1 Schistosome infections induce significant changes in the host biliary proteome.

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Abstract

Schistosomiasis is a disease caused by blood trematodes affecting man and animals that represents an important human health and veterinary problem. Main damages caused by this infection are a consequence of the host inflammatory reaction against the parasite eggs trapped inside the liver. Despite the hepatic pathology of schistosomiasis is very well known, there is no specific studies dealing with the schistosome infection effects on the biliary function. The purpose of this work was to analyse the changes induced by *Schistosoma bovis* infection in the biliary proteome. For this, whole gallbladders from *S. bovis*-infected and non-infected mice were dissected, homogenized and fractionated by differential centrifugation. The resulting protein fractions were resolved by SDS-PAGE, the gels were sliced, and the gel pieces analyzed by LC-MS/MS. Altogether, we identified 1,937 proteins, which were classified according to **their “protein class” and “molecular function”**, and then subjected to an **“Enrichment analysis”**. **The differences found in gallbladder proteomes between *S. bovis*-infected and non-infected mice are analysed.** We show that chronic schistosome infections cause significant changes in the biliary proteome that may produce physiological alterations and affect the therapeutic actions of drugs when administered to human patients and animals with schistosomiasis.

Key words: *Schistosoma bovis*, gallbladder, proteome.

1. INTRODUCTION

Schistosomiasis is a severe parasitic disease affecting humans and animals in many tropical and subtropical areas of the world that is caused by flukes of the genus *Schistosoma*. *Schistosoma bovis* is a blood-dwelling fluke of ruminants that is genetically and immunologically analogous to the important human pathogen *Schistosoma haematobium* [1]. Like human schistosomes, *S. bovis* cercariae penetrate the host body through the skin, reaching the skin's capillary vessels. Subsequently, they migrate through the blood stream towards the lungs, where remain for several days as schistosomulum larvae. Following this, the schistosomula travel to the portal vein and mesenteric vessels, where they finally develop into the adult stage [2]. At this location, the adult schistosome worms release large numbers of eggs, many of which pass through the gut wall to the gut lumen and then exit the host with faeces. However, a significant fraction (up to 50%) of the eggs do not pass through the intestinal mucosa towards the lumen and are carried by the portal vein blood flow mainly toward the liver sinusoids, where they remain trapped and induce granulomatous lesions and strong immune responses, which typically result in portal hypertension and hepatic fibrosis [3].

The murine model of schistosomiasis is characterised by portal fibrosis, with limited to absent injury of hepatic parenchyma, significant vascular damage, and an apparently normal liver function [4, 5]. Recently, it has been reported that during the chronic phase of schistosome infection in mice, the proteins associated with several liver metabolic functions (i.e., the citric acid cycle, the fatty acid cycle and the urea cycle) are markedly downregulated, while the proteins associated with stress responses, acute phase reactants and structural components are all significantly upregulated [6, 7].

In addition, important alterations in the histology of the bile ducts in response to entrapped eggs, including the formation of massive granulomas, have been reported, although neither the underlying pathomechanisms of these hepatic lesions nor the likely changes in bile composition as consequence of such hepatic lesions have been studied [8, 9].

Bile is a biological fluid with a complex composition (bile salts, phospholipids, cholesterol, bilirubin, inorganic salts and proteins) that is produced by hepatocytes and transferred through the intrahepatic bile ducts to the gallbladder, where it is stored and concentrated at an approximately ten-fold concentration. In response to meals, bile is released into the duodenum through the common bile duct [10, 11]. Bile serves diverse functions, ranging from metabolism to transport; it essentially helps to emulsify fats, facilitating their digestion and absorption and as an excretion route for xenobiotics (e.g. toxins and drugs) and endobiotics (e.g. cholesterol and bilirubin) [11].

Since bile is produced in the liver, it can be speculated that the pathological changes induced in the liver and the biliary system during schistosome infection would be reflected as alterations in bile protein composition, and therefore in bile function. In spite of this and its foreseeable physiological impact, there is no information concerning the changes that schistosomal hepatopathy may elicit in the composition of bile.

Accordingly, and bearing in mind the crucial functions of bile, in the present work we were prompted to investigate whether the *S. bovis* infection induces changes in the biliary proteome. To accomplish this, we applied a protocol aimed at reducing the complexity of bile samples, thereby avoiding the presence of molecules that interfere with protein analysis, such as bile salts and lipids [10]. For this, whole gallbladders from *S. bovis*-infected and non-infected mice were dissected, homogenized and fractionated by differential centrifugation. The resulting protein fractions were resolved

by SDS-PAGE, the gels were sliced, and the gel pieces analyzed by LC-MS/MS. Altogether, in infected and non-infected mice we identified 1,937 proteins, which were classified according to their “protein class” and “molecular function”, and then subjected to an “Enrichment analysis”. The differences found in gallbladder proteomes between *S. bovis*-infected and non-infected mice are discussed.

2. MATERIALS AND METHODS.

2.1. Experimental animals and infection

Forty NMRI mice weighing 30–35 g (Harlan Laboratories Models, S.L.) were used. These mice were distributed into two experimental groups of 20 mice per group: G0 group of non-infected mice and G1 group of mice infected with *S. bovis*.

Mouse infection with *S. bovis* was done by tail immersion with 200 *S. bovis* cercariae for 60 min. Four months later, when the infections were fully established, all mice were autopsied and their gallbladders extracted and stored at –80 °C until processing.

All animal manipulations were done according to the rules from the ethical and animal welfare Committee from the Institution where the experiments were conducted (IRNASA, CSIC), following the corresponding EU rules and regulations.

2.2. Gallbladder protein extracts for proteomic analyses

The mouse gallbladders were collected and grouped in batches of 5 gallbladders/batch and processed for the preparation of protein extracts. Each batch was resuspended in PBS (pH = 7.4) containing a cocktail of proteinase inhibitors (Roche Diagnostics) (PBS-I) and homogenized six times (2 min each) using an Ultra-Turrax

T10 disperser (IKA-Werke) at full power. The homogenates were sonicated for 3 min, and then subjected to a fractionation process by centrifugation to reduce the complexity of the protein composition of samples and facilitate their proteomic analysis. First, the homogenates were centrifuged at 17,000 $\times g$ and 4°C for 30 min and the resulting supernatants (S-1) and pellets (P-1) were recovered. Next, a fraction of the S-1 supernatants was centrifuged at 100,000 $\times g$ and 4°C for 60 min and the new supernatants (S-2) and pellets (P-2) were recovered. The pellets obtained in each centrifugation step were resuspended in 100 μl of PBS-I. The protein concentration of all the fractions (supernatants and pellets) was assessed using the BCA Protein Assay Reagent kit (Pierce).

Samples of 15 μg of each fraction (S-1, S-2, P-1 and P-2) were mixed with 4x Laemmli buffer [12], heated to 90°C for 3 min and centrifuged at 12,000 $\times g$ for 4 min. The protein samples were then resolved by SDS-PAGE in 10% polyacrylamide gels and stained either with Coomassie Blue (Coomassie Blue R-25 0.125%, methanol 50%, acetic acid 10%) or silver stain, using the PlusOne Silver Staining Kit (GE Healthcare).

The silver-stained gels were digitalized with the ChemiDoc system (Bio-Rad) and gel images were analysed with Image Lab software (Bio-Rad). The gel lanes stained with Coomassie blue were sliced into 3 pieces and these slices were sent to the Proteomic Service of the University of Valencia (Spain) for protein identification.

2.3. In-gel enzymatic digestion and liquid chromatography and tandem mass spectrometry (LC-MS/MS).

For each G0 and G1 group, two independent batches of five gallbladders each were subjected to enzymatic digestion and LC-MS/MS analysis. For all the samples, technical replicas were obtained by injecting each sample twice.

Briefly, gel slices were conditioned with 50% acetonitrile, dried and digested with sequencing grade trypsin (Promega) (**20 ng/μl in 25 mM NH₄HCO₃**) overnight at 37°C. The reactions were stopped with 10% trifluoroacetic acid (TFA) at a final concentration of 0.1%, and the supernatants were **filtered through a 0.22 μm filter and** dried by centrifugation in a vacuum. The concentration of peptides was estimated by UV spectrometry, assuming that a 1 mg/ml solution of proteins had an extinction coefficient of 1.1 absorbance units at 280 nm.

The peptides extracted following in-gel digestion **were resuspended in 6 μl of** 5% acetonitrile, 0.1% TFA, **and 5 μl of the sample was loaded onto** a trap column (**NanoLC Column, 3μ C18-CL, 350 μm×0.5 mm, Eksigen**) and desalted with 0.1% TFA at a flow rate of **3 μl/min for 5 min**. The peptides were then loaded onto an analytical column (**LC Column, 3μ C18-CL, 75 μm×12 cm, Nikkyo**) equilibrated in 5% acetonitrile and 0.1% formic acid. The peptides eluted were analyzed with a nanoESI-Q-TOF mass spectrometer (5600 TripleTOF, ABSciex) in information-dependent acquisition mode, in which a 0.25-s TOF MS scan from 350 to 1250 m/z was performed, followed by 0.05-s product ion scans from 100 to 1500 m/z on the 50 most intense 2–5 charged ions.

2.4. Database searching and protein identification

Database searches were performed in the NCBI *Mus musculus* database (35,149,712 sequences; 12,374,887,350 residues) using the Mascot v2.2 (Matrix Science) search engine. For the samples from the G1 group, an additional search was performed in a custom-made Trematoda_EST database (2,472,666 sequences; 396,860,374 residues) using the nucleotide sequences published in the NCBI_EST

database. This search was performed to evaluate the potential presence of proteins of parasite origin.

For the Mascot searches, the peak lists were generated directly from QSTAR wiff files by Mascot Daemon v. 2.2.2 (Matrix Science) with Sciex Analyst import filter options using the default parameters. Databases were searched using the following parameters: tryptic specificity, allowing one missed cleavage and a tolerance on the mass measurement of 70 ppm in MS mode and 0.6 Da for MS/MS ions. The carbamidomethylation of Cys was set as a fixed modification, and Met oxidation and Asn/Gln deamidation were set as variable modifications. The significance threshold was set at 0.05 and proteins required at least two unique significant peptides to be considered identified.

Redundant identifications were eliminated and the keratins and immunoglobulins found were excluded from the lists of identified proteins provided in the Results section.

2.5. Data analysis

Protein classification was performed according to the Gene Ontology (GO) hierarchy using the Universal Protein Resource (UniProt) retrieval system (<http://www.uniprot.org/>). **The “ID mapping” module for the** UniProt was used to transform the GI number to UniProt code, standardize proteins symbols, and associate them with corresponding gene names, gene ontology categories and IDs, molecular functions, subcellular location and tissue specificity.

The classification and analysis of the proteins identified was performed using the Panther classification system (<http://www.pantherdb.org/>) [13] and the “Enrichment Analysis” module of the WEB-based GENE SeT Analysis Toolkit (WebGestalt) (<http://5ht-dev.accre.vanderbilt.edu/webgestalt>) [14]. Enrichment analyses for the Gene Ontology categories (biological process and molecular function) and for the biological pathways in the Wikipathways database were carried out using the mmusculus_genome database as reference set, the hypergeometric test as a statistical method, and BH as a multiple test adjustment method [15]. The significant level for the GO enrichment analysis for biological process and molecular function was the top 10 categories, and for the Wikipathways enrichment analysis a significance of $p < 0.0001$ and a minimum number of 6 genes for each category were selected.

3. RESULTS

3.1. Gallbladder protein extracts.

Gallbladders from the G0 and G1 mice were homogenized in batches of 5 and these homogenates were fractionated by means of two consecutive centrifugations at 17,000 and 100,000 $\times g$, respectively. Thus, we obtained 4 fractions: supernatants S-1 and S-2 and pellets P-1 and P-2. The proteins in each of these fractions were resolved by SDS-PAGE and stained with silver for protein visualization.

Fig. 1 shows the results of this fractionation procedure, showing that the S-1 and P-1 fractions had complex band patterns that covered a broad range of molecular sizes, and also revealing some differences in protein composition between the G0 and G1 mouse groups localized around 52 kDa for S-1 and between 34 and 42 kDa for P-1.

Regarding fractions S-2 and P-2, their band patterns were less complex, although they still showed differences between the G0 and G1 mice (Fig. 1).

After fractionation, for each fraction (S-1, P-1, S-2 and P-2) all batches of gallbladders from the same mouse group (G0 or G1) showed identical band patterns to that of the homologous fraction in Fig. 1 (not shown), demonstrating the reproducibility of the procedure.

3.2. Proteins identified.

The results presented here are the sum of the identifications performed with Mascot in the NCBI nr *Mus musculus* database in the two gallbladder batches (5 gallbladder/batch) analyzed from each group of mice (G0 and G1). In order to simplify the comparative analysis between the G0 and G1 mice, these results do not include keratins, immunoglobulins or immunoglobulin-like proteins.

The proteomic analysis of all the gallbladder fractions –supernatants and pellets– allowed the identification, on the basis of at least two significant peptides, of a total of 1,937 non-redundant proteins: 1,589 in the gallbladder of non-infected mice (G0) and 1,489 in the gallbladder of mice infected with *S. bovis* (G1). Of these proteins, 348 were identified only in the gallbladder of the G1 group; 448 only in the G0 group, and 1141 in both groups of mice (Table 1 and Supplementary Table 1).

The number of non-redundant proteins identified in individual fractions ranged between 300 and 1069, the S-1 fractions showing the highest values, followed by the P-1, P-2 and S-2 fractions. The only exception was the P-2 fraction from the G0 group, in which up to 1,044 non-redundant proteins were identified (Table 1).

A search of the custom-made Trematoda_EST database with the samples from the G1 mice (infected with *S. bovis*) identified a total of 189 non-redundant hits. The

subsequent BLAST analysis of these sequences allowed us to identify 154 proteins and 35 hypothetical, unknown or non-characterized proteins (Supplementary Table 2). All these identified proteins, most of them belonging to highly conserved protein families such as actins, tubulins, heat shock proteins, histones, etc., had already been identified in the NCBI *Mus musculus* database, so that any of them could be considered of parasite origin.

The proteins identified in all the fractions from the same experimental group of mice, either G0 or G1, were grouped and analyzed together. The proteins were classified first by their “Protein Class” and “Molecular Function” using the Panther Classification System, followed by an “Enrichment Analysis” with the *Mus musculus* genome as the reference set.

Protein classification.

The “Protein Class” classification of the biliary proteomes from *S. bovis* infected and non-infected mice is shown in Fig. 2. For both groups of mice, G0 and G1, the proteins were grouped in the same 28 protein classes, showing a very similar distribution. The most numerous protein classes were oxidoreductases (17.6% in G0 and 15.7% in G1) and hydrolases (15.1% in G0 and 17.6% in G1), followed by transferases (13.1% in G0 and 11.8% in G1), nucleic acid-binding proteins (11.5% in G0 and 10.8% in G1), enzyme modulators (7.3% in G0 and 8.8% in G1), cytoskeletal proteins (9.0% in G0 and 8.6% in G1) and proteases (5.7% in G0 and 6.9% in G1). There were few differences in the protein class ratios between the G0 and G1 mice, being oxidoreductases and transferases slightly more numerous in the non-infected mice (G0), while hydrolases, proteases and enzyme modulators were slightly more enriched in the infected mice (G1).

An in deep analysis of the proteins classified as “**oxidoreductase proteins**” identified in both groups revealed certain particularities, detailed below. In this “**Protein Class**” 215 non-redundant proteins were included, of which 197 were identified in the G0 group and 174 in G1. In a comparative analysis of the proteins included in the “oxidoreductase proteins **class**” identified exclusively in each group, 41 in G0 and 18 in G1, the following were observed: (i) In both groups, G0 and G1, proteins involved in different metabolic processes (carbohydrate metabolism, glycogen metabolism, glycolysis, etc) were identified; (ii) 6 of the 7 proteins involved in immune response/inflammation/stress response, were identified only in the G1 group; (iii) proteins involved in lipid metabolism were only identified in the G0 group, and (iv) 8 of the 9 components involved in the respiratory electron transport chain, among which several members of the Cytochrome P450 family were included, were identified only in the samples from the G0 group (Supplementary Table 3).

Regarding these components of the Cytochrome P450 family (Cyp) in the gallbladders of groups G0 and G1, of the 39 proteins of this family identified jointly in both groups, all were identified in the G0 group but only 16 were present in the gallbladders of the infected mice (G1) (see Supplementary Table 1).

The additional “**molecular function**” classification of the identified proteins resulted in the following categories: catalytic, binding, structural, antioxidant, enzyme regulator, receptor, translation regulator, and transporter activity. In both groups of mice, G0 and G1, the most numerous categories were those involving catalytic (49.1% in G0 and 49.7% in G1), binding (21.3% in G0 and 21.4% in G1) and structural activity (11.7% in G0 and 10.9% in G1). The remaining categories showed remarkably lower ratios, ranging between 0.8 % and 5.8% in the G0 mice and 0.7 % and 5.5% in the G1

mice; in both mouse groups these ratios corresponded to proteins with antioxidant and transporter activity, respectively.

The proteins identified only in non-infected mice (G0), only in infected (G1) mice, and in both groups of mice (G0 and G1), classified according to their molecular function are summarized in Fig. 3. Proteins with catalytic function represented 46.3%, 50.0% and 49.6% of the identified proteins only in G0, only in G1, and in both groups, respectively. Thus, to avoid masking the relative abundance of the other groups of proteins represented, the catalytic activity category is not included in Fig 3. This figure shows no differences between G1 and G0 groups in the percentage of proteins with binding, antioxidant, receptor and transporter activity. By contrast, some differences were observed between both groups of animals in the percentages of proteins with structural (13.3% in G0 *versus* 7.9% in G1) and translation regulator activity (4.7% in G0 *versus* 0.8% in G1), which were both more numerous in G0 than in G1. Conversely, proteins with enzyme regulator activity were more numerous in the G1 group (8%) than in the G0 group (1.9%) (Fig. 3).

Enrichment analysis of the proteins identified.

We used the Web-based Gene Set Analysis Toolkit (WebGestalt) to detect the enriched biological pathways, biological processes and molecular functions associated with the bile proteins identified in G0 and G1 groups.

In the Fig. 4 we illustrate the ratio of enrichment of 48 biological pathways, with a significance of $p < 0.0001$ and a minimum number of 6 genes for each category. Up to 39 of these 48 pathways proved to be significantly enriched in the gallbladder proteomes of both groups of mice. For some of these 39 pathways, differences in their enrichment ratios were observed between the mouse groups, being typically higher in the G0 than in the G1 mice, and especially evident in the following pathways: oestrogen

metabolism (29.74% in G0 *versus* 16.33% in G1), glucuronidation (29.34% in G0 *versus* 22.46% in G1), mitochondrial beta-oxidation (41.98% in G0 *versus* 35.32% in G1) and oxidative stress (20.28% in G0 *versus* 13.61% in G1) (Fig. 4).

The remaining 9 pathways were differentially enriched in only one group. The circadian exercise, irinotecan pathway, retinol metabolism and TGF-beta receptor signalling pathway proved to be significantly enriched only in the G0 group, and the EGFR1 signalling pathway, the FAS pathway, the IL-6 signalling pathway and T Cell receptor signalling pathway were only enriched significantly in the G1 group.

Table 2 shows the proteins involved in a representative group of enriched pathways, e.g. the oestrogen metabolism, the irinotecan metabolism, the oxidative stress and the TGF-beta receptor signalling pathways. As can be seen, some protein families, such as the UDP-glucuronyl transferases, cytochrome P450, MAP kinase and NAD(P)H dehydrogenase, were involved in several biological pathways. The proportion of proteins identified in the G0 group with respect to those identified in G1, was always higher in the former. Accordingly, this proportion (n° of proteins identified in the G0 group with respect to those identified in G1) was as follows: 1.6 in the oestrogen metabolism pathway; 2.0 in the irinotecan pathway; 1.5 in oxidative stress, and 1.4 in the TGF Receptor Signalling Pathway.

Enrichment analyses for the Gene Ontology biological process and molecular function are represented in Fig. 5. The biological processes that proved to be significantly enriched in the gallbladder were all metabolic processes. The most outstanding differences between G0 and G1 were: (i) the higher number of proteins involved in the carbohydrate and monosaccharide metabolic processes identified in the infected mice (G1) and, (ii) the higher number of proteins involved in coenzyme and

cofactor metabolic processes identified in the G0 group with respect to the G1 mice (Fig. 5).

Regarding molecular functions, the group of proteins significantly enriched in the samples analyzed were those with a binding function and catalytic activity. As can be seen in Fig. 5, the most salient result was the greater number of genes with glutathione transferase activity present in the gallbladders of the G0 group as compared with G1. Overall, we identified up to 19 members of different classes (alpha, mu, kappa, pi and theta) of the glutathione S-transferase enzyme family, 15 in the gallbladders of infected mice and up to 19 in the gallbladders of non-infected mice (see Supplementary Table 1).

4. DISCUSSION

As stated in the introduction section, bile is a body fluid produced by the liver that has digestive and excretion functions and its formation depends on the structural and functional integrity of the bile-secretory apparatus [16, 17]. The main components of bile are bile salts, fatty acids and cholesterol, corresponding to a protein content of only 7%. The sources of proteins in bile include plasma, hepatocytes and the epithelium of the biliary tract and the gallbladder [17].

Regarding schistosomiasis, there is in fact no evidence indicating that schistosome infection elicits any kind of biliary disease. Indeed, only a few isolated cases of patients with schistosomal cholecystitis and associated with concomitant gallstones have been reported (less than 10 cases have been described in the medical literature) [18–21]. Nevertheless, considering that schistosome infections cause important liver lesions that also affect the bile secretory apparatus, it is highly likely that

biliary secretion would be affected in infected animals. Moreover, since some biliary proteins are produced by hepatocytes and the epithelium of the biliary tree, it could be surmised that such infections might also modify the bile protein content. Accordingly, and bearing in mind that there is little information about this aspect either, we were prompted to determine whether any kind of change in the proteome of the gallbladder might arise in mice infected with *S. bovis* for 4 months.

Regarding the composition of bile, although the chemical composition of human bile is well understood it is only recently when studies have begun to unravel its protein constituents. In the last 10 years, in parallel with the development of proteomic techniques, studies addressing human bile proteins have advanced considerably and we now have available a comprehensive catalogue of biliary proteins [11]. The aims of these studies have been to improve the understanding of the physiological processes involved in the regulation of the hepato-biliary system; to explore the involvement of bile proteins in health and disease, and to discover biomarkers for biliary tract malignancies [10, 11, 17, 22–24]. Such work has also served to optimize protocols for sample preparation and to solve the pitfalls involved in the proteomic analysis of bile samples that result from the massive concentration of interfering compounds [17].

In the present work we used a protocol to study the proteins present in bile samples similar to that described by Farina et al. [10], based on sample fractionation by serial centrifugation and acrylamide gel electrophoresis. The aim of this was to reduce the complexity of the bile samples before mass spectrometry and to limit unavoidable losses related to other additional purification methods [17]. In this study with *S. bovis* we analyzed complete gallbladders instead of bile samples, as has been the case in other studies addressing the human bile proteome, owing to the difficulty involved in collecting bile samples from mice in sufficient quantity without being contaminated

with tissue proteins. This problem was exacerbated in the group of mice infected with *S. bovis*, having these mice an apparent reduction in gallbladder volume (not shown), effect that has already been reported in patients with portal hypertension due to advanced *Schistosoma mansoni* infection [17].

After processing the gallbladders of the infected and non-infected mice, the small differences observed in the profile of the protein fraction bands (Fig. 1) afforded the first indications of possible modifications in the biliary proteome due to *S. bovis* infection. The analysis by LC-MS/MS of these fractions revealed 1589 and 1489 non-redundant proteins in mice not infected and infected with *S. bovis* respectively. Bearing in mind that we analyzed complete gallbladders, this number is compatible with the 812 proteins identified by Farina et al. [11] in a similar proteomic analysis of human bile.

Initially, the comparison of the biliary proteomes from non-infected and infected mice did not revealed important differences in either the number or the type of proteins identified, as inferred from the “Protein class classification” (see Fig 2). However, after a more detailed analysis of some groups of proteins, such as those included in the oxidoreductase class, together with the analysis of the enrichment of the wikipathways and Gene Ontology categories, we did detect significant differences between the mice infected and not infected with *S. bovis*. It should be noted that some of the differences observed in the protein composition between the G0 and G1 groups could in fact represent quantitative differences in the expression level of the proteins, since the least abundant proteins would be below the threshold of detection by MS.

The analysis and comparison of the respective proteomes revealed that several biological pathways and relevant molecular functions were underrepresented in the biliary proteome of the infected mice (G1 group). Thus, in the gallbladders of the infected mice we observed the expression of a significantly lower number of proteins

involved in several pathways, being especially relevant among them the irinotecan pathway, oestrogen metabolism and oxidative stress. We also observed a lower number of components with metabolic functions and of molecules with glutathione S-transferase activity (see Fig. 4 and 5). Irinotecan (IRI) is an anti-proliferative cytotoxic agent used to treat metastatic colorectal cancer that is metabolized in the liver and converted to SN-38, the active metabolite [26]. Additionally, the liver –and other peripheral tissues– is a site for the biosynthesis of oestrogens and it is also the main site for further biotransformation [27]. Three of the hepatic biological processes that are affected by *S. bovis* infection –namely irinotecan metabolism, the biosynthesis and biotransformation of oestrogens and oxidative stress– involve several components of the Cytochrome P450 family (CYP), glutathione S-transferase (GST) and the UDP glucuronyltransferase family (UGT).

Hepatic CYP, GST and UGT are three multigene families of enzymes that play a critical role in the metabolism of many drugs and xenobiotics [28–30]. It has been reported that the activities and levels of expression of these enzymes are altered by inflammatory stimuli, including helminthic infections [31]. In this sense, it has been shown that in chronic *S. mansoni* infection (late phase of schistosomiasis) the liver content and activity of some isoforms of CYP, GST and UDP are downregulated and these regulations seem to be associated with a greater induction of Th2 cytokines [32–35]. The biliary proteome of the mice infected with *S. bovis* studied in the present work reflects a situation similar to that described for the liver, where the numbers of CYP, GST and UGT isoforms are underrepresented with respect to normal values (16 CYP, 15 GST and 12 UGT isoforms in gallbladder in mice infected with *S. bovis*; 39 CYP, 19 GST and 19 UGT isoforms in gallbladder from non-infected mice) (see Supplementary Table 1). Our results corroborate those reported by other authors for the liver and show

that the elimination of drugs via both CYP and phase II of drug metabolism such as GST and UGT may be severely impaired during murine chronic schistosomiasis [35]. In fact, it has been demonstrated that chronic *S. mansoni* infection influence the pharmacokinetic profiles of some drugs, such as praziquantel, enpiroline and mefloquine, leading to delayed clearance [36, 37]. Accordingly, given that schistosomiasis is prevalent in countries where coinfection for diseases such as HIV or malaria is common, it is important to ask how schistosomiasis affects the enzymes responsible for the metabolism of drugs given to treat not only schistosomiasis but also these coinfections [35].

Regarding the signalling molecules and pathways significantly enriched in the G1 group of infected animals (Fig.4), several observations deserve further comment, including: (i) the upregulation of pathways involved in chronic inflammation induction such as the EGFR1 and the IL-6 signalling pathways, which are cross-connected and have important roles in the proliferation of key inflammatory hepatic cells. [38]; (ii) the upregulation of these two ways could in turn be related to the increase in the FAS-mediated apoptotic pathway also observed in our study, since inflammation is targeted once activated the apoptotic cascade [39]; (iii) the increase in the cytokine IL-6 can also be related to lipid metabolism disorders, in order to modulate them accordingly [40] and (iv) concerning the observed increase in the T-cell receptor signalling pathway, it is well established that schistosome infection promotes T-cell activation and a Th2-type inflammatory pathology, which requires host T-cell receptor signals, favouring parasite growth and development in the mammalian host [41, 42]. Then, the upregulation of this signalling pathway in the gallbladder and liver would favour the Th2-type cells that are critical during helminthic infections and support the deep effects of the parasite-induced inflammatory environment on the host.

In sum the present work reports the first data on the proteome of the mouse gallbladder and provides a comprehensive catalogue of biliary proteins that may be of great use in other studies addressing biliary physiology and pathology. We show that chronic schistosome infections cause significant changes in the biliary proteome that may produce physiological alterations and affect the therapeutic actions of drugs when administered to human patients and animals with schistosomiasis.

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 584

586 Figure captions

587

588 Figure 1. Silver-stained 10% polyacrylamide gels showing the protein fractions
589 obtained from gallbladder homogenates of non-infected (G0) and *Schistosoma bovis*-
590 infected mice (G1). Gel lanes were sliced into 3 pieces and the resulting gel slices were
591 digested with trypsin and analyzed by LC-MS/MS. S-1 and P-1, supernatant and pellet
592 from gallbladder homogenate centrifugation at 17,000g for 30 min. S-2 and P-2,
593 supernatant and pellet from centrifugation of S-1 at 100,000g for 1 h.

594

595 Figure 2. The proteins identified in the gallbladder from non-infected mice (G0) and
596 mice infected with *Schistosoma bovis* (G0) were classified in “**Protein classes**” using the
597 Panther Classification System. Bars represent the percentage of proteins in each protein
598 class relative to the total number of proteins in the group.

599

600 Figure 3. Classification according to their molecular function of the proteins identified
601 only in non-infected mice (G0), only in mice infected with *Schistosoma bovis* (G1) and
602 in both groups of mice (G0 and G1). Pie charts represent the percentages of proteins in
603 each category without including the proteins with catalytic activity.

604

605 Figure 4. Ratio of enrichment (R) of the 48 significantly enriched pathways associated
606 with proteins identified in the proteome of the gallbladders of non-infected (G0) and
607 *Schistosoma bovis*-infected (G1) mice. Significance criteria included $p < 0.0001$ and a
608 minimum number of 6 genes for each category.

609

610 Figure 5. Ratio of enrichment (R) of top 10 enriched biological processes and molecular
611 functions associated with proteins identified in the proteome of the gallbladders of non-
612 infected (G0) and *Schistosoma bovis*-infected mice (G1). Significance criteria included
613 $p < 0.0001$ and a minimum number of 6 genes for each category.

614

Table 1. Number of unique proteins identified in the fractions of the gallbladder homogenates. Redundant identifications, keratins and immunoglobulins have been excluded. G0, non-infected mice; G1, mice infected with *Schistosoma bovis*.

Gallbladder fractions	G0	G1
S-1 (17,000g supernatant)	998	1069
P-1 (17,000g pellet)	768	752
S-2 (10 ⁵ g supernatant)	300	379
P-2 (10 ⁵ g pellet)	1044	757
TOTAL	1589	1489

Specific and shared identifications	Only G0	G0 and G1	Only G1
	448	1141	348

Table 2

Table 2. Proteins identified in the gallbladder of *Schistosoma bovis* infected (G1) and non-infected (G0) biological pathways: Estrogen metabolism, Irinotecan pathway, Oxidative Stress and TGF-beta Receptor Signaling

Experimental group	Uniprot ID	Gene ID	Gene name	Protein name	Pathway
G0	P13745	14857	Gsta1	glutathione S-transferase, alpha 1 (Ya)	Estrogen metabolism
G0	P00184	13076	Cyp1a1	cytochrome P450, family 1, subfamily a, polypeptide 1	Estrogen metabolism
G0	Q8VCT4	104158	Ces3	carboxylesterase 3	Irinotecan pathway
G0	Q5FW80	394430	Ugt1a10	UDP glycosyltransferase 1 family, polypeptide A10	Irinotecan pathway
G0	P70691	22236	Ugt1a2	UDP glucuronosyltransferase 1 family, polypeptide A2	Irinotecan pathway
G0	Q8R0P3	394435	Ugt1a6b	UDP glucuronosyltransferase 1 family, polypeptide A6B	Irinotecan pathway
G0	Q61133	14872	Gstt2	glutathione S-transferase, theta 2	Oxidative Stress
G0	P09671	20656	Sod2	superoxide dismutase 2, mitochondrial	Oxidative Stress
G0	P28652	12323	Camk2b	calcium/calmodulin-dependent protein kinase II, beta	TGF-beta Receptor Signaling
G0	Q8K1M3	19087	Prkar2a	protein kinase, cAMP dependent regulatory, type II alpha	TGF-beta Receptor Signaling
G0	P07214	20692	Sparc	secreted acidic cysteine rich glycoprotein	TGF-beta Receptor Signaling
G0	Q6P5F9	103573	Xpo1	exportin 1, CRM1 homolog (yeast)	TGF-beta Receptor Signaling
G1	Q64669	18104	Nqo1	NAD(P)H dehydrogenase, quinone 1	Estrogen metabolism
G0, G1	B6VGH4	13077	Cyp1a2	cytochrome P450, family 1, subfamily a, polypeptide 2	Estrogen metabolism
G0, G1	P10649	14862	Gstm1	glutathione S-transferase, mu 1	Estrogen metabolism
G0, G1	Q6XL48	394433	Ugt1a5	UDP glucuronosyltransferase 1 family, polypeptide A5	Irinotecan pathway
G0, G1	Q6ZQM8	394432	Ugt1a7c	UDP glucuronosyltransferase 1 family, polypeptide A7C	Irinotecan pathway
G0, G1	Q62452	394434	Ugt1a9	UDP glucuronosyltransferase 1 family, polypeptide A9	Irinotecan pathway
G0, G1	Q64435	94284	Ugt1a6a	UDP glucuronosyltransferase 1 family, polypeptide A6A	Irinotecan pathway
G0, G1	P97494	14629	Gclc	glutamate-cysteine ligase, catalytic subunit	Oxidative Stress
G0, G1	P11352	14775	Gpx1	glutathione peroxidase 1	Oxidative Stress
G0, G1	P47791	14782	Gsr	glutathione reductase	Oxidative Stress
G0, G1	Q9JMH6	50493	Txnrd1	thioredoxin reductase 1	Oxidative Stress

G0, G1	Q9JLT4	26462	Txnrd2	thioredoxin reductase 2	Oxidative Stress
G0, G1	Q6PHZ2	108058	Camk2d	calcium/calmodulin-dependent protein kinase II, delta	TGF-beta Recept
G0, G1	Q9QZD9	54709	Eif3i	eukaryotic translation initiation factor 3, subunit I	TGF-beta Recept
G0, G1	P47811	26416	Mapk14	MAP kinase 14	TGF-beta Recept
G0, G1	P49817	12389	Cav1	caveolin 1, caveolae protein	TGF-beta Recept
G0, G1	Q02248	12387	Ctnnb1	catenin (cadherin associated protein), beta 1	TGF-beta Recept
G0, G1	P63017	15481	Hspa8	heat shock protein 8	TGF-beta Recept
G0, G1	P70168	16211	Kpnb1	karyopherin (importin) beta 1	TGF-beta Recept
G0, G1	Q9Z1Z2	20901	Strap	serine/threonine kinase receptor associated protein	TGF-beta Recept
G0, G1	Q922Z3	68015	Trap1	TNF receptor-associated protein 1	TGF-beta Recept

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 4

Figure 4

Figure 5

Figure 5