variability. Altogether, these insights emphasise that new frameworks may be required to more accurately assess the efficacy of interventions attempted to block *F. hepatica* development and to understand the complex host-parasite interactions in genetically heterogeneous organisms like sexually-reproducing parasites.

In this scenario, three-dimensional cell culture systems have emerged as a promising alternative to *in vivo* models, offering the potential to address specific research questions while adhering to the principles of Replacement and Reduction in animal experimentation. Among these, organoids—self-organising, multicellular structures that exhibit self-renewal and recapitulate key structural and functional aspects of their tissue of origin—represent a particularly compelling alternative (34–36). Compared to conventional two-dimensional, single-cell-type cultures, organoids more accurately mimic the complexity of native tissues, thereby providing a more physiologically-relevant model for investigating host-parasite interactions (35,36). Moreover, organoids can be generated from a diverse array of host species, including human, mouse, bovine, porcine, ovine, avian, feline, and canine origins. This is of particular relevance in the study of helminth infections, which are often highly host-specific and poorly modelled in the limited range of rodent hosts commonly used for *in vivo* research (35). In addition, the biological complexity of *in vivo* models frequently results in 'noisy' results due to uncontrolled inter-individual variability, which complicates data interpretation—a challenge observed in the present study and commonly reported in vaccine trials conducted by others against helminth infections. In this context, organoids and organoid-related models can be seen as a midpoint between overly simplistic single-cell systems and complex live animals (36), offering a more controlled and reproducible experimental platform to refine and validate hypotheses prior to conducting animal trials.

Taken together, the findings and reflections presented in this thesis provide a comprehensive view of the functional significance of the interaction between FhNEJ and the host fibrinolytic system in the successful establishment of these parasites within the mammalian organism. This work sets the foundation for future research on host-parasite relationships during early-stage fasciolosis, which might inspire the development of more effective therapeutic and control strategies against this widespread parasitic disease.

References

1. Diosdado A, Simón F, Serrat J, González-Miguel J. Interaction of helminth parasites with the haemostatic system of their vertebrate hosts: a scoping review. Parasite. 2022;29:35.
2. González-Miguel J, Siles-Lucas M, Kartashev V, Morchón R, Simón F. Plasmin in parasitic chronic infections: friend or foe? Trends Parasitol. 2016 Apr;32(4):325–35.
3. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. Br J Haematol. 2005 May;129(3):307–21.
4. González-Miguel J, Valero MA, Reguera-Gomez M, Mas-Bargues C, Bargues MD, Simón F, *et al.* Numerous *Fasciola* plasminogen-binding proteins may underlie blood-brain barrier leakage and explain neurological disorder complexity and heterogeneity in the acute and chronic phases of human fascioliasis. Parasitology. 2019 Mar;146(3):284–98.
5. Bernal D, de la Rubia JE, Carrasco-Abad AM, Toledo R, Mas-Coma S, Marcilla A. Identification of enolase as a plasminogen-binding protein in excretory-secretory products of *Fasciola hepatica*. FEBS Lett. 2004 Apr 9;563(1–3):203–6.
6. Nowak MA, Boerlijst MC, Cooke J, Smith JM. Evolution of genetic redundancy. Nature. 1997 Jul;388(6638):167–71.
7. Bhattacharya S, Ploplis VA, Castellino FJ. Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. J Biomed Biotechnol. 2012;2012:482096.
8. Chen SM, Zou Z, Guo SY, Hou WT, Qiu XR, Zhang Y, *et al.* Preventing *Candida albicans* from subverting host plasminogen for invasive infection treatment. Emerg Microbes Infect. 2020 Dec;9(1):2417–32.
9. MoonProt – A database for moonlighting proteins. Available from: http://www.moonlightingproteins.org/
10. Chen C, Zabad S, Liu H, Wang W, Jeffery C. MoonProt 2.0: an expansion and update of the moonlighting proteins database. Nucleic Acids Res. 2018 Jan 4;46(D1):D640–4.
11. Mani M, Chen C, Amblee V, Liu H, Mathur T, Zwicke G, *et al.* MoonProt: a database for proteins that are known to moonlight. Nucleic Acids Res. 2015 Jan 28;43(D1):D277–82.

12. Pompili S, Latella G, Gaudio E, Sferra R, Vetuschi A. The charming world of the extracellular matrix: a dynamic and protective network of the intestinal wall. Front Med. 2021 Apr 16;8:610189.

13. Nakagami Y, Abe K, Nishiyama N, Matsuki N. Laminin degradation by plasmin regulates long-term potentiation. J Neurosci. 2000 Mar 1;20(5):2003–10.

14. Ceruti P, Principe M, Capello M, Cappello P, Novelli F. Three are better than one: plasminogen receptors as cancer theranostic targets. Exp Hematol Oncol. 2013 Dec;2(1):1–11.

15. O'Kelly E, Cwiklinski K, De Marco Verissimo C, Calvani NED, López Corrales J, Jewhurst H, *et al.* Moonlighting on the *Fasciola hepatica* tegument: enolase, a glycolytic enzyme, interacts with the extracellular matrix and fibrinolytic system of the host. PLoS Negl Trop Dis. 2024 Aug;18(8):e0012069.

16. Srivastava AK, Guadagnin G, Cappello P, Novelli F. Post-translational modifications in tumor-associated antigens as a platform for novel immuno-oncology therapies. Cancers (Basel). 2022 Dec 26;15(1):138.

17. Cook K, Daniels I, Symonds P, Pitt T, Gijon M, Xue W, *et al.* Citrullinated α-enolase is an effective target for anti-cancer immunity. Oncoimmunology. 2018;7(2):e1390642.

18. Bøtkjaer KA, Byszuk AA, Andersen LM, Christensen A, Andreasen PA, Blouse GE. Nonproteolytic induction of catalytic activity into the single-chain form of urokinase-type plasminogen activator by dipeptides. Biochemistry. 2009 Oct 13;48(40):9606–17.

19. Becerro-Recio D, Serrat J, López-García M, Sotillo J, Simón F, González-Miguel J, *et al.* Proteomics coupled with *in vitro* model to study the early crosstalk occurring between newly excysted juveniles of *Fasciola hepatica* and host intestinal cells. PLoS Negl Trop Dis. 2022 Oct;16(10):e0010811.

20. Sugiki M, Yoshida E, Anai K, Maruyama M. Activation of single-chain urokinase-type plasminogen activator by a hemorrhagic metalloproteinase, jararafibrase I, in *Bothrops jararaca* venom. Toxicon. 1998 Jul;36(7):993–1000.

21. Di Maggio LS, Tirloni L, Pinto AFM, Diedrich JK, Yates Iii JR, Benavides U, *et al.* Across intra-mammalian stages of the liver fluke *Fasciola hepatica*: a proteomic study. Sci Rep. 2016 Sep 7;6:32796.

22. Lin L, Gårdsvoll H, Huai Q, Huang M, Ploug M. Structure-based engineering of species selectivity in the interaction between urokinase and its receptor. J Biol Chem. 2010 Apr;285(14):10982–92.

23. Shi Y, Toet H, Rathinasamy V, Young ND, Gasser RB, Beddoe T, *et al.* First insight into CD59-like molecules of adult *Fasciola hepatica*. Exp Parasitol. 2014 Sep;144:57–64.

24. Davey SD, Chalmers IW, Fernandez-Fuentes N, Swain MT, Smith D, Abbas Abidi SM, *et al. In silico* characterisation of the complete Ly6 protein family in *Fasciola gigantica* supported through transcriptomics of the newly-excysted juveniles. Mol Omics. 2022 Jan 17;18(1):45–56.

25. Mulvenna J, Sripa B, Brindley PJ, Gorman J, Jones MK, Colgrave ML, *et al.* The secreted and surface proteomes of the adult stage of the carcinogenic human liver fluke *Opisthorchis viverrini*. Proteomics. 2010 Mar;10(5):1063–78.

26. Chalmers IW, Fitzsimmons CM, Brown M, Pierrot C, Jones FM, Wawrzyniak JM, *et al.* Human IgG1 responses to surface localised *Schistosoma mansoni* Ly6 family members drop following praziquantel treatment. PLoS Negl Trop Dis. 2015;9(7):e0003920.

27. Cardoso FC, Macedo GC, Gava E, Kitten GT, Mati VL, de Melo AL, *et al. Schistosoma mansoni* tegument protein Sm29 is able to induce a Th_1-type of immune response and protection against parasite infection. PLoS Negl Trop Dis. 2008 Oct 1;2(10):e308.

28. Fazioli F, Blasi F. Urokinase-type plasminogen activator and its receptor: new targets for anti-metastatic therapy? Trends Pharmacol Sci. 1994 Jan 1;15(1):25–9.

29. Gong C, Jin Y, Wang X, Mao J, Wang D, Yu X, *et al.* Lack of S1PR2 in macrophage ameliorates sepsis-associated lung injury through inducing IL-33-mediated type 2 immunity. Am J Respir Cell Mol Biol. 2024 Mar;70(3):215–25.

30. Johansen FE, Kaetzel CS. Regulation of the polymeric immunoglobulin receptor and IgA transport: new advances in environmental factors that stimulate pIgR expression and its role in mucosal immunity. Mucosal Immunol. 2011 Nov;4(6):598–602.

31. Hanna REB, Moffett D, Forster FI, Trudgett AG, Brennan GP, Fairweather I. *Fasciola hepatica*: a light and electron microscope study of the ovary and of the development of oocytes within eggs in the uterus provides an insight into reproductive strategy. Vet Parasitol. 2016 May 15;221:93–103.

32. Hanna REB, Fairweather I, Robinson MW. Chapter 4. The reproductive system of *Fasciola hepatica*. In: Fasciolosis. 2nd ed. UK: CABI; 2022. p. 112–45.

33. Beesley NJ, Williams DJL, Paterson S, Hodgkinson J. *Fasciola hepatica* demonstrates high levels of genetic diversity, a lack of population structure and high gene flow: possible implications for drug resistance. Int J Parasitol. 2017 Jan;47(1):11–20.

34. Paužuolis M, Samperio Ventayol P, Neyazi M, Bartfeld S. Organoids as a tool to study the impact of heterogeneity in gastrointestinal epithelium on host-pathogen interactions. Clin Exp Immunol. 2024 Sep;218(1):16–27.

35. Duque-Correa MA, Maizels RM, Grencis RK, Berriman M. Organoids – new models for host-helminth interactions. Trends Parasitol. 2020 Feb;36(2):170–81.

36. Smith D, Price DRG, Faber MN, Chapuis AF, McNeilly TN. Advancing animal health and disease research in the lab with three-dimensional cell culture systems. Vet Rec. 2022 Jul;191(1):e1528.

Conclusions

Conclusions

1. *Fasciola hepatica* newly excysted juveniles (FhNEJ) interact with the host fibrinolytic system by expressing proteins on their tegument surface that bind to the zymogens plasminogen (PLG) and the precursor of the urokinase-type PLG activator (pro-u-PA) in a lysine-dependent and -independent manner, respectively, replicating the mechanism of interaction of these factors with their physiologic receptors.

2. The interaction between FhNEJ and host PLG is functionally relevant, as it leads to the generation of plasmin, the main fibrinolytic protease, in the presence of host-derived PLG activators.

3. The juvenile-specific cathepsins B2, B3, and L3 (FhCL3), as well as glutathione S-transferase and enolase, are identified as PLG- and/or pro-u-PA-binding proteins in the tegument of FhNEJ, highlighting a novel moonlighting function of these proteins.

4. FhNEJ express one or more proteases on their tegument surface capable of converting pro-u-PA into its catalytically-active form, bypassing the need of exogenous u-PA for the generation of plasmin from host-derived PLG. Thus, the interaction between FhNEJ and the host fibrinolytic system is bimodal: it involves PLG binding to promote plasmin generation and the activation of pro-u-PA.

5. FhNEJ express proteins on their tegument surface that bind to laminin, a major component of the intestinal extracellular matrix (ECM), potentially facilitating the adhesion of these parasites to the intestinal epithelium prior to trans-intestinal migration.

6. We demonstrate that the juvenile-specific FhCL3 is capable of degrading laminin and that the endogenous capacity of FhNEJ to degrade this ECM protein is enhanced by FhNEJ-induced plasmin generation from host PLG.

7. Plasmin generation from host-derived PLG, stimulated by FhNEJ in the pericellular space of mouse small intestinal epithelial cells, promotes u-PA secretion by host cells and stimulates collagen degradation and the differential expression of proteins related to ECM remodelling, fibrinolysis, and intestinal immunity.

8. Using a mouse model of early-stage fasciolosis, this thesis provides the first functional evidence that *F. hepatica* juveniles exploit host fibrinolysis to facilitate their migration through host tissues during the pre-hepatic phase of infection, revealing a key pathogenic mechanism and a potential therapeutic target.

List of abbreviations

List of abbreviations

2D-MS	Bidimensional electrophoresis coupled to mass spectrometry analysis
A2m	Alpha-2 macroglobulin
ACN	Acetonitrile
Actn4	Actinin-4
ADAM	A-desintegrin and metalloproteinase
ADCC	Antibody-dependent cellular cytotoxicity
Akt	Protein kinase B
AL	Activation loop
ALF	Angiostatin-like fragment
ANOVA	Analysis of variance
ATF	Amino-terminal fragment
ATP	Adenosine triphosphate
BM	Basement membrane
BSA	Bovine serum albumin
Cap1	Cyclase-associated protein 1
CBXP	Carboxipeptidase
Chst2	Carbohydrate sulfotransferase 2
CNS	Central nervous system
CO_2	Carbon dioxide
CpB	Carboxypeptidase B
Cpb2	Carboxipeptidase 2
CSIC	Spanish National Research Council
DC	Dendritic cell
DEP	Differentially expressed protein
DIA	Data-independent acquisition
DiACT	*Dirofilaria immitis* actin
DNA	Deoxyribonucleic acid
DPP	Dipeptidyl peptidase
DTT	Dithiothreitol
ECM	Extracellular matrix
EGF	Epidermal growth factor

ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESP	Excreted/secreted product
EV	Extracellular vesicle
FA	Formic acid
FABP	Fatty acid binding protein
FBAL	Fructose-bisphosphate aldolase
FC	Fold change
FDP	Fibrin(ogen) degradation product
FDR	False discovery rate
FEC	Faecal egg count
FhCB	*Fasciola hepatica* cathepsin B
FhCL	*F. hepatica* cathepsin L
FhENO	*F. hepatica* enolase
FhHDM	*F. hepatica* helminth defence molecule
FhKTM	*F. hepatica* kunitz-type molecule
FhLAP	*F. hepatica* leucine aminopeptidase
FhNEJ	*F. hepatica* newly excysted juvenile
FhNEJ-Som	Somatic-enriched protein fraction of *F. hepatica* newly excysted juveniles
FhNEJ-Teg	Tegument-enriched protein fraction of *F. hepatica* newly excysted juveniles
FhPrx	*F. hepatica* peroxiredoxin
FhSOD	*F. hepatica* superoxide dismutase
FhTrx	*F. hepatica* thioredoxin
FN	Fibronectin
FPR	Formyl peptide receptor
GALT	Gut-associated lymphoid tissue
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GI	Gastrointestinal
GO	Gene ontology
GPCR	G protein-coupled receptor
GPI	Glycosylphosphatidylinositol
GST	Glutathione S-transferase
H&E	Haematoxylin and eosin

H2B	Histone 2B
HRP	Horseradish peroxidase
HSP	Heat-shock protein
IBD	Inflammatory bowel disease
IEF	Isoelectric focusing
IFN-g	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgG1	Immunoglobulin G subclass 1
IL	Interleukin
IM	Interstitial matrix
IQR	Interquartile range
ISC	Intestinal stem cell
kDa	Kilodalton
KO	Knock-out
LBS	Lysine binding sites
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LM	Laminin
LU	Lymphocyte antigen 6 and urokinase-type plasminogen activator receptor
Ly6	Lymphocyte antigen 6
M (cell)	Microfold cell
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
mPSIEC	Mouse small intestinal epithelial cells
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MT-MMP	Membrane-type matrix metalloproteinase
MW	Molecular weight
NF-κβ	Nuclear factor κβ
NOD	Nucleotide-binding and oligomerization domain-like receptors
NP-40	Nonidet P40
NTD	Neglected tropical disease
OD	Optical density

PA	Plasminogen activator
PAI	Plasminogen activator inhibitor
PAp	Pan-apple domain
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline containing Tween20
PCR	Polymerase chain reaction
PG	Proteoglycan
Pgp	P-glycoprotein
pI	Isoelectric point
PI3K	Phosphatidylinositol 3 kinase
Pigr	Polymeric immunoglobulin receptor
PKC	Protein kinase C
PLG	Plasminogen
PLG-R	Plasminogen receptor
PLG-R_{KT}	Plasminogen receptor KT
Plm	Plasmin
PLS-DA	Partial least squares discriminant analysis
pro-MMP	Precursor of metalloproteinase
pro-u-PA	Precursor of the urokinase-type plasminogen activator
PRR	Pathogen-recognition receptor
PTM	Post-translational modification
Qsox1	Quiescin sulfhydryl oxidase 1
Rab3b	Ras-related protein Rab3b
RFU	Relative fluorescent units
S1pr2	Sphingosine 1-phosphate receptor 2
ScENO	Saccharomyces cerevisae enolase
scu-PA	Single-chain urokinase-type plasminogen activator
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
Sema3d	Semaphorin 3D
SMB	Somatomedin B
SWATH-MS	Sequential window acquisition of all theoretical mass spectra

TAA	Tumour-associated antigen
TAFI	Thrombin activatable fibrinolysis inhibitor
TCA	Tricarboxylic acid
TCBZ	Triclabendazole
TCBZ-R	Triclabendazole-resistant
TCBZ-S	Triclabendazole-sensitive
tcu-PA	Two-chain urokinase-type plasminogen activator
TFA	Trifluoroacetic acid
TFG-b	Transforming growth factor beta
Th_1	Type 1 T-helper cell immune response
Th_{17}	Type 17 T-helper cell immune response
Th_2	Type 2 T-helper cell immune response
TIMP	Tissue inhibitor of metalloproteinases
TJ	Tight junction
TLR	Toll-like receptor
t-PA	Tissue-type plasminogen activator
TXA	Tranexamic acid
u-PA	Urokinase-type plasminogen activator
u-PAR	Urokinase-type plasminogen activator receptor
WHO	World Health Organization
ZO	Zonula occludens
ε-ACA	Aminocaproic acid

Appendices

Javier González Miguel, Científico Titular en el Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), y Mar Siles Lucas, Profesora de Investigación en el Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC),

CERTIFICAN:

Que el presente trabajo titulado «The role of the fibrinolytic system in the migration of *Fasciola hepatica* juveniles across the host intestine» ha sido realizado bajo su dirección por Judit Serrat Fernández en el Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), y reúne, a su juicio, las condiciones de originalidad y calidad científica requeridas para su presentación y defensa para optar al Grado de Doctor por la Universidad de Salamanca.

Y para que así conste a los efectos legales y oportunos, expiden y firman el presente certificado en Salamanca, a 5 de junio de 2025.

GONZALEZ MIGUEL JAVIER - DNI [redacted]
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Javier González Miguel

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Mar Siles Lucas

La presente Tesis Doctoral está elaborada en el formato de compendio de artículos/publicaciones según la normativa aprobada por la Comisión Ejecutiva de la Escuela de Doctorado de la Universidad de Salamanca el 12 de julio de 2024 y consta de las siguientes publicaciones:

1. *Fasciola hepatica* juveniles interact with the host fibrinolytic system as a potential early-stage invasion mechanism.

Judit Serrat, David Becerro-Recio, María Torres-Valle, Fernando Simón, María Adela Valero, María Dolores Bargues, Santiago Mas-Coma, Mar Siles-Lucas, and Javier González-Miguel

PLoS Neglected Tropical Diseases, 2023 Apr 21;17(4):e0010936.
doi: 10.1371/journal.pntd.0010936

Factor de impacto JCR (2023): 3.4; Q1 (Parasitology, 9 de 45)

2. Molecular characterisation of the interplay between *Fasciola hepatica* juveniles and laminin as a mechanism to adhere to and break through the host intestinal wall.

Judit Serrat, María Torres-Valle, Marta López-García, David Becerro-Recio, Mar Siles-Lucas, and Javier González-Miguel

International Journal of Molecular Sciences, 2023 May 3;24(9):8165.
doi: 10.3390/ijms24098165

Factor de impacto JCR (2023): 4.9; Q1 (Biochemistry and Molecular Biology, 66 de 313)

3. Binding and cleavage of pro-urokinase by a tegument extract of *Fasciola hepatica* newly excysted juveniles activate the host fibrinolytic system.

Judit Serrat, María Torres-Valle, Carolina de Marco-Verissimo, Mar Siles-Lucas, and Javier González-Miguel

Veterinary Research, 2025 Jan 25;56(1):20.
doi: 10.1186/s13567-025-01449-4

Factor de impacto JCR (2023): 3.7; Q1 (Veterinary Sciences, 10 de 167)

4. Early host parasite interaction models reveal a key role for fibrinolysis in *Fasciola hepatica* intestinal migration.

Judit Serrat, Marta López-García, María Torres-Valle, Verónica Molina-Hernández, María Teresa Ruiz-Campillo, Mar Siles-Lucas, and Javier González-Miguel

Manuscrito en proceso de revisión.
doi: 10.21203/rs.3.rs-6822677/v1

Javier González Miguel, Científico Titular del Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), y Mar Siles Lucas, Profesora de Investigación en el Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC),

AUTORIZAN:

Que la Tesis Doctoral titulada "The role of the fibrinolytic system in the migration of *Fasciola hepatica* juveniles across the host intestine" sea presentada en la modalidad de compendio de artículos/publicaciones (Comisión Ejecutiva de la Escuela de Doctorado de la Universidad de Salamanca, 12 de julio de 2024).

Y para que así conste, a los efectos legales, expiden y firman el presente certificado en Salamanca, a 5 de junio de 2025.

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Mar Siles Lucas

Resumen

Las enfermedades tropicales desatendidas (ETDs) comprenden un grupo de veinte enfermedades infecciosas que afectan colectivamente a más de 1500 millones de personas en todo el mundo, especialmente en países de bajos ingresos. Aunque las ETDs no se asocian con tasas elevadas de mortalidad, provocan una morbilidad considerable que socava la productividad económica, perpetuando así el ciclo de la pobreza (1). Una proporción significativa de las ETDs está causada por parásitos helmintos, y estimaciones recientes indican que más de mil millones de personas que habitan regiones del África subsahariana, Asia y América Central y del Sur están infectadas con al menos uno de estos organismos (2). Los helmintos también afectan a una gran proporción de animales domésticos utilizados en la producción ganadera, representando así una amenaza para el bienestar animal y la seguridad alimentaria global. Esta situación es particularmente crítica en regiones de bajos ingresos, en las que las ETDs son más prevalentes y donde se prevé un mayor crecimiento demográfico en las próximas décadas, lo que subraya la necesidad urgente de desarrollar sistemas de producción alimentaria más sostenibles y eficientes que garanticen el cumplimiento de los estándares nutricionales globales. En este contexto, los rumiantes son clave para la seguridad alimentaria en regiones áridas y semiáridas del planeta, donde el terreno no es apto para el cultivo, ya que transforman pastos y forrajes —indigeribles para el ser humano—en alimentos de alta calidad y ricos en proteínas (3).

Una de las infecciones parasitarias más prevalentes en rumiantes es la fasciolosis, una enfermedad causada por trematodos helmintos del género *Fasciola*, incluyendo *F. hepatica* y *F. gigantica*. Debido a su notable capacidad de adaptación a nuevos hospedadores y entornos, *F. hepatica* presenta una distribución cosmopolita, con casos de infección documentados en todos los continentes, excepto en la Antártida (4). Dado que estos parásitos poseen un importante potencial zoonótico, y que los casos de infección en humanos se concentran especialmente en zonas rurales de países de bajos ingresos, la Organización Mundial de la Salud clasifica la fasciolosis humana como una ETD de transmisión alimentaria que requiere intervenciones específicas para su eliminación (5,6). Los hospedadores definitivos de *F. hepatica*, incluidos los seres humanos, así como animales domésticos y salvajes, se infectan mediante la ingestión de metacercarias que suelen estar adheridas a plantas acuáticas comestibles, como el berro silvestre. Una vez ingeridas, las metacercarias excistan en el duodeno y liberan los juveniles recién excistados (FhNEJ, por sus siglas en inglés), los cuales atraviesan la pared intestinal y migran a través del peritoneo y el parénquima hepático hasta alcanzar los conductos biliares hepáticos

mayores, donde los parásitos maduran hasta su fase adulta y producen huevos que se eliminan con las heces (7).

El tratamiento estándar para la fasciolosis es el triclabendazol (TCBZ), uno de los pocos antihelmínticos disponibles que es eficaz tanto contra las formas juveniles como adultas del parásito. No obstante, la aparición continua de cepas resistentes al TCBZ pone de relieve la necesidad urgente de desarrollar nuevas estrategias terapéuticas y de control frente a este parásito (8). En este sentido, la vacunación representa una alternativa prometedora al tratamiento farmacológico, dado su potencial de proporcionar un control rentable, sostenible y duradero de la fasciolosis. Hasta la fecha, se han realizado numerosos esfuerzos para desarrollar una vacuna contra *F. hepatica*, aunque ninguno de ellos ha alcanzado los niveles de protección requeridos para su comercialización. La mayoría de estos estudios se ha enfocado en combatir las formas adultas del parásito, las cuales son altamente eficaces en la evasión del sistema inmunitario y habitan en un nicho en gran medida inaccesible para los mecanismos de defensa del hospedador (9). En vista de esto, una estrategia vacunal dirigida a los estadios más tempranos de *F. hepatica* podría ofrecer un enfoque más exitoso para el control de la fasciolosis. Para ello, resulta fundamental profundizar en el conocimiento de la biología de las formas juveniles de *F. hepatica*, incluyendo una caracterización más profunda de las interacciones parásito-hospedador que favorecen su crecimiento, migración y supervivencia dentro del organismo de los mamíferos en los que se hospedan (3,10).

La migración tisular es una característica distintiva de los ciclos de vida de muchos parásitos helmintos que se ha conservado a lo largo de la evolución. Este proceso se ve facilitado tanto por la motilidad del parásito como por la acción de proteasas secretadas por el mismo que degradan componentes tisulares del hospedador que, de otro modo, obstaculizarían su migración. Además, los mecanismos endógenos que permiten la migración tisular se ven complementados por la capacidad de los parásitos de secuestrar la maquinaria proteolítica del hospedador mamífero. Un ejemplo paradigmático de este fenómeno es la interacción de parásitos tisulares y/o hematógenos con el sistema fibrinolítico del hospedador a través de la expresión de proteínas que reclutan el zimógeno plasminógeno y promueven su conversión en plasmina, la principal proteasa fibrinolítica (11,12). En la actualidad, se desconoce si las etapas juveniles de *F. hepatica* emplean una estrategia similar para facilitar su migración a través de los tejidos antes de establecerse definitivamente en los conductos biliares.

En base a estas consideraciones, el objetivo principal de esta tesis es ampliar el conocimiento sobre las relaciones parásito-hospedador que se establecen durante las etapas iniciales de la fasciolosis, con un enfoque particular en la posible interacción entre los FhNEJ y el sistema fibrinolítico del hospedador, así como el papel que esta interacción podría desempeñar en la migración de estos parásitos a través del intestino de su hospedador definitivo. Este conocimiento podría llevar a la identificación de moléculas parasitarias susceptibles de ser objeto de intervenciones terapéuticas o inmunológicas, lo que contribuiría al desarrollo de nuevas estrategias destinadas a impedir la maduración del parásito y bloquear la transmisión de la enfermedad entre las poblaciones afectadas.

Referencias

1. Hotez PJ, Molyneux DH, Fenwick A, Kumaresan J, Sachs SE, Sachs JD, *et al.* Control of neglected tropical diseases. N Engl J Med. 2007 Sep 6;357(10):1018–27.
2. Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. Helminth infections: the great neglected tropical diseases. J Clin Invest. 2008 Apr 1;118(4):1311–21.
3. Fitzpatrick JL. Global food security: the impact of veterinary parasites and parasitologists. Vet Parasitol. 2013 Aug 1;195(3–4):233–48.
4. Nyindo M, Lukambagire AH. Fascioliasis: an ongoing zoonotic trematode infection. Biomed Res Int. 2015;2015:786195.
5. World Health Organization. Ending the neglect to attain the Sustainable Development Goals: a road map for Neglected Tropical Diseases 2021-2030. Geneva: World Health Organization; 2020.
6. Pan American Health Organization (PAHO). Operational guidelines for the elimination of human fascioliasis as a public health problem in the Americas. Pan American Health Organization; 2024.
7. Siles-Lucas M, Becerro-Recio D, Serrat J, González-Miguel J. Fascioliasis and fasciolopsiasis: current knowledge and future trends. Res Vet Sci. 2021 Jan;134:27–35.
8. Kelley JM, Elliott TP, Beddoe T, Anderson G, Skuce P, Spithill TW. Current threat of triclabendazole resistance in *Fasciola hepatica*. Trends Parasitol. 2016 Jun;32(6):458–69.

9. Deslyper G, Doherty DG, Carolan JC, Holland CV. The role of the liver in the migration of parasites of global significance. Parasites Vectors. 2019 Dec;12(1):531.

10. González-Miguel J, Becerro-Recio D, Siles-Lucas M. Insights into *Fasciola hepatica* juveniles: crossing the fasciolosis rubicon. Trends Parasitol. 2021 Jan;37(1):35–47.

11. González-Miguel J, Siles-Lucas M, Kartashev V, Morchón R, Simón F. Plasmin in parasitic chronic infections: friend or foe? Trends Parasitol. 2016 Apr;32(4):325–35.

12. Diosdado A, Simón F, Serrat J, González-Miguel J. Interaction of helminth parasites with the haemostatic system of their vertebrate hosts: a scoping review. Parasite. 2022;29:35.

Conclusiones

1. Las formas juveniles recién excistadas de *Fasciola hepatica* (FhNEJ, por sus siglas en inglés) interaccionan con el sistema fibrinolítico de su hospedador definitivo mediante la expresión de proteínas en su superficie tegumentaria que se unen a los zimógenos plasminógeno (PLG) y al precursor del activador de PLG de tipo uroquinasa (pro-u-PA) de manera dependiente e independiente de lisina, respectivamente, replicando el mecanismo de interacción de estos factores con sus receptores fisiológicos.

2. La interacción entre FhNEJ y el PLG es funcionalmente relevante, ya que conduce a la generación de plasmina—la principal proteasa fibrinolítica—en presencia de activadores del PLG derivados del hospedador.

3. Las catepsinas específicas de juveniles FhCB2, FhCB3 y FhCL3, así como la glutatión S-transferasa y la enolasa, han sido identificadas como proteínas del tegumento de FhNEJ con capacidad de unión a PLG y/o pro-u-PA, lo que resalta una nueva función *moonlighting* de estas proteínas.

4. Los FhNEJ expresan una o más proteasas en su superficie tegumentaria capaces de convertir pro-u-PA en su forma catalíticamente activa, lo que permite la generación de plasmina a partir del PLG del hospedador. Así, la interacción entre FhNEJ y el sistema fibrinolítico del hospedador es bimodal: implica tanto la unión a PLG para promover la generación de plasmina como la activación de pro-u-PA.

5. Los FhNEJ expresan proteínas en su superficie tegumentaria que se unen a la laminina, un componente principal de la matriz extracelular (MEC) intestinal, lo que podría facilitar la adhesión de estos parásitos al epitelio intestinal antes de su migración transintestinal.

6. Se demuestra que la catepsina FhCL3, específica de las formas juveniles, es capaz de degradar la laminina y que la capacidad endógena de los FhNEJ para degradar esta proteína se ve potenciada por la generación de plasmina inducida por estos parásitos a partir del PLG del hospedador.

7. La generación de plasmina a partir del PLG del hospedador, inducida por los FhNEJ en el espacio pericelular de células epiteliales del intestino delgado de ratón, promueve la secreción de u-PA por parte de las células del hospedador y estimula la degradación de colágeno, así como la expresión diferencial de proteínas relacionadas con la remodelación de la MEC, la fibrinólisis y la inmunidad intestinal.

8. Mediante el uso de un modelo murino de fasciolosis, esta tesis aporta la primera evidencia funcional de que las formas juveniles de *F. hepatica* explotan el sistema fibrinolítico de su hospedador vertebrado para facilitar su migración a través de los tejidos durante la fase prehepática de la infección, revelando así un mecanismo patogénico clave y una potencial diana terapéutica.

Resúmenes de las publicaciones

CAPÍTULO 1. Las formas juveniles de *Fasciola hepatica* interaccionan con el sistema fibrinolítico del hospedador como posible mecanismo de invasión en las etapas tempranas de la infección

El trematodo *Fasciola hepatica* constituye el agente etiológico más ampliamente distribuido de la fasciolosis, una enfermedad parasitaria que afecta principalmente a humanos y rumiantes a nivel mundial. Durante la infección por *F. hepatica*, las formas juveniles recién excistadas (FhNEJ, por sus siglas en inglés) emergen en el duodeno del hospedador mamífero y migran hacia su localización definitiva, los conductos biliares intrahepáticos. Comprender los mecanismos mediante los cuales *F. hepatica* atraviesa la pared intestinal y migra hacia el hígado resulta crucial para el desarrollo de estrategias más eficaces contra la fasciolosis. La enzima central del sistema fibrinolítico de los mamíferos es la plasmina, una serina proteasa cuya amplia especificidad de sustrato—incluyendo componentes de las matrices extracelulares de los tejidos—es explotada por diversas especies parasitarias. El objetivo del presente estudio es determinar si los FhNEJ aprovechan las funciones del sistema fibrinolítico del hospedador como un mecanismo para facilitar su migración transintestinal. Con este fin, se obtuvo un extracto antigénico enriquecido en tegumento de FhNEJ (FhNEJ-Teg) *in vitro*, y posteriormente se analizó su capacidad para unirse al zimógeno plasminógeno (PLG) y potenciar su conversión a plasmina mediante ensayos inmuno-enzimáticos, cromogénicos y de inmunofluorescencia. Asimismo, se identificaron las proteínas con capacidad de unión a PLG presentes en FhNEJ-Teg mediante electroforesis bidimensional acoplada a espectrometría de masas, y dichas interacciones fueron validadas utilizando proteínas recombinantes. Nuestros resultados demuestran que el FhNEJ-Teg contiene proteínas capaces de unirse al PLG y de estimular su activación a plasmina, lo cual podría facilitar la migración de los parásitos a través de la pared intestinal y contribuir a su establecimiento dentro del hospedador mamífero. En conjunto, nuestros hallazgos enriquecen el conocimiento actual acerca las relaciones parásito-hospedador que se establecen durante las etapas iniciales de la fasciolosis, los cuales podrían ser aprovechados desde una perspectiva farmacológica y/o inmunológica para el desarrollo de estrategias de tratamiento y control más efectivas contra esta enfermedad de relevancia global.

CAPÍTULO 2. Caracterización molecular de la interacción entre las formas juveniles de *Fasciola hepatica* y la laminina como mecanismo de adhesión y penetración de la pared intestinal del hospedador

Fasciola hepatica es el principal agente etiológico de la fasciolosis, una enfermedad parasitaria zoonótica de creciente preocupación en el ámbito de la salud pública. Las metacercarias de *F. hepatica* son ingeridas por el hospedador y excistan en el intestino, liberando así las formas juveniles recién excistadas (FhNEJ, por sus siglas en inglés), las cuales atraviesan la pared intestinal y migran hacia los conductos biliares. Teniendo en cuenta que bloquear del desarrollo de *F. hepatica* es considerablemente más complejo una vez que los parásitos han atravesado la pared intestinal, centrar los esfuerzos terapéuticos hacia las fases inmaduras del parásito podría representar una estrategia más efectiva para el control de la fasciolosis. La matriz extracelular (MEC) intestinal está compuesta por una red de proteínas estructurales, entre ellas la laminina (LM) y la fibronectina (FN), que proporcionan soporte mecánico y actúan como barrera física frente a patógenos intestinales. En este estudio, se utilizaron ensayos inmunoenzimáticos y de inmunofluorescencia para evaluar la presencia de proteínas con capacidad de unión a LM y FN en un extracto antigénico enriquecido en tegumento de FhNEJ. Posteriormente, dichas proteínas fueron identificadas mediante electroforesis bidimensional acoplada a espectrometría de masas. Adicionalmente, se llevaron a cabo ensayos enzimáticos que, por primera vez, evidenciaron la capacidad de la catepsina L3—específica de los estadios juveniles—para degradar la laminina, y que dicha degradación se ve potenciada en presencia del plasminógeno del hospedador. Finalmente, un análisis proteómico reveló que la interacción con la laminina induce cambios en el repertorio proteico de los FhNEJ que podrían ser relevantes para el crecimiento y adaptación del parásito en el interior del hospedador mamífero. En conjunto, este estudio ofrece información valiosa sobre la interacción molecular entre FhNEJ y la MEC intestinal, lo cual podría conducir a la identificación de candidatos que podrían representar posibles dianas para el desarrollo de estrategias de control más eficaces contra la fasciolosis.

CAPÍTULO 3. La unión y escisión de la pro-uroquinasa por un extracto del tegumento de las formas juveniles recién excistadas de *Fasciola hepatica* activan el sistema fibrinolítico del hospedador

La plasmina, producto final del proceso de fibrinólisis, es una serina proteasa de amplio espectro que degrada componentes de la matriz extracelular (MEC), una función que es aprovechada por diversos patógenos para facilitar su diseminación a través del organismo. El trematodo *Fasciola hepatica* constituye el principal agente causal de la fasciolosis, una enfermedad de gran impacto en la ganadería y una zoonosis emergente en humanos. El éxito de la infección depende de la capacidad de las formas juveniles recién excistadas (FhNEJ, por sus siglas en inglés) para penetrar la pared intestinal del hospedador, un proceso que aún no se comprende en su totalidad. Estudios previos llevados a cabo en nuestro laboratorio han demostrado que los FhNEJ son capaces de unir plasminógeno (PLG), el zimógeno de la plasmina, en la superficie de su tegumento, lo que conduce a la generación de la proteasa catalíticamente activa en presencia de activadores de PLG derivados del hospedador y a la consiguiente degradación de la laminina, un componente central de la MEC intestinal. En el presente trabajo, describimos la interacción entre un extracto tegumentario de FhNEJ y el precursor del activador de PLG de tipo uroquinasa (pro-u-PA). Nuestros resultados demuestran que las catepsinas B3 y L3 de *F. hepatica*, así como la enolasa y la glutatión S-transferasa, median esta interacción, lo que sugiere un papel multifuncional o "moonlighting" para estas proteínas. Además, nuestros resultados revelan que el tegumento de los FhNEJ contiene alguna proteasa capaz de escindir y activar el pro-u-PA en su forma catalíticamente activa, lo cual incrementa la capacidad del parásito para generar plasmina a partir del PLG del hospedador. En su conjunto, nuestros resultados evidencian que los FhNEJ interaccionan con el sistema fibrinolítico del hospedador mediante múltiples mecanismos, lo que destaca el potencial de esta interacción como diana terapéutica para el bloqueo de la migración transintestinal de los FhNEJ y su establecimiento exitoso dentro del hospedador vertebrado.

CAPÍTULO 4. Modelos experimentales de interacción temprana entre parásito y hospedador destacan el papel fundamental de la fibrinólisis en la migración intestinal de *Fasciola hepatica*

Fasciola hepatica representa el agente etiológico más extendido de la fasciolosis, una enfermedad parasitaria que afecta a millones de rumiantes en todo el mundo y que constituye una infección zoonótica de importancia en salud pública. Tras la ingestión de metacercarias infectivas, los juveniles recién excistados de *F. hepatica* (FhNEJ) emergen en el duodeno y atraviesan la pared intestinal para iniciar una ruta migratoria que culmina con su establecimiento en los conductos biliares hepáticos. Se ha propuesto que la capacidad de los FhNEJ para explotar las actividades de amplio espectro de la plasmina del hospedador, la proteasa central del sistema fibrinolítico, constituye una estrategia utilizada por estos parásitos para atravesar la pared intestinal minimizando el gasto energético. Para investigar si la generación de plasmina inducida por los FhNEJ modula procesos relevantes para la migración parasitaria, incluidas la remodelación de la matriz extracelular (MEC) y la secreción de enzimas que la degradan, se estimularon células epiteliales intestinales murinas (mPSIEC) con FhNEJ y plasminógeno (PLG), el zimógeno de la plasmina. Las respuestas celulares mediadas por plasmina se examinaron adicionalmente mediante un análisis proteómico de las mPSIEC. En paralelo, se estudió la participación del sistema fibrinolítico en la migración de los FhNEJ mediante infecciones experimentales en ratones previamente tratados con un inhibidor farmacológico de la fibrinólisis. Nuestros resultados mostraron que la coestimulación de mPSIEC con FhNEJ y PLG produjo un aumento en la generación de plasmina en el espacio pericelular intestinal, lo que se asocia con una mayor degradación del colágeno y una secreción aumentada del activador de PLG de tipo uroquinasa. Además, mediante réplicas independientes de cultivos celulares y un riguroso análisis estadístico, se definió un conjunto de proteínas diferencialmente expresadas en mPSIEC tras la estimulación con FhNEJ y PLG. Estas proteínas participan en procesos biológicos relacionados con adhesión y migración celular, remodelación de la MEC, evasión de la respuesta inmunitaria y fibrinólisis. Finalmente, a pesar de la variabilidad observada entre experimentos, la inhibición farmacológica de la fibrinólisis previa a la infección con metacercarias de *F. hepatica* redujo la migración de los FhNEJ en ratones. En conjunto, este trabajo proporciona conocimientos sin precedentes sobre el papel del sistema fibrinolítico en la migración de los FhNEJ a través de los tejidos del hospedador, ampliando así nuestro conocimiento sobre las interacciones parásito-hospedador en las fases iniciales de la fasciolosis y señalando nuevas direcciones para futuras investigaciones en este ámbito.

Resum (en català)

Les malalties tropicals desateses (MTDs) constitueixen un conjunt de vint malalties infeccioses que afecten col·lectivament més de 1500 milions de persones arreu del món, especialment en països de renda baixa. Tot i que les MTDs no tenen associades elevades taxes de mortalitat, provoquen una morbiditat considerable que minva la productivitat econòmica i, per tant, contribueixen a perpetuar el cicle de la pobresa (1). Una proporció significativa de les MTDs són causades per paràsits helmints, i estimacions recents indiquen que més de mil milions de persones que viuen a l'Àfrica subsahariana, Àsia i Amèrica Central i del Sud estan infectades amb almenys un d'aquests organismes (2). A més, els paràsits helmints afecten una gran part dels animals domèstics utilitzats en ramaderia, representant així una amenaça per al benestar animal i la seguretat alimentària. Aquest fet és especialment important a les regions de renda més baixa, on les MTDs són més prevalents i on es preveu un major creixement demogràfic durant les pròximes dècades, fet que evidencia la necessitat d'establir sistemes de producció alimentària més sostenibles i eficients que garanteixin el compliment dels estàndards nutricionals globals. En aquest sentit, els remugants utilitzats en ramaderia són essencials per a la seguretat alimentària en regions àrides i semiàrides del planeta on el terreny no és apte per al conreu, ja que transformen pastures i farratges—indigeribles per a l'ésser humà—en aliments de qualitat i rics en proteïnes (3).

Una de les infeccions parasitàries més prevalents en remugants és la fasciolosi, una malaltia causada per trematodes helmints del gènere *Fasciola*; incloent-hi *F. hepatica* i *F. gigantica*. *F. hepatica*, degut a la seva extraordinària capacitat d'adaptació a nous hostes i entorns, presenta una distribució cosmopolita, amb casos d'infecció registrats a tots els continents excepte l'Antàrtida (4). Atès que aquests paràsits tenen un potencial zoonòtic important i que els casos d'infecció en humans es concentren principalment en àrees rurals de països de renda baixa, l'Organització Mundial de la Salut classifica la fasciolosi humana com una MTD transmesa per aliments que requereix intervencions específiques per a la seva eliminació (5,6). Els hostes definitius de *F. hepatica*, que inclouen l'ésser humà així com animals domèstics i salvatges, s'infecten en ingerir metacercàries, habitualment adherides a plantes aquàtiques com el créixens silvestre. Un cop ingerides, les metacercàries excisten al duodè i alliberen els anomenats juvenils recent excistats (FhNEJ, per les seves sigles en anglès), que travessen la paret intestinal i migren pel peritoneu i el parènquima hepàtic fins a arribar als conductes biliars hepàtics majors, on els paràsits adults maduren i produeixen ous que s'eliminen amb les femtes (7).

El tractament estàndard per a la fasciolosi és el triclabendazol (TCBZ), un dels pocs antihelmíntics eficaços contra les etapes juvenils i adultes del paràsit. No obstant això, l'aparició progressiva de paràsits resistents al TCBZ posa de manifest la necessitat urgent de noves estratègies terapèutiques i de control (8). En aquest context, la vacunació representa una alternativa prometedora al tractament farmacològic, donat el seu potencial d'oferir un control de la fasciolosi que sigui rendible, sostenible i a llarg termini. Fins a l'actualitat, s'han dut a terme nombrosos esforços per desenvolupar una vacuna contra *F. hepatica*, però cap d'ells ha assolit els nivells de protecció necessaris. La majoria d'aquests estudis s'han centrat en els paràsits adults, els quals són altament eficients en l'evasió del sistema immunitari i resideixen a una ubicació poc accessible per als mecanismes de defensa de l'hoste (9). Davant d'això, una estratègia vacunal dirigida a les etapes juvenils de *F. hepatica* podria oferir una via més efectiva per al control de la fasciolosi. Per tal d'aconseguir-ho, és essencial conèixer més detalladament la biologia d'aquests paràsits, incloent una caracterització més profunda de les interaccions hoste-paràsit que sustenten el seu creixement, migració i supervivència dins l'organisme de l'hoste mamífer (3,10).

La migració tissular és una característica distintiva dels cicles vitals de molts paràsits helmints que s'ha conservat al llarg de l'evolució. Aquest procés es veu facilitat tant per la motilitat dels paràsits com per l'acció de proteases secretades que degraden components tissulars de l'hoste que, altrament, obstaculitzarien la seva migració. A més, els mecanismes endògens que afavoreixen la migració tissular es complementen amb la capacitat dels paràsits d'apropiar-se de la maquinària proteolítica de l'hoste mamífer. Un exemple paradigmàtic d'aquest fenomen és la interacció de paràsits tissulars o hematògens amb el sistema fibrinolític de l'hoste mitjançant l'expressió de proteïnes capaces de reclutar el zimogen plasminogen i promoure la seva conversió a plasmina, la principal proteasa d'aquest sistema (11,12). Actualment, es desconeix si les etapes juvenils pre-hepàtiques de *F. hepatica* empren una estratègia similar per facilitar la seva migració tissular abans d'establir-se definitivament als conductes biliars.

En base a aquestes consideracions, l'objectiu principal d'aquesta tesi és ampliar el coneixement sobre les interaccions hoste-paràsit durant les etapes inicials de la fasciolosi, posant èmfasi en la possible interacció entre les FhNEJ i el sistema fibrinolític, així com el paper que aquesta interacció podria desenvolupar en la migració d'aquests paràsits a través de l'intestí del seu hoste definitiu. Aquest coneixement podria facilitar la identificació de molècules parasitàries susceptibles de ser objecte d'intervencions terapèutiques o immunològiques, contribuint així al desenvolupament de noves estratègies

que impedeixin la maduració del paràsit i interrompin la transmissió de la malaltia entre les poblacions afectades.

Referències

1. Hotez PJ, Molyneux DH, Fenwick A, Kumaresan J, Sachs SE, Sachs JD, *et al*. Control of neglected tropical diseases. N Engl J Med. 2007 Sep 6;357(10):1018–27.
2. Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. Helminth infections: the great neglected tropical diseases. J Clin Invest. 2008 Apr 1;118(4):1311–21.
3. Fitzpatrick JL. Global food security: the impact of veterinary parasites and parasitologists. Vet Parasitol. 2013 Aug 1;195(3–4):233–48.
4. Nyindo M, Lukambagire AH. Fascioliasis: an ongoing zoonotic trematode infection. Biomed Res Int. 2015;2015:786195.
5. World Health Organization. Ending the neglect to attain the Sustainable Development Goals: a road map for Neglected Tropical Diseases 2021-2030. Geneva: World Health Organization; 2020.
6. Pan American Health Organization (PAHO). Operational guidelines for the elimination of human fascioliasis as a public health problem in the Americas. Pan American Health Organization; 2024.
7. Siles-Lucas M, Becerro-Recio D, Serrat J, González-Miguel J. Fascioliasis and fasciolopsiasis: current knowledge and future trends. Res Vet Sci. 2021 Jan;134:27–35.
8. Kelley JM, Elliott TP, Beddoe T, Anderson G, Skuce P, Spithill TW. Current threat of triclabendazole resistance in *Fasciola hepatica*. Trends Parasitol. 2016 Jun;32(6):458–69.
9. Deslyper G, Doherty DG, Carolan JC, Holland CV. The role of the liver in the migration of parasites of global significance. Parasites Vectors. 2019 Dec;12(1):531.
10. González-Miguel J, Becerro-Recio D, Siles-Lucas M. Insights into Fasciola hepatica juveniles: crossing the fasciolosis rubicon. Trends Parasitol. 2021 Jan;37(1):35–47.
11. González-Miguel J, Siles-Lucas M, Kartashev V, Morchón R, Simón F. Plasmin in parasitic chronic infections: friend or foe? Trends Parasitol. 2016 Apr;32(4):325–35.

12. Diosdado A, Simón F, Serrat J, González-Miguel J. Interaction of helminth parasites with the haemostatic system of their vertebrate hosts: a scoping review. Parasite. 2022;29:35.

Conclusions (en català)

1. Les formes juvenils recentment excistades de *Fasciola hepatica* (FhNEJ) interaccionen amb el sistema fibrinolític de l'hoste definitiu mitjançant l'expressió de proteïnes a la seva superfície que s'uneixen als zimògens plasminogen (PLG) i al precursor de l'activador de PLG de tipus uroquinasa (pro-u-PA) de manera dependent i independent de lisina, respectivament, replicant el mecanisme d'interacció d'aquests factors amb els seus receptors fisiològics.

2. La interacció entre els FhNEJ i el PLG de l'hoste és funcionalment rellevant, ja que condueix a la generació de plasmina—la principal proteasa fibrinolítica—en presència d'activadors del PLG derivats de l'hoste.

3. Les catepsines específiques de les formes juvenils FhCB2, FhCB3 i FhCL3, així com la glutatió S-transferasa i l'enolasa, han estat identificades com a proteïnes del tegument dels FhNEJ amb capacitat de fixació al PLG i/o al pro-u-PA, destacant una nova funció *moonlighting* d'aquestes proteïnes.

4. Els FhNEJ expressen una o més proteases a la seva superfície tegumentària capaces de convertir el pro-u-PA en la seva forma catalíticament activa, evitant així la necessitat d'u-PA exogen per a la generació de plasmina a partir del PLG de l'hoste. Per tant, la interacció entre FhNEJ i el sistema fibrinolític de l'hoste és bimodal: implica tant la unió al PLG per promoure la generació de plasmina com l'activació del pro-u-PA.

5. Els FhNEJ expressen proteïnes a la seva superfície tegumentària que s'uneixen a la laminina, un component principal de la matriu extracel·lular (MEC) intestinal, fet que podria facilitar l'adhesió d'aquests paràsits a l'epiteli intestinal abans de la seva migració transintestinal.

6. Es demostra que la catepsina FhCL3, específica de les formes juvenils, és capaç de degradar la laminina, i que la capacitat endògena dels FhNEJ per degradar aquesta proteïna de la MEC es veu incrementada per la generació de plasmina induïda per aquests paràsits a partir del PLG de l'hoste.

7. La generació de plasmina a partir del PLG de l'hoste, induïda pels FhNEJ a l'espai pericel·lular de les cèl·lules epitelials de l'intestí prim de ratolí, promou la secreció d'u-PA per part de les cèl·lules de l'hoste i estimula la degradació de col·lagen, així com l'expressió diferencial de proteïnes relacionades amb la remodelació de la MEC, la fibrinòlisi i la immunitat intestinal.

8. Mitjançant l'ús d'un model murí de fasciolosi, aquesta tesi aporta la primera evidència funcional que les formes juvenils de *F. hepatica* exploten la fibrinòlisi de l'hoste per facilitar la seva migració a través dels teixits durant la fase prehepàtica de la infecció, revelant així un mecanisme patogènic clau i una possible diana terapèutica.