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Study of the cross-talk between *Fasciola hepatica* juveniles and the intestinal epithelial cells of the host by transcriptomics in an *in vitro* model

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ABSTRACT

Fasciolosis is a globally widespread trematodiasis with a major economic and veterinary impact. Therefore, this disease is responsible for millions of dollars in losses to the livestock industry, and also constitutes an emerging human health problem in endemic areas. The ubiquitous nature of *Fasciola hepatica*, the main causative agent, is one of the key factors for the success of fasciolosis. Accordingly, this parasite is able to subsist in a wide variety of ecosystems and hosts, thanks to the development of a plethora of strategies for adaption and immune evasion. Fasciolosis comprises a growing concern due to its high prevalence rates, together with the emergence of strains of the parasite resistant to the treatment of choice (triclabendazole). These facts highlight the importance of developing novel control measures which allow for an effective protection against the disease before *F. hepatica* settles in a niche inaccessible to the immune system. However, knowledge about the initial phases of the infection, including the migration mechanisms of the parasite and the early innate host response, is still scarce. Recently, our group developed an *in vitro* host-parasite interaction model that allowed the early events to be unveiled after the first contact between the both actors. This occurs shortly upon ingestion of *F. hepatica* metacercariae and the emergence of the newly excysted juveniles (FhNEJ) in the host duodenum. Here, we present a transcriptomic analysis of such model using an approach based on RNA sequencing (RNA-Seq), which reveals changes in gene expression related to proteolysis and uptake of metabolites in FhNEJ. Additionally, contact with the parasite triggered changes in host intestinal cells related to pseudogenes expression and host defence mechanisms, including immune response, among others. In sum, these results provide a better understanding of the early stages of fasciolosis at molecular level, and a pool of targets that could be used in future therapeutic strategies against the disease.

1. Introduction

Foodborne trematodiasis comprise a heterogeneous group of diseases caused by helminth parasites delivered to their definitive host through contaminated food or water. These are responsible for high morbidity in both humans and animals and constitute an emerging health issue, especially in low-income countries (Robinson and Sotillo, 2022). Despite their importance, which includes *ca.* 56 million people affected and substantial economic losses to livestock worldwide, these diseases remain poorly documented (Fürst et al. 2012). Generally, foodborne trematodes undergo a migratory cycle while renewing their antigenic repertoire that involves several host organs before settling into a definitive niche. This strategy confers these parasites with evolutionary advantages in terms of ability to avoid immune destruction by the vertebrate host (Ryan et al., 2020). Migration route of the fluke *Fasciola hepatica* begins with the ingestion of metacercariae. These excyst in the duodenum releasing the newly excysted juveniles (FhNEJ) that are able to cross the gut wall within 2–3 h after infection. Then, immature parasites crawl up the peritoneum towards the liver, where the migratory process continue until they find their definitive location, the biliary ducts, where *F. hepatica* reaches maturity (Siles-Lucas et al. 2021). The passage of the FhNEJ through the intestine is regarded as the

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'point of no return' in fasciolosis, as the success of this process appears to be the major determinant for the overall outcome of the infection (González-Miguel et al. 2021). Therefore, a thorough understanding of the events governing early host-parasite contact is of paramount importance for the effective treatment of the disease.

Over the last decades, different experimental models have been proposed aiming to reproduce in the laboratory the early stages of the contact between FhNEJ and their definitive host, using *ex vivo* (Van Milligen et al., 1998; Becerro-Recio et al. 2022a) or *in vitro* (Garcia--Campos et al., 2016; González-Miguel et al. 2020; Becerro-Recio et al. 2022b) approaches. Such models provide direct evidence of the passage of FhNEJ through the intestinal wall. Either alone or in combination with these models, -omic technologies have revealed themselves as a valuable tool for the study of *F. hepatica* (Cwiklinski et al. 2018) and other foodborne trematodes (Pakharukova et al. 2022). Thereby, these methodologies facilitate the molecular dissection of the host-parasite interface by examining the behaviour of hundreds or even thousands of biological features (*e.g.*, genes, proteins, metabolites) in a single experiment. This allows to identify key mechanisms for parasite migration and survival and to determine changes in their expression along time or in response to certain stimuli. Additionally, host responses can be similarly characterised in order to determine the processes triggered by the first contact with the parasite, as well as the incipient defensive mechanisms that occur early in the course of infection.

Recently, our group developed an *in vitro* proteomic approach on a co-culture consisting on mouse primary small intestinal epithelial cells (MPSIEC) together with FhNEJ. In this way, we performed analyses by sequential window acquisition of all theoretical mass spectra (SWATH-MS) to identify changes in protein expression as a consequence of parasite contact with the intestinal lumen (Becerro-Recio et al. 2022b). After stimulation, parasite and host cells showed a clear deviation towards down-regulation, with a total of 210 and 133 differentially expressed proteins in *F. hepatica* and mouse cells, respectively. These were involved in processes such as proteolysis and its regulation, antioxidant response or metabolic adaptation in FhNEJ, or ribosomal function, cytoskeletal regulation and cell adhesion processes in MPSIEC, among others. The present study shows a complementary approach at the transcriptomic level, aiming to identify changes in mRNA expression.

2. Materials and methods

2.1. MPSIEC cell culture

MPSIEC obtained from C57BL/6 mice (Cell Biologics) (ref. C57–6051) consisting on mouse primary small intestinal epithelial cells, were cultured in 60 mm diameter Petri dishes previously coated with 0.2% porcine gelatine, and incubated in epithelial cell medium (Innoprot) supplemented with 2% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% epithelial cell growth factor. Cells were cultured at 37 ºC in a humidified atmosphere containing 5% CO_2, and culture medium was replaced every 24–48 h. Whenever cells reached confluence, they were split in a 1:3 ratio. All the described experiments were performed using passage 1–5 cultures (Becerro-Recio et al. 2022b).

2.2. F. hepatica metacercariae in vitro excystment

F. hepatica Italian strain metacercariae, acquired from Ridgeway Research LTD (UK), were excysted *in vitro* using a methodology previously described in the literature (Hernández-González et al. 2010). Briefly, a sterile tube containing 10 ml of distilled water was supplemented with gaseous CO_2 and sodium dithionite (Sigma-Aldrich) to a final concentration of 0.02 M, whereupon the tube was incubated at 37 ºC for 5 min. At this point, the metacercariae were added to the mixture and incubated at 37 ºC for 1 h. After this, the metacercariae were washed thrice with distilled water and resuspended in an excystment medium containing Hank's balanced salt solution (Sigma-Aldrich), 0.03 M HEPES (Sigma-Aldrich) and 10% lamb bile (obtained from a local abattoir). Metacercariae were disposed into a 6-well plate and incubated at 37 ºC for 3–5 h, and during this time the excystment process was monitored under the microscope, collecting the FhNEJ with a micropipette as they were released from the metacercariae. Finally, the FhNEJ were washed twice with sterile PBS and immediately put into contact with the MPSIEC.

2.3. Co-culture model

The methodology used to replicate the host-parasite interaction was previously developed by our group (González-Miguel et al., 2020; Becerro-Recio et al. 2022b). In brief, passage 5 MPSIEC were cultured in 60 mm gelatin-coated plates (n = 3) and grown in epithelial cell growth medium (Innoprot) until cell confluence was reached. Then, freshly excysted FhNEJ were placed on MPSIEC culture plates at a rate of 500 parasites per plate. The co-culture was incubated for 3 h at 37 °C in a 5% CO_2 atmosphere, after which MPSIEC and FhNEJ were separated by successive washes with sterile PBS. Negative non-stimulated controls consisted of 3 batches of 500 FhNEJ and 3 confluent plates of passage 5 MPSIEC separately incubated under the same conditions (growth medium and incubation time, temperature and atmosphere) than the co-culture. All cultures were performed in triplicate (n = 3). Number of intestinal epithelial cells per plate and number of FhNEJ for *in vitro* stimulation experiments were settled to maximize the chances to have the majority of cells in contact with the FhNEJ and thus properly stimulated and to obtain enough stimulated host and parasite material for downstream analyses, although this specific host cells/parasite ratio could not reflect the *in vivo* situation.

2.4. Total RNA extraction and high-throughput RNA-Seq

Total RNA extraction from MPSIEC was performed using the RNeasy Mini kit (Qiagen), whereas for FhNEJ the miRNeasy Mini kit (Qiagen) was used together with the QIAshredder (Qiagen) cell homogenizer columns. In both cases, extraction was performed according to manufacturer's instructions including a DNaseI treatment step, and total RNA was stored at − 80 ºC until use. Quality of every RNA sample was assessed *via* the Thermo Scientific NanoDrop ND-1000 spectrophotometer so as to determine its concentration along with the A260/A280 and A260/A230 ratios, and further *via* the Agilent 2100 Bioanalyzer system in order to determine the RNA Integrity Number (RIN) of each sample. Only samples with a RIN > 7 were subjected to Illumina TruSeq RNA library preparation. Due to the particular nature of *F. hepatica* ribosomal RNA (Haçariz and Sayers, 2013), it was not possible to determine such parameter in some cases, where samples were sequenced without quality check.

RNA-Seq library preparation and sequencing were performed by Macrogen, Inc. (South Korea) facilities. MPSIEC samples were treated with the TruSeq Stranded mRNA kit (Illumina), while FhNEJ samples were processed with the TruSeq Stranded Total RNA with Ribo-Zero kit (Illumina). The purified RNA was then randomly fragmented, and the fragmented RNA was retrotranscribed and adaptor ligated at both ends. Finally, the fragments were spread in an appropriate flow cell, amplified by PCR and sequenced from both ends (paired-end sequencing). All samples were sequenced on an Illumina NovaSeq 6000 equipment. In MPSIEC, 2 × 150 bp reads; 30 M reads/sample were acquired, while in FhNEJ 2 × 150 bp reads; 40 M reads/sample were acquired. In the generation of fastq files with the resulting reads, the quality values of each nucleotide were expressed on the Phred+ 33 scale. The raw RNA-Seq read data were deposited and can be found in the European Nucleotide Archive (ENA) repository, through the accession number PRJEB57383.

2.5. Processing of RNA-Seq data

An overview of the computational analysis workflow is available at Supplementary Figure 1. A preliminary quality control of the sequencing process was performed on all samples using the FastQC v0.11.9 software (Wingett and Andrews, 2018), a quality control tool for high throughput sequence data which allowed to determine multiple general parameters from the reads so as to guide further analysis. Illumina sequencing adapters were then removed with the Trimmomatic v0.39 tool (Bolger et al. 2014), that was also used to trim the first 10 nucleotides of each read as they showed higher sequencing instability. Additionally, rRNA of *F. hepatica* samples was removed using SortMeRNA 4.3.2 (Kopylova et al. 2012), filtering against the consensus 18 S and 28 S rRNA sequences provided by the software. A summary of the quality control process was performed with the MultiQC v1.13 software (Ewels et al. 2016).

Once the processing of the reads was completed, these were mapped over the *Mus musculus* (GRCm39; https://www.ensembl.org/Mus_musculus/Info/Index) and *Fasciola hepatica* (PRJEB25283; https://parasite.wormbase.org/Fasciola_hepatica_prjeb25283/Info/Index) reference genomes (genome data), providing the corresponding GTF annotation files to enhance the performance of the process. The mapping was conducted using STAR 2.7.9a (Dobin et al. 2013), and the read count for each gene was computed with the HTSeq-Count software embedded into STAR.

2.6. Differential gene expression analysis and biological annotation

An Exploratory Data Analysis (EDA) was then performed by Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) using the *MixOmics* package from the Bioconductor suite (Rohart et al. 2017) to determine the overall degree of similarity among samples prior to a more specific analysis. Differential expression analysis was performed with the Bioconductor package *DESeq2* (Love et al. 2014), that normalized and transformed the read counts, as well as determining the up- and down-regulated genes after interaction between MPSIEC and FhNEJ. Only genes with p-value < 0.05 and present in at least one replicate for each experimental condition were considered as differentially expressed and were further analysed in subsequent steps. The statistical requirement was considered to be sufficiently robust, and therefore no minimum fold change threshold was considered. Graphical representation of this analysis was performed with R software, using the *ggplot2* package.

Once the list of differentially expressed genes (DEGs) in both MPSIEC and FhNEJ was obtained, the amino acid sequences encoded by the FhNEJ DEGs were obtained using the BioMart tool from WormBase ParaSite and annotated using Blast2GO 6.0 (Conesa et al. 2005) with the blastp algorithm against the NCBI protein database restricted to the *Platyhelminthes* phylum. In MPSIEC, the Bioconductor package *BiomaRt* was used to obtain the gene descriptions and associated Gene Ontology (GO) terms of all DEGs, and the corresponding sequences were translated and annotated using Blast2GO against a database containing the mouse proteome available at Uniprot (https://www.uniprot.org/proteomes/UP000000589). Graphical overview of the annotation results was performed using the ReViGO tool (Supek et al. 2011) for the GO categories Biological Process (BP) and Molecular Function (MF), using the Nodescore provided by Blast2GO as quantitative criteria.

3. Results

3.1. Quantitative analysis

An RNA-Seq strategy was used to determine the expression changes triggered by the host-parasite interaction between *F. hepatica* juveniles and intestinal epithelial cells in co-culture. The performance of the mapping process was similar in both FhNEJ and MPSIEC samples, with around 45% of the total reads mapping to a single locus. As for the read quality control, the Phred logarithmic scale gave values of 35–36 after trimming, even in the last positions, indicating that the sequencing quality is optimal (error between 0.1% and 0.01%; accuracy between 99.9% and 99.99%). After this, Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was performed for all replicates of FhNEJ and MPSIEC samples (Fig. 1). These charts showed that the control group of FhNEJ samples displayed a clear separation from stimulated samples. Additionally, the distance among replicates of the same condition was smaller especially along the horizontal axis, which gathered the highest amount of variability among samples. In MPSIEC samples, all control replicates were clustered together and clearly separated from stimulated samples, whereas one replicate from this group displayed some distance from the others on both horizontal and vertical axes.

As mentioned, no minimum fold change was considered for DEGs, in order to include all transcripts that were differentially expressed and could have biological relevance independently from their fold change magnitude. Although the total reads mapping was low, our analysis allowed the identification of a high number of DEGs in both samples, most of them (around 86%) corresponding to coding genes. A total of 342 DEGs were detected in FhNEJ samples (113 up-regulated and 229 down-regulated) (Fig. 2A) after stimulation, whereas 140 DEGs were found in MPSIEC samples (103 up-regulated and 37 down-regulated) (Fig. 2B).

3.2. Functional annotation

Functional annotation of the differentially expressed genes was performed to obtain their description, as well as their associated GO terms in the BP and MF categories, in order to determine the most relevant events triggered by the *in vitro* interaction.

In the case of FhNEJ, 296 of the detected DEGs corresponded to coding genes (111 up- and 185 down-regulated), whose nucleotide sequence was translated into amino acids and identified using the Blast2GO software. In MPSIEC, descriptions of DEGs were assigned using the *BiomaRt* R package, followed by an analysis analogous to that performed in FhNEJ, where the proteins encoded by DEGs were obtained, resulting in a total of 240 proteins (184 up- and 56 down-regulated). Full descriptions of the differentially expressed genes within the FhNEJ and MPSIEC samples are available in Supplementary Table 1 and 2, respectively. In addition, in order to facilitate the visualization of the results, the top 15 annotations with the highest or lowest fold change for both sample types, as an arbitrary amount of the more representative transcripts, are shown in Fig. 3.

For FhNEJ, 3 of the 15 top up-regulated transcripts were hypothetical proteins. Additionally, among the top 15 up-regulated transcripts in FhNEJ upon MPSIEC incubation, 4 are related to the regulation of gene expression. These include cilia- and flagella-associated protein 20, tetratricopeptide repeat protein, lipopolysaccharide-induced tumor necrosis factor-alpha factor and ubiquitin-conjugating enzyme E2. Transcripts potentially linked with tegument turnover included a putative glycosyltransferase 14 family member and a tegument antigen. The predicted protein sequence of the tegument antigen shows the motifs commonly found in the so-called Calcium Binding Proteins (CABP) of *F. hepatica* (Thomas and Timson, 2016). These include an EF-hand, a dynein complex and a calcium-binding motif. Nevertheless, the similarity between its predicted sequence and those CABP described in *F. hepatica* is low (data not shown). Other transcripts represented in the top 15 up-regulated molecules in FhNEJ included the neutral sphingomyelinase, the transmembrane protease serine 3, the epididymal secretory protein E1, a putative drug transporter potentially related to multidrug resistance (Catalano et al., 2022), the brain-specific serine protease 4 that could enable serine-protease activity, and the dynein light chain LC8-type, linked with the muscle contraction machinery (Becerro-Recio et al., 2022b). The top 15 down-regulated proteins in FhNEJ included 8 unidentified and hypothetical proteins, and the cell differentiation protein rcd1, which may play a role in FhNEJ cell

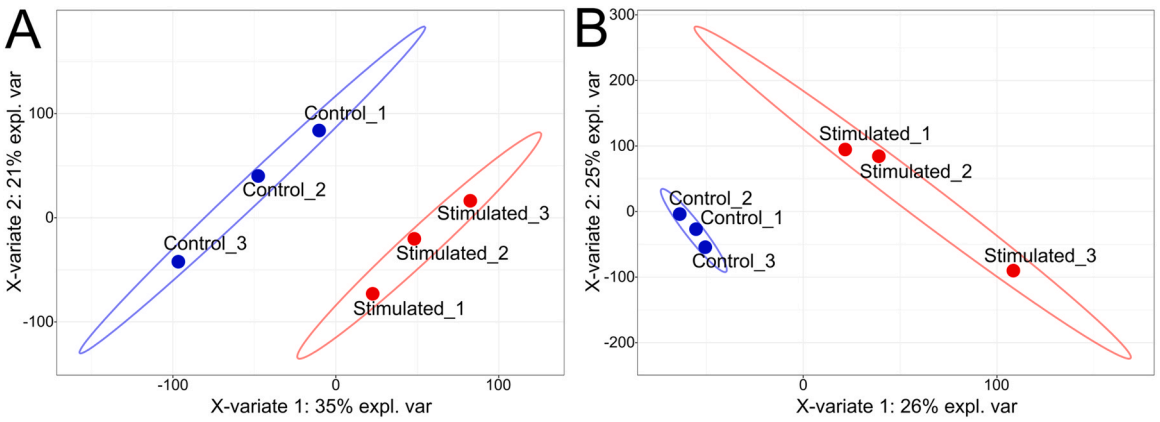

Fig. 1. Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) of FhNEJ (A) and MPSIEC (B) samples. Blue dots represent non-stimulated control samples, whereas red dots represent samples stimulated by co-culture for 3 h. The percentage of variability explained by each component is indicated in the corresponding axis, and the ellipse around each group of samples represents a 95% confidence interval for the modelled distribution.

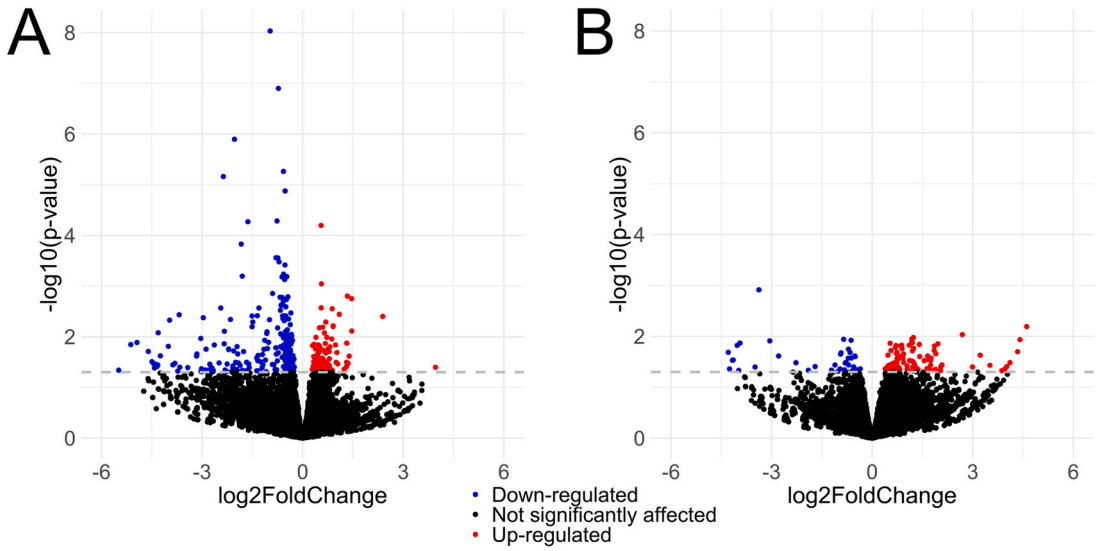

Fig. 2. Volcano plots showing the differential expression pattern of FhNEJ (A) and MPSIEC (B). Blue dots represent significantly down-regulated genes after stimulation and red dots represent up-regulated genes. The grey dotted line delimitates the statistical significance cut-off (p-value <0.05).

differentiation, the cell wall integrity and stress response component 1, a putative beta-arrestin, the arginine methyltransferase, the calcium-binding mitochondrial carrier protein SCaMG1, and the purine nucleoside phosphorylase.

For MPSIEC, 8 out of top 15 up-regulated and 9 out of top 15 down-regulated transcripts represent pseudogenes (Fig. 3). Additionally, the junction adhesion molecule 2, a pore-forming protein-like, the zinc finger protein 953, a hedgehog acyltransferase-like, the antigen identified by monoclonal antibody Ki 67, the shugoshin 1 and the FAM3 metabolism regulated signalling molecule 3 were found in the top 15 up-regulated proteins in MPSIEC. Among down-regulated proteins, the MFNG O-fucosylpeptidase 3-beta-N-acetylglucosaminyltransferase, the apical junction component 1, the EP300 interacting inhibitor of differentiation 3, the protein tyrosine phosphatase non-receptor type 7, the histidine ammonia lyase and the keratin 36 were found in MPSIEC.

Additionally, tables comparing the relative quantitation (fold change) and the absolute quantitation (read counts) for all the identified transcripts in both samples were generated (Supplementary Table 3). In this regard, absolute transcription rate of the most overexpressed genes in MPSIEC was not particularly high. In FhNEJ a high abundance of DEGs dynein light chain, tegumental antigen and epididymal secretory protein E1 was observed. Additionally, two highly expressed legumain isoforms were found, despite their relative change in expression fall outside the top 15 DEGs.

Secondly, the functional annotation was plotted using the ReViGO software, which displayed the most representative GO terms among DEGs found in the analysed samples. In FhNEJ, these GO terms in the BP category included macromolecule biosynthesis, signal transduction, proteolysis and cytoskeleton organisation (Fig. 4). Furthermore, the MF category within the DEGs in FhNEJ samples after MPSIEC stimulation was majorly represented by binding to different types of biomolecules, as well as peptidase and kinase activity (Supplementary Figure 2A).

In the case of MPSIEC, visualisation of the annotation using ReViGO showed several clusters of closely related BP. These included response to endogenous and abiotic stimuli, vesicle secretion and transport, and regulation of gene expression, as well as specific processes such as innate immune response, epithelial cell differentiation, or regulation of morphogenesis and cytoskeletal function (Fig. 5). Within the MF category, the most remarkable feature was a group of GO terms related to binding to different biomolecules, including receptor, signalling and cytoskeletal proteins, as well as catalytic activity (Supplementary Figure 2B). A detailed GO annotation of the BP categories found as up- and down-regulated in FhNEJ and MPSIEC upon interaction can be found in Supplementary Tables 4 and 5, respectively.

FhNEJ

296 DEG
111 Up-regulated
185 Down-regulated

Description	log2FC
cilia-and flagella-associated protein 20	2.39
Tetratricopeptide repeat protein	1.46
Lipopolysaccharide-induced tumor necrosis factor-alpha factor	1.39
hypothetical protein D915_004001	1.35
hypothetical protein D915_009891	1.33
Neutral sphingomyelinase	1.31
Transmembrane protease serine 3	1.31
putative drug transporter	1.31
putative glycosyltransferase 14 family member	1.24
Tegument antigen	1.09
Brain-specific serine protease 4	1.05
Epididymal secretory protein E1	1.02
ubiquitin-conjugating enzyme E2 A	1.00
dynein light chain LC8-type	0.98
hypothetical protein D915_009891	0.91
hypothetical protein D915_000771	-2.49
---NA---	-2.63
---NA---	-2.67
Cell differentiation protein rcd1	-2.70
Protein sidekick-1	-2.77
---NA---	-3.00
Cell wall integrity and stress response component 1	-3.04
---NA---	-3.16
unnamed protein product	-3.68
unnamed protein product	-3.87
putative beta-arrestin 1	-4.01
Arginine methyltransferase 1	-4.24
Calcium-binding mitochondrial carrier protein SCaMC1	-4.31
hypothetical protein D915_001150	-4.34
Purine nucleoside phosphorylase	-4.50

MPSIEC

240 DEG
184 Up-regulated
56 Down-regulated

Description	log2FC
predicted gene, 17795	4.61
predicted gene 17149	4.41
predicted gene 15892	4.34
ribosomal protein L31, pseudogene 6	4.11
junction adhesion molecule 2	4.01
pore forming protein-like	3.95
zinc finger protein 953	3.87
RIKEN cDNA 3000002C10 gene	3.51
predicted gene 12499	3.22
predicted gene, 19091	2.99
hedgehog acyltransferase-like	2.69
antigen identified by monoclonal antibody Ki 67	2.08
RIKEN cDNA 8430429K09 gene	2.04
shugoshin 1	2.04
FAM3 metabolism regulating signaling molecule D	1.96
RIKEN cDNA 4930473H19 gene	-1.21
predicted gene, 37233	-1.70
predicted gene 5802	-1.90
predicted gene, 38569	-2.27
MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	-2.79
predicted gene, 48106	-3.05
apical junction component 1	-3.38
RIKEN cDNA 2810403D21 gene	-3.49
predicted gene, 37477	-3.94
predicted gene 5779	-3.98
EP300 interacting inhibitor of differentiation 3	-4.03
protein tyrosine phosphatase, non-receptor type 7	-4.14
histidine ammonia lyase	-4.17
keratin 36	-4.25
expressed sequence AU020206	-4.29

Fig. 3. Differentially expressed genes after *in vitro* stimulation. The top 15 annotations with the highest (red) or lowest (blue) fold change are shown for both FhNEJ and MPSIEC.

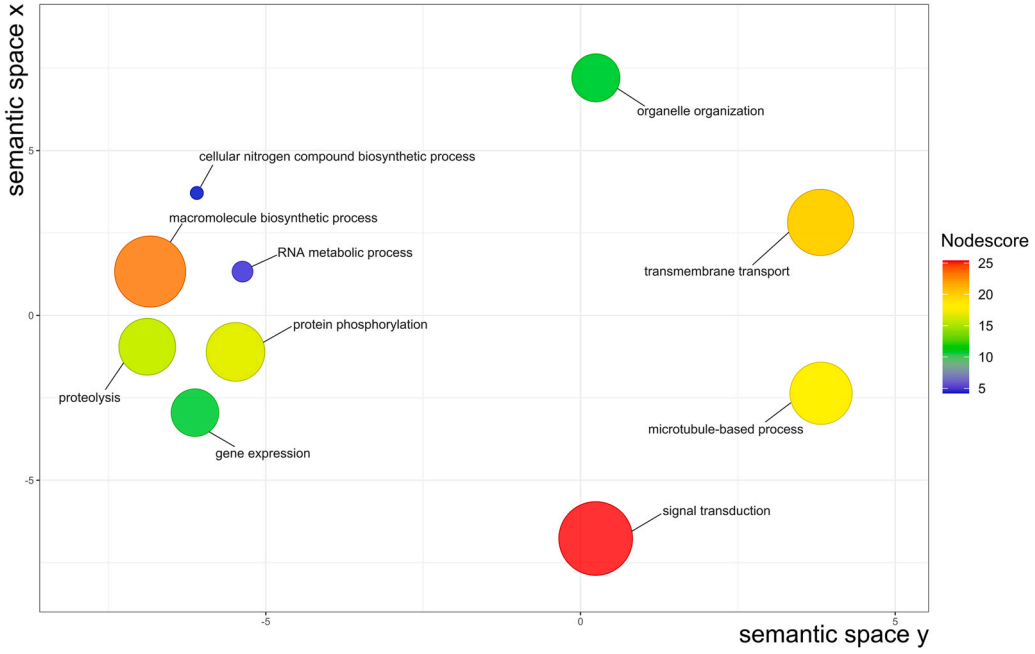

Fig. 4. ReViGO plots showing the most representative GO terms among DEGs found in FhNEJ samples, in the Biological Process category. The size and colour of each circle represents the Nodescore of each GO term, while the proximity between circles is proportional to the degree of similarity of the represented terms.

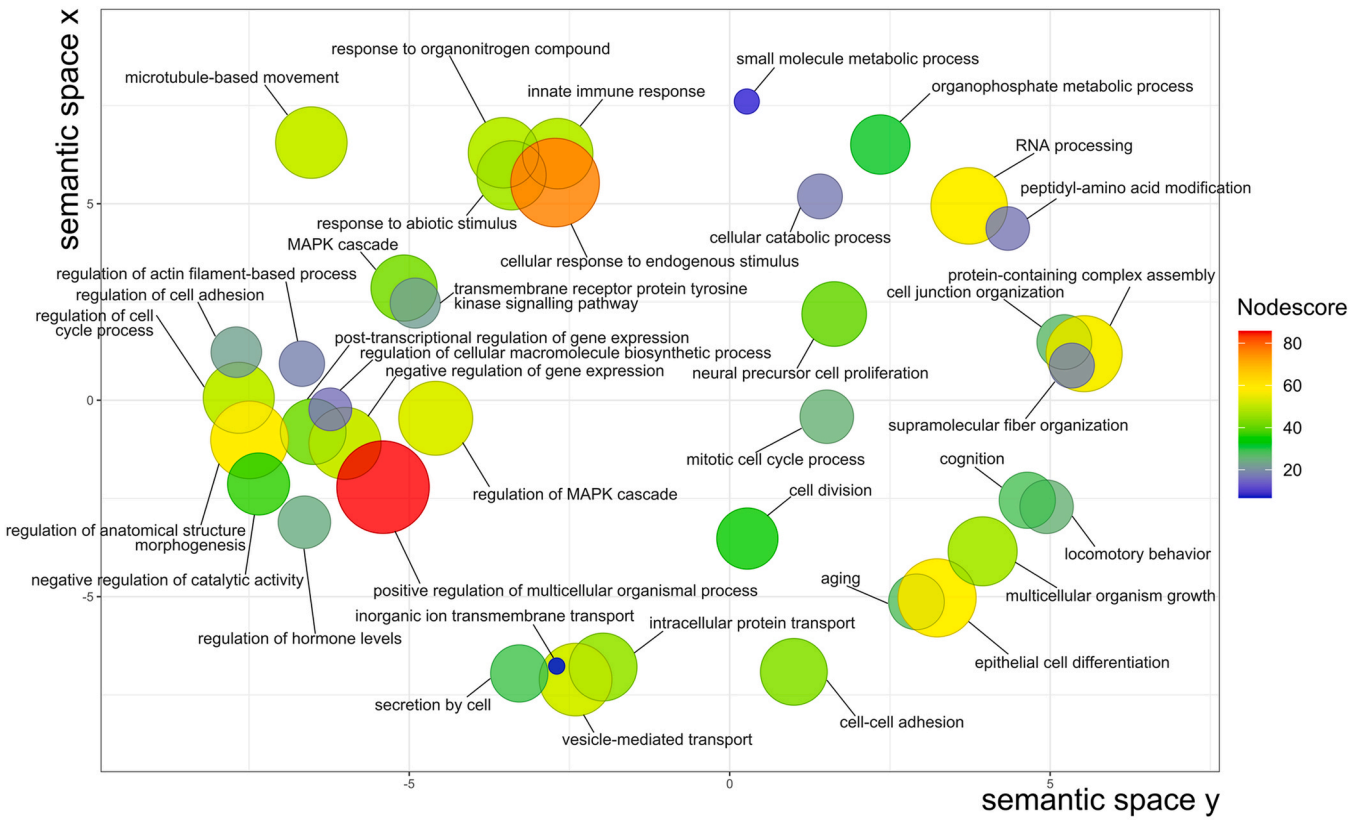

Fig. 5. ReViGO plots showing the most representative GO terms among DEGs found in MPSIEC samples, in the Biological Process category. The size and colour of each circle represents the Nodescore of each GO term, while the proximity between circles is proportional to the degree of similarity of the represented terms.

4. Discussion

The host-parasite relationships during the early phases of infection by *F. hepatica* in the vertebrate host have been scarcely studied at molecular level. Recently, we described the proteomic changes in an *in vitro* model that allows the interaction between FhNEJ and MPSIEC (Becerro-Recio et al., 2022b). These are the first host cells that the parasite meets upon excystment in the small intestine. Changes were studied by SWATH-MS, a proteomic approach that facilitates the quantitation of differentially expressed proteins among different samples. This methodology showed rapid changes in the protein expression pattern of FhNEJ in response to interaction with the host intestinal epithelial cells. MPSIEC also responded to parasite stimulus by showing alterations in the expression of proteins related to crucial biological processes. Here, we extend this analysis, evaluating the changes triggered by the host-parasite interaction in the transcriptome of MPSIEC and FhNEJ.

Identification of differentially expressed transcripts and their corresponding proteins was performed with the Blast2GO software. GO analysis of up-regulated transcripts in FhNEJ after MPSIEC stimulation showed that one of the dominant BP categories is proteolysis. The expression of proteases along the development of *F. hepatica* inside its definitive host has been found to be tightly regulated, due to their implications in a variety of processes crucial for parasite development, including parasite migration and feeding. Proteases are also specifically related to pathogenic and virulence phenomena (Cwiklinski and Dalton, 2022). Among the up-regulated molecules in this GO category, we found the transmembrane protease serine 3, described in other organisms as related to the initiation of proteolytic cascades and belonging to the S1 family of the SA clan of serine type peptidases, for which the prototype is chymotrypsin. Unfortunately, *F. hepatica* serine proteases are largely understudied in comparison with other groups of proteases, thus the biological and biochemical functions of these proteases are poorly understood. In other trematodes, this class of proteases seems to play a role in host-parasite interactions and parasite survival (Dvorak and Horn, 2018). Also linked with proteolysis, although not in the top 15 of over-expressed transcripts in FhNEJ after MPSIEC co-incubation, we identified the transcript of the peptidase legumain, an asparaginyl endopeptidase cysteine protease. Legumains have been found in the secretions of FhNEJ (Tkalcevic et al. 1995; Robinson et al. 2009). Later proteomic studies in *F. hepatica* metacercariae and juveniles also identified a range of legumains, suggesting that these peptidases are activated once the parasite reached the intestine (Cwiklinski et al. 2018). This assumption is compatible with the results obtained in our *in vitro* model showing a high abundance of legumain transcripts in FhNEJ after their interaction with the intestinal epithelial cells of the host. Importantly, legumains are involved in the activation of cathepsin L proteases by cleavage of their pro-peptides (Cwiklinski et al., 2022), allowing their proteolytic activity. Our previous proteomic results in this *in vitro* model showed that the interaction between FhNEJ and MPSIEC triggers a rapid protein expression change of FhNEJ in response to the host epithelial barrier, including cathepsins L3 and L4 (Becerro-Recio et al. 2022b). Due to the tightly regulated expression of legumains during the *F. hepatica* development and their role in activating cathepsins linked with collagenolytic and endopeptidase activities, the over-expression of peptidases detected here could be related to host invasion. In line with this, cathepsin B is also overrepresented in transcripts of FhNEJ after MPSIEC incubation.

As mentioned, four of the top 15 up-regulated transcripts in FhNEJ upon MPSIEC incubation are linked with the regulation of gene expression. Increased expression of these genes is consistent with increased cell proliferation in FhNEJ, thus most likely having a role in the rapid growth and transformation of the parasite inside its vertebrate host. During FhNEJ migration and development, the turnover of the tegument and the expression of different glycoconjugates on their

surface have been found of paramount importance for parasite survival and pathogenesis (McVeigh et al. 2018; González-Miguel et al. 2021). The tegument antigen and the glycosyltransferase found here in the top 15 transcripts of MPSIEC-stimulated FhNEJ could be associated with these processes. Importantly, the predicted protein of the tegument antigen transcript shows high similarity with the peptide sequences of the tegument antigens previously found by us as over-expressed in FhNEJ tegumental extracts both after interaction with MPSIEC *in vitro* (UniProt identifier A0A4E0RZ43) (Becerro-Recio et al. 2022b) and gut passage *ex vivo* (UniProt identifier A0A4E0RZ43) (Becerro-Recio et al. 2022a). Thus, this family of proteins seems to be localized to the FhNEJ tegument. Although the function of these molecules remains to be determined, it is worth mentioning that they are unique to trematodes. Thus, they are of interest in understanding their function in the biogenesis and rapid change of the T bodies that support the turnover of the tegument of *F. hepatica* inside the mammalian host (González-Miguel et al. 2021). Regarding the identified glycosyltransferase, this could be related to the activity required for protein glycosylation, a pattern that changes during the intra-mammalian parasite development (Cwiklinski et al. 2021). Carbohydrate-containing glycoconjugates are expressed on the surface of *F. hepatica* developing juveniles, and are released in their secretions, playing an important role during infection due to their immunomodulatory properties (Rodríguez et al., 2015).

Parasite trematodes do not synthesize lipids, including cholesterol. Trematodes need these molecules as energy source and they acquire them from the host (Cwiklinski et al., 2021). In this regard, among the 15 top overexpressed transcripts in FhNEJ, we found sphingomyelin, which attaches to cholesterol and helps to form other lipids and mobilizes the cell surface cholesterol to the interior of cells (Chatterjee, 1993). Additionally, we found the transmembrane protease serine 3, which shows an LDL receptor-like domain, mediating the endocytosis of cholesterol-rich low-density lipoprotein (Südhof et al., 1985). We also found the epididymal secretory protein E1, known as NPC2 (Niemann-Pick intracellular cholesterol transporter 2), already described in extracellular vesicles of *F. hepatica* (Cwiklinski et al., 2015). In this context, these proteins could assist FhNEJ in the uptake of metabolic substrates from the host, suggesting that the lipid energy source taken from the host is of great importance for this developmental stage of the parasite.

Our *in vitro* model also permitted us to examine transcriptional changes in the MPSIEC cells. This revealed fast and profound changes in MPSIEC gene expression after their interaction with FhNEJ. Notably, 8 out of top 15 up-regulated and 9 out of top 15 down-regulated transcripts represent pseudogenes. Interestingly, these genes did not tend to have high read counts in terms of absolute quantitation (Supplementary Table 3). Pseudogenes are regions of the genome that are similar to functional genes but are thought to be non-functional. However, some recent studies have suggested that pseudogenes have the potential to act in complex transcriptional and post-transcriptional modulation processes (Muro et al., 2011). Thus, pseudogenes are emerging as crucial regulators of development and disease in which are commonly regulated. However, systematic functional characterization and evolution of pseudogenes remain largely unexplored in mammals (Qian et al., 2022). Notwithstanding, there is growing evidence of the importance of the regulation of the transcription pseudogenes in pathological processes, including non-infectious and infectious diseases, that affect mammals (Cristiano, 2022).

Besides pseudogenes, up-regulated transcripts in MPSIEC upon incubation with FhNEJ included molecules potentially linked with host defence, as the Junction adhesion molecule 2 (JAM2), the Pore forming protein-like (PFP-L) and the FAM3 metabolism regulating signalling molecule D. JAM2 promotes lymphocyte trans-endothelial migration (Johnson-Léger et al. 2002), a process triggered by antigens of numerous infectious agents associated with the inflammatory response. Several mechanisms play a critical role in trans-endothelial migration, including disruption or loosening of adherens junctions (Muller, 2011). Noteworthy, the Apical junction component 1, predicted to be located in adherens junctions and involved in cell-cell junction organization, is one of the top 15 down-regulated transcripts in MPSIEC after incubation with FhNEJ. Thus, the cross-talk between the host intestinal epithelial cells and the FhNEJ results in the activation of signals related to trans-endothelial immune cells migration at intestinal level, that are crucial to fight against the parasite during the very early steps of infection. The triggering of these molecules in reference to inflammation by FhNEJ in MPSIEC could be related to pathogen-associated molecular patterns (PAMPs) released by the parasite. PAMPs have been described in *F. hepatica*, *e.g.* CL1 and GST (Dowling et al., 2010), but which specific molecules derived from FhNEJ could act as PAMPs in our settings should be further investigated.

Together with the triggering of this inflammatory mechanism, the up-regulation of the PFP-L in the intestinal epithelial cells could also result in the damage of FhNEJ in the gut lumen, since PFP are immune effectors that can target pathogens directly, resulting in formation of pores in cells and cell death. The PFP-L detected here is most similar in its amino acid sequence to the macrophage-expressed gene 1 protein-like or perforin-2, as indicated by alignment of its sequence with those available in GenBank through BLASTP (data not shown). Inside this family of proteins, perforin-2 could exert a similar and alternative function to the complement membrane attack complex (Krawczyk et al. 2020).

Potentially related to host defenses, the over-expression of FAM3D in mouse gastrointestinal tract is broadly linked with intestinal homeostasis, and more specifically with anti-microbial peptide production (Liang et al. 2020). Antimicrobial peptides (AMPs) are a class of small peptides that widely exist in nature and they are an important part of the innate immune system, showing a wide range of inhibitory effects against different pathogens, including parasites (Huan et al. 2020). Triggering of this molecule by FhNEJ in MPSIEC could result in the activation of additional defense mechanisms of MPSIEC against FhNEJ mediated by AMPs.

Other proteins found to be in the top 15 of over-expressed molecules in MPSIEC included proteins potentially related to DNA repair and chromatin organization (EP300, shugoshin, antigen identified by monoclonal antibody Ki 67), regulation of transcription (zinc finger protein 953) and post-translational modifications (hedgehog acyltransferase). Among the top 15 down-regulated proteins in MPSIEC, we also found proteins that could be linked with immune responses. The EP300 interacting inhibitor of differentiation 3 has been pointed out as important for the regulation of the function of Foxp3 Treg cells (Liu et al., 2013). Intriguingly, *F. hepatica* induces Foxp3 T cell over-expression in liver from infected sheep during early stages of infection (Pacheco et al., 2018). Additionally, the protein tyrosine phosphatase non-receptor type 7 plays a role in the negative regulation of T cell antigen receptor signal transduction (Saxena et al., 1998), and its down-regulation in MPSIEC cells upon FhNEJ interaction could have a connection with the enhancement of antigen presentation by T cells.

Comparison of the relative quantitation (fold change) and the absolute quantitation (read counts) for all the identified transcripts in both samples showed that absolute transcription rate of the DEGs transcripts in MPSIEC was not particularly high. Conversely, a high abundance of DEGs such as dynein light chain, tegumental antigen and epididymal secretory protein E1 was observed in FhNEJ. Additionally, we found two highly expressed legumain isoforms, despite their relative change in expression fall outside the top 15. This correlates with the high expression of these proteases described during the early stages of infection (Robinson et al., 2009). This comparison shows that DEGs do not necessarily represent the most abundant transcripts in this setting.

Finally, and despite the obvious difficulties in integrating these transcriptomic data with the previously published proteomic analysis using the same *in vitro* model by us (Becerro-Recio et al., 2022b), it is possible to establish some potential connections. Thus, there is a correlation in the differential expression of molecules belonging to some

protein families such as proteases (including legumain, cathepsins B, CL2 and 4, but not CL3) or epigenetic regulation proteins (histone acetyltransferase). On the contrary, no changes in the levels of metabolic enzymes, antioxidant proteins or protease inhibitors (observed in the proteomic approach) were found in the transcriptomic analysis. With regard to MPSIEC, both proteomic and transcriptomic approaches display expression alterations in common processes such as intracellular transport, cell adhesion and cell cycle regulation, as well as phosphorylation-mediated signalling. Notably, the decrease in ribosomal function in the cytosol, as the most prominent phenomenon in the proteomic study, is also present at the transcript level, represented by several ribosomal protein-coding genes with reduced expression after contact with the parasite. On the other hand, while at the protein level hardly any changes indicating the triggering of immune reactions were noticed, some transcripts related to this process were now found, most of which up-regulated. These include an interferon regulatory factor, suggesting that induction of a Th1-type response may be occurring during the initial stage of infection.

In summary, a high-throughput transcriptomic analysis coupled with an *in vitro* model of co-culture by FhNEJ and MPSIEC has been carried out for the first time to explore the host-parasite relationships in fasciolosis. This model allows the depiction of the interaction between the early stages of *F. hepatica* with a specific set of host cells facilitating the evaluation of the changes triggered by both organisms, among which we have found regulation of numerous transcripts specifically related to essential processes of the host-parasite relationship. The implementation of this model for other transcriptomic studies in fasciolosis will also allow more accurate comparisons with data sets produced by other authors, which currently entail complexity due to the different methodological approaches that are employed.

CRediT authorship contribution statement

David Becerro-Recio: Methodology, Investigation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Judit Serrat:** Methodology, Writing - review & editing. **Marta López-García:** Methodology, Writing - review & editing. **María Torres-Valle:** Project administration, Methodology, Writing - review & editing. **Francisco Colina:** Formal analysis, Data curation, Writing - review & editing. **Iván M. Fernández:** Formal analysis, Data curation, Writing - review & editing. **Javier González-Miguel:** Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Writing - original draft, Writing - review & editing. **Mar Siles-Lucas:** Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.109981.

References

Becerro-Recio, D., Serrat, J., López-García, M., Molina-Hernández, V., Pérez-Arévalo, J., Martínez-Moreno, Á., Sotillo, J., Simón, F., González-Miguel, J., Siles-Lucas, M., 2022a. 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. 16, 1–22. https://doi.org/10.1371/journal.pntd.0010766.

Becerro-Recio, D., Serrat, J., López-García, M., Sotillo, J., Simón, F., González-Miguel, J., Siles-Lucas, M., 2022b. 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. 16, 1–24. https://doi.org/10.1371/journal.pntd.0010811.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

Catalano, A., Iacopetta, D., Ceramella, J., Scumaci, D., Giuzio, F., Saturnino, C., Aquaro, S., Rosano, C., Sinicropi, M.S., 2022. Multidrug Resistance (MDR): A Widespread Phenomenon in Pharmacological Therapies. Molecules 27 (3), 616. https://doi.org/10.3390/molecules27030616. Jan.

Chatterjee, S., 1993. Neutral sphingomyelinase increases the binding, internalization, and degradation of low density lipoproteins and synthesis of cholesteryl ester in cultured human fibroblasts. J. Biol. Chem. 268 (5), 3401–3406.

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M., Robles, M., 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674–3676. https://doi.org/10.1093/bioinformatics/bti610.

Cristiano, L., 2022. The pseudogenes of eukaryotic translation elongation factors (EEFs): Role in cancer and other human diseases. Genes Dis. 9, 941–958. https://doi.org/10.1016/j.gendis.2021.03.009.

Cwiklinski, K., Dalton, J.P., 2022. Exploiting comparative omics to understand the pathogenic and virulence-associated protease: anti-protease relationships in the zoonotic parasites *Fasciola hepatica* and *Fasciola gigantica*. Genes 13 (10), 1854. https://doi.org/10.3390/genes13101854.

Cwiklinski, K., Drysdale, O., López Corrales, J., Corripio-Miyar, Y., De Marco Verissimo, C., Jewhurst, H., Smith, D., Lalor, R., McNeilly, T.N., Dalton, J.P., 2022 20. Targeting Secreted Protease/Anti-Protease Balance as a Vaccine Strategy against the Helminth *Fasciola hepatica*. Vaccines (Basel) 10 (2), 155. https://doi.org/10.3390/vaccines10020155.

Cwiklinski, K., Jewhurst, H., McVeigh, P., Barbour, T., Maule, A.G., Tort, J., O'Neill, S. M., Robinson, M.W., Donnelly, S., Dalton, J.P., 2018. 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. 17, 792–809. https://doi.org/10.1074/mcp.RA117.000445.

Cwiklinski, K., de la Torre-Escudero, E., Trelis, M., Bernal, D., Dufresne, P.J., Brennan, G. P., O'Neill, S., Tort, J, Paterson, S., Marcilla, A., Dalton, J.P., Robinson, M.W., 2015. The Extracellular Vesicles of the Helminth Pathogen, Fasciola hepatica: Biogenesis Pathways and Cargo Molecules Involved in Parasite Pathogenesis. Mol Cell Proteomics 14 (12), 3258–3273. https://doi.org/10.1074/mcp.M115.053934. Dec.

Cwiklinski, K., de Marco Verissimo, C., McVeigh, P., Donnelly, S., Dalton, J.P., 2021. Applying 'omics' technologies to understand *Fasciola* spp. biology. Chapter 11. In: John, P.Dalton (Ed.), Fasciolosis, 2nd ed.,. CABI Digital Library, pp. 338–378. eISBN: 978-1-78924-617-9.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T.R., 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21. https://doi.org/10.1093/bioinformatics/bts635.

Dowling, D.J., Hamilton, C.M., Donnelly, S., La Course, J., Brophy, P.M., Dalton, J., O'Neill, S.M., 2010. Major secretory antigens of the helminth *Fasciola hepatica* activate a suppressive dendritic cell phenotype that attenuates Th17 cells but fails to activate Th2 immune responses. Infect. Immun. 78 (2), 793–801. https://doi.org/10.1128/IAI.00573-09.

Dvorak, J., Horn, M., 2018. Serine proteases in schistosomes and other trematodes. Int. J. Parasitol. 48, 333–344. https://doi.org/10.1016/j.ijpara.2018.01.001.

Ewels, P., Magnusson, M., Lundin, S., Käller, M., 2016. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics 32, 3047–3048. https://doi.org/10.1093/bioinformatics/btw354.

Fürst, T., Keiser, J., Utzinger, J., 2012. Global burden of human food-borne trematodiasis: a systematic review and meta-analysis. Lancet Infect. Dis. 12, 210–221. https://doi.org/10.1016/S1473-3099(11)70294-8.

González-Miguel, J., Becerro-Recio, D., Sotillo, J., Simón, F., Siles-Lucas, M., 2020. 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. 278, 109028 https://doi.org/10.1016/j.vetpar.2020.109028.

Garcia-Campos, A., Baird, A.W., Mulcahy, G., 2016. Development of a versatile in vitro method for understanding the migration of *Fasciola hepatica* newly excysted juveniles. Parasitology 143, 24–33. https://doi.org/10.1017/S0031182015001481.

González-Miguel, J., Becerro-Recio, D., Siles-Lucas, M., 2021. Insights into *Fasciola hepatica* Juveniles: crossing the Fasciolosis Rubicon. Trends Parasitol. 37, 35–47. https://doi.org/10.1016/j.pt.2020.09.007.

Haçariz, O., Sayers, G., 2013. *Fasciola hepatica* - Where is 28S ribosomal RNA? Exp. Parasitol. 135, 426–429. https://doi.org/10.1016/j.exppara.2013.07.026.

Hernández-González, A., Valero, M.L., Pino, M.S., del, Oleaga, A., Siles-Lucas, M., 2010. Proteomic analysis of *in vitro* newly excysted juveniles from *Fasciola hepatica*. Mol. Biochem. Parasitol. 172, 121–128. https://doi.org/10.1016/j.molbiopara.2010.04.003.

Huan, Y., Kong, Q., Mou, H., Yi, H., 2020. Antimicrobial peptides: classification, design, application and research progress in multiple fields. Front. Microbiol. 11, 1–21. https://doi.org/10.3389/fmicb.2020.582779.

Johnson-Léger, C.A., Aurrand-Lions, M., Beltraminelli, N., Fasel, N., Imhof, B.A., 2002. Junctional adhesion molecule-2 (JAM-2) promotes lymphocyte transendothelial migration. Blood 100, 2479–2486. https://doi.org/10.1182/blood-2001-11-0098.

Kopylova, E., Noé, L., Touzet, H., 2012. SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. Bioinformatics 28, 3211–3217. https://doi.org/10.1093/bioinformatics/bts611.

Krawczyk, P.A., Laub, M., Kozik, P., 2020. To kill but not be killed: controlling the activity of mammalian pore-forming proteins. Front. Immunol. 11, 1–15. https://doi.org/10.3389/fimmu.2020.601405.

Liang, W., Peng, X., Li, Q., Wang, P., Lv, P., Song, Q., She, S., Huang, S., Chen, K., Gong, W., Yuan, W., Thovarai, V., Yoshimura, T., O'huigin, C., Trinchieri, G., Huang, J., Lin, S., Yao, X., Bian, X., Kong, W., Xi, J., Wang, J.M., Wang, Y., 2020. FAM3D is essential for colon homeostasis and host defense against inflammation associated carcinogenesis. Nat. Commun. 11 (1), 5912. https://doi.org/10.1038/s41467-020-19691-z.

Liu, Y., Wang, L., Predina, J., Han, R., Beier, U.H., Wang, L.C., Kapoor, V., Bhatti, T.R., Akimova, T., Singhal, S., Brindle, P.K., Cole, P.A., Albelda, S.M., Hancock, W.W., 2013. Inhibition of p300 impairs Foxp3^{+} T regulatory cell function and promotes antitumor immunity. Nat. Med 19 (9), 1173–1177. https://doi.org/10.1038/nm.3286.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 1–21. https://doi.org/10.1186/s13059-014-0550-8.

McVeigh, P., Cwiklinski, K., Garcia-Campos, A., Mulcahy, G., O'Neill, S.M., Maule, A.G., Dalton, J.P., 2018. In silico analyses of protein glycosylating genes in the helminth *Fasciola hepatica* (liver fluke) predict protein-linked glycan simplicity and reveal temporally-dynamic expression profiles. Sci. Rep. 8, 1–15. https://doi.org/10.1038/s41598-018-29673-3.

Muller, W.A., 2011. Mechanisms of leukocyte transendothelial migration. Annu Rev. Pathol. 6, 323–344. https://doi.org/10.1146/annurev-pathol-011110-130224.

Muro, E.M., Mah, N., Andrade-Navarro, M.A., 2011. Functional evidence of post-transcriptional regulation by pseudogenes. Biochimie 93, 1916–1921. https://doi.org/10.1016/j.biochi.2011.07.024.

Pacheco, I.L., Abril, N., Zafra, R., Molina-Hernández, V., Morales-Prieto, N., Bautista, M. J., Ruiz-Campillo, M.T., Pérez-Caballero, R., Martínez-Moreno, A., Pérez, J., 2018. *Fasciola hepatica* induces Foxp3 T cell, proinflammatory and regulatory cytokine overexpression in liver from infected sheep during early stages of infection. Vet. Res 49 (1), 56.

Pakharukova, M.Y., Zaparina, O., Baginskaya, N.V., Mordvinov, V.A., 2022. Global changes in gene expression related to *Opisthorchis felineus* liver fluke infection reveal temporal heterogeneity of a mammalian host response. Food Waterborne Parasitol. 27, e00159 https://doi.org/10.1016/j.fawpar.2022.e00159.

Qian, S.H., Chen, L., Xiong, Y.L., Chen, Z.X., 2022. Evolution and function of developmentally dynamic pseudogenes in mammals. Genome Biol. 23, 1–24. https://doi.org/10.1186/s13059-022-02802-y.

Robinson, M.W., Sotillo, J., 2022. Foodborne trematodes: old foes, new kids on the block and research perspectives for control and understanding host-parasite interactions. Parasitology 149, 1257–1261. https://doi.org/10.1017/S0031182022000877.

Robinson, M.W., Menon, R., Donnelly, S.M., Dalton, J.P., Ranganathan, S., 2009. An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*: Proteins associated with invasion and infection of the mammalian host. Mol. Cell. Proteom. 8, 1891–1907. https://doi.org/10.1074/mcp.M900045-MCP200.

Rodríguez, E., Noya, V., Cervi, L., Chiribao, M.L., Brossard, N., Chiale, C., Carmona, C., Giacomini, C., Freire, T., 2015. Glycans from *Fasciola hepatica* Modulate the host immune response and TLR-induced maturation of dendritic cells. PLoS Negl. Trop. Dis. 9, 1–26. https://doi.org/10.1371/journal.pntd.0004234.

Rohart, F., Gautier, B., Singh, A., Lê Cao, K.A., 2017. mixOmics: An R package for 'omics feature selection and multiple data integration. PLoS Comput. Biol. 13, 1–19. https://doi.org/10.1371/journal.pcbi.1005752.

Ryan, S., Shiels, J., Taggart, C.C., Dalton, J.P., Weldon, S., 2020. *Fasciola hepatica*-derived molecules as regulators of the host immune response. Front. Immunol. 11, 1–9. https://doi.org/10.3389/fimmu.2020.02182.

Saxena, M., Williams, S., Gilman, J., Mustelin, T., 1998. Negative regulation of T cell antigen receptor signal transduction by hematopoietic tyrosine phosphatase (HePTP). J. Biol. Chem. 273 (25), 15340–15344. https://doi.org/10.1074/jbc.273.25.15340.

Siles-Lucas, M., Becerro-Recio, D., Serrat, J., González-Miguel, J., 2021. Fascioliasis and fasciolopsiasis: current knowledge and future trends. Res. Vet. Sci. 134, 27–35. https://doi.org/10.1016/j.rvsc.2020.10.011.

Südhof, T.C., Goldstein, J.L., Brown, M.S., Russell, D.W., 1985. The LDL receptor gene: a mosaic of exons shared with different proteins, 815–282 Science 228 (4701). https://doi.org/10.1126/science.2988123.

Supek, F., Bošnjak, M., Škunca, N., Šmuc, T., 2011. Revigo summarizes and visualizes long lists of gene ontology terms. PLoS One 6. https://doi.org/10.1371/journal.pone.0021800.

Thomas, C.M., Timson, D.J., 2016. A mysterious family of calcium-binding proteins from parasitic worms. Biochem. Soc. Trans. 44, 1005–1010. https://doi.org/10.1042/BST20150270.

Tkalcevic, J., Ashman, K., Meeusen, E., 1995. *Fasciola hepatica*: rapid identification of newly excysted juvenile proteins. Biochem. Biophys. Res. Commun. https://doi.org/10.1006/bbrc.1995.2112.

Van Milligen, F.J., Cornelissen, J.B.W.J., Gaasenbeek, C.P.H., Bokhout, B.A., 1998. A novel *ex vivo* rat infection model to study protective immunity against *Fasciola hepatica* at the gut level. J. Immunol. Methods 213, 183–190. https://doi.org/10.1016/S0022-1759(98)00026-X.

Wingett, S.W., Andrews, S., 2018. Fastq screen: a tool for multi-genome mapping and quality control. F1000Research 7, 1–13. https://doi.org/10.12688/f1000research.15931.1.